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WHY DO BACTERIAL PROTOPLASTS BURST IN HYPOTONIC SOLUTIONS?

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SUMMARY

A study of the relative effectiveness of sugars, of short peptides and of amino acids as osmotic stabilizers for nonrespiring *Bacillus megaterium* protoplasts indicated that the protoplast membrane could act as a porous differential dialysis membrane in much the same manner as cellophane membranes do and that its effective porosity increased when it was stretched during osmotic swelling. The protoplast membrane also behaved as a highly extensible structure, in contrast to membranes such as those of erythrocytes and enormous protoplasts could be prepared by slowly dialyzing stabilizing solutes from protoplast suspensions. In effect, osmotic bursting of bacterial protoplasts could not be related to the ultimate tensile strength of maximally extended membranes. Rather, it appeared that when protoplasts swelled in hypotonic solutions, their surface membranes might become sufficiently stretched so that they admit stabilizing solutes. There would be then a rapid influx of solutes and water resulting in rapid stretching of the membrane and rupture due to a process of brittle fracture. Thus, bursting generally occurs without the membrane becoming fully extended.

We also found that urea and glycerol induced mechanical relaxation of protoplast membranes. This finding, together with our other findings concerning mechanical properties of protoplast membranes and the strengthening effect of formaldehyde treatment, suggests that the mechanically important membrane component is protein, possibly in the form of a microfibrillar array.

INTRODUCTION

Bacterial protoplasts, from which cell wall materials have been removed by treatment with enzymes, have been widely used as a source of membranes or membrane fragments and for studies of physiological processes such as solute transport. It is well known that bacterial protoplasts are osmotically sensitive and that, when they are not encased within the protective, rigid cell wall, they are stable only in media containing high concentrations of impermeant solutes. Despite the widespread use of wall-less protoplasts, there still remains an overall vagueness in current knowledge of the processes involved in their osmotic swelling and bursting.

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Probably the cells most thoroughly studied in regard to osmotic behavior are mammalian red blood cells. When placed in hypotonic media, red cells first change from biconcave discs to spheres. This change in shape involves an increase in cell volume but little or no change in surface area. If the suspending medium is sufficiently hypotonic, spherical erythrocytes will swell further, now with increasing surface area, until they hemolyze. Apparently the erythrocyte membrane has a very limited extensibility and can increase in surface area only about 28 % without mechanical failure[1]. This failure leads to formation of large holes with effective diameters greater than 120 Å in the surface membrane[2]. Once these holes are formed, internal solutes including hemoglobin are released from the cell, stress in the membrane is relieved and the membrane may eventually reseal to yield an osmotically sensitive ghost.

The erythrocyte membrane has viscoelastic properties so that red cells are more likely to undergo hemolysis when transferred rapidly, rather than slowly, to hypotonic media[3]. However, even with slow stretching, the surface membrane does not appear to be very extensible, and holes large enough to admit ferritin molecules of about 120 Å diameter are formed during "slow hemolysis"[2].

It is difficult to compare the osmotic responses of bacterial protoplasts with those of erythrocytes because of our limited knowledge of the former processes. In this paper, we interpret experimental results presented here and elsewhere[4–6] in order to obtain a clearer idea of the sequence of events in the osmotic bursting of *Bacillus megaterium* protoplasts and the pertinent physical properties of the protoplast membrane.

MATERIALS AND METHODS

Growth of bacteria and preparation of protoplasts

Cells of the asporogenous KM strain of *B. megaterium* were grown at 30° in an aerated 2 % Oxoid peptone broth (Colab Labs., Chicago Heights, Ill.) and were harvested by centrifugation in the cold. Protoplasts were obtained by treating cells suspended in 2.38 osmolal (2.00 molal) sucrose solution containing 0.04 M phosphate buffer (pH 7) with 0.4 mg per ml *N*-acetylmuramide glucanohydrolase (EC 3.2.1.17; lysozyme) which completely removes walls from cells of this strain of *B. megaterium*. At room temperature, removal of the walls was usually complete within 2 h, as judged by the change from cylindrical to spherical in the shape of the cell. We found that the osmotic fragility of protoplasts varied markedly, depending on the stage of the culture cycle at which cells were harvested. Therefore, to obtain consistent results, we harvested cells only from cultures in the final stage of exponential growth when the culture absorbance was between 1.85 and 2.10 and the total cell count was approx. $6 \cdot 10^8$ bacteria per ml. Absorbance was measured with a Beckman DU spectrophotometer, using light of 700-mμ wavelength and a 1-cm light path cuvette. The protoplasts obtained from these cells were more likely to survive rapid transfer to dilute sucrose solutions than were protoplasts obtained from cells harvested at other stages of the culture cycle.

Counting and sizing of protoplasts

Protoplasts were counted by use of a phase-contrast microscope (1500 × magnification) and a Petroff-Hausser counting chamber. The routine procedure was to count protoplasts in squares across the ruled area of the chamber, starting with the

uppermost left-hand square and moving two squares to the right and then down one for the next count and so on across the chamber. A second series of counts was made in the same way only starting at the lowermost right-hand square and proceeding to the left. A total of at least 20 squares was counted per determination. Then the chamber was emptied, cleaned and refilled for another count of the same sample. The total count was nearly always in excess of 100 protoplasts.

