

# Catalase, Superoxide Dismutase, and Virulence of *Staphylococcus Aureus*

## IN VITRO AND IN VIVO STUDIES WITH EMPHASIS ON STAPHYLOCOCCAL—LEUKOCYTE INTERACTION

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**ABSTRACT** Since oxygen-free polymorphonuclear neutrophils (PMN) cannot kill *Staphylococcus aureus* normally, the usual mechanisms for PMN bactericidal activity probably involve hydrogen peroxide or superoxide. Catalase can destroy hydrogen peroxide, and superoxide dismutase breaks down superoxide. Experiments were performed to study the influence of these enzymes (which are found in staphylococci) on virulence for mice or on leukocyte-bacterial interaction. 15 staphylococcal strains were injected i.p. into mice to quantitate virulence. There was good correlation between staphylococcal catalase activity and mouse lethality ( $r = 0.88$ ) but no correlation between staphylococcal superoxide dismutase activity and mouse lethality ( $r = 0.14$ ). Exogenous catalase (10,000 U/ml) increased the virulence of low-catalase staphylococci, but exogenous superoxide dismutase (200  $\mu\text{g}/\text{ml}$ ) did not alter the virulence of staphylococcal strains.  $\text{C}^{14}$ -labeled high-catalase or low-catalase staphylococci were ingested equally well by PMN, with or without the addition of exogenous catalase. A high-catalase staphylococcal strain was killed relatively poorly by PMN, and addition of exogenous catalase (but not superoxide dismutase) decreased the ability of PMN to kill a low-catalase strain. Iodination of bacterial proteins by PMN is related to hydrogen peroxide, and a high-catalase staphylococcal strain was iodinated only 63% as much as a low-catalase

strain. Addition of exogenous catalase decreased iodination of the low-catalase strain by 23%. These findings suggest that staphylococcal catalase protects intraphagocytic microbes by destroying hydrogen peroxide produced by the phagocyte. Thus, catalase may be a significant staphylococcal virulence factor.

### INTRODUCTION

Clinical and laboratory evidence exists to support the concept that there is marked variation in the virulence of strains of *Staphylococcus aureus* towards animals and man. Since polymorphonuclear neutrophils (PMN)<sup>1</sup> represent a major aspect of host defense against staphylococcal infection, we postulated that virulent staphylococci would have mechanisms to resist destruction by these phagocytes.

Oxygen-free PMN cannot produce hydrogen peroxide (1) and cannot kill *S. aureus* normally (2), and thus the usual mechanisms for killing of staphylococci by PMN probably involve either hydrogen peroxide (3) or superoxide (4), or both. Catalase is a heme protein enzyme found in *S. aureus* that decomposes hydrogen peroxide to water and oxygen. This enzyme's "purpose" in the bacterial cell may be to protect it from hydrogen peroxide-mediated leukocyte bactericidal mechanisms. Superoxide dismutase catalyzes the dismutation of superoxide radical ( $\text{O}_2^-$ ) to hydrogen peroxide and oxygen (5, 6). This enzyme is found in many bacterial species and may affect intraphagocytic bacterial survival by destroying superoxide.

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<sup>1</sup>Abbreviations used in this paper: HBSS, Hanks' balanced salt solution; PMN, polymorphonuclear neutrophils; TSB, trypticase soy broth.

