Living with Water Stress: Evolution of Osmolyte Systems

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Summary. Striking convergent evolution is found in the properties of the organic osmotic solute (osmolyte) systems observed in bacteria, plants, and animals. Polyhydric alcohols, free amino acids and their derivatives, and combinations of urea and methylamines are the three types of osmolyte systems found in all water-stressed organisms except the halobacteria. The selective advantages of the organic osmolyte systems are, first, a compatibility with macromolecular structure and function at high or variable (or both) osmolyte concentrations, and, second, greatly reduced needs for modifying proteins to function in concentrated intracellular solutions. Osmolyte compatibility is proposed to result from the absence of osmolyte interactions with substrates and cofactors, and the nonperturbing or favorable effects of osmolytes on macromolecular-solvent interactions.

The Darwinian fitness of water as the medium for life was first considered by L. J. Henderson (*1*, *2*), who in 1913 proposed reasons why only certain of the more abundant elements are found in living systems. In recent decades, discussions and experiments on biochemical evolution have focused almost exclusively on macromolecules—the changes occurring in nucleic acids and proteins.

The nature of the low molecular weight substances, both inorganic ions and small organic molecules, which comprise the bulk of the osmotically active solutes (osmolytes) present in all cells, has to a large extent been ignored. Yet these substances form part of the milieu for the biochemical reactions of living systems, and it is possible, therefore, that the solute composition of cells has been subject to stringent selection.

The strongest selective pressures exist for organisms that experience some form of environmental water stress, namely, high or fluctuating salinity, desiccation, or freezing. Organisms are found living from fresh water to saturated brines (~ 6*M* NaCl), and thus are confronted with potential problems of water gain or loss. Particularly among euryhaline osmoconformers, intracellular osmotic pressures may fluctuate greatly. Many terrestrial xerophytes and insects experience continuous or seasonal evaporative water loss, the latter often being accompanied by various forms of dormancy: estivation, seed or cyst production, or anhydrobiotic larvae. Finally, frost-tolerant species uniformly restrict ice formation to the extracellular fluids (*3*); the ice draws unfrozen water from the cells and concentrates the remaining intracellular fluid.

Under all of the above conditions, then, ratios of osmolytes to the water and macromolecules in cells are clearly altered. The central focus of this article is to examine the nature of the intracellular osmolytes that become concentrated during water stress, and to ask what is their distribution among prokaryotes, plants, and animals, and what are their influences on macromolecular function and structure. As we show, a striking convergent evolution of osmolyte systems has occurred, reflecting fundamental constraints on the kinds of solutes that are compatible with macromolecules. Thus, such an analysis allows one to bridge the foci of L. J. Henderson, on the one hand, and evolutionists concerned with the properties of macromolecules, on the other.

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Classes of Intracellular Osmolyte Systems and Their Distributions

The dominant types of solutes comprising cellular osmolyte systems in various water-stressed organisms are given in Tables 1 and 2. The first item of note is that the dominant solutes within cells are quite different from those outside, whether the latter be in the ambient water or in the extracellular body fluids (blood, hemolymph, or sap). Second, among eukaryotes, intracellular potassium ion concentration ($[K^+]$) varies little despite wide variations in osmotic pressure (Table 1) (*4*). Third, the major osmolytes in water-stressed eukaryotes are restricted to a few classes of low molecular weight metabolic products: polyhydric alcohols (polyols), such as glycerol and sucrose; free amino acids and amino acid derivatives (taurine and β-alanine); and urea and methylamines, such as trimethylamine-*N*-oxide (TMAO), betaine, and sarcosine. Finally, a high degree of convergent evolution has occurred (Table 2); each category of osmolyte system contains species that often belong to only distantly related phyla.

These facts raise a number of questions. First, why do cells accumulate energy-rich metabolites rather than more readily available inorganic ions such as K^+ and Na^+? The single exception to this rule is the ancient halophilic bacteria, which accumulate potassium ions to concentrations of several molar (Table 1). Second, why are certain metabolites accumulated in preference to others? For example, only a few types of amino acids are utilized by organisms of group B in Table 2. Third, why do osmolytes occur in certain combinations, and often in fairly strict proportions? For example, marine cartilaginous fishes and the coelacanth all use urea and methylamines in an approximately 2 to 1 ratio (Table 1). Finally, do these groups of solutes impose any special restrictions on the macromolecules of the organisms that use them? This question has special significance for osmoconformers experiencing wide osmotic fluctuations over short intervals, as in an estuary. If we consider all this from an evolutionary perspective, we can argue that the few classes of organic osmolyte systems found in nature share a common ability to provide environments "compatible" (*5*) for macromolecular structure and function. Before considering the molecular basis for compatibility and its consequences in evolution, we must first examine the effects and noneffects of different types of solutes on macromolecules.

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Inorganic Ions as Perturbants of Macromolecular Function

Although many biochemical functions require specific inorganic ions, increasing the concentrations of these ions above those typically found intracellularly often leads to disruption of function. For example, the deleterious effects of KCl and NaCl on the activities of four different enzymes from plants and animals from different habitats are shown in Fig. 1. Salts affect both catalytic rate (Fig. 1, a and c) and the apparent Michaelis constant, K_m (Fig. 1, b and d). Such data strongly suggest that high intracellular concentrations of salts during osmotic stress would seriously affect metabolic function (as well as the maintenance of proper transmembrane potentials). Adaptation to high intracellular ionic strength would probably require extensive amino acid substitutions in many different classes of cellular proteins. Furthermore, the strong concentration-dependence of the salt effects noted in Fig. 1 suggests that it would be difficult, if not impossible, to design a protein that maintains optimal functional abilities over a wide range of salt concentrations. Dependence on inorganic ions as primary osmolytes during water stress thus appears to be a suboptimal adaptation, especially compared with the alternatives we next consider.

Glycerol and Other Polyols

The enzyme glucose-6-phosphate dehydrogenase of two species of the euryhaline alga *Dunaliella* is unaffected by glycerol concentrations up to nearly 4 molal, in strong contrast to the deleterious effects of KCl and NaCl (Fig. 1a). Indeed, *Dunaliella viridis* accumulates this level of glycerol when grown in saturated salt solution. However, glycerol is not required for optimal activity. This observation, first made by A. D. Brown and colleagues (*5, 6*), led them to introduce the term "compatible solute." Such solutes are not obligatory for metabolism, in strong contrast to the ion requirement of halobacteria (see below).

