Roles of Cytoplasmic Osmolytes, Water, and Crowding in the Response of *Escherichia coli* to Osmotic Stress: Biophysical Basis of Osmoprotection by Glycine Betaine†

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ABSTRACT: To better understand the biophysical basis of osmoprotection by glycine betaine (GB) and the roles of cytoplasmic osmolytes, water, and macromolecular crowding in the growth of osmotically stressed *Escherichia coli*, we have determined growth rates and amounts of GB, K^+, trehalose, biopolymers, and water in the cytoplasm of *E. coli* K-12 grown over a wide range of high external osmolalities (1.02−2.17 Osm) in MOPS-buffered minimal medium (MBM) containing 1 mM betaine (MBM+GB). As osmolality increases, we observe that the amount of cytoplasmic GB increases, the amounts of K^+ (the other major cytoplasmic solute) and of biopolymers remain relatively constant, and the growth rate and the amount of cytoplasmic water decrease strongly, so concentrations of biopolymers and all solutes increase with increasing osmolality. We observe the same correlation between the growth rate and the amount of cytoplasmic water for cells grown in MBM+GB as in MBM, supporting our proposal that the amount of cytoplasmic water is a primary determinant of the growth rate of osmotically stressed cells. We also observe the same correlation between cytoplasmic concentrations of biopolymers and K^+ for cells grown in MBM and MBM+GB, consistent with our hypothesis of compensation between the anticipated large perturbing effects on cytoplasmic protein−DNA interactions of increases in cytoplasmic concentrations of K^+ and biopolymers (crowding) with increasing osmolality. For growth conditions where the amount of cytoplasmic water is relatively large, we find that cytoplasmic osmolality is adequately predicted by assuming that contributions of individual solutes to osmolality are additive and using *in vitro* osmotic data on osmolytes and a local bulk domain model for cytoplasmic water. At moderate growth osmolalities (up to 1 Osm), we conclude that GB is an efficient osmoprotectant because it is almost as excluded from the biopolymer surface in the cytoplasm as it is from native protein surface *in vitro*. At very high growth osmolalities where cells contain little cytoplasmic water, predicted cytoplasmic osmolalities greatly exceed observed osmolalities, and the efficiency of GB as an osmolality booster decreases as the amount of cytoplasmic water decreases.

Most living cells adapt to osmotic stress by accumulating intracellular osmolytes, and glycine betaine [*N*,*N*,*N*-trimethylglycine (GB)][1] is among the most commonly accumulated osmolytes in nature. The natural or engineered accumulation of betaine also increases the tolerance of crop plants to drought and temperature extremes (*1*). Other effects of GB on biological systems are also important; for example, accumulation of intracellular betaine may increase survival of pathogenic bacteria in chilled and dried foods and in the mammalian urinary tract (*2*) and can improve the yield of correctly folded proteins that are overexpressed in bacteria (*3*). To develop a better understanding of the biophysical basis of how GB protects cells from environmental stress, we have characterized the role of GB in adaptation of *Escherichia coli* to osmotic stress.

E. coli grows in minimal media over a very wide range of external osmolalities (from $\lesssim$0.02 to >2 Osm) by osmoregulation of amounts of cytoplasmic solutes, including transport of K^+, synthesis of putrescine^{2+} and/or glutamate and trehalose, and transport of osmoprotectants such as glycine betaine (GB) or proline if present in the growth medium (*2*, *4*, *5*). When *E. coli* is shifted from low to high osmolality, the initial passive response is loss of cytoplasmic water and turgor pressure. Subsequently, the amount of cytoplasmic putrescine decreases and amounts of cytoplasmic K^+, glutamate, and trehalose increase to cause partial restoration of the original amount of cytoplasmic water and original growth rate (*5*, *6*). For *E. coli* K-12 growing in MOPS-buffer minimal medium (MBM) above 0.3 OsM, both the growth rate and the amount of cytoplasmic water ($n_{H_2O}^{cyto}$) decrease strongly and approximately linearly with increasing

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[1] Abbreviations: GB, glycine betaine; Tre, trehalose; Pro, proline; KGlu, potassium glutamate; MOPS, 4-morpholinopropanesulfonate; MBM, MOPS-buffered medium; $\Delta\Pi$, turgor pressure; ϕ, osmotic coefficient; $n_{H_2O,b}^{cyto}$, cytoplasmic amount of bound cytoplasmic water; Σn_j, total amount of cytoplasmic osmolytes; Osm, osmolality; VPO, vapor pressure osmometry; K_p, partition coefficient.

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growth osmolality (Osm_{ex}) and cytoplasmic concentrations of biopolymers, K^+, glutamate, and trehalose all increase greatly (*5*, *7*). When GB is present in low concentrations in growth media, its uptake [by the osmotically regulated transport systems ProU and ProP (*6*)] greatly increases the growth rate and extends the range of high osmolalities over which *E. coli* grows (*8*, *9*); uptake of GB reduces cytoplasmic amounts of other osmolytes in *E. coli* (*10*) as well as in other bacteria (*2*, *11*) and in mammalian kidney cells (*12*), relative to cells cultured at the same osmolality in the absence of GB.

General biophysical questions raised by these observations include the following. What are the thermodynamic and molecular explanations of the action of GB as an osmoprotectant? Can *in vitro* data and analyses of osmotic properties of protein and nucleic acid solutions be used to interpret or predict *in vivo* osmotic behavior? What is the fundamental cytoplasmic determinant(s) of the growth rate of osmotically stressed cells? How can *E. coli* tolerate the wide ranges of concentrations of cytoplasmic solutes and biopolymers observed in growth at different osmolalities, given the strong dependences of the thermodynamics of noncovalent processes on concentrations of these solutes as a consequence of preferential interactions and macromolecular crowding?

To address these questions and evaluate proposed compensation mechanisms that allow *E. coli* to grow efficiently over a wide range of osmotic environments, we have characterized the cytoplasm of cells grown in MBM+GB above 0.3 Osm. For cells grown in MBM+GB over a range of high osmolalities (1.0−2.2 Osm) where growth is very slow or ceases entirely in MBM, we have quantified and analyzed cytoplasmic amounts of the major osmolytes, water, and biopolymers. We compare the cytoplasmic osmolality under these and previously studied growth conditions with that predicted from *in vitro* studies of these solutes in water and in concentrated protein solutions, and deduce from this comparison the thermodynamic basis of osmoprotection by GB. We observe the same correlation between the growth rate, amount of cytoplasmic water, and cytoplasmic K^+ concentration in MBM+GB and in MBM, and propose that the increase in macromolecular crowding that occurs with increasing osmolality provides fundamental explanations for both the reduction in growth rate and the insensitivity of cytoplasmic protein−DNA interactions to changes in cytoplasmic K^+ concentration.

OSMOTIC BEHAVIOR OF THE CYTOPLASM OF *E. COLI*

Assuming additivity of contributions of individual osmolytes, the osmolality of the *E. coli* cytoplasm can be predicted from the amounts of cytoplasmic osmolytes and *in vitro* osmometric and densimetry data. This analysis is developed below.

Water equilibrates across the cytoplasmic membrane and is not actively transported. Total cytoplasmic water ($n^{cyto}_{H_2O}$) can be empirically divided into amounts of "bound" water (defined empirically as that which cannot be removed by increasing the external osmolality of cells with NaCl) and "free" water on the basis of plasmolysis titrations of *E. coli* with NaCl (*7*, *10*, *13*). Free cytoplasmic water is defined as that which is extracted with NaCl ($n^{cyto}_{H_2O,f}$):

$$n^{cyto}_{H_2O,f} = n^{cyto}_{H_2O} - n^{cyto}_{H_2O,b} \quad (1)$$

Plasmolysis titrations reveal that the amount of bound or otherwise osmotically inactive water ($n^{cyto}_{H_2O,b}$) is ∼22 μmol/mg of dry weight [or ∼0.4 μL/mg of dry weight, independent of the osmolality of growth from at least 0.03−1.0 Osm (*7*, *10*, *13*)], similar to the amount observed in red blood cells by this method (*14*). This amount of bound water corresponds to ∼0.5 g of H_2O/g of cytoplasmic biopolymer in *E. coli* (*7*), and is within the range of *in vitro* values for water of hydration of proteins (*15*, *16*).

At a specified Osm_{ex} of growth or plasmolysis, the cytoplasmic osmolality Osm_{cyto} exceeds Osm_{ex} by $\Delta\Pi/(RT)$, where $\Delta\Pi$ is the turgor pressure:

$$Osm_{cyto} = Osm_{ex} + \Delta\Pi/(RT) \quad (2)$$

Cayley et al. (*7*, *10*, *13*) deduced that Osm_{cyto} was most simply represented as

$$Osm_{cyto} = -m^{\bullet}_{H_2O} \ln a^{cyto}_{H_2O} = \frac{m^{\bullet}_{H_2O}\phi_{cyto}\sum n^{cyto}_j}{n^{cyto}_{H_2O,f}} = \frac{\phi_{cyto}\sum n^{cyto}_j}{V^{cyto}_{H_2O,f}} \quad (3)$$

where, assuming a density of cytoplasmic water of 1 kg/L

$$V^{cyto}_{H_2O,f} = V^{cyto}_{H_2O} - V^{cyto}_{H_2O,b} \cong n^{cyto}_{H_2O,f}/m^{\bullet}_{H_2O} \quad (4)$$

In eq 3, $a^{cyto}_{H_2O}$ is the activity of water, ϕ_{cyto} is the osmotic coefficient of the cytoplasm, $m^{\bullet}_{H_2O} = 55.5$ mol/kg of H_2O, and $\sum n^{cyto}_j$ is the sum of the mole amounts of all osmotically significant cytoplasmic solute components.

