STABILIZATION OF PROTOPLASTS AND SPHEROPLASTS BY SPERMINE AND OTHER POLYAMINES

CELIA W. TABOR

National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service, Bethesda, Maryland

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ABSTRACT

TABOR, CELIA W. (National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.). Stabilization of protoplasts and spheroplasts by spermine and other polyamines. J. Bacteriol. **83**:1101–1111. 1962.—Spermine (10^{-3} M) or spermidine prevents lysis of lysozyme-produced protoplasts of *Escherichia coli* W, *E. coli* B, and *Micrococcus lysodeikticus* in hypotonic media. Spheroplasts prepared by the action of penicillin are also stabilized by these concentrations of spermine and spermidine, but the protection is not as complete. Streptomycin, polylysine, and Ca^{++} are also effective or partially effective stabilizers, but 1,4-diaminobutane, 1,5-diaminopentane, ornithine, Mg^{++}, and monovalent cations have no protective action at 10^{-3} M concentration, and only a slight effect at higher concentrations. The osmotic stability conferred on protoplasts by spermine is irreversible. However, the protective effect of polyamines against lysis is not accompanied by restoration of viability to lysozyme protoplasts. There is a marked reduction in the loss of ultraviolet-absorbing material from the protoplasts to the medium when 10^{-3} M spermine is present.

The naturally occurring polyamine, spermine, has been found to protect certain microorganisms from death due to exposure to hypotonic media. Mager (1955, 1959*a*) demonstrated that, when *Neisseria perflava* or *Pasteurella tularensis* is washed with water or when *Achromobacter fischeri* is suspended in dilute sodium chloride, the organisms lose their viability. However, very low concentrations of spermine added to the suspending medium completely protected these organisms. These findings suggested that spermine might serve as a stabilizing factor in other osmotically unstable systems such as the protoplasts and spheroplasts of *Escherichia coli*. Studies previously reported, both by Mager (1959*b*) and in a preliminary communication from our laboratory (Tabor, C. W., 1960*a*), have indeed demonstrated that low concentrations (10^{-3} M) of spermine added to water can stabilize these structures which would otherwise lyse in solutions of comparable tonicity.

In this paper, I shall present more detailed data on these experiments, using both protoplasts prepared by the lysozyme-ethylenediaminetetraacetate (EDTA) technique and spheroplasts prepared by the penicillin technique. I am also reporting studies on some of the characteristics of the stabilized protoplasts, particularly studies on their viability. In this paper, for convenience, the term "spheroplast" has been used to refer to the osmotically fragile forms produced by the action of penicillin on bacteria, and the term "protoplast" for the osmotically-fragile forms produced by lysozyme, even though there is still insufficient evidence for *E. coli* that the lysozyme-treated bacteria completely conform to the definition of protoplasts published by Brenner et al. (1958).

MATERIALS AND METHODS

C^{14}-spermine tetrahydrochloride was prepared by E. L. Jackson and S. M. Rosenthal (1960). 1,4-Diaminobutane dihydrochloride, 1,5-diaminopentane dihydrochloride, spermidine phosphate, and spermine tetrahydrochloride were commercial preparations. Spermidine trihydrochloride was prepared from the commercial spermidine phosphate. Spermidine phosphate (2 g) was passed through a Dowex 1 (OH^-) column (1.3 by 20 cm). The effluent was evaporated to dryness after acidification with excess hydrochloric acid, and the residual spermidine hydrochloride was recrystallized from ethanol-water.

Sucrose (1 liter, 1 M) was passed through a Dowex (50 (H^+) column (2.5 by 20 cm) to obtain

metal-free sucrose for use in the preparation of protoplasts.

All other chemicals were commercial preparations.

Micrococcus lysodeikticus (ATCC 4698) and *E. coli* B were obtained from the American Type Culture Collection. *E. coli* W was obtained from B. Davis.

Preparation of protoplasts by lysozyme. Protoplasts of *E. coli* were prepared by the technique described by Repaske (1958). *E. coli* was grown overnight in horse infusion broth at 37 C with shaking unless otherwise specified. Portions (8 ml) were harvested by centrifugation at room temperature; the cells were suspended in distilled water, and then recentrifuged. The pellet was then suspended in 4 ml of cold 0.5 M sucrose-0.03 M tris(hydroxymethyl)aminomethane (tris) chloride buffer (pH 8.0). All subsequent operations were carried out at 0 C. To this cell suspension, 4 μmoles of sodium EDTA (pH 8.0) and 15 to 25 μg of crystalline egg white lysozyme were added. To follow the action of the lysozyme, a sample of the suspension was centrifuged and the pellet was suspended in water. If protoplast formation was complete, the pellet was converted to a sticky viscid white material which was solubilized on stirring. If the pellet was suspended in 0.5 M sucrose, however, lysis did not occur.

All experiments were performed as soon as protoplast formation was complete (10 to 30 min after addition of lysozyme), since, on more prolonged exposure to lysozyme, even at zero degrees, the protoplasts underwent further changes; they appeared larger when examined by phase microscopy after several hours, and lost ultraviolet-absorbing material to the medium. It is important to note, as demonstrated by Repaske (1958), that the EDTA must be added at the same time as the lysozyme, that excess lysozyme or divalent ions can inhibit lysis, and that the cells must be freshly harvested after good aeration. Dowex 50-treated sucrose was used in the medium during the preparation of protoplasts.

To prepare protoplasts of *M. lysodeikticus*, cells were grown in Beers' (1955) liquid medium at 37 C with good aeration; 32 ml of culture were centrifuged. The cells were resuspended in 32 ml of water and collected again. The pellet was resuspended in 16 ml of 0.5 M sucrose-0.03 M tris Cl (pH 8), and 150 μg of lysozyme were added. The suspension was incubated at 25 C for 10 min.

