Some Effects of Spermine on *Escherichia coli*

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SUMMARY

Exponentially growing cultures of *Escherichia coli* were exposed to bactericidal concentrations of the polyamine spermine. The earliest changes noted were slowing of protein synthesis, stimulation of ribonucleic acid (RNA) synthesis, and increased potassium fluxes; these changes preceded a decline in viable number. Possible interrelationships among these early effects of spermine have been discussed, and these effects have been compared with those of another polycationic antibiotic, streptomycin.

Both glucose starvation and chloramphenicol treatment blocked the bactericidal action of spermine, but their protective mechanisms appeared different. Glucose starvation prevented the usual uptake of spermine by growing cells, whereas chloramphenicol treatment did not.

INTRODUCTION

The naturally occurring polyamine spermine [$NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] is known to be toxic toward a variety of microorganisms [see reviews by Tabor *et al.* (1), and Tabor and Tabor (2)], and appears to be the agent responsible for the antibacterial activity of human semen (3–5). Previous work has demonstrated that spermine is bactericidal, and that killing is enhanced by conditions promoting cell growth (6). In addition, metabolic studies have indicated that spermine can inhibit oxygen consumption, as well as enzyme induction and overall protein synthesis (7, 8).

The present paper describes a detailed study of the sequence of metabolic alterations produced by spermine in growing *Escherichia coli*. To facilitate comparison with another polycationic agent, streptomycin, our approach was patterned after an earlier study on the action of that drug (9). Accordingly, special attention was given to cell membrane function and the synthesis of macromolecules. Spermine was indeed found to resemble streptomycin, in causing early changes in protein synthesis, in RNA synthesis, and in transmembrane K fluxes; however, significant differences between the two agents were also found.

METHODS

Bacteria. Unless otherwise stated, *Escherichia coli*, strain B, was used. A spermine-resistant strain (B^{spm-r}) was derived by plating about 10^5 organisms on solidified medium $K_{0.3}$ containing 1 mg spermine per milliliter (see below), and selecting one of the resulting colonies for further culture.

Materials. Spermine tetrahydrochloride was purchased from Hoffmann-LaRoche, Inc., and concentrations are given as this salt. Chloramphenicol was a gift of Parke, Davis & Co. Nonradioactive amino acids and uracil were purchased from Calbiochem Co. ^{42}KCl was purchased from Iso/Serve Corp., and ^{14}C-labeled compounds were purchased from New England Nuclear Corp. Membrane filters (HA, 0.45 μ pore size) were a product of Millipore Filter Corp.

Media and growth conditions. Stock cultures were maintained on tryptic digest

agar slopes (tryptic digest, 1.5%; agar, 2%). Strain B^{spm-r} was kept on a 2% agar solid medium consisting of medium $K_{0.3}$ (see below) plus 0.2% glucose and spermine, 1 mg/ml.

Cells were grown at 37°, in bubbler tubes, in a low-potassium, low-phosphate, Tris-buffered medium, K_x, where X is the millimolar KCl concentration (10). Glucose was added to make a final concentration of 0.2% unless stated otherwise. Inocula were grown overnight in phosphate-buffered medium (9), harvested by filtration or centrifugation, and then diluted into medium K. Turbidity was followed for at least 1 hr before the start of an experiment, to ensure that growth was exponential.

Growth and viability measurements. Growth was determined turbidimetrically at 490 mμ, and viable number by plate counts on tryptic digest agar, all as described by Dubin *et al.* (9).

Radioactive counting procedures. Washed cells, or trichloroacetic acid precipitates of cells, were collected on membrane filters and assayed for radioactivity as previously described (9).

Estimation of protein and nucleic acid synthesis. Net synthesis of macromolecules was estimated by following the cumulative incorporation of ^{14}C-leucine or ^{14}C-uracil into cold trichloroacetic acid precipitates of cells; these data were usually plotted on a time scale proportional to the increase in turbidity of the control culture, so as to yield straight lines for the control incorporation (9).

