Factors Influencing the Antibacterial Action of Spermine and Spermidine on *Staphylococcus aureus*

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SUMMARY: Spermine and spermidine inhibited the growth of *Staphylococcus aureus* under both aerobic and anaerobic conditions. The activity of spermine was decreased by sera of different species; sheep and bovine serum showed most effect. This antagonistic effect was destroyed by heating the sera at 100° for 30 min. Sodium and potassium also decreased the antibacterial effect of spermine and spermidine. Spermine exerted a bactericidal effect on staphylococci in broth at 37° and at 6°, but not on washed organisms suspended in buffer at 6°. The dependence of the bactericidal effect of the bases seems to depend on some metabolic activity of the cocci.

The antibacterial action of spermine and spermidine was described previously (Gurevitch, Rozansky, Weber, Brzezinsky & Eckerling, 1951; Rozansky, Bachrach & Grossowicz, 1954; Rozansky, Razin & Grossowicz, 1954); Hirsch & Dubos (1952) studied the antimycobacterial effect of these substances. According to Hirsch (1953*a, b*) spermine itself was not antimycobacterial but a derivative of it, obtained by the action of an oxidase present in sheep and bovine sera, was the active agent. In the present paper evidence is presented that spermine itself is bactericidal against staphylococci.

METHODS

The technique described previously was used (Rozansky, Bachrach & Grossowicz, 1954). Spermine tetrahydrochloride and spermidine phosphate (Hoffman-La Roche Inc.) were used. The antistaphylococcal tests were made in Difco nutrient broth. The pH value of the medium was adjusted by adding sodium hydroxide or hydrochloric acid and was measured by a Beckman pH meter. Nutrient broth containing increasing twofold dilutions of spermine or spermidine was distributed into 2 ml. quantities in 100 × 12 mm. test tubes. The tubes were plugged with cotton-wool and autoclaved for 20 min. at 15 lb./sq.in.

Staphylococcus aureus strain 23 was the main test organism; 0.1 ml. of a 1/10⁴ dilution of a 24 hr. broth culture was used as inoculum. The tubes were incubated at 37°, unless otherwise specified. The antibacterial activity was expressed as the minimal concentration of spermine or spermidine which caused complete inhibition of growth visible to the naked eye. In experiments to test the bactericidal effect of spermine, a plate count technique was used.

Anaerobic tests were performed in a jar from which the air was evacuated and the remaining oxygen removed by pyrogallol and NaOH; reduced methylene blue was used as indicator of anaerobiosis.

RESULTS

Effect of spermine and spermidine under anaerobic conditions

To test whether the action of spermine and spermidine was affected by oxidative enzymes of the cocci or by the presence of oxygen in the medium, experiments under anaerobic conditions were performed. As *Staphylococcus aureus* strain 23 grew very poorly under anaerobic conditions, *S. aureus*, Oxford strain, was used and to obtain better growth, the broth (pH 7.4) was enriched with glucose to 0.5% (w/v). A similar experiment under aerobic conditions was run simultaneously.

Spermine and spermidine both showed the same degree of antibacterial activity under aerobic or anaerobic conditions. After 24 hr. incubation at 37°, 1.8×10^{-4} M-spermine (60 µg. spermine tetrahydrochloride/ml.) and 1.8×10^{-3} M-spermidine (1000 µg. spermidine phosphate/ml.) completely inhibited the growth of *Staphylococcus aureus*, Oxford strain. These results were confirmed in two other similar experiments, and with a different strain of *S. aureus*.

Effect of various unheated and heated sera

To evaluate any potential antibacterial role of spermine *in vivo*, it was desirable to investigate the influence of serum on the system. Since serum contains both heat labile components and heat stable components, the action of unheated and of heated sera was examined.

To test the effect of serum on the antistaphylococcal activity of spermine the following procedure was adopted: sera were diluted 1/4 with distilled water and sterilized by Seitz filtration. A parallel series of dilutions of serum was heated in boiling water for 30 min. Serial dilutions of spermine were made in broth containing the equivalent of 5% (v/v) of unheated or of heated serum. The pH value of the final mixtures was adjusted to 7.8. The tubes were inoculated and the results read after incubation at 37° for 48 hr.

