Scanning Microscopy

Volume 2 | Number 2 Article 22

2-14-1988

Solute Exclusion by Polymer and Protein-Dominated Water: Correlation with Results of Nuclear Magnetic Resonance (NMR) and Calorimetric Studies and Their Significance for the Understanding of the Physical State of Water in Living Cells

G. N. Ling
Pennsylvania Hospital

Scanning Microscopy, Vol. 2, No. 2, 1988 (Pages 871-884) 0891-7035/88$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

SOLUTE EXCLUSION BY POLYMER AND PROTEIN-DOMINATED WATER: CORRELATION WITH RESULTS OF NUCLEAR MAGNETIC RESONANCE (NMR) AND CALORIMETRIC STUDIES AND THEIR SIGNIFICANCE FOR THE UNDERSTANDING OF THE PHYSICAL STATE OF WATER IN LIVING CELLS

G. N. Ling*

Molecular Biology Department, Pennsylvania Hospital
Philadelphia, Pennsylvania 19107

(Received for publication May 02, 1987, and in revised form February 14, 1988)

Abstract

According to the polarized multilayer (PM) theory of cell water proteins with their backbones fully extended and their NHCO groups directly exposed to bulk water, polarize water in multilayers. Experimental testing of the theory led to a new understanding of the uniqueness of gelatin, due to its permanently maintained fully extended conformation and its ability to polarize the bulk phase water in multilayers with reduced solubilities for solutes in a size dependent manner (" size rule"). Other models which behave like gelatin are urea-denatured proteins, synthetic polymers like polyethylene oxide (PEO), and polyvinylpyrrolidine (PVP), but not native proteins. NMR studies showed that the majority of water molecules dominated by these polymers does indeed suffer rotational (and translational) motional restriction as predicted by the PM theory. In conjunction with ultra-high frequency dielectric studies but particularly quasielastic neutron scattering of both model systems (e.g., PEO) and living cells (i.e., brine shrimp cysts and frog muscle), this finding offers confirmation of the PM theory of living cell water and model systems.

Studies of the freezing point depression showed that the presence of as much as 50% of native proteins had no effect on the freezing point of water while inclusion of gelatin, PEO, etc., caused concentration-dependent lowering of the freezing temperature. These findings demonstrate the key role of polarized water in the phenomena of freezing point depression and the unusual ice forms seen in living cells.

KEY WORDS: Solute exclusion, polymers, cell water, proteins, freezing point depression, nuclear magnetic resonance (NMR), T_1, T_2, rotational correlation time, association-induction hypothesis, polarized multilayers

*Address for correspondence:
G. N. Ling, Molecular Biology Department, Pennsylvania Hospital, Eighth and Spruce Streets, Philadelphia, Pennsylvania 19107, USA
Phone No. 215-829-3261

Introduction

The basic unit of all living matter is the cell. To understand the living phenomena thus requires the correct knowledge of both the structure and functions of the constituent parts of the living cell. In acquiring this knowledge, modern electron microscopy has already made great contribution in many different ways. As an example, electron microscopy provided the pivotal evidence of the localized distribution of the major cell cation K in living cells. The finding has thrown serious doubts on the conventional theory of the living cells (the membrane-pump theory) which required K^+ be distributed freely and evenly in all cell water. This communication presents an alternative theory of the living cells (the association-induction hypothesis) in which the newly established localized distribution of cell K^+ is part and parcel of a coherent, comprehensive theory of living cells, a theory that agrees also with many other new discoveries including those concerning the unusual physical state of the most abundant component of all living cells, water.

The Bound Water Concept, Its Evidence and Apparent Disproof

Since long ago, biologists have repeatedly observed that the extruded cytoplasm of plant and animal cells does not mix with water (von Nägeli, 1855; Kühne, 1864).

This immiscibility led some investigators to the belief that water in the cell might be bound to the solid components of the cells (Fischer and Suer, 1935, 1938). In agreement with this view, scientists in the 1920's and early 1930's, demonstrated that water associated with biological materials often did not freeze at temperatures far below the freezing point of water (i.e., -20° C) (Rubner, 1922; Thoenes, 1925; Jones and Gortner, 1932). Another type of experimental data in support of the bound water concept was based on the assumption that bound water does not dissolve solutes and is thus " non-solvent". Thus if a known quantity of sucrose was added to a biological fluid, the freezing point depression would be lower than if all the water was available to dissolve sucrose. In agreement with the assumption, lower freezing point depressions

were observed (Newton and Gortner, 1922; Gortner, 1937).

These early efforts to demonstrate bound water in biological systems met with strong resistance. The first type of evidence cited against the existence of non-solvent bound water in living cells was the demonstration that urea distributes itself equally between water in muscle cells and in the external solution (Hill, 1930). Similarly ethylene glycol also distributes itself equally between water in erythrocytes and in water of the surrounding media (Mac Leod and Ponder, 1936). The second type of negative evidence was based on the fact that sometimes pure water could be supercooled to -20° C and the assertion that the presence of high concentrations of proteins in living cells and biological fluids made it not possible to accurately determine the freezing point of water. Thus the validity of cryoscopic demonstration of freezing point lowering was seriously doubted (Blanchard, 1940).

The impacts of these findings and arguments were so great that the concept of bound water and with it the colloidal approach to the understanding of living phenomena all but came to an end since the 40's. To many, colloidal chemistry appeared to be nothing more than the chemistry of macromolecules (Ferry, 1948).

Bound Water Concept Survived Under Different Management

The defeat of the champions of the colloidal approach to water in biological systems did not end the studies of water binding to proteins, a subject which was continued by protein chemists using purer proteins and with more precise methods. Concerning the fundamental question regarding the sites of hydration on the protein molecules two opposing theories were proposed. In one theory, proposed by Pauling (1945), only polar side chains offered the seat of hydration. (The backbone NHCO groups do not adsorb water.) Pauling also believed that only the first layer of water taken up by proteins is adsorbed; additional water taken up was only normal free liquid water condensed on the surface of the first layer. Based on Bull's data of water sorption on proteins (Bull, 1944) Pauling showed that by and large the amount of water sorbed on the first layer corresponds to the number of polar side chains of each protein examined.

An alternative theory was proposed earlier by Lloyd (1932). In this theory, both polar side chains and the imino and keto groups of the backbone are the seats of hydration. Strong support for this view was provided by Mellon, Korn, and Hoover (1948) who showed that polyglycine esters containing only polar groups in the form of backbone NHCO groups, nevertheless sorb a large quantity of water.

A Possible Solution of an Apparent Paradox

The theories of Pauling and Lloyd were mutually exclusive: in one the backbone NHCO group sorbs water; in the other they do not sorb water. Yet at the same time evidence continued to accumulate in support of both theories. Sorting out the relevant publications, it dawned on me that work supporting Pauling's theory came primarily from biochemists working mostly with purified, and often crystalline globular proteins. Those supporting Lloyd's theory as a rule came from industry with primary interests in fibrous proteins. Thus the paradox was clearly apparent and it might be resolved by postulating that in native globular proteins, especially at very low humidity where the first layer of water is sorbed, the seat of hydration is indeed primarily the polar side chains; in fibrous proteins, on the other hand, both polar side chains and backbone NHCO are the seats of water binding (Ling, 1972).

The Polarized Multilayer Theory of Cell Water

That gaseous molecules can adsorb in multilayers has been known for a long time. In 1929, de Boer and Zwikker proposed a quantitative "polarization theory" of multilayer adsorption on solid surfaces described by the following equation:

$$\log \left(\frac{p}{p_o}\right) = K_2 K_1^{\,a} + K_3, \qquad (1)$$

where p is the vapor pressure of the gas under study and p_o is the vapor pressure at full saturation under the same conditions. a is the amount of vapor sorbed. K_1, K_2, and K_3, are constants in a defined environment. Later Bradley (1936) derived a formally identical polarized multilayer adsorption isotherm. The difference between the two equations of identical form is that in Bradley's derivation, the permanent dipole moment of the gaseous molecules was taken into account. Since water has a large permanent dipole moment ($\mu = 1.87 \times 10^{-18}$ e.s.u.), which plays a key role in the building up of polarized multilayers (Brunauer et al., 1938), the Bradley isotherm is a more appropriate choice. Nevertheless the first derivation was due to de Boer and Zwikker (1929). Of particular interest in the application to water sorption, was de Boer and Zwikker's clear recognition of the important role of alternatingly positive and negative neighboring sites in producing stable multilayers of adsorbed molecules.

In 1965 the polarized multilayer theory of cell water was first introduced (Ling, 1965). In 1972, I suggested that it is the exposed positively charged NH (P) and negatively charged CO (N) groups of cell proteins that are the primary seats of multilayer adsorption of the cell water. Arranged in more or less parallel arrays these fully extended polypeptide chains constitute a matrix (called an NP-NP-NP system) that polarizes (all) the cell water into multilayers.