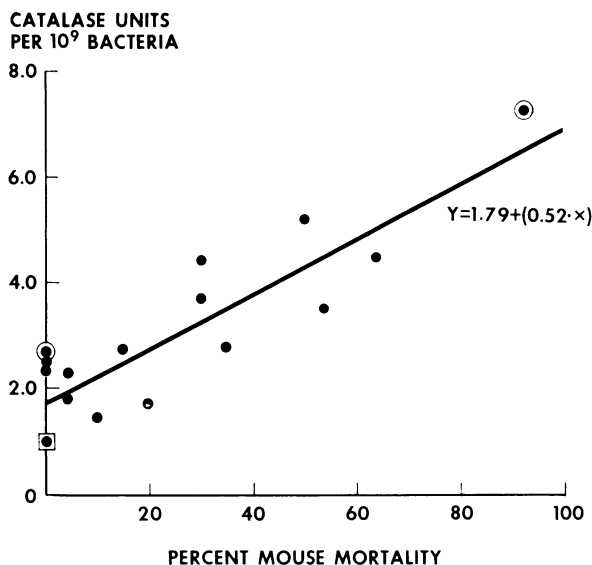


FIGURE 1 Catalase content and mouse virulence for 16 strains of staphylococci.  $10^9$  staphylococci were injected i.p. into mice and mortality at 24 h was compared with bacterial catalase content. Each point represents one staphylococcal strain. There was good correlation between catalase content of the bacteria and their lethality for mice ( $r=0.88$ , least squares curves fit;  $P < 0.001$ , Student's  $t$  test). The circled point on the right indicates the high-catalase, mouse-virulent *S. aureus* (Wood 46) and the circled point on the left indicates the low-catalase, avirulent, rifampin-resistant mutant of Wood 46. The boxed point represents a strain of avirulent *S. epidermidis*.

To see if catalase or superoxide dismutase is an important determinant of staphylococcal virulence, the lethality of 15 staphylococcal strains for mice was quantitated and correlated with the bacterial content of these enzymes. The effect of exogenous catalase or superoxide dismutase on mouse virulence of *S. aureus* strains was also examined.

In vitro studies were carried out to study the influence of bacterial catalase, superoxide dismutase, or exogenous enzymes on ingestion, iodination, and killing of staphylococci by human PMN.

#### METHODS

**Bacteria.** Strains of *S. aureus* used were mannitol-positive, DNase-positive, and coagulase-positive clinical isolates incubated for 18 h in trypticase soy broth (TSB) at 37°C before use.

A low-catalase mutant strain was obtained by incubating  $10^9$  *S. aureus* (the high-catalase, mouse-virulent, Wood-46 strain) in TSB with 10  $\mu\text{g}/\text{ml}$  of rifampin. After 18 h of incubation, subculture into TSB with 100  $\mu\text{g}/\text{ml}$  of rifampin resulted in a stable, highly rifampin-resistant organism (Staph R), avirulent for mice and low in catalase.

**Mouse virulence.** White male mice weighing 20–40 g (strain DUB/ICR Flow Laboratories, Inc., Rockville, Md.) were injected i.p. with 1 ml of an 18-h TSB culture of staphylococci containing  $10^9$  organisms. 20–60 animals were

tested with each staphylococcal strain. 24 h after injection, the number of animals surviving was noted. In some experiments either 10,000 U of bovine liver catalase (Sigma Chemical Co., St. Louis, Mo.) or 200  $\mu\text{g}$  superoxide dismutase (Truett Laboratories, Dallas, Tex.) were injected with the bacteria. Since catalase inactivated by heating formed visible clumps, catalase inactivated with trypsin (10 mg/ml), followed by heating at 70°C for 5 min was used in some experiments.

**Catalase assay.**  $10^9$  bacteria from an 18-h TSB culture were washed and resuspended in 10 ml of 0.9% saline. The suspension was iced and sonicated (model W1,400, Ultrasonic Systems, Inc., Farmingdale, N. Y.) for 5 min at a setting of six. 5  $\mu\text{l}$  of 30% hydrogen peroxide was added to 3.0 ml of Hanks' balanced salt solution (HBSS) at 37°C in the chamber of a polarographic oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio, model 53). 0.1 ml of the bacterial sonicate was added to the chamber, and the increase in oxygen saturation was recorded for 5 min. A standard curve of catalase versus increase in oxygen saturation per 5 min was constructed. Measured bacterial catalase content was expressed as sigma units per  $10^9$  organisms.

**Superoxide dismutase assay.**  $10^{10}$  washed bacteria in 1.5 ml saline were sonicated as described above. The dialyzed 35,000-g supernate was assayed for activity as an inhibitor of the reduction of cytochrome *c* by superoxide, as described by McCord and Fridovich (6) and modified by Salin and McCord (7). A standard curve was prepared with super-