In vitro, results of thermodynamic studies of solute−protein interactions are well-described using a local-bulk domain model in which solutes (e.g., denaturants, osmolytes, and Hofmeister salts) partition unevenly between free (bulk) water and the local domain (hydration water) at the protein surface; solutes at the protein surface displace water from the local (bound) water domain and are osmotically inactive. Solute−protein interactions can be characterized by solute-specific partition coefficients $K_{p,j}$ (*17*−*19*), defined most simply as the ratio of the local molal concentration of solute (in bound water) to its bulk concentration (in free water)

$$K_{p,j} = \frac{m^{loc}_j}{m^{bulk}_j} = \frac{(n_j/n_{H_2O})^{loc}}{(n_j/n_{H_2O})^{bulk}} \quad (5)$$

Partition coefficients quantifying the distribution of *E. coli* osmolytes between the surface of native BSA and bulk water have been determined from vapor pressure osmometry and densimetry data (*17*). If we assume additivity of contributions of individual small solutes to Osm_{cyto} and neglect the expected small osmotic contribution of cytoplasmic proteins, then interpretation of Osm_{cyto} in terms of two-component osmotic coefficients ϕ^o_j for individual solutes in water and local bulk partition coefficients ($K_{p,j}$) for these solutes in protein solutions yields

$$\mathrm{Osm}_{\mathrm{cyto}}^{\mathrm{calc}} \cong m^{\bullet}_{\mathrm{H_2O}} \sum_j \frac{\phi_j^{\mathrm{o}} n_j^{\mathrm{cyto}}}{n_{\mathrm{H_2O}}^{\mathrm{cyto}} - (1 - K_{\mathrm{p},j}) n_{\mathrm{H_2O,b}}^{\mathrm{cyto}}} = m^{\bullet}_{\mathrm{H_2O}} \sum_j \frac{\phi_j^{\mathrm{o}} n_j^{\mathrm{cyto}}}{n_{\mathrm{H_2O,f}}^{\mathrm{cyto}} + (K_{\mathrm{p},j}) n_{\mathrm{H_2O,b}}^{\mathrm{cyto}}} \quad (6)$$

In addition to osmolytes (including KGlu, trehalose, proline, and GB), other osmotically significant electroneutral components contributing to the sum in eq 6 include (i) the nucleic acid polyelectrolyte component, designated KNA, which behaves thermodynamically as if K^+ counterions are incompletely dissociated from nucleic acid (NA) phosphates, but nevertheless contributes significantly to $\mathrm{Osm}_{\mathrm{cyto}}$ (*7*), and (presumably) (ii) a composite metabolite component (designated KM) consisting of primarily univalent anionic metabolites (M^-) (*20*) and K^+ ions. The amount of the K^+/nucleic acid component (n_{KNA}) is given by the amount of nucleic acid phosphate charge not neutralized by associated Mg^{2+}, putrescine^{2+}, or other oligocations (*7*). In the absence of accurate determinations of amounts and osmotic coefficients of these components, here we combine these terms and estimate their value from published *in vivo* osmotic data at low osmolality, as described in Materials and Methods.

The assumption of additivity in eq 6 was tested and found to be applicable in interpreting osmolalities of polyelectrolyte solutions (*21*), and a similar assumption was also used to deduce that at low osmolality the *E. coli* cytoplasm behaved thermodynamically like a concentrated nucleic acid solution (KNA) containing a relatively small mole ratio of salt anions (primarily glutamate, also metabolites) to nucleic acid phosphate (*7*). Here we apply eq 6 to predict $\mathrm{Osm}_{\mathrm{cyto}}^{\mathrm{calc}}$, and compare it to the experimentally observed value of $\mathrm{Osm}_{\mathrm{cyto}}^{\mathrm{obs}}$ as a function of growth osmolality.

MATERIALS AND METHODS

Bacterial Growth. *E. coli* K-12 strain MG1655 (ATCC 47076) was grown in MOPS-buffered minimal glucose medium (MBM) (*22*), in MBM and 1 mM glycine betaine (MBM+GB), or in rich LB medium [1% (w/v) tryptone, 0.5% yeast extract, and 1% NaCl (*23*)] at 37 °C and 240 rpm on a gyratory shaker to midlog phase ($A_{550} \sim 0.5$, $\sim 4 \times 10^8$ cells/mL). When GB was added to MBM (basal osmolality of 0.1 Osm), its concentration was 1 mM except where noted. The osmolality of MBM was adjusted with NaCl and measured with a Wescor 5500 vapor pressure osmometer. Since growth at very high osmolality requires an inoculum grown in media of at least moderate osmolality (*24*), high-osmolality cultures were obtained by first growing cells from a freezer stock in LB with a moderate osmolality (0.5−0.8 M NaCl) overnight, followed by 1:100 dilution and growth overnight in minimal medium with NaCl at the experimental osmolality to obtain inocula for experimental cultures. Inocula were then diluted at least 100-fold into experimental media. Growth rates were determined from exponential fits of A_{550} (typically in the range of 0.1−0.4 absorbance unit) as a function of time for 50 mL cultures growing in 250 mL culture flasks.

Chemicals. [^{14}C]Glycine betaine (55 mCi/mmol) was obtained from American Radiochemical Labs, and ^3H_2O (1 mCi/g), [^{14}C]sucrose (621 mCi/mmol), and [^{14}C]inulin (9.4 mCi/mmol) were from Amersham. Stock solutions of [^{14}C]-sucrose and [^{14}C]inulin were purified before use as previously described (*13*). Bovine serum albumin (BSA), fraction V, was obtained from Sigma and purified as previously described (*17*). Yeast RNA (type III) was obtained from Sigma.

Measurement of the Amounts of Cytoplasmic Osmolytes. Amounts of K^+, trehalose, and GB in micromoles per milliliter of culture (obtained as described below) were normalized per milligram of cell protein by comparing the A_{550} of the culture at the time of harvest to a calibration curve of A_{550} versus the milligrams of cell protein per milliliter of culture. Plots of A_{550} versus milligrams of protein per milliliter of culture were independent of growth osmolality for cells grown in MBM+GB from 1 to 2.17 Osm, so a single calibration curve was used. Amounts of osmolytes per milligram of cell dry weight were calculated using the measured osmolality-independent ratio of protein to dry weight of 0.52 ± 0.03 (see below).

K^+. Cells were filter-harvested and analyzed by atomic absorption spectroscopy to determine the amounts of cytoplasmic K^+. In brief, triplicate 1 mL aliquots of cell cultures were filtered through 0.45 μm Millipore HAWP filters prewet with fresh medium lacking K^+ (wash buffer), warmed to 37 °C, and then rinsed with 3 × 1 mL of wash buffer. Washed filters were soaked with 5 mL of 1 N HNO_3 and 1 mg/mL CsCl for 30−60 min; the lysate was filtered through Gelman 0.45 μm Acrodisc filter units, and the filtrates were assayed with a Unicam 969 atomic absorption spectrometer. The intensity was corrected for the low background from filters alone, and solutions of KCl in 1 N HNO_3 and 1 mg/mL CsCl were used as a standard. The error in triplicate measurements was typically <5%.

Glycine Betaine (GB). The amount of cytoplasmic GB ($n_{\mathrm{GB}}^{\mathrm{cyto}}$) was determined by measuring the amount of [^{14}C]GB removed from the growth medium as described previously (*10*). Specifically, overnight cultures were diluted 1:1000 and grown in medium containing 0.5−1.0 mM [^{14}C]betaine to midlog phase. Triplicate 1 mL samples were harvested by centrifugation, and an aliquot from the top of the supernatant was added to 5 mL of scintillant and the number of counts per minute determined by scintillation counting. The fraction of [^{14}C]GB removed from the medium at the time of harvest was typically less than one-half of the amount originally present in the medium, with the cell pellet accounting for the remaining counts per minute. When such pellets from cells grown in MBM+GB at 2.17 Osm were washed by suspension in fresh medium (lacking GB) and recentrifuged, the supernatant contained a negligible number of counts per minute, indicating that [^{14}C]GB present in cell pellets is cytoplasmic (i.e., cannot be removed by washing). Amounts in moles of betaine in each sample were calculated from the specific activity of [^{14}C]GB in the culture. Triplicate measurements typically agreed to within ~5%. Our current value of $n_{\mathrm{GB}}^{\mathrm{cyto}}$ at 1.02 Osm is consistent with the trend in $n_{\mathrm{GB}}^{\mathrm{cyto}}$ with growth osmolality determined in the study presented here (Figure 2 below) but is ~20% smaller than that which we previously determined at 1.02 Osm (*10*), although current determinations of the mole amounts of K^+, trehalose, and water in cells grown under this condition agree with our previously published values.

Trehalose. The amount of cytoplasmic trehalose was determined as described previously (*25*). The amounts of trehalose in cell samples and standard solutions of trehalose were measured by the anthrone assay (*26*), which is sufficiently sensitive to detect 5 nmol of trehalose/mg of cell dry weight. Duplicate measurements for a given sample typically agreed to within ~9%, and averages of different samples typically agreed to within 7%.

Measurement of Amounts of RNA and Protein. To determine the amounts of RNA, aliquots of midlog phase culture were brought to 5% trichloroacetic acid (TCA), iced, and centrifuged at 4 °C for 10 min. Pellets were suspended in 5% TCA, heated for 20 min at 90 °C, iced, and centrifuged to remove insoluble material. (Samples precipitated with 0.5 N $HClO_4$, and then suspended and heated for 30 min at 70 °C in 0.5 N $HClO_4$, gave identical results.) The amount of RNA in the supernatant was quantified by the orcinol assay (*26*) using total yeast RNA as a standard. The concentration of yeast RNA standard was determined by measuring the absorbance at 260 nm of hydrolyzed samples brought to pH 8, using an extinction coefficient ϵ_{260} of 11.3 mM^{-1} cm^{-1} calculated from the base composition of total yeast RNA [mole fractions of 0.26, 0.28, 0.21, and 0.25 for A, G, C, and U, respectively (*27*)] and the absorbance of individual nucleotide monophosphates (*28*).

The total amount of protein in cells was determined by the method of Lowry et al. (*29*) using BSA as a standard. The amount of protein was determined with aliquots of suspensions prepared for the volume assay (see below) after dilution with water and sonically lysed, and was determined on aliquots of growing cultures that were brought to 170 μg/mL chloramphenicol, iced, pelleted by centrifugation at 10000*g* for 8 min at 4 °C, and then suspended in water and sonically lysed prior to the assay. (Aliquots of growing cultures that were precipitated with TCA, suspended in 0.1 N KOH, and diluted in water prior to the assay gave identical results.) Concentrations of BSA in standard solutions were determined by measuring the absorbance at 280 nm using an extinction coefficient calculated from its amino acid composition [$\epsilon_{280}^{1\%} = 6.5$ (*30*)], which is the same within uncertainty as the $\epsilon_{280}^{1\%}$ determined experimentally immediately after extensive lyophilization (*17*). In our previous determinations of the amount of cell protein (*7*, *10*), BSA standards were prepared gravimetrically using unlyophilized BSA; these were subsequently found to contain ~10% water. Correction for this brings our previously determined osmolality-independent protein:dry weight ratio for *E. coli* K-12 grown in MBM and MBM+GB (*7*, *10*) into agreement with that reported for *E. coli* B/r grown in minimal glucose medium [~0.55 mg/mg of dry weight (*31*)]. We assume that 80% of the total cell protein is cytoplasmic, consistent with previous work (*7*, *32*, *33*).

