Antibacterial Action of Oxidized Spermine

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SUMMARY

Spermine oxidized by partially purified serum amine oxidase inhibited the growth of a variety of Gram-positive and Gram-negative bacteria. This inhibition of growth was not antagonized by calcium ion, and occurred over a wide pH range. Inactivation increased with time and temperature of incubation. The antibacterial action was abolished by treating oxidized spermine with sodium borohydride. Oxidized spermine inhibited protein synthesis as shown by incorporation of ^{14}C -valine into cellular protein and by inhibition of β -galactosidase induction. Incorporation of ^{14}C -uracil into the nucleic acids of *Escherichia coli* was not inhibited by oxidized spermine.

INTRODUCTION

The naturally occurring polyamine spermine, $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$, inhibits the growth of various bacteria, mainly Gram-positive cocci (Rozansky, Bachrach & Grossowicz, 1954). On the other hand, spermine after oxidation by crude plasma or serum amine oxidase inhibits the growth of *Mycobacterium tuberculosis* (Hirsch & Dubos, 1952; Fletcher, Epstein & Jewell, 1953). Tabor & Rosenthal (1956) studied the oxidation of spermine by purified beef plasma amine oxidase. They found that the oxidation product inhibited the growth of *Escherichia coli*, *Staphylococcus aureus* and was toxic to *Trypanosoma equiperdum* and to human spermatozoa. The inactivation of various bacteriophages by oxidized spermine was also reported (Bachrach, Tabor & Tabor, 1963).

The mechanism of spermine oxidation by purified plasma amine oxidase has recently been elucidated (Tabor, Tabor & Bachrach, 1964); this reaction proceeds as follows:

The present paper deals with the study of factors affecting the antibacterial activity of spermine oxidized by purified serum amine oxidase. It will be shown that the oxidation products interfere with the growth of various bacteria and inhibit the synthesis of some bacterial proteins. A preliminary communication on this work has appeared (Bachrach & Persky, 1963).

METHODS

Organisms. All the bacterial strains used in this work were from the collection of the Department of Clinical Microbiology, Hebrew University-Hadassah Medical School, Jerusalem.

Media. Mycobacteria were grown in the medium described by Dubos & Davis (1946). Other bacteria were grown in nutrient broth (Difco) pH 7.4.

Chemicals. Spermine tetrahydrochloride was obtained from Mann Research Laboratories, Inc. (New York, U.S.A.). Spermidine trihydrochloride was the product of California Corporation for Biochemical Research (Los Angeles 32, U.S.A.). 3,3'-Diaminodipropylamine, glyoxal, and *n*-butyraldehyde were purchased from Eastman Kodak Company. Putrescine was obtained from Nutritional Biochemicals Corporation (Cleveland, 28, Ohio, U.S.A.). Sodium borohydride was purchased from British Drug Houses, Ltd. Amine oxidase was prepared from beef serum by the method of Tabor, Tabor & Rosenthal (1954), but omitting the last calcium phosphate gel step. The preparation was purified approximately 80-fold, with a specific activity of 100 spectrophotometric units/mg. of protein.

Spermine (10 μ mole) was incubated in a Warburg flask at 30° with: 100 μ mole of potassium phosphate buffer (pH 7.0); 0.3 ml. (300 spectrophotometric units) of amine oxidase; 150 units of catalase (Boehringer & Soehne, Mannheim, Germany) in a final volume of 3.0 ml. The reaction went to completion in 60–120 min. with the consumption of the theoretical amount of oxygen (reaction 1 above). The incubation mixture which contained 'oxidized spermine' was used immediately or kept at –20°.

Assay. Antibacterial activity was determined by twofold dilutions in 1.0 ml. nutrient broth. To each dilution of the antibacterial agent was added 0.05 ml. of a diluted bacterial suspension containing $2-10 \times 10^6$ organisms/ml. Results were recorded after incubation at 37° for 20 hr. Viable counts were made by plating, bacterial suspensions being diluted tenfold in nutrient broth. Samples from each dilution were added to nutrient agar kept at 45°, plates poured and the number of colonies was determined after incubation at 37° for 20 hr.

