Biophysical Characterization of Changes in Amounts and Activity of *Escherichia coli* Cell and Compartment Water and Turgor Pressure in Response to Osmotic Stress

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ABSTRACT To obtain turgor pressure, intracellular osmolalities, and cytoplasmic water activity of *Escherichia coli* as a function of osmolality of growth, we have quantified and analyzed amounts of cell, cytoplasmic, and periplasmic water as functions of osmolality of growth and osmolality of plasmolysis of nongrowing cells with NaCl. The effects are large; NaCl (plasmolysis) titrations of cells grown in minimal medium at 0.03 Osm reduce cytoplasmic and cell water to ~20% and ~50% of their original values, and increase periplasmic water by ~300%. Independent analysis of amounts of cytoplasmic and cell water demonstrate that turgor pressure decreases with increasing osmolality of growth, from ~3.1 atm at 0.03 Osm to ~1.5 at 0.1 Osm and to less than 0.5 atm above 0.5 Osm. Analysis of periplasmic membrane-derived oligosaccharide (MDO) concentrations as a function of osmolality, calculated from literature analytical data and measured periplasmic volumes, provides independent evidence that turgor pressure decreases with increasing osmolality, and verifies that cytoplasmic and periplasmic osmolalities are equal. We propose that MDO play a key role in periplasmic volume regulation at low-to-moderate osmolality. At high growth osmolalities, where only a small amount of cytoplasmic water is observed, the small turgor pressure of *E. coli* demonstrates that cytoplasmic water activity is only slightly less than extracellular water activity. From these findings, we deduce that the activity of cytoplasmic water exceeds its mole fraction at high osmolality, and, therefore, conclude that the activity coefficient of cytoplasmic water increases with increasing growth osmolality and exceeds unity at high osmolality, presumably as a consequence of macromolecular crowding. These novel findings are significant for thermodynamic analyses of effects of changes in growth osmolality on biopolymer processes in general and osmoregulatory processes in particular in the *E. coli* cytoplasm.

INTRODUCTION

Escherichia coli grows over more than a hundred-fold range of external osmolality (Osm), extending from as low as 0.015 Osm (Baldwin et al., 1995) up to ~1.9 Osm (McLaggan et al., 1990; Cayley et al., 1991) in minimal medium and up to ~3.0 Osm in rich medium (Record et al., 1998a). To grow over this range of external water activity ($1.0 > a_{H_2O} \gtrsim 0.95$, where $a_{H_2O} = e^{-Osm/55.5}$) requires a high degree of thermodynamic sophistication. In general, growing cells may adapt to changes in osmolality of the growth medium i) by making compensating changes in the intracellular osmolality by changing the amounts of water and/or solutes in the cytoplasm and periplasm, so that the osmolality difference ΔOsm and turgor pressure $\Delta\Pi = RT\Delta Osm$ across the cell wall are maintained, and/or ii) by allowing ΔOsm to change so that turgor pressure changes with external osmolality (Record et al., 1998a). *E. coli* makes large and systematic changes in the amounts of cell and cytoplasmic water and in the amounts of periplasmic and cytoplasmic solutes in response to changes in osmolality of growth (reviewed by Record et al., 1998a; Csonka and Epstein, 1996). How do these changes affect the activity of cytoplasmic water? Of particular relevance for the present study are observations that the amount of periplasmic membrane-derived oligosaccharide (MDO, heterogeneous anionic glucose oligomers that are too large to pass through pores in the outer membrane; Kennedy, 1996) increases as the osmolality of growth decreases for all wildtype *E. coli* K-12 strains examined (Kennedy, 1982; Kennedy and Rumley, 1988; Sen et al., 1988; Lacroix et al., 1989). Do these changes in amount of periplasmic MDO demonstrate that turgor pressure changes with osmolality of growth? Knowledge of turgor pressure as a function of osmolality of growth is the only way to determine the physiological range of cytoplasmic water activity, which, in turn, is needed for analyses of the thermodynamics of cytoplasmic biopolymer processes as a function of growth osmolality.

The activity of a small subset of genes and gene products varies with the osmolality of the growth medium, although the nature of the signal(s) controlling these osmoregulated changes is not well understood (Wood, 1999). Changes in turgor pressure have been considered as a possible osmoregulatory signal (Wood, 1999; Csonka and Epstein, 1996). Few estimates of turgor pressure of *E. coli* are available under any conditions, however, and the questions of whether turgor pressure exists only across the cell wall/

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Abbreviations used: $\Delta\Pi$, turgor pressure; Osm_{ex}, external osmolality; MDO, membrane-derived oligosaccharides; VLOM, very low osmolality medium; MBM, MOPS-buffered medium; DW, cell dry weight; $\bar{V}^{wa}_{cell}$, water-accessible cellular volume; $\bar{V}^{wa}_{cyto}$, water-accessible cytoplasmic volume; $\bar{V}^{wa}_{peri}$, water-accessible periplasmic volume; BDD, 1-bromododecane.

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outer membrane or across the inner (cytoplasmic) membrane and whether turgor pressure changes with osmolality of growth have been controversial and unresolved. Studies of osmotic and Donnan properties of nongrowing suspensions of the closely-related bacterium *Salmonella typhimurium* led Stock et al. (1977) to conclude that the cytoplasm and periplasm are isoosmotic, and that turgor pressure is maintained across the cell wall. However, studies of the osmoregulation of the expression of the *kdp*ABC operon in *E. coli* have been interpreted as indicating that changes in turgor pressure are sensed by the cytoplasmic membrane-bound kdpD sensor kinase (Laimins et al., 1981). Phase and electron microscopy studies of growing cells have led to the proposal that turgor pressure is maintained across the cytoplasmic membrane (Koch, 1995, 1998).

To address these issues, we have indirectly quantified the variation of turgor pressure with external osmolality by measuring the effects of osmolality of growth and of plasmolysis with NaCl on the volumes (i.e., amounts) of cell, periplasmic, and cytoplasmic water, and by analyzing the dependence on growth osmolality of the concentration of periplasmic MDO, calculated from published amounts of MDO and from our measurements of periplasmic volume. We interpret the observed changes in amounts or volumes of water and in concentration of periplasmic MDO in terms of simple physical models. We conclude that the periplasm and cytoplasm are isoosmotic and that *E. coli* systematically varies turgor pressure with external osmolality.

Background on passive and active responses of *E. coli* to changes in external osmolality

The cytoplasm of *E. coli* exhibits both passive and active responses to changes in external osmolality with NaCl or other cytoplasmic membrane-impermeable solutes (Record et al., 1998a). As an example of a passive response, consider a so-called plasmolysis titration (Cayley et al., 1991, 1992) in which a fresh, nongrowing cell suspension harvested from exponential growth in minimal medium at low osmolality (e.g., 0.1 Osm) is titrated with NaCl. Na^+ and Cl^- equilibrate across the outer cell membrane, subject to the Donnan distribution for ionic species, which results from the presence of outer-membrane-impermeable anions in the periplasm (Stock et al., 1977; Sen et al., 1988; and see below). Because the cytoplasmic membrane is impermeable to NaCl and incapable of supporting an osmotic pressure difference, the cytoplasm loses water to increase its osmolality to that of the periplasm; this passive response reduces the amount of cytoplasmic water at 1.0 Osm to ~30% of its original value without changing the amounts of any cytoplasmic solutes (Cayley et al., 1991; Record et al., 1998a). (Plasmolysis beyond 1.0 Osm results eventually in removal of all unbound [osmotically active] water from the cytoplasm.) To recover from the plasmolyzed state at 1.0 Osm and resume growth requires an active osmoregulated response, initiated by increased uptake of extracellular K^+, with the end result (in minimal growth medium without added osmoprotectants) that cytoplasmic amounts of K^+, glutamate$^-$ (and other organic anions), trehalose, and water increase, and the amount of cytoplasmic putrescine (2+) decreases (cf., Csonka and Epstein, 1996; Record et al., 1998a for reviews). We propose that the fundamental reason for the increases in amounts of cytoplasmic K^+, glutamate$^-$, and trehalose is to allow the cell to increase the amount of cytoplasmic water (by almost twofold over that characteristic of the nongrowing plasmolyzed state at 1.0 Osm) and thereby achieve the highest growth rate possible in a minimal medium without osmoprotectants at this osmolality (Cayley et al., 1991, 1992; Record et al., 1998a).

In the present study, we use suspensions of *E. coli* lacking external K^+ to prevent cells from adapting to osmotic stress, and analyze measurements of the passive changes in cell and compartment volumes in plasmolysis titrations of cells with NaCl to quantify their osmotic properties. This novel analysis provides the basis for our use of cell and cytoplasmic volume measurements to determine the osmotic properties and intracellular water activity of *E. coli* as a function of osmolality of growth.

The balloon model for turgor pressure of *E. coli*: why measurements of cell volume provide information about turgor

The bacterial cell wall has been considered balloon-like (Doyle and Marquis, 1994) because it can stretch under pressure. Outwardly directed turgor pressure (the osmotic pressure difference between the cell interior and the external medium) is the analog of the difference in air pressure, which inflates a balloon. Turgor pressure stretches the peptidoglycan of the *E. coli* cell wall (Woldringh, 1994 and references therein) elastically (Koch and Woeste, 1992) relative to the unstressed state existing in the absence of an osmotic pressure difference. *E. coli* has two compartments (periplasm and cytoplasm) with different permeabilities to solutes, so osmotic responses of both compartments must be considered to interpret effects of osmotic stress on cell volume. In this work, we provide additional evidence that the periplasm and cytoplasm are isoosmotic, in agreement with the conclusion of Stock et al. (1977) and Sen et al. (1988), which simplifies the physical situation. Thus, the balloon analogy is instructive, and serves as the qualitative basis for our model that changes in cell volume reflect changes in turgor pressure.

MATERIALS AND METHODS

Bacterial strain, growth media, buffers, and chemicals

All experiments were performed with *E. coli* K-12 strain MG1655. Cells were grown aerobically at 37°C in a very low osmolality MOPS (3-(*N*-

morpholino)-propanesulfonate)-buffered glucose minimal medium (VLOM; 0.03 Osm; Capp et al., 1996), in the MOPS-buffered glucose minimal medium (MBM; 0.1 Osm; Cayley et al., 1989), or in MBM with NaCl used to adjust osmolality of the growth media. Wash buffer is growth medium in which all K^+ (present as KH_2PO_4) and glucose were replaced by an isoosmotic amount of NaCl. Plasmolysis buffer is wash buffer with additional NaCl to increase the osmolality.

^3H_2O (1 mC_i/g) and [^{3}H] polyethylene glycol (1.51 mC_i/g) were obtained from DuPont (Boston, MA). [^{14}C] sucrose (621 mC_i/mmol), [^{14}C] inulin (9.4 mC_i/mmol), [^{14}C] urea (54.0 mC_i/mmol), and [^{3}H] sucrose (12.0 C_i/mmol) were obtained from Amersham (Arlington Heights, IL). All radiochemicals except ^3H_2O and [^{14}C] urea were purified of radiolabeled contaminants that interfere with volume measurements by preincubation with cell slurries as previously described (Cayley et al., 1991, 1992). NH_4OH (5.08 N in water), 1-bromododecane, and silicone oil were obtained from Aldrich (Milwaukee, WI).

