Scanning Microscopy

Volume 2 | Number 2 Article 24

2-14-1988

A Physical Theory of the Living State: Application to Water and Solute Distribution

G. N. Ling
Pennsylvania Hospital

Scanning Microscopy, Vol. 2, No. 2, 1988 (Pages 899-913) 0891-7035/88$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

A PHYSICAL THEORY OF THE LIVING STATE: APPLICATION TO WATER AND SOLUTE DISTRIBUTION

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

This review begins with a summary of the disproof of the membrane-pump theory and the alternative theory of the living cell, the association-induction (AI) hypothesis. Being alive in the AI hypothesis represents the maintenance of a high (negative) energy-low entropy state in which the two major components K^+ and water of the living cell are closely associated with the third major component of the living cells, proteins. K^+ is adsorbed singly on β- and γ-carboxyl groups and the bulk of cell water in multilayers on the exposed NHCO groups of fully extended polypeptide chains of cell proteins. These adsorptions account for both the constancy of cell K^+ and cell water per unit of cell proteins. ATP plays a key role in the maintenance of the cooperatively linked protein-ion-water assembly at the living state by its adsorption on key protein site and exercises the controlling influence through its strong inductive effects. Water polarized in multilayers demonstrates size-dependent exclusion of solutes, e.g., large (hydrated) Na^+ is excluded from water in living cells or model systems while smaller urea that fits into the dynamic water structure is not excluded. The confirmation of the polarized multilayer theory of cell water by nuclear magnetic resonance (NMR), dielectric, neutron scattering, and other studies not only reverses the conventional belief of the existence of the cell water as normal liquid water; it also gives a new definition to colloids.

KEY WORDS: Water, solute, theory, living state, Na pump, polarized multilayer, association-induction hypothesis, proteins, living cells.

*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

It is universally acknowledged that the basic unit of all life is the living cell. It is also widely known that though varying from one cell type to another, the chemical composition of each type of adult living cells is by and large quite constant*. The three most abundant components of the living matter are: water, proteins, and solutes of which K^+ (and Na^+) are the most prominent. The relative abundance is fixed for each cell type at a defined age and environment, containing primarily water but also an abundance of K^+ and little Na^+.

This fixity of the quantitative relationship among these three components of the living matter in fact comprises the constancy* of three relationships: (1) that between the two largest components, protein and water; (2) that between K^+ (Na^+) and water; and (3) that between K^+ (and Na^+) and proteins.

Until the early and middle 60's, primary interests of cell physiologists had centered around attempts to explain one of the three relationships: that between cell K^+-Na^+ and cell water. The most dominant and for some time outwardly successful efforts were under the banner of the membrane theory. It argued first that the membrane covering the cell is permeable only to water but impermeable to both K^+ and Na^+ or only to Na^+. The availability of radioactive tracers in the late 30's soon disproved these earlier concepts: the cell membrane is in fact quite permeable to both K^+, Na^+, and just about all solutes inside and outside living cells (Ling, 1984).

The Membrane-pump Theory

It was at about this time that Schoenheimer demonstrated the continuous flux of even the atoms making up the solid parts of the living cells, giving rise to the poetic concept of the living cell as a sort of 'flame' - wherein a steadiness in shape belies an unresting coming

*This " constancy" is not absolute but is that accepted in compiling a handbook, for example, providing data like the water content of tissue X & the K content of tissue Y (see Spector (1956)).

and going of its constituent parts (Schoenheimer, 1942). In harmony with this notion, an ancient idea regained increasing popularity, i.e., the concept of membrane pumps which traces its fundamental conceptual origin to Theodor Schwann (1810-1882), the founder of the cell theory (see Ling, 1988). For example, hypothetical pumps located in the cell membrane maintain a high concentration of K^+ in the cell by continually extruding Na^+ that would have displaced the cell K^+.

It was also about this time that the concept of the high energy phosphate bond was introduced. Thus two of the terminal phosphate bonds of adenosinetriphosphate (ATP) as well as the phosphate bond of some other organic phosphate like creatine phosphate (CrP) were thought to contain a large package of free energy utilizable in the performance of a large variety of biological work.

