STABILIZATION OF STREPTOCoccus FAECALIS PROTOPLASTS BY SPERLINE

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ABSTRACT

HAROLD, F. M. (National Jewish Hospital, Denver, Colo.). Stabilization of Streptococcus faecalis protoplasts by spermine. J. Bacteriol. 88:1416-1420. 1964.—Lysis of protoplasts of Streptococcus faecalis subjected to osmotic shock was prevented by the presence of 10^-9 M spermine and other divalent cations. Protein and nucleic acids were largely retained, but compounds of low molecular weight were discharged into the medium and the capacity for glycolysis was lost. Under these conditions, spermine was bound to the protoplasts. It could not be removed by washing with water or nonelectrolytes, but was displaced by salts, polyamines, and polycations. Removal of the spermine restored the osmotic fragility of the protoplasts, which could once again be protected from lysis by impermeant solutes. Protoplasts were also stabilized, in the absence of osmotic shock, by prolonged incubation with cations in 0.5 m sucrose. By either procedure, the protoplasts became resistant not only to osmotic lysis but also to sonic oscillation. It is concluded that the stabilization of protoplasts resulted from ionic binding of the cation to acidic sites on the external surface of the plasma membrane. This conferred upon the membrane additional mechanical strength, perhaps by the cross-linking of subunits, but did not alter its permeability to extracellular solutes.

Bacterial protoplasts, unlike intact cells, undergo osmotic lysis when exposed to hypotonic media; to remain stable, they must be protected by high concentrations of a nonpenetrating solute. Recently, however, Mager (1959) and Tabor (1962) found that osmotic lysis is prevented by low concentrations of certain cations, particularly spermine and related polyamines. Polyamines have been found to stabilize various biological systems (Tabor, Tabor, and Rosenthal, 1961), and inorganic divalent cations have long been used to enhance the stability of protoplasts; the physical basis of this phenomenon is not clear. The purpose of this communication is to present evidence that the stability of spermine-treated protoplasts in hypotonic media results from interaction of spermine with acidic groups located on the external surface of the plasma membrane, and to consider some physical and biological properties of stabilized protoplasts.

MATERIALS AND METHODS

Streptococcus faecalis ATCC 9790 was obtained from A. Abrams, University of Colorado. Bacteria were harvested from overnight cultures in Tryptone-yeast extract medium (Abrams, 1959), washed twice with water, suspended in 0.4 m sucrose-0.075 m maleate (pH 7), and converted to protoplasts by incubation with 200 µg/ml of lysozyme. Samples were diluted with water or solutions of various substances, and the loss of turbidity at 600 mÅ was taken as an index of protoplast lysis. In some experiments, the protoplasts were collected by centrifugation, suspended in 10^-3 m spermine hydrochloride (pH 7), and subsequently collected and resuspended in various media.

Protoplast membranes were prepared by osmotic shock or metabolic lysis (Abrams, McNamara, and Johnson, 1960). For measurement of glycolysis and uptake of metabolites, suspensions of protoplasts in buffered sucrose were supplemented with inorganic ions in the concentrations employed by Toennies and Shockman (1953): NH4+, 5 X 10^-3 M; Mg++, 0.8 X 10^-3 M; K+, 7 X 10^-4 M. Glycolysis was followed by titration to pH 7.2 with 0.02 N NaOH; the maleate level was lowered to 0.01 m. Nucleic acids and protein were determined by standard procedures (Harold, 1963). The effect of sonic irradiation on protoplasts and membranes was measured in a 10-ke Raytheon sonic oscillator. Spermine-C14 was purchased from New England Nuclear Corp., Boston, Mass.
RESULTS

Stabilization of protoplasts by spermine and other cations. When a suspension of protoplasts in sucrose was diluted tenfold with water, immediate lysis occurred as shown by the loss of turbidity. No lysis occurred upon equivalent dilution with \(10^{-3}\) M spermine (pH 7), and protoplasts thus subjected to osmotic shock in the presence of spermine could be repeatedly washed with water without lysing. In agreement with earlier reports (Tabor, 1962), spermine permitted retention of proteins and nucleic acids. However, if the protoplasts were first allowed to accumulate radioactive orthophosphate (Pi\(^{32}\)) or L-alanine-C\(^{14}\) and were then diluted into \(10^{-3}\) M spermine, virtually all the radioactivity was lost. The presence of spermine thus prevented gross lysis, but not the loss of constituents from the soluble pool (Table 1). Moreover, protoplasts stabilized by osmotic shock in \(10^{-3}\) M spermine completely lost the capacity for glycolysis. Glycolysis could be partially restored by addition of Pi, K\(^+\), Mg\(^{++}\), adenosine triphosphate, and nicotinamide adenine dinucleotide in relatively high concentrations (Wu and Racker, 1959), together with the supernatant fluid from the spermine shock.

