CMLS, Cell. Mol. Life Sci. 57 (2000) 1018–1032
1420-682X/00/0701018-15 $ 1.50 + 0.20/0
© Birkhäuser Verlag, Basel, 2000

**CMLS Cellular and Molecular Life Sciences**

# Intracellular osmotic action

**R. P. Rand[a,*], V. A. Parsegian[b] and D. C. Rau[b]**

[a]Department of Biological Sciences, Brock University, St. Catharines, ON, L2S 3A1 (Canada),
e-mail: rrand@spartan.ac.brocku.ca
[b]Laboratory of Physical and Structural Biology, National Institute of Child Health and Human Development,
National Institutes of Health, Bethesda (Maryland 20892-5626, USA), e-mail: aparsegi@helix.nih.gov,
donrau@helix.nih.gov

**Abstract.** Water often acts as a critical reactant in cellular reactions. Its role can be detected by modulating water activity with osmotic agents. We describe the principles behind this 'osmotic stress' strategy, and survey the ubiquity of water effects on molecular structures that have aqueous, solute-excluding regions. These effects are seen with single-functioning molecules such as membrane channels and solution enzymes, as well as in the molecular assembly of actin, the organization of DNA and the specificity of protein/DNA interactions.

**Key words.** Water; osmotic stress; channels; enzymes; DNA; actin.

## Introduction

Cells and their parts are sensitive osmometers. The full gamut of cellular machinery is a slave to the osmotic environment. Does it matter? Are there intracellular osmotic compartments that render molecular reactions sensitive to water activity, and therefore to solute activity? What are the solutes that can act osmotically?
As more isolated subcellular systems are interrogated, it becomes increasingly clear that most molecular reactions, and many molecular structures 'see' water not only as an indifferent solvent and space filler, but also as a small molecule which acts as a critical reactant. Here we describe an important method of interrogation, and the answers for a number of qualitatively different cellular and molecular processes.

## The interrogation

The idea behind 'osmotic stress' (OS) is nothing more than to treat water as just another small molecule, like any small 'solute', and to ask if changing its activity changes the behavior of a system [1, 2]. Water activity is lowered through the addition of any nonwater molecules. Any region from which a nonwater molecule is excluded will be 'osmotically stressed'. The lower activity makes it more difficult for that region to be maintained pure of the osmotic agent. That region will then be more likely to be 'dehydrated'. Figure 1 illustrates examples of species of compartments in cells that can be subject to such osmotic interrogation. The first systems to be interrogated osmotically were phospholipid assemblies [3], followed by DNA [4], polysaccharides [5] and protein systems [6, 7]. The behaviour of all systems examined in order to measure forces confirmed the ubiquity and commonality of hydration energetics [8]. We describe here examples showing an often surprising dependence of structure or of molecular activity upon water itself. Such common dependence shows that solutes of different sizes can exert different osmotic effects on different intracellular regions. Such is the extent of this dependence that one suspects the existence of a level of cellular activity that is mediated through water activity.

## The systems

Figure 2 illustrates the different systems we shall use to describe water's role in cellular activity. Membrane channel gating, DNA assembly and protein binding, soluble enzyme activity and molecular assembly demon-

* Corresponding author.

strate the ubiquity of OS sensitivity. The regions or compartments from which solutes are excluded are of two qualitatively different kinds. First, steric exclusion results when the solute is too large to enter the aqueous space. Second, preferential hydration of surfaces exposed to the stressing solution may occur. Preferential hydration, a term coined by Timasheff, is conceptually less intuitive. It means simply that the solute/water ratio near a surface is less than that in the bathing solution because that surface prefers to interact with water rather than solute. In that sense it is the opposite of preferential solute binding. Figure 3 illustrates this effect by plotting increasing solute and diminishing water concentrations as functions of increasing distance from the surface. The preferential exclusion of solute from the surface generates the compartment of preferential hydration that can be probed by osmotic stress. Preferential hydration may occur from simple steric exclusion from the surface (also termed depletion forces or crowding) or from an energetically more favorable interaction of the surface with water than with solute (called by Timasheff a surface tension mechanism of exclusion).

Solute exclusion for steric reasons and exclusion because of preferential hydration can coexist in any system. Figure 4 illustrates schematically how combinations of solute, the kind of surface and the conformation of that surface determine which compartments are stressed osmotically for any solute excluded from them.

Any purely osmotic interpretation of such effects requires that there be no change in binding of the solutes that are used to change the activity of water. The criteria for such an osmotic interpretation are elaborated by Timasheff [9] and by Parsegian et al. [1]. Specifically, (i) If the effect of added solute is due to solute binding rather than to osmotic stress, when solute $k_D \ll$ [solute], then the effect will vary with the solute activity, that is as log [solute]. At the dilute limit, osmotic action is linear with respect to solute concentration. The distinction can immediately be seen by plotting effect vs. log [concentration] or vs. [concentration] itself; (ii) osmotic action is less likely to depend strongly on solute identity than is action through solute binding. Measurements with widely different kinds of agents are useful here.

Dependence of the osmotic action on solute size and chemical nature can help to distinguish among the kinds of exclusion. Steric exclusion from cavities, channels, grooves or pockets will show little dependence on solute nature or size (once large enough to be excluded). An exclusion of solutes from exposed surfaces typically shows osmotic effects that are naturally sensitive to both size and chemical nature.

If these criteria are established, there should then be no confusion in interpretation [1, 9, 10].

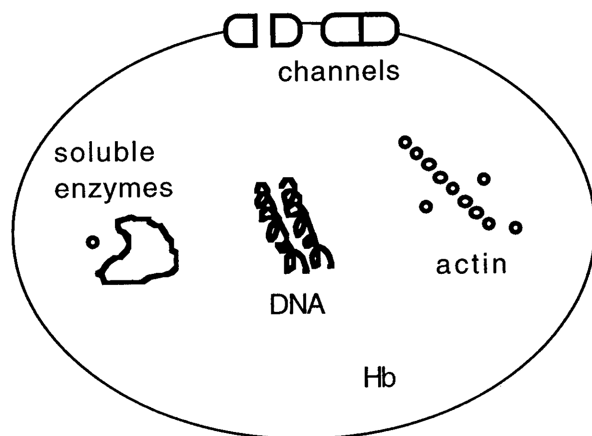



Figure 1. Schematic example of cellular components that can be probed using osmotic stress to determine the possible role of water in molecular assembly or molecular activity.

## Single-functioning molecules

### Channels

The first single molecules to be investigated osmotically were membrane channels [11–14]. The aqueous cavities within ionic channels are the easiest structures by which to conceive the ways of osmotic stress. A large set of ‘mesoscopic’ channels, alamethicin, voltage dependent anionic channel (VDAC) and porins, have been investigated. Imagine a cavity too small for entry of a large solute. It is as though the small space were bounded by a semi-permeable membrane (fig. 5A). To create such a space requires work to ‘purify’ the water that must be drawn away from the bathing solution to fill that space. Increasing concentrations of excluded solute require increasing work of purification. If there are two forms of the cavity-containing structure, open and closed, containing different volumes of solute-excluding waters, $V_{open}$ and $V_{closed}$, then the added work of cavity formation favors the form that requires fewer waters. In the language of osmotic pressure, an added osmotic pressure, $\Pi_{osmotic}$, of the solute changes the work needed to open the cavity by an amount equal to $\Pi_{osmotic} \times (V_{open} - V_{closed})$.

It is easy to see that large, completely excluded, solutes (fig. 5A) are not equivalent to small solutes that can enter the cavity (fig. 5B). There will usually be a smaller apparent change in the difference ($V_{open} - V_{closed}$) in the volume that is measured as accessible to the smaller solutes.

