Pergamon

Prog. Polym. Sci., Vol. 20, 1121–1163, 1995

0079-6700/95 $29.00

0079-6700(95)00015-1

HIGH AND LOW DENSITY WATER IN GELS

PHILIPPA M. WIGGINS*

Department of Medicine, Auckland University School of Medicine, Private Bag 92 019, Auckland, New Zealand

Keywords – Water, osmosis, polyelectrolytes, hydrophobic interaction.

CONTENTS

1. INTRODUCTION: WATER, THE SINGULAR LIQUID

We all give lip service to the contention that water is an unusual, perhaps a unique liquid but, for most of us, that is where our curiosity ends. What if it is unusual? It is the liquid in which all life originated and in which all living processes continue to take place; it is the liquid in which the properties of solutions and biochemical reactions have been characterised. It is a constant omnipresent background to much of science, the only truly ubiquitous liquid. Does it really matter to the non-specialist if it differs from other solvents? Why should we not continue to treat it as a passive continuum of unspecified characteristics in which all the interesting reactions of biochemistry and many of chemistry happen to take place?

The aim of this article is to draw to the attention of water specialist and non-specialist alike an unexpected complexity of aqueous solutions associated with polymers, including biopolymers. It will introduce the concept that, in the presence of small solutes, aqueous solutions and gels of polymers are self-organising entities which, as they become more complex, induce a high degree of order[1] which can become chaotic. These phenomena appear to occur at the level of single enzyme active sites as well as globally in intact cells. They are non-linear processes which reveal water to be not a constant background presence which can be ignored but an active, changeable participant in a variety of self-organising and self-limiting interactions.

1.1. *Some Anomalous Properties of Water*

Water is liquid over a much greater range of temperatures than other liquids. Figure 1 illustrates this for the hydrides of Group 6 of the periodic table, H_2O, H_2S, H_2Se and H_2Te. The higher molecular weight hydrides are liquid over a range of 15–47 °C, while liquid water, of course, exists over a range of 100 °C. More remarkable, however, is that the expected continuous change of melting points and boiling points from high to low molecular weights within the group terminates abruptly at H_2S; H_2O, if it followed the established pattern, would freeze between −90 and −100 °C and boil between −60

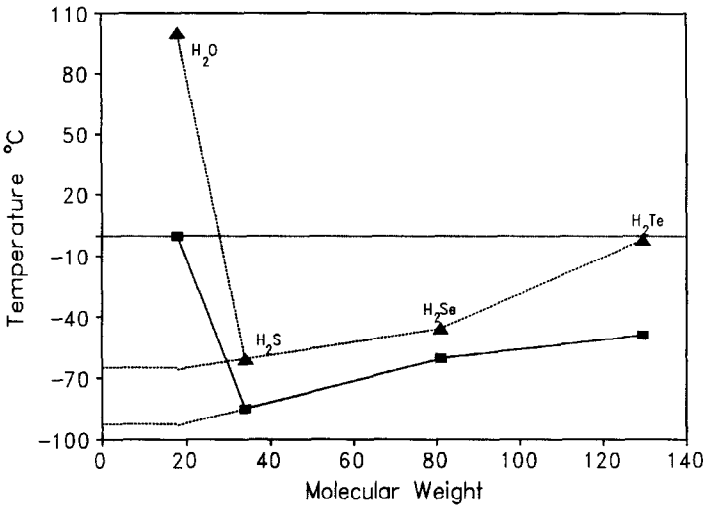

Fig. 1. Melting points and boiling points of hydrides of the elements of Group 6 of the periodic table, illustrating the anomalous behaviour of water.

and −70 °C. This is one clear and non-trivial anomaly. The boiling point of liquid water is elevated by approximately 160 °C above reasonable expectations. All other covalently-bonded hydrides and oxides are, like H_2S, H_2Se and H_2Te and unlike H_2O, gases at ambient temperatures.

Most liquids are less dense than the solids which melt to produce them; ice, however, is less dense than water. Moreover only H_2O and D_2O (heavy water) have a temperature of maximum density (4 and 11 °C respectively); other liquids decrease monotonically in density with increasing temperature.

Henderson[2] wrote in 1913, "As a solvent there is literally nothing to compare with water. In truth its qualifications are on this point so unique that nobody seems to have taken the trouble to gather together the evidence and, accordingly, beyond the bare assertion, a brief statement of the facts is not easy." He followed this with a rather detailed and most impressive statement of the facts as they were then known. He points to the composition of sea water and of serum as examples of the wealth of solutes which dissolve readily in water. We can now add to his list of the solvation triumphs of water that it is not, however, a universal solvent and its singular inability to dissolve hydrocarbon-like compounds freely is an important factor in the spontaneous assembly of phospholipid membranes[3] and folding of proteins.[4]

These, then, are just a few of the multitude of strange properties of water of which we are aware, and which a molecular theory of liquid water has to explain. They raise the real possibility that there may be yet other instances of unpredictable behaviour of the liquid of which we are not yet aware. It is only quite recently that the extreme deviations from normality of supercooled water[5–12] and water under tension[7,13–15] have been revealed. Some similar surprises will be discussed in subsequent sections.

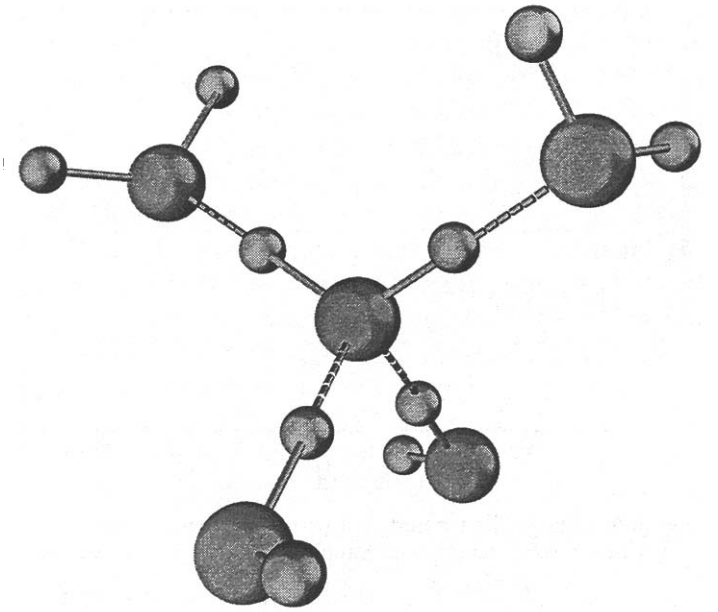

Fig. 2. A three-dimensional cluster of five water molecules as they occur in ice. The central molecule is hydrogen-bonded to its four nearest neighbours. Reproduced from *Cell Biochem. Function* (accepted) by permission of John Wiley & Sons Ltd.

1.2. *Models of Liquid Water*

Liquid water still eludes a molecular description which will account for even most of its dynamic and thermodynamic properties; this in spite of innumerable and continuing experimental and theoretical attempts to unravel its mysteries. It is one of the great chemical paradoxes that the most familiar chemical on earth is the least understood. It is, however, now generally accepted that the anomalous properties of liquid water are due in some manner to its three-dimensional hydrogen-bonded network which, in turn, results from its near-tetrahedral configuration and 1:1 ratio of its donor to acceptor groups.[16] Figure 2 illustrates how this network exists in Ice Ih, the ice that forms at ambient pressures. This represents a cluster of five water molecules mutually hydrogen-bonded. Each molecule has two negative poles (the lone pairs of electrons on the oxygen atom) and two positive poles on its hydrogen atoms. Since these poles are directed approximately toward the corners of a regular tetrahedron, attraction between poles of opposite sign on adjacent molecules results in long-range three-dimensional order in the crystal. Residual short-range order in the liquid is then, somehow, responsible for its anomalous behaviour. Other liquids (e.g. NH_3 and HF) can form intermolecular hydrogen-bonds, but only water and heavy water form them in three-dimensional arrays.

There have been two models proposed to account for the anomalies. Röntgen[17] proposed a mixture model in which water consisted of two different structural species in equilibrium with each other. This equilibrium, he suggested, was displaced by pressure and temperature, leading to the anomalous changes in density, including

the maximum in density at 4 °C, the decrease in compressibility with increasing temperature and the decrease in viscosity at 18 °C with increased pressure. All these had been well-documented by 1892. The alternative model is that of a random continuous hydrogen-bonded network of macroscopic extent[18] breaking and reforming on a timescale of 10^{-12} s and with a continuous spectrum of hydrogen-bond strengths. Stillinger[19] suggested that the anomalous properties of water arose from competition between bulky and compact arrangements of water molecules in the macroscopic network. He called these topological reformations "virtually a continuum of architectural possibilities rather than a discrete pair of options".

These are the two models between which experimentalists and theoreticians have tried to decide for most of this century. Theoretical advances have been hampered until recently by the complexity of mathematical descriptions of even the simplest models of water/water interactions. As Frank[20] pointed out in 1965, the interaction between two water molecules in the original formulation by Bernal and Fowler[21] (1933) consisted principally of an electrostatic attraction between a point negative charge on the oxygen of one molecule and a point positive charge on the hydrogen of another. He went on to say that it seemed a lot to expect that such a charge distribution would remain rigid in the liquid: it was much more likely that the molecules would polarise each other, making the charge distribution in the pair different from the sum of the distributions of single molecules. He added, "To attempt to take such mutual polarization into account is, unfortunately, to give up, at least for the present, the possibility of a manageable quantitative discussion."

1.3. *Computer Simulations of Liquid Water*

This obstacle in the way of theoretical advances has been partially overcome by computer simulations of populations of water molecules. Techniques such as Monte Carlo and molecular dynamics methods avoid the problems remaining in analytical theories of simple liquids.[22–28] Application of these techniques to the much more complex liquid, water, has led to significant advances in our understanding of the molecular structure and dynamics of its liquid state. While it is not possible to measure the energy of interaction between two water molecules, best-guess values can be assigned and their validity assessed by their ability to reproduce properties and behaviour of water which can be measured. In principle computer simulations are extremely powerful additions to the previous combination of experimental and theoretical approaches to the elucidation of liquid water structure. They can be designed to reproduce properties of the liquid which are not accessible experimentally: they obviate the need for analytical solutions of complex mathematical equations. Vedamuthu *et al.*[29] have pointed out that the best simulations use highly complex intermolecular potential functions which include electronic polarisation and flexibility of intramolecular bonds. In the meantime, however, this complexity severely limits sample size so that until much larger and faster computers are available, other aspects of liquid water structure must be neglected. Simulations have already successfully reproduced many measurable properties of the liquid and more and better results are confidently expected when larger computers can handle longer times and bigger

populations of molecules. Molecular dynamics simulations, based on percolation and random network theories, are consistent with the existence in liquid water of a continuous random network of mutually-bonded molecules which breaks and reforms extremely rapidly. A criticism of these statistical treatments is that they do not stress the undoubtedly important directional character of water/water bonding.[30,31] Again, however, this may merely be a temporary neglect dictated by the present deficiency in computer power. The ability of computer simulations to reproduce some measured properties of water has established random network theories as plausible explanations of water dynamics and structure.[18,19,26,28,32,33]

1.4. *A Modern Mixture Model of Liquid Water*

Recently the success of these simulations has rather overshadowed the simpler mixture model of liquid water.[17] Robinson and co-workers,[29,34] however, have argued persuasively for a modern version of Röntgen's model.[17] They suggest that density is the property of liquid water crucial to an understanding of its properties and behaviour and propose a mixture model in which two or more bonding configurations of water molecules exist. A bulky or capacious bonding form of low density such as occurs in Ice Ih and a dense form such as occurs in the most thermodynamically dense forms of ice. This model is not conceived to be a mixture of ices having long-range order, but as a rapidly fluctuating mixture of intermolecular bonding types found in the most stable polymorphs of ice. Perhaps the most important concept which emerges is that differences between the dense and capacious structures are not at the nearest-neighbour level, but occur instead in the outlying non-hydrogen-bonded next-nearest neighbour structure. Published density data over the range $-30\,^{\circ}C$ to $+70\,^{\circ}C$ were fitted to this model with six to seven decimal place precision. Assuming that the equilibrium between these structures is shifted by changes in both temperature and pressure, the authors accounted for many of the anomalies of liquid water extremely simply. Independent support for the model comes from the X-ray data of Bosio *et al.*,[35] who identified a major structural rearrangement of the second-neighbour shell, consistent with the proposal of Robinson and co-workers.[29,34] Earlier, Lumry *et al.*[16] had proposed, on thermodynamic grounds, two different microstates of water: "short-bond" forms with short, stiff near-linear hydrogen-bonds and "long-bond" forms with long, weak and bent hydrogen-bonds. The first was dominated by the low enthalpy of short hydrogen-bonds and the second was dominated by the high entropy resulting from motions of the water molecules on flexible hydrogen-bonds into the free volume not available to the "short-bond" forms. More recently, Libnau *et al.*[36] showed that infrared profiles of liquid water in the temperature range 2–96 °C could be interpreted as representing an equilibrium between two water structures differing in the average number of H-bonds per molecule. Benson and Siebert[37] also proposed a simple two-structure model for liquid water which, unlike random H-bond breaking models, could account with great precision for the anomalous heat capacity of water from 0 to 100 °C. Li and Ross[38] analysed inelastic neutron-scattering spectra for Ice I, finding two kinds of hydrogen-bonds of different strengths. A model in which strong and weak hydrogen-bonds in the ratio of

about 2:1 were randomly distributed throughout the network was able to reproduce the neutron spectrum. They speculated that if the same bimodal hydrogen-bonding existed in the liquid state, their model might be able to explain many anomalies. Chen[39] calculated virtual single-particle energy distributions from experimental data for the heat capacity of liquids at constant volume. This approach has an advantage over the enthalpy distribution from the heat capacity at constant pressure, because the pressure/volume term included in enthalpy calculations might be expected to obscure purely molecular characteristics. Chen found that methanol, ethanol, carbon disulphide, mercury and benzene exhibited a single-peaked distribution. Water, on the other hand, gave a bimodal distribution. Chen suggested that these two energy peaks (one in the neighbourhood of $4.2\,kJmol^{-1}$ and the other around $13\,kJmol^{-1}$) could represent two distinguishable states in the structure of water.

In addition to the many crystalline ices which exhibit long-range order, there are two amorphous ices, solids which, like liquids, have only short-range order.[40] These two amorphous ices differ in density: this is direct evidence that at these low temperatures there are high density and low density forms of a non-crystalline water substance; moreover the low density form can be converted into the high density form by pressure.[41]

This modern mixture model has great explanatory power, is attractive in its elegant simplicity and now appears to have substantial direct experimental support. Although it is not necessary for the discussion that follows of water associated with polymers, the model will frequently be invoked because of its clarity in illustrating the extremely simple mechanisms proposed. In particular, it offers a strategy for equilibration of populations of water molecules of different local chemical potential under such conditions that they are prevented from moving to abolish the chemical potential gradient. If, as assumed by Robinson and co-workers,[29,34] the equilibrium between low density water configurations and high density water configurations can be shifted by changes in either temperature or pressure, surely it can also, when necessary, adjust to abolish otherwise inflexible gradients in chemical potential of freely exchangeable assemblies of water molecules.

2. APPARENT OUT-OF-EQUILIBRIUM STATES OF WATER ASSOCIATED WITH POLYMERS

The properties of solutions of small solutes, such as electrolytes, sugars, alcohols, amino acids and other organic molecules are commonly characterised by extrapolation to infinite dilution, where the only interactions are water/water interactions and water/solute interactions. The assumption can also be made that the infinitely low concentration of solute does not significantly affect water/water interactions, simplifying the description of the system still more. In the real world of condensed phases of polymers (including, particularly, biopolymers) the state of infinite dilution is irrelevant to a description of the water/polymer/small solute system.[42–49] No longer is it legitimate to ignore the effect of other components on water/water interactions. It will be shown that polymer gels and solutions include regions of water molecules of different local chemical potential, necessitating, it is suggested, spontaneous local

changes in the density of water. Examples of conditions which appear likely to require this mode of equilibration of water are first identified; then follow descriptions of experimental tests of the proposal and characterisation of the different states of water which co-exist at the same temperature and pressure.