Polyols such as glycerol, mannitol, and sucrose are common cell osmolytes, and occur in many unicellular algae, certain salt-tolerant plants, and many insects exposed to freezing temperatures (Table 1). In each instance, polyols aid in cell water retention while remaining compatible with macromolecular function.

Free Amino Acids and Amino Acid Derivatives

Amino acids and their derivatives are the dominant solutes in such phylogenetically diverse organisms as salt-tolerant bacteria (but not the extreme halophiles), halophytes, marine invertebrates, and hagfishes (Table 1). As is noted above, only selected compounds in this category are utilized, suggesting

Table 1. Representative organisms using different osmolyte systems.

Group of organisms	Osmotic concentration (mmole/kg water)		Inorganic ions (mmole/kg water)		Amino acids	Organic osmolytes (mmole/kg water)			Urea	Polyols (molal)	Reference
	Cell	Environment	$[Na^+]$	$[K^+]$		Betaine	TMAO	Sarcosine			
Osmotic conformers											
Plants											
Unicellular algae											
Dunaliella viridis		4.25*M* NaCl								4.4	(*6*)
		2.55*M* NaCl								2.4	(*6*)
Multicellular species											
Triglochin maritima		0.2*M* NaCl			350						(*10*)
		Fresh water			40						(*10*)
Hordeum vulgari						*					(*19*)
Animals (muscle tissue)											
Invertebrates											
Balanus nubilus (barnacle)	1005	Seawater	45	169	503	82					(*55*)
Eriocheir sinensis (crab)	588	Fresh water	55	71	158	18	47				(*51*)
	1118	Seawater	144	146	341	14	75				(*51*)
Parastichopus sp. (echinoderm)	1246	Seawater	71	217	221	208					(*56*)
Sepia officinalis (mollusk)	1377	Seawater	31	189	483	108	86				(*57*)
Vertebrates											
Myxine glutinosa (hagfish)		Seawater	96	140	291		87		2		(*60*)
Latimeria (coelacanth)		Seawater	30	90			290		422		(*59*)
Squalus acanthias (dogfish)		Seawater	18	130		100	180		333		(*61*)
Dasyatis americana (ray)		Seawater	7	201			255		604		(*62*)
		50% seawater	30	171			186		377		(*62*)
Raja erinacea (ray)		Seawater	10	162	214		64	62	398		(*62, 77*)
		50% seawater	4	134	144		36	23	264		(*62,77*)
Eubacteria											
Klebsiella aerogenes		1*M* NaCl		625							(*63*)
Archaebacteria											
Halobacterium salinarium		3.4*M* NaCl	400–800	4500							(*5*)
		5.1*M* NaCl	400–800	7500							(*5*)
Osmotic regulator											
Animal: teleost fish											
Pleuronectes flesus		Fresh water	10	157	44		20				(*58*)
		Seawater	15	158	71		30				(*58*)

*Exact cytosol concentration not known due to contribution of vacuole to total cell volume.

that some, but not all, amino acids are compatible solutes.

This possibility has been explored by Clark and Zounes (*7*) and Bowlus and Somero (*8*). In Fig. 1, b to d, the effects of free amino acids and their derivatives on the function of three different enzymes from species of different environments are shown. The commonly occurring osmolytes glycine, alanine, proline, taurine, and β-alanine are found to be without major inhibiting or activating effects on enzymic activity, whether measured by catalytic rate or influences on K_m values. For example, the K_m of phosphoenolpyruvate (PEP) for the pyruvate kinase (PK) reaction of the intertidal crab *Pachygrapsus crassipes* is not significantly perturbed by proline and glycine concentrations of 1*M*, concentrations severalfold higher than the concentrations of individual free amino acids used in intracellular osmotic balance (approximately 50 to 100 m*M*) (*9*). In contrast, the K_m of PEP is strongly perturbed by the two basic amino acids arginine and lysine, which are not utilized as major osmolytes. Similarly, many salt-tolerant plants accumulate high concentrations of proline as the dominant intracellular osmolyte (Table 1), and Stewart and Lee (*10*) have shown that a diverse family of halophyte enzymes is insensitive to wide changes in proline concentration (Fig. 1c).

An important question about these free amino acid effects—or noneffects—is whether they are due strictly to the nonperturbing characteristics of the osmolytes or, alternatively, derive from special adaptations of the proteins of those organisms that use this class of organic osmolyte. One study that examined this alternative is shown in Fig. 1d. Lactate dehydrogenase (LDH) from skeletal muscle of tuna, a hypoosmotic regulator, has a K_m for its cofactor (reduced nicotinamide adenine dinucleotide, NADH), which is unaffected by high concentrations of compatible amino acids but is strongly perturbed by KCl and NaCl and by arginine and lysine. These and other data (*7*, *8*) are evidence that the predominant amino acid osmolytes are widely compatible with protein function, and thus obviate the need for special protein adaptations other than those concerned with regulation of free amino acid concentrations.

Since arginine is a strong perturbant of enzyme function (Fig. 1, b and d), it may appear paradoxical that many invertebrates use the muscle phosphagen, arginine phosphate, at levels up to 40 to 50 m*M* (*11*). Release of free arginine during vigorous muscle activity should inhibit enzymes like PK and LDH, whose function is critical for muscle glycolytic flux. Many invertebrates, however, contain the enzyme octopine dehydrogenase, that reductively combines free arginine with pyruvate to form octopine (Fig. 2), a product that is innocuous to enzymes (Fig. 1d) (*12*). Thus, in addition to regenerating NAD during muscle metabolism, the formation of octopine as a metabolic end product may have evolved partly on the basis of its being a compatible solute (*8*).

Table 2. Distributions of osmolyte systems.