Determinations of the Amount of Cytoplasmic Water, Water-Inaccessible Cell Volume, and Concentrations of Cytoplasmic Protein and RNA. Amounts of cytoplasmic water [$n_{H_2O}^{cyto}$ in micromoles per milligram of dry weight (DW)] were determined on fresh suspensions of centrifugally harvested cells using ^3H_2O and [^{14}C]sucrose as previously described (*13*). This yields the number of micromoles of cytoplasmic water per milliliter of suspension. Amounts of cell dry weight per milliliter of cell suspension were determined with aliquots of suspension that were centrifugally pelleted, suspended in water, and baked to a constant weight at 80 °C.

We observe by phase microscopy that most cells in exponential cultures in MBM+GB with 1.1 M NaCl (2.17 Osm) form long filaments, even if diluted into fresh 2.17 Osm medium and regown into the log phase several times. A small fraction of cells grown in MBM+GB with 0.9 M NaCl (1.77 Osm) form short filaments, whereas no filaments are observed in cultures in MBM+GB with ~0.7 M NaCl (~1.44 Osm), in LB with 1.1 M NaCl (~2.29 Osm), or in MBM (without GB) with 0.65 M NaCl (1.36 Osm). A small percentage of large round spheroplast-like cells were also sometimes observed at a high cell density in cultures grown in MBM+GB at 2.17 Osm. Cultures containing the spheroplast-like cells form soft pellets, even after centrifugation at 12000*g* for 30 min, which hampered accurate measurement of the intracellular volume and of the dry weight. Cultures grown in MBM+GB at 2.17 Osm were therefore harvested at an A_{550} of ~0.2−0.3 where <0.4% of the filaments adopted a spheroplast-like morphology.

The water-inaccessible cell volume V_{wi}^{cell} (in microliters per milligram of DW) was determined by subtracting the volume occupied by cytoplasmic water ($V_{H_2O}^{cyto} \cong n_{H_2O}^{cyto}/55.5$) from the sucrose-inaccessible cell volume as described previously (*7*). The cytoplasmic water-inaccessible volume V_{wi}^{cyto} was assumed to be 80% of V_{wi}^{cell} (*7*). The reproducibility within each triplicate determination was typically ~15%.

Cytoplasmic concentrations of protein and RNA are calculated by dividing their cytoplasmic amounts (in milligrams per milligram of DW) by the total cytoplasmic volume V_{tot}^{cyto} (in microliters per milligram of DW), where $V_{tot}^{cyto} = V_{H_2O}^{cyto} + V_{wi}^{cyto}$.

Validation of Methods. To address whether the fresh suspensions of centrifugally harvested cells used to quantify amounts of water and some osmolytes (see above) provide results relevant for growing cultures, we tested whether the amounts of water and osmolytes measured in fresh suspensions are the same as in growing cells in control experiments. Specifically, in the study presented here, we find that the amount of cytoplasmic [^{14}C]betaine measured in cells grown in MBM+GB at 2.17 Osm (assayed as described above) is identical within error before and after an additional cycle of cell suspension and centrifugation, indicating that centrifugation does not cause leakage of cytoplasmic betaine. We also found in previous studies that the amount of K^+ in fresh suspensions of centrifugally harvested and washed cells (harvest time of 45 min) grown at various osmolalities is identical within error to that in growing cells harvested by filtration (harvest time of ~10 s) (*7*), that the amounts of betaine, proline, and glutamate in fresh suspensions of centrifugally harvested cells grown at 1 Osm are identical within error to that in growing cells harvested by filtration (*10*), and that the amount of cytoplasmic water in fresh suspensions of centrifugally harvested cells grown at 0.3 Osm is identical within error to that in growing cells harvested rapidly by centrifugation through oil in microfuge tubes (harvest time of ~45 s) (*13*). Thus, the osmotic properties of our fresh suspensions of centrifugally harvested cells are apparently the same as those of growing cells.

Table 1: *In Vitro* Solute−Protein Partition Coefficients (K_p) and Two-Component Osmotic Coefficients (ϕ^o) as a Function of Molal Solute Concentration (m) for *E. coli* Osmolytes

solute	K_p[a]	ϕ^o [b]
betaine	0.46	$1 + 0.1768m - 0.0069m^2 + 0.0004m^3$
proline	0.72	$1 + 0.048m$
trehalose	0.53	$1 + 0.09m$
KGlu	0.51	1.76

[a] Partition coefficients K_p were determined as described in ref *17* from the densitometric determinations of Timasheff (*97*) of preferential interaction coefficients for interaction of GB with BSA, of proline with lysozyme, of NaGlu with BSA, and of trehalose with RNase. [b] Osmotic coefficients ϕ^o for betaine were obtained from nonlinear least-squares fitting of isopiestic distillation data (*98*) for betaine concentrations of $\lesssim 5$ *m*. Osmotic coefficients ϕ^o for other solutes were obtained from vapor phase osmometry for osmolyte concentrations of $\lesssim 1$ *m* (*17*).

Calculations of Contributions to Cytoplasmic Osmolality. Contributions of principal cytoplasmic osmolytes to osmolality were calculated from eq 6 using cytoplasmic mole amounts of these solutes and water determined for cells grown in MBM+GB in this study, together with *in vitro* literature values of two-component osmotic coefficients ϕ_j^o and values of local bulk partition coefficients K_p for individual solute−protein interactions calculated as described in ref *17* from literature values of preferential interaction coefficients determined by Timasheff and co-workers (see Table 1). These K_p values differ somewhat from those originally reported by us from analysis of vapor pressure osmometry (VPO) data (*17*). Improvements in both the determination of sample composition and data analysis yield VPO-determined values of K_p that are consistent with those cited in Table 1 (J. Cannon et al., work in progress). Table 1 lists values of ϕ_j^o and K_p for potassium glutamate (KGlu), trehalose, proline, and GB. No comparable information exists for the osmotic coefficient (ϕ^o_{KNA}) of the nucleic acid component, which is primarily ribosomal RNA, and the mole amount (n^{cyto}_{KM}), osmotic coefficient (ϕ^o_{KM}), and partition coefficient of the metabolite component (designated KM; see above). Therefore, we estimate the composite quantities ϕ^o_{KNA+KM} and $n^{cyto}_{KNA} + n^{cyto}_{KM}$ for these two components from previously published data from cells grown at 0.1 and 0.28 Osm, conditions under which the cytoplasm is well-modeled as a nucleic acid/salt solution (*7*, *34*). For these growth conditions, application of an electroneutrality condition to the cytoplasm predicts that $n^{cyto}_{KNA} + n^{cyto}_{KM} = n^{cyto}_{K} - n^{cyto}_{Glu}$; the osmotic coefficient ϕ^o_{KNA+KM} is then calculated from the cytoplasmic osmolality (Table 3) using eq 6. We arbitrarily assume a partition coefficient of unity for interactions of these components with cytoplasmic proteins, by analogy with the behavior of KCl (*19*).

Cells grown at high osmolality in the absence of proline or GB contain significant amounts of cytoplasmic MOPS from the uptake of external MOPS buffer (*22*). Amounts of charged and uncharged MOPS were estimated assuming that 70% of cytoplasmic MOPS ($pK = 7.2$) is anionic at the pH of the cytoplasm (~7.6); values of ϕ_j^o and K_p for KGlu and trehalose were used to estimate the contributions of the KMOPS and uncharged MOPS components, respectively, to cytoplasmic osmolality.

Because the total mole amount of cytoplasmic proteins is small (~0.01 μmol/mg of dry weight), we conclude that cytoplasmic proteins do not contribute to osmolality, even when the predicted large contribution of macromolecular crowding to the osmotic coefficient of proteins in concentrated solutions is taken into consideration (*35*), consistent with the very small contribution of proteins to the osmolality of frog myoplasm (*36*). Indeed, none of the approximations described in this section appear to have a significant effect on the semiquantitative conclusions of this analysis.

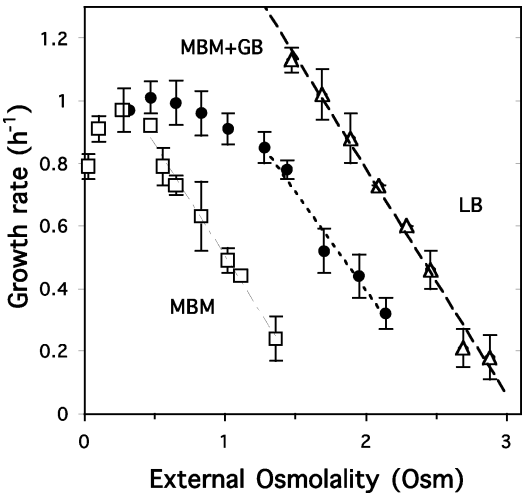

FIGURE 1: Dependence of the growth rate of *E. coli* K-12 strain MG1655 on the external osmolality in MOPS-buffered minimal medium (MBM, □), MBM with 1 mM glycine betaine (●), and LB medium (△). The external osmolality was adjusted with NaCl. Error bars show the standard deviation of three or more independent measurements of growth rate. The growth rates of cells in MBM are from Cayley et al. (*13*).

RESULTS

Effect of the Osmoprotectant Glycine Betaine on the Growth Rate of E. coli K-12. Figure 1 quantifies the striking ability of 1 mM glycine betaine (GB) in the medium to stimulate growth of *E. coli* K-12 in high-osmolality environments in MOPS-buffered minimal medium (MBM), and compares growth rates of cells in MBM+GB and in rich LB medium as a function of external (growth) osmolality (Osm_{ex}). While cells cultured in MBM with and without 1 mM GB exhibit the same maximum growth rate of ~1.0 h^{-1} at ~0.3 Osm, addition of GB greatly extends the upper limit of the range of osmolality where the growth rate remains within 10% of this maximum value from ~0.5 Osm (in MBM) to ~1 Osm. Above 1 Osm, the growth rate in MBM+GB decreases approximately linearly with increasing osmolality, a dependence which parallels that of *E. coli* grown in MBM (Figure 1) and is similar to the trend observed with other bacteria (*37*). The osmolality at which the growth rate approaches zero (estimated by linear extrapolation) increases from ~1.8 Osm (MBM) to ~2.6 Osm (MBM+GB). Growth of *E. coli* in LB medium is faster than growth in MBM+GB at all osmolalities (reaching ~2.1 h^{-1} at ~0.3 Osm; not shown) and extends the estimated high-osmolality limit of growth to ~3.1 Osm (Figure 1). Addition of 1 mM GB to osmotically stressed cells growing in LB with 1 M NaCl has no effect on the growth rate (not shown), presumably because of the presence of sufficient endogenous betaine in LB medium (*38*).