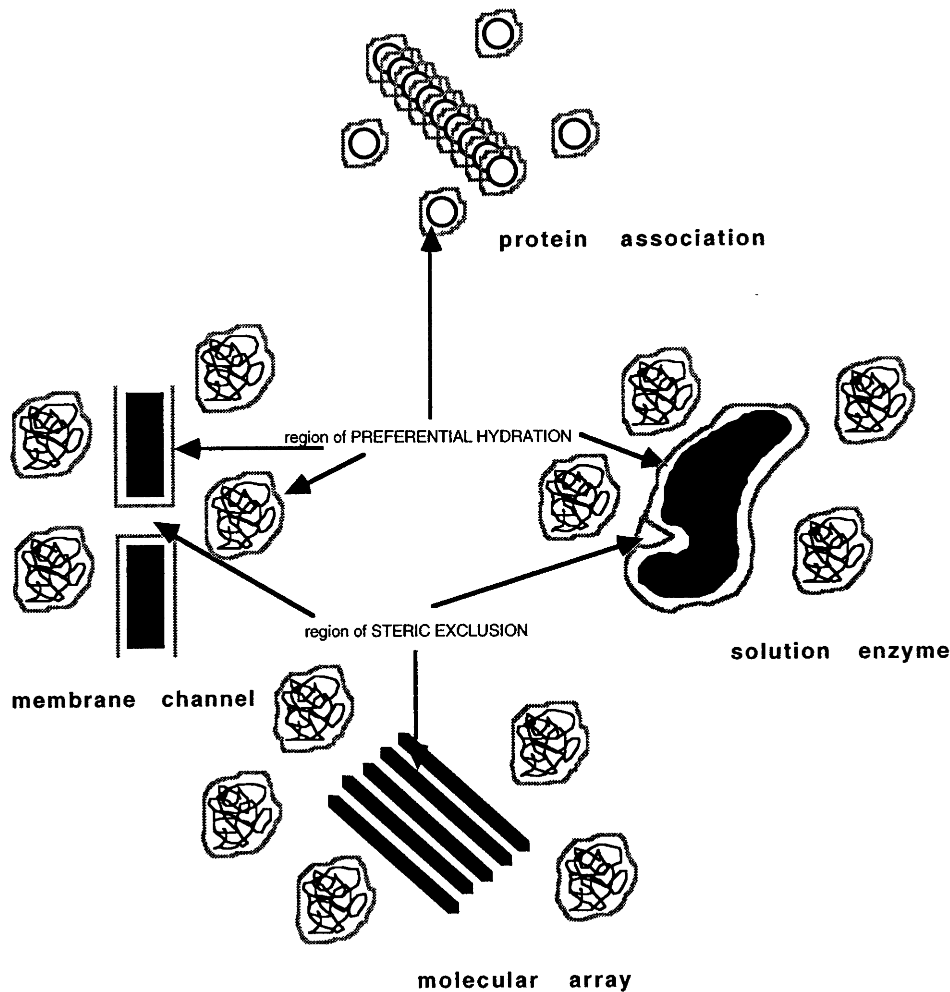



Figure 2. Illustration of the aqueous compartments around a variety of cellular systems that can be osmotically stressed as a result of exclusion of solute—the large squiggly molecules—from cavities, as a result of size (steric exclusion), or from surfaces, as a result of preferential hydration of those surfaces.

With ionic channels it is possible to watch the occurrence of open vs. closed states by tracking the amount of current flow permitted through them (fig. 6). Electrical current bursts in alamethicin channels in successively higher conductance states under control conditions are illustrated in figure 6A. Addition of the polymer poly(ethylene glycol) (PEG) to the bathing solutions on both sides of the channel-containing membrane suppresses channel opening, as shown in figure 6B.

Such suppression of channel formation by PEG can be used to determine the volume of water that must be drawn from the bathing medium in order for the channels to open. Figure 7 plots channel opening probability against the osmotic pressure of the PEG solution. In this case, each successive opening requires about 100 additional water molecules. PEGs of different sizes reveal different volumes of solute-inaccessible water (fig. 8). The difference ($V_{open} - V_{closed}$), which has a value of $\sim 100$ waters with large PEGs, falls to almost zero with the smallest PEGs [15].

## Enzymes in membranes and in solution

Osmotic responses similar to those shown by channels have been demonstrated for the membrane enzyme cytochrome c oxidase [16] and for the receptor rhodopsin [17], for regulation of coagulation factor $X_a$ [18] and for protein kinase C [19]. Analogous osmotic responses have also been found with several water-soluble enzymes. Among these are adenosine deaminase [20], hexokinase [21–23] and aspartate transcarbamylase [24].

## Hexokinase

Is water a reactant in the activity of a solution enzyme? Hexokinase is the first enzyme of glycolysis. It binds glucose within a cleft and phosphorylates it as shown in figure 9. This binding of glucose involves a loss of water according to

$$HK \cdot N_w^o + \text{glucose} \leftrightarrow HK \cdot \text{glucose} \cdot N_w^c + \Delta N_w$$

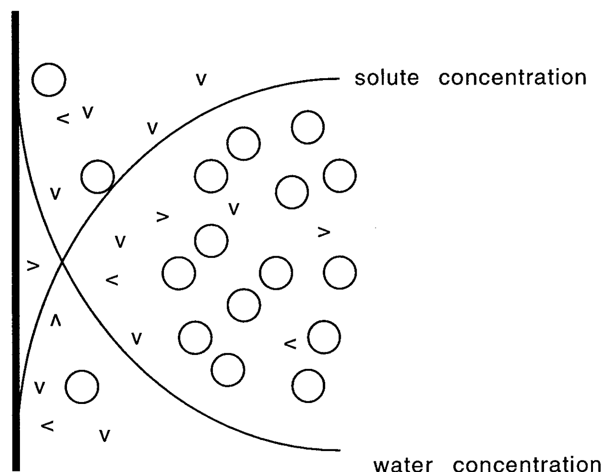



Figure 3. Illustration of the effect of preferential hydration whereby the solute concentration next to a surface is, for whatever reason, less than some distance from it. The water gradient is inevitably the reverse. Raising the bathing solute concentration acts to raise the energy of the surface.

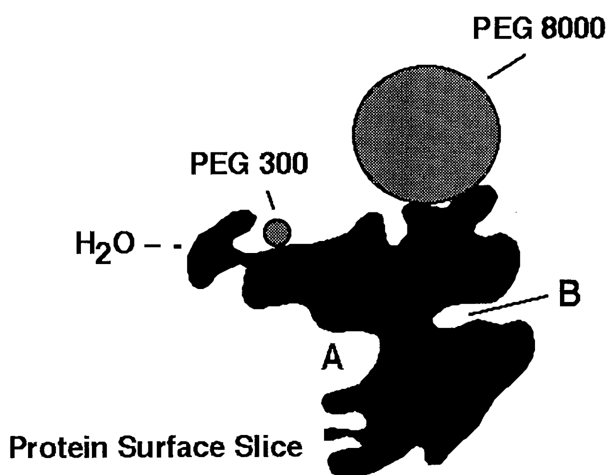



Figure 4. Both steric exclusion and preferential hydration act on proteins according to the combinations of solute size, the nature of the surface and the convolutions of the surface. Figure concept courtesy of Licata and Allewell [38].

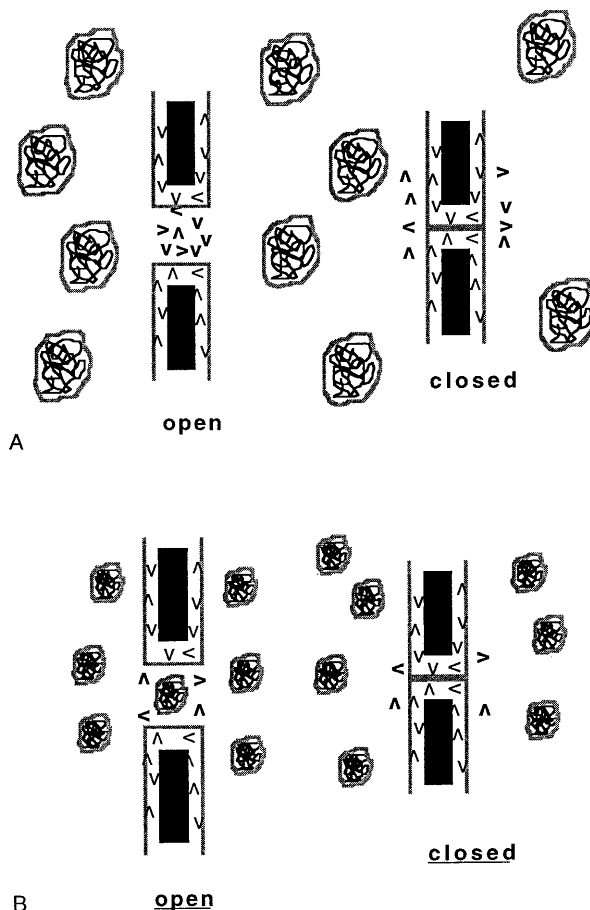



Figure 5. Illustrating the osmotic effect of solutes (squiggly molecules) on channel gating. Channels, open or closed, are associated with more or less water (v's), according to their size and degree of surface hydration outlined by the paler lines. *A* illustrates large solutes that are completely excluded from the open channel and act osmotically on the eight bold water molecules that fill the open channel. In *B* small solute molecules can enter the open channel and so act osmotically only on the four bold surface water molecules from which they are preferentially excluded.

where HK = the hexokinase molecule and $N_w$ indicates a given number of water molecules. Osmotic stress experiments were carried out to determine the value of $\Delta N_w = N_w^o - N_w^c$ in glucose binding according to this reaction [21, 23]. Large PEG molecules, with MW values between 1000 and 10 000, are excluded from all clefts around the protein and lower the water activity of the hexokinase environment. As shown in figure 10, the consequence of lower water affinity is increased affinity of the enzyme for glucose, as if water and glucose compete for the binding site. To what extent?

