The Journal of Experimental Biology 208, 2819-2830
Published by The Company of Biologists 2005
doi:10.1242/jeb.01730

Commentary

Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses

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Accepted 1 June 2005

Summary

Organic osmolytes are small solutes used by cells of numerous water-stressed organisms and tissues to maintain cell volume. Similar compounds are accumulated by some organisms in anhydrobiotic, thermal and possibly pressure stresses. These solutes are amino acids and derivatives, polyols and sugars, methylamines, methylsulfonium compounds and urea. Except for urea, they are often called 'compatible solutes', a term indicating lack of perturbing effects on cellular macromolecules and implying interchangeability. However, these features may not always exist, for three reasons. First, some of these solutes may have unique protective metabolic roles, such as acting as antioxidants (e.g. polyols, taurine, hypotaurine), providing redox balance (e.g. glycerol) and detoxifying sulfide (hypotaurine in animals at hydrothermal vents and seeps). Second, some of these solutes stabilize macromolecules and counteract perturbants in non-interchangeable ways. Methylamines [e.g. trimethylamine *N*-oxide (TMAO)] can enhance protein folding and ligand binding and counteract perturbations by urea (e.g. in elasmobranchs and mammalian kidney), inorganic ions, and hydrostatic pressure in deep-sea animals. Trehalose and proline in overwintering insects stabilize membranes at subzero temperatures. Trehalose in insects and yeast, and anionic polyols in microorganisms around hydrothermal vents, can protect proteins from denaturation by high temperatures. Third, stabilizing solutes appear to be used in nature only to counteract perturbants of macromolecules, perhaps because stabilization is detrimental in the absence of perturbation. Some of these solutes have applications in biotechnology, agriculture and medicine, including *in vitro* rescue of the misfolded protein of cystic fibrosis. However, caution is warranted if high levels cause overstabilization of proteins.

Key words: osmolyte, antioxidant, pressure, urea, trimethylamine oxide, hypotaurine, temperature, compatible solute, counteracting, compensatory.

Introduction: osmolytes in osmoconformers and osmoregulators

Water is widely regarded as the most important molecule of life, and an organism's ability to cope with changes in its internal water content is essential for survival. In particular, loss of internal water is a common threat, arising from evaporation into air, during the excretion of wastes or from osmosis into concentrated aqueous surroundings. The latter may occur in an external saline environment, by extracellular freezing, or from diseases that cause osmotic imbalances (e.g. diabetes and its associated hyperglycemia). Traditionally, organisms have been divided into two broad categories in terms of adaptations to water stress: osmoconformers, which usually use organic osmolytes to keep cellular osmotic pressure equal to that of the external fluid environment, and osmoregulators, which use ion transport to homeostatically regulate internal osmotic pressures.

Osmoconformers are most commonly found in the oceans and include most types of life other than most vertebrates and some arthropods. The salts (mainly NaCl) of ocean water yield an average osmotic concentration of ~1000 milliosmoles per liter (1000 mOsm), well above the ~300–400 mOsm created by the basic solutes found in most cells (K^+, metabolites, proteins, etc.). To prevent osmotic shrinkage, internal fluids of marine osmoconformers have about the same osmotic pressure as their environment (e.g. 1000 mOsm). However, while extracellular fluids in multicellular organisms are typically dominated by NaCl, the major osmotic components inside cells (which raise osmotic pressure above the basal level of 300–400 mOsm) are usually organic osmolytes (Fig. 1). These osmolytes can be up- or downregulated in many species to prevent osmotic shrinkage

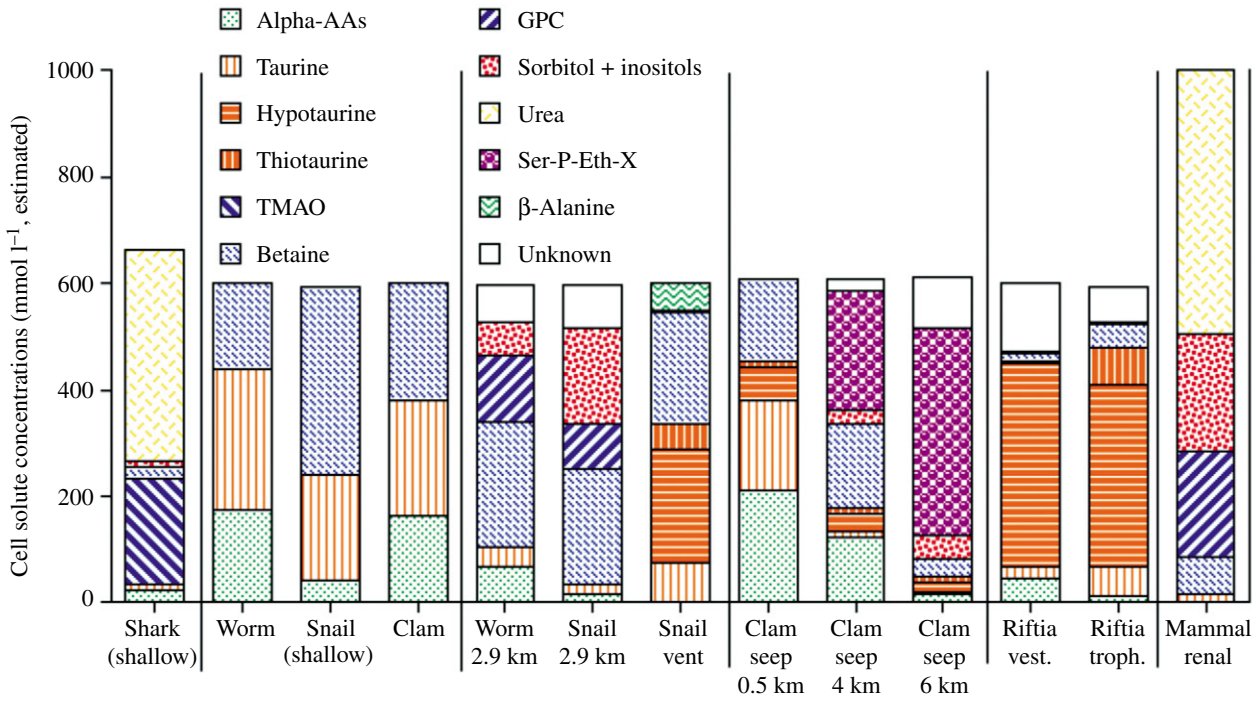

Fig. 1. Patterns of osmolyte distributions in marine animals and mammalian kidneys, shown as estimates of intracellular concentrations. Panels from left to right: (1) sharks and other elasmobranchs are dominated by urea and TMAO (data for *Squalus acanthias*); (2) shallow-water invertebrates, such as the polychaete worm *Glycera*, snail *Mitrella carinata* and clam *Saxidomus giganteus*, are typically dominated by taurine, betaine and α-amino acids (AAs) such as glycine; (3) invertebrates from 2.9 km depth, such as the polychaete worm *Glycera* and snail *Neptunea lyrata*, have less taurine and other amino acids and more *scyllo*-inositol, GPC, and unknowns, while a snail (*Depressigyra globulus*) from hydrothermal vents at 1.5 km depth has high levels of hypotaurine and thiotaurine; (4) vesicomyid clams (*Calyptogena* spp.) from sulfide seeps have hypotaurine and thiotaurine and show a depth-related increase in an unsolved serine–phosphoethanolamine solute (Ser-P-Eth-X) and an unknown methylamine; (5) vestimentiferan tubeworms (*Riftia pachyptila*) from hydrothermal vents at 2.6 km depth have high amounts of hypotaurine and an unknown methylamine, both in vestiment tissue (Vest.) and trophosome (Troph., location of sulfide-oxidizing microbial symbionts), which also has high levels of thiotaurine; (6) mammalian renal cells (inner medulla) have varying levels of sorbitol, *myo*-inositol, GPC, betaine and taurine (along with urea). Data from Peterson et al. (1992); Yin et al. (2000); Yancey et al. (2002); Fiess et al. (2002); Rosenberg et al. (2003).