Incorporation of ^{14}C -valine into bacterial protein. The incorporation of ^{14}C -valine into the protein of *Escherichia coli* was examined according to Levinthal, Keynan & Higa (1962) as follows. *E. coli* B was grown for 15–18 hr in 8.0 ml. quantities of Davis medium (Davis, 1949) supplemented with 0.5% (w/v) glucose. To each of these cultures, 10 ml. Davis glucose medium were added. The cultures were incubated for another 120 min. at 35°, and 0.33 μ mole ^{14}C -valine (G) (The Radiochemical Centre, Amersham, Buckinghamshire; specific activity 2.59 $\mu\text{c.}/\mu\text{mole}$) were then added. Samples containing 2.0 ml. of the incubation mixture were removed at various times, and added to 2.0 ml. of a solution containing 10% (w/v) trichloroacetic acid and 1% (w/v) Casamino acids (Difco Laboratories, Detroit, Michigan, U.S.A.). Samples were thoroughly shaken, kept at room temperature for 30 min. and then centrifuged at 3000g for 15 min. Each precipitate was then suspended in 1.5 ml. N-NaOH solution, and kept at room temperature for 30 min. Six ml. of a solution containing trichloroacetic acid and Casamino acid were then added to each suspension. The suspensions were heated in boiling water for 30 min., cooled to 20°–30° and filtered through 45 μ Millipore filters (Millipore

Filter Corp., Bedford, Mass., U.S.A.). The filters were dried, mounted on planchets and the radioactivity was determined by a Nuclear Chicago thin window gas flow counter.

Incorporation of ^{14}C -uracil into bacterial nucleic acid. Incorporation of ^{14}C -uracil into the nucleic acids of *Escherichia coli* was examined according to Levinthal *et al.* (1962). *E. coli* B was grown for 15–18 hr in 10 ml. volumes of Davis medium (Davis, 1949) supplemented with 0.5% (w/v) glucose. To each of these cultures, 10 ml. Davis glucose medium were added. The cultures were incubated for another 120 min. at 35° and 1.6 μmole ^{14}C -uracil (Tracerlab Inc. Waltham, Mass., U.S.A., specific activity 1.04 $\mu\text{c./}\mu\text{mole}$) were then added. Samples containing 2.0 ml. of the incubation mixture were removed at various times and added to 2.0 ml. of a solution containing 10% (w/v) trichloroacetic acid and 0.1% (w/v) uracil (Light & Co. Ltd., Colnbrook, Buckinghamshire). Samples were thoroughly shaken, chilled in an ice + water bath and then filtered through 45 μ Millipore filters previously soaked in a solution containing 5% (w/v) trichloroacetic acid and 0.05% (w/v) uracil. The filters were then washed 5 times with 8.0 ml. quantities of 5% trichloroacetic acid + uracil solution, dried, mounted on planchets and the radioactivity determined as above.

Induction of β -galactosidase. *Escherichia coli* w was inoculated into 50 ml. volumes of Davis medium (Davis, 1949) supplemented with 0.1% (w/v) of sodium succinate. After shaking at 35° for 15 hr sodium succinate was added to a final concentration of 0.5% (w/v) and incubation continued for another 60 min. The bacteria were harvested by centrifugation (9000*g* for 5 min.) and suspended in 12.5 ml. volumes of Davis medium supplemented with 0.5% (w/v) sodium succinate. Six ml. of this suspension were added to about 1000 μg . of 'oxidized spermine' in 1.8 ml. of 0.06*M*-sodium phosphate buffer (pH 7.0). After incubation at 35°, 0.5 ml. samples were removed at various times and added to 1.8 ml. of Davis medium + 0.5% succinate and 0.2 ml. of a 1×10^{-2} *M*-solution of methyl β -D-thiogalactoside (TMG; Sigma Chemical Co. St Louis 18, Mo., U.S.A.). The samples were incubated at 35° for 10 min., then 0.2 ml. of a 0.2% (w/v) chloramphenicol solution added. The test tubes were immediately chilled in an ice–water bath and the bacteria from each sample collected by centrifugation (9000*g* for 5 min.). To each precipitate containing induced bacteria were added 2.0 ml. of 0.05*M*-sodium phosphate buffer (pH 7.5) and one drop of toluene. After incubation at 37° for 30 min., 2.0 ml. of 1.5×10^{-3} *M*-*o*-nitrophenyl β -O-galactoside (ONPG) were added to each suspension. Incubation at 37° was continued for another 15 min. and the reaction was stopped by adding 2.0 ml. *N*- Na_2CO_3 solution to each sample. The intensity of the yellow colour formed was determined by using a Klett electrocolorimeter with a 42 filter.

