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Inhibition of Protein Synthesis by Spermine in Growing Cells of *Staphylococcus aureus*

MISCHA E. FRIEDMAN[1] AND URIEL BACHRACH

Department of Clinical Microbiology, Hadassah Medical School, Hebrew University, Jerusalem, Israel

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ABSTRACT

FRIEDMAN, MISCHA E. (Hebrew University, Jerusalem, Israel), AND URIEL BACHRACH. Inhibition of protein synthesis by spermine in growing cells of *Staphylococcus aureus*. J. Bacteriol. **92**:49–55. 1966.—*Staphylococcus aureus* SFL 9725 incorporated valine-C^{14} into cellular protein. This incorporation was inhibited by spermine when the *p*H of the culture was adjusted to 7.8, and the inhibition was antagonized partially by Mg^{++}. The incorporation of C^{14}-labeled leucine, phenylalanine, lysine, arginine, and possibly glutamic acid was inhibited to a much greater extent than that of alanine, glycine, or threonine. The uptake of spermine-C^{14} by *S. aureus* cells was rapid. More than 50% of the radioactivity resided in the soluble extract. The protein fraction of the soluble extract contained 99% of the recovered label, whereas the ribonucleic acid and deoxyribonucleic acid fractions contained little or no spermine-C^{14}.

The polyamine spermine, $NH_2(CH_2)_3NH \cdot (CH_2)_4NH(CH_2)_3NH_2$, was found by Herbst and Snell (9) to be a growth factor for *Haemophilus parainfluenzae*. Mager (11) demonstrated that this cationic compound is protective to bacterial spheroplasts and protoplasts. On the other hand, spermine inhibits the growth of *Staphylococcus aureus* (22), and Razin and Rozansky (20) later showed that spermine is bactericidal and that the adsorption of the polyamine by the cells decreases in the presence of high concentrations of hydrogen ion or other cations. Spermine is not detectable in *S. aureus* unless present in the growth medium. The same amount of polyamine is taken up per gram of cells at *p*H 8.0 or 6.0, and there is no evidence of its utilization (21). Spermine also acts as an antimutagen against induced mutation in *S. aureus* and other organisms (24), and Johnson and Bach (Bacteriol. Proc., p. 17, 1965), studying this effect in *Escherichia coli*, found the incorporation of spermine to be greater in spheroplasts than in whole cells. The cell walls incorporate only 1% of the radioactive label, and, of that bound to the spheroplasts, only 50% is exchangeable. Studies of the activity of spermine at the molecular level (3, 8, 12, 16) indicated a relationship between ribosomal material and spermine, and consequently protein synthesis.

[1] Permanent address: U.S. Army Biological Center, Fort Detrick, Frederick, Md.

Bachrach and Persky (1) demonstrated that oxidized spermine inhibits the incorporation of valine-C^{14} into cellular protein of *E. coli*. The effect of spermine on the incorporation of several C^{14}-amino acids by the whole-cell system of *S. aureus*, as well as studies on the incorporation and cellular distribution of labeled spermine, are reported here.

MATERIALS AND METHODS

Stock cultures and media. *S. aureus* SFL 9725 (a fresh isolate from human feces), obtained from D. Sompolinsky, Assaf Harofe Government Hospital, Zerifin, Israel, was coagulase-negative, mannitol-negative, nonhemolytic, and resistant to typing phages. According to Baird-Parker (2), this bacterium would not be classified as *S. aureus*, but as a member of *Staphylococcus* subgroup II. However, here the designation *S. aureus* SFL 9725 will be used. Stock cultures were maintained on Trypticase Soy Agar (BBL) supplemented with 0.001% thiamine and nicotinic acid, stored at 5 C, and transferred monthly. Broth media consisted of 1 or 2% Casitone (Difco), 0.2% glucose, 0.001% thiamine, and 0.001% nicotinic acid. The *p*H was 7.4. Inocula for growth studies consisted of cells harvested from 6-hr slants; denser inocula from overnight slants were used in amino acid incorporation studies. All incubations were at 37 C with shaking. Growth was measured in a Klett-Summerson colorimeter with either the red no. 66 or the green no. 54 filter. *E. coli* B from the stock culture collection of the Department of Clinical Microbiology was cultured in the medium of Davis (5) supplemented with 0.1% peptone.