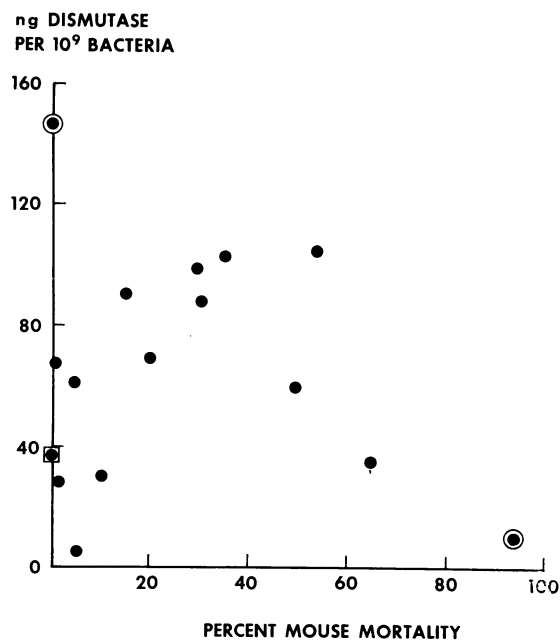


FIGURE 2 Superoxide dismutase content and mouse virulence for 16 strains of staphylococci.  $10^9$  staphylococci were injected i.p. into mice and mortality at 24 h was compared with bacterial superoxide dismutase content. Each point represents one staphylococcal strain. There was no correlation between superoxide dismutase content of the bacteria and their lethality for mice ( $r=0.14$ , least squares curves fit). The circled and boxed points are defined in the legend for Fig. 1.

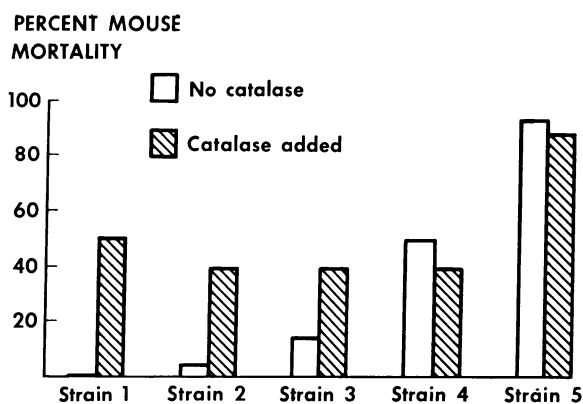


FIGURE 3 Effect of exogenous catalase on mouse virulence of *S. aureus*.  $10^8$  staphylococci were injected i.p. into mice and mortality at 24 h was compared with simultaneous experiments done with the addition of 10,000 U of catalase. The strains of low virulence 1, 2, and 3) showed marked enhancement of mouse lethality with the addition of catalase, while the more virulent strains (4 and 5) did not become more lethal to mice after the addition of catalase.

oxide dismutase. Measured bacterial superoxide dismutase content was expressed as nanogram superoxide dismutase activity per  $10^8$  bacteria.

**Catalase and superoxide dismutase activity in mouse peritoneal fluid.** 10,000 U of catalase or 200  $\mu$ g of superoxide dismutase in 1 ml 0.9% saline were injected i.p. into mice. Samples were removed for enzyme assay by washing the peritoneum of freshly killed mice with 3 ml of 0.9% saline at 0, 1, 8, 12, and 24 h after injection of enzyme. Catalase was measured as described above, and superoxide dismutase was assayed in the epinephrine-adrenochrome system described by Misra and Fridovich (8).

**Leukocyte bactericidal activity.** 10 ml of heparinized venous blood from normal donors was sedimented for 60 min with an equal volume of 3% dextran. The supernatant fluid containing the leukocytes was collected, erythrocytes remaining in the supernatant fluid were lysed with iced distilled water, and tonicity was restored with hypertonic saline. Leukocytes were then centrifuged at 200  $g$  for 12 min, and the resulting cell button was resuspended in HBSS with 10% autologous serum. 4 ml of suspension with  $5 \times 10^6$  bacteria and  $5 \times 10^6$  PMN/ml were tumbled at 37°C, and samples were removed at specified times for determination of total, supernatant, and sediment bacterial counts (9). Some experiments were done with the addition of 10,000 U of catalase/ml, and others were performed with 200  $\mu$ g of superoxide dismutase/ml.

