Hochachka and Mommsen (eds.). Biochemistry and molecular biology of fishes, vol. 1

CHAPTER 1

On the biochemistry and cell physiology of water

J.S. CLEGG * AND W. DROST-HANSEN **

* *Bodega Marine Laboratory, University of California, Davis, Bodega Bay, CA 94923, U.S.A., and ** Department of Chemistry, University of Miami, Coral Gables, FL 33124, U.S.A.*

Abstract: This chapter focuses on the most abundant molecule in all living systems. Without it enzymes do not function normally, DNA collapses into a tangled mess, and no dielectric continuum (solvent) exists, within which so many physiological processes take place. After brief discussion of pure water we focus here upon the cellular and molecular aspects; however it is quite evident that water participates at all levels of biological organization, from molecular to biosphere. We concentrate on the *aqueous phase* properties of cells and consider only briefly the importance of the primary hydration of intracellular solutes, a well studied and non-controversial issue. Most thought about cell biology has been built on the assumption that the structure and properties of intracellular water are not significantly different from those of pure water (or that in ordinary dilute aqueous solutions). That widely held assumption appears to be questionable in view of a large body of evidence to the contrary. Attention is paid to the large amount of data showing that the water adjacent to surfaces exhibits interesting and unusual physical properties compared to the ordinary bulk liquid. We make the case that the internal environment of cells is characterized by an enormous surface to volume ratio and argue that, on this basis alone, we should expect the water in cells to deviate in its properties. A number of examples are summarized which indicate that this expectation has been documented experimentally, but has thus far not been accommodated within popular prevailing paradigms. We consider some examples of the importance of these interesting properties of intracellular water to cell structure and function.

I. Introduction

Most active animal cells contain 75 ± 10% water by weight and volume — a well known fact. Perhaps more revealing is a consideration of mole ratios: for every 25,000 water molecules there are only about 100 inorganic ions and even fewer low molecular weight organics (intermediary metabolites, nucleotides, etc.) about 75 lipids and phospholipid molecules, and only one or two protein molecules (we can neglect nucleic acids in this exercise). Of course, the quintessential feature of intracellular water is that it is *the* primary solvent — the 'mother liquor'[100] — within which so much of the metabolic repertoire of cells occurs. At the same time, water is a major substrate for a variety of metabolic reactions (as well as a product) and the vital role played by water in macromolecular conformation is a well established fact. Indeed, the participation of water in cells is so pervasive that an understanding of virtually every cellular structure and its function would seem to require a detailed description of the participation of water. Be that as it may, relatively little attention is paid to the properties of water in cells. As Albert Szent-Györgyi[100] said: 'Biology has forgotten water, or never discovered it.' He also wrote that water is the last thing a deep sea fish would ever discover, a saying in keeping with this series.

As knowledge about the ultrastructure of animal cells has advanced, it has become increasingly evident that the aqueous phases within cells exist in the presence of an exceptionally high surface to volume ratio. Indeed, we will document later how extensive is this intracellular macromolecular surface area, and will advance the proposition that, as a result, the physical properties of at least a lot of the water in cells are altered with respect to those of ordinary aqueous solutions. Further, we will consider the consequences this may have for several aspects of cell physiology. In so doing, we are mindful of the fact that it is generally assumed that intracellular water is not different from pure water and that most consensus views of cell physiology are built on this assumption. Questioning widely held traditional belief might provoke in some a less than enthusiastic response; however, as Rudolf Arnheim[4] noted: '... if you try to make sure not to step on anybody's toes, you will have no space left to walk.' We begin with some general comments on pure water.

II. Some general comments on pure water

It is commonly emphasized that water is a most unusual liquid; moreover, it is also the only inorganic liquid to occur naturally on earth. Approximately one gram mole of grams of water exist on earth, in rough numbers, 10^{47} water molecules, and it is the only chemical compound on earth to occur naturally in all of its three phases: solid, liquid and vapor. More importantly, the properties of water differ, sometimes dramatically, from the properties expected for a compound of such low molecular weight: its melting and boiling points are high as is the heat of vaporization, and its heat capacity and dielectric permittivity are remarkably high.

Many other properties differ from those one would expect based on a comparison with other low molecular weight substances. The density of solid water is less than that of the liquid (at the melting point) and liquid water possesses a maximum in density near 4°C.

The explanation for the unusual properties of water is found in the ability of the water molecule to form hydrogen bonds (H-bonds). These bonds are tetrahedrally disposed around the molecule, leading to a very 'open' structure. In ordinary ice the number of nearest neighbors (n_x) is 4.00 and in liquid water this 'openness' is essentially retained with values for n_x around 4.2 to 4.4 in the range of temperatures and pressures of physiological interest.

Not withstanding nearly a century of study on the structure of water, a final, 'einwandfrei' description of the molecular architecture of water still escapes us. A monograph by Eisenberg and Kauzmann[44] provides a highly readable introduction to the structure and properties of water. A monumental summary of the state of water (and solutions) research during the late seventies and early eighties is available in the form of a seven volume exposition entitled 'Water — a Comprehensive Treatise,' edited by Felix Franks[52]. A brief but excellent introduction to research on water has been provided by Stillinger[99], a leading researcher on water, and more recent information is available in the volume edited by Packer[87].

Most current theories of pure water depict the structure as a disordered array of H-bonded water molecules such that a continuous 'lattice' of chains of water molecules can be found throughout the liquid. In all probability only a few water molecules are 'free' in the sense of not being hydrogen bonded to at least one other molecule, while only a small fraction of water molecules have all four possible bonds intact at any moment. Even in the momentary absence of an H-bond between a water molecule and its nearest neighbors, strong interactions are still operating (dipole-dipole, quadruple, and higher interactions).

It has proven difficult experimentally to elucidate liquid structure in general and the structure of water in particular. The only 'direct' methods of study are by scattering techniques, such as X-ray diffraction or neutron scattering. Such experiments at least provide a 'radial distribution function' (rdf) of the relative distances from a critical molecule to its various neighbors; however, to complete the structural analysis a model must be formulated and its 'goodness of fit' checked by the extent of agreement between the rdf and the calculated molecular positions based on the model. All other experimental structure studies are indirect, usually employing some sort of spectroscopy (IR, Raman, NMR, dielectric, etc.). None of these approaches delineate simultaneously the location of the molecules (coordinates) and their dynamics.

There exist essentially two classes of models for liquid water: mixture models and continuum models. The former category traces its origin to the end of the last century when several investigators proposed that liquid water might be a mixture of relatively bulky structured entities similar to ice in a medium of essentially monomeric, closely packed (i.e., 'dense') water molecules. In the continuum models H-bonds are not broken but merely allowed to bend and/or stretch in response to random thermal motion.

Kauzmann has long been an advocate of the continuum models for liquid water and has recently published another, rather detailed model[64]. This random network model is interesting and important in that it provides an equation of state of liquid water and successfully accounts for a variety of abnormal properties of water based primarily on (a) spectroscopic data, (b) data on the properties of ordinary, crystalline ice *I(h)*, and (c) information about the water molecule in the vapor phase. The theory predicts correctly such features of liquid water as the negative expansion coefficient at low temperature but the point of change-over from positive to negative is in error by about 50°C. Qualitatively the new theory predicts correctly a minimum in the isothermal compressibility but the value for the temperature of the minimum is off by 100°C while the absolute value for the compressibility coefficient is off by a factor of 3. Henn and Kauzmann's theory predicts the entropy of liquid water (at 0°C) to within about 1%, but the agreement between the calculated and observed specific heat is not nearly as satisfactory and the theory fails to predict the (very shallow) minimum near 30°C. Notwithstanding the less than good agreement between observed and predicted values for some of the properties discussed, the model is impressive because of its conceptual simplicity. The model makes reference directly to the liquid state only through the use of spectroscopic data and otherwise is based on data for the solid state and from the single water molecules in the vapor. Thus the success of the model lends credence to 'continuum' models of water.

