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Physiological and Genetic Responses of Bacteria to Osmotic Stress

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What am I, Life? A thing of watery salt
Held in cohesion by unresting cells,
Which work they know not why, which never halt . . .
John Masefield, Sonnet 14

INTRODUCTION

The osmotic strength of the environment is one of the physical parameters that determines the ability of organisms to proliferate in a given habitat. Although the ability to adapt to fluctuations in the external osmolarity is fundamental to the survival of organisms, the mechanisms responsible for osmotic adaptation have been elucidated only relatively recently. There are remarkable similarities between bacteria and plants in their cellular responses to osmotic stress, because organisms from both kingdoms accumulate the same set of cytoplasmic solutes upon exposure to conditions

of hyperosmolarity. Thus, it is likely that there will be close parallels in the mechanisms that these organisms employ to regulate responses to osmotic stress.

By osmotic stress I mean an increase or decrease in the osmotic strength of the environment of an organism, and I define osmotic regulation or osmoregulation as the active processes carried out by organisms to cope with osmotic stress. Various facets of osmoregulation in bacteria have been covered in recent articles: the constraints on bacterial growth by the physical parameters of the environment have been discussed by Ingraham (105) and Kogut and Russell (123), the interactions of biological macromolecules with the solutes accumulated by organisms in media of elevated osmolarity by Yancey et al. (275), the molecular biology of the accumulation of cytoplasmic osmolytes by LeRudulier et al. (138), and models for osmotic regulation of gene transcription by Higgins et al. (95). A review of potassium transport in bacteria was presented by Walderhaug et al. (263). A report of the proceedings of a workshop on the molecular basis of haloadaptation in microorganisms was published in *FEMS Microbiology Reviews* (vol. 39).

The question of how the osmolarity of the environment limits the proliferation of organisms can be broken down into two parts: what determines the ability of organisms to live in an environment of a specific osmolarity, and what are the processes that enable organisms to cope with changes in the external osmolarity? For example, bacteria can be found in environments of wide ranges of osmolarity, from mountain spring waters to saturated salt brines (99). Although the question of what factors determine the ability of each of these species to thrive in its particular niche is very important, at present the answers to it are more elusive than insights into the mechanisms of adaptation to changes in the osmolarity of the environment. In this review, I will confine myself to the second topic. I will concentrate mainly on osmotic regulation in *Escherichia coli* and *Salmonella typhimurium* for the reason that more is known about osmoregulation in these two species than in any other bacteria, but this choice is not intended to imply that osmotic regulation is intrinsically more interesting in these two bacteria than in other species.

THERMODYNAMIC BACKGROUND

Lipid membranes allow rapid diffusion of water molecules into or out of cells while presenting an effective barrier to most other biological molecules. Membranes that exhibit selective permeability for different substances are called semipermeable, and the osmotic properties of cells derive from this property of the membranes.

Osmotic Pressure

Consider a vessel that is divided into two identical compartments which are open to the atmosphere and are separated from each other by a vertical semipermeable membrane. Suppose that one of the compartments (designated compartment 1) is partially filled with pure water and the other compartment (compartment 2) is filled with an equal volume of aqueous solution of some molecule that cannot diffuse across the membrane. Because of the presence of the solute molecules, the concentration (or, strictly speaking, activity) of water in compartment 2 is less than that of pure water, and consequently water molecules will diffuse from compartment 1 into compartment 2, down the concentration (or activity) gradient. This movement of water will cause the level of water in compartment 1 to fall and to rise in compartment 2, setting up a hydrostatic pressure on compartment 2, the magnitude of which depends on the difference in the levels of the liquids in the two compartments. The diffusion of water into compartment 2 will continue until the excess pressure in compartment 2 is sufficient to counteract the tendency of water to diffuse into it. The pressure exerted on compartment 2 when the system reaches equilibrium is the osmotic pressure.

Osmotic Potential

Osmotic pressure is cumbersome to measure, because it has meaning only in the context of one solution separated from another solution by a semipermeable membrane. A more meaningful term describing the osmotic property of solution is osmotic potential (π), which is related to the activity (a) of the solvent by the equation:

$$\pi = (RT/\overline{V}) \ln a \quad (1)$$

where R is the universal gas constant, T is the absolute temperature, and $\overline{V}$ is the partial molar volume of the solvent (34, 230). By definition, the activity of a pure solvent is equal to 1 (34), so that the osmotic potential of any pure solvent is 0. Generally, the addition of solutes decreases the activity of solvents to a value of <1, and therefore the osmotic potential of solutions is usually negative. The utility of the osmotic potential is that it points out that when a solution is separated from a pure solvent by a membrane that is more permeable to the solvent than to the solute, the solvent molecules will move from the region of higher to lower osmotic potential.

In the subsequent discussion, I will use the term ideal solution to mean a solution in which the forces of interaction between the solute and solvent molecules are independent of the solute concentration. (This use of the term ideal solution is not as rigorous as the definition commonly given in physical chemistry texts [34], but it points out the fact that solutions behave like ideal ones when the solutes are present at low concentrations. For aqueous solutions, deviation from ideality can be quite serious. For example, at sucrose concentrations of >0.22 M, the osmotic potential at 20°C of aqueous sucrose solutions deviates by more than 10% from the calculated osmotic potential of ideal solutions of equal molarity [270].) For ideal solutions, the activity of the solvent equals the mole fraction of solvent. Let a_w, n_w, and n_s denote the water activity, the number of water molecules, and the number of solute molecules, respectively, in compartment 2 of the apparatus described above. If it is assumed that the solution in compartment 2 is sufficiently dilute that it can be considered ideal, then $a_w = n_w/(n_w + n_s)$, or $a_w = 1 - n_s/(n_w + n_s)$. If the solution is sufficiently dilute such that $n_s \ll n_w$, then $a_w \simeq 1 - n_s/n_w$. Let c_s denote the molar concentration (moles per liter) of the solute in compartment 2; $c_s = n_s/(n_s\overline{V}_s + n_w\overline{V}_w)$, where $\overline{V}_s$ and $\overline{V}_w$ are the partial molar volumes of the solute and water, respectively. For dilute solutions, $n_s\overline{V}_s \ll n_w\overline{V}_w$, and so $c_s \simeq n_s/n_w\overline{V}_w$. Thus, for dilute solutions, $a_w \simeq 1 - c_s\overline{V}_w$. Substituting for the activity of the solvent (water) in equation 1, the relationship $\pi \simeq (RT/\overline{V}_w) \ln(1 - c_s\overline{V}_w)$ is obtained. For solutions that are sufficiently dilute, $c_s\overline{V}_w \ll 1$, and under these conditions $\ln(1 - c_s\overline{V}_w)$ is approximately equal to $-c_s\overline{V}_w$ (34). Thus, the expression for the osmotic potential can be rewritten as

$$\pi \simeq -RTc_s \quad (2)$$

Although this relationship is applicable to ideal solutions with the solute present at low concentrations, it can be used

to calculate the total osmotic concentration, or osmolarity, of all the solutes as the quantity $-\pi/RT$. The quantity thus defined is the apparent concentration of solutes that would result in a given osmotic potential, assuming that the osmotic potential is related to the concentration of the solutes according to equation 2 throughout the range of concentrations.

Turgor Pressure

In the example used to illustrate osmotic pressure, the volume of the solution in the chamber containing the solute (chamber 2) was free to expand with the influx of water. But assume that a rigid cover is placed around the liquid in chamber 2 and that the semipermeable membrane is completely inelastic. In this case, the volume of the solution in chamber 2 would not be able to expand, and the pressure in the chamber would increase as a consequence of the tendency of water molecules to diffuse into it. This situation applies to bacterial and plant cells, which have rigid walls surrounding the cytoplasmic membrane. Thus, as water diffuses into these cells, the membranes can expand only as far as the interior side of the cell walls, and additional influx of water results in a buildup of pressure that is exerted by the cytoplasmic membrane on the walls. This pressure is the turgor pressure.

In practice, cells are not surrounded by pure water but by nutrient solutions of various osmolarities. The turgor pressure of the cells is the difference between the solute potential of the medium and that of the cell interior, and it can be calculated by the expression $P = (RT\overline{V}_w) \ln(a_{medium}/a_{cells}) \approx RT(c_{cells} - c_{medium})$, where P is the turgor pressure and a and c are, respectively, the water activity and total concentration of osmotically active solutes in the medium and the cells. (Several different units are used to express turgor pressure, which can be interconverted by the relationships: 1 atm = 1.013 bars = 1.013×10^6 dynes/cm^2 = 1.013×10^5 Pa = 760 mm Hg.)

OSMOTIC PROPERTIES OF CELLS

Relationship between Turgor Pressure and Cell Wall Expansion

The walls of bacteria and growing plant cells are not completely rigid, and the turgor pressure has been proposed to provide the mechanical force for the expansion of the cell walls during cell growth (120, 143). According to this model, the uptake or biosynthesis of osmotically active solutes causes an increase in the osmolarity of the cytoplasm, which is accompanied by influx of water into the cells, thus providing the necessary turgor pressure for expansion of the cell walls. Although the suggestion that turgor pressure is the driving force for cell wall expansion needs further substantiation (e.g., see reference 43 for a dissenting view), if it is correct, it would imply that the mechanisms that regulate the osmotic balance of organisms are central to the very process of cell growth.

Effects of Hyper- or Hypoosmotic Shock on Bacteria

Because the cytoplasmic membrane of bacteria is permeable to water but not to most other metabolites, hyper- or hypoosmotic shock causes an instantaneous efflux or influx of water, which is accompanied by a concomitant decrease or increase in the cytoplasmic volume, respectively. Since bacterial cell walls are rigid and can withstand pressures up to 100 atm (32), hypoosmotic shock generally results only in minor increases in cell volume (248). Hyperosmotic shock, on the other hand, causes considerable shrinkage of the cytoplasmic volume. This process, known as plasmolysis, can be detected as an instantaneous increase in the turbidity of the cultures upon introduction of the cells into media of increased osmolarity (121, 178, and references cited in reference 121). The kinetics and extent of plasmolysis are dependent on the magnitude of the increase in the osmolarity of the medium but not on the nature of the solutes used, provided that the solutes are excluded from the cytoplasm by the membrane. Molecules which can freely diffuse across the membrane, such as glycerol and ethanol, do not cause plasmolysis (178).

If the hyperosmotic shock is not too severe, plasmolysis is transient, and after some lag the cytoplasmic volume will increase as a result of osmotic adjustment by the cells. However, even after osmotic adaptation has taken place, the steady-state cytoplasmic volume of growing bacteria is dependent on the osmolarity of the medium, such that the cytoplasmic volume decreases with increasing external osmolarity (12, 13, 61, 134, 220, 253).

The exposure of organisms to conditions of hyperosmolarity results in a decrease in their cytoplasmic water activities. It seems likely that proteins and other biological macromolecules have evolved to function only within certain normal ranges of water activities, outside which some essential cellular function(s) becomes impaired. Sudden plasmolysis results in the inhibition of a variety of physiological processes, ranging from nutrient uptake (225, 226, 267) to deoxyribonucleic acid (DNA) replication (168). Sudden plasmolysis has been reported to be accompanied by an increase in the adenosine triphosphate (ATP) levels of the cells (202), possibly resulting from the inhibition of macromolecular biosynthesis.

Measurement of Turgor Pressure in Bacteria

The turgor pressure of bacterial cells can be calculated from the water activities of crude cell extracts (23, 178) or from the threshold osmolarities that induce plasmolysis (178, 248). A third method for the determination of turgor pressure, which is applicable only to organisms containing gas vacuoles, relies on measurement of the threshold external pressure that is required to collapse the gas vacuoles as a function of the osmolarity of the medium (122, 264). Each of these methods entails potentially serious errors, so that the values obtained for the turgor pressure of bacteria are to be regarded as only estimates, but the results of these measurements indicate that the turgor pressure of gram-positive bacteria is considerably greater than that of gram-negative bacteria, with values of 15 to 20 atm being observed for gram-positive and 0.8 to 5 atm for gram-negative organisms (105, 122, 266). Interestingly, the measurement of the collapse pressure of gas vesicles of halobacteria indicated that these organisms do not have a detectable turgor pressure (264, 265). (Halobacteria are archaebacteria which live in environments of extremely high salinity, and they might not experience fluctuations in the osmolarity of the environment as large as those experienced by organisms found in less saline environments. The cell walls of archaebacteria are considerably more flexible than those of eubacteria [265], but nevertheless, if the turgor pressure in halobacteria is actually nonexistent, the question of what provides the force for cell wall expansion in these organisms needs to be addressed.)

COMPATIBLE SOLUTES

Exposure of cells to high external osmolarity results in an efflux of water from the interior. The decrease in the internal water content brings about a reduction in the turgor pressure and a shrinkage of the cytoplasmic volume. As a consequence of the decrease in the cytoplasmic volume, the concentrations of all the intracellular metabolites increase and thus cause a reduction in the intracellular water activity. In the absence of active osmotic adjustment by the cell, the cytoplasmic volume would shrink until the water activity of the interior equaled that of the exterior. Because an elevation in the concentrations of various intracellular molecules may be inhibitory to cellular processes (e.g., inhibitors of specific enzymes may reach deleterious concentrations [10] or the increase in the concentrations of ions may become toxic [263]), passive alteration of the cell volume is not adequate for adaptation to changes in the osmolarity of the environment.

Instead of a passive volume regulation, organisms generally respond to osmotic stress by increasing the concentrations of a limited number of solutes. Thus, the water activity of the cell interior can be reduced, and consequently cell volume and turgor can be restored near their prestress values without an across-the-board increase in the concentrations of all cytoplasmic components. Since the molecules that are accumulated during conditions of osmotic stress are not greatly inhibitory to cellular processes, they have been termed compatible solutes (26).

The prominent compatible solutes found in bacteria are K^+ ions, the amino acids glutamate, glutamine, proline, γ-aminobutyrate, and alanine, the quaternary amines glycinebetaine and other fully *N*-methylated amino acid derivatives, and the sugars sucrose, trehalose (α-D-glucopyranosyl-α-D-glucopyranoside), and glucosylglycerol (67, 103, 217, 275). I will discuss some of these compatible solutes in detail below, but I can make a few generalizations about them here. First, as expected of compounds that are to be accumulated at high intracellular concentrations, compatible solutes are unable to cross the cell membranes rapidly without the aid of transport systems. Second, compatible solutes for the most part do not carry a net electrical charge near pH 7. This property can be rationalized to be beneficial, because uncharged molecules can be accumulated to high intracellular concentrations without greatly disturbing the structures of cellular macromolecules. However, K^+ ions and glutamate are noteworthy exceptions to this generalization, and these two solutes may not offer as effective protection against hyperosmotic stress as some of the uncharged metabolites (57, 253, 263).

