Role of Water in Some Biological Processes

PHILIPPA M. WIGGINS
Department of Medicine, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand

INTRODUCTION

The concept of a cell as a membranous bag of solution was first seriously challenged by Russian scientists, particularly by Troschin (118), whose monograph, Problems of Cell Permeability, was published in Russian in 1956, in German in 1958, and in Chinese in 1961. The English version was not published until 1966. Troschin’s thesis was that partitioning of solutes between the cell and extracellular solution was not determined solely by the permeability of the membrane, but that protoplasm itself preferentially accumulated some solutes and excluded others. Central to this idea was the concept that water in the protoplasmic gel was different from water in a simple aqueous solution.

When Ling (80) published his monograph, A Physical Theory of the Living State, in 1962, it was well received by Eastern scientists, who were familiar with Troschin’s rather similar ideas, but was received with considerable scepticism by most Western scientists, who had not yet heard of Troschin’s work. Ling proposed that most water inside a cell was polarized in multilayers on protein surfaces and was an extremely poor solvent for ions. K⁺ was accumulated by normally metabolizing cells because under those conditions the carboxyl groups of proteins preferentially associated with K⁺ ions rather than Na⁺. This theory, the association-induction hypothesis, had great explanatory power but ran counter to conventional wisdom because one of its main conclusions was that membrane-bound cation pumps did not exist and that distributions of all solutes were determined by a combination of selective association with proteins and modified solvent properties of intracellular water. It was in this climate of scepticism that the first nuclear magnetic resonance (NMR) measurements of water protons in tissues were made (19, 48). At first these measurements seemed to provide dramatic support for Ling’s hypothesis, because they appeared to indicate that the mobility of water inside cells was drastically reduced. This initial excitement, however, did not last and was not generally shared; it soon became clear that other interpretations of the NMR findings were possible. For example, the simplest explanation was one in which relatively few molecules, which were quite strongly immobilized, exchanged rapidly with normal water molecules; this is known as the two-fraction, fast-exchange model.

This illustrates a fundamental problem of using NMR of water protons to determine the properties of water in complex systems. Measurements are not hard to make, but their interpretation depends upon the model chosen to describe the state of water. Although the description of intracellular water in terms of a few bound molecules exchanging rapidly with normal liquid water may not correspond to reality, it cannot be dismissed without independent evidence that it is incorrect. NMR alone cannot provide that evidence.

Reception of the concept of modified intracellular water was not helped by the polywater incident. Anisimova et al. (1) described columns of water grown from the vapor phase in quartz capillaries; they called these columns, which had very strange properties, ortho water or anomalous water. Several western scientists took up this work with great enthusiasm, while the western press carried grave warnings about the dangers of converting the oceans to anomalous water, which Lippincott et al. renamed polywater (81, 82). Within a few years it became clear that the columns were not made of polymeric water, but were gels containing a great many impurities leached from the quartz capillaries in which
they had formed (8, 37, 81, 82, 104). This embarrassing episode inhibited serious consideration of concepts of structurally modified water and heightened scepticism about some interpretations of the NMR data on the properties of water inside cells.

There can be little doubt now that ATP-driven cation pumps do exist and that they are responsible for most of the asymmetric distributions of solutes across biological membranes (9, 32, 35, 47, 55, 57, 72, 76, 77, 102, 119, 120). This fact, however, does not itself preclude the possibility that intracellular water is different from normal water; the subject is still open and has some persuasive advocates, as well as many persuasive opponents. The strongest argument against the existence of unusual water inside cells has come from physical chemists, who have said that biological water cannot be different from normal water, so that any change in its structure or properties proposed for intracellular water must also be shown to apply to water outside cells. This is an unassailable argument, but it requires that we understand the particular constraints upon water structure that are experienced inside normally metabolizing cells. One obvious difference between cells and any nonbiological aqueous system is the very existence of ATP-driven cation pumps and other transport devices, which make osmotic equilibration of cells different from osmotic equilibration of any other real system, if only because most solutes are held out of equilibrium, but water must equilibrate.

In the past, those who have speculated that water inside cells might differ significantly from normal bulk-phase water have confined their attention almost exclusively to direct interactions between surfaces and solutes, on the one hand, and water molecules on the other (14–19, 28, 48, 80, 88, 92). Thus, water which is relatively immobilized on an ionic surface has been called bound water, while water which is far enough away from a surface that it is not expected to be under its influence is bulk water. This kind of approach is not capable of explaining much of the apparently anomalous solution chemistry of small ions and molecules in solutions of macromolecules, in gels, and inside cells. For example, Horowitz and co-workers (36, 57, 58) showed that the solvent properties of water in the cytoplasm of frog oocytes are different both from those of water in the nucleus and from those of normal water; Trantham et al. (117) measured a significantly lower self-diffusion coefficient of water in Artemia cysts than in bulk solution or in an agarose gel; Clegg (16) found that water in Artemia cysts was less dense than normal water at the same temperature; Timasheff et al. (116) showed that certain solutes are excluded from solvent adjacent to protein surfaces; Clark (13) found anomalous behavior of trimethylamine oxide in skinned muscle fibers; and Garlid (39) found that mitochondria at 0 to 4°C contained both normal water and water in which, for instance, urea had a partition coefficient of 1.5. These findings are not explicable in terms of bound and free water molecules, because they require that appreciable zones of water in the same solution of proteins or in the actomyosin lattice or inside cells or mitochondria have different solvent properties, different densities, and different mobilities. Unless those zones extended a significant distance from a surface, changes in their properties would not be detected experimentally. Is-raelachvili et al. (60, 62, 64) and Pashley et al. (95) have measured a long-range effect on water structure at hydrophobic surfaces. Wiggins and van Ryn (124) showed that this water did indeed have profoundly modified solvent properties over distances of 2 to 3 nm.

In contrast to this long-range effect, interactions between water molecules and ionic groups have usually been assumed to be short range, so that, as with small ions in solution, a few water molecules are relatively immobilized by a fixed-charge group on a surface, giving a sheath one molecule thick, and normal water structure resumes after a single layer of disordered molecules. Figure 1 illustrates in two dimensions this kind of hydration of charged groups. For simplicity, only two negatively charged groups on a surface are shown. Round each charge are four water molecules, most of which are oriented so that a hydrogen atom is pointing directly toward the negative charge. Water molecules of this restricted orientation cannot fit into the normal liquid water structure, so that outside these four molecules is a layer of randomly oriented molecules, merging immediately with the normal bulk liquid hydrogen-bonded structure. In addition (but not shown in Fig. 1), there are positively charged counter ions, with their oriented sheaths of water molecules, and a disordered zone one molecule thick, merging with normal water structure. The volume of water thus affected must be too small to have measurable effects on partitions of even the smallest solutes. If direct water-surface interactions are indeed short range, as seems probable, it becomes necessary to think of more subtle, indirect effects of surfaces on water structure. Pashley and co-workers (12, 61, 63, 92, 93, 94) and Parsegian and co-workers (79, 91) have measured a longer-range repulsive

FIG. 1. Two-dimensional arrangement of water molecules round two negatively charged sites on a surface. Four molecules are shown in contact with each charge, the majority of them with a positive hydrogen atom directed toward the negative charge. Outside those four molecules is a layer of randomly oriented molecules.
force which prevents two charged surfaces from coming together. They propose that this hydration force results from the energy required to dehydrate counter ions as the charged surfaces squeeze water out. There are highly specific effects of ions associated with this repulsive force, and there have been suggestions that there are indeed rather long-range perturbations of water (79, 91). This force, however, comes into operation only when two surfaces are very close together, and it may not be relevant to the properties of water and the state of ions at a single surface.

