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Nephelometric Determination of Turgor Pressure in Growing Gram-Negative Bacteria

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Gas vesicles were used as probes to measure turgor pressure in *Ancylobacter aquaticus*. The externally applied pressure required to collapse the vesicles in turgid cells was compared with that in cells whose turgor had been partially or totally removed by adding an impermeable solute to the external medium. Since gram-negative bacteria do not have rigid cell walls, plasmolysis is not expected to occur in the same way as it does in the cells of higher plants. Bacterial cells shrink considerably before plasmolysis occurs in hyperosmotic media. The increase in pressure required to collapse 50% of the vesicles as external osmotic pressure increases is less than predicted from the degree of osmotically inducible shrinkage seen with this organism or with another gram-negative bacterium. This feature complicates the calculation of the turgor pressure as the difference between the collapse pressure of vesicles with and without sucrose present in the medium. We propose a new model of the relationship between turgor pressure and the cell wall stress in gram-negative bacteria based on the behavior of an ideal elastic container when the pressure differential across its surface is decreased. We developed a new curve-fitting technique for evaluating bacterial turgor pressure measurements.

The surface stress theory postulates that cell wall enlargement responds to cell growth by a mechanism that maintains the turgor pressure of the cell (6, 10). In this mechanism, autolysin cleaves stress-bearing murein more rapidly when the stress is greater. The turgor pressure, which is due to the stress in the cell wall, is defined as the hydrostatic pressure across the cell wall that compensates for the difference in water activity between the cytoplasm and the suspending medium so that the electrochemical potential of water is the same on both sides. Alternative theories of cell growth and development (15, 16, 20) predict different patterns of fluctuations in internal osmotic pressure during the cell cycle. It therefore is important to know both the magnitude and the variability of the turgor pressure from cell to cell within a growing bacterial culture and how they change with physiological conditions.

Unfortunately, there are few satisfactory measurements of internal osmotic pressure in bacterial cells (30). The most promising method (27) can only be applied to the few microorganisms with gas vesicles. It measures turgor pressure as the difference between the applied pressure, above atmospheric pressure, required to collapse 50% of the gas vesicles in a population of suspended cells and that in cells whose turgor has been removed by large amounts of nonpenetrative solute in the medium. The measurement depends on the assumption that the turgor pressure of bacterial cells, P_t, drops to zero when the cells are immersed in a medium with an osmotic pressure high enough to relieve the stress on the cell wall by counterbalancing the internal osmotic pressure of the cell. Consequently, the calculated turgor pressure of the growing bacteria is given by

$$P_t = \overline{C} - C_a \quad (1)$$

where C_a is the apparent collapse pressure of cells growing in normal medium and $\overline{C}$ is the collapse pressure of the same cells in medium to which sufficient osmolyte has been added to cause plasmolysis. $\overline{C}$ is more fundamentally the mean collapse pressure of vesicles in free suspension. In this paper we retain the symbolism used by Walsby (29).

The present work uses a refined light-scattering method to assess the mean collapse pressure of gas vesicles in a population of cells under different physiological conditions and in different osmotic environments. Our purpose is to improve Walsby's method, estimate its reliability, and define its limitations in regard to measuring the turgor pressure and the variability of turgor pressure among cells in a growing population.

Gas vesicles have been found in several major groups of bacteria, including halophiles, cyanobacteria, and methanogens (29, 30). *Ancylobacter aquaticus* was chosen for these experiments because it has gas vesicles and is a gram-negative heterotroph. We hope that our initial results represented here may apply to other gram-negative organisms, e.g., *Escherichia coli*, which are so much more completely studied physiologically. The recent cloning of the gas vesicle protein gene (24) together with ongoing attempts to induce gas vesicle assembly in *E. coli* may eventually allow direct measurements of turgor in *E. coli* by using the methods described here.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *A. aquaticus* (17, 18, 25) M158, obtained from Alan Konopka, was grown in CAGV medium, which contains 1% Casamino Acids (vitamin free), 1% glucose, 2% Hutner modified salts, and 0.5% vitamins (12, 13). The growth rate of each subculture was monitored in a narrow 5-cm-light-path quartz cuvette at 660 nm with a Cary model 16 spectrophotometer. To follow growth of a culture turbidimetrically (5), it was necessary to collapse the vesicles before each optical density measurement. Vesicles were collapsed by the classic cork-and-bottle method (1). A sample of a growing culture of *A. aquaticus* was placed in a brass centrifuge tube shield filled to the top

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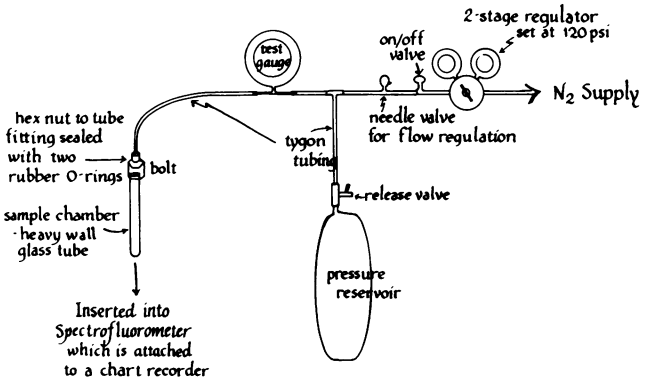


FIG. 1. Pressure system for nephelometry. The entire apparatus is a modification of a design by Walsby (27). See text.

(14 ml), so that no air bubbles remained between the silicone stopper and the surface of the liquid inside the shield. The stopper was struck briskly with a hammer at least three times to create a high momentary hydrostatic pressure inside the shield, thus collapsing all the vesicles inside the cells. The nephelometer described below and phase microscopy were used to be sure that this method collapsed all the vesicles in a sample.