Osmolyte system (occurrences)	Principal osmolytes	Reference
A. Polyhydric alcohols-polyols		
Cyanobacteria		
Synechococcus sp.	Glucosylglycerol	(*64*)
Fungi		
Saccharomyces rouxii	Arabitol	(*5*, *65*)
Asteromyces cruciatus	Arabitol, glycerol, mannitol	(*66*)
Lichens		
Lichina pygmeae	Mannosidomannitol	(*67*)
Unicellular algae		
Dunaliella spp.	Glycerol	(*6*)
Chlorella pyrenoidosa	Sucrose	(*68*)
Ochromonas malhamensis	Isofloridoside	(*52*)
Multicellular algae		
Fucus spp.	Mannitol	(*69*)
Vascular plants		
Gossypium hirsutum L.	Glucose, fructose, sucrose	(*70*)
Insects (freeze-tolerant or -resistant)		
Eurosta solidaginis (Diptera)	Glycerol, sorbitol	(*71*)
Bracon cephi (Hymenoptera)	Glycerol	(*72*)
Crustaceans		
Artemia salina (emerging larvae)	Glycerol, trehalose	(*78*)
Vertebrates		
Hyla versicola	Glycerol	(*79*)
B. Amino acids and amino acid derivatives		
Eubacteria		
Klebsiella aerogenes	Glutamic acid, proline	(*63*)
Salmonella oranienburg	Glutamic acid, proline	(*63*)
Streptococcus faecalis	γ-Aminobutyric acid, proline	(*63*)
Protozoa		
Miemiensis avidus	Glycine, alanine, proline	(*73*)
Vascular plants		
Spartina townsendii	Betaine	(*74*)
Atriplex spongiosa	Betaine	(*75*)
Aster tripolium	Proline	(*76*)
Mesembryanthemum nodiflorum	Proline	(*76*)
Invertebrates		
All phyla of marine invertebrates (see examples in Table 1)		(*9*)
Cyclostomes		
Myxine glutinosa (hagfish)	Various amino acids	(*60*)
Amphibia		
Bufo marinus	Various amino acids	(*21*)
C. Urea and methylamines		
Cartilaginous fishes (elasmobranchs and holocephalans: marine and estuarine): see examples in Table 1		
Coelacanth (*Latimeria chalumnae*)		(*59*)
D. Urea: estivating forms		
Mollusks		
Bulimulus dealbatus		(*22*)
Lungfishes: African and South American		(*24*)
Amphibians		
Scaphiopus couchi (spadefoot toad)		(*23*)
E. Inorganic ions		
Archaebacteria		
Halobacterium spp.	K^+	(*50*)

Urea and Methylamines

Urea occurs in various organisms, where it is an important product of nitrogen metabolism and is accumulated by some species as the major blood and intracellular osmolyte. In cartilaginous marine fishes and the coelacanth, urea averages approximately 400 m*M* (Table 1). This occurs despite the fact that urea, even at these relatively low concentra-

tions, has strongly perturbing effects on macromolecules (*13–15*). And, with few exceptions (*16*), the proteins of urea-rich fishes are as sensitive to urea perturbation as are the homologous proteins of species that do not use urea as a major osmolyte.

This apparent paradox concerning the use of urea as an osmolyte was resolved when the influences of the other major class of organic osmolyte found in most urea-rich species, the methylamines TMAO, betaine, and sarcosine, were studied. The work of Yancey and Somero (*13, 14*), shown in Fig. 3, a to c, demonstrated that the methylamine osmolytes were potent counteractants of urea perturbation of proteins. Moreover, these offsetting effects of urea and methylamines were greatest when the physiological ratio of the urea concentrations to that of the summed methylamines, approximately 2:1 in most species, was employed. Counteracting effects of methylamines and urea were found over a wide range of absolute concentrations, with the degree of counteraction depending only on the relative concentrations of the two types of osmolytes.

Methylamine-urea counteraction has been demonstrated for a number of different protein phenomena (*13, 14, 17*), including K_m values for several enzymes (Fig. 3a), the exposure of interior sulfhydryl groups of an enzyme (Fig. 3b), the reactivation of a denatured enzyme (Fig. 3c), and the development of tension in shark muscle fibers (*18*). Moreover, the counteracting effects are independent of the species source of the protein. Mammalian, teleost, amphibian, and elasmobranch proteins respond similarly in the presence of the counteracting solutes (*13, 14*), regardless of whether they experience these solutes in vivo. Thus, we believe, the adoption of this osmolyte system has obviated the necessity for development of specially modified proteins for function in the presence of high urea and methylamine concentrations, a conclusion similar to the one reached in our discussion of free amino acid osmolyte systems. In viewing urea and methylamine effects on proteins, it is important to realize that use of methylamines alone might be as deleterious as the use of urea alone, since high concentrations of methylamines could make some proteins too rigid to function effectively or could set K_m values at levels that are too low for optimal enzyme function.