Figure 10 shows the dependence of log $K_d$, the glucose binding constant, on water activity. The slope translates into a $\Delta N_w$ of approximately 325. That is, 325 fewer polymer-accessible waters are associated with hexokinase when one glucose molecule has bound than are present in the uncomplexed enzyme.

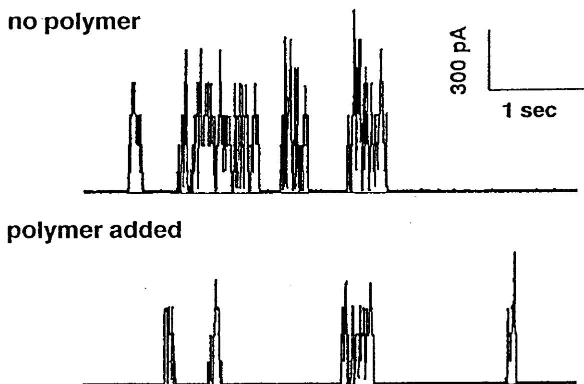



Figure 6. Records of single alamethicin channel gating currents showing several conductance states. The probability of open states is decreased by the presence of a polymer that is added to the bathing solution of the channel and excluded from it.

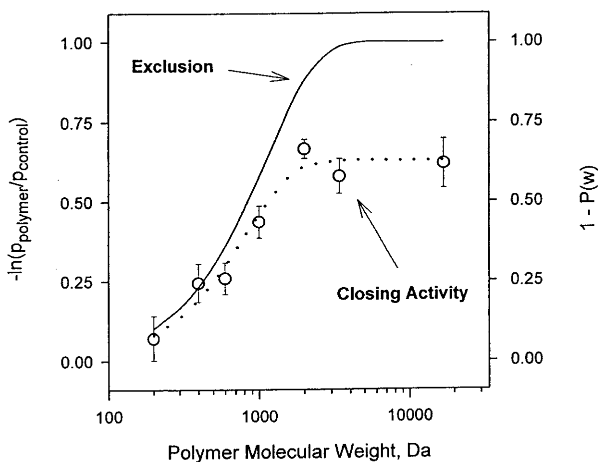



Figure 7. The osmotic sensitivity of alamethicin channel opening as a function of the size of the polymer used to exert osmotic stress. Left axis and open circles: Ratio of open to closed probabilities with and without polymer. Osmotic stress is progressively lost at smaller molecular weights (as suggested in the cartoon of fig. 5). Right axis: exclusion of the polymer as measured (solid line) by the occlusion seen (fig. 8) in mean channel conductance. P(w) is a proportionality factor for the probability of polymer entry [39].

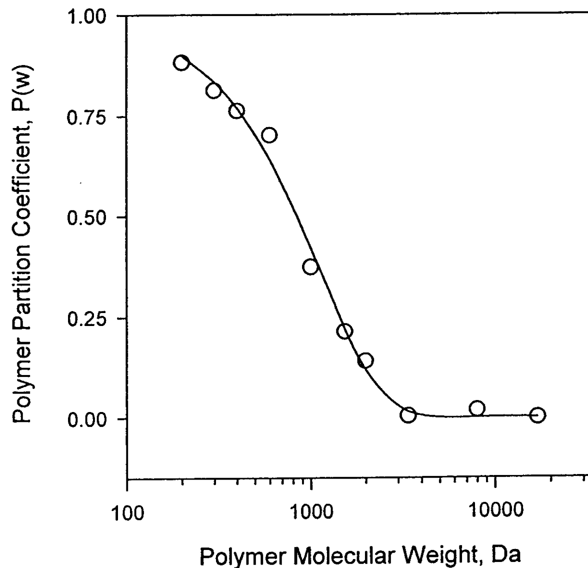



Figure 8. Polymer partition coefficient as a factor P(w) biasing admittance to the alamethicin pore measured from changes in pore conductance. The solid line is the same here and in figure 7 where it is used to show how the penetration of polymer correlates with the loss of osmotic action due to polymer exclusion.

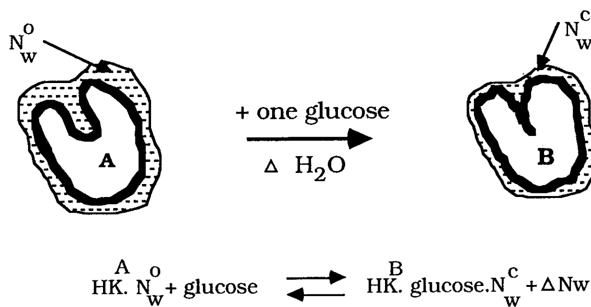



Figure 9. Reversible glucose binding reaction to hexokinase. Open (unbound) and closed (bound) conformations are shown associated with $N_w^o$ and $N_w^c$ water molecules that exclude large indifferent solute molecules that act osmotically on them. Osmotic stress experiments determine the difference between $N_w^o$ and $N_w^c$, i.e. $\Delta n_w$, by determining the osmotic dependence of glucose binding.

Figure 11 shows that as the dehydrating solute concentration in the enzyme environment increases, the numbers of waters 'displaced' upon glucose binding, as given by the slope, decrease considerably. At the highest osmotic pressures used, $\Delta N_w$ falls to as low a value as 25, a number that matches well the difference between the crystallographic structures of the open and the

closed states of hexokinase. Figure 12 shows the number of water molecules that are involved in the glucose-binding reaction at high and low osmotic pressures. Raising the osmotic pressure alone to the illustrated extent apparently dehydrates the protein by $\sim 300$ (325 − 25) water molecules (steps A–C, fig. 12). That is a lot of water. But is it energetically significant?

An energetic calculation requires the integration of the pressure-volume relation in the reaction involved. In the case illustrated by the steps A–C in figure 12, such a calculation gives the remarkably small value of approximately 1 kT. In other words hexokinase in solution can readily occupy all the hydrated conformational states between states A and C. The crystal structure conformation, even without glucose bound, is consistent with the substantially dehydrated state, C, as would be expected given the osmotic character of crystallization conditions.

What occurs for the osmotic dependence of the equilibrium binding of glucose is also true for the active enzyme. The osmotic dependence of the Michaelis-Menten constant for glucose shows that water acts as a competitive inhibitor for glucose to the same extent as in equilibrium binding, whereas $V_{max}$ is unchanged in all osmotic environments.

We conclude that the substrate-binding cavity of unbound hexokinase in solution is highly conformationally flexible, i.e. floppy. Its affinity for its glucose substrate is remarkably sensitive to the presence of large solute molecules around it.

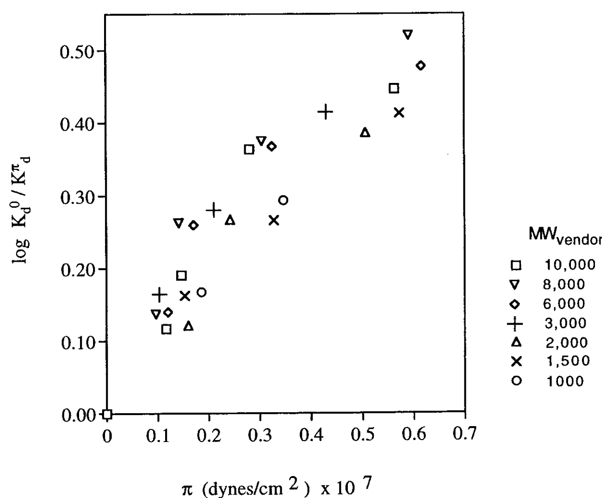



Figure 10. Dependence of the glucose binding constant, $K_d$, to hexokinase as it varies with osmotic pressure of the medium, controlled with PEGs of MW 1000–10,000. No difference is seen with MW. The slope of the best-fit linear line to these data shows that approximately 325 fewer water molecules are associated with the glucose-bound conformation of hexokinase [23].

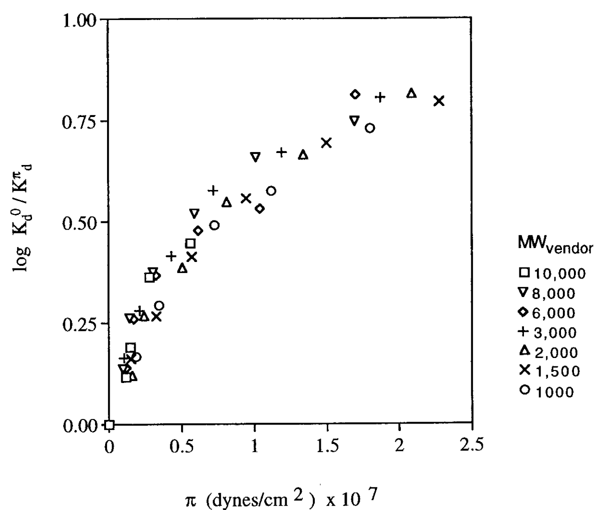



Figure 11. Dependence of the glucose binding constant, $K_d$, to hexokinase over a large range of osmotic pressures. At the highest pressures only 25 fewer water molecules are associated with the bound state.