The cell density was kept below 30 μg (dry weight) per ml by repetitive dilution to ensure that cells were in balanced growth for at least 20 generations, with an average doubling time of 4.1 h. The growth curve of strain M158 departs from linearity above 50 μg/ml. (In one case a stationary-phase culture was deliberately used.) Growing cultures were maintained in a 30°C temperature-controlled water bath, and air which had been saturated with charcoal-filtered water was bubbled through the cultures.

Apparatus. The optical system consisted of a model SPF-125 spectrofluorometer (Aminco, Silver Spring, Md.), which has a xenon light source. For this purpose both of the spectrofluorometer gratings were set at 550 nm, since these experiments did not involve separating the emission and excitation wavelengths of a fluorescent compound, but instead used the sensitivity of the fluorometer photomultiplier and its strong light source to monitor small changes in the light scattered at right angles from the incident beam.

The arrangement of the plumbing system is shown in Fig. 1. The sample holder was a custom-made thick-walled glass test tube capable of withstanding inside pressures of over 200 lb/in^2 (1,400 kPa). The tubes were made by Don Fowler, Indiana University Chemistry Department glass shop. The lip of the tube was designed to form a tight seal inside a 0.5-in. (1.27-cm) stainless-steel hex nut by using two butyl rubber O rings. The assembly, containing 3 ml of sample, was then attached through a hose adapter to a compressed nitrogen supply. The glass tube was placed in the fluorometer sample chamber and covered with a reinforced wooden box coated with matte black paint. The nitrogen tank was fitted with a two-stage pressure regulator, an on-off valve, and a micrometer needle valve which could be adjusted to a chosen constant flow resistance. The tubing was next connected to a large reservoir which functioned together with the needle valve to determine the rate of the pressure rise at the distal end of the system (total estimated volume of gas in the system, 920 ml). Finally, a test gauge (part no. 63-5612; Matheson, Joliet, Ill.; accuracy, ±0.25 lb/in^2) and/or pressure transducer (part no. PX300-150GOV; Omega, Stamford, Conn.; accuracy, ±0.75 lb/in^2) was connected in the line to serve as an accurate indicator of the actual pressure delivered to the sample. The change in the intensity of scattered light as pressure was applied to the sample was recorded with an X-Y plotter (model no. LY18100 Linseis, Princeton Junction, N.J.). The plotter readout was calibrated against the relative light intensity read on the spectrofluorometer meter and against the voltage output of the pressure transducer measured with a voltmeter (no. 350; Beckman Instruments, Inc., Fullerton, Calif.). In some experiments a chart recorder was used and pressure was marked on the trace. In these cases, the pressures at the quartiles and at the mean were interpolated from the calibration marks. In most experiments, the run was started by opening the on-off valve with the needle valve set at 7.0 and the pressure regulator set so that the pressure on the culture rose (in kilopascals) as $670(1 - e^{-1.485t})$, where t is measured in minutes. Under these conditions the average vesicle collapsed in 18 s in the absence of 0.5 M sucrose and in 35 s in the presence of 0.5 M sucrose.

Qualitative observations of light scattered by vesicles. Two samples of a growing culture were placed in glass test tubes. the vesicles in one of the samples were collapsed by pressurization by the cork-and-bottle method. Each tube was placed in front of the collimated beam of a helium-neon laser (wavelength, 632.8 nm) pointed at a white screen against the wall in a darkened room. The difference in the light refracted by the two samples was examined subjectively. Most of the light refracted by the pressurized sample was scattered in nearly the forward direction as expected for nonfilamentous bacteria which have no gas vesicles (3, 4, 7–9). However, most of the light scattered by the unpressurized cells emerged at angles greater than 20°. This demonstrates that the intensity of light diffracted at 90° by cultures of *A. aquaticus* is primarily due to light scattered from gas vesicles. Therefore, our instrument, which measures light at 90° from the primary beam, is less influenced by light scattering from the bacteria than are previously used instruments (28).

Selection of rate constant for pressure rise. To measure turgor pressure, the rate of increase of the applied pressure must be carefully chosen. If the pressure is increased too quickly, the value of C_a may be overestimated because of lags in the response time of the electrical system or because of differences between sucrose-treated and untreated cells in the lag before vesicles collapse at a particular pressure. Another possibility might be that plasmolysis at high sucrose concentrations is delayed briefly due to adhesion of the cell membrane to the wall. This would create a situation during which P_t would be temporarily negative rather than zero as assumed by the method (see appendix).

If the pressure is increased very slowly, the cells may reform collapsed vesicles (13) or reestablish the turgor pressure lost as a result of the collapse of the first few vesicles, or both (see appendix). Moreover, a slow pressure increase may allow dissolved gases to diffuse into the vesicles, increasing the partial pressures of these gases within the vesicles. Another possibility is that the cell may be sufficiently permeable to sucrose that the difference due to sucrose concentration may be diminished.

Although the photomultiplier measuring circuits of a spectrofluorometer require a finite time to respond completely after the light intensity is changed, the fluorometer is able to respond with a half-time of less than 1 s. This precludes the necessity of correcting any data reported here for instrument response time.

The effect of changing the rate at which the N_2 pressure

increased during a run was tested by comparing runs in which only the orifice setting of the needle valve was changed (Table 1). It can be seen that the effect of the rate of pressure increase on the collapse pressure of cells in unmodified medium is not important except at the largest orifice setting. This was confirmed by experiments (not shown) in which the pressure was raised in discrete steps applied at regular but various rates. In the presence of 0.75 osmolal sucrose, there was a larger effect of orifice setting; that is, a higher pressure is needed to collapse vesicles when the pressure rises quickly.

