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Osmotic Pressure in *Escherichia coli* as Rendered Detectable by Lysozyme Attack

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The enhanced susceptibility of plasmolyzed *Escherichia coli* to lysozyme attack was used to estimate the internal osmotic pressure of these cells under various conditions. Differences were detected between strains, culture media, stages in the growth cycle, and the osmotically active material used to produce plasmolysis. Lysozyme also was found to attack unplasmolyzed cells at 0 C and between 50 and 70 C.

The osmotic pressure of bacteria is believed to afford a measure of the concentration of small, free solute molecules contained within the semipermeable plasma membrane. This concentration helps establish the internal environment in which the chemistry of the cell takes place. Moreover, the differential osmotic pressure, that is, the difference between the osmotic pressure in the cell and that of the medium, will determine how firmly the plasma membrane is pressed against the rigid peptidoglycan layer. This differential osmotic pressure thereby may affect one or more of the several membrane-related activities.

Studies on the osmotic properties of *Escherichia coli* generally have involved varying the osmotic pressure of the medium and observing the response of the cells. Envelopes of *E. coli* cells are subject to alteration by osmotic stress. Hypotonicity leads to the release of small metabolites (11) and several envelope-bound enzymes (8) as well as to the formation of finger-like protrusions (2) and, perhaps, lysis (12). Hypertonicity leads to plasmolysis during which the plasma membrane shrinks away from the rigid peptidoglycan layer (5, 13). The osmotic pressure of the medium at which plasmolysis first becomes microscopically evident generally is cited as the internal osmotic pressure of the cell. It is tacitly assumed that the plasma membrane is free to shrink away from the cell envelope, at least in some regions. Values so obtained range from 2 atm for resting cells to 15 atm for rapidly growing cells (10). It is, of course, difficult and tedious to quantitate the prevalence of plasmolysis for large numbers of cells by microscope examination. Furthermore, several of the studies on plasmolysis have involved washed cells from which many osmotically active particles probably had been removed. Hence, the osmotic pressure of *E. coli* should not be considered a well-determined parameter.

Osmotic injury in the form of plasmolysis renders *E. coli* susceptible to lysozyme attack (3). Subsequent dilution of such cells in a hypotonic medium can result in the formation of spheroplasts or cell lysis. Lysis, in turn, is easily monitored by turbidity measurements. While this method is commonly utilized in the preparation of protoplasts or gently lysed cells, little attention has been given to the possibility of using the same technique to measure the internal osmotic pressure of *E. coli* or as an assay for certain injuries, osmotic or other, to the cell envelope. Thermal damage, for instance, would seem to be a likely candidate for detection. Thermal inactivation of cells is believed by some to involve a leaky plasma membrane (12), and microscopic observations have shown heat-induced alterations in the appearance of the outer membrane (6). Moreover, one might anticipate a role for internal osmotic pressure in thermal inactivation to the extent that outward pressure on a thermally weakened envelope effects irreversible changes

Osmotic pressure in *E. coli* cells subjected to various conditions and assayed by susceptibility to lysozyme attack are reported here. Differences were found between strains, culture media, stages in the growth cycle, and osmotically active materials used in the assay. Investigation of the effect of lysozyme on heated cells indicated an increased susceptibility to attack

between 50 and 70 C, in addition to which evidence was found for damage to the envelopes of some cells when placed at 0 C.

MATERIALS AND METHODS

E. coli strains B/r, B_{s-1}, and W3110 obtained from Stanley Person were grown at 37 C in 10, 15, or 25 ml of medium bubbled with air. Growth media used were nutrient broth (Difco) at 8 g/liter and Roberts C-minimal medium with 5% glucose (11).

Microscope observations were carried out with a Bausch and Lomb bright-field phase-contrast microscope at a magnification of ×900.

