Origins of the Osmoprotective Properties of Betaine and Proline in *Escherichia coli* K-12

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The amounts of cytoplasmic water and of all osmotically significant cytoplasmic solutes were determined for *Escherichia coli* K-12 grown in 3-(*N*-morpholino)propane sulfonate (MOPS)-buffered glucose-minimal medium containing 0.5 M NaCl in the presence and absence of the osmoprotectants betaine and proline. The goal of this work is to correlate the effects of osmoprotectants on the composition of the cytoplasm with their ability to increase the growth rate of osmotically stressed cells. At a concentration of 1 mM in the growth medium, betaine increases the growth rate more than does proline; choline, which is converted to betaine by *E. coli*, appears to have an intermediate effect on growth rate. The accumulation of either betaine or proline reduces the cytoplasmic amounts of K⁺, glutamate, trehalose, and MOPS (the major cytoplasmic osmolytes accumulated in the absence of osmoprotectants), so that at this external osmolarity the total amount of cytoplasmic solutes is essentially the same in the presence or absence of either osmoprotectant. More betaine than proline is accumulated, so the extent of replacement of cytoplasmic solutes is greater for betaine than for proline. Accumulation of these osmoprotectants is accompanied by a large (20 to 50%) increase in the volume of cytoplasmic water per unit of cell dry weight ($\bar{V}_{\text{cyto}}$). This effect, which appears to result from an increase in the volume of free water, $\bar{V}_f$ (as opposed to water of hydration, or bound water), is greater for betaine than for proline. Taken together, these results indicate that the molar effects of betaine and proline on water activity and on the osmotic pressure of the cytoplasm must be significantly larger than those of the solutes they replace. Cayley et al. (S. Cayley, B. A. Lewis, H. J. Guttman, and M. T. Record, Jr., *J. Mol. Biol.* 222:281–300, 1991) observed that, in cells grown in the absence of osmoprotectants, both growth rate and $\bar{V}_{\text{cyto}}$ decreased, whereas the amount of cytoplasmic K⁺ (n_{K^+}) increased, with increasing external osmolarity. We predicted that the observed changes in n_{K^+} and $\bar{V}_{\text{cyto}}$ would have large and approximately compensating effects on key protein-nucleic acid interactions of gene expression, and we proposed that $\bar{V}_f$ was the fundamental determinant of growth rate in osmotically stressed cells. The properties of cells cultured in the presence of betaine and proline appear completely consistent with our previous work and proposals. Accumulation of betaine and, to a lesser extent, proline shifts the set of linked physiological parameters (n_{K^+} , $\bar{V}_{\text{cyto}}$, growth rate) to those characteristic of growth at lower osmolarity in the absence of osmoprotectants. Models for the thermodynamic basis and physiological consequences of the effect of osmoprotectants on $\bar{V}_{\text{cyto}}$ and $\bar{V}_f$ are discussed.

Living cells adapt to osmotic stress by varying the intracellular concentration (i.e., thermodynamic activity) of osmotically active solutes (osmolytes). A variety of organisms accumulate the so-called osmoprotectants betaine and proline to prevent dehydration when placed under the stress of high-osmolarity environments (16, 22, 43, 45, 52). In *Escherichia coli* and *Salmonella typhimurium* grown in minimal medium, potassium is the primary osmolyte accumulated under conditions of osmotic stress. When betaine or proline is present in the growth medium, however, large amounts of the solute are transported into the cytoplasm by the osmotically regulated *proU* and *proP* gene products (reviewed in reference 16). The addition of low (≤ 1 mM) concentrations of betaine to the growth medium raises the upper limit of external osmolarity at which growth occurs (13), reduces the amount of time required for growth to resume after osmotic upshift (48), and increases the growth rate of osmotically stressed *E. coli* and *S. typhimurium* (36). The addition of proline causes qualitatively similar but less dramatic effects (16).

The solutes accumulated in the cytoplasm of *E. coli* K-12 strain MG1655 cultured in minimal medium of high osmolar-

ity buffered with 3-(*N*-morpholino)propanesulfonate (MOPS) are K⁺, glutamate, trehalose, and MOPS (12). The cytoplasmic amounts of each of these species are reduced by the accumulation of betaine (5, 11, 25, 41, 48). The mechanism by which the accumulation of betaine or proline increases the growth rate of cells subjected to osmotic stress is not understood, however. A variety of noncovalent interactions, especially those involving proteins and nucleic acids (38), and the activities of many enzymes (37, 51, 52) are adversely affected by increases in the concentration of salts in vitro. In contrast, the in vitro activities of many enzymes are largely independent of the concentrations of the compatible solutes betaine and proline (37, 43, 52). Since the accumulation of betaine and proline reduces the amount of K⁺ in growing cells, Sutherland et al. (48) proposed that accumulation of betaine may reduce the potentially deleterious effects of high concentrations of cytoplasmic K⁺ on macromolecules. Other mechanisms have also been considered. For example, Schoberte (42) proposed that a direct interaction of proline with proteins might mitigate the inhibitory consequences of cytoplasmic dehydration caused by osmotic stress.

Recently we measured the amounts of osmotically regulated solutes and water in the cytoplasm of *E. coli* grown in MOPS-buffered minimal media (MBM) (11) of various osmolarities (12). These data, together with the results of plasm-

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lysis (osmotic shrinking) experiments, led to the conclusion that the osmotic coefficient of the cytoplasm of cells grown at low to moderate osmolarities is quite low ($\phi \approx 0.5$ to 0.7). This osmotic nonideality of the cytoplasm was attributed in large part to coulombic interactions of potassium ions (K^+) with nucleic acids. Calculations based on polyelectrolyte theory confirmed the feasibility of this interpretation.

In the present paper, we extend these studies to an examination of the osmotic properties of the cytoplasm of *E. coli* K-12 grown in MBM-0.5 M NaCl (MBMS) supplemented with the osmoprotectant proline or betaine. (The presence of a supplement to MBM or MBMS is denoted by a dash and the concentration of the supplement.) We determined the effects of the accumulation of betaine or proline on the amount and properties of cytoplasmic water and solutes in *E. coli* K-12, and we use these data to discuss the ability of osmoprotectants to increase the growth rate of osmotically stressed cells.

MATERIALS AND METHODS

Strains, media, radiochemicals, and buffers. Cultures of *E. coli* K-12 strain MG1655 were grown aerobically at 37°C in MBMS. The osmolarity of MBMS was determined by using a Wescor 5500 vapor pressure osmometer to be 1.02 ± 0.01 osM before and after growth of cells. When proline, choline, or betaine was added, the extracellular concentration was 1 mM unless stated otherwise.

$[^{14}\text{C}]$ betaine (10 mCi/mmol) was a generous gift from L. Csonka. $[^3\text{H}]$ proline (35.2 Ci/mmol), $[^{14}\text{C}]$ choline chloride (56 mCi/mmol), $^3\text{H}_2\text{O}$ (1 mCi/g), $[^{14}\text{C}]$ taurine (115 mCi/mmol), $[^{14}\text{C}]$ sucrose (620 mCi/mmol), and $[^{14}\text{C}]$ inulin (11 mCi/mmol) were obtained from Dupont. ^{14}C -labeled taurine, sucrose, and inulin were purified by pretreatment with cells as described in Cayley et al. (12). $[^{14}\text{C}]$ taurine was also purified by high-performance liquid chromatography with a semipreparative column of Spherisorb SAX ($5\mu\text{m}$; Phase Separations Inc., Norwalk, Conn.) and distilled, deionized water as the eluent.