In 1970, Ling and Negendank demonstrated that in surviving frog muscle cells, 5% of the cell water is bound tightly and singly following a Langmuir type of adsorption isotherm. The remaining 95% of the cell water follows the Bradley multilayer adsorption (Ling and Negendank, 1970).

The Theory of Solute Exclusion from Water Existing as Polarized Multilayers

Na^+, sugars, and free amino acids are examples of solutes found, as a rule, at lower

concentrations inside the cell water than in the external medium. Early attempts to explain their exclusion from the cells in terms of impermeable cell membranes with limiting membrane pore size was disproved when radioactive isotopes made possible the demonstration of continual exchange of these solutes between the cell and its environment (Ling, 1984). Membrane pumps were then installed to keep Na^+ and other solutes at levels different from that found in the external medium. In due time, this hypothesis has also been shown to be untenable (see Ling, 1984, 1988a, b).

According to a new theory of the living cells called the association-induction (AI) hypothesis, which I first introduced in 1962 (Ling, 1962) and completed in 1965 (Ling, 1965) many solutes are maintained at low concentrations within the cells because they have low solubility in the cell water which exists in the state of polarized multilayers. Theoretically, there are two basic physical mechanisms; one or both may be operative to maintain a low concentration in the polarized water.

(i) Enthalpy mechanism: more energy (or more precisely, enthalpy) is required to dig a hole in the polarized water to accommodate the solute than the enthalpy gained in filling the hole left behind in the external solute. This mechanism is size dependent; the larger the solute molecule, the greater is the unfavorable enthalpy difference and hence the lower the solubility (for details, see Ling, 1984, p. 171).

(ii) Entropy mechanism: Water molecules in polarized multilayers are in general more stationary and also form stronger H-bonds. As a result, solutes dissolved in polarized water will be more restricted in their motional freedoms (especially in rotational motional freedom) than in normal liquid water. This loss of entropy also reduces solubility in the polarized water. Since the larger and more complex the molecules, the larger the share of rotational entropy, the entropy loss increases with molecular size and complexity.

Since both the entropic and enthalpic mechanisms of solute exclusion are size-dependent, the theory predicts what is called " the size rule", i.e., the degree of exclusion increases with increasing size of the molecules of similar type. Indeed very small molecules and molecules that can fit into the dynamic water structure of polarized multilayers may not be excluded at all.

For the quantitative representation of the solute exclusion properties, two parameters are introduced:

The equilibrium distribution coefficient or q-value represents the ratio of the equilibrium concentration of the solute in the polarized water over the concentration in the external normal liquid water.

The apparent equilibrium distribution coefficient or ρ-value represents the ratio of the equilibrium concentration of the solute in the cell over the concentration in the external water. If all of the solute is within cell water, the ρ-value equals the q-value. If some of the solute is adsorbed onto proteins, the ρ-value is greater than the q-value.

Experimental Confirmation of the Prediction of the Theory of Solute Exclusion

Since only protein molecules existing in the fully extended conformation with their NH and CO groups directly exposed to the bulk-phase water, can polarize the bulk phase water in multilayers; native proteins with their backbone NHCO groups locked in α-helical, β-pleated sheet or other inter- or intra-macromolecular H-bonds are not expected to have a great deal of influence on the solvency of the bulk phase water. On the other hand, if for structural reasons (e.g., gelatin, see below) or in response to secondary structure breakers (e.g., urea, guanidine HCl), a protein exists in the fully extended conformation, the q-values of large molecules (e.g., sucrose) or hydrated ions (e.g., Na^+ salts) will be reduced. In contrast, denaturants like sodium dodecyl sulfate which break tertiary structure but not α-helical or other secondary structure, are expected to have no or minimal effect on the q-values of sucrose, Na salts, etc.

All of these predictions have been experimentally verified. Since these experimental data have been published and repeatedly reviewed (Ling et al., 1980a, b); Ling, 1984; Ling and Ochsenfeld, 1987, and my companion paper in this volume) only a few key observations will be briefly mentioned here: (1) Gelatin. In 1861 Thomas Graham coined the term colloid, after gelatin (κολλα , glue or gelatin). One hundred and twenty years later we began to understand why gelatins differ from other proteins (i.e., an unusual amino acid composition comprising of nonhelix-forming proline, hydroxyproline and glycine leading to the maintenance of a permanent fully extended conformation) and what gelatin does that other proteins do not (i.e., polarize water in multilayers). As an illustration Fig. 1 shows the exclusion of Na citrate and sulfate by gelatin of different concentrations; (2) Polarized Water that Excludes Na^+ Salt and Sugar Does Not Exclude Urea or Ethylene Glycol. Earlier I have shown how the demonstration of equal distribution of urea and ethylene glycol between cell water and water in the external aqueous medium led to the widely accepted belief that there is no bound (nonsolvent) water in living cells (Hill, 1930; Mac Leod and Ponder, 1936). We have now shown that proteins denatured by 9 M urea have reduced solubility for sucrose, glycine, and Na salts (Ling et al., 1980a). Nevertheless the same water does not exclude urea (i.e., ρ-value for urea in urea-denatured protein solution equals 0.99). Other experimental studies indicate that water polarized by oxygen-containing polymers (see below) demonstrating strong exclusion for Na^+ salts, etc. does not exclude ethylene glycol or glycerol. In fact, their ρ-value in the polarized water was found to exceed 1.0 (Ling, to be published).

In conclusion, the near unity ρ-values for urea and ethylene glycol does not prove normalcy of cell water; they only disproved the as yet imperfect theory of bound water as possessing no solvency at all for all solutes. The new findings show that the solvency varies with the solute, generally following the " size rule"; (3)

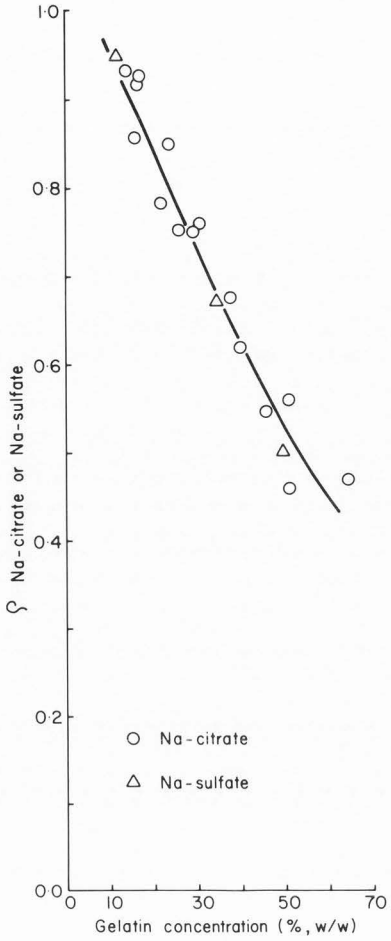
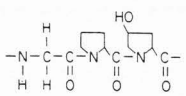
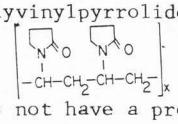


Fig. 1: The apparent equilibrium distribution coefficient (ρ-value) of Na citrate between various concentrations of gelatin solutions in the bags and in the external solution (37° C). The higher temperature was to keep the gelatin in the same fluid state as in other polymers. [from Ling and Ochsenfeld, 1983, by permission of Physiol. Chem. Phys. and Med. NMR].

Water Polarization by Oxygen-containing Synthetic Polymers. The striking features of gelatin in its amino acid composition are the possession of a large percentage of nonhelix-forming proline (11.7%), hydroxyproline (9.2%), and glycine (32.5%). The mechanism underlying glycine's well-known helix-breaking potential (Chou and Fasman, 1974) was unknown until recently, with the recognition of the role of inductive effect in the determination of secondary protein structure by its amino acid composition (Ling, 1986). However, the non-H-bond forming trait of proline and hydroxyproline is more straightforward, i.e., the lack of a proton on their peptide N atoms. Thus a triad of proline, hydroxyproline, and glycine has the following configuration



Now a polymer of glycine (only) has already been shown by Mellon et al. to adsorb water in multilayers. A synthetic polymer that may be regarded as a model of proline and hydroxyproline polymers is polyvinylpyrrolidone (PVP),



which also does not have a proton on the N atom of the pyrrole ring. Fig. 2 shows that indeed solutions of PVP, like gelatin, exclude Na citrate, only more effectively.