RESULTS

The antibacterial activities of various naturally occurring polyamines are given in Table 1. Spermine, $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}(\text{CH}_2)_3\text{NH}_2$, was the most potent agent and inhibited the growth of *Staphylococcus aureus* 6538 at 18 $\mu\text{g./ml.}$ nutrient broth. Spermidine $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_4\text{NH}_2$, and $\text{NH}_2(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$, 3,8'-diamino-dipropylamine were less active; 160 $\mu\text{g./ml.}$ were required to inhibit growth. Putrescine, $\text{NH}_2(\text{CH}_2)_4\text{NH}_2$, was not inhibitory even at 1000 $\mu\text{g./ml.}$ Spermine

oxidized by serum amine oxidase was as active as unoxidized spermine when tested against *S. aureus* at pH 7.4.

Previous experiments indicated that spermine is oxidized by serum amine oxidase to a dialdehyde (Tabor, Tabor & Bachrach, 1964). The carbonyl groups of the oxidation product seem to be responsible for the antibacterial action as indicated by the following experiment. 'Oxidized spermine' (0.6 μ mole) was added to 2.0 μ mole sodium borohydride and 7×10^7 *Staphylococcus aureus* organisms in 0.5 ml. nutrient broth. The number of viable bacteria was determined after 30 min. of incubation at 37°; about 90 % of the organisms were still viable. Only 0.01 % of the bacteria survived when oxidized spermine was added to the bacteria in the absence of sodium borohydride. Sodium borohydride is known to reduce carbonyl groups to the corresponding alcohols (Lyttle, Jensen & Struck, 1952).

Table 1. *Inhibition of growth of Staphylococcus aureus 6538 by various compounds*

Compounds were dissolved in 1.0 ml. volumes of nutrient broth (Difco; pH 7.4). Appropriate concentrations were prepared by twofold dilutions in nutrient broth. To each dilution were added $1-5 \times 10^6$ staphylococci. Results recorded after incubation at 37° for 20 hr.

Compound	Minimal inhibitory concentration (μ g./ml.)
Spermine	18
Spermidine	160
3,3'-Diaminodipropylamine	160
Putrescine	> 1000
Oxidized spermine	18*
<i>n</i> -Butyraldehyde	> 5000
Glyoxal	> 500

* Based on the amount of spermine added to the original incubation mixture.

The antibacterial action of oxidized spermine was not annulled after exposing *Staphylococcus aureus* to the oxidation product for 30 min. at 37° and then adding sodium borohydride.

Other aldehydes such as *n*-butyraldehyde and glyoxal did not affect the viability of *Staphylococcus aureus* even at concentrations of 5000 and 500 μ g./ml., respectively (Table 1). The antibacterial effect of oxidized spermine was not due to the serum amine oxidase preparation, which was also present in the incubation mixture. This enzyme alone, as well as catalase, had no effect on the viability of *S. aureus*.

Factors which affect the antibacterial action of oxidized spermine

Susceptibility of different bacteria. Spermine and spermidine are known to inhibit the growth of Gram-positive cocci, whereas Gram-negative bacteria are relatively resistant to their action (Rozansky *et al.* 1954). Oxidized spermine, however, inhibited the growth of the Gram-positive and the Gram-negative bacteria which were tested; the mycobacteria were also sensitive (Table 2).