The wash buffer (isotonic with the growth medium) and plasmolysis buffers lacked added K^+ and glucose and were prepared as previously described (12). The reported osmolarities $[\phi_{\text{ex}}(\Sigma C_j)_{\text{ex}}]$ of wash and plasmolysis buffers were calculated by using osmotic coefficients for solutions of NaCl determined by Robinson and Stokes (40). The calculated osmolarities agreed within ± 0.01 osM with determinations performed by osmometry on representative samples of these buffers.

Analytical methods. (i) **Measurement of cellular protein and dry weight.** The amounts of protein and the dry weights of cell samples were determined as previously described (12).

(ii) **Measurement of cytoplasmic osmolytes by ^{13}C and ^{14}N NMR spectroscopy.** The amounts of organic cytoplasmic solutes accumulated by *E. coli* grown in MBMS-1 mM proline and in MBMS-1 mM betaine were quantified by using ^{13}C nuclear magnetic resonance (NMR). Cells were grown to the midlog phase, harvested by centrifugation, and analyzed by natural-abundance ^{13}C NMR spectroscopy as previously described (11). The amount of betaine accumulated by cells grown in MBMS-1 mM betaine was also determined by ^{14}N NMR spectroscopy as described by Lewis et al. (27). The limit of detection of solutes by these methods is $\sim 0.03 \mu\text{mol/mg}$ of dry weight.

(iii) **Measurement of cytoplasmic potassium.** The amount of cytoplasmic K^+ in fresh cell slurries was determined by atomic absorption spectroscopy. Cells were grown in

MBMS-1 mM proline or MBMS-1 mM betaine to the midlog phase of growth, harvested by centrifugation, and prepared for atomic absorption spectroscopy as described by Richey et al. (39).

(iv) **Radiochemical measurement of cytoplasmic betaine.** The amount of betaine accumulated in cells grown in MBMS containing $[^{14}\text{C}]$ betaine was determined by two methods; the results were the same within the error in either method. The measurements reported below combine the results obtained by both methods.

In the first method, the removal of labeled betaine from the growth medium was measured by a method similar to that of Landfald and Strøm (24). For this procedure, a stationary-phase (overnight) culture was diluted $\sim 1:10^4$ with MBMS containing 0.20 mM $[^{14}\text{C}]$ betaine (0.05 to 0.1 mCi/mmol) and grown aerobically to an A_{550} of 0.5. Triplicate 1-ml samples were then spun for 45 s at $11,000 \times g$ in a Beckman microfuge. Then 100 μl of the supernatant from each sample was placed in a vial containing 5 ml of scintillation fluid, and the counts per minute in each sample were determined with a Beckman LS8000 scintillation counter. The amount of betaine accumulated by cells, n_{bet} (in micromoles per milligram of cell dry weight), was then calculated (25):

$$n_{\text{bet}} = [\text{bet}](1 - C_{\text{SN}}/C_m)/W \quad (1)$$

where C_{SN} is the specific activity of betaine in the supernatant (in counts per minute per milliliter), C_m is the specific activity of the medium, W is the dry weight of cells (milligrams per milliliter of culture at the time of harvest), and $[\text{bet}]$ is the initial concentration of betaine in the medium (0.2 mM).

The amount of betaine accumulated in cells grown in MBMS containing 0.2 mM $[^{14}\text{C}]$ choline (0.05 to 0.1 mCi/mmol) was also determined by this method. (Choline is a precursor of betaine accumulated in *E. coli* by osmotically regulated transport systems distinct from those mediating the accumulation of proline and betaine [47].)

The amount of intracellular betaine was also measured in cells harvested by filtration. Triplicate 1-ml samples of cells cultured as described above were filtered through prewetted 0.45- μm -pore-size Millipore HAWP filters, immediately rinsed with 2 ml of betaine-free medium warmed to 37°C , placed in vials containing 5 ml of scintillant, and counted with a Beckman LS8000 scintillation counter. The amount of intracellular betaine (micromoles per milliliter of culture) was determined by comparison of the counts per minute in the sample to the specific activity of the betaine in the medium. The amount of cytoplasmic betaine (micromoles per milligram of dry weight) was determined by reference to the amount of dry weight in parallel, nonradioactive cultures of the same A_{550} .

(v) **Amounts of cytoplasmic amino acids.** The steady-state amounts of cytoplasmic amino acids were determined as previously described (39). Cells were grown in MBMS or MBMS-1 mM proline to the midlog phase (A_{550} of ~ 0.5). Duplicate 5-ml samples of culture were filtered through prewetted 0.45- μm -pore-size Millipore HAWP filters and then immediately rinsed with 2 ml of wash buffer warmed to 37°C . The filters were then transferred to glass vials and extracted with 70% ethanol for 30 min at room temperature. Aliquots of the ethanol extract were then processed and assayed for the amount of amino acids with a Durrum D500 amino acid analyzer as previously described (39).

(vi) **Measurement of the volume of cytoplasmic and cellular water.** The steady-state volume of cytoplasmic water per

TABLE 1. Amounts of cytoplasmic osmolytes in cells grown in the absence and presence of proline or betaine^a

Osmolyte	Amt (μmol/mg [dry wt], mean ± SD) of osmolyte in cells grown in:		
	MBMS ^b	MBMS-1 mM proline	MBMS-1 mM betaine
K ⁺	0.95 ± 0.08	0.81 ± 0.12	0.76 ± 0.09
Glutamate	0.23 ± 0.03	0.21 ± 0.07	0.08 ± 0.03
Trehalose	0.36 ± 0.03	0.15 ± 0.01	≤0.02
MOPS	0.25 ± 0.03	0.07 ± 0.02	≤0.02
Proline	≤0.02	0.69 ± 0.03	≤0.02
Betaine	≤0.02	≤0.02	1.17 ± 0.04
Total	1.79 ± 0.10	1.93 ± 0.14	2.01 ± 0.10

^a Values are the means of three or more separate measurements. When more than one method was used to quantitate an osmolyte (Table 2), the tabulated values are the means of the amounts obtained by the different methods. The error in the total amount $[(\sum \eta_i)_{\text{cyto}}]$ was determined from the errors in the amounts of the individual osmolytes.

^b Data from Cayley et al. (12).

unit cell dry weight ($\bar{V}_{\text{cyto}}$) and the volume of cellular water per unit cell dry weight ($\bar{V}_{\text{cell}}$) of unplasmolyzed cells were measured by the method of Stock et al. (44) as described previously (12). $^3\text{H}_2\text{O}$ and [^{14}C]taurine were used to measure $\bar{V}_{\text{cyto}}$; $^3\text{H}_2\text{O}$ and [^{14}C]inulin were used to measure $\bar{V}_{\text{cell}}$. A volume of 1.5 liters of cells was grown to the midlog phase in MBMS containing 1 mM proline, 1 mM choline, or 1 mM betaine and harvested by centrifugation for 7 min at $7,000 \times g$ at room temperature. The cell pellets were washed by resuspending them in 200 ml of wash buffer and centrifuging them again. The washed pellets were resuspended in fresh wash buffer to a density of ~10 mg of dry cell weight per ml of slurry for determinations of $\bar{V}_{\text{cyto}}$ and $\bar{V}_{\text{cell}}$. Controls were performed in the presence and absence of betaine at 0.5 M NaCl to demonstrate that values of $\bar{V}_{\text{cyto}}$ determined with [^{14}C]taurine and [^{14}C]sucrose were the same within error, and that the $\bar{V}_{\text{cyto}}$ in taurine was independent of time of incubation with the radiolabel, in the range from 1 to 30 min.