Protoplast diameters were measured with a calibrated ocular micrometer. The values obtained were used to calculate average volumes on the assumption that protoplasts are spheres. Diameters of at least 150 protoplasts in the counting chamber were measured for each volume calculation, and the diameters of all the protoplasts in randomly chosen squares of the chamber were measured. Volumes calculated from direct microscopic measurements agreed with those calculated from photographic measurements. Photographs were used only occasionally because of the expense and because of the difficulty in deciding which protoplasts in the micrographs were sharply in focus.

Transfer of protoplasts to test solutions

Fresh protoplast suspensions were prepared daily and were chilled in an ice bath until needed. These initial suspensions were diluted in any of three ways: by direct addition of diluent with rapid mixing; by slow dropwise addition of diluent over a period of about 45 min with stirring by means of magnetic stirrers; or by putting the suspensions in cellophane dialysis sacks and then placing the sacks in diluent solutions for 18–24 h. The half-time for equilibration of sucrose across the cellophane sacks used was about 70 min, and the permeability coefficient of the membranes for sucrose was about 0.38 cm/h. Chilled diluents were used for all experiments, and both the dropwise addition of diluents and the dialysis were carried out in the cold.

Generally the dilution of the initial suspension was at least 40-fold. We considered that wall fragments in the initial suspension did not contribute significantly to final solution osmolalities in view of the finding of SALTON[7] that most of the fragments are macromolecules with molecular weights of 10000–20000.

Osmotic coefficients and aqueous diffusion coefficients

Osmotic coefficients used for calculating osmolalities of sucrose, glycerol and urea solutions were those given by SCATCHARD *et al.*[8]. Coefficients for glucose solutions were calculated from osmotic pressure data given in the *International Critical Tables*[9]. Coefficients for stachyose and raffinose solutions were calculated from data obtained by use of a vapor-pressure osmometer (Mechrolab, Mountain View, Calif.). Osmolalities of ribose and triglycine solutions were determined directly by use of a freezing-point osmometer (Advanced Instruments, Newton Highlands, Mass.) which was calibrated to give read-out in terms of mosmoles/kg of solution. Osmotic coefficients for amino acid or diglycine solutions were calculated with the aid of the experimentally derived equations of SMITH AND SMITH[10–13].

All aqueous diffusion coefficients (D) were taken from the tabulation of LONGSWORTH[14].

Chemicals

All amino acids and peptides were obtained from Mann Research Laboratories (New York, N.Y.) except proline which was obtained from Sigma Chemical Co.

(St. Louis, Mo.). Formaldedyde was obtained from Fisher Scientific Co. (Pittsburgh, Pa.) as a 37.2 % solution containing 10–15% methanol as a preservative. The concentration of methanol in protoplast suspensions to which formaldehyde was added was too low to have an appreciable effect on the protoplasts. Glycerol and urea were also purchased from Fisher Scientific Co. Reagent grade glucose and sucrose were used; all other sugars were of the highest quality and were supplied by Nutritional Biochemical Corp. (Cleveland, Ohio).

RESULTS

Effectiveness of sugars, amino acids and short peptides as osmotic stabilizers

The bursting of *B. megaterium* protoplasts during gradual lowering of the osmolality of their suspending medium is described in Fig. 1. Diluents containing different sugars were added dropwise to protoplast suspensions over a period of about 45 min, and the protoplasts were counted and sized immediately after dilution. Larger sugars proved to be more effective osmotic stabilizers in that protoplasts could adapt, without bursting, to more dilute solutions containing them. These results confirm and extend those of an earlier study[4] in which the osmolality of the suspending medium was lowered rapidly. In other slow-dilution experiments involving the use of diluents containing amino acids or short peptides, we again found that larger compounds were usually more effective stabilizers.

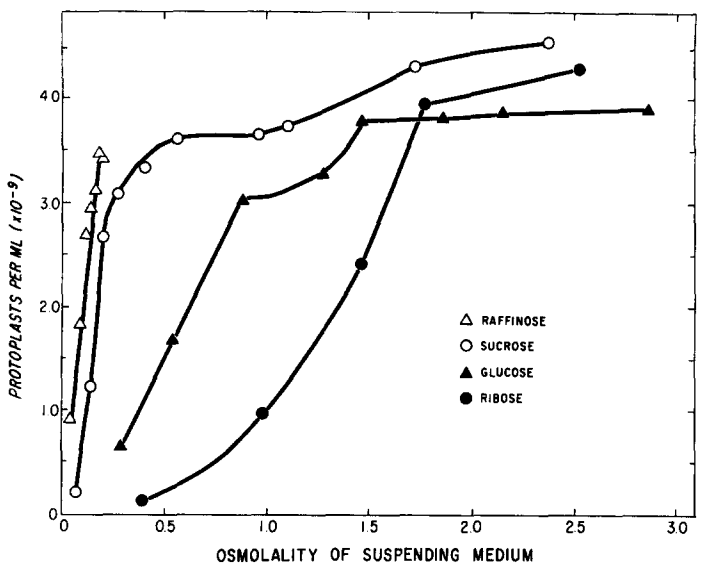

Fig. 1. Osmotic bursting of *B. megaterium* protoplasts in sugar solutions. The osmolality of protoplast suspensions initially prepared with 2.38 osmolal sucrose solution was lowered by slow, dropwise addition of chilled diluents containing raffinose (△), sucrose (○), glucose (▲) or ribose (●). All dilutions were at least 40-fold. The points shown represent mean values for four experiments with ribose or raffinose, five experiments with sucrose and seven experiments with glucose.