In nonvertebrate species, methylamines may counteract the effects of

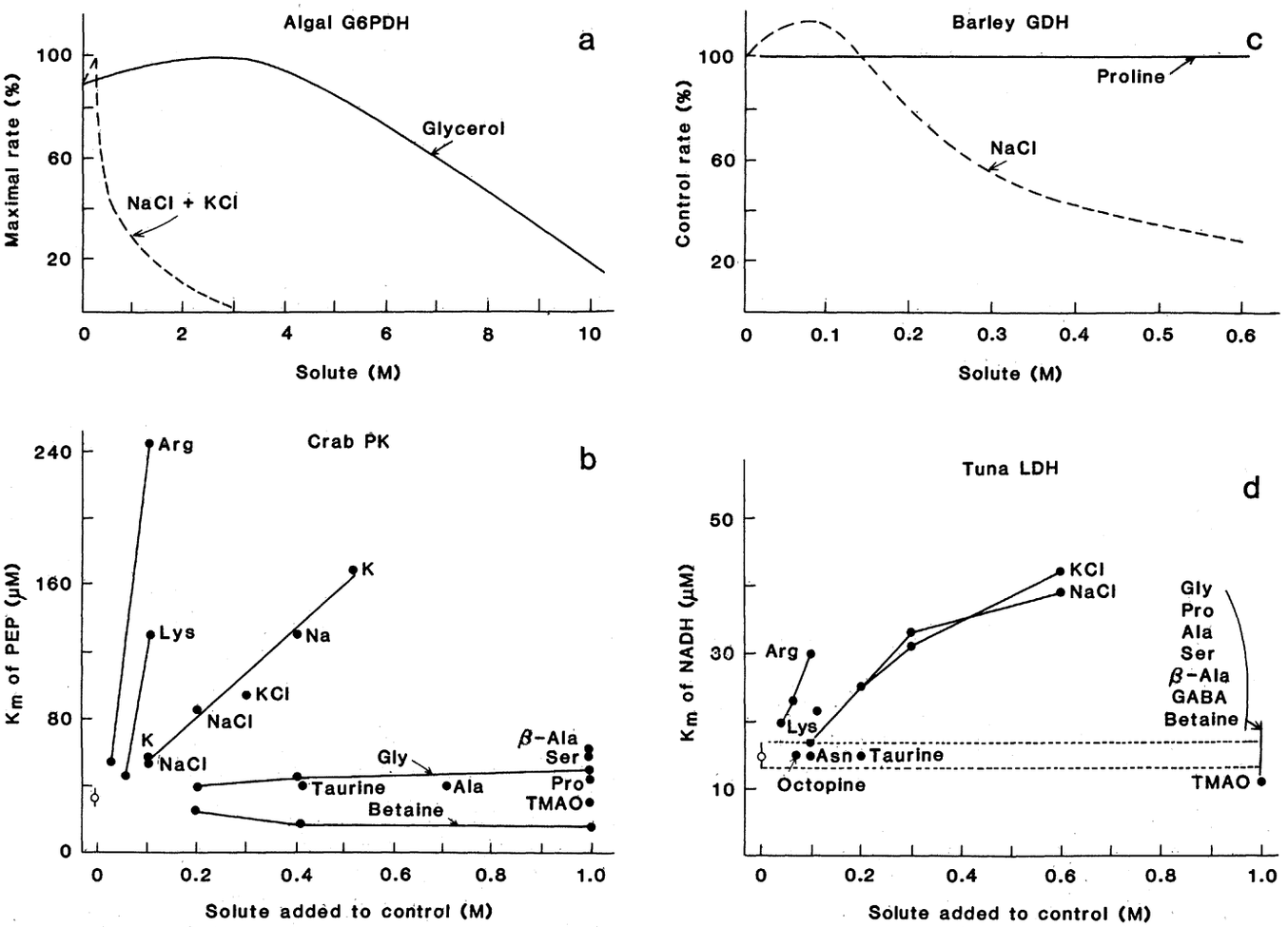

Fig. 1. (a) The effects of glycerol and an equimolar mixture of NaCl + KCl on the activity of glucose-6-phosphate dehydrogenase (G6PDH) of *Dunaliella viridis* [modified after Borowitkza and Brown (*6*); courtesy of *Archives of Microbiology*]. (b) The effects of NaCl, KCl, and organic osmolytes on the apparent Michaelis constant (K_m) of phosphoenolpyruvate (PEP) for pyruvate kinase (PK) of the crab, *Pachygrapsus crassipes*. The open symbol is the control (0.1*M* KCl) with 95 percent confidence limits shown by the vertical line. Abbreviations: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; β-Ala, β-alanine; GABA, γ-aminobutyric acid; Gln, glutamine; Glu, glutamate; Gly, glycine; GuCl, guanidinium chloride; Lys, lysine; Pro, proline; and Ser, serine [modified after Bowlus and Somero (*8*); courtesy of the *Journal of Experimental Zoology*]. (c) The effects of NaCl and proline on the activity of halophyte glutamate dehydrogenase (GDH) from the succulent halophyte *Triglochin maritima* [modified after Stewart and Lee (*10*); courtesy of *Planta*]. (d) The effects of salts and organic osmolytes on the apparent K_m of nicotinamide adenine dinucleotide (NADH) for the lactate dehydrogenase (LDH) reaction of bluefin tuna. Abbreviations are as in (a). The control value (open symbol) was obtained without added solutes. The dashed lines indicate 95 percent confidence intervals around the control value [modified after Bowlus and Somero (*8*); courtesy of the *Journal of Experimental Zoology*].

salts. For example, certain halophytes accumulate high concentrations of betaine (Table 1). Pollard and Wyn Jones (*19*) showed that betaine can offset salt-induced inhibition of plant enzymes (Fig. 3d), and that the degree of methylation of the nitrogen atom determines the effectiveness of a methylamine in this respect.

The methylamine concentrations in some species of urea-accumulating vertebrates living under osmotic stress are unknown. Examples are the mangrove swamp frog *Rana cancrivora* (Table 1) (*20*), and the toad *Xenopus laevis* (*21*). We predict that an approximate 2 to 1 ratio of the concentration of urea to that of summed methylamines also occurs in these species.

There are other animals, however, in which the accumulation of urea alone appears to be the osmotic strategy. This pattern of osmotic adaptation is noted particularly in animals undergoing a transition from an active to a dormant metabolic state in response to decreased water availability in the environment. Examples include the pulmonate snail *Bulimulus dealbatus* (*22*), the desert spadefoot toad *Scaphiopus couchi* (*23*), and the African lungfish (*24*). In all these cases, urea appears to be the dominant osmolyte that serves to aid in retention of body water. Urea may also play an additional role in estivating and hibernating species, however, by reversibly inhibiting certain enzymes of energy metabolism (*17, 25*). In the case of the glycolysis-regulating enzyme phosphofructokinase, as little as 25 m*M* urea acts to reversibly dissociate the active tetrameric form to inactive dimers, under the *p*H and temperature conditions typically found in hibernating mammals (*17*). Thus, transitions between torpid and active metabolic states may not require enzyme degradation and synthesis, but rather only readily reversible changes between inactive and active enzyme states in response to alterations in [urea], *p*H, and temperature (*17*).

Mechanisms of Solute Effects and Osmolyte Compatibility

Compatible and noncompatible solutes are distinguished on the basis of either of two properties. First, certain perturbing solutes interact specifically with ligands (substrates, cofactors, and modulators) and active sites, and therefore perturb enzyme function. Second, the manners in which compatible and perturbing solutes affect the hydration, solubility, and charge interactions of various protein groups (peptide backbone groups and amino acid side chains) may be fundamentally different, and these structural effects of course can be translated into functional manifestations.

Interactions with ligands and active sites. In some instances, perturbing solutes may act on ligands rather than by directly affecting protein structure. The salt-induced increase in the K_m of PEP for PK appears to be due to complex formation between K^+ and PEP, the complex being unable to bind to the enzyme (*26*). Similarly, the mechanism of arginine perturbation of K_m values of glycolytic enzymes (Fig. 1, b and d) may be due to its forming a complex with the phosphorylated ligands, PEP and NADH. Nine out of ten glycolytic enzymes have arginyl residues in their catalytic or regulatory sites (*27*), and the high affinity of arginine for phosphate groups (*28*) pertains as well for free arginine.

