Compatible and counteracting solutes: protecting cells from the Dead Sea to the deep sea

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Cells of many organisms accumulate certain small organic molecules – called compatible and counteracting solutes, compensatory solutes, or chemical chaperones – in response to certain physical stresses. These solutes include certain carbohydrates, amino acids, methylamine and methylsulphonium zwitterions, and urea. In osmotic dehydrating stress, these solutes serve as cellular osmolytes. Unlike common salt ions and urea (which inhibit proteins), some organic osmolytes are compatible; i.e.*, they do not perturb macromolecules such as proteins. In addition, some may protect cells through metabolic processes such as antioxidation reactions and sulphide detoxification. Other osmolytes, and identical or similar solutes accumulated in anhydrobiotic, heat and pressure stresses, are termed counteracting solutes or chemical chaperones because they stabilise proteins and counteract protein-destabilising factors such as urea, temperature, salt, and hydrostatic pressure. Stabilisation of proteins, not necessarily beneficial in the absence of a perturbant, may result indirectly from effects on water structure. Osmotic shrinkage of cells activates genes for chaperone proteins and osmolytes by mechanisms still being elucidated. These solutes have applications in agriculture, medicine and biotechnology.*

Keywords: osmolyte, antioxidant, pressure, urea, trimethylamine oxide, temperature, compatible, counteracting, compensatory, chaperone

Introduction

The 21st century has often been predicted to be the 'era of molecular biology', in which major scientific advances will arise from studies of the structure, function and information content of biological macromolecules (DNA and proteins and their integration into genomes and proteomes). As important as this work is, we should not overlook the most common molecules of life, the cellular 'micro-

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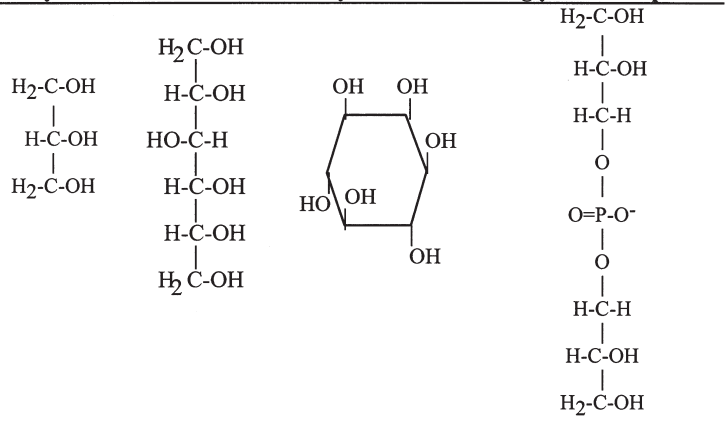
molecules': water, electrolytes, organic metabolites, and other small solutes. The folding of a macromolecule into its proper three-dimensional conformation, the assembly of multimolecular complexes, and reactions engaged in by macromolecules are affected not only by the macromolecules' own structures, but also by the surrounding water and solutes. Among the latter are a variety of small organic molecules accumulated to high concentrations by cells as adaptations to a variety of physical stresses. At a minimum, these solutes provide some form of protection against a stressor while not perturbing macromolecules even at high concentrations. For this reason they are often called *compatible solutes*[1]. They are typically small carbohydrates, amino acids and derivatives, and methylamine and methylsulphonium solutes (also urea, which, however, is not a compatible solute) (Figure 1). Though such solutes have been known for decades, research continues to find new types, uses (both in nature and in practical application) and mechanisms of action. In addition, it is increasingly apparent that the term 'compatible' is somewhat misleading, because some of these solutes have properties which may be harmful unless a perturbant is present. Thus, for reasons that will be discussed, other terms are also used for these molecules, including *counteracting solutes*[2], *compensatory solutes*[3] and *chemical chaperones*[4].

Fig. 1 opposite. *Major categories of compatible and counteracting solutes, with selected examples within each group. For all but urea, dozens of different molecules have been found within each category. TMAO is trimethylamine oxide, GPC is glycerophosphorylcholine; DMSP is dimethylsulphonylproprionate. A: Carbohydrates are uncharged sugars and some charged forms such as diglycerol phosphate in certain archaea. B: Amino acids and derivatives are zwitterions, and some charged forms (not shown) such as β–glutamate in some prokaryotes. C: Methylamine and methylsulphonium zwitterions have methyl groups on nitrogen and sulphur atoms, respectively. Methylamines that are fully substituted with methyl groups are called 'betaines,' the most widespread being glycine betaine (trimethylglycine; found in all kingdoms of life). D: Urea is a major osmolyte in marine cartilaginous fishes (sharks, skates, rays, chimaeras), the coelacanth, and some frogs. It also builds up in some lungfish, frogs and gastropods to help conserve body water during estivation. In the mammalian kidney, urea is concentrated (up to several molar in desert rodents) both as a waste product and as an osmotic agent to help resorb water.*

A. CARBOHYDRATES

POLYOLS | **CYCLITOLS** | **ANIONIC POLYOLS**

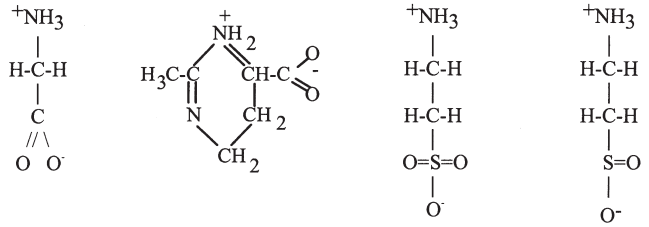
Glycerol | **Sorbitol** | **myo-Inositol** | **Diglycerol Phosphate**