Preparation of spheroplasts with penicillin. Spheroplasts of *E. coli* W were prepared according to a modification of Lederberg's (1956) method. Horse infusion broth cultures were grown overnight with shaking at 37 C. Then, 4 ml of the fully-grown culture were added to 12 ml of synthetic medium (Vogel and Bonner, 1956) containing 0.5 M sucrose, 0.2% glucose, and 18 mg of penicillin; the cells were then incubated without shaking at 37 C for 165 min. The formation of spheroplasts could be demonstrated by lysis of the cells on suspension in water, as well as by their appearance in the phase microscope. If the spheroplasts were allowed to incubate longer, gradual lysis occurred even in sucrose; therefore, all studies were performed 165 to 180 min after the exposure to penicillin. Samples (1 ml) of the spheroplast suspension were centrifuged, and the pellets were suspended in 1 ml of the solutions to be tested. Optical densities were read at 650 mμ in a model DU Beckman spectrophotometer.

Determination of viability. The viability of the protoplasts, i.e., the number undergoing conversion to cells upon plating, was determined by dilution with horse infusion medium containing 0.5 M sucrose, and plating samples of a suitable dilution on horse infusion agar plates. Colonies were counted at 24 hr. The best yield of viable protoplasts was observed when the sucrose in the diluting medium came from lots containing low concentrations of metal. The horse infusion-sucrose medium used for dilution gave a significantly better yield of viable protoplasts than 0.5 M sucrose alone, or the inorganic salts medium (Vogel and Bonner, 1956); no improvement in viability was observed when 0.01 M $MgCl_2$-0.5 M sucrose or when 0.001 M spermine-0.5 M sucrose was used. The final plate contained horse infusion agar without added sucrose, since the addition of sucrose at this stage markedly slowed the growth rate and did not increase the yield of colonies.

Assay conditions. All protoplast or spheroplast suspensions were maintained at 0 C, except during centrifugations. Cells were subjected to the same procedures as protoplasts or spheroplasts when comparable data were obtained with cells.

RESULTS

Effect of adding polyamines to the suspending medium. The first studies were concerned with the protection of protoplasts against lysis in hypotonic media conferred by the addition of polyamines to the suspending medium. Protoplasts were prepared by treating *E. coli* or *M. lysodeikticus* with lysozyme as described.

When *E. coli* cells were suspended in water, the optical density observed at 650 mμ was the same as the optical density observed when cells were suspended in sucrose (Table 1). When protoplasts of *E. coli* prepared by the lysozyme procedure were collected and resuspended in 0.5 M sucrose, the optical density was the same as when an equal number of whole cells was suspended in sucrose. However, when these protoplasts were suspended in water at the same concentration, rapid and complete lysis occurred. Observation with the phase microscope showed only transparent material, presumably due to cell membranes or walls. This lysis was associated with a marked fall in the optical density at 650 mμ (Table 1). If lysozyme protoplasts were suspended in a 10^{-3} M solution of spermine hydrochloride instead of water, there was essentially no change in the microscopic appearance, and little change in the optical density at 650 mμ. Spermine (10^{-4} M) gave only slight protection, while lower concentrations were ineffective; high spermine concentrations (10^{-2} M) produced aggregation of the protoplasts. Spermidine trihydrochloride (10^{-3} M) and 10^{-3} M $CaCl_2$ were also effective stabilizers. 1,4-Diaminobutane hydrochloride, 1,5-diaminopentane hydrochloride, and $MgCl_2$ (10^{-3} M) all showed only a slight effect. In this experiment, streptomycin sulfate (10^{-3} M) and quinacrine hydrochloride (10^{-3} M), and in other experiments triethylenetetramine hydrochloride (10^{-3} M), arcaine (10^{-3} M), poly-L-lysine (182 μg/ml), and protamine sulfate (200 μg/ml) were also effective stabilizers. However, these also produced aggregation of the protoplasts; dispersion was incomplete, thus rendering the optical density measurement relatively inaccurate. Thus, the polyamines, the divalent cation Ca^{++}, and several strongly basic compounds were effective in preventing lysis. Diamines and Mg^{++} were slightly effective. Monovalent cations and basic amino acids, however (K^+, Na^+, NH_4^+, lysine, and ornithine), were essentially without any stabilizing effect at 10^{-2} M or even at 10^{-1} M concentrations; higher concentrations were not tested.

The stabilization of *M. lysodeikticus* protoplasts by polyamines and other bases was also

TABLE 1. *Protoplast stability in various solutions**

Suspending medium	Optical density (650 mμ)	
	E. coli W	*M. lysodeiklicus*
Cells		
0.5 M Sucrose	0.92	
Water	0.89	0.34
Protoplasts		
0.5 M Sucrose	0.97	0.06
Water	0.12	0.02
0.001 M Spermine HCl	0.63	0.26
0.001 M Spermidine HCl	0.45	0.04
0.001 M 1,4-Diaminobutane HCl	0.19	0.03
0.001 M 1,5-Diaminopentane HCl	0.14	0.02
0.001 M Triethylenetetramine HCl	0.20	
0.001 M Agmatine SO_4	0.18	
0.001 M $MgCl_2$	0.26	
0.001 M $CaCl_2$†	0.59	0.03
0.01 M KCl	0.16	
0.01 M NaCl	0.16	
0.01 M NH_4Cl	0.17	
0.01 M L-Ornithine HCl	0.19	
0.01 M L-Lysine HCl	0.19	0.03
0.001 M Quinacrine HCl	0.51	
0.001 M Streptomycin SO_4	0.62	0.11
0.001 M Polylysine HCl		0.35

* *E. coli* cells grown overnight in horse infusion broth at 37 C were converted to protoplasts by the lysozyme-EDTA procedure. Samples (1 ml) of the cells or of the protoplasts were centrifuged, and the pellets were resuspended in 2 ml of the medium indicated. A fall in optical density at 650 mμ indicates lysis. *M. lysodeikticus* cells were grown overnight in Beers' (1955) medium at 37 C with shaking. Protoplasts were prepared as described in the text; 1-ml samples were centrifuged, and the pellets were resuspended in 2 ml of spermine or other suspending medium. Polylysine was 10^{-3} M with respect to the lysine moiety.