Incorporation of C^{14}-amino acids into bacterial protein. The incorporation of C^{14}-amino acids into the protein of whole cells of *S. aureus* or *E. coli* was examined by the method of Levinthal et al. (10) as follows. *S. aureus* was grown in 1% Casitone medium until the optical density (540 mμ) reached 0.3 to 0.35 (approximately 3 hr). After the *p*H of the culture was adjusted to 7.8 with 1 N NaOH, labeled amino acid (2 μc/20 ml of medium if L isomer, 4 μc/20 ml if DL isomer) was added. Spermine was added simultaneously with the C^{14}-amino acid. Samples of 2.0 ml of the incubated mixture were removed at various times and pipetted into 2.0 ml of 10% trichloroacetic acid prepared in 1% Casamino Acids. Samples were well mixed, kept in ice for 30 min, and centrifuged at 12,000 × *g* for 15 min. Each precipitate was suspended in 1.5 ml of 1 N NaOH and kept at room temperature for 20 min; then 6.0 ml of the 10% trichloroacetic acid solution was added, and the mixture was heated at 90 to 95 C for 30 min. After being cooled to room temperature, the suspensions were filtered through membrane filters (0.45-μ pore size; Millipore Filter Corp., Bedford, Mass.) previously soaked in 5% trichloroacetic acid in 1% Casamino Acids, washed twice with 10-ml quantities of the 5% trichloroacetic acid solution, and dried. The filters, mounted in planchets, were counted for radioactivity with a Nuclear-Chicago thin-window gas-flow counter.

The radioactivity of the various fractions of the cell was determined by following the fractionation procedure of Park and Hancock (19) after a log-phase culture had been incubated for 20 min in the presence of glycine-C^{14} and spermine.

Incorporation and distribution of labeled spermine. When the density of the *S. aureus* culture reached 0.3 to 0.35, the *p*H was adjusted to 7.8, and spermine-C^{14} tetrahydrochloride (0.25 μc/20 ml of medium) was added. At various time intervals, 2.0-ml samples were diluted in 10.0 ml of cold distilled water. The suspensions were filtered immediately through membrane filters which had been treated previously with 0.001 M polylysine (molecular weight, 3,300; YEDA Research and Development Co., Rehoveth, Israel), washed with additional amounts of cold water, dried, mounted on planchets, and counted. Distribution studies were carried out with log-phase cells that had been incubated for 25 min in the presence of 0.0138 μc of spermine-C^{14} (1.13 μg/ml of medium). The cells were washed five times with cold 0.85% saline by centrifugation at 12,000 × *g* for 10 min at 4 C in a Sorvall RC-2 centrifuge, resuspended in saline, and disrupted by treatment for 90 min in a Raytheon 10-kc sonic oscillator; the soluble extract was then treated in the manner described by Matthaei and Nirenberg (17) for the preparation of S-30 and S-100 supernatant solutions and 100,000 × *g* particulate matter. The cell debris was separated from whole cells by repeated centrifugation at 200 × *g* for 2 to 3 min at a time. All washes were with 0.85% saline. Radioactivity of these materials was calculated per microgram of ribonucleic acid (RNA). After deproteinization by phenol extraction, the S-100 fluid was applied to a methylated albumin column (15), and the effluent fractions containing RNA and deoxyribonucleic acid (DNA) were located by ultraviolet absorption at 260 mμ in a Hitachi Perkin-Elmer spectrophotometer. The radioactivity of the protein and the collected effluents was measured in a Packard Tri-Carb liquid scintillation spectrometer, with the samples dissolved in the scintillation liquid of Davidson and Feigelson (4).