B. AMINO ACIDS AND DERIVATIVES

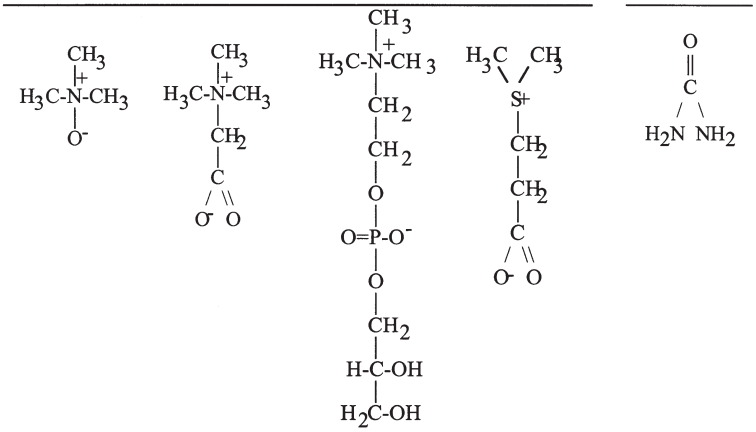
α-TYPE | **CYCLIC TYPE** | **β- TYPES**

Glycine | **Ectoine** | **Taurine** | **Hypotaurine**



C. METHYLAMINE, METHYLSULPHONIUM SOLUTES | **D. UREA**

TMAO | **Glycine Betaine** | **GPC** | **ß-DMSP**



Compatible and counteracting solutes in osmotic stress

To illustrate the use of these micromolecules in nature, consider their best studied role – that of *organic osmolytes*. Osmolytes are small molecules accumulated by cells of many organisms subject to dehydrating osmotic stress (*i.e.*, loss of cellular water). By causing shrinkage of cells, this stress concentrates all cellular molecules, creating inhibitory levels of inorganic ions and altering reaction rates. Dehydration arises from evaporation of water into air, from excretory functions that require water (*e.g.*, removing wastes and salts via the mammalian kidney), and from osmosis into concentrated solutions. The latter arises externally from saline waters (including the oceans, which have salt concentrations much higher than those found in most cells), internally from dehydrating diseases (*e.g.*, diabetes mellitus, in which high blood glucose concentrations causes dehydration), and both externally and internally from freezing (in which solutes are concentrated around ice crystals). The purpose of osmolytes is to reduce or eliminate water loss by elevating cellular osmotic pressure. Cells will also release these solutes if they are subjected to osmotic swelling. Thus osmolytes help maintain cell volume by preventing shrinking and swelling.

When and where are organic osmolytes used? When exposed to a dehydrating force, organisms may respond in a variety of ways. First, cells may simply shrink passively, which typically results in inhibition or death. Second, if an organism is mobile, it might seek a different environment (e.g., a burrow). Third, some multicellular organisms – called *osmoregulators* – have specialised organs (such as a gill or kidney) which mimimise changes in internal osmotic pressure. This is not a common strategy; in the oceans, for example, so-called 'higher' vertebrates (from bony fish to mammals) are among the few organisms that have evolved this 'homeo-osmotic' ability (Figure 2, Cod).

Fourth, individual cells (often within a multicellular organism) may regulate their own volumes with osmolytes. This process is widespread in nature and includes most marine organisms, which are called *osmoconformers* because their internal osmotic pressures are approximately equal to that of the environment (Figure 2, Skates, Clams, Snails, Worm, and *Riftia*). Also, in osmoregulators and their terrestrial descendants, a specialised organ that concentrates salts may itself have to cope with high osmotic pressures. Consider the mammalian kidney. Long considered as exemplary osmoregulators, mammals are now known to have high levels of organic osmolytes in their renal medullary cells, which are exposed to high osmotic pres-

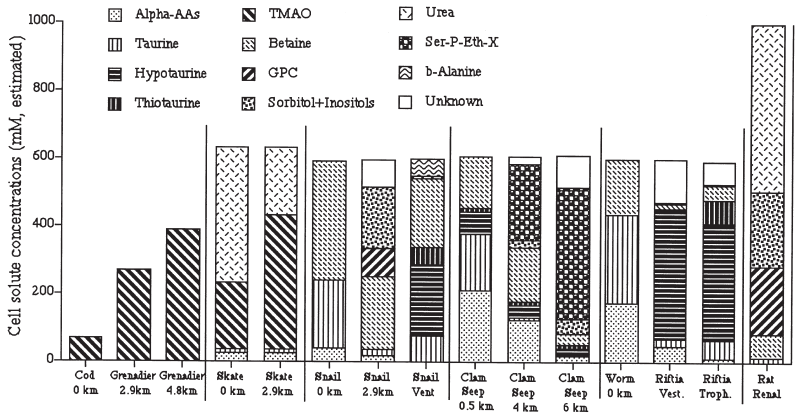


Fig. 2. *Estimates of major organic osmolytes in cells of various marine animals and mammals, based on data from the author's laboratory*[2,8,17,21,29,30]*. Normal cell components not shown include potassium, metabolites, proteins, etc., which typically yield another 300–400 mOsm. Cod and grenadiers are related bony fish (Gadiformes family), with grenadiers being only found in the deep sea. Bony fish are normally classified as osmoregulators and thus have low organic osmolyte levels, as exemplified by the cod.*

sures due to the urine-forming mechanism (Figure 2, Rat Renal). Furthermore, when osmoregulation begins to fail in a severely dehydrated mammal, other cells such as those in the brain and heart will begin osmoconforming using organic osmolytes[5].

In multicellular osmoconformers, extracellular fluids typically have NaCl as the primary osmolyte. In addition, cells undergoing shrinkage stress may use inorganic ions as osmolytes for small and short-term osmotic adjustments. However, this is not a long-term solution for a very important reason: inorganic ions at high concentrations within cells cause a wide variety of perturbing effects, including disruption of protein structure and breakage of DNA[6]. In contrast, most organic osmolytes do not create these problems; on the contrary, as discussed later, some actually protect macromolecules. Thus, organic osmolytes are preferentially used intracellularly rather than inorganic ions for most long-term osmotic stresses. In a marine osmoconformer, these solutes may be the dominant organic component of the entire organism. While the oceans have at osmotic pressure of about 1000 mOsm (milliosmoles per liter), cells of osmoconformers may have only about 300–400 mOsm from inorganic ions and the basic cell solutes (metabolites, proteins, etc.). The remaining 600 mOsm or more can be due to organic osmolytes.

Compatible and counteracting solutes in other stresses

Solutes identical or similar to organic osmolytes are also accumulated in some organisms for purposes other than preventing osmotic shrinkage, most notably during anhydrobiosis (in which organisms enter an extremely dehydrated dormant state)[7], and possibly under hydrostatic pressure[8]. These micromolecules appear to be used primarily for stabilising membranes and/or macromolecules.