For measurement of the $\bar{V}_{\text{cyto}}$ of hyperosmotically shocked cells, cells grown in MBMS-1 mM betaine were prepared as described above, except that the final density of the cells was ~60 mg (dry weight) per ml of slurry. Aliquots were then diluted sixfold into plasmolysis buffer (wash buffer containing from 0.575 to 2.4 M NaCl), such that the diluted cell samples contained ~10 mg (dry cell weight) per ml and from 0.56 to 2.0 M NaCl. The $\bar{V}_{\text{cyto}}$ of the plasmolyzed cells was then promptly measured with $^3\text{H}_2\text{O}$ and [^{14}C]taurine as previously described (12).

RESULTS

Amounts of cytoplasmic solutes in cells grown in the presence and absence of proline or betaine. When *E. coli* K-12 strain MG1655 is grown in MBM or MBM-0.1 M NaCl, K⁺ is the primary cytoplasmic solute (12). For cells grown in MBMS, K⁺ remains the most abundant cytoplasmic solute, but significant quantities of other osmotically regulated cytoplasmic solutes (trehalose, glutamate, and MOPS) are present. In cells grown at this external osmolarity (1.02 osM), the cytoplasmic amount of each organic solute is about three- to fourfold less than that of K⁺ (Table 1).

The amounts of all of the major cytoplasmic osmolytes are significantly altered when cells cultured in MBMS are supplemented with the osmoprotectant proline or betaine at 1 mM. In cells grown in MBMS-1 mM proline, K⁺ is still the most abundant solute, although there is less of it than in cells grown in MBMS (Table 1). The amount of cytoplasmic proline is nearly as great as that of K⁺. Although trehalose, MOPS, and glutamate are all detectable in cells grown in MBMS-1 mM proline, the amounts of trehalose and MOPS are significantly less than those in cells grown in MBMS. This result confirms the finding of Dinnbier et al. (17) that the accumulation of proline reduces the steady-state amount of trehalose in osmotically stressed cells.

For cells grown in MBMS-1 mM betaine, betaine is the most abundant cytoplasmic solute. The only other organic solute observed by ^{13}C NMR spectroscopy in these cells was a relatively small amount of glutamate. The intracellular synthesis of trehalose and the transport of exogenous MOPS by *E. coli* are therefore fully repressed by the accumulation of betaine, in agreement with previous results (11, 25, 41).

Although the composition of the pool of cytoplasmic osmolytes in MBMS-grown cells is significantly altered by the accumulation of osmoprotectants, the total amount of osmolytes (in micromoles per milligram of dry weight) is only slightly affected (Table 1). The amount of each osmoprotectant accumulated by cells grown in MBMS is nearly balanced by an equivalent reduction in the steady-state amounts of the other osmolytes relative to those in unsupplemented cultures.

To increase our confidence in the accuracy of our measurements of cytoplasmic solutes, we determined the amounts of glutamate, proline, and betaine by several different methods (Table 2). The amount of cytoplasmic betaine accumulated by cells grown in MBMS-1 mM betaine was determined by using ^{13}C NMR spectroscopy, ^{14}N NMR spectroscopy, and the two radiochemical techniques described in Materials and Methods. These methods differ in the analytical methods of quantification and the procedures

TABLE 2. Amounts of cytoplasmic osmolytes determined with different techniques^a

Medium supplement ^b	Osmolyte assayed	Amt (μmol/mg [dry wt], mean ± SD) of osmolyte as determined with:				
		Radiochemistry	Amino acid analysis	^{13}C NMR	^{14}N NMR	Avg
None	Glutamate		0.24 ± 0.03	0.22 ± 0.03		0.23 ± 0.03
Proline	Glutamate		0.26 ± 0.03	0.16 ± 0.02		0.21 ± 0.07
Proline	Proline		0.67 ± 0.07	0.71 ± 0.06		0.69 ± 0.03
Choline	Betaine ^c	0.79 ± 0.11		0.91 ± 0.10 ^d	0.94 ± 0.04 ^d	0.88 ± 0.08
Betaine	Betaine	1.16 ± 0.10		1.21 ± 0.04	1.13 ± 0.04	1.17 ± 0.04

^a Values are the means of two or more separate determinations.

^b Cells were grown in MBMS with the indicated supplements at 1 mM.

^c Choline is completely converted into betaine in the cytoplasm of osmotically stressed cells (24).

^d Results of a single experiment.

used to prepare cell samples. For example, the procedure to prepare samples for analysis by NMR includes two anaerobic centrifugation steps and takes about 45 min to complete. In contrast, cells were harvested aerobically by rapid filtration (requiring 5 to 10 s to complete) for one radiochemical method. Likewise, the amounts of cytoplasmic glutamate and proline were measured by both amino acid analysis (where cells were harvested by filtration) and ^{13}C NMR spectroscopy. In all but one case, the amount of each osmolyte detected by different techniques agreed within the uncertainty limits ($\sim\pm 10\%$) of each assay (Table 2). The amount of glutamate in cells grown in MBMS–1 mM proline measured by amino acid analysis and ^{13}C NMR spectroscopy differed by $0.10\ \mu\text{mol/mg}$ (dry weight), which exceeds the combined experimental uncertainties of $0.05\ \mu\text{mol/mg}$ (dry weight). However, the magnitude of this single discrepancy is a relatively small fraction ($\sim 5\%$) of the total amount of cytoplasmic solutes in cell grown in MBMS–1 mM proline. We therefore feel that the uncertainties reported in Table 1 are reasonable and that the uncertainty in the total amount of cytoplasmic osmolytes is probably less than $\pm 10\%$. The agreement of the results obtained by NMR spectroscopy and the other methods also suggests that the measured amounts of organic osmolytes are independent of the method used to harvest cells. A similar observation was made by Nakajima et al. (35), who found that the amounts of cytoplasmic K^+ determined in cells harvested by filtration or by centrifugation are identical.

Accumulation of osmoprotectants increases $\bar{V}_{\text{cyto}}$ and the growth rate of cells grown in MBMS. The steady-state $\bar{V}_{\text{cyto}}$ of cells grown in MBMS–1 mM osmoprotectant is higher than that of cells grown in MBMS alone (cf. Fig. 1 and Table 3). For cells grown in MBMS containing 1 mM betaine, $\bar{V}_{\text{cyto}}$ is $\sim 48\%$ larger than that in unsupplemented cultures and $\sim 22\%$ larger than that in cultures supplemented with 1 mM proline. Because $\bar{V}_{\text{cyto}}$ is larger in the presence of betaine than in the presence of proline, the total analytical concentration [$(\sum n_i)_{\text{cyto}}/\bar{V}_{\text{cyto}}$, a free-volume molar or approximate molal concentration in moles of solute per liter of cytoplasmic water-accessible volume] of solutes in cultures supplemented with betaine ($1.15 \pm 0.08\ \text{mol liter}^{-1}$) is significantly less than that in proline-supplemented cultures ($1.34 \pm 0.12\ \text{mol liter}^{-1}$), which is in turn less than that in unsupplemented cultures ($1.52 \pm 0.11\ \text{mol liter}^{-1}$). Because the thermodynamic nonideality of intracellular solutes is significant, however, cytoplasmic concentrations of solutes cannot be used directly to calculate the osmotic properties of the cell interior (see Discussion) (12).