Analytical methods. Materials to be assayed colorimetrically for RNA were extracted first by the method of Schneider (23), and the extracts were subjected to the orcinol test of Drury (6).

Radiochemicals. L-Valine-*1*-C^{14} (specific activity, 168 mc/mmole), DL-valine-*1*-C^{14} (3.7 mc/mmole), DL-leucine-*1*-C^{14} (36.6 mc/mmole), and uniformly labeled L-lysine-C^{14} (7.5 mc/mmole), L-arginine-C^{14} (7.7 mc/mmole), L-glutamic acid-C^{14} (6.35 mc/mmole), and L-threonine-C^{14} (5.47 mc/mmole) were obtained from the Radiochemical Centre, Amersham, England. D-Alanine-*1*-C^{14} (8.0 mc/mmole), glycine-*1*-C^{14} (10.2 mc/mmole), and uniformly labeled L-alanine-C^{14} (90 mc/mmole) were purchased from Volk Radiochemical Co., Skokie, Ill. L-Phenylalanine-*1*-C^{14} (177 mc/mmole) was obtained from Schwarz Bio Research, Inc., Orangeburg, N.Y., and spermine-C^{14} tetrahydrochloride (4.25 mc/mmole) was purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Effect of spermine on the growth of S. aureus. The growth of strain SFL 9725 was inhibited 50% by approximately 90 μg/ml of spermine and completely by approximately 120 μg/ml, if the inhibitor was present at the time of inoculation (Fig. 1). The media for these tests were adjusted initially to *p*H 7.5. Stationary cultures were inhibited completely in the presence of approximately 50 μg/ml of spermine.

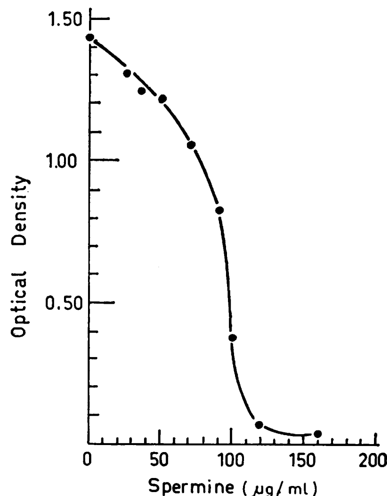

FIG. 1. *Effect of spermine on growth of Staphylococcus aureus SFL 9725. Organisms were grown in 10-ml cultures in 150-ml Erlenmeyer flasks with shaking in a water bath (37 C).*

Incorporation of valine-C^{14}. Almost all of the counts from valine-C^{14} incorporated into growing log-phase cells in 10 min were found in trichloroacetic acid-insoluble material, indicating an incorporation into a high molecular weight polypeptide (Table 1). The addition of spermine to growing cells did not inhibit growth.

TABLE 1. *Incorporation of valine-C^{14} into growing log-phase cells of Staphylococcus aureus SFL 9725*[a]

Sample	Counts per min per 2 ml of culture	Per cent recovery
Control culture	642	
+ spermine (90 μg/ml)	416	
+ spermine (279 μg/ml)	212	
Trichloroacetic acid-washed culture (A)	629	100
+ spermine (B)	348	88
+ spermine (C)	178	95
Trichloroacetic acid wash A	28	
Trichloroacetic acid wash B	20	
Trichloroacetic acid wash C	24	

[a] At density 0.3 (540 mμ), 14 μg of DL-valine-C^{14} (4 μc) was added to 20 ml of culture adjusted to *p*H 7.8 with 1 N NaOH simultaneously with the spermine. After 10 min of additional incubation with shaking in a water bath (37 C), samples were removed and treated according to Levinthal et al. (10). Trichloroacetic acid-washed cultures were washed twice with 3 ml of 10% trichloroacetic acid in 1% Casamino Acids by centrifugation before being given the Levinthal treatment. The first 5% trichloroacetic acid washes after the heating of the cells at 95 C were collected.