Effect of pH value. The inhibition of bacterial growth by oxidized spermine was not dependent on the pH value of the culture medium. Table 3 shows that oxidized

Table 2. *Susceptibility of various bacteria to the action of oxidized spermine*

Oxidized spermine was dissolved in nutrient broth (Difco; pH 7.4). Appropriate dilutions were prepared by twofold dilutions in nutrient broth. To each dilution were added $1-5 \times 10^8$ organisms of the test organism. Results were recorded after incubation at 37° for 20 hr. Mycobacteria were grown in Dubos medium and the results were recorded after incubation at 37° for 14 days.

Organism	Minimal inhibitory concentration ($\mu\text{g./ml.}$)
<i>Staphylococcus aureus</i> 4996	18*
<i>S. aureus</i> 6538	18
<i>Bacillus subtilis</i>	18
<i>Escherichia coli</i> C	20
<i>Proteus vulgaris</i>	20
<i>Salmonella paratyphi</i>	20
<i>Shigella flexneri</i>	20
<i>Pseudomonas aeruginosa</i>	18
<i>Serratia marcescens</i>	18
<i>Mycobacterium tuberculosis</i> , strain BCG	12
<i>M. tuberculosis</i> H ₃₇ R _v	12

* Based on the amount of spermine added to the original incubation mixture.

Table 3. *Inactivation of Staphylococcus aureus 6538 by oxidized spermine and polyamines at various pH values*

Experimental conditions as given in Table 1, except that nutrient broth at various pH values was used.

pH value	Oxidized spermine	Spermine	Spermidine	3,3'-Diamino-dipropylamine
Minimal inhibitory concentration ($\mu\text{g./ml.}$)				
5.5	40*	.	.	.
6.0	20	.	.	.
6.5	20	75	630	630
7.4	20	18	315	315
8.3	20	2	18	18
9.0	20	.	.	.

* Based on the amount of spermine added to the original incubation mixture; . = not tested.

spermine 20 $\mu\text{g./ml.}$ inhibited the growth of *Staphylococcus aureus* in a range from pH 6.0 to 9.0. The antibacterial activity of some polyamines is given for comparison. At pH 8.3 the antibacterial activity of spermine was about 40 times that observed at pH 6.5. A similar effect of pH value was noted when spermidine and 3,3'-diamino-dipropylamine were used (Table 3).

Effect of media. *Staphylococcus aureus* was grown in nutrient broth (Difco) at 37° for 20 hr, the cocci harvested at 4° and washed three times with cold 0.15M-NaCl. Samples of the cocci were then suspended in 0.15M-NaCl, 0.15M-NaCl+0.05M-glucose, or nutrient broth (Difco). Oxidized spermine was added to the suspensions which were then incubated at 37° for 60 min. Samples (0.5 ml.) were taken and diluted 1/200 in cold 0.15M-NaCl and the number of viable bacteria counted by

plating. Table 4 shows that oxidized spermine was toxic to *S. aureus* suspended in 0.15M-NaCl. Only growing multiplying *S. aureus* was affected by intact spermine, which was not toxic to washed cocci suspended in NaCl solution.

Cations such as Ca^{2+} or Mg^{2+} are known to antagonize the antibacterial action of spermine (Razin & Rozansky, 1959). The antibacterial action of oxidized spermine, on the other hand, was not antagonized by calcium ion even at 0.1M when tested against *Staphylococcus aureus* in nutrient broth.

Table 4. *Effect of medium on the toxicity of oxidized spermine*

Staphylococcus aureus 6538 was grown in nutrient broth and washed with 0.15M-NaCl. Cocci were then suspended in the various media containing 180 μg .* of 'oxidized spermine' or 0.2 ml. of 0.15M-sodium chloride, and incubated at 37° for 60 min.; total volume 0.4 ml. The number of viable cocci was determined by plating, the results being recorded after incubation at 37° for 20 hr.