Measurement of amounts of cellular and cytoplasmic water in nongrowing cell suspensions

Volumes of cell water ($\bar{V}^{wa}_{cell}$) and cytoplasmic water ($\bar{V}^{wa}_{cyto}$) in units of μL per mg cell dry weight (μL/mg DW) of fresh nongrowing cell suspensions were obtained from comparisons of the volume of cell pellets accessible to ^3H_2O to that of ^{14}C inulin (a polymer that is outer-membrane impermeable) or ^{14}C sucrose (which freely diffuses into the periplasm but is not transported into the cytoplasm) using the method of Stock et al. (1977) as described previously (Cayley et al., 1991, 1992). (These volumes are equivalent to amounts of water in mg water/mg DW assuming a density of intracellular water of 1.0 g/mL). Briefly, cells were harvested from exponential growth (at $\sim 3 \times 10^8$ cells/mL, $<$0.2 mg DW/mL) by centrifugation at 7,000 $\times$ *g* for 8 min. Cell pellets were suspended in isoosmotic wash buffer, recentrifuged, resuspended to a final cell density of $\sim$5 mg DW/mL with wash buffer and swirled periodically for the $\sim$30 min typically needed to complete a series of measurements. Three μC_i/mL of ^3H_2O and 0.2 μC_i/mL of either ^{14}C sucrose or ^{14}C inulin were then added per mL of suspension, after which the samples were immediately centrifuged at 12,000 $\times$ *g* for 30 s (a sufficient time to pellet cells completely). The cpm in samples of the supernatant and cell pellets were then assayed by dual isotope scintillation counting and used to determine $\bar{V}^{wa}_{cell}$ and $\bar{V}^{wa}_{cyto}$ (in μL/mg DW) as described previously (Cayley et al., 1991). Measurements of $\bar{V}^{wa}_{cyto}$ immediately after harvest and 40 min after harvest were the same, demonstrating that the amount of cytoplasmic water did not vary during the time needed to perform a series of measurements. In addition, $\bar{V}^{wa}_{cyto}$ was independent of time of incubation of ^{14}C sucrose in suspensions before volume assay for at least 10 min, the longest time tested. Control experiments showed no significant differences in volumes determined with ^{3}H PEG instead of ^{14}C inulin or with ^{14}C taurine instead of ^{14}C sucrose or ^{3}H sucrose, indicating the absence of any specific interactions of these probes with cell components. Bubbling suspensions with O_2 or incubation with 5 mM fructose, 11 mM glucose or 1.3 mM KH_2PO_4 for five min prior to assay did not affect $\bar{V}^{wa}_{cyto}$, showing that any deprivation of oxygen or nutrients that occurred during preparation of suspensions did not lower $\bar{V}^{wa}_{cyto}$. All steps after cell growth were performed at room temperature.

In NaCl plasmolysis titrations to determine the passive responses of $\bar{V}^{wa}_{cell}$ and $\bar{V}^{wa}_{cyto}$ to increases in external osmolality, suspensions of cells grown at 0.03 Osm (in VLOM) or at 0.83 Osm (in MBM+0.4 NaCl) were assayed as described above except that, immediately before addition of radiochemicals, samples were diluted fivefold with plasmolysis buffer to achieve the desired range of final NaCl concentrations and a final cell density of $\sim$5 mg DW/mL.

Most previously published values of $\bar{V}^{wa}_{cyto}$ (Cayley et al., 1991, 1992) referred to in this paper were determined using ^{14}C taurine in place of ^{14}C sucrose. McLaggan and Epstein (1991) found that taurine can be accumulated with a K_M of $\sim$30 mM at high osmolality by strains of growing *E. coli* defective in the osmotically regulated accumulation of cytoplasmic trehalose. However, in addition to controls that previously demonstrated that neither purified ^{14}C taurine (typically used at a concentration of 0.28 mM) nor ^{14}C sucrose is accumulated by nongrowing suspensions of our wild-type strain under the conditions of our volume assays (Cayley et al., 1991, 1992), we observe that 1) $\bar{V}^{wa}_{cyto}$ of suspensions measured with purified ^{14}C sucrose or ^{14}C taurine for cells grown in MBM+0.5 M NaCl are the same within error, 2) dilution of ^{14}C taurine to 1 mM with unlabeled taurine does not alter measured values of $\bar{V}^{wa}_{cyto}$ of cells grown in MBM+0.2 M NaCl, and 3) variation of the concentration of ^{14}C taurine from 0.1 mM to 1 mM does not significantly affect measured values of $\bar{V}^{wa}_{cyto}$ of cells plasmolyzed with 1 M NaCl. Use of taurine therefore introduced no systematic errors in determinations of $\bar{V}^{wa}_{cyto}$.

We also tested whether the centrifugal harvest method used to prepare suspensions caused physiological changes relative to growing cells. Upon completion of a volume assay with suspensions of cells grown at 0.1 Osm, samples examined in a phase microscope exhibited motility, indicating that these suspensions retained and were capable of utilizing endogenous energy reserves. Cell viability (the number of colony forming units determined by plating on LB agar) and $\bar{V}^{wa}_{cyto}$ also remained constant over the course of an assay, as determined by comparison of these values before and after a series of volume measurements. Moreover, cell suspensions had not entered stationary phase by completion of a typical volume assay, because suspended cells resumed a normal growth rate immediately, with no observable lag, upon dilution into fresh growth medium, and the thermotolerance of centrifugally-harvested suspensions grown at 0.1 Osm (determined by the reduction in viability with time after heating at 48°C; see Hengge-Aronis et al., 1991) was identical to that of growing cells and not enhanced as it was in cells grown into stationary phase at 0.1 Osm (data not shown).

Measurements of amounts of cellular and cytoplasmic water and of protein in growing cells

Volumes of water in growing cells were determined by measuring the distribution of radiolabeled probes in pellets of cultures harvested by brief centrifugation through oil. Briefly, 2 μC_i/mL of ^{14}C inulin, ^{14}C sucrose, or ^3H_2O was added to midlog phase cultures growing in MBM (0.1 Osm, an osmolality at which the density of cells is $\sim$1.08–1.09 g/mL; Baldwin et al., 1995) immediately before centrifuging 1.4 mL aliquots in microfuge tubes containing 200 μL of 1-bromododecane (BDD; ρ = 1.038 g/mL) at 12,000 $\times$ *g* for at least 90 s. (Centrifugation for less than $\sim$90 s incompletely pelleted cells, whereas results were independent of centrifugation times longer than 90 s). After removing 100 μL of supernatant from each tube, the remaining supernatant and BDD was carefully removed by aspiration, the pellets suspended in 100 μL of water, and the cpm in the supernatant and pellet samples determined by scintillation counting. (The cpm in the suspended pellets ranged from $\sim$1,000 cpm for samples containing ^{14}C inulin up to $\sim$2,000 cpm for samples containing ^3H_2O, and no cpm was detected in the discarded BDD). The amount of protein in each sample was determined by comparing the A_{550} of the culture at the time of assay to a standard curve of mg protein/mL of culture versus A_{550} determined separately on multiple cultures grown in MBM, using the assay of Lowry et al. (1951) with BSA as standard as previously described (Cayley et al., 1991). Values were normalized to the dry weight of samples using measurements of the protein/dry weight ratio determined separately. Volumes of cytoplasmic and cellular water (in μL/mg dry weight) were then calculated as for suspensions by subtracting the sucrose- or inulin-accessible volumes, respectively, from the water-accessible volume. In experiments to control for the effects of centrifugation of suspensions through BDD, the procedure used for assaying volumes of suspensions was used except that, after radiochemical addition, samples were centrifuged in tubes containing 200 μL of BDD for 120 s.

Preliminary experiments showed that the error in the measurements of volumes of growing cells assayed as above was much lower in cells centrifuged through BDD than in cells centrifuged in the absence of BDD. Preliminary experiments also showed that the firmness of pellets and reproducibility of results obtained with cells spun through BDD ($\rho = 1.038$ g/mL) was greater than for silicon oil ($\rho = 1.050$ g/mL). Evaporation of ^3H_2O was an insignificant source of error because the membrane-permeable solute ^{14}C urea (which readily equilibrates into cytoplasmic water; Mitchell and Moyle, 1956) gave results that were identical to those obtained with ^3H_2O as long as sufficient time (~90 s) was allowed for urea to diffuse into the cytoplasm of cells before centrifugation of samples through BDD.

Calculations of periplasmic MDO concentration and of the contribution of MDO to turgor pressure

To calculate concentrations of periplasmic MDO at different osmolalities of growth, molar amounts of MDO in *E. coli* K-12 from the data of Kennedy (1982) and Lacroix et al. (1989) were divided by the corresponding amounts of free water in the periplasm obtained from our volume measurements. Kennedy (1982) and Lacroix et al. (1989) measured the relative amounts of MDO (in cpm/mg DW, using ^3H-glycerol to label MDO) as a function of growth osmolality varied with NaCl. Values of the radioactivity of the extracted MDO taken from Fig. 1 of Kennedy (1982) were corrected for non-MDO material by subtracting the reported osmotically-invariant amount of non-MDO radioactivity in MDO extracts. Lacroix et al. (1989) reported relative amounts of MDO determined after purification from non-MDO-labeled material. The relative amounts of MDO from Kennedy (1982) were normalized to nmol MDO/mg DW using the value of 136 nmol MDO glucose/mg DW determined by Kennedy and coworkers in cells grown at 0.07 Osm and their assumption of 9 glucose/MDO (Rumley et al., 1992). The relative amounts of MDO from LaCroix et al. (1989) were converted to nmol MDO/mg DW using their value of 125 ± 20 nmol MDO glucose/mg DW determined in cells grown at 0.07 Osm and by assuming 9 glucose/MDO. (The principal MDO species have 8–9 glucose monomers; Kennedy, 1996).

To calculate periplasmic concentrations of MDO (C_{MDO}, expressed as moles of MDO per L of free periplasmic water), the amount of periplasmic water $\bar{V}^{wa}_{peri}$ (in μL/mg cell DW) as a function of osmolality were obtained by interpolation of the linear fit (cf., Fig. 3 *B* below) of measurements of $\bar{V}^{wa}_{peri}$ determined in this study and in Cayley et al. (1991). Values of $\bar{V}^{wa}_{peri}$ were converted to volumes of free periplasmic water ($\bar{V}^{wa}_{peri,f}$) by subtracting an osmolality-independent estimate of the volume of bound water of hydration of periplasmic biopolymers ($\bar{V}^{wa}_{peri,b} = 0.09$ μL/mg cell DW) obtained by assuming that periplasmic biopolymers are hydrated to the same extent as cytoplasmic biopolymers (0.5 g H_2O/g biopolymer; Cayley et al., 1991, 1992) and that the protein/DW ratio of the periplasm and cytoplasm are the same. Periplasmic protein was taken as 20% of cell protein (Ames et al., 1984; Cronan et al., 1987).

The contribution of periplasmic MDO (concentration C_{MDO} and apparent valence Z^{app}_{MDO}) to turgor pressure across the cell wall as a function of salt concentration was calculated from a Donnan equilibrium analysis, in which the osmotic pressure difference across the cell wall is ascribed to anionic MDO in the periplasm and to the unequal distribution of salt ions between the periplasm and the external solution required for periplasmic electroneutrality. For this calculation, the ionic composition of the minimal growth medium with added NaCl was approximated as that of a univalent salt with an anion concentration C_X equal to the sum of the anion concentrations in the growth medium (primarily Cl^- and the $MOPS^-$ anion). Then the MDO contribution to turgor pressure ($\Delta\Pi_{MDO}$) is given by

$$\Delta\Pi_{MDO} \cong RT[C_{MDO} + (Z^{app}_{MDO}{}^2 C^2_{MDO} + 4C^2_X)^{0.5} - 2C_X], \quad (1)$$

where $R = 0.0821$ liter atm mol^{-1} deg^{-1} and T is Kelvin temperature. In Eq. 1, nonideality effects are neglected. Subject to this approximation, Eq. 1 is valid over the entire range of concentration ratios C_{MDO}/C_X, and gives the correct limiting results $\Delta\Pi_{MDO} = RTC_{MDO}$ for $C_X \gg C_{MDO}$ and $\Delta\Pi_{MDO} = RT(|Z^{app}_{MDO}| + 1)C_{MDO}$ for $C_{MDO} \gg C_X$.

Other methods

The osmolalities of the growth media, wash buffer, and low-osmolality plasmolysis solutions (≤0.1 Osm) were measured using a Wescor model 5520 vapor pressure osmometer. Osmolalities of other plasmolysis buffers were calculated assuming additivity of osmotic contributions from the wash buffer and added NaCl. The latter contribution was calculated using osmotic coefficients of NaCl from Robinson and Stokes (1959). This procedure was verified to be accurate to within 1% for representative samples measured by osmometry. All fittings were performed using the program NONLIN (Johnson and Frasier, 1985; Straume et al., 1991), a nonlinear functional-form, least-squares fitting program. All errors reported for fittings were obtained from NONLIN using a 67% confidence probability.

