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Shrinkage of Growing *Escherichia coli* Cells by Osmotic Challenge

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The immediate response of growing *Escherichia coli* to changing external osmotic pressure was studied with stopped-flow turbidimetric measurements with a narrow-beam spectrophotometer. It is shown theoretically that in such a photometer rod-shaped bacteria have an apparent absorbance which is proportional to the inverse of the surface area. The apparent optical density, corrected for effects of alteration of the index of refraction of the medium, increased continuously as the external osmotic pressure was raised. Because of the short time scale of the measurements, the turbidity increases could result either from shrinkage of the cells or from plasmolysis, or both, but not from growth or metabolic adaptation. With low concentrations of pentose such that the external osmotic pressure was not greater than that inside the cells, plasmolysis would not occur and, consequently, only shrinkage of the previously stretched sacculus remains to account for the observed optical effects. Taking the osmotic pressure of the growing cells as 5 atmospheres (506 kPa), the turbidity changes correspond to the murein fabric having been stretched 20% beyond its unstressed equilibrium area during growth under the conditions used.

A knowledge of the amount of strain in the wall of normally growing bacteria is important in understanding how the cell enlarges. In particular, such measurements can provide a critical test of the recently proposed surface stress theory of bacterial morphogenesis (14–16, 18, 19; A. L. Koch and I. D. J. Burdett, J. Gen. Microbiol., in press). This theory accounts for the shapes of a variety of bacteria in terms of responses to an increase in turgor pressure stretching the wall and favoring the enzymatic enlargement of the stress-bearing peptidoglycan wall.

The stretched state of the wall and the osmotic pressure of bacteria are interrelated by the biophysical properties of the stress-bearing peptidoglycan. The osmotic pressure has been measured by equilibrium vapor pressure techniques (28, 36) and by nonpenetrable volumes measurements with probes such as dextran or inulin which do not pass through the outer membrance or sucrose that does not penetrate the cytoplasmic membrane (24, 25, 36). Since very dense suspensions must be used, there are severe difficulties with both kinds of measurements due to the shift of the physiological state of the organisms to anaerobiosis. However, the results of such studies suggest that the osmotic pressure differential in *Escherichia coli* is from 3 to 5 atmospheres (304 to 506 kPa), corresponding to 3 to 5 bar or an osmotic pressure of 0.11 to 0.19 osmolal.

The method developed here allows for the calculation of the mean surface area changes from the turbidities measured in a narrow-beam spectrophotometer. The shrinkage of cells when mixed with osmotically active substances was followed by stop-flow without the complications of slower adaptation of the organism in response to the osmotic challenge. It was found that the surface area of the cells decreased progressively with no threshold as the osmotic pressure differential (and turgor pressure) was decreased.

MATERIALS AND METHODS

The organism used was strain AX655, a derivative of *E. coli* K-12 capable of filamentation when grown at an elevated temperature (2); it is F^- *thr leu thi arg proA his gal xgl ara mtl lac rpsL sep*(Ts). This nonmotile strain was a gift of Conrad Woldringh and James Walker. In all cases, the organism was grown at 25°C with aeration in a broth consisting of 10 g of tryptone (Difco Laboratories) and 5 g of NaCl per liter.

Stopped-flow apparatus. The experimental setup consisted of the aerated, growing culture in one vessel and the same broth amended with an osmotic additive in another vessel. Dipped into these containers were narrow-bore, plastic tubes which led to 1 ml of Manostat mini-pet semiautomatic pipettes. The Manostats were mounted so that by sliding a wooden block equal volumes were ejected from both syringes. The outputs of the syringes were forced through thin Teflon tubing into the spectrophotometer. There they were joined by a T-tube immediately connected to a flow-through cell in the cuvette holder within the Cary model 16 spectrophotometer. The absorbancy trace at 660 nm was recorded. In some, but not all, circumstances the readings were quite stable over many minutes.

Control cultures. Experiments in which the organisms had been grown at 42°C for two generations served as one type of control for optical artifacts. Because division had been blocked, the mean cell size was about four times larger than the mean cell size of the normal cell populations and had approximately the same diameter. The biomass, estimated turbidimetrically, increased exponentially at the time of harvest. Another control consisted of early-stationary-phase cultures of the same organisms grown at 25°C. These cells were somewhat smaller than the exponentially growing cells.