† An effect of Ca^{++} on protoplast stability was demonstrated by Repaske (1958). He showed that Dowex 50-treated *E. coli* cells could be converted by lysozyme to protoplasts without the EDTA which is needed with untreated cells. Under these circumstances the formation of protoplasts was inhibited by the addition of Ca^{++} or other divalent ions.

studied. Samples (1 ml) of a *M. lysodeikticus* protoplast suspension were centrifuged, and the pellets were suspended in 2 ml of the solutions indicated in Table 1. Protoplasts of *M. lysodeikticus* were protected most completely by polylysine; however, agglutination occurred at this concentration. Spermine also gave good protection against lysis, but spermidine, 1,4-diaminobutane, 1,5-diaminopentane, and calcium were without any observable effect (Table 1).

The effect of low concentrations of the polyamines and other bases on the osmotic fragility of spheroplasts produced by the penicillin procedure was investigated. Optical densities at 650 mμ are given in Table 2. These spheroplasts lysed if suspended in water, as indicated by the marked fall in optical density at 650 mμ. If they were resuspended in 0.5 M sucrose, however, essentially no fall in optical density occurred. Spermine hydrochloride and streptomycin sulfate gave the greatest protection, but streptomycin also resulted in clumping. Even though a marked fall in optical density occurred with spermidine or calcium ions, indicating that the protection was much less than that due to spermine, nevertheless a minor degree of protection was consistently found in a number of experiments.

TABLE 2. *Spheroplast stability in various solutions**

Suspending medium	Optical density (650 mμ)
	E. coli B
Water	0.07
Spermine HCl	0.21
Spermidine HCl	0.12
1,4-Diaminobutane HCl	0.09
1,5-Diaminopentane HCl	0.08
Streptomycin SO_4	0.19
$CaCl_2$	0.12

* Spheroplasts were prepared by adding 4 ml of an overnight horse infusion broth culture of *E. coli* B to 12 ml of synthetic medium (Vogel and Bonner, 1956), containing 0.5 M sucrose. To this, 0.16 ml of 0.1 M glucose and 18 mg of penicillin were added, and the cultures were incubated at 37 C for 165 min. In the experiment listed, 1-ml samples were centrifuged immediately and the pellets were resuspended without delay in 1 ml of 10^{-3} M solutions of spermine or other bases to be tested. The optical densities of the suspensions at 650 mμ were read promptly. The optical density of the original spheroplast suspension was 0.21.

In summary, I have shown that, in general, the most effective protection against lysis of protoplasts or spheroplasts in hypotonic medium is obtained with the basic compounds, spermine, spermidine, poly-L-lysine, quinacrine, and streptomycin, and by the divalent cation Ca^{++}. Diamines, Mg^{++}, monovalent cations, and dibasic amino acids had little or no protective effect.

Effect of preliminary exposure to low concentrations of polyamines on subsequent lysis in water. The above experiments demonstrated that protoplasts and spheroplasts of *E. coli* do not lyse in suspending media containing low concentrations of polyamines. Once the protoplasts have been exposed to these low concentrations of polyamines, they do not lyse even if subsequently suspended in water (Table 3). The protective effects of preliminary exposure to these compounds against subsequent lysis in water were in general similar to the results obtained in Table 1. When a sample of the same protoplast preparation was suspended in water without previous exposure to spermine, the optical density at 650 mμ fell to 0.01.

In a comparable experiment with *E. coli* B, the effects of the polyamines were similar to those observed with *E. coli* W (Table 3).

Similar but less complete protection was found when penicillin spheroplasts of *E. coli* W had been exposed to low concentrations of spermine before exposure to water. Optical densities at 650 mμ are given in Table 4. The highest optical density was observed with polylysine hydrochloride, but this was complicated by gross clumping of the spheroplasts which could not be dispersed. Spermine tetrahydrochloride was a moderately effective stabilizer; spermidine trihydrochloride and streptomycin sulfate were somewhat less effective. 1,4-Diaminobutane dihydrochloride, 1,5-diaminopentane dihydrochloride, calcium chloride, magnesium chloride, ammonium chloride, sodium chloride, and L-lysine hydrochloride were without significant effect at the concentrations tested.

In other experiments, I found that once the protoplasts had been exposed to spermine, even repeated centrifugation and resuspension in water resulted in no change in the corrected optical density at 650 mμ (Table 5).

Effect of preliminary exposure of bacteria to polyamines before the addition of lysozyme or penicillin. In the above series of experiments,

TABLE 3. *Stability of protoplasts in water after preliminary exposure to spermine and other bases**

Compounds tested for stabilizing activity	Optical density in water (650 mμ)	
	E. coli W	*E. coli* B
Cells	0.20	0.25
Protoplasts		
None	0.01	0.04
Spermine HCl	0.13	0.14
Spermidine HCl	0.11	0.14
1,4-Diaminobutane HCl	0.07	0.10
1,5-Diaminopentane HCl	0.04	0.06
$CaCl_2$	0.12	0.11
$MgCl_2$	0.00	
NH_4Cl	0.01	
NaCl	0.00	
Lysine HCl	0.02	
Streptomycin SO_4†	0.12	
Poly-L-lysine HCl†‡	0.21	

* Protoplasts of *E. coli* W or *E. coli* B were prepared in sucrose-tris buffer with lysozyme and EDTA; 10 min after the addition of lysozyme, 1-ml samples were made 10^{-3} M with respect to spermine or other base by addition of 0.01 ml of a 10^{-1} M solution. The samples were then centrifuged, and the pellets were resuspended in 2 ml of water. Optical densities were read at 650 mμ immediately after the suspensions were prepared. The cultures used for these experiments were grown in the synthetic medium (Vogel and Bonner, 1956).