RESULTS

Variation of the volume of cell water of *E. coli* in NaCl titrations of cells grown at very low (0.03) and moderately high (0.83) osmolality

Figure 1 plots the volume of cell water ($\bar{V}^{wa}_{cell}$) per unit DW of *E. coli* grown at 0.03 Osm (in VLOM) and during the subsequent course of plasmolysis titrations with NaCl. At the growth osmolality of 0.03 Osm, $\bar{V}^{wa}_{cell} = 2.96 \pm 0.10$ μL/mg DW. Numerically, values of $\bar{V}^{wa}_{cell}$ correspond to amounts of cell water in mg water/mg DW, assuming a density of 1 mg/μL. Figure 1 shows that addition of NaCl reduces $\bar{V}^{wa}_{cell}$ of these cells monotonically to a high osmolality minimum value (designated $\bar{V}^{wa}_{cell,min}$) of 1.9 ± 0.05 μL/mg DW, attained within error at plasmolyzing osmola-

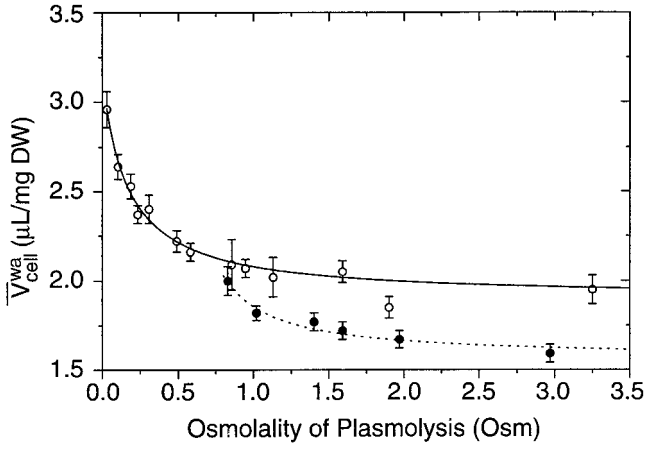

FIGURE 1 Reduction in water-accessible cellular volume $\bar{V}^{wa}_{cell}$ of suspensions of cells grown at 0.03 Osm (in VLOM; ○) and at 0.83 Osm (in MBM+0.4 M NaCl; ●) with increasing plasmolyzing osmolality adjusted with NaCl. Each point shows the average (±1 SD) of 2–7 independent determinations, each performed in triplicate on separate cultures. The curves are empirical hyperbolic best fits to the data, and were used to estimate $\bar{V}^{wa}_{cell,min}$.

lities in excess of ~1 Osm. Figure 1 also plots the behavior of $\bar{V}^{wa}_{cell}$ in plasmolysis titrations of cells grown at 0.83 Osm (in MBM+0.4 M NaCl), at which osmolality the amount of periplasmic MDO is very low (Kennedy, 1982; Lacroix et al., 1989). The initial value of $\bar{V}^{wa}_{cell}$ is much smaller for cells grown at 0.83 Osm than at 0.03 Osm (cf., Fig. 1 and Table 1), in agreement with previous observations (summarized in Table 1) that $\bar{V}^{wa}_{cell}$ decreases with increasing osmolality of growth (Richey et al., 1987; Larsen et al., 1987; Cayley et al., 1991). Notably (cf., Fig. 1), after plasmolysis to any osmolality greater than 0.83 Osm, the amount of water remaining in cells grown at 0.83 Osm is always significantly less than the amount of water in cells grown at 0.03 Osm. Figure 1 shows that, for cells grown at 0.83 Osm and titrated with NaCl, increasing external osmolality reduces the volume of cell water to a high osmolality minimum value $\bar{V}^{wa}_{cell,min}$ of 1.56 ± 0.05 μL/mg DW. This plateau value is significantly less than the value of $\bar{V}^{wa}_{cell,min}$ obtained from plasmolysis titrations of cells grown at 0.03 Osm (1.90 ± 0.05 μL/mg DW).

To verify that the variation of $\bar{V}^{wa}_{cell,min}$ with osmolality of growth is only the result of changes in the volume of water per cell, and not in the amount of dry weight per cell, we examined whether the dry weight per cell varies with osmolality of growth. Table 1 lists the growth rate and mass of protein per viable cell for cultures grown from 0.1 to 1.0 Osm. Whereas the growth rate exhibits a maximum near 0.28 Osm, the amount of protein per viable cell shows no systematic variation with osmolality and has an average value of 0.41 ± 0.03 pg per cell, which is within the range of published values for *E. coli* B/r (Bremer and Dennis, 1996). Because the protein/DW ratio of cells grown over this range of conditions is 0.68 ± 0.07, independent of osmolality (Cayley et al., 1991), the dry weight per cell is therefore also constant over the range of osmolalities and growth rates examined.

Table 1 also lists volumes of water per viable cell, calculated from determinations of the amount of protein per viable cell, the protein/DW ratio, and the volume of cell water per mg DW. The calculated amount of H_2O per cell decreases from ~1.8 fL for cells grown at 0.03 Osm to ~1.1 fL for cells grown at 1.0 Osm (Table 1). For cells grown at 0.1 Osm (in MBM), the volume of water per cell is ~1.5 fL. Because the water-inaccessible volume of these cells is 0.63 ± 0.09 μL/mg DW (or ~0.4 fL per cell), independent of osmolality (Cayley et al., 1991), therefore, the total volume of the average cell grown at 0.1 Osm is ~1.9 fL per cell. The corresponding dimensions of a cell with this volume, assuming a cylindrical shape with hemispherical ends and an overall 2:1 length:width ratio, is approximately 2.2 μm × 1.1 μm, consistent with accepted dimensions of *E. coli* (Neidhardt et al., 1990).

Changes in water-accessible cytoplasmic and periplasmic volume during NaCl plasmolysis titrations of *E. coli* grown at very low osmolality (0.03 Osm)

Figure 2 plots the volumes of cytoplasmic and periplasmic water of *E. coli* grown at 0.03 Osm and subsequently plasmolyzed with NaCl. The initial volumes of water in these compartments are $\bar{V}^{wa}_{cyto}$ = 2.53 ± 0.08 μL/mg DW and $\bar{V}^{wa}_{peri} \equiv \bar{V}^{wa}_{cell} - \bar{V}^{wa}_{cyto}$ = 0.43 ± 0.13 μL/mg DW. As the concentration of NaCl is increased, $\bar{V}^{wa}_{cyto}$ decreases monotonically to an apparent plateau volume of ~0.4 μL/mg DW, approached at plasmolyzing osmolalities in excess of 3 Osm. (For reference, the curve for the reduction in $\bar{V}^{wa}_{cell}$

TABLE 1 Dependence on growth osmolality of volume accessible to water, growth rate, and amounts of protein and water per *E. coli* cell

Growth Osmolality (Osm)	Growth rate* (generations/hr)	$\bar{V}^{wa}_{cell}$* (μl/mg DW)	Protein/cell† (pg/cell)	H_2O/cell‡ (fL/cell)
0.03	0.84 ± 0.07	2.96 ± 0.10	ND	1.77 ± 0.14
0.1	0.91 ± 0.04	2.52 ± 0.06	0.39 ± 0.02	1.52 ± 0.12
0.28	1.00 ± 0.10	2.45 ± 0.11	0.43 ± 0.04	1.48 ± 0.13
0.56	0.79 ± 0.06	ND	0.41 ± 0.01	ND
0.65	0.73 ± 0.03	2.06 ± 0.05	0.41 ± 0.01	1.24 ± 0.10
0.83	0.63 ± 0.11	1.99 ± 0.05	0.40 ± 0.04	1.20 ± 0.09
1.0	0.49 ± 0.04	1.87 ± 0.12	0.42 ± 0.05	1.13 ± 0.11
1.0 + 1 mM proline§	0.56 ± 0.05	ND	0.41 ± 0.03	ND

*Growth rates and amounts of cell water $\bar{V}^{wa}_{cell}$ for cells grown at 0.03 Osm, 0.1 Osm and 0.83 Osm were determined in this study; the other values of growth rate and $\bar{V}^{wa}_{cell}$ are from Cayley et al. (1991).

†Amounts of protein per viable cell in picograms are the average (±1 SD) of approximately five measurements, each performed in triplicate. Viable cell counts were determined by dilution plating samples on LB agar. The average of all measurements at all osmolalities is 0.41 ± 0.03 pg/cell.

‡The volume of water per cell in femtoliters was calculated using the average amount of protein per cell (0.41 ± 0.03 pg), the ratio of protein to dry weight of cells grown under these conditions (0.68) and tabulated values of $\bar{V}^{wa}_{cell}$.

§Proline is an osmoprotectant, accumulated from the medium, that increases the growth rate of osmotically stressed cells (Cayley et al., 1992; Csonka and Epstein, 1996).

ND, not determined.

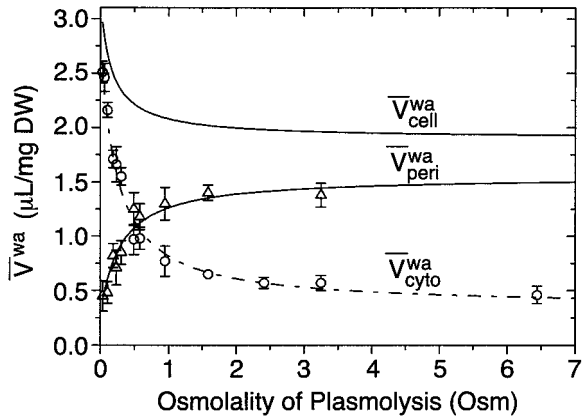

FIGURE 2 Reduction in water-accessible cytoplasmic volume (○) and increase of water-accessible periplasmic volume (△) of cells grown at 0.03 Osm (in VLOM) with increasing plasmolyzing osmolality adjusted with NaCl. Each point shows the average (±1 SD) of 6–12 measurements performed on 2–4 cultures. The curve through the $\bar{V}^{wa}_{cyto}$ data is an empirical best-fit hyperbolic function. The corresponding fit to the $\bar{V}^{wa}_{cell}$ data for cells grown at 0.03 Osm from Fig. 1 is shown for comparison. Periplasmic data points were obtained by subtraction of cytoplasmic volumes from cell volumes. The periplasmic volume curve was determined by subtraction of the fitted curves for cell and cytoplasmic volumes.

with plasmolyzing osmolality from Fig. 1 is also shown in Fig. 2.) Plasmolysis of cells grown at 0.03 Osm affects $\bar{V}^{wa}_{cyto}$ much more than $\bar{V}^{wa}_{cell}$, reducing $\bar{V}^{wa}_{cyto}$ to less than 20% of its initial value, whereas $\bar{V}^{wa}_{cell}$ is reduced to 60% of its original value. Consequently $\bar{V}^{wa}_{peri}$ (the difference between $\bar{V}^{wa}_{cell}$ and $\bar{V}^{wa}_{cyto}$) increases during these plasmolysis titrations by more than 300%, to a plateau value of approximately 1.45 ± 0.07 μL/mg DW. This increase in $\bar{V}^{wa}_{peri}$ indicates that outer-membrane impermeable periplasmic solutes (including MDO) are diluted to about 30% of their initial concentration upon plasmolysis with high concentrations of NaCl. The observed variations in cell and compartment volumes in NaCl plasmolysis titrations are larger but otherwise quite analogous to those reported by Stock et al. (1977) for sucrose plasmolysis titrations of cells grown at 0.14 Osm.

Volume measurements on fresh cell suspensions are applicable to growing cells

K^+-deprived suspensions of cells were used in the plasmolysis titrations to study the passive response of cells to osmotic stress, because increased uptake of K^+ from the medium is the initial response required for adaptation of *E. coli* to hypertonic shock (Csonka and Epstein, 1996). The suspensions also lacked glucose to prevent new synthesis of organic osmolytes, further ensuring that plasmolyzed cells are unable to respond to increases in external osmolality by active osmoregulated mechanisms, and, instead, exhibit only the passive response of loss of cell water. The absence of an active response in plasmolyzed cell suspensions was verified by our observation (data not shown) that the amount of cytoplasmic water in cells after plasmolysis with 1 M NaCl remained unchanged for at least 15 min, the longest time tested.