Turbidity theory. Because bacteria have dimensions comparable to the wavelength of visible light and are usually suspended in medium whose index of refraction is only slightly lower than the cytoplasm, the theory for light scattering falls in a region where the Rayleigh-Gans theory is appropriate (10–13, 17, 21). To calculate the light-scattering properties of suspensions of rod-shaped bacteria, extensive numerical calculations are necessary to make corrections for the interference between light scattered from different parts of each bacterium. The corrections depend on the size, shape, and orientation, as well as on the distribution, of matter within the cell.

For any assumed geometry, the light scattered at an angle, θ, can be calculated by finding the appropriate $P(\theta)$ function. Functions applicable to spheres, shells, ellipsoids of revolution, and cylinders are available and, although complicated, can be calculated readily with computers. These functions

also depend on size, orientation, wavelength, and index of refraction of the medium. The dependency is such that several factors can be combined by defining x as $(4\pi r/\lambda')\sin(\theta/2)$ and expressing P as a function of x instead of as a function of θ. In the expression for x, r is the radius of a sphere with the same volume as that of the cell and λ' is the wavelength of light in the aqueous medium ($= \lambda/n$, where n is the index of refraction). This substitution of the variable reduces the problem of calculation greatly.

The problem at hand requires averaging suitable P-functions for rod-shaped organisms of a range of sizes over all orientations in space. Fortunately, I had earlier prepared tables of the functions appropriate for rod-shaped bacteria (11). The $P(x)$ values were obtained as the average between the functions for a cylinder and those for an ellipsoid of revolution, averaged in turn over all orientations in space and finally averaged over a twofold range of axial ratios. Of course, in the last averaging process the higher abundance of newborn cells relative to cells about to divide (20) was taken into account. Two tables of $P(x)$ values were prepared; one is for the case of birth length twice the width, and the other is for the case of birth length four times the width. The two size ranges, 2/1 to 4/1 and 4/1 to 8/1, span the major size variation of a population of growing and dividing rods.

For the application to the turbidity measurement it is necessary to compute the integral of the scattered light over all angles to calculate the light that does not fall on the detector. This is because turbidimetric observations measure the light that is redirected from the forward direction that therefore fails to be collected on the phototube.

The turbidity is $K \int_o^\pi P(\theta)(1 + \cos^2\theta)\sin(\theta)d\theta$. The $(1 + \cos^2\theta)$ factor takes into account the perpendicular and parallel components of unpolarized light; the $\sin(\theta)d\theta$ factor takes into account the area of an annular ring swept out between the angles of θ and $\theta + d\theta$ from the forward direction. K is given by $4\pi^3(dn/dc)^2q^2\nu n_o^2/2.3\lambda^4$, where n_o is the index of refraction of the medium, q is the dry mass of the particle, dn/dc is the index of refraction increment, ν is the number of particles per ml, and λ is the wavelength of light in vacuo. $P(\theta)$ also depends on the size and wavelength as well as on the angle of observation. The computer successively chose values of θ from 0 to 180°C in 0.5° increments and calculated the value of x from $x = (4\pi r/\lambda')\sin(\theta/2)$. It then calculated $P(\theta)$ by interpolation from the tabulated values of $P(x)$. Finally, it calculated the integral by numerical summation.

Three curves are shown in Fig. 1 for theoretical populations, each spanning a twofold range of volumes. The most pertinent is marked 2/1 to 4/1 since this is about the range of proportions of length to width of the *E. coli* strain studied here. Also included is a curve for axial ratios spanning 4/1 to 8/1. It is not much different than the first curve; it would be expected to apply to the control population division blocked for a generation time. A third curve is shown for a hypothetical situation in which a spherical organism grows exponentially in mass to twice the birth mass (1.26 times the birth radius) and instantly divides. This curve might apply to certain mycoplasmas.