**Iodination of protein by leukocytes.** Minor modifications of the method of Root and Stossel (10) were employed.  $5 \times 10^6$  PMN were incubated with  $5 \times 10^8$  preopsonized live *S. aureus* and 0.2  $\mu$ Ci of  $\text{Na}^{125}\text{I}$  (1.9  $\mu$ mol). In some experiments 10,000 U of catalase/ml were added to the reaction mixtures. The reaction was stopped after 20 min, and the washed trichloroacetic acid precipitates were counted in a Beckman 300 gamma counter (Beckman Instruments, Fullerton, Calif.).

**Phagocytosis of labeled bacteria.** Modifications of the methods of Downey and Diedrich (11) were employed. Staphylococci were grown in TSB with 10  $\mu$ Ci/ml of  $\text{C}^{14}$ -labeled amino acid mixture (New England Nuclear, Bos-

ton, Mass.). After 18 h incubation bacteria were washed in saline, and  $5 \times 10^8$  bacteria/ml were added to  $5 \times 10^6$  PMN/ml, as described in the section on leukocyte bactericidal activity. Mixtures were tumbled at 37°C, and 0.2 ml samples were removed at 0, 5, 10, and 20 min. These samples were placed in 4 ml of iced HBSS with 10% fetal bovine serum and centrifuged at 100  $g$  for 5 min at 4°C. The cell buttons were washed, digested with Protosol (New England Nuclear, Boston, Mass.), suspended in counting solution, and counted in a Beckman LS 250 scintillation counter (Beckman Instruments Inc., Fullerton, Calif.). In some experiments 10,000 U of catalase/ml were added to the leukocyte-bacterial suspensions.

## RESULTS

**Mouse virulence and bacterial catalase content.** The data are shown in Fig. 1. There was good correlation between catalase content of the bacteria and the lethality of strains for mice ( $r = 0.88$ , least squares curves fit).

**Mouse virulence and bacterial superoxide dismutase content.** The data are shown in Fig. 2. There was no

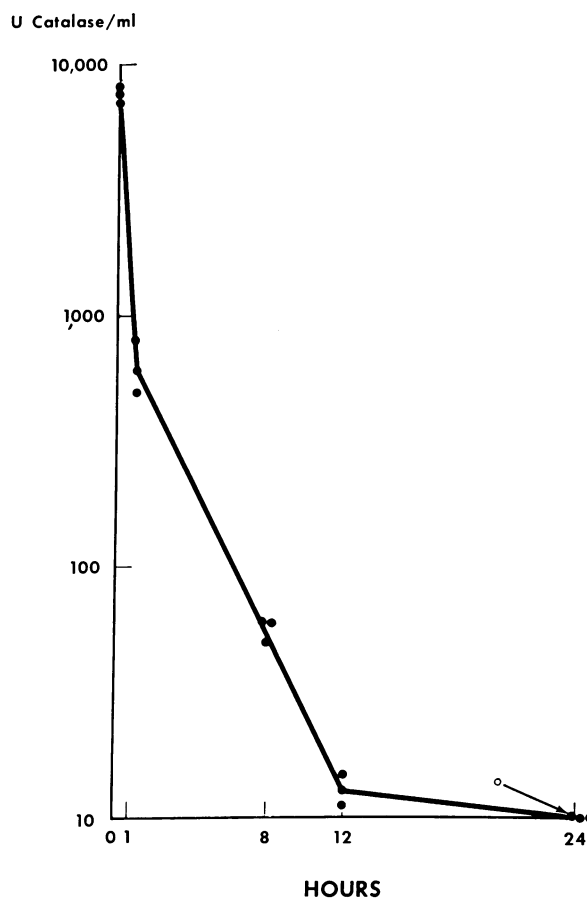


FIGURE 4 Catalase activity in mouse peritoneal fluid. Residual enzyme activity was determined after i.p. injection of 10,000 U of catalase. Each point represents a determination on a different mouse.