Although osmolytes and related solutes usually fall in one of just a few chemical categories, dozens of different types within these categories (except for urea) have been reported (Figure 1 shows only a few examples). What can explain this diversity? The uses of these solutes including recent findings will now be reviewed in more detail.

Compatibility

The basic concept of osmolyte compatibility was first developed using the single-celled alga *Dunalliela*, which inhabits saline lakes such as the Dead Sea, where it faces an osmotic pressure five times that of seawater! These cells osmoconform primarily by accumulating glycerol. Experiments with enzymes showed that, while inorganic ions (especially NaCl) at high levels disrupt protein function, glycerol did not inhibit, even at concentrations of several molar[1] (Figure 3, compatible). Other studies have shown that this compatibility property occurs with proteins from low-salinity organisms without organic osmolytes, leading to an important hypothesis: non-perturbing effects of osmolytes are general features of protein-solute interactions, not the result of specific adaptations in protein structure[2,9]. Thus, the compatibility hypothesis states that certain organic solutes are used to regulate cell volumes because of this universal property.

The hypothesis in its simplest form predicts that most osmolytes are functionally interchangeable. Thus, perhaps the reason osmolytes vary among organisms is simply due to different diets or metabolisms that are unrelated to osmotic stress. For example, the common occurrence of non-nitrogenous carbohydrate and sulphonium osmolytes in some photosynthesisers may have evolved in response to nitrogen limitation. Genetic engineers have used this concept in attempting to create crop plants that can grow, for example, in saline water, using bacterial osmolyte genes[10]. Interchangeability may often be correct; however, the idea has not been extensively tested. Nor does it explain a number of patterns in nature, for example, why

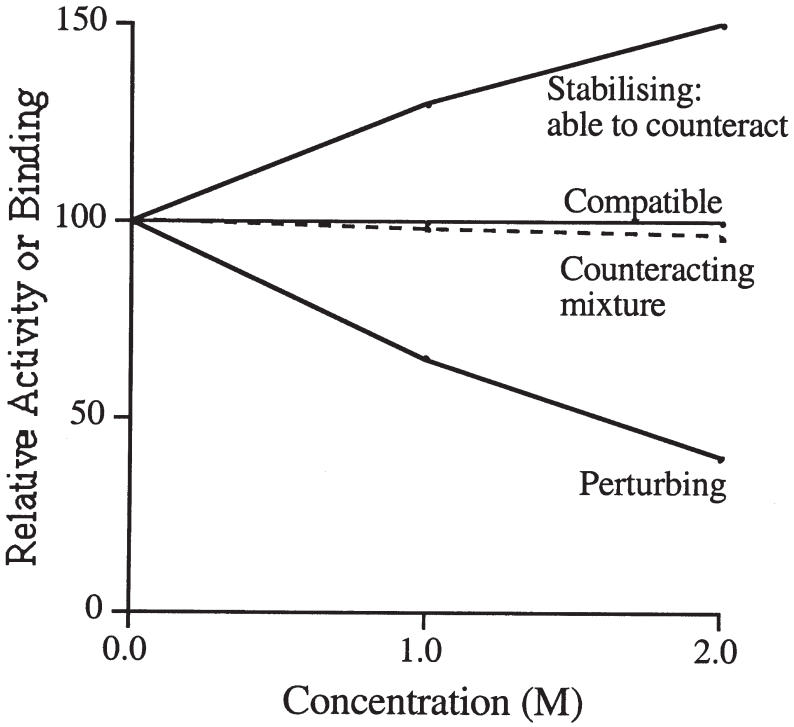


***Fig. 3**. Schematic diagrams of compatible and counteracting effects on proteins, based on real examples. Although concentration is shown on the abscissa, for some perturbants such as temperature and pressure, the factor is different, but the plot would be relatively similar.*

many organisms use mixtures of osmolyte types. Medullary cells of the mammalian kidney accumulate the carbohydrates myo-inositol and sorbitol, the methylamines GPC and glycine betaine, and the amino-acid taurine (Figure 1) to osmoconform to the often high osmotic pressures found in the renal medulla [6,11]. If these solutes are all interchangeably compatible, why are there so many and of different types?

As indicated earlier, the term 'compatible' is misleading, at least for some solutes. While much remains to be learned about the variety of osmolytes and related solutes in nature, the diversity in part appears to result from unique properties of some of these solutes, properties which may be helpful only with certain stresses. These cytoprotective properties fall into two broad categories: protective metabolic reactions, and counteraction of destabilising forces on macromolecules.

Metabolic cytoprotection and compatibility

Some compatible solutes may not be metabolically passive, but rather may be involved in non-osmotic reactions that can protect cells in various ways other than (or in addition to) osmotically. These are as follows (summarised in Table 1):

Antioxidation

In some cases, osmolytes and related solutes may be compatible (i.e., they do not perturb protein structures) while simultaneously serving as antioxidants. For example, it has been found that cyclic polyols (such as inositol, Figure 1) and polyols such as mannitol, which accumulate in many plants during osmotic stress and also in the mammalian kidney, may also scavenge free radicals generated during dehydrating conditions[12]. Taurine (Figure 1), which will be discussed later, is reported in some studies to have antioxidant functions[13], though its chemical structure does not suggest this ability, and the evidence is equivocal. A related solute, hypotaurine with its reactive sulphur atom (Figure 1), is clearly a strong antioxidant, able to bind OH radicals (which bond to the sulphur atom, converting hypotaurine into taurine)[14]. Hypotaurine is found at osmotically significant levels in mammalian reproductive fluids, where it acts as an osmolyte and may protect sperm and eggs from oxygen radicals[15]. It is also a major osmolyte in certain marine animals, as discussed next.