It was shown previously (10) that the turbidity of uniform populations of spherical cells of the size range and index of refraction of bacteria is approximately proportional to $1/r^2$ or to the reciprocal of the surface area. This is shown as the fourth curve (Fig. 1). In all four cases there is adherence to the $1/r^2$ law, as indicated by the curves going through the origin in a linear fashion on this type of plot. When r becomes small, the turbidity levels off and becomes independent of size. This happens when the particles are so small

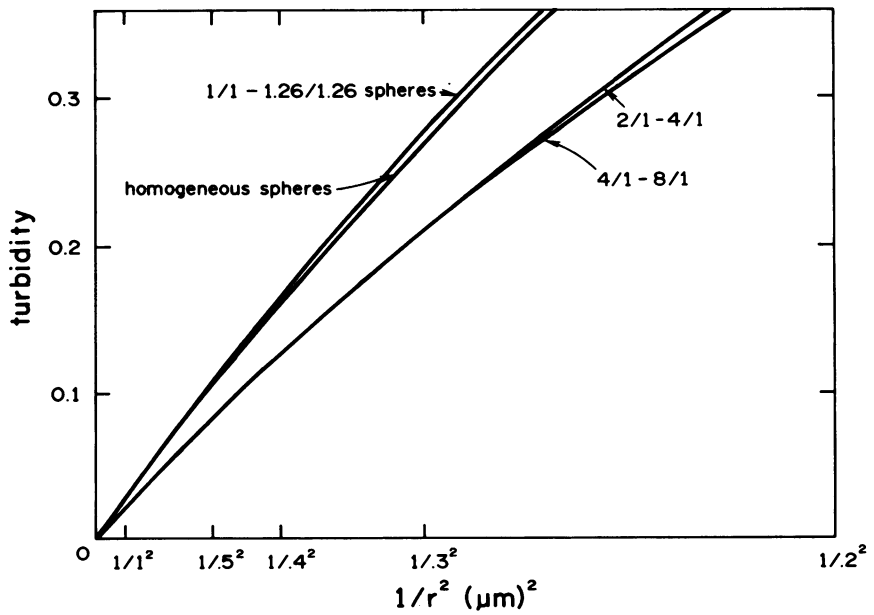

FIG. 1. Theoretical relationships of turbidity to the reciprocal of the equivalent radius squared. An asynchronous exponential population of randomly oriented rod-shaped cells having constant radii and growing in mass twofold over the cell cycle was modeled. Two degrees of asymmetry, 2/1 to 4/1 and 4/1 to 8/1, were considered. The ordinate is the turbidity calculated as indicated in the text; it must be divided by K, calculated from the expression given in the text, to give the actual turbidity. The calculations were carried out for an assumed wavelength of light in vacuo of 660 nm. The abscissa is the square of the reciprocal of the radius of a sphere having the same volume as the mean volume of the rod-shaped or spherical cells. Another curve shows the dependency of turbidity for a cellular population of spherical cells which divide by binary fission but are spherical for most of the life cycle. The remaining curve shows the turbidity of populations of spheres, all of the same radius.

that they are no longer in the Rayleigh-Gans range but have dimensions smaller than the wavelength of light and obey the Rayleigh law instead. However, down to an equivalent radius of 0.3 μm, the turbidity is proportional to r^{-2}. More exactly, the expressions down to $r = 0.15$ μm for the turbidity for rod-shaped organisms are $K(0.0226r^{-2} - 0.0025r^{-4})$ and $K(0.0236r^{-2} - 0.0003r^{-4})$ for the 2/1 to 4/1 and the 4/1 to 8/1 cases, respectively. It must be emphasized that these expressions only apply to cells in the size range of bacteria and would not apply to larger single-cell organisms such as most yeasts, protozoa, or algae.

This inverse square law in turn means (see reference 9) that when a population of cells is caused to swell or shrink with only the addition or loss of water, the absolute turbidity changes in proportion to $1/r^2$. It also means that growing populations with different mean sizes would have turbidities proportional to r^4 or volumes to the 4/3 power.

RESULTS

In interpreting the results of studies in which a nonpenetrating solute is added, a correction must be made for the index of refraction change due to the osmoticum. To reduce the magnitude of this correction, I have used the smallest carbohydrates that are not actively taken up by the bacteria. The pentoses, arabinose and xylose, were the osmotica used in Fig. 2 and 3, but studies with ribose gave similar results. The correction was applied simply by making similar measurements with the addition of a large-molecular-weight substance, Ficoll, which affects the index of refraction as do the smaller saccharides but does not have an appreciable osmotic affect. Alternately (and with negligible difference), the correction was applied from the known index of refraction increment for carbohydrates, amino acids, etc. (0.00136 per g/100 ml), and the known average index of refraction of the bacteria (5% greater than the aqueous environment).