Osmotic effects. Samples (5 ml) of cultures in exponential phase were placed in 12-ml heavy-wall centrifuge tubes (Sorvall). The initial absorbance (~0.2) of the suspensions in these tubes was determined at 425 nm with a Bausch and Lomb spectrophotometer, model 20, after which the cells were centrifuged at 8,000 × *g* at room temperature for 3 min. The supernatant fluid was decanted and the inside walls of the tubes were blotted to remove most of the remaining liquid. The tubes were than placed in a bath at the appropriate temperature. Room temperature was approximately 25 C; a stable 11 C bath was obtained with running cold tap water; and the 0 C bath consisted of an ice slurry.

Two to three minutes were allowed for the pellet to reach the desired temperature. The desired sucrose or salt solution (0.1 ml), maintained at the same temperature as the pellet, was added to the tubes, and the pellets were suspended by a few seconds of shaking on a Vortex mixer. A 0.1-ml sample of egg white lysozyme [56 μg/ml in 0.0005 M tris (hydroxymethyl)aminomethane (Tris) at pH 7.2] was added and the tube was reshaken. After an additional 30 to 60 s in the bath the tubes were removed and the cells were diluted. This dilution was accomplished with 5 ml of nutrient broth at 25 C for cells grown in nutrient broth and with 5 ml of double-distilled water for cells grown in the C-minimal medium. The absorbance was measured immediately following dilution and again after 5 to 10 min. It was found that little change occurred after 5 min. Results were expressed in terms of the ratio of final absorbance/initial absorbance which, multiplied by 100, is per cent initial absorbance. The mean values of 3 to 8 separate measurements were plotted as a function of solute concentration along with error bars indicating estimates of the standard deviation of the mean (16).

One departure from this procedure began with cells grown in nutrient broth to an initial absorbance of ~0.65 when the osmotic pressure of cells from cultures after the exponential phase was measured. For another set of experiments cells grown in C-minimal medium were washed twice with double-distilled water before being subjected to sucrose and lysozyme. The initial absorbance was measured prior to pelleting the cells after the second suspension in water. The lysozyme applied to these washed cells was dissolved in 0.01 M Tris at pH 7.2.

High-temperature effects. Cells to be subjected to high temperature were grown in C-minimal salts to a concentration of 1.5 to 2 × 10^8 cells/ml (absorbance ≃0.4). A 2-ml amount of this suspension was added to a preincubated test tube containing 8 ml of C-minimal medium lacking the $MgCl_2$ and Na_2SO_4 but with lysozyme and ethylenediaminetetracetic acid (EDTA) added to give final concentrations of 56 μg/ml and 4%, respectively. The tubes were removed at intervals from the bath and the absorbance was measured at 425 nm. The first readings were made 20 s after adding the cells to the hot medium and these served as the values for initial absorbance. The measurement process required removing the tubes from the bath for about 15 s per reading.

RESULTS

Osmotic pressure and differential pressure. The decrease in absorbance of lysozyme-treated *E. coli* B/r after subjection to various concentrations of sucrose at room temperature (~25 C) is indicated in Fig. 1. Figure 2 shows the two positions in the growth cycle from which samples were taken. Plasmolysis and lysis first became evident in exponential phase cells at about 0.2 M sucrose and by 0.5 M sucrose the maximum amount of lysis had been attained. These results agreed with those reported by Birdsell and Cota-Robles (3), who used a similar technique, as well as with those of Scheie (13) who noted the percentage of cells plasmolyzed by counting under a microscope. The residual absorbance, even at high concentrations of sucrose, might be accounted for in one or more ways. Ghosts of either cells or spheroplasts contributed a small amount to the absorbance. On the other hand, up to 20% of the cells may have been resistant to plasmolysis; or, it is possible that some of the cells were permeable to the sucrose after lysozyme attack and did not lyse; or perhaps the outer portions of the cell envelope on some of the cells, even if plasmolyzed, were impermeable to lysozyme and, consequently, the lysozyme still could not reach its substrate. Microscope observation of exponential-phase cells treated with 0.8 M sucrose and lysozyme indicated the presence of both rods and spheres after dilution into nutrient broth; hence, more than one of the above must have been involved.

E. coli B/r cells from cultures after exponential phase were less susceptible to plasmolysis and lysozyme attack for a given concentration of sucrose. Fifty percent lysis was not exhibited until 1.0 M sucrose was used, at which point the maximum amount of lysis probably had not been reached.