Osmoremedial Mutations

The three-dimensional structures of biological macromolecules are dependent on physical properties such as temperature, pH, and water activity of their environment, and cellular components evolved to have at least partial activity over the entire range of concentrations of compatible solutes that are maintained by organisms in the ecological niches where they can proliferate. However, there are a number of examples of mutant proteins whose structures are abnormally sensitive to the solute composition of the cytoplasm, which is in part determined by the external osmolarity. Such mutant proteins are nonfunctional when the cells are grown in media of low osmotic strength but regain at least partial activity when the cells are grown in media of elevated osmolarity. The mutations resulting in this phenotype have been designated osmoremedial. Mutations of this type are common; Hawthorne and Friis (90) found that in *Saccharomyces cerevisiae*, about 16% of all auxotrophic mutations were osmoremedial. Usually, the phenotypic defect in osmoremedial mutants can be overcome by any nontoxic solute (16, 20, 66, 77, 90, 128, 155, 162, 166, 228, 247, 262). However, in contrast to the findings of all other researchers, Kohno and Roth (124) reported that only ionic solutes could overcome the defect in osmoremedial *his* mutants of *S. typhimurium*. Kohno and Roth found that NaCl was the most effective solute for the correction of the osmoremedial mutations they examined and that other salts could bring about some reversal of the mutant phenotype, but their effectiveness was allele specific. Glycerol, which can traverse bacterial membranes by passive diffusion, was also ineffective as an osmoremedial agent with most *S. typhimurium* mutants.

The osmoremedial phenotype is associated with slight alterations in the amino acid sequence of the affected proteins, as indicated by the observation that many temperature-sensitive mutations are also osmoremedial (16, 20, 77, 157, 166, 228, 237, 247). Kohno and Roth (124), who carried out the most comprehensive study of osmoremedial mutations, reported that of more than 100 temperature-conditional (heat or cold sensitive) *his* mutations in *S. typhimurium*, all were osmoremedial. These researchers demonstrated that histidinol dehydrogenase from the osmoremedial *hisD6585* mutant could be stabilized in vitro by the addition of a number of salts. However, the in vivo osmoremedial efficacy of various salts did not correspond to their in vitro abilities to stabilize the enzyme. Because some of the affected enzymes of the osmoremedial mutants could be stabilized in vitro by one or another solute, the most plausible explanation for the osmoremedial phenotype is that the increase in the concentration of one or more of the compatible solutes brought about by the elevated external osmolarity enhances the stability of the mutant proteins. However, since various solutes at equivalent osmolarities did not necessarily bring about equal in vivo correction (124), it is not clear whether differences in the in vivo response of osmoremedial mutants to different solutes are due to major differences in the composition of compatible solutes elicited by specific external osmolytes, or whether the osmoremedial phenotype is the result of subtle changes in the concentration of some intracellular metabolite(s) that might be differentially elicited by various extracellular solutes.

The osmoremedial phenotype was initially thought to be an indication of a defect in membrane-associated proteins (128, 237). However, this is not necessarily the case, as osmoremedial mutations affecting a number of soluble enzymes have been isolated (66, 90, 124, 247, 262). Kohno and Roth (124) demonstrated that osmoremedial mutations can affect multimeric as well as monomeric soluble proteins. Since osmotic correctability depends on the alteration of the solute environment of the cellular compartment where the proteins are found, it is an interesting question whether it would be possible to obtain osmoremedial mutations in genes encoding periplasmic or outer membrane proteins, but thus far no such mutant proteins have been described.

Mutations Conferring Sensitivity to Hyperosmotic Stress

Conceivably, it might be possible to obtain mutations that confer increased sensitivity to hyperosmotic stress. Although this is a very interesting aspect of the field of

osmoregulation, very little is known about mutations that confer this phenotype. In limited number of cases, differential sensitivities of various cellular processes to osmotic stress have been observed. For example, nitrogen fixation in *Klebsiella pneumoniae* (136) and the conjugal transfer of F plasmid in *E. coli* (239) are more sensitive to hyperosmotic stress than overall cell growth. Mutations which block the pathway for trehalose synthesis result in sensitivity to osmotic stress (74, 221) (see below). Recently, Rod et al. (221) reported that wild-type *E. coli* K-12 and several commonly used cell lines derived from it carry an amber codon in some unknown gene which renders these strains sensitive to osmotic stress, compared with wild-type *S. typhimurium*, *E. coli* B, or *E. coli* K-12 derivatives harboring amber suppressor mutations. These authors found that the osmosensitive *E. coli* K-12 cell lines were impaired in their ability to synthesize trehalose and to take up proline from the growth medium under conditions of osmotic stress, and therefore they concluded that the amber codon in these strains affects some gene involved in osmotic adaptation. The observations of Rod et al. raise a potential complication in the interpretation of results on osmoregulation obtained with *E. coli* K-12, because depending on the pedigree of the cell lines used by different groups, the experimental organisms could have major differences in their responses to osmotic stress.

In *E. coli*, mutations affecting membrane structure which confer sensitivity to acriflavine also result in sensitivity to hyperosmotic media (190). *E. coli* mutants defective in K^+ uptake are also sensitive to hyperosmotic stress (78). In *S. typhimurium*, a deletion in the *his* attenuator (*hisO1242*), which caused constitutive expression of the histidine-biosynthetic enzymes, was found to result in osmosensitivity (188). The osmosensitivity is due to the increased formation of the products of the *hisF* and *hisH* genes, but the specific reason for this phenotype has eluded explanation. Interestingly, proline and glycinebetaine can overcome the osmosensitivity associated with the *hisO1242* mutation (S. Fletcher and L. N. Csonka, unpublished results).

Sodium ions are kept at a very low intracellular concentration in enteric bacteria (35, 61) as a result of the functioning of an Na^+/H^+ antiport system. Mutations that inactivate the Na^+/H^+ antiporter confer sensitivity to Na^+ or Li^+ ions (76), but this sensitivity is not associated with a generalized sensitivity to osmotic stress. Lusk and Kennedy (147) described a mutation that altered the phospholipid composition of the membrane and caused Na^+ sensitivity, but the connection between the sodium sensitivity and the alteration in lipid metabolism in the affected strain has not been explained.

Compatible Solutes

Compatible solutes can be accumulated by bacteria by de novo synthesis or by transport from the culture medium. There are differences in the effects of various compatible solutes on the osmotic stress tolerance of cells, because some can elicit a dramatic stimulation of the growth rates of the cells in media of high osmolarity when they are added to the culture, whereas others have no detectable effects on the growth of the cells. The reason for this difference between the two types of compatible solutes is not understood, but it may be that solutes which can alleviate osmotic inhibition can be accumulated to higher internal levels by transport or are less toxic at high internal concentrations than solutes which do not have similar effects. Alternatively, it may be that the substances which can palliate osmotic inhibition have some special interactions with cellular macromolecules resulting in increased stability of these macromolecules in cells grown in media of high osmolarity (233). Regardless of the molecular mechanism responsible for their ability to overcome osmotic inhibition, solutes which have this property are sometimes called osmoprotectants.

Potassium ions. Potassium ions are the most prevalent cations in the cytoplasm of bacteria (40, 263), and consequently they serve as one of the major intracellular osmolytes that maintain turgor. The intracellular concentration of K^+ in a wide assortment of bacterial species has been found to be nearly proportional to the osmolarity of the growth medium (38–40, 61, 164, 205, 216, 257), and there is a positive correlation between the intracellular content of this cation and the ability of bacteria to tolerate conditions of high osmolarity (40).

The major contributions to the understanding of the role of K^+ in osmoregulation emerged through the work of W. Epstein and his colleagues. Epstein and Schultz (61) found that in exponentially growing *E. coli*, the steady-state intracellular concentration of K^+ increased from 0.15 to 0.55 M as the osmolarity of the medium was increased from 0.1 to 1.2 osm. Increased accumulation of K^+ was elicited only by high concentrations of solutes that could not diffuse across the cell membrane (e.g., glucose, sucrose, and NaCl), and the intracellular concentration of K^+ was dependent only on the osmolarity of the medium, regardless of the solute used. However, accumulation of K^+ could not be elicited by glycerol (61), which diffuses freely across the cell membrane (178). These results suggest that the signal for enhanced K^+ accumulation is not the decrease in the intracellular water activity per se, but the loss of turgor or possibly the reduction in cytoplasmic volume.

Dinnbier et al. (52) and Ohwada and Sagisaka (201) recently reported that accumulation of K^+ was only transient after hyperosmotic shock and that 30 min after the exposure of *E. coli* to hyperosmotic media, the cells actually excreted K^+. These researchers concluded that the intracellular K^+ concentration in bacteria that have undergone complete osmotic adaptation was not very different from the prestress value (see also reference 223). The reason for this apparent contradiction of the results of Epstein and Schultz (61) is unclear but it needs to be resolved because a central role has been proposed for K^+ as an osmoregulatory signal (57, 253; see below).

Studies with a radioactive isotope of potassium demonstrated that during steady-state growth of *E. coli*, there is a rapid exchange of intracellular and extracellular potassium (62, 169), indicating that the intracellular concentration of this ion is determined by both its rate of uptake and its rate of efflux. Meury et al. (171) investigated the effects of hyper- or hypoosmotic shock on the rates of K^+ influx and efflux in cells that were starved for a carbon source and found that a sudden increase in the osmolarity of the medium caused an increase in K^+ influx, at a rate that was negatively correlated with the K^+ concentration of the cells but was independent of the magnitude of the increase in the osmolarity of medium. Exposure of the cells to media of lower osmolarity stimulated K^+ efflux, at a rate that was proportional to the decrease in the osmolarity of the medium. The stimulation of both the influx and efflux processes in response to exposure to media of hyper- or hypoosmolarity, respectively, occurred very rapidly and did not require an energy source. The mechanism of regulation of these two processes is not known, but it has been proposed to involve a direct effect of turgor on the proteins that mediate K^+ entry and exit (57).

High levels of glutathione were required for the retention of K^+ (170), and the concentrations of this metabolite were found to increase with osmotic stress (187).

E. coli has a number of K^+ transport systems of which two have been extensively characterized: Trk, which has a relatively low affinity for K^+ (K_m, 1.5 mM), and Kdp, which has a much higher affinity (K_m, 2 μM) (263). The former system is expressed constitutively, but its activity is enhanced in response to hyperosmotic shock (57, 171, 263). The latter is subject to transcriptional regulation (see below), and its activity is also stimulated by hyperosmotic shock in a manner similar to that observed with the Trk system (218).

E. coli mutants with defects in the Kdp system were isolated as strains showing growth inhibition with 0.02 mM K^+ (58). A second round of mutagenesis of *kdp* mutants followed by selection for derivatives requiring at least 0.1 mM K^+ resulted in the identification of seven additional loci, *trkA*, *trkB* (or *kefB*), *trkC* (or *kefC*), *trkD*, *trkE*, *trkG*, and *trkH*, whose function is required for K^+ uptake or retention in media containing high concentrations of this cation (59, 263). Originally it was thought that these genes govern the expression of at least three independent low-affinity K^+ transport systems, but more recently Epstein and Laimins (60) suggested that they specify components of the Trk system. Subsequently, Bakker et al. (11) demonstrated that the *trkB* (*kefB*) and *trkC* (*kefC*) gene products are involved in K^+ efflux but not in K^+ uptake. The roles of the *trkD*, *trkE*, *trkG*, and *trkH* loci and the possible interactions of the K^+ influx and efflux systems have yet to be resolved.

The components of the Kdp system are encoded by the *kdpABC* operon (238), which is under positive transcriptional control by the *kdpD* and *kdpE* gene products (57, 210). Hesse et al. (94) determined the nucleotide sequence of the *kdpABC* operon and found that the predicted amino acid sequence of the *kdpA* protein has extensive similarity to a mammalian sarcoplasmic Ca^{2+} ATPase. This result, together with the observation that some *kdpA* mutations were found to cause decreased affinity of the Kdp system for K^+, suggests that the *kdpA* gene product is responsible for the binding of K^+ during its transport (238).

Glutamate and glutamine. The cytoplasmic levels of glutamate increase in most procaryotes after exposure to media of high osmolarity (22, 28, 100, 153, 164, 257, 276). In gram-negative bacteria, osmotic stress can elicit greater than 10-fold increases in the levels of glutamate, so that this amino acid can account for more than 90% of the free amino acids in some organisms grown in media of elevated osmotic strength (257). Richey et al. (220) reported that the steady-state intracellular glutamate concentration in exponentially growing *E. coli* was directly proportional to the osmolarity of the growth medium. The levels of glutamine also increase in response to osmotic stress in gram-negative bacteria (28, 45, 136, 257), but because glutamine is present at much lower levels than glutamate, it is probably not important for the maintenance of cytoplasmic osmolarity. Since glutamine is a precursor of glutamate in most bacteria (258), its accumulation in response to osmotic stress may be a consequence of the increased need for the synthesis of glutamate. The basal levels of glutamate are generally 8- to 10-fold greater in gram-positive than in gram-negative bacteria, and although there is an increase in the levels of glutamate in most gram-positive bacteria in response to osmotic stress, the relative increases are much less and occur much more slowly than in gram-negative organisms (4, 28, 164, 257). Osmotic stress does not affect glutamate levels in the gram-positive *Planococcus* and *Staphylococcus* species, but rather results in dramatic increases in glutamine (and/or alanine in the case of *Staphylococcus* spp.) (4, 118, 172).