Figures 2A and 3A show the effect of various osmotica in changing the average area of the cells in the suspension, inferred from the reciprocal of the corrected turbidity. The same response was seen both for the three types of bacterial cultures considered and when measurements were made either with the stopped-flow apparatus at 5 s after mixing (Fig. 2A) or in an ordinary cuvette at 1 min after mixing (Fig. 3A). There is more scatter of the points in the former case, but basically the same trend is shown. The shrinkage of surface area was roughly proportional to the changes in the osmotic pressure differential. There is no indication of a threshold or discontinuity in the response in the 0.1- to 0.2-osmolal region.

The data also have been plotted in the usual manner for studies of the osmotic swelling and shrinkage of membrane-bound systems in which the limiting membrane is able to enlarge and contract readily (Fig. 2B and 3B). Thus, the volume calculated from the optical measurements was plotted versus the reciprocal of the external osmotic pressure (the osmotic pressure of the broth was taken as 0.167 osmolal). For a van't Hoff-Boyle osmometer, the slope and intercept of the graph were used to calculate the deviation from ideality and from the osmotically inactive volume. The data do not fit a straight line, but a regression line would indicate a ca. 30% inactive volume.

DISCUSSION

To use the theoretical relationships developed here, the turbidimetric measurements must be made in an absolute photometer (10–13, 17, 21, 40), that is, one in which the incident beam of light is very narrow and the angle that is

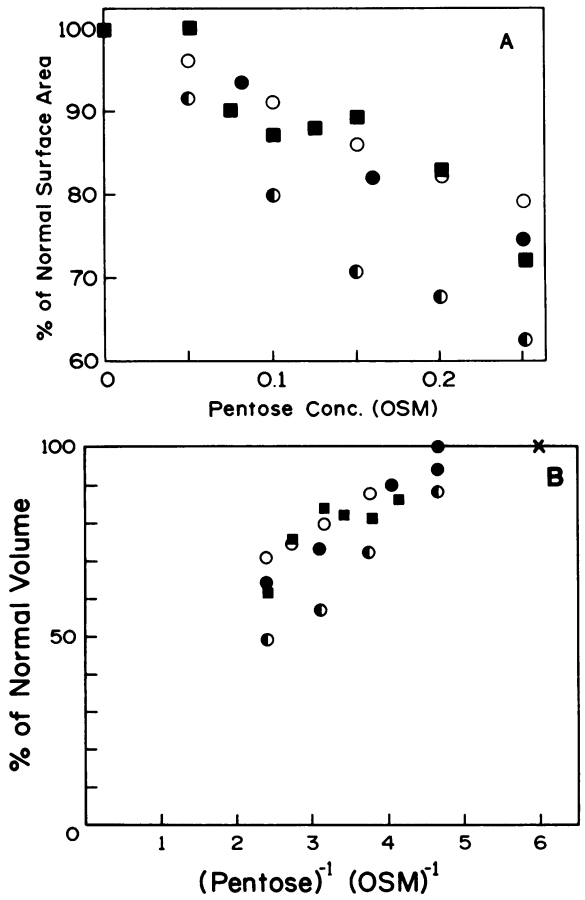

FIG. 2. Effect of external osmotic pressure on the (A) surface area and (B) volume of bacteria. Measurements were made with the stopped-flow apparatus 5 s after mixing. The absorbance data were corrected for the index of refraction changes. The decrease in average surface area was calculated as proportional to the reciprocals of these values. The volumes were calculated as the 3/2 power of the surface area. Symbols: ●, exponentially growing cells with added xylose; ■, exponentially growing cells with added arabinose; ○, stationary cells with added xylose; ◐, filamentous cultures with added xylose. The symbol X in (B) corresponds to the volume taken as 100% measured at the osmotic pressure of the nutrient broth.

intercepted by the light detector is very small. The Cary model 16 spectrophotometer has a narrow beam and was used on this study. My previous results have shown (12) that it has a sufficiently narrow beam to accurately measure the true turbidity without introducing the artifacts characteristic of non-narrow-beam instruments.

Although the theory for the light scattering of spherical cells with the optical properties of bacteria was developed much earlier, it is only now that the turbidity of cultures of rod-shaped organisms has been calculated. It is shown that the theory that was derived for a population of spheres of the same size applies as accurately to the asynchronous mixture of normal rod-shaped bacteria taken from a growing culture in Brownian motion equilibrium as it does to homogeneous spheres.