Sulphide/sulphate detoxification

Large concentrations of two sulphur-containing solutes – hypotaurine (Figure 1) and thiotaurine – have been reported in some marine invertebrates living at hydrothermal vents and cold seeps. The solutes were initially found in the giant vestimentiferan tubeworms (Riftia) and vesicomyid clams[16]. These taurine derivatives are osmolytes in the sense that they are responsible for much of the

Table 1 *Summary of protective properties of compatible solutes through metabolic reactions*

Cytoprotective property	Compatible solute in nature	Organism example
Antioxidation	Polyols; hypotaurine; taurine	Mammal
Redox balancing	Proline, glycerol, ß-alanine betaine	*Dunalliela*
Sulphide detoxification/storage	Hypotaurine	*Riftia*
Energy reserve	Glucose, trehalose, etc.	Frog (freezing)
Predator repellent	DMSP, trans-hydroxyprolinebetaine	Diatom
Sulfate detoxification	Choline-O-sulfate	Saltmarsh plant
Ca^{++} modulation	Taurine?	Mammal

osmotic pressure of cells in these osmoconformers, effectively replacing the osmolytes – mainly taurine and glycine – that dominate in shallow non-vent and non-seep worms and clams[8,17] (Figure 2, Seep Clams and Riftia). But these solutes appear to have another role beyond osmotic balance. Organisms at vents and seeps are exposed to high levels of H_2S, which is toxic to animals due to its reaction with iron in respiratory and electron-transport proteins. The conundrum is that the gas is a primary energy source for some microbes, some of which are sulphide-oxidising symbionts housed within the tubeworms and clams where they provide essential energy compounds such a sugars. What reason might there be for using these solutes as major osmotic constituents in place of common compatible solutes? Hypotaurine and thiotaurine are interconvertable as follows (where $HS^{\cdot}$ is a sulphide radical):

$$NH_3^+\text{-}CH_2\text{-}CH_2\text{-}SO^-_2 \text{ (hypotaurine)} + HS^{\cdot} \leftrightarrow NH_3^+\text{-}CH_2\text{-}CH_2\text{-}SO^-_2\text{-}SH \text{ (thiotaurine)}$$

Thus, it has been proposed that these solutes protect the animal from sulphide radicals, and/or they store sulphide for future use by the symbionts, sequestering it in a nontoxic (compatible) form[16]. Indeed, hypotaurine is high in all tissues of the tubeworms and clams, but thiotaurine occurs in non-trace amounts only in symbiont-bearing tissues, which are the gills in vesicomyid clams and the trophosome in vestimentiferans[16]. This favours the storage hypothesis. Also, these animals may not need to make thiotaurine in other tissues because they possess circulatory proteins (hemoglobin in *Riftia*) that bind sulphide for safe transport from the environment to the tissue with the symbionts.

As a further test of this idea, we examined two vent animals – a snail and a limpet – that do not have internal symbionts, but which graze on free-living vent microbes. We found that both have high levels of both hypotaurine and thiotaurine in their bodies (Figure 2, Vent Snail), and that the ratio of thiotaurine to hypotaurine decreases in animals held in the laboratory without sulphide[18]. This suggests that these animals use the solutes for detoxification throughout their bodies. Presumably they do not have a circulatory protein for sulphide transport.

Redox balance

Some osmolytes are not actively protective in themselves, but their synthesis may be. Recall glycerol, the archetypic compatible solute discussed earlier as an osmolyte in *Dunalliela* in the Dead Sea. Glycerol synthesis requires the use of NADH. This may be essential

for maintaining cellular redox balance by regeneration of NAD^+ during anaerobic metabolism[19]. Proline synthesis in water-stressed plants may also serve this purpose[10].

Defense

The methylsulphonium solute DMSP (Figure 1) is widespread in marine algae, in which (at least in some species) it is regulated to track external salinity; i.e., it is an osmolyte. Laboratory studies show that it is relatively compatible with cell proteins, but that does not explain why it is used rather than other common osmolytes such as glycine or taurine or glycerol. The answer may lie in its catabolism. DMSP can be broken down into a gas, DMS (dimethylsulphide) and acrylate, which appear to repel grazers such as copepods[20].

Energy storage

Some carbohydrates such as glucose and trehalose (accumulated in some organisms during freezing and anhydrobiosis, respectively) can serve as immediate sources of energy after an organism emerges from a stress-induced dormancy.

Uncertain functions

Finally, some compatible solutes resist full explanations for their non-osmotic roles. Taurine (Figure 1) is perhaps the most intensely studied, and most enigmatic, compatible solute in this regard. This non-protein amino acid is a major, often the dominant, osmolyte in many marine invertebrates such as worms, snails, anemones and clams in shallow waters[8]. It is not clear why this is. Perhaps it serves to detoxify sulfate (SO_4^{2-}), which is relatively high in seawater; or perhaps it is merely just a compatible solute with no other role, favoured because it is an amino acid is not needed for protein synthesis. Curiously, our recent studies have found that taurine concentrations decline exponentially with depth in clams (Figure 2, Clams) and probably other marine invertebrates; *e.g.*, clams from 6.4 km depth have virtually no taurine[21]. It is not apparent why this pattern should exist.

Taurine is also relatively high in mammalian heart and brain cells, where it clearly serves as an osmolyte during severe dehydration[13]. Seemingly unrelated to its osmotic role, it is also essential for early neural development in mammals. Some mammals can synthesise taurine while others must obtain it from their diets; cats in the wild, for example, acquire taurine from their carnivorous diets, but domesticated housecats must be fed cat food containing taurine if

they are not to develop neural problems. However, it is not clear how taurine exerts its developmental effects. Taurine is said to be cytoprotective by acting as an antioxidant, a calcium modulator, a synaptic neuromodulator, and a membrane stabiliser, but evidence for most of these effects is inconclusive, probably because the effects are indirect (*e.g.*, by taurine affecting the actions of other compounds) rather than as direct actions of the taurine molecule itself. We and others have found that brain contents of taurine decline with age in mammals, the significance of which is also unclear[5] (this decline is the largely unjustified rationale for including taurine in most of the new popular 'energy' drinks).

The metabolic roles of taurine and other compatible solutes summarised in Table 1 indicate that, while most or all of these solutes are compatible in the sense that they probably do not disturb macromolecules, they are not interchangeable. This has implications for practical applications (about which more will be said later).