Amounts of Osmolytes in E. coli Grown at High Osmolalities in MBM+GB. To understand the thermodynamic

Table 2: Cytoplasmic Amounts of Osmolytes, Water, and Biopolymers in Cells Grown in MBM+GB at High Osmolality[a]

	1.02 Osm[b] without GB	1.02 Osm[c] with GB	1.44 Osm with GB	1.77 Osm with GB	2.17 Osm with GB
betaine (μmol/mg of DW)	0	0.91 ± 0.05	1.22 ± 0.08	1.51 ± 0.1	1.72 ± 0.17
K^+ (μmol/mg of DW)	0.95 ± 0.08	0.81 ± 0.05	0.80 ± 0.05	0.82 ± 0.05	0.74 ± 0.06
trehalose (μmol/mg of DW)	0.36 ± 0.03	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.08 ± 0.02
$n_{H_2O}^{cyto}$ (μmol/mg of DW)	65.5 ± 3.3	93.2 ± 7.2	83.3 ± 3.9	72.7 ± 5.0	57.2 ± 15.5
protein (mg/mg of DW)	0.47 ± 0.05	0.39 ± 0.04	0.42 ± 0.02	0.41 ± 0.03	0.42 ± 0.02
RNA (mg/mg of DW)	0.13 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01

[a] All measurements are the average (±standard deviation) of measurements performed in triplicate on at least three independent cultures. [b] For cells grown in MBM at 1.02 Osm, $n_{MOPS} = 0.25 \pm 0.03$ μmol/mg of DW and $n_{glut} = 0.23 \pm 0.03$ μmol/mg of DW (*7*). [c] For cells grown in MBM+GB at 1.02 Osm, $n_{MOPS} < 0.03$ μmol/mg of DW and $n_{glut} = 0.08 \pm 0.03$ μmol/mg of DW (*10*).

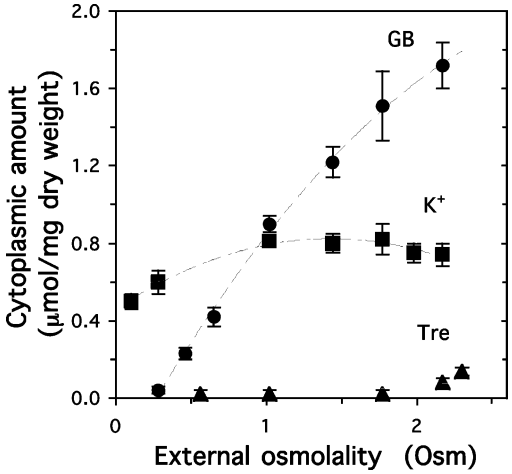

FIGURE 2: Amounts of cytoplasmic osmolytes (in micromoles per milligram of DW) as a function of external osmolality in *E. coli* grown in MBM+GB. Amounts of K^+ (■), trehalose (▲), and betaine (●) plotted are the mean (±standard deviation) of triplicate measurements performed on at least three independent cultures, except for the amount of K^+ at 2.0 Osm where only one triplicate determination was performed. Amounts of K^+ at 0.28 (*7*) and 0.1 Osm (this work) were determined in cells grown in MBM, but should not be affected within error by addition of GB since negligibly small amounts (~0.04 μmol/mg of DW) of cytoplasmic GB are accumulated under these low-osmolality growth conditions.

basis for the extraordinary ability of GB to stimulate the growth of osmotically stressed *E. coli*, we determined cytoplasmic amounts of GB and other osmolytes as a function of Osm_{ex} in cells grown in MBM+GB to compare with previous results in MBM. At 0.3 Osm, where the growth rate is maximal in MBM and addition of 1 mM GB has no effect on the growth rate (Figure 1), only a minimal amount (0.04 μmol/mg of DW) of cytoplasmic GB is detected (Figure 2). The amount of cytoplasmic GB increases approximately linearly with increasing Osm_{ex} from 0.3 to 1 Osm, and the growth rate remains within 10% of its maximal value. Above 1 Osm, the amount of cytoplasmic betaine increases less strongly with increasing growth osmolality and the growth rate decreases more strongly with increasing osmolality. At 2.17 Osm, the osmoprotectant GB represents ~20% of cell dry weight of growing *E. coli* cells. In nongrowing, desiccated organisms that survive drying [i.e., anhydrobiotes], trehalose represents up to ~20% of the dry weight (*39*).

Figure 2 indicates that the rate of increase of the amount of betaine with increasing osmolality ($dn_{GB}^{cyto}/dOsm_{ex}$) above 1 Osm in MBM+GB is reduced significantly as compared to lower osmolality, indicating that amounts of other osmolytes must increase and/or that the amount of cytoplasmic water must decrease as the growth osmolality increases. For *E. coli* K-12 growing in MBM without added osmoprotectants, we previously found that cytoplasmic amounts of K^+, glutamate, MOPS, and trehalose all increase significantly with increasing growth osmolality (*7*). In contrast, Figure 2 shows that the amount of cytoplasmic K^+ in cells grown in MBM+GB from 1 to 2.17 Osm remains relatively constant and the amount of trehalose only increases at the highest osmolalities that were examined (≥2.17 Osm). Amounts of cytoplasmic GB, K^+, and trehalose as a function of growth osmolality in MBM+GB are listed in Table 2. For *E. coli* K-12 grown in MBM+GB at high osmolality, no cytoplasmic MOPS and only a small amount (0.08 μmol/mg of DW) of cytoplasmic glutamate are observed (*10*). The amount of cytoplasmic glutamate must be similarly small in cells grown at higher osmolality in MBM+GB, because any significant change from that observed at 1.02 Osm would require a corresponding change in the amount of cytoplasmic K^+ (*40*, *41*). Since only the amount of cytoplasmic GB increases significantly with increasing Osm_{ex} above 1 Osm, we next examined whether the decrease in dn_{GB}^{cyto} with increasing Osm_{ex} (Figure 2) is accompanied by a reduction in the amount of cytoplasmic water with increasing osmolality of growth.

Cytoplasmic Amounts of Water and Biopolymers in Cells Grown in MBM+GB with Water. In principle, the accumulation of osmolytes could be sufficient to keep the cell volume constant over a broad range of growth osmolalities. Indeed, the osmoregulatory mechanisms of some mammalian cells prevent long-term changes in cell volume after shifts in osmolality (*42*). However, we find that the large increase in the amount of cytoplasmic osmolytes (predominantly betaine) with increasing osmolality for *E. coli* grown in MBM+GB (Table 2 and Figure 2) is insufficient to maintain the amount of cytoplasmic water independent of the osmolality of the growth medium. Indeed, we observe that $n_{H_2O}^{cyto}$ in cells grown in MBM+GB decreases ~60% as Osm_{ex} increases from 1 to 2.17 Osm (Table 2), a trend previously observed for *E. coli* grown at lower osmolalities (*7*, *25*, *43*), for other bacteria (*37*), and for yeast (*44*, *45*).

The amounts of cytoplasmic water and osmolytes are related by eq 7, obtained from eqs 2−4:

$$n_{H_2O}^{cyto} = \{55.5\phi_{cyto}\sum n_j^{cyto}/[Osm_{ex} + \Delta\Pi/(RT)]\} + n_{H_2O,b}^{cyto} \quad (7)$$

According to eq 7, at a specified high osmolality of growth [where $Osm_{cyto} \cong Osm_{ex}$ because $\Delta\Pi/(RT)$ is relatively small in comparison to Osm_{ex}], the amount of cytoplasmic water $n_{H_2O}^{cyto}$ is determined primarily by the total mole amount of

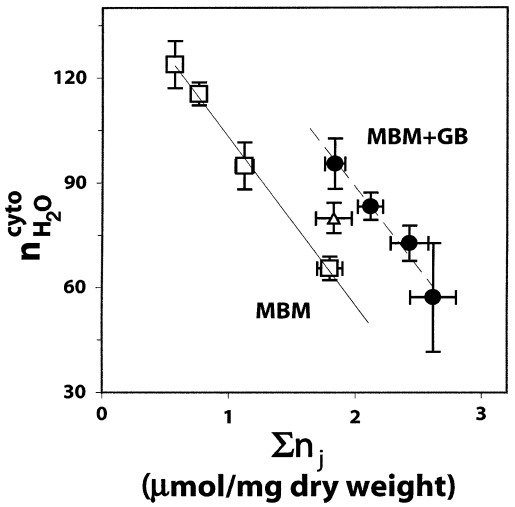

FIGURE 3: Amount of cytoplasmic water (in micromoles per milligram of DW) as a function of the total amount of cytoplasmic osmolytes ($\sum n_j^{cyto}$) in *E. coli* grown in MBM (□), MBM+GB (●), and MBM with 1 mM proline (△) at various osmolalities. Species contributing to $\sum n_j^{cyto}$ are GB, proline, KGlu, trehalose, and MOPS. The data for cells grown in MBM and in MBM with 1 mM Pro are from Cayley et al. (*7*, *10*).

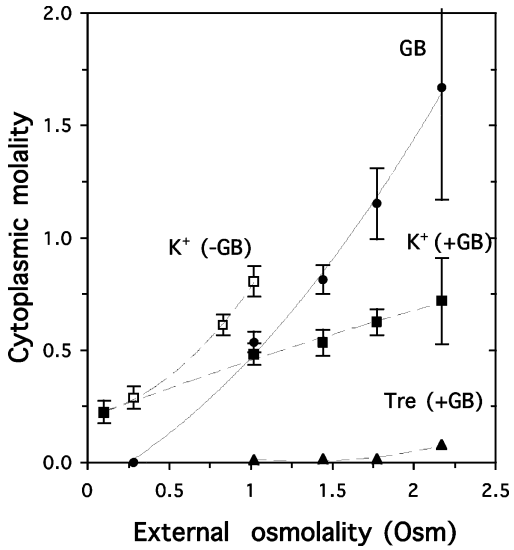

FIGURE 4: Concentrations of cytoplasmic betaine and K^+ as a function of growth osmolality. The cytoplasmic molalities of betaine (●), K^+ (□), and trehalose (▲) in cells grown in MBM+GB and of K^+ (■) in cells grown in MBM were calculated by dividing their amounts (in micromoles per milligram of DW) by the total amount of cytoplasmic water (in microliters per milligram of DW) under each growth condition. The data for cells grown in MBM above 0.1 Osm are from ref *7*. Errors were obtained by propagating the uncertainties in the amount of osmolytes and water.

osmolytes $\sum n_j^{cyto}$ and by the cytoplasmic osmotic coefficient ϕ_{cyto} characteristic of the osmolyte composition of these cells. Figure 3 plots $n^{cyto}_{H_2O}$ as a function of $\sum n_j^{cyto}$ (calculated from the amounts of osmolytes in Table 2 with the assumption that $n_{Glu} = 0.08$ μmol/mg of DW, independent of Osm_{ex}, above 1 Osm) for cells grown in MBM+GB and compares these results with those obtained previously in MBM. In both media, $n^{cyto}_{H_2O}$ decreases with increasing $\sum n_j^{cyto}$; for the ranges that were investigated, the best-fit lines for each data set are approximately parallel and the data in MBM+GB are significantly offset to a higher $n^{cyto}_{H_2O}$. (In MBM+GB at lower osmolality, values of $n^{cyto}_{H_2O}$ must approach those determined in MBM.) The thermodynamic origin of the large increase in $n^{cyto}_{H_2O}$ for cells grown in MBM+GB as compared to MBM at 1.02 Osm [where $\sum n_j^{cyto} \sim 1.8$ μmol/mg of DW in both the presence and absence of GB; Table 2 (*10*)] must be a large increase in ϕ_{cyto} (cf. eq 7) accompanying the changes in the amounts of individual cytoplasmic osmolytes that result from addition of 1 mM GB to MBM (see Discussion). For cells grown at 1.02 Osm in MBM with 1 mM proline, where $\sum n_j^{cyto}$ (indicated by the triangle in Figure 3) also equals ~1.8 μmol/mg of DW (*10*), the value of $n^{cyto}_{H_2O}$ is intermediate between those obtained in MBM and MBM+GB at this osmolality, indicating that ϕ_{cyto} (and hence $n^{cyto}_{H_2O}$) is smaller in cells grown in MBM with proline than in MBM with GB.

