JOURNAL OF BACTERIOLOGY, June 1969, p. 949-955
Vol. 98, No. 3

Printed in U.S.A.

Killing and Lysis of Gram-negative Bacteria Through the Synergistic Effect of Hydrogen Peroxide, Ascorbic Acid, and Lysozyme

T. E. MILLER

Trudeau Institute, Medical Research Laboratories, Saranac Lake, New York 12983

Received for publication 22 January 1969

A mixture of hydrogen peroxide and ascorbic acid has been found to generate an antibacterial mechanism which is active against gram-negative bacteria. It results in bacterial death and renders the organism sensitive to lysis by lysozyme. Under the conditions used, horseradish peroxidase did not augment the antibacterial effect. It is suggested that the effector mechanism involves the generation of short-lived free radicals which disturb the integrity of the cell wall. This effect alone might kill bacteria by interfering with selective permeability, but in the presence of lysozyme a further bactericidal activity is accomplished by complete disruption of the cell. It is proposed that a transient antibacterial system such as that described could exist within phagocytic cells. Free radicals would be formed through the interaction of certain oxidizable substances and hydrogen peroxide, which is produced during the enhanced metabolic activity that accompanies ingestion of bacteria. Such a system would help to explain why macrophages, which are apparently devoid of preformed bactericidins, are nonetheless very efficient in killing most phagocytosed bacteria.

Elie Metchnikoff, a pioneer in the field of immunology, was the first to emphasize the importance of phagocytes in the host-parasite relationship. He demonstrated that after the ingestion of a living microorganism by phagocytic cells a digestive and destructive action accomplished the complete dissolution of the microbe (11). He was not able to characterize the digestive ferment acting on the microbes or even to determine whether enzymic attack was the process responsible for their death.

Cohn, in his studies on bacterial inactivation within macrophages, demonstrated that death preceded digestion. He also noted that lysozyme-sensitive bacteria were degraded more readily in both polymorphonuclear leukocytes and macrophages (3). Lysozyme is the bacteriolytic enzyme shown by Fleming in 1922 (10) to lyse suspensions of certain bacterial species. Its lytic action is brought about by the enzymatic hydrolysis of the β-1,4-glycosidic linkages in the peptidoglycans which are responsible for the rigidity of the bacterial cell (14, 32). This substrate is known to exist in the cell walls of both gram-positive and gram-negative bacteria, but not always in an accessible location. Since the cell wall of the gram-positive *Micrococcus lysodeikticus* is composed almost exclusively of the peptidoglycan, this organism is exquisitely sensitive to lysis by lysozyme. In the case of gram-negative bacteria, however, the surface layers, containing lipopolysaccharides, proteins, and lipids, so obscure the substrate as to make it inaccessible to the enzyme and, therefore, resistant to lysis. It is known, however, that in some circumstances gram-negative bacteria may be rendered sensitive to lysozyme. Thus, under extreme conditions of H-ion concentration, such as *p*H 3.5 (32) or 9.0 (34), or in conjunction with the chelating compound ethylenediaminetetraacetate (29), lysozyme exerts a lytic effect on gram-negative bacteria. These, however, are artificial conditions which are unlikely to be employed by phagocytes in their attack on bacteria. A more natural bacteriolytic system, consisting of specific antiserum, complement, and lysozyme, was shown to degrade lysozyme-resistant gram-negative bacteria (1, 23). However, destruction of bacteria within phagocytes can occur in the absence of a classical antibody molecule (2, 19).

From this brief review, it is obvious that a need exists for a better understanding of the mechanisms which would explain the degradation of bacteria within phagocytic cells (3). One

approach to this problem is to create a lytic system in vitro which makes use of conditions and substances that are known to exist in phagocytic cells. The system would have to be capable of acting on surface layers of gram-negative bacteria so as to uncover the substrate of lysozyme. For this purpose, hydrogen peroxide and ascorbic acid were selected for study because they have been shown to exert a degradative effect on lipids, carbohydrates, and proteins (12, 15, 22, 24, 37); moreover, they are known to be present in leukocytes (13, 27, 28, 36).