The accumulation of glutamate and glutamine was observed in several species of bacteria grown in media devoid of exogenous amino acids (257), indicating that in these cases the increase in the level of the two amino acids was due to an enhancement of their rate of synthesis. However, no attempts have been made to determine to what extent the increase in glutamate or glutamine levels is due to stimulation of their synthesis or inhibition of their turnover. It is also not clear whether the uptake of these two amino acids is regulated by the osmolarity of the medium. There are two alternative pathways of synthesis of glutamate from ammonia and α-ketoglutarate: a single-step reaction catalyzed by glutamate dehydrogenase, and a two-step sequence involving glutamine synthetase and glutamate synthase (258). The only characterization of the relative importance of the two pathways of glutamate synthesis for osmoregulation has been carried out with *Vibrio harveyi*, in which Gurevich et al. (83) found that hyperosmotic stress resulted in a decrease in glutamate dehydrogenase and an increase in glutamate synthase activity. Tempest et al. (257) noted that the activity of glutamate dehydrogenase of *Klebsiella aerogenes* was sensitive to pH, with a very sharp optimum occurring near pH 8, and that the exposure of the cells to osmotic stress elicited a rapid exit of protons from the cytoplasm. These researchers proposed that the alkalinization of the cytoplasm activated glutamate dehydrogenase, thereby resulting in increased synthesis of glutamate. Measures (164) observed that K^+ at high concentrations stimulated glutamate dehydrogenase activity in several gram-positive and gram-negative bacteria and therefore suggested that the accumulation of K^+ under conditions of osmotic stress is the regulatory signal for the synthesis of glutamate. There have been no studies reported on the possible transcriptional control of either glutamate synthase or glutamate dehydrogenase by osmotic stress.

The accumulation of glutamate per se is not required for osmotic stress tolerance, because the *S. typhimurium* mutants that overproduce proline did not have increased glutamate levels after being exposed to media of high osmolarity but were nevertheless more tolerant of osmotic stress than the wild-type strain (47).

Electrolyte balance of the cytoplasm. In order to maintain the membrane potential of *E. coli* at its steady-state value of −120 to −150 mV (35), the uptake of K^+ during osmotic stress must be balanced by the accumulation of equivalent anions or expulsion of cations. Although glutamate is the most abundant anion in bacteria, its concentration in *E. coli* is only about 50% of that of K^+ in media of high osmolarity (52, 61, 220), and therefore other osmotically regulated processes are needed to ensure that the accumulation of K^+ will not dissipate the membrane potential.

Tempest et al. (257) and Castle et al. (35) reported that osmotic stress results in alkalinization of the cytoplasm. On the basis of the increase in the cellular pH determined by Castle et al. (35) and the buffering capacity of the *E. coli* cytoplasm (241), Richey et al. (220) calculated that the increase in the cytoplasmic pH would represent the expulsion of a sufficient number of protons to account for the remaining 50% of the K^+ taken up. The protons extruded in the course of K^+ transport probably derive from the ionization of cytoplasmic ampholytes rather than from the organic acid products of carbon sources, because not only are the concentrations of such products insufficient to balance the

K^+ (131) but the increased uptake of K^+ upon osmotic stress occurs even in carbon source-starved cells (171).

Munro et al. (187) found that exposure of the cells to hyperosmotic shock resulted in rapid excretion of putrescine. These researchers proposed that since putrescine is a divalent cation, each molecule of putrescine could be traded for two K^+ ions, and thus the osmotic strength of the cytoplasm could be increased without disturbing the overall concentration of ions. Munro et al. (187) found that the putrescine pool in *E. coli* grown in media of low osmolarity was 0.15 μmol/mg of protein, so that this solute could balance the accumulation of 0.30 μmol of K^+ per mg of protein, which is the approximate level in cells grown in media with an osmotic strength of 0.6 osm (220). However, there is some discrepancy in the reported values for the putrescine content of cells grown in media of low osmolarity: Neidhardt (195) found that this metabolite was only present at 0.034 μmol/mg of protein, and it was not detected by Larsen et al. (131) in their nuclear magnetic resonance measurements of the abundance of cytoplasmic solutes. Therefore, the role of putrescine in the response to osmotic stress needs further clarification.

From these considerations, it would appear that the accumulation of glutamate and the efflux of protons are sufficient to provide the charge balance for the K^+ accumulation that occurs upon osmotic stress. However, it should be noted that contrary to the results of Tempest et al. (257) and Castle et al. (35), Dinnbier et al. (52) found that hyperosmotic shock elicited only a transient increase in the cytoplasmic pH (which corresponded to the time of rapid K^+ uptake), but after osmotic adaptation the cytoplasmic pH returned to its prestress value. Since these researchers also found that glutamate levels were only 50% of the K^+ levels during the entire course of osmotic adaptation, they suggested that the cells must accumulate some uncharacterized anion to balance the net K^+ taken up in response to exposure to hyperosmotic conditions. In any event, whether or not the uptake of K^+ is balanced by the extrusion of protons or by the accumulation of anions, the need to maintain electroneutrality in cells grown in media of high osmolarity raises questions as to which of the above processes are the primary ones that respond to osmotic control and how their regulation is coordinated.

γ-Aminobutyrate. Measures (164) reported that the levels of γ-aminobutyrate increase in response to osmotic stress in a variety of bacteria, but because he used complex media for the growth of the cells, his experiment did not clarify whether the accumulation of this compound was due to increased synthesis or uptake. Accumulation of γ-aminobutyrate upon osmotic stress was not seen in enteric bacteria grown in minimal medium (45, 47, 134), so the increase in γ-aminobutyrate levels reported by Measures was probably the result of uptake from the medium rather than synthesis. Mutations that abolish the transport or catabolism of γ-aminobutyrate have been obtained in *E. coli* (167), but their effect on osmotic stress tolerance has not been determined.

Trehalose. Trehalose has been found to be synthesized in a number of bacteria, including *E. coli* (70, 131, 148, 217), in response to osmotic stress. The intracellular concentration of trehalose in *E. coli* equals about 20% of the osmolar concentration of solutes in the growth medium. However, because mutations in *E. coli* that resulted in tyrosine overproduction also caused trehalose accumulation (200), the production of trehalose may respond to other signals besides the osmotic strength of the growth medium. One report (223) suggested that *E. coli* accumulated high concentrations of glucose in response to osmotic stress, but probably the accumulation of this sugar is an artifact of hydrolysis of trehalose during the extraction (221).

The synthesis of trehalose entails the condensation of glucose 6-phosphate and uridine diphosphate-glucose, yielding trehalose 6-phosphate, with subsequent dephosphorylation to trehalose. Giæver et al. (74) demonstrated the presence of a trehalose 6-phosphate synthetase activity in *E. coli* and *S. typhimurium* grown in media of high osmolarity, but could not detect a trehalose phosphate phosphatase activity in these cells. The formation of the trehalose 6-phosphate synthetase is under osmotic control, because Giæver et al. (74) found that there was a marked increase in the specific activity of this enzyme when the cells were exposed to osmotic stress. However, an allosteric control is probably also exerted over the activity of one of the enzymes of trehalose synthesis, because these researchers noted that an *E. coli* mutant which expressed trehalose 6-phosphate synthetase constitutively nevertheless synthesized high levels of trehalose only in media of elevated osmolarity.

Mutations which result in an impairment in the accumulation of trehalose result in increased sensitivity to osmotic stress. Giæver et al. (74) isolated such mutations in *E. coli* at two loci, *otsA* and *otsB*, and found that the affected strains were unable to synthesize trehalose-6-phosphate synthetase in response to hyperosmotic stress. Mutations in the *galU* gene, which encodes glucose-1-phosphate uridylyl transferase, also block the synthesis of uridine diphosphate-glucose and result in the impairment of synthesis of trehalose (74, 221). However, it should be noted that the *galU* mutations also block the synthesis of constituents of the lipopolysaccharide and other sugar polymers whose formation is under osmotic control, such as membrane-derived oligosaccharides (116) and the extracellular polysaccharide M antigen (5), so the effects of the *galU* mutations are not specific to the inhibition of trehalose synthesis. Exogenous glycinebetaine, which was found to suppress the accumulation of trehalose in the wild-type cells (52, 131), can overcome the osmosensitivity of the *galU*, *otsA*, and *otsB* mutants (74).

E. coli can grow with trehalose as the sole carbon source, and it has a periplasmic trehalase for the extracellular hydrolysis of this sugar. The structural gene for trehalase, *treA*, is part of an operon that contains other genes for the transport and metabolism of trehalose. Interestingly, exposure of the cells to osmotic stress was found to result in a 10-fold induction of trehalase and other enzymes of trehalose degradation (21). The increased synthesis of the enzymes of trehalose catabolism in response to osmotic stress is difficult to rationalize, but perhaps it provides a fine-tune control of the intracellular trehalose levels as a result of the simultaneous operation of both the biosynthetic and degradative pathways.

Osmoprotectants

Proline. One of the most remarkable observations in the field of osmoregulation was reported by Christian in 1955 (38, 39); that in *Salmonella oranienburg*, exogenous proline could alleviate the growth inhibition imposed by osmotic stress. This finding demonstrated that osmotic stress tolerance is not necessarily dependent on the interactions of a large array of gene products but can be reduced to simple phenomena, such as the accumulation of a compatible solute.

Bacteria can accumulate proline to high intracellular concentrations by increased net synthesis or by enhanced up-

take from the medium. Measures (164) found that osmotic stress resulted in large increases in the intracellular levels of proline in a large variety of bacteria. However, as discussed above, this study does not illuminate whether the accumulation of any metabolite is due to increased synthesis, enhanced transport, or diminished degradation. Many species of gram-positive bacteria are able to increase the proline pool size upon exposure to osmotic stress in the absence of exogenous proline (28, 257), suggesting that the synthesis or the degradation of proline in these organisms is under osmotic control. The mechanism responsible for this regulation has not been studied. However, *Staphylococcus aureus* can accumulate high concentrations of proline in response to osmotic stress as a result of transport but not by increased synthesis (4, 125). In general, gram-negative bacteria achieve high intracellular concentrations of proline during osmotic stress only by enhanced transport. The enteric organisms *S. typhimurium*, *E. coli*, and *K. pneumoniae* have very low intracellular levels of proline when they are grown in media lacking proline, and osmotic stress has no effect on the rate of proline synthesis or degradation (24, 45, 47, 91, 134); these organisms are entirely dependent on the presence of exogenous proline to be able to accumulate it to high concentrations during osmotic stress. In *E. coli*, the intracellular level of proline taken up by active transport was found to be proportional to the osmotic strength of the medium (25).

E. coli and *S. typhimurium* have three independent proline transport systems: PutP, ProP, and ProU (271). The PutP system is required for the transport of proline when this metabolite is used as a carbon or nitrogen source (154, 214); it is not important for the transport of proline as an osmoprotectant, because mutants which lack it are stimulated normally by proline in media of elevated osmolarity (46, 78, 175) and because its activity is not affected by hyperosmotic stress (56, 82). However, the other two systems, ProP and ProU, are responsible for the accumulation of proline to high levels under conditions of hyperosmotic stress.

The ProP system was first described as a minor proline permease in *S. typhimurium* and *E. coli* mutants lacking the PutP system (6, 165, 214, 246). Exposure of the cells to osmotic stress resulted in stimulation of the activity of this permease, mainly as the result of some posttranslational modification of one of its components (30, 56, 82, 175, 176). Kaback and Deuel (112) reported that the rate of proline transport by cell-free *E. coli* membrane vesicles was stimulated by exposure to buffers of high osmolarities, and recently Milner et al. (176) demonstrated that this effect was due to stimulation of the activity of the ProP system. There is a two- to threefold elevation in the steady-state level of transcription of the structural gene(s) of the ProP system upon osmotic stress, which also contributes to its increased activity (30, 56, 79, 111). The activity of this system is also enhanced in response to amino acid starvation (6, 82) by a mechanism that is not understood. The ProP system has a rather low affinity for proline (K_m, 0.3 mM) in cells grown in media of low osmolarity (6), but whether the osmotic enhancement of its activity is due to an increase in its V_{max} or a decrease in the K_m for proline has not been determined.

The existence of the ProU system was inferred from the observation that proline was able to function as an osmoprotectant for *proP putP* double mutants of *S. typhimurium* (46). Furthermore, such double mutants were resistant to the proline analogs L-azetidine-2-carboxylate and 3,4-dehydro-DL-proline in media of low osmolarity but became sensitive to both in media of osmotic strength ≥0.3 osm. Lastly, proline auxotrophic mutants that also lack the PutP and ProP systems require ≥1 mM concentrations of proline for maximal growth because of the impairment of proline transport, but they can grow normally with as little as 0.15 mM proline in media of elevated osmolarity. These observations suggested that there is a third proline permease, the ProU system, that functions only in media of elevated osmolarity (46). The activity of this permease is enhanced by osmotic stress by at least 100-fold as a result of an increase in the steady-state level of transcription of the *proU* operon (14, 31, 55, 56, 78). The ProU system also has a relatively low affinity for proline, with the K_m estimated at 0.2 mM (15, 56).

The existence of multiple transport systems for a substrate, as is the case with proline, raises the question of whether some control mechanism coordinates the operation of the independent porters. For example, if two or more transport systems which could accumulate a substrate to different intracellular concentrations were functioning simultaneously, then it might be possible that the substrate taken up by one porter could leak out via another one, resulting in a futile cycle. In the case of the PutP system, there seems to be a direct coupling between the PutP transport protein and proline dehydrogenase (154), because *putP* mutants are unable to use proline as a nitrogen or carbon source even under conditions of osmotic stress, in spite of the fact that the intracellular proline concentration is 500-fold greater than the extracellular concentration as a result of transport via the ProP and ProU systems (V. J. Prange and L. N. Csonka, unpublished results). The question of whether some higher-order mechanism is coordinating the activities of the three proline transport systems is an interesting one, but it has not been addressed sufficiently.

Christian's observation that exogenous proline can alleviate osmotic stress (38, 39) was the rationale for the isolation of proline-overproducing mutations resulting in enhanced tolerance to osmotic stress in *S. typhimurium* (45) and *Serratia marcescens* (252). In wild type *S. typhimurium* and *E. coli*, the main control over the synthesis of this imino acid is exerted by allosteric inhibition by proline of γ-glutamyl kinase, which is the first enzyme of the proline-biosynthetic pathway (48, 133). Mutations resulting in proline overproduction and enhanced osmotic stress tolerance were shown to be located in the *proB* gene, the structural gene for γ-glutamyl kinase (151). In the case of the allele (*proB74*) that gave the most pronounced osmotic stress tolerance, the mutation proved to be a single-base-pair change (49) that resulted in a 100-fold loss of sensitivity of γ-glutamyl kinase to feedback inhibition by proline (243). The proline-overproducing *proB74* mutation has been transferred from *S. typhimurium* into other enteric bacteria, in which it resulted in enhanced osmotic stress tolerance similar to that in its original host (108, 136). The *S. typhimurium* proline-overproducing mutants had one unexpected characteristic: their intracellular proline levels increased upon hyperosmotic stress even in the absence of exogenous proline (45). This apparent regulation of the intracellular proline level is probably due to enhanced retention of this metabolite by the cells in media of high osmolarity as a result of increased activity of the ProU and ProP systems (47).