2.1. *Hydrophobic or Weakly Hydrogen-Bonding Surfaces*

A hydrophobic or weakly hydrogen-bonding surface restricts the hydrogen-bonding potential of water molecules to which it is immediately adjacent. Relative to molecules surrounded by other water molecules, these waters can make fewer and weaker hydrogen-bonds. Moreover, since the participation of a given water molecule in one hydrogen-bond strengthens other hydrogen-bonds that it makes,[50] weak hydrogen-bonding persists over several layers of molecules out from the surface. There is thus a zone of molecules in a state of higher enthalpy than more distant molecules. The only way that water can move to abolish or diminish its gradient in chemical potential is by the association of two such surfaces with each other, a process which squeezes out some or all high enthalpy water.[51] This happens with small hydrophobic solutes and is one example of the "hydrophobic interaction".[16,52–57] When, however, hydrophobic surfaces cannot associate to squeeze out high enthalpy water, either the two populations of water molecules stay out of equilibrium, in spite of the fact that they can exchange freely, or the high energy water decreases its local chemical potential to equal that of the rest of the water. It has been suggested that it does so by expanding and doing pressure/volume work.[58–63] In terms of the modern mixture model[29,34] the high enthalpy water expands by an adjustment of the equilibrium in favour of the lower density configurations.

This is not a commonly accepted interpretation of hydrophobic hydration and the hydrophobic effect. Franks,[43] for example, attributed it to a loss of degrees of freedom of water molecules hydrating the apolar solute, without any change in the number of water–water bonds or in the hydrogen-bond energy. This appears as a decrease in entropy of water and may persist over a few layers. It must still follow from this, however, that there are apparently out-of-equilibrium populations of water molecules, in this case, because of the lower entropy of the hydrophobically hydrating molecules. The consequent association of apolar solute molecules is consistent with the need of the system to lower its overall free energy if it can.

The proposed mechanism of expansion of hydrophobically hydrating water molecules, whether by displacing an equilibrium between high and low density configurations or by expanding a random network of molecules, makes testable predictions: if the singular properties of liquid water (Section 1.1) are due to its three-dimensional hydrogen-bonding (Section 1.2) it follows that any change in the number or strength of its hydrogen-bonds must, to some degree, change all its properties. At the same temperature and pressure, a less dense form of water might be expected to have stronger hydrogen-bonds, higher viscosity, lower mobility, higher refractive index, lower reactivity[64] and changed solvent properties. This may be the origin of Drost-Hansen's "vicinal water".[65,66]

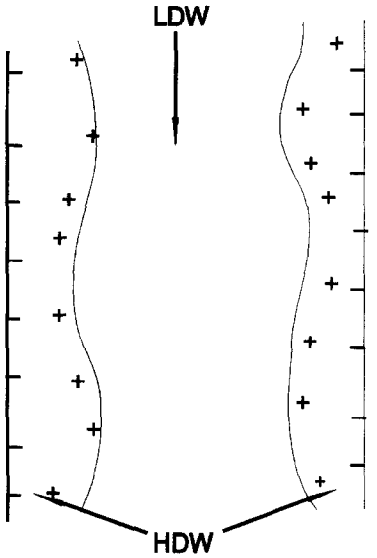

Fig. 3. Expectations of the distribution of low density and high density water in a polyelectrolyte solution or gel.

2.2. *Charged Polymeric Surfaces*

Figure 3 illustrates a cavity, lined by charged surfaces, in a gel or solution of a polyelectrolyte. Again, there are contiguous populations of water molecules apparently out of equilibrium. The activity of water in the double layers is lower than that of the rest of the water, whether or not there is excess electrolyte present. Water is prevented from moving to abolish the gradient in its activity by the high electrostatic field which holds the counter-ions in the vicinity of the fixed charges. Again, therefore, exchangeable populations of water molecules must stay in an out-of-equilibrium steady state or both regions of water must adjust their local densities to compensate for their differences in activity.

2.3. *Relationship to Classical Osmotic Theory*

Classical osmotic theory applied to charged gels in contact with aqueous solutions assumes that the lower activity of internal water (which is solvent for the counter-ions) is counter-balanced by the pressure exerted on it by the gel matrix.[67] This pressure increases the chemical potential of internal water (with its low activity) to equal that of external water (with its higher activity). This, however, does not solve the problem of the two internal aqueous zones of different activity (see Fig. 3); they are subjected to the same pressure and must, therefore, remain out-of-equilibrium. There is, however, another pressure, acting selectively on the low activity water in the double layer. This is the pressure of water prevented by the high electrostatic field from diffusing into the double layer to dilute the counter-ions. According to van't Hoff's classical osmotic theory[68] this is the osmotic pressure difference between the two solutions and should increase the activity of water in the double layers to equal that of the rest of the water

in the system. There are thus, apparently, two ways in which water in Fig. 3 can come to equilibrium.

It has been argued, however,[61] that pressures encountered in osmotic systems are too low to have the substantial effect on chemical potential required for equilibration between regions of different activity. Water is incompressible by pressures encountered in osmotic systems. For example, at 25 °C a pressure of 484 atm is required to decrease water's specific volume by 2%.[69] The osmotic pressure of a typical extracellular solution in mammalian tissues is only 7.5 atm, which would decrease the specific volume of water by an insignificant 0.03%. If the volume of water does not change significantly, neither entropy nor enthalpy are much affected, so that it is difficult to understand how osmotic pressure can have a major effect upon the chemical potential of water. It was, therefore, proposed that the osmotic pressure merely stops water moving; that the classical osmotic pressure equation:

$$\pi V = -RT\ln a,$$

where π is the osmotic pressure of a solution of water activity, a, describes a steady state, not an equilibrium state of water. Water, then, has to equilibrate by changing its local density. Whether in the scheme of Fig. 3 water equilibrates by changing its density or not is a matter for experiment to decide (see Section 6).

2.4. *Osmotically Stressed Water*

A gel in contact with a solution loses water when a soluble polymer too large to penetrate its interstices is added. Depending upon the dynamics of the gel/water system (see Section 3.1), that loss may be sufficient to equalise water activity inside and outside the gel, or it may stop short of that equilibration, when residual water, in a state of higher activity than external water must either stay out of equilibrium or decrease its density by displacement of the LDW/HDW equilibrium. Wiggins and van Ryn[63] found that the density of water in Biogel P-100 beads decreased down to 0.96 g ml^{-1} with increasing osmotic stress imposed by polyethylene glycol 20 M. In a very important series of experiments, Parsegian and Rand and co-workers have applied osmotic stress to many polymeric systems.[70–81]

In interpreting their results they assume that water moves until its activity is equalised in the two compartments. This must often be true, but there remains the possibility that in some cases (as with Biogel P-100 stressed by PEG 20 M) there may be constraints which prevent complete equilibration of water in this way. These constraints include rate-limitation by the polymeric material which moves too slowly to allow complete water movement during the time-course of the experiment (see Sections 4.1, 6.2.1, 6.2.2 and 6.2.4) or the existence of a state of lower overall free energy with excess water remaining in the polymer phase.

3. IDENTIFICATION OF LOW DENSITY WATER IN POLYAMIDE BEADS

Gel beads of the Biogel-P series (BioRad Ltd) have been used extensively to characterise low density water at a weakly hydrogen-bonding surface. They were

Table 1. Entropies of hydration of some ions in $J K^{-1} mol^{-1}$

Ion	ΔS_{hyd}
Mg^{2+}	−162
Ca^{2+}	−100
H^+	−22
HPO_4^{2-}	8
Na^+	38
Cl^-	77
K^+	80
NH_4^+	91
$H_2PO_4^-$	111
HCO_3^-	117
NO_3^-	169

chosen as being quality-controlled preparations resembling proteins, but with resistance to bacterial degradation. The surfaces carry only weakly hydrogen-bonding groups (>N–H, >C=O, end-amide groups and some carboxyl groups, their hydrolysis products). There are also stretches of short hydrocarbon chains. The smaller-pored members of the series have pore diameters of 2 nm or less so that all internal water is close enough to the weakly-bonding surface to be perturbed by it. The interface between the two putative populations of water molecules of different density is at the mouth of a pore, making it possible, in principle, to equilibrate solutions with the gel beads and determine the solvent properties of low density water. From these experiments[58,63] and similar ones using cellulose acetate films[82] the selective solvent properties of low density water at a weakly hydrogen-bonding surface have been established qualitatively: low density water selectively accumulates univalent anions (NO_3^-, HCO_3^-, $H_2PO_4^-$, Cl^-, in decreasing rank order), large univalent cations (NH_4^+, K^+), glucose, amino acids, urea and compatible solutes[83,84] (betaine, trimethylamine oxide and some polyols); it excludes small, highly hydrated cations (Mg^{2+}, Ca^{2+}, H^+, Na^+), highly hydrated anions (HPO_4^{2-}) and hydrophobic molecules. Table 1 shows the entropies of hydration of some of these ions of interest,[85] suggesting that the solvent properties of low density water are at least partially entropy-driven. The gain in entropy on solution of an ion increases as the intrinsic entropy of the solvent decreases. This would explain the increase in selective partitioning of ions into low density water from top to bottom in Table 1.

Compatible solutes, which will be discussed in Sections 6 and 8, are examples of a striking convergent evolution, being found in plants, bacteria and animals. They are synthesised in some cells and selectively taken up by others when an external solution increases in osmolality. They have the property of stabilising and protecting enzymes which are inactivated by high electrolyte concentrations.[83,84]

3.1. *The Slow Development of Low Density Water*

One of the most important variables has proved to be the time for which gel beads are treated with aqueous solutions. Water in cellulose acetate films had taken some

days to develop the infrared spectrum characteristic of low density water.[86] The explanation for this can be found in the peculiar dynamics of water/polymer mixtures. High molecular weight polymers form glasses below a characteristic temperature, their glass transition temperature (T_g).[42–49,87] Addition of water, the most effective and ubiquitous plasticiser for glassy polymers, lowers T_g progressively from perhaps 200 °C for a large polymer to below 0 °C, when the water content is high enough. When the polymer/water system is in the glassy state diffusion is extremely slow and effectively does not take place at all within the time-frame of a normal experiment. Below T_g there is a rubbery state in which water is rapidly imbibed by the pores in the beads, but its penetration and total mixing with the polymeric material is still slow. Presumably this is because, although water is liquid and has more-or-less its normal mobility, its rate of diffusion from one place to another is limited by the rate at which the tangled or even cross-linked polymer chains can move to make room for it. Therefore the final equilibration or arrival at a steady state of water in the gel beads is not determined by the brief time of self-diffusion of water over the small distances involved (μm); it has turned out to take days rather than seconds. This was realised quite early in studies of both cellulose acetate films and polyamide beads; 6 days were taken as the safe time for equilibration, but it was only after a series of unreproducible experimental results that the time relationships of gel/water/solute systems were properly investigated. These relationships will be described first as they help to understand both earlier and later experiments.

3.2. *"Equilibration" Experiments*

Dry gel (usually Biogel P-2 to P-6) was weighed into a screw-topped Pierce vial with a Teflon septum. A constant volume of solution was put in and the vials rotated slowly, duplicate vials being taken off for analysis at timed intervals. The solution contained ^{14}C-polyethylene glycol 3350 (PEG 3350) which was too big to penetrate the water in the gel during the time of the experiment and acted as external solution marker. The internal volume of the gel was calculated at each time.[62] When the solution contained 10 mol m^{-3} KNO_3 the internal volume of the gel increased steadily from 3.5 to 4.5 ml g^{-1} dry gel over a period of 17 days. Over all this time the concentration of external K^+ did not change significantly: i.e. the bead was swelling with the entire external solution, 10 mol m^{-3} KNO_3. When the external solution contained no added solute or 100 mol m^{-3} NaCl or 10 mol m^{-3} $MgCl_2$ internal volume became constant in 1–2 days. It must be emphasised that only internal volume and external concentrations of solutes were measured in these experiments; other changes may have been taking place without being detected. These results explained many earlier experiments with both cellulose acetate films[82,88] and Biogel beads[58,63] in which the apparent partition co-efficient of K^+ salts was always close to unity, in spite of the fact that other observations suggested strongly that they were selectively accumulated into low density water. In the present experiments KNO_3 was selectively accumulated into the pores of the beads, generating a gradient in water activity which was abolished by subsequent movement of water, restoring the partition co-efficient of KNO_3 to unity. This was selective uptake because the swelling did not take place in the

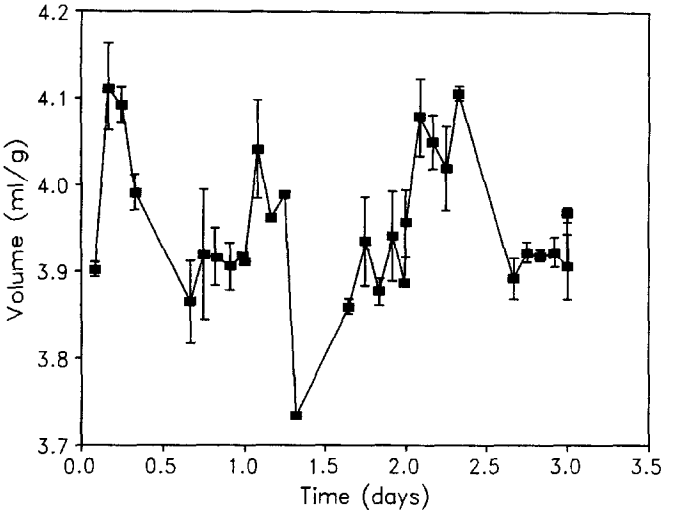

Fig. 4. Oscillations with time of the internal volume of Biogel P-6 beads rotating in Dulbecco's Modified Minimum Eagle Culture Medium (D-MEM), consisting of: 110 mol m^{-3} NaCl, 5.4 mol m^{-3} KCl, 25 mol m^{-3} $NaHCO_3$, 1.8 mol m^{-3} $CaCl_2$, 0.81 mol m^{-3} $MgSO_4$, 0.9 mol m^{-3} NaH_2PO_4, 5.6 mol m^{-3} D-glucose and low concentrations of amino acids.

presence of other solutes which had been shown to be excluded from low density water.

The next series of experiments used combinations of solutes: K^+-like solutes were selectively accumulated into low density water and Na^+-like solutes were specifically excluded. In the presence of 13 such combinations of mixed solutes, the internal volume of the gel oscillated with a time-period of one or more days. The simplest solution to induce oscillations was 5 mM K^+ phosphate, pH 7: $H_2PO_4^-$ was the accumulated solute and HPO_4^{2-} the excluded solute. Figure 4 illustrates one such result in which the external solution was a Dulbecco's Modified Minimum Eagles Culture Medium (D-MEM), consisting of: 110 mol m^{-3} NaCl, 5.4 mol m^{-3} KCl, 25 mol m^{-3} $NaHCO_3$, 1.8 mol m^{-3} $CaCl_2$, 0.81 mol m^{-3} $MgSO_4$, 0.9 mol m^{-3} NaH_2PO_4, 5.6 mol m^{-3} D-glucose and low concentrations of amino acids.

The excluded solutes in this culture medium were NaCl, $CaCl_2$ and $MgCl_2$, while the accumulated solutes were KCl, $KHCO_3$, KH_2PO_4, amino acids and glucose. Figure 4 illustrates the reason for the inverted commas around the word equilibration in the heading to this section. In systems of polymer, water and solutes of both kinds equilibrium was not reached.

3.3. *What Makes the Internal Volume Oscillate?*

There are two keys to understanding these experiments and the general phenomenon which they illustrate. They will be given here as assumptions which are validated in subsequent experiments (see Section 4.1).