Over the past 15 years, a large number of papers have appeared dealing with computer simulation of water structure. Simulations are based on accepting a reasonable expression for the pair-wise interaction of water molecules, namely the pair potential (energy) function, $u(r)$. Two types of approaches are possible: a Monte Carlo technique and the molecular dynamics method. In the first approach the energy change is calculated which results from a small displacement of the molecules (from the positions chosen for the 'first instance') from the sum of $u(r)$ over all the pairs of molecules. Only those displacements which lead to lower total energy are accepted. Ultimately, the positions of maximum likelihood are obtained and expressed in terms of the rdf which in turn may be compared to the rdf obtained from scattering experiments. In the molecular dynamics method the molecules are supplied with energy and the resulting motions monitored. At fixed time intervals (say 10^{-12} s) the coordinates of the molecules are obtained and the path to equilibrium may thus be determined. As a result, the calculations provide both equilibrium thermodynamic properties as well as time-dependent aspects (for instance, the self-diffusion coefficient).

Much has been learned from simulation studies and the results are surely valuable hints as to what the structure of liquid water may be. Because of computational limitations, the typical sample of water molecules used in these analyses is about 500. If these molecules formed a small droplet the radius would be 5 water molecules and half of these would be from the 'outside layer.' Thus one must expect some dramatic surface effects (even if periodic or cyclic boundary conditions are employed in order to minimize this effect). Furthermore, as discussed below, many of these calculations are based on a pair-wise potential energy function, probably a serious limitation.

A particularly intriguing model of water has been proposed by Stanley and Teixeira[9,56,97,98] especially aimed at elucidating water structure at low temperature. The model is based on the idea of percolation theory, i.e., interconnectivity on a 'lattice.' The structure is that of an 'infinite' H-bonded network, continuously being restructured. At any moment some bonds are strained or broken; within the network are 'patches' of lower (local) density and lower entropy than the overall (global) values. The spatial positions of the various types of 'connectedness' are not randomly distributed but are correlated. In particular the structure contains tiny 'patches' of four-bonded molecules. Among other things, the model allows calculations of the isothermal compressibility, specific heat and thermal expansion coefficient. The size of the patches increases with decreasing temperature. A novel feature is that rather than a lower cut-off value in energy for hydrogen bond interactions, any attraction is considered a state of H-bonding. An analysis of the resulting H-bond distributions suggests the lifetimes of the H-bonding fit a power law, implying that the H-bonds do not have a characteristic lifetime; as would be expected intuitively, the lifetimes increase rapidly as the temperature is lowered, particularly below 0°C. Regrettably the model is based on computer simulations involving only 216 molecules (which still requires a great deal of computing time) and no attempt has been made so far to simulate the influence of a confining wall, thus still leaving open the question of the predicted nature of interfacial water.

Many other impressive computer simulations have indeed been made in efforts to model the structure of liquid water. However, these calculations usually are based on pair-additivity of the potentials for the H-bonded water molecules, so the possibility exists that subtle effects may escape the theoretician as no means are provided to build-in the possibility of extensive cooperativity — an aspect which Henry Frank[51] has so eloquently stressed. Very likely, this is the crux of the problem of interfacially modified water which will be referred to here as 'vicinal water.' If nothing else, the thermal anomalies in the properties of vicinal water strongly implicate cooperativity on a large scale — a *collective* behavior of water molecules which no existing potential function is yet able to reproduce. The cooperativity reflects non-pair-additivity and it does not seem plausible that 'effective' potential energy functions can be devised which will remedy the specific lack of a detailed understanding of many-body interactions in water. Brave attempts to allow for cooperativity have been made by Finney, Barnes and coworkers[50].

A particularly readable account of computer ('machine') simulations of water is provided by Barnes[5]. This article is also one of the first to report on progress in the problem of computer simulations for water at interfaces — i.e., spatially confined water. The approach included allowance for cooperativity among the water molecules: 'The cooperative nature of hydrogen-bonding should not be visualized as a mere strengthening of the bond networks, but as part and parcel of their existence.'

It appears that we are still quite far from reaching a consensus as to the structure of pure water. In view of this it is hardly surprising that we know even less about the structure of water near interfaces, a question of direct importance

when considering the properties (and structure) of intracellular water. It seems appropriate to first examine the conditions under which intracellular water exists; that is, we should ask what the intracellular environment consists of, and what the water 'sees'.

III. Intracellular architecture of eukaryotes

Everyone knows that cells are not 'bags of enzymes'; yet, there does not seem to be a consensus on the extent to which the interiors of cells are 'organized'. A variety of organelles (membrane bounded and otherwise) exist in eukaryotic cytoplasm, as do the classical cytoskeletal elements. However, the extent of interconnection existing between these and other cytoplasmic structures (including the plasma membrane) and the nucleus is a matter yet to be determined. The traditional (textbook) view is that these structures are mostly 'suspended' in a concentrated solution — 'the cytosol' — consisting of inorganic ions, metabolites, adenine and other nucleotides, and macromolecules, chiefly proteins of which many are enzymes of intermediary metabolism. In this view, most cytoplasmic water would find itself as part of this highly concentrated 'cytosol' (a very ambiguous term[20]) often referred to as a 20% protein solution. A similar picture could be drawn for the crowded aqueous phase of the nucleus (the 'nucleoplasm') and the interiors of membrane-bound cytoplasmic organelles[20].

An alternative to this description has been advanced in which the vast majority of the macromolecules are organized into complexes, and even small solutes such as certain inorganic ions, nucleotides and metabolites are also envisaged to participate in this organization. This paradigm is perhaps best visualized in the form of the microtrabecular lattice (MTL) proposed by Keith Porter on the basis of high voltage electron microscopy[90]. Fig. 1, donated by Porter, illustrates this image of cytoplasmic organization. It is worth noting that, while some believe the MTL to be an artifact of the preparative procedures of microscopy (see 10,74,93) evidence from *non-microscopic* studies on intact (or almost intact) cells, in general, supports the existence of the MTL as a real structure. Because this evidence has been the subject of extensive, and we believe compelling recent reviews[6,15,20,72,106], that effort will not be repeated here. Although many details are lacking, we find this 'organized description' to be most consistent with existing evidence. Thus, if this paradigm is accepted, then the great majority of the intracellular water finds itself in a *dilute* solution, containing relatively few macromolecules and other solutes. One might expect, therefore, that this water would behave like that in an ordinary bulk aqueous phase. However, as pointed out, the aqueous volume in between ultrastructurally recognized structures is in very close proximity to macromolecular surfaces (Fig. 1). Estimates of the surface area of the MTL have been carried out by image analysis of HVEM photographs[57]. This area is so vast, about 100,000 μm^2 for cultured mammalian cells, that at least 50% of the total water finds itself within 50 Å from some surface[20,22]. Indeed, estimates from fluorescence recovery after photobleaching measurements on the diffusion of fluorescently

labeled probes (Ficoll and dextrans) suggest that the fractional surface area may be even greater than indicated from HVEM analysis[8,79]. Thus, the influence of surfaces on the properties of water near them becomes an issue of some importance.

IV. Water near interfaces

1. Vicinal water

Vicinal water is defined as water (or aqueous solution) the structure of which is modified by proximity to an interface but excluding chemically 'bound' water directly on the surface (the water of primary hydration). As for the depth of the structurally modified layer, Adamson[1] has eloquently argued "that a 'quiescent' liquid/air surface is actually in a state of violent agitation in the molecular scale with individual molecules passing rapidly back and forth between it and the bulk regions on either side." The depth of this disturbed layer is probably of the order of 50 Å (about 15 water layers.) While structurally modified, these surface layers are not considered 'vicinal' (in the sense to be delineated below). However, in the presence of a monolayer (or even with just partial coverage) deep surface structure modifications occur, possibly extending over distances of many hundred molecular layers (see refs. 3, 67, 68). While the pure air/water interface does not show any evidence of vicinal water structuring, surface tension measurements made by the capillary rise method have shown unexpected thermal anomalies, for instance near 30°C. [The first set of reliable data showing such an anomaly goes back to a German investigator named Brunner in a paper from 1847 (see refs. 33, 34). The anomaly appears as an inflection point in the surface tension versus temperature; because the surface tension is a free energy, the temperature derivative is an entropy of surface formation.] One of us has shown[33,34] that for water in narrow glass capillaries there is an increase in the entropy of about a factor of 2 over a temperature interval of only 3 or 4°C near 30°C. This effect is considered to be a manifestation of a relatively long-range vicinal restructuring of the water, originating from the glass surface and this vicinal water structure undergoes some type of transition, probably a higher order phase transition, around 29–32°C.

The proximity to a solid surface appears to induce long-range structuring of the proximate water and the structures thus induced undergo no less than four thermal transitions between the freezing and boiling point of water. The characteristic, critical temperatures occur near 14–16°C, 29–32°C, 44–46°C and 59–62°C (i.e., essentially equidistantly spaced at 15°C intervals).

