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Effect of Catecholamines on the Bactericidal Activity of Polymorphonuclear Leukocytes[1]

DIANE QUALLIOTINE, LAWRENCE R. DECHATELET, CHARLES E. McCALL, AND M. ROBERT COOPER

The Departments of Biochemistry and Medicine, The Bowman Gray School of Medicine, Winston-Salem, North Carolina 27103

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Epinephrine, norepinephrine, and dihydroxyphenylalanine at 8 mM concentrations prevented iodination of zymosan by intact neutrophils and decarboxylation of L-alanine by leukocyte sonic extracts. The same concentration of epinephrine also reduced bactericidal activity of the leukocyte against *Staphylococcus aureus*, *Enterobacter cloacae*, and *Proteus rettgeri* without decreasing phagocytosis of bacteria. Spectral studies indicated that epinephrine interferes with the myeloperoxidase-mediated reactions by competing for available H_2O_2 via its enzymatic oxidation to adrenochrome. These findings support a mechanism in which H_2O_2 plays an important role in the bactericidal activity of the leukocyte.

The phagocytic process of neutrophils initiates a sequence of biochemical and morphological changes which normally lead to the death of the ingested organism. Among the biochemical events are three well-defined metabolic phenomena, including an increase in hexose monophosphate shunt (HMS) activity, an increase in oxygen consumption, and an increase in hydrogen peroxide production (11). The exact relationship between these metabolic phenomena and the actual killing mechanism is a subject of considerable study and controversy. That these increases are intimately involved with the phagocytic and bactericidal activity of the leukocytes is evidenced by the fact that leukocytes of patients with chronic granulomatous disease of childhood lack the normal increments in H_2O_2 production, HMS activity, and O_2 consumption upon phagocytosis; this is accompanied by a defect in killing of ingested bacteria (9). Furthermore, neutrophils of a patient with a complete glucose-6-phosphate dehydrogenase deficiency showed no increase in HMS during ingestion and an impaired bactericidal activity (1).

Production of H_2O_2 during an increment in the HMS after phagocytosis has been implicated in the actual bactericidal system of the leukocyte (10). This may be bactericidal through the H_2O_2-myeloperoxidase-halide interactions, which result in iodination of the microbial cell wall (12), or through the production of aldehydes from the decarboxylation of amino acids (24). A third bactericidal mechanism involving H_2O_2 has been described which is independent of myeloperoxidase and requires, instead, the presence of ascorbic acid (5).

We recently reported that epinephrine, norepinephrine, and dihydroxyphenylalanine stimulate the oxidative metabolism of the leukocyte, namely, the HMS activity and O_2 consumption (20). In the present communication, we have investigated the effects of these drugs on the myeloperoxidase-mediated reactions as they relate to the bactericidal activity of the leukocyte.

MATERIALS AND METHODS

Isolation of leukocytes. Venous blood was collected from apparently healthy volunteer donors, and leukocytes were isolated by a method previously described (2). White-cell samples were counted, differentials were performed by conventional methods, and concentrations were adjusted by addition of the appropriate buffer. Cell viability was determined by staining with 1% trypan blue dye.

Formation of aldehyde. The procedure of Sbarra (24) was used, wherein the decarboxylation of L-alanine is taken as a measure of aldehyde formation. Normal human neutrophils from 100 ml of venous blood were suspended in 6 ml of phosphate-buffered saline (16) and subjected to sonic treatment three times, for 15 seconds each time with cooling in between, with a Branson sonifier with a power output of 20 w. Protein content of the broken cell preparations was estimated by the procedure of Gornall et al. (6) by using bovine serum albumin as a standard. The sonic extract was used in the assays without centrifugation or dilution.

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Each flask contained 2.61×10^{-3} M H_2O_2 in 1.67×10^{-2} M phosphate buffer (*p*H 5.5), 1.65×10^{-3} M L-alanine-*1*-^{14}C (New England Nuclear Corp., Boston, Mass.) containing 0.25 μCi of ^{14}C, and 0.1 M KCl. L-epinephrine and L-norepinephrine were made as soluble as their hydrochloride salts by addition of 1 N HCl; the catecholamines and DL-β-3,4-dihydroxyphenylalanine (DOPA) were dissolved in the H_2O_2-phosphate solution and brought to *p*H 5.5 with NaOH. All biochemicals were products of Sigma Chemical Co., St. Louis, Mo. The decarboxylation reaction was initiated by the addition of 25 μliters of sonic extract, bringing the total volume to 3.0 ml. Incubation was at 37 C for 1 hr; $^{14}CO_2$ liberated during the course of the reaction was trapped in a center well containing 0.50 ml of hyamine hydroxide. Radioactivity was quantitated in a liquid scintillation counter. Each value was determined in triplicate in each experiment. Purified human myeloperoxidase, kindly supplied by Julius Schultz, was substituted for the sonic extract in some experiments.