3.3.1. *Reversion of Low Density Water to Normal*

The first is that, as discussed in Section 2, when water is prevented from diffusing from one region to another to abolish a gradient in its activity, it equilibrates by changing its local density. This is apparently a general phenomenon, so that when, under conditions such that water cannot move from outside to inside, low density water in a Biogel bead selectively accumulates a solute, decreasing its activity relative to that of external water, it re-equilibrates by increasing its local density and reverting toward normal density, structure and solvent properties. This can be expressed more simply in terms of the modern mixture model. The equilibrium between high density water configurations (HDW) and low density water configurations (LDW) is displaced inside the pore in favour of LDW to compensate for its higher enthalpy. When that water accumulates a solute to higher than external concentrations, its activity decreases, necessitating a reversion of the HDW/LDW equilibrium back toward its normal position.

3.3.2. *Reinforcement of Low Density Water*

Solutes excluded from low density water, on the other hand, decrease external water activity relative to internal water activity and, in the absence of net water movement, the HDW/LDW equilibrium shifts in favour of LDW.

3.3.3. *Dynamics of Gel/Water/Solutes*

The second important principle lies in the different dynamics of polymer, water and small solutes. The polymer is in the rubbery state in which diffusion is not so slow as to be impossible in the time-frame of these experiments but, nevertheless, very slow compared with the self-diffusion of water and solutes.[49,87] These dynamics give rise to the non-freezing water that is observed in differential scanning calorimetry. The water is prevented from diffusing freely to the growing ice front by the extremely slow movement of the polymer.[89]

3.3.4. *Water Dynamics*

Water is in a liquid state of reduced mobility when it is in its low density form, but able to exchange between gel and external solution at a rate nearer to that of its normal self-diffusion, than to that of the polymer mobility. When internal water changes its density up or down (by, for example, displacement of the equilibrium between HDW and LDW) the change is initiated at the interface between the two populations of water molecules and propagates through the internal compartment at a rate comparable, presumably, to the self-diffusion of water. Net movement of water into or out of the gel, however, is very slow because it must await movement of the polymer chains.

3.3.5. *Small Solutes*

Small solutes are different again. Their self-diffusion is of the same order as that in simple aqueous solution so that they, too, can exchange readily between internal and

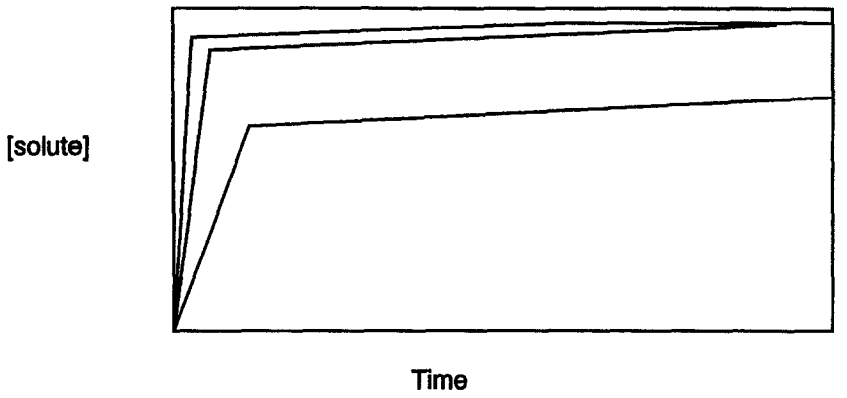

Fig. 5. The approach to equilibrium of a small solute in gel water. The extremely rapid phase, during which demands made upon the polymer network are negligible, is followed by an extremely slow phase, the rate of which is limited by the ability of the polymer chains to move to accommodate the small solute. The rate of the first phase decreases with increasing solute size.

external solutions. They differ from water, however, in that they can also contrive to engage in net movements into or out of the gel without severe limitations imposed on their rate of diffusion by the dynamics of the polymer. This is simply because, relative to water, they are present in such low concentrations that their movement requires less adjustment from the polymer; when some solutes are moving in and, simultaneously, others out it requires no adjustment of the polymer at all. Nevertheless, Ginzburg and Cohen[90] found that elution of small neutral solutes from a Dowex cation exchange resin depended upon their molar volumes. The larger the molar volume, the more was the solute excluded from the gel. This suggests that during the relatively short time of elution, solutes were prevented from equilibrating in the gel water by the resistance of the polymeric network to movement. This resistance would be least for the solutes which occupied least volume. Figure 5 illustrates the approach to equilibrium of a small solute in gel water. There is an extremely rapid phase, during which demands made upon the polymer network are negligible, followed by an extremely slow phase, the rate of which is limited by the ability of the polymer chains to move to accommodate the small solute. Finally equilibrium is reached. A solute occupying a larger volume reaches the slow phase at a lower internal concentration and is therefore slower to come to equilibrium; in the bottom curve equilibrium is not reached during the time of the experiment.

Ling and co-workers,[91–95] using equilibrium dialysis over periods of 2–3 days, have measured the concentrations of many electrolytes and non-electrolytes in protein and polymer solutions, finding linear relationships between external and internal concentrations. The most important determinant of the ratio of these concentrations was the size of the molecule: Ling's "size rule" states that the smaller the solute, the larger its relative concentration in the polymer solution. Ling and co-workers interpreted the ratios as equilibrium distribution ratios. An alternative explanation, however, is that the measurements were made on the slow part of the uptake curve in Fig. 5, when

equilibrium was not yet reached. Smaller solutes reached higher concentrations before the slow phase started, and, therefore, appeared to have higher partition co-efficients than larger solutes. Ling also showed that the conformation of the polymer was a crucial factor in the degree of exclusion of small solutes. Native proteins had little effect but solutes were strongly excluded from solutions of denatured proteins and long-chain random coil polymers. This is consistent with Ling's Polarized Multilayer Theory of intracellular water[96] but might also be explained by differences in water/polymer mixing. Random coil and extended polymers mix intimately with water. Globular proteins, on the other hand, which assume rather tightly folded conformations of low surface area exclude all but a few water molecules.[4]

Troshin[97] reached similar conclusions in 1956, when he "equilibrated" sugars with co-acervates and red blood cells for 15–18 h, finding that the internal concentration was lower than the external concentration and that it decreased with increasing molecular weight.

3.3.6. *Addition of Water to Dry Gel*

These, then, are the dynamic constraints which determine the sequence of events when dry gel is put in contact with a solution (as in Fig. 4). The gel rapidly imbibes water which then, relatively slowly, plasticises the polymer by penetrating into all its interstices and becoming an integral part of the rubbery state. During this slow process water begins to wet the poorly hydrogen-bonding surfaces, its density decreases, changing its solvent properties. Solutes which accumulate selectively into normal water begin to move out of the gel, while solutes which accumulate selectively into low density water begin to move into the gel. As this exchange proceeds rapidly, without time for significant water movement, the small solutes tend toward an equilibrium distribution between normal and low density water. Water is unlikely to be in equilibrium following this exchange, which alters both its internal and external activity. In Fig. 4 the residual gradient in water activity at this point was clearly inwardly directed because the internal volume increased with time. As water followed the accumulated solutes into the gel, internal solutes were diluted and external solutes concentrated until water activity was the same inside and out. At this point water stopped moving. Continued uptake of solutes then decreased internal water activity and, rapidly, internal water reverted to normal density, structure and solvent properties. Solutes distributed randomly between the two compartments; internal water was again in a state of high enthalpy and was squeezed out by movement of the polymer matrix. The low mobility of the polymer made this a slow process; while it was happening internal water again decreased its density and a cycle began again.

Although this treatment has used sequential unitary processes to describe the phenomenon, it is really a single continuous non-linear process with overlapping reactions occurring at widely different rates. The slow movement of water into or out of the gel allowed time for transient changes in water density, producing metastable states of water. If the movement of water were yet much slower it would be kinetically frozen in these metastable states.

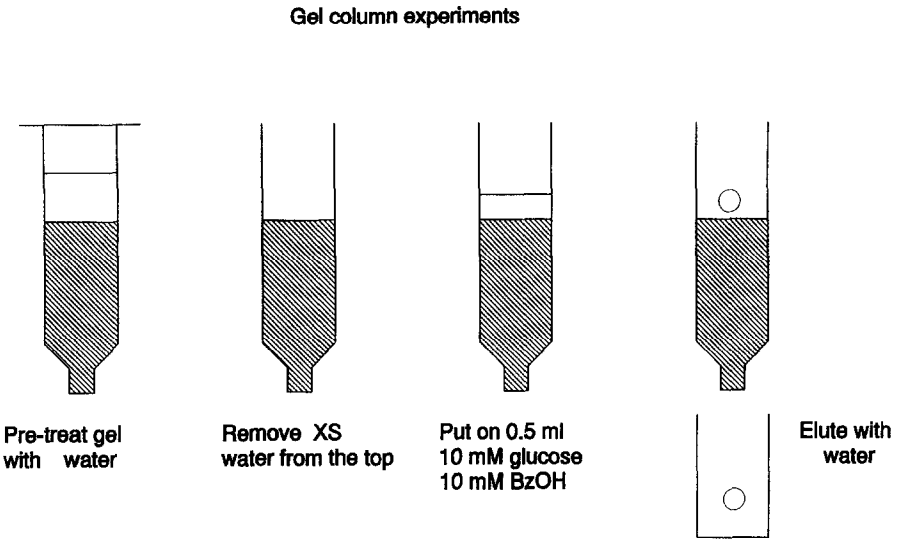

Fig. 6. Sequential steps in the column elution experiments.

4. COLUMN EXPERIMENTS

It became clear with these experiments that equilibration was not possible with mixed solutes (or, indeed, even with single solutes) and that the only way to investigate the phenomenon was to treat it as a process and to design experiments to extract information about the effects of added solutes and of dynamical conditions on a specific process. Figure 6 illustrates the extremely simple experimental technique used: 1 g washed, dried P-6 gel in a column was mixed into a slurry with water or an aqueous solution, the column capped top and bottom and left for various time intervals for the development of low density water. Excess water was removed from the top of the column, 0.5 ml sample, containing a probe of low density water, put on and eluted at constant flow rate (0.5 ml min^{-1}) with water or a solution. The rationale for this technique is that during the brief time during which the probe distributed itself between a pore and the external solution as it passed down the column, net movement of water was negligible and the only movements possible were exchange of water between internal and external compartments, making and breaking of low density water and diffusion of solutes. The process that was followed was diffusion of probe solute into and out of the aqueous compartments in the gel column.

4.1. *Time for Maximal Changes in Water Density*

Following treatment of gel columns with water for different intervals of time, a standard elution experiment was performed. The sample contained glucose (labelled with ^{14}C) as a positive probe of low density water and benzyl alcohol (BzOH) as a negative probe. Fractions were collected and analysed for glucose, by counting in an LKB 1217 RackBeta β-scintillation counter, and for BzOH by measurement of the absorbance at 250 nm in a Perkin-Elmer 124 double-beam spectrophotometer. A

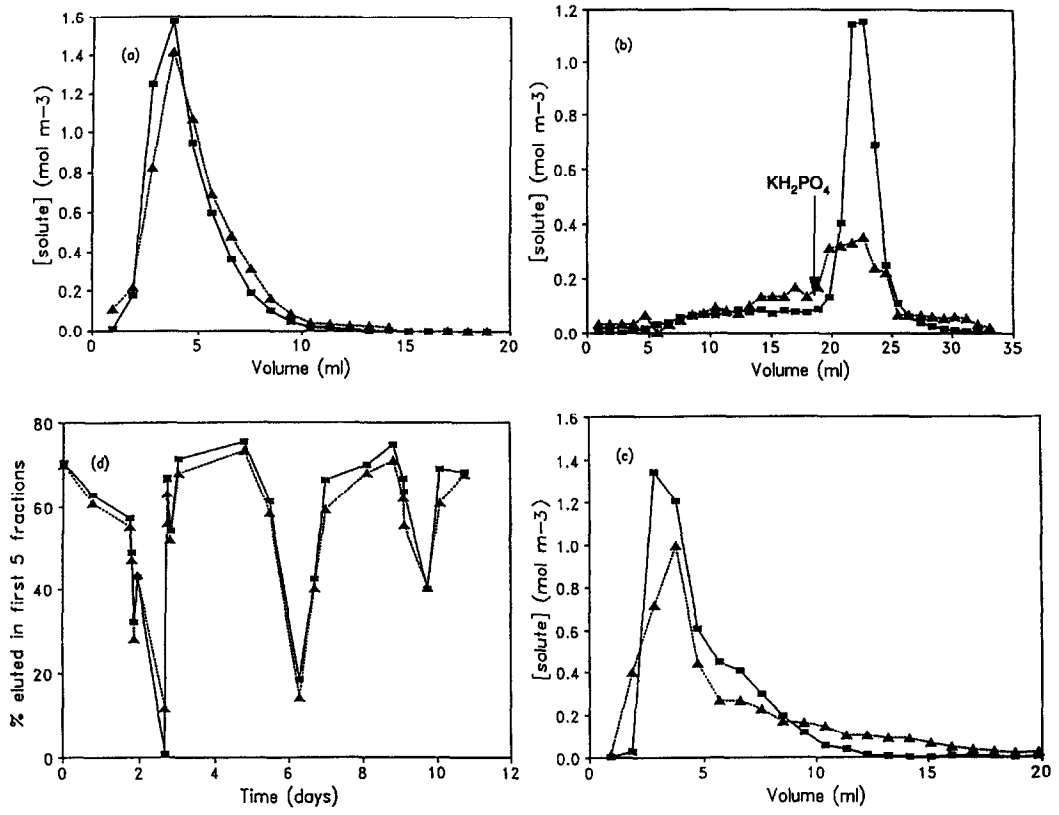

Fig. 7. Elution of 1 ml of 10 mol m^{-3} ^{14}C-D-glucose, 10 mol m^{-3} BzOH from a P-6 column after various times of pre-treatment of the column with water. (a) 1 h; (b) 64.4 h; (c) 68.5 h; (d) the percentage of each probe eluted in the first five fractions as a function of time of pre-treatment.

column of the same age as the experimental column was routinely eluted with water in order to determine the absorbance due to NH_4^+ from the gel which was always present in the fractions. This absorbance was negligible at 250 nm, but significant at 210 nm.

Figure 7 shows some representative results: after pre-treatment of the column in water for 1 h almost all of both probes eluted in a single peak centred at approximately 4 ml, glucose slightly ahead of the alcohol. This suggests that very little low density water had formed in 1 h and/or that which had formed was not, as yet, very different from normal water. The slight displacement of the two probes is probably indicative of differences in their molar volumes as discussed in Section 3.3. After 64.4 h of pre-treatment with water, low density water had increased so much, either in volume or in selectivity, that very little of either probe eluted in the first 5 or even 20 ml. They were recovered by elution with KH_2PO_4, which is avidly accumulated by low density water, restoring it to its normal density, structure and solvent properties and releasing both glucose and BzOH.

After 68.5 h of pre-treatment with water, substantial fractions of both probes eluted in the first peak, indicating a considerable collapse of low density water since 64.4 h.

Tails of retained probes were evident, but the elution pattern resembled that of the 1 h treatment more closely than that of the 64.4 h treatment. Displacement of the two probes relative to each other, however, was different from that in the absence of low density water. The final frame of Fig. 7 shows the percentage of each probe eluted in the first five fractions over periods up to 11 days. It reached a minimum in just less than 3 days and thereafter exhibited damped oscillations. This unexpected result is specific to this polyamide gel (see Section 6.2.2). It has been included because it makes some useful points and explains a very large number of previous irreproducible results. End amide groups hydrolyse slowly when the gel is in contact with water.[98] As the concentration of NH_4^+ increased in Fig. 7 the gel was converted into an oscillating system (like that of Fig. 4) with NH_4^+ as the accumulated solute and H^+, already present as the counter-ion to end COO^- groups, as the excluded solute. Previously (see Section 3.2, when only internal water content was measured these oscillations were not seen, presumably because water movements in response to the relatively low concentrations of ions were too slight. The increasing presence of NH_4^+ was confirmed by the routine measurements of the absorbance at 210 nm in fractions eluted from a gel in the absence of BzOH. These experiments are interesting as a direct measurement of the growth and decay of low density water; it is clearly a much more sensitive method than measurement of the internal volume of the gel.