or swelling if the osmotic concentration of the environment changes.

Osmoregulators, which in the oceans include vertebrates other than hagfish, coelacanths and elasmobranchs (sharks, skates, etc.), are quite different. Such animals typically have regulatory organs (e.g. gills, kidneys) that work to keep internal body fluids at ~400 mOsm or less in marine species, obviating the need for organic osmolytes. This is the pattern inherited by terrestrial vertebrates, which typically have ~300 mOsm body fluids. (The brine shrimp *Artemia* in desert salt lakes is another example of a strong osmoregulator.) However, there are major exceptions to this generalized pattern, with some osmoregulators utilizing organic osmolytes in certain situations. For example, in mammals (considered to be exemplary osmoregulators), cells of the kidney medulla osmoconform to the high osmotic concentrations in that organ's extracellular fluid. And, as will be discussed later, osmoregulating fishes in the deep sea have very high levels of an organic solute that is a major osmolyte in some osmoconformers.

Organic solutes similar or identical to organic osmolytes are also accumulated by some organisms in thermal and anhydrobiotic stresses, and possibly under high hydrostatic pressure. These solutes are typically (and sometimes misleadingly) called 'compatible' solutes, based on the concept that they do not perturb cellular macromolecules even when the solutes are at high concentrations (Brown and Simpson, 1972). However, as will be discussed, many of these solutes have cytoprotective properties, such as antioxidation and stabilization of proteins, that go beyond simple compatibility and that vary from solute to solute.

Types of organic osmolytes

Many different small molecules are known to serve as organic osmolytes and other compatible solutes. As has been extensively reviewed previously (Yancey et al., 1982; Yancey, 2001), these solutes fall into a few major chemical categories (Fig. 2): small carbohydrates including sugars (e.g. trehalose), polyols (glycerol, inositols, sorbitol, etc.) and derivatives (such as *o*-methyl-inositol); amino acids (glycine, proline, taurine, etc.) and derivatives (e.g. ectoine); methylamines [such as *N*-

trimethylamine oxide (TMAO) and glycine betaine] and methylsulfonium solutes including dimethylsulfonopropionate (DMSP); and urea. Except for urea (used only by relatively few types of animals), these categories are widespread in occurrence; for example, glycine betaine is found in every kingdom of life, and taurine is widespread among marine animals and some mammalian organs. Carbohydrate osmolytes occur in archaea, fungi, algae, plants and mammalian kidneys, and possibly deep-sea invertebrates. Sugars and polyols are usually the dominant solutes accumulated in organisms that tolerate or avoid freezing, such as terrestrial plants, insects, amphibians and some polar fishes. Also, many organisms use mixtures of osmolyte categories; e.g. the mammalian kidney uses the polyols *myo*-inositol and sorbitol, the methylamines glycerophosphorylcholine (GPC) and glycine betaine, and the amino acid taurine (the organ also has high urea as both a waste product and an osmotic agent that helps concentrate the urine) (Fig. 1, mammal renal bar). What selective forces have resulted in this widespread use of organic osmolytes, with the (metabolically less costly) inorganic ions usually not used?

The basic compatibility hypothesis

As noted earlier, organic osmolytes are typically called compatible solutes based on the hypothesis that these solutes (other than urea) do not interact with macromolecules in detrimental ways; thus, they can be safely up- and downregulated with little impact on cellular functions (Brown and Simpson, 1972; Yancey et al., 1982). This is in stark contrast to inorganic ions, which at high concentrations typically bind to and destabilize proteins and nucleic acids. Indeed, exposure of some cells to high NaCl medium can produce breaks in DNA (Kültz and Chakravarty, 2001).

In concert with the compatibility hypothesis, most osmolytes are neutral (either zwitterionic or lacking charges) at physiological pH, although some bacterial and archaeal osmolytes are anionic (e.g. diglycerol phosphate; Fig. 2) and are paired with K^+ to achieve neutrality (Martin et al., 1999). In its simplest form, the compatibility hypothesis also suggests that organic osmolytes are interchangeable, i.e. that a cell can be osmotically protected with a variety of compatible osmolytes whether it normally uses them or not. There is evidence to support this, as illustrated by the following examples. (1) Growth of *Escherichia coli* in saline growth media can be improved with a variety of osmolytes, some not used naturally by the bacterium, added to the medium (Hanson et al., 1991). (2) One cultured line of mammalian kidney medullary cells (PAP-HT25) in hyperosmotic culture medium uses primarily sorbitol as an osmolyte. When production of sorbitol is inhibited, cell growth decreases in parallel with declining cell sorbitol content (inhibition had no effect in normal medium, in which these cells use little sorbitol; Yancey et al., 1990a). However, addition of glycine betaine (normally absent) to the medium largely restores viability (Moriyama et al., 1991). (3) With