McLaggan and Epstein (30) recently showed that *E. coli* accumulates taurine via the *proP* and *proU* transport systems after a shift of growing cells to high osmolarity with aeration in the presence of glucose. Milner et al. (32) showed that glucose was required for the function of the *proP* transport system. Because our cell slurries lack a carbon source and are not aerated, significant transport of taurine is unlikely to occur under our conditions. This expectation is confirmed by control experiments, which demonstrate that values of $\bar{V}_{\text{cyto}}$ determined with sucrose and taurine as the probes are the same within uncertainty of either experiment (data not shown). No effect of the nature of the probe solute is observed in MBMS with or without 1 mM betaine. In addition, we find that values of $\bar{V}_{\text{cyto}}$ measured with taurine for cells grown in MBMS with or without 1 mM betaine are constant from 1 to 30 min after the addition of the radiochemical (data not shown), demonstrating that taurine is not significantly accumulated by cells under the experimental

conditions employed here. This finding does not contradict the conclusion of McLaggan and Epstein (30) that taurine is not a suitable tracer in transport and bioenergetic determinations at high osmolarity, since these studies are usually performed on aerated cells in the presence of a carbon source.

As indicated by the term osmoprotectant, accumulation of cytoplasmic betaine or proline increases the growth rate of osmotically stressed *S. typhimurium* and *E. coli* (15, 16, 36). Values of $\bar{V}_{\text{cyto}}$ for *E. coli* K-12 grown in MBMS containing betaine or proline are plotted versus the growth rate of those cells in Fig. 1, which also includes the best linear fit to the data of Cayley et al. (12) for osmotically stressed cells grown in MBM–NaCl without osmoprotectants. Also included in Fig. 1 are data for cells grown in MBMS–1 mM choline. Choline is completely converted into betaine in the cell (24), but the amount of betaine accumulated in the cytoplasm in this strain of *E. coli* is less with choline as a precursor than when betaine is supplied directly in the medium (Table 2). For this case, both the growth rate and $\bar{V}_{\text{cyto}}$ appear to be intermediate between those obtained for cells grown in MBMS in the presence of betaine and those for cells grown in MBMS in the presence of proline.

For cells grown in MBM with added NaCl in the absence or presence of osmoprotectants, there appears to be a universal linear relationship between $\bar{V}_{\text{cyto}}$ and the growth rate over the range of osmolarities of the growth media of 0.3 to 1.1 osM. The growth rate and $\bar{V}_{\text{cyto}}$ of cells grown in MBMS–1 mM betaine are about the same as those of cells grown in MBM– $\sim 0.22\ \text{M}$ NaCl, whereas the growth rate and $\bar{V}_{\text{cyto}}$ of cells grown in MBMS–1 mM proline are comparable to those of cells grown in MBM– $\sim 0.4\ \text{M}$ NaCl. As we previously observed (12), extrapolation of the data in Fig. 1 to zero growth rate yields an estimate of the minimum volume of cytoplasmic water required for growth of approximately $0.5 \pm 0.2\ \mu\text{L/mg}$ (dry weight).

The $\bar{V}_{\text{cell}}$ in cells grown in MBMS–1 mM betaine ($2.32 \pm 0.17\ \mu\text{L/mg}$ [dry weight]), measured by the method of Stock et al. (44) with ^{14}C inulin and $^3\text{H}_2\text{O}$, is also significantly larger than the $\bar{V}_{\text{cell}}$ of cells grown in MBMS (1.87 ± 0.12

FIG. 1. Relationship between $\bar{V}_{\text{cyto}}$ and the growth rate of osmotically stressed *E. coli*. The $\bar{V}_{\text{cyto}}$ of cells grown in MBMS alone ($\circ$) or in MBMS with 1 mM proline ($\square$), 1 mM choline ($\diamond$), or 1 mM betaine ($\triangle$) are plotted as a function of growth rate (in generations per hour). Each value is the mean ± 1 standard deviation of 3 to 10 separate measurements. The solid line is the weighted linear least-squares fit to corresponding data from reference 12 for cells grown in MBM containing 0.2 to 0.55 M NaCl.

FIG. 2. The relationship between $\bar{V}_{\text{cyto}}$ and the amount of cytoplasmic potassium in osmotically stressed cells (n_{K^+}) is plotted as a function of $\bar{V}_{\text{cyto}}$ of cells grown in MBMS with 1 mM betaine (Δ) or 1 mM proline ($\square$). The solid line is the linear least-squares fit to corresponding data from reference 12 for cells grown in MBM containing up to 0.5 M NaCl ($\circ$).

$\mu\text{L/mg}$ [dry weight]). However, the volume of water in the periplasm ($\bar{V}_{\text{peri}} = \bar{V}_{\text{cell}} - \bar{V}_{\text{cyto}}$) of cells cultured in the presence of betaine ($0.59 \pm 0.20 \mu\text{L/mg}$ [dry weight]) is the same within error as that in unsupplemented cultures ($0.69 \pm 0.13 \mu\text{L/mg}$ [dry weight]).

Effect of the accumulation of osmoprotectants on the relationship between $\bar{V}_{\text{cyto}}$ and n_{K^+} in osmotically stressed cells. It has previously been reported for *E. coli* and *S. typhimurium* that, as the concentration of NaCl in the growth medium is increased, $\bar{V}_{\text{cyto}}$ decreases (12, 25, 39) and the amount of cytoplasmic K^+ (n_{K^+}) increases (12, 16, 18, 48). These two trends lead to an apparently linear relationship with a negative slope (Fig. 2). (The solid line represents the fit to data from Cayley et al. [12].) Also shown in Fig. 2 are the corresponding points for cells grown in MBMS-1 mM betaine and MBMS-1 mM proline. It is striking that the effect of the accumulation of osmoprotectants on $\bar{V}_{\text{cyto}}$ and n_{K^+} is indistinguishable within error from the effect of varying the external osmolarity in the absence of osmoprotectants. The physiological parameters $\bar{V}_{\text{cyto}}$, n_{K^+} , and growth rate therefore appear to be linked, so that they change concertedly in response to changes in external osmolarity or the accumulation of osmoprotectants.

Plasmolysis titrations of cells grown in MBMS-1 mM betaine. Cayley et al. (12) used measurements of $\bar{V}_{\text{cyto}}$ during plasmolysis titrations with NaCl-based plasmolysis buffers to obtain information about the thermodynamic state of cytoplasmic solutes and water in cells grown in minimal media of low to moderate osmolarity. For the present investigation, we performed similar measurements on cells grown in MBMS-1 mM betaine and then resuspended in hypertonic plasmolysis buffers with added NaCl. The resuspension conditions were designed to ensure that plasmolyzed cells cannot accumulate solutes. Consequently, $\bar{V}_{\text{cyto}}$ decreases as the osmolarity of the plasmolysis buffer increases.

The osmotic coefficient of the cytoplasm, $\phi_{(ii)}$, is calculated from the slope of a plot of $\bar{V}_{\text{cyto}}$ as a function of the reciprocal osmolarity of the plasmolysis buffers (see Fig. 3) (12):

$$\bar{V}_{\text{cyto}} = \bar{V}_b + \phi_{(ii)}(\Sigma n_j)_{\text{cyto}}[\phi_{\text{ex}}(\Sigma C_j)_{\text{ex}}]^{-1} \quad (2)$$