Glycinebetaine. A second important osmoprotectant compound accumulated by bacteria under conditions of hyperosmolarity is glycinebetaine (*N,N,N*-trimethylglycine). Cyanobacteria and some other CO_2-fixing procaryotes are able to carry out de novo synthesis of glycinebetaine (70, 103, 216) but most other bacteria are unable to do so, and therefore they are dependent on the transport of this com-

TABLE 1. Effectiveness of proline and glycinebetaine as osmoprotectants in various bacteria

Family or group and species	Alleviation of osmotic inhibition[a]		Reference(s)
	Proline	Glycinebetaine	
Enterobacteriaceae			
Escherichia coli	+	++	134
Salmonella typhimurium	+	++	134
Salmonella oranienburg	+	Unknown	39, 40
Klebsiella pneumoniae	+	++	134
Serratia marcescens	+	+	252
Erwinia carotovora	Unknown	+	54
Pseudomonadaceae			
Pseudomonas aeruginosa	±	++	L. Csonka (unpublished)
Pseudomonas mendocina	+	+	L. Csonka (unpublished)
Pseudomonas stanieri	−	−	L. Csonka (unpublished)
Pseudomonas stutzeri	+	+	L. Csonka (unpublished)
Pseudomonas pseudoacaligenes	+	−	L. Csonka (unpublished)
Rhizobiaceae			
Rhizobium meliloti	−	+	135
Rhizobium trifolii	−	−	135
Rhizobium japonicum	−	−	135
Hedysarum coronarium	−	+	135
Vibrionaceae			
Vibrio cholerae	−	−	L. Csonka (unpublished)
Vibrio natriegenes	−	+	L. Csonka (unpublished)
Vibrio pelagius	−	−	L. Csonka (unpublished)
Vibrio percolans	−	+	L. Csonka (unpublished)
Thiobacilli			
Thiobacillus ferrooxidans	+	+	117
Thiobacillus thiooxidans	+	+	Kieft et al.[b]
Others			
Alcaligenes eutrophus	−	−	L. Csonka (unpublished)
Clostridium pasteurianum	−	−	267
Mycoplana bullata	Unknown	+	54
Pediococcus soyae	Unknown	+	229
Streptomyces griseus	+	Unknown	119

[a] Symbols: +, stimulation in media of high osmolarity; −, lack of stimulation in media of high osmolarity; ++, greater efficacy of glycinebetaine than of proline as an osmoprotectant; ±, weak stimulation. The studies were conducted with bacteria grown in defined medium containing high concentrations of NaCl.

[b] T. L. Kieft, S. Corbett, and M. Jarpe, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, I121, p. 192.

pound for its accumulation. Sakaguchi (229) first reported that exogenous glycinebetaine can alleviate the growth inhibition due to hyperosmolarity in *Pediococcus soyae*; subsequently, Rafaeli-Eshkol and Avi-Dor (213) found that glycinebetaine can stimulate the respiration rate of a halophilic bacterium, Ba-1, in media of elevated NaCl concentration. More recently, LeRudulier and Bouillard (134) observed that this compound is a potent osmoprotectant for members of the family *Enterobacteriaceae*, and Perroud and LeRudulier (209) found that the intracellular concentrations of glycinebetaine maintained by *E. coli* were proportional to the osmolarity of the medium. The transport of glycinebetaine was observed to be stimulated by osmotic stress in *Enterobacteriaceae* (see below), cyanobacteria (184), and *Lactobacillus acidophilus* (101). Although the ability to respond to exogenous glycinebetaine or proline as an osmoprotectant is widespread among bacteria, not all species are able to do so. Table 1 presents a summary of the ability of a number of bacterial species to be stimulated by proline or glycinebetaine in media of inhibitory osmolarity. As can be seen, some species respond equally to both osmoprotectants, some (including *Enterobacteriaceae*) are stimulated more dramatically by glycinebetaine than by proline, and some are stimulated by neither compound. Glycinebetaine is not used as a carbon or nitrogen source by enteric bacteria (134). It can be used as both by *Rhizobium meliloti*, but only in media of low osmolarity. Growth of this microbe in media of high osmolarity results in inhibition of the catabolism of glycinebetaine (244).

In the course of characterization of the uptake of glycinebetaine in *S. typhimurium* and *E. coli*, Cairney et al. (30, 31) and May et al. (163) discovered that the transport of glycinebetaine is mediated by the ProP and ProU systems. This observation is intriguing in view of the fact that these two transport systems also mediate the uptake of proline, even though proline and glycinebetaine do not seem to be very similar in structure. The ProU system is probably more important than the ProP system for the transport of glycinebetaine, because the ProU system has K_m of 1 μM for this quaternary amine, compared with a K_m of 44 μM for ProP (30, 31). The ProU system of *E. coli* and *S. typhimurium* contains a periplasmic glycinebetaine-binding protein of 31 kilodaltons (kDa) (96, 163). This protein was purified from *E. coli* and was shown to have a K_D of 1.4 μM for glycinebetaine (15). The purified glycinebetaine-binding protein had no detectable affinity for proline, suggesting that the higher

in vivo activity of the ProU system with glycinebetaine than with proline as a substrate could be the consequence of the relative affinities of the binding protein for these two substrates.

Cairney et al. (31) reported that *S. typhimurium* mutants lacking both the ProU and the ProP systems were almost completely defective in glycinebetaine transport activity, and therefore they concluded that the ProP and ProU systems were the only two porters mediating the uptake of glycinebetaine. Cairney et al. (31) did not report whether mutations in the ProP and ProU systems abolished the stimulatory effect of glycinebetaine in media of high osmolarity. However, LeRudulier and Bouillard (134) and a colleague and I (55) observed that glycinebetaine can alleviate osmotic inhibition in *proU proP* double mutants. This was found to be true for every *proU proP* double mutant we tested, including strains with transposon insertions in both the *proP* and *proU* loci (J. A. Gutierrez and L. N. Csonka, *in* J. H. Cherry, ed., *Biochemical and Physiological Mechanisms Associated with Environmental Stress Tolerance in Plants*, in press). Growth studies indicated that *proP proU* double mutants experienced a long lag after inoculation into media containing 0.8 to 1.0 M NaCl plus 1 mM glycinebetaine, but once the strains began to grow, their growth rates were similar to that of the wild-type strain. Furthermore, the steady-state levels of glycinebetaine in *proP proU* double mutants grown in the presence of [^{14}C]glycinebetaine in media of high osmolarity were very similar to those observed in *proP*$^+$ or *proU*$^+$ strains (Gutierrez and Csonka, in press). Thus, our observations suggest that there is an additional glycinebetaine permease besides the ProP and ProU systems in *S. typhimurium*.

S. typhimurium mutants that were not stimulated by glycinebetaine in media of elevated osmolarity have been isolated (55). Because these mutants responded normally to stimulation by proline in media of elevated osmolarity, we concluded that the mutations in the strains affect the osmoprotectant ability of glycinebetaine but not of proline. The mutations (designated *bet*) were mapped roughly to 70 to 80 map units of the *S. typhimurium* chromosome (M. Haskell and L. N. Csonka, unpublished results), suggesting that they are not allelic to either the *proP* or the *proU* locus, which are at 93 and 57 map units, respectively. The [^{14}C]glycinebetaine transport activities of *proU*$^+$ *proP*$^+$ *bet* mutants were comparable to those of *bet*$^+$ control strains (Gutierrez and Csonka, in press), and so the *bet* mutations do not impair glycinebetaine uptake. At present, a satisfactory understanding of the *bet* mutations is lacking, but they may affect some cellular function that is required for growth in media of high osmolarity in the presence of glycinebetaine.

Choline. Although enteric bacteria are unable to synthesize glycinebetaine from glucose or other carbon sources, *E. coli* K-12 can convert choline to glycinebetaine under conditions of osmotic stress, so that choline is also an osmoprotectant for *E. coli* (130, 250, 251). The formation of glycinebetaine from choline entails two oxidation steps, with glycinebetaine aldehyde as the intermediate (130). In *E. coli*, a single enzyme, which has both choline and glycinebetaine aldehyde dehydrogenase activities, is encoded in the *betA*$^+$ gene (3). This enzyme is membrane bound and its activity is coupled to electron transport, so that it requires some terminal electron acceptor, such as O_2 (130). Because of this requirement for an electron acceptor, choline cannot be used as an osmoprotectant anaerobically (130). *E. coli* also possesses a nicotinamide adenine dinucleotide-linked glycinebetaine aldehyde dehydrogenase activity, specified by the *betB*$^+$ gene. The uptake of choline is mediated by two transport systems, one of which has a K_m of 8 μM and the other a K_m of 1.5 mM; the former is encoded in the *betT*$^+$ gene, and the structural gene for the latter has not been defined. The *betA*$^+$, *betB*$^+$, and *betT*$^+$ genes are clustered at 7.5 min on the *E. coli* chromosome. *S. typhimurium* lacks corresponding genes and therefore is unable to use choline as an osmoprotectant; introduction of the three *bet* genes of *E. coli* on a cloning vector into *S. typhimurium* conferred the ability to respond to choline as an osmoprotectant (3).

Eshoo (63) analyzed the transcriptional regulation of the *bet* genes of *E. coli* by using strains carrying transcriptional fusions of the *lacZ* gene to the *betA*, *betB*, and *betT* genes and found that osmotic stress elicited a 7- to 10-fold increase in the transcription of these three genes. The addition of choline to the media of high osmolarity resulted in an additional threefold increase in the expression of the *bet-lacZ* fusions. Maximal transcription of the three *bet* genes is dependent on the presence of O_2 (63), in accord with the observation that choline cannot be converted to glycinebetaine anaerobically (130).

Other osmoprotectants. A number of structural analogs of proline and glycinebetaine also have osmoprotecting effects. Dimethylthetin (*S*,*S*-dimethyl-sulfoniumacetate) is as effective as glycinebetaine as an osmoprotectant for *E. coli* (37). Other compounds that are less effective are stachydrine (*N*,*N*-dimethylproline), β-butyrobetaine, L-pipecolate (131, 137), and 5-hydroxy-L-pipecolate (79). *N*,*N*-Dimethylglycine and *N*-methylproline also have a slight osmoprotectant activity for *K. pneumoniae* (138). Glutamate betaine (*N*,*N*,*N*-trimethyl glutamate) was reported to be synthesized in cyanobacteria under conditions of osmotic stress (217), but there are no reports on the ability of this compound to alleviate osmotic stress when supplied exogenously.

OSMOREGULATION OF THE PERIPLASMIC SPACE

In gram-negative bacteria, there is a compartment between the cytoplasmic membrane and the peptidoglycan cell wall, known as the periplasm or periplasmic space, which houses a number of hydrolases for macromolecular nutrients, binding proteins for metabolites, and receptors for chemotactic signals (204). The periplasmic space occupies approximately 20 to 40% of the total volume of *E. coli* and *S. typhimurium* (248), and it is maintained as a separate compartment during steady-state growth at all conditions of osmolarity (220). Because solutes of ≤500 Da can readily diffuse into the periplasmic space through porin proteins located in the outer membrane (196), there are unique problems in the maintenance of the osmotic potential of the periplasm.

Membrane-Derived Oligosaccharides

The periplasmic space of enteric bacteria contains large quantities of highly anionic polysaccharides, known as membrane-derived oligosaccharides, which are too large to diffuse through the porin proteins (115, 116). In *E. coli*, these molecules consist of 6 to 12 glucose units which are held together by β-1,2 or β-1,6 linkages, and they carry *sn*-1-phosphoglycerol, phosphoethanolamine, and *O*-succinyl side chains. Their average molecular mass is about 2,300 Da, and their average charge is −5 (115). *Agrobacterium tumefaciens* and *Rhizobium* species also contain high-molecular-mass β-1,2 glucans, which probably function to maintain the turgor pressure of the periplasm of these organisms (173).

The presence of these anionic polymers in the periplasm gives rise to an electric potential across the outer membrane. This potential, known as the Donnan potential, results in the accumulation of cations at a higher concentration in the periplasm than in the medium, resulting in hydrostatic pressure in the periplasmic space (115). Stock et al. (248) originally demonstrated the existence of a Donnan potential across the outer membrane, and their observations were confirmed by Sen et al. (236).

Kennedy (115) discovered that synthesis of the membrane-derived oligosaccharides was subject to osmotic regulation so that they are synthesized maximally in media of low osmolarity and increasing osmolarity results in a reduction in their synthesis. This observation was in accord with an earlier finding of Munro and Bell (186), who reported that osmotic stress inhibited the turnover of phosphatidylethanolamine, which serves as the precursor of the phosphoethanolamine residue in the membrane-derived oligosaccharides. In *E. coli*, the enzymes involved in the formation of membrane-derived oligosaccharides are expressed constitutively, suggesting that the osmotic control of synthesis of these polysaccharides entails regulation of the catalytic activity of at least one of these enzymes (116). Mutations in the *mdoA* or *mdoB* gene block the synthesis of membrane-derived oligosaccharides (65, 116). Strains carrying these mutations nevertheless do not show any growth defects in media of high or low osmolarity. Kennedy (116) argued that the absence of sensitivity of *mdoA* and *mdoB* mutants to media of low osmolarity indicates that there is an alternative mechanism besides the synthesis of membrane-derived oligosaccharides for maintaining the osmotic potential of the cytoplasm which has not yet been discovered.

OSMOTIC CONTROL OF TRANSCRIPTION

Clark and Parker (41) carried out a two-dimensional electrophoretic analysis of the effect of the osmolarity of the growth medium on the protein composition of *E. coli*. They found that only three proteins were synthesized at preferentially higher rates in response to hyperosmotic shock, and none were synthesized at detectably increased rates when the cells were exposed to hypoosmotic shock. Although, as shall be seen, this result is an underestimate of the number of proteins whose synthesis is under osmotic control, it nevertheless points out that there are only few genes whose transcription is osmotically regulated. In the next section, I will address the transcriptional regulation of some of these genes.

kdp

Initially, Rhoads et al. (219) reported that the *kdp* operon of *E. coli* is repressed by high concentrations of exogenous K^+. The conclusion was modified by Laimins et al. (129) as a result of their experiments analyzing the transcriptional regulation of *kdp-lacZ* fusions, which revealed that the *kdp* operon could be induced even in the presence of excess K^+ by hyperosmotic shock. Induction could be brought about by high concentrations of any ionic or nonpolar solutes that were excluded by the membrane, but not by glycerol. However, Laimins et al. (129) observed that exposure of the cells to hyperosmolarity in media containing concentrations of K^+ sufficient for the cells to maintain turgor resulted in only a transient burst of elevated transcription of the *kdp* operon and expression of the operon returned to its basal value 30 min after the hyperosmotic shock. There seems to be an interplay between hyperosmotic shock and the intracellular K^+ concentration in the regulation of expression of the *kdp* operon, because K^+ limitation can cause permanent induction of the operon even in media of low osmolarity (78, 129, 253).