**INDIRECT EFFECTS OF CHARGED SURFACES ON WATER STRUCTURE**

Figure 2 illustrates the simplest case of an indirect effect of a charged surface upon properties of water both near to and far from the surface. This represents a solution of a negatively charged polyelectrolyte, such as DNA, RNA, hyaluronic acid, chondroitin sulfate, or any other of the legion of soluble charged biopolymers. This polyelectrolyte is in its sodium form: each fixed charge on a molecule is balanced by an Na⁺ ion. Some cations are directly associated with their corresponding negative charges, but others are free in solution (Fig. 2a). As the large ions diffuse through solution, they carry their Na⁺ ions with them. Now if one could sample a region of solution immediately adjacent to the surface of a polyanion, remove it, and measure its freezing-point depression or its vapor pressure in an osmometer, the osmometer would register a very high osmolality. If, on the other hand, one could sample a region of solution in between molecules, such as the one with a circle round it in the figure, and measure its osmolality in the microscopic osmometer, the osmolality would be that of essentially pure water, i.e., zero. In other words, water adjacent to the polyelectrolyte molecules contains a high concentration of solutes, while water in a contiguous region, has no solutes. How can these two regions of water equilibrate? The usual treatment of osmotic equilibrium tells us that if water in one compartment has a lower concentration of solutes than water in an adjacent compartment, then, in the absence of a pressure difference between the compartments, water should flow into the more concentrated solution until the two osmolalities are equal. This makes the activity of water (which is its concentration multiplied by an activity coefficient) the same in the two compartments. There is no pressure opposing such a flow in a true solution, so perhaps water does do that, and the true picture of the solution of polynions and counter ions in aqueous solution is that shown in Fig. 2b. In this case, all samples of solution have the same osmolality and water is in equilibrium. This, however, is an impossible solution to the problem. We have now generated negatively charged molecules swimming in a sea of positive charge. Such gross separation of charge in a highly conducting solution like this one would require amounts of energy found only in high-energy physics laboratories. Figure 2c offers an alternative possibility. Here, all the Na⁺ ions are associated with fixed charges, and none are left in free solution. Again, water is in equilibrium throughout the solution. Na⁺, however, tends to dissociate from anions in solutions: Na₂SO₄, sodium acetate, and both sodium phosphates are completely dissociated in aqueous solution, so that if the fixed charges on the polyan are sulfates, carboxyl groups, or phosphate groups, Na⁺ ions are unlikely to be completely associated. Imposition of such a high degree of order on the ions is also very costly. Clearly it is not legitimate to consider the requirements of water in isolation; the whole system (polynions, counter ions, and water) must come to its state of lowest free energy, which is neither that illustrated in Fig. 2b nor that illustrated in Fig. 2c. It is a compromise, similar to that shown in the original diagram (Fig. 2a), in which some Na⁺ ions are complexed and others are free, so that neither gross separation of charge nor impossibly rigid order is imposed. Using X-ray standing waves, Bedzyk et al. (4) have recently measured the thickness of the diffuse layer of ions adjacent to phospholipid head groups and found it to be 5.8 nm at pH 6.8. This result shows that some counter ions are indeed in solution and that the zone of water of low activity extends an appreciable distance from the charged surface. It is then in that compromise arrangement that water must equilibrate. The condition for equilibrium distribution of water is that its chemical potential must be the same throughout. The chemical potential of water depends upon its activity and molar volume (the volume occupied by one mol of water) and on pressure and temperature. When the activity of water in each region is fixed and pressure and temperature are the same throughout, water has only one degree of freedom left: it can change its molar volume. The foregoing can be
expressed in the following way. Water in a region away from the surface of the polyanion is in a state of higher chemical potential than water at the surface, because it contains fewer solutes. Therefore, it decreases its chemical potential by doing work of expansion against the prevailing pressure. As the same time, because the state of lowest free energy of the whole system requires adjustments from all components, water adjacent to the polyanion must increase its chemical potential; it does this by allowing the prevailing pressure to compress it. Thus, water equilibrates by increasing in density where the concentration of solutes is high and decreasing in density where the concentration of solutes is low.

The indirect force which is operating on water in this simplest of systems must be regarded as a force which operates on a whole assembly of water molecules but is not directly experienced by individual molecules. Collectively, water molecules move apart from one another to decrease their local free energy, or collectively they move together to increase their local free energy. In this way water can equilibrate between compartments of different osmolality, and in so doing it generates contiguous regions of solution of different densities.

This phenomenon, which is probably extremely common, especially in biological systems, has not previously been appreciated. This is hardly surprising, because direct measurement of any property of water in the relatively simple solution illustrated in Fig. 2a gives an average value. For example, measurement of the density of water in such a solution would yield a value not significantly different from that of normal water and would not reveal the fact that it represented the average value of dense water surrounding the polyanions and low-density water between the polyanions. To probe these different populations of molecules separately, it is necessary to turn to the gel state of matter.

THE GEL STATE OF MATTER

Most polyelectrolytes, at high enough concentrations, form gels. The gel state of matter has remarkable properties. For example, an agarose gel or a gel of hyaluronic acid may contain 99.9% (by weight) water. That water can escape from the gel in response to applied pressure or increased temperature or just by evaporation. It is in the liquid state, and, on a molecular scale, most of it is far away from any of the gel material which contains it. Interstices in the matrix of the polymeric material are far too big to restrict the movement of water. Why does it stay there? Why does it not flow out from between the macromolecules and assume its free liquid state, which is its normal state at room temperature and pressure? Retention of water by such a gel is one example of a natural tendency for components of a system to mix as thoroughly as they can. In a simple solution, solvent and solute molecules mix thoroughly. In Fig. 2, mixing of polyanions, counter ions, and water was almost complete, with the single constraint that counter ions had to stay near the polyanion. In a gel there is the same tendency for components to mix, but because there is some tangling or cross-linking of macromolecules, mixing can take place only by movement of water into the gel interstices. Therefore, the dry gel imbibes water until mixing is optimal under the constraints of the system. Liquid water is restrained by the small amount of gel material because of this powerful force of mixing, which, again, is a force operating on the whole assembly of molecules rather than on individual molecules. To separate water from the gel, work must be done against this force.

Figure 3 represents a cavity inside a charged gel equilibrated with a dilute solution of electrolyte. The arrangement of charges and counter ions is similar to those in Fig. 2a. Charges are fixed to the surfaces, and counter ions are restrained to occupy regions of solution near the surfaces. They cannot stray far without separating charge unacceptably, and they cannot all associate with the negative charges without imposing too rigid an order on the system. Again, therefore, there are regions of solution in this gel-external solution system with different concentrations of solutes and therefore different activities of water. Water adjacent to the surfaces has a low activity; water in the middle of the cavity and external water have higher activity. Even in the presence of additional ions, this inequality of water activity in different regions persists. This leads to the expectation that, as in Fig. 2a, these different regions of water equilibrate by changing their local density. If that is true, water adjacent to the surfaces has higher than normal density, while water in the center of the cavity and in the external solution has lower than normal density. Partial separation of the different water populations between gel and external solution makes experiment easier.

This, it must be pointed out, is not the usual treatment of charged gels, which are believed to be examples of the operation of the Gibbs-Donnan membrane equilibrium (103).

Gibbs-Donnan Membrane Equilibrium

Figure 4 illustrates the usual representation of the Gibbs-Donnan membrane equilibrium. It consists of two compartments separated by a membrane which is permeable to small ions and water but not to the large polyanion \( \text{P}^{\text{n}}^- \), which is therefore confined to compartment 1. Donnan and Harris (27) showed theoretically that if activity coefficients of ions are the same in both compartments, the concentration of small diffusible ions is always higher in the compartment containing \( \text{P}^{\text{n}}^- \). Therefore, they said, water must flow from compartment 2 into compartment 1 down its concentration gradient. This flow can be prevented by application of a suitable pressure to compartment 1, so that water has the same chemical potential throughout. There is, however, a fallacy in this argument, because the true picture of compartment 1 is that of Fig. 2a, with the addition of relatively sparse ions occupying the spaces between the polyanions. If a pressure is applied to the solution of Fig. 2a, it prevents
influx of water across a permeable membrane, but, because that pressure is experienced equally by the water of low activity surrounding the polyions and the water of higher activity between the polyions, it can do nothing to help equilibration of these two populations of water molecules. Water is still not in equilibrium throughout the entire system, and local changes in density are still mandatory.