Biopolymers. Amounts of cell protein and RNA (in milligrams per milligram of DW) in *E. coli* grown in MBM+GB from 1.02 to 2.17 Osm are listed in Table 2. Over this range, neither amount is detectably dependent on osmolality; biopolymer contributions to dry weight of the cell are 52 ± 3% protein and 14 ± 2% RNA. The weight ratio of RNA to protein (0.27) is comparable to that reported for *E. coli* K-12 strain AB1157 grown in a glucose minimal medium (*46*). We find that amounts of biopolymers determined in cells grown in MBM+GB at high osmolality are the same within uncertainty as those determined in MBM at a lower osmolality (*7*), even though the contribution to cell dry weight from osmotically variable small solutes (including osmolytes, putrescine, and membrane-derived oligosaccharides) varies over this range.

Cytoplasmic Concentrations of Glycine Betaine, K^+, and Trehalose. Cytoplasmic molal concentrations of GB, K^+, and trehalose in cells grown in MBM+GB, calculated from the analytical data for these solutes and cytoplasmic water in Table 2, are plotted in Figure 4 as a function of growth osmolality and compared with values calculated from previously published data in MBM. The cytoplasmic concentration of GB increases strongly with increasing osmolality of growth in MBM+GB, especially above 1 Osm where $n^{cyto}_{H_2O}$ decreases and n^{cyto}_{GB} increases with increasing Osm_{ex}. Cytoplasmic concentrations of GB and K^+ are 0.54 and 0.48 M at 1.02 Osm and 1.67 and 0.72 M at 2.17 Osm, respectively. (The increase in the cytoplasmic K^+ concentration with increasing Osm_{ex} in MBM+GB is entirely the result of the reduction in the steady state amount of cytoplasmic water.) By comparison, in cells grown at 1.02 Osm in MBM without added osmoprotectant, the K^+ concentration is 0.81 M. The reduction in the cytoplasmic K^+ concentration upon uptake of GB is the consequence of two effects. (1) GB replaces K^+ salts, and (2) this replacement leads to uptake of more cytoplasmic water, thus diluting all cytoplasmic solutes. The concentration of trehalose in cells grown in MBM+GB is always small except at the highest osmolality of growth examined. Although trehalose is only a small fraction of the osmolyte pool at 2.17 Osm, the trehalose concentration is nearly 0.1 M.

Comparison of Predicted and Observed Cytoplasmic Osmolalities. The contributions of individual solute components to cytoplasmic osmolality were calculated with eq 6 from the amounts of cytoplasmic solutes and water reported in Table 2, utilizing literature values of two-component

Table 3: Contributions of Osmolytes to Cytoplasmic Osmolality Calculated Using *in Vitro* Thermodynamic Data[a]

	0.1 Osm	0.28 Osm	0.56 Osm	1.02 Osm	1.02 Osm with Pro	1.02 Osm with GB	1.44 Osm with GB	1.77 Osm with GB	2.17 Osm with GB
GB or Pro	0	0	0	0	0.53	0.69	1.11	1.73	2.87
Tre and MOPS[b]	0	0	0.04[c]	0.45	0.14	0.01	0.02	0.02	0.10
KGlu and KMOPS[b]	0.05	0.12	0.31[c]	0.74	0.37	0.10	0.11	0.13	0.17
KNA and KM	0.15	0.18	0.24	0.36	0.30	0.33	0.39	0.43	0.50
Osm_{cyto}^{calc}	0.20	0.30	0.59	1.55	1.34	1.13	1.62	2.31	3.63
Osm_{cyto}^{obs} [d]	0.18	0.32	0.59	1.03	1.04	1.05	1.47	1.79	2.18

[a] Abbreviations: MOPS, neutral morpholinopropanesulfonate; KMOPS, K^+ salt of anionic MOPS; KM, K^+ salt of anionic metabolites; KNA, K^+ salt of nucleic acid. The standard deviation of values of Osm_{cyto}^{calc}, calculated by propagating the uncertainties in cytoplasmic osmolalities (each calculated with eq 6 from the uncertainties in the amounts of osmolytes and cytoplasmic water), was on average ~13% except for cells grown at 2.17 Osm with GB, where the large uncertainty in the amount of free water (Table 2) results in an uncertainty of ~40%. [b] MOPS is transported by *E. coli* grown at 1.02 Osm in MBM and in MBM with 1 mM proline (*10*). Contributions to osmolality of neutral and anionic cytoplasmic MOPS to Osm_{cyto}^{calc} were estimated as described in the text. [c] Amounts of neutral and anionic MOPS in cells grown in MBM at 0.56 Osm were estimated by interpolation of the data of Cayley et al. (*7*). [d] Values of Osm_{cyto}^{obs} were obtained from eq 2, using the tabulated growth osmolalities and estimates of turgor pressures ($\Delta\Pi$). At or below 1.0 Osm, values of $\Delta\Pi$ were obtained from ref *13*. Above 1.0 Osm, values of $\Delta\Pi$ (always <1 atm) were estimated from cell volumes (S. Cayley, unpublished results) and the volumetric elastic modulus (*13*).

(solute and water) osmotic coefficients ϕ_j^o and of local bulk partition coefficients K_p determined for interactions of these solutes with native proteins (all listed in Table 1). In these calculations, the amount of water in the local domain at the biopolymer surface was set equal to the previously determined amount of bound cytoplasmic water [$n_{H_2O,b}^{cyto} \cong 22$ μmol/mg of DW, independent of growth osmolality (*13*)]. These predicted contributions to cytoplasmic osmolality, obtained as described in Materials and Methods, are given in Table 3. The combined contribution of the K^+/nucleic acid (KNA) and K^+/metabolite (KM) electroneutral components (cf. Table 3) was estimated using an osmotic coefficient ($\phi_{KNA+KM}^o = 0.78 \pm 0.10$) obtained from the fitting of eq 6 to previously published analytical and osmotic data in MBM at low osmolality [0.10 and 0.28 Osm (*7*)] and a combined amount of the nucleic acid and metabolite components ($n_{KNA} + n_{KM}$) estimated from the difference between the amounts of K^+ and glutamate, as described in Materials and Methods. At high osmolality in MBM+GB, where the amount of cytoplasmic K^+ does not change significantly with Osm_{ex} (Figure 2), we assume that the amount of glutamate is also independent of Osm_{ex}. If the amount of the KM component is assumed to be the same in MBM and MBM+GB at all osmolalities, and is equated to ~0.19 μmol/mg of DW [estimated from the difference between the amounts of cytoplasmic K^+ and glutamate which are free to efflux from *E. coli* cells transiently permeabilized by osmotic downshock (*41*)], then amounts of the nucleic acid component (KNA) as a function of osmolality are the same within uncertainty as those estimated by Cayley et al. (*7*). Also, if it is assumed that the osmotic coefficients of KM and KGlu are the same, then decomposition of ϕ_{KNA+KM}^o (assuming additivity) yields an estimate of ϕ_{KNA}^o in the range of 0.1−0.3, as expected for a strong polyelectrolyte like RNA (*7*).

Table 3 compares predictions of cytoplasmic osmolality (Osm_{cyto}^{calc}, calculated from eq 6) with "observed" cytoplasmic osmolalities (Osm_{cyto}^{obs}), obtained with eq 2 from the growth osmolality and the turgor pressure $\Delta\Pi$, estimated as described in Table 3. Above ~1 Osm, $\Delta\Pi$ is less than 1 atm (*13*), so the difference between Osm_{ex} and Osm_{cyto}^{obs} is less than 0.04 *m*, and therefore not significant for this calculation.

Differences between calculated and observed cytoplasmic osmolality are plotted as a function of growth osmolality in Figure 5A. Because ϕ_{KNA+KM}^o is obtained from analysis of the low osmolality (0.1 and 0.28 Osm) data, Osm_{cyto}^{calc} is required to be equal within uncertainty to Osm_{cyto}^{obs} in this regime. Calculated and observed cytoplasmic osmolalities agree within uncertainty at 0.56 Osm in MBM and at 1.02 Osm in MBM+GB. However, at or above 1.02 Osm, values of Osm_{cyto}^{calc} exceed Osm_{cyto}^{obs}; the deviation increases with increasing osmolality and at 1.02 Osm is somewhat more pronounced in MBM than in MBM+GB. At this osmolality, where we (*10*) previously compared the osmotic properties of cells grown in MBM, MBM with 1 mM proline, and MBM+GB, Osm_{cyto}^{calc} decreases from ~1.5 Osm in MBM to ~1.3 Osm in MBM with proline and ~1.1 Osm in MBM+GB, a progression which mirrors the progressive increase in the amount of cytoplasmic water from MBM to MBM with proline to MBM+GB (see Table 2). The most significant deviations of Osm_{cyto}^{calc} from Osm_{cyto}^{obs} occur for 2.17 Osm in MBM+GB (for which Osm_{cyto}^{calc} is 1.7 times as large as Osm_{cyto}^{obs}) and 1.02 Osm in MBM (for which Osm_{cyto}^{calc} is 1.5 times larger than Osm_{cyto}^{obs}). These are growth conditions in which there is little cytoplasmic water (Table 2; *7*, *10*). According to our two-domain model of cytoplasmic water (*7*, *10*, *13*), only ~67% of cytoplasmic water is free (bulk) water in *E. coli* grown at 1.77 Osm in MBM+GB and at 1.02 Osm in MBM, and only ~60% is free at 2.17 Osm in MBM+GB.

