Osmotic Behavior of Bacterial Protoplasts:
Temperature Effects

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Among protoplasts released from cells of Bacillus megaterium grown at 20, 30, or 37 C, osmotic swelling in NaCl solution at a given external osmotic pressure was greatest for protoplasts from cells grown at 20 C and least for protoplasts from cells grown at 37 C. Protoplasts from cells grown at lower temperatures were also less stable to osmotic shock and lysed at higher external osmotic pressures than did protoplasts from cells grown at higher temperatures. But for cells grown at any one temperature, osmotic stabilization was itself temperature dependent so that the higher the ambient incubation temperature, the higher the osmotic pressure needed to prevent lysis of a given fraction of the input protoplast population. However, comparison of the osmotic stability of protoplasts from cells grown at different temperatures at various ambient incubation temperatures revealed that, except at 5 C where no differences were discerned, protoplasts from cells grown at lower temperatures still lysed at higher osmotic pressures than did those from cells grown at higher temperatures. The apparent internal osmolality (28 to 31 atm) did not vary significantly among whole cells from the three growth temperatures. Therefore, the observed differences in osmotic behavior could not be attributed to changes in internal osmotic pressure. Rather, it seemed likely that the differences were due to changes in membrane properties.

Bacterial protoplasts are osmotically active membrane-bound bodies which shrink, swell, or lyse in response to forces exerted on them by the fluid in which they are suspended. Within moderate ranges of external osmotic pressure, protoplasts behave as perfect osmometers (11, 16) and follow the Lucké and McCutcheon (15) modification of the van't Hoff-Boyle equation: $\Pi(V - V_o) = k$, where $\Pi$ is the osmotic pressure of the surrounding medium, $V$ is the total cell volume, $V_o$ is the osmotically inactive volume, and $k$ is a constant. Protoplasts are analogous to mechanical osmometers in that their response to osmotic forces is determined by the interaction of the three components of the osmometer system: the internal phase, the external phase, and the intervening membrane barrier.

Most previous studies have been concerned with the effects of perturbations in the suspending medium, or external phase. Incubation temperatures of 50 C or more (20), hydrogen ion concentration (19), surface tension (21), and stabilizing solute size and conformation (5, 24) are all important external factors which influence the osmotic behavior of protoplasts. Relatively little is known about the osmotic properties of the cell cytoplasm, or internal phase. However, by use of water vapor equilibrium studies, Mitchell and Moyle (18) and Bateman et al. (2) have estimated cellular internal osmotic pressures. Gram-negative bacteria (Escherichia coli, Serratia marcescens) had apparent internal osmotic pressures from 4 to 7 atm, which were low compared to the 20 to 30 atm values obtained for the one gram-positive bacterium investigated (Staphylococcus aureus). We took a more direct approach to determine an apparent internal osmotic pressure of the gram-positive rod, Bacillus megaterium.

The selectively permeable membrane which separates the internal compartment from the external compartment constitutes the third part of the osmometer. Membrane permeability and physical properties play major roles in the responses of bacterial protoplasts to osmotic stress. The limiting membrane regulates swelling and lysis in hypotonic media in that, in a mechanical fashion, swelling appears to in-
crease the effective membrane "pore" size. If the membrane stretches enough to permit stabilizing solute molecules to enter the protoplast, there is a rapid influx of solutes and water which results in rapid stretching of the membrane and subsequent lysis of the protoplast (5).

The chemical composition of microbial cells, including their limiting membranes, is known to be dramatically affected by changes in culture growth temperature. For example, the lipids of *B. megaterium* contain relatively more saturated fatty acids when the cells are grown at 30°C than when they are grown at 23°C (9). Changes in membrane stability or membrane permeability in other cells grown at elevated temperatures have been related to increased saturation of fatty acids in these cells (4, 13).

Temperature-induced changes in membrane composition which affect mechanical strength or permeability, or both, should alter the osmotic behavior of bacterial protoplasts. Indeed, the osmotic behavior of protoplasts from *B. megaterium* was found to be altered by changes in growth temperature. Protoplasts isolated from cells grown at higher temperatures were generally more resistant to osmotically induced swelling and lysis than were those isolated from cells grown at lower temperatures. Since the apparent internal osmotic pressure of the cells was unaffected by growth temperature, membrane changes appeared to be responsible for the observed alterations in osmotic behavior.

