Water in Biological Systems

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The object of this article is to draw attention to some of the major areas of investigation of the state of water in biochemical and biological systems. Deductions about the role of water in such systems are somewhat speculative, but the situation might be improved.

THERE is considerable evidence that water does not just function as an inert solvent for biochemical reactions but plays a major role in the mechanistics of biological processes. Perhaps the best known example of this is the concept of hydrophobic bonding, as applied to the explanation of native biopolymer conformations; this was first invoked[1,2] to account for the fact that the non-polar amino-acids, for example in myoglobin[3] and haemoglobin[4], are resident in the interior of the protein molecule, out of contact with the aqueous environment.

It has been known for some time that the introduction of non-polar moieties into water is accompanied by unfavourable entropy changes which may originate from changes in the hydrogen bonding and/or the arrangement of water molecules in the vicinity of the solute molecules[5]. This phenomenon, hydrophobic hydration, has been studied by various physical techniques, and all the results indicate that both the diffusional and intramolecular modes of water are markedly affected[6]. As a first approximation the formation of a hydrophobic bond between non-polar residues can be regarded as a partial reversal of this process and is thus thermodynamically favoured.

As Scheraga pointed out[7], the primary contribution to the strength of this hydrophobic bond derives from changes in the "structure" of water when the non-polar groups interact with one another. It is this fact which ensures that, in spite of the high degree of chemical diversity among the amino-acid sequences of proteins such as haemoglobin and myoglobin, their three dimensional (tertiary) structures are so similar.

Before proceeding any further, the meaning of the term "structure", as applied to liquid water, must be clarified. While it is obvious that long lived structures do not exist in a liquid, nevertheless, the physical properties of water indicate a certain degree of intermolecular order, reminiscent of that found in ice. On a suitable time scale, that is 10^{-11} s, ordered arrangements of water molecules, or structures, may well exist, and their lifetimes and vibrational and rotational modes are very sensitive to changes in pressure and temperature and to the presence of solutes. Quantum mechanical calculations suggest[8] that H-bonding of water molecules is a cooperative process, and it is probably this cooperativity, rather than the extensive character of any structural units, which is responsible for this sensitivity to environmental changes. One important consequence of this is that long range interactions are possible between hydrophobic solute molecules, and they can be detected in dilute solutions[9].

Another striking demonstration of the important role of water in biological systems is that the substitution of D_2O for H_2O in the growth medium leads to alterations of almost every biological trait of a cell[10] and even to complete inhibition of seed germination in some cases[11]. The kinetics of reactions which depend on acid-base catalysis by water, for example, some enzyme reactions[12], may thus be retarded to such an extent that the overall metabolic balance is severely disturbed. A decrease by a factor of 2–3 in the rate constant of proton transfer reactions is to be expected[13] for such isotope effects. The effect on the subunit aggregation in allosteric proteins, however, can be much greater than the influence on the rate of the enzyme catalysed reaction[14].

There has developed recently an increasing interest in the state of water in biological systems. A good deal of attention has been paid to water in muscle and brain[15,16] and some results have also appeared for water in hair[18], in bacterial spores[19] and in plant tissues[20]. The use of oriented fibres has permitted detailed investigations to be made of the hydration of collagen[21,22] and DNA[23]. All these investigations have been based on nuclear magnetic resonance (NMR) spectroscopy. This approach is invaluable in studies of the aqueous component of a complex system, because the water proton NMR signal is so much narrower than the proton signals from the biopolymers and therefore easily distinguished from them. X-ray analysis, on the other hand, is of little use with these systems because of the relatively high mobility of the water molecules.

What is Not Known

Polar hydrophilic solutes can interact with water to form hydrogen bonds. For example, the hydration of polysaccharides in solution, as deduced from NMR spectra of the aqueous D_2O component, can be correlated[24] simply with the number of OH groups in the repeating unit of the polymer. In the gel state, however, where there is a more rigid polysaccharide structure, probably containing double helical segments[25], some overlap of hydration shells seems to occur with repeating units which are sufficiently hydrated. Similarly, for polypeptides, such as polylysine and polyglutamic acid which possess H-bonding groups in the side chain, there is a noticeable effect[26] due to the interaction of water with the polymer, whereas, at equivalent concentrations, lysine and glutamic acid in solution show no observable effects. Moreover, the lifetime of the hydration structure in polyglutamic acid and polyadenylic acid is about 10^{-4} s, for it is only destroyed when the fluctuations in the helix-coil interconversion occur at the maximum rate, that is, at the mid point of the helix-coil transition. Evidence that the interaction between water and biopolymers is long lived supports the view that such interactions are a mediating property with respect to biopolymer conformations in solution. A recent application of this NMR approach, however, makes it clear that the interpretation of experimental data may not be as unambiguous as formerly supposed[27]. It has been suggested that the NMR effects observed from the deuteron relaxation studies which we have mentioned may just be a measure of the number of exchangeable protons on the polymer.