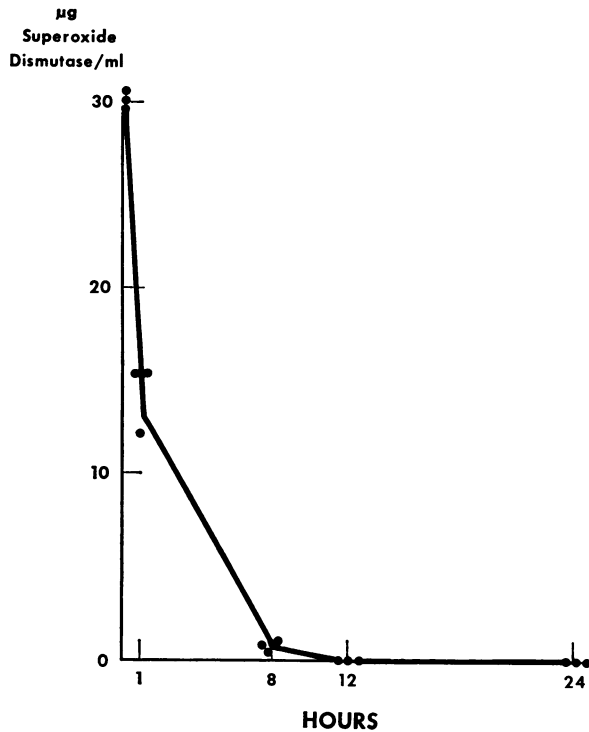


FIGURE 5 Superoxide dismutase activity in mouse peritoneal fluid. Residual enzyme activity was determined after i.p. injection of 200  $\mu\text{g}$  of superoxide dismutase. Each point represents a determination on a different mouse.

correlation between superoxide dismutase content of the bacteria and lethality of strains for mice ( $r = 0.14$ , least squares curves fit).

*Effect of exogenous catalase or superoxide dismutase on virulence of staphylococci for mice.* The amount of enzyme used in the in vitro and in vivo studies was calculated from the data of Chang (12), who showed that approximately  $10^{-19}$  ml of media was swept into the phagocytic vacuole when one particle was ingested by one cell. To achieve an amount of enzyme in the phagocytic vacuole equivalent to that reached when a high-catalase-containing staphylococcal organism is ingested, 10,000 U of catalase/ml was utilized. This resulted in approximately  $1.0 \times 10^{-8}$  U of catalase/vacuole per ingested particle. The high-catalase staphylococcal strain contains  $0.8 \times 10^{-8}$  U of catalase/bacterium.

Since the staphylococcal strain with the highest superoxide dismutase content had  $146 \times 10^{-9}$  ng/ bacterium, a concentration of 146  $\mu\text{g}$  superoxide dismutase/ml would result in approximately this amount of enzyme swept into each phagocytic vacuole. 200  $\mu\text{g}/\text{ml}$  of superoxide dismutase was used in the in vivo and in vitro experiments. Intraperitoneal injections of catalase or superoxide dismutase did not kill any mice.

Five staphylococcal strains were studied by injecting bacteria (with and without 10,000 U of catalase) i.p. into mice. As shown in Fig. 3, catalase increased the lethality of the low-catalase, less virulent strains but did not increase the lethality of the more virulent higher-catalase-containing strains. Inactivated catalase did not alter mouse virulence of staphylococci.

In contrast, i.p. injection of 200  $\mu\text{g}$  of superoxide dismutase along with any of the strains of staphylococci did not alter mouse mortality.

*Catalase and superoxide dismutase activity in mouse peritoneal fluid.* Enzyme activity rapidly disappeared from the peritoneal fluid. No superoxide dismutase activity could be found by 12 h, and no catalase activity by 24 h (Fig. 4 and 5). However, the assays employed were relatively more sensitive for catalase than for superoxide dismutase.  $3.7 \times 10^8$  staphylococci/ml contain the minimal amount of catalase detectable, in contrast to