where $(\Sigma n_j)_{\text{cyto}}$ is the total amount of cytoplasmic solutes, ϕ_{ex} is the external osmotic coefficient, and $(\Sigma C_j)_{\text{ex}}$ is the concentration of solutes in the plasmolysis buffer. The volume (per unit dry weight) of bound or osmotically inactive water, $\bar{V}_b$, in the cytoplasm is obtained from the intercept of this type of plot. The quantity $\phi_{(ii)}$ is the osmotic coefficient of the cytoplasm defined with respect to a reference state in which all solutes are assumed to be randomly mixed in the volume of free cytoplasmic water ($\bar{V}_f = \bar{V}_{\text{cyto}} - \bar{V}_b$) and excluded from the volume $\bar{V}_b$. In this reference state, the total amount of each solute would contribute to the ideal osmotic pressure (12). Thus, $\phi_{(ii)}$ reflects the nonideality of cytoplasmic solutes including effects of their accumulation into $\bar{V}_b$ or their nonrandom mixing in $\bar{V}_f$. Figure 3 shows that $\bar{V}_{\text{cyto}}$ (in microliters per milligram of dry weight) of cells grown in MBMS-1 mM betaine and suspended in hypertonic plasmolysis buffer of variable osmolarity varies linearly with the reciprocal of the external osmolarity $\{[\phi_{\text{ex}}(\Sigma C_j)_{\text{ex}}]^{-1}\}$. The intercept of the weighted linear least-squares fit of the data ($\bar{V}_b$) is $0.40 \pm 0.05 \mu\text{L/mg}$ (dry weight), and the slope $[\phi_{(ii)}(\Sigma n_j)_{\text{cyto}}]$ is $1.39 \pm 0.09 \mu\text{mol/mg}$ (dry weight) (Table 3). The linearity of this plot implies that $\phi_{(ii)}$ and $\bar{V}_b$ are independent of the extent of plasmolysis, as noted previously (12) for cells grown without added osmoprotectant at lower osmolarities.

To interpret the intercept of Fig. 3 as $\bar{V}_b$ and the slope as $\phi_{(ii)}(\Sigma n_j)_{\text{cyto}}$, the amount of cytoplasmic osmolytes $(\Sigma n_j)_{\text{cyto}}$ must be independent of the extent of plasmolysis (19, 20). Since hyperosmotic shock increases the rate of uptake and the amounts of cytoplasmic K^+ (17, 18) and betaine (9, 10, 32), cell samples were washed and assayed in buffers lacking added K^+ and betaine to prevent the accumulation of these solutes by plasmolyzed cells. Glucose was also omitted from the wash buffer and plasmolysis buffers to prevent cells from synthesizing intracellular trehalose or glutamate. As a control to verify that these steps were sufficient to prevent plasmolyzed cells from accumulating osmolytes, the $\bar{V}_{\text{cyto}}$ of cells diluted into plasmolysis buffer containing 1 M NaCl was

FIG. 3. Plot of $\bar{V}_{\text{cyto}}$ of cells grown in MBMS-1 mM betaine versus reciprocal osmolarity of the plasmolysis buffer. Cells were plasmolyzed by dilution into plasmolysis buffer (wash buffer containing a final concentration of 0.56 to 2.0 M NaCl). $\bar{V}_{\text{cyto}}$ was measured as described in Materials and Methods. Each point represents the mean ± 1 standard deviation of 6 to 12 separate determinations made on two to four independent cultures. Values of $1/\phi_{\text{ex}}(\Sigma C_j)_{\text{ex}}$ were calculated from the composition of the plasmolysis buffers as described in the text and checked by direct measurement. The line is the weighted linear least-squares fit to the individual determinations of $\bar{V}_{\text{cyto}}$.

TABLE 3. Volumes of water and osmotic coefficients in the cytoplasm of *E. coli* grown at high osmolarity^a

Growth medium	Mean $\pm$ SD					
	$\bar{V}_{\text{cyto}}$	$\bar{V}_b$	$\bar{V}_f$	$(\Sigma n_i)_{\text{cyto}}^b$	$\phi_{(ii)}(\Sigma n_i)_{\text{cyto}}$	$\phi_{(ii)}$
MBMS	1.18 $\pm$ 0.06	(0.40 $\pm$ 0.04) ^c	0.78 $\pm$ 0.07	1.79 $\pm$ 0.10	0.89 $\pm$ 0.08 ^d	$\sim$ 0.50 $\pm$ 0.05
MBMS-1 mM proline	1.44 $\pm$ 0.08	(0.40 $\pm$ 0.04) ^c	1.04 $\pm$ 0.09	1.93 $\pm$ 0.14	1.18 $\pm$ 0.10 ^d	$\sim$ 0.61 $\pm$ 0.07
MBMS-1 mM betaine	1.75 $\pm$ 0.08	0.40 $\pm$ 0.05 ^e	1.35 $\pm$ 0.09	2.01 $\pm$ 0.10	1.39 $\pm$ 0.09 ^f	0.69 $\pm$ 0.06

^a Values are from three or more individual cultures, each determined in triplicate. Volumes are measured in microliters per milligram (dry weight). $\bar{V}_b$ and $\phi_{(ii)}$ are defined by equation 2.

^b Data are from Table 1.

^c Assumed value (weighted average from reference 12).

^d Estimated from $\bar{V}_{\text{cyto}}$ for a turgor pressure ($\Delta\pi$) of ca. 3.0 atm, a $\bar{V}_b$ of 0.40 $\pm$ 0.04 $\mu\text{l}/\text{mg}$ (dry weight), a T of 310 K, and an R of 0.0821 liters atm per mol per K.

^e From the y intercept of Fig. 3.

^f From the slope of Fig. 3.

measured at different times after dilution. We found that the $\bar{V}_{\text{cyto}}$ of these cells did not change significantly as a function of time after plasmolysis for at least 20 min (data not shown), the longest time tested. Since an increase in $(\Sigma n_i)_{\text{cyto}}$ should lead to an increase in the $\bar{V}_{\text{cyto}}$ of plasmolyzed cells, this result indicates that $(\Sigma n_i)_{\text{cyto}}$ is unaffected by plasmolysis and reinforces the interpretation of the linear fit in Fig. 3 that $\bar{V}_b$ and $\phi_{(ii)}$ are constant over the range of osmolarities of the plasmolysis titration.

The value of $\bar{V}_b$ in cells grown in MBMS containing 1 mM betaine (0.40 $\pm$ 0.05 $\mu\text{l}/\text{mg}$ [dry weight]) is the same, within error, as that measured in cells grown in the low-osmolarity media MBM and MBM-0.1 M NaCl (0.42 $\pm$ 0.06 and 0.38 $\pm$ 0.07 $\mu\text{l}/\text{mg}$ [dry weight], respectively [12]). The amount of bound water in the cytoplasm therefore appears to be independent of the osmolarity of the growth medium and thus independent of the type and amount of cytoplasmic solutes. Because $\bar{V}_{\text{cyto}}$ is greatly increased by the accumulation of betaine while $\bar{V}_b$ apparently remains constant, the percentage of cytoplasmic water that is bound decreases from $\sim$ 34% for cells grown in MBMS in the absence of exogenous osmoprotectants to $\sim$ 22% for cells grown in MBMS-1 mM betaine. The ratio $\bar{V}_f/\bar{V}_b$ increases from $\sim$ 2.0 to $\sim$ 3.4.