Inhibition of valine-C^{14} incorporation by spermine and effect of pH. The rapid incorporation of valine-C^{14} by log-phase cells of *S. aureus* SFL 9725 was inhibited by spermine, but only if the *p*H of the culture was 7.6 or higher at the time of the addition of spermine (Fig. 2). Following a slight incorporation for the first 5 min, uptake ceased with 200 to 400 μg/ml of spermine. On the other hand, the incorporating system of *E. coli* was completely resistant to 209 μg/ml at *p*H 7.8 or 8.0.

Effect of time of addition of spermine. When spermine (280 μg/ml) was added 5 min before the addition of valine-C^{14} to a culture adjusted to *p*H 7.8, the incorporation of radioactive material barely reached 50 counts per min after 25 min (Fig. 3). This represents an approximate 2-fold increase in the effect of spermine over that observed when the inhibitor and valine-C^{14} were added together, and a 10-fold increase when the spermine was added 5 min after the addition of the valine.

Reversal of spermine. Mg^{++} in the form of $MgCl_2$ caused a slight reversal of the effect of spermine, 2.3 mg/ml reducing the inhibition caused by 265 μg/ml of spermine from 96 to 78%. No advantage was found by using an increased concentration of $MgCl_2$ or by replacing $MgCl_2$ with $MgSO_4$. Mn^{++} did not reverse the

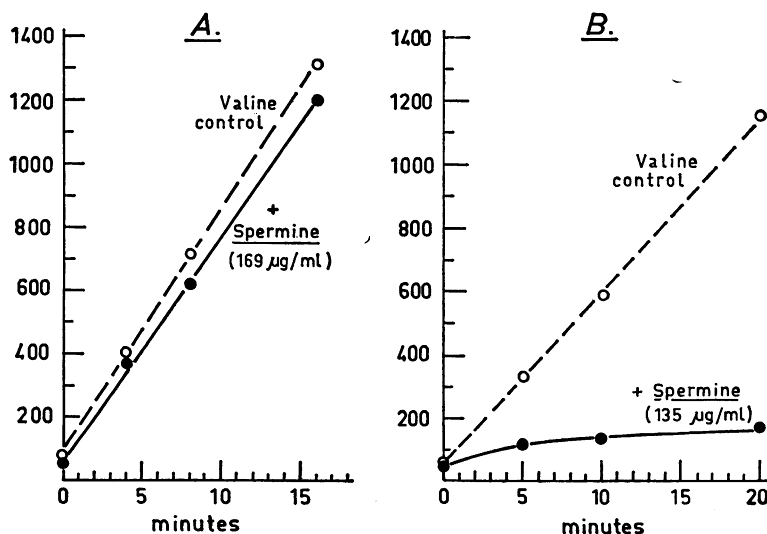

FIG. 2. *Effect of spermine on incorporation of valine-C^{14} into growing log-phase cells of Staphylococcus aureus SFL 9725. (A) No pH adjustment. (B) pH of culture adjusted to pH 7.8 with concomitant addition of spermine and DL-valine-C^{14} (0.18 μc or 5.5 μg/ml). Organisms were grown in 20-ml cultures in 150-ml Erlenmeyer flasks with shaking in a water bath (37 C).*