Among the commonly utilized organic osmolytes, none has a positive charge that could form a complex with the generally negatively charged cell metabolites. Only octopine (Fig. 2) has a net charge, but it is negative (*8*). Polyols, urea, and TMAO are uncharged, and the other methylamines and amino acids are zwitterions in the physiological *p*H range (Fig. 2).

When we consider the second proposed basis for solute system compatibility, we move to somewhat more tenuous ground. The mechanisms of solute effects on macromolecular structure are rather poorly understood, and considerable debate exists over whether those effects are mediated by direct solute-macromolecule interactions or, alternatively, by solute-induced changes in water structure which, in turn, affect macromolecular stability (*29*). We approach these questions as empirically as possible.

Comparisons with Hofmeister series phenomena. We first call attention to a similarity between certain compatible solutes and those ions empirically found to favor the native states of macromolecules. That all neutral salts do not have equal effects on the structure and solubility of proteins and other colloids was pointed out by Hofmeister in 1888 (*30*). He and others since have noted that the same ranking of cations and anions—shown at the top of Fig. 2—applies generally to all macromolecular conformational changes: namely, gross denaturation (such as helix-coil transitions of proteins); aggregation-disaggregation, including polymerization and salting-out behaviors; and subtle functional effects on catalysis (*29, 30*). Furthermore, the

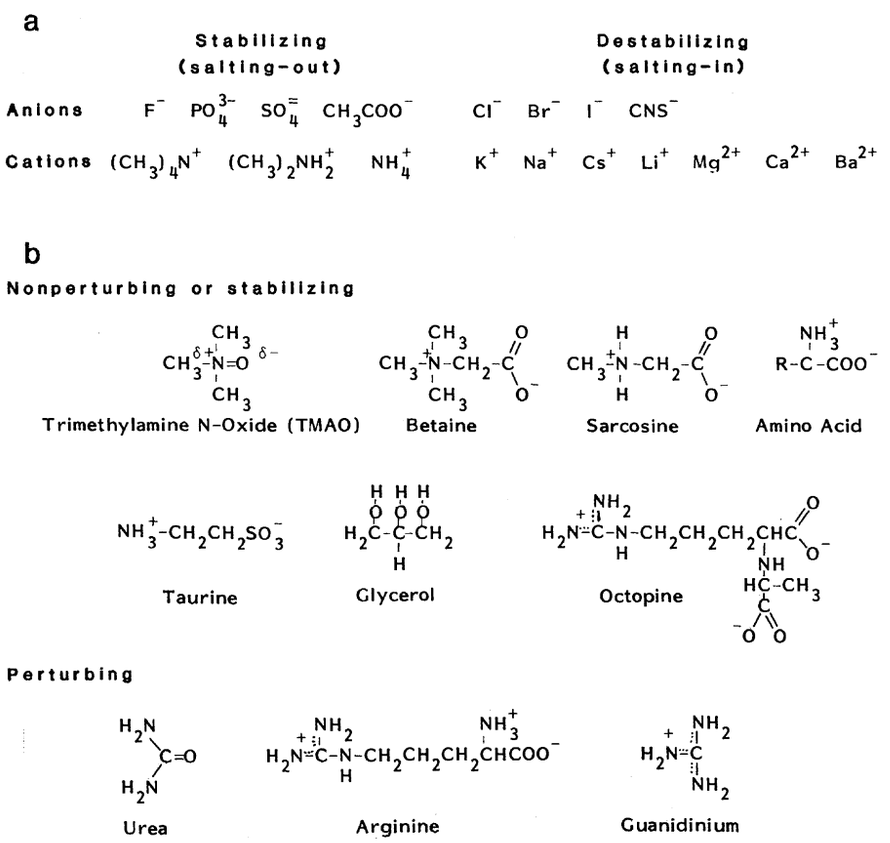

Fig. 2. (a) The sequences of the ions of the Hofmeister series (*29, 30*). (b) Common intracellular solutes having stabilizing or perturbing effects on macromolecular structure and function.

effects of ions are algebraically additive. For example, the destabilizing effect of I^- can be offset by addition of an appropriate amount of a stabilizing ion such as SO_4^{2-} or NH_4^+. Initially, relatively stable systems (such as ribonuclease) that are perturbed only at high ionic strengths ($\geq 1M$) were examined (*29*). Recent studies on more delicate systems with much lower stabilization free energies show them to be sensitive to far lower neutral salt concentrations (a few tenths of a mole per liter), while exhibiting the same Hofmeister series ranking and additivity effects (*31*).

Clark and Zounes (*7*) were the first to point out the striking similarity between ions that favor native macromolecular structure and the functional groups of many organic osmolytes accumulated during water stress (Fig. 2). Amino acids resemble ammonium acetate, taurine resembles ammonium sulfate, and the methylamines resemble the quaternary ammonium ions. We, therefore, propose that organic osmolytes are at least partially selected because they have stabilizing effects on macromolecules parallel to those of favorable ions of the Hofmeister series.

Data presented in Fig. 4, a and b, support this hypothesis. With the exception of the compatible solute, proline, all of the commonly accumulated amino acid and methylamine osmolytes increase the melting temperature (T_m) of ribonuclease, whereas the T_m is decreased by urea and arginine, and by the structurally similar guanidinium ion (Fig. 2). We again note Hofmeister-like algebraic additivity in about the same 2:1 ratio between urea and methylamines as was observed earlier for kinetic properties (Fig. 3a) and structural transformations (Fig. 3, b and c). We also note that, as in the Hofmeister series where the degree of methylation of the nitrogen atoms of substituted ammonium ions increases their ability to stabilize macromolecules (Fig. 2) (*32*), the same is true of organic methylamines. Among these, fully methylated TMAO is most stabilizing. Likewise, the ability of methylated glycine derivatives to offset the macro-