† Clumping of the particles was grossly visible. These were broken up as well as possible by vigorous shaking before reading.

‡ In other experiments, the addition of 0.01 ml of either protamine sulfate (20 mg/ml) or triethylenetetramine hydrochloride (10^{-1} M) to 1-ml samples of *E. coli* W protoplasts also protected the protoplasts against subsequent lysis in water. Clumping of the protoplasts occurred with these substances. Poly-L-lysine solutions were 10^{-3} M with respect to the lysine moiety.

polyamines were added only after the lysozyme or penicillin had acted on the cells. Results now to be described suggest that prior treatment of cells with polyamines prevents the formation of osmotically-fragile protoplasts by added lysozyme (Table 6). The prior treatment of cells with spermine or spermidine prevented the development of the osmotic fragility observed in the preceding experiments. With preliminary exposure of the cells to 1,4-diaminobutane, however, there was only a partial protection. When examined with the phase microscope, cells treated first with spermine and then with lysozyme-EDTA retained their normal shape, size, and motility. However, viability studies (see below) indicated that there had been some alteration in these cells.

In a comparable group of experiments, in which spermine was added to *E. coli* before the formation of penicillin spheroplasts, spermine had only a moderately protective effect against osmotic lysis (Table 7).

It is of interest that Brown (1960) found that spermine inhibits the lysis of cell walls isolated from a halophilic bacterium by an autolytic enzyme present in the walls.

Effect of spermine on the loss of ultraviolet-absorbing material to the medium from lysozyme protoplasts. A loss of ultraviolet-absorbing materials to the medium occurred over an 18-hr period at 2 C, even when the protoplasts were collected by centrifugation and suspended in 0.5 M sucrose (Table 8). The loss of ultraviolet-absorbing material was not accompanied by comparable lysis of the cells; i.e., the suspension remained opaque, and in the phase microscope

TABLE 4. *Stability of spheroplasts in water after preliminary exposure to spermine and other bases**

Compounds tested for stabilizing activity	Optical density in water (650 mμ)
None	0.05
Spermine HCl	0.12
Spermidine HCl	0.07
1,4-Diaminobutane HCl	0.03
1,5-Diaminopentane HCl	0.04
Streptomycin SO_4	0.07
$CaCl_2$	0.04
$MgCl_2$	0.02
Poly-L-lysine HCl	0.28
Lysine HCl	0.04
NH_4Cl	0.04
NaCl	0.04

* Samples (1 ml) of penicillin spheroplasts in sucrose were adjusted to 10^{-3} M concentration of the substances to be tested. The spheroplasts were then collected by centrifugation, and were resuspended in 1 ml of water. Optical densities were read at 650 mμ immediately after the suspensions were prepared. The optical density of the spheroplast suspension at the end of the incubation with penicillin was 0.26.

TABLE 5. *Effect of repeated suspensions in water on the optical density (OD) of spermine-treated protoplasts**

Suspending medium	Vol	OD of suspension (650 mμ)	OD of suspension (corrected for dilution)
	ml		
A Sucrose-tris + spermine (10^{-3} M)	2	1.10	1.10
First H_2O suspension	4	0.54	1.08
Second H_2O suspension	4	0.54	1.08
Third H_2O suspension	4	0.49	0.98
B Sucrose-tris	2	1.10	1.10
First H_2O suspension	4	0.01	0.02

* Protoplasts of *E. coli* W were prepared in sucrose-tris. In group A, a sample was made 10^{-3} M in spermine, and then resuspended in water three times. In group B, a sample of the protoplast suspension was centrifuged without exposure to spermine, and taken up in water.

TABLE 6. *Effect of prior treatment with polyamines on the production by lysozyme of osmotic fragility of Escherichia coli W**

Polyamine present during lysozyme treatment	OD (650 mμ) in sucrose	OD (650 mμ) in water
None	1.02	0.11
Spermine hydrochloride	—	0.82
Spermidine hydrochloride	—	0.78
1,4-Diaminobutane hydrochloride	—	0.43

* *E. coli* W cell suspensions in sucrose-tris were made 10^{-3} M in spermine, spermidine, or 1,4-diaminobutane. Then lysozyme and EDTA were added; 30 min after the lysozyme addition, a 1-ml sample was centrifuged, and the pellet was resuspended in 2 ml of water. The optical density was read at 650 mμ.

the protoplasts appeared unchanged except for an increase in the degree of swelling. If the protoplasts were stored for 18 hr at 2 C in 10^{-3} M spermine, with or without 0.5 M sucrose, much less ultraviolet-absorbing material appeared in the medium.

Binding of polyamines by cells and protoplasts. Experiments were performed with C^{14}-spermine and C^{14}-1,4-diaminobutane to compare the uptake of polyamines by *E. coli* W cells and

TABLE 7. *Effect of prior treatment with spermine on the formation of osmotically-fragile spheroplasts from Escherichia coli W by penicillin**

Polyamine present during penicillin treatment	OD (650 mμ) 165 min after penicillin	
	Spheroplast suspension	Spheroplasts centrifuged and suspended in water
None	0.19	0.08
Spermine HCl	0.28	0.14

* Cells were diluted with fresh medium as described under Methods. To one tube, penicillin alone was added. A second tube was made 2×10^{-3} M in spermine just before the penicillin was added. A control culture without penicillin was run simultaneously. These were allowed to incubate for 165 min at 37 C. At this time the optical density (650 mμ) of the control culture in the absence of penicillin was 0.26.