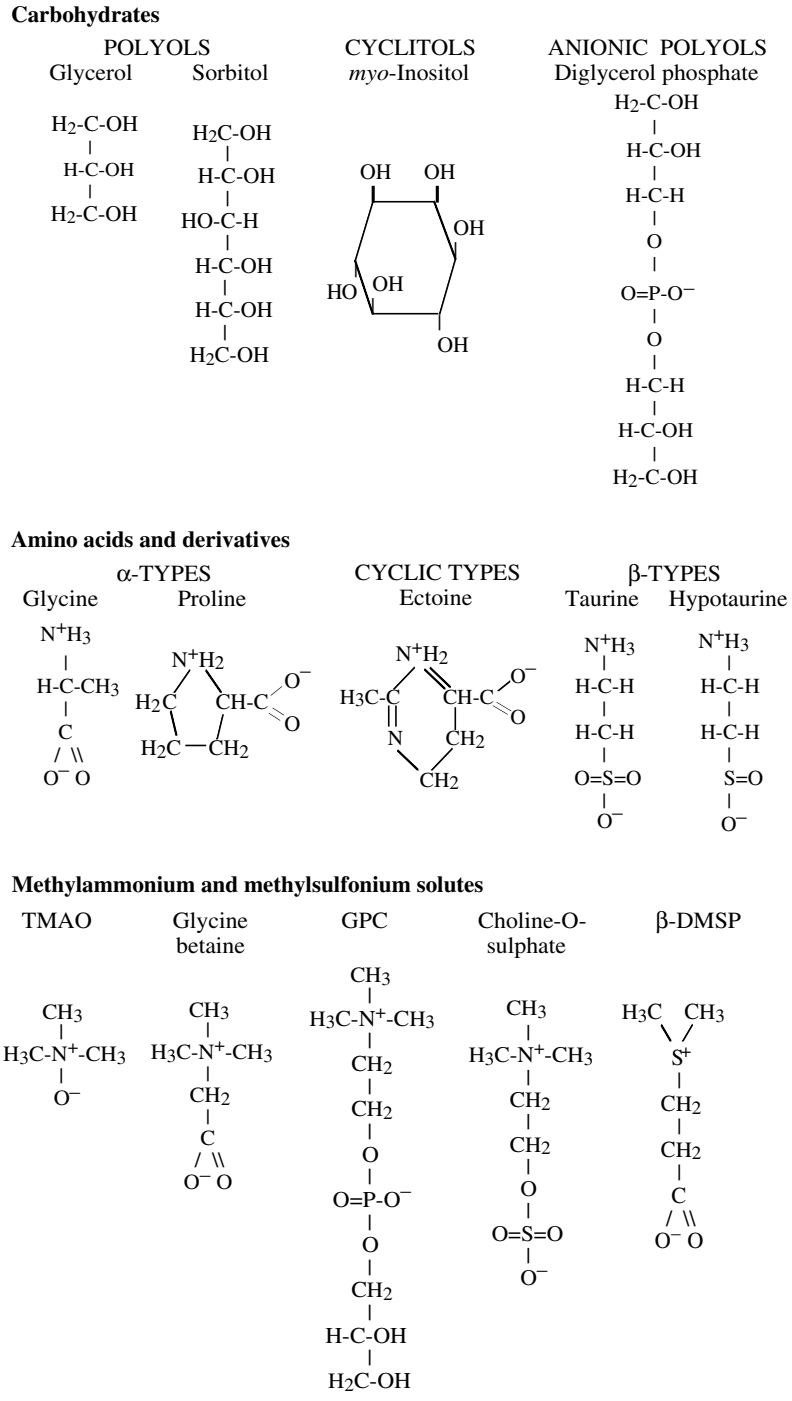

Fig. 2. Examples of organic osmolytes in three of the four major categories; the fourth category, urea, is not shown. TMAO, trimethylamine *N*-oxide; GPC, glycerophosphorylcholine; DMSP, dimethylsulfonoproprionate.

Table 1. *Summary of protective properties of osmolytes through metabolic reactions*

Cytoprotective property	Compatible solutes in nature
Antioxidation	Hypotaurine; DMSP; polyols (e.g. water-stressed plant)
Redox balancing, hypoxia	Proline, β-alanine betaine, glycerol (e.g. *Dunaliella* alga)
Sulphide detoxification/storage	Hypotaurine (e.g. hydrothermal vent tubeworm, *Riftia*)
Sulphate detoxification	Choline-O-sulphate (e.g. mangrove plant)
Energy reserve	Glucose, trehalose, etc. (e.g. frozen wood frog)
Predator repellent	DMSP, *trans*-hydroxyprolinebetaine (e.g. diatom)
Ca^{2+} modulation	Taurine? (e.g. mammalian neuron)

normal cells of rat renal medulla, in both primary cultures (Rohr et al., 1999) and *in vivo* (Yancey et al., 1990b), inhibition of sorbitol synthesis triggered a compensating increase in glycine betaine, such that there were no short-term osmotic imbalances or apparent damage. These are but a few of many examples.

These experiments suggest that some osmolytes (even from different chemical categories) are functionally interchangeable. Thus, perhaps the reason osmolytes vary among organisms is simply due to different diets or metabolisms that are unrelated to water stress. This may often be the case; for example, the widespread use of (non-nitrogenous) carbohydrate and sulfonium osmolytes in photosynthesizers may arise from nitrogen limitation. However, long-term effects of interchanging osmolytes have not been adequately tested. Also, the compatibility concept does not readily explain why there is such an enormous variety of organic osmolytes, found in all kingdoms of life; Fig. 2 shows only a few examples of the dozens of different known organic osmolytes. Nor does it explain why many organisms employ a complex mixture of osmolytes. As will become apparent, much remains to be learned about the reasons for this variety, but it may result from unique properties of some osmolytes, properties that may be helpful only with certain stresses. These cytoprotective properties fall into two broad categories: (1) protective metabolic reactions and (2) counteraction of destabilizing forces on macromolecules.

Metabolic protection

It is becoming clear that some osmolytes and related solutes are not metabolically inert but rather engage in unique reactions that can protect cells in various ways other than osmotically. Taurine (Fig. 2) is perhaps the most intensely studied, and most mysterious, compatible solute in this regard. This sulfur-based, non-protein amino acid is a major, often the dominant, osmolyte in many marine invertebrates such as bivalves in shallow waters (Fig. 1, clam shallow bar). It is not clear why this is, nor why taurine contents in at least some marine invertebrates decrease with depth in the oceans (Fig. 1, shallow, 2.9 km and seep bars; Pruski et al., 2000a; Fiess et al., 2002). Taurine is also relatively high in mammalian heart and brain cells, where it can serve as a major osmolyte in severe dehydration (reviewed in Miller et al., 2000; Olson et al., 2003). It is also essential for mammalian neural development in ways that may not be related to osmotic balance. Neonatal cats, for example, become blind if raised without taurine due to improper retinal development. However, it is not certain how taurine exerts its developmental effects. The compound is said to be cytoprotective by acting as an antioxidant, a calcium modulator, a synaptic neuromodulator and a membrane stabilizer (Schaffer et al., 2003). Most of these effects appear to be indirect (e.g. by taurine affecting the actions of other compounds) rather than direct actions of the taurine molecule itself. Brain taurine contents decline with age in mammals, with other solutes such as glutamate becoming important as osmolytes (Trachtman et al., 1995; Miller et al., 2000). This decline, which is not understood, appears to be the basis for including taurine in most of the new so-called ‘energy’ drinks. There is still much not understood about this solute, something that drinkers of the taurine-rich energy drinks should consider.

The metabolic effects of taurine and other osmolytes are summarized in Table 1, and metabolic roles of other osmolytes that are better understood are discussed in more detail below.