**MATERIALS AND METHODS**

**Organism and growth conditions.** Cells of *B. megaterium* strain KM were grown in 2% Oxoid peptone (Flow Laboratories, Rockville, Md.), pH 7.0, with vigorous aeration. Cells were grown at 20, 30, or 37°C to the phase of declining growth rate. Under these conditions, the cells did not form spores. Growth was monitored by means of a Spectronic 20 spectrophotometer (Bausch and Lomb) at 700 nm, equipped with 1.1-cm light path cuvettes, with distilled water used as a blank. An optical density of 2.0 corresponded to 0.98, 1.00, and 0.95 mg of cells (dry wt)/ml for cells grown at 20, 30, and 37°C, respectively. Cells were harvested by centrifugation when they reached a concentration between 1.1 and 1.4 mg of cells (dry wt)/ml.

**Preparation of protoplasts.** Cells harvested as described were suspended in 1.016 osmolal (0.771 M) sucrose solution to a concentration of 5.0 mg of cells (dry wt)/ml. N-acetylmuramidase glucanohydrolase (EC 3.2.1.17; lysozyme; Sigma Chemical Co., St. Louis) was then added to a final concentration of 200 μg/mg of cells (dry wt). Lysozyme was added in buffered solution (0.01 M sodium phosphate, pH 7.0) which contained 25 mg of lysozyme/ml. At room temperature, protoplast release was completed in 20 min, as judged by the change from cylindrical to spherical cell shape seen by phase-contrast microscopy. These suspensions are referred to as primary protoplast suspensions.

**Counting procedures.** Colony-forming units were enumerated by the spread plate technique. Chilled 1.2% peptone (Difco Labs, Detroit) was used as the diluent. There were 3.7 x 10⁴, 3.2 x 10⁴, and 4.8 x 10⁴ colony-forming units per mg of cells (dry wt) for cells grown at 20, 30, and 37°C, respectively.

Total cell numbers were counted after fixation in 6% (vol/vol) formaldehyde (Fisher Scientific Co., Fair Lawn, N.J.). Cells were then counted in a Petroff-Hauser counting chamber. A Leitz-Ortholux phase-contrast microscope was used for all direct counts. A magnification of 970 diameters was used for counting whole cells. Under these conditions, cross walls between cell pairs were readily observed. For each of four batches of cells, the counting chamber was filled four times, and at least 200 cells were counted for each chamber filling. There were 6.2 x 10⁴, 5.9 x 10⁴, and 7.0 x 10⁴ cells per mg of cells (dry wt) for cells grown at 20, 30, and 37°C, respectively. Protoplasts were counted in a similar fashion. After fixation in 6% (vol/vol) formaldehyde, protoplasts were counted at a magnification of 1,600 diameters. The counting chamber was refilled at least twice for at least three batches of cells grown at each of the three temperatures. Over 100 protoplasts were counted per chamber filling except where lysis was nearly complete and few protoplasts were available for enumeration.

**Assessment of osmotic behavior.** Osmotic swelling and shrinking of protoplasts were measured by a modification of the procedure of Marquis (16). At room temperature, 0.5 ml of primary protoplast suspension was added to 4.5 ml of various NaCl solutions of known osmotic pressures. Unless otherwise stated, after 30 min at room temperature the optical densities at 650 nm (absorbancy, *A*₆₅₀) of the resultant protoplast suspensions were measured in a Spectronic 20 spectrophotometer. For a given sample of protoplasts, the decrease in *A*₆₅₀ as a function of decreasing osmolality of the suspending medium was taken to reflect an increase in protoplast volume. For study of osmotically induced lysis, the suspensions were incubated for 30 min at 5, 20, 30, or 37°C, and the number of remaining protoplasts was assessed by direct count.

**Determination of apparent internal osmotic pressure.** Cells were suspended in 0.01 M sodium phosphate buffer, pH 7.0, to a concentration of 20 to 25 mg of cells (dry wt) per ml of suspension and treated with 1 mg of lysozyme per ml of suspension until lysis was complete. This crude lysate was then centrifuged at 30,000 x g for 30 min to remove large particulate matter, primarily membrane fragments and nucleic acids which adhere to the membrane. After appropriate dilution in 0.01 M sodium phosphate buffer (pH 7.0), the osmotic pressure of the supernatant fluid was determined by use of a freezing-point osmometer (Advanced Instruments, Inc., Needham Heights, Mass.) which was calibrated to give readout in milliosmoles per kilogram of solvent. The resultant osmotic pressure values were corrected for...
the contributions from spent medium components, lysozyme, solubilized cell wall material, and buffer.