Medium	Oxidized spermine	Number of viable cocci
Sodium chloride (0.15M)	{ -	9×10^8
	{ +	6×10^1
Sodium chloride (0.15M) +	{ -	2×10^8
glucose (0.05M)	{ +	0
Nutrient broth (Difco)	{ -	4×10^8
	{ +	0

* Based on the amount of spermine added to the original incubation mixture.

Effect of temperature. *Staphylococcus aureus* was grown in nutrient broth at 37° for 20 hr, the cocci harvested at 4°, washed three times with cold 0.15M-NaCl and resuspended in nutrient broth + oxidized spermine. Samples were incubated at various temperatures for 60 min. Samples (0.5 ml.) were then diluted 1/200 in cold 0.15M-NaCl solution, and a viable count made by plating. Table 5 shows that oxidized spermine was not very toxic to *S. aureus* incubated at 4° and 25°; incubation at 30° or 37°, however, led to complete inactivation of the cocci.

Table 5. *Effect of temperature on the toxicity of oxidized spermine*

Experimental conditions were as in Table 4 except that the staphylococci were suspended in 0.15M-NaCl. The numbers of viable cocci, after incubation for 60 min. at the temperatures indicated, were determined by plate counts, the results being read after incubation of the plates at 37° for 20 hr.

Temperature	Oxidized spermine	Control (no spermine)
Number of viable bacteria		
4°	3.7×10^8	5×10^8
25°	1.8×10^8	5.3×10^8
30°	0	4.5×10^8
37°	0	5.7×10^8

Effect of exposure time. *Staphylococcus aureus* was grown, harvested and washed as above. The washed organisms were resuspended in nutrient broth + oxidized spermine and incubated at 37°. Samples (0.5 ml.) were diluted 1/200 in cold 0.15M-NaCl after various incubation times, and a viable count made by plating. Figure 1 shows that there was a linear relationship between the time of exposure and the log of number of survivors.

Inhibition of protein synthesis by oxidized spermine. The wide spectrum of the antibacterial activity of oxidized spermine suggested that a mechanism common to various micro-organisms is the site of the antibacterial action. The effect of oxidized spermine on the synthesis of proteins and ribonucleic acids was therefore examined. Protein synthesis was studied by (a) incorporation of ^{14}C -valine into trichloroacetic acid (TCA)-insoluble products; (b) induction of β -galactosidase.

Incorporation of ^{14}C -valine into bacterial protein. *Escherichia coli* B incorporated ^{14}C -valine into their protein (Fig. 2). This reaction was fast; about 50 % of the total

Fig. 1

Fig. 2

Fig. 1. Effect of incubation time on the inactivation of *Staphylococcus aureus* by oxidized spermine.

Fig. 2. Incorporation of ^{14}C -valine into the protein of *Escherichia coli* B. ● — ●, incorporation system containing an equivalent of 5 mg. dry wt. bacteria/ml.; x — x, incorporation system containing oxidized spermine 120 $\mu\text{g.}/\text{ml.}$ and equiv. of 5 mg. dry wt. bacteria/ml.

radioactivity was incorporated within the first minute of incubation and incorporation was complete after 10 min. A considerable inhibition of incorporation was noticed when oxidized spermine (final concentration 120 $\mu\text{g.}/\text{ml.}$) was added to the bacterial suspension at zero time together with ^{14}C -valine. During the first minute of incubation, the incorporation of ^{14}C -valine into the bacterial protein was only 15 % of that obtained in the control experiment. After 2 min. of incubation, the incorporation was also about 15 % of that obtained in the control. After 20 min. of incubation, the incorporation of ^{14}C -valine in the presence of oxidized spermine was similar to that obtained in the control experiment within 1 min.