To test directly whether the amounts of cell and cytoplasmic water in cell suspensions and in growing cells are the same, we used a radiochemical method to measure volumes of growing cells harvested by brief centrifugation through BDD oil as described in Methods. Table 2 shows that the water-accessible cell and cytoplasmic volumes of mid-log phase cells growing at 0.1 Osm are the same, within error, as in fresh suspensions, indicating that fresh suspensions have the same osmotic properties as growing cells (and see Discussion). Table 2 also shows that centrifugation of cells through BDD per se does not perturb the amount of intracellular water, because cell and compartment volumes of suspensions assayed by centrifugation through BDD are the same as suspensions assayed without BDD. A bathing atmosphere of medium must therefore accompany cells as they sediment through BDD, a conclusion verified by our observation that the extracellular (i.e., inulin-accessible) volume in pellets of samples spun through BDD contained ~1.8 μL extracellular water/mg total cell protein. Although significantly less than the ~2.5 μL extracellular water/mg protein of samples of pellets spun in the absence of BDD, the 1.8 μL/mg protein greatly exceeds that estimated for a monolayer coverage (~0.02 μL/mg protein) of a smooth surface with the dimensions of the *E. coli* cell.

The equivalence of the amounts of cell and cytoplasmic water in fresh suspensions and growing cells validates our use of suspensions in this study. Measurements on suspensions are preferable to measurements of growing cells because they are of higher accuracy (see Table 2), in large part because the cell density of log phase cultures is ~25-fold lower than that of suspensions. Moreover, to measure only the passive response of cells to osmotic stress, plasmolysis

TABLE 2 Water-accessible volumes (μL H_2O/mg DW) of the cell and compartments of growing cells and of centrifugally harvested suspensions

	$\bar{V}^{wa}_{cell}$	$\bar{V}^{wa}_{cyto}$	$\bar{V}^{wa}_{peri}$
Growing cells spun through oil*	2.54 ± 0.10	2.11 ± 0.20	0.43 ± 0.23
Suspensions†	2.54 ± 0.06	2.19 ± 0.11	0.35 ± 0.13
Suspensions spun through oil‡	2.60 ± 0.05	2.26 ± 0.12	0.34 ± 0.13

*Values of $\bar{V}^{wa}_{cell}$ and $\bar{V}^{wa}_{cyto}$ are the mean (±1 SD) of measurements performed on four and five separate cultures, respectively, each grown at 0.1 Osm in MBM and assayed in triplicate.

†Suspensions of centrifugally harvested cells were prepared and assayed as described in Methods. The reported values are the mean (±1 SD) of results obtained in this study combined with those of Cayley et al. (1991) performed under identical conditions.

‡Volumes of suspensions spun through oil were assayed as for samples without oil except that samples were assayed by centrifugation through 200 μL of bromododecane oil. The value of $\bar{V}^{wa}_{cell}$ is the average (±1 SD) of a single triplicate measurement. The value of $\bar{V}^{wa}_{cyto}$ is the average (±1 SD) of measurements performed on four separate cultures, each assayed in triplicate.

titrations must be performed on nongrowing samples lacking extracellular K^+, because the initial active response to the increase in external osmolality of increased uptake of K^+ from the medium (Csonka and Epstein, 1996) begins immediately after external osmolality increases (Epstein and Schultz, 1965).

Variation of periplasmic MDO concentration with osmolality of growth: implications for turgor pressure

Figure 3 *A* summarizes amounts of MDO calculated by us as described in Methods from the data of Kennedy (1982) and Lacroix et al. (1989) for *E. coli* K-12 strains grown at various osmolalities. Amounts of MDO decrease monotonically from ~15 nmol MDO/mg DW at 0.07 Osm to very low levels at high osmolality. Figure 3 *B* shows our determinations of the volume of free periplasmic water $\bar{V}^{wa}_{peri,f}$, obtained from the difference between measured volumes of cell and cytoplasmic water as a function of osmolality of growth as described in Methods. At least above 0.3 Osm, $\bar{V}^{wa}_{peri,f}$ increases slightly as osmolality of growth increases, in agreement with previous measurements of $\bar{V}^{wa}_{peri}$ (Richey et al., 1987; Larsen et al., 1987). The experimental uncertainty is too large to allow us to conclude whether a different behavior occurs at lower osmolality, and, consequently, we have fit all these data to a line for purposes of interpolation. Interpolated values of $\bar{V}^{wa}_{peri,f}$ (Fig. 3 *B*) were used with determinations of n_{MDO} (Fig. 3 *A*) to estimate concentrations of MDO (c_{MDO}; see Methods) in the free water of the periplasm as a function of the osmolality of growth. Figure 3 *C* shows that c_{MDO} decreases from ~0.05 mol/L at a growth osmolality of 0.07 Osm to ~0.003 mol/L at a growth osmolality of 0.8 Osm. Uncertainties in absolute MDO concentrations are approximately ±35%; this uncertainty, although large, has no effect on the semiquantitative conclusions obtained from analyses of these results below. (The trend in relative amounts of MDO is known to higher accuracy; Lacroix et al., 1989). In particular, the contribution of MDO (which Kennedy [1996] concluded are free and not bound to other periplasmic components) to turgor pressure across the cell wall may be estimated from these c_{MDO} and from the concentration of anions of the medium, as described in Methods and analyzed below.

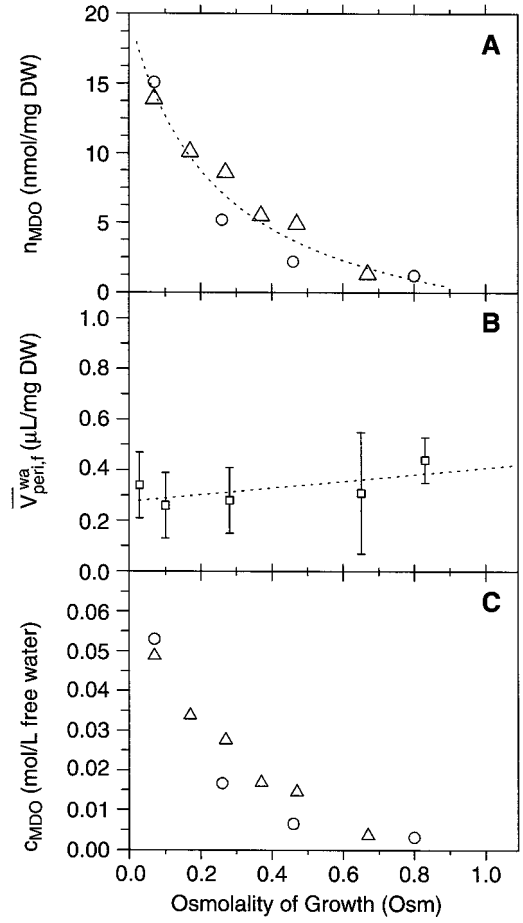

FIGURE 3 Reduction of the amount and concentration of periplasmic MDO with increasing osmolality of growth. *Panel A* plots the amounts of MDO (n_{MDO}) in nmol/mg cell DW calculated as described in Methods from the analytical data of Kennedy (1982; ○) and Lacroix et al. (1989; △) versus growth osmolality. The curve is the empirical best hyperbolic fit to all the data. *Panel B* plots the volumes of free periplasmic water $\bar{V}^{wa}_{peri,f}$ (determined as described in Methods) in μL/mg cell DW versus osmolality of growth. The line is the best linear fit to the data and is shown for purposes of interpolation. *Panel C* shows the decrease in concentration of periplasmic MDO (c_{MDO}) (in mol/L free periplasmic water) calculated from values of n_{MDO} (*A*) and interpolated values of $\bar{V}^{wa}_{peri,f}$ (*B*) with increasing osmolality of growth.

ANALYSIS

Analysis of $\bar{V}^{wa}_{cyto}$ in plasmolysis titrations of cells grown at low osmolalities (0.03 Osm to 0.28 Osm) demonstrates that turgor pressure decreases with increasing osmolality of growth

For cells grown at 0.1 and 0.28 Osm, Cayley et al. (1991) quantitatively analyzed the behavior of the volume occupied by cytoplasmic water $\bar{V}^{wa}_{cyto}$ in plasmolysis titrations to obtain both the volume occupied by bound water of hydration $\bar{V}^{wa}_{cyto,b}$ and the amount of osmotically-significant solutes $\phi_{cyto}(\Sigma\, n_j)_{cyto}$ (see below) in the cytoplasm. Here, we extend this analysis to determine turgor pressure of these cells and of cells grown at 0.03 Osm. Turgor pressure is fundamentally related to the difference between the osmolality of the cytoplasm (Osm_{cyto}) and the external medium (Osm_{ex}) by

$$\Delta\Pi = RT(Osm_{cyto} - Osm_{ex}). \quad (2)$$

The osmolality of the cytoplasm is related to the molar amount of cytoplasmic solutes $(\Sigma\, n_j)_{cyto}$ and to the volume

occupied by free (unbound) cytoplasmic water ($\bar{V}^{wa}_{cyto,f}$) by

$$Osm_{cyto} = \phi_{cyto}\left(\sum n_j\right)_{cyto}\Big/\bar{V}^{wa}_{cyto,f}, \quad (3)$$

where ϕ_{cyto} is the osmotic coefficient of the cytoplasm (Cayley et al., 1991). In Eq. 3,

$$\bar{V}^{wa}_{cyto,f} \equiv \bar{V}^{wa}_{cyto} - \bar{V}^{wa}_{cyto,b}, \quad (4)$$

where $\bar{V}^{wa}_{cyto,b}$ is defined as the volume occupied by bound cytoplasmic water (expected to be primarily water of macromolecular hydration; Cayley et al., 1991; Record et al., 1998a). Combining Eqs. 2–4 gives

$$\bar{V}^{wa}_{cyto} = \bar{V}^{wa}_{cyto,b} + \phi_{cyto}\left(\sum n_j\right)_{cyto}\Big/(\Delta\Pi/RT + Osm_{ex}). \quad (5)$$

Plots of $\bar{V}^{wa}_{cyto}$ versus $1/Osm_{ex}$ for data obtained in NaCl plasmolysis titrations of cells grown at 0.1 Osm and 0.28 Osm (Cayley et al., 1991) and at 1.02 Osm (Cayley et al., 1992) are linear for all plasmolyzing NaCl concentrations investigated, although the initial (unplasmolyzed) value of $\bar{V}^{wa}_{cyto}$ at 0.1 Osm deviates from the best fit line. Interpreted using Eq. 5, this linear behavior indicates most simply that $\Delta\Pi/RT$ is negligible relative to Osm_{ex} in the range of plasmolyzing NaCl concentrations investigated, and that both $\phi_{cyto}(\Sigma n_j)_{cyto}$ and $\bar{V}^{wa}_{cyto,b}$ are independent of plasmolyzing NaCl concentration (Cayley et al., 1991). A similar plot of the $\bar{V}^{wa}_{cyto}$ data from Fig. 2 versus Osm^{-1}_{ex} for cells grown at 0.03 Osm is highly nonlinear (Fig. 4), indicating most simply that residual turgor pressure is significant relative to $RTOsm_{ex}$ at low NaCl concentrations. To simplify determination of $\phi_{cyto}(\Sigma n_j)_{cyto}$ and $\bar{V}^{wa}_{cyto,b}$, we therefore linearly fit $\bar{V}^{wa}_{cyto}$ versus $1/Osm_{ex}$ for cells plasmolyzed to an osmolarity exceeding 1 Osm to Eq. 5 assuming turgor pressure is zero. (Above 1 Osm, $\bar{V}^{wa}_{cell}$ ceases to vary significantly with Osm_{ex}, from which we deduce that residual turgor pressure is insignificant relative to $RTOsm_{ex}$). These data are well fit by a line ($r^2 = 0.99$) with best-fit slope $\phi_{cyto}(\Sigma n_j)_{cyto} = 0.31 \pm 0.04$ μmol/mg DW and intercept $\bar{V}^{wa}_{cyto,b} = 0.45 \pm 0.03$ μL/mg DW, labeled *A* in Fig. 4. (Fitting these data assuming cells grown at 0.03 Osm exhibit a small residual turgor pressure (~0.4 atm) at high plasmolyzing osmolalities (see below) yields insignificantly different values of $\bar{V}^{wa}_{cyto,b}$ and $\phi_{cyto}(\Sigma n_j)_{cyto}$.) The value of $\bar{V}^{wa}_{cyto,b}$ for cells grown at 0.03 Osm is within error of previous determinations of $\bar{V}^{wa}_{cyto,b}$ of cells grown at higher osmolality (see Table 3). The value of $\phi_{cyto}(\Sigma n_j)_{cyto}$ is approximately equal to that for cells grown at 0.1 Osm (Cayley et al., 1991), consistent with our previous conclusion (Capp et al., 1996; see also Record et al., 1998a) that the primary difference in the pool of cytoplasmic osmolytes in these cells is that cells grown at 0.03 Osm have ~0.04 ± 0.02 μmol/mg DW more putrescine(2+) and, thus, ~0.08 ± 0.04 μmol/mg DW less K^+ than cells grown at 0.1 Osm.