Further evidence of the importance of choosing the rate of increase of applied pressure is the following observation. When the rise in pressure was stopped for 5 min at a pressure which immediately collapsed about 20% of the vesicles, a significant fraction of the vesicles continued to collapse at an exponentially decreasing rate (data not shown). After about 5 min, almost no further decrease in light intensity could be detected. When the pressure increase was resumed, the remaining vesicles collapsed as expected. However, the value of C_a obtained when the pressure treatment was interrupted was significantly lower than when the experiment was run with no interruption at the same orifice setting. This effect was also evident with sucrose-treated samples. The magnitude of the difference of estimates of C_a varied with the interruption pressure and the sucrose osmolality. We estimate that these effects could cause variations of about 15 kPa in the value of turgor pressure. Because of these results, we chose to perform the bulk of the experiments by using the rate of rise in pressure increase produced in the system when the micrometer needle valve was set at 7.0. We feel that this procedure avoids both a too fast and a too slow rate of rise in pressure. A single setting is not, however, a perfect compromise. This is because it takes longer to reach the pressure collapsing 50% of the vesicle when C_a is high, i.e., when sucrose is present, and at this point the pressure in our present apparatus would be rising more slowly. Consequently, a larger orifice should be used for the sucrose treatment to achieve the same rate of pressure increase at the point of 50% collapse. However, to completely eliminate this factor we would need a precise knowledge of the delay in collapse after a critical pressure is applied to each vesicle.

Reassembly of vesicles. Collapsed vesicles reassemle spontaneously given sufficient time (12, 13), although this process can be inhibited by either chloramphenicol or rifampin (13). Konopka et al. (12, 13) argue that protein from collapsed vesicles is reutilized, although new protein synthesis is required. To ensure that no significant reassembly of vesicles interfered with our experiments, all of the vesicles in a growing culture were collapsed by applying 700 kPa of pressure for 1 min, the pressure was then released, and the subsequent increase in light scattering was monitored with the spectrofluorometer until a plateau was reached after about 100 min. Only about 55% of the original intensity of light scattering was recovered in this nonaerated culture. A total of 1% of this total recovery occurred within 7 min, 5% occurred within 14 min, and 10% occurred within 40 min. Reassembly may be faster or more complete in an aerated culture, but our interest was in determining the rate of reassembly in the sample chamber of the nephelometer under actual experimental conditions. Since our other experiments were usually completed in less than 3 min, we concluded that reassembly of vesicles did not affect the results.

TABLE 1. Effect of orifice setting on collapse pressure with and without sucrose

Orifice setting	Without sucrose		With sucrose (0.75 osmolal)	
	C_a[a] (kPa)	Time to C_a[b] (s)	C_a (kPa)	Time to C_a (s)
4	253	82	403	135
7	265	16	491	34
10	259	11	507	26
14	300	11	596	28

[a] C_a, Applied pressure above atmospheric causing collapse of 50% of the gas vesicles.
[b] Time from the start of pressure increase until 50% of the light scattered by vesicles was lost.

TABLE 2. Effect of growth on C_a in sucrose-augmented medium

Time in presence of sucrose	C_a[a] (kPa) in medium with the following sucrose concn (osmolal):		
	0	0.2	0.3
<3 min	334	438	459
48 h	331	347	373

[a] C_a, Applied pressure above atmospheric pressure that causes a 50% loss in light scattering by the vesicles within the cells.

Isopestic NaCl and sucrose. In principle, any osmotic agent could relieve the turgor pressure, but there might be secondary effects such as those due to the partial permeability of the cells to some agents (2). We compared results obtained by using isopestic solutions (i.e., solutions having the same osmolality) of NaCl and sucrose. These substances, of course, have quite different chemical and physiological activities. A 5-ml portion of culture was added to 5 ml of medium containing 0.1169 g of NaCl or 1.2285 g of sucrose, producing a precise 0.2 osmolal increase in NaCl or sucrose concentration over the osmolality of the growth medium. These values were calculated by using the tables of Robinson and Sinclair (19). This osmolality approximately halves the turgor pressure of these cells under these conditions. Any difference that these two quite diverse substances might have in lowering turgor pressure would have been most apparent and would have resulted in different values of C_a. However, the applied pressures required to collapse 50% of the vesicles in the NaCl and sucrose treatments did not differ significantly from each other. Two replicates of sucrose-treated cells had a mean 50% collapse pressure of 457 kPa, and two replicates of NaCl-treated cells had a mean 50% collapse pressure of 450 kPa. The two controls, in which 5 ml of unaltered medium was used to dilute 5 ml of culture, had a mean 50% collapse pressure of 266 kPa, showing that turgid cells require lower applied pressures to cause vesicle collapse.

Growth on sucrose. Allowing cultures to grow during several doublings in medium containing 0.2 or 0.3 osmolal sucrose raised the collapse pressure slightly (Table 2), but the increase was much less than it is immediately after sucrose is added to a growing culture. Cells grown in the presence of high concentrations of sucrose had fewer vesicles per cell than the control cells. Cultures to which 1 osmolal sucrose was added grew very slowly and had no vesicles that were visible with a phase microscope.

Time in sucrose. Sucrose was chosen as an osmotic agent in most experiments because when added at a high concentration it does not enter gram-negative cells readily (2, 22). It may be taken up by the cell over time, however, since some

gram-negative bacteria have transport systems for sucrose or extracellular enzymes for reducing sucrose to more readily transportable forms. We hoped that the response of the cell to high sucrose concentrations as an osmotic challenge would not change over the time required to measure collapse pressures. To validate this assumption for our organism, we diluted a growing culture with medium containing sucrose to make the final concentration of 0.200 osmolal. Collapse curves were begun immediately and 20 min after the samples were mixed with sucrose. C_a values obtained for the delayed samples were 96 kPa lower than those taken immediately. Since it takes some time from mixing until the instant that pressure was applied, the C_a may have changed either because of accommodation to the change in external osmotic pressure by the cell or because the vesicles may be sensitive to changes in their immediate chemical environment. Based on the known operational lag (2 min), a linear back extrapolation indicated that the value of C_a would have decreased only 9.6 kPa during a typical experimental run as compared with the value it would have if the collapse curve could have been measured instantaneously. This decline of C_a is 2.2% of the measured 50% collapse pressure. It might be argued that the accommodation was very rapid in the first few seconds after the addition of sucrose but then became more gradual. In this case a linear back extrapolation would be invalid.