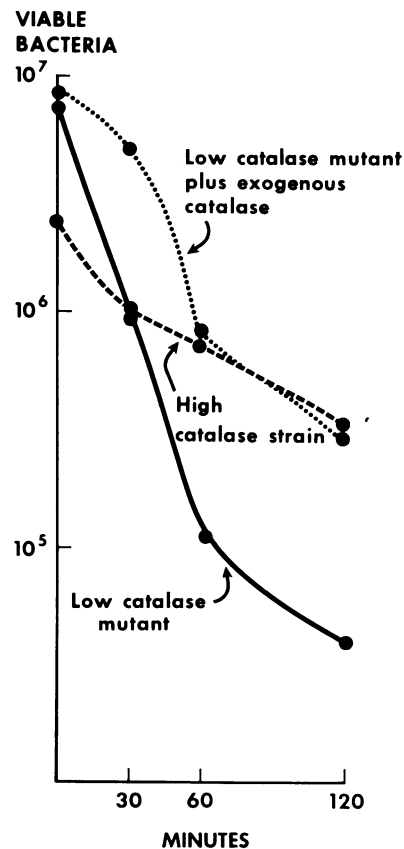


FIGURE 6 Killing of staphylococci by leukocytes. Equal numbers of staphylococci and human neutrophils were incubated at  $37^\circ\text{C}$  with tumbling, and bacteria surviving at several time periods were quantitated. 10,000 U of catalase/ml was added to a low-catalase staphylococcus-PMN suspension and this resulted in decreased killing of the organism by PMN.

$9 \times 10^8$  staphylococci/ml, which contain the minimal amount of superoxide dismutase detectable by our assays.

**Leukocyte bactericidal activity.** The mouse-virulent, high-catalase Wood-46 strain of *S. aureus* was killed less effectively at 2 h than the low-catalase avirulent mutant strain of Wood 46. The addition of 10,000 U of catalase/ml decreased the killing of the low-catalase mutant strain (Fig. 6). However, the addition of 200  $\mu$ g/ml of superoxide dismutase did not change the rate or degree of killing of the low catalase mutant.

**Phagocytosis of labeled bacteria.** Ingestion by PMN of the high-catalase mouse-virulent Staph W (strain Wood 46) was equal to that of the low-catalase avirulent Staph R (rifampin-resistant mutant of Wood 46). The

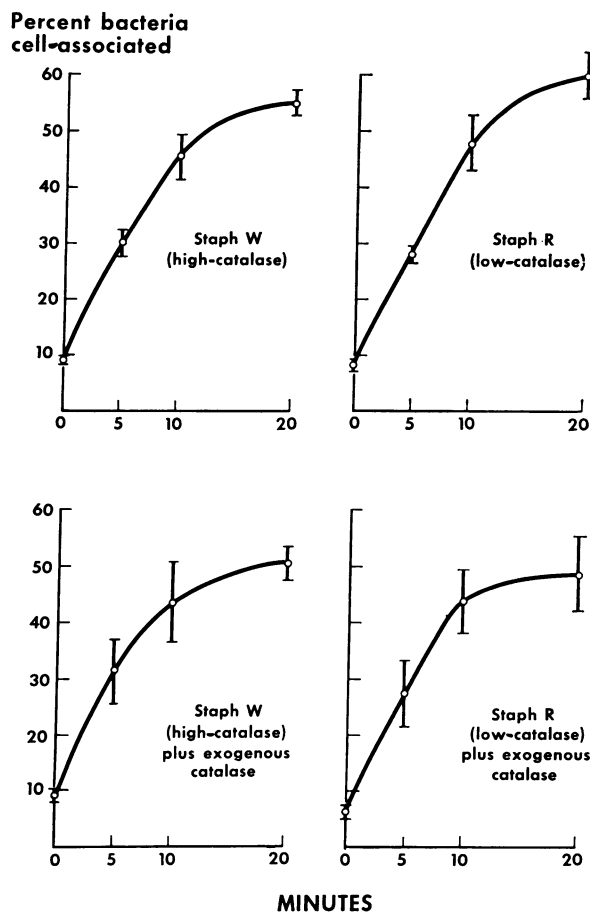


FIGURE 7 Phagocytic uptake of  $C^{14}$ -labeled bacteria.  $C^{14}$ -labeled bacteria were incubated with PMN and cell-associated microorganisms were separated by differential centrifugation and quantitated by liquid scintillation counting. Means and SEM are shown:  $n=5$  for bacteria alone and  $n=10$  for experiments with catalase. There were no significant differences in bacterial uptake at any time ( $P > 0.07$ , Student's  $t$  test).