## Hemoglobin

The loading of four oxygens onto hemoglobin requires that $\sim 60$ water molecules be drawn away from the bathing solution [25]. Any of a number of different solutes added to the solution shifts the famous S-shaped oxygen-loading curve to the right—a direction that requires a greater pressure of oxygen for a given level of oxygen bound (fig. 13). The appearance of this shift is like that created by preferential binding of any effector. Here the effector is water. When $p_{50}$, the pressure of oxygen needed to load hemoglobin to 50% of saturation, is plotted against the chemical potential of water, a linear relationship is obtained (fig. 14). The slope is a measure of the change in the number of waters bound to hemoglobin when oxygen binds.

Remarkably, the observed osmotic sensitivity of hemoglobin is the same for a wide range of neutral solutes, including different-sized polyethyleneglycols, the monosaccharide glucose, the disaccharide sucrose, the tetrasaccharide stachyose and glycine. When $p_{50}$ values are plotted against the activities of the same solutes, there is no such clean linear dependence. This fact, together with the insensitivity of the water effect to solute identity, suggests that solute is acting on water in sterically inaccessible pockets.

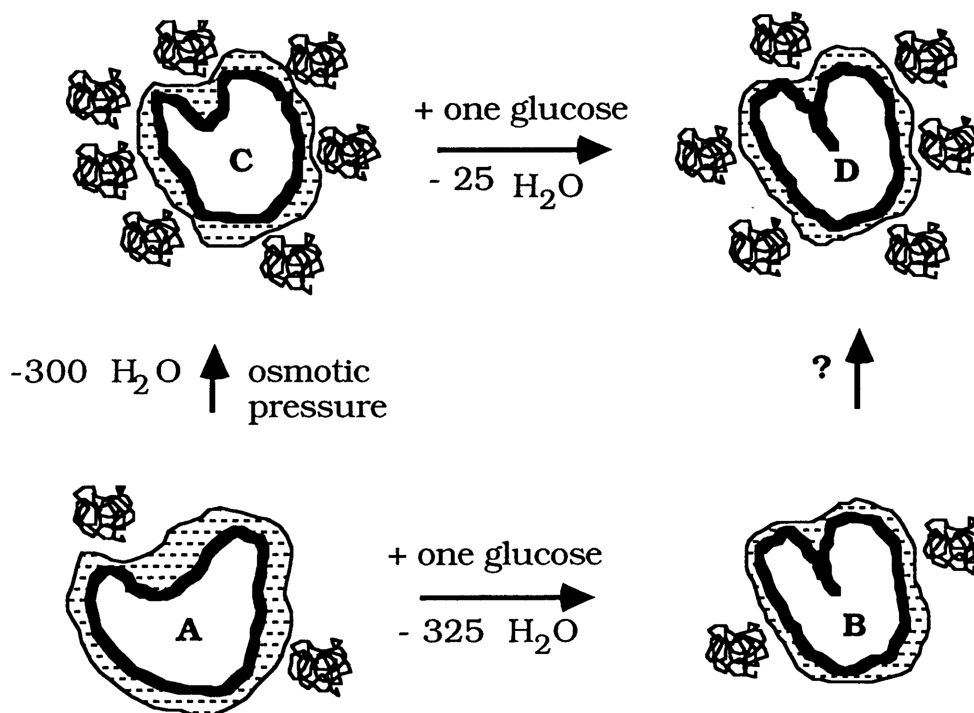



Figure 12. Schematic representation of the osmotic stress results on glucose binding to hexokinase. Glucose binding induces the detectable fluorescence change associated with the conformational change required for catalysis (A–B and C–D). For PEG of MW 1000–10,000, and for low osmotic pressures, the decrease in numbers of waters associated with glucose binding, $\Delta N_w$, is $325 \pm 10\%$. $\Delta N_w$ decreases with osmotic pressure to approximately 25. The larger MW PEGs, excluded from aqueous compartment around the protein, act to osmotically shrink that compartment (A–C) by 300 water molecules in the absence of glucose binding. There are no data to indicate what $\Delta N_w$ for the step B–D might be, although it is likely to be very small for these two closed glucose-bound states.

This is not true for any solute whatsoever. The effect of chloride on oxygen affinity can be explained by a direct binding action, as well as an indirect, osmotic, action [25]. The plot of $p_{50}$ against chloride activity is slightly curved. If chloride is added to hemoglobin together with varying amounts of a neutral solute so that the water activity is kept fixed, then the resulting '$Cl^-$ only' effect gives a pleasingly straight line. This corresponds to the binding of one ion, as shown in figure 15. When the osmotic pressure of the chloride solution is taken into account, it becomes clear that, if added in the absence of other solutes, $Cl^-$ simultaneously binds directly and works osmotically against the addition of the 60 water molecules.

Osmotic action is also seen in the binding of oxygen to *Scaphurca* hemoglobin dimers. Here, the binding of oxygen involves a displacement of bound waters, as shown in figure 16. Greater osmotic stress therefore draws waters away from the binding site and facilitates $O_2$ binding. The difference of six solute-inaccessible waters correlates nicely with the waters seen crystallographically [26]. Analogous to hexokinase, the structure of the oxy form of this Hb can be stabilized by osmotic stress without actual oxygen binding.

## Molecular assembly

### Actin polymerization

One expects that protein association may involve dehydration of the contacting surfaces. Such dehydration can be energetically costly. The familiar and ubiquitous G-F polymerization reaction of actin is shown in figure 17. When it is carried out at increasing levels of several different solutes, the results show that about 14 fewer water molecules are associated with the Ca-ATP-actin complex when it is in the polymer (F) form [27].

The reaction can now be written as

$$\text{G-actin} \cdot 14(H_2O) + \text{F-actin}_n \leftrightarrow \text{F-actin}_{n+1} + 14\ H_2O$$

Remarkably, Mg-ATP-actin, a more closed structure than Ca-ATP-actin, shows no such net changes in associated water. It is as if the waters that leave Ca-ATP-actin come from a closure of the cleft associated with polymerization.

Hexokinase, like actin and other kinases, has the common structural motif of a substrate binding site in a deep cleft. But unlike hexokinase, Ca-ATP-actin does not lose such large amounts of water when its cleft closes. The difference may be that with ATP bound, actin is far more compact than the substrate-free and floppy hexokinase. As the first in a series of enzymatic and structural steps by actin, the ATP-binding step involves very little change in net hydration.

**DNA/protein interactions**

The osmotic stress technique has been used to measure changes in water binding accompanying the DNA binding of several drugs [28, 29] and of several proteins: *Escherichia coli* gal repressor [30], *E. coli* CAP protein [31], Hin recombinase [32], Ultrabithorax and Deformed homeodomains [33], *E. coli* tyr repressor [34], EcoRI [32, 35] and the Sso7d protein [36].