To account for the observed transcriptional regulation of the *kdp* operon, Laimins et al. (129; see also references 57 and 263) proposed that the turgor pressure of the cell is the only signal that regulates the expression of this operon. They suggested that the effects of K^+ limitation on *kdp* expression are indirect, because reduction in the intracellular K^+ concentration results in a loss of turgor, which then would cause induction of the *kdp* operon. The increased uptake of K^+ through the Trk and Kdp systems would restore turgor and thus result in repression of the *kdp* operon.

The transcription of the *kdpABC* genes is under positive control of the *kdpD* and *kdpE* proteins, which are involved in sensing the turgor and transferring the signal to the *kdpABC* promoter. The KdpD product has been proposed to span the periplasmic space and to form contacts with both the inner and outer membranes (57, 263). It might sense changes in cell turgor by measuring the distance between the inner and outer membranes and then transmit this signal to the KdpE protein, which would then act as a transcriptional activator of the *kdpABC* operon in response to the loss of turgor.

Although the idea that turgor is the sole regulatory signal for expression of the *kdpABC* operon is attractive because it proposes homeostatic control of turgor pressure, some experimental results appear to be inconsistent with it. Gowrishankar (78) and Sutherland et al. (253) found that different types of solutes do not have equal effects on the expression of the *kdp* operon, because nonpolar solutes, such as glucose, caused a transient induction of the operon whereas the ionic solutes NaCl and $(NH_4)_2SO_4$ resulted in a permanent induction even in media containing high concentrations of K^+. In a detailed analysis of this phenomenon, Sutherland et al. (253) observed that during steady-state growth, the expression of the *kdp* operon as a function of the exogenous K^+ concentration was identical in a medium of low osmolarity and in media of high osmolarity containing 0.44 M sucrose or 0.3 M choline chloride. In all three cases, the *kdp* operon was fully repressed at K^+ concentrations $\geq$5 mM, and it was expressed at progressively higher levels as a function of decreasing K^+ concentrations below this threshold. However, Sutherland et al. (253) found that in cells growing exponentially in the presence of 0.3 M NaCl, the *kdp* operon was fully repressed only at K^+ concentrations $\geq$50 mM, and at any K^+ concentration below 50 mM steady-state expression of the operon was higher in cells grown in the presence of 0.3 M NaCl than in the other three media, in agreement with the results of Gowrishankar (78). Sutherland et al. (253) suggested that this result does not contradict the turgor control model of *kdp* expression but rather might have been the consequence of the fact that the high concentrations of Na^+ (and presumably NH_4^+) either inhibit the Trk system or stimulate K^+ efflux and thus result in decreased cell turgor. It should be noted that since 0.3 M NaCl does not cause markedly greater growth inhibition than osmotically equivalent concentrations of sucrose or other solutes, the cellular turgor in the presence of high concentrations of NaCl is probably not very different from the turgor in the presence of the other solutes. (It is possible that the inhibition of K^+ uptake by Na^+ and NH_4^+ results in a small decrease in turgor which has no effect on cell growth but is nevertheless sufficient to derepress *kdp*.) However, as Gowrishankar (80) pointed out, the observation made by

Sutherland et al. (253) that the expression of the *kdp* operon as a function of the extracellular K^+ concentration in cells grown in medium of low osmolarity or in the presence of 0.44 M sucrose was identical to that in cells grown in the presence of 0.3 M choline chloride is more difficult to reconcile with turgor being the only regulatory signal for *kdp* expression. Choline is efficiently oxidized to glycinebetaine in media of high osmolarity (130), and glycinebetaine is a more effective compatible solute than K^+ (253). If the expression of *kdp* were dependent solely on turgor, then the threshold for *kdp* repression would be expected to occur at a lower K^+ concentration in the presence of 0.3 M choline chloride than in the presence of 0.44 M sucrose or in media of low osmolarity. Thus, these observations raise questions about the hypothesis that turgor is the sole regulator of *kdp* expression and suggest that this operon is at least in part regulated by some factor that is related directly or indirectly to the availability of K^+ (80).

proU

Transcriptional regulation. Studies with *lac* (14, 30, 31, 55, 56, 78) and *phoA* fusions (84) demonstrated that hyperosmotic stress elicits a several hundred-fold induction of transcription of the *proU* operon of *E. coli* and *S. typhimurium*. As is the case with *kdp* operon, induction of *proU* can be triggered by solutes that are excluded by the membrane but not by substances that can freely traverse this permeability barrier. However, unlike the *kdp* operon, the *proU* operon is always induced permanently as long as the cells are exposed to media of high osmolarity (56, 78, 253), whereas the *kdp* operon is induced only temporarily (129). The steady-state level of transcription of *proU* is a sigmoidal function of the osmolarity in the medium, with ~0.2 osm being the threshold osmolarity for induction and ~1.0 osm resulting in maximal expression (31, 78).

A detailed analysis of the kinetics of induction of *proU* in *S. typhimurium* performed by Jovanovich et al. (111) revealed three stages in the process. After exposure of the cells to hyperosmotic shock, there was a 15- to 20-min lag before increased transcription of *proU* was detectable. Next, there was rapid stimulation of transcription of the *proU* operon at a differential rate that greatly exceeded the steady-state differential rate observed in cells growing in a medium of equal osmolarity. Finally, the differential rate of transcription of *proU* gradually decreased to the steady-state value. Although the kinetics of induction of *kdp* and *proU* operons have not yet been analyzed under identical conditions, Jovanovich et al. (111) concluded that the two processes are sufficiently similar to suggest that they may be regulated by a common signal.

Structure of the *proU* operon. Southern blot analysis of the DNA of two *S. typhimurium* strains carrying *proU*::Tn*10* insertions and 12 strains carrying *proU-lacZ* transcriptional fusions generated by phage Mu d1 revealed that the size of the *proU* locus is at least 2.6 kilobase pairs (kbp) (D. Overdier and L. N. Csonka, unpublished results). Because a *proU*::Tn*10* insertion which is close to the *proU* promoter proved to be polar on the expression of the *proU-lacZ* fusion that is most distal from the *proU* promoter, we concluded that the 2.6-kbp *proU* region is transcribed as a single messenger RNA (mRNA). In an attempt to identify the structural gene for the glycinebetaine-binding protein, we screened all available mutants carrying *proU* insertions (2 Tn*10*'s, the 12 Mu d1's that were mapped by Southern blot analysis, and an additional 13 Mu d1's that are in the incorrect orientation for transcription of the *lac* genes) and found that the glycinebetaine-binding protein was absent from all of them (S. A. Fletcher, M. M. Ederer, and L. N. Csonka, unpublished results). The simplest interpretation of these results is that the structural gene for the glycinebetaine-binding protein is the last gene within the 2.6-kbp region we analyzed. We cloned the *proU-lacZ* fusion that is most proximal to the promoter and determined the nucleotide sequence of the 5′ end of the *proU* operon. This analysis revealed that the gene closest to the probable transcription start site of the *proU* operon has considerable amino acid homology to the HisP and MalK proteins (L. Csonka, D. Overdier, and E. Olson, unpublished data), which are two inner membrane components of the histidine and maltose transport systems, respectively (2).

Gowrishankar et al. (81) cloned the *proU*$^+$ operon of *E. coli* and demonstrated by Tn*1000* mutagenesis and complementation tests that it contains at least two genes, *proU* and *proV*. Because Tn*1000* mutations in *proV* were polar on the *proU* gene, these researchers concluded that *proV* is located upstream of *proU* within the operon. Interestingly, Gowrishankar et al. found that on high-copy-number plasmids, the *proV*$^+$ gene by itself without the other gene(s) of the *proU* operon resulted in enhanced sensitivity of the strains to osmotic stress. They also reported that the presence of the entire *proU*$^+$ operon on high-copy-number plasmids resulted in a 12-fold reduction in the induced levels of β-galactosidase in strains carrying a chromosomal *proU-lacZ* fusion. This effect was not due to the titration of some regulatory protein by the large number of copies of the *proU* region, because plasmids which carried only the *proV* gene under control of its native promoter without the other gene(s) of the operon did not diminish the expression of the chromosomal *proU-lacZ* fusion. Gowrishankar et al. (81) suggested that the cytoplasmic levels of proline or glycinebetaine accumulated by the ProU system regulate the expression of the *proU-lacZ* fusions.

May et al. (163) isolated several *proU-lacZ* fusions in *E. coli* and subsequently Barron et al. (15) demonstrated that one of these fusions [Φ(*proU-lacZ*)*2*] had joined the N-terminal portion of the glycinebetaine-binding protein to β-galactosidase. Faatz et al. (64) also cloned the *proU*$^+$ operon of *E. coli*, and on the basis of comparison of the restriction maps of the plasmids carrying the *proU*$^+$ operon and Φ(*proU-lacZ*)*2*, they concluded that the gene designated *proV* by Gowrishankar et al. (81) is the structural gene for the glycinebetaine-binding protein. Because of the size of the chromosomal DNA present on the plasmid carrying Φ(*proU-lacZ*)*2*, Faatz et al. (64) concluded that *proV* is the first gene of the *proU* operon. This conclusion is inconsistent with our DNA sequencing results; this discrepancy could be due to different organization of the *proU* operon in *E. coli* and *S. typhimurium* or to the fact that the structural genes for the inner membrane component and the glycinebetaine-binding protein of the ProU transport system might be in separate operons.

Models for *proU* regulation. Since the *proU* operon is expressed at a high level as long as the conditions of high osmolarity are maintained, transient loss of turgor alone cannot be the regulatory signal for the expression of this operon, as has been proposed for *kdp*. At least in principle, the osmotic strength (or the water activity) of the cytoplasm could be the signal for the transcription of *proU*, but the fact that solutes that can freely diffuse across the cytoplasmic membrane do not induce *proU* indicates that some other signal governs the transcriptional regulation of this operon.

Because the steady-state concentration of K^+ increases with increasing external osmolarity, Epstein (57) and Sutherland et al. (253) proposed that the intracellular concentration of this cation might be the regulatory signal for the induction of *proU*. To the extent that the experimental conditions used for the measurement of the kinetics of accumulation of K^+ elicited by hyperosmotic stress in *E. coli* K-12 or *E. coli* B (52, 201) are comparable to those used for the determination of the induction profile of *proU* in *S. typhimurium* (111), the fact that these two processes take place approximately in synchrony is in support of this hypothesis. Also in accord with this proposal, Sutherland et al. (253) found that severe reduction of intracellular K^+ levels (due to growth of cells in K^+-deficient media or the presence of a *kdp* mutation) specifically impaired the osmotic induction of *proU-lacZ* fusion, in comparison to other operons or overall protein synthesis. In further support of this model, Sutherland et al. (253) observed that the addition of glycinebetaine resulted in an approximately 25% reduction in the K^+ concentration of cells growing in 0.3 M NaCl, and it caused a more than threefold reduction in the level of transcription of a *proU-lacZ* fusion. However, Sutherland et al. reported that even though exogenous proline caused very nearly the same reduction as glycinebetaine in the K^+ concentration of cells grown in 0.3 M NaCl, it nevertheless brought about a considerably smaller decrease than glycinebetaine in the expression of *proU-lacZ* fusion. Since it is not fully settled whether osmotic stress causes merely a transient (52, 201) or permanent (61, 220) increase in the K^+ levels of *E. coli*, no compelling proposals can yet be made for possible regulatory roles of this cation in osmotic adaptation.

In an attempt at identifying components required for *proU* regulation, Druger-Liotta et al. (53) isolated over 60 mutants of *S. typhimurium* that expressed this operon at elevated levels in the absence of osmotic stress. The mutations in each of these strains proved to be closely linked to *proU* and *cis*-dominant, suggesting that they were probably alterations in the sites required for the transcriptional control of the operon. The failure to generate mutations in a gene encoding a *trans*-acting regulatory protein for *proU* could have been due to the fact that mutations inactivating this protein might be lethal. Alternatively, it might be that the transcriptional control of *proU* is brought about entirely by *cis*-acting signals contained in the *proU* promoter region, without the involvement of any specific regulatory proteins. Several mechanisms are conceivable for such a control mechanism. For example, the binding of ribonucleic acid (RNA) polymerase to the *proU* promoter may be highly sensitive to the electrolyte concentration of the cytoplasm, or perhaps regulation of the *proU* operon may be affected by a transcription termination or attenuation mechanism, whose efficiency is determined by the osmolarity of the cytoplasm.

DiBlasio and Vinopal (Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, K123, p. 214) isolated a temperature-sensitive lethal mutation in *E. coli* that resulted in constitutive expression of the *proU* operon at the permissive temperature. This mutation proved to be closely linked to the *topA* gene (the structural gene for DNA topoisomerase I), suggesting that the transcriptional regulation of the *proU* operon may be affected by alterations in the supercoiling of the *proU* promoter brought about by changes in the intracellular osmolarity. Higgins et al. (97) also proposed this model, and to test it, they determined the in vitro effects of osmotic stress on the DNA supercoiling. They found that osmotic stress brought about a marked increase in the number of negative supercoils in plasmids with ColE1 or pACYC origins of replication. The supercoiling of the DNA of *E. coli* and *S. typhimurium* is in part determined by the opposing actions of gyrase (which introduces negative supercoils) and topoisomerase I (which removes negative supercoils). In support of the model that supercoiling regulates *proU* expression, Higgins et al. (97) found that the addition of inhibitors of gyrase (novobiocin and nalidixic acid) or the introduction of mutations impairing the activity of gyrase resulted in inhibition of the expression of *proU*. Conversely, mutations in the structural gene for topoisomerase I (*topA*) resulted in elevated expression of *proU*. Lastly, by selecting mutants expressing *proU-lacZ* fusions at high levels in the absence of osmotic stress, Higgins et al. (97) obtained strains with increased DNA supercoiling; the mutations responsible for these phenotypes mapped to two loci, *topA* and a newly identified locus, *osmZ*.