The fallacy inherent in the Gibbs-Donnan membrane equilibrium also applies to the usual treatment of distributions of ions and water in gels. This proposal dates back to Procter and Wilson (100) in 1916 and has not been questioned since (49). Gels, they said, act as their own membrane by confining the fixed charges to one compartment. It follows that a gel must exert a pressure on the internal solution, otherwise the gel would imbibe more and more water until it dispersed. This treatment, however, ignores the fact that there are three populations of water molecules in the gel-external-solution system. If pressure is exerted by the gel matrix on the solution inside the cavity, so that the region of water immediately adjacent to the surface is in equilibrium with the external water, water in the center of the cavity, which experiences the same pressure, has a lower chemical potential than external water. Pressure changes alone cannot solve this problem; densities must also change. It is doubtful, in fact, whether any pressure differences exist at all. In Fig. 2a, which illustrates an essentially similar state of affairs, there was no pressure difference; pressure differences have been invoked because Donnan and Harris (27) had shown that pressure prevented net flow of water into compartment 1 in Fig. 4. As was pointed out above, however, the remarkable thing about gels is that they imbibe so much water, not that they finally reach an endpoint in their inhibition. It seems more probable, therefore, that charged gels imbibe water until mixing is optimal and that ions and water then equilibrate under those constraints. Water comes to equilibrium by collective movements to adjust its local densities; equilibrium distributions of ions and other solutes will be discussed below.

**Density of Water in Gels**

Using density bottles, Wiggins and Van Ryn measured the average density of solutions of polyethylene glycol 20 M (PEG 20M) when they had been equilibrated with Bio-Gel P-100 (R. M. Wiggins and R. T. van Ryn, Biophys J., in press). This polymer, of average molecular weight 20,000 and effective hydrodynamic diameter 7.6 nm, is too big to enter the pores of the gel, which, therefore, imbibes pure water from the external solution. At equilibrium, water inside the gel contains no solutes under these conditions, while external water contains a higher concentration of PEG 20M than the original solution did. Water equilibrates by collective movement of molecules apart from one another in the interstices of the gel and by collective movement of molecules together in the external solution. Wiggins and Van Ryn found that the average density of the PEG solution decreased with increasing weight of gel, suggesting that water inside the gel had a lower density than normal. Estimates of the density of internal water showed that it was lower than the normal density at the prevailing temperature and that it decreased with increasing concentration of PEG in the external solution. Values as low as 0.96 were obtained. The same value was obtained by Garrigos et al. (40) for water "bound" inside the rather large hydrophobic hole of the S1 myosin monomer in aqueous solution. They pointed out that this was equivalent to a mixture of 53% ice and 47% water. The magnitude of the force which induces water to expand to this degree is remarkable. Negative pressures of the order of 1,000 atm (1.013 × 10^5 kPa) would be required to achieve a similar degree of expansion (30).

These experiments showed that waters of different densities can be detected in gels and that it is in the region of lower osmolality that its density decreases. If water merely changed its density, this phenomenon would be of little interest, but there are considerable changes secondary to changes in density which make this an extremely important phenomenon in all rather condensed aqueous systems containing charged gels or polyelectrolytes. Included in this category are all cells and their organelles, cell walls, the interstitium, and connective tissue.

**THE UNIQUENESS OF WATER**

Water is singular as a liquid because of its ability to form three-dimensional networks of molecules, mutually hydrogen bonded (5, 7, 30, 34, 41, 43, 115, 124). This results from the fact that each water molecule has four fractional charges directed in three-dimensional space toward the corners of a regular tetrahedron. Two of these are positive (the hydrogen atoms), and two are negative (the lone pairs of electrons on the oxygen atom). The consequence of this distribution of positive and negative poles is that each water molecule can make up to four weak bonds (hydrogen bonds) with neighboring water molecules, a positive pole of one molecule being attracted to a negative pole of another. Figure 5 shows a two-dimensional projection of the crystal structure of ice. Here, each water molecule is hydrogen bonded to four others and all the hydrogen bonds are linear; i.e., each hydrogen atom lies on a straight line between two O atoms and is covalently bonded to one and hydrogen bonded to the other. These straight bonds are strong. Ice is characterized by its extremely open structure (its density at 0°C is 0.92). When ice melts, some hydrogen bonds are broken and molecules infiltrate the empty regions to form the more compact (density, ca. 1.0) liquid water. For the purpose of this discussion, the most important property of liquid water is that its molecules are now too close together to make many of the strong, straight, hydrogen bonds that characterized ice. Few hydrogen atoms lie on straight lines between oxygen atoms, and the bent bonds which do form are relatively weak. Therefore, although hydrogen bonding is still continuous throughout the liquid (7), the weakness of the bonds allows the structure to be disrupted by thermal energy extremely rapidly: water is a rather fluid liquid.

It should be clear now that the changes in density of water that have been measured in gels and proposed for polyelectrolyte solutions are accompanied by changes in the strength.
of water-water hydrogen bonding. This is the source of the secondary effects of changed density that have great functional significance. Figure 6 illustrates changes in water structure that might be expected to result from changes in its density. Dense water (Fig. 6a) is characterized by extremely bent and weak hydrogen bonds; there are many unbonded molecules. This water should be extremely reactive, because it is rich in the lone pairs of electrons and free OH groups which are the reactive centers of water molecules (115). Its viscosity should also be low. Low-density water (Fig. 6b), on the other hand, has many icelike straight hydrogen bonds; it should be inert because of the paucity of reactive centers, and its viscosity should be higher. One can imagine a continuous spectrum of water structures between these two extremes.

FIG. 5. Two-dimensional projection of the crystal structure of ice.

FIG. 6. Hypothetical two-dimensional projections of the structure of liquid water in which molecules have collectively moved together (a) and of liquid water in which molecules have collectively moved apart (b).

Water as a Solvent

Biologically, the most important secondary effects of increases or decreases in the density of water are changes in its ability to dissolve solutes. Water is normally a good solvent for charged and polar solutes and a relatively poor solvent for hydrocarbons. Henderson (50) discussed the evolutionary significance of this particular fitness of water to support life.

Water and Ions

Water dissolves ions readily, because the polar molecules orient themselves round ions (as illustrated in Fig. 1) and, in so doing, partially neutralize charges so that positive and negative ions can exist as separate entities in a dilute solution without forming ion pairs. To acquire their stabilizing sheath of water molecules, ions must compete with water molecules, which need to make as many hydrogen bonds with one another as possible. Clearly it is easier for ions to be hydrated by dense, weakly bonded, reactive water than by low-density, strongly bonded, inert water, and the more water molecules a particular ion needs to stabilize it in aqueous solution, the more it will prefer dense water to open water. On the other hand, the broken region of water molecules, surrounding the layer immediately adjacent to the ion (Fig. 1), favors the solution of ions in highly bonded water, because the relative disorder generated in this zone is greater when the bulk of the water is highly ordered (Fig. 6b) than when the bulk of the water is already disordered (Fig. 6a). This factor becomes dominant as the size of the ion, and therefore the size of the disordered water zone, increases. These opposing factors result in very selective partitioning of ions between two aqueous phases of different density (119, 120, 124; Wiggins and van Ryn, in press). Figure 7 summarizes results of experiments on the distributions of ions between two aqueous phases of different densities in gels (124; Wiggins and van Ryn, in press). Small, highly hydrated cations tend to accumulate in the more...
Increasing density of water

\[ \text{Mg}^{2+} > \text{Ca}^{2+} > \text{H}^+ >> \text{Na}^+ \]

\[ \text{K}^+ < \text{Rb}^+ < \text{Cs}^+ < \text{NH}_4^+ \]

\[ \text{ATP}^3 >> \text{ATP}^2 = \text{ADP}^2 = \text{HPO}_4^{2-} \]

\[ \text{H}_2\text{PO}_4^- < \text{Cl}^- < \text{Br}^- < I^- \]

FIG. 7. Rank orders of partitioning of ions between two aqueous compartments containing water of different densities.

dense phase, while larger, singly charged cations prefer the less dense phase. Na\(^+\) and K\(^+\), which behave very similarly in normal aqueous solutions, prefer to partition in opposite cation gradients discussed in the Hofmeister series (56).