Protoplasts stabilized in this manner were resistant not only to osmotic shock but also to sonic disintegration (Fig. 1). Also, unlike the original protoplasts, they did not lyse upon addition of n-butanol (up to 15%, v/v) or 0.05% Triton x 100.

A variety of cations could replace spermine in preventing gross lysis. Spermine, spermidine, streptomycin, protamine, polylysine (molecular weight 2,500 and 150,000), Hg\(^{++}\), and other heavy metals were effective at \(10^{-4}\) M. (Molarities of the polypeptides are given in terms of the monomer.) Cadaverine, putrescine, Ca\(^{++}\), Mg\(^{++}\), and the methyl esters of lysine, arginine, and histidine stabilized at \(10^{-4}\) M, whereas monovalent cations and free amino acids did not. Varying degrees of clumping were encountered, particularly with the polypeptides. In general, it appears that stabilizing cations must carry a net positive charge of two or more.

Restoration of osmotic fragility to spermine-stabilized protoplasts. Protoplasts diluted with \(10^{-3}\) M spermine-C\(^{14}\) bound approximately 100 to 200 \(\mu\)moles of spermine per gram (dry weight) of original cells. As was observed by Tabor (1962), these protoplasts could be washed repeatedly with water without loss of the bound C\(^{14}\) or of osmotic stability. However, bound C\(^{14}\) was displaced when the protoplasts were washed with electrolyte solutions, particularly polyanions, and also exchanged readily with certain polyanions.

TABLE 1. Effect of spermine on the loss of protoplast constituents by osmotic shock*

<table>
<thead>
<tr>
<th>Treatment of protoplasts</th>
<th>OD(_{660})</th>
<th>Protein</th>
<th>Nucleic Acids</th>
<th>Acid-soluble Pi</th>
<th>Alkaline C(^{14})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not diluted............</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Diluted with 0.4 M sucrose</td>
<td>100</td>
<td>98</td>
<td>99</td>
<td>77</td>
<td>94</td>
</tr>
<tr>
<td>Diluted with water</td>
<td>17</td>
<td>28</td>
<td>28</td>
<td>2</td>
<td>21</td>
</tr>
<tr>
<td>Diluted with 10(^{-4}) M spermine</td>
<td>95</td>
<td>92</td>
<td>89</td>
<td>3</td>
<td>21</td>
</tr>
</tbody>
</table>

* A suspension of protoplasts in buffered sucrose (see Materials and Methods) was incubated for 20 min at 37 C with 2.5 mg/ml of glucose and either 5 \(\mu\)g/ml of Pi\(^{32}\) or 2 \(\mu\)g/ml of alanine-C\(^{14}\), to label the acid-soluble pool. After tenfold dilution as indicated, the protoplasts were centrifuged, washed once with 0.4 M sucrose, and analyzed. Retention of constituents is expressed as percentage of the original amount.

FIG. 1. Sonic disintegration of spermine-stabilized protoplasts and membranes. Normal and stabilized protoplasts were washed with 0.5 M sucrose and resuspended in the same medium. Normal and spermine-treated membranes were washed and resuspended in water. The membranes were sonically treated at 10 kc, 50% power output. At intervals samples were diluted with 0.5 M sucrose or water for turbidimetry.
It thus appears that the permeability properties of the plasma membrane are not altered by spermine or by its subsequent removal.

Since stabilization of protoplasts by osmotic shock with 10^-3 M spermine results in the loss of osmotically active constituents of low molecular weight, it would be expected that after restoration of osmotic fragility a lower concentration of impermeant solute would be required for osmotic protection. This prediction is confirmed by the results shown in Fig. 3; similar observations were made with DL-alanine and NaCl as osmotic stabilizers.