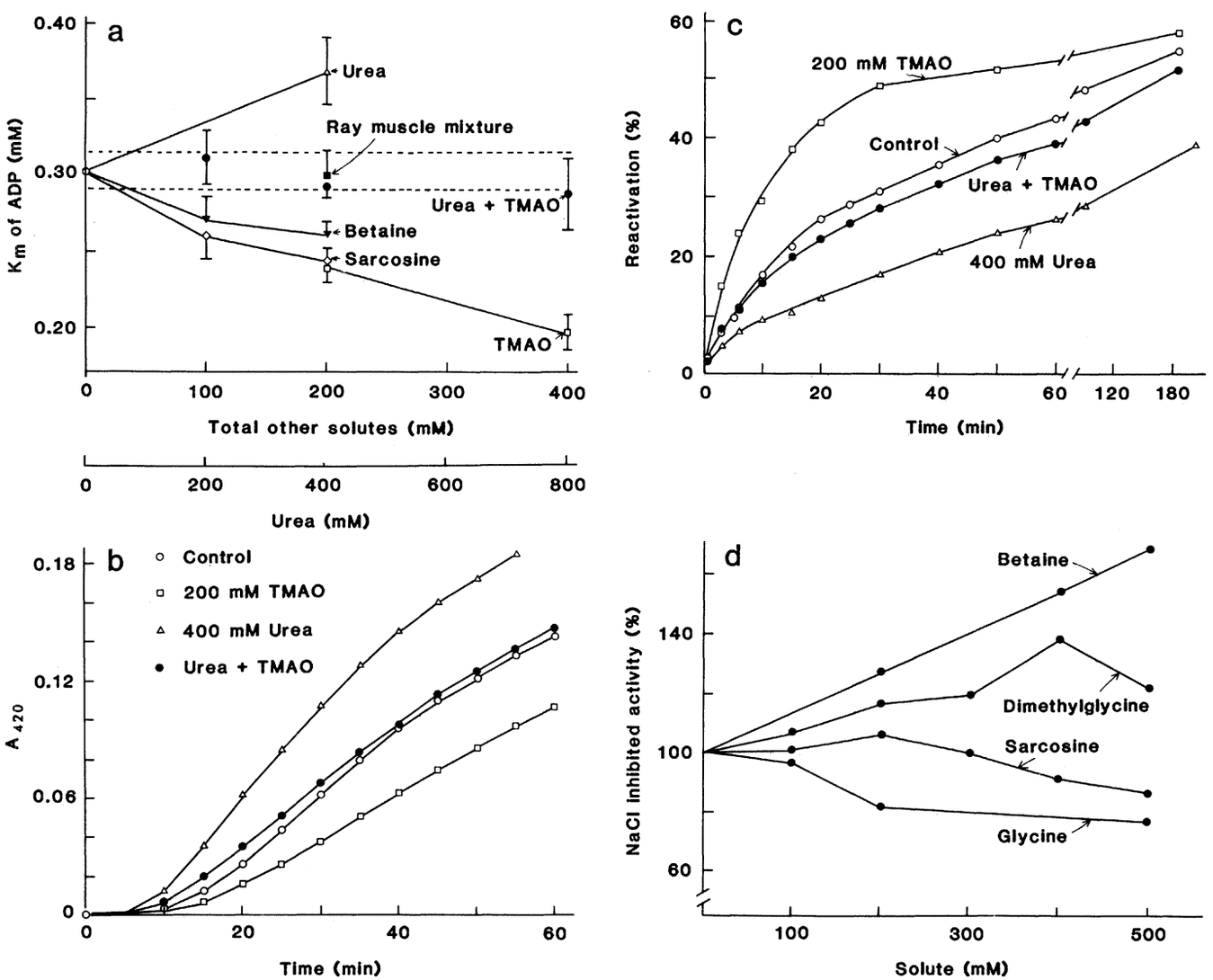

Fig. 3. (a) The effects of urea and methylamine solutes, alone and at a 2:1 ratio, on the apparent K_m of adenosine 5′-diphosphate of pyruvate kinase from the round stingray *Urolophis halleri*. The ray muscle mixture contained: 400 m*M* urea + 65 m*M* TMAO + 55 m*M* sarcosine + 50 m*M* β-alanine + 30 m*M* betaine [modified after Yancey and Somero (*14*); courtesy of the *Journal of Experimental Zoology*]. (b) The counteracting effects of urea and TMAO on the labeling of −SH groups of bovine glutamate dehydrogenase by the sulfhydryl reagent 4-chloro-7-nitrobenzofurazan [modified after Yancey and Somero (*13*); courtesy of the *Biochemical Journal*]. (c) The counteracting effects of urea and TMAO on the recovery of activity of acid-denatured lactate dehydrogenase purified from the white shark *Carcharodon carcharias* [modified after (*13*); courtesy of the *Biochemical Journal*]. (d) The effects of successively methylated derivatives of glycine on the inhibition of barley malate dehydrogenase by 300 m*M* NaCl (the activity in the presence of salt alone is 100 percent) [modified after Pollard and Wyn Jones (*19*); courtesy of *Planta*].

molecular destabilizing effects of neutral salts depends on their degree of methylation (Fig. 3d).

Neutral salts differentially affect macromolecular solubility in the same sequence that they affect tertiary protein structure (Fig. 2). Hence, stabilizing ammonium sulfate has long been used as a satisfactory precipitant of native proteins (*33*). More recent studies reveal a parallel between the ability of neutral salts to stabilize tertiary structure and their ability to foster higher levels of subunit assembly (*34*). The work of Hand and Somero (*17*) with the tetrameric enzyme phosphofructokinase (PFK) indicates that TMAO strongly favors the aggregation of PFK into polytetramers (Fig. 4c). Also TMAO stabilizes the functional tetrameric form of PFK, reducing its dissociation into nonfunctional dimers (*17*). This effect is achieved at TMAO concentrations that are five- to tenfold lower than required for a stabilizing ion (F^-) of the Hofmeister series (Fig. 4d). The capacities of various methylamines to stabilize the PFK tetramer again correlate with the extent of methylation of the nitrogen atom (*17*).

In studies of another aggregating system, skinned barnacle muscle, Clark *et al.* (*35*) showed that the disruption of native myofilament architecture induced by neutral salts (KCl + NaCl) can be prevented by simultaneous addition of TMAO. Selected amino acids and glycerol also produced this result, but urea did not. Indeed, urea disrupts myofilaments in frog muscle (*36*).

In reviewing the above, we find that the additivity effects seen with (i) urea plus methylamine systems, (ii) the offsetting by methylamines of salt inhibition, and (iii) the dependence of methylamine effects on the degree of nitrogen atom methylation (Fig. 3d and Fig. 4b) are phenomenologically parallel to classical Hofmeister series effects. Salt actions on enzymes also show Hofmeister series rankings with respect to catalytic activity (*37*), influences on K_m (the apparent Michaelis constant) values (*38*), and alterations of the enzyme hydration changes that accompany catalysis (*39*). In conclusion, we consider it probable, although by no means proved, that certain of the compatible organic osmolytes act to stabilize biopolymers by mechanisms similar to those invoked to explain Hofmeister series effects.