The effect of the various sera is indicated in Table 1. All the unheated sera at 5% (v/v) antagonized the action of spermine; sheep and bovine sera decreased the activity 16- and 8-fold, respectively; guinea-pig and rabbit sera were much less active; the sample of human serum showed an intermediate effect. On the other hand, heated sera (human, sheep, guinea-pig, rabbit) did not affect the action of spermine; only the heated bovine serum retained a little antagonistic activity.

To simulate conditions in the animal body more closely, the effect of undiluted serum was tested. Samples of sera of the same species were pooled, sterilized by Seitz filtration and put into tubes, the first receiving 1.8 ml., and the following tubes 1 ml. of serum. Spermine tetrahydrochloride was added to the first tube to give a final concentration of 2 mg./ml.; decreasing concentrations of spermine were then obtained in successive tubes by transferring 1 ml. amounts from one tube to the next. All tubes were inoculated, and the results are read after 48 hr. of incubation at 37°. The results are shown in Table 1. Undiluted serum of man, guinea-pig and rabbit decreased the

antibacterial activity of spermine against *Staphylococcus aureus* slightly, as compared with sheep and bovine serum; 60–125 μg . spermine.4HCl/ml. being inhibitory in the sera of man, guinea-pig and rabbit, while *c.* 10 times this amount of spermine was necessary to inhibit growth in sheep and bovine sera.

Table 1. *The effect of heated and unheated sera of various species in annulling the toxicity of spermine for Staphylococcus aureus*

Inoculum used: *c.* 10^4 organisms/ml. Heated serum was serum diluted 1/4 with distilled water and heated for 30 min. in boiling water. All media were adjusted to pH 7.8. Final concentration of sera 5 % (v/v).

The results were read after 48 hr. incubation at 37° , a positive inhibitory effect on growth meaning no growth of the test organism visible to the naked eye. The coefficient of inhibition of spermine activity by added serum (last column) is the degree of decrease in spermine activity as compared with the control without added serum (= 1); 16 = 16-fold decrease, etc.

Sera added to medium	Minimal concentration of spermine.4HCl which inhibited growth in presence of serum (μg ./ml.)	Coefficient of inhibition of spermine activity by serum
Unheated		
Sheep	500	16
Bovine	250	8
Rabbit	60	2
Guinea-pig	60	2
Human	125	4
Heated		
Sheep	30	1
Bovine	60	2
Rabbit	30	1
Guinea-pig	30	1
Human	30	1

Action of spermine on staphylococci suspended in buffer

While some antibacterial agents affect multiplying organisms only, spermine and spermidine have been shown to be bactericidal at 37° and at 6° (Rozansky, Bachrach & Grossowicz, 1954; Rozansky, Razin & Grossowicz, 1954). This killing effect was tested on organisms suspended in a nutrient medium in which even at 6° metabolic activities might still proceed to some extent. The effect of spermine on washed cells suspended in buffer was therefore tested. A 24 hr. broth culture of the staphylococcus was centrifuged, the organisms washed three times and diluted with saline to $1/10^4$; 1 ml. of this suspension was added to 10 ml. of either Sørensen buffer (M/15, pH 7.8) or broth adjusted to pH 7.8. Spermine was added to both series to a concentration of 3×10^{-4} M, and controls without spermine were included. The suspensions were kept at 6° and after the intervals indicated in Fig. 1 the surviving organisms were counted by plating. From Fig. 1 it can be seen that organisms in broth + spermine at 6° died rapidly, with only 2% surviving after 6 hr. and practically none at 24 hr., but there was almost no bactericidal effect on organisms kept in buffer +

spermine. On prolonged incubation (72 hr.) a decrease of about 50% was noticed in the control tubes and no greater mortality occurred in buffer+spermine.

Fig. 1. Effect of spermine on washed cells of *Staphylococcus aureus* at 6°. The pH value of all media was 7.8. The number of surviving bacteria was determined by plate count. Initial inoculum *c.* 10^6 cells/ml. —x—, cells suspended in Difco nutrient broth+spermine. 4HCl 100 μ g./ml.; —x—, cells suspended in phosphate buffer+spermine. 4HCl 100 μ g./ml.; —Δ—, cells suspended in Difco nutrient broth (no spermine); —○—, cells suspended in phosphate buffer (no spermine).