Although Higgins et al. (97) demonstrated convincingly that supercoiling influences the level of transcription of *proU*, their data did not show that the osmotic control of *proU* transcription is necessarily exerted by supercoiling. For example, the gyrase inhibitor novobiocin had different effects in *S. typhimurium* and *E. coli* on the induction of *proU*. In the former organism, this antibiotic increased the basal level of *proU* expression slightly and reduced the induced level about sixfold, resulting in an overall eightfold decrease in the induction ratio, whereas in the latter bacterium, it caused an approximately twofold reduction in both the basal and the induced levels of *proU*, thus not influencing the induction ratio at all. Also, the supercoiling of various mutants was not correlated with the level of *proU* expression, because in some instances different mutants with equal DNA supercoiling expressed *proU* at unequal levels. Furthermore, there were inconsistencies in the effects of *topA* mutations in *S. typhimurium*; one allele (*topA2770*) resulted in about a threefold increase in the basal expression of *proU*, whereas another allele (Δ*topA2771*) resulted in a fivefold decrease. Higgins et al. (97) suggested that the Δ*topA2771* deletion may result in excessive supercoiling of the DNA and thus interfere with the expression of *proU*. However, they reported that the *proU* operon could be induced by osmotic stress even in the Δ*topA2771* strain, so if supercoiling did in fact regulate the transcription of *proU*, then it could not already have been excessive in the Δ*topA* strain in media of low osmolarity.

Supercoiling has far-ranging effects on the recognition of a large number of promoters by RNA polymerase, since over 80% of random promoters in *E. coli* and *S. typhimurium* exhibited altered efficiencies of transcription upon treatment with the gyrase inhibitor coumermycin A (110). It is noteworthy that anaerobiosis alters DNA supercoiling (274). Mutations in the genes for the two subunits of gyrase were found to cause decreased transcription of the *hisR* gene and thus indirectly result in constitutive synthesis of the enzymes of the histidine pathway (227). The *osmZ* mutations have been shown to be highly pleiotropic (97). Thus, to the extent that osmotic stress modulates the supercoiling of the chromosome, it should affect the expression of a large number of genes whose transcription is sensitive to supercoiling. On the contrary, as discussed below, there are only a few genes whose expression is subject to osmotic control. While Higgins et al. (97) clearly demonstrated that the transcription of the *proU* promoter is dependent on the supercoiling, the question of whether the osmotic control of the transcription of the *proU* promoter is affected primarily by supercoiling needs to be explored further.

proP

Initial studies involving *proP-lacZ* fusions indicated that there was only a minor (<3-fold) induction in the transcription of the *proP* locus during steady-state growth in media of high osmolarity (30, 56, 79). However, when Jovanovich et al. (111) examined the transcriptional control of *proP* during adaptation to hyperosmotic shock, they found that the kinetics of induction of *proP* were also triphasic. Initially there was a 15- to 20-min lag before induction of the *proP-lacZ* fusion was detectable. Then there was a rapid induction of the *proP* gene, with the differential rate of its expression increasing 17-fold. After an additional 25 min, the differential rate of expression of the gene slowly decayed to a value that was only 1.4-fold above the basal level. Jovanovich et al. (111) pointed out that the kinetics of induction of the *proP* locus were similar to those of the *proU* and *kdp* operons during the initial stages of adaptation to hyperosmotic stress.

ompF and *ompC*

Gram-negative bacteria contain a number of porin proteins embedded into the outer membrane that act as channels to facilitate the diffusion of small hydrophilic molecules across this outermost permeability barrier of the cells (189, 196). *E. coli* and *S. typhimurium* have two major porins, the OmpF and OmpC proteins, whose production is regulated by a variety of environmental conditions, such as osmolarity of the medium (17, 144, 242, 259), carbon source (235), and temperature (146). The total cellular levels of the OmpF and OmpC proteins are fairly constant, but the relative proportion of the two varies, so that conditions that favor the synthesis of OmpF result in repression of OmpC, and vice versa. Media of low osmolarity, poor carbon sources, and low temperature increase the levels of OmpF and diminish the levels of OmpC, and conversely media of high osmolarity, good carbon sources, and high temperature cause a reduction in the levels of OmpF and an increase in the levels of OmpC.

Structures of the OmpF and OmpC proteins. The porin proteins of *E. coli* are very similar both in their amino acid sequences and in the nucleotide sequences of their structural genes (180). The DNA homology between the *ompC*$^+$ and *ompF*$^+$ genes of *E. coli* is sufficient to permit the in vivo formation of hybrid genes consisting of various portions of the N-terminal end of the *ompF* gene fused to the corresponding C-terminal portion of the *ompC* gene that yield fully functional protein products (198). The channels formed by the OmpF and OmpC proteins are composed of trimers of the respective monomeric subunits. The OmpF channel has a slightly larger cut-off than the OmpC porin for the size of the molecules that they can pass through the outer membrane, with the estimated pore diameters of the OmpF and OmpC channels being 1.13 to 1.16 nm and 1.02 to 1.13 nm, respectively (89, 196).

Positive-controlling OmpR and EnvZ proteins. The synthesis of the OmpC and OmpF porins is dependent on the OmpR and EnvZ proteins, which together act as positive regulatory factors for the transcription of the *ompC* and *ompF* genes. The OmpR protein is the actual transcriptional activator for both porin structural genes; the EnvZ protein, which is embedded into the cytoplasmic membrane (68, 141), has been proposed to be the osmosensor that modifies the OmpR protein, activating the transcription of the *ompF* gene in media of low osmolarity and of the *ompC* gene in media of high osmolarity (88).

The *ompR* and *envZ* genes constitute an operon (designated the *ompB* locus), with *ompR* being the promoter-proximal gene. Although the two genes are transcribed as a single mRNA, the OmpR protein is present in great excess of the EnvZ protein, probably because the *envZ* gene lacks an efficient Shine-Dalgarno sequence (42, 139, 140, 182, 254, 272). The OmpR and EnvZ proteins are also required for the transcriptional activation of the genes of a tripeptide permease (*tppB*) of *S. typhimurium*, (75) and of an outer membrane protease (*opr*) of *E. coli* (36). The expression of a plasmid-encoded gene for microcin B17 (*mcb*) is dependent on the OmpR protein (92), and it presumably also requires the EnvZ protein, but this has not yet been demonstrated.

The EnvZ and OmpR proteins belong to a group of bacterial regulatory proteins that show an intriguing amino acid sequence similarity (224, 249). This group consists of pairs of regulatory proteins that also include the PhoB and PhoR proteins (controlling the phosphate regulon of *E. coli*), the NtrB and NtrC proteins (for the regulation of the genes of nitrogen metabolism in *Enterobacteriaceae*), the CpxA and SfrA proteins (regulating the synthesis of membrane proteins in response to toxic compounds in *E. coli*), the VirA and VirG proteins of *Agrobacterium tumefaciens* (regulating the expression of other *vir* genes required for infection of plant cells), the DctB and DctD proteins of *Rhizobium leguminosarum* (serving as the transcriptional regulators of a dicarboxylic acid permease), and the CheA and CheY proteins of *E. coli* (which regulate the direction of rotation of the flagellar motor during chemotaxis). The first member of each of the above pairs of regulatory proteins is the sensor of an environmental signal (e.g., osmolarity in the case of EnvZ, phosphate availability in the case of PhoB), and it transduces the information to the second partner of the pair, which acts as the positive effector of the respective cellular response. For all these pairs of proteins except CheA and CheY, the modification of the second member of each pair results in the transcriptional activation of the genes of the corresponding regulons. In the case of the CheY protein, its modification by CheA determines whether the flagella rotate clockwise or counterclockwise. The conversion of the NtrC and the CheY proteins into the active form has been shown to involve their phosphorylation by the NtrB (114, 197) and the CheA proteins (93, 273), respectively. Recently, it was demonstrated that the EnvZ protein can be phosphorylated in vitro (102), and therefore transformation of the OmpR protein into the activator form for the transcription of the *ompF* and *ompC* genes probably involves its phosphorylation by EnvZ. The OmpR protein makes direct contacts with RNA polymerase in activating the transcription of the *ompC* and *ompF* genes, as indicated by the observation that mutations in the *rpoA* gene (which encodes the α subunit of RNA polymerase) can affect the OmpR-dependent transcription of the *ompC* and *ompF* genes (71, 161).

Analysis of the *ompR* and *envZ* genes has been very complicated, and thus far has not fully resolved the roles of their products in the regulation of the synthesis of the OmpC and OmpF proteins. Null mutations (deletions, transposon insertions, and nonsense mutations) were isolated in the *ompR-envZ* operon of *E. coli* and *S. typhimurium* (19, 29, 72, 73, 75, 127, 240). Nonsense mutations or transposon insertions in *envZ* abolished the synthesis of the OmpC protein under all conditions of osmolarity but permitted the synthesis of low levels of OmpF that was still dependent on the OmpR protein. Null mutations that affect only the *ompR* or both the *ompR* and the *envZ* genes virtually abolished the

synthesis of the OmpF and the OmpC porins under all growth conditions.

Missense mutations in the two regulatory genes can result in almost any phenotype with respect to the expression of the two porins, so that *ompR* or *envZ* mutations causing $OmpC^-$ $OmpF^-$, $OmpC^-$ OmpF-constitutive [OmpF (Con)], or OmpC-constitutive [OmpC(Con)] $OmpF^-$ phenotypes or various gradations of expression of one or the other porin have been reported (86–88, 142, 192, 231, 260). "Reverse osmoregulation" of the OmpF gene (i.e., its high-level expression in media of high osmolarity and low level expression in media of low osmolarity) can be conferred by mutations in both the *ompR* and the *envZ* genes (127, 191). Several so-called pleiotropic *envZ* mutations have been isolated that confer an $OmpF^-$ OmpC(Con) phenotype and also result in diminished synthesis of unrelated envelope proteins, such as the MalE, LamB, PhoA, and PhoE proteins, and an iron permease (145, 268, 269). Null mutations in *ompR* or *envZ* do not alter the synthesis of any of these other envelope proteins, implying that the OmpR and EnvZ proteins do not function directly in the transcriptional control of their formation (33, 71, 72, 240, 261).

Three interesting exceptions to the generalization that expression of the OmpF and OmpC porins is dependent on a functional EnvZ protein have provided insights into the mode of action of the OmpR and EnvZ proteins. First, Matsuyama et al. (160) isolated an *ompR* allele (*ompR77*) that suppressed the defect in the expression of the various envelope proteins caused by a pleiotropic *envZ* allele (*envZ11*). The OmpR77 protein could correct the defect only in strains carrying the *envZ11* allele but not in strains carrying other *envZ* mutations, indicating that there is probably a direct interaction between the EnvZ and OmpR proteins. Second, overproduction of the $OmpR^+$ protein (from the $ompR^+$ gene on pBR322) resulted in the constitutive synthesis of OmpC, even in strains carrying an *envZ*::Tn*10* mutation (240). Third, there is an *ompR* mutation [Φ(*ompR-lacZ*)*11*, a fusion of almost the entire *ompR* gene to *lacZ*] which conferred an OmpF(Con) $OmpC^-$ phenotype even on strains carrying an *envZ*::Tn*10* mutation (19, 240). The last two observations imply that it is not necessary for the EnvZ protein to interact directly with the *ompC* promoter to activate its transcription.

The transcriptional regulation of the *ompF* and *ompC* genes was explained by a model in which the OmpR protein acts as the transcriptional activator of the *ompC* and *ompF* genes (88). The OmpR protein needs to be modified by the EnvZ protein (probably by phosphorylation) for it to function as an efficient activator of transcription of the *ompC* and *ompF* genes. The OmpR protein can be in two (or more; see reference 240) conformations or states of modification, of which one favors transcription of the *ompF* gene at low osmolarity and the other favors transcription of the *ompC* gene at high osmolarity. The interconversion of the OmpR protein between the various forms is mediated by the EnvZ protein, whose activity is dependent on the osmolarity of the growth medium. Since the EnvZ protein is present at much lower levels than OmpR, it probably acts catalytically to modify OmpR. In the original form of the model, Hall and Silhavy (88) proposed that the OmpR protein can be interconverted by the action of the EnvZ protein between a monomeric and a multimeric form, with the monomeric form activating transcription of *ompF* and the multimeric form activating transcription of *ompC*. Although there are genetic complementation results indicating that the OmpR protein exists as a multimer (88, 142, 193), in view of subsequent experimental results, a two-state model for the modification of OmpR by EnvZ is inadequate to explain all the roles of OmpR as a transcriptional activator, and the original model formulated by Hall and Silhavy (88) will probably prove to be an oversimplification (240).

***cis*-Acting regions required for transcriptional regulation of *ompF* and *ompC*.** The function of the OmpR protein in activating the transcription of the *ompF* gene was analyzed by Inokuchi et al. (107) and Ostrow et al. (206) in experiments in which various portions of the promoter region of the *ompF* gene of *E. coli* were fused to a promoterless *lacZ* gene, and the effects of osmotic stress and *ompR* or *envZ* mutations on the expression of β-galactosidase were determined. Inokuchi et al. constructed plasmids in which a DNA fragment carrying the *ompF* promoter region, from nucleotide −111 to nucleotide +123, was fused to the *lacZ* gene. (Nucleotide +1 refers to the transcription start site of operons; negative numbers indicate nucleotide positions upstream and positive numbers indicate nucleotide positions downstream of the transcription start site.) Inokuchi et al. (107) found that the synthesis of β-galactosidase from this construct responded to osmotic control and was dependent on functional $OmpR^+$ product in a manner that was similar to that seen for expression of the chromosomal *ompF* gene, and therefore they concluded that the *cis*-acting sites required for the OmpR-dependent osmotic control of the *ompF* gene are present in this 234-nucleotide-long region. Ostrow et al. (206) carried out a similar analysis but arrived at somewhat different conclusions about the *cis*-acting regions that are required for the osmoregulation of *ompF*. These researchers constructed chimeric genes in which portions of the *ompF* promoter region, extending from nucleotide −240 to various points in the coding sequences for the *ompF* gene, were joined to a promoterless *lacZ* gene. Each of these constructs could express the *lacZ* gene in an $OmpR^+$- and $EnvZ^+$-dependent manner. However, expression of the *lacZ* gene was independent of the osmolarity of the medium and was similar to the repressed levels seen in wild-type cells in media of high osmolarity. The latter result suggested that some region within 240 nucleotides upstream of the transcription start site of the *ompF* gene is necessary for the EnvZ- and OmpR-dependent transcription of the gene but is not sufficient for osmotic control. Ostrow et al. constructed additional plasmids in which a ~1,200-bp DNA fragment that is normally upstream of nucleotide −240 was introduced into the corresponding position in the above plasmids. The presence of this additional fragment restored more normal osmotic control to the *lacZ* gene, indicating that some sequences in the region from −1200 to −240 nucleotides upstream of the OmpR-binding site are required for osmotic control of expression of the *ompF* gene. The reason for the discrepancy between the results of Inokuchi et al. and Ostrow et al. is not clear, but it may be due to the fact that the former group used constructs on high-copy-number plasmids, whereas the latter group placed the gene fusions on phage λ inserted into the chromosome in single copy.