TABLE 8. *Loss of ultraviolet-absorbing material from protoplasts during storage at 2 C for 18 hr**

Suspending medium	Absorption of supernatant solution (260 mμ)	Visible lysis
Cells		
0.5 M Sucrose-0.03 M tris (pH 8)	0.33	None
Protoplasts		
Water†	1.50	Complete
Water-0.001 M spermine‡	0.13	None
0.5 M Sucrose-0.03 M tris (pH 8)	1.68	None
0.5 M Sucrose-0.03 M tris (pH 8)-0.001 M spermine	0.46	None

* Cells or protoplasts of *E. coli* W were stored in the suspending medium at 2 C for 18 hr. The suspension was centrifuged at 0 C and the 260-mμ absorption of the supernatant fluid was determined. For this experiment, the cells were grown in a synthetic medium plus glucose (Vogel and Bonner, 1956). In other experiments, the addition of 10^{-3} M spermine to cells suspended in sucrose did not diminish the amount of 260-mμ material lost from cells to the suspending medium.

† This represents the amount of 260-mμ absorbing material released on complete lysis of approximately 1 mg of protoplasts.

‡ Similar results were obtained if 5×10^{-4} M spermine or 10^{-3} M spermidine was present instead of 10^{-3} M spermine.

TABLE 9. *Binding of C^{14}-spermine and C^{14}-1,4-diaminobutane by Escherichia coli cells and protoplasts**

E. coli	Suspending medium	Total C^{14} added	Total C^{14} in *E. coli*	Total C^{14} in supernatant solution
		count/min	*count/min*	*count/min*
Cells	0.001 M C^{14}-spermine HCl†	2,600	1,600	1,000
	H_2O wash		1,560	40
	H_2O wash		1,560	0
Protoplasts	0.001 M C^{14}-spermine HCl†	2,600	1,300	1,300
	H_2O wash		1,280	20
	H_2O wash		1,270	30
Cells	0.001 M C^{14}-1,4-diaminobutane HCl**	680	280	400
	Sucrose wash‡			40
	Sucrose wash			40
Protoplasts	0.001 M C^{14}-1,4-diaminobutane HCl**	680	280	400
	Sucrose wash‡			30
	Sucrose wash			0

* Samples (0.5 ml) of cells or protoplasts in 0.5 M sucrose-0.03 M tris (pH 8) were centrifuged, and the pellets were resuspended in 1 ml of 0.001 M spermine hydrochloride containing 2,600 counts per min per ml of C^{14}-spermine hydrochloride, or in 1 ml of 0.001 M 1,4-diaminobutane hydrochloride containing 680 counts per min per ml of C^{14}-1,4-diaminobutane hydrochloride. After centrifugation, the supernatant solution was separated from the pellet and a sample was plated, dried, and counted. The count/min in the pellet were calculated by difference. The spermine-treated pellet was suspended in 1 ml of water and recentrifuged; the pellet was again suspended in fresh water, and recentrifuged. Samples of each supernatant solution were plated, dried, and counted. No gross evidence of lysis occurred during this experiment. The 1,4-diaminobutane-treated pellet was resuspended twice in 0.5 M sucrose-0.03 M tris HCl buffer (pH 8.0), centrifuged, and the count/min of each supernatant solution were determined.

† No sucrose was present.

‡ Sucrose (0.5 M) was used in this experiment, since in other experiments we have observed a lower optical density when protoplasts pretreated with 1,4-diaminobutane were suspended in water than when the same protoplasts were suspended in sucrose.

** The C^{14}-1,4-diamobutane hydrochloride was dissolved in 0.5 M sucrose–0.03 M tris chloride (pH 8).

protoplasts. When cells were suspended in 0.001 M C^{14}-spermine, the uptake equaled approximately 50 μmoles per g, wet wt (Table 9). This was not due to exchange, since by methods previously described (Tabor, Rosenthal, and Tabor, 1958) I found that the spermine concentration of *E. coli* cells which had been grown in horse infusion broth was less than 0.4 μmole per g (wet wt).

When protoplasts which had been prepared with lysozyme from the same cells were suspended in 0.001 M C^{14}-spermine, a comparable amount of spermine was taken up by the protoplasts. This spermine was tightly bound to both the protoplasts and cells, since on repeated suspension in water, only a small percentage of the counts appeared in the water. However, the bound spermine in the protoplasts could exchange with spermine in the medium; when protoplasts were suspended in 0.1 M spermine, the counts appeared in the medium.

When cells were suspended in 0.001 M C^{14}-1,4-diaminobutane in 0.5 M sucrose, a smaller percentage of the C^{14}-1,4-diaminobutane was taken up from the medium. On repeated suspension in 0.5 M sucrose, most of the C^{14} was retained by the cells (Table 9). This represents about 25 μmoles per g (wet wt). A comparable culture of *E. coli* W grown in horse infusion broth contained less than 10 μmoles of 1,4-diaminobutane per g (wet wt); therefore, exchange could not account for all of the uptake of counts. Similar results were obtained when lysozyme protoplasts were suspended in C^{14}-1,4-diaminobutane. The uptake of spermine by whole cells has been described for *Staphylococcus aureus* (Razin and Rozansky, 1959; Rosenthal and Dubin, 1960) and for *E. coli* (Dubin and Rosenthal, 1960). The uptake of spermidine has been described for protoplasts of *Bacillus subtilis* (Bachrach and Cohen, 1961). My results are in agreement with the reported observations.