Such sorting of ions between regions of different water densities is reminiscent of the more familiar sorting of ions across biological membranes. There is no doubt that membranes have active-transport devices, such as ATP-driven cation pumps, which contribute to the extreme concentration gradients of ions across membranes, but, to some degree, this mechanism of selection of ions by populations of water molecules of differing structure might also contribute. As discussed in the section on bioenergetics (below), mechanisms of coupling of ATP hydrolysis to cation transport, and of dissipation of cation gradients to synthesis of ATP, can be explained with great economy in terms of transient changes in water structure inside enzyme-binding cavities (123).

Water and Hydrocarbons: Hydrophobic Effect

Water molecules are in a state of high energy if they fail to make the maximal number of hydrogen bonds possible either with one another or with solutes or surfaces (34). Wiggins and van Ryn (124; in press) pointed out that water molecules adjacent to a hydrocarbon surface or to a hydrocarbon moiety of a solute are in a state of higher energy than molecules farther away from the surface because they are unable to make hydrogen bonds on that side. Since hydrogen bonding is cooperative, weak hydrogen bonding is transmitted through several layers of water molecules, generating the rather long-range effect described by Israelachvili et al. (60, 62, 64). This is similar, in some respects, to the effects of polyelectrolytes on water, both close to the surface of the polyelectrolyte and farther away. Water adjacent to the polyelectrolyte is in a state of low free energy because of the high concentration of solutes; therefore, it increases in density and increases its chemical potential. Water at a hydrophobic surface is in a state of high energy because it makes fewer and weaker hydrogen bonds than does water in the bulk of the solution. Once more there are two populations of water molecules, this time of different energy, which can equilibrate at constant temperature and pressure only by changing their density. Thus, collectively, molecules adjacent to the hydrophobic surface move apart and decrease their chemical potential, while molecules in the bulk of the solution move together and increase their chemical potential. Again, it must be pointed out that this is not a generally accepted mechanism for the hydrophobic effect, an extensively investigated phenomenon which still lacks a molecular explanation (5).

The most interesting feature of the hydrophobic effect is that nonpolar molecules in aqueous solution tend to aggregate, squeezing out water. This is the reason for self-assembly of lipid bilayers, without which cells could not function as they do. Again, this process can be regarded as a mechanism for the solution of apolar molecules to achieve its state of lowest free energy; each component of the system has to contribute to this final state in which water must have the same chemical potential throughout, although different assemblies of water molecules have different energies. Figure 8 illustrates three possible configurations of an aqueous solution of apolar molecules. In Fig. 8a each molecule is surrounded by a zone of water molecules, which are in a state of higher energy than the rest of the water in the system, unless they expand and decrease their local density relative to populations of molecules between the apolar solutes. Energetically, this is a rather costly configuration, because although expansion of the zone of water adjacent to the solute molecules decreases the energy of water molecules, it increases the order of the water lattice unacceptably (Fig. 6) (90). In Fig. 8b the apolar molecules have formed dimers, decreasing the total amount of ordered water. This
saving, however, is offset to some degree by the order induced by pairing of the apolar solutes, which were more randomly distributed in Fig. 8a. Figure 8c illustrates an intermediate configuration, which is a compromise. The solutes are aggregating, but they retain some water between them. This reduces the cost involved in squeezing all the water out in Fig. 8b and yet decreases the cost involved in expansion of as much water as was necessary in Fig. 8a. The configuration assumed by a real solution of apolar molecules will be that which results in its lowest total free energy; it cannot be predicted from these qualitative descriptions. Figure 8, however, does offer an explanation for the aggregation of apolar solutes in watery systems.

SUMMARY OF POSSIBLE INDIRECT EFFECTS OF BIOPOLYMERS ON WATER

Biopolymers characteristically present to water both charged and hydrophobic surfaces. Globular proteins in solution, for example, bury much of their hydrophobic surface area internally, but expose approximately half of it externally to water (34). Nearly all charged and hydrophilic groups, which endow the large molecules with solubility in water, are on the outer surface. Inevitably, therefore, those patchy surfaces generate patchy interfacial water structures (121): reactive zones of dense water grow round the charged groups with their relatively high concentration of counter ions, while inert zones of low-density water grow round the hydrophobic regions. Timasheff et al. (116) measured the distribution of solutes in protein solutions by equilibrium dialysis. They found that sucrose, glucose, glyceral, and structure-stabilizing amino acids were preferentially excluded from the solvent adjacent to the protein surface owing to perturbation of solvent structure by contact with the protein surface. These findings suggest that, consistent with theoretical predictions and experiments on gels, appreciable zones of water had modified solvent properties.

When a dilute protein solution achieves its state of lowest free energy, equilibration of water between the different surface zones and the bulk water far from the surfaces involves significant changes in density of the surface water zones only, because changes in the density of the very much larger volume of water between protein molecules would be energetically unfavorable (Wiggins and van Ryn, in press). When, however, the solution concentrates and finally forms a gel, with like most cells a water content of perhaps 2 to 4 g/g (dry weight), the surface zones of water become comparable in volume to water between proteins, and all water probably participates in the equilibration process by changing its density. Whether the average density of water inside a cell is greater or lower than normal then depends upon the balance of osmotic and hydrophobic constraints operating in different regions of the cell. Clegg (16) found, using a variety of techniques, that the average density of water in Artemia cysts was 0.97, whereas Hansson-Mild et al. (45), using an isotopic exchange method, found that the density of water in amphibian eggs was significantly higher than the normal density of water. Although this difference might be due to technique, it is also likely that it is due to difference in cell type, macromolecular composition, and mechanisms of volume regulation, which will be discussed in the next section.

REGULATION OF CELLULAR VOLUME AND PROPERTIES OF INTRACELLULAR WATER

The classical treatment of regulation of cellular volume of mammalian cells under normal metabolic conditions was reviewed in 1987 (83). Because water can cross plasma membranes relatively rapidly, it is reasonably assumed that it must be in equilibrium between intracellular and extracellular compartments. Since, moreover, plasma membranes of animal cells are probably too fragile to support a pressure gradient, it is also assumed that pressures are the same in the intracellular and extracellular compartments. The conclusion drawn from these assumptions is that the activity of water must be the same in the intracellular and extracellular solutions; in other words, that the (experimentally immeasurable) osmolality of the intracellular solution must be equal to the (measurable) osmolality of the extracellular solution. When the two assumptions and the conclusion drawn from them are put into the form of the classical van't Hoff equation, the following relationship between cellular volume and extracellular osmolality is obtained: $V - V_b = k/\text{OsM}_w$, where $V$ is the total volume of the cell, $V_b$ is the volume of the cell which is osmotically inactive, $k$ is a constant, and OsM$_w$ is the osmolality of the extracellular solution. Thus, $V - V_b$ is the volume of cellular water which responds classically to changes in extracellular osmolality (26). When mammalian cells are exposed to hyper- or hypo-osmotic solutions, they immediately lose or gain water, respectively, but the extent of this loss or gain is frequently much smaller than that described by the above equation; i.e., $V_b$ appears to be greater than the volume of cellular dry matter (a difficult quantity to measure) and apparently includes some of the cellular water. This water has been described as osmotically inactive water, bound water, or compartmentalized water. It often varies with metabolic conditions. Dupre and Hempling (29), for example, showed that the absolute and relative amounts of apparently osmotically inactive water varied widely over the cell cycle of Ehrlich acites tumor cells. They pointed out that there was no satisfactory way of calculating concentrations of intracellular ions in terms of these changing amounts of water in which they were presumably dissolved. Similarly, Hinke (53) found that 25% of water in the muscle fiber of the giant barnacle was not acting as a solvent for electrolytes. Even erythrocytes appear to have some water which is not osmotically active (101). Interpreting this as an abnormal osmotic coefficient of hemoglobin (127) does not add to our understanding at a molecular level, but does suggest that it is not a property of cells specifically but is common to concentrated solutions of hemoglobin inside and outside cells.