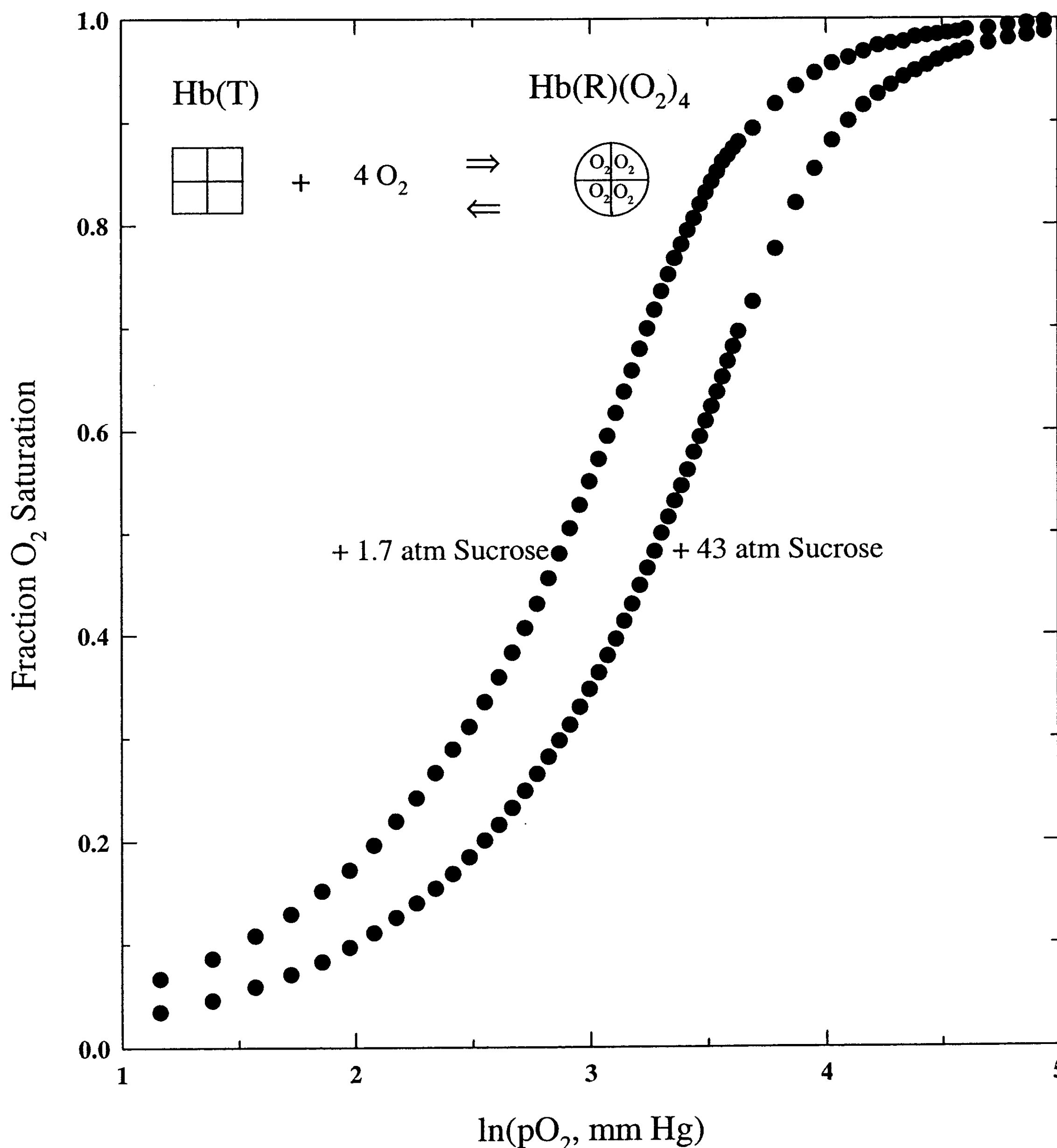



Figure 13. A 'water effect' on hemoglobin. There is a shift in the oxygen uptake curve upon the addition of osmotic stress.

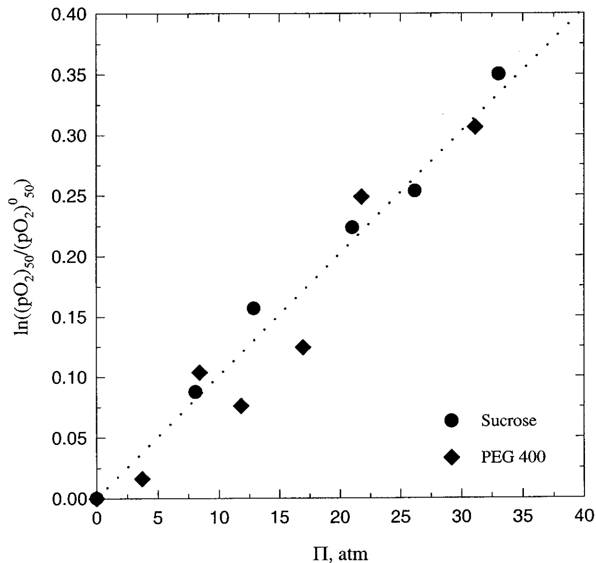



Figure 14. Shift in $(pO_2)_{50}$, the oxygen pressure needed for 50% loading of oxygen onto hemoglobin, vs. Π, osmotic pressure of added neutral solutes. The slope, independent of neutral solute identity, gives the amount of water that must be wrested from the bathing solution as hemoglobin goes from the deoxy to fully oxy state.

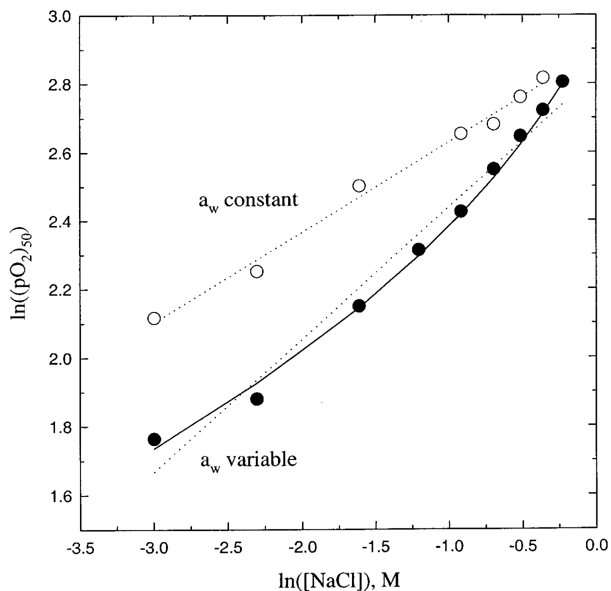



Figure 15. Shift in $(pO_2)_{50}$, of hemoglobin vs. salt concentration. For $a_w$ = constant (straight line, open circles), water activity is kept fixed by adjusting with added sucrose. For $a_w$ = variable (curved solid line, solid circles), water activity varied with added salt; curvature is due to double action of salt – directly through $Cl^-$ binding and indirectly through osmotic stress.

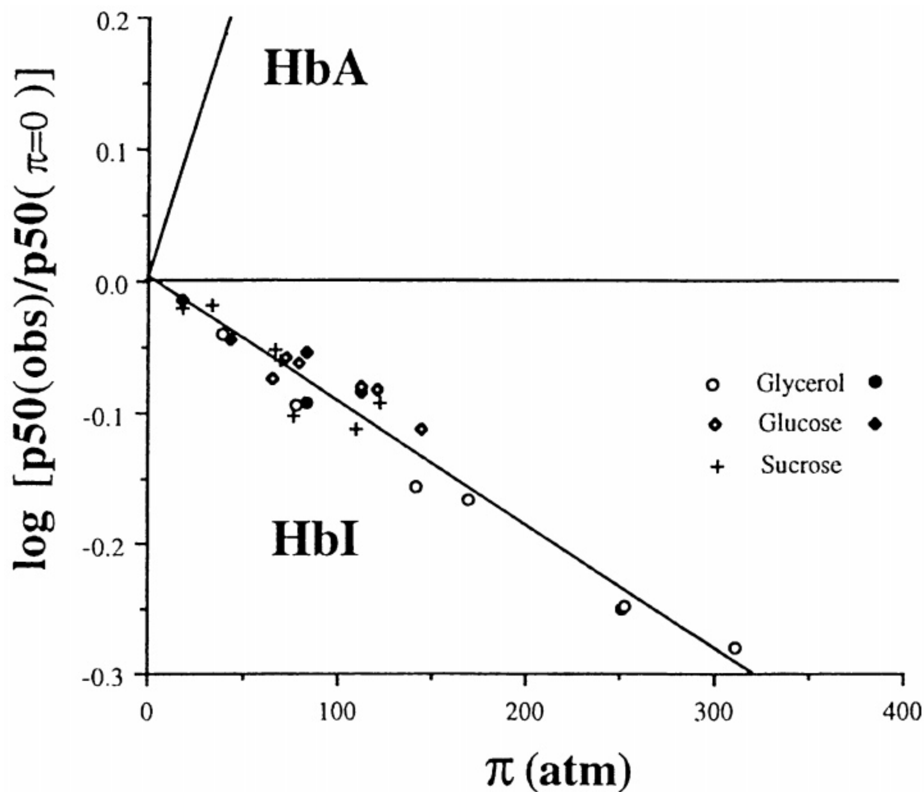
Osmotic agents can stabilize the intimate association of a restriction endonuclease with its specific DNA target as compared with a nonspecific sequence as indicated schematically in figure 18. The free-energy difference between the complex of EcoR1 with nonspecific DNA (containing two-or-more-error sequences) and the EcoR1 complex with its recognition sequence is linearly dependent upon the water chemical potential in the solution (fig. 19). The osmotic dependence indicates that at 20 °C the nonspecific complex sequesters about 110 more waters than the specific EcoR1 complex. The plot in figure 19 is linear and insensitive to solute identity used to set the water chemical potential. These factors are important for distinguishing an indirect solute effect on water activity from direct solute binding. The insensitivity of the difference between specific and nonspecific EcoR1 complexes to solute size or to their chemical nature implies that the water retained by the nonspecific complex is sequestered in a cavity that most likely lies at the DNA-protein interface and is sterically solute-inaccessible (fig. 18).