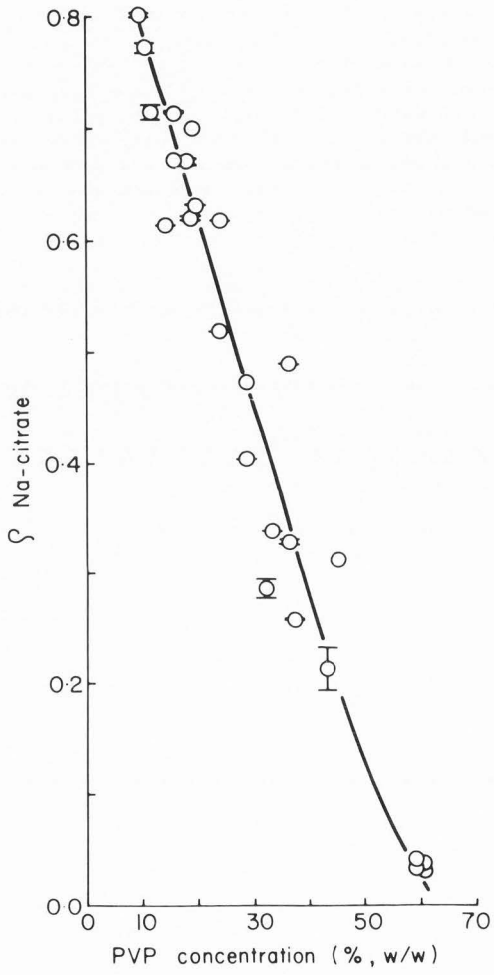


Fig. 2: The apparent equilibrium distribution coefficient (ρ-value) of Na citrate between various concentrations of PVP solutes in the bag and in the external solution at various concentrations of PVP (25° C). [from Ling and Ochsenfeld, 1983, by permission of Physiol. Chem. Phys. and Med. NMR].

The ability of PVP solutions to exclude large solutes has the following additional significance: (1) Solvency Reduction Does Not Depend on the Assumption of a Gel or Coacervate State. Unlike gelatin, PVP does not form gels, but is in the form of a viscous solution. The effectiveness of

PVP solution to exclude large solutes therefore shows that gel-formation (which I at one time believed to be an integral part of solute exclusion mechanism, Ling, 1972) and " coacervate" formation (which Troshin (1966) associated with solute exclusion) are both nonessential; (2) Solvency Reduction Propoduced by NO-NO-NO System: The behaviors of PVP are paralleled by a number of other oxygen-containing synthetic polymers, including polyethylene oxide (PEO) $(-CH_2CH_2O-)_n$, polyethylene glycol (PEG) $H(OCH_2CH_2)_nOH$ and

polyvinylmethyl ether (PVME) $(-CH(OCH_3)-CH_2-)_n$, with a

similar strong ability to sorb and polarize in multilayers large amounts of water. Solutions of oxygen-containing polymers like PVP, PEO, and PVME are not NP-NP-NP systems but are all NO-NO-NO systems, since the negative oxygen atoms are separated from their immediate oxygen neighbors not by positive P sites but by neutral or vacant (O) sites (see Ling and Ochsenfeld, 1983); (3) The Size Rule: Solute exclusion by oxygen-containing synthetic polymers, like their living cell counterparts, obey the " size rule" (Ling et al., 1980b; Ling, 1984).

Nuclear Magnetic Resonance (NMR) Characteristics of Polarized Water in Living Cells and Model Systems

When water molecules are placed in a steady magnetic field, and an oscillating electromagnetic field of the proper frequency is applied in a perpendicular direction to the static magnetic field, the hydrogen nucleus of the water molecule will undergo a transition into an excited state. If the oscillating electromagnetic field of suitable frequency is now suddenly turned off, the excited hydrogen nuclei will return to equilibrium with the environment (referred to as the " lattice") with a first order exponential time course. The time constant of this exponential decay is called spin lattice relaxation time or T_1.

If the static magnetic field is held constant, the hydrogen nuclei are excited only if the applied perpendicular electromagnetic field matches the natural or Larmor frequency (ω_o) of the hydrogen atom. Theoretically, one might expect that the energy absorption will occur at a sharply defined frequency. In fact, it is a wider band of frequencies, due largely to Heisenberg's Uncertainty Principle. For gaseous and nonviscous liquids the observed signal width (or line width) along a changing frequency scale is of the order of T_1^{-1}. However in solid ice, the line width is much wider. If the $\upsilon_{\frac{1}{2}}$ represents the linewidth at the half maximum height of the proton signal, then

$$T_2 = \frac{1}{\pi \upsilon_{\frac{1}{2}}} , \qquad (2)$$

where T_2 is another relaxation time called spin-spin relaxation time. The reason that the linewidth is so much broader and T_2 so much shorter than T_1 in solid ice than in water vapor or liquid is the much larger spread of the Larmor frequencies of the different protons of the sample. This arises from contributions to the applied static magnetic field by local magnetic fields produced by the rigidly held neighboring hydrogen nuclei. In gaseous or liquid water, the tumbling and constant motion of the neighboring protons cancel out this local field effect.

Water molecules in the gaseous or liquid state move rapidly; water molecules in the solid ice state move much more slowly. A parameter used to measure this difference is the rotational correlation time τ_c, which equals the time needed for a molecule to rotate through an angle equal to 1 radian (the angle at the center of a circle subtended by an arc equal in length to the radius of the circle).

Fig. 3 shows the theoretical relationship between T_1, T_2, and τ_c. Note that T_1 has a minimum; its position varies with the field frequency. Below this minimum, T_1 and T_2 are equal; above it, T_1 and T_2 are different.

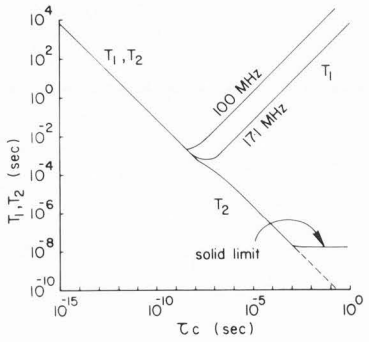


Fig. 3: Theoretical curves of T_1 and T_2 of water proton at various rotational correlation times, τ_c calculated from the equations of Bloembergen et al. (1948) as modified by Kubo and Tomita (1954). [from Ling and Murphy, 1983, by permission of Physiol. Chem. Phys. and Med. NMR]

Testing the Polarized Multilayer Theory of Cell Water by NMR

According to the polarized multilayer theory of cell water, virtually all cell water exists in the state of polarized multilayers and as such the water molecules suffer rotational (and translational) motional restriction (Ling, 1965, 1972, 1984). The NMR correlation time τ_c, measures the rotational motion. As shown in Fig. 3, a different τ_c gives rise to a different T_1 and T_2. The determination of T_1 and T_2 thus in theory offers a means of testing the polarized multilayer theory of cell water.

With this specific purpose in mind Cope (1969) and Hazlewood et al. (1969) began their NMR studies of cell water. In agreement with results of earlier studies of Odeblad (1957) and others, Cope and Hazlewood found that T_1 and T_2 of water in living cells were different from those of normal liquid water. From a large variety of

living tissues studied, T_1 was found to be often reduced by a factor of 5 to 10, while T_2 was reduced by a factor from 50 to 100 from the T_1 and T_2 of normal liquid water under similar conditions which are about 3 seconds (see below) (for extensive compilation of data published before 1979, see Hazlewood, 1979).

At first look, the disparity between T_1 and T_2 measured suggests that the τ_c of cell water is very much to the right of the T_1 minimum given in Fig. 3. The very low T_2 then suggests that τ_c is so long as to approach that of solid ice (2.5×10^{-6} at -3° C, see Steinemann and Gränicher, 1957; Glasel, 1972, p. 242). More careful thinking, however, shows that this would be unreasonable. The translational self-diffusion coefficient of H_2O in solid ice (Ice I) is one million times slower than in liquid water (Eisenberg and Kauzman, 1969, p. 121 and p. 218). Thus if water in living cells is quantitatively like solid ice, the translational diffusion coefficient of water in living cells must also be similarly reduced. In fact, the translational self-diffusion coefficient of water in living cells is lower than that in liquid water by only a factor of 2 to 3 (Hazlewood, 1979; Ling, 1984, p. 391).