To test for this possibility we measured the C_a values immediately after the addition of chilled sucrose under conditions where the temperature rapidly became and remained at 12°C. Since any accommodation process should depend on metabolism or active transport or both, its rate should be 10-fold or more slower at the lower temperature. For the value recorded immediately, the 2-min lag would correspond to an operational delay of about 12 s at 30°C. The C_a was 487 ± 13 kPa. This, of course, must be corrected for the effect of temperature on the collapse pressure. This effect was estimated from the difference of the C_a values from experiments at 12 and 30°C with no sucrose added. After correction for the effect of temperature on C_a, the value of C_a was 400 kPa, comparable to the data presented below for 0.2 osmolal sucrose.

Experiments were also performed with different orifice settings to see if time in sucrose would change the effect caused by the rate of use of pressure reported above. The rise in C_a with rate of pressure rise (Table 1) was unaffected by up to 20 min of pretreatment with a high sucrose concentration. We therefore concluded that variations in the time of exposure to sucrose during experiments did not significantly affect our results.

Adding sucrose to eliminate the turgor pressure is the method which has most often been used by previous workers (27, 29). Because of the effects outlined above, we decided to use an alternative method as supporting evidence that our turgor measurements were accurate. We therefore used ampicillin to disrupt the cell envelope and eliminate turgor.

Other controls. Additional experiments (not shown) demonstrated other features of the system: (i) the highest applied pressure used was sufficient to collapse all vesicles; (ii) the collapse pressure changed very little as the cell cultures entered the stationary phase of growth; and (iii) the lack of aeration during the time required for an experiment affected the turgor pressure very little. In addition, we felt that we could ignore the possibility that dissolved gases could diffuse to the vesicles during the time required to apply the external pressure. Because diffusion would have to occur through several centimeters of fluid, it would be negligible in the time it took to perform the measurement.

Calculation of SD of vesicle collapse pressure from a single collapse curve. In addition to the 50% collapse pressure of a sample derived by finding the midpoint between the initial and final intensity of light scattering corresponding to 0 and 100% collapse of the vesicles, the pressures which produced 25 and 75% decreases in light intensity ($P_{25\%}$ and $P_{75\%}$) were determined. These pressures were used to calculate the standard deviation (SD) of the collapse pressure of the vesicles in the population of cells. The calculation was based on the assumption that the collapse curve is the integral of the Gaussian normal distribution. In this case $SD = (P_{25\%} - P_{75\%})/(2 \times 0.6745)$. The factor 0.6745 in this expression converts the difference between the first or third quartile and the mean of a normal distribution into the SD. This factor was taken from a table of the normal probability integral.

RESULTS

Collapse pressure in the presence of various concentrations of sucrose. Samples from a growing culture of *A. aquaticus* were diluted with prepared solutions of sucrose in CAGV medium so that the final osmolality of sucrose in each case was precisely known. Collapse curves were obtained immediately after addition of the sucrose solution (or of fresh medium when a control sample was run). Diluents were kept at 30°C before use. The 50% collapse pressures determined from these curves are plotted against the final osmolality in the sample shown in Fig. 2. It can be seen that as the osmolality of sucrose increases, C_a increases in a curvilinear relationship. The predictions of general models are indicated by lines A through E. These models are described under Discussion.

Fifty percent collapse pressure in the presence of ampicillin. Four replicate experiments were performed in which ampicillin (500 μg/ml) was added to a growing culture. After about 20 min, collapse curves were made; this was the time when the majority of the cells were at the verge of lysing (data not shown). The collapse curves obtained were biphasic. An example has been superimposed on its untreated control in Fig. 3. The collapse curves of ampicillin-treated cultures were broader than curves obtained from either sucrose-treated or untreated cells. This suggested that two distinct populations of cells were present. Other kinetic studies (data not shown) indicated that the proportion of cells in the culture which had been disrupted by ampicillin quickly plateaued. This subpopulation was designated component II and was characterized by a higher and fixed C_a value. After 20 min, component II comprised 84% of the population and had a mean C_a of 478 kPa (Table 3). It is assumed that the value of C_a for component II is an estimate of $\bar{C}$. This time was chosen for the estimation of $\bar{C}$ because after lysis the vesicles gradually disappear when observed in a microscope or in a nephelometer. They may therefore exist in a weakened state after liberation from the cell and be subject to proteolysis, etc.

Turgor pressure calculation by the Walsby method. Results are given in Table 4 for two types of samples: cells in growth medium and cells in medium to which 0.5 to 1 osmolal sucrose had just been added. An estimate of turgor pressure, 187 kPa, was calculated as the difference between the collapse pressures of the two groups on the presumption that the turgor pressure had been reduced to zero by all of the sucrose treatments.