The apparent difference between the two probes, glucose and BzOH, is so slight that their selection as positive and negative probes of low density water needs justifying. Their retardation by the gel increased and decreased together. They were both eluted from the gel when low density water reverted to normal with solutions of KH_2PO_4, NH_4HCO_3 or NH_4NO_3 as eluants. Previous experiments[61] showed that the essential difference between them appeared when they were put on the gel separately: glucose showed no retardation, while BzOH was significantly retarded. Only when they were put on together was glucose also retarded, as it was in Fig. 7.

4.1.1. *Glucose*

When $10\,mol\,m^{-3}$ glucose was put on a gel previously treated with water, glucose concentrated selectively into the low density gel water, decreasing its activity relative to that of external water. In the absence of net water movement, gel water responded by increasing in density back toward its normal value, so that accumulated glucose diffused out again.

As glucose moved down the column all internal low density water reverted to normal. In general a solute which is selectively taken up by low density water converts that water back to normal structure and solvent properties, unless low density water is protected or the concentration of solute is extremely low. This is the mechanism of release of glucose by KH_2PO_4, NH_4HCO_3 or NH_4NO_3. It follows that any probe of low density water is very likely to demolish it, making it impossible to determine whether or not it was there in the first place. This is one of many reasons why low density water has eluded detection. In the experiments of Ginzburg and Cohen[90] (see Section 3.3.5) all solutes (sucrose, glucose, glycerol,

ethylene glycol and formamide) eluted in sharp symmetrical peaks, showing no sign of the presence of low density water, in spite of the fact that all were, in the present sense, probes of low density water.

4.1.2. *Benzyl Alcohol*

BzOH, on the other hand, is a typical hydrophobic molecule which adsorbs from aqueous solution to a hydrophobic surface. This is an example of the hydrophobic interaction, believed to be driven not by attractive interactions between the hydrophobic molecule and the surface, but by the unfavourable water/hydrophobic molecule interaction.[16,52,54–56] Retention of BzOH by the P-6 gel, therefore, is not surprising but the requirement for the presence of low density water, demonstrated by the release of BzOH with glucose on elution with KH_2PO_4, NH_4HCO_3 or NH_4NO_3 gives a new dimension to this interaction. When the P-6 gel was pre-treated with a solution of 100 mol m^{-3} BuOH, BzOH was not retarded, indicating that, in addition to low density water, it needed unoccupied adsorption sites. It was shown previously[63] that alcohols increase the selectivity of low density water, partly by adsorbing to the surface and making it more hydrophobic, and partly by creating a water activity gradient by means of their accumulation in the external solution. When BzOH alone was put on a water-washed P-6 gel[61] it rapidly partitioned between gel water and normal water, with some adsorbed to the gel surface and an external concentration higher than the internal concentration. As the column was eluted with water some BzOH diffused out of the gel, decreasing the external water activity, so that low density water inside the gel expanded further and some BzOH was retarded by the gel. This sequence of events was repeated as BzOH moved down the column.

4.1.3. *Glucose and Benzyl Alcohol Together*

When the mixture was put on the gel previously washed with water, both solutes partitioned between low density water and external water, but this time the presence of BzOH protected low density water so that the column retained both some glucose concentrated in low density water and some BuOH adsorbed to the hydrophobic surfaces. Again, these processes were repeated as the solutes moved down the column, outward diffusion of BzOH protecting low density water into which some glucose was concentrated.

As water in the gel became more selective with time, more BzOH adsorbed, further increasing the retention both of glucose and itself. The difference, however, was that BzOH was retained on the surface and glucose in solution. If the hydrophobic interaction is driven by unfavourable water/apolar molecule interactions, it is, perhaps, not surprising to find that it becomes stronger as water adjacent to the hydrophobic surface decreases in density and exacerbates those unfavourable interactions, whether they arise from enthalpy increase or entropy decrease of water adjacent to the hydrophobic surface or both.

4.1.4. *Summary of Conclusions from Figs 4 and 7*

Assumptions which were stated in Section 3.3 have been validated by Figs 4 and 7, which are representative of a very large number of experiments. The specificity of solute behaviour cannot be attributed to selective binding to the gel matrix because the osmotic changes in Fig. 4 showed clearly that the solutes were in solution in water; moreover, the kinds of interactions of different solutes are incompatible with binding to the solid phase. BzOH caused retention of glucose which was not retained alone. It must, therefore, be the gel water which sometimes retains glucose and sometimes does not, and is induced to release both glucose and BzOH by KH_2PO_4, NH_4HCO_3 or NH_4NO_3.

When water can move to abolish a gradient in its chemical potential it does so (Fig. 4), even if extremely slowly. When it is prevented from moving, the HDW/LDW equilibrium is displaced in the direction of more LDW to decrease its chemical potential and in the direction of more HDW to increase its chemical potential. These changes are rapid because they require very little movement of the polymer molecules but merely a small change in the density of water already in intimate contact with them; this change travels as fast as the self-diffusion of water. Thus in the presence of glucose alone water in the gel had time to revert to its normal equilibrium position, allowing random distribution of glucose, before the eluting solution carried water and solutes past the pore. On the other hand the first displacement of the HDW/LDW equilibrium, while the column was pre-treated with water, developed extremely slowly because its rate was limited by movement of the polymeric material to accommodate water. Finally, these experiments have shown that solutes which accumulate selectively into low density water precipitate its reversion to normal water unless (1) it is protected by the presence of an excluded solute (NaCl, etc. in Fig. 4 and BzOH in Fig. 7) or (2) there is time during the experiment for water to move to abolish its activity gradient (Fig. 4).

5. MICRO-OSMOSIS

The phenomenon described in Sections 3 and 4 has been given the name micro-osmosis.[61,62] It is much more than a freakish aberration of water which can be safely ignored: evolution has had to overcome it, circumvent it or, more probably, harness it, as optimal conditions for biochemical processes emerged. In employing low density water to perform chemical work and work of transport[99,59,100] evolution has had to ensure that while it is performing that work it is protected by suitable solutes, so that its collapse does not take place until the work is complete, when its protection is immediately removed. Enzymes and receptor/ligand interactions are well-known to be self-limiting systems in which a resting state is converted to an active state, which reverts to the resting state when and not before the reaction is complete. Micro-osmosis has many attributes which make it an extremely strong candidate for such processes:

(1) It is a continuous non-linear process involving many reactions of widely different rates.

(2) It is initiated at the interface between two populations of water molecules of different local chemical potential (such as the mouth of a hydrophobic active site of an enzyme or receptor).

(3) It takes the form of local changes in density of water as the two populations adjust toward equilibrium, starting at that interface and propagating in both directions from it; it is thus vectorial, as are cation transporting enzymes and motor molecules.

(4) As water changes its density it changes its solvent properties: when it decreases its density in a hydrophobic cavity it activates small cations (H^+, Na^+, Ca^{2+}, for example, the cations which are actively transported by membrane-bound enzymes).[101–106]

(5) As water changes its density small solutes begin to diffuse into their preferred aqueous environments, creating fresh gradients in water activity.

(6) When solute movement increases the changes in the densities and solvent properties of the two water populations, it stabilises them and generates highly ordered asymmetrical distributions of solutes.

(7) When solute movement reverses previous changes, restoring a single compartment of water of normal density and solvent properties, micro-osmosis is self-limiting and generates temporal and spatial oscillations.

(8) Neither water nor solute necessarily ever comes to equilibrium.

(9) If there is time for water to move during the micro-osmotic event it can do pressure/volume work (eg the mechanical work of motor molecules such as myosin).

(10) It can be converted from one mode of operation to another by a flux of a suitable solute.

6. HIGH AND LOW DENSITY WATER IN POLYELECTROLYTE GELS AND SOLUTIONS

In Section 2.2 it was suggested that charged polymeric surfaces should generate both high density water and low density water.

6.1. *Viscosity of Dextran Sulphate Solutions*

The first experimental approach to this problem was measurement of the effect of small solutes added to a solution of dextran sulphate of average MW 500,000. The viscosity of these solutions would obviously be largely determined by the slowness of movement of the large polymer molecules, but it was thought possible that there might be an additional contribution from the viscosity of water present in the solution. Figure 8 shows some representative results. Addition of NaCl more than halved the viscosity of 10% dextran sulphate in its sodium form. Addition of $MgCl_2$ had a similar effect.[63] BuOH also decreased the viscosity, although less drastically, while betaine (and urea) increased it.

Inspection of Fig. 3 shows that, by analogy with experiments on small-pored Biogel-P gel beads, there should be two populations of water molecules of different activity in these solutions, setting the scene for micro-osmosis, starting at the interface between them and proceeding in both directions, displacing the HDW/LDW equilibrium toward HDW in the double layer where water activity is low and toward LDW

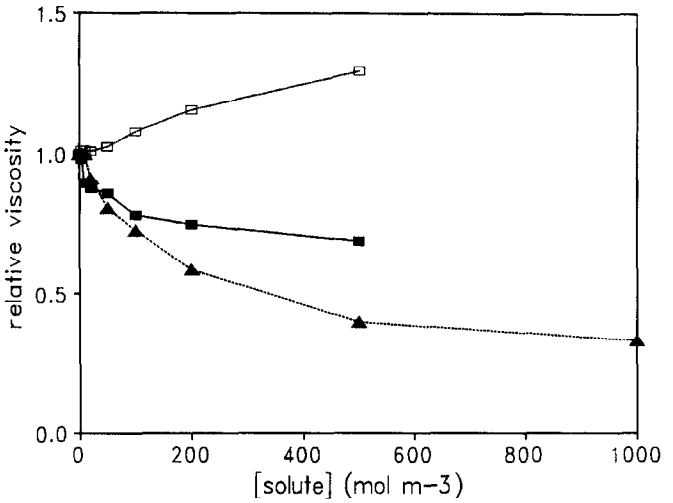

Fig. 8. Effects of added solutes upon the viscosity of 10% dextran sulphate solutions. □: betaine; ■: *n*-BuOH; ▲: NaCl.

in between polyions where the water activity is high. Characterisation of states of water in such a solution is more difficult than it was for the polyamide gel or films of cellulose acetate, where the boundary between the two populations of water molecules was at the mouth of a pore. In a solution or even a gel of a polyelectrolyte the two populations occupy a single compartment, so that any global measurement of a water-related property can give only an average value. Viscosity is such a measurement. On average, therefore, electrolytes decrease viscosity and compatible solutes increase it. Since NaCl and BuOH were relatively excluded from low density water in polyamide beads, it can be assumed that they will both be relatively accumulated into the high density water of the dextran sulphate solution, while betaine is accumulated into low density water. Whereas, in the polyamide gel bead system movement of water to abolish a gradient in its activity was extremely slow, it seems probable that, once the dextran sulphate/water has been given time to reach a steady state of distribution of water throughout the polymer material (approximately 24 h), internal movement of water can take place rapidly without waiting for adjustment from the polymer molecules. Therefore accumulation of solute selectively into one of the two populations of water molecules is probably followed by movement of water. In the presence of NaCl or BuOH, apparently, high density water grows at the expense of low density water, while in the presence of betaine, low density water grows at the expense of high density water. This suggests that the dynamics of polymer/water systems are not entirely dominated by the slow movement of the polymers. They also depend upon the dynamical properties of water associated with the polymer, dynamical properties which depend upon the hydration characteristics of all small solutes present.

Raman spectroscopy of water in dextran sulphate solutions is another technique which gives an average property of all water molecules present. Siew *et al.*[107] found that weak hydrogen-bonding was dominant and increased with the concentration of dextran sulphate, but was less than expected on the basis of the number of sulphate

Table 2. Glucose eluted in the first 6 ml of water from a Dowex AG 50W-X16 column pre-treated with water. There was no further change after 13 days. Each value is the mean and standard deviation of duplicate elutions

Time (days)	H^+	Na^+	K^+	Ca^{2+}	Mg^{2+}
1	98±2%	95±2%	92±1%	87±3%	85±1%
13	96±2%	86±1%	75±2%	86±2%	89±6%

groups and their effect on the spectrum of the –OH stretch band of water, in solutions of Na_2SO_4 and sodium methyl sulphate, suggesting that other factors, increasing hydrogen-bond strength, were present. The several components of the average changes in water properties have been identified by other experiments which specifically probe either high density or low density water.

6.2. *Experiments Using a Strong Cation Exchange Column*

Dowex AG 50W-X16, a high capacity strong cation exchange resin, with functional groups R–SO_3^- on a matrix of styrene divinyl benzene, has been tested for the presence of high and low density water using the probes (^{14}C-D-glucose and BzOH) that were used in polyamide columns. The experimental method was essentially the same as that illustrated in Fig. 6. The gel (5 g) in its H^+ form was converted to its Na^+, K^+, Ca^{2+} or Mg^{2+} form with 4 bed volumes of NaOH, KOH, $CaCl_2$ or $MgCl_2$ (mol dm^{-3}) then washed with 4 bed volumes of water.

6.2.1. *Time-Course of Development of Low Density Water*

Development of low density water was followed by means of a standard elution of 0.5 ml of 10 mol m^{-3} ^{14}C-glucose with water at constant flow rate of 0.5 ml min^{-1}. Table 2 summarises the results. After pre-treatment of the columns with water for 1 h very little glucose was retarded by the gel but the amount retarded (presumably in low density water) increased with change of cation in the order $H^+ < Na^+ < K^+ < Mg^{2+} = Ca^{2+}$. During 13 days of pre-treatment with water, only Na^+ and K^+ columns changed significantly in their degree of selectivity for glucose, so that the rank order of retention of glucose became $K^+ > Na^+ = Ca^{2+} = Mg^{2+} > H^+$. After 13 days there was no further change in the elution patterns; presumably, water had penetrated the gel as far as possible.

The rank order of effectiveness of counter-cations in generating low density water was different from their rank order of partitioning into low density water in cellulose acetate films or Biogel beads: $Mg^{2+} < Ca^{2+} < H^+ < Na^+ < K^+$. Effectiveness of the univalent cations showed an inverse correlation with their degree of hydration. Apparently, the higher the residual charge on the hydrated cation the closer was it held to the fixed charge. This thinned the double layer and concentrated the counter-ions. Thus LDW became most selective for glucose when K^+ was the counter-ion. Divalent cations are more complex. They are much more highly hydrated than any univalent cation but, nevertheless, each hydrated cation must have a greater residual charge even than K^+. The fact that retention of glucose with time of pre-treatment of

the column did not increase in the presence of these two counter-cations suggests that they were exerting more than one effect. It must be emphasised that in all these experiments there is no way of distinguishing between an increase in volume of low density water and an increase in its selectivity. One possibility is that, as populations of water molecules of high and low activity developed with time in the Ca^{2+} and Mg^{2+} columns, a preferred way of lowering the overall free energy of the system was to squeeze out some of the high activity water, just as high enthalpy water is squeezed out in the hydrophobic interaction (see Section 2.1). This would have the effect of decreasing the volume but not the selectivity of the residual low density water. Parsegian and co-workers have identified or inferred a temperature-dependent attractive force between collagen triple helices[80] and in Mn^{2+}-condensed DNA double helices,[79] which resembled the hydrophobic interaction in that it increased with increasing temperature from 5 to 35 °C and involved an increase in entropy. Might the "squeezing" out of high energy water be the origin of that force? This effect would increase with increasing temperature because, while expansion of high activity water is accompanied by a favourable decrease in enthalpy which is not temperature sensitive, it is opposed by a decrease in entropy. Since the term $T\Delta S$ increases with temperature, a squeezing out of high activity water must be increasingly preferred to its expansion as a means of equalising the chemical potential in the two zones inside the gel. It is accompanied by an increase in entropy. At lower temperatures extremely low density water can form with a smaller entropic cost. Might this, also, be the mechanism of low temperature denaturation of proteins? As the temperature decreases hydrophobic portions of surface can remain in contact with water because the $T\Delta S$ term involved in water expansion has decreased until the loss of entropy in assembly of compact proteins becomes dominant in the free energy of folding. High temperature denaturation of proteins, on the other hand, occurs at a temperature at which water/water hydrogen-bonding in the bulk liquid is already so weak that molecules adjacent to a hydrophobic surface have only slightly elevated enthalpy. Again, there will be a temperature at which expansion of hydrophobically hydrating water is so slight that it is thermodynamically favoured relative to the compact conformation of the folded protein.