Stabilisation and counteraction

Metabolically protective solutes are probably compatible in the classic sense; that is, they do not perturb macromolecules as a result of their high concentration, while they simultaneously protect cells through certain metabolic reactions. However, other osmolytes and related micromolecules may not be strictly compatible. Numerous studies have shown that these types of solutes can stabilise macromolecular structures and often enhance activities (Figure 3, Stabilising-able to counteract); indeed, at high enough concentrations, almost all so-called compatible solutes exhibit this property[2]. This led to the term 'chemical chaperones' noted earlier, to connote similarity to molecular chaperones[4], the highly studied stress proteins such as heat shock proteins (HSPs) which help prevent stress-induced denaturation and aggregation of other cell proteins. In fact, as will be discussed later, the roles of chemical and molecular chaperones may overlap in some situations.

Importantly, not all osmolytes and related solutes are equal as stabilisers, with some not exhibiting stabilisation significantly at physiological concentrations. Moreover, for solutes that do stabilise well at such concentrations, this property is not necessarily beneficial by itself. In nature, stabilising ability seems to be used only when there are stresses which directly destabilise macromolecules and membranes, making use of the fact that ability to stabilise can give the ability to *counteract* destabilising forces (Figure 3, Counteracting mixture). (Similarly, molecular chaperones such as HSPs are

also not increased to high concentrations unless there is an inducing stress. So-called constitutive HSPs that are always present are at low levels.) These stresses, summarised in Table 2, are as follows:

Perturbing solutes: urea and salts

Some organic osmolytes are able to counteract effects of other solutes that destabilise macromolecules and ligand interactions. Urea (Figure 1) is such a perturbant. It is (seemingly paradoxically) the major organic osmolyte in shallow marine cartilaginous fishes (ureosmotic animals), and a highly concentrated waste produce in mammalian kidneys. At the concentrations found in these animals, urea should be toxic. The solution to this paradox seems to lie in other osmolytes found in these animals, mainly the methylamines trimethylamine N-oxide (TMAO) and GPC (Figure 1). These methylamines are not compatible in the non-perturbing sense, rather they exhibit strong enhancement of protein stability and ligand binding at physiological concentrations. For TMAO, this property is additive with urea's effects such that they counteract completely at about a 2:1 urea-TMAO ratio, similar to physiological levels, about 400:200 mM in shallow-water cartilaginous fishes[2]. Counteraction of urea by TMAO and other methylamines has been demonstrated with a variety of protein systems from different organisms, with muscle fibers, with living cells[22], and recently with bacterial tRNA[23]. These studies show that counteraction is universal; i.e., it occurs whether a macromolecular system is from a urea-accumulating tissue or not. TMAO is usually a better stabiliser than other osmolytes including glycine betaine. Methylamines can also offset some effects of inhibitory inorganic ions such as Na^{+}[24].

Anhydrobiosis

A number of unrelated organisms routinely undergo severe drying, yet remain viable in a dormant state. Eukaryotic examples include

Table 2 *Summary of protective roles of stabilising/counteracting solutes through stabilisation of macromolecules and membranes*

Stabilising property	Counteracting solutes in nature	Organism example
Counteract urea	Methylamines, especially TMAO	Elasmobranch
Increase thermostability	Trehalose, anionic polyols; ectoines?	Vent archaea
Protect in freezing	Carbohydrates	Frog
Preserve in dry state	Carbohydrates, especially trehalose	Baker's yeast
Counteract inorganic ions	Methylamines; others?	Salt marsh plant
Counteract hydrostatic pressure	TMAO; others?	Deep-sea fish

brine shrimp, baker's yeast, and tardigrades. Disaccharides, most notably trehalose, commonly build up in these organisms, but not as an osmolyte since over 95% of cellular water is lost in the dormant stages. Trehalose appears to bind to macromolecules and membranes, in essence replacing water molecules, through hydrogen bonding with its many hydroxyl groups. Furthermore, trehalose vitrifies (*i.e.*, forms a glass-like state) in the dry state, and does so more effectively than many other sugars. Both of these effects maintain viability of large biomolecules (although in a non-functional state)[7]. Furthermore, trehalose is a non-reducing sugar which, unlike glucose and some other monosaccharides, does not engage in 'browning' (Maillard) reactions which can damage proteins during drying[25]. There is no general term (parallel to 'osmolyte') for solutes like this. (Perhaps we should call them 'anhydrolytes'!)

The efficacy of trehalose in anhydrobiotic preservation has been primarily demonstrated in vitro. In vivo, however, the situation is more complex. Trehalose decidedly does accumulate to high levels in many anhydrobiotic organisms, but not all. For example, bdelloid rotifers undergo reversible anhydrobiosis without accumulating trehalose or similar solute. Also, bacteria genetically engineered to produce counteracting solutes, and then subjected to desiccation in the laboratory, did not survive being dried out when engineered for trehalose production, but exhibited some viability when engineered for production of hydroxyectoine, an amino acid derivative . The uncertainty raised by these observations remains unresolved[25].

Freezing

During freezing, solutes are concentrated by extracellular ice-crystal formation, leading to cellular dehydration. Many freeze-resistant organisms accumulate carbohydrates such as glucose or sorbitol; these serve both as compatible osmolytes, which reduce water loss, and as cryoprotectants, which lower the internal freezing point. Some studies suggest organisms accumulate two different types of cryoprotectants: basic compatible solutes such as glycerol, and membrane stabilisers such as proline and trehalose. The latter may bind to head groups of membrane phospholipids, replacing water molecules (akin to the actions of trehalose in anhydrobiosis)[26].

High temperature

Almost all natural osmolytes and related solutes can increase protein thermal stability in vitro, although for most of these micromolecules, non-physiologically high concentrations are required. However, cer-

tain carbohydrates 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 sugar can protect enzymes from thermal denaturation [27]. A species of hyperthermophilic archaeon from a hydrothermal vent accumulates ß-mannosylglycerate (paired with K^+ for electroneutrality) with osmotic stress, and di-myo-inositol phosphate and K^+ with thermal stress[28]. The former is therefore an osmolyte, while the latter is something else with no general term (perhaps 'thermolyte'?). Another vent archaeon has high levels of diglycerol phosphate (Figure 1). Both trehalose and anionic solutes such as these sugar phosphates (paired with K^+) can stabilise proteins at high temperatures (even boiling in some cases), while other solutes of this type are much less effective. In one study, counteraction was effective on proteins of archaea, yeast, and mammals, again showing the universal nature of these types of interactions[28].