As stated earlier, it is known that degradation of bacteria within phagocytic cells follows the bactericidal event (3). It was of interest, therefore, to investigate whether the act of uncovering the lysozyme substrate of these gram-negative bacteria was in itself bactericidal. The antibacterial activity of hydrogen peroxide as a topical antiseptic has been recognized for decades (7). Also, the in vitro bactericidal activity of hydrogen peroxide in conjunction with ascorbic acid has been reported (8, 9). Recently, hydrogen peroxide has been implicated in the killing of bacteria inside phagocytic cells (13, 16, 21). According to a current hypothesis, the synthesis of hydrogen peroxide is coupled with the oxidation of reduced nicotinamide adenine dinucleotide phosphate produced through the hexose monophosphate shunt (30), a metabolic event which is augmented after the ingestion of bacteria by the cell (20). However, the exact mechanism of bacterial inactivation in all the above cases is far from clear.

This paper demonstrates that a mixture of hydrogen peroxide and ascorbic acid generates an antibacterial mechanism which appears to depend upon free radical formation. It results in bacterial death and renders the organism sensitive to lysis by lysozyme.

MATERIALS AND METHODS

Determination of lytic activity. The components used in the system were added to Bausch and Lomb Spectronic-20 colorimeter tubes. They were incubated at 37 C and, at intervals, the optical densities (OD) were measured at 540 nm.

Determination of bactericidal activity. The components used in this system were added to Falcon plastic tubes containing bacteria at a final concentration of 10^8 to 10^9/ml. The mixtures were incubated for 30 min at 37 C. They were then diluted in Hanks' balanced salt solution and were plated on Trypticase soy-agar plates.

Materials. The hydrogen peroxide used was a 30% (v/v) solution of s. p. grade Superoxol from Merck & Co., Inc., Rahway, N.J. L-Ascorbic acid was obtained from Eastman Organic Chemicals, Rochester, New York, and from Merck and Co. Horseradish peroxidase (HOPD grade) and lysozyme (Lys) were obtained from Worthington Biochemical Corp., Freehold, N.J. Sodium thiosulfate was obtained from J. T. Baker Co., Phillipsburg, N.J.

The gram-negative organisms employed in this study were *Salmonella pullorum*, *S. enteritidis*, and an untyped *Escherichia coli* from a human isolate. They were grown in Trypticase soy broth (BBL). Organisms in the log phase of growth were washed and suspended in the buffer to be employed for the experiment at a concentration which gave the desired OD at 540 nm when added to the reaction mixtures.

RESULTS

Lytic effect of H_2O_2, ascorbic acid, and lysozyme. In the following experiments, the reaction mixtures contained combinations of acetate (*p*H 5.0) or phosphate buffers (*p*H 6.2 or 7.0) at 50 to 100 μmoles/ml: *S. pullorum*, 2×10^8 to 6×10^8/ml; H_2O_2, 1.0 to 10.0 μmoles/ml; ascorbic acid (AA), 0.1 μmole/ml; and Lys and horseradish peroxidase (HRP), both at 10 μg/ml. The various mixtures were incubated at 37 C for 60 min. OD at 540 nm was measured after 0, 30 and 60 min.

The results of Fig. 1 reveal that little or no change occurred in OD in any of the control mixtures. In contrast, the combination of H_2O_2, AA, and Lys showed a significant decrease in OD indicative of lysis. The inability of HRP to enhance this effect was unexpected, since it is known to catalyze the reaction between H_2O_2 and AA (40). The same lytic phenomenon was also found with *S. enteritidis* and *E. coli*.

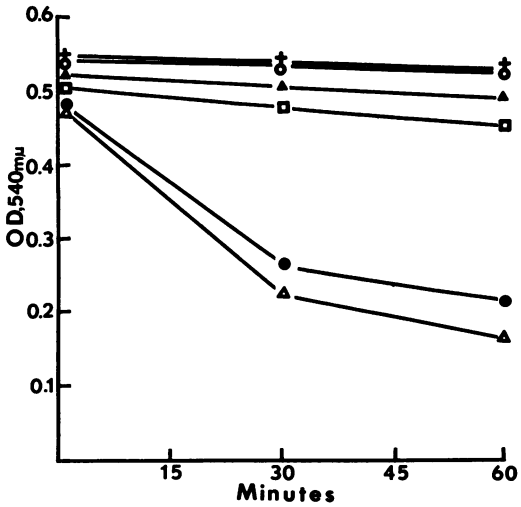

FIG. 1. *Changes in OD of a suspension of S. pullorum incubated at 37 C in the presence of buffer control (+); Lys and AA (○); H_2O_2 and Lys (▲); H_2O_2 and AA (□); H_2O_2, AA, Lys, and HRP (●); H_2O_2, AA, and Lys (△).*

Effect of pH on the lytic activity of H_2O_2, AA, and Lys. In the foregoing experiment, lysis of bacteria occurred at *p*H 6.2. However, the H-ion concentration within phagocytes might be expected to range between neutrality and the acidic *p*H which develops within digestive vacuoles. Accordingly, the lytic activity of H_2O_2, AA, and Lys were compared at *p*H 5.0, 6.2, and 7.0. Since the previous experiment had suggested a slight decrease in OD with H_2O_2 and AA, this mixture was also tested at the same H-ion concentrations (Fig. 2).