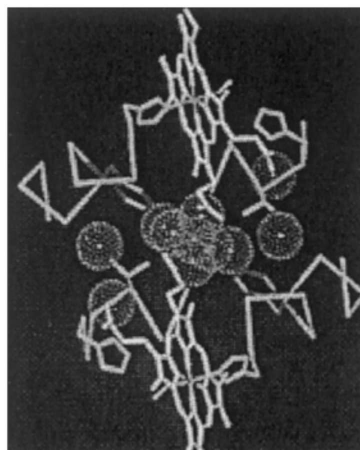
**DNA assemblies**

The packing of long, semiflexible DNA requires elaborate machinery in cell nuclei. In prokaryotes, too, there are packaging proteins, including those that crystallize DNA when cells are in distress [37]. When subject to osmotic stress in vitro, DNA packs in several liquid crystalline arrays whose structure is revealed by X-ray diffraction (fig. 20A). To hold DNA in a hexagonal array at the density pictured, which is typical of its density in a viral head (fig. 20B) requires approximately 100 atmospheres of osmotic pressure). The work of creating these arrays has been measured and, when possible, converted into measurements of forces between pairs of molecules [8]. Such measurements, for example see figure 21, give accurate estimates of the kinds of pressures under which DNA is held in viruses. Remarkably, these in vitro measurements give results equivalent to those obtained in vivo with DNA overexpressed in *E. coli* [37]. These measurements show how well the ideas of osmotic stress used in vitro can translate into thinking about its action within the cell.

**The importance of understanding water competition**

The number of workers using osmotic stress and the emerging number of systems showing water dependence are growing. We have constructed a Web site to collate this work and to provide osmotic pressure



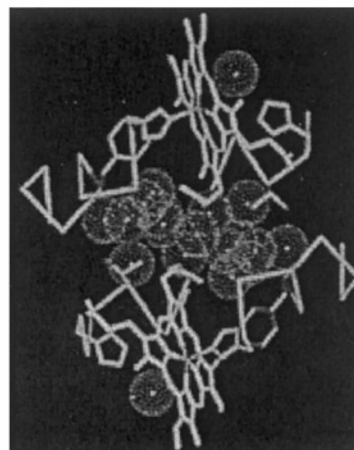




Figure 16. Shift in $(pO_2)_{50}$, the oxygen pressure needed for 50% loading of oxygen onto human HbA (detailed in figure 14) [40] and dimer HbI [26] vs. Π, the osmotic pressure of added neutral solutes. The slope, independent of neutral solute identity, gives the amount of water that must be taken from the bathing solution in the case of HbA, or released to the bathing solution in the case of HbI, by oxygen as these molecules go from the deoxy to fully oxy state. The latter number agrees nicely with the water seen in the crystallographic structures of HbI indicated by the molecular models of HbI adapted from Royer [26].

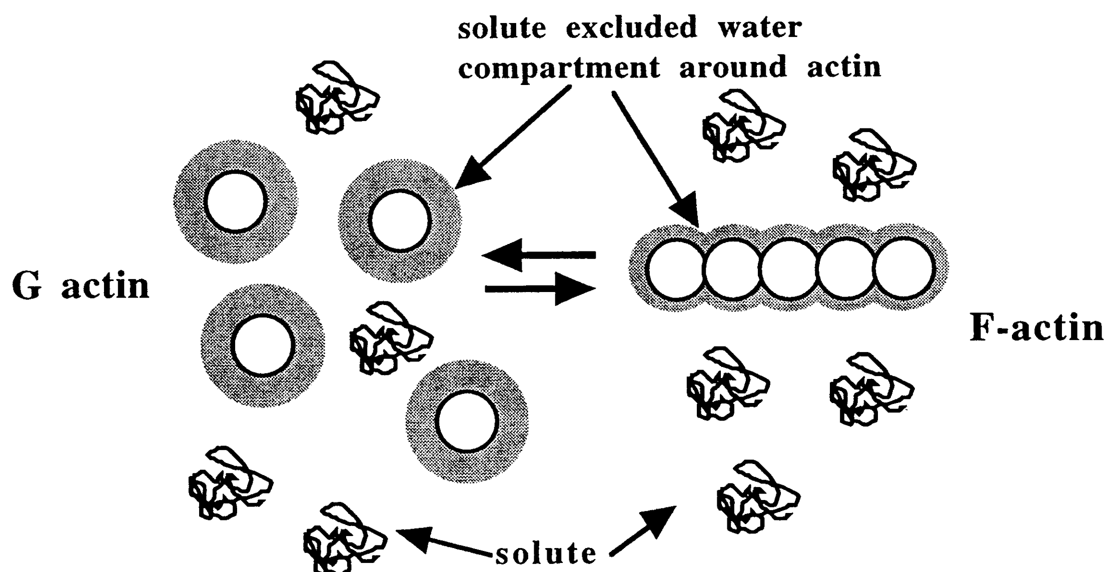



Figure 17. Illustration of the equilibrium between the critical concentration of G-actin monomers and F-actin polymers, each surrounded with a solute-excluded aqueous compartment indicated in grey. Dependence of the critical concentration on osmotic pressure shows that that aqueous compartment on polymerization decreases by about 14 water molecules for Ca-F-actin but not for Mg-F-actin.

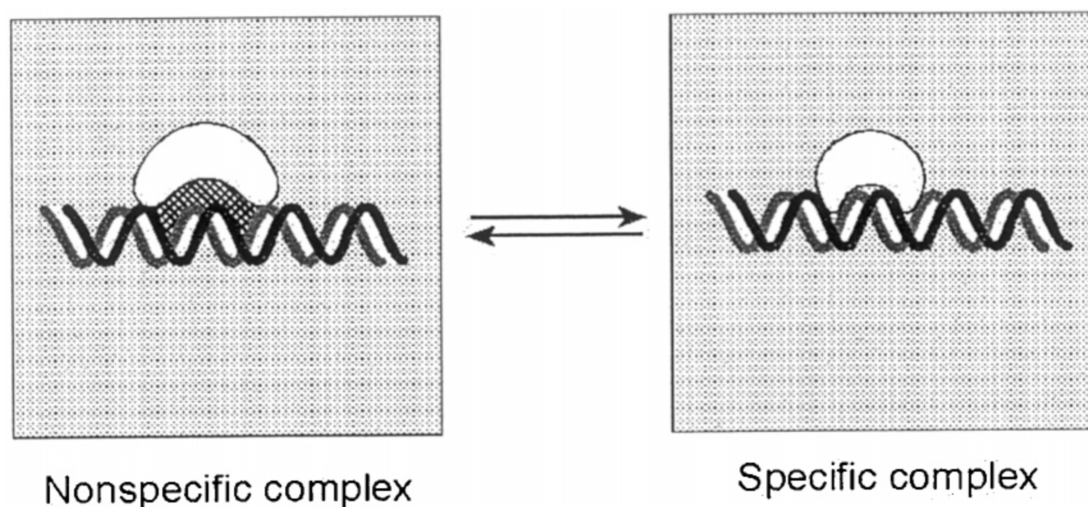



Figure 18. A schematic representation of the difference in sequestered water between EcoRI specifically bound to its recognition sequence and the nonspecific protein-DNA complex. The crystal structure [41] shows that the specific complex is characterized by direct protein-DNA contacts with no intervening water. From the osmotic dependence of the difference between specific and nonspecific binding of EcoRI, the nonspecific complex retains practically a full layer of water at the interface between DNA and protein surfaces (shown as the cross-hatched area). From the insensitivity of the number of sequestered waters to solute size and chemical nature, it is probable this water is sterically excluded from solutes.

data for performing the experiments at http://aqueous.labs.brocku.ca/osfile.html. This could serve as a useful forum for exchange of information within this community.

As a 'ligand' or 'effector' water is important because of its large numbers rather than its wide range of activities. It is also important because what we call hydration depends upon the competitive affinity of a macromolecule (or a region of a macromolecule) for water compared with its affinity for the many other solutes in the bathing solution. This competition is realized in many ways.

Waters near the surface of a protein are not available to dissolve other solutes. This may be because the protein strongly perturbs the water so that it cannot dissolve other molecules; or it may be that some solutes are too big to enter the thin surface region. In either case, there is a 'preferential hydration' of the protein relative to that particular solute. The cost of forming that protein surface will go up with added solute, in direct proportion to the number of water molecules that can no longer act as a solvent to that solute. These waters have been drawn away from the solution by the protein. The extra cost of drawing away that water is proportional to the extra osmotic pressure from the added solute or 'osmolyte'.