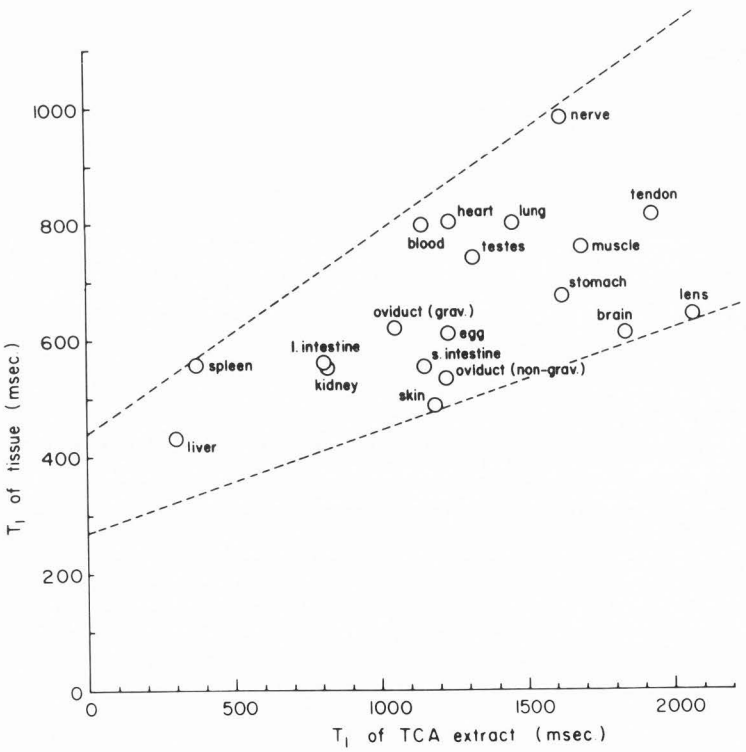


Fig. 4: Relation between spin lattice relaxation time (T_1) of water protons of fresh tissues normalized to a uniform 80% water content and the T_1 of hot trichloroacetic acid extracts of the tissues. "Blood" refers to separated blood cells, mostly erythrocytes. Each point is the average of four determinations. Correlation coefficient measured is +0.58. [from Ling, 1983a, by permission of Physiol. Chem. Phys. and Med. NMR]

Similar reasonings then led to the growing belief at one time that the low and different T_1 and T_2 values observed had little to do with the physical state or motional freedom of the bulk of cell water. Rather, the reduced T_1 and T_2 are the consequence of rapid exchange of either water protons or magnetic energy between the bulk of normal liquid cell water and some minor fraction of tightly bound water (for review of this view, see Cooke and Kuntz, 1974; Kuntz and Zipp, 1977). The widely different T_1 and T_2 of water protons in different normal tissues as well as the differences between cancer cells and their normal counterparts (Damadian, 1971) were thought to be simply due to their different water contents, both intracellular and extracellular (Weisman et al., 1972; Inch et al., 1974; Eggleston et al., 1978).

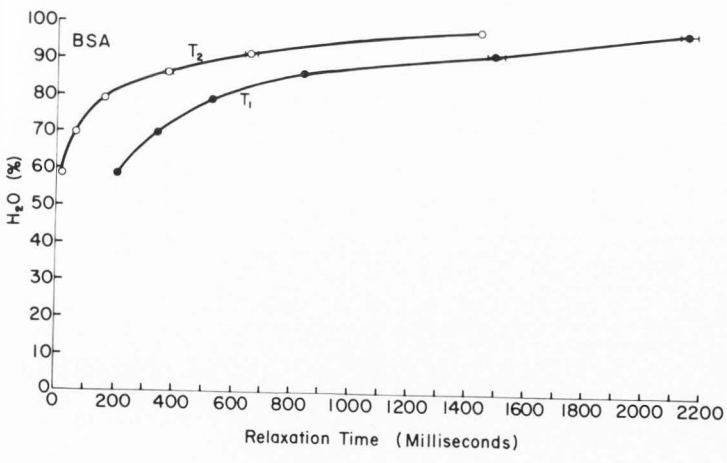
In 1980 Ling and Tucker presented evidence that while unquestionably T_1 and T_2 of the water proton of living cells varies with the cell water contents, the differences in T_1 and T_2 between different normal tissues and between cancer cells and normal tissues persist when all of their water contents are manipulated to equal each other by exposure to hypo- or hypertonic Ringer solutions. Ling and Tucker then pointed out that at constant water contents, the different T_1 and T_2 of different tissues, normal tissues, and cancer cells could be due to (1) different proteins and possibly water tightly bound to them; (2) different amounts of paramagnetic ions present in the cells, and/or (3) differences in the physical state (motional freedom) of the bulk of cell water. Experiments were then designed to test for the presence of these factors: (1) The Role of Paramagnetic Ion Contents in Normal vs. Cancer Cells. Having established that 10% trichloroacetic acid does not liberate a significant amount of bound paramagnetic ions (e.g., Fe in hemoglobin), Ling (1983a) compared the T_1 of the TCA extracts of 19 frog tissues with the T_1 of the fresh tissues normalized to a uniform water content of 80%. The data reproduced in Fig. 4 yields a linear correlation coefficient (r) of +0.58, which is significant at the 1% level in a two-tailed test. Since r^2 is roughly equal to the percentage contribution of the correlated parameter to the phenomenon observed (Snedecor and Cochran, 1980, p. 181), about 30% of the T_1 variation among the 19 frog tissues is due to the TCA extractable ions*. The important

*The TCA extracts contain essentially no proteins. The only components that are high enough in concentration to influence water proton relaxation are "free" paramagnetic ions including copper, iron, and manganese.

	Sample	Tissue	T_1 (msec)	T_2 (msec)
A.	P	ovalbumin (no ashes)	1325±10	470±7
B.	A	muscle	550±4	266±4
	B	liver	109±3	67±1
	C	spleen	49.5±1.0	30±0.7
	D	kidney	176±2	116±1
	E	heart	93±3	65±1
	F	lung	91±3	65±1
	G	brain	441±7	245±4
C.	H	LSA	710±17	354±3
	I	Meth A	938±10	361±4
	J	hepatoma 134	853±18	388±15
	K	sarcoma 180	970±20	373±6
	L	P815	950±10	405±7
	M	Ehrlich	910±17	330±7

Table 1: NMR relaxation times, T_1 and T_2 of pure ovalbumin solutions and ovalbumin solution containing the dissolved ashes of normal mouse tissues and ascites cancer cells. T_1 and T_2 were measured on a coherent CPS-2 NMR pulse spectrometer (Spin Lock, Ltd., Port Credit, Canada) operating at a resonance frequency of 17.1 MHz. T_1 was determined with 180°-τ-90° pulse sequence; T_2 with Carr-Purcell spin echo method. Sample temperature was 25° C. T_1 and T_2 values are average of three determinations ± S.E. Ovalbumin was from Nutritional Biochemical Co., Chagrin Falls, OH, and by ashing shown to contain no significant amount of paramagnetic impurities. All cancer cells were in the ascites form: LSA (lymphoma); Meth A (fibrosarcoma); sarcoma 180 (pleomorphic leukocyte sarcoma); P815 (mast cell leukemia); Ehrlich (mammary adenocarcinoma). [from Ling, 1983b, by permission of Physiol. Chem. Phys. and Med. NMR]

Fig. 5: T_1 and T_2 of water proton in aqueous solutions of bovine serum albumin plotted against their water contents. [from Ling and Murphy, 1983, by permission of Physiol. Chem. Phys. and Med. NMR]



discovery that the T_1 and T_2 of the water proton of cancer tissues differ from those of normal tissues by Damadian (1971) led eventually to the development of magnetic resonance imaging (MRI) as a noninvasive diagnostic tool of great power. In evaluating the significance of the longer T_1 and T_2, Damadian pointed out that his findings agree with Ling's polarized multilayer theory of cell water and an early suggestion of the late Albert Szent-Györgyi that water in cancer cells is less structured than in normal cells (Szent-Györgyi, 1957, footnote on p. 57). Table 1, taken from Ling (1983b) shows that a major cause of the longer T_1 and T_2 seemed to be lower concentrations of paramagnetic ions in cancer cells than in normal tissues. This finding contradicted the then widely held belief that paramagnetic ions have minimum effect on T_1 and T_2 of water proton in living cells; (2) The Role of Proteins and a Small Quantity of Tightly Bound Water. On preceding pages of this review, I pointed out that as a rule, native proteins like bovine serum albumin (BSA) had little influence on the solvency of Na^+ salts, sucrose, and glycine. Therefore to all intents and purposes, solutions of BSA are largely normal liquid water or at least water having properties resembling normal liquid water in as far as solvency for sucrose, Na^+ salts, etc. Yet as shown in Fig. 5 in a solution containing 20% BSA completely freed of paramagnetic impurities the T_1 and T_2 are markedly shorter than that of normal liquid water. Indeed both the low values of T_1 and T_2 and the widely different values of T_1 and of T_2 leading to T_1/T_2 ratios much larger than unity bear strong resemblance to the T_1 and T_2 characteristics of living cells. These resemblances thus demonstrate that proteins do indeed have the power to provide the means of causing rapid relaxation of water protons; (3) The Role of Multilayer Polarization of Water in T_1, T_2, and τ_c of Water Protons. In the preceding section, I have shown that water content, paramagnetic ions, diamagnetic proteins (and possibly a minute amount of tightly bound water) all contribute to the relaxation rates of water protons, lowering the T_1 and T_2 of the system measured. Now we come back to the crucial question that NMR was once intended to answer: Does the bulk of cell water suffer loss of rotational (and translational) freedom in consequence of interaction with the fully extended protein chains as proposed in the polarized multilayer theory of cell water? Unfortunately NMR cannot provide a straightforward answer because there is no conceivable way of removing the cell proteins (and paramagnetic ions) which interfere with determination of the true T_1, T_2, and τ_c of the polarized water (if it exists), without destroying the cells.

