Antimicrobial Activity of Catalase at Acid pH (34263)

S. J. KLEBANOFF

USPHS Hospital, Seattle, Washington 98114; and Research Training Unit, Department of Medicine, University of Washington, Seattle, Washington 98105

An antimicrobial system which consists of the neutrophil peroxidase, myeloperoxidase, an appropriate oxidizable cofactor such as thiocyanate, iodide, bromide, or chloride ions and H_2O_2 has been described (1–6). The antimicrobial effect of the iodide-dependent system is associated with, and may therefore be a consequence of, the iodination of the microorgansims (3). The phagocytosis of bacteria by intact leukocytes is associated with the conversion of iodide to a form which is trichloracetic acid precipitable and water insoluble, and the fixed iodide can be localized radioautographically in the cytoplasm of the leukocyte in association with the ingested microorganisms (3). The demonstration of the iodination reaction in the intact leukocyte under these conditions suggests that the myeloperoxidase-mediated antimicrobial systems may be operative in the cell. Further support for peroxidase involvement in the antimicrobial activity of the leukocyte comes from studies of H_2O_2 generation by normal leukocytes (7–11) and by leukocytes which are defective in bactericidal capacity (12–16) and from studies on the abnormal fungicidal properties of peroxidase-negative neutrophils (17). The myeloperoxidase-mediated antimicrobial systems are inhibited by a number of agents which include inhibitors of peroxidase such as cyanide or azide, certain reducing agents, alternate iodine acceptors, and catalase. The latter substance is of special interest since it may be present in leukocytes and can be formed by a number of microbial species.

The classical distinction between peroxidase and catalase is based on the catalysis by the former of the oxidation of a number of substances by H_2O_2, and the degradation of H_2O_2 by the latter to oxygen and water. Catalase can thus compete with peroxidase for the available H_2O_2 and, as a result, peroxidase-catalyzed reactions are, in general, inhibited by catalase. This distinction between peroxidase and catalase, however, is not absolute. Keilin and Hartree (18, 19) first demonstrated that catalase can catalyze the oxidation of a number of substances by H_2O_2 if the concentration of H_2O_2 is maintained at low steady-state concentrations. Under these conditions, the oxidizable substance can successfully compete with H_2O_2 for oxidation by the H_2O_2–catalase enzyme–substrate complex. The usual decomposition of H_2O_2 by catalase is due to the high affinity of the H_2O_2–catalase enzyme–substrate complex for H_2O_2. The ability of catalase to utilize H_2O_2 in peroxidatic reactions prompted a study of the microbicidal activity of catalase.

Materials and Methods. Lactobacillus acidophilus (ATCC No. 4357) was grown on Lactobacillus Selective (LBS) medium and broth [Baltimore Biological Laboratories (BBL) Baltimore, Md.] and *Escherichia coli* (ATCC No. 11775), *Staphylococcus aureus* (strain 502A), *Serratia marcescens*, and *Candida tropicalis* on Trypticase soy agar and broth (BBL). A 16-hr culture in broth was washed 2 times with water and suspended in water to the required absorbency at 540 mμ just prior to use. The microorganisms were incubated with the components indicated in the legends for 60 min at 37° in an Eberbach water bath shaker oscillating 120 times per min and the viable cell count was determined by the pour plate method as previously described (3). The crystalline beef liver catalase (Worthington Chemical Corp., Freehold, N.J.; 53,560 units/mg) and glucose oxidase (Sigma Chemical Co., St. Louis, Mo.; Type V, 4 mg and 870 units/ml) were dialyzed overnight against water prior to use. Galactose oxidase (20 units/mg) was ob-

TABLE I. Antimicrobial Effect of Catalase.[a]

Supplements	Viable cell count (organisms/ml)				
	S. aureus	*L. acidophilus*	*E. coli*	*S. marcescens*	*C. tropicalis*
None	2.4×10^6	4.5×10^6	4.3×10^6	3.2×10^6	2.6×10^5
Catalase + iodide + glucose + glucose oxidase	3.0×10^3	0	2.0×10^2	8.0×10^4	1.0×10^2
+ iodide + glucose	1.9×10^6	3.2×10^6	5.0×10^6	4.0×10^6	1.1×10^5
+ iodide + glucose oxidase	1.7×10^6	1.4×10^6	3.6×10^6	5.6×10^6	1.5×10^5
+ glucose + glucose oxidase	2.0×10^6	3.9×10^6	2.7×10^6	3.6×10^6	9.3×10^4
Iodide + glucose + glucose oxidase	2.1×10^6	4.0×10^6	4.2×10^6	3.8×10^5	1.9×10^5
Catalase + iodide + glucose + glucose oxidase (heated)	2.7×10^6	3.2×10^6	4.7×10^6	5.2×10^6	1.1×10^5
Catalase (heated) + iodide + glucose + glucose oxidase	2.4×10^6	4.9×10^6	5.3×10^6	4.2×10^6	2.3×10^5

[a] The reaction mixture contained sodium lactate buffer, pH 4.5, 30 μmoles (pH 5.0 for *S. marcescens*); *S. aureus*, 2.6×10^6 organisms/ml; *L. acidophilus*, 4.6×10^6 organisms/ml; *E. coli*, 4.8×10^6 organisms/ml; *S. marcescens*, 4.0×10^6 organisms/ml; *C. tropicalis*, 2.5×10^5 organisms/ml, water to a final volume of 0.5 ml and the supplements as follows: catalase, 22 μg; sodium iodide, 0.01 μmole (0.1 μmole for *C. tropicalis*); glucose, 5 μmoles; glucose oxidase, 0.004 μg. Catalase and glucose oxidase were heated at 100° for 10 min where indicated.

tained from Worthington Chemical Corp.