Unlike the hydrophobic, or apolar, hydration phenomenon which has already been mentioned, hydrophilic interactions would be expected to be highly orientation dependent. Warner[28] has pointed out that in many organic molecules the spacing of ether, ester, hydroxy, or carbonyl oxygen atoms is 4.8 Å, which is almost identical with the next nearest neighbour oxygen spacing in a hypothetical ice lattice, expanded to 25°. Thus, provided there are remnants of an ice-like structure in liquid water, cooperative hydrogen bonding could take place without strain to organic molecules such as biotin, 1,4-quinone, β-D-glucose, and triglycerides. Similarly, the polypeptide chains of some proteins, for example, insulin, can adopt strain-free conformations in which all the carbonyl oxygen atoms are spaced 4.8 Å apart. These observations have led to speculations regarding the function of water as a possible "structural cement" in connexion with the association of subunits of tobacco mosaic virus (TMV)[28] and lipid-protein interactions in biological membranes[29]. Self diffusion coefficient measurements on a 4% solution of TMV, evaluated for very brief jump times, have established[30] that only 3% of the water should be considered as highly immobilized. This amounts to four to five water molecules per amino-acid residue in TMV, and so it leaves open the possibility that monolayers of ordered water separate the hydrophilic surfaces of the subunits. In the case of TMV, the argument hinges[28] on whether the X-ray data, which seem to be inconsistent with Warner's hexagonal peptide model, are sufficient to invalidate his proposal.

There is some experimental evidence for this particular concept of hydrophilic hydration, in that one or two interesting correlations have been found[28] between the 4.8 Å spacing of oxygen atoms and chemical and biological reactivity or organic compounds. Our own studies of the physical properties of dilute solutions of carbohydrates which also possess the 4.8 Å spacing of oxygens have shown quite clearly that water can discriminate between molecules as similar as α and β-methyl pyranosides which only differ in the position (axial against equatorial) of one hydroxy group. It is also clear, however, that the water-solute interaction is of a short range nature in this case, unlike that observed for hydrophobic solutes[9]. This does not necessarily rule out its importance in systems such as membranes where the hydrophilic surfaces may be close together[26], but much remains to be done to substantiate this hydrophilic hydration hypothesis. Investigations of its general applicability are needed, with particular emphasis on macromolecular surfaces[31].

New structures are another unknown quantity. It is generally assumed that the structure of water in a region of hydrophilic hydration resembles that of ice I (hexagonal ice), and it has been suggested that, in the vicinity of hydrophobic groups, water adopts a clathrate-type structure[32] such as exists in crystalline gas hydrates[33]. Other structures have, however, also been proposed for water in contact with hydrophilic surfaces. In hydrated collagen, it has been suggested[34,35] that water molecules are arranged in a pentagonal cage network; and the much publicized polywater, prepared by the condensation of water vapour in quartz capillaries[36], has recently been assigned[37], although on scant experimental evidence, a polymer structure in which most oxygen atoms are bonded to only three hydrogen atoms. In other cases, such as water in muscle, the aqueous component probably exists in at least two varieties of differing molecular mobility[15,17] which, in turn, are each thought to contain two or more subfractions[15,17]. There is, however, insufficient evidence even to begin to speculate on possible structures.

Role of Water

Much remains to be learnt about the role of water. As far as the structure and thermodynamics of macromolecules are concerned, NMR studies have shown that in certain systems the relaxation rate of water is raised, that is, the water becomes more solid-like in its motions. Certain predictions can therefore be made about the properties of systems containing such dynamically modified water.

For example, the lipoprotein component of membranes would be expected to induce such modifications in water motions. Whatever the modified molecular configuration may be, the transport properties of any ions present will undoubtedly vary from their behaviour in bulk water. A comparison of ionic transport properties in water and ice[38] shows that any influence which promotes water "structure" would, on the one hand, reduce the mobility of alkali metal ions, and, on the other, enhance the mobility of protons and hydroxyl ions. The slowing down of proton exchange in aqueous solutions containing ions which break up water structure has been established[39]. The relevance of such phenomena to processes involving ionic transport across membranes has been discussed by Klotz[40].

Having introduced a generalization to the effect that proteins influence water structure, it is necessary to underline the assumptions implicit in such a statement. In what circumstances can proteins be said to have a specific interaction with water? This is an important consideration, because it may relate to the effectiveness of the reciprocal interaction between water and protein, and so to the role of water in controlling protein conformation. The point must be made that anhydrous proteins do not exist, and that the very term protein refers to a polypeptide+water system. The same is true for other biopolymers, and it is known that native conformations can only be maintained in the presence of a critical amount of water which varies with the polymer species.