Influence of sodium and potassium chlorides

Spermine and spermidine are bases and act probably at anionic sites on the bacterial cell. The effect of sodium and potassium ions on the action of spermine and spermidine was therefore examined. Increasing concentrations of sodium chloride, potassium chloride, sodium phosphate or sodium sulphate, were made in broth and twofold dilutions of spermine and spermidine were provided. Table 2 shows that the salts antagonized the antibacterial action of spermine and spermidine. A 10-fold increase in the concentration of sodium chloride was accompanied by a 32-fold decrease of spermine, and an 8- to 16-fold decrease of spermidine activity. Potassium chloride antagonized the effects of spermine and spermidine in the same way.

Table 2. *Minimal molar concentrations of spermine and spermidine which inhibit growth of Staphylococcus aureus in Difco nutrient broth containing various concentrations of sodium and potassium salts*

The pH value of the medium was 7.7. Size of inoculum: c. 10^4 cells/ml. Results were read after 48 hr. incubation at 37°. Inhibition means no visible growth.

Concentration of salt (%, w/v)	NaCl		KCl		Na ₂ HPO ₄ ·12H ₂ O		Na ₂ SO ₄	
	Spermine	Spermidine	Spermine	Spermidine	Spermine	Spermidine	Spermine	Spermidine
	Minimal concentration (M) which inhibited growth							
0.25	4.5×10^{-5}	2.2×10^{-4}	9×10^{-5}	4.5×10^{-4}	4.5×10^{-5}	4.5×10^{-4}	4.5×10^{-5}	2.2×10^{-4}
0.5	4.5×10^{-5}	4.5×10^{-4}	4.5×10^{-5}	4.5×10^{-4}	4.5×10^{-5}	4.5×10^{-4}	4.5×10^{-5}	4.5×10^{-4}
1	1.8×10^{-4}	4.5×10^{-4}	1.8×10^{-4}	1.8×10^{-3}	7.5×10^{-4}	1.8×10^{-3}	3.6×10^{-4}	9×10^{-4}
2.5	1.5×10^{-3}	1.8×10^{-3}	1.5×10^{-3}	3.7×10^{-3}	1.5×10^{-3}	3.7×10^{-3}	1.5×10^{-3}	3.7×10^{-3}

DISCUSSION

Hirsch (1953*a, b*) with *Mycobacterium tuberculosis* found that the antimycobacterial activity of spermine was due, not to the substance itself, but to a derivative of it produced by a thermolabile enzyme, spermine oxidase. This enzyme was present in sheep serum but not in human serum. This enzyme mechanism appears not to operate in the antistaphylococcal action of spermine for the following reasons: (i) The antistaphylococcal tests were performed in an autoclaved medium in which any thermolabile enzyme would have been destroyed. (ii) Spermine and spermidine were active against staphylococci under anaerobic conditions, thus excluding the formation of a derivative activated by oxygen. (iii) Sheep serum was not necessary for the action of spermine and spermidine against staphylococci; on the contrary, serum diminished the antistaphylococcal effect. Sheep and bovine sera exhibited a strong antispermine effect, whereas human, rabbit and guinea-pig sera were considerably less antagonistic. Since the antispermine effect of serum was almost completely destroyed by heating in boiling water for 30 min., it is possible that a protein, perhaps an enzyme, might be responsible for the inactivation of spermine by serum.

Spermine and spermidine are polyamines of pronounced basic reaction and their antibacterial effect may be that of cationic poisons. In this respect they resemble the basic thymus peptide (Hirsch, 1954), polylysine (Katchalski, Bichowski-Slomnitzki & Volcani, 1953) and triethanolamine and similar amines (MacLeod & Onofrey, 1954). The antibacterial effect of spermine and spermidine, and of polylysine and triethanolamine, was antagonized by sodium and potassium. This reversal of spermine toxicity might be visualized as a cation exchange whereby the base spermine is replaced by non-toxic sodium and potassium ions (Massart, 1948). The bactericidal effect of spermine and spermidine was not limited to multiplying staphylococci, but there was no bactericidal action on washed staphylococci suspended in phosphate buffer, i.e. there was a clear difference in susceptibility between resting and non-resting organisms. The non-sensitivity of the organisms suspended in buffer

might be due either to their inability to take up spermine or spermidine in the absence of an energy-yielding substrate, or to the absence of some specific metabolic process capable of being blocked by spermine.

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