Hydrostatic pressure in the deep sea

We have recently proposed another type of counteraction in the deep sea, where high hydrostatic pressure destabilises protein structure and ligand binding by trapping layers of dense water around highly polar and charged groups. Although some proteins have evolved resistance to pressure effects, many have not or have done so incompletely. Some counteracting solutes may help. In shallow marine animals, TMAO (Figure 1) is either absent or found at less than 100 mmol kg^{-1} wet wt. (except in ureosmotic fish such as sharks). However, deep-sea teleost fishes (osmoregulators which might be predicted to have low organic osmolyte levels), as well as certain crustaceans, skates and other osmoconforming animals, have as much as 300 mmol kg^{-1} TMAO, which would indicate more than 400 mM within cells (Figure 2, Grenadiers and 2.9 km Skates). Most strikingly, TMAO contents increase linearly with depth, in bony fishes down to 4.8 km, both among and within species[8,29,30].

In deep-sea osmoconformers, TMAO is an osmolyte because it essentially replaces the common osmolytes of shallow relatives, namely glycine in shrimp and urea in skates. Recall that the latter type of animal, like other elasmobranchs, has about a 2:1 urea-TMAO ratio in shallow species. This pattern reverses with depth, such that a skate from 3 km depth has a 1:2 urea-TMAO ratio (Figure 2, Skates)[29]. In teleost, the increase in TMAO with depth simply increases the total internal osmotic pressure in these animals (Figure 2, Grenadiers). The solute will certainly act as an osmolyte in this case, but it may not be regulated for this purpose.

Since hydrostatic pressure is the only environmental factor that is linear with depth, we hypothesised that TMAO might be a pressure counteractant. Indeed, experiments revealed that TMAO (better than other common osmolytes such as glycine and glycine betaine) is able to offset pressure inhibition in numerous ways. These include: (1) stability of several homologues of lactate dehydrogenase (LDH) from deep, shallow and terrestrial vertebrates, (2) polymerisation of actin from a deep-sea fish; (3) enzyme-substrate binding for LDH and pyruvate kinase from deep and shallow animals; and (4) growth of living yeast cells[8].

We and others have suggested alternative hypotheses to explain the high TMAO in deep-sea animals. These hypotheses include temperature (*e.g.*, perhaps TMAO counteracts cold-denaturation of proteins); diet; increased buoyancy (TMAO solutions are lighter than seawater), energy savings (through reduction of osmotic gradients)[29]; and a byproduct of lipid metabolism[31]. However, none of these ideas readily explain the highly linear pattern and why such possible roles would not occur in shallow animals as well.

Other researchers have found that some sugars and polyols can counteract pressure destabilisation of bacterial enzymes[32]. This is a concern for the food industry which is increasingly using hydrostatic pressure for sterilisation. This also brings up the possibility that other osmolytes might counteract pressure in deep-sea life.

Regarding that possibility, some deep-sea animals (echinoderms, some mollusks, polychaetes, vestimentiferans, *etc.*) do not have TMAO, probably due to absence of synthesis pathways and dietary sources. However we found that all of these animals (at least the species examined) have high levels of osmolytes different from those of shallow species; moreover, these osmolytes are potentially counteracting solutes, including the polyol *scyllo*-inositol, and other methylamines including glycine betaine, GPC, and several unsolved methylamines (*e.g.*, Figure 2, 2.9 km Snails)[8]. Also, vesicomyid seep clams from 2 to 6.4 km depth contain an unsolved serine-phosphate-ethanolamine compound which increases linearly with depth, forming over 60% of the osmolyte pool of the deepest species (Figure 2, Seep Clams)[21]. This solute contains phosphate, a known stabilising anion, and an undetermined moiety currently being investigated.

Other researchers have recently found that some deep-sea bacteria accumulate the osmolyte ß-hydroxybutyrate in correlation with exposure to hydrostatic pressure as well as to osmotic pressure[33]. The investigators coined the term *piezolyte* for such solutes. However, it is not yet known whether ß-hydroxybutyrate, the serine-

phosphate-ethanolamine unknown, or *scyllo*-inositol can counteract the effects of pressure.

Mechanisms of stabilisation by counteracting solutes

As noted earlier, compatible and counteracting concepts are based on universal solute-macromolecule effects, *i.e.*, ones that do not involve special adaptations in macromolecules that have evolved in the presence of the solutes[2]. The mechanisms are not fully known, but universal water-solute-macromolecule interactions are clearly involved. Destabilisers such as some inorganic ions and urea generally bind to proteins, causing them to unfold because more this exposes more groups that undergo the thermodynamically favourable binding with the destabiliser (Figure 4, right). The stabilising/counteracting solutes work through more than one mechanism. First, recall that some stabilisers probably bind to macromolecules but in a manner that replaces water molecules while maintaining viable structure (as in anhydrobiosis). Second, the solutes used as

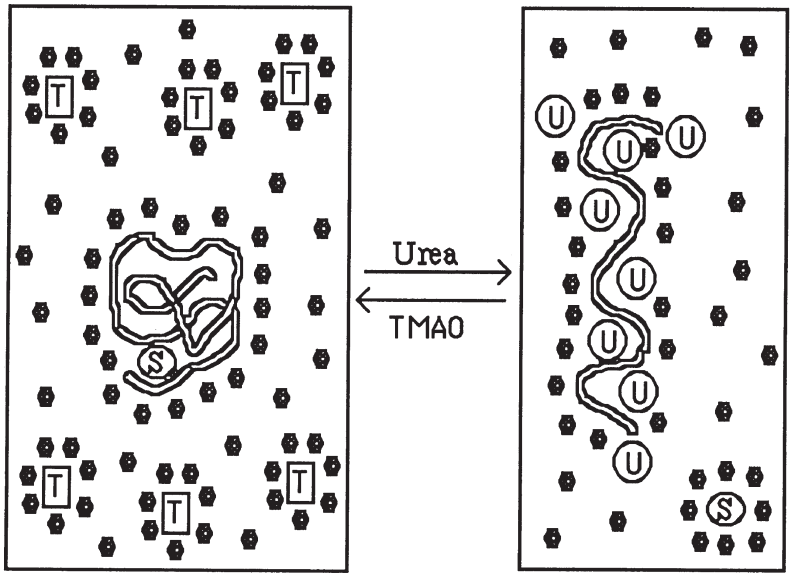


Fig. 4*. Simplified representations of water molecules (small circles) and a protein (long strand). with TMAO (T), a strong stabilising solute, and urea (U), a denaturing solute. Urea binds to the protein and so favours unfolding. TMAO enhances water structure and interferes with water interaction with the peptide backbone; this favours folding which minimises exposure of the backbone to the water-TMAO solution.*

osmolytes do not bind to proteins; rather, they are notably excluded from the layer of water molecules adjacent to a protein's surface (Figure 4, left)[34]. This *preferential exclusion*, in turn, may result from one or more mechanisms, two of which are proposed to be as follows:

Steric exclusion

Any large dissolved solute takes up more space than a water molecule, and if binding strongly to water molecules and not attracted to a protein, the solute will be less able to pack next to it than water. This geometric exclusion model may apply to carbohydrate osmolytes, whose hydroxyl groups may allow them to interact well with bulk water but not with water at protein surfaces[34].