Iodination reaction. Iodination of zymosan particles was measured by a modification of the method of Klebanoff (12). A solution of zymosan particles at a concentration of 2.5×10^9 particles/ml was prepared by the procedure of Hirsch (8). The assay was performed as previously reported (15) with several modifications. Total volume of the standard incubation mixture was increased to 1.0 ml to facilitate addition of the drugs. When used, L-epinephrine and L-norepinephrine were dissolved in phosphate-buffered saline with the addition of HCl, neutralized with NaOH, and delivered in a volume of 0.3 ml. The assay was incubated for 60 min in a Dubnoff metabolic shaker (88 oscillations/min) at 37 C. Protein was precipitated, washed, and counted as previously described (15). A standard tube, containing 0.1 ml of $^{125}I^-$ in 3 ml of H_2O and 2 ml of 5% trichloroacetic acid, was counted, and results were normalized to a standard count of 100,000 counts/min. A blank with all reagents except leukocytes was subtracted from the experimental values (19), giving a net total uptake. All assays were done in duplicate.

Bactericidal assay. *Staphylococcus aureus*, *Enterobacter cloacae*, *Proteus rettgeri*, and *Escherichia coli* were cultured overnight in soy broth, centrifuged, washed once, and suspended in Hanks balanced salt solution (Difco Laboratories, Detroit, Mich.) to a standard optical density (0.08 at 525 nm) in a Coleman spectrophotometer. L-epinephrine was freshly prepared in Hanks balanced salt solution and neutralized to *p*H 7.0. Leukocytes were suspended in Hanks balanced salt solution and adjusted to a concentration of 1.2×10^7 cells/ml. The complete reaction mixture contained in a final volume of 3.0 ml: bacteria, 1.2 ml; fresh human AB serum (3), 0.6 ml; L-epinephrine, 0.6 ml; and leukocytes, 0.6 ml. The mixtures were preincubated in 15-ml, conical, plastic centrifuge tubes for 10 min at 37 C in a shaker bath before addition of bacteria, and then were incubated at the same temperature with tumbling at 55 rotations/min.

At 0, 60, and 120 min of tumbling, 1-ml samples were diluted 10-fold and sonically treated for 30 sec by using a Branson sonifier with a power output of 20 w to disrupt the leukocytes. The total number of viable bacteria was determined by the plate dilution technique of Maaløe (14). Preliminary experiments demonstrated that the sonication procedure did not affect the viability of the bacteria. In each experiment, tests were done in duplicate.

Phagocytosis assay. Phagocytosis of microorganisms was quantitated by measuring leukocyte uptake of radiolabeled bacteria. *S. aureus* and *E. coli* were incubated overnight at 37 C with 50 μCi of uniformly ^{14}C-labeled protein hydrolysate in 5 ml of Trypticase Soy Broth. Bacteria were killed the following day in a boiling-water bath for 20 min and were washed three times with 0.9% saline. The bacterial suspension was then diluted to a standard optical density (0.075 at 525 nm) and stored at 4 C in 15-ml samples.

For the phagocytic assay, leukocytes were suspended at 5×10^6 cells/ml in Hanks balanced salt solution with 10% fresh AB serum. L-epinephrine was freshly prepared at various concentrations in Hanks balanced salt solution, with the addition of HCl, and was neutralized with NaOH. To minimize clumping, the bacterial suspension was sonically treated three times for 15 sec immediately before use. The assay media contained 1.0 ml of Hanks balanced salt solution (with various concentrations of epinephrine), 5×10^6 leukocytes, and 1.0 ml of the bacterial suspension. The control samples were preincubated for 10 min with 1.0 ml of 0.04 mM sodium fluoride before the addition of bacteria. The experimental samples were preincubated with epinephrine for 10 min, after which bacteria were added to initiate phagocytosis. The reaction was halted after 15 min by addition of sodium fluoride and cooling. The cells were separated from extracellular bacteria by centrifugation at $100 \times g$ for 10 min in the cold. The cell pellet, containing ingested bacteria, was washed three times with 2 ml of 10% fetal calf serum in phosphate-buffered saline by repeated suspension and centrifugation. The washed pellets were dried overnight at 55 C and digested with 0.5 ml of 0.2 N NaOH for 4 hr. The solutions were then neutralized with 0.2 ml of 3% glacial acetic acid, 0.5 ml of distilled water was added, and 1-ml samples were counted in 10 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) by using a Packard Tricarb scintillation counter.