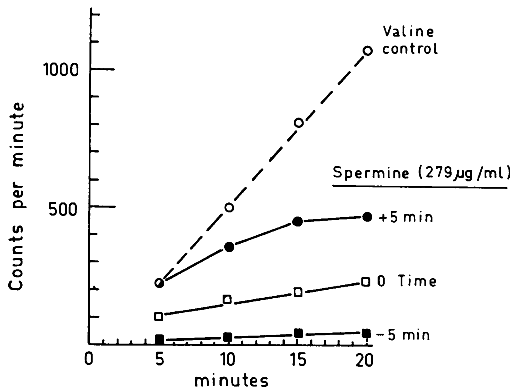

FIG. 3. *Effect of time of addition of spermine on incorporation of valine-C^{14} into growing log-phase cells of Staphylococcus aureus SFL 9725. Spermine was added before, with, and after the addition of L-valine-C^{14} (0.1 μc or 0.07 μg/ml) to a 20-ml culture at pH 7.8. The organisms were incubated with shaking in a water bath (37 C).*

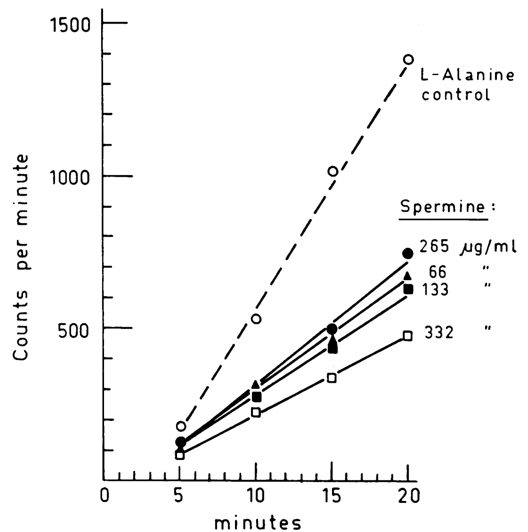

FIG. 4. *Effect of spermine on incorporation of L-alanine-C^{14} into growing log-phase cells of Staphylococcus aureus SFL 9725. The spermine and the L-alanine-C^{14} (0.1 μc or 0.1 μg/ml) were added together to 20 ml of culture at pH 7.8. Organisms were incubated with shaking in a water bath (37 C).*

inhibition by spermine, but Ca^{++} reduced the inhibitory effect of spermine by 50%. This observation, however, was not pursued further, since Ca^{++} caused heavy precipitation in Casitone cultures at *p*H 7.8. Attempts to antagonize spermine with NH_4^+ ions were without consistent success.

Effect of spermine on incorporation of other C^{14}-amino acids. Although log-phase cells incorporated leucine-C^{14} and phenylalanine-C^{14} to a lesser degree than valine-C^{14}, spermine had the same inhibitory effect. On the other hand, incorporation of label from D-alanine, L-alanine, or glycine by *S. aureus* cells did not cease after an initial uptake, but proceeded linearly at a slower rate than that of the control. An inhibition of 51 to 66% was observed after 20 min of incubation (see curve for L-alanine, Fig. 4). The incorporation of glycine-C^{14} into cell wall mucopeptide (residue) was reduced approximately the same extent as was the total incorporation into the cell (Table 2). In addition, almost twice as much "free" glycine (cold trichloroacetic acid fraction) was released from the cells grown with spermine as from those cultured in its absence. The behavior of spermine in regard to the incorporation of label from lysine, arginine, and to a great extent glutamic acid was similar to that shown with valine (Table 3). The incorporation of threonine-C^{14}, however, was affected very little by spermine (Fig. 5).

Spermine incorporation and cellular distribution. The incorporation of spermine-C^{14} by multiplying log-phase cells was rapid; the fastest rate occurred in the first 2 to 4 min (Fig. 6). The incorporation seemed to be antagonized by polylysine; however, use of this compound for such experiments was limited, since it caused a precipitation even in sterile Casitone medium.