Results. Data in Table I demonstrate the bactericidal and fungicidal effect of catalase, iodide, glucose, and glucose oxidase and indicates the requirement for each component of the reaction mixture. Lactate buffer, pH 4.5, was employed when *S. aureus, L. acidophilus, E. coli,* or *C. tropicalis* were the test organisms. However, when *S. marcescens* was employed, buffer at pH 5.0 was used because of the fall in viable cell count observed at the more acid pH in the absence of any additions. A higher concentration of iodide (0.1 μmole rather than 0.01 μmole/0.5 ml of reaction mixture) was required when *C. tropicalis* was the test organism. The antimicrobial effect of catalase, iodide, glucose, and glucose oxidase was decreased by preheating either catalase or glucose oxidase at 100° for 10 min. Glucose and glucose oxidase could be replaced by another H_2O_2-generating enzyme system (galactose and galactose oxidase) but not by reagent H_2O_2 (0.1–0.00001 μmole) added *in toto* at the beginning of the incubation.

Curves in Fig. 1 demonstrate the effect of pH on the antimicrobial effect of catalase. *E. coli* was employed as the test organism. Similar results were obtained with the other organisms although there were some differences in acid sensitivity. *E. coli* was unaffected by incubation for 60 min in buffer at pH levels between 4.0 and 7.0 in the absence of the catalase-mediated antimicrobial system. The viable cell count fell, however, when the pH was decreased below 4.0. When catalase, iodide, glucose, and glucose oxidase were added to the reaction mixture, a bactericidal effect was observed at pH levels below 5.5 with complete killing at pH 4.0. The catalase–iodide–glucose–glucose oxidase antimicrobial system was inhibited by azide, cyanide, ascorbic acid, reduced glutathione, cysteine, 3-amino-1,2,4-triazole, methimazole, propylthiouracil, ergothioneine, thiocyanate, thiosulfate, formate, ethanol, NADH, NADPH, and tyrosine at a final concentration of 0.001 *M* (Table II).

Discussion. The properties of the catalase and peroxidase-mediated antimicrobial systems have certain similarities and differences. Both systems have an acid pH optimum, require H_2O_2 and they have a number of inhibitors in common. Myeloperoxidase and

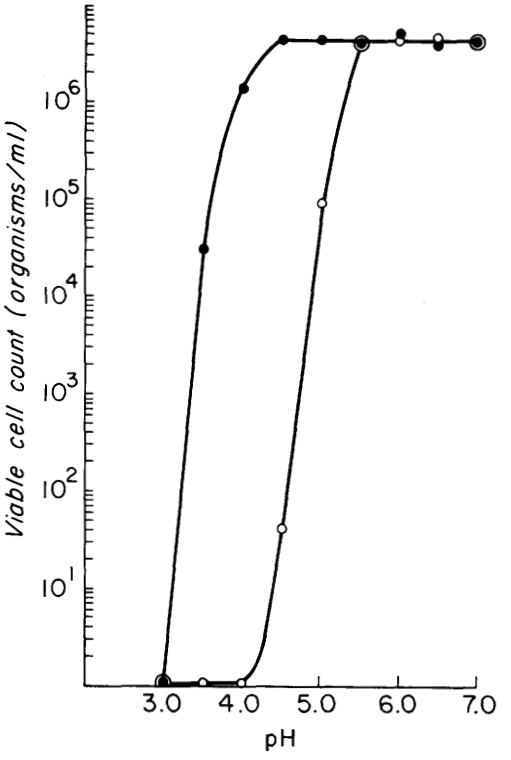

FIG. 1. Effect of pH: the reaction mixture contained either lactate (pH 3.0–6.0) or phosphate (pH 6.5–7.0) buffer, 30 μmoles; *E. coli*, 4.4 × 10^6 organisms/ml; and water to a final volume of 0.5 ml. One half of the tubes contained no further additions (—●—), and the remainder (—○—) the complete antimicrobial system as in Table I.

lactoperoxidase, however, are considerably more effective than catalase as the catalyst of the iodide-dependent antimicrobial system. When catalase is employed, the H_2O_2 generating system cannot be replaced by reagent H_2O_2 added *in toto* at the beginning of the reaction which suggests that, as with other peroxidatic reactions catalyzed by catalase, the catalase-mediated antimicrobial system requires the maintenance of low steady-state concentrations of H_2O_2. Reagent H_2O_2 can be employed in the peroxidase-mediated system. It is presumed that the antimicrobial effect of catalase under these conditions is due to the conversion of iodide by peroxidation from a weak to a strong antimicrobial agent. A similar mechanism has been proposed to account for the antimicrobial effect of the peroxidase–iodide–H_2O_2 system (3). The antimicrobial effect of the peroxidase-mediated system is associated with the iodination of the microorganisms (3). Similarly, crystalline catalase can catalyze the iodination reaction at acid pH when combined with iodide ions, a H_2O_2 generating system such as glucose and glucose oxidase and a suitable iodine acceptor molecule.