Myeloperoxidase-mediated oxidation of epinephrine. The rate of oxidation of epinephrine to adrenochrome was determined from the absorbance at 460 nm. Leukocyte sonic extracts were prepared in phosphate-buffered saline, and protein content was determined by the biuret method of Gornall et al. (6) by using bovine serum albumin as a standard. The reaction was carried out in cuvettes containing 9×10^{-4} M H_2O_2 in phosphate buffer (0.01 M, *p*H 6.0) and various concentrations of epinephrine. When used, sodium azide was dissolved in 0.01 M phosphate buffer and delivered in a 0.5-ml volume. The reaction was initiated by addition of sonic extract or purified human myeloperoxidase, bringing the total volume to 3.0 ml. Rate of reaction was followed at 15-sec intervals with a Gilford recording spectrophotometer. With each

concentration of epinephrine, a control without enzyme was included.

The absorption spectra of the reaction products were obtained with a Beckman DB recording spectrophotometer and compared with standard spectra of pure epinephrine and adrenochrome.

RESULTS

Formation of aldehyde. The effects of 8 mM epinephrine, norepinephrine, and DOPA on the aldehyde reaction are shown in Table 1. Two controls were included in each experiment to illustrate the requirement for chloride ion in the H_2O_2-myeloperoxidase-halide reaction. The addition of each drug to the complete reaction mixture results in a marked inhibition of aldehyde formation; activities observed in the presence of drugs were even lower than those of chloride-deficient controls. A similar inhibition was observed when purified human myeloperoxidase was substituted for the sonically treated leukocyte suspension.

Iodination reaction. Table 2 illustrates the effects of the three compounds on iodination of zymosan in intact human neutrophils. Net iodination was calculated by subtracting activities (counts/min) observed in the absence of leukocytes from those observed in the presence of cells. Epinephrine, norepinephrine, and DOPA at 8 mM virtually abolished net iodination of particles by neutrophils, with epinephrine being the most effective inhibitor. No significance is attached to the variations of values in the absence of leukocytes.

Effects of epinephrine on bacterial clearance. Figure 1 demonstrates that 8 mM epinephrine significantly reduced the bactericidal activity of leukocytes against *S. aureus*, *E. cloacae*, and *P. rettgeri*, a serum-sensitive bacteria. This effect was observed at both 60 and 120 min of incubation with the hormone. Each point is the mean of two experiments (in duplicate) carried out on different days with different leukocyte donors. Epinephrine was also found to inhibit clearance of a serum-sensitive strain of *E. coli*, but considerable variation was obtained which appeared to be related to the source of serum. Epinephrine had no adverse effect on phagocyte viability at 0, 60, or 120 min, as measured by the exclusion of 1% trypan blue dye, and did not demonstrate bactericidal activity when incubated with bacteria in the absence of cells.

The addition of epinephrine (either 8 or 0.8 mM) to a bactericidal cell-free system, as described by Klebanoff (12), results in a total inhibition of bactericidal activity (data not shown).

Phagocytosis assay. Epinephrine at three concentrations had no inhibitory effect on net phagocytosis of heat-killed, ^{14}C-labeled *E. coli* or *S. aureus* (Table 3). In experiments with *S. aureus*, cells were not preincubated with epinephrine; instead, bacteria were added to cells simultaneously with epinephrine. The significance of the apparent stimulation of *E. coli* ingestion is doubtful because there was relatively low uptake in controls.

Oxidation of epinephrine. Addition of leukocyte sonic extracts or of purified human myeloperoxidase to a mixture of H_2O_2 and epinephrine resulted in formation of a colored product which absorbed at 460 nm (Table 4). The omission of epinephrine or enzyme from the system resulted in no color formation, indicating an absolute requirement for these components. H_2O_2 is also required for significant reaction.

A concentration of 5 mM sodium azide, an inhibitor of myeloperoxidase, reduced the absorbance nearly 90% at 5 and 10 min of reaction.