At *p*H 5.0, no lytic activity was observed in the H_2O_2 + AA + Lys mixture. In contrast, lysis was virtually complete within 15 min at *p*H 7.0; whereas at the intermediate *p*H, progressive lysis occurred over the entire period of incubation.

In the absence of Lys, H_2O_2 + AA produced a small but maximal change in OD at *p*H 7.0. From this data, it was not readily apparent whether this decreased OD indicated partial lysis or a change in the shape of the bacterial cells. The latter might be expected if the surface components of the cell are modified under the influence of H_2O_2 + AA, so that Lys-substrate becomes exposed. This possibility was examined in the following experiment.

Uncovering the lysozyme substrate with H_2O_2 and AA. Reaction mixtures were set up containing bacteria and H_2O_2 or bacteria, H_2O_2, and AA. The concentration of each was identical to those used in the previous experiments. The mixtures were incubated for 30 min, centrifuged to remove reagents, and resuspended in buffer containing Lys at 10 μg/ml. A normal reaction mixture was also prepared at this time; it contained bacteria, Lys, H_2O_2, and AA. The reaction mixtures were then reincubated for 60 min at 37 C. OD was read at intervals of 15 min.

The results in Fig. 3 show that bacterial cells were modified by pretreatment with H_2O_2 and AA. Upon resuspension, they were found to have been rendered susceptible to the enzymic action of Lys. H_2O_2 alone did not sensitize the cells in this way.

It will be noted that OD at zero-time were not uniform. This was due to a dilution effect in tubes from which AA had been omitted, but in those containing H_2O_2 and AA it was due to the rapidity with which lysis occurred after the addition of Lys.

Relationship between bacteriolytic and bactericidal activities at neutral and acid pH. The results in Table 1 demonstrate that a powerful bactericidal effect is generated in a mixture consisting of H_2O_2, AA, and Lys. It was much greater at *p*H 7.0 than at *p*H 5.0, and it was impaired, rather than enhanced, by the addition of HRP, as was noted previously in bacteriolysis. For this reason, HRP was omitted from all subsequent

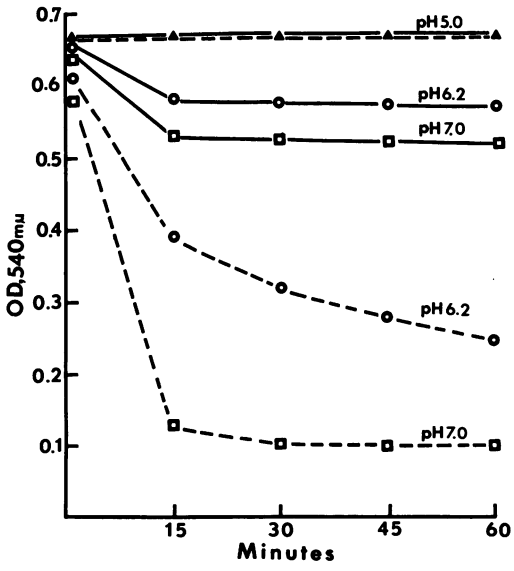

FIG. 2. *Effect of pH on the lysis of S. pullorum incubated at 37 C in the presence of: H_2O_2 and AA (solid lines); H_2O_2, AA, and Lys (broken lines).*