It appears that vicinal water is induced by proximity to most (or all) 'solid interfaces' regardless of the detailed specific chemical nature of the surface. This puzzling result is referred to as the 'paradoxical effect' (see refs. 40, 41, 48). The phrase 'solid surfaces' is used in this context in a most general sense, from mica or quartz plates, mineral grains and membranes to large macromolecules (above a certain 'critical' molecular weight) and possibly some types of aggregates, such as

glycogen particles and micelles. The paradoxical effect is an important consideration as it is experimentally far more difficult to study the intracellular aqueous environment of intact cells than to study physico-chemically relatively well-defined model-systems. If we are able to demonstrate that vicinal water occurs near *any* aqueous phase/solid interface, then we may conclude that vicinal water must also be induced by some (or all) of the 'surfaces' within cells, such as membranes and those of the cytomatrix (Fig. 1).

We are not concerned in this paper with the more traditional aspects of the hydration of the cell constituents. While this aspect is, of course, of immense direct

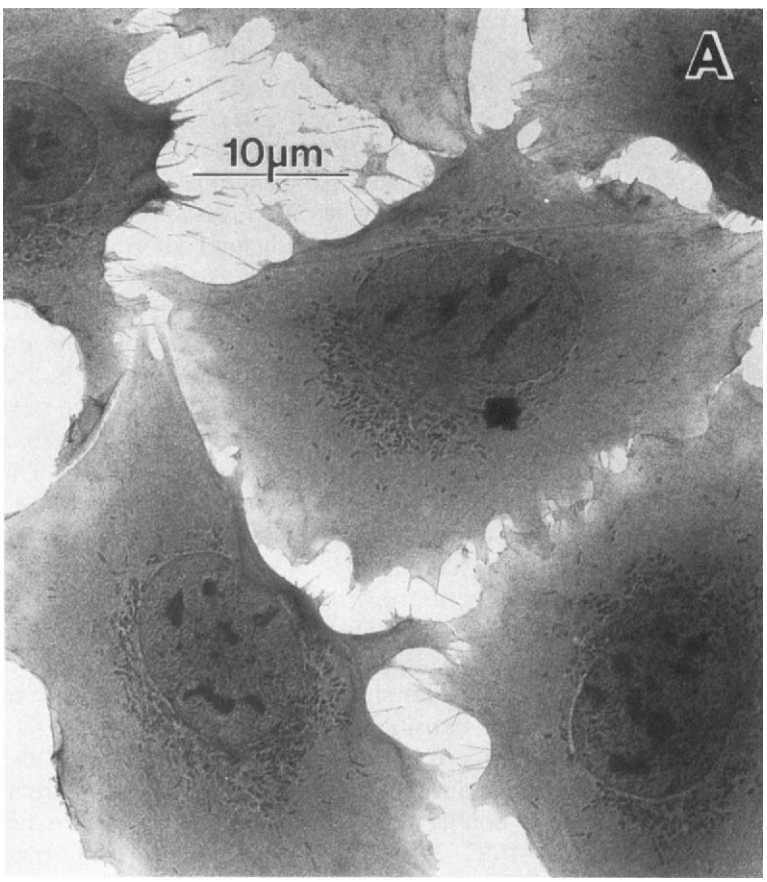

Fig. 1. High-voltage electron photomicrograph of cultured NRK cells (rat kidney) provided by K.R. Porter. A illustrates several individual cells, and B shows a small region of the cytoplasm magnified 90 times with respect to A. T (one of the numerous microtrabeculae), MT (microtubule), ER (endoplasmic reticulum), M (mitochondrion), P (polyribosome). The asterisks locate volumes between detectable cytoplasmic structure, which are presumably dilute aqueous solutions. Reference to details of preparative procedures can be found in the review by Porter[90]. The essential feature of this image of cells is that the cytoplasm is a 'unit structure', in which virtually all the formed elements are interconnected structurally and functionally.

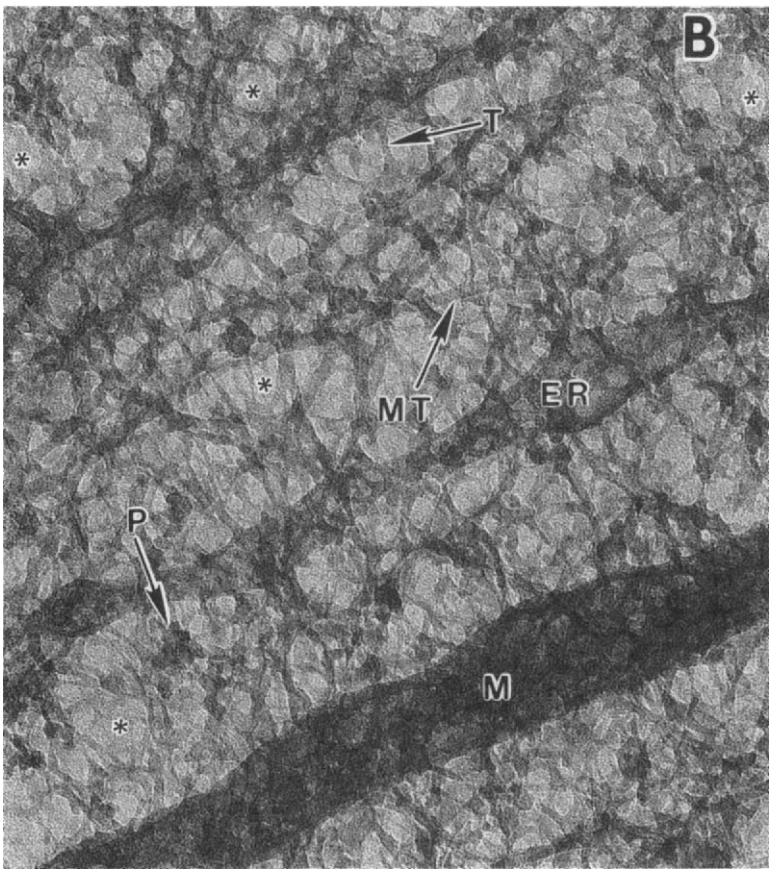

Fig. 1. (continued).

importance to macromolecular structure and function (see refs. 11, 54, 58, 86, 92) the main point of the present paper is that 'vicinal hydration' occurs at distances of at least 50 Å from the actual surface, and it is this aqueous phase upon which we focus our attention here.

Extensive discussions of the structure and properties of water at interfaces can be found in papers and reviews by various authors and the reader is referred to selected papers by Deryaguin and coworkers (see refs. 17, 27–30). Israelachvili et al.[69–71], Peshel and coworkers[89], Low[77] and Etzler[45], Etzler and Drost-Hansen[32–43;45–48]. For an objective and informative review of water at solid surfaces see also the classic paper by Clifford[18]. That paper examines in detail a vast literature on aqueous interfacial phenomena; a major conclusion is that surface induced changes in water structure may occur but that it is not likely that any such structures will extend more than 100Å from the surface. Clifford speculates that one possible mechanism for creation of interfacial water structures (different from the bulk structure) might be local geometric constraints preventing

the full development of large structured elements of bulk water. A similar mechanism might explain the 'paradoxical effect': truly external geometric constraints might occur with any solid, non-smooth surface and thus account for vicinal water being induced by ionic, hydrophilic and hydrophobic surfaces, — i.e., regardless of the chemical nature of the surface. Given the enormous geometric complexity of a cell, the aqueous cytoplasm would be the obvious place to expect to find vicinal water.

For an excellent recent review of the types of forces which operate at interfaces in water, see the paper by Evans and Ninham[49]. These authors specifically delineate the role that physico-chemical insight and surface and colloid chemistry is likely to play in the description of biological systems. Their review progresses from general considerations of hydrophobic effects as the prime cause of aggregation in general to theories of micellar aggregation, in particular, and the question of vesicle stability. In their review Evans and Ninham provide a concise enumeration of the forces operating in colloidal systems; the authors note that classical theories were not able to deal successfully with some colloidal systems, such as clay swelling, interactions between air bubbles in salt solutions, or the characteristic spacing of lamellar lecithin multilayers. The difficulties are related to the relatively short range stabilizing forces, referred to as hydration forces or the structural component of the disjoining pressure. These authors also summarize the nature of hydration forces, hydrophobic forces and secondary hydration forces, and speculate on the likely role in cell biology of these forces. The monograph by Israelachvili[69] is highly recommended as an introduction to this topic.