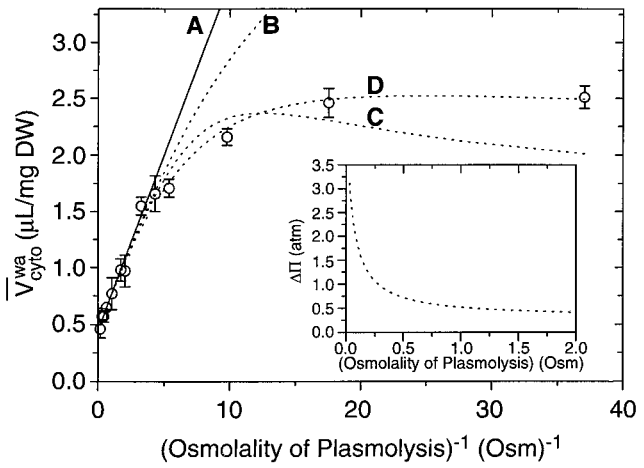

FIGURE 4 Effects of periplasmic MDO on $\bar{V}^{wa}_{cyto}$ in a NaCl plasmolysis titration of cells grown at 0.03 Osm (VLOM). Contributions to turgor pressure $\Delta\Pi$ from the Donnan osmotic pressure of periplasmic MDO as a function of Osm_{ex} were calculated with Eq. 1 using a lower bound estimate of n_{MDO} = 15 nmol/mg DW (estimated by extrapolation of Fig. 3 *A* as described in the text), interpolated values of $\bar{V}^{wa}_{peri,f}$ from Fig. 3 *B*, and several choices of MDO valence Z^{app}_{MDO}. These contributions to $\Delta\Pi$ were used in Eq. 6 to predict the behavior of $\bar{V}^{wa}_{cyto}$ versus $1/Osm_{ex}$, where Osm_{ex} was varied with NaCl in a plasmolysis titration (cf. Methods). *Curve A* shows the linear fit to the data of cells plasmolyzed to ~1 Osm and above (see text). *Curve B* shows the fit assuming $Z^{app}_{MDO} = 0$ to calculate $\Delta\Pi$; *curve C* shows the fit assuming $Z^{app}_{MDO} = -3$ to calculate $\Delta\Pi$; *curve D* is the best fit of Eq. 6 to the data and corresponds to the situation in which $|Z^{app}_{MDO}|$ is initially 2 in unplasmolyzed cells and increases to 3 as the concentration of NaCl increases (see text). *Inset*: Reduction in turgor pressure of cells grown at 0.03 Osm (VLOM) with increasing osmolality of plasmolysis. Turgor pressure was predicted from the concentration of periplasmic MDO by Eq. 1 with a value of Z^{app}_{MDO} that varied with [NaCl] as in *curve D*.

The linearity of the high-osmolality region of the plasmolysis plot in Fig. 4 indicates that $\bar{V}^{wa}_{cyto,b}$ and $\phi_{cyto}(\Sigma n_j)_{cyto}$ for cells grown at 0.03 Osm are independent of plasmolyzing osmolality. Because we use nutrient-deprived suspensions to ensure that the sum of the amount of cytoplasmic solutes $(\Sigma n_j)_{cyto}$ is constant throughout these plasmolysis titrations (see above), ϕ_{cyto} is also constant at high plasmolyzing osmolalities. We assume that ϕ_{cyto} does not vary at lower plasmolyzing osmolality, which is consistent with the K^+-nucleic acid polyion model used to interpret ϕ_{cyto} (Cayley et al., 1991). Because $\bar{V}^{wa}_{cyto,b}$ is independent of growth osmolality (see Table 3), we assume it is also independent of low plasmolyzing osmolality. With these plausible assumptions, the turgor pressure of these cells before or in the initial stages of plasmolysis may be estimated from experimental values of $\bar{V}^{wa}_{cyto}$ using

$$\Delta\Pi = RT\left(\phi_{cyto}(\sum n_j)_{cyto}/\bar{V}^{wa}_{cyto,f} - Osm_{ex}\right). \quad (6)$$

From the value of $\bar{V}^{wa}_{cyto}$ of unplasmolyzed cells (2.53 ± 0.08 μL/mg DW) and the values of $\phi_{cyto}(\Sigma n_j)_{cyto}$ and $\bar{V}^{wa}_{cyto,b}$

TABLE 3 Amounts of cytoplasmic water and osmotic properties of *E. coli* grown at different osmolalities*

Osmolality of Growth Medium	$\bar{V}^{wa}_{cyto}$	$\bar{V}^{wa}_{b,cyto}$*	$(\phi \Sigma n_j)_{cyto}$	$\Delta\Pi^{\dagger}$ (atm)	$\varepsilon^{\ddagger}$ (atm)
0.03 Osm (VLOM)	2.53 ± 0.08	0.45 ± 0.03	0.31 ± 0.04	3.1 ± 0.4	4.9 ± 0.9
0.10 Osm (MBM)[§]	2.19 ± 0.11	0.40 ± 0.02	0.29 ± 0.01	1.5 ± 0.3	3.4 ± 0.7
0.28 Osm[§] (MBM+0.1 M NaCl)	2.08 ± 0.06	0.38 ± 0.08	0.52 ± 0.07	0.7 ± 1.1	1.7 ± 2.7

*The average and standard deviation of the values shown plus the value of $\bar{V}^{wa}_{b,cyto}$ determined for cells grown at 1.02 Osm in MBM+0.5 M NaCl+1 mM betaine (0.40 ± 0.05 μL/mg DW; Cayley et al., 1992) is 0.41 ± 0.03 μL/mg DW.

†Turgor pressure was calculated as the difference $\Delta\Pi = \Pi_{cyto} - \Pi_{ex} = RT[(\phi \Sigma n_j)_{cyto}/\bar{V}^{wa}_{f,cyto}) - Osm_{ex}]$, where $R = 0.0821$ L atm mol^{-1} K^{-1}, $T = 310$ K, and $\bar{V}^{wa}_{f,cyto} = \bar{V}^{wa}_{cyto} - \bar{V}^{wa}_{b,cyto}$.

‡Values of the volumetric elastic modulus ε were calculated from Eq. 7 using $\bar{V}^{tot}_{cell,0} = 2.19 \pm 0.03$ μL/mg DW, values of $\bar{V}^{tot}_{cell}$ from Fig. 1, and the tabulated $\Delta\Pi$.

§Values of $\bar{V}^{wa}_{cyto}$ and $\bar{V}^{wa}_{b,cyto}$ (μL/mg DW) and $(\phi \Sigma n_j)_{cyto}$ (μmoL/mg DW) for cells grown at 0.10 Osm and 0.28 Osm are from Cayley et al. (1991) except for the value of $\bar{V}^{wa}_{cyto}$ for cells grown at 0.1 Osm, which was obtained by combining data obtained in the present study with data reported in Cayley et al. (1991).

obtained above and tabulated in Table 3, the calculated turgor pressure of cells grown at 0.03 Osm is $\Delta\Pi = 3.1 \pm 0.4$ atm. This value significantly exceeds the turgor pressures calculated from Eq. 6 from the published values of $\phi_{cyto}(\Sigma n_j)_{cyto}$ and $\bar{V}^{wa}_{cyto,b}$ for cells grown at 0.1 Osm (MBM; $\Delta\Pi = 1.5 \pm 0.3$ atm) and at 0.28 Osm (MBM+0.1 M NaCl; $\Delta\Pi = 0.7 \pm 1.1$ atm, i.e., $\Delta\Pi \lesssim 1.8$ atm; see Table 3 and Cayley et al., 1991).

A limitation of our plasmolysis titration method seen in these results is that the propagated error becomes comparable to the turgor pressure itself for growth osmolalities above 0.1 Osm, because the absolute error in $\phi_{cyto}(\Sigma n_j)_{cyto}/\bar{V}^{wa}_{cyto,f}$ increases and the turgor pressure decreases with increasing osmolality. Hence, other approaches (developed below) are needed to establish whether turgor pressure varies with osmolality of growth above 0.1 Osm.

Contribution of MDO to periplasmic osmolality and turgor pressure as functions of the osmolality of growth and of plasmolysis

We evaluated periplasmic MDO concentration as functions of osmolality of plasmolysis and growth from the amounts of MDO and volumes of unbound periplasmic water in Fig. 3. These values were then used in Eq. 1 to quantify the effect of periplasmic MDO concentration C_{MDO}, MDO apparent valence Z^{app}_{MDO}, and external salt (anion) concentration C_X on $\Delta\Pi$. These calculations, described below, make a strong case for our conclusions that 1) the periplasm and cytoplasm are isoosmotic under all conditions, 2) the turgor pressure exerted across the cell wall at any condition of plasmolysis is primarily determined by C_{MDO}, Z^{app}_{MDO}, and C_X, and 3) periplasmic MDO concentration and turgor pressure decrease together with increasing osmolality of growth or of plasmolysis with NaCl.

Donnan analysis of effects of residual turgor from periplasmic MDO on the initial stages of a plasmolysis titration of cells grown at 0.03 Osm

In Fig. 4, the linear fit of the high osmolality data, where the effects of residual turgor are minimized as discussed above, is designated *A*. The large systematic deviations of the data of Fig. 4 from line *A* at low Osm_{ex} (i.e., high Osm_{ex}^{-1}) are in the direction expected if cells grown at 0.03 Osm have a large residual turgor pressure at low plasmolyzing osmolality.

To assess whether the residual turgor pressure calculated from the amount of periplasmic MDO can predict $\bar{V}^{wa}_{cyto}$ over the entire range of plasmolyzing osmolalities in Fig. 4 (and thus to test whether the cytoplasm and periplasm are isoosmotic), we estimated the amount of MDO in cells grown at 0.03 Osm by extrapolation of the empirical fit to the data of Fig. 3 *A* to be ~18 ± 3 nmol MDO/mg DW. A conservative lower-bound estimate of n_{MDO} (15 nmol MDO/mg DW) was then used to estimate residual turgor from the Donnan model (Eq. 1) for two choices of MDO apparent valence. Curve *B* represents the case $Z^{app}_{MDO} = 0$, corresponding to the situation in which MDO bear no net charge (for example, as a result of Mg^{2+} binding). Although clearly Curve *B* is an improvement over line *A*, it does not fit the $\bar{V}^{wa}_{cyto}$ plasmolysis data below 0.2 Osm. Curve *C* was calculated assuming $Z^{app}_{MDO} = -3$, corresponding to the situation in which there is no cation binding to MDO and therefore MDO have their full structural charge (the primary species of MDO have an approximate structural charge of −3; Kennedy, 1996). Curve *C* provides a better fit than curve *B* to the $\bar{V}^{wa}_{cyto}$ data at 0.1 and 0.2 Osm, but deviates at lower osmolality, predicting values of $\bar{V}^{wa}_{cyto}$ that are significantly smaller than those measured at 0.03 and 0.05 Osm. (The discrepancy increases if higher values of n_{MDO} or Z^{app}_{MDO} are assumed.) Despite this, Curve *C* demonstrates unambiguously that the Donnan osmotic pressure contribution of negatively charged MDO is more than sufficient to account for the effects of turgor pressure on $\bar{V}^{wa}_{cyto}$. We then let Z^{app}_{MDO} vary with Osm_{ex}, and calculated the values of Z^{app}_{MDO} that yield the observed values of $\bar{V}^{wa}_{cyto}$ (Curve *D*). We find that Z^{app}_{MDO} in Eq. 1 must be smaller in magnitude at the low osmolality of growth than after plasmolysis with high concentrations of NaCl. To fit the $\bar{V}^{wa}_{cyto}$ data of Fig. 4 requires that $|Z^{app}_{MDO}| \cong 2$ at the growth osmolality of 0.03 Osm, but that $|Z^{app}_{MDO}| = 3$ upon plasmolysis of these cells with ≥0.05 M NaCl, an increase in $|Z^{app}_{MDO}|$ which could result from dissociation of

bound Mg^{2+} (or other oligovalent cations) from MDO with increasing NaCl concentration.