In addition, rates of synthesis were measured by the incorporation of radioactivity during short, precisely timed, exposures ("pulses") to labeled precursor. This measurement is more sensitive to rate changes than is measurement of cumulative incorporation, and also minimizes the possible influence of any metabolic turnover on the apparent synthetic rate. Pulse measurements also facilitated studies on RNA, as opposed to nucleic acid as a whole, since less than 5% of a 2-min uracil pulse appeared in deoxyribonucleic acid (DNA), compared to about 15% for cumulative incorporation measurements. Conditions for a pulse were adjusted so that after a lag of 5–10 sec, incorporation was linear throughout the 1- or 2-min duration of the pulse (see 11–13).

Uptake of ^{14}C-spermine was determined by collecting cells on membrane filters and washing them three times with 3 ml of medium at room temperature. Washing six times with medium, or three times with medium containing nonradioactive spermine, 1 mg/ml, did not remove more label than the standard procedure.

Potassium studies. The methods for determining potassium content, efflux, and influx, were as described previously (9, 10), except that in all cases intracellular ^{42}K was measured by counting washed cells, rather than culture filtrate. The $T_{1/2}$ (half-time of efflux) for control cultures was 8–10 min, in agreement with earlier studies using this medium (10). This value did not vary with the potassium concentration of the resuspension medium over the range of 0.3–3.3 mM.

RESULTS

The Bactericidal Effect of Spermine

Under our conditions the minimal bactericidal concentration of spermine was approximately 500 μg/ml; below this concentration, growth slowed but never stopped. Levels of 750–1000 μg/ml were used in most of the experiments to be described.

Grossowicz *et al.* (6) and Razin and Rozansky (7) noted that glucose starvation would protect *Staphylococcus aureus* from the bactericidal effect of spermine; this was the case with *E. coli* as well. We have found that chloramphenicol also blocks the bactericidal action of spermine. Concentrations of 200 μg/ml protected completely; lower concentrations afforded partial protection. The effect of the lowest concentration tested, 1 μg/ml, is shown in Table 1. However, the results of studies on ^{14}C-spermine uptake suggested that glucose starvation and chloramphenicol treatment protected by different mechanisms. The uptake of spermine by a control culture (Fig. 1) amounted to about 60 μmoles per gram wet weight of cells, in rough agreement

TABLE 1

Effect of chloramphenicol on the bactericidal action of spermine

At zero time, portions of a culture growing exponentially in medium $K_{0.3}$ were diluted 1:2 into tubes of fresh, warm $K_{0.3}$ plus chloramphenicol (1 μg/ml) and/or spermine (1 mg/ml) as indicated. Viability was determined as described in the text.

Time (min)	Viable number per ml, × 10^{-6}		
	Chloramphenicol	Spermine	Chloramphenicol + spermine
8	265	245	250
55	390	290	320
105	700	125	260

with earlier studies (7, 14, 15). This uptake was markedly depressed by glucose starvation, but was virtually unaffected by chloramphenicol treatment. Chloramphenicol must therefore protect via some intracellular action, presumably involving the protein-synthesizing system, whereas glucose starvation might protect by slowing protein synthesis and/or by preventing the uptake of an effective amount of spermine.

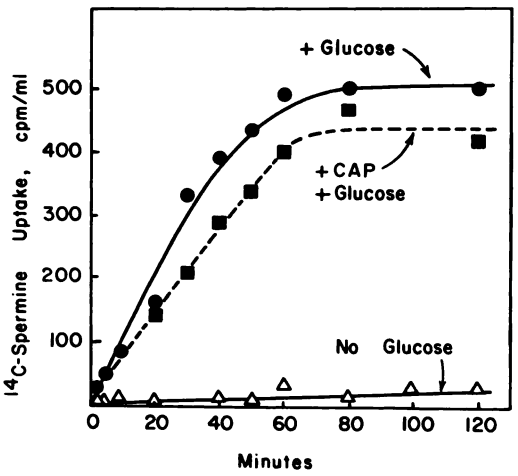

FIG. 1. *Effect of glucose starvation and chloramphenicol treatment on the uptake of ^{14}C-spermine*