Since differences between Osm_{cyto}^{calc} and Osm_{cyto}^{obs} are largest under growth conditions where the amount of cytoplasmic water $n_{H_2O}^{cyto}$ is small, we plotted the ratio of observed to predicted cytoplasmic osmolality ($Osm_{cyto}^{obs}/Osm_{cyto}^{calc}$) as a function of $n_{H_2O}^{cyto}$ in Figure 5B. For *E. coli* grown in the presence and absence of osmoprotectants, $Osm_{cyto}^{obs}/Osm_{cyto}^{calc}$ decreases monotonically from unity when $n_{H_2O}^{cyto}$ is reduced to less than ~70 μmol/mg of DW and extends toward zero as free water is eliminated. This correlation indicates that the efficiency of cytoplasmic osmolytes as osmolality boosters decreases as the amount of cytoplasmic water (and the ratio of free to bound water) decreases. A likely explanation of this correlation is that local:bulk concentration ratios (partition coefficients) of cytoplasmic solutes increase under

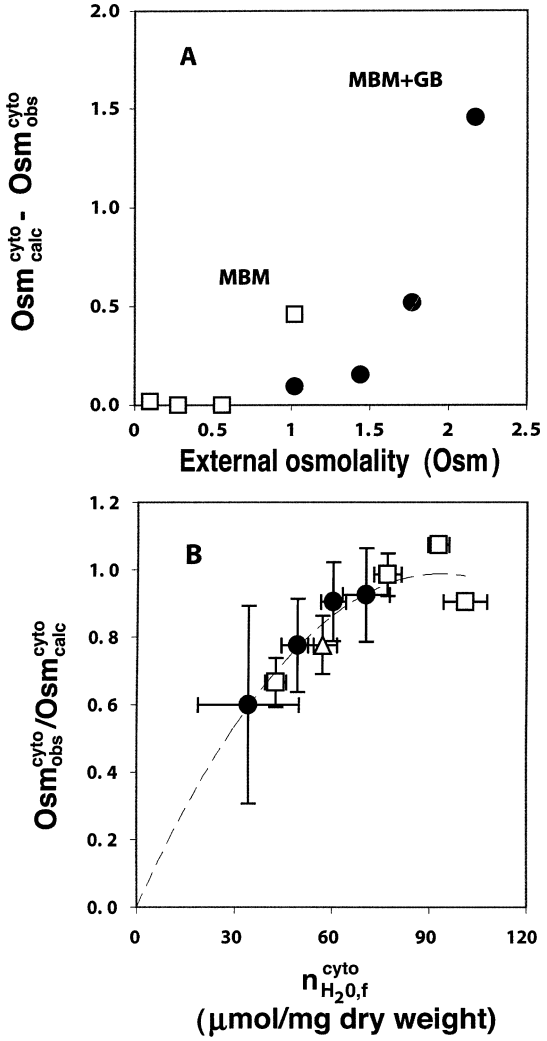

FIGURE 5: Comparison of observed (Osm^{obs}_{cyto}) and calculated (Osm^{calc}_{cyto}) cytoplasmic osmolalities. Panel A shows differences between values of Osm^{calc}_{cyto} and Osm^{obs}_{cyto} (from Table 3) as a function of growth osmolality, and panel B shows the $Osm^{calc}_{cyto}/Osm^{obs}_{cyto}$ ratio as a function of the amount of cytoplasmic water for cells grown in MBM (□), MBM with 1 mM Pro (△), and MBM+GB (●). Errors were obtained by propagating the uncertainties in the amounts of water and osmolytes.

conditions in which the solute concentration is high and where free water is not in great excess over local (bound) water. (We are currently examining whether a parallel situation occurs *in vitro*.) Other correlations between the amount of free water and osmotically regulated cell properties are introduced below.

Relationships between Cell Properties and the Amount of Cytoplasmic Water. (1) Correlation with Growth Rate. Both the amount of cytoplasmic water $n^{cyto}_{H_2O}$ and the growth rate of *E. coli* vary strongly with growth osmolality and with the presence or absence of osmoprotectants (Table 2 and Figure 1). A plot of growth rate versus $n^{cyto}_{H_2O}$ (Figure 6), comparing the data of this study for cells grown at high osmolality in MBM+GB with previously published data for cells grown at lower osmolalities in MBM and at 1.02 Osm in MBM with proline, extends and supports our previous finding (*47*) that these quantities are indeed correlated. As the amount of cytoplasmic water increases, the growth rate increases. From eq 7, $n^{cyto}_{H_2O}$ in growing cells at a given

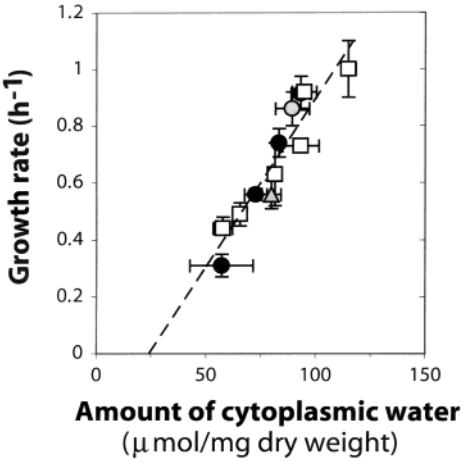

FIGURE 6: Empirical linkage between the growth rate and the amount of cytoplasmic water in osmotically stressed *E. coli*. Growth rates (h^{-1}) are plotted vs the corresponding amounts of cytoplasmic water $n^{cyto}_{H_2O}$ (in micromoles per milligram of DW) in cells grown in MBM [□ (data from ref *7*)], MBM+GB (●), and MBM+1 mM Pro [gray circle (*10*)] and MBM + 1 mM choline [gray triangle (*10*)] at various high osmolalities. The line is the weighted linear least-squares fit to the data.

growth osmolality is entirely determined by the amounts of cytoplasmic solutes and their osmotic nonideality (ϕ^o_j). The amounts of KGlu, MOPS, and trehalose that accumulated to increase the cytoplasmic osmolality of cells growing in MBM are incapable of maintaining an osmotic balance with the growth medium without a large reduction in the amount of cytoplasmic water, as compared to lower growth osmolality (*7*). Replacement of these solutes with GB or proline, each of which is observed to cause larger increases in osmolality than isomolal concentrations of KGlu or trehalose (*17*), allows the cytoplasm to retain more water for the same mole amounts of cytoplasmic solutes (Figure 4), which correlates (Figure 6) with an increase in growth rate. The question of how increasing $n^{cyto}_{H_2O}$ increases the growth rate is considered in Discussion.

(2) Correlation with Cytoplasmic Concentrations of Biopolymers and K^+. Table 2 shows that the contribution of protein and RNA to the cytoplasmic dry weight for *E. coli* K-12 grown in MBM is 0.55 ± 0.03 mg/mg of DW, independent of osmolality and osmoprotectant. The cytoplasmic biopolymer concentration ($C_{biopolymer}$) is calculated from this result using the relation for the total cytoplasmic volume of $V^{cyto}_{tot} = V^{cyto}_{H_2O} + V^{cyto}_{wi}$, where V^{cyto}_{wi} is the water-inaccessible cytoplasmic volume (see Materials and Methods) and $V^{cyto}_{H_2O} \cong n^{cyto}_{H_2O}/55.5$. For cells grown in MBM+GB above 1 Osm, we find $V^{cyto}_{wi} = 0.58 \pm 0.11$ μL/mg of DW, independent of growth osmolality and not significantly different than the osmolality-independent value of V^{cyto}_{wi} previously determined in *E. coli* K-12 cultured in MBM lacking betaine [0.50 ± 0.09 μL/mg of DW (*7*)]. Since the amount of biopolymers and the water-inaccessible cytoplasmic volume are independent of osmolality of growth, changes in $C_{biopolymer}$ result entirely from changes in the amount of cytoplasmic water. Figure 7A shows that $C_{biopolymer}$ decreases approximately linearly from ~330 to ~220 mg/mL as $n^{cyto}_{H_2O}$ increases from approximately 60 μmol/mg of DW [characteristic of cells grown at ~1.1 Osm in MBM and at ~2.1 Osm in MBM+GB (Table 2)] to approximately 110 μmol/mg of

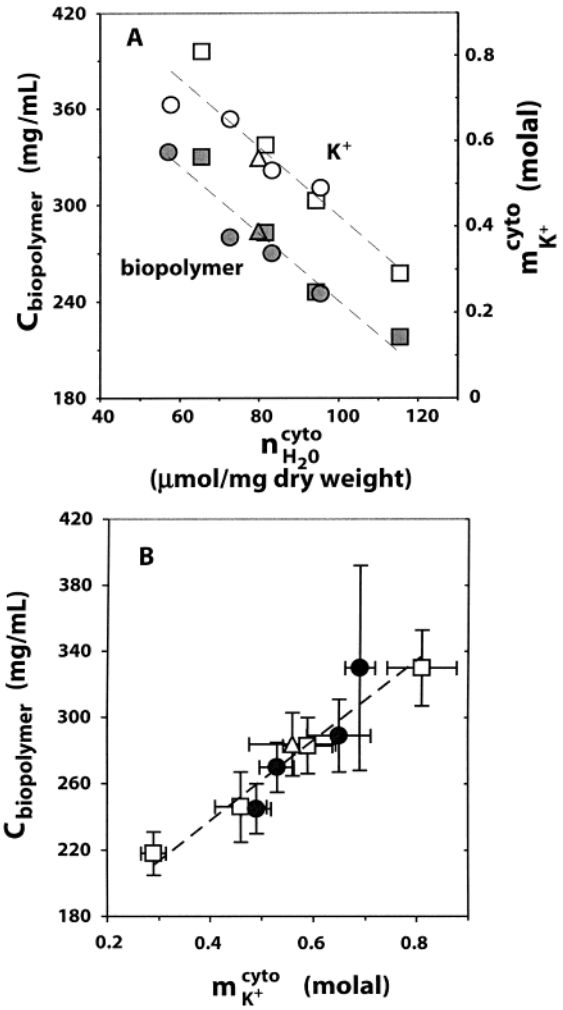

FIGURE 7: Correlations of cytoplasmic concentrations of K^+ and biopolymers and the amount of cytoplasmic water ($n^{cyto}_{H_2O}$) in *E. coli* grown at different osmolalities. In panel A, cytoplasmic biopolymer weight concentrations ($C_{biopolymer}$, filled symbols) and K^+ molalities ($m^{cyto}_{K^+}$, empty symbols) are plotted vs $n^{cyto}_{H_2O}$ for cells grown in MBM (□ and ■), MBM+GB (○ and ●), and MBM with Pro (△ and ▲). Values are the mean of at least three separate triplicate determinations. The lines shown are linear least-squares fits to the data. Panel B plots the cytoplasmic concentrations of K^+ vs the biopolymer concentrations from panel A. Uncertainties (omitted from panel A for clarity but typically ±10 and ±12% for biopolymer and K^+ concentrations, respectively) were propagated from the uncertainties in the amounts of cytoplasmic biopolymers, K^+, and water.