6.2.2. *Time-Course of Development of High Density Water*

Figure 9 shows the results of eluting 0.5 ml of a sample containing 10 mol m^{-3} ^{14}C-glucose and 10 mol m^{-3} BzOH with water at a constant flow rate of 0.5 ml min^{-1}. The Dowex AG 50W-X16, in its K^+-form, had been pre-treated with water from 1 h to 4 days. These elutions show the strong retardation of BzOH, which can either adsorb to a hydrophobic surface in the presence of low density water, or be selectively concentrated into high density water. After pre-treatment for 1 h there was little retardation of either glucose or BzOH but with increasing times retention of both solutes grew. Figure 10 shows the decreasing percentages of each solute eluted in the first 15 ml; BzOH was more strongly retained than glucose. Changes continued for 12 days (compare Table 2). These gels did not oscillate in content of low density water with time

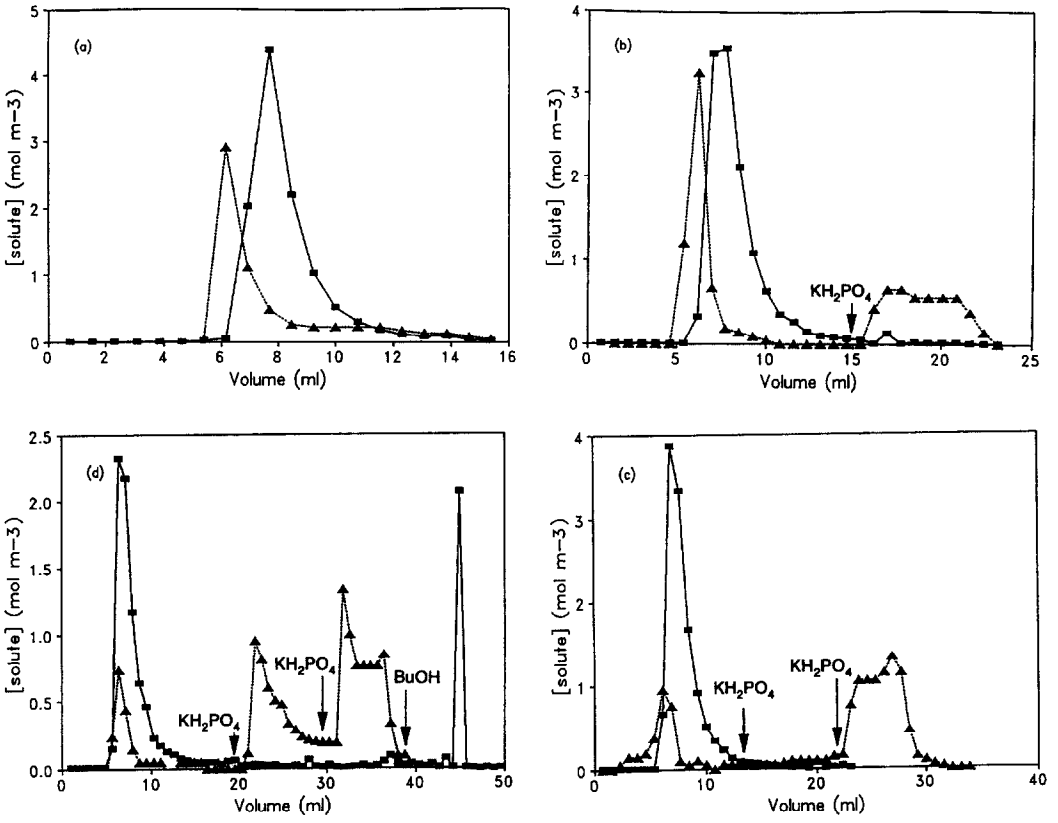

Fig. 9. Elution of 1 ml of 10 mol m^{-3} ^{14}C-D-glucose (■) and 10 mol m^{-3} BzOH (▲) with water from a Dowex AG 50W-X16 column after various times of pre-treatment with water. (a) 1 h; (b) 1 day, residual BzOH eluted with KH_2PO_4 after fraction 14; (c) 3 days, residual BzOH eluted slowly with KH_2PO_4 at fraction 13, but eluted after standing overnight from fraction 23; (d): 4 days, some residual BzOH eluted immediately with KH_2PO_4, the rest eluted after standing overnight; residual glucose was eluted with 200 mol m^{-3} BuOH.

(compare Fig. 7) but both high and low density water formed extremely slowly because intimate mixing of polymer and water was rate-limited by movement of the polymer.

The most remarkable difference between this gel and the P-6 gel, on which identical elutions were performed, lies in the solutions which released the two solutes. KH_2PO_4, NH_4HCO_3 and NH_4NO_3 all released both solutes from P-6, but in the highly charged gel they all released only BzOH. After 3 or 4 days KH_2PO_4 released all BzOH only after overnight treatment of the column. No glucose release was precipitated by KH_2PO_4, rather glucose continued to leak out very slowly during the massive release of BzOH. After 4 days some residual glucose was finally released by elution with 200 mol m^{-3} *n*-BuOH (or 100 mol m^{-3} NaCl). Even then, 15% of the original glucose remained on the gel and could only be eluted by neutralising the gel with HCl (mol dm^{-3}).

It had been concluded (Section 4.1.4) that KH_2PO_4 released both solutes from the P-6 column by precipitating reversion of low density water to its normal density and solvent properties. BuOH, on the other hand was of the class of solutes which stabilise low density water in the P-6 column and protect it against reversion. This paradox can

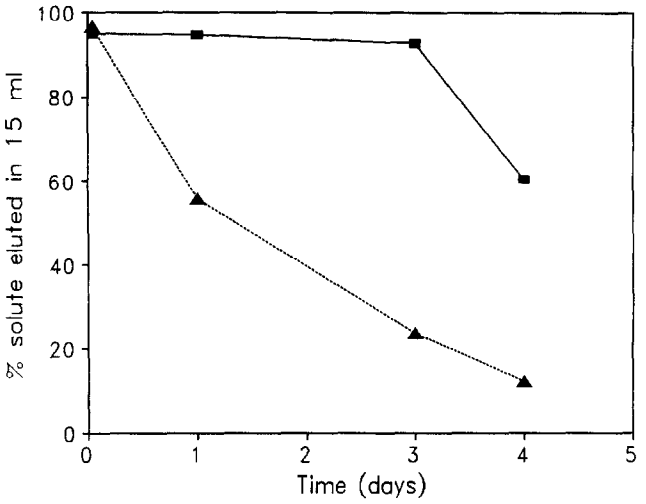

Fig. 10. Percentage of glucose and BzOH eluted from the column of Fig. 9 in the first 15 ml.

be resolved if, as speculated in Section 6.1, water can move relatively rapidly within an internal compartment of a charged gel because significant movement of the polymer is not required. When KH_2PO_4 was the eluant of the polyamide column, it diffused selectively into the low density water already containing glucose at a high concentration. During the rapid elution, polymer movement was too slow to allow net influx of water from the eluting solution to abolish this osmolality gradient and low density water reverted rapidly to normal, releasing glucose and, presumably, KH_2PO_4. In the ion exchange column, on the other hand, although net water movement from the eluting solution could not dilute the glucose and KH_2PO_4 accumulated into low density water between the double layers, water from the double layers could; during elution with KH_2PO_4 movement of internal water diluted glucose which, therefore, did not elute and concentrated BzOH which did. BuOH as eluant, on the other hand, drew water from the LDW into the HDW zone, concentrating residual glucose, some of which eluted. If BzOH had been adsorbed to hydrophobic sections of surface, it would have eluted with glucose, as in Fig. 7.

6.2.3. *Thickness of the Double Layer*

The thickness of the double layer has generally been estimated by a solution of the Poisson equation; Bedzyk *et al.*[108] however, measured it using standing X-rays. Further measurements would be of great interest to see whether, as suggested here, thickness also depends critically upon the hydration properties of both electrolytes and non-electrolytes present. Figure 11 illustrates the changes in thickness of the double layer which appear to take place during elution of glucose and BzOH from a Dowex cation exchange column. Similar changes can be invoked to account for the effects of small solutes on the viscosity of dextran sulphate solutions (Fig. 8).

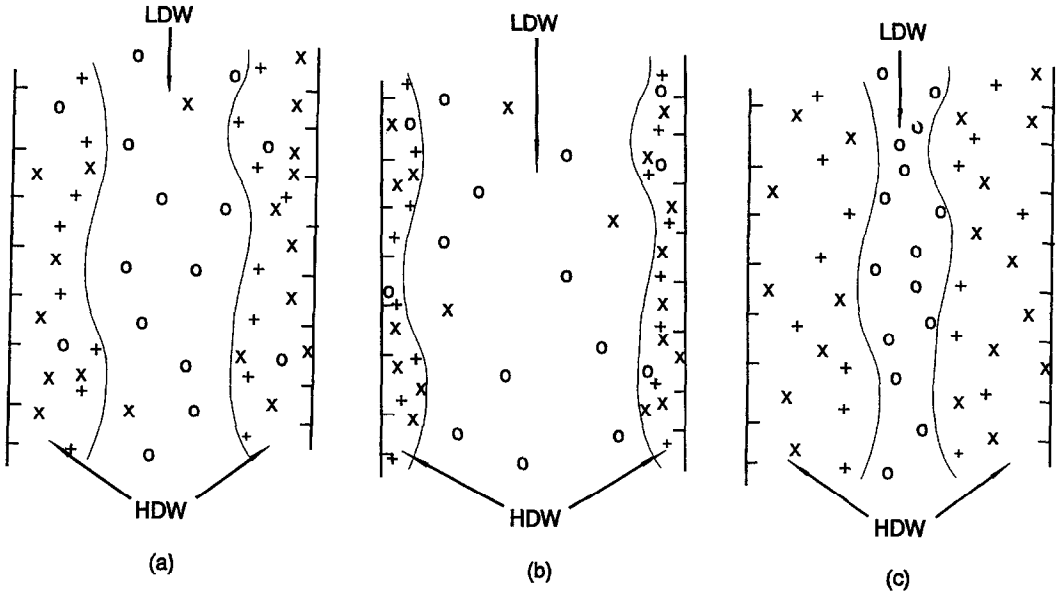

Fig. 11. Scheme illustrating partition of glucose (o) and BzOH (x) between low density and high density water during their elution from a Dowex cation exchange resin. (a) After elution with water some glucose remained in solution in LDW; some BzOH remained in HDW. Separation was not complete: there was a low concentration of glucose in HDW and of BzOH in LDW. (b) During elution with KH_2PO_2 which went selectively into LDW, drawing water from the double layer. This concentrated BzOH which eluted and diluted glucose which remained in the enlarged LDW region. In addition to enlarging the LDW zone KH_2PO_4 decreased the magnitude of the osmolality gradient between the two contiguous solutions so that the water equilibrium shifted back toward LDW in the double layer and back toward HDW in the rest of the water. This somewhat decreased the selectivity of water in each region, allowing slightly more glucose in HDW and elution of all BzOH. (c) During elution with *n*-BuOH, which went selectively into HDW, diluting BzOH and concentrating glucose which eluted. This time the selective solvent properties of both regions were increased, so that LDW retained some glucose.

6.2.4. *Protection of Low Density Water by BuOH in Dowex*

In Fig. 10 BuOH eluted glucose from low density water because when it was added glucose was already sequestered at a high concentration in low density water. Its further concentration by loss of water to the double layer allowed it to elute. Figure 12 shows, however, conditions under which BuOH can protect low density water. When 1 ml of $10\,mol\,m^{-3}$ K^+ L-glutamate, pH 7 (another probe of low density water) was eluted with water from 10 g of a water-treated column in its K^+ form, all the probe eluted in a single sharp early peak. No low density water survived its passage down the column. When, however, the column, pre-treated with water, was then washed with two bed volumes of $200\,mol\,m^{-3}$ BuOH, K^+ L-glutamate eluted relatively slowly and incompletely. This retention of L-glutamate can be explained in terms of Fig. 11. After pre-treatment with *n*-BuOH double layers would be in the state illustrated in Fig. 11(c); i.e. excess water would be available to follow K^+ L-glutamate into low density water so that its concentration remained low enough to preclude its

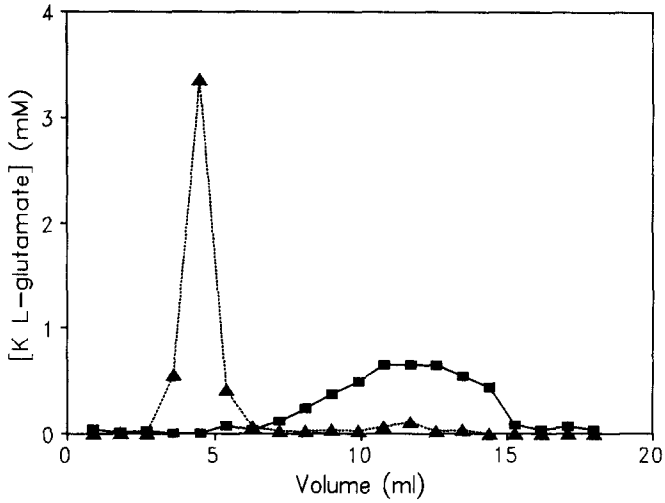

Fig. 12. Protection of low density water in a Dowex AG 50W-X16 column by pre-treatment with 200 mol m^{-3} *n*-BuOH. ▲: elution of 1 ml of 20 mol nm^{-3} K^+-L-glutamate with water from a column pre-treated with water; ■: elution of 1 ml of 20 mol m^{-3} K^+-L-glutamate with 200 mol m^{-3} *n*-BuOH from the same column pre-treated with water and then with two bed volumes of 200 mol m^{-3} *n*-BuOH.

precipitous elution. This is a remarkable example of retention of a negatively charged solute by a negatively charged gel.

6.2.5. *Protection of Low Density Water by Betaine in Dowex*

Figure 13 illustrates protection of low density water by betaine, a plot which illustrates the non-linearity of micro-osmosis. This column was pre-treated and eluted with

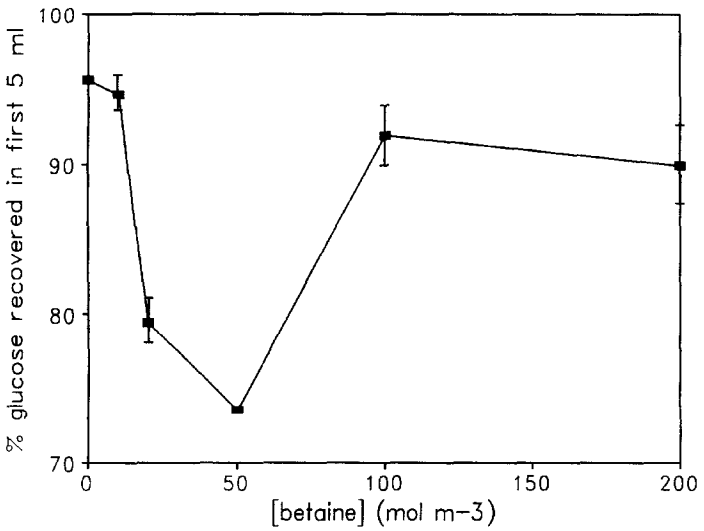

Fig. 13. Protection of low density water in a Dowex AG 50W-X16 column by pre-treatment and elution with betaine.

the appropriate concentration of betaine which was also contained in the sample of 10 mol m^{-3} glucose. Maximal protection of low density water was obtained at 50 mol m^{-3} betaine, thereafter it diminished. The non-linear response to betaine results from the three effects that betaine has on the populations of water in the gel compartment: (1) it draws water from the double layers into the regions between them, thus increasing the volume of LDW at the expense of HDW and increasing retention of glucose; (2) growth of low density water round the three methyl groups on betaine further increases the retention of glucose by LDW; (3) it decreases the osmolality gradient between the two regions, allowing both LDW and HDW to revert toward normal density and decreasing retention of glucose. This threefold consequence of the presence of a typical compatible solute in a polyelectrolyte gel anticipates its complex biological activity, which will be discussed in Section 8.1.