Cells from a culture growing in medium $K_{0.3}$ were collected on a membrane filter, washed with glucose-free medium $K_{0.3}$, and resuspended in 30 ml of the same. Ten-milliliter aliquots were added to 5 ml of warm medium $K_{0.3}$ plus ^{14}C-spermine to make 1 mg/5.5 × 10^4 cpm/ml; glucose and chloramphenicol (CAP) (final concentrations of 0.2% and 200 μg/ml, respectively) were present as indicated.

The Effect of Spermine on Protein Synthesis

Spermine caused an early inhibition of protein synthesis as measured by the cumulative incorporation of ^{14}C-leucine, and the magnitude of this effect increased with increasing drug concentration (Fig. 2, top).

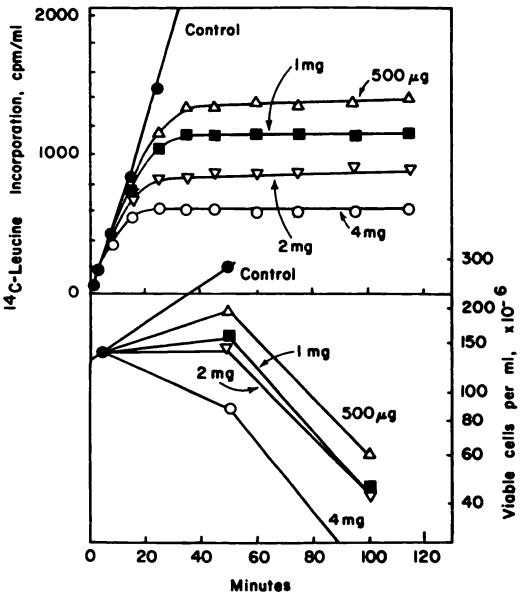

FIG. 2. *Effect of spermine on protein synthesis and cell viability*

At zero time, samples of a culture growing in medium $K_{0.3}$ were added to tubes containing fresh, warm medium with ^{14}C-leucine, 20 μg/2.4 × 10^4 cpm/ml, plus spermine to make the final concentrations (per ml) indicated. ^{14}C-Leucine incorporation and viable number were determined as described in the text.

In the experiment illustrated viable counts were also done, and these indicated that the slowing of protein synthesis preceded cell death. The lag between these two effects was demonstrated more clearly when rates of protein synthesis were measured by "pulsed" incorporation of ^{14}C-leucine (see Fig. 4, below). Similar kinetics were observed with uptake of ^{14}C-labeled phenylalanine, valine, isoleucine, and arginine.

The Effect of Spermine on Nucleic Acid Synthesis

Figure 3 represents an experiment showing the effect of spermine on cumulative ^{14}C-uracil incorporation into DNA and into total nucleic acids. There was an early stimulation of total nucleic acid synthesis without a change in DNA synthesis; later, total nucleic acid synthesis was inhibited. Spermine was also found to stimulate the incorporation of ^{32}P-phosphate, as well as ^{14}C-guanine, showing that the stimulation of uracil incorporation was not due to synthesis of uracil-rich RNA or to preferential use of exogenous uracil.