TABLE 2. *Effect of spermine on the incorporation of glycine-C^{14} into fractions of growing log-phase cells of Staphylococcus aureus SFL 9725*[a]

Fraction	Counts per min per 2 ml of culture	
	Without spermine	With spermine
Whole cells	27,672	12,354
Cold trichloroacetic acid	192	340
Aqueous ethyl alcohol-soluble	34	34
Hot trichloroacetic acid	954	500
Trypsin-solubilized	5,534	304
Residue	17,352	9,458

[a] At density 0.3 (540 mμ), glycine-C^{14} (0.009 μc/ml) and spermine (195 μg/ml) were added together to 20 ml of culture adjusted to *p*H 7.8 with 0.5 N NaOH. After 20 min of incubation with shaking in a water bath (37 C), 2.0-ml samples were removed for counting of whole cells, and the remainder of the culture was treated according to Park and Hancock (19).

When log-phase cells were grown for 25 min

TABLE 3. *Effect of spermine on the incorporation of C^{14}-amino acids into growing log-phase cells of Staphylococcus aureus SFL 9725*[a]

C^{14}-amino acid (μc/ml)	Spermine	Counts per min per 2 ml of culture	Inhibition
	μg/ml		*%*
DL-Valine (0.19)	0	981	83
	135	172	
DL-Leucine (0.19)	0	386	
	135	80	79
L-Phenylalanine (0.097)	0	328	
	135	53	84
L-Lysine (0.095)	0	362	
	133	108	70
	332	66	82
L-Arginine (0.095)	0	604	
	133	145	76
	332	65	89
L-Glutamic acid (0.095)	0	891	
	133	252	72
	332	136	85

[a] At density 0.3 (540 mμ), C^{14}-amino acid and spermine were added together to 20 ml of culture adjusted to pH 7.8 with 1 N NaOH. After 20 min of incubation with shaking in a water bath (37 C), 2.0-ml samples were removed and treated according to Levinthal et al. (10).

in the presence of 0.0138 μc of spermine-C^{14} and then disrupted by sonic oscillation, 50% or more of the radioactivity recovered was found in the soluble cell extract. The 100,000 × g particulate matter contained a total of 3,210 counts per min, and the S-100 supernatant fluid, 134,200 counts per min. If radioactivity was calculated as counts per minute per microgram of RNA, values of 15 were obtained for both the particulate and supernatant fractions. The specific radioactivity content of the whole cells was 44 counts per min per μg of RNA. After chromatography of the S-100 material through a column of methylated albumin, the separated RNA and DNA fractions showed little radioactivity; 99% of the recovered label was found in the protein fraction obtained by extraction with phenol.

DISCUSSION

The inhibition of growth of *S. aureus* by spermine, first demonstrated by Rozansky et al. (22), was confirmed for strain SFL 9725. In addition, spermine was shown to inhibit the incorporation of valine-C^{14} by multiplying log-phase *S. aureus* cells. After a short period during which a small amount of radioactivity was taken up by the cells, the incorporation of labeled valine stopped.

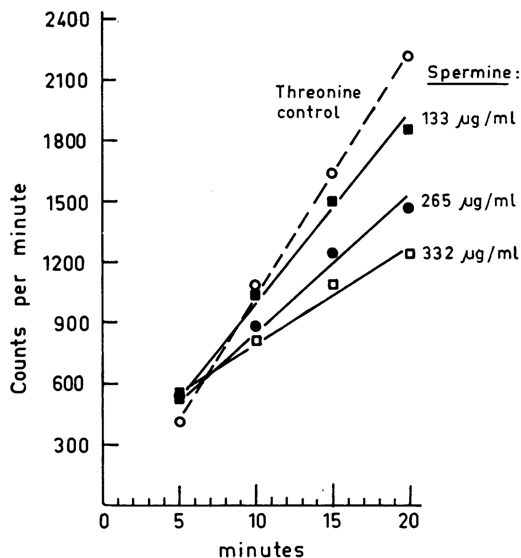

FIG. 5. *Effect of spermine on incorporation of threonine-C^{14} into growing log-phase cells of Staphylococcus aureus SFL 9725. The spermine and the L-threonine-C^{14} (0.1 μc or 2.1 μg/ml) were added together to 20 ml of culture at pH 7.8. Organisms were incubated with shaking in a water bath (37 C).*