TABLE 1. *Effects of 8 mM epinephrine, norepinephrine, and dihydroxyphenylalanine (DOPA) on the aldehyde reaction of human leukocytes*

Conditions	With leukocyte sonic extract[a] (counts/min)	With purified human myeloperoxidase[b] (counts/min)
Control, − KCl	349	289
Control, + KCl	4,063	25,312
+ Epinephrine	115	284
+ Norepinephrine	129	286
+ DOPA	164	254

[a] The reaction was initiated with 0.025 ml of sonic extract containing 0.060 mg of protein. Each value represents the mean of three determinations.

[b] The reaction was initiated with 0.010 mg of purified enzyme. Values are the means of closely agreeing duplicates.

TABLE 2. *Effects of 8 mM epinephrine, norepinephrine, and dihydroxyphenylalanine (DOPA) on iodination of zymosan ingested by leukocytes*

Condition	Corrected counts/min[a]		
	+ Leukocytes	− Leukocytes	Net iodination[b]
Control	38,818	1,268	37,550
+ Epinephrine	1,774	1,879	−105
+ Norepinephrine	5,663	4,948	715
+ DOPA	3,775	3,406	369

[a] Counts per minute were normalized to a standard count of 100,000. All values were means of duplicate samples.

[b] Net iodination is value (counts per minute) without leukocytes subtracted from value in presence of leukocytes.

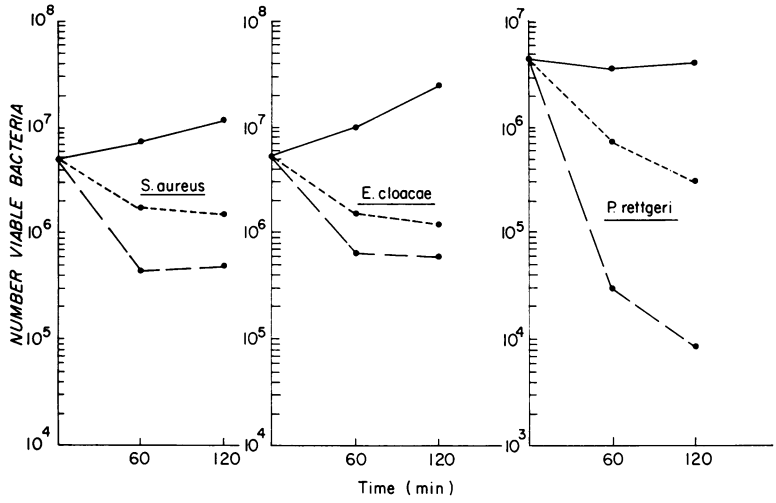

FIG. 1. *The effect of 8 mM L-epinephrine on bactericidal activity of leukocytes against Staphylococcus aureus, Enterobacter cloacae, and Proteus rettgeri. Solid line, bacteria and serum (except in the case of serum-sensitive P. rettgeri in which solid line represents bacteria alone); dashed line, bacteria, serum, and cells; dotted line: bacteria, serum, cells, and epinephrine. Values represent means of two experiments, in duplicate, carried out on different days.*

TABLE 3. *Effect of epinephrine on phagocytosis of ^{14}C-labeled, heat-killed bacteria by human leukocytes*[a]

Condition	Net counts/min in pellet[b]	
	E. coli	*S. aureus*
Control	7,824	34,468
+ 8 mM Epinephrine	13,428	29,447
+ 0.8 mM Epinephrine	13,160	37,667
+ 0.08 mM Epinephrine	9,704	42,691

[a] In experiments with *S. aureus*, cells were not preincubated with epinephrine.

[b] Determined by subtracting counts per minute in pellet at zero time from counts per minute in pellet after a 15-min incubation. All values are the means of closely agreeing duplicates.

TABLE 4. *Myeloperoxidase-mediated reaction of epinephrine with H_2O_2*

Condition[a]	Absorbance (460 nm)	
	5 min	10 min
Complete system, sonic extract	0.290	0.365
Complete system, myeloperoxidase	0.376	0.481
− Sonicate or myeloperoxidase	0.017	0.016
− H_2O_2	0.072	0.095
− Epinephrine	0.000	0.002
+ 5 mM Azide	0.038	0.042
+ 1 mM Azide	0.068	0.072
+ 0.5 mM Azide	0.091	0.119

[a] Complete system contained 8×10^{-3} M epinephrine, 9×10^{-4} M H_2O_2 in 0.01 M phosphate buffer, and 0.025 ml of sonic extract containing 0.069 mg of protein. In one experiment, 2.5 μg of purified human myeloperoxidase was substituted for leukocyte sonic extract.