Antioxidation

In some cases, osmolytes may be compatible (i.e. they do not perturb protein structures), while simultaneously being active cytoprotectants by serving as antioxidants. For example, it has been found that cyclitols (cyclic polyols; Fig. 2) and polyols such as mannitol, which are used by many plants for water retention, may also scavenge free radicals generated during drought and cold and other stresses; proline and betaine (also common osmolytes in plants) were not effective (Orthen et al., 1994; Shen et al., 1999). Taurine has already been mentioned; it cannot scavenge reactive oxygen species (ROS) but rather seems to enhance other cellular antioxidant functions. However, taurine can directly bind HOCl (a reactive molecule produced by mammalian leukocytes) to form *N*-chlorotaurine (Schaffer et al., 2003). Glycine betaine has also been implicated in reducing lipid peroxidation in plants (reviewed in Cushman, 2001). Finally, DMSP (Fig. 2), a major osmolyte of marine algae, also has antioxidant properties (Sunda et al., 2002).

Of all solutes accumulated at relatively high concentrations in some situations, hypotaurine, with its reactive sulfur atom (Fig. 2), is one of the strongest antioxidants, able to scavenge OH radicals (which bond to the sulfur atom, converting hypotaurine into taurine) as well as HOCl (Aruoma et al., 1988). Hypotaurine is known to occur at osmotically

significant levels in two situations: mammalian reproductive fluids (where it appears to act as an osmolyte and may protect sperm and eggs from oxygen radicals; Setchell et al., 1993) and marine animals living in sulfide-laden waters (see Sulfide detoxification, below). Why it is not used extensively elsewhere is not clear, but it is possible that using a strong antioxidant in the absence of radicals could lead to cell damage in some way. Thus, it may be accumulated primarily for its antioxidation properties in specific situations, with an osmotic role being a secondary one.

Redox balance and hypoxia protection

Some osmolytes are not actively protective in themselves, but their synthesis may be. Glycerol, the archetypical compatible solute (Brown and Simpson, 1972), accumulates in certain water-stressed yeasts and algae to high levels (up to several molar in species in salt ponds and lakes). Glycerol has been shown to be largely compatible with protein function, but its synthesis also requires the use of NADH. This may be essential for maintaining cellular redox balance (by regeneration of NAD^+) during anaerobic metabolism; indeed, mutant yeasts unable to make glycerol are not only highly sensitive to osmotic stress but also accumulate excessive NADH and thus cannot grow (Ansell et al., 1997). Glycerol may also help reduce radical oxygen production (Shen et al., 1999). Proline accumulation as an osmolyte in water-stressed plants may also be primarily for maintaining redox states, rather than for (or in addition to) compatibility or stabilizing properties (reviewed in Cushman, 2001).

Other osmolytes may protect cells during hypoxia by other mechanisms. β-alanine betaine, a major osmolyte in several species of salt-marsh plants, appears to replace glycine betaine (found in related plants). Unlike glycine betaine, β-alanine betaine requires no direct use of oxygen to produce it, possibly favoring its use in hypoxic muds of salt marshes (Hanson et al., 1994). Recently, high cellular levels of trehalose in fruit flies and transfected mammalian cells have been found to confer enhanced resistance to hypoxia. However, this effect has attributed to protein stabilization (Chen and Haddad, 2004), the second cytoprotective category that will be discussed later.

Sulfide/sulfate detoxification

Large concentrations of two taurine derivatives – hypotaurine (Fig. 2) and thiotaurine – have been reported as major organic components of marine invertebrates living at hydrothermal vents and cold seeps (reviewed in Pruski et al., 2000a). We have shown that these solutes are osmolytes in the sense that they create much of the osmotic pressure of cells, and because they effectively replace the common osmolytes (e.g. taurine, glycine) of non-vent and non-seep invertebrates (Fig. 1, clam seep and Riftia bars; Yin et al., 2000; Fiess et al., 2002). But they may have another role. Vents and seeps emit high quantities of H_2S, a gas that is toxic to animals but that is a primary energy source for some microorganisms. Initially, the two taurine derivatives were found in animals (vestimentiferan tubeworms, vesicomyid clams) that house sulfide-oxidizing microbial symbionts. Pruski et al. (2000a) hypothesized that the solutes either protect from sulfide radicals and/or store and transport sulfide (for future use by the symbionts) nontoxically, as follows:

$$\text{(hypotaurine) } {}^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}SO_2^{-} + HS^{\cdot} \rightarrow {}^{+}NH_3\text{-}CH_2\text{-}CH_2\text{-}SO_2^{-}\text{-}SH \text{ (thiotaurine)} .$$

As evidence for the storage function, hypotaurine is high in all tissues in these animals, but thiotaurine has been found only in non-trace amounts in symbiont-bearing tissues: gills in vesicomyid clams and trophosome in vestimentiferans. This led to a proposal that thiotaurine is a marker of symbiosis (Pruski et al., 2000b). Studies in our laboratory suggest the hypotaurine–thiotaurine reaction has a greater, body-wide cytoprotective role against sulfide in some species: we found that two species of vent gastropods without endosymbionts have both hypotaurine and thiotaurine as major osmolytes (Fig. 1, snail vent bar) and that the ratio of thiotaurine to hypotaurine decreases in animals held in the laboratory without sulfide (Rosenberg et al, 2003).

A different form of sulfur detoxification may be involved in some mangrove plants (angiosperms). Species of *Aegialitis* mangroves use choline-O-sulfate as their primary osmolyte. It has been proposed that the synthesis of this solute serves to detoxify sulfate, a major anion in seawater that can be inhibitory at high concentrations (Hanson et al., 1994). Plants are more vulnerable than animals to inhibitory ion accumulation since most do not have excretory tissues. The methylsulfonium osmolyte DMSP (Fig. 2) may serve a similar role in marine algae.

Other metabolic roles, and compatibility revisited

Other important metabolic and protective functions have been attributed to some osmolytes. Other possible functions for taurine have already been mentioned (see Table 1). Certainly, carbohydrate osmolytes such as glucose, sorbitol and trehalose (commonly accumulated with temperature stress such as freezing) can serve as immediate sources of energy after an organism emerges from a stress-induced dormancy. Defense against predators is another possible function of some osmolytes. DMSP (Fig. 2), widespread in marine microalgae, can be broken down into a gas, DMS (dimethylsulfide), and acrylate, which may serve to repel grazers such as copepods (Wolfe, 2000; Van Alstyne and Houser, 2003). In some terrestrial plants, hydroxyprolinebetaine is accumulated in water stress; an isomer of this (*trans*-4-hydroxy-L-prolinebetaine) is a strong inhibitor of animal acetylcholine esterase and therefore may deter herbivores (Hanson and Burnet, 1994).

The active metabolic roles of osmolytes that have been presented here indicate that many compatible solutes are not interchangeable, which has significant implications for practical applications (more will be said on this later). However, the basic compatibility concept is still probably correct in the sense that most or all of these compounds should not bind to and perturb most macromolecules.