Stabilization of protoplasts without osmotic shock. All the results described above were obtained with protoplasts stabilized by dilution with 10^-3 M spermine, a procedure involving osmotic shock. It was subsequently found that protoplasts incubated at 37°C with 10^-3 M spermine or 10^-2 M CaCl_2 in 0.5 M sucrose-0.02 M maleate (pH 7) progressively acquired stability in the course of 30 min. Binding of spermine-C^14 under these conditions occurred to the extent of about 60 μmoles per g (dry weight) of original cells; this is less than half the amount bound after osmotic shock, presumably because in the latter procedure it is lost.

Table 2. Displacement of bound spermine-C^14 from protoplasts*

<table>
<thead>
<tr>
<th>Medium</th>
<th>Per cent C^14 retained</th>
<th>Osmotic fragility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>100</td>
<td>No</td>
</tr>
<tr>
<td>Sucrose, 0.5 M</td>
<td>83</td>
<td>No</td>
</tr>
<tr>
<td>Glycerol, 1 M</td>
<td>92</td>
<td>No</td>
</tr>
<tr>
<td>NaCl, 0.2 M</td>
<td>44</td>
<td>Yes</td>
</tr>
<tr>
<td>Na_2SO_4, 0.2 M</td>
<td>36</td>
<td>Yes</td>
</tr>
<tr>
<td>Sodium phosphate, 0.2 M</td>
<td>36</td>
<td>Yes</td>
</tr>
<tr>
<td>pH 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenosine triphosphate</td>
<td>38</td>
<td>Yes</td>
</tr>
<tr>
<td>Inorganic polyphosphate, chain length = 50, 2 \times 10^{-3} M in 0.5 M sucrose</td>
<td>31</td>
<td>Yes</td>
</tr>
<tr>
<td>Spermine, 2 \times 10^{-3} M</td>
<td>19</td>
<td>No</td>
</tr>
<tr>
<td>Streptomycin, 2 \times 10^{-3} M</td>
<td>68</td>
<td>No</td>
</tr>
<tr>
<td>CaCl_2, 2 \times 10^{-3} M</td>
<td>85</td>
<td>No</td>
</tr>
<tr>
<td>Protamine, 2 \times 10^{-3} M (monomer)</td>
<td>15</td>
<td>No (clumped)</td>
</tr>
<tr>
<td>Polylsine, 2 \times 10^{-3} M (monomer) (molecular weight, 2,500 and 150,000)</td>
<td>3</td>
<td>No (clumped)</td>
</tr>
</tbody>
</table>

* Protoplasts were suspended in 10^-3 M spermine-C^14, centrifuged, washed once with water, and resuspended in the solutions shown [0.6 mg (dry weight) per 2 ml]. After 15 min, 1-ml samples were filtered through Millipore filters, and the filters were washed and counted. Osmotic fragility was tested on separate samples by centrifugation and resuspension in water.

Cations (Table 2). Nonelectrolytes such as sucrose or glycerol did not displace spermine-C^14.

Stabilized protoplasts washed with salts or polyamions were found to have recovered osmotic fragility; subsequent resuspension in water caused lysis. This effect was most conveniently studied by addition of inorganic polyphosphate to a suspension of the protoplasts. Polyphosphate induced lysis of an aqueous suspension of spermine-stabilized protoplasts (Fig. 2). Impermeant solutes (Abrams, 1959) such as sucrose, sodium chloride, and DL-alanine afforded osmotic protection; glycerol and urea, which penetrate quickly into S. faecalis protoplasts, did not prevent lysis. Washing of stabilized protoplasts with nonelectrolytes, or exchange of spermine for other polyvalent cations, did not restore osmotic fragility.

FIG. 2. Osmotic protection of spermine-stabilized protoplasts in the presence of polyphosphate. Protoplasts were stabilized by osmotic shock with 10^-3 M spermine, washed, and resuspended in the media shown. At 0 min, inorganic polyphosphate (2 \times 10^{-3} M phosphorus; chain length, 80) was added to all, and turbidity was measured at intervals.
Stabilization of *S. faecalis* protoplasts by agitation with spermine. Partial lysis of some protoplasts exposed additional binding sites. Protoplasts stabilized by incubation with spermine in buffered sucrose retained the capacity for glycolysis, metabolic swelling (Abrams, 1959), and energy-dependent uptake of Pi. When protoplasts were centrifuged and resuspended in water, gross lysis did not occur, but compounds of low molecular weight were lost into the medium. Increased resistance to disintegration by sonic oscillation and butanol was again observed, as was the displacement of C14-spermine by polyanions and polycations.