Failing a direct answer to the key question, one can provide straightforward answers to a somewhat different but relevant question, " Does water that has lost its solvency for solutes normally excluded by water in living cells suffer rotational

(and translational) motional restriction according to the AI hypothesis?" We can now answer this question because we knew only recently that at least four synthetic oxygen-containing polymers exist which reduce the solvency of the bulk-phase water but do not cause rapid relaxation of the bulk-phase proton through a rapid exchange mechanism such as that provided by paramagnetic ions and by proteins.

Fig. 6 shows a plot of T_1 and T_2 against the water content of different concentrations of PVP. Note that whereas in the case of BSA, T_1 becomes progressively larger and larger than T_2 as the protein content increases, the T_1 and T_2 of PVP solution remained very close to each other with increase of PVP concentration. Table 2 shows that all four polymers exhibit a more or less constant ratio of T_1/T_2 throughout the entire concentration range of polymers studied, and that this ratio is essentially the same as that of normal liquid water (Table 3).

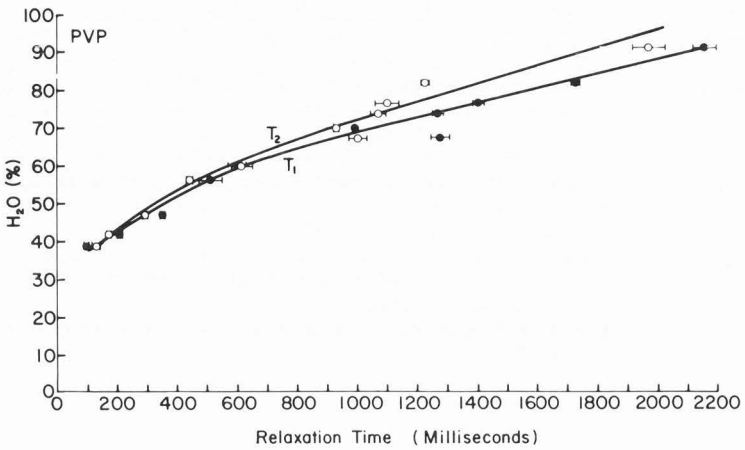


Fig. 6: T_1 and T_2 water protons in aqueous solutions of polyvinylpyrrolidone (PVP) against their water contents. [from Ling and Murphy, 1983, by permission of Physiol. Chem. Phys. and Med. NMR]

BSA		Gelatin		PVP		PEO		PVME	
% H_2O	T_1/T_2	% H_2O	T_1/T_2	% H_2O	T_1/T_2	% H_2O	T_1/T_2	% H_2O	T_1/T_2
98.0	1.49	89.5	1.63	90.7	1.10	95.9	1.37	91.2	1.32
91.7	2.27	74.9	2.54	81.6	1.41	90.8	1.41	83.0	1.30
86.5	2.25	59.3	4.67	76.4	1.27	87.4	1.54	75.4	1.27
79.2	3.30	47.2	9.29	69.9	1.06	77.1	1.40	65.9	1.10
70.0	5.27	42.4	5.50	67.1	1.28	77.8	1.39	59.4	1.11
59.0	11.50	35.6	12.50	59.9	0.98	66.4	1.45	40.4	1.01
		24.8	13.00	41.8	1.20	56.3	1.20	26.8	1.82
				39.0	0.74	39.9	1.20		

Table 2: The ratios of T_1/T_2 for BSA, gelatin, PVP, PEO, and PVME at various water contents. To save space, every 4th set of data from PVP and every 2nd set of data from PEO were deleted. These deleted data are in essence similar to those retained. [from Ling and Murphy, 1983, by permission of Physiol. Chem. Phys. and Med. NMR]

Let us now return to Fig. 3, where T_1 and T_2 are plotted against the rotational correlation time τ_c. The constancy of the T_1/T_2 ratio means that we are on the left hand side of the T_1 minimum. Once this is decided, it is a simple matter to find the τ_c corresponding to each set of T_1 and T_2 values.

However, as such these T_1, T_2, and τ_c values are not the final answers we seek because these T_1 and T_2's are the average of two kinds of water, normal liquid water with a T_1 of about 2.6 seconds; and polymer-altered water, the T_1 (or T_2) of which can be calculated from the T_1 (or T_2) observed only if we know how much of the water in a polymer solution is polymer-altered and how much is normal liquid water.

Fortunately, one can obtain this set of figures by making the reasonable assumption that if a polymer solution shows a ρ-value for Na citrate of say 0.4, then 40% of the water is polymer dominated with zero solvency for Na citrate. 60% is then normal liquid water, content. With this assumption, then, data provided by solvency studies of the polymer solution, illustrated in Fig. 1, can be combined with the observed T_1 data from NMR studies to determine the T_1 of the polymer-affected water with the aid of the Zimmerman-Brittin equation (Zimmerman and Brittin, 1957):

$$T_{obs}^{-1} = \sum_{j=1}^{n} P_i T_i^{-1}, \qquad (3)$$

where P_i is the probability of finding water of the ith kind, which may be equated to the proportion of water of that type. T_i is the relaxation time of that type of water. For T_1 in the polymer solution,

Distilled Water	pH	T_1 (msec)	T_2 (msec)	T_1/T_2
Air	6.6	2550	1740	1.47
N_2	6.6	3200	2100	1.52
Air	3.5	2550	2150	1.21
N_2	3.5	3250	2700	1.20
Air	10.6	2600	2200	1.30
N_2	10.6	3325	2650	1.28

Table 3: T_1 and T_2 of water protons of distilled water at different pH's in air or nitrogen. [from Ling and Murphy, 1983, by permission of Physiol. Chem. Phys. and Med. NMR]

$$(T_1)^{-1}_{obs} = X_{n.l.}\,(T_1)^{-1}_{n.l.} + X_{PAW}\,(T_1)^{-1}_{PAW}, \qquad (4)$$

where $(T_1)_{obs}$ is the observed T_1 of the polymer solution of a certain concentration. $X_{n.l.}$ is the fraction of water that is normal liquid water, and $(T_1)_{n.l.}$ its corresponding T_1 (i.e., 2.6 sec.); X_{PAW} is the fraction of polymer-affected water in the total water content and is equal to $(1 - \rho_{Na\text{-}citr.})$; $(T_1)_{PAW}$ is the T_1 of the polymer affected water we are seeking.

Once the $(T_1)_{PAW}$ has been obtained, a comparison with the data of Fig. 6, for example, will allow us to calculate the τ_c of the polymer-affected water in that specific case.

Table 4 illustrates the data in our final evaluation of PEO-dominated water. Note that the τ_c increases from about 1 x 10^{-11} to 2.7 x 10^{-11} second with increase of PEO content from 7.5% to 50%. Now the τ_c of normal liquid water is 3 x 10^{-12} sec. One finds that the polymer-affected water, which has lost its solvency for Na citrate, does indeed suffer a restriction of its rotational (and translational) motional freedom such that its rotational correlation time has lengthened by a factor of from 3 to 19 depending on the concentration of PEO.

Our next effort was to find out if the observed relaxation rate, $(T_1)^{-1}$ of living cells like the frog muscle can accommodate the relaxation due to both the bulk phase water with T_1 similar to that of $(T_1)_{PAW}$ just determined and the minor fraction of rapidly relaxing water associated with paramagnetic ions and diamagnetic proteins. Such a calculation was made and shown to fit the observed T_1 easily (Ling and Murphy, 1983). With this result in hand, it is obvious that the earlier conclusion that the observed NMR relaxation times pointed to the presence of the bulk of cell water as normal liquid water was not warranted. Nor, however, can we say that the short T_1 and T_2 of the water proton by itself has provided unequivocal proof that the bulk of cell water is like that of the Na citrate-nonsolvent water in the polymer-water system examined.

The unavoidable interference due to paramagnetic " contaminants" and diamagnetic proteins makes NMR relaxation times incapable of providing unequivocal data about the motional freedom of water molecules in highly hydrated cells like the frog muscle - a problem which may be less thorny in the case of much less hydrated Artemia cyst cells (Seitz et al., 1980).

Fortunately, there are other ways of determining the rotational and translational motional freedom of water molecules in living cells. These include high frequency dielectric dispersion methods which yield a dielectric relaxation time, τ_D, which is related to NMR correlation time, τ_c by the simple relation $\tau_D = 3\,\tau_c$. The most recent studies of Clegg and coworkers of brine shrimp cysts with a water content of about 58% suggest that they contain no free water. The τ_D measured was about twice as long as that of normal liquid water (Clegg et al., 1984). Unfortunately, as pointed out by Trantham et al. (1984) a dielectric relaxation measurement (like NMR T_2) might also be made to look more like normal liquid water than it truly is due to rapid exchange and domination by a minute amount of free water in the sample. Luckily there is yet another even more reliable method of measuring the rotational and translational freedom of water in living cells, i.e., the method of quasi-elastic neutron scattering (QENS). The study of Trantham et al. (1984) yielded a rotational diffusion coefficient of water in brine shrimp cysts 13 times slower than that of normal liquid water and a translational diffusion coefficient 4 times slower. Just as significant was the demonstration that water in a 30% PEO solution exhibits very similar QENS behaviors (Rorschach, 1985). In frog muscle with much higher water content (80%), the rotational diffusion coefficient was recently estimated at about twice as long as normal liquid water dominated by PEO and other oxygen-containing polymers (i.e., about 3 times longer).