If one plots graphically the solution osmolalities at which some fraction, say 25 %, of the initial protoplast population bursts in solutions of the four sugars of Fig. 1

against the aqueous diffusion coefficients (D) of the solutes, a nearly linear relationship is seen (Fig. 2A). The stabilizing effectiveness of these sugars appears to be inversely related to their aqueous diffusion coefficients, which in turn are inversely related to molecular sizes[15]. A similar relationship is also seen (Fig. 2A) for the short peptides and certain amino acids.

However, peptides were more effective stabilizers than were sugars with similar diffusion coefficients; triglycine was a better stabilizer than was glucose, and diglycine was better than ribose. CRAIG[16] previously reported that sugars pass more easily through cellophane dialysis membranes than do peptides with nearly identical diffusion coefficients; for example, he found that triglycine passed more slowly through a cellophane membrane than did glucose. Short peptides behave as elongate cylinders in solution[17], whereas small sugar molecules behave as spheres[18]. Since spherical molecules have larger diffusion coefficients than do nonspherical molecules of the same volume[19], an elongate triglycine molecule should be smaller than a spherical glucose molecule, even though they have nearly the same aqueous diffusion coefficient. However, because of rotational diffusion, elongated molecules have greater difficulty passing through pores than do spherical ones of the same volume[20]. Thus, molecular shape as well as size appeared to be important in the passage of solutes across the protoplast membrane and in osmotic stabilization.

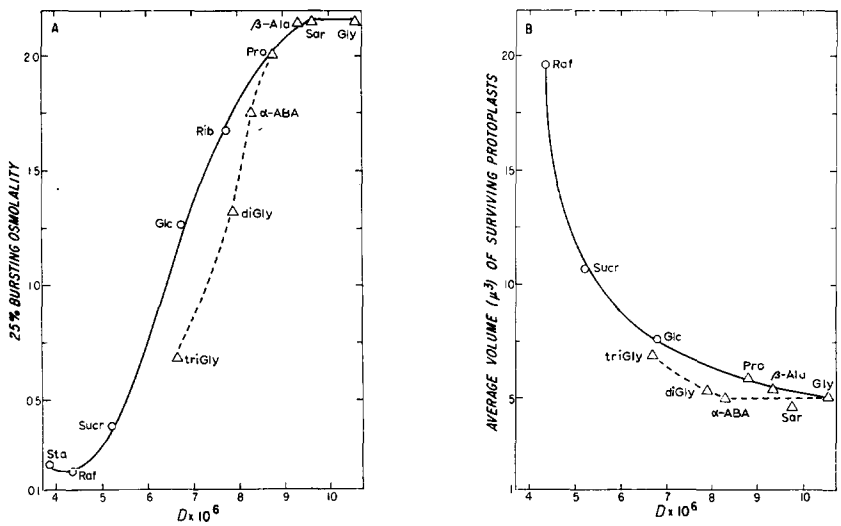

Fig. 2. Osmotic bursting (A) and swelling (B) of *B. megaterium* protoplasts related to aqueous diffusion coefficients of stabilizing solutes. The osmolality of protoplast suspensions was lowered slowly by dropwise addition of diluents containing sugars (○) or amino acids and short peptides (△). The volumes in B are average survivor volumes for suspensions in which 25% of the initial population had burst. Counting and sizing were carried out immediately after dilution. Abbreviations: Sta, stachyose; Raf, raffinose; Sucr, sucrose; Glc, glucose; Rib, ribose; triGly, triglycine; diGly, diglycine; α-ABA, α-aminobutyric acid; Pro, proline; β-Ala, β-alanine; Sar, sarcosine; Gly, glycine. All points represent means of four experiments except Sta and triGly, two experiments; Sucr and β-Ala, five experiments; Glc, seven experiments.

In these experiments, stachyose did not prove to be a better stabilizer than raffinose (Fig. 2A) as it had in previously reported[4] experiments involving rapid dilution of protoplast suspensions. We also tested polyethylene glycols as stabilizers, but

the results were difficult to interpret because individual glycol preparations contain molecules in a range of molecular sizes and because even unswollen protoplasts appeared to be permeable to these compounds which have an appreciable degree of lipid solubility. For example, Carbowax 4000 (Union Carbide Corp., New York, N.Y.) which contains molecules with weights in the 3000–3700 range proved to be a poorer stabilizer than did raffinose (25 % bursting osmolalities of 0.70 and 0.18, respectively), and protoplasts exposed to high concentrations of Carbowax appeared cytologically abnormal with large vacuoles. The results in Fig. 2A do suggest, however, that there may be a limit to the extent that stabilizing effectiveness increases with increasing molecular size.

The average volumes of surviving protoplasts presented in Fig. 2B indicate that protoplasts swell to larger volumes before bursting in solutions of larger molecules such as raffinose than they do in solutions of smaller molecules such as glycine. In a number of experiments, we measured the average sizes of membrane ghosts as well as of surviving protoplasts and found that the ghosts had average diameters only slightly larger (6–9 %) than those of intact protoplasts. Most of the ghosts, which were only faintly visible in the phase microscope, appeared to be nearly spherical with single torn or damaged regions. However, the surface areas of ghosts were still difficult to calculate accurately because of irregularities in shape and because of the possibility that the damaged membrane might have been dimpled. Certainly bursting did not seem to result in major mechanical collapse or contraction of the membrane.