6.3. *Low and High Density Water in an Anion Exchange Resin*

Dowex AG 1-X8 resin, a strong anion-exchanger, has functional groups R–$CH_2N^+(CH_3)_3$ on a matrix of styrene divinyl benzene. The resin (10 g) in its Cl^- form was pre-treated with water for times up to 12 days; the same standard elution experiment was performed, using 1 ml of 10 mol m^{-3} ^{14}C-glucose, 10 mol m^{-3} BzOH. Figure 14 shows representative results. After pre-treatment for 1 h almost all of both solutes eluted with water. Thereafter, practically no BzOH and decreasing amounts of glucose eluted with water. Up to 2 days of pre-treatment with water, BzOH eluted with 100 mol m^{-3} KH_2PO_4 (or NH_4HCO_3 or NH_4HCO_3), but by the ninth and twelfth days, which showed similar patterns of elution (Fig. 15), not even 100 mol m^{-3} NH_4NO_3, which is avidly accumulated into low density water, released it: neither did 200 mol m^{-3} *n*-BuOH nor HCl (mol dm^{-3}) release either solute. Only when the gel was neutralised with KOH did all residual solutes elute.

The similarity between these oppositely charged gels is striking. Both retained more BzOH than glucose and both released it with KH_2PO_4. Both lost all selectivity when they were neutralised, showing that the presence of charges on the polymers was necessary for their selective properties but that the sign of that charge was not important. Just as *n*-BuOH protected low density water on a negatively charged column so that a negative ion was retained (Fig. 12), the same concentration of *n*-BuOH allowed retention of positively charged lysine by the positively charged column. Cl^--Dowex showed greater selectivity than K^+-Dowex, probably for the same reason offered for the different selectivities of H^+, Na^+ and K^+-Dowex cation exchanger. Both the functional groups on the gel matrix (R–$CH_2N^+(CH_3)_3$) and the counter-anions (Cl^-) are lightly hydrated so that the residual electrostatic attraction is very high, thinning the double layer and intensifying the selectivity of both LDW and HDW.

7. HOW MICRO-OSMOSIS HAS HITHERTO EVADED DETECTION

Experimentalists generally find only what they are looking for. Experimentalists with a bent for scientific survival do not look for a phenomenon unless there are

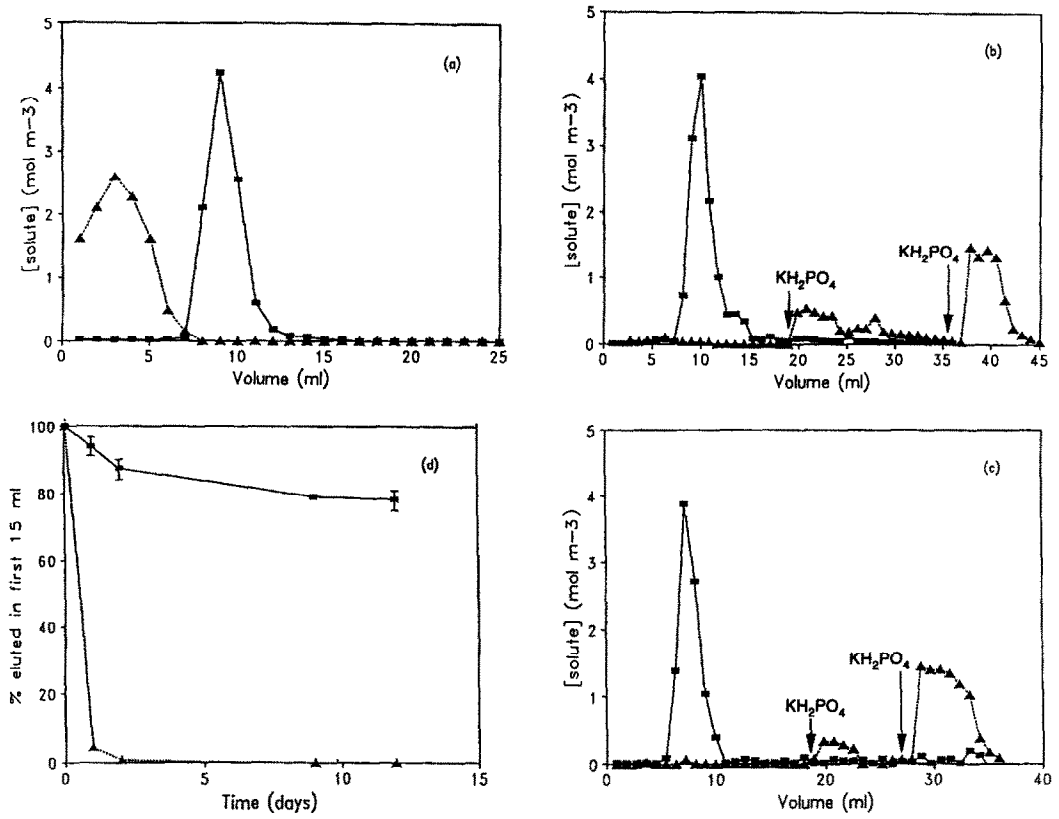

Fig. 14. Elution of 1 ml of 10 mol m^{-3} ^{14}C-D-glucose (■) and 10 mol m^{-3} BzOH (▲) with water from a Dowex AG 1-X8 column after various times of pre-treatment with water. (a) 1 h; (b) 1 day, some residual BzOH was eluted immediately with KH_2PO_4 and the rest following standing overnight; (c) 2 days, similar to 1 day; (d) the percentage of each solute eluted by water in the first 15 ml, as a function of time of pre-treatment of the column with water.

compelling reasons for suspecting that it might exist. Such motivation has been lacking because of our long-standing beliefs about equilibration of water: viz. that if water does not respond to osmotic stress by moving down its activity gradient, there must be a pressure (whether it is measurable or not) which relieves that stress and brings water to equilibrium. There has therefore been no incentive to examine the consequences of a change in density of water as a response to stress.

Development of both high density and low density water is so slow both at hydrophobic surfaces and at charged surfaces (Figs 7, 8, 11) that most experiments are too brief to detect them (e.g. Plesner and Malmgreen-Hansen[88] found no evidence for selectivity toward K^+ in cellulose acetate films). In the real world of the intracellular environment, however, biopolymers are made and spend their lifetimes in water, so that conditions for micro-osmosis are ever present.

Low density water is extremely labile. In particular it is often abolished rapidly by the very solutes which it selectively accumulates, solutes which are used as probes for its existence. Hence the irony that in a single measurement the experimenter often

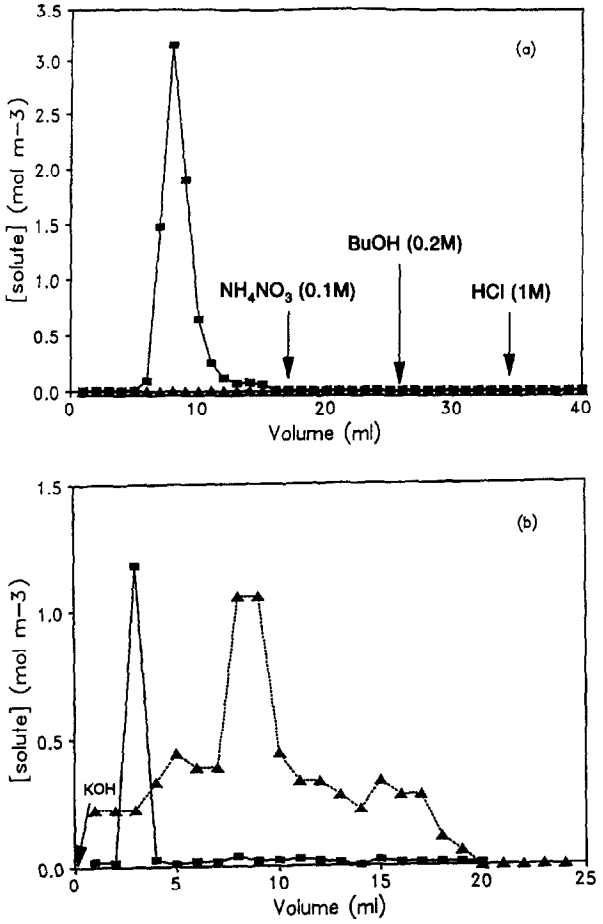

Fig. 15. (a) Elution of 1 ml of 10 mol m^{-3} ^{14}C-D-glucose (■) and 10 mol m^{-3} BzOH (▲) with water followed by NH_4NO_3, *n*-BuOH and HCl from a Dowex AG 1-X8 column after 9 or 12 days of pre-treatment of the column with water. (b) Elution of both solutes following neutralisation of the same column with KOH mol dm^{-3}.

cannot be sure whether low density water existed in a gel and the experimental probe demolished it or whether it was never there at all.

In gels and solutions of polyelectrolytes, extracellular and intracellular matrices, low density and high density water co-exist in a single compartment so that most measurements of a global water-related property give only an average value which, by itself, does not indicate whether or not it is an average of extremes. Raman[107,109] and infrared[110] spectroscopy, nuclear magnetic resonance,[111–113] electron spin resonance[114–117] and dielectric spectroscopy[118] are examples of techniques which yield an average quantity, the interpretation of which is extremely model dependent. This problem can only be resolved by doing additional experiments which probe low density and high density water selectively, taking into account the dynamics of the particular experimental model used.

Low density water in polyamide gels releases its accumulated solute in the presence of other more strongly accumulated solutes (chaotropic solutes). In highly charged gels it releases solutes in the presence of excluded solutes (salts of small cations and hydrophobic molecules).

In all its manifestations micro-osmosis is non-linear, extremely sensitive to starting conditions and sometimes chaotic.[119] Reproducibility of experimental results is not always straightforward.

8. MICRO-OSMOSIS IN CELLS AND ENZYMES

Motivation to look for water of changed solvent properties has, however, been strong in this laboratory for 25 years. It arose from a reading of the biological literature,[86] which seemed to require that the physical chemistry of aqueous solutions needed some major modification if it were to account for events in the aqueous compartments of cells. Although more and more sophisticated techniques for studying the properties of water have emerged in recent years, practical problems associated with investigating the properties of intracellular water in living cells have not eased. Accordingly, model systems which appear to mimic aspects of the intracellular environment, as described in this review, have been used. The difficulties encountered and the inordinate time that has elapsed in elucidating the properties of water in such apparently straightforward systems as charged and polyamide gels justify this approach. It still depends entirely, however, on the assumption that the gels used embody the essence of the intracellular environment.

8.1. *Micro-Osmosis in Cells and Tissues*

Both intracellular and extracellular matrices are highly charged polyelectrolyte gels carrying more negative than positive charges;[59] internally, K^+ and Cl^- are the principal counter-ions while externally Na^+ and Cl^- predominate. By analogy with Dowex W50-X16 and Dowex AG 1-X8 both matrices should contain regions of high density and low density water. This analogy may seem inappropriate because the negative charges in cells are carboxyl groups, while in Dowex W50-X16 they are sulphate groups. Since, however, the presence of high density and low density water has been shown to be independent of the sign of the fixed charges, it is unlikely to be critically dependent upon their chemical nature. In addition to counter-ions and low concentrations of metabolites, resting cells contain compatible solutes[83,84,120–127] such as betaine, TMAO, amino acids, sorbitol and taurine, which accumulate selectively into low density water, increasing its volume at the expense of high density water and further increasing its viscosity as more low density water forms round their hydrophobic moieties (see Figs 8 and 14). The particular counter-ions which are most effective in generating extreme forms of low density and high density water are already selected by the Na,K-ATPase,[101,128,103] a membrane-bound enzyme which uses metabolic energy to transport three Na^+ outward for two K^+ inward, generating an intracellular environment which is practically free of inorganic electrolyte other

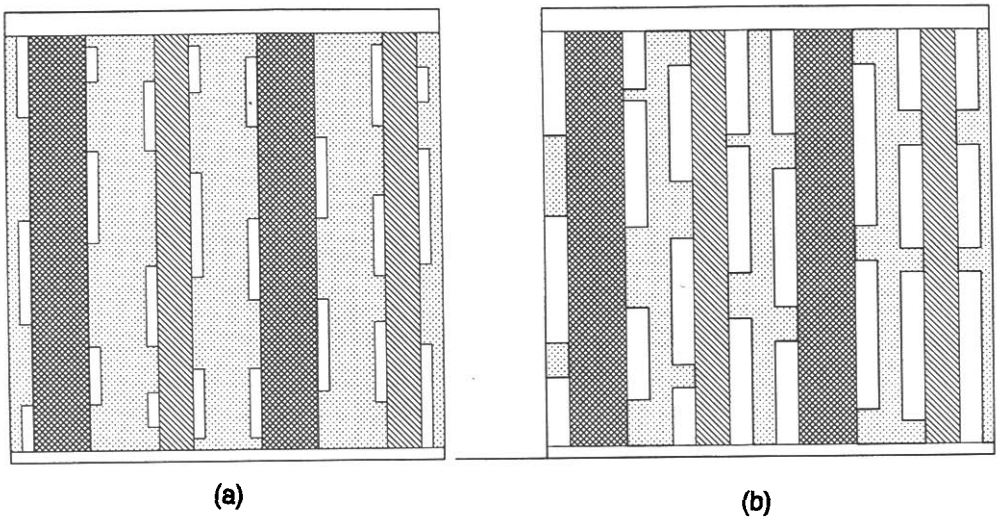

Fig. 16. Representation of the state of water in a muscle fibre: cross-hatching, thick filaments; diagonal hatching, thin filaments; clear, high density water; stippled, low density water. (a) The resting fibre. (b) The active fibre.

than the counter-ions which are predominantly K^+ and Cl^-, Ca^{2+} which is almost all sequestered in intracellular stores[104,129] and Mg^{2+} complexed to organic anions.

A resting cell has, in general, a low metabolic rate,[130] which is anticipated for the environment just described. It is activated by influx of ions, usually Na^+ or Ca^{2+}, through ion channels traversing proteins embedded in the plasma membrane. Electrophysiologists measure these ion fluxes as changes in voltage across the plasma membrane.[131] Although it has been well established that cell activation is triggered by influx of ions, the precise mechanism is unknown. It is proposed, here, that the ions accumulate selectively in high density water in double layers, increasing its fluidity and increasing its volume by drawing water from low density regions. As with dextran sulphate solutions the overall effect of ions is to decrease the viscosity of the cytoplasmic compartment. Diffusion of metabolites becomes faster, movement of polymers is easier, enzyme reactions, which all involve conformational changes, accelerate. Whatever the particular function of that cell may be, it is now active: for example muscle fibres are activated by an influx of Na^+ which, by generating high density water, allows release of Ca^{2+} from the sarcoplasmic reticulum, increasing still more the fluidity and volume of high density water. The actomyosin-ATPase is activated, forcing the thick and thin filaments to slide past each other in the low resistance medium. This is a very oversimplified account of the process, but is intended only to stress the roles of ions, compatible solutes and water. Figure 16 illustrates the two states of water in a resting and an active muscle fibre.

The muscle fibre must now revert to its resting inert state before the excess concentrations of electrolytes have irreversibly deleterious effects. This is perhaps the most important feature of micro-osmosis in muscle cells. When the environment is made sufficiently fluid for the filaments to slide, the many mechanisms which serve to return

the muscle fibre to its resting state are also activated. The Ca-ATPase returns Ca^{2+} to the sarcoplasmic reticulum,[104,105,129] the Na,K-ATPase transports excess Na^+ outward across the plasma membrane in return for K^+.[101,103,128] Water returns to the low density zone and cytoplasmic viscosity increases back to its resting level. Synchronisation of these several functions enables cells to live dangerously on the edge of chaos,[1] surrounded by ions which are destructive if their entry is uncontrolled, but which are essential for normal cellular performance.