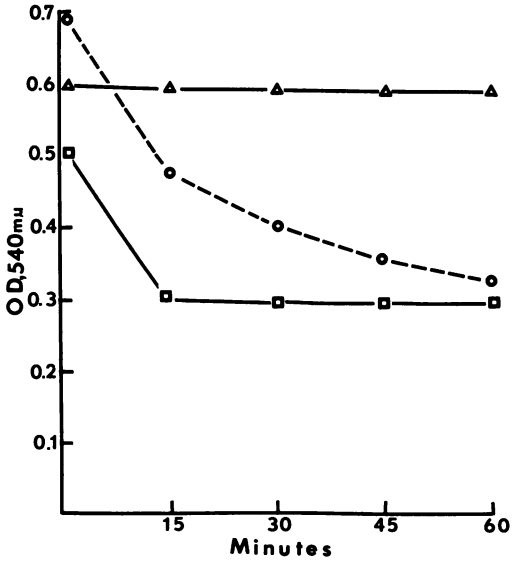

FIG. 3. *Sensitization of S. pullorum by H_2O_2 and AA to Lys activity. Solid lines indicate bacteria pretreated with the following reagents for 30 min at 37 C: H_2O_2 alone (△); H_2O_2 and AA (□). Cells were then resuspended in buffer containing Lys. A control lytic tube (○) contained H_2O_2, AA, and Lys added at zero-time.*

experiments. With one exception, the data of Table 1 show that control mixtures were bactericidally inert. The control which showed activity contained HRP, AA, and H_2O_2. It, too, was more effective at *p*H 7.0. Since HRP did not augment either the bacteriolytic or bactericidal (Table 1) activities of Lys + AA + H_2O_2, it was of interest to study bacterial survival in the presence of AA and H_2O_2, even though this combination was shown to be devoid of bacteriolytic activity.

The data in Table 2 indicate that the combination of H_2O_2 and AA results in a powerful antibacterial system which is further accentuated by the addition of Lys, especially at *p*H 7.0. Figure 4 shows that a positive correlation exists between H-ion concentration and the bactericidal or bacteriolytic activities of the complete reaction mixture. At the higher *p*H, both lytic and cidal activities were more intense. It seemed inconsistent, however, that no bacteriolysis occurred at a *p*H 5.0, despite the presence of a marked bactericidal effect. If the "uncovering" of the lysozyme substrate is involved in the bactericidal event, the absence of a lytic effect at *p*H 5.0 could perhaps be explained in terms of the *p*H optimum for Lys. The following experiment was performed in an attempt to clarify this point.

TABLE 1. *Bactericidal activity of H_2O_2, AA, and Lys at acidic and neutral pH values*

Reagent[a]	No. of viable salmonellae per milliliter at	
	*p*H 5.0	*p*H 7.0
None	5.5×10^8	4.5×10^8
HRP + Lys + AA	5.1×10^8	4.6×10^8
HRP + Lys + H_2O_2	2.1×10^8	4.4×10^8
HRP + AA + H_2O_2	5.6×10^7	3.2×10^4
HRP + Lys + AA + H_2O_2	1.1×10^7	2.2×10^4
Lys + AA + H_2O_2	2.4×10^5	9.0×10^2

[a] Concentrations were as stated in text; incubations were at 37 C for 30 min.

TABLE 2. *Enhancing effect of Lys on the bactericidal activity of H_2O_2 and AA*

Reagent[a]	No. of salmonellae per milliliter at	
	*p*H 5.0	*p*H 7.0
None	2.8×10^8	3.2×10^8
H_2O_2 + AA	9.6×10^4	1.3×10^4
H_2O_2 + AA + Lys	3.9×10^4	3.0×10^2

[a] Incubation was at 37 C for 30 min.

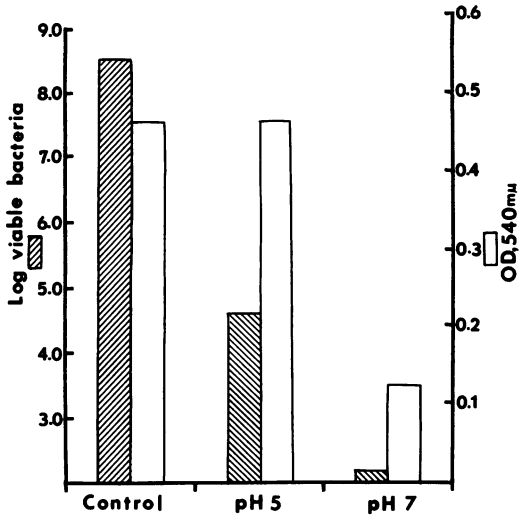

FIG. 4. *Effect of pH on bactericidal (hatched bars) and bacteriolytic (open bars) activities of mixtures containing H_2O_2, AA, and Lys at concentrations indicated in the text. The control tubes contained the bacteria in buffer alone. Incubation was at 37 C for 30 min.*

Three different reaction mixtures were prepared. Tubes 1 and 2 contained bacteria in acetate buffer at *p*H 5.0; tube 3 contained H_2O_2 and AA at the same *p*H. All tubes were incubated for 30 min at 37 C. The bacteria were then removed by centrifugation and resuspended at *p*H 7.0 as follows: tube 1 received Lys as a control; tube 2 received H_2O_2, AA, and Lys; and tube 3 received Lys alone. All tubes were then reincubated at 37 C for an additional 30 min. OD at 540 nm was measured at 0, 15, and 30 min. All reagent concentrations were those used previously.