Results such as those in Fig. 1 make it clear that all cells in a culture did not have the same osmotic pressure. Nevertheless, there is some

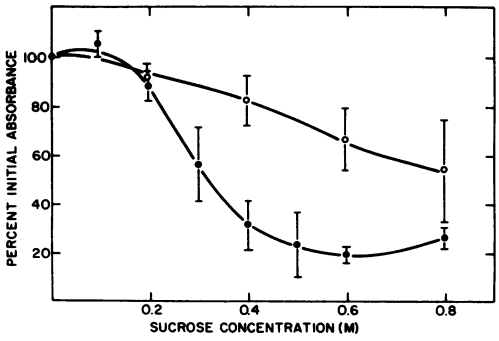

FIG. 1. *Lysozyme-produced change in absorbance for cells of E. coli B/r grown in nutrient broth and treated at 25 C with various concentrations of sucrose. Initial absorbance of 0.2 (●) and 0.65 (○).*

advantage to selecting some single value for comparative purposes. Traditionally, the sucrose concentration at which plasmolysis is observed was used but may not be the most appropriate value. A more suitable choice would be the osmotic pressure of the sucrose solution at which there was a 50% decrease in final absorbance. From Fig. 1 this would correspond to 0.31 M sucrose, or 7.6 atm (1) for exponential phase *E. coli* B/r. Considering that the medium in which these cells were grown had an osmotic pressure of about 30 mosmol, or 0.7 atm (13), it follows that the differential pressure (the difference between internal and external osmotic pressure) in these cells grown in nutrient broth was approximately 6.9 atm.

By the same criterion, cells with an initial absorbance of 0.65 had an osmotic pressure of almost 35 atm. Microscope observation of these cells after subjection to 1 M sucrose confirmed that many cells were not plasmolyzed and that the plasmolysis in evidence was not as severe as that produced in exponential-phase cells with 1 M sucrose (14). Rods, spheres, and ghosts of both were visible after dilution.

Similar data for strains B_{s-1} and W3110 in exponential phase are presented in Fig. 3. The osmotic pressure, using the above criterion, in B_{s-1} was 0.21 M sucrose or 5.0 atm and that in W3110 was 0.41 M sucrose or 10.5 atm. It may be significant that strain W3110 cells normally appeared to have a smaller cross section than either B/r or B_{s-1} cells; thus their cytoplasm may have been more concentrated.

In Fig. 4 data are shown for strain B/r grown in Roberts C-minimal medium and for strain B/r grown in C-minimal medium but washed twice before being subjected to sucrose and lysozyme. The cells grown in C-minimal medium had an osmotic pressure of 17 atm (0.6 M sucrose), more than twice that of cells grown in nutrient broth. Since C-minimal medium has an osmotic pressure of about 7.2 atm (13), the differential pressure was almost 10 atm. This is 40% higher than the differential pressure for the same cells grown in nutrient broth. Washed cells, on the other hand, showed a markedly lower osmotic pressure, which illustrates one of the hazards of obtaining information of an osmotic nature from washed cells.

A decrease in the final absorbance of cells grown in C-minimal medium was observed even with the sucrose treatment deleted. This is believed to have been due to a hypotonic osmotic shock, brought about when the cells were resuspended in the lysozyme medium,

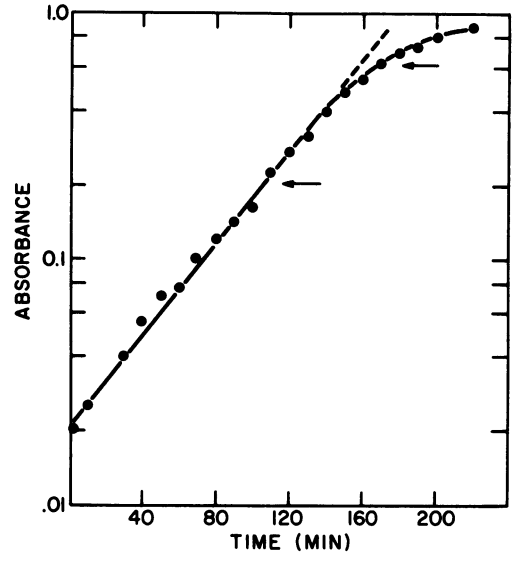

FIG. 2. *Growth of E. coli B/r in nutrient broth at 37 C. Arrows indicate times at which cells were removed for treatment with sucrose and lysozyme.*