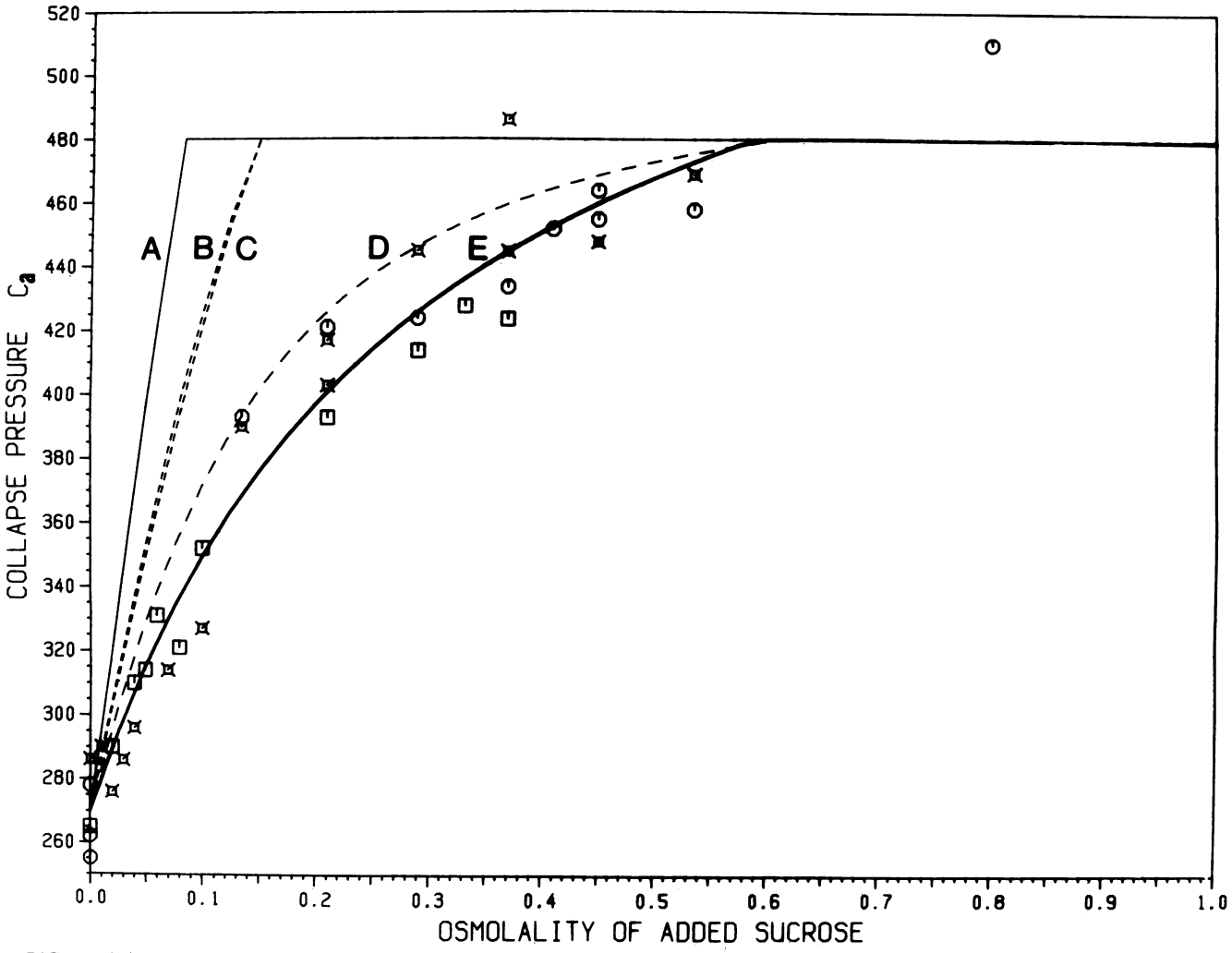


FIG. 2. Collapse pressures versus medium osmolality. The points represent three sets of experiments performed on three different days, in which samples from a growing culture were diluted with concentrated sucrose solutions to the final osmolalities indicated. The collapse pressure, C_a, is the midpoint of a collapse curve at which 50% of the light refracted by vesicles had been diminished. Curves A through E are theoretical functions which fit the data to various degrees. They are explained in detail in the appendix.

Estimate of the variability of turgor pressure. The variability of collapse pressure of the gas vesicles, calculated from the pressures collapsing 25 and 75% of the vesicles in medium of high sucrose osmolality, is given in Table 4 as 86 kPa. This is the average of the estimates from 10 collapse curves. Presumably the variation within a sucrose-treated sample reflects only differences in collapse pressures between individual vesicles, because the turgor pressure of all cells becomes zero in the presence of the sucrose. The variability measured in a population of growing cells also includes the variability of turgor pressure from cell to cell and was somewhat larger (91 kPa). Based on the theory of propagation of errors, an SD of turgor pressure from cell to cell (SD_{P_t}) of 30 kPa was calculated (Table 4). This cell-to-cell variability and its SD are only approximations, but they suggest that the variation of turgor pressure among cells within the population is not large.

DISCUSSION

Figure 2 shows the applied pressure that collapses 50% of the gas vesicles of the growing bacterium as a function of the concentration of sucrose added to the medium. The experimental results are consistently reproducible and quite different from the predictions of several models. The simplest model is that the bacterial cell wall is rigid and that $\bar{C}$ is the same for all cells regardless of physiological condition. Constant volume implies that the internal osmotic pressure does not change as the external osmotic pressure is increased due to water flux.

The fit to this model is labeled A in Fig. 2. (For the mathematical relationships, see the appendix.) The observed initial slope (590 kPa/osmolal) is considerably less (23%) than predicted for a rigid container (2,520.5 kPa/osmolal). This suggests that the cell wall is elastic and that as the external osmotic pressure is raised, the cell contracts and the cytoplasm is concentrated. Shrinkage explains only a portion of the difference between curve A and the data points. The predictions of the model employed by Walsby (29), which assumes that the volumetric elastic modulus is constant as the turgor pressure decreases, are marked B and D. The curves marked C and E are the predictions of the Hooke's law model with a constant value of Young's modulus (see appendix). Curves B and C are fitted to the