The results in Fig. 5 demonstrate that sensitization of bacteria occurred at *p*H 5.0 in the presence of H_2O_2 and AA, but owing to the inactivity of Lys at this *p*H, bacteriolysis was absent. This is evidenced by the fact that the sensitized bacteria underwent immediate lysis when resuspended in buffer containing Lys at *p*H 7.0.

The foregoing experiments suggest a common basis for the bacteriolytic and bactericidal effects produced by H_2O_2, AA, and Lys insofar as the act of bacterial sensitization to Lys is identical in both.

Nature of the products responsible for bacterial death or sensitization to lysozyme. The results to this point have demonstrated that bacterial lysis occurs when the interaction of H_2O_2 and AA has rendered the cell sensitive to Lys. However, they do not indicate the nature of the products responsible for this sensitization. The following experiments were performed in an attempt to resolve this question.

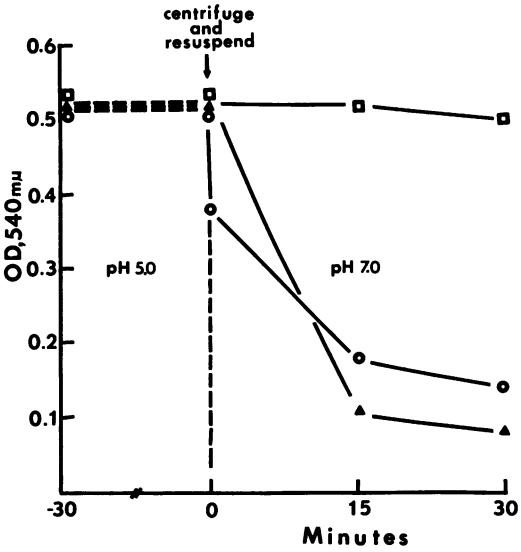

FIG. 5. *Salmonellae sensitized by H_2O_2 and AA at pH 5 to enzymatic lysis by Lys at pH 7. Pretreatment period was for 30 min at 37 C. The experimental conditions were pretreatment of bacteria in buffer at pH 5, followed by Lys in buffer at pH 7 (□); buffer at pH 5, followed by H_2O_2, AA, and Lys at pH 7 (▲); H_2O_2 and AA at pH 5, followed by Lys at pH 7 (○).*

A reaction mixture containing H_2O_2 and AA would be expected to produce end products of water and the oxidized form of AA, dehydroascorbic acid. Therefore, a test was made to determine whether dehydroascorbic acid at the equivalent molar concentration would serve to sensitize bacteria to the lytic action of Lys. It was completely inactive in this respect, suggesting the involvement of some other product of the reaction between H_2O_2 and AA. The preincubation of H_2O_2-AA-Lys, before the addition of bacteria, would be expected to reveal the presence of this sensitizing substance if it were stable. However, the bacteria were shown not to be sensitized under these conditions. The same results were obtained when Lys and bacteria were added to a preincubation mixture of H_2O_2 + AA. Likewise, the data in Table 3 demonstrate that the preincubated mixture of H_2O_2, AA, and Lys were devoid of bactericidal activity. The aforementioned experiments strongly suggested the involvement of some unstable intermediary product of the interaction between H_2O_2 + AA. It has been shown by Yamazaki et al. (40) that unstable free radicals are generated in mixtures of AA and H_2O_2. The possible involvement of free radicals in the sensitization process was investigated in the following experiment.

Inhibition of the bactericidal system by a free radical scavenger. Although free radicals can be directly demonstrated by electron paramagnetic resonance spectroscopy (40), proof of their involvement in reactions described in this study requires a more indirect approach. For example, one criterion of a reaction which depends upon free radical formation is its inhibition by radical trapping agents (35). Sodium thiosulfate, which has been used as a free radical scavenger (37), was therefore included in the mixture of H_2O_2, AA, and Lys at a concentration of 0.2 μmoles/ml. The results in Table 4 indicate that bactericidal activity was completely inhibited by sodium thiosulphate, presumably by the trapping of free radicals before they could act upon the bacterial surface.