8.1.1. Rigor Mortis *and Cell Death*

When a muscle fibre fails to recover from its activated state, it depletes its ATP levels, so that ions which have been kept out of equilibrium by ATP-dependent processes can move across the membrane or from intracellular stores toward their equilibrium distributions. There is massive release of proteins, sustained contracture, the muscle becomes hard and rigid and loses irreversibly its normal activity. This has been attributed to uncontrolled entry of Ca^{2+}.[132] Ca^{2+}, with its high charge density and (relative to Mg^{2+}) low hydration free energy is a preferred counter-ion to fixed charges on the Dowex W50-X16 resin, and presumably to carboxyls in the cytoplasm. With loss of intracellular K^+ and gain of Ca^{2+} the intracellular environment can be expected to change, as described for DNA double helices and collagen triple helices in Section 6.2.1. Ca^{2+} as counter-ion induces an extreme gradient in water activity between double layers and the rest of the cytoplasmic water. At 38 °C, the unfavourable term $T\Delta S$ is high so that a probable response of the cell is to squeeze out some of the high activity water. Because water plasticises the myofilaments, its loss must render the intracellular compartment, extremely viscous, hard and irreversibly inactive. This is how *rigor mortis* is described by muscle physiologists.

Cells also have the ability to programme their own death by apparently multiple mechanisms following influx and sustained raised intracellular concentrations of Ca^{2+}.[133–136] There is protein and RNA synthesis, breakdown of the nuclear envelope, DNA fragmentation, plasma membrane disruption and irreversible depletion of ATP. Whitfield[137] called calcium the killer in the programmed suicide mechanism of differentiated senescent cells or functionally superfluous cells. Damage to so many different aqueous polymeric structures suggests a common mechanism which might be replacement of Ca^{2+} for K^+ as counter-ion to DNA and cytoplasmic proteins, leading to the *rigor* state. Other functions such as protein and RNA synthesis could be activated in the high density water remaining. There is also the possibility that non-enzymic random DNA cleavage might take place in this extremely reactive form of high density water, with its many free OH groups and lone pairs of electrons, the reactive centres of water.[64]

8.1.2. *Measurements of Properties of Intracellular Water*

Most measurements of properties of water in cells have been made during the resting state, because the active state is too short-lived. These results confirm that, on average, intracellular water has lower density than normal water,[138–140] lower

rate of self-diffusion,[141–143] slower motional properties,[144,145] different solvent properties[84,146–148] and higher microscopic viscosity.[114] Some of the earliest nuclear magnetic resonance studies showing changed motional properties of water in muscle were those of Cope[149] and Hazelwood *et al.*[150] As predicted, all of these measurements reveal rather modest changes in the average properties of intracellular water, consistent with the co-existence of high and low density regions. The average, however, points to a preponderance of low density water.

Other experimenters have found changes in the average properties of intracellular water with fertilisation of sea urchin eggs,[151,152] with cell cycle phase[153,154] and under freezing and osmotic stress.[155] Hansson-Mild *et al.*[156] found high density water in amphibian eggs, and Hazelwood *et al.*[156] showed that the mobility of muscle water decreased in the maturing neonatal rat muscle and that at the same time the sodium content declined from very high to normal levels. These observations are not in conflict with one another: they serve to confirm that intracellular water assumes different states under different sets of conditions.

8.2. *Micro-Osmosis in Ion Channels, Receptors and Enzymes*

Active sites of enzymes, ligand binding sites of receptors and entrances of ion channels are all housed in very small, rather hydrophobic clefts in which water probably resembles that in polyamide beads; i.e. it is of lower than normal density, higher viscosity and has selective solvent properties. Mechanisms of the processes which occur in these small cavities must involve water in ways which are not applicable to similar processes in normal aqueous solution.[157]

8.2.1. *Ion Channels*

The patch clamp technique is used to characterise ion channels through proteins in plasma membranes.[158] Although many channels open only in response to specific hormones there are also both anion and cation channels which open and close spontaneously. A channel is often visualised as having a rather wide funnel-shaped water-filled entrance compartment and a narrow region which determines its selectivity. It is highly probable that these small hydrophobic cavities normally contain viscous, low density water which prevents entry of Ca^{2+} and Na^{+} and greatly slows down entry of K^{+} and anions. This, then, might be the closed state of channels, whatever their selectivity. Such a channel opens when spontaneous concentrative influx of HCO_3^-, $H_2PO_4^-$ or Cl^-, together with K^{+}, precipitates reversion of low density water to its normal viscosity and solvent properties, allowing free access to all ions. Its open state lasts only until low density water reforms. The kinetics of a channel of this kind depend upon how much water, rate-limited by the dynamics of the protein embedded in the membrane, enters during its opening phase. Open and closed times could be as short as microseconds or longer than milliseconds.

8.2.2. *Voltage-Gated Channels*

Some channels open when the resting membrane potential, which is negative inside

Table 3. Numbers of groups strongly attracted into low density water on some common neurotransmitters

Neurotransmitters	*MW*	$-CO_2^-$	$-NR_3^+$	Other
ξ-amino-butyric acid	103.12	1	1	K^+, Cl^-
Glycine	75.07	1	1	—
Acetylcholine chloride	146.28	—	1	Cl^-
K-glutamate	186.13	2	1	K^+
Noradrenaline	169.2	—	1	Cl^-
Serotonin	176.21	—	2	$2Cl^-$
Adrenaline	183.2	—	1	Cl^-
Dopamine	153.18	—	1	Cl^-
Adenosine triphosphate	507.21	—	5	$5Cl^-$, $-HPO_4^{3-}$
Adenosine	267.24	—	5	$5Cl^-$
Histamine	111.15	—	3	$3Cl^-$
Taurine	125.14	—	1	$-SO_3^-$

the cell is lowered. A spontaneously opening channel employing the anion-driven opening mechanisms discussed above opens more frequently when the electric field opposing anion movement is lowered. This would appear as voltage-gating.

8.2.3. *Neurotransmitters and Hormones*

Neurotransmitters are small molecules that are released at synapses between nerve cells, bind to receptors on the recipient cell and open a specific ion channel so that information in the form of an ion flux passes from one nerve cell to another. Tables 3 and 4 list some common neurotransmitters and neuroactive peptides together with the numerous $-NH_3^+$ and $-CO_2^-$ groups and counter-ions which attract them selectively into low density water. It is known that these molecules interact specifically with receptors, because they can be blocked by antagonistic molecules. They must do more, therefore, than merely enter the cavity, restore low density water to its normal

Table 4. Numbers of groups strongly attracted into low density water in some neuroactive peptides. The final column is the percentage of the total number of amino acid residues which carry either $-CO_2^-$ or $-NR_3^+$

Peptide	$-CO_2^-$	$-NR_3^+$	Percentage
Cholecystokinin 8	3	1	50
Cholecystokinin 4	2	1	75
β-endorphin	3	9	38
Leu-enkephalin	1	1	40
Met-enkephalin	1	1	40
Angiotensin	2	3	63
Somatostatin 14	1	4	36
Substance P	3	3	55
Substance K	2	2	40
Neuromedin K	3	2	50
Bradykinin	1	3	44
Bombesin	3	3	43
Vasopressin	1	2	33
Oxytocin	1	1	22

solvent properties so that ions can enter the cavity and pass through an open channel into the neighbouring cell. The following sequential steps are suggested for their action:

(1) they enter the low density water, drawn in by the groups which as single ions would partition preferentially into it;
(2) they bind to specific binding sites in the cavities;
(3) counter-ions brought in with them lower the water activity, causing it to revert toward its normal solvent properties;
(4) ions enter the cavity and those selected by the specific filter pass through the channel into the cell;
(5) the transmitter molecule dissociates from its binding site and diffuses out because the water which selected it no longer exists and part of its binding energy was the favourable free energy of hydration of the NH_4^+ and COO^- groups and their counter-ions;
(6) water in the cavity resumes its low density state.[100]

Hormones circulate in the bloodstream and bind to their specific receptors from extremely low concentrations. Many of them carry $-COO^-$ and $-SO_4^-$ groups which would allow them to elicit a biological response by means of a flux of ions in a manner similar to that described for neurotransmitters.

These are typical self-limiting micro-osmotic processes; the initiating molecule enters the cavity, changes the properties of water which drew it in, allowing a flux of ions which elicits the biological response just before the initiating molecule dissociates and diffuses out. The timing is crucial.

8.2.4. *Enzymes that do Work*

Enzymes that do work have been discussed elsewhere in some detail but, at the time, there was no clear evidence that concentrative uptake of a selected solute resulted in reversion of low density water to normal density, structure and solvent properties. That evidence now exists, making the previous explanations more plausible.[59,99] There is considerable independent evidence for the direct involvement of water in the mechanisms of these enzymes, much of it from the laboratory of de Meis and concerning the Ca^{2+}-transporting ATPase.[158–164] Oplatka and co-workers have also suggested that water is a "key player" in muscle contraction.[165–167]

9. CONCLUSIONS

If, during the time-course of an experiment, water can move to abolish a gradient in its chemical potential, it does so. When it is prevented from moving by an opposing pressure, an electric field or the inability of a polymer to move during that time to accommodate water movement, it assumes a metastable state: exchangeable populations of water molecules of different chemical potential adjust, locally, the equilibrium between low density and high density water configurations, compensating for the difference in chemical potential and achieving local equilibrium. Over a longer period

during which water can move, it passes through a series of such metastable states to a final state of lower free energy.

The transient metastable states can be long-lived if the polymer/water system is in a state of extremely low viscosity. Even when they are short-lived they can be detected by suitably designed experiments and can be harnessed to separate small solutes.

Low density water exists at weakly hydrogen-bonding or hydrophobic surfaces and is a prerequisite for adsorption of hydrophobic molecules. Low and high density water have been found to co-exist in polyelectrolyte solutions or gels, justifying suspension of the belief that the osmotic pressure of a solution increases the chemical potential of its water to equal that of the pure solvent. Changes in density are still apparently necessary. Moreover, since these accommodating changes in density appear to be common, at least in polymer/water systems, it is likely that there are other examples which have not yet been identified. For example, the metastable states of water in polymers described by Slade and Levine[46–49,87] and Franks,[42–45] may well be states of water of changed density; i.e. water, unable to move to equilibrate with the ambient water vapour pressure because of the dynamics of the polymer, may raise or lower its chemical potential by displacement of the LDW/HDW equilibrium. If the density is lowered it increases the already high viscosity of the polymer/water system; if the density is raised it accounts for the remarkable residual mobility of some water which does not freeze.

Finally, these collective movements of water molecules offer a different insight into the hydrophobic interaction, the attractive hydration force,[79,80] and mechanisms of high and low temperature denaturation of proteins.

ACKNOWLEDGEMENTS

This work was funded by a grant from the Health Research Council of New Zealand.

REFERENCES

1. S. A. Kaufmann, *The Origins of Order*, 1st edn, Oxford University Press, Oxford (1993).
2. L. J. Henderson, *The Fitness of the Environment*, The Macmillan Company, Boston, MA (1913).
3. R. N. Robertson, *The Lively Membranes*, Cambridge University Press, Cambridge (1983).
4. J. L. Finney, *Water a Comprehensive Treatise* (F. Franks Ed.), Vol. 6, pp. 47–122, Plenum, New York (1979).
5. G. D'Arrigo, G. Maisano, F. Maliamace, P. Migliardo and F. Wanderlingh, *J. Chem. Phys.* **75,** 4264–4270 (1981).
6. C. A. Angell, *Water A Comprehensive Treatise* (F. Franks Ed.), Vol. 7, pp. 1–81, Plenum Press, New York (1982).
7. C. A. Angell, *Nature* **331,** 206–207 (1988).
8. L. Green, D. J. Durben, G. H. Wolf and C. A. Angell, *Science* **249,** 649–652 (1990).
9. J. Teixeira, C. Alba-Simionesco and C. A. Angell, *Prog. Colloid Polym. Sci.* **84,** 117–121 (1991).
10. Y. Xie, K. F. Ludwig, G. Morales, D. E. Hare and C. M. Sorensen, *Phys. Rev. Lett.* **71,** 2050–2053 (1993).
11. D. E. Hare and C. M. Sorensen, *J. Chem. Phys.* **93,** 6954–6961 (1990).
12. D. E. Hare and C. M. Sorensen, *Chem. Phys. Letts.* **190,** 605–608 (1992).
13. S. J. Henderson and R. J. Speedy, *J. Phys. E. Sci. Instrum.* **13,** 778–782 (1980).
14. R. J. Speedy, *J. Phys. Chem.* **86,** 982–991 (1982).

15. Q. Zheng, D. J. Durben, G. H. Wolf and C. A. Angell, *Science* **254,** 829–832 (1991).
16. R. Lumry, E. Battistel and C. Jolicoeur, *Faraday Symp. Chem. Soc.* **17,** 93–108 (1982).
17. W. C. Röntgen, *Ann. Phys. Chem.* **45,** 91–97 (1892).
18. F. Sciortino, A. Geiger and H. E. Stanley, *J. Chem. Phys.* **96,** 3857–3865 (1992).
19. F. H. Stillinger, *Science* **209,** 451–457 (1980).
20. H. S. Frank, *Fed. Proc.* **24,** S1–S11 (1965).
21. J. D. Bernal and R. H. Fowler, *J. Chem. Phys.* **1,** 515–548 (1933).
22. A. Rahman and F. H. Stillinger, *J. Chem. Phys.* **33,** 3336–3359 (1971).
23. S. Swaminathan, S. W. Harrison and D. L. Beveridge, *J. Am. Chem. Soc.* **100,** 5705–5712 (1978).
24. A. T. Hagler and J. Moult, *Nature* **272,** 222–226 (1978).
25. J. C. Owicki and H. A. Sheraga, *J. Am. Chem. Soc.* **99,** 7403–7412 (1977).
26. A. Geiger and H. E. Stanley, *Phys. Rev. Lett.* **49,** 1749–1752 (1982).
27. F. H. Stillinger and T. A. Weber, *Science* **225,** 983–989 (1984).
28. F. Sciortino, P. H. Poole, H. E. Stanley, and S. Havlin, *Phys. Rev. Lett.* **64,** 1686–1689 (1990).
29. M. Vedamuthu, S. Singh and G. W. Robinson, *J. Phys. Chem.* **98,** 2222–2230 (1994).
30. P. Postorino, R. H. Tromp, M. -A. Ricci, A. K. Soper and G. W. Neilson, *Nature* **366,** 668–670 (1993).
31. P. G. Kusalik and I. M. Svishchev, *Science* **265,** 1219–1221 (1994).
32. R. L. Blumberg, H. E. Stanley, A. Geiger and P. Mausbach, *J. Chem. Phys.* **80,** 5230–5241 (1984).
33. F. Sciortino, A. Geiger and H. E. Stanley, *Nature* **354,** 218–221 (1991).
34. G. W. Robinson, , S. Singh and S. -B. Zhu, *Modern Nonlinear Optics, Part 3* (M. Evans and S. Kielich Eds.), pp. 627–729, John Wiley (1993).
35. L. Bosio, S. -H. Chen, and J. Teixeira, *Phys. Rev.* **A27,** 1468–1475 (1983).
36. F. O. Libnau, J. Toft, A. A. Christy and O. M. Kvalheim, *J. Am. Chem. Soc.* **116,** 8311–8316 (1994).
37. S. W. Benson, and E. D. Siebert, *J. Am. Chem. Soc.* **114,** 4269–4276 (1992).
38. J. Li and D. K. Ross, *Nature* **365,** 327–329 (1993).
39. C. -H. Chen, *J. Phys. Chem.* **98,** 7906–7914 (1994).
40. V. Buch, *J. Chem. Phys.* **93,** 2631–2639 (1990).
41. O. Mishima, L. D. Calvert and E. Whalley, *Nature* **314,** 76–78 (1985).
42. F. Franks, *Water*, revised 1st edn, The Royal Society of Chemistry, London (1984).
43. F. Franks, *Biophysics and Biochemistry at Low Temperatures*, Cambridge University Press, Cambridge (1985).
44. F. Franks, *Adv. Exp. Med. Biol.* **302,** 1–19 (1991).
45. F. Franks, *Dev. Biol. Stand.* **74,** 9–18 (1992).
46. L. Slade and H. Levine, *Crit. Rev. Food Sci. Nutr.* **30,** 115–360 (1991).
47. L. Slade and H. Levine, *Adv. Exp. Med. Biol.* **302,** 29–101 (1991).
48. L. Slade and H. Levine, *Carbohydr. Polym.* **21,** 105–131 (1993).
49. L. Slade and H. Levine, *Advances in Food and Nutrition Research* (J. E. Kinsella Ed.), Vol. 38, Academic Press, San Diego, CA (1994).
50. P. Barnes, J. L. Finney, J. D. Nicholas and J. E. Quinn, *Nature* **282,** 459–464 (1979).
51. R. M. Pashley, P. M. McGuiggan and D. F. Evans, *Science* **229,** 1088–1089 (1985).
52. A. Ben-Naim, *Hydrophobic Interactions*, Plenum, New York (1980).
53. E. Eisenberg and A. D. McLachlan, *Nature* **319,** 199–203 (1986).
54. K. P. Murphy, P. L. Privalov and S. J. Gill, *Science* **247,** 559–561 (1990).
55. K. A. Sharp, A. Nicholls, R. Friedman and B. Honig, *Biochemistry* **30,** 9686–9697 (1993).
56. C. J. Cramer and D. G. Truhlar, *Science* **256,** 213–216 (1992).
57. R. N. Pribble and T. S. Zwier, *Science* **265,** 7579 (1994).
58. P. M. Wiggins and R. T. van Ryn, *Biophys. J.* **58,** 585–596 (1990).
59. P. M. Wiggins, *Microbiol. Rev.* **54,** 432–449 (1990).
60. P. M. Wiggins, *Trends Electrochem.* **3,** 129–140 (1994).
61. P. M. Wiggins, *N.Z. Sci. Rev.* **51,** 79–84 (1994).
62. P. M. Wiggins, *Langmuir*, **11,** 1984–1986 (1995).
63. P. M. Wiggins, R. T. van Ryn, and G. M. C. Ormrod, *Biophys. J.* **60,** 8–14 (1991).
64. M. C. R. Symons, *Acc. Chem. Res.* **14,** 179–187 (1981).
65. W. Drost-Hansen, *The Biophysics of Water* (F. Franks and S. Mathias Eds.), pp. 163–169, John Wiley, New York (1982).