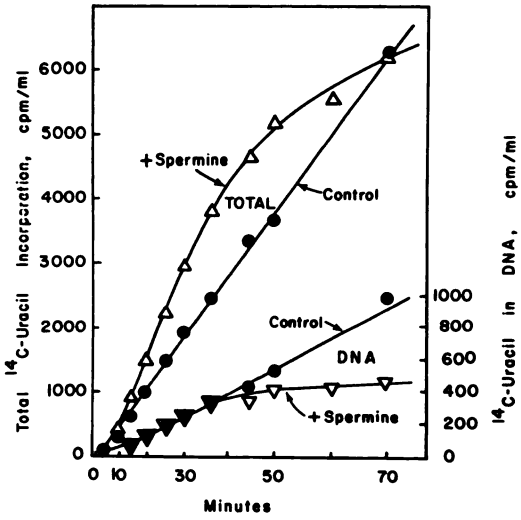

FIG. 3. *Effect of spermine on nucleic acid synthesis*

A culture growing exponentially in medium $K_{0.6}$ was divided into two portions, and each was diluted 1:2 into fresh, warm medium $K_{0.6}$ plus ^{14}C-uracil to make a final concentration of 5 μg/1.5×10^4 cpm/ml. One culture also contained spermine to make 1 mg/ml. Incorporation into DNA and total nucleic acids was determined as described in the text.

When the timing of spermine's effects on pulsed incorporation of ^{14}C-leucine and ^{14}C-uracil, and on viable number, was studied in a single experiment (Fig. 4), the results were in agreement with the separate experiments described above. Note in particular (a) the early onsets of inhibition of protein synthesis and stimulation of RNA synthesis; (b) cessation of all protein synthesis by 30 min, when there was still considerable RNA synthesis and viable number was just starting to fall; (c) continuing RNA synthesis through a 95% fall in viable number, in the virtual absence of protein synthesis. Spermine regularly induced a rate of RNA synthesis about double that of the control; here this occurred at about 20 min after exposure to spermine.

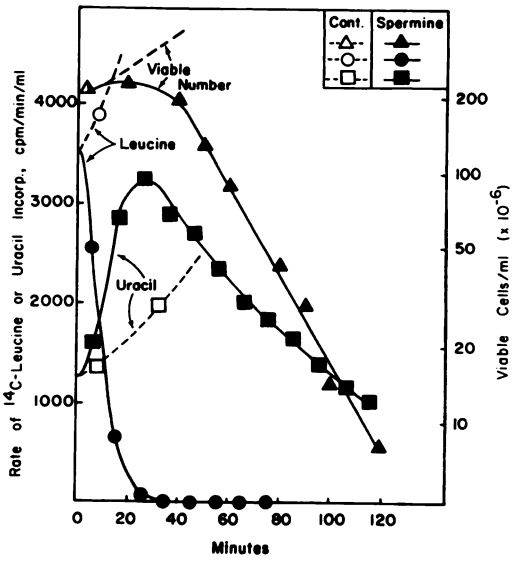

FIG. 4. *Sequence of effects of spermine on viable number, and rates of RNA and protein synthesis*

At zero time, 20 ml portions of growing culture were added to each of two tubes containing 10 ml of fresh, warm medium $K_{0.3}$; one tube contained in addition spermine to make 1 mg/ml. Rates of protein and RNA synthesis were measured by 1-min pulses on 1-ml samples of each culture, the final concentrations being: ^{14}C-leucine, 0.2 μg/1.2×10^4 cpm/ml; ^{14}C-uracil, 0.5 μg/6.0×10^4 cpm/ml.

To test the possibility that spermine might stimulate RNA synthesis *directly*, the effects of spermine and chloramphenicol on RNA synthesis were compared. It is thought that the only direct action of chloramphenicol is the inhibition of protein synthesis, and that the resulting accumulation of charged (aminoacyl) transfer RNA may account for the stimulation of RNA synthesis by chloramphenicol (16). If

spermine stimulates RNA synthesis *directly*, the effect should be detectable in a culture where protein synthesis is already maximally inhibited. This in fact proved to be the case.