Table 2. *Summary of protective roles of counteracting solutes through stabilization of macromolecules and membranes*

Stabilising property	Counteracting solutes in nature
Counteract urea inhibition	Methylamines, especially TMAO (e.g. shark)
Increase thermostability	Trehalose, anionic polyols (e.g. vent archaeon)
Protect membranes in freezing	Trehalose; proline (e.g. frozen insect)
Preserve in dry state	Carbohydrates, especially trehalose (e.g. dried yeast)
Counteract inorganic ion inhibition	Methylamines (e.g. salt marsh plant)
Counteract hydrostatic pressure	TMAO; other solutes? (e.g. deep-sea fish)

Stabilization and counteraction

The basic compatibility concept is misleading in another way. Numerous studies, often unrelated to research on natural compatible solutes, have shown that these types of solutes can stabilize macromolecular structures (proteins, membranes) in a variety of conditions. However, not all compatible solutes are equal in this regard (although nearly all are stabilizers at high concentrations). Moreover, this property is not necessarily a benefit in and of itself (as will be discussed later). In nature, stabilizing ability seems to be used only when there are stresses that directly destabilize macromolecules and membranes. These stresses include perturbing solutes, anhydrobiosis, high temperature, freezing and high hydrostatic pressure (Table 2).

Perturbing solutes: urea and salts

Some organic osmolytes are able to offset, or 'counteract', effects of solutes that also build up in osmotic stress and that perturb macromolecules. Urea is such a perturbant. It is a highly concentrated waste produce in mammalian kidneys and urine, and it is the major organic osmolyte in marine elasmobranch fishes (ureosmotic animals) (Fig. 1, shark and mammal renal bars). At concentrations in these fishes and mammalian kidneys (e.g. several hundred millimolar), urea destabilizes many macromolecular structures and inhibits functions such as ligand binding. However, these animals have other osmolytes, mainly methylamines such as TMAO and GPC (Figs 1, 2). These solutes do not exhibit simple compatibility, but rather show strong enhancement of protein activity and stability at physiological concentrations. For TMAO, this property is additive with urea's effects such that they counteract, most effectively at about a 2:1 urea:TMAO ratio (Fig. 3A), which is similar to physiological levels (roughly 400:200 mmol l^{-1} in shallow-water elasmobranch fishes; Fig. 1, shark bar). Counteraction of urea has been extensively confirmed in a variety of protein systems (reviewed by Yancey, 2001) and has also been recently demonstrated for nucleic acids in the form of bacterial tRNA (Fig. 3B; Gluick and Yadav, 2003). TMAO is usually a better stabilizer than other osmolytes, including glycine betaine and glycerol (Ortiz-Costa et al., 2002; Russo et al., 2002; Yancey et al., 2004; Kumar et al., 2005), perhaps explaining why TMAO is preferred in ureosmotic fishes. Like basic compatibility, counteraction occurs whether a protein is from a urea-accumulating tissue or not (e.g. bacterial tRNA noted above).

Accumulation of high levels of urea also occur in some amphibians, especially estivating frogs. However, they do not appear to accumulate significant amounts of counteracting osmolytes (Withers and Guppy, 1996). Inhibition by urea may actually be useful during estivation. However, at least some of their enzymes are more resistant to urea than are enzymes of other species (Grundy and Storey, 1994; Fuery et al., 1997). Thus, as originally noted for elasmobranch proteins (Yancey et al., 1982), there may be more than one way to adapt to high urea. Perhaps the metabolic cost of making counteracting osmolytes is disfavored in estivation.

Methylamines can also offset some perturbing effects of salts. Methylated derivatives of glycine (sarcosine, dimethylglycine and glycine betaine) can counteract NaCl inhibition of a plant enzyme's activity, with protection increasing with degree of methylation (Pollard and Wyn Jones, 1979). Many other studies show counteraction of salt inhibition by methylamines, including complex cellular systems (reviewed by Yancey, 2001).

Anhydrobiosis

Disaccharides, most notably trehalose, commonly build up in anhydrobiotic dormant organisms (e.g. baker's yeast, resurrection plants, tardigrades). However, these sugars do not exhibit non-interactive compatibility and are not osmolytes since the organisms lose most of their water. Rather, these solutes appear to bind to macromolecules and membranes, in essence replacing water molecules and maintaining the basic structure of these large biomolecules. Moreover, trehalose forms a glass-like state (i.e. it vitrifies) in the dry state, which also helps preserve cellular structures. Trehalose appears to be better than other biological sugars in forming a protective vitrified state (Crowe et al., 1998). Trehalose also is a non-reducing sugar, which, unlike glucose and some other monosaccharides, does not engage in 'browning' (Maillard) reactions that can damage proteins during drying (reviewed by Tunnacliffe and Lapinski, 2003).

Although *in vitro* experiments have clearly established the efficacy of trehalose, recent studies are questioning its role in anhydrobiosis in nature. In particular, bdelloid rotifers have been found to undergo reversible anhydrobiosis without accumulating trehalose or similar solute (Tunnacliffe and Lapinski, 2003). The issue raised by these observations remains unresolved.

Freezing

Freezing is another stress faced by many ectotherms in which specific small solutes play a role. Strategies to survive

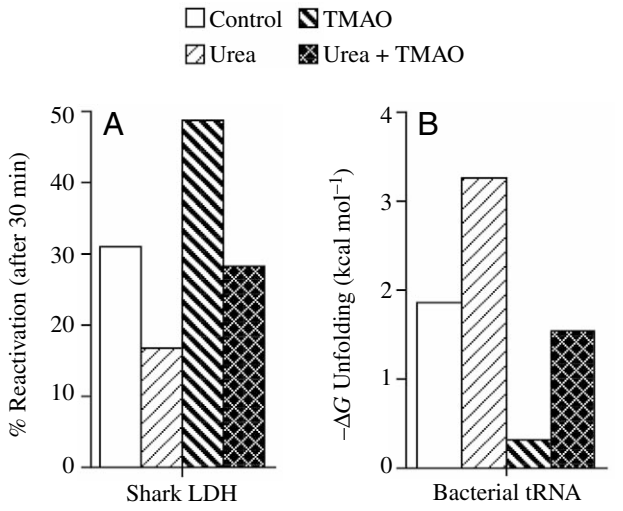

Fig. 3. Old and new examples of counteraction between urea and trimethylamine *N*-oxide (TMAO). (A) Extent of refolding of denatured great white shark A_4-lactate dehydrogenase (LDH) in physiological buffer with no osmolytes (control), 400 mmol l^{-1} urea, 200 mmol l^{-1} TMAO or combined urea:TMAO (2:1) (data from Yancey and Somero, 1979). (B) Free energy (ΔG) of unfolding of *E. coli* $tRNA^{fmet}$ in physiological buffer with no osmolytes (control), 2 mol l^{-1} urea, 1 mol l^{-1} TMAO or combined urea:TMAO (2:1) (data from Gluick and Yadav, 2003).

body temperatures below freezing fall into two categories: freeze avoidance and freeze tolerance. Avoiders (whose body fluids do not freeze) use a variety of mechanisms such as non-colligative antifreeze proteins, reduced nucleation sites and supercooling. Many avoiders also accumulate (in all body fluids) high levels of colligative antifreezes, or cryoprotectants, which are typically compatible carbohydrates such as glycerol. Well-studied model animals that use glycerol include gall moth (*Epiblema scudderiana*) caterpillars (Storey and Storey, 1996) and rainbow smelt, which, unlike most teleost fish, is nearly an osmoconformer due to the accumulation of glycerol as an antifreeze (Raymond, 1992).