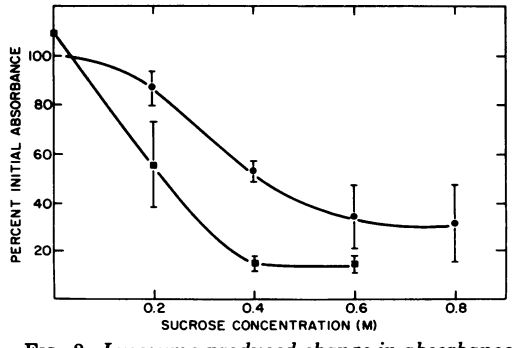

FIG. 3. *Lysozyme-produced change in absorbance for cells of E. coli W3110 (●) and E. coli B_{s-1} (■) grown in nutrient broth and treated at 25 C.*

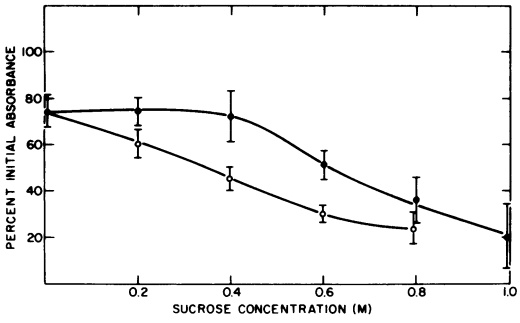

FIG. 4. *Lysozyme-produced change in absorbance for cells of E. coli B/r grown in Roberts C-minimal medium (●) and E. coli B/r grown in C-minimal medium but washed twice with water before treatment* (○).

that injured the envelope and left it susceptible to lysozyme attack.

Salts also are osmotically active and one expects them to produce plasmolytic effects similar to those caused by sucrose. Figure 5a shows the decrease in absorbance produced by resuspending *E. coli* B/r cells (grown in nutrient broth) in various concentrations of NaCl or $MgCl_2$ prior to adding lysozyme. Since dissociation of salts produces a greater effect than does sucrose for a given molar concentration, Fig. 5b presents the same results but with salt concentrations expressed in terms of the concentration of sucrose that would give the same freezing point depression (1). There was little difference between effects produced by NaCl and sucrose. $MgCl_2$ was not as effective in potentiating cell lysis although microscope examination of cells suspended in 0.6 M $MgCl_2$ showed them to be extensively plasmolyzed. Thus, lack of lysis was not due to absence of plasmolysis. Moreover, the $MgCl_2$-treated cells were predominantly rod shaped after the final dilution which suggests that lysozyme did not reach its substrate. Mg^{2+} is believed to be important to the integrity of the outer membrane (9), and it is conceivable that the excess Mg^{2+} in the medium caused the outer membrane to become less permeable to the lysozyme.

Deplasmolysis. Plasmolysis is known to be a transient phenomenon, lasting only a few minutes in 0.4 M sucrose (13). Lysozyme attack was used in an attempt to follow deplasmolysis. Pellets of *E. coli* B/r grown in nutrient broth were suspended in 0.2 or 0.4 M sucrose and then incubated at 37 C for various intervals before lysozyme was added. No increase in final absorbance could be detected for periods in sucrose of up to 30 min, although cells observed under phase optics had lost all visible signs of plasmolysis within 10 min. Presumably, the restoration of the original cytoplasmic space did not entail a return to normal contact between the plasma membrane and the remainder of the cell envelope.

Deplasmolysis takes place even faster if nutrients are present when the cells are subjected to sucrose (15). Figure 6 indicates that the injury to B/r cells grown in nutrient broth and suspended in sucrose plus nutrient broth was slightly greater than what would have been expected on the basis of the additional osmotic pressure due to the nutrient broth. Microscope observation showed no signs of plasmolysis so, again, lysozyme attack indicated the existence of an injury in the cell envelope persisting beyond the time at which recovery was assumed to have taken place.