In summary, QENS studies have shown that the bulk of cell water indeed suffers rotational (and translational) motional restriction as predicted by the polarized multilayer theory of cell water. Results of the QENS studies are in full harmony with those from high frequency dielectric dispersion and NMR studies.

Thus far, no QENS studies have been made comparing the motional freedom of water of normal cells and cancer cells. Fortunately the theory of solute exclusion in polarized multilayered water offers different and highly sensitive ways of determining the motional restriction of bulk phase with the aid of the estimate of the q-value of dynamic water structure probes, i.e., a battery of nonmetabolizable pentoses. In normal tissues, the q-values for all these pentoses tend to be similar and > 1. In all cancer cells studied, on the other hand, the q-values of the pentoses are equal to or very close to unity (Ling, 1984, p. 340-343). Together these

PEO						
Polymer Content (%)	H_2O Content (%)	T_1 (msec)	ρ_{Na} citrate	Minimal Polymer Altered Water (%)	T_1^{PAW} (msec)	τ_c^{PAW} (sec)
7.5	92.5	2280	0.87	13	1250	1.02×10^{-11}
10	90	2110	0.73	27	1400	9.2×10^{-12}
15	85	1800	0.51	49	1360	9.4×10^{-12}
20	80	1540	0.39	61	1220	1.06×10^{-11}
30	70	1110	0.22	78	947	1.36×10^{-11}
40	60	780	0.13	87	707	1.81×10^{-11}
50	50	520	0.08	92	487	2.67×10^{-11}

Table 4: The T_1^{PAW} and τ_c^{PAW} of estimated polymer-altered water (PAW) in PEO-water system. The amounts of PAW were obtained from the ρ-values for Na citrate and were taken from Ling and Ochsenfeld (1983). [from Ling and Murphy, 1983, by permission of Physiol. Chem. Phys. and Med. NMR]

data indicate that the dynamic polarized multilayered structure of highly malignant cancer cells is indeed looser than in most normal living cells.

The Freezing and Thawing Behaviors of Normal and Polarized Water

In the preceding section, I have shown how with the introduction of the polarized multilayer theory of cell water and the predicted and experimentally confirmed size-dependent solute exclusion, the nonexclusion of urea and ethylene glycol can no longer be regarded as evidence of free cell water. Rather these findings are fully in harmony with the new theory of cell water being in the state of polarized multilayers quite distinct from normal liquid water. In this section, I shall present results of new experimental studies that would invalidate the arguments that once reviewer Blanchard used so effectively to put down the colloidal approach to cell water properties. Here the new perspective was provided by the availability of more refined instruments for the study of freezing and thawing of aqueous systems unknown to both the proponents of " bound water" concepts and those against it in the 1930's.

Native Proteins at Concentrations as High as 50 % Do Not Cause Unusual Freezing Behavior of Water

Using a scanning calorimeter of great sensitivity one can measure the heat given off or adsorbed in a minute sample weighing a few milligrams in weight.

Fig. 7 shows the cooling thermogram of different concentrations of bovine hemoglobin solutions. The freezing temperature as well as the rate of freezing were not distinctly different from one another in the presence of hemoglobin from a concentration of 5% up to as high as 50%! The addition of silver iodide seeding crystals raised by a few degrees the freezing temperature in a uniform manner. In the presence of AgI crystals, the freezing temperature of solutions of five other native proteins (BSA, γ-globulin, β-lactoglobulin, egg albumin, and protamine sulfate) from 5% to 50% in concentration also produced no change in the freezing temperature or rate of freezing.

The implication of these findings is that the arguments once used by Blanchard and others against earlier " bound water" concept (based on failure of water in biological systems to freeze at -20° C) can no longer be considered valid. Presence of mechanical barriers even as high as that provided by 50% protein had no significant effect on freezing temperature.

Fig. 8 shows the profoundly different behavior of solutions containing different concentration of the polymer, polyvinylmethyl ether (PVME) in the presence of AgI. Note that the freezing point becomes lower and lower with increase of PVME concentration until at 65%, no freezing could be observed. Even at -50° C. the unfrozen 65% PVME solution was not solid (vitreous) but remained a highly viscous liquid (Ling and Zhang, 1983; Zhang and Ling, 1983).

The freezing behavior of PVME solution was shared by not only PEO and PVP, but by all the proteins that reduced the solvency of bulk phase water for Na salts, sugar, and free amino acids. These include gelatin and urea- and guanidine-HCl-denatured native proteins, but not sodium dodecyl sulfate (SDS)-denatured proteins, which as mentioned earlier, did not have an effect on the secondary structure of protein nor on the solvency of the bulk phase water containing the SDS-denatured proteins.

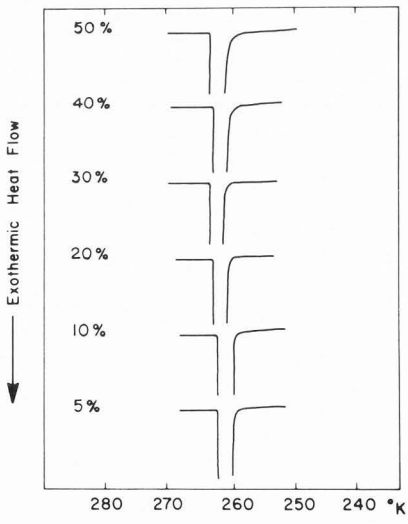


Fig. 7: Cooling thermograms of various concentrations of native bovine hemoglobin solutions without AgI crystals. [from Ling and Zhang, 1983, by permission of Physiol. Chem. Phys. and Med. NMR]

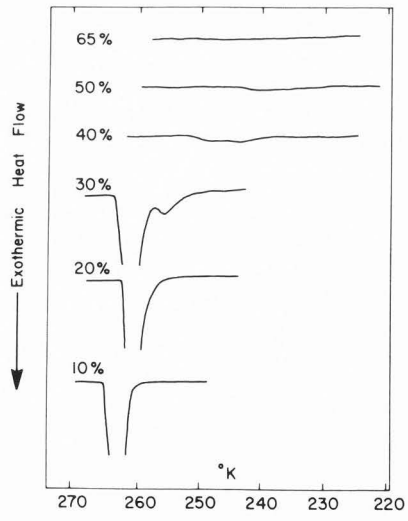


Fig. 8: Cooling thermograms of various concentrations of PVME with AgI crystals. [from Ling and Zhang, 1983, by permission of Physiol. Chem. Phys. and Med. NMR]

Why Should Water in Living Cells Freeze If It Exists As Polarized Multilayers

If the cut end of a supercooled muscle cell is touched with an ice crystal one or more single branchless spikes will rapidly grow along the direction of the orientation of the myofilaments, straight when the muscle is in its usual resting orientation, helical, if the muscle fiber has been twisted. At no time does the spike form branches (Chambers and Hale, 1932; Miller and Ling, 1970; Ling, 1984, p. 284-285).

It is well known that normal ice has hexagonal symmetry. Hexagonal ice formation persists in solutions of glycerol (6 M), sucrose (50%), and even bovine serum albumin (35%) (Luyet and Rapatz, 1956). Of a variety of solutions these authors investigated only two showed marked deviations. In these cases the ice formed showed a lobed club-like shape of uniform width and devoid of the extensive feather-like secondary and tertiary branching seen in normal ice. Luyet and Rapatz called this unusual ice form " irregular dendrites". The two substances that they found to cause the formation of irregular dendrites were: gelatin and PVP. As mentioned above gelatin and PVP, like PVME, PEO, and urea-denatured proteins showed pronounced ability to cause the freezing point depression of the bulk phase water; 35% native bovine serum albumin does not.

There is thus full accord between the ability of a polymer of proteins existing in the fully extended conformation to lower the freezing point and suppress freezing altogether (at high concentration) and its ability to suppress normal branching in ice formed. Since the ice spike formed in muscle cells is the extreme form of nonbranching ice, proteins in the living muscle cells must also exist in the fully extended conformation, like PVP and gelatin, rather than like native bovine serum albumin with most of its backbone NHCO groups locked in α-helical and other intra- and intermolecular H-bonds.

Acknowledgements

The foregoing work was supported by NIH Grants 2-R01-CA16301 and 2-R01-GM11422, Office of Naval Research Contract N00014-85-K-0573 and Pennsylvania Hospital General Research Support.