Azide inhibition was seen to be concentration dependent. Figure 2 compares the time course of the myeloperoxidase-mediated reaction with two concentrations of epinephrine. The reaction with 8 mM epinephrine showed greater color development and a more rapid rate of reaction, even at 6 min of incubation, than did the lower concentration of the hormone.

To further investigate the nature of this reaction and to identify its products, experiments were carried out with purified human myeloperoxidase, epinephrine, and H_2O_2. The reaction products of this mixture were analyzed spectrophotometrically by scanning the 240- to 540-nm region. Epinephrine is known to be spontaneously oxidized to adrenochrome, taking on a pink coloration (7); therefore, standard spectra of both compounds were run (Fig. 3A and B). Spectrophotometric analysis of the reaction products after incubation with 8 mM epinephrine shows, in addition to the epinephrine peak at approximately 280 nm, a shoulder at 300 nm and a new absorbancy band at 485 nm (Fig. 3C). A similar shoulder and band are observed in the presence of 0.8 mM epineph-

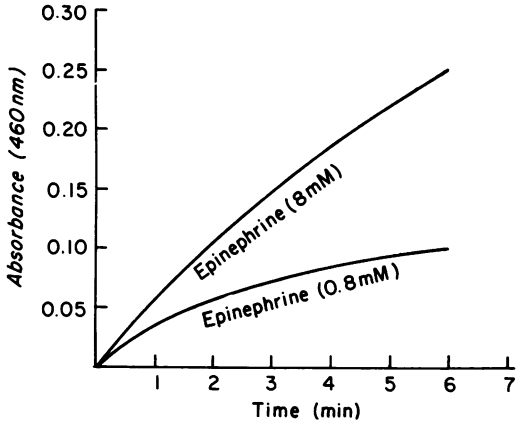

FIG. 2. *Myeloperoxidase-catalyzed reaction of epinephrine with H_2O_2. Controls run in the absence of myeloperoxidase had no absorbance; reactions were initiated by the addition of 2.5 μg of purified human myeloperoxidase.*

rine (Fig. 3D). These spectra characterize the oxidation of epinephrine to adrenochrome (25).

DISCUSSION

The in vitro addition of epinephrine, norepinephrine, or dihydroxyphenylalanine to human neutrophils results in a stimulation of the oxidative metabolism of the cells. Specifically, these drugs increase HMS activity as well as oxygen consumption of both phagocytizing and nonphagocytizing leukocytes. The HMS stimulation by epinephrine has been demonstrated to be independent of cyclic adenosine 5'-monophosphate and related to adrenochrome formation. Adrenochrome, the oxidation product of epinephrine, seems to mediate the shunt stimulation by oxidizing reduced form nicotinamide adenine dinucleotide phosphate and thus increasing the supply of rate-limiting reduced nicotinamide adenine dinucleotide phosphate (20).

Several reports in the literature have suggested a close interrelationship between the oxidative metabolism of the leukocyte and its bactericidal capacity (12, 13, 23). The present study demonstrates, however, that epinephrine, at a concentration which significantly stimulates oxygen consumption and HMS activity, greatly reduces the leukocytes' ability to kill at least three strains of bacteria (*S. aureus*, *E. cloacae*, and *P. rettgeri*). There is no corresponding decrease in phagocytosis of bacteria; in fact, with *E. coli*, there may be a slight stimulation of uptake. Differences in uptake between *E. coli* and *S. aureus* may be due to either inherent differences in opsonization and uptake of the bacteria or impaired phagocytosis of *E. coli* because of clumping of cells during the preincubation period with epinephrine. In the experiment with *S. aureus*, bacteria were added immediately to cells and epinephrine, without preincubation.