Osmotically inactive water need not be invoked to interpret any of these experiments, if the assumption that intracellular osmolality equals extracellular osmolality is dropped. This has not been regarded as an assumption, because systems in which water can equilibrate at constant temperature and pressure with different activities had not been described. The results shown in Fig. 2, however, and the experiments on gels (124; Wiggins and van Ryn, in press) have shown that activities of exchangeable populations of water molecules can indeed differ at constant temperature and pressure, provided that densities also differ. It is possible to retain the underlying assumptions that water equilibrates across plasma membranes and that lipid bilayers cannot withstand a difference in hydrostatic pressure, without making the additional assumption that activities must be equal. In the discussion of polyelectrolyte gels, it was pointed out that water could not equilibrate by application of pressure alone: local changes in density were also necessary. Therefore, even if there are pressure differences generated by connective-tissue elements such as collagen fibers (97), by the cytoskeleton (65, 85), or by the rigid cell wall of plant
or bacterial cells (26), it is inevitable that populations of water molecules with different activities and densities and therefore different solvent properties, viscosities, and reactivities must coexist in the cytoplasm and organelles of all cells.

**Secondary Changes in Volume Following Osmotic Shock**

Cells which, physiologically, are exposed to media of changing osmolality, have a rich diversity of mechanisms for returning their volumes toward normal, following the initial rapid loss or gain of water (54, 96). For example, a monolayer of confluent kidney cells in culture appeared to activate selective ion and amino acid transport pathways (105); mouse hepatocytes apparently regulate their volumes by adjusting K⁺ conductance (61); a perfused proximal nephron restored its volume toward normal, following hyposmotic perfusion, by loss of K⁺ salt plus water (71); turtle colon (42) increased its basolateral K⁺ conductance; and interleukin-2-stimulated T lymphocytes (78) modified both K⁺ and Cl⁻ conductances. It is noteworthy, and will be discussed below, that many cells select K⁺ as the cation which they gain or lose in order to regulate their volumes. Yancey (128) showed that urea, methyamines, and polyols were the principal osmolytes which adjusted to regulate volume in the kidneys of laboratory mammals.

Some reviews (112, 129) have highlighted the extraordinary convergent evolution of intracellular organic solutes that are synthesized or taken up to balance extracellular hyperosmolality. Polyhydric alcohols, free amino acids, and combinations of urea and methylamines are the three commonest types of the so-called compatible solutes.

Even bacteria, which normally occupy a volume defined by the rigid cell wall, respond to hyperosmotic solutions, first by expressing genes for K⁺ transport and second by expressing genes for transport of betaine and similar compatible solutes. Expression of genes for K⁺ uptake results directly from an increase in extracellular osmotic pressure, but expression of the betaine transport genes is apparently induced by the increase in ionic strength following the primary uptake of K⁺ (52). The presence of glycine betaine, proline betaine, and sorbitol in human urine have been shown to protect *Escherichia coli* against hypertonic NaCl (10, 11).

Cells would not go to such lengths to avoid permanent swings in water content and intracellular ionic strength unless those conditions were deleterious to the maintenance of normal structure, function, and metabolic control. Somero (112) has shown that although many enzymes are inhibited by increasing concentrations of simple electrolytes, they continue to function normally in concentrations of compatible solutes up to 1 M. Clearly this is a good functional explanation for the use of compatible solutes as osmolytes, but its molecular mechanism has not been elucidated in detail.

**State of Water in a Hypothetical Mammalian Cell with Only Ions as Osmolytes**

Wilson (126) and Leaf (77) suggested, independently, that normally metabolizing mammalian cells maintain a constant steady-state volume because the rate at which Na⁺ ions leak into the cell is equal to the rate at which they are pumped out of the cell by the Na⁺,K⁺-ATPase. A cell and its extracellular solution, therefore, constitute a double-Donnan system, in which the excess osmolality of the intracellular solution, with its impermeant charged macromolecules and counter ions, is balanced in the external solution by the excess osmolality due to NaCl. The water content of a cell, according to this model, is determined by the osmolality of the extracellular solution and the activity of the Na⁺,K⁺-ATPase, together with variable leak pathways (54). This treatment implicitly assumes that the distribution of water is determined simply by the requirements of water alone. As was pointed out in the discussion of Fig. 3, however, the equilibrium distribution of water between a gel and the external solution is determined largely by the force of mixing of macromolecular elements and external solution, and ions and water must then equilibrate, if they can, under those constraints. Ions and other solutes which are actively transported cannot equilibrate across plasma membranes, but water and other readily permeant small solutes do; the constraints of mixing now include the asymmetric distributions of the actively transported ions. Since water can equilibrate with different activities in contiguous compartments, it is no longer necessary to assume that the Na⁺,K⁺-ATPase pumps the intracellular osmolality down to a level which is identical with that of the extracellular solution (approximately 300 mosM). It might be either lower or higher. Whatever its average value, however, it can be expected that it will vary from region to region within a cell. K⁺ ions, which, typically, have an apparent total intracellular concentration of approximately 150 mM, are present at much higher levels than Na⁺ (approximately 10 mM) and Cl⁻ (approximately 5 mM). In order that macroscopic electroneutrality may be maintained, the balancing charge must be located on intracellular proteins and other polyelectrolytes. It is therefore to be expected that the small diffusible ions are all relatively accumulated into regions of solution immediately adjacent to the fixed charged groups, so that if ions are the only intracellular osmolytes, osmolality in those regions is much higher than in the regions between polyelectrolytes (compare Fig. 2 and 3). It follows that the bulk of cytoplasmic water is sandwiched between the extracellular solution and the solution surrounding the intracellular polyelectrolytes, both of which have higher osmolality. In vivo, the extracellular compartment is itself a gel with its own tendency to imbibe water, so that all compartments are of comparable volume and must all participate in the changes in water density which are necessary for its equilibration. Water near the polyion surfaces and water in the extracellular gel must increase in density, whereas cytoplasmic water between polyion surfaces must decrease in density.

Wiggins and van Ryn (in press) performed an experiment to mimic cytoplasmic water by equilibrating charged gels with dilute electrolyte solutions containing an impermeant solute (PEG 20M). The impermeant solute (like the Na⁺,K⁺-ATPase on plasma membranes) kept the external solution at a higher osmolality than the internal solution. When the water content of the gel at equilibrium was in the range 2 to 4 g/g (dry weight) (similar to the range of water contents of cells), the time course of the experiments indicated that the microviscosity of some of the water was extremely high indeed (Wiggins and van Ryn, in press). The fact that no cells, except an ancient and primitive halobacterium, use electrolytes alone to balance the extracellular osmolality (129) suggests strongly that they need to avoid such extreme differences in contiguous populations of intracellular water molecules. Armitage et al. showed that although rabbit corneal epithelium could tolerate a high external osmolality of sucrose, gross damage occurred when the hyperosmotic agent was NaCl (2). As discussed in connection with Fig. 6,
such changes in water-water hydrogen bond strength are accompanied by changes in viscosity, reactivity, and solvent properties.

Compatible Solutes

Compatible solutes such as betaine, trimethylamine oxide, urea, and amino acids accumulate preferentially into the less dense of two contiguous aqueous regions. Wiggins and van Ryn (124) showed that urea, by selectively accumulating into low-density water in hydrophobic pores, made that water less K⁺ selective. In other words, accumulation of urea decreased the activity of the low-density water, which therefore had less need to expand in order to equilibrate with external water. Uptake of urea by cells, therefore, would partially balance the excess osmolality in regions of the cytoplasm adjacent to charged polyions, thus restoring both extremes of water structure toward normal. Urea by itself, however, is not selected as an osmolyte. This is probably because its partition coefficient is not very different from unity, so that unacceptably high concentrations are needed. Compatible solutes, on the other hand, also accumulate into the less dense of two populations of water molecules, presumably because of the hydration properties of the polar ends of the molecules. Their accumulation does not destroy the low-density water structures as does urea, K⁺, and NH₄⁺ accumulation, because compatible solutes carry with them a significant hydrophobic moiety, which, itself, generates regions of low-density water. Clark (13), for example, showed that the self-diffusion coefficient of water decreased in the presence of increasing concentrations of compatible solutes. She concluded that large numbers of water molecules, perhaps as many as 100, were moderately affected in their translational motion by each organic solute molecule Clark also found that skinned muscle fibers swelled in the presence of increasing concentrations of mixed salts, whereas, according to the classical Gibbs-Donnan membrane equilibrium theory, the fiber should lose water as ionic strength increases and the repulsive force between like charges decreases. Moreover, when trimethylamine oxide (TMAO) was added to a constant concentration of electrolytes, the fiber lost water as the concentration of TMAO increased. Both findings can be explained in terms of coexisting populations of water molecules with different solvent properties.