References

Blanchard KC (1940) Water, free and bound. Cold Springs Harbor Symp. Quant. Biol. 8: 1-8.

Bloembergen N, Purcell EM, Pound RV (1948) Relaxation effects in nuclear magnetic resonance absorption. Phys. Rev. 73: 679-712.

Bradley S (1936) Polymolecular adsorbed films: Part I. The adsorption of argon on salt crystals at low temperature and the determination of surface films. J. Chem. Soc. 1936: 1467-1473.

Brunauer S, Emmett PH, Teller E (1938) Adsorption of gases in multimolecular layers. J. Amer. Chem. Soc. 60: 309-319.

Bull H (1944) Adsorption of water vapor by proteins. J. Amer. Chem. Soc. 66: 1499-1507.

Chambers R, Hale HP (1932) Formation of ice in protoplasm. Proc. R. Soc. London Ser. B, 110: 336-339.

Chou PY, Fasman GD (1974) Conformational parameters for amino acids in helical, sheet and random coil regions calculated from proteins. Biochem. 13: 211-222.

Clegg JS, Mc Clean VER, Szwarnoski S, Sheppard RJ (1984) Microwave dielectric measurements (0.8-70 GHz) on Artemia cysts at variable water contents. Phys. Med. Biol. 29: 1409-1419.

Cooke R, Kuntz ID (1974) The properties of water in biological systems. Ann, Rev. Biophys. Bioengr. 3: 95-126.

Cope F (1969) Nuclear magnetic resonance evidence using D_2O for structured water in muscle and brain. Biophys. J. 9: 303-319.

Damadian R (1971) Tumor detection by nuclear magnetic resonance. Science 171: 1151-1153.

de Boer JH, Zwikker C (1929) Adsorption als folge von polarizatien die adsorptionsisotherme. Z. Physik Chem. B3:407.

Eggleston JC, Saryan LA, Hollis DP (1978) Nuclear magnetic resonance investigation of human neoplastic and abnormal nonneoplastic tissues. Cancer Res. 35: 1326-1332.

Eisenberg D, Kauzman W (1969) The Structure and Properties of Water, Oxford University Press, NY

Ferry JD (1948) Protein gels. Adv. Prot. Chem. 4: 1-78.

Fischer MH,Suer WJ (1935) Base-Protein-Acid compounds. Arch. Pathol. 20: 683-689.

Fischer MH, Suer WJ (1938) Physicochemical state of protoplasm. Arch. Pathol. 26: 51-60.

Glasel JA (1972) Nuclear magnetic resonance studies on water and ice. In: Water. A Comprehensive Treatise, Vol. 1, Franks F (ed) Plenum Press, NY, 215-254.

Gortner RA (1937) Selected Topics in Colloid Chemistry, Cornell University Press, Ithaca, NY, Chap. 8.

Graham T (1861) Liquid diffusion applied to analysis. Philos. Trans. R. Soc. London 151: 103-124.

Hazlewood CF (1979) A view of the significance and understanding of the physical properties of cell associated water. In: Cell Associated Water, Drost-Hansen W, Clegg JS (eds) Academic Press, NY, 165-259.

Hazlewood CF, Nichols BL, Chamberlain NF (1969) Evidence for the existence of a minimum of two phases of ordered water in skeletal muscle. Nature 222: 747-750.

Hill AV (1930) The state of water in muscle and blood and the osmotic behavior of muscle. Proc. R. Soc. London Ser. B. 106: 477-505.

Inch WR, Mc Credie JA, Knispel RR, Thompson RT, Pintar MM (1974) Water content and proton spin lattice relaxation time for neoplastic and nonneoplastic tissues from mice and humans. J. Natl. Cancer Inst. 52: 353-356.

Jones ID, Gortner RA (1932) Free and bound water in elastic and non elastic gels. J. Phys. Chem. 36: 387-436.

Kubo R, Tomita K (1954) General theory of magnetic resonance absorption. J. Phys. Soc. Japan 8: 888-919.

Kühne W (1864) Unterschungen über das protoplasma und die contractilität. Z. Physik. Chem. 1: 158-179.

Kuntz ID, Zipp A (1977) Water in biological systems. New Eng. Med. J. 297: 262-266.

Ling GN (1962) A Physical Theory of the Living State: The Association-Induction Hypothesis, Blaisdell, Waltham, MA. Chapter 8.

Ling GN (1965) The physical state of water in living cell and model systems. Ann. NY Acad. Sci. 125: 401-417.

Ling GN (1972) Hydration of macromolecules. In: Water and Aqueous Solutions, Structure, Thermodynamics, and Transport Processes. Horne A (ed) Wiley-Interscience, NY 663-699.

Ling GN (1983a) Evidence for a significant role of paramagnetic ions in the observed NMR relaxation rates of living tissues. Physiol. Chem. Phys. and Med. NMR 15: 505-510.

Ling GN (1983b) NMR relaxation times of water protons in normal tissues and cancer cells: The role of paramagnetic ions. Physiol. Chem. Phys. and Med. NMR 15: 511-513.

Ling GN (1984) In Search of the Physical Basis of Life, Plenum Publishing Corp., NY.

Ling GN (1986) The role of inductive effect in determination of protein structure. Physiol. Chem. Phys. and Med. NMR 18: 3-16.

Ling GN (1988a) A physical theory of the living state: Application to water and solute distribution. Scanning Microsc. In press.

Ling GN (1988b) A Revolution in Physiology of the Living Cell: Its Completion and Beyond. (contact the author for details)

Ling GN, Murphy RC (1983) Studies on the physical state of water in living cells and model systems. II. NMR relaxation times of water protons in aqueous solutions of gelatin and oxygen-containing polymers which reduce the solvency of water for Na^+, sugars, and free amino acids. Physiol. Chem. Phys. and Med. NMR 15: 137-154.

Ling GN, Negendank W (1970) The physical state of water in frog muscles. Physiol. Chem. Phys. 2: 15-33.

Ling, GN, Ochsenfeld MM (1983) Studies on the physical state of water in living cells and model systems. I. The quantitative relationship between the concentration of gelatin and certain oxygen-containing polymers and their influence upon the solubility of water for Na^+ salts. Physiol. Chem. Phys. and Med. NMR 15: 127-136.

Ling GN, Ochsenfeld MM (1987) Studies on the physical state of water in living cells and model systems. VI. Concentration-dependent sustained volume changes of dialysis sacs containing aqueous solution of native and denatured protein, telatin, and oxygen-containing polymers immersed in solutions of Na salt and of sugar and sugar alcohol. Physiol. Chem. Phys. and Med. NMR 19: 177-192.

Ling GN, Tucker M (1980) Nuclear magnetic resonance relaxation and water contents in normal mouse and rat tissues and in cancer cells. J. Natl. Cancer Inst. 64: 1199-1207.

Ling GN, Ochsenfeld MM, Walton C, Bersinger TJ (1980a) Mechanism of solute exclusion from cells: The role of protein-water interaction. Physiol. Chem. Phys. 12: 3-10.

Ling GN, Walton C, Bersinger TJ (1980b) Reduced solubility of polymer-oriented water for sodium salts, amino acids, and other solutes normally maintained at low levels in living cells. Physiol. Chem. Phys. 12: 111-138.

Ling GN, Zhang ZL (1983) Studies on the physical state of water in living cells and model systems. IV. Freezing and thawing point depression of water by gelatin, oxygen-containing polymers and urea-denatured proteins. Physiol. Chem. Phys. and Med. NMR 15: 391-406.

Lloyd DJ (1932) Colloidal structure and its biological significance. Biol. Rev. Cambridge Philos. Soc. 7: 254-273.

Luyet B, Rapatz F (1956) Patterns of ice formation in some aqueous solutions. Biodynamica 8: 1-67.

Mac Leod J, Ponder E (1936) Solvent water in the mammalian erythrocyte. J. Physiol. 86: 147-152.

Mellon EF, Korn AH, Hoover SR (1948) Water absorption of proteins. III. Contribution of the peptide group[2]. J. Amer. Chem. Soc. 70: 3040-3044.

Miller C, Ling GN (1970) Structural changes of intracellular water in caffeine-contracted muscle cells. Physiol. Chem. Phys 2:495.

Newton R, Gortner RC (1922) A method for estimating hydrophilic colloid content of expressed plant tissue fluids. Bot. Gaz. 74: 442-446.

Odeblad E (1957) Studies on vaginal contents and cells with proton magnetic resonance. Ann. NY Acad. Sci. 83: 189-206.

Pauling L (1945) The adsorption of water by proteins. J. Amer. Chem. Soc. 67: 555-557.

Rorschach HE (1985) Water in polymers and Artemia cysts: Relation of neutron scattering and NMR results. In: Water and Ions in Biological Systems, Pullman A, Vasilescu V, Packer L (eds), Plenum Press, NY, 729-736.