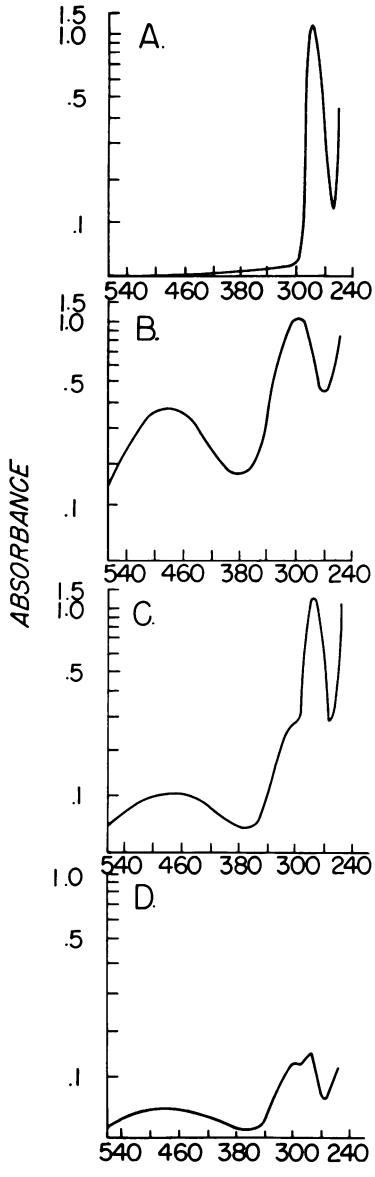

FIG. 3. *Spectra of pure epinephrine and adrenochrome and of the reaction products of myeloperoxidase-mediated oxidation of epinephrine. A, Epinephrine (0.4 mM); B, adrenochrome (0.1 mM); C, product of reaction between 8 mM epinephrine, 0.9 mM H_2O_2, and 10 μg of myeloperoxidase; D, same as C, but with 0.8 mM epinephrine. Products of reactions were diluted 1 to 20 to obtain readable spectra.*

Aldehyde formation and iodination of bacterial cell walls, both of which are highly bactericidal in

vitro (12, 18), were completely blocked by 8 mM epinephrine, norepinephrine, and DOPA. Inhibition of the aldehyde reaction also occurred when purified human myeloperoxidase was substituted for sonically treated leukocyte preparations.

Because epinephrine interferes with both H_2O_2-myeloperoxidase-halide reactions, one can postulate that it acts in one of the following ways: (i) by directly inhibiting the myeloperoxidase enzyme or (ii) by competing for one of the substrates of the reactions. It is unlikely that epinephrine inhibits the reactions by interfering with the halide because iodination depends on the presence of iodide, and aldehyde formation requires chloride. Spectrophotometric studies have revealed that epinephrine is oxidized to a colored product (which absorbs at 460 nm) in the presence of myeloperoxidase and H_2O_2. There is an absolute requirement for all three components for the reaction to occur. Sodium azide, an inhibitor of myeloperoxidase, blocks color formation significantly at three concentrations. Comparisons of spectra of the reaction products with standard spectra of pure epinephrine and adrenochrome identified the colored product as adrenochrome.

These findings indicate that epinephrine is probably not a direct inhibitor of myeloperoxidase, because the enzyme must be active to catalyze oxidation of the hormone to adrenochrome, but, rather, that epinephrine interferes with myeloperoxidase-mediated reactions by competing for available H_2O_2. The concentration of H_2O_2 in the aldehyde reaction assay is approximately 3 mM, whereas H_2O_2 concentrations of the iodination and bactericidal assays are certainly much lower, because these assays rely on endogenous production of the H_2O_2. Epinephrine was effective in the inhibition of iodination of zymosan, aldehyde formation, and bactericidal activity at 8 mM, a concentration which could easily account for complete disappearance of H_2O_2 in these systems.

A previous communication reported that ascorbic acid could inhibit both the iodination of zymosan in intact neutrophils and the decarboxylation of L-alanine without affecting the clearance of two organisms (15). Three possible explanations for these observations were offered. First, H_2O_2-myeloperoxidase-halide interactions are not important for intraleukocytic bactericidal activity. Second, the leukocyte is able to compromise by actively destroying bacteria despite a block in two of its major bactericidal mechanisms. Third, although ascorbic acid alone does not affect bacterial viability, within the cytoplasm of the neutrophil it might indirectly enhance bactericidal activity and thus compensate for the block induced in iodination and aldehyde formation. Such a reaction with ascorbic acid and lysozyme has been suggested (17).

The present results with epinephrine seem to support this third possibility. Like ascorbate (4), epinephrine can cause an increase in HMS activity and O_2 consumption. Also, like ascorbate, epinephrine can inhibit both of the myeloperoxidase-mediated reactions. However, because the oxidation of epinephrine does not give rise to an intermediate with bactericidal activity, the addition of this compound to a suspension of phagocytizing neutrophils does result in a significant inhibition of bacterial clearance.

These results together suggest that the generation of hydrogen peroxide is an important weapon in the armamentarium of the neutrophil and that, once the H_2O_2 is generated, it may take part in a number of different reactions which inactivate the phagocytized bacteria. It seems quite possible that all of these H_2O_2-dependent reactions operate simultaneously in the intact cell, and the total bactericidal activity represents the sum of these reactions in addition to a number of other killing mechanisms which are not mediated by H_2O_2 (21, 22, 26).

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