**Osmotic pressure and osmolality.** Osmotic pressure, \( \Pi \), is often expressed by the equation: 
\[
\Pi = \nu RTc \phi,
\]
where \( \nu \) is the number of particles (ions) produced by the dissociation of the solute, \( T \) is the absolute temperature, \( c \) is the solute concentration, and \( \phi \) is the osmotic coefficient of the solute. The coefficient, \( \phi \), corrects for deviations from ideality. Osmolality is equivalent to molality times \( \phi \). A solution containing 1 osmol/kg has an osmotic pressure of 22.4 atm at 25 C. Osmotic pressures of NaCl or sucrose solutions were calculated from data in a *Handbook of Chemistry and Physics*. Osmotic coefficients were those given by Scatchard, Hamer, and Wood (23).

**RESULTS**

**Osmotic swelling and shrinking.** Protoplast volume is inversely proportional to optical density (11, 16). Hence, high value of the reciprocal of optical density is indicative of large protoplast volume. When transferred to hypertonic NaCl solutions, protoplasts swelled proportionately as the osmotic pressure of their suspending medium was lowered (Fig. 1). Relatively short incubation periods, up to 90 min, at room temperature did not markedly alter the character of the swelling response at moderate osmotic pressures. Swelling in solutions of high osmotic pressure, 2.86 to 4.00 osmolar NaCl (0.35 to 0.25 osmolar\(^{-1} \)), may have been enhanced by increased incubation time. In this range, the extent of protoplast swelling appeared to be greater after 90 min of incubation than after only 5 min, but the absolute changes in \( A_{450} \) were small and of questionable significance. Beyond this initial region, swelling was not much different unless the incubation was greatly prolonged. After 240 min, swelling in osmotic concentrations below 2.22 osmolar (above 0.45 osmolar\(^{-1} \)), was so extensive that the protoplasts lysed. Consequently, in all further experiments an incubation time of 30 min was used.

The growth temperature of the parental culture influenced the osmotically induced swelling of protoplasts suspended in NaCl solutions at room temperature (Fig. 2). Swelling profiles over a wide range of osmotic pressures showed the similar trend of increasing reciprocal optical density \( (1/A_{450}) \) as a function of decreasing osmotic pressure. Protoplasts derived from cells grown at 20 or 30 C reached a minimal volume, i.e., minimum \( 1/A_{450} \), at an osmotic pressure between 3.3 and 4.1 osmolar NaCl (between 71 and 92 atm). Protoplasts obtained from cells grown at 37 C did not reach a minimal volume in the range of osmotic pressures investigated. Apparently, protoplasts from cells grown at 20 C swelled to a much greater extent in NaCl solutions than did protoplasts liberated from cells grown at the higher temperatures. Swelling of protoplasts from cells grown at 30 or 37 C did not appear significantly different. Over the range in which the swelling response appeared linear (2.34 to 1.09 osmolar or 0.427 to 0.917 osmolar\(^{-1} \)), the slopes of the least-squares linear regression lines, in units equivalent to volume increase per osmotic pressure decrease,
were 1.15, 0.64, and 0.55, respectively, for protoplasts obtained from cells grown at 20, 30, and 37°C. Direct counts indicated that little or no lysis occurred over the range of osmotic pressures investigated here.

**Osmotic lysis.** The temperature at which the protoplast suspensions were incubated profoundly affected the threshold for the onset of osmotic lysis (Fig. 3). In all cases, protoplasts were much more sensitive to osmotic lysis in suspending media at 37°C than in suspending media at 5°C. The osmolality required to stabilize 50% of the input protoplasts, herein referred to as the S_{50} value, increased markedly with increasing incubation temperature (Table 1). For protoplasts from cells grown at 20°C, the S_{50} values increased from 0.592–0.704 to 0.806–0.957 as the incubation temperature increased from 5–20 to 30–37°C. The responses of protoplasts from cells grown at 30 or 37°C were influenced in a similar fashion by increasing incubation temperatures (Table 1; Fig. 4). Furthermore, at any given incubation temperature above 5°C, increases in growth temperature of the parental culture decreased the S_{50} values, i.e., protoplasts from cells grown at a high temperature were less fragile than protoplasts isolated from cells grown at a low temperature. For example, at an incubation temperature of 30°C (Fig. 4C) protoplasts from cells grown at 20°C had an S_{50} value of 0.806 osmolar (1.24 osmolar⁻¹), whereas protoplasts from cells grown at 30°C had an S_{50} value of 0.725 osmolar (1.38 osmolar⁻¹), and those from cells grown at 37°C required only 0.690 osmolar (1.45 osmolar⁻¹) to stabilize 50% of the input protoplasts. Protoplasts were most stable at low incubation temperatures and least stable at high incubation temperatures, regardless of the temperature at which the parental culture had been grown.