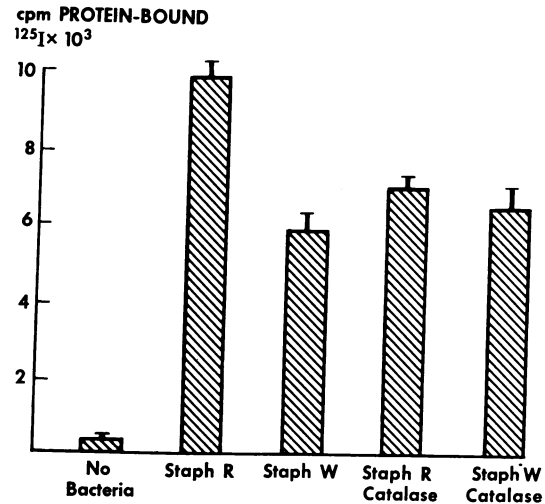


FIGURE 8 Protein iodination by leukocytes.  $5 \times 10^6$  PMN were incubated with  $5 \times 10^8$  preopsonized live high-catalase (Staph W) or low-catalase (Staph R) *S. aureus* in the presence of  $0.2 \mu$ Ci of  $^{125}I$ . Results of six experiments are expressed as means plus SEM. Staph R stimulated protein iodination more than Staph W ( $P = 0.0015$ ). Exogenous catalase reduced iodination of Staph R ( $P = 0.021$ , Student's  $t$  test).

addition of exogenous catalase did not alter ingestion of the staphylococci (Fig. 7).

**Protein iodination by leukocytes.** Iodination was greatest when the low-catalase avirulent mutant strain (Staph R) was incubated with PMN. Addition of exogenous catalase to this strain reduced iodination to levels noted with the high-catalase virulent strain (Staph W) (Fig. 8).

## DISCUSSION

There is evidence that the ability of staphylococci to survive inside phagocytes is related to bacterial virulence (13). The hydrogen peroxide-myeloperoxidase-halide system has been shown to be a potent oxygen-dependent mechanism for killing of organisms ingested by phagocytes (3). Since PMN in an anaerobic environment cannot kill *S. aureus* normally (2), oxygen-dependent metabolic activity is probably important for normal PMN bactericidal activity.

Staphylococci contain the enzyme catalase, which breaks down hydrogen peroxide to water and oxygen, and this enzyme could serve to protect ingested staphylococci from the lethal effects of hydrogen peroxide produced by PMN. Gelsa (14) showed that there was a direct relationship between the catalase content of staphylococci and other factors he considered "virulence-related," such as pigment, coagulase, fibrinolysin, and lecithinase. Amin and Olson (15) found that increased bacterial catalase activity correlated with increased survival of staphylococci in hydrogen peroxide solutions and also

showed that there was an increase in levels of bacterial catalase after growing the organisms in hydrogen peroxide-containing media. Studies with strains of *Brucella abortus* suggested a positive correlation between catalase content and virulence of the organisms (16). This relationship was not found to be true with strains of *Brucella melitensis* and *Brucella suis*, however (17).

It has recently been postulated that superoxide may also be important in the killing of bacteria by phagocytes (4, 18, 19). If superoxide is important for phagocyte bactericidal activity, one would expect that superoxide dismutase, an enzyme that changes superoxide to hydrogen peroxide and oxygen, might be directly correlated with bacterial virulence. Yost and Fridovich (20) suggest that *E. coli* resistance to whole blood bactericidal activity may be directly related to bacterial superoxide dismutase. Johnston, Keele, Webb, Kessler, and Rajabopalan found that superoxide dismutase "bound" to latex particles inhibited leukocyte bactericidal activity (21). However, Klebanoff (22), using an in vitro system, found that superoxide dismutase inhibited bactericidal activity only with concentrations greater than 88 µg/ml. He felt that inhibition seen with these high concentrations was due to nonspecific protein effect.

Our studies failed to show any correlation between staphylococcal superoxide dismutase content and virulence of these organisms for mice. Furthermore, while exogenous catalase did enhance staphylococcal mouse lethality, exogenous superoxide did not. The very rapid peritoneal clearance of the superoxide dismutase may be a partial explanation for its lack of effect on mouse mortality in vivo. While neither catalase nor superoxide dismutase altered ingestion of bacteria by PMN, only catalase impaired the ability of PMN in vitro to kill intracellular staphylococci.

These findings suggest that staphylococcal catalase protects intraphagocytic microbes by destroying hydrogen peroxide produced by the phagocyte. Thus catalase may be a significant bacterial virulence factor.

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