Ascorbic Acid: Preferential Lysis and Catalase Induction in Antibiotic-Resistant Strains of *Staphylococcus aureus*

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Abstract □ Experiments revealed that antibiotic-sensitive staphylococci produce more catalase per unit viable cell than the antibiotic-resistant strains. Ascorbic acid induces catalase activity in the antibiotic-resistant strains. In addition to the variations in catalase formation, ascorbic acid preferentially lyses antibiotic-resistant but not sensitive staphylococci in the exponential phase of growth. By using this criterion, a series of 36 hospital strains of antibiotic-resistant and sensitive strains were differentiated ($p < 0.005$) by ascorbic acid after a 90-min. incubation period.

Keyphrases □ Ascorbic acid—preferential lysis and catalase induction in antibiotic-resistant *Staphylococcus aureus* □ *Staphylococcus aureus*, antibiotic resistant—ascorbic acid-induced catalase activity and preferential lysis □ Antibiotic-resistant staphylococci—ascorbic acid-induced catalase activity and preferential lysis

The antimicrobial action of ascorbic acid is attributed to hydrogen peroxide resulting from the decomposition of ascorbic acid (1). It is well known that microorganisms overcome this lethal action of hydrogen peroxide by producing catalase. Indeed, ascorbic acid has been reported (2) to induce catalase in an antibiotic-resistant strain of *Staphylococcus aureus*. These authors also observed that catalase activity in cultures adapting to antibiotics *in vitro* gradually diminished or even stopped. It is, therefore, likely that the established antibiotic-resistant strains of staphylococci may produce less catalase than the antibiotic-sensitive strains. If this is the case, then these two groups of staphylococci may respond differently to ascorbic acid with regard to growth and/or catalase activity; antibiotic-resistant and sensitive staphylococci could then be distinguished on this basis. The present investigation was aimed at determining if catalase activity, response to ascorbic acid, and antibiotic resistance in staphylococci were associated.

EXPERIMENTAL

Test Organisms—*S. aureus* strain oxford and the phage propagating strain PS84 were used as typical representatives of antibiotic-sensitive and resistant staphylococci, respectively. In addition, 34 hospital strains of staphylococci were tested. These strains were maintained by serial passage on nutrient agar (oxoid).

Culture Media—Tryptone soya broth (oxoid) was used for all experiments. Ascorbic acid (2.5 mg./ml.) was dissolved in this broth, and the pH was adjusted to 7.3 with sodium hydroxide. This ascorbic acid broth was sterilized by membrane filtration, while tryptone soya broth was autoclaved at 15 lb. pressure for 15 min.

Antibiotic Sensitivity—All cultures were tested for antibiotic sensitivity using the multodisks (oxoid) on nutrient agar plates. Strains PS84 and oxford were also tested by the tube dilution method.

Growth Curve—Four sterile McCartney bottles (A, B, C, and D) were used in the growth curve experiment. Bottles A and B contained tryptone soya broth (20 ml. each); Bottles C and D contained tryptone soya broth fortified with ascorbic acid (2.5 mg./ml.) neutralized to pH 7.3. All transfers were made in a laminar work station. Bottles A and C were inoculated with *S. aureus* (oxford strain) grown overnight in tryptone soya broth. The absorbance of the final medium in these bottles after inoculation was 0.06 at 560 nm.[1]. Similar inoculations were made in Bottles B and D with *S. aureus* (PS84 strain). A blank set of sterile tryptone soya and ascorbic acid broths was also included. All bottles were incubated at 37° under agitation. Aliquots from each bottle were aseptically removed at 0, 1, 2, 3, and 4 hr. during incubation to determine the number of viable organisms, absorbance, and catalase activity.

Table I—Catalase Activity in Oxford and PS84 Strains of *S. aureus*

	Medium			
	Tryptone Soya Broth (pH 7.3)		Tryptone Soya Broth plus 2.5 mg./ml. Ascorbic Acid (pH 7.3)	
S. aureus	Oxford	PS84	Oxford	PS84
Rate of multiplication (generations/hour)	4.5	1.7	3.7	1.3
Catalase activity[a]:				
Milligrams hydrogen peroxide	8.350	5.601	2.811	7.110
Percent	100	67	100	309
Catalase activity[b]	100	100	67	126
χ^2 test		11.82 ($p < 0.001$)		

[a] Per 10^8 colony-forming units up to 4 hr. of growth. [b] Expressed as percent of catalase activity in tryptone soya broth.

Measurement of Growth—Viable counts were made by plating suitable dilutions of the sample in 0.1-ml. amounts in nutrient agar plates. Colonies formed after 16 hr. of incubation at 37° were counted.

The absorbance of each sample was measured at 560 nm.[1], using tryptone soya broth and ascorbic acid broth blanks as controls.

Estimation of Catalase—Catalase activity was estimated iodometrically (3). An aliquot of 1.0 ml. of each culture was added to 10.0 ml. of 0.1 *N* hydrogen peroxide contained in a conical flask. Five minutes later, the enzyme was inactivated by adding 2.0 ml. of 2 *N* sulfuric acid. This was followed by 5 ml. of freshly prepared 10% aqueous solution of potassium iodide and 2–3 drops of 15% ammonium molybdate. Liberated free iodine was then titrated against 0.1 *N* sodium thiosulfate. Blank readings were obtained by titrating the uninoculated broth; sulfuric acid was added to hydrogen peroxide prior to adding the broth for this determination. Catalase activity in each sample, computed from the amount of sodium thiosulfate required, was then expressed as milligrams hydrogen peroxide decomposed in 5 min. Catalase activity was also expressed per 10^8 colony-forming units. This value was calculated by dividing the observed catalase activity by the actual number of colony-forming units per milliliter determined by the viable count method described.