Freeze tolerators, by contrast, let their extracellular fluids freeze with the aid of ice nucleators; however, intracellular fluids typically do not freeze due to the presence of, once again, colligative cryoprotectants such as glycerol, trehalose and sorbitol. In this situation, cells shrink somewhat due to increasing extracellular concentrations caused by ice formation. However, shrinkage is limited by the compatible solutes serving as osmolytes as well as antifreezes. Model animals include gall fly (*Eurosta solidaginis*) larvae and intertidal barnacles, which use glycerol, wood frogs (*Rana sylvatica*), which use glucose, and New Zealand alpine wetas (*Hemideina maori*), which use trehalose (Baust and Lee, 1982; Storey and Storey, 1996; Neufeld and Leader, 1998). Carbohydrates are also found as cryoprotectants in many plants.

Thus, small carbohydrates have been selected as colligative antifreezes independently in different taxa and strategies. In addition, certain amino acids such as proline also accumulate in some freeze-tolerant animals (Storey and Storey, 1996; Neufeld and Leader, 1998), but not to levels that would suggest an antifreeze function. In fact, there is some evidence that cryoprotectants fall into two categories with distinct roles. First, the carbohydrates such as glycerol act as both colligative antifreezes, and, in freeze tolerance, as osmolytes (i.e. they reduce loss of cellular water), while at the same time being compatible with macromolecules. Non-carbohydrate solutes might be substituted for this role, but carbohydrates may be preferred as the easiest to both synthesize and transport across membranes rapidly. They also form a ready energy source for use upon emergence from freezing.

By contrast, a second group of cryoprotectants may have stabilizing functions that other solutes do not. In particular, proline and trehalose appear to bind to head groups of membrane phospholipids, in effect replacing water molecules. Thus, they can stabilize membranes during cell shrinkage (Rudolph and Crowe, 1985; Storey and Storey, 1996).

High temperature

Almost all natural osmolytes and other compatible solutes can increase protein thermal stability *in vitro*; although for most osmolytes, this occurs only at non-physiologically high concentrations. However, certain carbohydrate solutes may be used in living organisms to counteract temperature disruption of proteins. For example, heat stress induces accumulation of trehalose in yeast, in which the disaccharide can protect enzymes from thermal denaturation (Singer and Lindquist, 1998).

Hyperthermophilic archaea from marine hydrothermal vents accumulate β-mannosylglycerate, di-*myo*-inositol phosphate and K^+ at high temperatures and salinities. One species has high levels of diglycerol phosphate at high temperatures (Fig. 2; Martin et al., 1999). Both trehalose and anionic osmolytes such as these sugar phosphates (paired with K^+) can stabilize proteins at high temperatures (even boiling in some cases), while other osmolytes are much less effective. One study showed this type of counteraction to be effective on proteins of archaea, yeast and mammals, suggesting a universal ability (Santos and da Costa, 2002).

Hydrostatic pressure in the deep sea

The most recent example of counteraction has been found in the deep sea, where high hydrostatic pressure destabilizes protein structure and ligand binding. Although some proteins appear to have evolved pressure resistance, many have not or have done so incompletely (Siebenaller and Somero, 1989). Our recent studies suggest that some osmolytes can help with pressure. In shallow marine animals, TMAO (Fig. 2) is either absent or found at less than 100 mmol kg^{-1} wet mass (except in ureosmotic fish such as sharks). However, deep-sea teleost fishes (osmoregulators usually thought to have low organic osmolyte levels), as well as certain crustaceans, skates and other osmoconforming animals, have up to 300 mmol kg^{-1} TMAO, increasing with depth (Fig. 4A). Initially, we found the

increase in TMAO down to 3 km depth (Gillett et al., 1997; Kelly and Yancey, 1999); recently, we have found that the pattern extends linearly down to 4.8 km both among different species and within the same species (Fig. 4A; Yancey et al., 2004). In osmoconformers, high levels of TMAO essentially replace the common osmolytes of shallow relatives, e.g. glycine in shrimp, urea in skates, which, in a species from 3 km depth, had a 1:2 urea:TMAO ratio rather than the typical 2:1 ratio of shallow elasmobranchs (Rajids, Fig. 4A). A similar pattern has been confirmed for some sharks (Treberg and Driedzic, 2002).

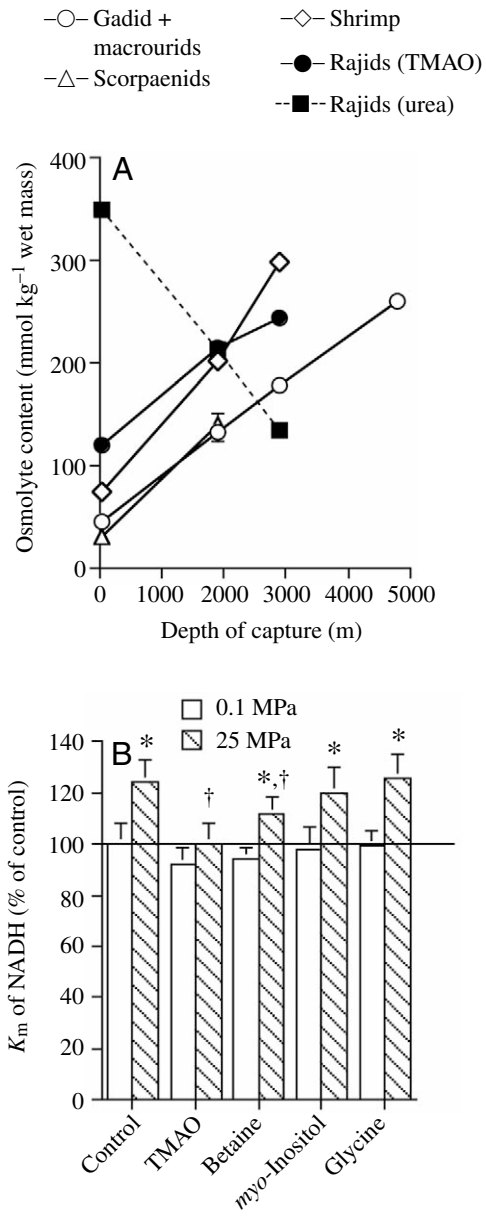

Fig. 4. Trimethylamine *N*-oxide (TMAO) as a possible pressure counteractant in deep-sea animals (see also Fig. 1 for other deep-sea osmolytes). (A) Contents of TMAO (and urea in rajids, as shown) in muscles as a function of depth in shrimp, rajids (skates) and teleost fishes: gadid (cod) and related macrourids (grenadiers), plus scorpaenids (rockfish) (data from Kelly and Yancey, 1999; Yancey et al., 2004). (B) Effect of 250 mmol l^{-1} osmolytes on NADH K_m of A_4-lactate dehydrogenase (LDH) from deep-sea grenadier (*Coryphaenoides armatus*). Measurements were made at atmospheric pressure (0.1 MPa) and 250 atmos (25 MPa), showing that TMAO counteracts pressure better than other common solutes. *Significant increase compared to 0.1 MPa water control; †significant decrease compared to 25 MPa water control (modified from Yancey et al., 2004).