**Apparent internal osmotic pressure.** The variations in osmotic behavior could have resulted from differences in the internal osmotic pressures of the protoplasts from cells grown at different temperatures. Thus, it was necessary to determine apparent internal osmotic pressures of cells grown at each temperature. Cell lysates were prepared as described and their osmotic pressures were measured at various dilutions. To determine the osmotic pressure in the lysate attributable to internal cell solutes, a series of corrections for the contributions of contaminating materials had to be applied to the raw values. (Detailed information concerning absolute osmotic pressure values and corrections are available from the authors.)

According to Scherrer and Gerhardt (24), the intercellular space in a pellet of B. megaterium cells is 21.6% of the total pellet volume. In our system, spent medium that had been trapped in this space contributed 2.4% of the raw osmotic pressure value. Similarly the correction factor for the buffer plus lysozyme solution was 38.4%. To assess the contribution of cell wall degradation products to the raw lysate, the osmotic pressure of lysozyme-degraded wall was measured and found to show a linear relationship to wall concentration. Lysozyme degradation of 1 g of walls in 1 kg of solution would result in the release of 1.44 mosmol of solutes. In our system, then, wall degradation products accounted for 8.6% of the observed osmotic pressure. After these corrections were applied, the contribution of internal cell solutes was 50.6% of the osmotic pressure of the raw lysate.

Least-squares regression analysis revealed a linear relationship between the corrected osmotic pressures and the original cell concentrations (Fig. 5). In milliosmoles per gram of original cell dry weight (± one standard deviation), the values for cells grown at 20, 30, and 37°C were 1.64 ± 0.09, 1.80 ± 0.11, and 1.67 ± 0.15.

### Table 1. Influence of temperature on protoplast stability in NaCl solutions

<table>
<thead>
<tr>
<th>Incubation temp (°C)</th>
<th>S_{50} (mosmol/kg)</th>
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<tr>
<td>5</td>
<td>592</td>
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<td>20</td>
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<tr>
<td>30°C</td>
<td>667</td>
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<tr>
<td>37°C</td>
<td>725</td>
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* Concentration of NaCl that will stabilize 50% of an input protoplast population.

* Growth temperature.

![Figure 3](http://jb.asm.org/Downloadedfrom)
PROTOPLASTS AND TEMPERATURE

Fig. 4. Influence of growth temperature on protoplast lysis at four incubation temperatures. Growth temperatures were 20 (O), 30 (□), or 37 C (△). Incubation temperatures of 5, 20, 30, and 37 C are represented in parts A, B, C, and D, respectively.

Fig. 5. Least-square regression lines relating the apparent internal osmotic pressure to the relative concentration of cytoplasmic solutes in cell lysates. Symbols: O, cells grown at 20 C, means of determinations on 5 batches of cells; □, cells grown at 30 C, individual values from 5 batches of cells; △, cells grown at 37 C, means of determinations on 4 batches of cells. Bars represent ±1 standard deviation from the means obtained from determinations on the cells grown at 37 C.

respectively. Student's t test of the data indicated that the observed differences were not statistically significant.

With the assumption that growth temperature does not markedly influence the water content of the cells, the above values can be used to calculate apparent internal osmotic pressures. According to Gerhardt and Judge (10), intact B. megaterium cells are, by weight, 78.5% water and 21.5% dry material. Salton's (22) figures indicate that roughly 20% of the dry weight is due to cell wall components. Hydrated walls swell to an extent such that 92.1% of their wet weight is due to water (10). A 4.65 g wet weight quantity of cells will contain 1 g of dry matter, of which 0.2 g is wall material and 0.8 g is protoplast components. Of the 3.65 g of water in the wet pellet, 2.33 g is cell wall water and only 1.32 g is protoplast water. It was assumed that the water of hydration associated with the membrane was negligible. Hence, the internal or protoplast water is 62.0% of the protoplast wet weight and is equivalent to 1.32 times the whole cell dry weight. Consequently, the osmoles of internal cell solutes from 1 kg of cell dry matter would be contained in 1.32 kg of protoplast water. Or, the internal osmoles per kilogram of protoplast water will be 75.7% of the internal osmoles provided by a kilogram of cell dry material. The above values for cells grown at 20, 30, and 37 C then give, respectively, 1.24, 1.36, and 1.26 osmol/kg of protoplast water,
which correspond to internal osmotic pressures of 27.8, 30.5, and 28.2 atm. These values are somewhat higher than the range of 0.5 to 1.0 osmol/kg (11.2 to 22.4 atm) reported for *B. megaterium* (16) but are in agreement with 20 to 30 atm generally quoted for gram-positive bacteria (18).