The Na pump concept was further elaborated when it was discovered that the membranes of various cells contain an enzyme which hydrolyzes ATP in the presence of both K^+ and Na^+ (Skou, 1960). The activity of this K, Na activated ATPase was shown to exhibit a similar sensitivity to the inhibitory effect of the cardiac glycoside, ouabain, as the outward flux rate of Na^+ from cells does (Bonting, 1970). It was therefore suggested that this K^+-Na^+ activated ATPase was in fact either the entirety or an important component of the Na pump. A vast amount of effort has been and still is being invested in the development of this theory, even though mounting evidence indicates that the basic idea may be untenable.

In the early 50's, I discovered that frog muscle could maintain its normal K^+ and Na^+ concentration for many hours, long after its respiration was inhibited by nitrogen and cyanide and glycolysis by Na iodoacetate in addition to cooling to 0^o C, which should itself slow down outward pumping of Na^+ more than its inward leakage (Ling, 1952). Furthermore the outward flux rate of the poisoned muscle - which in terms of the membrane-pump theory must be virtually all due to pumping - was unchanged in the chilled and poisoned muscles (Ling, 1952). The essence of these findings was later confirmed by Keynes and Maisel (1954) and by Conway et al. (1961). This is where these two groups' work on this subject ended.

I thought at that time (Ling, 1952) that even though all active metabolism in these poisoned muscles had been brought to a halt, the cell might still have enough energy to operate the Na pump since muscle tissues all contain high concentrations of both ATP and CrP. With this in mind, I first made sure that besides respiration, glycolysis, and the store of ATP and CrP, the cell had no,as yet,undiscovered new sources of energy. Having satisfied myself on this account (see Ling, 1962; Ling et al., 1973) I spent a good part of the three years 1953 to 1956 in studying the phenomenon. Results of the last three sets of experiments presented in 1962 are reproduced in Table 1, which represents a balance sheet between the maximally available energy - mostly in the form of decrease of the concentration of ATP and CrP during a period of time when Na^+ was (supposedly) being pumped at a rate carefully measured - and the minimally needed energy to pump these many moles of Na^+ against an essentially normal (measured) outside positive-electrical potential (i.e., the resting potential) and an outside high Na^+-concentration gradient. The results showed that if the cell utilizes energy for one and only one effort alone (i.e., to pump Na^+), the minimally needed energy is from 15 to 30 times the maximally available energy.

In the 25 years following, my findings and conclusions have not been challenged in print; their essence has been twice confirmed (Jones, 1965; Minkoff and Damadian, 1973). Three remedial postulations to keep down the energy need

Table 1

Energy Balance Sheet for the Na^+ Pump in Frog Sartorius Muscles (0^o C)[a,b]

Date	Duration (hr)	Rate of Na^+ exchange integrated average (moles/kg per hr)	$\psi + E_{Na}/F$ integrated average (mV)	Minimum rate of energy required for Na^+ pump (cal/kg per hr)	Maximum rate of energy delivery (cal/kg per hr)	$\left[\frac{\text{Minimum required energy}}{\text{Maximum available energy}}\right]$
9-12-56	10	0.138	111	353	11.57 (highest value, 22.19)	3060%
9-20-56	4	0.121	123	343	22.25 (highest value, 33.71)	1542%
9-26-56	4.5	0.131	122	368	20.47 (highest value, 26.10)	1800%

[a]The minimum rate of energy delivery required to operate a Na^+ pump according to the membrane-pump theory was calculated from integrated values of the measured rates of Na^+ exchange and the energy needed to pump each mole of Na^+ ion out against the measured electrical and concentration gradients. The maximum energy delivery rate was calculated from the measured hydrolysis of CrP, ATP, and ADP, the only effective energy sources available to the muscles, which had been poisoned with IAA and [b]nitrogen.

[b]From Ling (1962).

have all been experimentally disproven. They are Ussing's exchange diffusion, sequestration of Na^+ in the sarcoplasmic reticulum, and Glynn's non-energy consuming pump (see Ling et al. 1979; Ling, 1988).

However, even this great disparity is not the end but only the beginning of the energy problem facing the membrane-pump theory.

The Na pump is only one of the untold number of pumps needed at the plasma membrane because virtually every soluble component of the cell and its environment requires pumps, and there are also other solutes - which the cell could not have been exposed to in its evolutionary history for which the cell can maintain non-equilibrium distributions and which thus also require pumps.