From these results, it is apparent that amines are taken up and bound tightly by some part of the *E. coli* not destroyed by lysozyme; the bound spermine is in an exchangeable form.

TABLE 10. *Effect of spermine on the viability of Escherichia coli W protoplasts**

Addition	Viable count/ml at hours after lysozyme			
	0	0.5	4.5	24.0
No addition	2×10^9	2×10^7	1×10^7	2×10^4
Spermine added‡ (5×10^{-4} M)		2×10^7†	2×10^7	1×10^7

* Protoplasts were prepared in 0.5 M sucrose—0.03 M tris HCl by the routine lysozyme procedure. A sample of the suspension was made 5×10^{-4} M in spermine 0.5 hr after the lysozyme addition. Viable cell counts were made by dilution of samples at 0 C in horse infusion broth—0.5 M sucrose, and plating a sample on horse infusion agar plates. Assays were made before the addition of lysozyme (zero time) and at various times after the addition of lysozyme. Colonies were counted after 24 hr incubation of the plates at 25 C.

† Essentially the same results were obtained in other experiments with 10^{-3} M spermine.

‡ Added 0.5 hr after addition of lysozyme.

If spheroplasts suspended in sucrose are treated with C^{14}-spermine, about half of the C^{14} is absorbed by the spheroplasts; if they are then resuspended in sucrose, the C^{14}-spermine is gradually lost to the medium. Although this could mean that the binding is less firm than with protoplasts, it seems more likely that the loss is due to the gradual lysis of the spheroplasts referred to above.

Effect of spermine on the viability of protoplasts and spheroplasts. Previous workers have shown that when bacteria are converted to protoplasts, the cells lose their viability, i.e., their ability to reproduce (Weibull, 1958). I have found a 95 to 99% loss of viability 30 min after the cells were treated with lysozyme. In the experiment reported in Table 10, for example, the recovery of viable cells from *E. coli* protoplasts was 1% after 30 min of incubation with lysozyme. Concentrations of polyamines which protected lysozyme protoplasts of *E. coli* against osmotic lysis did not restore viability of the protoplasts. Efforts to increase the yield of viable cells by allowing protoplasts to incubate at 0 C or at 37 C in horse infusion broth-0.5 M sucrose were not successful; the addition of 10^{-3} M spermine or 10^{-3} M spermidine to the diluting medium did not increase the yield of viable cells.

Although the survival at 0.5 hr may be due to cells unaffected by lysozyme, the following data suggest that it is due to viable protoplasts. In a comparable experiment, a sample of a protoplast suspension was centrifuged, taken up in twice the volume of water, and then diluted in horse infusion-sucrose medium for plating; the viable count was less than 10^4 per ml. One sample was diluted directly in horse infusion-sucrose medium; the viable count was 1×10^7 per ml. The poor recovery of viable cells from protoplasts even under conditions where there was no lysis may be explained by leakage of essential materials from the protoplasts to the surrounding medium, by a further action of lysozyme on protoplasts, or by failure of the protoplasts to synthesize certain critical compounds. Attempts to restore viability by correcting possible metabolic deficiencies in the protoplasts, i.e., by addition of α,ϵ-diaminopimelic acid, N-acetylglucosamine, or adenosine triphosphate to the diluting medium (in 5×10^{-3} M to 5×10^{-4} M concentration), did not improve the yield of viable protoplasts.

If protoplasts were allowed to stand at 0 C in the lysozyme-EDTA medium, a continued fall in viability occurred (Table 10). When, however, spermine was added to a sample of protoplasts in the lysozyme-EDTA medium (final spermine concentration, 5×10^{-4} M), this fall was inhibited. Thus, after 24 hr at 0 C the viable cell count of the untreated protoplast suspension fell to $\frac{1}{1,000}$ the count observed at 30 min after lysozyme, while that of the spermine-treated suspension fell only to $\frac{1}{2}$ of the 30-min count. Spermidine (10^{-3} M) was also effective in preventing the progressive fall in viability with time.

By the addition of polyamines to a cell suspension just before the addition of lysozyme, the fall in viability which occurred after lysozyme treatment in the absence of spermine was markedly decreased. These experiments were carried out in 0.03 M tris HCl buffer (pH 8) without sucrose, to intensify lysis of unstable forms. With 10^{-3} M spermine, spermidine, or 1,4-diaminobutane in the medium during lysozyme action, 16 to 26% of the initial viability was observed at 30 min after exposure to lysozyme (Table 11). These are to be compared with the 5% of the initial viability obtained with protoplasts formed in the absence of any of the amines in this experiment. The fall in optical

TABLE 11. *Optical density and viability of Escherichia coli cells after the simultaneous addition of spermine and lysozyme**

Amine added	OD (650 mμ) at 0.5 hr	Per cent of original OD	Viable count/ml at 0.5 hr	Per cent of original cell count
None	0.11	11	1.7×10^8	5
Spermine	0.82	82	6×10^8	17
Spermidine	0.78	78	9×10^8	26
1,4-Diamino-butane	0.43	43	5.5×10^8	16
Original culture	1.01		3.5×10^9	

* Cell suspensions in 0.03 M tris HCl buffer (pH 8.0), without sucrose, were made 10^{-3} M in spermine, spermidine, or 1,4-diaminobutane. A control tube containing the cell suspension without added amine was also prepared. All the tubes were then treated immediately with EDTA and lysozyme at 0 C for 30 min. Samples of the incubation mixture were diluted and plated 30 min after the addition of lysozyme, as in Table 10. Colonies were counted after 24 hr of growth at room temperature. Cell count of the starting culture used was 3.5×10^9 cells/ml. Optical densities of the protoplast suspensions at 650 mμ were observed at 0.5 hr after the lysozyme addition.

density after lysozyme treatment of cells pretreated with spermidine or spermine was slight, indicating that lysis did not account for the loss of viability just described. Examination of these cells by phase microscopy showed that they resembled cells in shape, size, and motility. Thus, prior treatment of cells with polyamines prevents the development of osmotic fragility, and partially prevents the loss of viability observed after lysozyme treatment.