DISCUSSION

These studies suggest that the oxidation of ascorbic acid by hydrogen peroxide gives rise to a very potent antibacterial mechanism which is mediated by free radicals. This is manifested by bacterial death or by bacteriolysis in the presence of lysozyme.

It is known that free radicals are quite nonspecific in their attack (18, 31). In this case, it is assumed that they modify the outermost layer of the bacterial cell, allowing lysozyme to reach its substrate. It is consistent with this view that *n*-butyl alcohol, a lipophilic solvent, also renders gram-negative bacteria susceptible to lysozyme

TABLE 3. *Lack of bactericidal activity upon preincubation of reagents before addition of bacteria*

Reagent[a]	No. of viable salmonellae per milliliter
None	4.7×10^8
Lys + AA	5.3×10^8
Lys + H_2O_2	4.5×10^8
AA + H_2O_2	6.6×10^8
Lys + AA + H_2O_2	5.7×10^8

[a] Reagent mixtures without bacteria were incubated for 30 min at 37 C. The salmonellae were then added and incubated for 30 min at 37 C.

TABLE 4. *Inhibition of H_2O_2-AA-Lys bactericidal system with a free radical scavenger (ST)*[a]

Reagents	No. of viable salmonellae per milliliter
None	4.4×10^8
H_2O_2 + AA + Lys + ST	5.3×10^8
H_2O_2 + AA + Lys	5.5×10^2

[a] ST = sodium thiosulfate.
[b] Incubation was 37 C for 30 min.

(25, 26). It is possible, therefore, that free radicals produce a breach in the cell wall, with resulting loss in selective permeability and consequent death. The electron microscope may be able to reveal the physical changes which occur in cells exposed to H_2O_2 and AA, and an internal radioactive label, such as ^{32}P, may be useful in detecting a permeability defect. These techniques have been used to great advantage in analyzing the immune bacteriolytic system (1, 23) by Spitznagel and Wilson (33, 39).

Although the bactericidal activity of hydrogen peroxide and ascorbic acid has been reported by earlier workers (8, 9), it was necessary to reexamine this action to relate it to the bacteriolytic effect described in this paper. There is, moreover, an apparent discrepancy between these observations and those reported recently by other workers (16, 17, 20, 21), who have shown optimal bactericidal activity of H_2O_2 and myeloperoxidase at acidic reactions, whereas in the presence of lysozyme, optimal bacteriolytic and bactericidal activities were found at neutral *p*H in this study. This conflict was partially resolved by the finding that sensitization, but not bacteriolysis, occurred at *p*H 5.0. This was unexpected, since lysozyme is said to be active from *p*H 3.5 to pH 10.0 (26). However, Fleming (10) and Thompson (38) have reported optimal lysozyme activity at neutral *p*H. It is possible, therefore, that the more vigorous bactericidal activity of the hydrogen peroxide-myeloperoxidase system at low H-ion concentrations (16, 21) may be accounted for by the *p*H optimum of this enzyme rather than that of lysozyme.