Highly contracted protoplasts in very hypertonic solutions did not discriminate well among stabilizers on the basis of their molecular sizes and shapes, so that compounds as small as glycine and sarcosine proved to be effective long-term stabilizers (Fig. 2). The limiting contracted volume of about 5 μ^3 for protoplasts in concentrated solutions of glycine, sarcosine or α-aminobutyric acid (Fig. 2B) is only slightly greater than the minimum osmotically dehydrated volume of 4.7 μ^3 previously reported[5] for similar protoplasts prepared in this laboratory. Highly contracted protoplasts remained permeable to water, and they swelled readily when placed in dilute solutions. Moreover, they regained their capacity to discriminate among compounds such as sucrose, glucose and ribose.

We considered that the behavior of highly contracted protoplasts might be related to their initial size and porosity when prepared in 2.38 osmolal sucrose solution. However, transfer of the protoplasts to approx. 4 osmolal sucrose solution prior to the transfers to amino acid solutions did not significantly alter their osmotic responses.

Further, we considered that the cytoplasm of highly contracted protoplasts might have gelled so that surface membranes were no longer required for the cells to retain their size and shape. However, the sensitivity of highly contracted protoplasts to membrane-disrupting agents such as propanol, butanol or cetyldimethylbenzylammonium chloride was essentially the same as that of protoplasts suspended in 1.11 or 2.38 osmolal sucrose solutions.

In all, the results presented in Fig. 2 are consistent with the view that the protoplast membrane behaves like extensively studied[2] cellophane dialysis membranes and is perforated by aqueous channels which can effectively dilate when the membrane is stretched. There is one apparent inconsistency in Fig. 2; surviving protoplasts in solutions of peptides or α-aminobutyric acid were somewhat smaller than those in solutions of glucose, proline or β-alanine even though the osmolalities for 25 % bursting

were greater in the latter solutions. This difference may reflect minor leakage of glucose, proline or β-alanine into swelling protoplasts or some interaction of peptides and α-aminobutyric acid with the membrane. However, a number of factors make it difficult to interpret these small volume differences.

Protoplasts did not appear to be in complete osmotic equilibrium with their environment; for example, as shown in Fig. 3, they tended to contract, then swell during long-term storage. It was shown in a previous study[5] that nonrespiring protoplasts release solutes such as inorganic phosphate during the initial stages of storage, and presumably differential solute loss leads to contraction.

Another complicating factor was the tendency of protoplasts to undergo spontaneous contractions in certain dilute solutions. Again, contraction was probably due to selective loss of solutes smaller than the stabilizing solute. An example is described in Fig. 4. If the osmolality of protoplast suspensions containing approx. 0.19 osmolal raffinose was slowly lowered to 0.16, the protoplasts swelled and some of them burst. However, if the dilution was sufficient to lower the ambient osmolality below 0.16, bursting again occurred, but the surviving protoplasts were smaller than they were initially. The irregularities in the curves of Fig. 1 appeared to be related to this type of contraction[21].

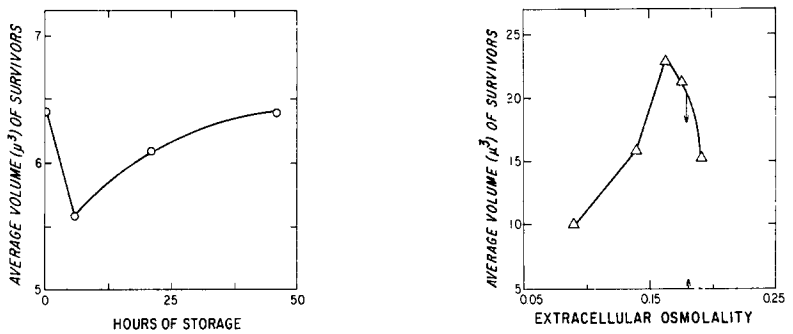

Fig. 3. Contraction and swelling of bacterial protoplasts during long-term storage in 1.33 osmolal sucrose solution. The suspension was stored in the refrigerator at about 4°. None of the protoplasts burst during the initial phase of storage characterized by cell contraction, and it was not until well into the phase characterized by cell swelling that bursting in the population became extensive.

Fig. 4. Contraction of protoplasts in dilute raffinose solutions. Diluents were added slowly to protoplast suspensions initially containing 0.19 osmolal raffinose, and the protoplasts were sized immediately after dilution. The arrow indicates the 25% bursting osmolality.

Extensibility of the protoplast membrane

Why do protoplasts burst when the osmolality of their suspending medium is lowered? Previously reported work[5] indicated that rapid osmotic swelling resulted in brittle fracture of the protoplast membrane. But slow swelling produced much less of this type of fracture, and protoplast membranes could become very extended if stretched slowly. In order to find out just how extensible the bacterial protoplast membrane is, we placed protoplast suspensions in cellophane dialysis sacks and submerged them in cold distilled water or in dilute sucrose solution in large flasks. The systems were refrigerated overnight so that sucrose, the stabilizing solute, could diffuse slowly through the dialysis tubing, thereby reducing the osmolality of the protoplast suspension.