Osmophobicity

According to recent extensive data, preferential exclusion for TMAO arises from a thermodynamic repulsion between this stabiliser and the peptide backbone, explaining how TMAO's effect can be universal[35]. Proteins will tend to fold up more compactly in the face of this 'osmophobic'[35] effect to minimise the number of unfavorable interactions between the stabilising solute and the peptide backbone (Figure 4, left). Osmophobicity may arise from TMAO-water interactions, resulting perhaps from the fact that the TMAO molecule combines a strong zwitterionic dipole with a hydrophobic (methylated) end. New studies indeed show that TMAO enhances water structure , causing greater organisation and stronger hydrogen bonding among water molecules near it[36]. Possibly the peptide bond of proteins is less able to interact with (*i.e.*, be hydrated by) the organised water around TMAO than bulk water.

The 'yin and yang' of cytoprotective solutes

As mentioned several times already, stabilisation and counteraction are not necessarily another aspect of compatibility, as it is often portrayed. The counteracting-osmolytes hypothesis was initially based on the urea-TMAO mixture of cartilaginous fishes[2]. Two questions posed at the time were: why is there a mixture rather than pure urea or TMAO, and why is the ratio fairly consistent? The hypothesis originally suggested that a mixture of urea and TMAO at a specifc ratio is more beneficial than either solute alone, since TMAO alone might 'overstabilise' proteins, e.g., making them too rigid for optimal function , causing ligand binding to be too tight, and/or causing

proteins to precipitate[2]. This concept has not received much focus, but there is evidence supporting it, as follows:

Evidence in nature

Strong stabilisers such as TMAO and trehalose are reported to be high in organisms only when there is an obvious perturbant present, mostly notably urea, hydrostatic pressure, and heat. Recall the linear increase in TMAO with depth in marine animals (Figure 2): If high TMAO is beneficial to deep-sea animals as an (interchangeable) compatible solute, for buoyancy, as a useful chaperone, or by reducing osmotic gradients in osmoregulators, why isn't it used more extensively by shallow animals? Notably, the only known exceptions to this in shallow waters are the ureosmotic fishes. It is possible that the energy required to make TMAO creates a cost-benefit tradeoff that selects against its use in shallow waters, but this seems unlikely given that the deep-sea is severely energy limiting compared to shallow waters.

As another example, the mammalian renal medulla appears to regulate one of its methylamine osmolytes, GPC, to maintain a constant ratio to the concentration of urea, which is high both extracellularly and intracellularly in high protein diets. However, GPC is not regulated to follow renal salt concentration in salt-loaded or dehydrated animals, in which extracellular but not intracellular NaCl levels are high[11]. Again, if this methylamine is interchangeably compatible, or a useful general chaperone, why is it not at high levels when extracellular salt but not urea is high in the kidney?

Some metabolic protectants seem also to remain at low levels unless an appropriate stress is present. For example, hypotaurine, which is probably the most reactive antioxidant of all the compatible solutes, is not used extensively in nature (at least at high concentrations). If antioxidation power were always beneficial to cells, then those that must have organic osmolytes for osmotic balance might be expected to use it routinely (in place of taurine, for example).

Evidence from experiments

Test with living cells suggest that some stabilisers are harmful by themselves. Using cultured mammalian renal cells, we found that adding high urea or glycine betaine alone to the medium greatly reduced cell growth. However, adding both partly or fully restored normal growth [22].

Stabilising solutes at high concentrations can in fact be detrimental to protein function. For example, TMAO inhibits some enzymes[2],

and it can enhance formation of non-functional protein aggregates[37], including ß-amyloid formation[11]. High trehalose concentrations, induced by temperature stress in yeast, protect yeast enzymes at high temperatures, but rather strikingly inhibit them at normal temperatures. This was memorably called the 'the yin and yang of trehalose'[27].

These observations do support the idea that many counteracting solutes are harmful in the absence of a perturbant. In other words, they are not compatible in the classic sense.

Regulation of osmolytes

For many of the organisms using compatible and counteracting solutes, little is known about how the solutes are regulated, and complete steps from sensing of the stress to activation of appropriate genes have not been fully elucidated for any system. The most extensive studies have been done on bacteria, yeast and mammalian renal cells. Only the latter will be briefly reviewed here. During an initial water loss following exposure to a hypertonic solution, renal cells regulate volume with inorganic ions while upregulating molecular chaperones of the HSP type. The HSPs may provide initial protection for cell proteins against the effects of higher levels of intracellular ions (see reference 6 for a review of these studies). More slowly, genes for proteins involved in organic osmolyte regulation are induced and osmolytes begin to accumulate (Figure 5). As the organic osmolytes begin to accumulate, levels of HSP begin to decline, presumably because cell proteins are no longer perturbed (Figure 5)[6,11]. Thus there is coordination in the use of molecular and chemical chaperones. Perhaps the HSP chaperones maintain temporary viability of proteins, but not in a functional form, while the organic osmolytes restore normal function. Traditionally, these studies have been done with a single-step osmotic shock, an event that does not happen in a normal kidney in vivo. New studies in which renal cells were more gradually (and realistically) exposed to increasing osmolarity reveal that induction of HSP and osmolyte genes is even more pronounced[6].