Taken together, the semiquantitative calculations in Fig. 4 show that a reasonable estimate of the amount and apparent valence of periplasmic MDO is sufficient to satisfy the condition $Osm_{cyto} = Osm_{peri}$. We therefore propose that the Donnan osmotic pressure of charged MDO is the primary determinant of turgor pressure for cells grown at 0.03 Osm and conclude that the periplasm and cytoplasm are isoosmotic, in agreement with the findings of Stock et al. (1977) and Sen et al. (1988) (see Discussion).

Role of MDO as a determinant of the extent of cell wall stretch and cell volume in plasmolysis titrations

For cells grown at 0.03 Osm, the turgor pressure calculated from c_{MDO} by Eq. 1 over the course of a plasmolysis titration does not reach zero, even at high osmolalities of plasmolysis. Plasmolyzed cells retain residual turgor pressure because the large amount of MDO in the periplasm of cells grown at 0.03 Osm is diluted no more than fourfold by plasmolysis. (The volume occupied by periplasmic water increases from 0.43 μL/mg DW to a maximum of ~1.45 μL/mg DW; see Fig. 2.) The inset to Fig. 4 plots the turgor pressure $\Delta\Pi$ predicted from C_{MDO} and Z^{app}_{MDO} as a function of plasmolyzing osmolality, on the basis of the variation of Z^{app}_{MDO} with NaCl concentration, which yields Curve *D* of Fig. 4. From the maximum value of $\bar{V}^{wa}_{peri}$ obtained at high plasmolyzing osmolality and the lower bound value of n_{MDO} estimated for cells grown at 0.03 Osm (~15 nmol/mg DW), we calculate that cells grown at 0.03 Osm retain at least 0.3 atm of residual turgor pressure at the highest plasmolyzing osmolality (6 Osm) employed. (This calculation is independent of the choice of Z^{app}_{MDO} because the Donnan contribution of salt ions to turgor pressure is negligible in cells suspended in plasmolyzing media of high [NaCl].)

If the large amount of MDO in cells grown at 0.03 Osm at high plasmolyzing osmolality results in retention of significant residual turgor at high plasmolyzing osmolalities, then cells grown at high osmolality (a growth condition where cells contain very low amounts of MDO; see Fig. 3 *A*) should exhibit negligible residual turgor and completely unstretched cell walls after extensive plasmolysis with NaCl, and thus have a lower minimum cell volume ($\bar{V}^{wa}_{cell,min}$) than cells grown at 0.03 Osm. This prediction was confirmed by our observation that $\bar{V}^{wa}_{cell,min}$ of cells grown at 0.83 Osm is significantly lower than that of cells grown at 0.03 Osm (see Fig. 1), consistent with our conclusion that the amount of MDO is a primary determinant of periplasmic osmolality and therefore of turgor pressure and cell wall stretch in *E. coli* K-12.

Contribution of MDO to turgor pressure as a function of growth osmolality

Figure 5 plots predicted turgor pressures of growing *E. coli* as a function of osmolality of growth. Figure 5 predicts a monotonic decrease in $\Delta\Pi$ from ~3 atm for cells grown at 0.03 Osm to less than 0.5 atm for cells grown at 0.8 Osm. Included in this figure are values of $\Delta\Pi$ at low growth osmolality (0.03–0.28 Osm) determined from measurements of $\bar{V}^{wa}_{cyto}$ in plasmolysis titrations (cf., Table 3) and values of $\Delta\Pi$ predicted from contributions of outer membrane-impermeable periplasmic solutes, including: the Donnan contribution of the concentration of periplasmic MDO (calculated assuming that the MDO apparent valence Z^{app}_{MDO} (Eq. 1) changes the same way with [NaCl] for growth and for plasmolysis of cells grown at 0.03 Osm); and an estimate of the contribution of periplasmic proteins (and any other outer membrane-impermeable periplasmic solutes) to turgor pressure, obtained from the analysis of the cell volume data (see below). The latter effect is approximately 0.3 atm, and is therefore predicted to become the dominant contribution to $\Delta\Pi$ in cells growing above 0.8 Osm.

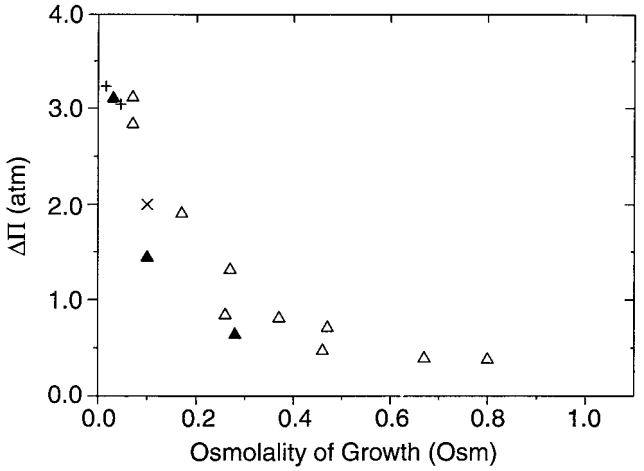

FIGURE 5 Reduction in turgor pressure ($\Delta\Pi$) with increasing osmolality of growth. (△) Turgor pressure calculated with Eq. 6 from the cytoplasmic water-accessible volume $\bar{V}^{wa}_{cyto}$ determined for cells grown at 0.03, 0.10, and 0.28 Osm, using the values of $\phi_{cyto}(\Sigma\, n_j)_{cyto}$ and $\bar{V}^{wa}_{b,cyto}$ in Table 3. Uncertainties in these values are ±(0.3–1) atm (Table 3). (▲) Turgor pressures calculated with Eq. 1 from the values of c_{MDO} from Fig. 3 *C* and from the variation of Z^{app}_{MDO} with [NaCl] inferred from the best-fit curve D of Fig. 4; uncertainties in these values are approximately ±35%. (+) Turgor pressures determined by measuring collapse pressures of gas vacuoles in *Microcystis* sp. grown at 15 mOsm and 45 mOsm (Reed and Walsby, 1985). (×) Turgor pressure determined by measuring collapse pressures of gas vacuoles in *Ancylobacter aquaticus* grown at 0.1 Osm (Koch and Pinette, 1987), which has an error of ~15%. The osmolalities of the media used by Reed and Walsby (1985) and Koch and Pinette (1987) were estimated from their composition.

Relationship between cell volume and turgor pressure

As an independent method of quantifying turgor pressure to compare with our estimates based on analysis of periplasmic MDO concentration, we applied an empirical relationship between changes in cell volume and changes in turgor

pressure previously used to characterize turgor–volume relationships in plant (Zimmermann, 1978; Cosgrove, 1988) and microbial (Walsby, 1980; Reed and Walsby, 1985) cells.

Changes in turgor pressure ($\Delta\Pi$) and the corresponding changes in total cell volume ($\Delta\bar{V}_{cell}^{total}$) have been related by the empirical equation (Broyer, 1952; Phillip, 1958; Zimmermann, 1978)

$$\Delta\Pi = \varepsilon\Delta\bar{V}_{cell}^{total}/\bar{V}_{cell,0}^{total}, \quad (7)$$

where ε is the volumetric elastic modulus (Cosgrove, 1988), $\Delta\bar{V}_{cell}^{total} = \bar{V}_{cell}^{total} - \bar{V}_{cell,0}^{total}$, and $\bar{V}_{cell,0}^{total}$ is the total cell volume in the zero-turgor reference state. Total cell volumes are defined as the sum of the water-accessible cell volume ($\bar{V}_{cell}^{wa}$) and the water-inaccessible cell volume ($\bar{V}_{cell}^{wi}$)

$$\bar{V}_{cell}^{total} = \bar{V}_{cell}^{wa} + \bar{V}_{cell}^{wi}. \quad (8)$$

For the conditions of the present study, $\bar{V}_{cell}^{wi} = 0.63 \pm 0.09$ μL/mg DW, independent of external osmolality (Cayley et al., 1991). The empirical elastic modulus ε is apparently independent of both $\bar{V}_{cell}^{total}$ and of turgor pressure over a range of volumes and pressures for some plant cells (Cosgrove, 1988) and for the gram negative cyanobacterium *Microcystis* (where turgor pressure can be measured directly from the pressure that must be applied to collapse cytoplasmic gas vacuoles; Reed and Walsby, 1985). However, ε can vary up to 20-fold with cell volume and $\Delta\Pi$ in some plant cells (Zimmermann, 1978) and, given the absence of quantitative studies of the elasticity of the cell wall of intact *E. coli* cells, ε cannot be assumed to be constant.

Analysis of behavior of cell volume in plasmolysis titrations

The decrease in cell volume with increasing osmolality of plasmolysis of cells grown at 0.03 Osm (VLOM) and 0.83 Osm (MBM+0.4 M NaCl) from Fig. 1 is replotted in Fig. 6 *A* according to Eq. 7. To calculate $\Delta\bar{V}_{cell}^{total}/\bar{V}_{cell,0}^{total}$ for both data sets, we used the same value of $\bar{V}_{cell,0}^{total}$ (2.19 ± 0.10 μL/mg DW), calculated by adding $\bar{V}_{cell}^{wi}$ to the minimum value of cell water ($\bar{V}_{cell,min}^{wa}$) reached at high osmolalities in the plasmolysis titration of cells grown at 0.83 Osm (1.56 ± 0.05 μL/mg DW), because these cells have very low levels of MDO and should therefore have negligible residual turgor when plasmolyzed with high concentrations of NaCl. From Fig. 6 *A* and Eq. 7, we conclude that $\Delta\Pi/\varepsilon$ decreases from approximately 0.6 to 0.18 with increasing osmolality of plasmolysis of cells grown at 0.03 Osm, and that $\Delta\Pi/\varepsilon$ decreases from approximately 0.2 to zero for cells grown at 0.83 Osm. Inasmuch as we and others have demonstrated that turgor pressure decreases during a plasmolysis titration of bacterial cells (cf., Fig. 4, *inset*; Reed and Walsby, 1985; Koch and Pinette, 1987), this reduction in $\Delta\Pi/\varepsilon$ in plasmolysis, at least in part, reflects a reduction in $\Delta\Pi$ with increasing osmolality of plasmolysis. To assess if ε also varies with

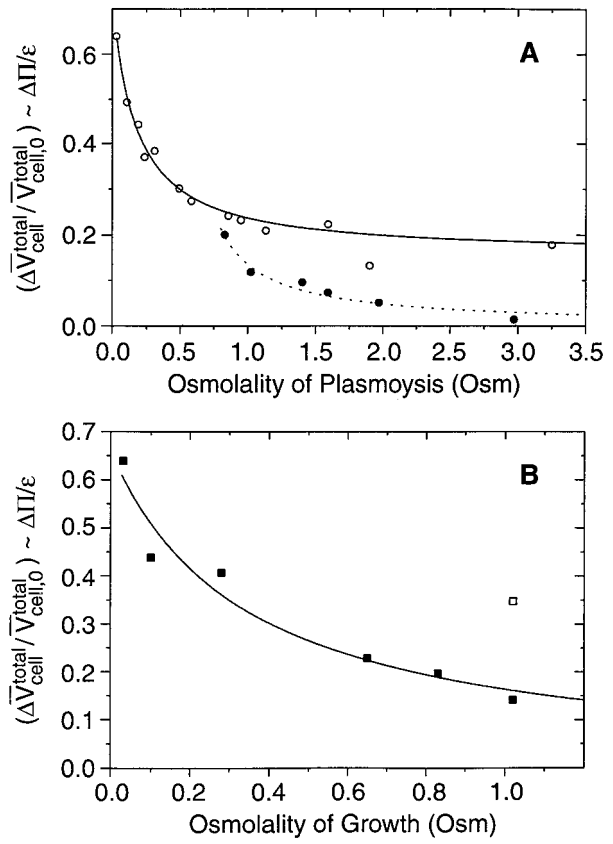

FIGURE 6 (*A*) Reduction in $\Delta\bar{V}_{cell}^{total}/\bar{V}_{cell,0}^{total} = \Delta\Pi/\varepsilon$ (Eq. 7) with increasing osmolality of plasmolysis for cells grown at 0.03 Osm (○) or 0.83 Osm (●). The difference in total cell volume between stressed and unstressed state is $\Delta\bar{V}_{cell}^{total} = \bar{V}_{cell}^{total} - \bar{V}_{cell,0}^{wa}$, where $\bar{V}_{cell}^{total} = \bar{V}_{cell}^{wa} + \bar{V}_{cell}^{wi}$ and $\bar{V}_{cell,0}^{total} = 2.19$ μl/mg DW (see text). The curves through the data are empirical best-fit hyperbolic functions. (*B*) Reduction in $\Delta\bar{V}_{cell}^{total}/\bar{V}_{cell,0}^{total} = \Delta\Pi/\varepsilon$ with increasing osmolality of growth. The curve is an empirical best fit hyperbolic function to the points calculated from the values of $\bar{V}_{cell}^{wa}$ for cells grown in minimal medium shown in Table 3 (■). The value of $\Delta\bar{V}_{cell}^{total}/\bar{V}_{cell,0}^{total}$ for cells grown at 1.02 Osm with 1 mM of the osmoprotectant betaine (□) was calculated using the value of $\bar{V}_{cell}^{wa}$ reported by Cayley et al. (1992).

osmolality of plasmolysis, we compared the variation of $\Delta\Pi$ (from Fig. 4, *inset*) and $\Delta\Pi/\varepsilon$ (from Fig. 6 *A*) for cells grown at 0.03 Osm as a function of plasmolyzing osmolality. From this comparison, we calculate that ε initially decreases with increasing osmolality of plasmolysis from ~4.9 atm at 0.03 Osm to ~2.5 atm at 0.4 Osm and plateaus at ~2 atm at higher plasmolyzing osmolalities. (This variation of ε generates Curve *D* in Fig. 4 when Eq. 7 is used to calculate turgor pressure).