Although the theoretical calculations take into consideration a number of factors (size range, frequency of various size classes, and orientation), they do not take into account other factors. The justification for omitting these factors is the finding that the equivalent mean radius is the important

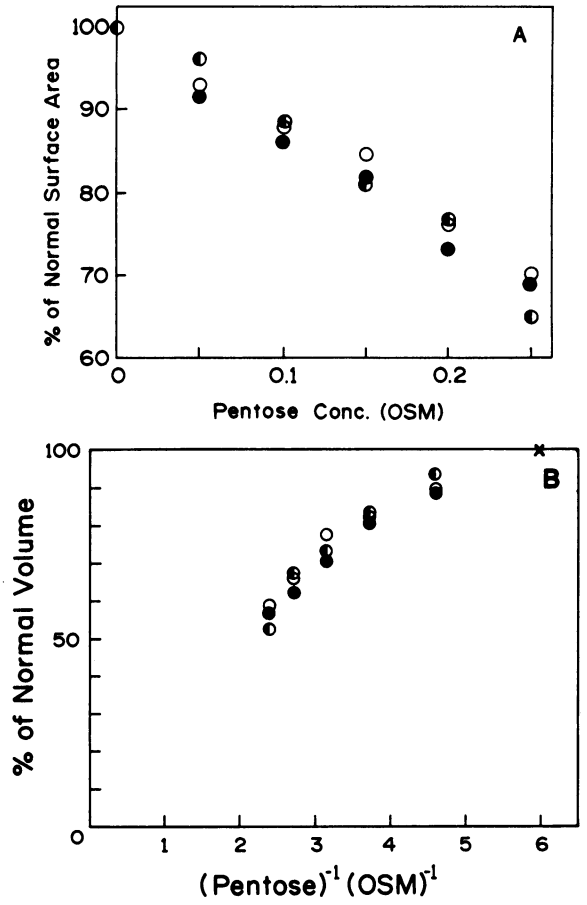

FIG. 3. Effect of external osmotic pressure on the surface area of the bacteria after 1 min. The conditions were as in Fig. 2, but measurements were made 1 min after mixing in a 1-cm cuvette. The turbidity of the organisms, under these conditions, had a more stable absorbance at this time, and somewhat more precise readings were obtained than with the stopped-flow apparatus. Xylose was used as the osmoticum. Symbols: ●, exponential phase cells; ○, stationary phase; ◐, filaments.

factor and that the ratio of length to width is much less significant. Therefore, more minor deviations between the bacterial suspension and the mathematical idealization, if taken into account, would certainly give only negligible changes. These neglected differences include the fact that: (i) the shape of the organism is a little different than the average between a ellipsoid and a flat-ended cylinder; (ii) the radius of the cells is not precisely constant during the cell cycle but decreases a few percent during the elongation phase and rebounds during division (38); (iii) the nearly divided cells (showing a deep constriction) are of a significantly different shape than are nondividing cells, although they represent only a few percent of the population (41); and (iv) the index of refraction is not constant throughout the cell but varies in the membranes, the periplasmic space, and the region of the cell containing the bulk of the DNA. Our previous work (10, 11, 17) shows that these effects would be minor in *E. coli* growing in rich medium.

The osmotic pressure differential across the bacterial cells in the studies reported here was decreased by adding pentoses and other sugars, and the immediate turbidity changes were analyzed. Turbidity changes occur very rapidly when the osmotic pressure of the medium is altered (1, 26). This is because the index of refraction changes instantaneously and because the permeation of water through the cell envelope is very fast (the half-time is a few milliseconds). However, a good deal of the literature (3, 4, 7–9, 22, 27, 31–34) shows that there are two factors that result in slow, further changes in the turbidity: (i) plasmolysis as the cytoplasmic membrane is partially detached from the outer membrane and (ii) deplasmolysis as the consequence of accumulation of ions from the medium, such as potassium, proline, and glutamic acid (3, 7–9, 22, 27, 32, 37). Some of the literature suggest that the plasmolysis is not instantaneous but takes several minutes (30, 32). Deplasmolysis may take 20 min or longer. In the studies reported here, the measurements were sufficiently fast that metabolic recovery of the bacteria also can be neglected. Cyanobacteria, algae, and higher plants also show biphasic responses to changes in the osmotic pressure of their environment (35, 39, 43).