As stated earlier, the primary purpose of these studies was to provide an in vitro model that would explain the digestion of gram-negative bacteria inside phagocytes (3). In the model studied, bacterial inactivation consisted of two steps. The organism was first rendered sensitive to lysozyme by H_2O_2 and AA; the second step involved bacteriolysis in the presence of Lys. Of great interest was the observation that the sensitization step was accompanied by bacterial death. Thus, death preceded digestion in the system, a sequence of events which is consistent with Cohn's conclusions regarding bacterial inactivation within phagocytes (3). Cohn also demonstrated that lysozyme-sensitive bacteria were degraded more readily in both polymorphonuclear leukocytes (PMN) and macrophages. But, unlike the PMN, not all mononuclear phagocytes contain lysozyme (4, 5), nor have they been shown to contain any of the other well-characterized antibacterial substances (6). Presumably, they also lack peroxidase (4), the catalyst of an antibacterial system involving H_2O_2 (16, 17, 21). Nonetheless, both types of phagocytes are capable of killing a broad spectrum of ingested bacteria (2, 3, 19). Since bacterial death was shown to occur in the present studies in the absence of Lys or peroxidase, a similar process of bacterial inactivation could occur in macrophages even if both of these enzymes are lacking. This does not preclude the possibility that the same mechanism also operates in granulocytes which do contain Lys and peroxidase. It could be argued that phagocytosis of the bacterium results in the synthesis of excess H_2O_2 as a result of the enhanced metabolic activity which is known to accompany particle uptake (28, 30). In the presence of AA or any other oxidizable substance which can liberate free radicals, the integrity of the bacterial cell wall would be lost. This alone might account for the intracellular death of a majority of organisms. The additional influence of enzymic attack on substrates exposed in a disorganized cell wall could serve to increase the vulnerability of the remainder.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant AI-07809 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. Amano, T., S. Inai, Y. Seki, S. Kashiba, K. Fujikawa, and S. Nishimura. 1954. Studies on immune bacteriolysis. I. Accelerating effect on immune bacteriolysis by lysozyme-like substance of leucocytes and egg-white lysozyme. Med. J. Osaka Univ. 4:401–415.
2. Blanden, R. V., G. B. Mackaness, and F. M. Collins. 1966. Mechanisms of acquired resistance in mouse typhoid. J. Exp. Med. 124:585–600.
3. Cohn, Z. A. 1963. The fate of bacteria within phagocytic cells. I. The degradation of isotopically labeled bacteria by polymorphonuclear leucocytes and macrophages. J. Exp. Med. 117:27–42.
4. Cohn, Z. A. 1965. The metabolism and physiology of the mononuclear phagocytes, p. 323–353. *In* B. W. Zweifach, L. Grant, and R. T. McCluskey (ed.), The inflammatory process. Academic Press Inc., New York.
5. Cohn, Z. A., and B. Benson. 1965. The *in vitro* differentiation of mononuclear phagocytes. III. The reversibility of granule and hydrolytic enzyme formation and the turnover of granule constituents. J. Exp. Med. 122:455–466.
6. Cohn, Z. A., and J. G. Hirsch. 1965. Phagocytic cells, p. 215–237. *In* R. Dubos and J. G. Hirsch (ed.), Bacterial and mycotic infections of man, 4th ed. J. B. Lippincott Co., Philadelphia.
7. Edsall, G., and H. L. Ley, Jr. 1965. The prevention of infections, p. 913–948. *In* R. J. Dubos, and J. G. Hirsch (ed.), Bacterial and mycotic infections of man. 4th ed. J. B. Lippincott Co., Philadelphia.
8. Ericsson, Y., and H. Lundbeck. 1955. Antimicrobial effect *in vitro* of the ascorbic acid oxidation. I. Effect on bacteria, fungi, and viruses in pure culture. Acta Pathol. Microbiol. Scand. 37:493–506.
9. Ericsson, Y., and H. Lundbeck. 1955. Antimicrobial effect *in vitro* of the ascorbic acid oxidation. II. Influence of various chemical and physical factors. Acta Pathol. Microbiol. Scand. 37:507–527.
10. Fleming, A. 1922. On a remarkable bacteriolytic element