Rand and Parsegian[91] have provided a thorough review of the hydration forces operating between phospholipid bilayers, much of this research coming from the laboratories of these authors. They point out the experimental and interpretive difficulties associated with such studies but, on balance, arrive at the conclusion that water is influenced over 10–30 Å from the bilayer surfaces.

Thus far we have concentrated on 'vicinal water', the particular view developed by one of us (W.D.-H.). As mentioned, the concept of vicinal water envisions modified water structures at interfaces, induced by mere proximity to the surface regardless of its specific chemical nature and extending over distances far larger than normally considered (say 30 to hundreds of molecular diameters). The most characteristic feature of this vicinal water is the occurrence of no less than four narrow temperature ranges in which the structural properties of the interfacial water changes more or less abruptly. The transitions near 30 and 45°C may notably affect the physiology of mammals, birds and many other species including many microorganisms[32,36]. The 15°C anomaly is also expected to affect the physiology of many marine organisms[36]. Some characteristic properties of vicinal water are listed in Table 1 (together with the corresponding bulk values). The table is taken from a recent paper[43], in which are listed the various publications from which the information has been compiled.

A number of other investigators have, over the years, presented their own descriptions of the nature of intracellular water and we take these up next. Although differing in detail, sometimes markedly, they all arrive at the conclusion

TABLE 1

A comparison of some properties of pure and vicinal water [a]

Property	Bulk	Vicinal
Density (g/cm^3)	1.00	0.97
Specific heat (cal/kg)	1.00	1.25 ± 0.05
Thermal expansion coefficient ($°C^{-1}$)	$250 \cdot 10^{-6}$ (25°C)	$300–700 \cdot 10^{-6}$
(adiab.) Compressibility coefficient (Atm^{-1})	$45 \cdot 10^{-6}$	$60–100 \cdot 10^{-6}$
Excess sound absorption (α / v^2) $cm^{-1} \cdot s^2$	$7 \cdot 10^{-17}$	$\sim 35 \cdot 10^{-17}$
Heat conductivity (cal/sec)/cm^2/°C/cm)	0.0014	~ 0.01–0.05
Viscosity (cP)	0.89	2–10
Energy of activation ionic conduction (kcal/mol)	~ 4	5–8
Dielectric relaxation frequency (Hz)	$19 \cdot 10^9$	$2 \cdot 10^9$

[a] See text for references to original citations.

that most (or all) of cell water differs importantly from that in dilute solution, and emphasize the considerable importance this could have to cell structure and function. Our coverage must be brief, so we refer the reader to books (6,15,85,87,106), all of which contain many additional references to the literature.

2. The NMR titration model

Ivan Cameron, Gary Fullerton and their colleagues in a series of studies applied nuclear magnetic resonance (NMR) spectroscopy to a variety of cells, tissues and macromolecules and have developed the idea of multiple water compartments in them (for recent coverage and reference to earlier work see refs. 14 and 110). Their approach involves the study of the motional behavior or water as a function of total water content of the system (achieved in cells by air-drying or by osmotic manipulation using impermeant solute addition, or medium dilution.) In some cases (*Xenopus* oocytes, mammalian lens) they conclude that *all* of the cell water is significantly perturbed (different) whereas in other systems (erythrocytes, sarcoma cells, sea urchin eggs) only about 50% seems to behave that way. Although one might question whether these severe manipulations of water content are excessively unphysiological and might produce 'artifacts', we believe they make a strong case for the existence of large fractions of cell water whose properties differ from those of dilute aqueous solutions. These differences are attributed to water-surface interactions within the cells under study, and the different hydration compartments are defined on the basis of distinct hydrogen bonding mechanisms.

3. The Work of Hazlewood and Rorschach

We should stress that the first work revealing altered motional properties of cell water by NMR was done concurrently on skeletal muscle by Cope[26] and Hazle-

wood *et al.*[61]. Hazlewood and his many colleagues, most notably Rorschach, have, since that time, produced a vast amount of evidence indicating that virtually none of the water in cells has motional properties similar to those of pure water. This group has studied a wide variety of cells and tissues, including malignant ones, and has utilized quasi-elastic neutron scattering (QNS) as well as NMR. Their publications are so numerous that only a few selected papers and reviews can be cited here[60–63,83,94,95]. Their work has also been covered in a number of recent books (see refs. 6, 85). This pioneering work has been challenged repeatedly but, in every case to our knowledge, the objections to their conclusions have been answered adequately and usually convincingly. It is our view that Hazlewood, Rorschach and coworkers have proven that the translational and rotational motions of the vast majority of animal cell water are indeed markedly slower than pure water, implying if not requiring that the structure is also different. Their work also stresses the enormous significance of these findings to cell biology, and laments the lack of attention paid to the subject.

4. The association–induction hypothesis

Hazlewood and colleagues have usually interpreted their results in the context of Gilbert Ling's association-induction hypothesis (AIH). First formulated in the late 1950s (see the massive book by Ling[76]) the major features of the AIH are as follows:

Virtually all of the water in cells is considered to exist as polarized multilayers arising from fixed charges on extended protein surfaces. The water multilayers exclude ions and other solutes to varying degree, and the contributions of membrane processes (active transport) are considered to be negligible. Ions are also associated with fixed charges on cellular macromolecules, notable proteins, the degree of binding for a given ion being influenced by a number of factors. 'Cardinal sites' exist on these particular proteins which, when filled with specific adsorbents such as ATP, initiate cooperative interactions within the protein–ion–water system. Hormones and regulatory cyclic nucleotides are included in the list of cardinal adsorbents. Binding at the cardinal site leads to cooperative alterations and the selective accumulation of K^+ over Na^+, and also generates the polarized multilayers of water; ATP splitting and the removal of ADP results in a movement of the system to a lower energy state in which the ion selectivity is lost, as is the polarization of water.

This hypothesis has been supported strongly by some, most notably by the extensive work of Hazlewood (as mentioned), and others (84,85). Nevertheless, the scientific community as a whole has been reluctant to accept the AIH. At least part of this reluctance seems to stem from less than compelling evidence for the actual existence of polarized multilayers of water in cells and for the extended proteins that generate them and selectively bind ions. While one might disagree with details of Ling's hypothesis it is not so easy to dismiss the objections he has raised against the validity of 'the traditional view.' Whether or not Ling is correct

remains to be seen, in our opinion. Certainly, if he is correct, cell biology must undergo a revolution.

5. The reference phase technique

Horowitz, Paine and colleagues (see refs. 66, 88) have also provided experimental evidence that the traditional view of cellular water requires modification. They have developed an elegant method for examining the solvent properties of intracellular water *in situ* called the reference phase technique. A microdrop of gelatin sol (the reference phase) containing an appropriate radioactive solute is injected into the cytoplasm of amphibian oocytes and made to gel by temperature reduction within the physiological range for these cells. After diffusion equilibrium is achieved the cells are placed at $-160°$ to $-190°$, and the reference phase, nucleus, and selected areas of cytoplasm are microdissected at $-45°C$, and then analyzed for solute and water contents. The properties of the solute and water in the reference phase are considered to be the same as those of the surrounding Ringer's salt solution (hence, a 'reference'). Therefore, it can be predicted that if the traditional view is correct, then cytoplasmic concentrations must equal reference phase concentrations for the solute under study. However, that is not the result they observed for several ions and a number of non-metabolized compounds, indicating that cytoplasm exhibits solvent properties that differ distinctly from those of bulk water. They propose that the aqueous interior of cells is not a single homogeneous phase, and that total cell concentrations of solutes do not accurately reflect actual solute concentration distributions within different parts of cells; cytoplasmic concentrations can *not* be considered equal to chemical activities because of the occurrence of intracellular binding and the altered solvent properties of the cytoplasm.

These studies have important consequences to current views on cell physiology, the most obvious being solute transport. Since it is the electrochemical gradient across any cellular membrane that determines the direction and driving force for transport, current estimates of the energy requirements for, and even the direction of transport are likely to be less than accurate. Because Na^+ and K^+ concentrations in the nucleus differ appreciably from those of the cytoplasm and since these distributions appear to be determined by differences in binding and/or solvent properties the possibility arises that changes in binding and/or solvent properties of the cytoplasm could be intimately involved with the regulation of these cations in the nucleus. It should also be recognized that the data indicate that these cells do maintain certain solutes and ions against electrochemical gradients between cytoplasm and the cell exterior; therefore, the authors propose that active transport functions are performed by the plasma membrane in this system, and that altered solvent properties and ion binding are not the whole story.