Since hydrostatic pressure is the only environmental factor that is linear with depth, we hypothesized that TMAO might counteract pressure effects. Indeed, TMAO (but not other common osmolytes) *in vitro* was able to offset pressure inhibition of (1) stability of several homologues of lactate dehydrogenase, (2) polymerisation of actin, (3) enzyme-substrate binding for two enzymes and (4) growth of living yeast cells (Yancey and Siebenaller, 1999; Yancey et al., 2002, 2004). One example is shown in Fig. 4B. Other hypotheses to explain the high TMAO in deep-sea animals, such as diet, buoyancy, energy savings (Kelly and Yancey, 1999) and byproduct of lipid storage (Seibel and Walsh, 2002), do not readily explain the highly linear pattern. Thus, TMAO may not be serving primarily as an osmolyte but rather as a pressure counteractant.

Other researchers have found that some sugars and polyols can counteract pressure destabilization of bacterial enzymes (Saad-Nehme, 2001), a concern for the food industry, which is increasingly using pressure for sterilization. These findings raise the possibility that other osmolytes might help counteract pressure in nature.

We have recently found that some deep-sea animals (echinoderms, some mollusks, polychaetes, vestimentiferans, etc.) do not have TMAO, probably because their taxa lack the biosynthesis pathways. However, all have high levels of potentially stabilizing (and often unusual) osmolytes, including the polyol *scyllo*-inositol, and other methylamines, including glycine betaine, GPC and several unsolved methylamines (Fig. 1, 2.9 km bars) (Fiess et al., 2002; Yancey et al., 2002). Also, vesicomyid clams from 2–6.4 km depth contain an unsolved serine-phosphate-ethanolamine compound that increases linearly with depth, forming over 60% of the osmolyte pool of the deepest species (Fig. 1, clam 4 km and 6 km bars; Fiess et al., 2002). Since organic phosphates (e.g. diglycerol phosphate, GPC) have been found to be stabilizers of proteins in other situations, perhaps this compound is also a stabilizer.

Deep-sea bacteria have been found to accumulate the osmolyte β-hydroxybutyrate in correlation with exposure to hydrostatic pressure as well as to osmotic pressure (Martin et al., 2002). The investigators proposed the term ‘piezolyte’ for solutes that are accumulated at high pressure. (This suggests that parallel terms such as ‘thermolyte’, ‘cryolyte’ and ‘anhydrolyte’ might be considered!) Whether the serine-phosphate or β-hydroxybutyrate can offset the effects of pressure is unknown. However, a recent study on marine bacteria has shown that adaptation to salinity synergistically enhances survival at high pressure, suggesting that some

osmolytes may protect against both stresses in these microorganisms (Kaye and Baross, 2004).

Mechanisms of stabilization

The compatible and counteracting hypotheses predict that solute–macromolecule effects are universal, i.e. stabilization should occur with proteins or membranes from any organism regardless of whether it uses osmolytes or not (Wyn Jones et al., 1977; Yancey et al., 1982). How can these effects be universal, given the great diversity in macromolecular structures? The mechanisms are not fully known, but universal water–solute–macromolecule interactions are involved for many osmolytes and related solutes. Destabilizers such as some salt ions and urea generally bind to proteins, causing them to unfold because this exposes more groups that undergo thermodynamically favorable binding with the destabilizer (Fig. 5C). By contrast, many stabilizing solutes do not bind to proteins; indeed, they are excluded from a protein's hydration layer (the water molecules adjacent to a protein's surface) (Timasheff, 1992). Recently termed the 'osmophobic' effect by Bolen and Baskakov (2001), exclusion arises from an apparent repulsion between stabilizers and the peptide backbone, explaining how this effect can be universal. Because of this repulsion, proteins will tend to fold up more compactly, since this will reduce exposure of the peptide-bond backbone to thermodynamically unfavorable interactions with the stabilizing solute (Fig. 5A,B).

Why are stabilizing solutes repelled by the protein backbone? New studies by Bennion and Daggett (2004) show that TMAO enhances water structure (Fig. 5), causing greater organization through stronger hydrogen bonding among water molecules. (By contrast, urea weakens water–water hydrogen bonding.) Possibly, the peptide bond of proteins is less able to interact with (i.e. be hydrated by) the organized water around TMAO than with bulk water.

Other stabilizers may work through more direct interactions, as discussed earlier for membrane interactions of trehalose and other solutes used in anhydrobiosis and freezing. Taurine has also been reported to bind to membranes through ionic interactions (Schaffer et al., 2003). The charged osmolytes of hyperthermophiles (mannosylglycerate, diglycerol phosphate; Fig. 2) appear to enhance native protein conformations through electrostatic interactions, in addition to preferential exclusion (Faria et al., 2004).

The 'yin and yang' of cytoprotection

Are stabilization and counteraction simply another aspect of compatibility, as it is often portrayed? Not necessarily. At its inception, the 'counteracting-osmolytes' hypothesis proposed that a mixture of urea and methylamine is more beneficial than either solute alone, since a methylamine such as TMAO might 'overstabilize' proteins, e.g. making them too rigid for optimal function or causing them to precipitate (Yancey et al., 1982). This concept has not received much attention, but there is evidence supporting it, as follows.

(1) Strong stabilizers such as TMAO and trehalose appear to be high in organisms only when there is a perturbant present (e.g. urea, pressure, high temperature). The pattern of increasing TMAO with depth in marine animals (Fig. 4A)