Of the fibrous proteins investigated so far, from the point of view of hydration structure, collagen has received the most attention. The water is thought to form a pentagonal structure which is bonded to the triple helix. However, the latest view[41] of this system is that the three dimensional structure of the water may not be determined by its interaction with the protein, and the suggestion is made that, whenever water is restricted in a channel of molecular dimensions, as it is in this case, it adopts a particular structure which is not present in the bulk liquid. It is certainly established that when water is constrained by microscopic glass capillaries, its vapour pressure is considerably lower than would be expected on geometric grounds, and consequently its liquid structure is more highly organized[42]. This suggestion of the presence of capillary water in fibrous proteins emphasizes the need for more definitive experiments to test the relevance of structured water in biological systems.

One useful approach which has been taken[43] in the case of solutions of macromolecules involves thermodynamic investigations. Thus, DNA, egg albumen and chymotrypsinogen were seen to exhibit a common thermal denaturation behaviour, indicating that changes in water structure provided the common factor. A similar idea has also been advanced[44,45] to explain why the effect of aqueous anions on the stability of the native structures of biopolymers follows a common sequence, the Hofmeister, or lyotropic, series, for both proteins and nucleic acids. More work along these lines is needed.

At this point it is as well to draw attention to the two schools of thought on the means by which water exercises its thermodynamic control of the conformation of macromolecules. Klotz[1] has argued in favour of apolar hydrate formation, which involves the formation of water cages about hydrophobic groups and is accompanied by a favourable enthalpy change. On the other hand, Kauzmann[2] and Scheraga[7] favour the view that the apolar bands exist between adjacent hydrophobic groups in the biopolymer, and, as mentioned earlier, such a process will be entropy controlled. In the case of myoglobin and haemoglobin, at least in the crystalline state[3,4], the number of exposed hydrophobic residues has been shown by X-ray analysis to be very small,

which supports the latter hypothesis. In lysozyme[46], however, and particularly in subtilisin[47], numerous hydrophobic side chains are situated on the molecular surface, so that a decision between the two theories is not clear cut. In solution systems, it remains to be seen which idea is the more credible.

These problems are not trivial. Since the phenomenon of tertiary and quaternary structure in proteins, and thus of enzyme activity, seems[2,48,49] to be related to interactions of water with non-polar amino-acid side chains, further investigations are clearly indicated.

There is a fair amount of evidence[50] to suggest that general acid-base catalysis is more important for difficult reactions than for processes in which the reactants have marked affinity for each other. This observation may be relevant to enzyme-catalysed reactions, since most biological substrates are non-activated. In fact, since it seems unlikely that any single catalysed reaction step is sufficient to account for the high efficiency of such reactions, the concept of concerted general acid-base catalysis is even more appealing[51]. Thus one or more molecules of water may serve the function of either general acid or general base catalyst[52] and would not appear in the rate law. Indeed, it has been suggested that in the mutarotation of sugars, water acts simultaneously as acid and base catalyst. Such speculations merit investigation in the context of enzyme catalysis.

Control of Cell Processes

It has been argued[53] that the entire water in the living cell exists in "polarized multilayers", and freezing experiments provide evidence for this[54]. They indicate that, within a muscle cell, ice crystals only grow in the direction of the fibre and, if the fibre is previously twisted, then this will be reflected in the ice spikes[55]. The results of a thermodynamic study suggest[53] that intracellular sodium ions are bonded into this multilayer water structure and that their concentration is lower than in the external medium because of the unfavourable entropy of ionic transfer to the intracellular environment. NMR studies[56,57] of the intracellular sodium ion of muscle have confirmed that the molecular motion of 70% of the sodium ions is restricted to such an extent that it is said to be in a complexed state. Some of the criticisms which were subsequently levelled at these NMR experiments have recently been re-evaluated by independent workers and the original findings confirmed[58].

The implications of such experiments are that the asymmetric distribution of solutes between intra and extracellular environments might be accounted for without recourse to the concept of specific solute pumps, which so far have been a generally accepted ingredient of cellular behaviour[59]. The state of intracellular water seems crucial to these arguments. It has been found[60] that the diffusion coefficients of various solutes in an intracellular environment are a factor of two lower than in aqueous solution, but the fact that the diffusivities of some cations, anions and non-electrolytes are reduced to the same extent is in direct conflict with the concept[56] of selective ion binding by the macromolecular cell components.