Thermal effects. The lysis produced by treating *E. coli* B/r with sucrose and lysozyme at different temperatures is indicated in Fig. 7. Data not shown for 37 C closely resembled

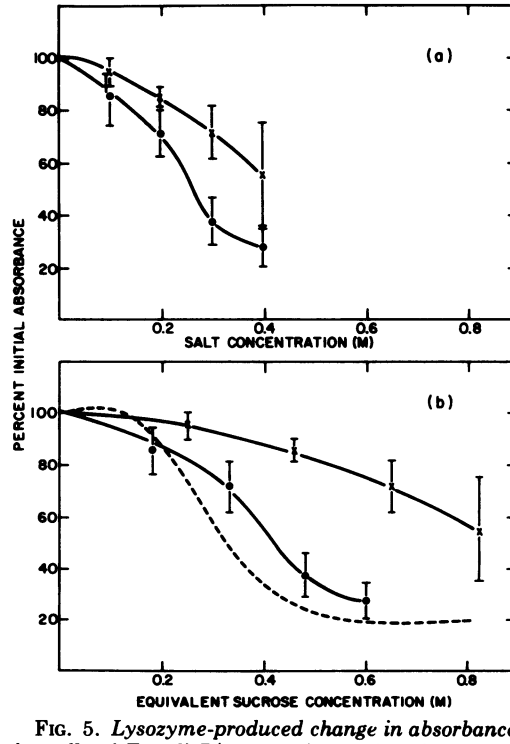

FIG. 5. *Lysozyme-produced change in absorbance for cells of E. coli B/r grown in nutrient broth and treated at 25 C with NaCl (●) and with $MgCl_2$ (X). (a) Salt concentration expressed in molarity; (b) salt concentration expressed in terms of molarity of sucrose producing the same freezing point depression. Dotted line represents data from Fig. 1 for cells treated with sucrose.*

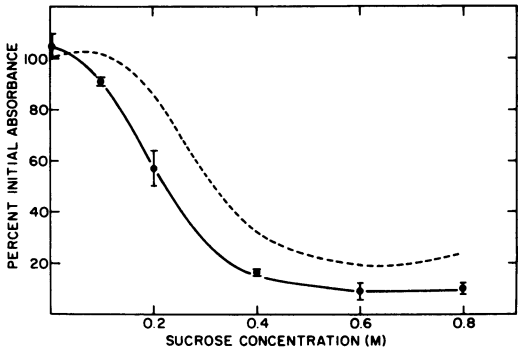

FIG. 6. *Lysozyme-produced change in absorbance of cells of E. coli B/r grown in nutrient broth and treated at 25 C with sucrose in the presence of nutrient broth. Dotted line shows the data from Fig. 1 for cells treated with sucrose.*

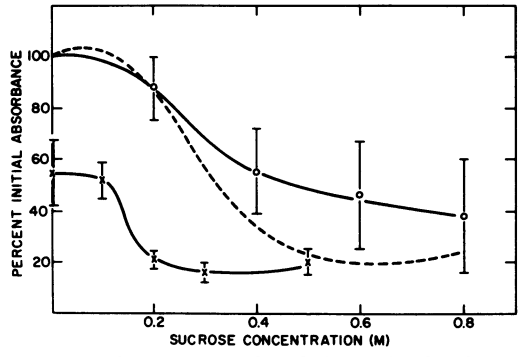

FIG. 7. *Lysozyme-produced change in absorbance for cells of E. coli B/grown in nutrient broth and treated with sucrose at 11 C (O) and at 0 C (×). Dotted line represents data from Fig. 1 in which cells were treated at 25 C.*

those for 25 C. The most striking feature, one exhibited by all three strains, was that at 0 C no sucrose was needed for lysozyme to cause lysis. Resuspension of the cooled cells in nutrient broth with no lysozyme present did not produce any decrease in absorbance, nor was any noted for similarly treated cells grown in C-minimal medium. Between 11 and 0 C the envelope of *E. coli* cells grown in nutrient broth underwent some alteration that increased susceptibility to lysozyme attack. This effect is currently under investigation.