The experiments presented here do not demonstrate the specific changes that take place in the cells exposed simultaneously to spermine and lysozyme. By appearance and osmotic fragility they are indistinguishable from untreated cells; however, approximately 80% have been rendered nonviable. These results can be explained by partial inhibition of lysozyme action by spermine, or by complete stabilization of the cell shape in the presence of spermine, even if the lysozyme were not inhibited. My experiments do not distinguish between these two possibilities. Other criteria for protoplast formation that have been used with other organisms, e.g., the appearance of reducing sugar and of free *N*-acetylglucosamine after lysozyme treatment, were not satisfactory in these studies. *E. coli* W incubated for 24 hr without lysozyme gave a positive reducing sugar test by the Park and Johnson (1949) procedure, while even 24 hr incubation with lysozyme liberated no free *N*-acetylglucosamine, i.e., less than 10 μg/ml by the Reissig, Strominger, and Leloir (1955) method. The appearance of free reducing groups when intact *E. coli* were incubated at 37 C in the presence of tris buffer (pH 8) could reflect leaking. Under these conditions, I also observed that there was a loss of 260 mμ-absorbing material from these cells, which equaled half the amount lost if a comparable suspension of cells underwent complete lysis.

The viability of *E. coli* spheroplasts depended on the medium in which the cells were converted to spheroplasts. Under my standard conditions, without added magnesium, I observed 10% viability at 150 min after the addition of penicillin. When the medium contained 10^{-3} M $MgCl_2$ (as described by Lederberg, 1956), I found, in agreement with Lederberg's results, a marked increase in viability (over 50%). In both cases, little or no effect was observed upon the addition of 2×10^{-3} M spermine during the preparation of the spheroplasts or 10^{-3} M spermine at 150 min.

DISCUSSION

Primosigh et al. (1961) have shown that the wall of *E. coli* consists of three layers, an outer lipoprotein layer, a middle lipopolysaccharide layer, and an inner mucopeptide layer. They consider that the rigidity of the cell wall can be attributed to the innermost layer, the outer two layers being flexible. When the inner rigid layer is not present or functioning, osmotic changes can give rise to swelling and ultimately to rupture of the cell.

Protoplasts are cells which have a defective rigid wall because of the enzymatic action of lysozyme on the mucopeptide layer (Primosigh et al., 1961). Spheroplasts are thought to have defective rigid layers because of penicillin inhibition of synthesis of the mucopeptide layer (Park and Strominger, 1957). The stabilization of protoplasts and spheroplasts by spermine may be due to a functional correction of these defects, or to an effect or spermine on the outer layers. Primosigh et al. (1961) point out that there is on the mucopeptide an extra carboxyl group due to diaminopimelic acid. It is possible that this might bind the polyamines tightly, and

thus prevent the loss of rigidity. On the other hand, a strong affinity of spermine for phospholipid has been described (Razin and Rozansky, 1959). The binding of spermine to these outer layers might overcome the repulsive forces of sulfate or phosphate groups which are situated in close proximity to each other. In this connection, the binding of spermine by the protoplast is tight; C^{14}-spermine studies have demonstrated that repeated suspension of these protoplasts in water fails to wash out significant amounts of the labeled spermine. The effect of polyamines on the cell wall offers one possible explanation of the ability of polyamines to inhibit leakage of ultraviolet-absorbing materials from protoplasts. Alternatively, the stabilizing effect of polyamines on nucleic acids (see below) or the inhibition of nucleases, described by Keister (1958) and by Herbst and Doctor (1959), may be responsible for this inhibitory effect on leakage. Although many other basic compounds can also stabilize protoplasts and spheroplasts, it is of interest that the polyamines occur naturally in high concentration in many bacteria.

The stabilization of mitochondria (Tabor, C. W., 1960*a*,*b*; Herbst and Witherspoon, 1960), of *E. coli* ribosomes (Cohen and Lichtenstein, 1960; Zillig, Krone, and Albers, 1959; Hershko, Amoz and Mager, 1961; Colbourn, Witherspoon, and Herbst, 1961), of bacteriophage T_5 (Tabor, H., 1960), of π obtained from bacteriophage T_2 (Fraser and Mahler, 1958), of calf thymus deoxyribonucleic acid (DNA) (Mahler, Mehrotra, and Sharp, 1961; Tabor, H., Biochemistry, *in press*), of *B. subtilis* transforming DNA (Tabor, H., 1961), and of T_2 DNA (Mahler et al., 1961) by polyamines has recently been reported from several laboratories. In some of these instances, it seems most likely that the strong affinity of nucleic acids for polyamines (Razin and Rozansky, 1959; Keister, 1958) may be the important factor in the mechanism of stabilization in these systems. An antioxidant effect of the polyamines has also been observed for unsaturated fatty acids (Tabor, C. W., 1960*c*), which might be important in some of these systems. Although the present studies have not resulted in any single unifying theory of action of polyamines, it is possible that the polyamines serve as physiological stabilizers within the cell.

LITERATURE CITED

BACHRACH, U., AND I. COHEN. 1961. Spermidine in the bacterial cell. J. Gen. Microbiol. **26**:1–9.

BEERS, R. F., JR. 1955. Submerged culture of *Micrococcus lysodeikticus* for large-scale production of cells. Science **122**:1016.