TABLE I

SWELLING AND BURSTING OF *B. megaterium* PROTOPLASTS DURING RAPID OR SLOW LOWERING OF AMBIENT SUCROSE OSMOLALITY

Protoplasts were initially prepared in 2.38 osmolal sucrose solution.

Procedure for lowering osmolality	*25% bursting osmolality*	*Average volume of survivors (μ^3)*
Direct transfer of protoplasts to dilute solutions	1.40	6.6
Dropwise addition of diluent over 45 min	0.38	10.7
Dialysis dilution	0.07	22.8

TABLE II

SURFACE AREAS OF PROTOPLASTS IN DILUTE SUCROSE SOLUTIONS

Procedure for exposing protoplasts to dilute solutions	*Final sucrose osmolality*	*Survivors (%)*	*Average surface area (μ^2)*	*Relative surface area (%)*
Dropwise addition of diluents over 45 min*	2.38	100	13	100
	0.56	80	21	161
	0.14	5	28	216
Dialysis dilution*	0.56	100	22	169
	0.40	99	28	216
	0.07	72	39	300
Enzymatic removal of cell wall in dilute sucrose solutions	0.54	100	36	277
	0.27	100	52	400

* Protoplasts were initially prepared in 2.38 osmolal sucrose solution.

We found that very few protoplasts burst during this procedure; in fact, it was difficult to dilute the suspensions sufficiently to obtain a reliable 25% bursting osmolality (Table I). The protoplasts swelled to enormous sizes during dialysis dilution, and calculations indicated that their surface areas increased by as much as 3-fold on the average without mechanical failure of the surface membrane (Table II).

When the walls of cells suspended in dilute sucrose solutions were removed slowly by lysozyme digestion, the emerging protoplasts were able to adapt to very hypotonic solutions and they could swell to even larger sizes than could protoplasts subjected to dialysis dilution (Table II). This result may indicate that mesosomal membranes were retained and incorporated into the protoplast surface membrane when protoplasts were released from nonplasmolyzed cells in dilute media, whereas they were lost, as described previously[22,23], when protoplasts were released from plasmolyzed cells in 2.38 osmolal sucrose solutions. We were unable to see mesosomes protruding from the surfaces of protoplasts prepared in 2.38 osmolal sucrose solution or in more dilute media. On the other hand, the results may indicate only that mechanical stress applied to the membrane during swelling differs in the two situations.