**Binding of spermine-C14 to the plasma membrane.** The experiments discussed above suggest that spermine and other cations interact with the surface of the protoplast, i.e., the plasma membrane. This conclusion is supported by experiments on the localization of bound spermine-C14. Protoplasts were diluted into 10^-3 M spermine-C14, washed, resuspended in 0.5 M sucrose, and disrupted by sonic treatment at maximal power output. Upon centrifugation of the lysate, 80% of the C14-spermine was sedimented with the membrane fraction at 25,000 X g in 15 min.

Additional information was obtained from studies on membrane ghosts, consisting largely of intact plasma membranes (Abrams et al., 1960). When these structures were suspended in neutral 10^-3 M spermine-C14, 30 to 40 μmoles were bound to the amount of membranes equivalent to 1 g of original cells. Membranes treated in this manner exhibited increased stability to sonic oscillation (Fig. 1). Bound spermine was once again displaced by electrolytes. *S. faecalis* membranes contain acidic groups, mostly in the form of nucleic acid and lipid phosphate (Abrams, Nielsen, and Thaemert, 1964; Ibott and Abrams, 1964), both of which are known to interact with spermine (Razin and Rozansky, 1959). Removal of phospholipids from the membranes by repeated extraction with ethanol-ether reduced the capacity for adsorption of spermine-C14 by 50 to 80%; digestion with nucleases or with trypsin (which removed two-thirds of the protein associated with the membrane fraction) had no effect.

**Discussion**

The results outlined above strongly support the suggestion of Mager (1959) that stabilization of protoplasts by spermine and other cations ultimately depends upon their ionic binding to acidic groups on the external surface of the plasma membrane. The best evidence for the surface localization of spermine comes from the finding that spermine is displaced by inorganic salts, polyanions, and polycations, and that the protoplasts then recover their original osmotic fragility. Inorganic salts such as sodium chloride serve as osmotic stabilizers, and it can therefore be inferred that they are excluded by the plasma membrane. Although direct evidence is not available, it seems most probable that charged macromolecules such as inorganic polyphosphate, protamine, and polylysine likewise cannot penetrate into the interior of the protoplasts. Thus, the displacement of bound spermine by these substances, particularly by polyvalent cations, is most readily understood as an ion-exchange reaction occurring at the surface of the protoplasts.

Protoplast stabilization by cations can thus be regarded as a manifestation of the cation-exchange properties of the plasma membrane, which have also been implicated in the binding of cationic antibiotics (Newton, 1956) and many heavy metals (Passow, Rothstein, and Clarkson, 1961). In no case has the chemical identity of the binding sites been established. The present results
are consistent with cation binding to phospholipids, but the direct evidence is meager. Electrophoretic studies with protoplasts and membranes (Few, Gilby, and Seaman, 1960) have provided support for the view that the phospholipid molecules are at least partly shielded by a protein layer. If phospholipids constitute the cation-binding sites, the presence of a protein layer might retard the diffusion of spermine and thus account for the finding that stabilization requires either osmotic shock or prolonged incubation at 37 C. In any event, interaction of the membrane with polynvalent cations confers upon it increased stability, not only to osmotic shock but also to mechanical stress and to dissolution by organic solvents. The requirement for multivalent cations suggests that cross-linking of some kind is involved, perhaps between subunits of the membrane matrix, such as those described in mitochondria (Green and Fleischer, 1963).

When spermine-stabilized protoplasts are subjected to osmotic shock, the bulk of the protein and nucleic acid is retained, but constituents of low molecular weight escape. The loss of the solute compounds, which account for most of the osmotic pressure (Mitchell and Moyle, 1956), may well be an important element in the osmotic stability of the protoplasts. Apart from this transient leakiness of the membrane, its permeability properties appear to be unchanged by the presence of the cation or by its subsequent removal. Since the loss of intracellular ions and coenzymes prevents glycolysis (restoration of glycolysis is accompanied by lysis), the biological utility of stabilization by spermine appears questionable. However, Gooder (1964) found that the fraction of \( S. faecalis \) protoplasts surviving after osmotic shock is significantly increased by spermine. Thus, the present results in no way contradict the hypothesis of Tabor et al. (1961) that the widespread distribution of polyamines among living things reflects their function as natural stabilizers of anionic structures.

Acknowledgments

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Literature Cited