Analysis of behavior of cell volume as a function of growth osmolality

It is well known that $\bar{V}_{cell}^{wa}$ decreases with increasing growth osmolality (Richey et al., 1987; Larsen et al., 1987; Cayley et al., 1991; and see Table 1). Figure 6 *B* plots both our new

and published (Cayley et al., 1991) cell volume data according to Eq. 7. Figure 6 *B* shows that $\Delta\bar{V}_{cell}^{total}/\bar{V}_{cell,0}^{total}$ decreases monotonically from a value of ~0.6 for cells grown at 0.03 Osm to ~0.15 for cells grown at 1.0 Osm. The reduction in $\Delta\bar{V}_{cell}^{total}/\bar{V}_{cell,0}^{total} = \Delta\Pi/\varepsilon$ in Fig. 6 *B* with increasing growth osmolality is similar to the behavior of $\Delta\Pi$ calculated from cytoplasmic and periplasmic data in Fig. 5, indicating that the reduction in $\bar{V}_{cell}^{total}$ with increasing osmolality of growth is principally the result of the reduction in $\Delta\Pi$. Values of ε calculated from $\bar{V}_{cell}^{total}$ by Eq. 7, using values of $\Delta\Pi$ obtained from analysis of $\bar{V}_{cyto}^{wa}$ (cf., Table 3) or from analysis of periplasmic MDO (Fig. 5) differ only marginally given the large uncertainties, but are consistent both in magnitude and in direction of change with osmolality with the values of ε calculated from the plasmolysis data in Fig. 6 *A*. As a working hypothesis, we therefore propose that ε decreases with increasing growth osmolality from approximately 5 atm at 0.03 Osm to a plateau value of approximately 2 atm above 0.4 Osm. Whether this decrease in ε is most fundamentally the result of the increase in NaCl concentration, or of the decrease in cell volume and turgor pressure, remains to be determined.

Assuming that the variation of ε with osmolality obtained from analysis of plasmolysis titrations (Fig. 6 *A*) also applies to growing cells, then ε for cells growing at 0.83 Osm is ~2 atm. From this value of ε and values of $\Delta\bar{V}_{cell}^{total}/\bar{V}_{cell,0}^{total}$ (and hence $\Delta\Pi/\varepsilon$) from Fig. 6 *B*, we estimate that the initial turgor of cells grown at 0.83 Osm is ~0.4 atm. The corresponding turgor estimated from the concentration of periplasmic MDO at this osmolality (see Fig. 3 *C*) is ~0.15 atm. This difference indicates that periplasmic proteins or outer-membrane impermeable solutes in addition to MDO contribute to periplasmic osmolality and therefore contribute an additional ~0.3 atm to turgor pressure in cells grown at high osmolality. This value was added to the turgor calculated from the concentrations of MDO to obtain the predictions shown in Fig. 5.

DISCUSSION

Osmolalities of the periplasm and cytoplasm are equal

From our analyses of the contributions of anionic MDO to periplasmic osmolality in growth and in plasmolysis, we conclude that the periplasm and cytoplasm are always isoosmotic, and that the osmolality of both compartments always exceeds that of the external environment, especially for cells grown at low osmolality, where the amount of MDO is largest. The Donnan osmotic contribution of periplasmic MDO, together with a small contribution from periplasmic biopolymers, determines the osmotic pressure difference (turgor pressure) across the cell wall. We show that the turgor calculated from our estimate of the amount of periplasmic MDO is indeed capable of predicting $\bar{V}_{cyto}^{wa}$ over the course of the plasmolysis titration of cells grown at 0.03 Osm, confirming that the periplasm and cytoplasm are isoosmotic. Because turgor pressure stretches the cell wall, our observation that the cell wall is more stretched in plasmolyzed cells containing large amounts of MDO (i.e., growing at 0.03 Osm) than in cells with small amounts of MDO (i.e., growing at 0.83 Osm) confirms our hypothesis that the periplasm and cytoplasm are isoosmotic and that the amount of MDO determines turgor pressure.

Our findings are in agreement with those of Stock et al. (1977), who found that the osmolality of the periplasm, estimated from the Donnan distribution of periplasmic ions in *S. typhimurium*, is the same within error as the osmolality of the cytoplasm estimated from analysis of plasmolysis titrations of cell suspensions. Sen et al. (1988) extended these findings by showing that the amount of periplasmic MDO was sufficient to predict the observed Donnan potential maintained across the outer membrane in *E. coli* grown at low osmolality. Our conclusion that the Donnan ion distribution contributes to turgor pressure and cell-wall stretch is also consistent with the finding of Alemohammad and Knowles (1974) that use of NaCl to increase the osmolality of suspensions of *E. coli* reduced the water-accessible cell volume to a greater extent than an equiosmolal concentration of the nonelectrolyte sucrose. Taken together, these results indicate that negatively charged MDO and the resultant Donnan ion distribution are significant determinants of periplasmic osmolality, that the periplasm and cytoplasm are isoosmotic, and that *E. coli* maintains turgor pressure across the cell wall and not across the cytoplasmic membrane.

An alternative view is that turgor pressure is maintained across the cytoplasmic membrane in growing cells (i.e., that $Osm_{cyto} > Osm_{peri}$; Koch, 1995, 1998). Because a significant periplasmic space separates the cytoplasmic membrane from the peptidoglycan, a necessary consequence of this alternative view is that periplasmic biopolymers must form an incompressible matrix or gel which presses against the peptidoglycan, thereby stretching the cell wall. Supporting this proposal is the observation that the periplasmic space has a uniformly smooth and narrow width (see Schwarz and Koch, 1995 and references therein) in electron micrographs of cryofixed and freeze substituted *E. coli*. However, this method has been criticized because it causes cell shrinkage (Woldringh, 1994). Furthermore, our observation that, in the initial stages of a NaCl plasmolysis titration, *E. coli* cells grown at 0.03 Osm still have residual turgor and stretched cell walls but have significantly larger periplasmic water volumes than unplasmolyzed cells (see Figs. 1 and 2) is inconsistent with cell-wall stretch originating from the cytoplasmic membrane pressing an incompressible periplasmic matrix pressing into the cell wall. That periplasmic proteins have measurable translational diffusion coefficients (Brass et al., 1986), whereas cytoplasmic proteins are translationally immobilized when most free water is removed from cytoplasm by hyperosmotic shock (Jacobson and

Wojcieszyn, 1984), also suggests that periplasm must have significant amounts of free water, a conclusion supported by the finding of Stock et al. (1977) that the amount of periplasmic water is reduced in cells osmotically stressed with high concentrations of the outer-membrane impermeable solute sodium polyglutamate. (Indeed, the accumulation of MDO at low osmolality provides a mechanism for retention of free water in the periplasm; see below). We therefore conclude that periplasm and cytoplasm must be isoosmotic, and that turgor is exerted across the cell wall. Presumably, the outer membrane can withstand an outwardly directed turgor pressure because it is tethered to the cell wall peptidoglycan by lipoproteins and porins.

The use of cell suspensions (for example by Stock et al., 1977; Cayley et al., 1991, 1992; and the present study) to measure osmotic properties of *E. coli* has been criticized as being irrelevant to growing cells (Koch, 1995, 1998). This criticism would be valid if the cytoplasmic pools of osmolytes and/or metabolites of these cells could leak or become depleted relative to growing cells during harvest of suspensions. Neither of these potentially serious scenarios apparently occurs, however. Cells centrifugally harvested from growth at both low and high osmolalities by the conditions used in this study have the same amounts of the primary osmolytes (K^+, glutamate, betaine, and proline) as rapidly filter-harvested growing cells (Cayley et al., 1991, 1992) and do not detectably leak cytoplasmic K^+ for at least an hour after harvest (Richey et al., 1987). The osmotic contribution from cytoplasmic metabolites is also presumably not significantly altered by our harvest procedure because the most abundant metabolites in *E. coli* are anionic, and anions must be retained in some form in the cytoplasm to neutralize the charge of cytoplasmic K^+. Fresh suspensions do retain the osmotic properties of growing cells, as demonstrated by our observation that the cell and cytoplasmic volumes of growing cells and of fresh suspensions are the same (see Table 2). Thus, we conclude that our results are relevant for growing cells and in agreement with the conclusion of Stock et al. (1977) that the periplasm and cytoplasm are isoosmotic.

Turgor pressure decreases with increasing osmolality of plasmolysis or growth; implications for the activity and activity coefficient of cytoplasmic water

Independent analyses of measurements of cytoplasmic volume, cellular volume and the concentration of periplasmic MDO all indicate that turgor pressure decreases with increasing osmolality of plasmolysis and of growth (Table 3 and Fig. 5). Our conclusion that turgor pressure decreases with increasing osmolality of plasmolysis is in agreement with direct measurements showing that turgor pressure of cytoplasmic gas vacuole-containing bacteria decreases during plasmolysis titrations (Koch and Pinette, 1987; Reed and Walsby, 1985). The decrease in turgor pressure of *E. coli* with increasing osmolality of growth also has precedent in plants, where turgor pressure can decrease significantly with increasing growth osmolality (Cosgrove, 1993). A nonosmotic variability of turgor pressure also has been previously reported in growing bacteria: measurements of collapse pressures of cytoplasmic gas vacuoles in the phototrophic gram-negative bacterium *Anabaena flos-aqua* have shown that turgor pressure varies by at least 2 atm as a function of light intensity (Kinsman et al., 1991).

Our estimates of turgor pressure for *E. coli* are comparable to those measured directly in gram-negative bacteria containing gas vacuoles (Reed and Walsby, 1985; Koch and Pinette, 1987; see Fig. 5), and to a previous estimate of turgor pressure from volume measurements of *S. typhimurium* (Stock et al., 1977), all of which indicate $\Delta\Pi$ is $\sim$2–4 atm in cells grown at $<$0.15 Osm. Koch and Pinette reported that the turgor pressure of the gram negative heterotroph *Microcystis* grown at low osmolality ($\sim$0.1 Osm), estimated from measurements of the pressures needed to collapse cytoplasmic gas vesicles, is $\sim$2 atm. From their reported vesicle-collapse pressures for cells growing at higher osmolality ($\sim$0.4 Osm), we estimate that the turgor pressure of cells growing at $\sim$0.4 Osm ($\sim$0.9 atm) is lower than in cells grown 0.1 Osm, a trend consistent with Fig. 5. Given the recent functional expression of gas vacuoles cloned from *Bacillus megaterium* into *E. coli* (Li and Cannon, 1998), gas vacuole collapse-pressure measurements could provide a way to directly test our conclusion that turgor pressure of *E. coli* decreases as growth osmolality increases.