There is need for an interdisciplinary approach. It is always tempting to suggest that one obstacle to further progress is that physiologists and biochemists normally do not consider the behaviour of the aqueous component of complex biological systems and, therefore, miss what may be the common factor in the control of a number of biological processes. On the other hand, it is because physicists and physical chemists have, in general, turned a blind eye to biological systems that concepts such as solute pumps have not been subjected to many critical investigations by sophisticated modern techniques, even though it has been claimed that the total energy needed to operate all the pumps essential to maintain existing intracellular solute concentrations far exceeds the metabolic energy available to the cell[10,53]. An alternative to the osmotic theory of cell swelling has also been proposed[61], which relies on the concept of hydration of cell proteins, in an attempt to provide an explanation of the changes in cellular volume which occur when the membrane is permeable to external solutes as well as to water. The development and impetus of theories such as these rely on the physical scientists directing some of their effort to these problems in order to evaluate the extent to which these are valid alternatives or indeed useful supplements to the traditional views on cellular behaviour[62].

A surprising number of the publications referred to emanate, at least in part, from medical schools in the United States. This illustrates the strong biochemical and biological support from which this type of work benefits. In the biophysics departments in the United Kingdom, the major research effort is concentrated on X-ray diffraction studies, which have led to exciting discoveries of biopolymer structures, but which are not well suited to a study of dynamic behaviour or to a characterization of the aqueous component of the system. Certainly the preliminary work on the state of water could most profitably be carried out by biophysicists or biophysical chemists. Since biologists do not seem to relish being converted to biophysicists[63], it is unlikely that, left to themselves, they would initiate such work.

The efforts of physical chemists have been directed almost exclusively towards studies of model systems. One must, however, always beware of too hasty a choice of system. In this sense, aqueous solutions of synthetic polypeptides have been shown not to serve as good thermodynamic models for globular proteins[64]. In some cases the systems selected for investigation have been unusual, to say the least. A study of the effects of modifying the water-gluten interactions in doughs[65] has shown promise in relation to the action of anaesthetics, depressants and stimulants on the nervous system. A start has also been made on what might be called real systems. The function of water in controlling membrane permeability[66,67] and narcosis[68,69] is receiving attention. An elegant investigation has been reported[70] of the state of intracellular water and the change in membrane permeability resulting from nerve action. It is in areas such as these, including studies of the chemistry of those protein reactions which may reflect the role of water[71], that the liaison between biochemists and biophysicists is invaluable.

Other Problems

Practical problems also include the state of water in biological systems. The techniques most useful here are those which can, in principle, focus on one component of a multicomponent system. The methods of NMR, dielectric and possibly ultrasonic relaxation fall into this category. At the same time, of course, it is useful in biopolymer systems to look for corresponding changes in the state of the polymer. Very high resolution NMR spectroscopy (220 MHz) is finding some applications here[72], an interesting example being that it can detect some difference in the conformation of the cyano complex of myoglobin in H_2O and D_2O solutions (reported by B. Sheard at the international conference on magnetic resonance in biological systems, Oxford, 1970). The recent introduction of time domain techniques to study dielectric relaxation[73,74] should ensure that investigations of the simultaneous reorganization of both the aqueous and other components of solutions become more widespread in the future.

The role of water poses other practical problems. Results of relaxation studies are often discussed[75,76] in terms of the short range interaction between water and a solute, as specific hydration, or bound water. It has been suggested that this approach is not as useful to the elucidation of the role of water in solutions as is a measure of the overall change in water structure accompanying a transformation of the system. Thus, such diverse processes as the transfer of

electrolytes or non-electrolytes from H_2O[77] to other solvent media, the binding of negatively charged inhibitors to α-chymotrypsin[78] and the effect of ethanol on transition I of ribonuclease A[79] exhibit what is known as compensation behaviour. The enthalpy change, ΔH, is linearly related to the entropy change, ΔS, with a proportionality constant or "compensation temperature" in the range 250–320 K. This property of compensation is believed to be unique to aqueous solution process[80]. It cannot originate from specific hydration of the solutes. It seems to be related to a property of water which is sensitive to long range interactions. Thermodynamic measurements, depending as they do on time-averaged interactions, seem to offer a more sensitive probe of the modulating influence of water on solute properties than do the spectroscopic methods.

Any proposal model must be consistent with the thermodynamics of the system. To make a quantitative estimate of the contribution played by the aqueous component, the thermodynamics of all the processes occurring simultaneously must first be evaluated. This rules out *in vivo* studies at the moment, because of a lack of the detailed knowledge required to make sense of them[81]. Thus the major thermodynamic effort must first be focused on biochemical rather than biological systems.

Commercial calorimeters are available with adequate sensitivity, and the calorimetric determination of equilibrium constants[82] offers the possibility of determining free energies, in addition to enthalpies and heat capacities, without incurring further cost of additional equipment.

We have presented some evidence that the motions of water molecules in biological systems differ from those in bulk water. It remains to be seen to what extent the structure of water is also modified. It is not anticipated that much progress can be expected in this area, while the intermolecular nature of bulk water itself is still a matter of lively controversy[83].

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