DW [characteristic of cells grown in MBM (and MBM+GB) at the optimal growth osmolality of ~0.3 Osm]. In addition, Figure 7A shows that the molal concentration of cytoplasmic K^+ ($m^{cyto}_{K^+}$) under all growth conditions is also described well as an approximately linear function of $n^{cyto}_{H_2O}$ with the same slope as the plot of $C_{biopolymer}$ versus $n^{cyto}_{H_2O}$. Figure 7B shows the resulting correlation of cytoplasmic concentrations of biopolymers and K^+, which we propose is fundamental to understanding the otherwise paradoxical lack of effect of changes in $m^{cyto}_{K^+}$ on protein−nucleic acid interactions (see Discussion).

DISCUSSION

Evidence for Two Domains (Local and Bulk) of Water in Protein Solutions and the Cytoplasm and for Partitioning of Osmolytes between These Domains. The cytoplasm of *E. coli*, in common with the cytoplasm of eukaryotic cells, protein crystals (*48*), and with both dilute and concentrated protein solutions (*17*, *35*, *49*), behaves thermodynamically as if it has two domains of water: bulk (free) water and local (bound) water of hydration of cytoplasmic biopolymers (*5*, *7*). The amount of bound (local domain) water ($n^{cyto}_{H_2O,b}$) in the cytoplasm of *E. coli* has been operationally defined as that which is not removed in a titration of nongrowing cells with NaCl, a solute which does not cross the cytoplasmic membrane: $n^{cyto}_{H_2O,b} \cong 22$ μmol/mg of DW, corresponding to a volume of bound cytoplasmic water of ~0.4 μL/mg of DW, independent of osmolality of growth up to at least 1 Osm (*13*). This amount of bound water corresponds to ~0.5 mg/mg of cytoplasmic biopolymer (*7*), which is within the range of estimates of amounts of water hydrating biopolymers *in vitro* (*15*, *16*). Significantly, when the amount of intracellular water in *E. coli* or other organisms is reduced to ≲0.4 μL/mg of DW, observed changes in physiology and/or physical properties of cells occur. For example, at relative humidities where the amount of cytoplasmic water is less than 0.4 μL of H_2O/mg of DW, the death rate of aerosolized *E. coli* increases greatly (*50*), a phenomenon attributed to the loss of bound water (*51*). In seeds containing less than 0.4 μL of H_2O/mg of dry weight, the cytoplasmic viscosity (determined from dielectric relaxation measurements of water) increases exponentially (*52*). Metabolic activity is greatly inhibited in Artemia cysts (*53*) and plant seeds (*54*) when the amount of water is reduced to ~0.3−0.6 μL/mg of DW. Finally, extrapolation of the weighted linear least-squares fit of the observed correlation between $n^{cyto}_{H_2O}$ and the growth rate of *E. coli* to zero growth rate (see Figure 6) yields an estimate of the minimum amount of cytoplasmic water required for growth (~0.5 μL/mg of DW) that is the same within error as $n^{cyto}_{H_2O,b}$.

Together, these observations indicate the utility of a simple two-domain model for cytoplasmic water: a local domain of water of hydration of the cytoplasmic biopolymer surface and a bulk domain. Solutes partition between these two domains; only the solute in the bulk domain contributes to osmolality. This simple two-domain model provides quantitative interpretations of NaCl titrations of *E. coli* (*7*, *10*, *13*), of thermodynamic properties of protein solutions (*17*), and of effects of osmolytes, denaturants, and other solutes on protein processes (*18*, *19*, *49*). Thermodynamic measurements on aqueous solutions of protein and solute, interpreted using the local bulk water model, show that local bulk partition coefficients K_p (cf. eq 5; *17*) for GB and other *E. coli* osmolytes and native protein are in the range of ~0.4−0.7 (Table 1), indicating local concentrations of all these osmolytes are significantly lower than in bulk solution. If the local domain is a monolayer of water at the surface of the protein, then the local concentration of GB near the protein surface is <50% as large as its bulk concentration. This strong exclusion from the vicinity of the native protein surface may rationalize the use by cells of these osmolytes to increase cytoplasmic osmolality (*55*).

Betaine as an Osmoprotectant: Deductions Drawn from Changes in the Amounts of Cytoplasmic Osmolytes and Water. Glycine betaine is more effective than other *E. coli* osmolytes in increasing osmolality (i.e., reducing H_2O

activity) in water, in concentrated protein solutions *in vitro* (ref *17* and Table 1), and in the cytoplasm of *E. coli* (ref *10* and this work). Addition of 1 mM GB to cells grown in MBM at 1 Osm causes the amount of cytoplasmic water ($n_{H_2O}^{cyto}$) to increase significantly for a constant total amount of cytoplasmic osmolytes $\sum n_j^{cyto}$ (Figure 3 and ref *13*). Addition of GB also increases the steady state cell volume of other osmotically stressed bacteria (*37*).

Since neither the amount of bound cytoplasmic water ($n_{H_2O,b}^{cyto}$) nor the turgor pressure ($\Delta\Pi$) of *E. coli* decreases upon uptake of GB (*10*, *13*), the observation that uptake of betaine increases $n_{H_2O}^{cyto}$ must originate from a very large increase (~45%) in the osmotic coefficient of the cytoplasm (ϕ_{cyto}). Although the osmotic coefficient of a two-component aqueous GB solution is relatively high [the osmolality of 1 *m* GB in water is ~10% higher than that of 1 *m* trehalose and ~30% higher than that of 0.5 *m* KGlu (*17*)], these differences in osmotic coefficients by themselves do not account for more than 25% of the observed increase in $n_{H_2O}^{cyto}$. Likewise, the somewhat greater exclusion of GB than of KGlu or trehalose from the local domain of water at the native protein surface (as exemplified by the solute−protein partition coefficients K_p in Table 1) cannot by itself explain the greatly different effects of these osmolytes on $n_{H_2O}^{cyto}$. Possibly *E. coli* osmolytes have greater differences in their interactions with other cytoplasmic surfaces (such as nucleic acids and membrane) than with the protein surface, although studies are needed to test this proposal.

For *E. coli* growing under conditions of moderate but not extreme osmotic stress, where the amount of cytoplasmic water is relatively large (e.g., in MBM at 0.56 Osm and in MBM+GB at 1 Osm), use of the local bulk domain model and *in vitro* data for the osmotic properties of *E. coli* solutes in water and in protein solutions, together with the assumption of additivity of contributions to cytoplasmic osmolality (eq 6), provides a reasonably accurate quantitative calculation of cytoplasmic osmolality (Figure 5A). Under conditions of more extreme osmotic stress, however, this calculation overestimates cytoplasmic osmolality to an extent that increases as the amount of free cytoplasmic water decreases (Figure 5B). GB and other cytoplasmic osmolytes therefore appear to become less completely excluded from biopolymer surface when the cytoplasmic ratio of bulk water to local water becomes small and the concentrations of osmolytes and biopolymers become very high.

In vitro, addition of GB favors self-assembly processes that remove the biopolymer surface from water, in proportion to the extent that GB is excluded from that surface; *in vitro* examples include formation of *lac* repressor−*lac* operator complexes (ref *56* and manuscript in preparation by J. Hong et al.) and protein folding (refs *55* and *57* and manuscript in preparation by D. J. Felitsky and M. T. Record, Jr.). *In vivo* examples of stabilizing effects on proteins brought about by the presence of GB include the observation that a conditional lysA mutant of *E. coli* is viable only when grown at moderate Osm_{ex} in the presence of GB (*58*), and that whereas dnaK deletion mutants of *E. coli* cultured in minimum medium at 42 °C are nonviable, their plating viability is partially restored if they are grown with GB (*59*). (Complicating this interpretation is the fact that other osmotically linked variables, including the concentrations of KGlu and biopolymers and the expression of osmotically regulated genes, also change upon uptake of betaine.)

Proposed Compensation between Perturbing Effects of Macromolecular Crowding and Cytoplasmic K^+ Concentration on Protein−Nucleic Acid Interactions. Macromolecular crowding affects the kinetics and equilibria of a wide range of processes. *In vitro*, crowding reduces biopolymer diffusion rates and thermodynamically drives biopolymer assembly processes (*60*−*63*). In *E. coli* grown in MBM+GB, the cytoplasmic concentration of proteins and nucleic acids increases from ~245 mg/mL at 1 Osm to ~330 mg/mL at 2.17 Osm (Figure 7). The crowding effect of such high biopolymer concentrations is thought to drive association of glycolytic (*64*) and PTS enzymes (*65*) and condensation of the nucleoid (*66*) in *E. coli*, and may contribute to folding the flagella synthesis regulatory protein flgM, which is intrinsically unstructured in dilute solution but gains structure in *E. coli* and in concentrated protein solutions (*67*). That decreasing $n_{H_2O}^{cyto}$ (i.e., increasing biopolymer concentration) actually increases the magnitude of the effects of macromolecular crowding *in vivo* is consistent with the observation that the stability of a monomeric lambda repressor variant in *E. coli* increases upon removal of cytoplasmic water by osmotic stress (*68*) because protein stability is thought to increase in crowded (*69*, *70*) and/or confined (*71*) environments. Changes in the cytoplasmic biopolymer concentration may also play a role in volume regulation of red blood cells (*72*) and in osmoregulation of the activity of transporter ProP in *E. coli* (*73*). Finally, the behavior of osmoremedial mutants (conditional lethal mutants that grow at high but not low osmolality) has been attributed to the increase in the effect of crowding on *in vivo* processes as $n_{H_2O}^{cyto}$ decreases with increasing osmolality (*7*).