There are three populations of water molecules in skeletal muscle which must equilibrate by changing their densities and reactivities. (i) Water surrounding the highly charged regions of the filaments contains a high concentration of counter ions and must increase its density; this water becomes weakly bonded. (ii) Zones of water adjacent to the hydrophobic moieties of the filaments must decrease in density; this water becomes strongly bonded. (iii) Since the filaments are very highly charged and the distance between them not great, water between the filaments might participate in the equilibration process by decreasing its density; this water, too, becomes rather strongly bonded. As the concentration of ions increases, they accumulate more and more into the first, weakly bonded water region, which grows in size as, to some extent, water accompanies the extra ions: the myofilament lattice swells. TMAO, on the other hand, accumulates selectively into regions (ii) and (iii). This affects local water properties in two ways. First, the increase in osmolality of these regions offsets, to a degree, the excess osmolality of region (i), so that with increasing concentration of TMAO the properties of the three regions tend to converge. Water in region (i) increases in hydroton bond strength and decreases in volume. Second, the hydrophobic association of TMAO molecules with one another and with hydrophobic regions of the surfaces squeezes some high-energy water out between molecules (Fig. 8). Such association must be greater in water which is already more strongly hydrogen bonded than normal. Therefore, despite the increase in osmolality in regions of relatively low-density water, the lattice loses water from all regions of solution.

Cytoplasmic Organization and Water Structure

It is well established that the folded conformation of proteins in solution is determined largely by the properties of water (34). If cytoplasmic water exists in different hydrogen-bonded states in different regions of the cell, both protein conformations and aggregation-disaggregation states are likely to be affected. Hydrophobic solutes (other than compatible solutes) accumulate preferentially into weakly bonded water (124). It follows that the buried hydrophobic moieties of proteins can more readily emerge, and the protein unfold, when the surrounding water is dense and weakly bonded. Conversely, stabilization of the native folded conformation and aggregation of monomeric proteins by hydrophobic interactions are promoted by low-density, strongly bonded water.

There is much evidence that enzymes in the cytoplasmic compartments of cells are organized into structures, rather than freely diffusing in solution (38, 66). For example, Srere (113) reviewed evidence for complexes of sequential metabolic enzymes and for channeling of metabolites, so that the product of one reaction goes directly to the active site of the next enzyme without entering the aqueous phase of the cytoplasm. Clegg (14, 15), using the microtrabecular lattice model of Porter et al. (98, 99) for the cytoplasm, has suggested that enzymes are associated with an F-actin filament of the microtrabecular lattice and that there are very few enzymes in solution in the cytoplasmic water. He cites evidence from centrifugal stratification of unicellular eucaryotes, which produced, among others, a soluble phase which contained no macromolecules. When, on the other hand, cells were disrupted by incubation with dextran sulfate (74, 75) gave intermediate results (17). More recent experiments of Clegg and co-workers (17, 18, 65a) have shown that glycolysis in L-929 cells is little affected by reduction of their water content by 85%. Moreover, sonicated cells showed a strong dependence of glycolytic rate on cell density and a distinct lag before glycolysis became linear; intact cells, on the other hand showed no dependence on cell density, and the rates of glycolysis were linear. Cells made permeable by incubation with dextran sulfate gave intermediate results (7). These findings were interpreted as evidence that in intact cells enzymes of the glycolytic pathway are spatially organized and that their organization is destroyed by sonication. Readers are referred to the excellent review by Clegg (14) for detailed evidence supporting the model, which is illustrated in Fig. 9. The essential features are that enzymes are associated with an F-actin filament, leaving in the interstices of the lattice a solution containing ions and some metabolites, but no proteins. Clegg suggested that there were three kinds of water: water bound to surfaces, vicinal water with properties somehow modified by the surface (28), and perhaps ordinary aqueous solution farther from the filaments.
He also speculated that the association of enzymes with the filament might be due to modified solvent properties of the vicinal water, rather than direct binding to actin. He suggested that the water vicinal to the actin filament was more structured than normal water.

**Spatial Distribution of Cytoplasmic Ions**

Application of the concepts that have been developed in this article leads to a different assignment of water properties, but supports the general features of Fig. 9. A G-actin molecule has 21% negatively charged and 11.9% positively charged amino acids. Most of these charged residues must be in contact with water, so that the F-actin filament is a highly charged, high-molecular-weight polyelectrolyte and must generate the kinds of water structure described in the discussions of Fig. 1 and 2. Immediately adjacent to the charged groups on the protein surface are some bound water molecules, hydrating the charges (Fig. 1). Then, adjacent to each charged group is a counter ion (Fig. 2) with its own bound water of hydration. Counter ions are mostly in solution, highly concentrated in a zone of water surrounding the filaments. The amino acid composition of actin is representative of many intracellular proteins. Taking values of the percentage of amino acids that are either positively or negatively charged for 123 intracellular proteins listed in the *Handbook of Biochemistry* (110), the mean percentage of negatively charged amino acids is 20.8 \( \pm \) 4.3 and that of positively charged amino acids is 13.1 \( \pm \) 2.4. The variability from protein to protein is surprisingly small. If, from the same tables, one estimates the average molecular weight of amino acids as 126.4, then the amounts of charge per kilogram of dry protein in a cell are of the order of 1.6 mol of negative charge and 1.01 mol of positive charge. As discussed above, typical values of apparent ionic concentrations in cells are 150 mM K\(^+\), 10 mM Na\(^+\) (and low concentrations of Ca\(^{2+}\), Mg\(^{2+}\), and amines), and 5 mM Cl\(^-\) (and low concentrations of phosphate, other P-containing anions, and bicarbonate). With a water content of 3 kg/kg (dry weight), these concentrations of ions provide approximately 0.5 mol of counter cations and 0.2 mol of counter anions per kg (dry weight). The significance of this very approximate calculation is that cells do not contain enough diffusible inorganic ions to balance the total positive and negative charges on their proteins. The balance must be made up of H\(_2\)O\(^+\) and OH\(^-\).

If, as the evidence cited by Clegg (14) suggests, the solution in the interstices of the microtrabecular lattice is protein free, because proteins are preferentially associated with the filaments, it is very probable that it is also ion free, because, in order to preserve macroscopic electroneutrality,
all ions must partition preferentially into regions near actin and its associated enzymes.

Figure 10a illustrates the distribution of ions in the interstices of a microtubular lattice of a hypothetical cell with only ions as osmolytes. Each rodlike structure represents an F-actin filament with its associated enzymes. Positive and negative ions (whether they are K⁺ and Cl⁻ or H₂O⁺ and OH⁻) accumulate preferentially into zones of water surrounding the charges on the filaments. In response to this excess concentration of solute particles, water equilibrates by increasing its density in regions near charges on the filaments and decreasing it in regions near hydrophobic patches of the filaments and between filaments. The lines represent demarcations between zones of water of different densities.

Preferential association of proteins with the lattice is to be expected because both their hydrophobic patches and their charged patches of surfaces partition into weakly bonded water. In this cell with only ions as osmolytes, however, extreme forms of water which are deleterious to enzyme stability and function coexist. Water adjacent to the filament is probably so weakly bonded that internal hydrophobic moieties emerge and enzymes unfold and denature.

Figure 10b represents (again in an oversimplified way) the distribution of ions and compatible solutes in the microtubular lattice of a cell which contains not only ions but also compatible solutes as osmolytes. Ions still accumulate near the filament-enzyme surfaces, but now this excess osmolality is offset, to a degree, by accumulation of compatible solutes into the strongly bonded regions adjacent to hydrophobic patches of surface and in between filaments. Water near the charges on the filaments can equilibrate with a less drastic increase in density and reactivity, while the rest of the water maintains a rather open structure, because of the hydrophobic portions of its dissolved solutes. A difference in structure and solvent properties between the zones still exists, but now it is a difference between a weakly bonded but nearly normal structure near the filaments and expanded, unreactive water between filaments. Enzymes still accumulate into the more reactive water associated with the filaments, but they do not denature.