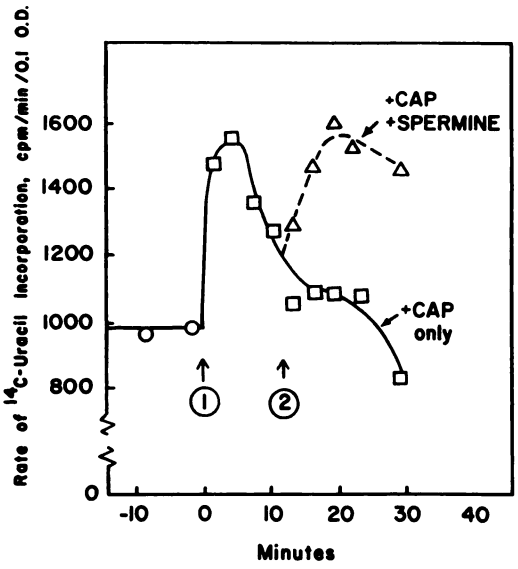

FIG. 5. *Stimulation of RNA synthesis by spermine in chloramphenicol-treated cells*

At zero time (arrow *1*), 20 ml of a growing culture were added to 6.6 ml of medium $K_{0.2}$ containing chloramphenicol (CAP), final concentration 500 μg/ml. After 12 min, equal portions of this chloramphenicol-treated culture were added to tubes containing 1.5 volumes of fresh medium $K_{0.2}$ (arrow *2*); one tube also contained spermine, 1 mg/ml. Rates of RNA synthesis were measured by means of ^{14}C-uracil pulses as in the experiment reported in Fig. 4. To correct for effects of growth and of dilution, these rates have been expressed in terms of incorporation per 0.1 O.D. unit.

In a representative experiment (see Fig. 5), an exponentially growing culture was first treated with a high level of chloramphenicol. Total inhibition of protein synthesis was presumably almost immediate (17), and a marked stimulation of RNA synthesis resulted. When the rate of RNA synthesis had begun to fall, the culture was subdivided and one portion was exposed to spermine. RNA synthesis was stimulated even in these cells, where no protein synthesis had occurred for over 10 min. In a variant of this experiment, cells were exposed *directly* to a high concentration of chloramphenicol and spermine, and the *initial* rate of RNA synthesis was found to be greater than when they were exposed to chloramphenicol alone. In analogous experiments, streptomycin failed to stimulate RNA synthesis in chloramphenicol-treated cells. This conforms with previous indications that the effects of streptomycin and chloramphenicol on RNA synthesis are similar (13).

Membrane Studies

Spermine resembles another polycationic antibiotic, streptomycin, in that it appears to stabilize bacterial membranes under certain conditions (14, 18–20). Since streptomycin can also *damage* the permeability barrier of growing bacteria (9, 21), it was of interest to determine whether spermine would likewise cause membrane damage under some circumstances.

Preliminary results indicated that relatively gross membrane damage—as manifested by cell lysis and increased permeability to nucleotides and a synthetic β-galactoside (see 9)—did occur in spermine-treated cells, but only after loss of viability had begun. Furthermore, these changes could not be demonstrated unless the cells were resuspended in drug-free medium, presumably because of the stabilizing effects of spermine on the cell envelope.

A more subtle derangement of membrane function was, however, demonstrated by the study of potassium metabolism in spermine-treated cultures. Figure 6 illustrates the effects of spermine on cellular potassium content and on unidirectional potassium efflux. One sees an initial transient dip in potassium content, followed by a rise roughly in parallel with turbidity, and later by a slow loss of cellular potassium. This late loss was variable, not occurring until 2 hr after exposure to spermine in some experiments. Cells removed from the spermine-treated culture after 1 min showed no increase in potassium efflux (Fig. 6, top), but cells removed after 16 min did, the half-time of efflux ($T_{1/2}$)