We observe the same correlation of the concentrations of biopolymers and of K^+ in cells grown in MBM+GB at different osmolalities that we previously observed for cells grown in MBM (Figure 7B), consistent with our hypothesis (*7*, *47*) that perturbing effects of changes in crowding compensate for opposing effects of changes in K^+ concentration on the binding of proteins to DNA. Although the rate and extent of complex formation between *lac* repressor and *lac* operator DNA and between RNA polymerase and the λP_R promoter exhibit large negative power dependences on the concentration of K^+ *in vitro* (*74*, *75*), changes in external osmolality have no effect on the normalized levels of *lac* repression or of expression of λP_R *in vivo* for cells grown in the absence of betaine (*43*), despite the large changes in the concentration and activity of cytoplasmic K^+ (*7*). More generally, relative strengths of *in vivo* binding of most transcription-regulating proteins to DNA are apparently unaffected by changes in cytoplasmic K^+ concentration since the relative levels of expression of most mRNAs in *E. coli* are unaffected by increasing osmolality (*76*). We proposed that the apparent insensitivity of protein−nucleic acid interactions to increasing KGlu concentrations *in vivo* occurs because concomitant increases in biopolymer concentration (i.e., increased crowding nonideality) with increasing growth osmolality provide an offsetting favorable thermodynamic contribution to binding (*7*, *47*). And, although the change in the thermodynamic activity of K^+ (and not K^+ concentration *per se*) should be the fundamental thermodynamic variable

for describing the effect of changing salt concentration on the binding of proteins to DNA *in vivo*, the activity coefficient of cytoplasmic K^+ is likely to depend primarily on its own concentration and on nucleic acid concentration, and not on GB concentration. The universal linkage of the cytoplasmic concentrations of biopolymers and K^+ shown in Figure 7B therefore supports our proposal that they have offsetting effects on protein−DNA interactions *in vivo*.

Since betaine does not affect the linkage between cytoplasmic concentrations of K^+ and biopolymers, we conclude that the *in vivo* effect of increasing the betaine concentration on the affinity of proteins for DNA is small relative to the effects of increasing crowding or K^+ concentration. In support of this conclusion, we note that the increase in crowding as growth osmolality increases from 0.1 to 1 Osm is predicted to increase the activity coefficient of RNA polymerase in *E. coli* by ∼1000-fold (*7*), and the activity coefficient of *lac* repressor is predicted to be more than 1000-fold higher in the cytoplasm of *E. coli* than in dilute solution (*32*). Likewise, reducing the KGlu concentration from 0.6 to 0.2 M increases the *in vitro lac* repressor−*lac* operator equilibrium binding constant ∼500-fold (*75*). In contrast to these large perturbing effects of changes in the concentration of K^+ and biopolymers, studies of several protein−DNA interactions (including binding of the *lac* repressor to the *lac* operator) have shown that addition of betaine at concentrations of up to 2 M increases the binding constant by less than 1 order of magnitude (refs *56* and *77*−*79* and manuscript in preparation by J. Hong, et al.

Proposed Roles of Macromolecular Crowding and Betaine in Affecting the Growth Rate of Osmotically Stressed Cells. Why does the growth rate of *E. coli* decrease with increasing osmolality? Existing proposals have considered the energetic cost of osmolyte accumulation and the inhibitory effect of increasing cytoplasmic K^+ concentration. Alternatively, we propose that the amount of free cytoplasmic water is the fundamental determinant of growth rate in osmotically stressed cells, based on the universal correlation between $n_{H_2O}^{cyto}$ and the growth rate in osmotically stressed *E. coli* for all conditions we have examined (Figure 6). [Notably, growth rate and cell volume are correlated for osmotically stressed yeast cells (*44*).] In this proposal, the accumulation of betaine by osmotically stressed cells increases the growth rate by increasing the amount of free cytoplasmic water.

Why does a reduction in $n_{H_2O}^{cyto}$ decrease the growth rate? The increase in crowding that must accompany a reduction in the amount of cytoplasmic water could thermodynamically drive aggregation of cytoplasmic biopolymers into nonfunctional assemblies [as with crowding-induced formation of inactive tetramers from active subunits of glyceraldehyde-3-phosphate dehydrogenase *in vitro* (*60*)]. Alternatively, since biopolymer diffusion coefficients decrease strongly with increasing macromolecule concentration (*80*, *81*) and the rate of association of biopolymers can become diffusion-limited in sufficiently crowded solutions (*60*), the increase in crowding with increasing growth osmolality could reduce the growth rate if the rate of some essential process requiring diffusion is diffusion-limited (*47*). Since crowding generally reduces diffusion rates of large proteins more than those of small proteins *in vitro* (*80*) and *in vivo* (*61*, *82*, *83*), any growth rate-limiting diffusional process most likely would involve large biopolymers. For example, while the diffusion coefficient of green fluorescent protein (GFP, 27.5 kDa) is 11-fold slower in *E. coli* than in dilute solution, tetrameric β-galactosidase fused to GFP (∼540 kDa) does not diffuse at a detectable rate in *E. coli* (*82*). Even diffusion of the small fluorescent probe BCECF (623 Da) is greatly affected by changes in cell volume of eukaryotic fibroblasts (*84*).

Since diffusion coefficients of proteins vary exponentially with protein concentration at moderate concentrations *in vitro* (and vary more steeply than exponentially at higher concentrations) (*81*, *85*), our proposal that processes requiring diffusion of cytoplasmic biopolymers may limit the growth rate predicts that growth rate will vary exponentially (or more strongly) with cytoplasmic biopolymer concentration. Figure 6 reveals an approximately linear correlation between growth rate and biopolymer concentration. However, a plot of the logarithm of the growth rate versus biopolymer concentration for the data of Figure 6 (not shown) is equally linear with a similar correlation coefficient as the linear fit to Figure 6 ($R^2 = 0.86$ vs $R^2 = 0.89$). This finding is consistent with the proposal that the kinetics of some biopolymer diffusional process(es) limit the growth rate in the highly concentrated cytoplasm of *E. coli* grown at high osmolality.

The high bioenergetic cost of osmolyte accumulation has also been proposed to explain why increasing growth osmolality decreases the growth rate of *E. coli* (see references in ref *8*). However, osmotic stress may not place a large bioenergetic burden on *E. coli* because growth rate and cell yield are not correlated in glucose-limited batch cultures grown at different osmolalities in minimal medium with betaine (*8*). Another measure of the bioenergetic cost of synthesis of glutamate and trehalose from glucose is provided by the number of moles of ATP consumed per mole of osmolyte synthesized calculated on the basis of known biosynthetic pathways (*86*−*88*). Table 4 compares these ATP costs in *E. coli* with those of transport of K^+ and betaine (based on literature studies of energy requirements of

Table 4: ATP Requirements [micromoles of ATP consumed (+) or formed (−) per micromole of osmolyte] for the Accumulation of Osmolytes by *E. coli* Grown Aerobically in Glucose Minimal Medium

osmolyte	glucose used	ATP equivalents[a]	NAD(P)H[b]	total ATP
trehalose[c]	2	3	0	3
glutamate[d]	1	−1	−3	−7
K^+, MOPS or betaine[e]	0	2	0	2

[a] ATP equivalents includes all molecules with high-energy phosphate bonds (i.e., UTP, phosphoenolpyruvate, etc.). [b] The reducing power of each NADH and NAD(P)H is sufficient to produce two ATP molecules in *E. coli* (*87*). [c] Trehalose synthesis requires formation of UDP-glucose from UTP and Glu-6-P, which condenses with Glu-1-P to form trehalose (*99*). [d] Values show the requirements for the overall synthesis of glutamate from glucose and NH_4^+ catalyzed by glutamate dehydrogenase in the reaction α-ketoglutarate + NH_4^+ + H^+ + NADPH → glutamate + H_2O + $NADP^+$, where the anapleuorotic synthesis of α-ketoglutarate requires one glucose and produces one ATP equivalent and four NAD(P)H molecules (*87*). Accumulation of ammonia occurs by facilitated diffusion via the Amt transport protein and requires no energy (*100*) for our growth conditions. [e] These values apply for K^+ accumulated by the high-affinity Kdp transport system and betaine (and MOPS) accumulated by the ProU transport system of *E. coli*, both members of the ABC transport family, which we assume hydrolyzes two ATP molecules to ADP and P for each substrate molecule that is transported (*101*, *102*).

osmolyte transport systems; see the footnotes of Table 4). By this approach, we calculate that trehalose synthesis requires more ATP than transport of K^+ or betaine, which in turn requires more ATP than glutamate synthesis (Table 4). From these calculations, we conclude that accumulation of osmolytes consumes more ATP in *E. coli* grown at 1.02 Osm in MBM+GB (~2.9 μmol of ATP equivalents/mg of DW) than in MBM (~1.9 μmol of ATP equivalents/mg of DW). By this measure, the bioenergetic cost of osmolyte accumulation is not a primary determinant of the growth rate of osmotically stressed cells.

Deleterious effects of high cytoplasmic concentrations of K^+ have also been proposed as the fundamental origin of the reduction in the growth rate of *E. coli* at high osmolality (*89*, *90*). Indeed, we find that K^+ concentration and growth rate are correlated in *E. coli* for all conditions we have examined, since concentrations of K^+ and growth rates are both linearly correlated with the amount of cytoplasmic water (Figures 6 and 7). Furthermore, the extent of binding of *E. coli* proteins to DNA and activities of some plant and animal enzymes decrease strongly with increasing NaCl concentration in dilute solution (*75*, *91*, *92*). However, these observations are not universal characteristics of *E. coli* enzymes; a limited survey revealed none with a steep dependence of enzyme activity on NaCl concentration *in vitro* (*93*). Moreover, since salt affects the activity of some enzymes by inducing dissociation into subunits, changes in crowding *in vivo* may reduce the magnitude of the effect of changes in salt concentration on enzyme activity, just as proposed for binding of proteins to DNA. For example, adding high concentrations of macromolecular crowding agents reduces the extent of NaCl-induced dissociation of active oligomers of plant phosphoenolpyruvate carboxylase into inactive subunits (*94*). Furthermore, activities of enzymes *in vitro* are typically more sensitive to the nature and concentration of the anion than the cation of 1-1 salts (see, for example, ref *95*), and the position of chloride in the Hofmeister series indicates it is generally more inhibitory than glutamate (*74*). For example, the activity of *E. coli* trehalose-phosphate synthase at high K^+ concentrations is significantly increased by replacement of chloride with glutamate (*96*). Of other *E. coli* proteins, only activities of DNA binding proteins have been systematically investigated as a function of KGlu concentration. In general, although a large increase in the strength of DNA binding is observed upon replacement of KCl or NaCl with KGlu at a constant salt concentration, the level of binding nevertheless decreases strongly with increasing KGlu concentrations (*74*, *75*). However, the effect of increasing KGlu concentrations on protein−DNA interactions in *E. coli* may well be compensated by the concomitant increase in crowding, as discussed above. Thus, our working hypothesis is that the preference of *E. coli* for betaine over K^+ as an osmolyte is not primarily to relieve K^+ inhibition of cytoplasmic processes but reflects the ability of replacement of K^+ by betaine to increase the amount of cytoplasmic water, and consequently the growth rate.

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