Inhibition of β -galactosidase induction. Further evidence for the effect of oxidized spermine on protein synthesis was provided by enzyme induction. Suspensions of

Escherichia coli w, in Davis medium + succinate, were incubated with oxidized spermine (final concentration 180 $\mu\text{g./ml.}$) for various periods. Methyl β -D-thiogalactoside (TMG) was then added to induce the formation of β -galactosidase. Figure 3 shows that *E. coli*, pre-incubated with oxidized spermine, was induced to a small degree only. Inhibition of induction was proportional to incubation time with oxidized spermine. When the pre-incubation was for 30 min., induction was inhibited by about 75 %; about 95 % inhibition of β -galactosidase formation was obtained with *E. coli* exposed to oxidized spermine for 90 or 120 min. Control experiments showed that treatment of *E. coli* w with oxidized spermine had no effect on the oxidation of glucose (measured by the manometric method of Warburg), neither was there any inhibition in the breakdown of β -galactosides by oxidized spermine when β -galactosidase-induced bacteria were used. These control experiments thus rule out a possible indirect effect of oxidized spermine on the metabolism of β -galactosides.

Effect of oxidized spermine on incorporation of ^{14}C -uracil into bacterial nucleic acids. Incorporation of ^{14}C -uracil into bacterial nucleic acid was studied as described in Methods. Oxidized spermine (180 $\mu\text{g./ml.}$), added to a suspension of *Escherichia coli* B and ^{14}C -uracil, had no significant effect on the incorporation (Fig. 4).

Fig. 3

Fig. 4

Fig. 3. Effect of oxidized spermine on the induction of β -galactosidase in *Escherichia coli* w. Bacterial suspensions (equiv. 5 mg. dry wt./ml.) were pre-incubated with oxidized spermine (180 $\mu\text{g./ml.}$) for various times. Methyl- β -D-thiogalactoside was added and β -galactosidase activity (expressed in Klett units) then determined.

Fig. 4. Incorporation of ^{14}C -uracil into the nucleic acids of *Escherichia coli* B. ● — ●, control system; × — ×, incorporation system containing oxidized spermine 180 $\mu\text{g./ml.}$

DISCUSSION

The antibacterial activity of the naturally occurring polyamines is apparently due to their cationic nature. These polyamines are known to form complexes with acidic components of the bacterial cell, such as DNA, RNA (Felsenfeld & Huang, 1960) or phospholipids (Razin & Rozansky, 1959), and are readily adsorbed to bacterial constituents (Bachrach & Cohen, 1961; Tabor, 1962). The antibacterial action of 'oxidized spermine' seems to be of a different nature. The carbonyl groups of the newly formed dialdehyde seem to be responsible for the toxic effect. This is borne out by the fact that sodium borohydride, which reduces the dialdehyde to the corresponding alcohol, abolished the toxic activity. Cations, such as Ca^{2+} , did not interfere with the antibacterial action of oxidized spermine, pointing to a non-electrostatic linkage between the toxic compound and the bacterial cell. The fact that oxidized spermine inhibited bacterial growth over a wide pH range also favours a non-electrostatic linkage.

The ability of oxidized spermine to inhibit the growth of a variety of bacteria is of interest, and suggests that a mechanism common to all susceptible organisms is damaged. Such a mechanism cannot involve synthesis of cell walls, which are different in the various organisms. Results presented in this paper show that oxidized spermine inhibits protein synthesis, whereas incorporation of ^{14}C -uracil into bacterial nucleic acids is unaffected. The mechanism of this inhibition is yet to be elucidated. It is possible that interaction between oxidized spermine and bacterial nucleic acids is the cause of inhibition of protein synthesis. It is reasonable to speculate that the carbonyl groups in the molecule of the oxidation product react with the amino groups of the purines and pyrimidines in the bacterial nucleic acids with the formation of Schiff bases. The formation of such compounds would not be dependent of the hydrogen ion concentration of the medium and on the presence of cations. Furthermore, such a mechanism could also explain the previously reported inactivation of various bacteriophages by oxidized spermine (Bachrach, Tabor & Tabor, 1963). We have recently found that oxidized spermine penetrates into coliphages of the T-uneven series and forms complexes with their DNA. This modified DNA is injected into the host bacterial cell after infection, but does not lead to the formation of new phages (Bachrach & Leibovici, 1964).

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