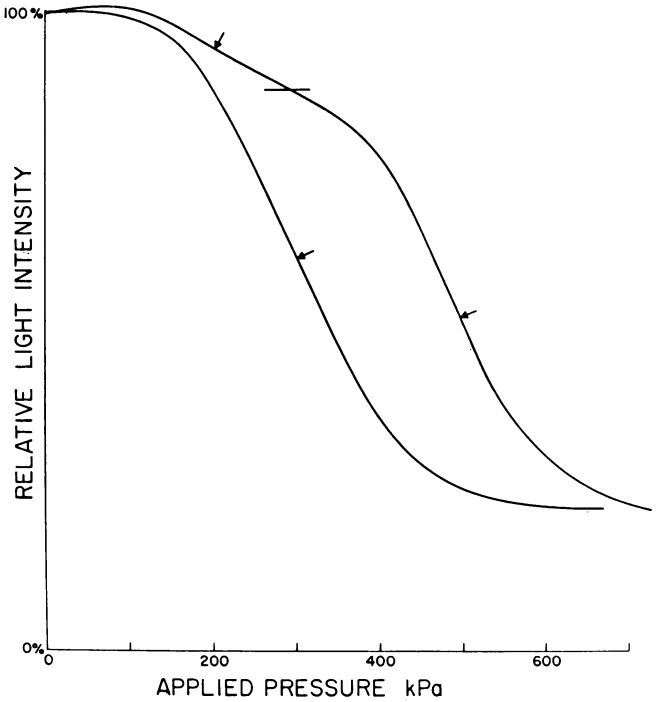


FIG. 3. Collapse curve obtained after ampicillin treatment. These curves represent the decrease in light refracted by the vesicles in a control (no drug) and a sample to which 500 μg of ampicillin per ml had been added 20 min before pressure was applied to the sample, as described in Materials and Methods. The collapse curve of the control sample has a 50% collapse pressure, C_a, of 304 kPa. It follows a smooth S-shaped curve, indicating that the collapse pressures of individual vesicles within the cells of the sample are normally distributed. The curve to the right, which is typical of the curves we obtained from ampicillin-treated cultures, is biphasic, indicating that two environments for the vesicles are present. The arrows indicate the 50% point for the components. The 50% collapse pressures of components I and II were 196 and 493 kPa, respectively. The horizontal line indicates an estimate of the relative proportions of the two components.

assumption that the turgor in the growing cell increases the volume to 1.76 times the relaxed turgor-free volume. Thus, when either model is fitted to be consistent with a degree of elasticity found in our studies of *E. coli* (11), the fit is poor. Curves D and E are for the same two models for larger values of k (see appendix). Curve E was obtained by minimizing the sum of the squared deviations (SSD) of the Hooke's law model from the experimental data. The factor obtained corresponds to the case of a turgid cell with a volume 7.3-fold greater than that in its relaxed configuration. This large expansion factor is contrary to our microscopic observation.

The minimization procedure was not applicable to the Walsby model. This was because the SSD became progressively larger as larger values of k were tried and no minimum was found. The line shown as curve D had nearly twice the SSD as did curve E, although it corresponds to a similar degree of volume expansion of the growing cell (7.3 times that of the turgor-free cell). The value of k that gives the same SSD as does curve E corresponds to the growing cell being expanded thousands of times above the volume when the turgor is dissipated. On this basis we feel that the Walsby model is inapplicable.

There are a number of other possibilities to explain a gradual increase in C_a with increasing external osmotic pressure instead of an abrupt plateau when P_t equals zero. One is that the turgor pressure varies very much from cell to cell even when the sample has been drawn from a culture in balanced growth. The computer program was modified to minimize the squared deviation between a model considering variability and the observed data. In the fitting process it was assumed that Young's modulus is the same as in curve C but that the turgor pressure is highly variable and is normally distributed from cell to cell in the population. The best fit derived by the computer requires that the SD_{Pt} be 105 kPa (computer results not shown). From the analysis of the widths of collapse curves with and without sucrose given

TABLE 4. Preliminary estimate of the mean turgor pressure and its SD from cell to cell in the population

Medium (value measured)	Collapse pressure (kPa)	
	Mean ± SD	Mean of SD[a] ± SD
Without sucrose (C_a; n = 6)	272 ± 13.2	91 ± 7.6
With ≥0.5 osmolal sucrose ($\bar{C}$; n = 10)	459 ± 25.0	86 ± 8.8
Difference (turgor pressure, P_t)	187 ± 28.3[b]	30 ± 11.6[c]

[a] The SD of the collapse pressure of individual vesicles in a sample was estimated from the pressures corresponding to quartiles of the collapse curve (see Materials and Methods).

[b] $P_t = \bar{C} - C_a$; SD = $(13.2^2 + 25.0^2)^{0.5}$

[c] Assuming that the variation in turgor pressure from cell to cell is independent of the variation of the collapse pressure of individual vesicles, then $SD_{Pt} = (91^2 - 86^2)^{0.5}$. The SD of this quantity is given by $(7.6^2 + 8.8^2)^{0.5}$.

TABLE 3. Effect of ampicillin treatment on the 50% collapse pressure[a]

C_a of growing culture (kPa)	Ampicillin-treated culture			
	Time of treatment (min)	C_a (kPa) of:		% of cells in component II
		Component I	Component II	
297	25	207	462	84
304	22	196	493	84
295	23	186	479	82
291	20	203	479	84
296 ± 5.4[b]	22.5 ± 2.1[b]	198 ± 9.2[b]	478 ± 12.7[b]	83.5 ± 1.0

[a] Four separate growing cultures were studied. For each a collapse curve was prepared for cells removed from the culture. A second portion of each culture was aerated for 20 to 25 min with 500 μg of ampicillin per ml, and a collapse curve was prepared. (This is the time when the cells are just beginning to lyse.) The collapse curves were analyzed as shown in Fig. 3. Component I corresponds to unlysed bacteria with a progressively higher turgor pressure. Component II corresponds to vesicles in cells that have lysed sufficiently to lose turgor pressure or to liberate vesicles. The latter C_a values provide an estimate of $\bar{C}$.

above, the pressure has an SD_{P_t} of 30 ± 11.6 kPa. It is doubtful that SD_{P_t} could be as large as 130 kPa. The assumption of such a high degree of variability of the P_t of cells would also be contradicted by the smaller degree of variability found by the examination of individual cells under a microscope (Pinette and Koch, unpublished data).