Mastro and Keith (84) estimated the viscosity of the aqueous cytoplasm of transformed and untransformed cells by using a spin probe. They concluded that under physiological conditions the viscosity calculated from both rotational and translational diffusion was two to three times that of water. The spin probe used was 2,2,5,5-tetramethyl-3-methanol pyrroline-N-oxyl, a predominantly hydrophobic molecule which would be likely to partition preferentially into the more weakly bonded of two regions of water. The derived viscosities were therefore weighted averages, with the weighting favoring the more fluid aqueous regions. This suggests that water in the interstices of the microtubular lattice is of rather low density and of higher viscosity than Mastro and Keith concluded.

This picture of the cytoplasm differs from Clegg’s model (Fig. 9) in that it suggests that the bulk of cytoplasmic water between filaments of the microtubular lattice is of lower density and higher viscosity than normal, while water closer to the charged patches of the lattice has more normal properties. Substrates would channel from one enzyme to the next when the product of one reaction stayed preferentially in the zone of more reactive water. All cations, in the rank order Mg²⁺ > Ca²⁺ > H⁺ > Na⁺ > K⁺ > NH₄⁺, and most molecules with substantial hydrophobic moieties come into this category. Compatible solutes, for which accumulation of the polar grouping on the molecule into strongly bonded water predominates over accumulation of the hydrophobic moiety into weakly bonded water, are exceptions.

**Why K⁺ as Predominant Intracellular Cation?**

With the exception of some simple nonnucleated erythrocytes (31, 55, 108, 109), all cells accumulate K⁺ relative to Na⁺ (70). Even the primitive halobacteria contain approximately 4 M KCl when their external solution is approximately 4 M NaCl (43). Cells of vertebrates use the resulting sodium gradient to drive secondary active transport...
WATER AND BIOENERGETICS

Active Transport

Although the evidence suggests that cells contrive to avoid extremes of water structure in the bulk of their aqueous compartments, it is probable that nature has exploited those same extremes to generate forces for active transport or for the performance of chemical work. Wiggins and MacClement (123) pointed out that evolution had selected cation pumps and other enzymes with active sites buried in small, rather hydrophobic water-filled cavities, thus predetermining that reactions would all take place in abnormal water. Figure 11 illustrates an improved version of the proposed mechanism of the Ca-ATPase, incorporating the concept of osmotically induced high-density, weakly bonded, reactive water at charged surfaces.

Four transient states of the enzyme binding cavity are illustrated. In Fig. 11a there are two specific Ca\(^{2+}\)-binding sites near the apex of the binding cavity. In the resting state of the enzyme they are unoccupied, but are neutralized by four K\(^+\) ions in solution. Two partially hydrated Mg\(^{2+}\) ions are rather weakly bound to specific sites nearer the mouth of the cavity. As it is depicted in Fig. 11a, each Mg\(^{2+}\) retains a single positive charge and is neutralized by a Cl\(^-\) ion (such finer details, of course, are speculative, but serve to illustrate one physicochemically plausible way in which the model might operate). The remaining negative charge on the surface of the cavity is the aspartic acid group, which is subsequently phosphorylated by ATP. It also has a counter

FIG. 11. Configuration of four hypothetical states of the water-filled binding cavity of the Ca-ATPase of sarcoplasmic reticulum, during coupling of Ca\(^{2+}\) transport to ATP hydrolysis. (a) The cavity contains an excess concentration of counter ions; its water is fluid and weakly bonded. Ca\(^{2+}\) and MgATP enter and bind. (b) The phosphorylated enzyme has no charge left; water expands away from the hydrophobic surfaces and activates the Mg\(^{2+}\) and then the Ca\(^{2+}\) ions, which diffuse out through a channel. (c) K\(^+\) ions reenter, to neutralize the regenerated charges, as Ca\(^{2+}\) ions leave; water is again reactive and fluid and hydrolyzes the aspartyl phosphate. (d) With empty binding sites, the cavity is restored to its state in panel a.

of sugars (73), amino acids (33), H\(^+\) (54), and Ca\(^{2+}\) (7), which might be sufficient justification for the considerable expenditure of energy required to generate it. Other cells and mitochondria, however, which use proton gradients for secondary active transport, also accumulate K\(^+\) relative to Na\(^+\) (52, 67, 68, 87). Many intracellular enzymes have an absolute requirement for K\(^+\), and many function better with K\(^+\) rather than Na\(^+\). A possible explanation for the universality of selection of K\(^+\) ions is their greater affinity for the less dense, more strongly bonded of two aqueous compartments. In a cytoplasm with the ionic distribution depicted in Fig. 10 the requirements of electroneutrality prevail over the preference of K\(^+\) ions for the less dense water, and they are forced into the weakly bonded water adjacent to the charged segments of the filaments and enzymes. These K\(^+\) ions are more likely than Na\(^+\) ions to form ion pairs with the negative sites on the proteins, not because they have greater intrinsic affinity for the carboxyl groups (as Ling [80] has proposed), but because they have less affinity for the weakly bonded water. If some counter ions associate with their negative charges, the excess osmolality in the surrounding solution decreases. Water surrounding the residual counter ions in solution can then equilibrate with the rest of cytoplasmic water with a less drastic increase in density and hence reactivity. It appears that selective accumulation of K\(^+\) might be another strategy (like accumulation of compatible solutes) by which cells avoid inducing extreme forms of water.
cation. The rest of the surface of the cavity consists of hydrophobic moieties. In this state the cavity is extremely highly charged and has a high concentration of counter ions adjacent to the charged groups. For example, if the volume of the cavity in this state is 10 nm³, the excess concentration of solutes as a result of the presence of seven counter ions is approximately 1.2 M. To equilibrate with water external to the cavity, in the face of this considerable difference in osmolality, water molecules inside the cavity must collectively move together, increasing the local density of water, so that water-water hydrogen bonding is weakened. This state of water facilitates inward diffusion of Ca²⁺ to occupy its binding sites, both because the water is fluid and diffusion is rapid and because Ca²⁺ ions partition preferentially into it. Two Ca²⁺ ions bind near the apex, neutralizing those charges; since counter cations are no longer required, four K⁺ ions diffuse out. With the much lower negative charge in the cavity, magnesium ATP⁺ can more readily enter and phosphorylate the aspartic acid residue. This results in loss of another K⁺ ion, because Mg²⁺ remains complexed to the phosphoryl group.

In Fig. 11b the surfaces of the cavity have changed from being predominantly charged to being predominantly uncharged and hydrophobic. Some water has left the cavity with the K⁺ ions, reducing its volume; what water remains is in a state of higher energy than external water, because it cannot make hydrogen bonds with the hydrophobic surface. It equilibrates with external water by expanding, increasing the strength of water-water hydrogen bonds, and losing its ability to hydrate small cations. Expansion starts at the mouth of the cavity and moves up toward the apex, pushing the still partially hydrated Mg²⁺ ions off their binding sites and up toward the apex. When they are overtaken by the front of low-density water, they displace Ca²⁺ ions from their binding sites. This displacement reaction is possible because Mg²⁺ is more highly hydrated than Ca²⁺ and therefore has a much lower affinity for the highly bonded water.

In Fig. 11c the displaced Ca²⁺ ions, which are in a state of high activity in the strongly bonded water, diffuse spontaneously out through a specific channel which has opened at the apex and K⁺ ions move in to maintain electroneutrality. Until now the phosphoryl group has been protected from hydrolysis because it has been surrounded by low-density unreactive water. With the influx of K⁺ ions, however, and release of Mg²⁺ from the Ca²⁺-binding sites, the cavity once more has a higher concentration of solutes than the external solution; it takes up more water, and that water compacts and becomes highly reactive. The phosphoryl group is hydrolyzed, the channel closes, and the enzyme returns to its initial state (Fig. 11d). This sequence of events is consistent with experimental results (47, 123).