6. 'Pore water'

In a remarkable series of recent studies (see refs. 107–109) Philippa Wiggins and her colleagues have developed the view that the structure of water confined to

small volumes or pores (in contact with a bulk aqueous phase) can be altered by osmotic pressure gradients across the imaginary interface between the pore water and bulk phase. They find that the pore water exhibits different specificities for the critical inorganic ions, Na^+ and K^+, due to its altered structure. Likewise, different ions influence the structure (and properties) of pore water. One of several interesting features of this proposal is that altered water structures can arise in these small, confined volumes *without* the influence of water–surface interactions (of course, the presence of surfaces exists in these systems as well). These authors stress the implications of their results to the issues of osmotic balance, cell volume regulation and the properties of water in small membrane-bound organelles. Thus, although arrived at through a different experimental and conceptual approach, this research adds substantially to other evidence that at least much of intracellular water exhibits different density and solvent properties compared to the bulk liquid. Wiggins has contributed greatly to our understanding of intracellular water, and we urge the interested reader to study her original work — it is creative, and full of provocative ideas on the subject.

7. The cluster model

John Watterson has taken a more theoretical approach to the question of cell water structure, drawing from the literature on cell ultrastructure and the physics and biology of osmotic pressure (102–104). He begins with the idea that dynamic groupings of water molecules result from cooperative intermolecular binding and develops the notion that, at any instant, an unbroken interconnection exists, percolating from one side of the 'cluster' to the other. One of the most interesting features of his 'Cluster Model' is that water is envisaged as *the* coordinating vehicle in cells. In his words[105]:

'Subcellular movement takes place as though directed by an underlying co-ordination, implying a unifying principle which links metabolic chemical energy with macroscopic mechanical forces in a two- way partnership. This principle is clearly one of structure existing throughout subcellular space, and of all subcellular components, I think that water is the only one capable of fulfilling this role.'

Watterson's model is difficult to summarize briefly, and the interested reader is encouraged to examine his papers in detail to obtain the flavor of his argument as well as its substance. We believe Szent-Györgyi would enjoy Watterson's views since he once wrote[100] that 'Life is water dancing to the tune of solids.'

8. The properties of water in Artemia cells

Since the early 1970s an interdisciplinary, cooperative research program has been aimed at describing the physical properties of water in the cysts of *Artemia*, a useful model system for this purpose since they have the natural ability to reversibly lose (and regain) virtually all their cellular water. The cysts contain about 4000 cells with essentially no extracellular space, and their ultrastructure, biochemistry and development have been studied extensively (see several recent

TABLE 2

Some properties of pure water and the water in cysts of *Artemia* [a]

Parameter [b]	Pure water	Cyst water	Pw/Cw
NMR			
t_1	3000	275	11
t_2	1750	53	33
D	2.4	0.4	6
QNS			
D	2.5	0.75	3
γ	1	4	0.3
MD			
ϵ' (2 GHx)	78	40	2
ϵ' (35 GHz)	23	16	1.5
γ	8	10 –25	0.5
α	< 0.02	0.5	< 0.4
M–V	1.000	0.966	1.04

[a] Cysts were at their maximum water content (about 1.4 g/g) except for MD studies in which they contained 1.2 g/g.

[b] NMR = Nuclear magnetic resonance; QNS = quasi-elastic neutron scattering; M–V = mass–volume measurement; t_1 and t_2 are 'relaxation times,' in milliseconds; D, the self-diffusion coefficient of water, in cm^{-5} cm^2/s; γ, correlation times in 10^{-12} s; ϵ', the dielectric permittivity; α, the 'spread parameter' for dispersion over the frequency range 0.8–70 Ghz;, the density in g/cm^3, derived from mass–volume measurements (M–V). Sources for these data are given in Clegg[20,22].

books[12,81,101]). The rationale behind this research has been to probe the properties of cell water by applying as many techniques as possible to the same system in the hope that the weaknesses of each will, as it were, cancel out. It is not possible here to summarize all of the results; most of the work has been recently reviewed[20,21]. However, we have concluded from these studies that little (if any) of the water in these cells exhibits the kinetic and thermodynamic properties of the water in ordinary aqueous solutions. Table 2 tabulates some of these results for fully hydrated cysts, whose cells contain about 1.6 g H_2O/g dry mass (typical for yolky embryonic cells). While the 'unusual' nature of this system may raise doubts in the minds of some about the general applicability of the results, we have argued otherwise[21]. Finally, we note that concurrent studies on the metabolism of these cells have generated an hypothesis describing potential relationships between enzyme organization and surface-associated water, the 'vicinal water network model'[19].

V. Intracellular microviscosity

We consider this topic under a separate heading because it illustrates how seldom the properties of cell water are taken into account when interpreting experimental data. Many estimates of intracellular microviscosity have been published, the values ranging from 3–15 cP (centipoise). These data, obtained most recently by

electron spin resonance (see ref. 82) and fluorescence methods (see refs. 31, 79) have been analyzed in detail, particularly with regard to potential artifacts due to binding of the probe utilized. To our knowledge the enhanced microviscosity of cell water (compared to the pure liquid of 1 cP) has not been attributed to the possible difference in structure between cell water and pure water. In fact, two recent and highly sophisticated studies[31,78] do not even mention the possibility that an altered structure of intracellular water might account for part or all of the observed high values for intracellular microviscosity. It seems reasonable to propose that the reduction in cell water rotation and translation, and other evidence for enhanced hydrogen-bonding in intracellular water (see previous sections) might be a major cause for the increased microviscosity values obtained in intact cells.

VI. Cell volume, ion binding and cell water

It is a rather remarkable fact that cells somehow 'know' what their volume should be under ordinary physiological circumstances. Equally remarkable, it seems that we do not know precisely how they measure this parameter. A vast literature has been produced on the matter of cell volume and its regulation (for recent literature see the book edited by Kleinzeller[72], and the reviews of Chamberlin and Strange[16], and Hoffmann and Simonsen[65]). In virtually all these studies (but see Macknight[80] for a very thoughtful and objective exception) it is assumed that the properties of intracellular water are identical to those of the external medium and that cell volume is achieved and maintained by balancing the external and internal osmotic pressure through accumulation or release of inorganic ions (notably Na^+, K^+, and Cl^-) and/or compatible solutes such as primary amines and polyhydroxyalcohols (see Somero[96]). There is certainly good evidence for that contention. However, we note that according to this view the *properties* of cell water are not even considered in explanations for cell volume and its regulation. In this regard it is important to note that an increasing body of evidence indicates that a substantial fraction of the inorganic ions in cells may not contribute to the colligative properties of cytoplasm — that is, they are 'bound'. This evidence comes from a number of different authors (many of whom have no 'ax to grind') and a wide array of studies, ranging from NMR and electron microprobe studies, to fluorescence ratio imaging, ion efflux and electron microscopy (see books[76,85], and articles[14,25,59,63] for literature coverage). Although doubt exists concerning the precise fraction of free versus bound ions, it appears reasonable to suspect that on the order of one-half the total inorganic ion complement is 'bound' in the sense that this fraction is not freely diffusing in cells. This has considerable significance to the issue of cell volume and the properties of cell water. Thus, in the case of mammalian cells, Cl^-, K^+, and Na^+ are considered to be the overwhelming contributors to intracellular osmotic pressure. Indeed, the sum of these three ions, roughly 250 mM, is commonly thought to balance most of the external osmotic pressure (about 300 mOsm/kg water). But if about 50% of these ions do not contribute to intracellular osmotic pressure then we must ask how the cell can be

at osmotic equilibrium? Negendank[85] has considered this question at some length, and has argued that the answer must involve the properties of cell water, a contention with which we agree[23]. The implication of this analysis is that solutes dissolved in cell water may exert higher osmotic pressure than those same solutes in the external aqueous phase. An inescapable consequence of this explanation is that the structure of cell water must also be different. Given the evidence on ion binding, and the absence of significant mechanical resistance to cell volume changes near 'osmotic equilibrium' in animal cells, there seems to be no other reasonable explanation (see, however, the interesting analysis by Lechene[75], and by Bereiter-Hahn and Strohmeier[7]). It will be interesting to see what the future holds for these proposals, and for the traditional explanation of cell volume and its regulation.