However, gram-negative bacteria have only a very thin stress-bearing layer: the peptidoglycan content is so small that the sacculus must be only one molecular layer thick, with the peptide bridges in an extended conformation (5, 6, 23, 29, 42; Koch and Burdett, in press). Since the inner and outer membranes are composed of molecules that interact via secondary bonds, these organelles will not aid in supporting a continuous stress but rather will creep. Evidently the glycan with its cross-linked covalent bonds is sufficiently strong to resist the turgor pressure of 3 to 5 bars. However, it means that the bacterium cannot behave as an ideal van't Hoff-Boyle osmometer since there will be an upper limit to the elastic stretch and a lower limit of the minimum surface that it can achieve without wrinkling. Moreover, the well-established phenomenon of phasmolysis of *E. coli* (31, 33) is contradictory to an object that shrinks and swells, dependent on the external osmotic pressure only. However, *E. coli* cells also cannot behave as many walled algae and plant cells do. When subjected to increasing concentrations of solute, these cells show little decrease in size, but when the turgor pressure becomes negative, plasmolysis occurs and the cytoplasmic volume decreases, but the volume enclosed by the cell wall changes little. If this were the case for gram-negative bacteria, in which the turbidity of bacteria is due mainly to the contribution of the cytoplasm (11, 17), the calculated surface area shown in Fig. 2A and 3A or the volume shown in Fig. 2B and 3B should remain constant until the internal osmotic pressure was neutralized and then progressively decrease as the external osmotic pressure was increased further.

The actual results were somewhat closer to those expected from an osmometer. The mean cell volumes have been plotted against the reciprocal of the total external osmotic pressure (Fig. 2B and 3B). On this type of plot an ideal osmometer yields a straight line whose *Y*-intercept is the fraction of an osmotically inactive volume. Therefore, one interpretation of the results is in accord with the hypothesis that the *E. coli* cell behaves as a van't Hoff-Boyle osmometer with a ca. 30% inactive volume.

Because of the chemical structure of murein and the existence of plasmolysis, it is suggested that an intermediate situation exists. The wall of the growing gram-negative rod is stretched beyond its stress-free conformation during normal growth. As the external osmotic pressure is raised, the entire cell shrinks. Eventually, the wall regains its most compact molecular conformation. The further increase in the external pressure leads both to crenation of the wall as the cytoplast contracts and to plasmolysis. Presumably, plasmolysis oc-

curs secondarily, permitting the sacculus to recover a non-strained normal-appearing shape.

Assuming that the osmotic pressure inside a growing cell is ca. 5 atmospheres (506 kPa) or 0.2 osmolal (28, 36) the results of Fig. 2A and 3A indicate that the mean area is decreased to ca. 80% when the external osmotic pressure is increased by this amount, causing the hydrostatic pressure to be fully relaxed. This 20% decrease corresponds (if the wall is isotropic in the two surface dimensions) to the wall having been expanded during normal growth 11.8% [$= 1/\sqrt{0.8} - 1)\ 100$] in linear dimension beyond the relaxed state. If there is some asymmetry, 11.8% is the geometric mean. This estimate is in good agreement with our estimate of the degree of shrinkage in length which was observed in the phase microscope when filaments of the strain used here were treated with sodium dodecyl sulfate (A. L. Koch and S. Lane, unpublished data).

The present work shows that osmotic pressure relations in the gram-negative bacteria are different than the relationship in algae and higher plants (35, 43). In the latter case, the wall, composed of cellulose and other polymers, is so rigid that under normal turgor it is only slightly strained. Thus there is no change in the cytoplasmic dimensions or in the shape until the external osmotic pressure has been increased enough to make the pressure differential negative and to cause the cytoplasmic membrane to pull away from the wall, causing plasmolysis. Although plasmolysis also occurs with gram-negative bacteria (30, 32), what has been demonstrated here is that there is an additional and separate phenomenon of shrinkage when the turgor pressure is reduced but is still positive. Although this was inherent in the results of Alemohammad and Knowles (1) and although they did conclude that the cell envelope exhibits some elasticity, they did not conclude, as is done here, that optical density changes cannot be attributed to plasmolysis and must therefore be attributed to shrinkage. Consequently, the wall of normally growing bacteria is under tension, causing the wall to be strained.

Our results are similar to those of Walsby (39), on the cyanobacterium *Anabena flos-aquae*. He measured the effect of osmotic and hydrostatic changes on the light scattering due to gas vacuoles. He found that the elastic modulus, $V\Delta P/\Delta V$, was much smaller than that of higher plants (43) and was ca. 11 bar. It is ca. 9 bar for *E. coli* from the present study.

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