Rubner M (1922) Uber die wasserbindung in kolloidenmit besonderer berücksichtigung des querges-treiften muskels. Abh. Preuss. Akad. Wiss. 3: 70-100.

Seitz PO, Hazlewood CF, Clegg J (1980) Proton magnetic resonance studies on the physical state of water in Artemia cysts. In: The Brine Shrimp Artemia, Physiology, Biology, and Molecular Biology, Personne G, Sorgeloos P, Rolls O, Jaspers E (eds) Universa, Wetteren, Belgium, 2: 545-555.

Snedecor GW, Cochran WG (1980) Statistical Methods, 7th ed., Iowa State University Press, Ames, Iowa. p. 181.

Steinemann A, Granicher H (1957) cited in: Glasel JA (1972) Nuclear magnetic resonance studies on water and ice. In: Water: A Comprehensive Treatise, Vol. 1, Franks F (ed) Plenum Press, NY 215-254, Table VI.

Szent-Györgyi A (1957) Bioenergetics, Academic Press, NY, p. 57

Thoenes F (1925) Untersuchungen zur frage der wasserbindung in kolloiden und tierschen geweben. Biochem Z. 157: 174-185.

Trantham EC, Rorschach HE, Clegg JS, Hazlewood CF, Nicklow RM, Wakabayashi N (1984) Diffusive properties of water in Artemia cysts

as determined from quasi-elastic neutron scattering spectra. Biophys. J. 45: 927-938.

Troshin AS (1966) The Problem of Cell Permeability, Pergamon Press, NY. Chap. 1-9.

von Nägeli C (1855) Pflanzenphysiologische Untersuchungen, Vol. 1, von Nägeli C, Nägeli IC (eds) Zurich, 120.

Weisman ID, Bennett LH, Maxwell LR, Woods MW, Burk D (1972) Recognition of cancer in vivo by nuclear magnetic resonance. Science 178: 1288-1290.

Zhang ZL and Ling GN (1983) Studies on the physical state of water in living cells and model systems. V. The warming exothermic reaction of frozen aqueous solution of polyvinylpyrrolidone, poly (ethylene oxide), and urea-denatured proteins. Physiol. Chem. Phys. and Med. NMR 15: 407-416.

Zimmerman JR, Brittin WE (1957) Nuclear magnetic resonance studies in multiple phase systems: Lifetime of a water molecule in an adsorbing phase on silica gel. J. Phys. Chem. 61: 1328-1333.

Discussion with Reviewers

C.F. Hazlewood: In your presentation you commented extensively on the failure of cell to produce enough energy to operate the sodium pump alone. How do you account for such a large discrepancy between your calculations and those of the membrane-pump-camp? It is argued by some that the ATP turn over is not adequately taken into account in your calculations. Would you please comment on this latter criticism?
Author: The energy need of the Na pump calculated by Levi and Ussing (1948), by Harris and Burn (1949), and by Keynes and Maisel (1954) was that of normal cells in the normal environment and these calculations were not intended to put the Na pump hypothesis to a rigorous test. In contrast, my calculation was the result of experiments designed rigorously to test if the cell commands enough energy to operate the Na pump. For this purpose I completely blocked respiration with pure nitrogen plus cyanide, and glycolysis with iodoacetate (IAA) backed up with actual lactate analyses at the beginning and the end of the experiment to catch the minute glycolytic activity that had escaped the inhibiting effect of IAA. Since there is no additional energy source besides respiration, glycolysis, and the store of ATP, ADP, and creatine phosphate (CrP), this blockage of respiration and glycolysis made it possible for us to determine precisely how much energy is maximally available to the cell over a period of time if one measured the total changes in the content of ATP, ADP, and CrP at the beginning of the experiment (after the complete arrest of respiration and glycolysis) and at the end of the period studied.

The success of our experiment rests on the ability of isolated frog muscle to maintain its normal K^+ and Na^+ concentrations unchanged for many hours (e.g., 7.7 hours, see Table 4.4 in Ling, 1984) after the full arrest of respiration and glycolysis at 0° C.

Since all active metabolism that regenerates ATP has been completely blocked by the combined action of the poisons used, we have created a " closed system" in which there is no ATP turnover except among CrP, ATP, and ADP themselves. Since all three components were analyzed at the beginning and at the end of the experiment, all available sources of the so-called " high energy phosphate bonds" have been taken into account. The accusation that I have ignored ATP turnover could only come from those who had not read the publication since so much space was devoted to describing the blockage of ATP regeneration (Ling, 1962).

C.F. Hazlewood: It appears that you are proposing the existence of matrix protein that is responsible for the development of the polarized multilayers of water. What is this matrix protein? Does the matrix protein exist in the extended form and, if so, how is it related to the cytoskeletal structures of the cell?
Author: The matrix protein exists in the fully stretched state with a large portion of its backbone NHCO groups directly exposed to and polarizing the bulk of cell water and it is present pervasively in the cell. The identity of the matrix protein or proteins is still not known for certain, even though more and more evidence points to the major protein of the cell, e.g., hemoglobin in erythrocytes. Our current working hypothesis is that for hemoglobin to function as the matrix protein it must combine with a fixed protein component in the cell. Thus if all intracellular proteins (hemoglobin and nonhemoglobin) of human erythrocytes are removed, reintroduction of pure hemoglobin does not restore to the cells their ability to reaccumulate K^+ and to extrude Na^+ in the presence of ATP. However, when pure hemoglobin is reintroduced into ghosts that have retained part of their intracellular nonhemoglobin (and some small amount of hemoglobin) reaccumulation of K^+ and extrusion of Na^+ occurs in the presence of ATP. Since the cytoplasmic component most resistant to extraction includes actin, this and other cytoskeletal proteins may indeed offer anchorage for as well as serve the key function of " auxiliary cardinal adsorbents" on hemoglobin. While this is our working hypothesis, much future work is required to pursue this approach.

W. Negendank: Is it really reasonable to assume that the polymer solutions contain two discrete kinds of water - solvent and nonsolvent, when analyzing the NMR relaxation times and correlation time? Doesn't this contradict part of the theory of polarized multilayers, which explains variation of q-values of different solutes without making such an assumption? Would it be preferable to pose this issue as a " thought experiment" or a " limiting condition" ?
Author: This is a very relevant question. In the original paper of Ling and Murphy (1983) we somewhat arbitrarily assumed that in a polymer solution, which exhibits a ρ-value of, say, 0.45 for Na citrate, contains 45% apparently normal

liquid water (ANLIW). The remaining 55% of the water is called " polymer affected water" (PAW). Our more recent work with the aid of a battery of molecular probes of different molecular weights (rather than a single one, e.g., Na citrate) provides support for the basic assumption that in these polymer solutions there are indeed two classes of water. However, the fraction of ANLIW has been found to be much smaller than the fraction of Na citrate solvent water.

Why should there be two fractions of water in the polymer solution is also an interesting question. One possible explanation is that the phenomenon reflects an uneven distribution of the polymers in the solution: Domains of higher (entangled) polymer concentration distributed in regions of lower polymer concentration containing high proportion of ANLIW. If so, the fraction of ANLIW may be expected to decrease with the molecular weight of the polymer until a plateau is reached beyond which further increase of the molecular weight of the probe does not cause further decrease of the q-value of the probe molecule. This limiting q-value defines the size of the fraction of ANLIW in a particular polymer solution.

W. Negendank: To what extent is the theory of water polarized in multilayers contradicted by concepts of hydrogen bonding in liquid water? Can this be resolved by a more dynamic model of water?

Author: I don't think that there is any true contradiction between the model of water existing in the state of polarized multilayers and an H-bonded liquid water structure. Since we still do not truly understand the structure of liquid water, an H-bonded structure is largely an extrapolation from ice structure and it is so complex that at this moment it is difficult if not impossible to cope with it quantitatively for our purposes. The treatment of water as dipoles trades some details (which we cannot benefit from) for a much simpler model that can give us quantitative predictions that can be experimentally tested. Indeed the choice of the dipole model of the H-bonded model bears resemblance to the choice of French Impressionist painters who discovered that a forest could be more faithfully portrayed by ignoring certain details unessential for the total picture. Treatment of cell water as polarized multilayers of water dipoles allows us to get to the essential properties of the bulk water by ignoring unessential details which an H-bonded structure offers.

References

Harris EJ, Burn GP (1949) The transfer of sodium and potassium ions between muscle and the surrounding medium. Trans. Farad. Soc. 45: 508-528.

Keynes RD, Maisel GW (1954) The energy requirement for sodium extrusion from a frog muscle. Proc. R. Soc. London Ser.B, 142: 383-392.

Levi H, Ussing HH (1948) The exchange of sodium and chloride ions across the fibre membrane of the isolated frog sartorius. Acta Physiol. Scand. 16: 232-249.