Growth Response (Absorbance) of Antibiotic-Resistant and Sensitive Staphylococci to Ascorbic Acid—The basic standardization to determine the minimal amounts of ascorbic acid, the type of culture media, the amounts of inoculum, the minimum period of incubation, *etc.*, was made with the oxford and PS84 strains of *S. aureus*. Up to 2–3 hr. of incubation of the ascorbic acid broth did

[1] A Spectronic-20 was used.

Table II—Growth[a] of Oxford and PS84 Strains of *S. aureus* in Tryptone Soya Broth Containing 2.5 mg./ml. Ascorbic Acid, pH 7.3

Time of Incubation, hr.	Absorbance		Colony-Forming Units	
	Oxford	PS84	Oxford	PS84
1	100	84	100	92.0
2	120	70	104	96.5
3	112	77	104	96.5
4	105	94	108	96.5

[a] Expressed as percent of growth observed in corresponding tryptone soya broth.

not cause significant darkening. Furthermore, these strains completed their exponential growth within 3 hr. of incubation under agitation. The test was based on the comparison of the absorbance formed in tryptone soya broth and the ascorbic acid broth. Consequently, at zero hour the inocula were adjusted so as to obtain identical absorbances in both media. The cultures were then incubated at 37° and absorbance was read at 560 nm. at varying time intervals. The absorbance in the ascorbic acid broth was expressed as the percent absorbance of that observed in the corresponding tryptone soya broth culture. Viable counts of the PS84 and oxford strains were made at each observation during the growth.

RESULTS AND DISCUSSION

The response of the antibiotic-resistant and sensitive staphylococci to ascorbic acid was measured by using four different parameters: (*a*) rate of growth, (*b*) catalase activity, (*c*) catalase induction, and (*d*) susceptibility to ascorbic acid.

Under the experimental conditions described, the PS84 strain multiplied at a significantly lower rate than the oxford strain (Table I). Such behavior of antibiotic-resistant strains was previously reported (4). What is unknown, however, is the significant difference in the catalase activity of antibiotic-resistant and sensitive staphylococci. In the present study, 10^8 colony-forming units of the antibiotic-resistant PS84 strain produced 67% of the catalase formed by 10^8 colony-forming units of the antibiotic-sensitive oxford strain in tryptone soya broth. In ascorbic acid broth, however, the catalase activity of the PS84 strain was 309% of that of the oxford strain per 10^8 colony-forming units. These differences ($\chi^2 = 11.82$, $p = 0.001$) indicate that the antibiotic-resistant PS84 strain exhibited an impaired catalase activity as compared with the antibiotic-sensitive oxford strain. Increased catalase production may then be a characteristic of antibiotic-sensitive organisms.

When ascorbic acid was added to the medium, however, the PS84 strain formed catalase in considerably larger amounts. Is it then likely that antibiotic-resistant staphylococci growing in the presence of ascorbic acid become increasingly susceptible to an antibiotic? This, indeed, is the case. Synergistic inhibition of *S. aureus* with ascorbic acid and penicillin was previously reported (5). The author has also observed synergistic action between ascorbic acid and several antibiotics *in vitro* on the PS84 strain[2]. Furthermore, ascorbic acid acting singly and in combination with sulfamethoxazole-trimethoprim inhibits *Pseudomonas aeruginosa* but not *Streptococcus faecalis* (6).

[2] These data will be published elsewhere.

Table III—Differential Response to Ascorbic Acid by Antibiotic-Resistant and Sensitive Strains of Staphylococci after 90 min. of Growth *In Vitro*

	Antibiotic-Resistant Strains	Antibiotic-Sensitive Strains
Number of strains tested	28	8
Mean absorbance[a] in ascorbic acid broth	78	117
Standard deviation	3.7417	8.4853
t test	11.1 ($p < 0.005$)	

[a] Expressed as percent absorbance of the corresponding tryptone soya broth culture.

The variations in the catalase activity were also associated with the susceptibility of the strains to ascorbic acid (Table II).

In the presence of ascorbic acid, the absorbance of the oxford strain was 100% or more of the control throughout the observation period; the absorbance of the PS84 culture was well below 100% of the control throughout. Viable count determinations were not sensitive enough to highlight these differences, although the pattern was suggested. The data show preferential lysis of some antibiotic-resistant cells during exponential growth in the presence of ascorbic acid. It might then be said that exponentially growing antibiotic-resistant cells are sensitive to the lytic effect of ascorbic acid while antibiotic-sensitive staphylococci are resistant to it. Whether or not such a generalization is applicable to hospital strains of staphylococci was then investigated with 36 strains (Table III).

The data in Table III reveal that antibiotic-sensitive hospital strains of staphylococci also produced a significantly higher absorbance, like the oxford strain, than the antibiotic-resistant strains in the presence of ascorbic acid (2.5 mg./ml.).

In conclusion, this investigation reveals that antibiotic-resistant staphylococci produce significantly less amounts of catalase and are preferentially lysed by ascorbic acid during exponential growth. Antibiotic-sensitive staphylococci exhibit the opposite pattern. Further work would bear out the importance of this observation.

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