found in tissues and secretions. Proc. Roy. Soc. Ser. Biol. Sci. 93:306–317.
11. Hirsch, J. G. 1965. Phagocytosis. Ann. Rev. Microbiol. 19: 339–350.
12. Hunter, F. E., Jr., A. Scott, P. E. Hoffsten, F. Guerra, J. Weinstein, A. Schneider, B. Schutz, J. Fink, L. Ford, and E. Smith. 1964. Studies on the mechanism of ascorbate-induced swelling and lysis of isolated liver mitochondria. J. Biol. Chem. 239:604–613.
13. Iyer, G. Y. N., M. F. Islam, and J. H. Quastel. 1961. Biochemical aspects of phagocytosis. Nature 192:535–541.
14. Jolles, P. 1964. Recent developments in the study of lysozymes. Angew. Chem. Int. Ed. Engl. 3:28–36.
15. Jungeblut, C. W. 1941. Studies on the inactivation of diphtheria toxin by vitamin C (L-ascorbic acid). J. Infec. Dis. 69:70–80.
16. Klebanoff, S. J. 1967. Iodination of bacteria: a bactericidal mechanism. J. Exp. Med. 126:1063–1078.
17. Klebanoff, S. J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. J. Bacteriol. 95:2131–2138.
18. Leach, S. J. 1954. The mechanism of enzymic oxidoreduction. Advan. Enzymol. 15:1–47.
19. Mackaness, G. B. 1964. The immunological basis of acquired cellular resistance. J. Exp. Med. 120:105–120.
20. McRipley, R. J., and S. J. Sbarra. 1967. Role of the phagocyte in host-parasite interactions. XI. Relationship between stimulated oxidative metabolism and hydrogen peroxide formation and intracellular killing. J. Bacteriol. 94:1417–1424.
21. McRipley, R. J., and A. J. Sbarra. 1967. Role of the phagocyte in host-parasite interactions. XII. Hydrogen peroxide-myeloperoxidase bactericidal system in the phagocyte. J. Bacteriol. 94:1425–1430.
22. Mengel, C. E., H. E. Kann, Jr., and W. D. Meriwether. 1967. Studies of paroxysmal nocturnal hemoglobinuria erythrocytes: increased lysis and lipid peroxide formation by hydrogen peroxide. J. Clin. Invest. 46:1715–1723.
23. Muschel, L. H., W. F. Carey, and L. S. Baron. 1959. Formation of bacterial protoplasts by serum components. J. Immunol. 82:38–42.
24. Noller, C. R. 1957. Chemistry of organic compounds, p. 381. 2nd ed. W. B. Saunders. Co., Philadelphia.
25. Noller, E. C., and S. E. Hartsell. 1961. Bacteriolysis of *Enterobacteriaceae*. I. Lysis by four lytic systems utilizing lysozyme. J. Bacteriol. 81:482–491.
26. Noller, E. C., and S. E. Hartsell. 1961. Bacteriolysis of *Enterobacteriaceae*. II. Pre- and co-lytic treatments potentiating the action of lysozyme. J. Bacteriol. 81:492–499.
27. Nungester, W. J., and A. M. Ames. 1948. The relationship between ascorbic acid and phagocytic activity. J. Infec. Diseases 83:50–54.
28. Paul, B., and A. J. Sbarra, 1968. The role of the phagocyte in host-parasite interactions. XIII. The direct quantitative estimation of H_2O_2 in phagocytizing cells. Biochim. Biophys. Acta 156:168–178.
29. Repaske, R. 1946. Lysis of gram-negative bacteria by lysozyme. Biochim. Biophys. Acta 22:189–191.
30. Roberts, J., and Z. Camacho. 1967. Oxidation of NADPH by polymorphonuclear leucocytes during phagocytosis. Nature 216:606–607.
31. Robertson, W. 1961. The biochemical role of ascorbic acid in connective tissue. Ann. N.Y. Acad. Sci. 92:159–167.
32. Salton, M. R. J. 1957. The properties of lysozyme and its action on microorganisms. Bacteriol. Rev. 21:82–99.
33. Spitznagel, J. K., and L. A. Wilson. 1966. Normal serum cytotoxicity for P^{32}-labeled smooth *Enterobacteriaceae*. I. Loss of label, death, and ultrastructural damage. J. Bacteriol. 91:393–400.
34. Spizizen, J. 1962. Preparation and use of protoplasts, p. 122–134. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. V. Academic Press Inc., New York.
35. Staudinger, H., B. Kerkjarto, V. Ullrich, and Z. Zybrzycki. 1964. A study on the mechanism of microsomal hydroxylation, p. 815–833. *In* T. E. King, H. S. Mason, and M. Morrison (ed.), Oxidases and related redox systems. John Wiley and Son, Inc., New York.
36. Stephens, D. J., and E. E. Hawley. 1936. The partition of reduced ascorbic acid in blood. J. Biol. Chem. 115:653–658.
37. Sundblad, L., and E. A. Balazs. 1966. Chemical and physical changes of glycosaminoglycans and glycoproteins caused by oxidation-reduction systems and radiation, p. 229–250. *In* R. W. Jeanloz and E. A. Balazs (ed.), The amino sugars, vol. 2B. Academic Press Inc., New York.
38. Thompson, R. 1940. Lysozyme and its relation to the antibacterial properties of various tissues and secretions. Arch. Pathol. 30:1096–1134.
39. Wilson, L. A., and J. K. Spitznagel. 1968. Molecular and structural damage to *Escherichia coli* produced by antibody, complement, and lysozyme systems. J. Bacteriol. 96:1339–1348.
40. Yamazaki, I., H. S. Mason, and L. Piette. 1960. Identification by electron paramagnetic resonance spectroscopy of free radicals generated from substrates by peroxidase. J. Biol. Chem. 235:2444–2449.