BRENNER, S., F. A. DARK, P. GERHARDT, M. H. JEYNES, O. KANDLER, E. KELLENBERGER, E. KLIENEBERGER-NOBEL, K. MCQUILLEN, M. RUBIO-HUERTOS, M. R. J. SALTON, R. E. STRANGE, J. TOMCSIK, AND C. WEIBULL. 1958. Bacterial protoplasts. Nature **181**:1713–1715.

BROWN, A. D. 1960. Inhibition by spermine of the action of a bacterial cell-wall lytic enzyme. Biochim. et Biophys. Acta **44**:178–179.

COHEN, S. S., AND J. LICHTENSTEIN. 1960. Polyamines and ribosome structure. J. Biol. Chem. **235**:2112–2116.

COLBOURN, J. L., B. H. WITHERSPOON, AND E. J. HERBST. 1961. Effect of intracellular spermine on ribosomes of *Escherichia coli*. Biochim. et Biophys. Acta **49**:422–424.

DUBIN, D. T., AND S. M. ROSENTHAL. 1960. The acetylation of polyamines in *E. coli*. J. Biol. Chem. **235**:776–782.

FRASER, D., AND H. R. MAHLER. 1958. Effects of diamines on the protoplast-infecting agent derived from T_2 bacteriophage. J. Am. Chem. Soc. **80**:6456.

HERBST, E. J., AND B. P. DOCTOR. 1959. Inhibition of ribonucleic acid degradation in bacteria by spermine. J. Biol. Chem. **234**:1497–1500.

HERBST, E. J., AND B. H. WITHERSPOON. 1960. Inhibition of spontaneous and induced swelling of mitochondria by spermine. Federation Proc. **19**:138.

HERSHKO, A., S. AMOZ, AND J. MAGER. 1961. Effect of polyamines and divalent metals on *in vitro* incorporation of amino acids into ribonucleoprotein particles. Biochem. Biophys. Research Communs. **5**:46–51.

JACKSON, E. L., AND S. M. ROSENTHAL. 1960. The preparation of C^{14}-labelled spermine and C^{14}-labelled spermidine. J. Org. Chem. **25**: 1055–1056.

KEISTER, D. L. 1958. Some aspects of the metabolic function of the naturally occurring amines, p. 33. Ph.D. thesis, University of Maryland, College Park.

LEDERBERG, J. 1956. Bacterial protoplasts induced by penicillin. Proc. Natl. Acad. Sci. U.S. **42**:574–577.

MAGER, J. 1955. The influence of osmotic pressure on the polyamine requirement of *Neisseria perflava* and *Pasteurella tularensis* for growth in defined media. Nature **176**:933–934.

MAGER, J. 1959*a*. Spermine as a protective agent against osmotic lysis. Nature **183**:1827–1828.

MAGER, J. 1959*b*. The stabilizing effect of spermine and related polyamines on bacterial protoplasts. Biochim. et Biophys. Acta **36**:529–531.

MAHLER, H. R., B. D. MEHROTRA, AND C. W. SHARP. 1961. Effects of diamines on the thermal transition of DNA. Biochem. Biophys. Research Communs. **4**:79–82.

PARK, J. T., AND M. J. JOHNSON. 1949. A submicrodetermination of glucose. J. Biol. Chem. **181**:149–151.

PARK, J. T., AND J. L. STROMINGER. 1957. Mode of action of penicillin. Science **125**:99–101.

PRIMOSIGH, J., H. PELZER, D. MAASS, AND W. WEIDEL. 1961. Chemical characterization of mucopeptides released from the *E. coli* B cell wall by enzymic action. Biochim. et Biophys. Acta **46**:68–80.

RAZIN, S., AND R. ROZANSKY. 1959. Mechanism of the antibacterial action of spermine. Arch. Biochem. Biophys. **81**:36–54.

REISSIG, J. L., J. L. STROMINGER, AND L. F. LELOIR. 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. **217**:959–966.

REPASKE, R. 1958. Lysis of gram-negative organisms and the role of versene. Biochim. et Biophys. Acta **30**:225–232.

ROSENTHAL, S. M., AND D. T. DUBIN. 1960. Acetylation of polyamines by Staphylococcus. Federation Proc. **19**:2.

TABOR, C. W. 1960*a*. The stabilizing effect of spermine and related amines on mitochondria and protoplasts. Biochem. Biophys. Research Communs. **2**:117–120.

TABOR, C. W. 1960*b*. Effects of spermine, putrescine, and cadaverine on mitochondria. Federation Proc. **19**:139.

TABOR, C. W. 1960*c*. The inhibitory effect of spermine on fatty-acid-induced lysis of protoplasts and erythrocytes, and on cytochrome c-induced fatty acid oxidation. Abstr. Ann. Meet. Am. Chem. Soc., p. 50c.

TABOR, H. 1960. Stabilization of bacteriophage T_5 by spermine and related polyamines. Biochem. Biophys. Research Communs. **3**:382–385.

TABOR, H. 1961. The stabilization of *Bacillus subtilis* transforming principle by spermine. Biochem. Biophys. Research Communs. **4**:228–231.

TABOR, H., S. M. ROSENTHAL, AND C. W. TABOR. 1958. The biosynthesis of spermidine and spermine from putrescine and methionine. J. Biol. Chem. **233**:907–914.

VOGEL, H. J., AND D. M. BONNER. 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.

WEIBULL, C. 1958. Bacterial protoplasts. Ann. Rev. Microbiol. **12**:1–26.

ZILLIG, W., W. KRONE, AND M. ALBERS. 1959. Untersuchungen zur Biosynthese der Proteine, III. Beitrag zur Kenntnis der Zusammensetzung und Struktur der Ribosomen. Z. physiol. Chem., Hoppe-Seyler's **317**:131–143.