It should be emphasized that catalase under most experimental conditions, e.g., in the presence of relatively high concentrations of H_2O_2 or at pH levels above 5.0 would be expected to inhibit H_2O_2-dependent antimicrobial systems. However, since the conditions of pH and H_2O_2 generation which are believed to be operative in the intact leukocyte following phagocytosis (7–11, 20) may closely approximate the optimum conditions for the catalase-mediated antimicrobial system, it cannot be assumed that catalase formed either by the leukocyte or by the ingested microorganism would have an inhibitory effect on a H_2O_2-dependent antimicrobial system in the intact cell. Indeed, catalase may contribute to the antimicrobial effect.

Summary. Crystalline catalase exerts an antimicrobial effect when combined with io-

TABLE II. Effect of Inhibitors.[a]

Inhibitors	Viable cell count (organisms/ml)
None	7.0 × 10^2
Azide	3.4 × 10^6
Cyanide	3.7 × 10^6
Ascorbic acid	2.4 × 10^6
Reduced glutathione	2.2 × 10^6
Cysteine	2.3 × 10^6
3-Amino-1,2,4-triazole	5.7 × 10^5
Methimazole	2.2 × 10^6
Propylthiouracil	2.0 × 10^6
Ergothioneine	2.5 × 10^6
Thiocyanate	3.1 × 10^6
Thiosulfate	3.2 × 10^6
Formate	3.5 × 10^6
Ethanol	1.9 × 10^6
NADH	2.6 × 10^6
NADPH	4.0 × 10^6
Tyrosine	2.0 × 10^6

[a] The reaction mixture was as described in Table I except that *E. coli*, 4.2 × 10^6 organisms/ml was employed and the inhibitors (0.5 μmole) were added as indicated.

dide ions and a H_2O_2 generating system. The antimicrobial effect is optimal at pH levels below 5.0 and is inhibited by azide, cyanide, ascorbic acid, reduced glutathione, cysteine, 3-amino-1,2,4-triazole, methimazole, propylthiouracil, ergothioneine, thiocyanate, thiosulfate, formate, ethanol, NADH, NADPH, and tyrosine. The influence of catalase, of either leukocytic or microbial origin, on the antimicrobial activity of the leukocyte is considered.

I gratefully acknowledge the excellent technical assistance of Mrs. Ann Waltersdorph and Miss Joanne Fluvog. These studies were supported in part by PHS grant AI 07763 from the National Institute of Allergy and Infectious Diseases.

1. Klebanoff, S. J. and Luebke, R. G., Proc. Soc. Exptl. Biol. Med. **118**, 483 (1965).

2. Klebanoff, S. J., Clem, W. H., and Luebke, R. G., Biochim. Biophys. Acta **117**, 63 (1966).

3. Klebanoff, S. J., J. Exptl. Med. **126**, 1063 (1967).

4. McRipley, R. J. and Sbarra, A. J., J. Bacteriol. **94**, 1425 (1967).

5. Klebanoff, S. J., J. Bacteriol. **95**, 2131 (1968).

6. Lehrer, R. I. and Cline, M. J., Clin. Res. **17**, 121 (1969).

7. Iyer, G. Y. N., Islam, D. M. F., and Quastel, J. H., Nature **192**, 535 (1961).

8. Rechcigl, M. and Evans, W. H., Nature **199**, 1001 (1963).

9. Roberts, J. and Camacho, Z., Nature **216**, 606 (1967).

10. Paul, B. and Sbarra, A. J., Biochim. Biophys. Acta **156**, 168 (1968).

11. Zatti, M., Rossi, F., and Patriarca, P., Experientia **24**, 669 (1968).

12. Selvaraj, R. J. and Sbarra, A. J., Nature **211**, 1272 (1966).

13. McRipley, R. J. and Sbarra, A. J., J. Bacteriol. **94**, 1417 (1967).

14. Holmes, B., Page, A. R., and Good, R. A., J. Clin. Invest. **46**, 1422 (1967).

15. Bachner, R. L. and Karnovsky, M. L., Science **162**, 1277 (1968).

16. Klebanoff, S. J. and White, L. R., New Engl. J. Med. **280**, 460 (1969).

17. Lehrer, R. I. and Cline, M. J., Clin. Res. **16**, 331 (1968).

18. Keilin, D. and Hartree, E. F., Proc. Roy. Soc. (London), Ser. B **119**, 141 (1936).

19. Keilin, D. and Hartree, E. F., Biochem. J. **39**, 293 (1945).

20. Rous, P., J. Exptl. Med. **41**, 399 (1925).

Received May 21, 1969. P.S.E.B.M., 1969, Vol. 132.