There is extensive biochemical evidence that the OmpR protein binds to the promoter regions of the *ompC* and *ompF* genes. The *cis*-acting sites required for the initiation of transcription of the *ompC* and *ompF* genes were initially localized by nucleotide sequence analysis of deletions or point mutations that resulted in diminished, enhanced, or OmpR-independent expression of the *ompC* and *ompF* genes (50, 106, 179, 207, 256). Two groups purified the OmpR protein to homogeneity and demonstrated that it specifically binds to the promoter regions of the *ompC* and *ompF* genes

(109, 199). Deoxyribonuclease I protection experiments indicated that the $OmpR^+$ protein binds to a region from nucleotides −41 to −103 upstream of the major transcription start site of the *ompC* gene (149, 150) and to nucleotides −51 to −111 upstream of the *ompF* start site (183, 199). Mizuno et al. (183) also performed the binding studies with two missense mutant OmpR proteins that were purified to homogeneity. One of these mutant proteins, OmpR2, which results in an $OmpC^-$ OmpF(Con) phenotype, did not bind to the *ompC* promoter and showed reduced areas of contact with the *ompF* promoter. The second mutant protein, OmpR3, which confers an OmpC(Con) $OmpF^-$ phenotype, had unaltered patterns of binding to both the *ompC* and the *ompF* promoters. Because the OmpR3 protein can bind the *ompF* promoter normally, Mizuno et al. (183) proposed that the failure of the OmpR3 protein to activate the transcription of the OmpF porin may be due to loss of interaction with the EnvZ protein or some other factor. In the above binding studies, the wild-type OmpR protein seemed to bind equally to both the *ompC* and the *ompF* promoters, even though the in vivo expression of these genes varies inversely in response to the osmolarity of the medium. Thus, one implication of these results is that the binding of the OmpR protein by itself is insufficient for proper transcriptional control of the two porins. Since these studies were carried out in the absence of EnvZ protein, they did not illuminate the mechanism by which the OmpR protein is converted among the various forms that favor the transcriptional activation of the *ompC* over the *ompF* gene or vice versa.

Posttranscriptional control of OmpF and OmpC synthesis. Upstream of the *ompC* gene there is a promoter read in the opposite direction from the *ompC* promoter that initiates the synthesis of a 174-nucleotide-long RNA. This RNA, designated *mic*RNA, is highly complementary to the *ompF* mRNA in its 5′ region, spanning the translation initiation site. Introduction of the gene for the *mic*RNA on high-copy-number plasmids resulted in almost complete inhibition of the in vivo synthesis of the OmpF protein (181). Since expression of the *mic*RNA is regulated coordinately with the OmpC protein, Mizuno et al. (181) proposed that an additional layer of control over expression of the OmpF protein involves inhibition of the translation of the *ompF* mRNA by hybridization to the antisense *mic*RNA. However, the function of the *mic*RNA in cells carrying the *mic* gene in single copy is unclear: deletion of the *mic* gene did not result in a detectable effect on the steady-state levels of the OmpF protein under conditions of either low or high osmolarity (159). The *mic*RNA was proposed to be involved in a fine tuning of the transient rates of synthesis of OmpF during adaptation to hyper- or hypoosmotic shock (1). Recently, Misra and Reeves (177) reported that mutations at the *tolC* gene (which governs sensitivity to colicin E1) resulted in greatly increased synthesis of the *mic*RNA, which in turn caused inhibition of the synthesis of OmpF.

Regulatory interactions in the synthesis of envelope proteins. In addition to the above control mechanisms, there seems to be another, more general form of regulation of synthesis of a number of envelope proteins. Fiedler and Rottering (65) found that *mdoA* mutations resulted in increased levels of the OmpF protein and decreased levels of the OmpC protein in cells grown in medium of low osmolarity. These researchers suggested that the membrane-derived oligosaccharide concentration of the periplasm may be the signal sensed by the EnvZ protein, but they did not demonstrate that the effect of the *mdoA* mutation on the OmpF and OmpC levels is exerted at the level of transcription of the *ompF* and *ompC* genes. The induction of the LamB protein by maltose results in a reduction in OmpC levels (51), the synthesis of the LC porin in strains lysogenic for phage PA-2 brings about a reduction in the levels of both the OmpC and OmpF porins (69, 212), some *ompF* mutations result in high levels of OmpC (207), and conversely some *ompC* mutations cause elevated synthesis of OmpF (232). The mechanism of this concerted control of envelope protein synthesis is not understood.

Overview of OmpF and OmpC regulation. The control of the expression of porin proteins has been most extensively studied in enteric bacteria, and there is very scant information about the possible osmoregulation of porins in other gram-negative species. The osmotic strength of the medium was found to have an effect on the levels of two outer membrane proteins (OmpT and OmpU) in *Vibrio cholerae* (174), but it is not clear whether these proteins are analogous to the OmpF and OmpC proteins of *E. coli*. Although elaborate mechanisms control the relative levels of the OmpC and OmpF porins in enteric bacteria, the adaptive significance of this regulation is not obvious. Mutations in either the *ompC* or the *ompF* gene alone do not confer obvious growth defects under laboratory conditions. Small increases in resistance to certain antibiotics were observed in strains that had reduced levels of OmpF, in accord with the observation that the larger pore size of the OmpF porin than of the OmpC porin allows the more rapid diffusion of antibiotics across the outer membrane (196). Since conditions of high salinity and high temperature favor the synthesis of OmpC over OmpF, Nikaido and Vaara (196) conjectured that the OmpC porin may be synthesized preferentially when enteric bacteria are present in the intestinal tracts of their animal hosts, where nutrients are likely to be present at higher concentrations, and the OmpF porin is synthesized preferentially when they are in external environments, where the temperature and salinity are lower and nutrients are likely to be more dilute.

Other Genes Whose Expression Is under Osmotic Control

In addition to the operons discussed above, there are only a few other genes whose transcription is known to be influenced by the osmolarity of the medium. Osmotic stress was found to cause a slight decrease in the basal levels of several enzymes of the *mal* regulon (29, 78) and a fourfold increase in the expression of the *phoA* gene (261). Transcription of the *otsA* and *otsB* genes, whose products are required for the synthesis of trehalose during conditions of osmotic stress, is induced sixfold upon exposure of the cells to media of high osmolarity (74). As already discussed, the genes of the *bet* regulon of *E. coli*, which encode proteins for the transport of choline and its oxidation to glycinebetaine, are induced about 10-fold by hyperosmotic stress (63).

Gutierrez et al. (84) screened ~3×10^4 *E. coli* colonies mutagenized with Tn*phoA* for osmoregulated expression of alkaline phosphatase, and they identified 11 loci whose transcription was enhanced by osmotic stress. Gutierrez et al. (84) established that one of the Tn*phoA* insertions was in the *proU* operon and a second one was in the *ompC* gene. Subsequently, Boos et al. (21) showed that a third osmoregulated *phoA* fusion obtained by Gutierrez et al. (84) was in the *tre* operon, and it abolished the synthesis of the periplasmic trehalase. The genes mutagenized by the other insertions isolated by Gutierrez et al. were not identified. The above *phoA* fusions exhibited a 3- to 45-fold induction of alkaline phosphatase upon hyperosmotic stress, except for

the *proU-phoA* fusions, which caused an induction of 700-fold (84).

Is There a Global Osmosensing Regulatory Protein?

As the above discussion indicated, about 20 or so genes whose transcription is either enhanced or diminished by osmotic stress have been identified by *lac* or *phoA* fusions in *E. coli* and *S. typhimurium*. Since only dispensable genes could be targeted by this method, it is possible that osmoregulated genes which encode essential proteins have been overlooked. Nevertheless, as the two-dimensional electrophoretic analysis of Clark and Parker indicated (41), it is unlikely that a large number of osmoregulated proteins have been overlooked in *E. coli* or *S. typhimurium*.

The question arises of whether there are regulatory elements common to the osmoregulated operons. The OmpR and EnvZ proteins, which regulate the expression of the OmpC and OmpF porins, are not involved in the osmotic control of transcription of *proU* (31, 163) or the other osmotically regulated genes identified by Gutierrez et al. (84). The similarities in the initial kinetics of induction of the *kdp* (129), *proU*, and *proP* (111) loci suggest that these three loci might be regulated at least in part by a common signal during osmotic adaptation, such as the accumulation of an osmotic stress alarmone or loss of turgor, but this possibility needs to be examined further. At present, there is insufficient basis to decide whether there is a single underlying mechanism for this transcriptional control.

MAJOR UNRESOLVED PROBLEMS IN OSMOREGULATION

I have covered most of the current research topics in osmoregulation, and clearly this is a relatively unexplored field in which most of the research projects are in the initial stages of discovery. There are a number of additional fundamental questions about osmoregulation which I have not addressed but which are likely to provide the most interesting growing points of the field.

Why Glycinebetaine (or Proline)?

The ability of glycinebetaine and proline to overcome the inhibitory effects of osmotic stress in bacteria, together with the fact that these two compounds are also accumulated by plants during osmotic stress (67), raises the question of what is special about these two compounds. Two alternative hypotheses were proposed to account for this remarkable property of these osmoprotectants. According to the first hypothesis, proline and glycinebetaine have special interactions with proteins which protect proteins from denaturation in the presence of high concentrations of electrolytes. The second one states that these two compounds are merely inert compatible solutes that are used to maintain cell turgor in media of high osmolarity.

The suggestion that the special properties of osmoprotectants derive from their interactions with proteins was made for proline by Schobert (233). She proposed that the aliphatic portion of the proline ring binds to the nonpolar residues on proteins via hydrophobic interactions, and because the highly charged carboxyl and imino residues of proline would then be directed toward the water, this interaction would result in coating of the proteins with a hydrophilic shell that would enhance their solubility. Support for the conclusion that proline has unusual interactions with proteins was provided by the observation of Schobert and Tschesche (234) that proline at a concentration of 5 to 6 M could enhance the solubility of insulin, β-lactoglobulin, and bovine albumin. Presumably, the ability of proline to increase the solubility of proteins is a manifestation of its capacity to stabilize them in environments that would otherwise lead to their denaturation.

However, for the most part the effects of proline on protein solubility were slight, and they were manifested only at proline concentration above 5 M. Schobert and Tschesche (234) did observe that proline at this high concentration brought about a 170-fold enhancement of the solubility of insulin, but at concentrations below 5 M it had only negligible effects. While organisms are able to accumulate proline during osmotic stress, this metabolite has not been found at the extremely high concentrations that are required for the special interactions with proteins that were proposed by Schobert and Tschesche. For example, in *S. typhimurium*, proline at concentrations of less than 1 M could alleviate the inhibitory effects of osmotic stress (45).

Although Schobert's proposal is intriguing, it is contradicted by observations which suggest that the special properties of organic osmoprotectants derive precisely from the fact that they do not interact with proteins. Several groups (9, 44, 104, 194, 208, 211) found that organic compounds that are commonly used as osmolytes by organisms can enhance the stability of proteins or membranes in environments of low water activity or high temperature. Albeit these results appear to be consistent with Schobert's hypothesis that the osmoprotectants interact directly with proteins (or other macromolecules), the available evidence points to the conclusion that the effective osmoprotectants tend to avoid protein surfaces. In an attempt to determine the effects of polyols or amino acids (glycine, serine, alanine, β-alanine, α-aminobutyrate, proline, and glycinebetaine) protein structure, Arakawa and Timasheff (7–9) found that these compounds did not affect the partial molar volumes of proteins in aqueous solutions, indicating that they did not interact with proteins. Arakawa and Timasheff (9) suggested that proteins are stabilized by solutes which are excluded from their surfaces because the surface area of denatured proteins is generally greater than that of native proteins and therefore solutes that are excluded from protein surfaces tend to favor the native conformation. These authors also noted that the organic compounds that are excluded from protein surfaces are generally uncharged at physiological pH and thus have minimum electrostatic interactions with proteins. The observation that the osmoprotectants glycinebetaine and proline suppress the osmotic accumulation of K^+ in enteric bacteria (253) is consistent with the notion that these compounds are less toxic to cellular processes than K^+ and are accumulated preferentially over K^+ by the cells as a means of maintaining turgor.

At present, much of the experimental evidence supports the view that organic osmolytes are merely inert osmotic balancers whose main function is to provide cell turgor. However, there is one unsatisfactory aspect to this conclusion, for it does not illuminate why, of all possible organic compounds, proline and glycinebetaine seem to be the preferred organic osmolytes. Several amino acids are excluded from protein surfaces to the same extent as proline and glycinebetaine (8, 9) and are not toxic to enzyme activity at high concentrations (245, 275). Thus, it is intriguing that *E. coli* and *S. typhimurium*, which are very versatile in using a myriad of organic compounds as carbon or nitrogen sources, confine themselves to proline and glycinebetaine as exoge-

nous osmoprotectants. (It is also somewhat surprising that these organisms are restricted to using these two metabolites as osmoprotectants only when they are available exogenously. Since proline and glycinebetaine, or its precursor, choline, are plant constituents, they might be abundant in the diet of the animal hosts of these bacteria, and for reasons of energy economics, the cells may rely on uptake rather than synthesis for their accumulation to high levels.)

The ability of a given compound to serve as an osmoprotectant for an organism could depend in part on the presence of efficient transport systems that can accumulate it to concentrations approximating the osmolarity of the medium. In this regard, it is curious that glycinebetaine is much more potent as an osmoprotectant for enteric bacteria than proline (134). As suggested above, the preference for glycinebetaine over proline might be due to the greater affinity of the ProU system for the former substance over the latter. However, proline has very nearly the same effect on suppressing the osmotic accumulation of K^+ as glycinebetaine (253), and the intracellular concentrations of proline and glycinebetaine are very similar in cells grown in media of high osmolarity (J. A. Gutierrez and L. N. Csonka, unpublished results). For any compound to be an acceptable compatible solute, it must not have any excessive inhibitory effects on any metabolic processes. It may be that proline at high concentrations is more toxic for one or more biochemical steps than glycinebetaine, but whether this is actually the case needs to be established.