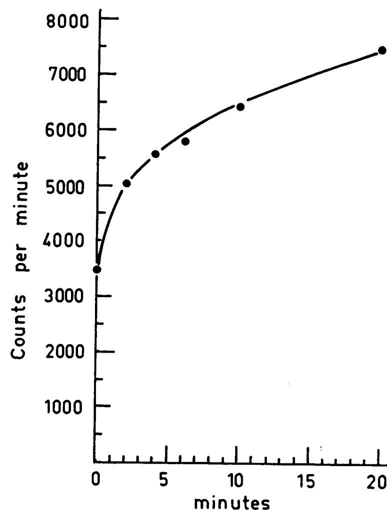

FIG. 6. *Incorporation of spermine-C^{14} tetrahydrochloride (0.0125 μc or 1.0 μg/ml) into growing log-phase cells of Staphylococcus aureus SFL 9725. The culture, adjusted to pH 7.8, was incubated with shaking in a water bath (37 C).*

This was true also for labeled leucine, phenylalanine, arginine, lysine, and possibly glutamic acid, but not for L- or D-alanine, glycine, or threonine. With this latter group, a constant rate of incorporation was observed even in the presence of 332 μg/ml of spermine. These observations may reflect a possible effect spermine has on the penetration of amino acids into *S. aureus*. Also, the lack of complete inhibition of the incorporation of alanine and glycine may be explained as a failure of spermine to affect the system incorporating these amino acids into cell wall mucopeptide (26). However, study of various fractions of the cell obtained after incorporation of glycine-C^{14} indicates that this is not the case with this amino acid. In addition, the uptake of lysine, which is also part of the mucopeptide, was affected by spermine in the same manner as valine.

The effect of *p*H on spermine inhibitory activity and the role of Mg^{++} or polylysine in reversing this activity points to the cationic nature of the polyamine. The lack of complete reversal by Mg^{++} may have been due to the alkaline *p*H at which spermine activity is tested or to the possibility that the incorporated spermine was bound tightly to the polynucleotides of the cell (7).

S. aureus cells in the log phase incorporated spermine-C^{14} rapidly; cells in 1 ml of culture bound 0.14 μg of labeled spermine after 20 min. At least 50% of the bound spermine-C^{14} was in the particulate matter obtained after centrifugation at 100,000 $\times$ *g*. This finding is in agreement with the observation of Cohen and Lichtenstein (3), who found that spermine is tightly bound to bacterial ribosomes. Analysis of the 100,000 $\times$ *g* supernatant fraction on a methylated albumin column indicates that spermine was not bound to soluble RNA or DNA. Yet, redistribution of spermine-C^{14} during the phenol extraction has to be considered, although this treatment should not lead to a dissociation of the spermine-nucleic acid complexes (25).

No definite explanation as to the mode of antibacterial action of spermine can be given as yet. However, the reports of Mager et al. (12) and Friedman and Weinstein (8) strongly suggest that one activity may be similar to that of streptomycin, in which genetic miscoding occurs, resulting in the formation of incomplete or nonfunctional protein that could be lethal to the cell. It is noteworthy that the code words of the amino acids (18) which were inhibited by spermine consist of either two adenylic or two uridylic acids. It is tempting to speculate that spermine is bound to these nucleotides or to the corresponding nucleotides in the anticodons of the soluble RNA. The finding of Mandel (14) and Mahler and Mehrotra (13) that the effect of spermine on the melting temperature of DNA is a function of its adenine-thymine content also supports the view that adenylic and thymidylic acids (and possibly uridylic acid) are related to the biological activity of spermine. Obviously, a more direct explanation of the role of spermine in these reactions would have been obtained with a cell-free staphylococcal incorporating system utilizing synthetic messenger RNA. These studies will have to await the solution of the problems concerned with obtaining 70*S* ribosomes from staphylococci (27).

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