VII. Some additional consequences of the properties of cell water

The preceding section certainly indicates that our thinking about at least one aspect of cell physiology would have to be altered appreciably if, as we suggest, the properties of cell water are not the same as those in the external medium. But there are other consequences worth noting:

1. Much of what is known about macromolecular function in cells is based on data obtained *in vitro*, almost always in highly dilute aqueous solutions. That approach is very convenient, but if intracellular water differs from that in test tubes, as we believe it does, then information obtained *in vitro* may not allow us to construct (or better 'reconstruct') an accurate description of these molecules and their activities when they operate *within* cells. We agree with Albrecht-Buehler[2] that reductionistic approaches are powerful, but they can never alone provide us with a reliable description of cells. His review of this issue is powerful and highly recommended.

2. It is widely accepted that direct interactions between macromolecules and their surrounding water of hydration play critical roles in their structure and function. There is no debate about this issue, and it seems very likely that water plays subtle but important roles in metabolism through water–enzyme interactions. However, to understand those roles we must know the details of the aqueous microenvironment in which this activity occurs.

3. Available evidence suggests, to us at least, that the solvent properties of at least a large fraction of the total cell water, notably in cytoplasm, differ from those of ordinary aqueous solutions. On this basis, some contribution to the uneven distribution of certain solutes across the plasma membrane, as well across membranes *within* cells (organelles), could arise from such solvent differences. In addition, small metabolites might 'partition' between various intracellular aqueous phases[55]. Even protein distribution within cells could be influenced in this fashion. A speculative 'model' on the organization of enzymes in the aqueous cytoplasm includes the possibility that a loose association of enzymes with the cytomatrix may

be driven by water interactions involving their respective surfaces, similar to those involved in association through hydrophobic interactions[19].

4. Assembly–disassembly processes are influenced by the properties of the aqueous phase within which they occur. Such mechanisms could be critical to enzyme–enzyme associations and the dynamic turnover of the cytomatrix, and possibly other cell structures. Watterson's[105] analysis, speculative though it may be, is worth careful study in this regard.

5. Many molecular interactions in cells involve electrostatic interactions which are, of course, very sensitive to the dielectric properties of the aqueous phase in which they occur. Thus, the possibility that the dielectric permittivity of cell water is reduced (Table 2, 24) relative to dilute solutions, may be of some importance.

6. A reasonably good correlation exists between modifications in the cytomatrix and changes in the amount and properties of cell water, both of which commonly, although not always, accompany cell transformation by viruses or carcinogens. While that may be fortuitous, it is notable that the usual observation is a reduction in cytomatrix surface area and an increase in the amount of cell water that has 'bulk-like' properties (see refs. 22, 60). That is consistent with the proposed relationship between the cytomatrix and its effects on the properties of the surrounding aqueous environment, and vice versa. It has also not escaped our attention[19,20,25] that many of the metabolic changes accompanying the transformation process are associated with 'soluble' enzymes which, in the view of some of us, are not really 'soluble' at all but are instead part of the water-cytomatrix system.

7. Without the concept of vicinal water, and its characteristic thermal anomalies at several different temperatures of physiological interest, it is difficult to see how a large body of usual thermal responses of organisms can be explained completely. On the other hand, accepting the thermal transitions in the vicinal water structures allow for relatively facile explanations of (sometimes dramatic) complex thermal responses of organisms including some very abrupt thermal death limits, selection of body temperatures and multiple temperature growth optima.

VIII. Concluding comments

Because the traditional (consensus) view on intracellular water considers it to be identical to that in dilute aqueous solutions we have chosen to concentrate here on the body of evidence that is not in accord with that opinion. As George Bernard Shaw put it: 'An idea that no one believes cannot be proved too often.'

Our position has been that the scientific community, most of whose research is not directly concerned with the matter, should be informed that alternative positions, based on substantial evidence, have been taken by a number of investigators. Perhaps more importantly, we believe that the consequences of altered cell water to virtually every aspect of cell structure and function could be extremely important, and would have great impact on the way we view cells and their activities. If nothing else, we hope that more attention will be given to the matter: there is sufficient reason to be concerned about paradigms of cell biology built on

the assumption that the intracellular aqueous phase(s) is no different than, essentially, pure water. At the same time we recognize that the body of existing evidence, only some of which has been dealt with here, has apparently not been sufficiently compelling to change matters, or simply overlooked. Thus, we also recognize that the assumption of ordinary cell water has, thus far, worked surprisingly well in the development of contemporary thought in cell biology. Is this assumption a reasonable one? Can we consider paradigms built upon it as adequate first-approximations, as almost right? Perhaps so, but we are reminded of the saying by Mark Twain that 'the difference between the right... and the almost right... is the difference between lightning and lightning bug.'

Acknowledgments. We thank Professor Keith Porter for provision of HVEM photomicrographs and Diane Cosgrove for her usual skill and patience with manuscript preparation. The expense of publication came from NSF Grant DCB 8820347 (to J.S.C.). Most importantly, we dedicate this paper to Professor Carlton Hazlewood who, for over 25 years, has championed the importance of intracellular water and dedicated his research to its study.

IX. References

1. Adamson, A.W. *Physical Chemistry of Surfaces*. New York: Wiley, 1982.
2. Albrecht-Buehler, G. In defense of 'nonmolecular' cell biology. *Int. Rev. Cytol.* 120: 191–241, 1990.
3. Alpers, W. and H. Hühnerfuss. Molecular aspects of the system water/monomolecular surface film and the occurrence of a new anomalous dispersion regime at 1.43 GHz. *J. Phys. Chem.* 87: 5251–5258, 1983.
4. Arnheim, R. *Observations on Psychology, the Arts, and the Rest*. Berkeley: University of California Press, 1989, p. 369.
5. Barnes, P. Machine Simulation of Water. In: *Progress in Liquid Physics* edited by C.A. Croxton. New York: Wiley, 1978, pp. 391–428.
6. Benga, G. *Water Transport in Biological Membranes* Vol. 1. Boca Raton: CRC Press, 1989, p. 1–223.
7. Bereiter-Hahn, J. and R. Strohmeier. Hydrostatic pressure in metazoan cells in culture. In: *Cytomechanics*, edited by J. Bereiter-Hahn, O.R. Anderson and W.E. Reif. Berlin: Springer-Verlag, 1987, p. 261–272.
8. Blum, J.J., G. Lawler, M. Reed, and I. Shin. Effect of cytoskeletal geometry on intracellular diffusion. *Biophys. J.* 56: 995–1005, 1989.
9. Blumberg, R.L., H.E. Stanley, A. Geiger, and P. Mausbach, Connectivity of hydrogen bonds in liquid water. *J. Chem. Phys.* 80: 5230–5241, 1984.
10. Bridgman, P.C. and T.S. Reese. The structure of cytoplasm in directly frozen cells. I. Filamentous meshworks and the cytoplasmic ground substance. *J. Cell Biol.* 99: 1655–1660, 1984.
11. Brooks, C.L. and M. Karplus. Solvent effects on protein motion and protein effects on solvent motion. *J. Mol. Biol.* 208: 159–181, 1989.
12. Brown, R.A., P. Sorgeloos, and C.N.A. Trotman. *The Biology of Artemia*. Boca Raton: CRC Press, 1990, in press.
13. Cameron, I.L., W.E. Hardman, K.E. Hunter, C. Haskin, N.K.R. Smith, and G.D. Fullerton. Evidence that a major portion of cellular potassium is bound — a review paper. *Scan. Microsc.* 4: 89–102, 1990.
14. Cameron, I.L., P. Merta, and G.N. Fullerton. Osmotic and motional properties of intracellular water as influenced by osmotic swelling and shrinking of *Xenopus* oocytes. *J. Cell. Physiol.* 142: 592–602, 1990.
15. Cañedo, L.E., L.E. Todd, L. Packer, and J. Jaz. *Cell Function and Disease*. New York: Plenum, 1988, pp. 1–524.