Because turgor pressure is very low at high osmolality of growth, the activity of cytoplasmic water a_w^{cyto} is only very slightly less than the external water activity a_w^{ex} at high osmolality. (For example, at 1.0 Osm where $\Delta\Pi \cong 0.3$ atm, the osmolality difference ΔOsm $\cong$ 0.013 and $a_w^{cyto}/a_w^{ex} \cong$ 0.99977.) This result is surprising because the amount of cytoplasmic water (and, in particular, the amount of free cytoplasmic water) decreases strongly with increasing osmolality of growth (cf. Table 3 and Cayley et al., 1991; Record et al., 1998a). Comparison of activities and mole fractions of cytoplasmic water indicates that the activity coefficient of cytoplasmic water ($\gamma_w^{cyto} = a_w^{cyto}/X_w^{cyto}$) increases with increasing osmolality of growth, and exceeds unity at high osmolality. For example, for cells grown at 1.0 Osm, we determined the molar amount of cytoplasmic water $n_w^{cyto} \cong 65.6 \pm 3.3$ μmol/mg DW and a lower bound for the amount of osmotically significant cytoplasmic solutes $(\Sigma\, n_j)_{cyto} = 1.79 \pm 0.10$ μmol/mg DW (Cayley et al., 1991). Therefore, at 1.0 Osm, an upper bound on the mole fraction of cytoplasmic water is $X_w^{cyto} \leq 0.97$. (If only free cytoplasmic water is used in this calculation, then the corresponding bound is $X_{w,free}^{cyto} \leq 0.964$.) From $\Delta\Pi \cong 0.3$ atm at 1.0 Osm, we calculate $a_w^{cyto} = 0.959$. Hence, the activity coefficient $\gamma_w^{cyto} \gtrsim 1.01$; if only free water is used in the

calculation, $\gamma_{w,free}^{cyto} \gtrsim 1.02$. Extrapolation of the trends in n_w^{cyto} and $(\Sigma\, n_j)_{cyto}$ to higher osmolality of growth predicts much larger positive derivations from ideality; we predict that $\gamma_w^{cyto} \approx 1.04$ at 1.5 Osm (and $\gamma_{w,free}^{cyto} \approx 1.2$), and are currently testing these predictions. These unusual effects on the activity coefficient of cytoplasmic water must be a thermodynamic consequence of macromolecular crowding, which causes thermodynamic activities of cytoplasmic biopolymers to exceed their concentrations by large amounts; biopolymer activity coefficients are predicted to increase greatly, which increase with increasing osmolality of growth (Cayley et al., 1991; Guttman et al., 1995).

Cell wall elasticity and the volumetric elastic modulus of E. coli

The peptidoglycan sacculus of *E. coli* determines cell shape and expands as a result of turgor pressure. Koch and Woeste (1992) have shown that isolated peptidoglycan sacculi can stretch elastically with little if any hysteresis to about four times (~400%) their unstretched volume, consistent with molecular modeling studies of the extent to which the peptide bridges of peptidoglycan can extend under stress (Labischinski and Maidhof, 1994). We find (cf. Fig. 1) that the increase in $\bar{V}_{cell}^{total}$ at 0.03 Osm from its high osmolality plateau value is approximately 40%, which corresponds to only 10% of the range over which Koch and Woeste (1992) observed reversible stretching. The change in $\bar{V}_{cell}^{total}$ with osmolality, therefore, constitutes a relatively small deformation, which is likely to be elastic (and see Doyle and Marquis, 1994). In the elastic regime, a reduction in turgor pressure should result in a corresponding reversible reduction in the extent of cell-wall stretch, which we measure directly as a reduction in cell volume.

The volumetric elastic modulus (Eq. 7) is an empirical parameter previously used to quantify pressure–volume relations of bacterial (Reed and Walsby, 1985; Walsby, 1980) and many plant (Zimmermann, 1978; Cosgrove, 1988) cells. Analysis of the variation of $\bar{V}_{cell}^{total}$ with osmolality of growth and of plasmolysis using Eq. 7 indicates that, in both cases (Fig. 6, *A* and *B*, respectively), $\Delta\Pi/\varepsilon$ decreases with increasing osmolality. Our proposal for *E. coli* that ε decreases as NaCl concentration increases and $\bar{V}_{cell}^{wa}$ decreases is consistent with the behavior of ε deduced for plant (Zimmermann, 1978; Cosgrove, 1988) and bacterial (Reed and Walsby, 1985; Walsby, 1980) cells. Given recent progress in applying atomic force microscopy to examine the elasticity of biological materials (Vinckier and Semenza, 1998), it may be possible to directly test this prediction regarding ε. Although the reduction in ε with increasing NaCl or decreasing $\bar{V}_{cell}^{wa}$ is only marginally outside of experimental uncertainty, clearly our analysis provides no basis for proposing that ε could increase with increasing osmolality of plasmolysis or growth. Hence, the reduction in $\Delta\Pi/\varepsilon$ with increasing osmolality must be interpreted as a decrease in $\Delta\Pi$.

The values of ε we report for *E. coli* (~2–5 atm) are at the low end of the range of ε determined in plants (5–500 atm; Zimmermann, 1978), consistent with the expectation that the mostly monolayered (Labischinski and Maidhof, 1994) cell wall of *E. coli* should be less rigid than the multilayered cell walls of plants. Previous estimates of ε in cyanobacteria with Eq. 7 (11–13 atm; Reed and Walsby, 1985; Walsby, 1980), based on direct measurement of the pressure required to collapse cytoplasmic gas vesicles but indirect estimates of volumes, are higher than for *E. coli*. Whether the differences in ε measured in these cyanobacteria and *E. coli* reflect differences in the elasticity of their cell walls or differences in the methods used to estimate ε remains to be established.

Implications of the variation of turgor pressure with osmolality of growth

It is widely believed that cells with walls require turgor pressure for cell expansion, although Money (1997) reported an exception. Studies with plant cells have shown that both turgor pressure and growth rate can decrease as the osmolality of the growth medium increases (Cosgrove, 1993). However, this correlation is not evidence for a direct functional dependence of growth rate on $\Delta\Pi$. To test for such a dependence directly, Zhu and Boyer (1992) changed the turgor pressure in the alga *Chara corallina* by injecting or removing cell solution at constant Osm_{ex}, and observed that growth rate is independent of turgor pressure unless turgor pressure is lowered below a threshold (~0.4 atm in their study) at which point growth ceases. Whether *E. coli* also has a threshold turgor is unclear, but our conclusion that turgor is <0.5 atm in cells grown at high osmolality suggests that *E. coli* needs only a small turgor pressure for growth. However, even low turgor pressure can stretch the cell wall significantly. For example, plasmolysis of cells grown at 0.83 Osm significantly reduces cell volume (Fig. 1), even though the initial turgor pressure we estimate for these cells is less than ~0.5 atm (see Analysis). The ability of low levels of turgor to stretch the cell wall is consistent with our inference that the elastic modulus of the cell wall of *E. coli* decreases with increasing osmolality of plasmolysis or growth (and hence with decreasing cell volume and turgor pressure). If a stretched cell wall is required for cell-wall synthesis in growing bacteria (see Wood, 1999 and Höltje, 1998), then the parallel reduction in ε with the reduction in $\Delta\Pi$ as osmolality of growth increases may have physiological relevance as a mechanism to ensure that cells growing with low turgor will have sufficiently stretched cell walls to permit new cell-wall synthesis.

At low osmolality, turgor pressure is an unavoidable consequence of the fact that the osmolality of the cytoplasm exceeds that of the external growth medium, primarily be-

cause of the osmotic contributions of the K^+ required for electroneutrality of cytoplasmic nucleic acids (Record et al., 1998a) and metabolites. At low osmolality, MDO are required to elevate the osmolality of the periplasm to that of the cytoplasm. If the osmolality of the periplasm were less than that of the cytoplasm, the cytoplasmic membrane would expand and compress periplasmic components into the cell wall, thereby eliminating the free periplasmic water presumably required for diffusion. We propose that the high concentration of periplasmic MDO and the relatively high turgor pressures of cells grown at low osmolality is fundamentally a consequence of the need to maintain a functional periplasmic volume.

At high external osmolality, the challenge to the cytoplasm from its osmotic environment has reversed, from the need to reduce the amounts of cytoplasmic osmolytes to the need to increase them to increase the amount of cytoplasmic water. As the osmolality of minimal growth media is increased, the amount of cytoplasmic K^+ increases, and the growth rate and amount of cytoplasmic water decreases (Cayley et al., 1991). From these observations, we concluded that osmolality-dependent changes in the amounts of cytoplasmic K^+, water, and growth rate are linked for all growth conditions examined (Cayley et al., 1992; Record et al., 1998b). The linkage provides a resolution of the paradox that, although increasing K^+ concentration dramatically reduces the extent of protein–DNA interactions in vitro, increases in K^+ concentration have no effect on gene expression in vivo (Cayley et al., 1991). In this explanation, the reduction in $\bar{V}^{wa}_{cyto}$ with increasing osmolality of growth greatly increases the extent of macromolecular crowding, which we propose must compensate for the effect of increasing concentration of cytoplasmic K^+ on the equilibria and rates of protein–nucleic acid interactions (Record et al., 1998b). In support of this proposal, we observe that accumulation of the osmoprotectant betaine by osmotically stressed cells increases growth rate and $\bar{V}^{wa}_{cyto}$ and reduces cytoplasmic K^+ concentration (Cayley et al., 1992). The observations that the amount of MDO and turgor pressure (and possibly the elastic modulus of the cell wall) decrease with increasing osmolality of growth in minimal media suggest that these periplasmic and cell-wall parameters may also be linked to growth rate in cells above 0.28 Osm. If correct, this proposal indicates a thermodynamic sophistication and coordinated regulation of the volumes of the cytoplasm and periplasm of *E. coli.*

Because accumulation of the osmoprotectant glycine betaine increases the volume of cells growing at high osmolality (Cayley et al., 1992), Eq. 7 predicts that $\Delta\Pi/\varepsilon$ and, hence, the turgor pressure of cells grown at high osmolality in the presence of betaine are higher than that in cells grown in its absence. When added to the medium of cells growing at high osmolality, betaine is transported into the cytoplasm by the osmotically regulated porters proU and proP (Csonka and Epstein, 1996) and increases the amount of cytoplasmic (Meury, 1994; Cayley et al., 1992) and cellular (Cayley et al., 1992) water. From the value of $\bar{V}^{wa}_{cell}$ (2.32 ± 0.17 μL/mg DW) of *E. coli* K-12 grown at 1 Osm+betaine (in MBM + 0.5 M NaCl + 1 mM betaine; Cayley et al., 1992), the value of $\Delta\Pi/\varepsilon$ calculated from Eq. 7 is 0.35, significantly higher than the corresponding value of cells grown at 1 Osm in the absence of betaine (see Fig. 6 *B*). Hence, assuming that accumulation of betaine does not decrease ε, Eq. 7 predicts that the turgor pressure of cells grown at 1 Osm+1 mM betaine is higher than that of cells grown without betaine. In light of our proposal that the amount of MDO and turgor pressure are linked to growth rate, it will be of interest to test the effect of accumulation of betaine on the amount of MDO or other turgor-generating periplasmic solutes.

Our study has implications for the mechanism of osmoregulation of gene expression. Studies of expression of the *lac*Z gene fused to the *kdp*ABC operon (encoding the high-affinity K^+ transport system of *E. coli*) led to the proposal that turgor pressure regulates expression of the *kdp*ABC operon (Laimins et al., 1981). For cells growing in media with high concentrations of K^+, an increase in osmolality caused a transient increase in *kdp*ABC expression, which returned to the prestress level as growth resumed at the new osmolality. Because the KdpD sensor kinase (which is proposed to regulate *kdp*ABC gene expression by responding to changes in turgor pressure; Malli and Epstein, 1998) is in the cytoplasmic membrane and we conclude that turgor pressure is exerted across the cell wall, presumably KdpD does not sense turgor pressure directly but may respond to the consequences of water efflux and loss of turgor (e.g., changes in solute concentration, crowding or other biophysical properties of the cytoplasm or cytoplasmic membrane; Wood, 1999).

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