It is also possible that $\bar{C}$ varies from cell to cell. It is clear that the collapse pressure varies widely from vesicle to vesicle; i.e., the mean $\bar{C}$ was 480 kPa, and its SD was 86 kPa. On the reasonable assumption that the strengths of vesicles vary at random, we can calculate the variation between cells of C averaged over the vesicles within a cell. This can be done with the formula for the SD of the mean, i.e., $SD/\sqrt{n}$, where n is the number in a sample. Since there are approximately 100 vesicles within a cell, $n = 100$ and the SD of the mean values of cells is given by $86/(100)^{0.5}$, or 9 kPa. This is a source of variation sufficiently small to be neglected. It may be possible that some cells have only, or mainly, weak vesicles and that other cells in the population have only, or mainly strong vesicles. But this would not contribute to the gradual nature of the rise of the collapse curves.

Still another possibility is that the pressure required to collapse a vesicle increases as the cytoplasm becomes concentrated as the external osmotic pressure is made higher. The local environment of the vesicles would then contain a higher concentration of proteins and a higher ionic strength. These effects would be most clearly evident at high sucrose concentrations. The C_a estimated from 26 collapse curves of cells in 0.6 to 3 osmolal sucrose was 523 ± 47 kPa. This mean is higher than the 480 kPa estimated from the data of Fig. 2. Moreover, it is higher than the estimate of $\bar{C}$ obtained by allowing the cell wall to rupture with ampicillin (474 kPa; Table 4).

These possibilities lead to an overestimate of $\bar{C}$ when too high a sucrose concentration is used. On the other hand, $\bar{C}$ may be underestimated when too low a concentration of sucrose is used. If a concentration just sufficient to lower the turgor pressure to zero is used, the cells may adapt to the osmotic challenge rapidly. We believe that the adaptation can be so fast that the speed with which we can generate a collapse curve is insufficient to prevent underestimation of $\bar{C}$. The experimental result presented here suggests that the use of 0.4 to 0.8 osmolal sucrose with the rate of pressure increase employed in this study avoids both pitfalls.

The turgor pressure derived from the observations made here is the first measurement obtained by using a gram-negative heterotroph under conditions of balanced exponential growth. The most recent work in this area (22) can be faulted because the measurements were performed after the cells were harvested and greatly concentrated (and no doubt made anaerobic). Our recent studies (Pinette and Koch, unpublished data) of *A. aquaticus*, in which the collapse of vesicles within individual cells was measured under a microscope, attempted to measure the variability of turgor pressure among cells and led to the conclusion that there is no significant correlation of turgor pressure with cell size. No conclusion with respect to the absolute value of the mean turgor pressure was drawn, although a mean value of $\bar{C}$ of 500 kPa, consistent with the present findings, was obtained. We found a value for P_t of 110 kPa, which is considerably lower than the estimate from the present study or that provided to us by A. E. Walsby (personal communication). We suggest that the plasmolysis of the cells might be impeded because the cells are bound to the surface of the glass capillary, and although the water must leave the cells rapidly (21), they may be in a state of negative turgor pressure (Fig. 4). It is essential that vesicles be under neither a positive nor a negative hydrostatic pressure for P_t to be properly estimated. The experiments described here indicate that this kind of error, and a variety of other errors discussed above, does not cause artifactual results with our present method. Actually, the turgor pressure estimate must be increased by the osmotic pressure of the growth medium, but the osmolality of the medium for the growth of this aquatic organism is negligible.

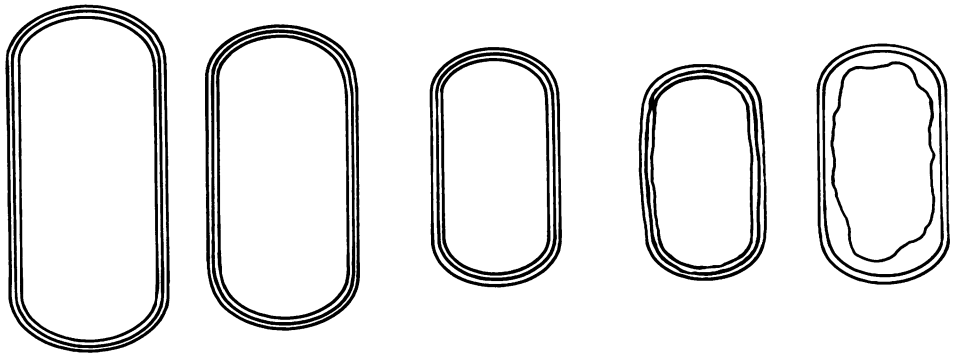
With regard to the accuracy of our estimate of the turgor pressure, from 60 collapse curves obtained from exponentially growing balanced cultures of strain M158 over an 8-month period, C_a was 285.4 kPa with an SD of 23.4 kPa. Some of this variability can be attributed to experimental error. Since C_a is composed of the difference between $\bar{C}$ and P_t (equation 1), it is possible that $\bar{C}$ varies from culture to culture. If this is so, then less of the variance will be left over for the variance of P_t. However, even if all of the variation of our measurement is attributable to variation in P_t, the turgor pressure estimate obtained by using the computer model (210 kPa) has a standard error of only $23.4 \text{ kPa}/\sqrt{60} = 3$ kPa.

APPENDIX

Our results with *A. aquaticus* are quite similar to those obtained by Walsby with *Anabaena flos-aquae* (26, 27, 29, 30). Both organisms have elastic cell walls, so both experience a smaller reduction in turgor pressure than expected based upon the amount that the external osmotic pressure is increased. This is because both organisms shrink and concentrate the cell contents when placed in medium of high osmolality. The shrinkage is much larger than it is for the cells of higher plants. Because the cell wall of higher plant cells is almost rigid, C_a increases by almost the same amount as the external osmolality is increased (14). Thus, if the bacterial cell walls were rigid, C_a would increase 2,520.5 kPa for every 1 osmolal increase in external osmolality, as in the initial part of curve A in Fig. 2. If the external osmotic pressure were made high enough, no further increase in the osmotic pressure of the medium would affect the observed 50% collapse pressure and C_a would equal $\bar{C}$. Consequently, for the case of a population of identical bacterial cells, all with identical values of $\bar{C}$ and P_t, C_a would rise directly with the increase in external osmotic pressure until the turgor pressure was reduced to zero, after which C_a would remain constant at $\bar{C}$ (Fig. 2, curve A). This prediction is quite different from the actual experimental results.