16. Chamberlin, M.E. and K. Strange. Anisosmotic cell volume regulation: a comparative view. *Am. J. Physiol.* 257:C159-C173, 1989.
17. Churev, N.V. and B.V. Deryaguin. Inclusion of structural forces in the theory of colloids and films. *J. Coll. & Interface Sci.* 103: 542–553, 1985.
18. Clifford, J. Properties of water in capillaries and thin films. In: *Water — a Comprehensive Treatise*, Vol. 5, edited by F. Franks. New York: Plenum, 1975, pp. 75–132.
19. Clegg, J.S. Metabolism and the intracellular environment: the vicinal-water network model. In: *Cell-Associated Water*, edited by W. Drost-Hansen and J.S. Clegg. New York: Academic Press, 1979, p. 363–413.
20. Clegg, J.S. Properties and metabolism of the aqueous cytoplasm and its boundaries. *Am. J. Physiol.* 246:R133-R151, 1984.
21. Clegg, J.S. On the physical properties and potential roles of intracellular water. In: *Regulation of Cell Metabolism*, edited by G.R. Welch and J.S. Clegg. New York: Plenum, 1986, pp. 41–55.
22. Clegg, J.S. On the internal environment of animal cells. In: *Microcompartmentation*. Boca Raton: CRC Press, 1988, pp. 1–16.
23. Clegg, J.S. L-929 cells under hyperosmotic conditions: water, Na+and K+. *Cell Biophys.* 13: 119–132, 1988.
24. Clegg, J.S., S. Szwarnowski, V.E.R. McClean, R.J. Sheppard, and E.H. Grant. Interrelationships between water and cell metabolism in *Artemia* cysts. X. Microwave dielectric studies. *Biochim. Biophys. Acta* 721: 458–468, 1982.
25. Clegg, J.S. and M.B. Barrios. The 'cytosol': a neglected and poorly understood compartment of eukaryotic cells. In: *Cell Function and Disease*, edited by L.E. Cañedo, L.E. Todd, L. Packer and J. Jaz. New York: Plenum, 1988, pp. 159–170.
26. Cope, F. Nuclear magnetic resonance evidence using D_2O for structured water in muscle and brain. *Biophys. J.* 9: 303–319, 1969.
27. Deryaguin, B.V. Recent research into the properties of water in thin films and micro-capillaries. In: *The State and Movement of Water in Living Organisms*, XIX Sympos. Soc. Exp. Biol. Cambridge: Cambridge Univ. Press, 1964, pp. 55–60.
28. Deryaguin, B.V. and N.V. Churev. Structural component of disjoining pressure. *J. Coll. Interface Sci.*, 1974, 49: 249–255.
29. Deryaguin, B.V., V.V. Karasev and E.N. Khromova. Thermal expansion of ordinary and heavy water in the pores of titanium dioxide. *J. Coll. Interface Sci.* 78: 274, 1980.
30. Deryaguin, B.V., V.V. Karasev and E.N. Khromova. Thermal expansion of water in fine pores. *J. Coll. Interface Sci.*, 109: 586–587, 1986.
31. Dix, J.A. and A.S. Verkman. Mapping of fluorescence anisotropy in living cells by ratio imaging. Application to cytoplasmic viscosity. *Biophys. J.* 57: 231–240, 1990.
32. Drost-Hansen, W. Temperature anomalies and biological temperature optima in the process of evolution. *Naturwiss.* 43: 512, 1956.
33. Drost-Hansen, W. Aqueous interfaces — methods of study and structural properties. Part II, In: *Chemistry and Physics of Interfaces*, edited by S. Ross. Washington: American Chemical Society, 1965.
34. Drost-Hansen, W. The effects on biological systems of higher-order phase transitions in water. *Ann. N.Y. Acad. Sci.* 125 (Art. 2): 471–501, 1965.
35. Drost-Hansen, W. Allowable thermal pollution limits – a physico-chemical approach. *Chesapeake Sci.*, 10: 281–288, 1969.
36. Drost-Hansen, W. Structure and properties of water at biological interfaces. In: *Chemistry of the Cell Interface*, Vol. B., edited by H.D. Brown. New York: Academic Press, 1971, pp. 1–184.
37. Drost-Hansen, W. Effects of pressure on the structure of water in various aqueous systems. *S.E.B. Symp. Proc.* 26: 61–101, 1972.
38. Drost-Hansen, W. Molecular aspects of aqueous interfacial structure. *J. Geophys. Res.* 77: 5132–5146, 1972.
39. Drost-Hansen, W. Water at biological interfaces — structural and functional aspects. *Phys. Chem. Liq.* 7: 243–346, 1978.
40. Drost-Hansen, W. Role of vicinal water in cellular evolution. In: *Water and Ions in Biological Systems*, edited by A. Pullman, V. Vasilescu and L. Packer. New York, Plenum Press, pp. 523–534, 1985.
41. Drost-Hansen, W. Anomalous volume properties of vicinal water and some recent thermodynamic (DSC) measurements relevant to cell physiology. In: *Water and Ions in Biological Systems*, edited by A. Pullman, V. Vasilescu and L. Packer. New York: Plenum, pp. 289–294, 1985.

42. Drost-Hansen, W. and M. Lavergne. Discontinuities in slope of the temperature dependence of the thermal expansion of water. *Naturwiss.* 43: 511, 1956.
43. Drost-Hansen, W. and J.L. Singleton. Our aqueous heritage. I. Evidence for vicinal water in cells. II. Role of vicinal water in cells. In: *Foundations of Medical Cell Biology*, edited by E.E. Bittar. Greenwich, CN: JAI Press, in press.
44. Eisenberg, D. and W. Kauzmann. *The Structure and Properties of Water*. Oxford: Clarendon, 1969.
45. Etzler, F.M. A statistical thermodynamic model of water near solid interfaces. *J. Coll. Interface Sci.* 92: 43–56, 1983.
46. Etzler, F.M. Enhancement of hydrogen bonding in vicinal water: heat capacity of water and deuterium oxide in silica pores. *Langmuir* 4: 878–883, 1988.
47. Etzler, F.M. and W. Drost-Hansen. A role for vicinal water in growth, metabolism and cellular organization. *Adv. Chem. Am. Chem. Soc.* 188: 485–497, 1980.
48. Etzler, F.M. and W. Drost-Hansen. Recent thermodynamic data on vicinal water and a model for their interpretation. *Croatica Chemica Acta* 56: 563- 592, 1983.
49. Evans, D.F. and B.W. Ninham. Molecular forces in the self-organization of amphiphiles. *J. Phys. Chem.* 90: 226–234, 1986.
50. Finney, J.L. Towards a molecular picture of liquid water. In: *Biophysics of Water*, edited by F. Franks and S. Mathias. New York: Wiley, 1982, pp. 73–95.
51. Frank, H.S. Structural models. In: *Water — a Comprehensive Treatise, Vol. 1*, edited by F. Franks. New York: Plenum, 1972, pp. 515–543.
52. Franks, F. (editor). *Water — a Comprehensive Treatise, Vols. 1–7*. New York: Plenum Press, 1972–1982.
53. Reference deleted.
54. Frauenfelder, H. and E. Gratton. Protein dynamics and hydration. *Methods Enzymol.* 127: 207–216, 1986.
55. Garlid, K.D. Aqueous phase structure in cells and organelles. In: *Cell-Associated Water*, edited by W. Drost-Hansen and J.S. Clegg. New York: Academic Press, 1979, pp. 293–362.
56. Geiger, A. and H.E. Stanley. Low-density 'patches' in the hydrogen-bond network of liquid water. Evidence from molecular-dynamics computer calculations. *Phys. Rev. Lett.* 49: 1749–1752, 1982.
57. Gershon, N.D, K.R. Porter, and B.L. Trus. The cytoplasmic matrix: its volume, surface area and the diffusion of molecules through it. *Proc. Natl. Acad. Sci. U.S.A.* 82: 5030–5035.
58. Goldanskii, V.I. and Y.F. Krupyanskii. Protein and protein-bound water dynamics studied by Rayleigh scattering of Mössbaner radiation (RSMR). *Quart. Rev. Biophys.* 22: 39–92, 1989.
59. Harootunian, A.T., J.P.Y. Kao, B.K. Eckert, and R.Y. Tsien. Fluorescence ratio imaging of cytosolic free Na^+ in individual fibroblasts and lymphocytes. *J. Biol. Chem.* 264: 19458–19467, 1989.
60. Hazlewood, C.F. A view of the significance and understanding of the physical properties of intracellular water. In: *Cell-Associated Water*, edited by W. Drost-Hansen and J.S. Clegg. New York: Academic Press, 1979, pp. 165–260.
61. Hazlewood, C.F., B.L. Nichols, and N.F. Chamberlain. Evidence for the existence of a minimum of two phases of ordered water in skeletal muscle. *Nature*, Lond. 222: 747–750, 1969.
62. Hazlewood, C.F. and M. Kellermayer. Ion and water retention by permeabilized cells. *Scan. Microsc.* 2: 267–273, 1988.
63. Hazlewood, C.F. and M. Kellermayer. The state of potassium in skeletal muscle and in non-muscle cells. *Scan. Microsc.*, 3: 1241–1245, 1989.
64. Henn, A.R. and W. Kauzmann. Equation of state of a random network, continuum model of liquid water. *J. Phys. Chem.* 93: 3770–3783, 1989.
65. Hoffmann, E.K. and L.O. Simonsen. Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* 69: 315–382, 1989.
66. Horowitz, S.B. and D.S. Miller. Solvent properties of ground substance studied by cryomicrodissection and reference phase techniques. *J. Cell Biol.* 99: 172s–179s, 1984.
67. Hühnerfuss, H. Molecular aspects of organic surface films on marine water and the modification of water waves. *La Chimica L'Industria* 65: 97–101, 1983.
68. Hühnerfuss, H. and W. Wolfgang. The thermal anomaly of relaxation effects in monomolecular surface films. *J. Coll. Interface Sci.* 107: 476–480, 1983.
69. Israelachvili, J. *Intermolecular and Surface Forces*. New York: Academic Press, 1985.
70. Israelachvili, J. and G.E. Adams. Measurement of forces between two mica surfaces in aqueous electrolyte solutions in the range 0–100 nm. *J. Chem. Soc.* 74: 975–1001, 1977.
71. Israelachvili, J. and B.W. Ninham. Intermolecular forces – the long and the short of it. *J. Coll. Interface Sci.*, 57: 14–25, 1977.