Implication by other investigators (6, 12) for participation of the cell envelope in thermal inactivation at temperatures greater than 50 C suggested that an increase in susceptibility to lysozyme might be found at similar temperatures. Cell suspensions heated to 55 C for 10 min, cooled to room temperature, and then treated as above did not respond with a decrease in absorbance. Although this was consistent with a similar finding by Harries and Russell (7), it merely indicated that after cooling no increased susceptibility could be detected. Cells grown in C-minimal medium and then heated in C-minimal medium lacking Mg^{2+} but containing lysozyme did suffer a decrease in absorbance. Figure 8 shows the enhancement of this effect with EDTA present at a concentration of 0.4%. Some loss in absorbance was noted, even without lysozyme or EDTA, and probably resulted from thermally induced lysis, which is consistent with other suggestions of such lysis (12). Results of heating and treating the three strains, B/r, B_{s-1}, and W3110 are shown in Fig. 9. Strain B_{s-1}, reportedly more thermally sensitive than B/r (4), showed less sensitivity to lysozyme attack. Perhaps the cause of such increased thermal sensitivity was a more easily disrupted envelope which became leaky and therefore not subject to hypotonic lysis after lysozyme attack. No lysis was detected in cells heated to 70 C. This may have been due to heat fixation of the cells after which they could no longer respond osmotically. Measurements of similar effects in nutrient broth were not possible as the lysozyme formed a precipitate in the broth that prevented accurate measurement of the absorbance of the cells.

DISCUSSION

Determination of osmotic pressure by lysozyme attack has distinct advantages over techniques utilizing microscopy. A quantitation is possible that would require considerable time

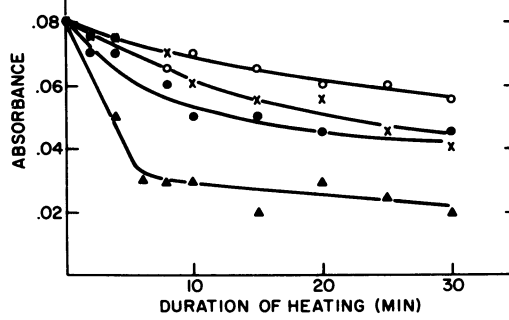

FIG. 8. *Absorbance of E. coli B/r grown in C-minimal medium and heated at 55 C in the presence of C-minimal medium (O), C-minimal medium minus the $MgCl_2$ and with EDTA added (●), C-minimal medium plus lysozyme (×), and C-minimal medium minus $MgCl_2$ plus EDTA and lysozyme (▲).*