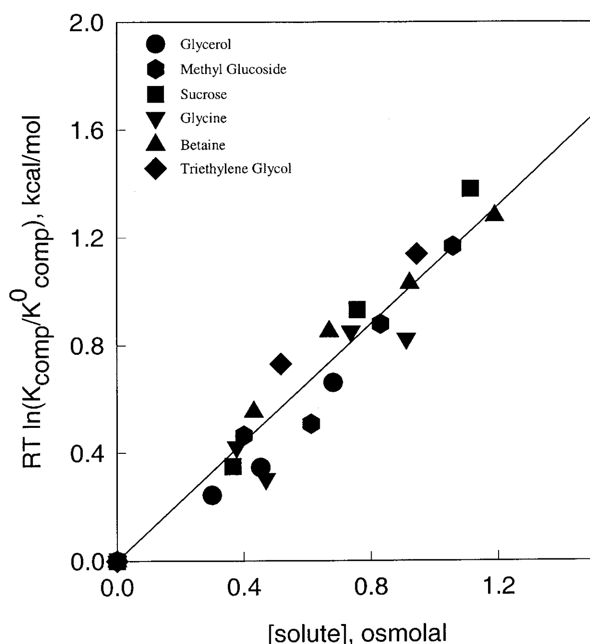



Figure 19. Competitive binding free energies scale linearly with osmotic stress. The slope of the lines translates into a release of about (110 ± 15) water molecules for the transfer of EcoRI from poly(dI − dC) · poly(dI − dC) to the specific site. The slight dependence of $\Delta n_w$ on the solute identity indicates a steric exclusion of these solutes from a well-defined, water-filled space, most probably associated with the nonspecific complex.

Waters in a cavity may be available to the smallest solutes but not to excluded large species. Added small solute will not act osmotically, but large solutes will. The extra cost of forming the cavity from addition of large solute will be the extra osmotic pressure imparted by it multiplied by the volume of water from which the large solute is prevented access.

In this context 'osmotic' takes on a variable meaning. It is not likely that there will be significant gradients of the actual chemical potential of water in different parts of a cell. This would make no more sense than that there would be significant thermal gradients. Rather, there is a different osmotic response of different large molecules to different osmotic agents. The analogy is that parts of the cell have different specific heats that determine the different amounts of heat they acquire consequent upon changes in temperature.

Whatever the variability of osmotic stress, its focus is the work of transfer of water during the transition between states of a macromolecule. Discrimination between solutes becomes a means whereby the cell or the experimenter can use these solutes to control different kinds of change in conformation. Cells do not usually vary their temperature or the chemical potential of cell water (which equilibrates rapidly with the external medium). But cells do regulate and vary widely the set of small molecules that control macromolecular association and reaction. The response of specific processes to osmotic stress therefore informs us about the control of intracellular processes.

## Technical note

In the simplest reaction scheme A ↔ B between two states A and B, the probabilities of their occurrence follow the same kind of thinking as with thinking about a chemical reaction. These probabilities depend upon the difference $(G_B - G_A)$ in work needed to go between the two states. This difference is measured in units of the thermal energy $kT$ per molecule (or RT per mole) that drives the reaction either way. In reaction language, the ratio of probabilities (as concentrations) of A and B go as $\ln(K_{eq}) = -(G_B - G_A)/kT$.

If these two states have different volumes, $V_A$ and $V_B$, of solute-excluding water, an added osmotic pressure $\Pi_{osmotic}$ of the excluded non-binding solute creates an additional work $\Pi_{osmotic}(V_B - V_A)$ needed to go between the two forms. Then $K_{eq}$ changes with $\Pi_{osmotic}$ as

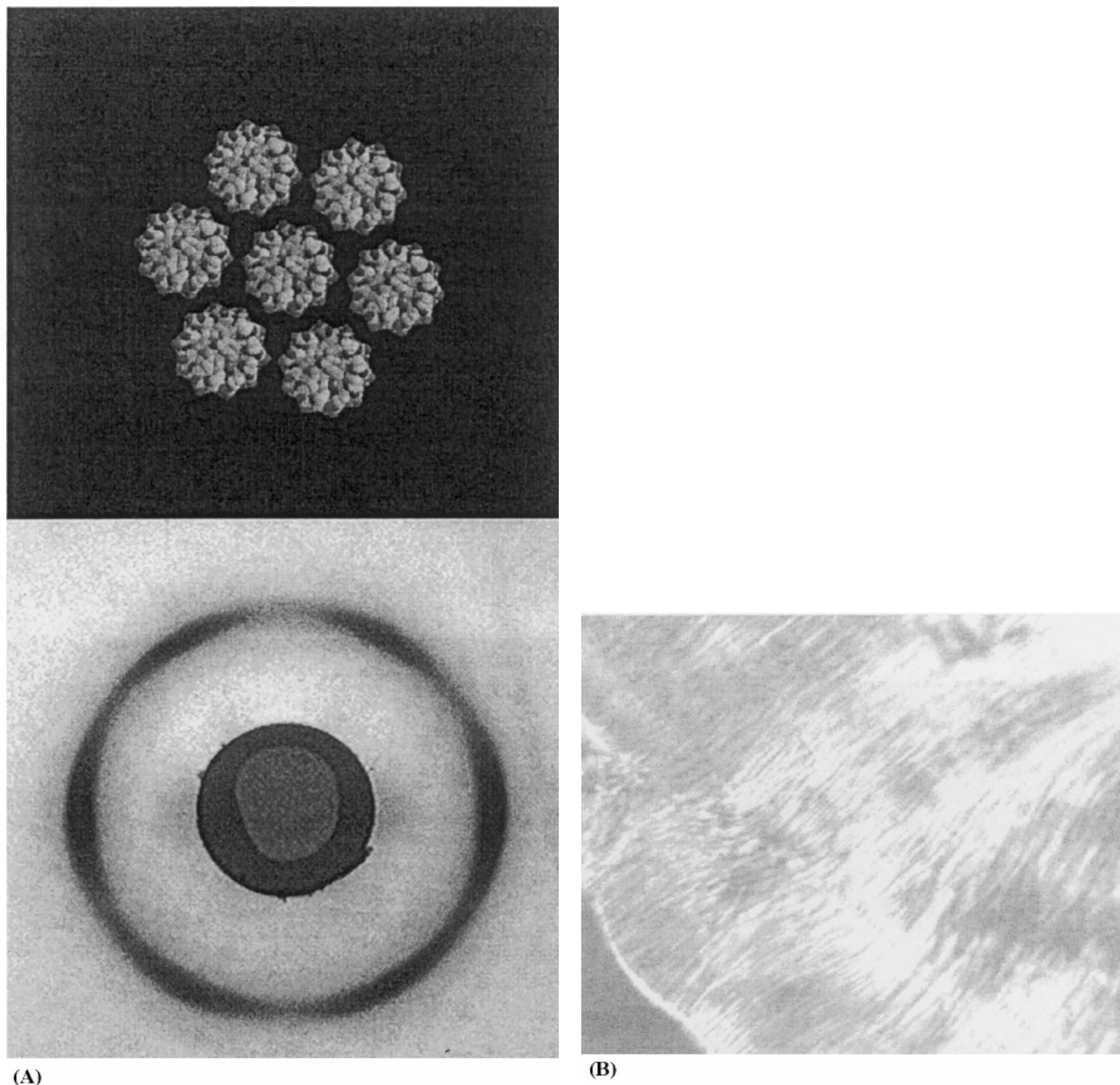



Figure 20. DNA phases seen by X-ray diffraction textures and by polarization microscopy. (*A*) High-density hexagonally packed exposed 'end-on' to X-rays. [42]; (*B*) low-density 'cholesteric' phase seen through birefringence. These regimes correspond to the two regimes of intermolecular forces shown in figure 21 and were made on the same samples on which intermolecular forces were measured (fig. 20B, R. Podgornik et al., unpublished photograph).

$$\ln(K_{eq}) = -(G_B - G_A)/kT - \Pi_{osmotic}\,(V_B - V_A)/kT.$$

A plot of $kT \ln(K_{eq})$ against $\Pi_{osmotic}$ gives a difference $(V_B - V_A)$ in aqueous volumes that are inaccessible to the added solute.

N.B. Any nonlinearity in this plot warns that a strictly osmotic interpretation of the result might not be valid. The volumes $V_B$ and $V_A$ may themselves be deformed under osmotic stress, or, more important, the solute may not be acting in the ideally osmotic way that this formalism assumes.

To determine the number of waters rather than a volume of waters, divide $(V_B - V_A)$ by the 30-Å$^3$ volume of a water molecule.