The attractive features of this model of active transport are that it is both simple and versatile. Moreover, it offers a plausible mechanism for a conformational change (72, 76, 106) of an enzyme to activate a bond cation. The existence of charged groups, which specifically bind substrate or cation in a very small, predominantly hydrophobic cavity, sets the scene for transient switches of water structure, first from dense, weakly bonded, fluid, reactive water to open, strongly bonded, viscous, inert water, and second, when cations have been transported across the membrane, back to its original state. These switches are accompanied by extreme changes in hydrating ability. Both kinds of water are necessary for optimal activity of a pump. Fluid, reactive water allows rapid influx of Ca²⁺ ions and ATP to initiate a cycle; strongly bonded water activates the Ca²⁺ ions and protects the phosphoenzyme from hydrolysis until transport of two Ca²⁺ ions has taken place. The final switch to reactive water accelerates dephosphorylation of the enzyme so that another cycle can begin.

This single force for transport can then be harnessed specifically by individual ATPases, each of which has binding sites which select the unique cation to be transported outward. These cations are all small and highly hydrated (Ca²⁺, H₂O⁺, and Na⁺) (32, 35, 47, 51, 67, 102). K⁺, which partitions preferentially into low-density water, is transported inward by both the Na,K-ATPase and the H,K-ATPase. Kyte (77) has pointed out that these ATPases are so similar in composition, structure, and function that they probably share a common ancestor. Perhaps they also share a common mechanism.

**Chemical Work**

Many biopolymers (proteins, polynucleotides, polysaccharides) and smaller molecules (ATP, ADP, AMP) spontaneously hydrolyze in aqueous solution. This hydrolysis is normally extremely slow, so that the molecules have a long lifetime and require an enzyme to accelerate their hydrolysis. The significance of this spontaneous hydrolysis, however, is that condensation with formation of polymers from monomeric units, or ATP from ADP and P, cannot take place to an appreciable extent. Nevertheless, all these condensation reactions are, in fact, carried out by enzymes, which, like cation pumps, somehow use the energy of another reaction to drive the thermodynamically unfavorable condensation reaction.

Again, transient changes in active-site water structure offer a simple mechanism for enzymes which perform chemical work. In the example of the Ca-ATPase given above, the phosphorylated intermediate of the enzyme was protected from hydrolysis during the active transport step, when it was in contact with inert, unreactive water. When that water switched back to its highly reactive state, the phosphoenzyme hydrolyzed. This suggests that changes in the reactivity of water might be used by enzymes either to accelerate or to reverse hydrolysis reactions. Synthesis of ATP by disipation of a proton gradient through an ATP-synthase could be formulated in the following steps. (i) The rather hydrophobic cavity contains negative binding sites for protons and positively charged sites for ADP and P. The charged binding sites all have counter ions in solution; the water is weakly bonded and reactive. (ii) ADP and P bind, neutralizing the fixed positive charges, with efflux of some counter anions together with water. The cavity narrows, and a proton channel opens across the membrane. (iii) Protons diffuse spontaneously through the channel and bind to their binding sites, thus obliterating the residual negative fixed charges. Counter cations diffuse out, more water is lost, and the residual water, now housed in a hydrophobic cavity, expands and becomes strongly bonded and inert. ADP and P can now spontaneously condense because the water molecule produced in the reaction is effectively removed from the system by its strong bonding to other water molecules. O'Connor and Wiggins (89) pointed out that changes in chemical potential of species participating in the ATP-ADP equilibrium, when water switched to its low-density state, might also help to drive the reaction in the direction of synthesis of ATP. (iv) A proton is consumed in this reaction, regenerating a negative charge and causing influx of a K⁺ ion and water. At the same time the positive binding site for P is
regenerated, followed by influx of Cl⁻ and water. As the internal osmolality increases, water relaxes from its strongly bonded state and ATP dissociates, regenerating another fixed charge. The second proton, which entered the cavity from across the membrane, exchanges with K⁺ from the mitochondrial solution, and the enzyme reverts to its ground state, with its cavity filled with weakly bonded reactive water.

Clearly many of the details of this scheme may be wrong, but it illustrates the kind of sequence of events that could result in synthesis of ATP powered by switches in water structure accompanying dissociation of a proton gradient. De Meis et al. (21–25) have for many years suggested that water is somehow involved in the reaction mechanism of the Ca-ATPase, both in its Ca⁺⁺-transporting mode and in its ATP synthetic mode. Their many elegant experiments with model systems strongly support this concept. Water has also been shown to be directly involved in light-induced processes in plant thylakoids (3), in the activation thermodynamics of alkaline phosphatase (44), and in the partial reactions of the Ca-ATPase of sarcoplasmic reticulum (122, 125).

CONCLUSION

These few examples of transient or steady-state changes in water structure, induced either osmotically or hydrophobically by surfaces, illustrate the explanatory power of this newly described biological force. It is not only very common (operating wherever there are large molecules which are either charged or hydrophobic or both), but also very powerful, being able to induce a degree of contraction or expansion of water which would otherwise require positive or negative pressures of the order of 1,000 atm (30).

The generation of this force depends upon several factors, most of which are unique to water. The first important factor, which is common to all liquids, is the powerful tendency for components of a system to mix as completely as possible. When this force is overridden by a second force, such as an electrostatic force which prevents counter ions from moving far from their polion, the solvent must equilibrate by changing its partial molar volume. The aspects of the force which are unique to water are as follows. First, water is the only liquid which forms three-dimensional networks of molecules extending throughout its volume. Second, the strength of the hydrogen bonds which link this network increases as molecules move apart, and the bonds straighten. Third, and this is an important aspect of water solution chemistry, when, at constant temperature and pressure, water can equilibrate only by decreasing its density, its own three-dimensional structure opposes that change; thus, for a given change in chemical potential, the change in density must be larger than would be required for a normal liquid. This can be understood by referring to Fig. 6. When water changes from its dense, weakly bonded state, to its open, strongly bonded state, there is a decrease in its energy term because molecules make stronger hydrogen bonds, and this helps to decrease its chemical potential. However, at the same time, the liquid changes from a rather random structure to a much more ordered structure. Such an increase in order increases the chemical potential of water. As Pain (90) has pointed out, this second term, which opposes the decrease in chemical potential with expansion, is numerically greater than the first term, which helps it. It follows that for water (and water only), a relatively small change in chemical potential requires quite large increases in molar volume. The secondary changes in the properties of water are correspondingly great. The reverse should apply to an increase in the chemical potential of water by compaction: a smaller increase in density than that required for a normal liquid should suffice.

Other biological examples in which high- and low-density populations of water molecules might contribute to structure or function abound. Morel (86) pointed out that the equilbrium spacing of the myofilament lattice seems to require a contribution from water structure. Damodaran (20) suggested that water plays a role in preserving the structural stability of ribosomes. Ion channels through membranes have funnellike entrances which are of such dimensions that they are unlikely to contain normal water; Zimmerberg and Parsegian (130) showed that application of an osmotic pressure gradient across a voltage-gated channel caused it to remain closed at higher than normal voltages. They interpreted their results exclusively in terms of channel size, but it is likely that part of the decrease in conductance resulted from the presence inside the channel of water which, relative to the water in the channel’s normal state, was of lower density and higher viscosity. Kinases which transfer a phosphate group from ATP somehow contrive to prevent it being transferred to water; perhaps the water surrounding the transferred group is unreactive. Receptor-binding sites are frequently found in water-filled, rather hydrophobic cavities (46); the antigen-binding region of immunoglobulins is water filled, hydrophobic, and charged. Soluble, sulfated, carboxylated, or phosphorylated polymers are ubiquitous, often in association with gels. A soluble sulfated mucus, for example, is associated with a solid mucus gel with considerable hydrophobic components. Exploration of the properties of water associated with these various biopolymers should be revealing. It is safe to predict that is will also be confusing and frustrating, because when two extreme forms of water coexist at a single polymer surface, or in a single binding cavity, direct elucidation of their individual properties is fraught with difficulties. This experimental block is one reason why controversy has surrounded the properties of water in gels and cells for so long and why a significant biological force has remained hidden.

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