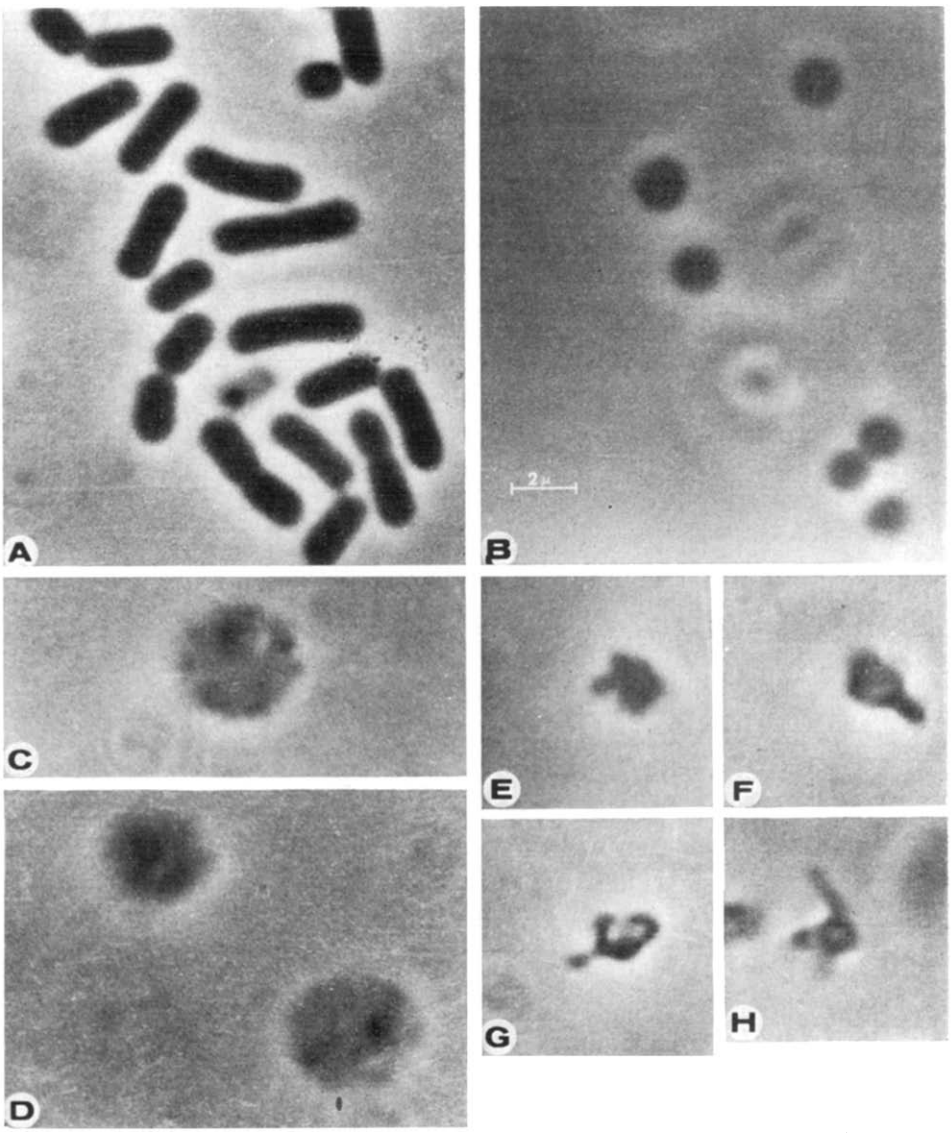

Fig. 5. Swelling and contraction of *B. megaterium* protoplasts. A. Whole cells. B. Protoplasts in 2.38 osmolal sucrose solution. C and D. Grossly swollen protoplasts transferred to 0.07 osmolal sucrose solution by dialysis dilution. E, F, G and H. Grossly swollen protoplasts which were made to contract by slowly transferring them to 2.38 osmolal sucrose solution. A phase-contrast microscope giving 1000 × magnification was used for all photography, and the bar indicating 2 μ applies to all of the photographs.

The grossly swollen protoplasts obtained by dialysis dilution could be readily distinguished microscopically from membrane ghosts. Swollen protoplasts were osmotically sensitive and could be made to contract by transferring them to concentrated sucrose solutions. Streamers or membrane tags which protrude from the protoplast surface were often seen (Fig. 5), and the contracted protoplasts generally were not

spherical. Grossly swollen protoplasts appeared faint in the phase microscope but appeared darker when they were made to contract. Membrane ghosts appeared even fainter and damaged; they did not appear dark in concentrated sucrose solutions. Certainly, it seemed that grossly swollen protoplasts must have retained some intracellular solutes. Unfortunately, centrifugation caused them to disintegrate, so that they could not be readily separated from their suspending medium.

It was difficult to size accurately grossly swollen protoplasts which had been made to contract because of the membrane tags mentioned above. However, rough estimates indicated that they were, if anything, larger in 2.38 osmolal sucrose solution than were freshly prepared protoplasts. Thus extensive swelling appeared to be accompanied by irreversible stretching of the membrane, since one would expect swollen-contracted protoplasts to be smaller than fresh protoplasts because of solute leakage during overnight storage.

Relaxing effects of glycerol and urea on protoplasts

Glycerol alone was not an effective stabilizer for protoplasts, but it did enhance the stabilizing capabilities of other solutes. An example is presented in Table III.

TABLE III

SPARING EFFECT OF GLYCEROL FOR OSMOTICALLY STRESSED PROTOPLASTS

Protoplasts prepared in 2.38 osmolal sucrose solution were transferred to the solutions indicated by slow, dropwise addition of diluent. The average initial volume of the protoplasts was 6.6 μ^3.

Final osmolality of medium into which protoplasts were transferred			*Number of protoplasts per ml* $\times 10^{-9}$	*Average volume* (μ^3)
Sucrose	*Glycerol*	*Total*		
0.89	0	0.89	1.52	9.3
0.89	0.43	1.32	1.80	15.3
0.89	0.85	1.74	2.56*	15.7
0.89	1.69	2.58	2.08*	17.3
0.89	3.38	4.27	2.40*	17.1

* Indicates no bursting during transfer.

TABLE IV

RELAXATION OF PROTOPLASTS EXPOSED TO UREA

Protoplasts were prepared in 2.38 osmolal sucrose solution and were transferred to the solutions indicated by slow, dropwise addition of diluents.

Sucrose osmolality of medium to which protoplasts were transferred	*Urea concn. in medium (M)*	*Number of protoplasts per ml* $\times 10^{-9}$	*Survivors* (%)	*Average volume* (μ^3)
2.38	0	8.60	100	5.1
2.38	3.0	9.08	106	5.7
0.89	0	5.24	61	9.5
0.89	1.5	6.12	71	11.7

Addition of glycerol to diluents spared protoplasts from bursting during gradual (45 min) lowering of the sucrose osmolality of their suspending medium from 2.38 to 0.89. Surprisingly, glycerol also caused the protoplasts to swell more than they would normally have done. This swelling effect, which was repeatedly observed, clearly was not an osmotic effect because, even if the protoplasts were highly permeable to glycerol, they should have been no larger than they were in glycerol-free media.

A similar effect occurred in stabilizing media to which urea was added (Table IV). Protoplasts were consistently larger in sucrose-*plus*-urea solutions than they were in solutions containing only sucrose at the same concentrations. Protoplasts burst when transferred to solutions containing only urea before they could be observed microscopically. Thus, both urea and glycerol seem to produce a relaxation of the protoplast membrane with concomitant reduction of protoplast sensitivity to osmotically induced membrane stress.