Direct interaction versus solvent-mediated effects. The next question that arises is whether compatible and noncompatible solutes act directly on the protein, as suggested, for example, by Robinson and Jencks (*40*) for the action of urea on a polypeptide, or whether they act on the solvent properties of the surrounding water, as discussed, for example, by Franks (*41*). These two possibilities are difficult to distinguish experimentally and are not necessarily mutual-

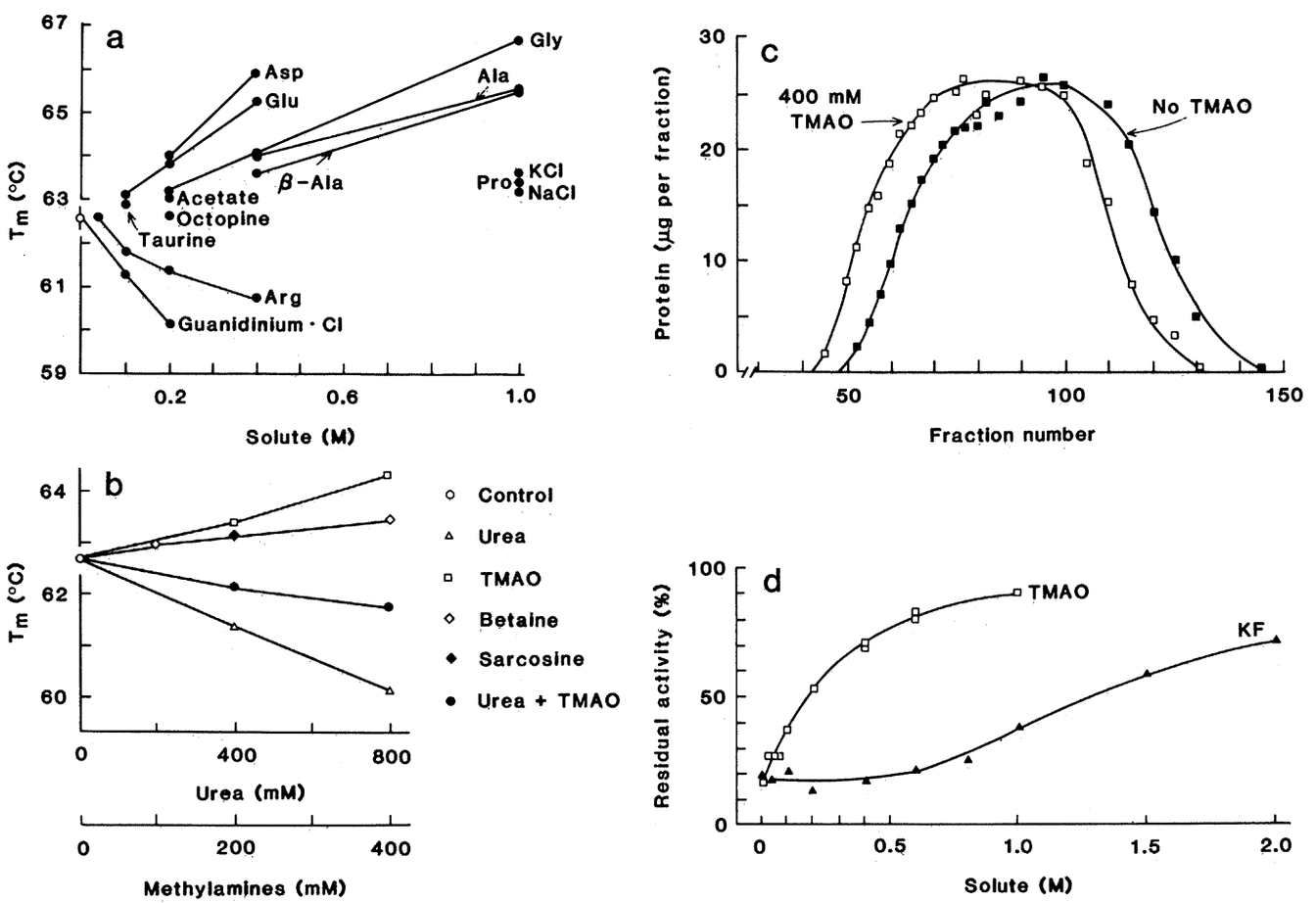

Fig. 4. (a) The effects of different organic solutes on the thermal transition (melting) temperature (T_m) of bovine pancreatic ribonuclease. The control value (no added solutes) is shown by the open symbol (±95 percent confidence intervals). Abbreviations are as in Fig. 1a [modified after Bowlus and Somero (*8*); courtesy of the *Journal of Experimental Zoology*]. (b) The effects of urea and methylamine osmolytes, alone and at a 2:1 ratio, on the T_m of bovine pancreatic ribonuclease [modified after Yancey and Somero (*13*); courtesy of the *Biochemical Journal*]. (c) Frontal-elution gel filtration of phosphofructokinase (0.2 mg/ml) on Sepharose 6B in the presence and absence of 400 m*M* TMAO [modified after Hand and Somero (*17*); courtesy of the *Journal of Biological Chemistry*]. (d) The relative effectiveness of TMAO and KF in reducing the *p*H-induced cold inactivation of phosphofructokinase. Experiments were initiated by addition of enzyme (*p*H 8.0) to the incubation medium (0.1*M* phosphate buffer, 0.2 m*M* dithiothreitol, and TMAO or KF) at 6°C to a final *p*H of 6.5 and a protein concentration of 20 μg/ml. The percent residual activity at the end of a 60-minute incubation was plotted against solute concentration (*17*).

ly exclusive. In theory, osmolytes may affect charged, polar, or nonpolar groups on a protein in such a way as to decrease or increase their affinities for the solvent relative to their affinities for other intra- or intermolecular moieties. The range of these possibilities has been discussed previously (*29, 42*). Here we consider only a few additional pieces of evidence.