72. Jones, D.P. *Microcompartmentation*. Boca Raton: CRC Press, 1988, pp. 1–261.
73. Kleinzeller, A. *Cell Volume Control: Fundamental and Comparative Aspects in Animal Cells*. New York: Academic Press, 1987, pp. 1–281.
74. Kondo, H. Reexamination of the reality or artifact of the microtrabeculae. *J. Ultrastruct. Res.* 87: 124–130, 1984.
75. Lechene, C. Cellular volume and cytoplasmic gel. *Biol. Cell.* 55: 177–180, 1985.
76. Ling, G.N. *In Search of the Physical Basis of Life*. New York: Plenum Press, 1984, pp. 1–791.
77. Low, P.F. Nature and properties of water in montmarillonite-water systems. *J. Soil. Sci.* 43: 174–191, 1979.
78. Luby-Phelps, K., D.L. Taylor, and F. Lanni. Probing the structure of cytoplasm. *J. Cell Biol.* 102: 2015–2022, 1986.
79. Luby-Phelps, K., F. Lanni, and D.L. Taylor. The submicroscopic properties of cytoplasm as a determinant of cellular function. *Ann. Rev. Biophys. Chem.* 17: 369–396, 1988.
80. MacNight, A.D.C. Volume maintenance in isomatic conditions. *Curr. Top. Memb. Transp.* 30: 3–43, 1987.
81. Macrae, T.H., J.C. Bagshaw, and A.H. Warner. *Biochemistry and Cell Biology of Artemia*. Boca Raton: CRC Press, 1989, pp. 1–264.
82. Mastro, A.M. and D.J. Hurley. Diffusion of a small molecule in the aqueous compartment of mammalian cells. In: *The Organization of Cell Metabolism*, edited by G.R. Welch and J.S. Clegg. New York: Plenum Press, 1986, pp. 57–74.
83. Misra, L.K., E.E. Kim, C.F. Hazlewood, and L.W. Dennis. Magnetic resonance relaxation times and imaging in the pathophysiology of muscle. In: *Cell Function and Disease*, edited by L.E. Cañedo, L.E. Todd, L. Packer, and J. Jaz. New York: Plenum Press, 1988, pp. 421–444.
84. Negendank, W. A cooperative transition theory applied to the kinetics of ion exchanges in cells. *Cell Biophys.* 13: 93–117, 1988.
85. Negendank, W. and L. Edelmann. *The State of Water in the Cell*. Chicago: Scanning Microscopy, 1988, pp. 1–114.
86. Ooi, T. and M. Oobatake. Intermolecular interactions between protein and other molecules including hydration effects. *J. Biochem.* 104: 440–444, 1988.
87. Packer, L. Biomembranes. Part O. Protons and Water. *Methods Enzymol.* 127: 1–830, 1986.
88. Paine, P.L. Diffusive and nondiffusive proteins *in vivo*. *J. Cell Biol.* 99: 188s–195s.
89. Peschel, G. and P. Belouschek. The problem of water structure in biological systems. In: *Cell-Associated Water*, edited by W. Drost-Hansen and J.S. Clegg. New York: Academic Press, 1979, pp. 3–52.
90. Porter, K.R. Structural organization of the cytomatrix. In: *The Organization of Cell Metabolism*, edited by G.R. Welch and J.S. Clegg. New York: Plenum Press, 1986, pp. 9–26.
91. Rand, R.P. and V.A. Parsegian. Hydration forces between phospholipid bilayers. *Biochim. Biophys. Acta* 988: 351–376, 1989.
92. Richards, W.G., P.M. King, and C.A. Reynolds. Solvation effects. *Protein Engineering* 2: 319–327, 1989.
93. Ris, H. The cytoplasmic filament system in critical point dried whole mounts and plastic embedded sections. *J. Cell Biol.* 100: 1474–1479, 1985.
94. Rorschach, H.E. and C.F. Hazlewood. Protein dynamics and the NMR relaxation time T1 of water in biological systems. *J. Magn. Reson.* 70: 79–88, 1986.
95. Rorschach, H.E.D.W. Beardon, C.F. Hazlewood, D.B. Heidorn, and R.M. Nicklow. Quasi-elastic scattering studies of water diffusion. *Scan. Microsc.* 1: 2043–2049, 1987.
96. Somero, G. Protons, osmolytes, and fitness of internal milieu for protein function. *Am. J. Physiol.* 251:R197–R213, 1986.
97. Stanley, H.E. and J. Teixeira. Interpretation of the unusual behaviour of H_2O at low temperatures: tests of a percolation model. *J. Chem. Phys.* 73: 3404–3422, 1980.
98. Stanley, H.E. and J. Teixeira. Are concepts of percolation and gelation of possible relevance to the behaviour of water at very low temperatures? *Ferroelectrics* 30: 213–226, 1980.
99. Stillinger, F.H. Water revisited. *Science* 209: 451–457, 1980.
100. Szent-Györgyi, A. Biology and the pathology of water. *Persp. Biol. Med.* 13: 239, 1971.
101. Warner, A.H., T.H. MacRae, and T.C. Bagshaw. *Cellular and Molecular Biology of Artemia Development*. New York: Plenum Press, 1989, pp. 1–431.
102. Watterson, J.G. Does solvent structure underlie osmotic mechanisms? *Phys. Chem. Liq.* 16: 313–316, 1987.
103. Watterson, J.G. Solvent cluster size and colligative properties. *Phys. Chem. Liq.* 16: 317–320, 1987.
104. Watterson, J.G. A role for water in cell structure. *Biochem. J.* 248: 615–617, 1987.

105. Watterson, J.G. The role of water in protein function. *Progr. Mol. Subcell. Biol.*, in press.
106. Welch, G.R. and J.S. Clegg. *The Organization of Cell Metabolism*. New York: Plenum Press, 1986, pp. 1–389.
107. Wiggins, P.M. Water structure in polymer membranes. *Prog. Polym. Sci.* 13: 1–35, 1988.
108. Wiggins, P.M. and B.A.E. MacClement. Two states of water found in hydrophobic clefts: possible contribution to mechanisms of cation pumps and enzymes. *Int. Rev. Cytol.* 108: 249–303, 1987.
109. Wiggins, P.M. and R.T. Van Ryn. Changes in ion selectivity with changes in density of water in gels and cells. *Biophys. J.*, 58: 585–596, 1990.
110. Zimmerman, S., I.L. Cameron, and A.M. Zimmerman. The relaxation state of water in sea urchin eggs. In: *The Cell Biology of Fertilization*, edited by H. Schatten and G. Schatten. New York: Academic Press, 1989, pp. 319–340.