The osmolyte genes include AR (for aldose reductase, an enzyme which makes sorbitol), BGT1 (for betaine-GABA transporter, which brings glycine betaine into the cell), and SMIT (for sodium-*myo*-inositol transporter, which brings that polyol into the cell). All these genes have promoter regions containing a response element called TonE (tonicity element) or ORE (osmotic response element). In turn, the response elements are activated by a binding protein (BP) called TonEBP or OREBP[6]. Still lacking is an understanding of the sensing

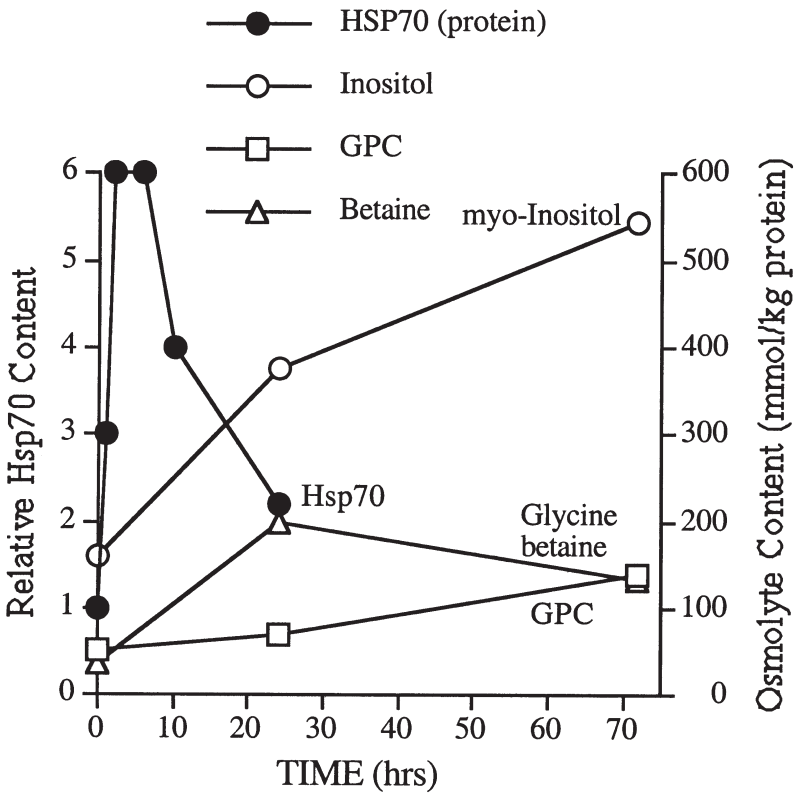


Fig. 5. *Time course of changes in cell contents of heat-shock protein Hsp70 and the osmolytes shown in Madin-Darby canine kidney cells in culture exposed to medium increased at time 0 from normal 290 mOsm to 500 mOsm*[11].

and signaling mechanism that result in TonEBP binding to the promoters. Some studies have shown that gene activation correlates well with intracellular osmotic strength, which would increase during initial osmotic stress from rising levels of inorganic ions[6]. However, a new study contradicts this. Human 293T cells were subjected to water loss by hypertonic solution and by desiccation, both of which raise intracellular osmotic strength. Paradoxically, only hypertonic stress (and only from Na salts and mannitol, but not from Kcl or sorbitol) resulted in activation of AR, BGT1 and SMIT; desiccation had no effect[38]. Other signals involved in activation of TonEBP have been found, but a complete sequence of signal events is still being sought[6].

Genomic analysis is now being brought to bear on this issue with renal cells and with a new model system – the nematode *C. elegans*, a well characterised genetic and developmental model animal whose genome has been sequenced. These worms have been made to survive in 500 mM NaCl, similar to seawater. The worms initially shrink upon exposure to the high salt, but then restore normal volume through the accumulation of glycerol. Northern blot analysis revealed an increase in transcription for glycerol 3-phosphate dehydrogenase, a key enzyme in glycerol production. Future studies will employ DNA microarrays to look for other genes involved[39].

Practical applications of compatible and counteracting solutes

Properties of osmolytes make them potentially useful in molecular biology and biotechnology, such as stabilisation of laboratory reagents and pharmaceuticals. These applications and others in non-living systems have been reviewed elsewhere[11]. For living systems, a number of agricultural and medical applications are being explored. Crop plants are being engineered to accumulate a variety of so-called compatible solutes for water-related stresses (salinity, drought)[10]. Welch and colleagues have suggested that chemical chaperones might be used medically to rescue misfolded proteins in many human diseases[4]. *In vitro*, this idea has some support. Prions (the scrapie type) have been made to fold into non-harmful forms in the presence of TMAO[4]. Recently, we found that addition of various mammalian osmolytes and TMAO can restore function of a cystic fibrosis mutant protein in cultured cells[40]. Another group found that dietary trehalose enhanced survival of mice with a disease similar to that of human Huntington's. The brains of these animals had fewer of the protein clumps that characterise this devastating disease[41].

However, caution is warranted in all these usages with living cells. If in fact many of these solutes engage in powerful metabolic reactions or have strong stabilising properties, then they could be harmful if used at high concentrations without a perturbant to offset.

Conclusions

Compatible and counteracting solutes, because they can protect cells in universal fashion, should increase the rate of evolutionary adaptation. That is, it is presumably simpler to evolve the ability to accumulate a solute that broadly protects cell macromolecules than the alternative, *i.e.*, evolution of macromolecular structure (involving thousands

of genes) to preserve function in a stress situation[2]. Some of these micromolecules are probably compatible with macromolecular function while at the same time are able to provide cytoprotective metabolic reactions. Others are stabilisers of macromolecules through universal water-micromolecule-macromolecule interactions, used to counteract specific perturbants of macromolecules. Some organisms may take advantage of several roles simultaneously. For example, cells of the mammalian renal medulla, in the face of high extracellular NaCl and intracellular urea, regulate their volumes using methylamines and polyols. The former (or at least GPC) may counteract urea[11], while the latter are compatible solutes with possible antioxidant functions (reactive oxygen species are known to be higher in hypertonic stress[6]).

In many instances, non-osmotic protective roles for these solutes have been well documented, though much remains to be learned about the mechanisms involved. But in other cases the selective rationales for the patterns and types of these solutes in many organism remain speculative or are not known. Much more research needs to be done, both for the basic knowledge and for potential practical applications.

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