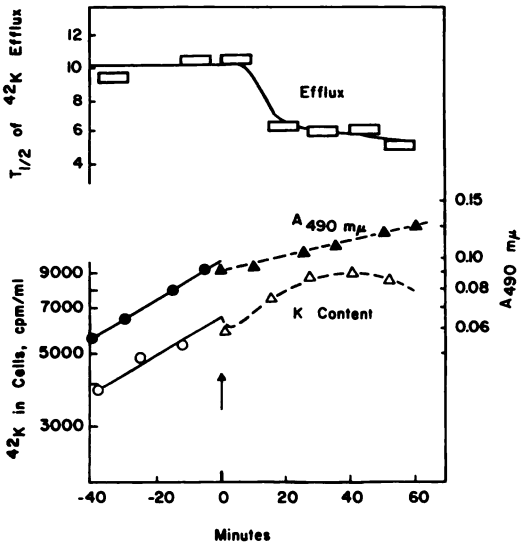

FIG. 6. *Effect of spermine on potassium content and efflux*

Before the experiment had begun, cells had grown for approximately 2.5 generations in medium $K_{0.37}$ containing ^{42}KCl, 0.1 μC/ml. At zero time, the culture was treated with spermine, 1 mg/ml. Efflux was determined by removing samples of culture at intervals and resuspending the cells in nonradioactive medium $K_{0.3}$ (lacking spermine); the $T_{1/2}$ was then estimated by the rate of loss of radioactivity from these subcultured cells [method A of Dubin *et al.* (9)]. Potassium content was measured on 0.5-ml samples.

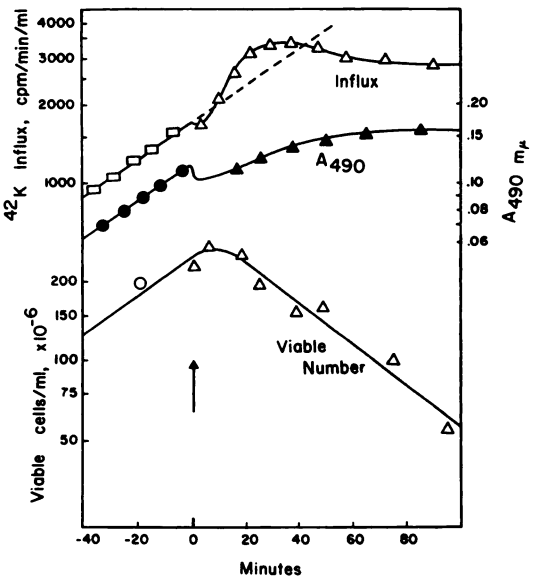

FIG. 7. *Effect of spermine on viability and potassium influx*

A culture growing in medium $K_{0.3}$ was treated at zero time with 1 mg/ml of spermine. Influx was determined by pipetting 2-ml samples of culture into 5 ml of warm medium $K_{0.3}$ containing added ^{42}KCl, to make a final concentration of 5.8×10^5 cpm/0.35 μmole/ml. The influx subcultures for the spermine-treated cells also contained spermine, 1 mg/ml. The dotted line represents the expected increase in influx for the control culture.

falling to 6 min from a control value of 10 min. This change persisted throughout the duration of the experiment. Unlike the grosser indices of membrane integrity mentioned above, the altered $T_{1/2}$ was not affected by the presence of spermine in the resuspension medium.

The development of an increased potassium efflux before a decline in potassium content suggested that the potassium influx was also increased. This was confirmed by direct measurement of influx (Fig. 7). There was regularly an initial depression of influx, which may be related to the initial fall in potassium content described above. Following this, influx accelerated to a rate greater than that of the control, and continued virtually unchanged throughout a 75% fall in viable number. The increased influx was seen whether or not spermine was present in the influx subcultures; the effect must therefore persist in the absence of spermine for at least the several minutes over which the influx measurements are made.

The sequential relationship between the effect of spermine on potassium fluxes and the effect on pulsed incorporation of ^{14}C-leucine was also studied. However, in three experiments, the onsets of the two effects could not be clearly separated in time, both starting approximately 10 min after exposure to spermine at 1 mg/ml.