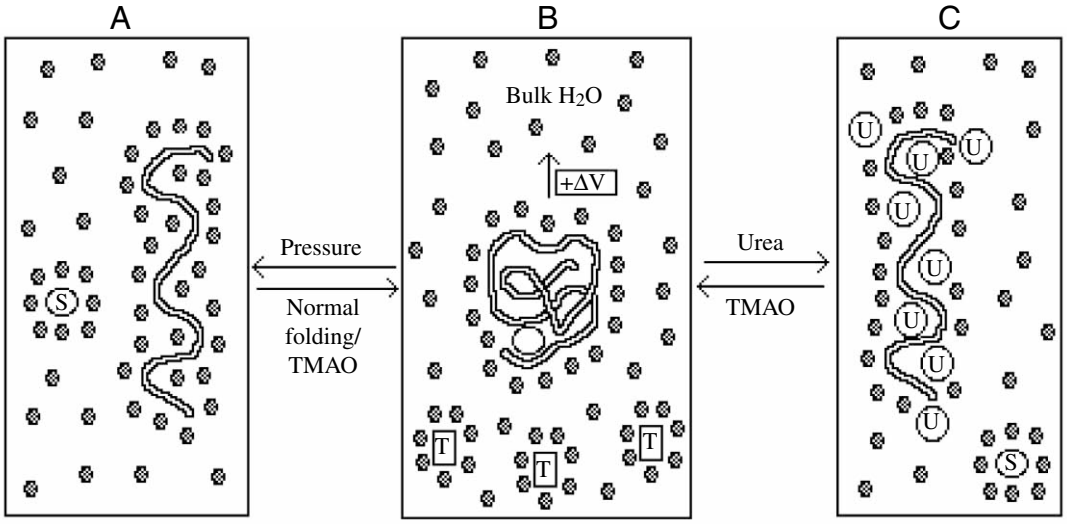

Fig. 5. Model of trimethylamine *N*-oxide (TMAO), urea and pressure effects on protein folding, based on basic pressure effects (Siebenaller and Somero, 1989), counteracting effects (Yancey et al., 1982) and osmolyte physicochemical studies of Timasheff (1992), Bolen and Baskakov (2000) and Bennion and Daggett (2004). Small spheres represent water molecules. (A) An unfolded protein and/or substrate (S) with hydration layers at a higher density than that of bulk water. (B) Thus, upon folding and/or ligand binding, there is a net expansion (+Δ*V*) as water molecules are released into bulk water during folding. If this is the case, hydrostatic pressure will inhibit folding and/or binding (A). (C) Addition of urea (U) enhances unfolding since that maximizes favorable binding interactions. In B, TMAO (T) is surrounded by its own structured water layer, which disfavors exposure of the protein's peptide backbone and of the substrate to bulk water. TMAO thus favors folding and binding, reducing the total order (higher in A and C).

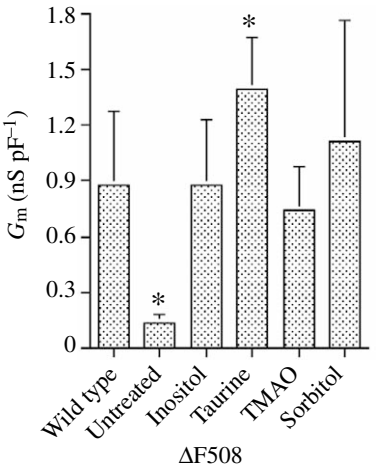

Fig. 6. Specific ion conductance (G_m) of the wild-type and ΔF508 mutant cystic fibrosis transmembrane-conductance regulator (CFTR) in forskolin-stimulated transfected mouse fibroblast cells. The untreated mutant cells have very low conductance compared with the wild type, while cells exposed to 300 mmol l^{-1} of the indicated osmolytes had conductance rates as great or greater than the wild type (modified from Howard et al., 2003). *Significant difference compared to wild type.

illustrates this: if high TMAO is beneficial to deep-sea animals, why isn't it used more extensively by (non-ureosmotic) shallow animals? As another example, the mammalian renal medulla appears to regulate one of its methylamine osmolytes, GPC, to maintain a constant ratio to urea, rather than for osmotic stress alone (Peterson et al., 1992). Again, if this methylamine is a simple compatible solute, why not use it at high levels under all water-stress conditions? Perhaps the costs of synthesis and retention create a tradeoff in the use of some osmolytes, but perhaps the compounds are harmful in the absence of a perturbant.

(2) Methylamines at high concentrations can be detrimental to protein function in the absence of a perturbant, at least *in vitro*. For example, TMAO inhibits some enzymes (Yancey et al., 1982), and it can enhance formation of non-functional protein aggregates (Devlin et al., 2001), including β-amyloid formation (Yancey, 2001).

(3) Using cultured renal cells, we found that adding high urea or glycine betaine alone at high concentrations to the medium greatly reduced cell growth. However, adding both partly or fully together restored normal growth (Yancey and Burg, 1990).

(4) In yeast, high trehalose concentration, induced by temperature stress, protects enzymes at high temperatures, but rather strikingly inhibits them at normal temperatures. Yeasts that cannot eliminate their trehalose suffer when they return to normal temperatures. This has been termed 'the yin and yang of trehalose' (Singer and Lindquist, 1998). This phrase nicely captures the important conclusion arising from these observations, namely that some 'compatible' solutes may be harmful in the absence of a perturbant.

(5) Hypotaurine is one of the most reactive antioxidants of all known compatible solutes, and yet its use in nature at high concentrations is rare. Perhaps it is too reactive for ordinary antioxidant needs.

(6) Some cryoprotectants such as dimethylsulfoxide and ethylene glycol, which can protect protein structure in freeze–thaw cycles, will denature proteins at higher temperatures. This may be due to the fact that hydrophobic interactions increase with temperature, such that these solutes may be excluded from proteins at low but not high temperatures (Arakawa et al., 1990).

Practical applications of osmolytes

As has been reviewed elsewhere (Cushman, 2001; Yancey, 2001), properties of osmolytes are becoming increasingly useful in molecular biology, agriculture and biotechnology. For example, Welch and colleagues have suggested that stabilizing osmolytes, which they call 'chemical chaperones', might rescue misfolded proteins in human diseases (Welch and Brown, 1996). Recently, we found that addition of various mammalian osmolytes and TMAO can indeed restore function of one form of cystic fibrosis mutant protein (Fig. 6; Howard et al., 2003). TMAO can also prevent misfolding of prion proteins (Bennion et al., 2004). Crop plants are also being engineered to accumulate a variety of so-called compatible solutes for stress conditions (Cushman, 2001). Some of these solutes, especially taurine and sometimes inositol and glycine betaine, are major ingredients of a number of energy or sports drinks. However, caution is warranted in all these usages. If, in fact, many of these solutes have unique metabolic reactions and/or stabilizing properties, then they could cause harmful reactions or protein aggregates if used where their non-osmotic properties are not needed.

Unanswered questions and conclusions

Much remains to be learned about the evolution of osmolyte systems. Water–solute–protein interactions are still incompletely understood, for example. Regardless of the mechanisms of osmolyte function, it seems clear that their universal properties should speed up adaptation to water stress conditions relative to the alternative, i.e. evolving macromolecular structures to preserve function in a concentrated ion solution (Yancey et al., 1982). However, it should be noted that the possible co-evolution of protein structures with cellular osmolyte compositions has received little study. Also, non-osmotic protective roles for osmolytes have been well documented in some instances; but in other cases the selective rationales for osmolyte patterns and types in many organisms remain speculative or are not known. Further studies on unique properties of osmolytes need to be conducted.

In conclusion, a variety of other stresses (oxidative, protein-perturbing, etc.) can co-occur with water stress, and many osmolytes probably have unique properties that protect cells

from these disturbances, either through cytoprotective metabolic reactions such as antioxidation or stabilization of macromolecules through water–solute or solute–macromolecule interactions. Understanding these properties will help greatly in elucidating both basic biochemical adaptations and the practical use thereof and may be particularly important if some protective properties of osmolytes are harmful in the absence of a perturbant to offset. If this view is correct, the term 'compatible solute' is often inappropriate. Instead, osmolytes and related solutes should be called 'compatible' only when they clearly have no effect on macromolecules and should be called 'counteracting' when they are used in nature to offset a perturbant. Or, perhaps, the more recent term 'compensatory solute' (Gilles, 1997) should be adopted.

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