Effects of formaldehyde on protoplasts

Formaldehyde is a fixative commonly used for biological materials; it reacts with the various nitrogen-containing groups of proteins and can form intermolecular and intramolecular crosslinks (methylene bridges) of varying length between these groups[24]. Formaldehyde treatment increased the resistance of protoplasts to osmotic bursting (Table V) and appeared to have two distinguishable effects on membrane physical properties. Protoplasts suspended in concentrated sucrose solutions and exposed to 3% formaldehyde for relatively short periods of time (15 min) swelled to unusually large volumes when the osmolality of their suspending medium was subsequently lowered. For example, as indicated in Table V, protoplasts treated in this manner swelled to average volumes of 51.2 μ^3 (surface area of about 63 μ^2) during slow reduction of their environmental osmolality from 1.89 to 0.27. Maximum average volumes and surface areas for untreated protoplasts in 0.27 osmolal sucrose solution in any of our experiments, including those involving direct release of protoplasts into dilute sucrose solutions, were 35 μ^3 and 52 μ^2, respectively.

On the other hand, if formaldehyde treatment was prolonged, protoplasts became very resistant to osmotic swelling, and, as indicated in Table V, protoplasts treated with 3% formaldehyde for 120 min swelled from their initial average volume of

TABLE V

EFFECTS OF FORMALDEHYDE ON THE OSMOTIC RESPONSES OF *B. megaterium* PROTOPLASTS

Initially the treated suspensions contained $7.3 \cdot 10^9$ protoplasts per ml in 1.89 osmolal sucrose solution *plus* 3% formaldehyde. The average, initial protoplast volume before dilution was 4.9 μ^3.

Period of treatment with 3% formaldehyde (min)	*Protoplasts surviving slow reduction of ambient sucrose osmolality from 1.89 to 0.27 (%)*	*Average volume (μ^3)*
0	15	—
15	104	51.2
30	119	21.7
60	120	5.9
120	110	6.1

4.9 μ^3 to a final volume of only 6.1 μ^3 when the ambient osmolality was reduced from 1.89 to 0.27.

The experiments described here were carried out with unbuffered formaldehyde solutions, so that the final pH of the protoplast suspensions was about 5.8. When the formaldehyde solutions were buffered with phosphate, the time required for "fixation" was longer, but the two effects of formaldehyde were still evident.

DISCUSSION

The experimental results presented in this paper clearly show that the bacterial protoplast membrane can act as a differential dialysis membrane and discriminate among hydrophilic solutes in much the same manner as do cellophane membranes. CRAIG AND KONIGSBERG[25] found that the effective pore size of cellophane membranes can be increased by mechanical stretching and decreased by acetylation. Similarly, the permeability of protoplast membranes is altered by stretching, so that swollen protoplasts require larger osmotic stabilizing solutes than do unswollen ones.

CRAIG[20], CRAIG AND ANSEVIN[26] and CRAIG AND PULLEY[27] found that cellophane dialysis membranes, which discriminate among sugars such as sucrose, raffinose and stachyose on the basis of molecular size (or diffusion coefficient), were relatively non-selective for smaller sugars and amino acids. Likewise, membranes capable of discriminating among xylose, arabinose and glucose showed little selective permeability for amino acids. More highly acetylated, lower porosity membranes did discriminate among amino acids, and, surprisingly, molecular charge did not appear to have a major effect on the passage of amino acids through these acetylated membranes.

In our experiments the degree of membrane stretching could not be held constant because of osmotic swelling and shrinking. Protoplasts could discriminate among molecules ranging from raffinose to proline. But apparently the membrane could not be sufficiently compressed by osmotic withdrawal of cytoplasmic water to make it selective for molecules as small as β-alanine, sarcosine or glycine. Highly contracted protoplasts, which were osmotically stabilized by these small amino acids, remained permeable to water, to urea and to glycerol; although the permeability of the cells to these latter compounds may have been affected by their relaxing action on bacterial membranes.

The bursting response of protoplast populations suggested that the permeability of individuals to any particular stabilizing solute changed rather abruptly during swelling. Protoplasts in populations transferred to dilute solutions either burst during the transfer procedure or did not burst for many hours, or even days, after transfer. In fact, following the initial transfer to dilute media, protoplasts tended to shrink for a time and then to swell only after some hours. Thus, the series of events leading to osmotic bursting did not appear to include slow diffusion, over a period of hours, of solutes into the protoplasts. The most reasonable view of bursting seems to be that swelling protoplasts may become permeable to a particular stabilizing solute due to dilation of membrane pores. Then the resultant inflow of solute lowers the cytoplasmic water activity so that additional water enters the cell; more swelling ensues with further membrane stretching, further dilation of pores and consequent increases in permeability. As more and more water enters accompanied by solute, the process cascades and finally leads to bursting.

It is also reasonable, however, to ask why protoplasts burst at all when their membranes are stretched. Our findings regarding the extensibility of the protoplast membrane suggest that osmotic bursting is not interpretable in terms of the ultimate tensile strength of maximally stretched membranes. Rather, since ghost size and survivor size are closely related and since it is possible to prepare enormous protoplasts by dialysing away stabilizing solutes, it appears that osmotic bursting is due mainly, or exclusively, to brittle fracture of membranes which are not fully extended.

The susceptibility of many polymers to brittle fracture has been well documented; for example, fibers of poly(ethylene terephthalate) can be extended to as much as 5 times their initial length when the rate of stretching is 100% extension per min, but they fracture after only 15% extension when stretched at a rate of about 500% per min (ref. 28). Keratin fibers also show stress–relaxation during slow stretching, and the process in wool fibers is thought to involve disulfide interchange reactions[29].