Resistance

E. coli B^{spm-r} was derived from strain B as described; its growth was unaffected by the highest concentration of spermine tested, 10 mg/ml. On exposure to ^{14}C-spermine, 1 mg/ml, this strain took up only

about 20% as much radioactivity in 70 min as did the parent (sensitive) strain, although its mass increase was about twice that of the parent. Resistance in this case, as in an earlier study on *S. aureus* (7), therefore appears to be due to decreased permeability. B^{spm-r} was normally sensitive to streptomycin, both in the presence and in the absence of spermine.

DISCUSSION

The present results have demonstrated certain similarities between the action of spermine on *E. coli,* and that of another polycationic antibiotic, streptomycin. Both agents are bactericidal, and have early effects on protein and RNA synthesis, as well as on potassium fluxes.

The effect of streptomycin on protein synthesis has been carefully studied in cell-free systems, where it causes extensive misreading of the genetic code (23, 24). *In vitro* studies with spermine have also demonstrated such misreading (25), as well as an overall inhibition of protein synthesis (26–28). However, the relationships among misreading, inhibition of protein synthesis, and cell death have not been fully elucidated for either streptomycin or spermine.

It has been postulated that streptomycin exerts its lethal action on whole cells by causing misreading during protein synthesis, and that the resulting defective proteins interfere with certain vital functions (23). The parallel decline in rate of protein synthesis and viable number in streptomycin-treated cells tends to support this mechanism (9, 21). In the case of spermine, the prevention of killing with chloramphenicol, which also occurs with streptomycin (29), is in good accord with the "misreading hypothesis." On the other hand, the fact that spermine slows overall protein synthesis well *before* a decline in viable number suggests that, if spermine does kill by causing misreading, this misreading is not directly related to the overall inhibition of protein synthesis.

The early stimulation of RNA synthesis by spermine is probably a complex effect. Like other agents with presumed direct effects on the protein synthesizing system (e.g., chloramphenicol, streptomycin) spermine might be expected to cause a secondary, but early, stimulation of RNA synthesis (9, 16). The implication of the present work—that spermine may have an additional, direct effect on RNA synthesis —is supported by recent *in vitro* studies. These have shown that spermine, but not streptomycin, will directly stimulate the DNA-dependent RNA polymerase (30–32).

The present results are also in general agreement with the earlier findings that (a) RNA accumulates in spermine-inhibited *E. coli* (8), and (b) a naturally occurring amine, as yet unidentified, stimulates RNA synthesis in *B. subtilis* (33).

The increase in both influx and efflux of potassium in spermine-treated cells is an unusual, but not unique, effect. A balanced increase in potassium fluxes has been observed in encephalomyocarditis virus-infected L cells (34), as well as in *E. coli* after apparent derepression of the potassium carrier by potassium depletion (22).

Perhaps the most apparent explanation for our potassium flux results is that spermine, as has been suggested for streptomycin (9), can cause a subtle type of membrane damage which allows an increased outward diffusion of potassium. Unlike streptomycin-treated cells, such spermine-damaged cells might retain sufficient potassium-concentrating ability to compensate for this increased potassium leakage. However, recent studies have suggested that the bulk of the normal potassium efflux in *E. coli* is carrier mediated, and is tightly coupled to influx (22). It is possible that the early effect of spermine on potassium fluxes represents a specific effect on the potassium carrier, rather than membrane damage in the usual sense.

In conclusion, this study has shown that the effects of spermine on macromolecule synthesis and membrane function are in some respects similar to those of another polycationic antibiotic, streptomycin. The two drugs differ in that (a) spermine-treated cells remain viable for a considerably longer period after the early metabolic changes than do streptomycin-treated cells;

(b) spermine may have a direct stimulatory effect on RNA synthesis not shared by streptomycin; and (c) the increased potassium efflux in spermine-treated cells is balanced for some time by an increased potassium influx. A complete understanding of the possible interrelationships among polycationic antibiotics, and among their various metabolic effects and their lethal action, must await further study.

ACKNOWLEDGMENTS

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