In studies on skinned muscle fibers, Clark *et al.* (*35*) observed that negative charges on myofilament proteins increase with increasing ionic strength of the bathing medium, as is expected for ion-induced proton dissociation (*43*). Increasing ionic strength led to greater swelling of the myofilament lattice and eventually to myofilament disruption. With the simultaneous addition of increasing amounts of TMAO, however, although there was no diminution of protein charge, excess ions accumulated in the muscle fiber and lattice swelling was suppressed. This suggests that TMAO-induced cation association at fixed charge sites took place. In the case of myofilament proteins then, at least some of the osmolyte effects may be mediated directly at the biopolymer surface, or by way of the first layer of hydration water.

In contrast, there is evidence that certain osmolytes may act via changes in the solvent properties of water. For example, Gekko and Timasheff (*44*) have shown that the ordered vicinal water around proteins largely excludes glycerol. Thus, addition of glycerol to a protein solution thermodynamically favors a minimization of protein-solvent (water plus glycerol) interactions, that is, protein-protein interactions (subunit aggregation and native conformation) are favored. In addition, osmolytes may influence the translational motion of water molecules. When either the viscosity or the self-diffusion coefficient of water is measured in solutions of biologically predominant salts like KCl, these parameters are negligibly affected by physiologically realistic concentrations of the salt (below 1*M*) (*45*). In contrast, compatible organic osmolytes at the same concentrations have significant effects on the translational motion of water molecules in that a decrease of about 20 percent at 1*M* solute concentration is common (*46*). A similar observation was made by Goldammer and Hertz (*47*) on mixtures of water and organic liquids. They showed, however, that this effect is not due to the formation of long-lived hydration spheres in these mixtures. Zeidler (*48*) suggests that organic solutes are able to induce hydrogen-bonding of solvent molecules over long distances. Furthermore, in solutions containing TMAO (0.5*M*) and various concentrations of KCl, the K^+ activity is reduced by 30 percent, and Cl^- activity by about 5 percent (*49*). Clearly, major interactions between at least some organic osmolytes and neutral salts are possible in aqueous solutions, but the role of these interactions in affecting biopolymer conformations and enzyme activities (note Fig. 3d) remains to be determined.

Evolutionary Implications

The fact that such phylogenetically diverse organisms as bacteria, unicellular algae, vascular plants, invertebrates, and vertebrates utilize a small family of organic osmolytes (Table 2) suggests that strong selective pressures are associated with this striking example of convergent evolution. One way to understand these selective factors involves briefly examining an "exception which proves the rules" elaborated in the preceding discussions of compatible solute systems.

The halobacteria are a dramatic exception to the osmotic strategies found in all other contemporary organisms. This ancient group of prokaryotes conforms osmotically in environments where salt (largely NaCl) concentrations reach saturating levels. Intracellular K^+ concentrations of approximately 7 molal have been recorded in species of *Halobacterium* (Table 1) (*50*). This raises the question of the costs and benefits of this osmotic strategy, and why natural selection in other osmotically conforming species has not led to adoption of the halophile strategy.

Many enzymes of species other than halobacteria are strongly inhibited by KCl and NaCl at concentrations greater than 0.1 to 0.2*M*. To achieve salt tolerance, the proteins of halobacteria have undergone massive amino acid substitutions, involving enrichment in aspartyl, glutamyl, and weakly hydrophobic residues (*50*). These substitutions enable halophile proteins to have the proper conformational states and, therefore, functional properties at—but only at—high K^+ concentrations. Many enzymes of halobacteria absolutely require salt concentrations of 1*M* or greater to attain their peak activities (*50*), and the halobacteria are thus strictly confined to only those environments that always have suitably high ambient salinities. By contrast, a *Dunaliella* species without such broadly modified proteins can grow well in either saturated brine or very dilute solutions, due in large measure to the fact that the internal osmotic pressure is controlled by a compatible solute (glycerol).

Thus, by accumulating compatible solutes or families of counteracting solutes, osmotically concentrated organisms may achieve simultaneously a genetically more "simple" and a temporally far more flexible adaptive mechanism in the face of cyclic water stress. Through the use of organic osmolytes, adaptive changes in proteins, which are determined by changes in the DNA sequences coding these proteins, may be minimized. Rather than modifying hundreds to thousands of different proteins to cope with high or widely fluctuating intracellular salt concentrations, organisms that use compatible solutes have altered but a relatively small fraction of their macromolecular repertoire. Systems needing modification presumably include the enzymes controlling the synthesis and degradation of the organic osmolytes themselves. At least some of the enzymes responsible for osmolyte accumulation in water-stressed species are, in fact, regulated by small changes in salt concentration (*51, 52*). Pathways involved in osmolyte removal from the organism to the external environment may likewise require modification. For example, regulating the passage of urea into the urine and controlling urea flux through the gills are both key elements in the osmotic adaptations of marine cartilaginous fishes (*53*). Changes also may be required in the K_m values of enzymes that use one or more of the organic osmolytes as substrates. All such changes associated with controlling the concentrations of the organic osmolytes, and with coping with high levels of osmolytes that play multiple metabolic roles, appear minor when viewed against the backdrop of the massive changes in protein sequence undergone by the halobacteria.

In summary, we propose that the repeated adoption of a few classes of organic osmolytes by phylogenetically diverse organisms encountering varied types of water stress is a reflection of two important phenomena. One is the ubiquitous set of physicochemical interactions between solutes, water, and macromolecules that establish which types of solutes are compatible with macromolecular structure and function. The second is a phenomenon that might be termed "genetic simplicity." Through the use of compatible solute systems, proteins are able to work in the presence of high or variable solute concentrations, and the modification of vast numbers of proteins is avoided. Thus, it is unnecessary for a *Dunaliella* or estuarine crustaceans to contain the genetic information

for high-salt and low-salt forms of a given type of enzyme. Instead, a single form of a given protein can be used over a wide range of osmotic concentrations when these concentrations are adjusted with compatible solutes. A similar argument applies on a broader, evolutionary time scale. Thus, for example, colonization of habitats that confront the organism with the threat of desiccation will involve relatively small amounts of genetic change (such as in the regulatory systems mentioned above) when compatible solute systems are used for osmoregulation. Only the metabolically simple (*50*) halobacteria, which evolved during the heyday of genetic diversity among primitive prokaryotes (*54*), appear to have been capable of modifying their proteins to conform to the constraints of high internal salt concentrations. All other organisms facing water stress possess a solute adaptation strategy that has, as its hallmarks, a minimal requirement for genetic change and a high degree of flexibility in allowing the organism to conform to wide ranges of external salinity.

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