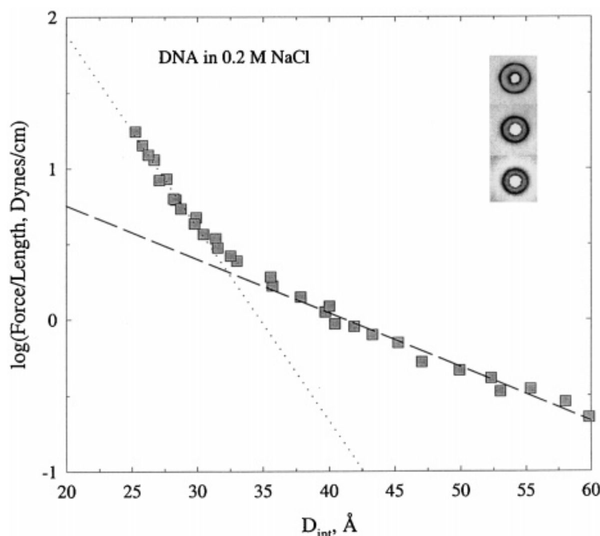



Figure 21. Force per unit length vs. interaxial spacing of parallel DNA double helices. Because the DNA diameter is 20 Å, $D_{int}$ = 20 Å is the limit of contact, zero separation between molecular surfaces. The exponentially varying force seen here has two regimes. At $D_{int} < 32$ Å, tightly packed DNA shows an exponential force decay distance of $\sim 3.3$ Å independent of or only weakly dependent on salt concentration. For $D_{int} > 32$ Å, a more slowly decaying force has a characteristic decay distance that depends on salt concentration. Inset: Hexagonal order seen by X-ray diffraction. Tightly packed hexagonal (top) and loosely packed (bottom) corresponding to samples seen by X-ray diffraction and polarization microscopy in figure 20A and B, respectively.

*Acknowledgements*. R.P.R. thanks the Natural Sciences and Engineering Research Council of Canda for financial support and Ms. Nola Fuller for many years of expert discussions and technical support. D.C.R. and V.A.P. thank Sergey Bezrukov for intensive conversations and for visually pleasing updates of his original figures on ionic channels.

1 Parsegian V. A., Rand R. P. and Rau D. C. (1995) Macromolecules and water: probing with osmotic stress. Methods Enzymol. **259:** 43–94
2 Parsegian V. A., Rand R. P., Fuller N. L. and Rau D. C. (1986) Osmotic stress for the direct measurement of intermolecular forces. Methods Enzymol. **127:** 400–416
3 Parsegian V. A., Fuller N. L. and Rand R. P. (1979) Measured work of deformation and repulsion of lecithin bilayers. Proc. Natl. Acad. Sci. USA **76:** 2750–2754
4 Rau D. C., Lee B. and Parsegian V. A. (1984) Measurement of the repulsive force between polyelectrolyte molecules in ionic solution: hydration forces between parallel helices. Proc. Natl. Acad. Sci. USA **81:** 2621–2625
5 Rau D. C. and Parsegian V. A. (1990) Direct measurement of forces between linear polysaccharides xanthan and schizophyllan. Science **249:** 1278–1281
6 Leikin S., Rau D. C. and Parsegian V. A. (1994) Direct measurement of forces between self-assembled proteins: temperature-dependent exponential forces between collagen triple helices. Proc. Natl. Acad. Sci. USA **91:** 276–280
7 Kornblatt J. A., Kornblatt M. J., Bon Hoa G. H. and Mauk A. G. (1993) Responses of two protein-protein complexes to solvent stress: does water play a role at the interface. Biophys. J. **65:** 1059–1065
8 Leikin S., Parsegian V. A., Rau D. C. and Rand R. P. (1993) Hydration forces. Ann. Rev. Phys. Chem. **44:** 369–395
9 Timasheff S. N. (1998) Proceed. Natl. Acad. Sci. **95:** 7363–7367
10 Parsegian, V. A., Rand R. P., and Rau D. C. (2000). Osmotic stress, crowding, preferential hydration, and binding. A comparison of perspectives. Proc. Natl. Acad. Sci. USA **97:** 3987–3992
11 Rayner M. D., Starkus J. G., Ruben P. C. and Alicata D. A. (1992) Voltage-sensitive and solvent-sensitive processes in ion channel gating. Biophys. J. **61:** 96–108
12 Vodyanoy I., Bezrukov S. M. and Parsegian V. A. (1993) Probing alamethicin channels with water-soluble polymers: size-modulated osmotic action. Biophys. J. **65:** 2097–2105
13 Zimmerberg J., Bezanilla J. F. and Parsegian V. A. (1990) Solute inaccessible aqueous volume changes during opening of the potassium channel of the squid giant axon. Biophys. J. **57:** 1049–1064
14 Zimmerberg J. and Parsegian V. A. (1986) Polymer inaccessible volume changes during opening and closing of a voltage-dependent ionic channel. Nature **323:** 36–39
15 Bezrukov S. M. and Vodyanoy I. (1993) Probing alamethicin channels with water-soluble polymers: effect on conductance of channel states. Biophys. J. **64:** 16–25
16 Kornblatt J. A. and Bon Hoa G. H. (1990) A nontraditional role for water in the cytochrome c oxidase reaction. Biochemistry. **29:** 9370–9376
17 Mitchell D. C. and Litman B. J. (1999) Effect of protein hydration on receptor conformation: decreased levels of bound water promote metarhodopsin II formation. Biochemistry **38:** 7617–7623
18 McGee M. P. and Teuschler H. (1995) J. Biol. Chem. **270:** 1570–1574
19 Giorgione J. R. and Epand R. N. (1997) Biochemistry **36:** 2250–2256
20 Dzingeleski G. D. and Wolfenden R. (1993) Hypersensitivity of an enzyme reaction to solvent water. Biochemistry **32:** 9143–9147
21 Rand R. P., Fuller N. L., Butko P., Francis G. and Nicholls P. (1993) Measured changes in protein solvation with substrate binding and turnover. Biochemistry **32:** 5925–5929
22 Reid, C. (1995) The influence of water on the glucose affinity of hexokinase. In: Biological Sciences, p. 162, Brock University, St. Catharines, Canada
23 Reid C. and Rand R. P. (1997) Probing protein hydration and conformational states in solution. Biophys. J. **72:** 1022–1030
24 LiCata V. J. and Allewell N. M. (1997) Functionally linked hydration changes in *E. coli* aspartate transcarbamylase and its catalytic subunit. Biochemistry **36:** 10161–10167
25 Colombo M., Rau D. C. and Parsegian V. A. (1992) Science **256:** 655–659
26 Royer W. E. J., Pardanani A., Gibson Q. H., Petereson E. S. and Friedman J. M. (1996) Ordered water molecules as key allosteric mediators in a cooperative dimeric hemoglobin. Proc. Natl. Acad. Sci. USA **93:** 14526–14531
27 Fuller N. and Rand R. P. (1999) Water in actin polymerization. Biophys. J. **76:** 3261–3266
28 Sidorova N. Y. and Rau D. C. (1999) J. Biomol. Struct. Dyn. **17:** 19–31
29 Qu X. and Chaires J. B. (1999) J. Am. Chem. Soc. **121:** 2649–2650
30 Garner M. M. and Rau D. C. (1995) Water release associated with specific binding of gal repressor. EMBO J. **14:** 1257–1263
31 Vossen, K. M., Wolz R., Daugherty M. A., and Fried M. G. (1997). Role of macromolecular hydration in the binding of the *Escherichia coli* cyclic AMP receptor to DNA. *Biochemistry* 36: 11640–11647
32 Robinson C. R. and Sligar S. G. (1993) Molecular recognition mediated by bound water: a mechanism for star activity of the restriction endonuclease *Eco* RI. J. Mol. Biol. **234:** 302–306
33 Li L. and Matthews K. S. (1997) Biochemistry **36:** 7003–7011
34 Poon J., Bailey M., Winzor D. J., Davidson B. E. and Sawyer W. H. (1997) Biophys. J. **73:** 3257–3264

35 Sidorova N. Y. and Rau D. C. (1996) Differences in water release for the binding of *Eco*R1 to specific and nonspecific DNA sequences. Proc. Natl. Acad. Sci. USA **93:** 12272–12277
36 Lundback T., Hansson H., Knapp S., Ladenstein R. and Ladenstein H. T. (1998) Thermodynamic characterization of non-sequence-specific DNA-binding by the Sso7d protein from *Sulfolobus solfataricus*. J. Mol. Biol. **276:** 775–786
37 Wolf S. G., Daphna D., Arad T., Finkel S. E., Kolter R. and Minsky A. (1999) Nature **400:** 83–85
38 Licata V. and Allewell N. M. (1998) Methods Enzymol. **295:** 42–62
39 Parsegian V. A., Bezrukov S. M. and Vodyanoy I. (1995) Watching small molecules move: interrogating ionic channels using neutral solutes. Biosci. Rep. **15:** 503–514
40 Colombo M. F., Rau D. C. and Parsegian V. A. (1992) Protein solvation in allosteric regulation: a water effect on hemoglobin. Science **256:** 655–659
41 McClarin J. C., Frederick C. A., Wang B.-C., Greene P., Boyer H. W. and Rosenberg J. M. (1986) Science **234:** 1526–1541
42 Podgornik R., Strey H. H., Gawrisch K., Rau D. C., Rupprecht A. A. and Parsegian V. A. (1996) Bond orientation order, molecular motion and free energy of high-density DNA mesophases. Proc. Natl. Acad. Sci. USA **93:** 4261–4266