DISCUSSION

Effects of accumulation of osmoprotectants on the amounts of osmotically regulated cytoplasmic solutes. Cayley et al. (12) showed that K^+ , MOPS, trehalose, and glutamate are the only solutes that accumulate to osmotically significant levels in the cytoplasm of *E. coli* K-12 grown in MBMS. Other than proline and betaine, these are also the only solutes detected with ^{13}C NMR spectroscopy, ^{14}N NMR spectroscopy, and amino acid analysis in *E. coli* K-12 grown in MBMS containing osmoprotectants (Table 1) (27). Furthermore, trehalose and glutamate are the only organic solutes (other than proline or betaine) that have been detected in extracts of a variety of *E. coli* strains grown in minimal medium of high osmolarity containing proline or betaine by using high-performance liquid chromatography (31), gas-liquid chromatography (41), and proton NMR (25, 41). Nevertheless, these results cannot formally exclude the possibility that the accumulation of betaine increases the accumulation of inorganic osmolytes such as the anions chloride and sulfate. This possibility, however, is unlikely for several reasons. First, accumulation of betaine generally inhibits the induction of other mechanisms by which *E. coli* may adapt to osmotic stress. The present work demonstrates that the accumula-

tion of betaine reduces the amount of MOPS and K^+ transported from the medium into the cytoplasm and reduces the amount of glutamate and trehalose synthesized in the cytoplasm of cells grown in MBMS. The high levels of expression of osmotically regulated genes in cells grown in media of high osmolarity are reduced by the accumulation of betaine (16). Moreover, in cells cultured in the absence of betaine, the cytoplasmic amounts of inorganic ions other than K^+ appear to be independent of external osmolarity (12). Therefore, it seems unlikely that the accumulation of betaine could be accompanied by significant accumulation of inorganic anions. Second, the charge imbalance between K^+ and negatively charged MOPS and glutamate is unaffected by the accumulation of osmoprotectants (Table 1). Although the amounts (per unit of cell dry weight) of K^+ , glutamate, and MOPS are reduced in cells grown in MBMS-1 mM betaine relative to cells grown in MBMS, the excess cationic (+) charge of osmolytes in these media is the same within error: 0.54 $\pm$ 0.09 $\mu\text{mol}(+)/\text{mg}$ (dry weight) in cells grown in MBMS, 0.53 $\pm$ 0.14 $\mu\text{mol}(+)/\text{mg}$ (dry weight) in cells grown in MBMS-1 mM proline, and 0.68 $\pm$ 0.08 $\mu\text{mol}/\text{mg}$ (dry weight) in cells grown in MBMS-1 mM betaine. (These calculations assume that cytoplasmic MOPS is 70% anionic [11].) The amounts of Mg^{2+} and nucleic acid, the other major ionic species in the cytoplasm, also do not appear to vary with external osmolarity (12, 23, 49). The invariance of the excess cationic charge of osmotically regulated cytoplasmic solutes with accumulation of osmoprotectants argues against a variation in the cytoplasmic amounts of inorganic ions other than K^+ . We therefore assume that Table 1 is a complete list of the osmolyte species accumulated in significant amounts by *E. coli* K-12 strain MG1655 grown in MBMS in the presence of osmoprotectants.

Physiology of osmoprotection by betaine and proline. Comparison of the amounts of cytoplasmic solutes ($n_{i,\text{cyto}}$; Table 1), $\bar{V}_{\text{cyto}}$ (Table 3), and growth rates (Fig. 1) yields a very striking observation: the increase in growth rate that results from accumulation of the osmoprotectants betaine and proline and the accompanying changes in n_{K^+} and $\bar{V}_{\text{cyto}}$ are the same as those obtained by a reduction in osmolarity in the absence of an osmoprotectant (12) (Fig. 2). Apparently, growth rate, n_{K^+} , and $\bar{V}_{\text{cyto}}$ are a linked set of parameters in cells grown under the stress of high-osmolarity environments. What is the fundamental significance of this relationship between $\bar{V}_{\text{cyto}}$, growth rate, and n_{K^+} ? What parameter most fundamentally determines growth rate as a function of osmolarity? Most proposals regarding the mechanism of osmoprotection of proline and betaine in *E. coli* have focused either on purported beneficial influences of the osmo-

protectants on cellular macromolecules or deleterious effects of K^+ that are mitigated by replacement of K^+ by the osmoprotectants. In the following discussion, we first review several of these previous suggestions and then introduce an alternative. This alternative proposal is consistent with our previous hypothesis (12) that changes in K^+ activity (a_{K^+}) and $\bar{V}_{cyto}$ as a function of external osmolarity (in cells grown in the absence of osmoprotectants) are coupled in order to achieve homeostasis of gene expression by compensation between effects of a_{K^+} and macromolecular crowding on protein-nucleic acid interactions. The compensation between a_{K^+} and $\bar{V}_{cyto}$ in cells grown at high osmolarity occurs at the expense of $\bar{V}_f$. We previously proposed that $\bar{V}_f$ is fundamentally correlated with growth rate in osmotically stressed cells (12). Here we extend that proposal to the situation where osmoprotectants are present, and we show that this fundamental correlation is also consistent with the effects of the accumulation of osmoprotectants on osmotically stressed cells.

(i) **Previous proposals on mechanisms of osmoprotection.** Cairney et al. (9, 10) and Milner et al. (32) studied the kinetics of the transport of proline and betaine into the cytoplasm of *E. coli*. They found that, although both proline and betaine are substrates of the *proU* and *proP* transport systems, betaine is the preferred substrate of at least the *proU* system. Based on these results, Milner et al. (32) proposed that *E. coli* should accumulate more betaine than proline. Our measurements directly confirm this proposal. We find that the growth rate and the amount of osmoprotectant (in micromoles per milligram of dry weight) in cells grown in MBMS-1 mM betaine are greater than those of cells grown in MBMS-1 mM proline. Furthermore, for cells grown in the presence of choline, both the growth rate and the amount of osmoprotectant accumulated are intermediate between those of cells grown with proline and betaine (Table 2, Fig. 1). Larsen et al. (25) observed that γ -butyrobetaine is less effective than proline betaine in increasing the growth rate of osmotically stressed *E. coli* and that cells accumulate less γ -butyrobetaine than proline betaine. Hence, it appears that the growth rate of osmotically stressed cells is directly related to the amount of osmoprotectant accumulated. Our measurements suggest that this is not principally a direct favorable effect of the osmoprotectant, but rather that accumulation of an osmoprotectant leads to a reduction in other cytoplasmic solutes (K^+ , trehalose, glutamate, MOPS) and an increase in $\bar{V}_{cyto}$. The question of whether intrinsic differences in the osmoprotective properties of betaine and proline exist is not addressed by the present data.

High concentrations of betaine stabilize the native structure of proteins in vitro against thermal and chemical denaturation (3, 52) and can reverse the inhibitory effects of high concentrations of salt or urea on the enzymatic activity of proteins in vitro (29, 36, 52). The fact that cells that accumulate high concentrations of the protein denaturant urea also accumulate compensatory amounts of protein-stabilizing methylamines like betaine (4, 6, 52) suggests that betaine may stabilize proteins against chemical denaturation in vivo as well. Furthermore, concentrations of proline and betaine up to 1 M do not inhibit the enzymatic activity of many proteins in vitro (37, 43, 52). Hence, the accumulation of high cytoplasmic concentrations of these osmoprotectants should be compatible (8) with enzyme function in vivo.

Since the accumulation of betaine reduces the amount of cytoplasmic K^+ in *E. coli*, Sutherland et al. (48) proposed that the accumulation of betaine stimulates the growth rate of osmotically stressed cells by reducing the potentially

deleterious effects of high concentrations of cytoplasmic K^+ on enzymatic activity. In particular, any increase in K^+ concentration in vitro dramatically reduces the affinity of most proteins for DNA (38). High concentrations of K^+ inhibit the activities of many enzymes (37, 51, 52). Because Figures 1 and 2 show that n_{K^+} and the growth rate are linearly related under all conditions studied here, the present data cannot eliminate cytoplasmic K^+ concentration as the primary regulator of growth rate. However we have proposed that, as the osmolarity of the growth medium increases, the potentially deleterious effect of increasing K^+ concentration or, more precisely, a_{K^+} on specific protein-DNA interactions in vivo is compensated for by the effect of the increased concentration of macromolecules (and therefore increased macromolecular crowding) resulting from the decrease in $\bar{V}_{cyto}$ (12). If crowding and a_{K^+} exert balanced effects on gene expression, then a_{K^+} appears unlikely to be the fundamental determinant of growth rate. In general, osmotic stress reduces the growth rate of many plant and microbial species, even though a number of these organisms appear to utilize solutes other than K^+ as their primary cytoplasmic osmolyte (22, 52). Thus, the potentially inhibitory effects of increases in the concentration of cytoplasmic K^+ cannot provide a general explanation of the growth-inhibitory effect of osmotic stress on most organisms.