66. J. S. Clegg, and W. Drost-Hansen, *Biochemistry and Molecular Biology of Fishes* (P. W. Hochachka and T. P. Mommsen Eds.), Vol. 1, pp. 1–23, Elsevier Science, Oxford (1991).
67. F. Helfferich, *Ion Exchange*, McGraw-Hill, New York (1962).
68. J. H. van't Hoff, *Z. fur Chemie.* **1,** 481–508 (1887).
69. D. Eisenberg and W. Kauzmann. *The Structure and Properties of Water*, The Clarendon Press, Oxford (1969).
70. J. Zimmerberg and V. A. Parsegian, *Nature* **323,** 36–39 (1986).
71. R. P. Rand and V. A. Parsegian, *Biochim. Biophys. Acta* **988,** 351–376 (1989).
72. R. Podgornik, D. C. Rau and V. A. Parsegian, *Macromolecules* **22,** 1780–1786 (1989).
73. J. Zimmerberg, F. Bezanilla and V. A. Parsegian, *Biophys. J.* **57,** 1049–1064 (1990).
74. R. P. Rand, N. Fuller, V. A. Parsegian and D. C. Rau, *Biochemistry* **27,** 7711–7722 (1988).
75. R. P. Rand, N. L. Fuller, P. Butko, G. Francis and P. Nicolls, *Biochemistry* **32,** 5925–5929 (1993).
76. D. C. Rau and V. A. Parsegian, *Science* **249,** 1278–1281 (1990).
77. V. A. Parsegian, R. P. Rand and N. L. Fuller, *J. Phys. Chem.* **95,** 4777–4782 (1991).
78. M. F. Colombo, D. C. Rau and V. A. Parsegian, *Science* **256,** 655–659 (1992).
79. D. C. Rau and V. A. Parsegian, *Biophys. J.* **61,** 260–271 (1992).
80. S. Leikin, Rau, and V. A. Parsegian, *Proc. Natl Acad. Sci.* **91,** 276–280 (1994).
81. M. F. Colombo, D. C. Rau and V. A. Parsegian, *Proc. Natl Acad. Sci.* **91,** 10517–10520 (1994).
82. P. M. Wiggins and R. T. van Ryn, *J. Macromol. Sci. Chem.* **A23,** 875–902 (1986).
83. P. H. Yancey, M. E. Clark, S. C. Hand, R. D. Bowlus and G. N. Somero, *Science* **217,** 1214–1222 (1982).
84. M. E. Clark, *Curr. Top, Membr. Transp.* **30,** 251–271 (1987).
85. H. L. Friedman and C. V. Krishnan, *Water a Comprehensive Treatise* (F. Franks Ed.), Vol. 3, pp. 1–118, Plenum Press, New York (1973).
86. P. M. Wiggins, *Prog. Polym. Sci.* **13,** 1–35 (1988).
87. H. Levine and L. Slade, *Water Relationships in Food*, Plenum Press, New York (1991).
88. I. Plesner and B. Malmgren-Hansen, *Chem. Soc. Faraday Trans.* **90,** 2381–2393 (1994).
89. A. R. Khare and N. A. Peppas, *Polymer* **34,** 4736–4739 (1994).
90. B. Z. Ginzburg and D. Cohen, *Trans. Faraday Soc.* **60,** 185–189 (1963).
91. G. N. Ling, *Scanning Microsc.* **2,** 871–884 (1988).
92. G. N. Ling, *Physiol. Chem. Phys. Med. NMR* **20,** 281–292 (1988).
93. G. N. Ling and W. Hu, *Physiol. Chem. Phys. Med. NMR* **20,** 293–307 (1988).
94. G. N. Ling and M. M. Ochsenfeld, *Physiol. Chem. Phys. Med. NMR* **20,** 309–317 (1988).
95. G. N. Ling and M. M. Ochsenfeld, *Chem. Phys. Med. NMR* **21,** 19–44 (1989).
96. G. N. Ling, *A Physical Theory of the Living State*, Blaisdell Publishing Company, New York (1962).
97. A. S. Troshin, *Problems of Cell Permeability*, Pergamon Press, London (1966).
98. L. Fischer, *Gel Filtration Chromatography*, 2nd edn, Elsevier/North Holland Biomedical Press, Amsterdam (1980).
99. P. M. Wiggins and B. E. A. MacClement, *Int. Rev. Cytol.* **108,** 249–303 (1987).
100. A. Renwick and P. M. Wiggins, *FEBS Lett.* **297,** 1–3 (1992).
101. A. Schwartz, G. E. Lindenmayer and J. C. Allen, *Pharm. Rev.* **27,** 1–134 (1975).
102. P. A. Doris, *Proc. Soc. Exp. Biol. Med.* **205,** 202–212 (1991).
103. J. C. Skou and M. Esmann, *J. Bioenerg. Biomembr.* **24,** 249–261 (1992).
104. M. Tada, T. Yamamoto and Y. Tonomura, *Physiol. Rev.* **58,** 1–79 (1978).
105. A. K. Grover and I. Khan, *Cell Calcium* **13,** 9–17 (1992).
106. C. Y. Kwan, *Can. J. Physiol. Pharmacol.* **70,** 501–508 (1992).
107. D. C. W. Siew, R. P. Cooney, M. J. Taylor and P. M. Wiggins, *J. Raman Spectrosc.* **25,** 727–733 (1994).
108. M. J. Bedzyk, G. M. Bommarito, M. Caffrey and T. L. Penner, *Science* **248,** 52–56 (1990).
109. D. C. W. Siew, G. M. Clover, R. P. Cooney and P. M. Wiggins, *J. Raman Spectrosc.* **26,** 3–8 (1995).
110. K. Bartel, W. Mantele, F. Siebert and W. Kreutz, *Biochim. Biophys. Acta* **248,** 52–56 (1985).
111. D. E. Callahan, S. F. Deamond, D. C. Creasy, T. L. Trapane, S. A. Bruce, P. O. Ts'o and L. S. Kan, *Magn. Reson. Med.* **22,** 68–80 (1991).
112. D. N. Wheatley, A. Redfern and R. P. Johnson, *Physiol. Chem. Phys. Med. NMR* **23,** 199–216 (1991).

113. T. Iwama, H. Yamada, S. Era, M. Sogami, T. Andoh, N. Sakai, K. Kato, K. Kuwata and H. Watari, *Magn. Reson. Med.* **24,** 63–63 (1992).
114. A. M. Mastro and A. D. Keith, *J. Cell. Biol.* **99,** 180s–187s (1984).
115. P. M. Wiggins and G. A. Bowmaker, *Bioelectrochem. Bioenerg.* **17,** 473–487 (1987).
116. P. M. Wiggins, R. T. van Ryn and G. A. Bowmaker, *Bioelectrochem. Bioenerg.* **17,** 457–471 (1987).
117. S. I. Nikonova, A. M. Nikonova, T. E. Shumilova and IuA. Kisliakov, *Biofizika* **36,** 511–515 (1991).
118. U. Kaatze, *Phys. Med. Biol.* **35,** 1663–1681 (1990).
119. J. Gleick, *Chaos Making a New Science*, Viking Penguin, New York (1987).
120. A. Garcia-Perez and M. Burg, *Hypertension* **16,** 595–602 (1990).
121. M. C. Lai, K. R. Sowers, D. E. Robertson, M. F. Roberts and R. P. Gunsalus, *J. Bacteriol.* **173,** 5352–5358 (1991).
122. E. A. Galinski and A. Oren, *Eur. J. Biochem.* **198,** 593–598 (1991).
123. C. L. Winzor, D. J. Winzor, L. G. Paleg, G. P. Jones and B. P. Naidu, *Arch. Biochem. Biophys.* **296,** 102–107 (1992).
124. D. E. Robertson, D. Noll and M. F. Roberts, *J. Biol. Chem.* **267,** 14893–14901 (1992).
125. M. C. Lai and R. P. Gunsalus, *J. Bacteriol.* **174,** 7474–7477 (1992).
126. J. A. Pocard, L. T. Smith, G. M. Smith and D. Le-Rudulier, *J. Bacteriol.* **176,** 6877–6884 (1995).
127. A. Garcia-Perez and M. Burg, *J. Membr. Biol.* **119,** 1–13 (1991).
128. P. M. Wiggins, *J. Theor. Biol* **99,** 665–679 (1982).
129. P. M. Wiggins, *J. Theor. Biol* **99,** 645–664 (1982).
130. H. Blum, J. A. Balaschi and R. G. Johnson, *J. Biol. Chem* **266,** 10254–10259 (1991).
131. W. Hoppe, W. Lohmann, H. Markl and H. Ziegler, *Biophysics*, 2nd edn, Springer, Berlin (1983).
132. P. M. Grinwald and W. G. Nayler, *J. Mol. Cell. Cardiol.* **13,** 867–880 (1981).
133. L. Santos-Argumedo, C. Teixeira, G. Preece, P. A. Kirkham and R. M. Parkhouse. *J. Immunol.* **151,** 3119–3130 (1993).
134. M. Brini, M. Murgia, L. Pasti, D. Picard, T. Pozzan, *EMBO. J.* **12,** 4813–4819 (1993).
135. B. Chen, D. G. Cyr and B. F. Hales, *Teratology* **50,** 1–12 (1994).
136. M. F. Flajnik, *Bioessays* **16,** 671–675 (1995).
137. J. F. Whitfield, *Crit. Rev. Oncol.* **3,** 55–90 (1992).
138. J. S. Clegg, *Cell* **6,** 153–169 (1984).
139. J. S. Clegg, *J. Cell. Biol.* **99,** 167s–171s (1984).
140. J. S. Clegg, *J. Physiol.* **246,** R133–R151 (1984).
141. P. Seitz, D. C. Chang, C. F. Hazelwood, H. E. Rorschach and J. S. Clegg, *Arch. Biochem. Biophys.* **210,** 517–524 (1981).
142. H. E. Rorschach, C. Lin and C. F. Hazelwood, *Scanning Microsc.* **5,** S1–9 (1991).
143. E. C. Trantham, H. E. Rorschach, J. S. Clegg, C. F. Hazelwood, R. M. Nicklow. *Biophys. J.* **45,** 927–938 (1984).
144. I. L. Cameron, P. Merta and G. D. Fullerton, *J. Cell. Physiol.* **142,** 592–602 (1990).
145. I. L. Cameron, L. A. Cox and G. D. Fullerton, *J. Cell. Physiol.* **149,** 365–374 (1991).
146. , T. J. Century, I. R. Fenichel and S. B. Horowitz, *J. Cell. Sci.* **7,** 5–13 (1970).
147. S. B. Horowitz and P. L. Paine, *Biophys. J.* **25,** 45–62 (1979).
148. K. D. Garlid, *Colloq. Int. Cent. Natl Rech. Sci.* **246,** 317–321 (1975).
149. F. W. Cope, *Biophys. J.* **9,** 303–319 (1969).
150. C. F. Hazelwood, B. I. Nichols and B. F. Chamberlain, *Nature* **222,** 747–750 (1969).
151. P. J. Merta, G. D. Fullerton and I. L. Cameron, *J. Cell. Physiol.* **127,** 439–447 (1986).
152. I. L. Cameron, W. E. Hardman, G. D. Fullerton, M. Kellermayer, A. Ludany and A. Miseta, *J. Cell. Physiol.* **69,** 742–746 (1991).
153. C. A. Belfi, S. V. Medendorp and F. Q. Ngo, *Magn. Reson. Med.* **22,** 379–393 (1991).
154. A. M. Dupré and H. G. Hempling, *J. Cell. Physiol.* **97,** 381–396 (1978).
155. P. T. Beall, *Biophysics of Water* (F. Franks and F. S. Mathias Eds.), pp. 323–326, John Wiley, Chichester (1982).
156. C.F. Hazelwood, B. L. Nichols, D. C. Chang and B. Brown, *Hopkins Med. J.* **128,** 118–131 (1971).
157. K. Hansson-Mild, S. Lovtrop and E. Forslind, *J. Exp. Biol.* **83,** 305–314 (1979).
158. J. V.Wu, C. T. Rubinstein and P. Shrager, *J. Neurosci.* **13,** 5153–5163 (1993).
159. L. de Meis, *Biochim. Biophys. Acta* **973,** 333–349 (1989).
160. L. de Meis and V. A. Suzano, *FEBS Lett.* **232,** 73–77 (1988).

161. L. de Meis, O. B. Martins and E. W. Alves, *Biochemistry* **19,** 4252–4261 (1980).
162. L. de Meis, M. S. Otero, O. B. Martins, E. W. Alves, G. Inesi and R. Nakamoto. *J. Biol. Chem.* **257,** 4993–4998 (1982).
163. L. de Meis, M. I. Behrens, J. H. Petreski and M. J. Polito, *Biochemistry* **24,** 7783–7789 (1985).
164. R. V. Nayudu and L. de Meis, *FEBS Lett.* **255,** 163–166 (1989).
165. A. Oplatka, H. Gadasi, R. Tirosh, Y. Lamed, A. Muhirad and N. Liron, *J. Mechanochem. Cell Motility* **2,** 295–306 (1974).
166. A. Oplatka, *FEBS Lett.* **355,** 1–3 (1994).
167. R. Tirosh, W. Z. Low and A. Oplatka, *Biochim. Biophys. Acta* **1037,** 274–280 (1990).