Protoplast membranes seem to fracture because critical stresses develop in them during rapid stretching. KATCHALSKY *et al.*[3] showed that erythrocytes could adjust to solutions of lower tonicity if they were transferred slowly rather than rapidly, and so the erythrocyte plasma membrane appeared to have viscoelastic properties. They felt that hemolysis pores formed because of tangential stresses in stretched membranes, and they calculated that the red cell membrane increased in surface area only about 13% before fracturing, even when a dialysis dilution procedure was used for transfer. Later, RAND AND BURTON[1] also demonstrated that the erythrocyte membrane fractured after very little stretching, when the surface area had increased about 27%, but they felt that hemolysis occurred because of critical strains (increases in surface area) rather than critical stresses. In all, it appears that the erythrocyte plasma membrane has very limited extensibility in contrast to the bacterial protoplast membrane.

Limited swelling capacity may not be characteristic of all animal cells. For example, PATINKIN AND DOLJANSKI[30] found that Landschütz ascites cells and lymphocytes could increase in surface area by 275 and 180%, respectively. Since these cells have highly convoluted surface membranes, an increase in surface area may involve only unfolding of membranes with little or no stretching. The bacterial protoplasts that we used appeared to be devoid of mesosomes and membrane tags, and it was previously shown[5] that membrane tension is developed during osmotic swelling of these cells.

Although brittle fracture of protoplasts appears to be stress induced, we feel that dilation of membrane pores is primarily related to membrane strain and only secondarily to membrane stress. To assess the relationship between membrane strain and pore dilation, we considered protoplast populations in which 25% bursting had occurred, either in 0.18 osmolal raffinose solution or 1.26 osmolal glucose (see Fig. 2). The average surface areas of the protoplasts in these populations were calculated to be 35.1 and 18.7 μ^2, respectively, so that the ratio of surface areas is 35.1/18.7, or 1.88. We then considered that the cross-sectional area of a spherical molecule is a good indicator of the ease with which the molecule can diffuse into membrane pores and therefore of its ability to serve as an osmotic stabilizer. Cross-sectional areas of raffinose and glucose molecules, calculated from the data of SCHULTZ AND SOLOMON[18], are respectively 113 and 55 Å^2, so that the ratio of cross-sectional areas is 113/55, or 2.05. Consequently, it appeared that an 88% increase in surface area resulted in about a doubling of average, effective pore area, and pore dilation could have been the result simply of

pore stretching without any tearing of the membrane fabric. Essentially the same conclusion can be reached by comparing the appropriate ratios for protoplasts in sucrose solutions with those for protoplasts in either raffinose or glucose solutions.

Since grossly swollen protoplasts could be made to contract osmotically by slowly adding sucrose to their suspending medium across a dialysis membrane, they must have been impermeable to sucrose, at least in some degree. Apparently, stress–relaxation or viscous flow in the membrane led to contraction of the membrane pores. Presumably the pores never closed completely because the protoplasts remained permeable to water. In fact, it has recently been suggested[31] that pores with effective radii of about 4 Å may be fundamental structural features of most biological membranes.

In all, the physical properties of protoplast membranes described in this paper, especially their extensibility and their susceptibility to brittle fracture, indicate that the basic mechanically important membrane component is protein. This view is supported by the finding that urea and glycerol induce relaxation of the membrane. It is difficult, though, to speculate on the reactions involved in this relaxation because of current uncertainty as to whether these compounds, which are protein denaturants, affect protein structure by disrupting protein–protein hydrogen bonds[32] or by interfering with hydrophobic bonding[33,34]. The relaxing and strengthening effects of formaldehyde on protoplasts also point to proteins as the main mechanical structures of the membrane.

Individual protein fibers and microfibrils have been found[35] to have a wide range of extensibilities; for example, silk fibers are practically inextensible, whereas elastin fibers can be stretched without fracture to somewhat more than twice their native length. However, our results show that untreated protoplast membranes can increase in surface area by at least 300 %, *e.g.*, when protoplasts swelled from the highly contracted volume of 5 μ^3 (surface area of about 13 μ^2) to volumes as large as 23 μ^3 (surface area of about 39 μ^2). This unusually high degree of extensibility suggests that membrane proteins could be in the form of a net or meshwork which would have greater capacity to stretch without breaking than would single protein fibers or microfibrils. Further, the susceptibility of the membrane to brittle fracture during rapid stretching is reminiscent of the behavior of collagen fibers[36], in which mechanical stress can induce crystallization in microfibrils with resulting brittleness.

On the basis of our results, presented here and elsewhere[4–6], we can put together a view, albeit a somewhat speculative one, of the events leading to osmotic bursting of bacterial protoplasts. When protoplasts are placed in hypotonic media, they swell due to influx of water, their membranes become stretched and membrane pores are dilated. If the pores become large enough to admit stabilizing solutes, the sort of cascading process described earlier could occur with rapid swelling of the cells. During this rapid swelling, mechanical stresses may become sufficiently great to cause brittle fracture of the membrane, release of intracellular solutes and relief of membrane tension. In our experiments, the empty, membranous ghosts did not reseal to yield closed vesicles.

Thus, osmotic bursting of bacterial protoplasts differs in many respects from hemolysis of erythrocytes, and it appears that the bacterial protoplast membrane is physically different from the erythrocyte membrane despite their similar appearances in electron micrographs.

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