Schoberte (42) proposed that the osmoprotective properties of proline result from a direct interaction between nonpolar surfaces of proline and of cytoplasmic proteins. In this model, amphiphilic proline forms bridges between hydrophobic patches of the protein and bulk water, effectively reducing the hydrophobicity of the surface of cytoplasmic proteins and increasing their solubility. However, several lines of reasoning suggest that proline may not function in this way in osmotically stressed *E. coli* (16). For example, Arakawa and Timasheff have shown a net exclusion of proline from the vicinity of proteins in vitro (3). Furthermore, the correspondence between the quantitative results obtained by NMR and by analytical methods for betaine, proline, and glutamate (Table 2) implies that none of these solutes is tightly site-bound to cytoplasmic macromolecules. Estimates of $(\sum n_j)_{cyto}$ and $\phi_{(ii)}$ for cells grown in MBMS and MBMS-1 mM proline (see below) also suggest that proline is more excluded from the surface of cytoplasmic molecules than are the solutes present in cells grown in MBMS alone. It is therefore likely that proline does not significantly associate with proteins in the cytoplasm of *E. coli* under the conditions we have examined. In addition, NMR relaxation studies suggest that betaine and trehalose do not interact significantly with macromolecules in the cytoplasm of cells grown in MBMS or MBMS-1 mM betaine (27). It is possible, however, that proline associates with some proteins under conditions of very low water activity. Similarly, trehalose is thought to substitute for water under conditions of extreme dehydration, binding specifically to the polar groups of dry phospholipids (14, 26). Whether proline and trehalose bind to dehydrated membranes or proteins of *E. coli* remains to be examined, however.

(ii) **Accumulation of betaine may increase the growth rate of *E. coli* by increasing the volume of cytoplasmic water.** Cayley et al. (12) observed that the growth rate and $\bar{V}_{cyto}$ are linearly related in hyperosmotically stressed *E. coli* K-12 grown in MBM-NaCl without osmoprotectants. Figure 1 demonstrates that this linear relationship between $\bar{V}_{cyto}$ and the growth rate is generally applicable to cells cultured in the presence and absence of osmoprotectants. (It should be noted that the relationship between $\bar{V}_{cyto}$ and the growth rate

reported here is restricted to the specific case of *E. coli* growing under hyperosmotic stress in MOPS-buffered minimal medium with glucose as the carbon source [12].) We therefore propose that $\bar{V}_{\text{cyto}}$ (or, more specifically, $\bar{V}_f$) is the fundamental determinant of growth rate in cells subjected to hyperosmotic stress, and that the increase in $\bar{V}_f$ that accompanies the accumulation of betaine or proline is the key to the mechanism of their osmoprotection in *E. coli*. In support of this model, growth appears to cease where $\bar{V}_f$ approaches zero (Fig. 1) (12).

In our model, the price paid by the cell for using K^+ as an osmolyte is the concomitant decrease in $\bar{V}_f$ (and therefore in growth rate) that apparently compensates for the deleterious effects of increases in $[K^+]$ on gene expression. The reduction in a_{K^+} that accompanies accumulation of betaine is coupled to an increase in $\bar{V}_f$, so that the equilibria and kinetics of protein-nucleic acid interactions are approximately independent of external osmolarity. At present, the mechanism by which *E. coli* balances a_{K^+} and $\bar{V}_f$ is unknown. To our knowledge, the occurrence (or absence) of similar phenomena in other organisms has not been examined.

Why is the amount or volume of free water the fundamental determinant of growth rate in osmotically stressed cells? The nonspecific aggregation of proteins or other biopolymers into nonfunctional assemblies, driven by macromolecular crowding, may be an important factor limiting the growth rate with decreasing $\bar{V}_f$. Alternatively, one might invoke the direct consequences of the reduction in the amount of free water for cytoplasmic processes. Since water is a stoichiometric participant in many covalent and noncovalent cellular processes, with an apparently large stoichiometry in the case of macromolecular noncovalent interactions (38), it is possible that changes in the amount of free water affect growth rate by their effect on the equilibria and/or kinetics of those processes. However, cytoplasmic water activity is the fundamental thermodynamic variable of relevance for equilibrium and kinetic analyses. If turgor pressure does not change significantly with external osmolarity or the addition of an osmoprotectant, then the intracellular water activity must change in parallel with the extracellular water activity, assuming the molar volume of intracellular water is unaffected. Since the addition of millimolar concentrations of osmoprotectants does not affect the extracellular water activity, it appears unlikely that the effect of osmoprotectants on $\bar{V}_f$ and on the growth rate is the result of changes in intracellular water activity. Other possible ways in which $\bar{V}_f$ may control growth rate include effects of macromolecular crowding on rates of diffusion of proteins and metabolites through bulk cytoplasmic water. Both theoretical and experimental studies (33, 34) have shown that the rate of diffusion of proteins is significantly lower in crowded solutions of macromolecules than in dilute solutions. For example, Muramatsu and Minton (34) measured the diffusion of several tracer proteins in solutions containing up to 200 mg of background species of proteins per ml. They found that the rate of diffusion of proteins decreased dramatically as the concentration of background protein increased, and they concluded that the rate of diffusion of macromolecules through the concentrated cytoplasm of living cells may be several orders of magnitude slower than that in a dilute solution. That this finding is relevant *in vivo* is suggested by the observation of Brass et al. (7), who found that the lateral diffusion coefficient of fluorescently labeled proteins in the periplasm of *E. coli* was 0.1% of that measured in water. Accordingly, the rate of bulk (i.e., three-dimensional) diffusion of proteins may be sufficiently retarded in the crowded

cytoplasm of osmotically stressed *E. coli* that the rate of assembly of quaternary structures (e.g., multienzyme complexes) may limit the growth rate at a low $\bar{V}_f$.

Thermodynamic analysis of the effect of accumulation of betaine on the volume of cytoplasmic water ($\bar{V}_{\text{cyto}}$). The generalization of equation 2 to the situation where a growing cell maintains the osmotic pressure difference (turgor pressure $[\Delta\pi]$) across its boundary is

$$\bar{V}_{\text{cyto}} = \frac{\phi_{(ii)}(\Sigma n_j)_{\text{cyto}}}{\frac{\Delta\pi}{RT} + \phi_{\text{ex}}(\Sigma C_j)_{\text{ex}}} + \bar{V}_b \quad (3)$$

where R is the gas constant and T is absolute temperature. For cells grown in MBMS, the addition of 1 mM betaine does not significantly change $\phi_{\text{ex}}(\Sigma C_j)_{\text{ex}}$. Four possible thermodynamic sources of the 50% increase in $\bar{V}_{\text{cyto}}$ that accompanies the accumulation of betaine by cells grown in MBMS may therefore be formulated from equation 3: (i) the amount of osmotically active cytoplasmic solutes $(\Sigma n_j)_{\text{cyto}}$ might increase; (ii) $\Delta\pi$ might decrease; (iii) $\bar{V}_b$ might increase; and (iv) $\phi_{(ii)}$ might increase, i.e., betaine might be significantly more ideal (in the thermodynamic sense) than the solutes it replaces (trehalose, MOPS, and some K^+ and glutamate). Combinations of these effects would also of course be possible.