Walsby's interpretation of quite similar data from *A. flos-aquae* (29) assumes, as we do, that the cell shrinks as the external osmotic pressure increases. This happens because the stress in the wall changes in proportion to the induced change in turgor pressure. However, Walsby's model states that the change in cell volume is directly proportional to a change in turgor pressure. Alternatively, our model proposes that the change in cell volume is a consequence of the elastic behavior of the cell wall, which obeys Hooke's law, i.e., that Young's modulus, the ratio of stress in the wall to the strain produced, is constant. Consequently, the linear dimension of the cell wall changes with the net pressure acting upon it in proportion to $(1 + kP)$, where k is proportional to the reciprocal of Young's modulus and P is the turgor pressure at a specific concentration of external osmolyte. Therefore, the volume of the cell changes in proportion to $(1 + kP)^3$. Walsby's assumption is that the volume is proportional to $(1 + kP)$ raised to the first power, and we assume that it is proportional to the cube of this quantity. In Walsby's treatment (29), k would be identified with the reciprocal of a constant volumetric elastic modulus (14).

The relationships of internal and external osmotic pressure to turgor pressure are diagrammed in Fig. 4. From the second column of Fig. 4, it can be seen that at external osmotic pressures below the point of incipient plasmolysis

Stage	Normal	Shrinkage	Incipient Plasmolysis	Negative Turgor[a]	Plasmolysis Membrane Separation
Environment	Growth Medium	+ Osmolyte	++ Osmolyte	+++ Osmolyte	+++ Osmolyte
					
External Osmotic Pressure	$\pi_e = 0$	$\pi_e = +$	$\pi_e = ++$	$\pi_e = +++$	$\pi_e = +++$
Turgor Pressure	P_t	P	0	negative	0
Relative Volume of Cytoplasm[b]	$(1 + kP_t)^3$	$(1 + kP)^3$	1	1	< 1
Internal Osmolarity[b]	$\frac{\pi i}{(1 + kP_t)^3}$	$\frac{\pi i}{(1 + kP)^3}$	πi	πi	πi

[a]Before plasmolysis, but after the osmotic pressure outside becomes greater than that inside, the turgor pressure is negative.

[b]The formulae apply for the Hooke's law model. The cubic exponent should be dropped for Walsby's model.

FIG. 4. Shrinkage and plasmolysis in a gram-negative bacterium. The morphological and pressure relationships as the external osmotic pressure is raised are indicated. The external osmotic pressure, π_e, is approximately zero in our growth medium. The turgor pressure, P_t, decreases and becomes zero at the point of incipient plasmolysis. During this process the volume of the cell decreases by $(1 + kP_t)^3$. At still higher concentrations, the turgor pressure becomes negative, but it then falls to zero when plasmolysis ensues. The volume within the cell membrane then shrinks further, and the wall returns to its relaxed state and to the size it had at incipient plasmolysis.

$$P = \pi_i/(1 + kP)^3 - \pi_e \quad (2)$$

where π_e is the osmotic pressure in the external medium and π_i is the osmotic pressure in the internal cytoplasm when the turgor pressure is zero and no plasmolysis has taken place. Assuming that plasmolysis takes place quickly at any external osmotic pressure above the point of incipient plasmolysis, the turgor pressure becomes zero

$$P = 0 \quad (3)$$

Using equations 2 and 3, we can predict the experimental data which relate C_a to π_e from assumed values of P_t, $\bar{C}$, and k for either Walsby's or our model.

To analyze our experimental data, however, we require a means of estimating P_t, $\bar{C}$, and k. To do this, we fitted each model to our data by minimizing the SSD of the observed data from the predicted data by systematically varying one or more of these three parameters. This minimization was done with a BASIC computer program and is shown by curves D and E in Fig. 2.

The program first solves for the value of π_i from the relationship

$$P_t = \pi_i/(1 + kP_t)^3 \quad (4)$$

It then solves for P. As long as π_e is less than π_i the program solves for P where P_t is the turgor pressure in the absence of added osmolyte, iteratively from equation by a modified regula falsi procedure (23). Otherwise it sets P to zero. The program was also used to fit Walsby's model (equations 2 and 4 without the cubic exponent) to our data. In this case, the new form of equation 2 is a quadratic equation which was solved directly. Because of the ability to use the regula falsi procedure, it will be easy to adapt the computer program to other relationships between turgor pressure and volume expansion of the sacculus that may be more appropriate once the mechanical properties of the gram-negative wall are known in more detail.

The dependence of the volume contained by the sacculus on turgor pressure should be more complex than in either model. Certainly the Hooke's law treatment must be applicable at low osmotic pressures when the sacculus is not wrinkled, and when the strain is not so high as to approach the elastic limit. The turgor pressure will then be proportional to the stress, the strain will be proportional to the stress, and equation 2 will apply. However, when the strain approaches the elastic limit of the sacculus, a unit pressure change will give a smaller linear strain because the possible modes of expansion have been exploited in all directions in the plane of the surface.

To take a variability in turgor pressure into account, the computer program was modified to compute the P_t averaged over 11 turgor classes, such that the central class corresponded to the mean turgor pressure and the others corresponded to the wings of the normal distribution. The two extreme classes correspond to cells that have turgor pressure 2 SD from the mean or greater. In the averaging process, the classes were weighted according to the frequencies of the normal distribution. The best-fitting curve required that the SD_{P_t} from cell to cell within the growing population is 105 kPa. Even this best fit does not approach the experimental data very closely, and for this reason it is not shown.

In summary, models and computer programs to describe the behavior of cells with gas vesicles under external applied pressure and osmotic pressure have been developed. They take into account two possible ways that the cell envelope may respond to pressure, and they provide a way to take into account the variation of the turgor pressure among the cells.

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