Why Are DNA-Protein Interactions Much More Sensitive to Electrolyte Concentration In Vitro Than In Vivo?

Several studies indicated that both the in vitro binding affinities and rates of binding of transcriptional regulatory proteins to their target sites on DNA are extremely sensitive to the electrolyte concentration of the buffers used (215). This sensitivity stems from the fact that at physiological pH, DNA is a very highly charged anion and therefore is surrounded by cations. Thus, the binding of proteins to DNA results in the displacement of cations from the surface area of the DNA that contacts the protein. The binding of a protein to a DNA can be represented by the reaction: (binding site $\cdot$ nM^+) + protein $\rightleftharpoons$ (binding site $\cdot$ protein complex) + nM^+, where n is the number of cations (M^+) released from the surface of the DNA for each protein bound. Although this formula is an oversimplification (because it does not include possible displacement of anions from the protein or water molecules from either the DNA or the protein), it nevertheless serves to illustrate why binding of proteins to DNA should be very sensitive to the concentration of ions. At equilibrium, the concentrations of the components of the above reaction are determined by the relationship [(binding site $\cdot$ protein complex) $(M^+)^n$]/[(binding site $\cdot$ nM^+) (protein)] = K. Therefore, the ratio (binding site $\cdot$ protein complex)/(binding site $\cdot$ nM) will vary with $(M^+)^{-n}$, and since for many DNA-binding proteins n is greater than 5, small fluctuations in the cation concentration can result in huge changes in the concentration of the DNA-protein complex. (Because the binding of proteins to DNA is usually accompanied by the displacement of cations, increasing electrolyte concentration generally diminishes the formation of a given DNA complex. However, when two or more proteins are involved in a particular interaction, e.g., the binding of several transcriptional activators or repressors to the same region, the overall effects of fluctuations of ion concentration may be very complex.) The predicted sensitivity of DNA-protein interactions to the concentration of ions is borne out by in vitro experimental results. For example, Mossing and Record (185) found that the binding of the Lac repressor to the *lac* operator was inversely proportional to the fifth power of the Na^+ concentration of the buffer, and Roe et al. (222) observed that the affinity of RNA polymerase to the phage λ p_R promoter during the formation of open (transcriptionally active) complexes varied inversely with the 15th power of the K^+ concentration. The concentration of ions not only alters the affinities of binding of proteins to DNA; it has equally dramatic effects on their rates of binding. In the case of the association of RNA polymerase with promoters, it is actually the rate of formation of open complexes rather than the affinity of binding that determines in vivo promoter strengths. Roe et al. (222) found that the pseudo-first-order time constant of formation of open complexes between RNA polymerase and λ p_R promoter varied in inverse proportion with the ninth power of the Na^+ concentration. Since the intracellular K^+ concentration of *E. coli* can vary from 0.15 to 0.55 M depending on the osmolarity of the medium (61, 220), in view of the "exquisite" sensitivity of DNA-protein interactions to the in vitro electrolyte concentration, one would expect that osmotic stress might have equally dramatic in vivo effects on the transcription of nearly all genes. However, the growth rate of cells shows only gradual inhibition as the osmolarity of the medium is increased (see, for example, references 39 and 40). In addition, as discussed above, there are only a few operons whose transcription is altered by the osmolarity of the medium. Thus, the remarkable in vitro dependency of DNA-protein interactions on the ion concentration is not mirrored by similar in vivo sensitivity.

The reason for the unexpectedly small effects of the exogenous osmotic strength on in vivo gene expression is unclear, but Richey et al. (220) gave a number of possible explanations. (i) Perhaps in the cell the chromosome is entirely covered by nonspecific DNA-binding proteins, so that it is not exposed to K^+. In this case, the attachment of specific DNA-binding proteins, such as *lac* repressor or RNA polymerase, would entail the displacement of other nonspecific binding protein(s) and thus not be sensitive to the cellular cation concentration. (ii) The cellular composition of the low-molecular-weight ions, especially anions, has not been fully determined. Since the DNA-protein binding studies have been conducted in buffers of simplified composition, the in vitro conditions might not adequately resemble the in vivo ones and may have resulted in errors in the determination of the equilibrium binding constants and rates of protein-DNA interactions. In support of this possibility, Leirmo et al. (132) found that using glutamate instead of Cl^- as the prevalent anion greatly enhanced the in vitro association rates or affinities of DNA-protein interactions. (iii) A large fraction of all DNA-binding proteins, including RNA polymerase, are bound to nonspecific sites on the DNA, so that there is competition for these proteins between their specific and nonspecific binding sites. Since the binding to the nonspecific sites is more sensitive to the ion concentration than the binding to the specific sites, Richey et al. (220) suggested that the decreased affinity of proteins for their specific binding sites with increasing intracellular K^+ concentrations may be compensated for by an increase in the free protein molecules that become available as a result of their release from the nonspecific binding sites. Since these possibilities have not yet been explored sufficiently, the question of how the DNA-protein interactions are buffered in vivo against the adverse effects of fluctuations in the

cellular ion concentration remains one of the challenges for the field of osmoregulation.

How Is the Osmotic Potential of the Periplasm Regulated?

The periplasmic space constitutes a substantial portion (20 to 40%) of the cell volume in *E. coli* (220, 248), and in exponentially growing cells its volume is not greatly affected by the osmolarity of the growth medium (220). In electron micrographs, the cytoplasmic membrane appears to be in contact over most of its surface area with the periplasmic space (204), except for a limited number of sites where it seems to be attached to the outer membrane (18). Hobot et al. (98) proposed that the periplasm contains a gellike substance made up of loosely crosslinked polysaccharides joined to the peptidoglycan, which may maintain the proper distance between the cytoplasmic membrane and the cell walls.

Stock et al. (248) determined the magnitude of the Donnan potential across the outer membrane of *E. coli* grown in media of low osmolarity, and they concluded that it was sufficiently high that the periplasm is iso-osmotic with the cytoplasm. These results were subsequently corroborated by Sen et al. (236). It must be pointed out that because of the experimental difficulties involved in measurement of the Donnan potential and the osmolarity of the cytoplasm, it is possible that both of these values are subject to large experimental errors. However, if the conclusion of Stock et al. (248) is correct, it implies that there cannot be a pressure differential across most of the cytoplasmic membrane (i.e., those areas that are in contact with the cytoplasm on one side and the periplasm on the other side). If, on the other hand, the reported value for the Donnan potential or the osmotic potential of the cytoplasm is erroneous, the cytoplasm could be under greater hydrostatic pressure than the periplasm, which would be manifested as a turgor pressure exerted by the cytoplasmic membrane on the periplasmic gel. At present, there is insufficient information available on the structural properties of this substance to determine whether it is sufficiently incompressible to withstand such pressure.

Although the Donnan potential results in a higher concentration of cations in the periplasm than in the culture medium, the osmolarity of the periplasm will asymptotically approach the osmolarity of the medium with increasing ion concentrations in the growth medium (152, 236, 248). This means that although the Donnan potential can generate a hydrostatic pressure for the periplasm in media of low cation concentrations, it is progressively less able to do so as the exogenous cation concentration is increased. Because the osmolarity of the growth medium does not have any major effects on the periplasmic volume in exponentially growing cells (220), and because high concentrations of electrolytes repress the synthesis of membrane-derived oligosaccharides (116), one might expect that some other high-molecular-weight polymers besides membrane-derived oligosaccharides would generate the Donnan potential when the cells are grown in media containing high ion concentrations. Stock et al. (248) and Sen et al. (236) determined the Donnan potential only in cells grown in media of relatively low electrolyte concentrations, and it will be important to measure this parameter in cells grown in the presence of high concentrations of ions.

Use of the Donnan potential for maintenance of the osmotic potential of the periplasm poses another intriguing question. Uncharged molecules are distributed at equal concentrations between the periplasm and the culture medium and thus do not alter the Donnan potential across the outer membrane. On the other hand, electrolytes at high concentrations discharge the Donnan potential, but at equal osmolarities the effects of univalent ions are different from those of divalent ions, as are the effects of trivalent ions (152, 236, 248). Yet another effect on the osmotic potential of the periplasm might be exerted by solutes that are too large to pass through the porins into this compartment. Any fluctuations in the osmotic potential of the periplasm are likely to be rapidly transmitted to the cytoplasm, and therefore one would expect that the addition of these different types of solutes might have different effects on the osmotic regulation of the cytoplasm. Furthermore, protons will be partitioned unequally across the outer membrane as a result of the Donnan potential. The addition of high concentrations of electrolytes would therefore disturb the pH of the periplasm and conceivably alter, at least transiently, the pH of the cytoplasm. The interplay between the regulation of the osmotic potential of the periplasm and of the cytoplasm has not been examined in sufficient detail and remains one of the most interesting unresolved problems in the field of osmoregulation.

What Are the Osmoregulatory Signals?

The response of microorganisms to changes in the external osmolarity can be divided into three phases. First there is rapid shrinkage or swelling of the cytoplasmic volume as a result of the efflux or influx of water on the respective hyper- or hypoosmotic shock, which is followed by biochemical readjustment of the cells to restore turgor or volume to levels compatible with growth, and finally growth is resumed under the new conditions (27). It is not difficult to imagine that transient fluctuations in the turgor could be sensed by membrane-bound proteins that monitor the structure of the membrane or that alterations in the intracellular volume could be detected by cytoplasmic proteins that respond to the concentrations of key signal molecules. The KdpD and KdpE proteins, which control the expression of the *kdp* operon (57, 129), constitute one membrane-associated turgor-sensing system, but ion channels or transport systems that are activated by deformation of membranes caused by fluctuations in pressure (85, 126, 158, 203) could be other examples of this type of regulatory apparatus.

The growth of bacteria in media of high osmolarity results in increased transport or synthesis of a few compatible solutes and enhanced transcription of a limited number of genes that encode proteins involved in osmotic stress tolerance. As Epstein (57) suggested, the K^+ level of the cells could be the primary signal for the regulation of some or perhaps all of the cellular processes that are under osmotic control. The regulatory role of K^+ may be direct, or indirect as may be the case with the *proU* operon, where K^+ could exert its regulatory role on transcription via its proposed effects on supercoiling (253). However, even if K^+ is the primary osmoregulatory signal, it is not clear what provides the signal for accumulation of the anions that are required to balance the K^+. Furthermore, to the extent that one or more cellular processes under osmotic control are regulated by signals other than K^+, it will be necessary to identify the signals in these cases.

One conceivable signal for the osmotic control of some cellular processes could be the osmolarity, or water potential, of the cytoplasm. As discussed above, this has to be ruled out for the regulation of the *kdp* and *proU* operons,

because their induction cannot be triggered by glycerol or other solutes that can diffuse across the cell membrane. A second possible signal could be the cellular turgor. According to the model that turgor provides the direct force for cell wall expansion (143), incremental growth of the cells will result in a slight decrease in turgor. The hypothesis of Epstein (57) linking K^+ transport to turgor suggested that a homeostatic mechanism restores turgor as a result of increased K^+ uptake. Since with increasing osmolarity, higher intracellular levels of K^+ are required to maintain turgor at a constant value, this hypothesis can explain the positive correlation between the intracellular K^+ levels and the external osmolarity.

However, the proposed relationship between turgor and K^+ uptake entails two conceptual difficulties. First, since the steady-state K^+ concentration of cells is approximately proportional to the osmolarity of the medium (61), one can calculate that the net rate of K^+ uptake is greater in cells grown in high-osmolarity media than in low-osmolarity media. Therefore, if the rate of K^+ uptake is regulated by turgor, one would expect that the turgor of the cells in the former case would be lower than in the latter. This does not seem to be substantiated by measurements of the concentrations of the osmotically active solutes of the cells (52, 131, 220). (It is possible, though that because of the large experimental errors in the measurement of turgor pressure, small changes in turgor, which may nevertheless be sufficient to regulate K^+ transport, may be undetectable.) The possibility that the periplasmic space of gram-negative bacteria is isoosmotic with the cytoplasm, as suggested by the experiments of Stock et al. (248), raises a more serious challenge to the model that turgor pressure can regulate cellular processes. Assuming that this conclusion is valid, there could be transient differences between the hydrostatic pressure of the periplasm and the cytoplasm during plasmolysis, but once osmotic adaptation has taken place, there cannot be a pressure differential on the two sides of cytoplasmic membrane. Consequently, proteins that are contained entirely within the cytoplasmic membrane (such as the Trk or ProP permease and the EnvZ protein) cannot monitor the turgor pressure of these cells. (It could be argued that any chemical reaction that is associated with a change in the volume of the reactants versus the products is sensitive to hydrostatic pressure, and hence there could be pressure-sensing proteins in the cytoplasm, membrane, or periplasm. This possibility is very unlikely to account for osmoregulation because the fluctuation in the turgor of *E. coli* is less than 5 to 10 atm, and the conformations of proteins are not likely to be influenced sufficiently by pressure fluctuations of this magnitude to be of importance [156].)

It should be pointed out that there are precedents for osmotic control without turgor. The observation by Milner et al. (176) that activity of the ProP system in membrane vesicles is stimulated by hyperosmotic shock indicates that turgor is not the regulatory signal for this process, because in the absence of inelastic walls, membranes cannot be under any turgor pressure. When erythrocytes are diluted into hypo- or hyperosmolar buffers, they undergo a transient swelling or shrinking, respectively, but with time they regain their original volume as a result of the proper uptake or excretion of Na^+ and Cl^- ions (126). Although animal cells, which do not have cell walls, cannot maintain a turgor pressure, they nevertheless have a mechanism for the maintenance of a constant volume in the face of hyper- or hypoosmotic challenge. It is not clear what the regulatory signal is in the case of erythrocytes, but since changes in volume are associated with changes in concentrations of metabolites, a plausible signal might be the concentration of a specific molecule(s). There are very rapid fluctuations in the cellular volume of bacteria during plasmolysis, so changes in the concentration of some metabolite could be an alternative signal in addition turgor changes for the initiation of the processes of osmotic adaptation. Full characterization of the primary signals and the regulation of the osmolarity of the periplasm and the cytoplasm of bacteria remain the most exciting unknown areas in the field of osmotic regulation.

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