The quantitative studies presented in Table 1 show that explanation i makes at most a small contribution to the effect of the accumulation of betaine on $\bar{V}_{\text{cyto}}$, since accumulation of betaine increases $(\Sigma n_j)_{\text{cyto}}$ by only 12%, a marginally significant change which is far less than the 70% increase required to produce the observed increase in $\bar{V}_{\text{cyto}}$. Explanation ii cannot contribute significantly, because the turgor pressure of *E. coli* grown at low osmolarity is only ~3 atm (ca. 300 kPa) (44), whereas a decrease in turgor pressure of ~10 atm (ca. 1,000 kPa) upon addition of betaine to cells cultured in MBMS would be required to explain the change in $\bar{V}_{\text{cyto}}$. Also, a variety of indirect observations suggest that the accumulation of betaine may actually increase turgor pressure (5, 21, 46).

We cannot formally rule out explanation iii, since we have not yet measured $\bar{V}_b$ in cells grown in MBMS lacking betaine. The limiting case of $\bar{V}_b = 0$ for cells grown in MBMS without betaine, together with the slight changes in $(\Sigma n_j)_{\text{cyto}}$ that we report here, could in principle account for most of the reduction in $\bar{V}_{\text{cyto}}$ of osmotically stressed cells cultured in the absence of betaine. However, we believe this possibility to be quite unlikely because the values of $\bar{V}_b$ measured in cells under three different osmotic conditions are all the same within error (cf. reference 12 and Table 3).

By this process of elimination, we conclude that $\phi_{(ii)}$ is the thermodynamic parameter that changes most upon accumulation of osmoprotectants. In other words, the molar effects of betaine and proline on water activity and on the osmotic pressure of the cytoplasm are significantly larger than those of the solutes that they replace. The slope of the plasmolysis plot for cells grown with betaine (Fig. 3) is equal to the product $\phi_{(ii)\text{bet}}(\Sigma n_j)_{\text{cyto}}$. Table 3 gives this value, along with the predicted values of the slopes of the plots for cells grown in MBMS alone (where trehalose is the primary organic osmolyte) and in MBMS–1 mM proline. These latter quantities were calculated from equation 3 by using measured values of $\bar{V}_{\text{cyto}}$ and assuming that the turgor pressure is 3 atm and that $\bar{V}_b$ is 0.40 $\mu\text{l/mg}$ (dry weight) under all conditions. Using the values of $(\Sigma n_j)_{\text{cyto}}$ from Table 1, we estimate the

following osmotic coefficients from these slopes: $\phi_{(ii)\text{bet}} \equiv 0.69 \pm 0.06$, $\phi_{(ii)\text{pro}} \equiv 0.61 \pm 0.07$, and $\phi_{(ii)\text{MBMS}} \equiv 0.50 \pm 0.05$.

In vivo osmotic coefficients may be determined by the extent of exclusion of osmolytes and osmoprotectants from bound water. The thermodynamic analysis described above (equation 3) is based on a reference state for the osmotic coefficient $\phi_{(ii)}$ in which the cytoplasmic solutes are completely excluded from $\bar{V}_b$. We interpret $\bar{V}_b$ as the volume of water of hydration of cytoplasmic macromolecules, membrane constituents, and solutes (12). Our choice of reference state is justified by the observation that the osmolytes accumulated by *E. coli* are preferentially excluded from the water of hydration of proteins in aqueous solution (1–3, 28). By this definition, then, any accumulation of solutes into $\bar{V}_b$ gives rise to osmotic nonideality [$\phi_{(ii)} < 1$].

For cells grown in MBMS (1.02 osM), $\bar{V}_b$ is a large fraction (~34%) of $\bar{V}_{\text{cyto}}$ (Table 3). Therefore, changes in the preferential exclusion of a solute from water of hydration in the cytoplasm will significantly affect its concentration in the volume of free water. Differences in the degree of exclusion from $\bar{V}_b$ of betaine or proline on the one hand and the solutes which they replace (i.e., trehalose, K^+ , glutamate, and MOPS) on the other are thus a plausible explanation for the differences in the apparent values of $\phi_{(ii)}$. This explanation is entirely consistent with the in vitro results of Arakawa and Timasheff (reviewed by Timasheff [50]), whose direct measurements of preferential interaction coefficients have demonstrated a high degree of preferential exclusion of betaine and other osmoprotectants from the water of protein hydration. Based on the results presented in this paper, we therefore propose that betaine and proline are excluded from $\bar{V}_b$ to a greater extent than are trehalose, K^+ , MOPS, and/or glutamate in the cytoplasm of *E. coli*.

Concluding comments. The work presented here represents the first simultaneous measurement of the steady-state amounts of all osmotically significant cytoplasmic solutes and water in *E. coli* K-12 grown at high osmolarity in the presence and absence of the osmoprotectant betaine or proline. The addition of 1 mM betaine to a culture growing in 1.02 osM MBMS results in extensive accumulation of betaine and a dramatic increase in growth rate. The accumulation of betaine is accompanied by replacement of trehalose, MOPS, and some K^+ and glutamate but has little effect on the total amount of cytoplasmic solutes; however, $\bar{V}_{\text{cyto}}$ increases greatly (~50%). The accumulation of proline also occurs at relatively constant total amount of cytoplasmic solutes but has a lesser effect than accumulation of betaine on growth rate and $\bar{V}_{\text{cyto}}$ (~20%). The rank order of the capacity of cells to accumulate osmoprotectants (betaine > choline > proline) is the same as the order of extent of replacement of potassium glutamate and trehalose, the order of increase in $\bar{V}_b$, and the order of increase in growth rate and in power as an osmoprotectant.

The molar contributions of betaine and proline to the osmotic pressure of the cytoplasm appear to be greater than those of the solutes they replace. The thermodynamic origin of this effect appears to be the greater degree of exclusion of betaine and proline from $\bar{V}_b$ relative to those of the osmolytes they replace (trehalose, K^+ , MOPS, glutamate). Because $\bar{V}_b$ is a large fraction (23 to 34%) of the total volume of water in the cytoplasm under the conditions studied here, relatively small differences in the degree of exclusion of the different osmolytes from the water of hydration of cytoplasmic macromolecules are consistent with changes in $\bar{V}_{\text{cyto}}$ of the magnitude observed here.

The present results, together with those previously obtained for cells grown in the absence of exogenous osmoprotectants (12), suggest that the growth rate, $\bar{V}_{\text{cyto}}$, and n_{K^+} are a tightly linked set of parameters in *E. coli* grown under the stress of high osmolarity. Although we cannot rule out other mechanisms, we propose that $\bar{V}_{\text{cyto}}$ (more specifically, $\bar{V}_f$) is the fundamental determinant of the growth rate of cells cultured at high osmolarity and that the increase in $\bar{V}_f$ that accompanies the accumulation of osmoprotectants is the fundamental origin of the concomitant increase in growth rate. $\bar{V}_f$ increases because accumulation of osmoprotectants occurs without a net change in $(\Sigma n_i)_{\text{cyto}}$ and because in the cytoplasmic milieu the osmoprotectants are thermodynamically more ideal than the solutes that they replace. (We previously proposed [12] that $\bar{V}_{\text{cyto}}$ [and hence $\bar{V}_f$] and n_{K^+} are linked by the need to preserve approximate constancy of the extents and rates of the protein-nucleic acid interactions of gene expression.) The molecular bases of the apparent constancy of $(\Sigma n_i)_{\text{cyto}}$ and of the apparent linkages among $\bar{V}_f$, n_{K^+} , and growth rate in the absence and presence of osmoprotectants remain to be determined.

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