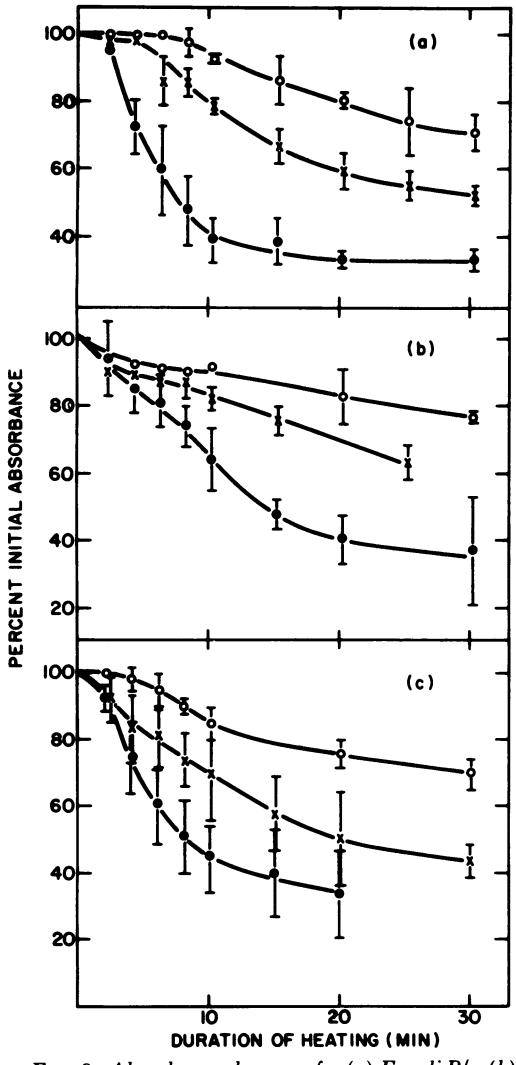

FIG. 9. *Absorbance decrease for (a) E. coli B/r, (b) E. coli B_{s-1}, and (c) E. coli W3110 grown in C-minimal medium and heated in C-minimal medium minus the $MgCl_2$ and Na_2SO_4 but in the presence of lysozyme and EDTA at 51 C* (○), *53 C* (×), *and 55 C* (●).

and effort with a microscope. In addition, lysozyme apparently can be used to detect osmotic damage not visible with a light microscope.

The osmotic pressure of bacteria often has been used by investigators to produce or prevent major changes in the cell. This includes most studies of plasmolysis, hypotonic shock, and the conversion of cells to an osmotically sensitive form. Values such as those reported here should assist future pursuit of similar studies. At the same time, one might inquire as to how osmotic pressure is used by the cell. Does it have a causative or regulatory role, or is it merely the result of other processes or properties more fundamental? Differences have been pointed out h[illegible] between strains and growth media that, while they do not provide an answer, do suggest that such a line of investigation may now be worth pursuing.

One of the most interesting results is the apparent range of osmotic pressures exhibited in every situation presented. Some cells growing in nutrient broth plasmolyzed in the presence of just over 0.1 M sucrose while others required 0.5 M sucrose. This corresponds to a five-fold difference either in the internal concentration of osmotically active particles as assumed here, in the permeability of *E. coli* to sucrose, or in the degree to which the plasma membrane is fastened to the cell wall. Moreover, there were alsays some cells that did not lyse even after being subjected to 1.0 M sucrose. No other physical parameter comes to mind that exhibits such a variation in an exponential-phase culture. A study of the osmotic pressure in synchronized cells might prove instructive.

The data also suggest that osmotic pressure plays a role in thermal inactivation. Cells with a high internal osmotic pressure (grown in C-minimal medium) lysed in the presence of lysozyme and EDTA at temperatures similar to those associated with ribonucleic acid (RNA) degradation, leakage of metabolites (12), and deoxyribonucleic acid (DNA) strand breaks (18). Some lysis occurred even without lysozyme or EDTA, which reinforces the suggestion made elsewhere that up to 20% of those cells maintained in a salt medium may lyse at these temperatures while washed cells heated in water do not (12). Washing cells in water leads to the release of metabolic pools (11) and a decrease in osmotic pressure as reported here. It follows that the differential osmotic pressure would likewise be reduced.

A plausible hypothesis for thermal death might include some critical temperature at which a portion of the rigid cell wall is weakened so that it can no longer restrain the plasma membrane which ruptures, whereupon enough cytoplasmic material is released to equalize the internal and external pressures. The point of rupture would provide the entry point for lysozyme. The blebs which de Petris (6) found in the outer membrane of some heated cells may have been formed at the site of such a rupture and one could acount for EDTA-enhanced lysis by noting that, if the EDTA removes portions of the outer membrane as believed (9), the point of rupture would

become more rapidly accessible to attack by lysozyme. Furthermore, it also has been reported that *Aerobacter aerogenes* cells heated in water are more resistant to inactivation than are those heated in a growth-supporting medium (17).

One might reasonably expect a lesion in the membrane to heal once the pressure differential is removed. However, subsequent degradation of RNA may again increase the internal osmotic pressure, to the point of producing lysis, providing the integrity of the peptidoglycan layer had been destroyed. According to such an hypothesis one would expect lysozyme to be ineffective when added after cells had been cooled. What remains, of course, is the question as to which of the various heat-induced phenomena is the primary cause of thermal inactivation.

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