**DISCUSSION**

Protoplasts from *B. megaterium* cells grown at a given temperature were less stable to osmotic shock at higher ambient incubation temperatures than at lower ones (Table 1). Abrams (1) has reported that protoplasts of *Streptococcus faecalis* swell in the presence of a metabolizable substrate. At low substrate concentrations the swelling was reversible; at high concentrations the protoplasts swelled and lysed. The swelling response was eliminated by decreasing the concentration of K\(^+\) and con-comitantly increasing the Na\(^+\) concentration. Because we used sodium salts in the suspending medium and because the amount of residual sucrose in any of the test solutions was low, it seems unlikely that metabolic swelling contributed significantly to the observed results.

Protoplasts from cells grown at higher temperatures were more stable regardless of the incubation temperature (Fig. 4), with the sole exception of 5°C. Thus, growth temperature did influence the capacity of cells to respond to changes in environmental osmotic pressure. Because the apparent internal osmotic pressure did not vary with growth temperature (Fig. 5), it appears that membrane changes were responsible for the observed differences in osmotic behavior. The fundamental membrane changes that underlie these behavioral variations are probably chemical and result in alterations in membrane permeability or mechanical strength, or both.

It is well known from studies of a large number of bacterial species that the composition of lipids is indeed altered by variations in the growth temperature (6, 8, 9, 13, 25). In general, higher growth temperatures result in greater saturation of phospholipid fatty acids (4, 6, 13) which tends to decrease membrane permeability (7). For example, from lipid studies with *E. coli*, Haest et al. (13) concluded that when the growth temperature is elevated, bacteria compensate for the thermodynamic increase in permeability by reducing the degree of unsaturation of the phospholipid side chains.

Such lipid changes may also increase the mechanical strength of the membrane. According to Chang and Matson (4), Arrhenius plots of yeast cell thermostability revealed that transition points, indicative of phase changes, occurred at lower temperatures when the cells were grown at 26°C than when they were grown at 40°C. The yeasts grown at the higher temperature had more saturated fatty acids which would allow closer packing of membrane lipid residues. Consequently, they proposed that the effective bonding in the membrane was increased, resulting in a structure that was more stable to thermal stress.

Possible contributions of membrane proteins to the control of diffusive permeability have largely been ignored. Apparently no study reporting correlations between lipid changes and permeability changes has also included an examination of possible changes in membrane proteins. In fact, most of the evidence comes from work with liposomes formed solely of lipid material extracted from the cells. However, membrane stretching during osmotic swelling does influence the behavior of protoplasts through the development of membrane tension (16) and by increasing the effective porosity of the membrane (5). Reagents such as formaldehyde that normally cross-link proteins profoundly influence protoplast swelling, as does urea, a protein denaturant which effects a relaxation of membrane structure. Therefore, Corner and Marquis (5) suggested that protein network was the major structural component of the protoplast membrane. Ultrastructural evidence for this view was reported by Grula et al. (12), who found that extraction of all the lipids from the protoplast membrane left intact a protein meshwork which retained the typical "unit-membrane" appearance. A recently proposed model for biological membranes in general embodies the principle of an underlying protein network backbone (26).

It may also be that lipid changes per se are not the sole governing factor in temperature-induced permeability changes. For example, the lipids in the membrane might function not only as part of the permeability barrier but also as plasticizers of the protein network. Plasticizers are small molecules commonly added to synthetic polymers to improve various properties such as flow or drawing. The action of both urea and glycerol in effecting membrane relaxation has been likened to polymer plasticization (5), as have the actions of many small molecules on collagen (17). Presumptive evidence that lipids do behave as plasticizers can be obtained from the work of Blais and Geil (3). They examined, in the electron microscope, the ability of the erythrocyte membrane to deform under direct stretching. Normal membranes could be deformed into long fibers, whereas
lipid-depleted membranes lost this ability and deformed by tearing rather than by drawing into fibers. If lipids were to function as plasticizers, changes in the lipids would certainly alter the response of the membrane to stretching and, ultimately, would alter permeability.

The prevailing view, then, is that lipids control the permeability of cells and, therefore, temperature-induced changes in the lipid components are responsible for the differences in permeability manifested by cells grown at different temperatures. The results reported here are consistent with this idea because the higher the growth temperature, the more saturated the fatty acid residues in cells of B. megaterium (9). However, it should be pointed out that the role of lipids may be less direct than presently thought and that protein changes may make an important contribution to the observed permeability changes. Resolution of these questions awaits detailed chemical analyses of both protein and lipid changes in membranes from cells grown at different temperatures.

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LITERATURE CITED