The Na pump and the many other pumps also need to be postulated (and some were) at the surface of subcellular particles, which due to their much larger surface area require correspondingly more energy. Thus the surface of liver mitochondria is 20 X that of the liver cell's plasma membrane (Lehninger, 1964); the surface of the sarcoplasmic reticulum has been estimated at 50 X that of the muscle plasma membrane (Peachey, 1965).

Finally, I want to return to the energy deficiency for the Na^+ pump figure of 15 to 30 times as the energy being required to pump Na^+ in poisoned frog muscle at 0° C. Virtually all of the energy available (e.g., 98%, see Ling, 1962, p. 202) was calculated from the decrease of ATP and CrP concentration during the period of time of continual Na pumping, and the assumption that ATP and ADP contain, respectively, two or one high energy phosphate bonds, each containing -14 Kcal/mole of utilizable free energy, and the assumption that each CrP contains -12 Kcal/mole of similar utilizable energy. Are these assumptions still valid? The answer is No!

In their definitive review on the subject of " High Energy Phosphate Bond Concept", George and Rutman (1960) clearly demonstrated that the widely accepted standard free energy (ΔF^o) of -14 Kcal/mole of the two ATP phosphate bonds was not standard free energy at all! All standard free energy should be determined under the condition where each reactant must be at standard condition (i.e., 1 M). Since H^+ is given off during ATP hydrolysis and thus also a reactant, to determine the true ΔF^o, the H^+ concentration should also be 1 M and the pH of the medium should be 0. In the actual study, a pH of 7 was maintained. As a result, the free energy change observed is largely driven by the Le Chatelier principle to liberate more H^+. Indeed George and Rutman showed that most of the -14 Kcal/mole measured is due to this component described as $\emptyset\ RT\ \partial n\ [H^+]$, where R and T are the gas content and absolute temperature, $[H^+]$ is the H^+ concentration of the medium, and $\emptyset$, a constant, varies from 0 to 1 as the pH increases from 5 to 9. The energy component of the phosphate bond of ATP is in fact lower than that of AMP conventionally regarded as " low energy". It may be mentioned that the work of Podolsky and Morales (1956), using highly sophisticated calorimetric method, showed that the enthalpy of ATP hydrolysis is not the -12 Kcal/mole, as one time believed to be, but only -4.7 Kcal/mole. These findings preceded and are of course in full harmony with George and Rutman's conclusions.

By taking these facts into account, one may say that even the 15 to 30 times figure represents a gross underestimation of the energy disparity. (For still more evidence against the same theory, see Ling, 1984).

Along with these developments, the technology of preserving cells at very low temperature improved, culminating in the successful rearing of a human baby from a human embryo once frozen at near absolute zero temperature. Since at such a low temperature all chemical reactions come to a halt, life, if it were truly flame-like, would have irrevocably come to an end. The fact that life did not come to an end but continued, adds still more evidence that the continuation of life does not necessarily depend on pumping or other ceaseless chemical activities.

The Association-Induction (AI) Hypothesis

In 1962, a new concept of life was introduced, defined not by ceaseless chemical activities like that of a flame but by the maintenance of the living matter in a high (negative)* state called the living state. Functional activities are transient reversible assumptions of a lower (negative) energy state; death, a permanent, irreversible one.

Simple Models of the High Energy* States

When the moon and sun are properly aligned, one observes high tides. The higher level of water reached at the beach can be dammed up and with the aid of a turbine one can transform the potential energy of the water into work. Here it is the gravitational field that has created the high energy state.

A randomly strewn pile of iron filings is not affected by a chain of soft nails loosely tethered end to end. However, if one pole of a strong magnet is brought into contact with the end of one of the terminal nails, the nail will be magnetized, which will in turn magnetize the nail next to it and the process continues until the loosely joined chain becomes more rigidly attached together (Figure 1). In the process, the iron filings will no longer remain randomly distributed but become magnetized themselves and in so doing assume more organized patterns of distribution. Here the high energy and closely associated state of the assembly of nail and iron filings is created by magnetic polarization emanating from the strong magnet.

The High Energy Living State. According to the association-induction (AI) hypothesis, a theory of the living cell I first introduced in 1962, the major components of the living cell

*Here and elsewhere, high energy means high negative energy; low energy means low negative energy. This definition is chosen in order to avoid misunderstanding which might also occur in the analogous case of describing a couple deeply in love as demonstrating a minimal attraction.

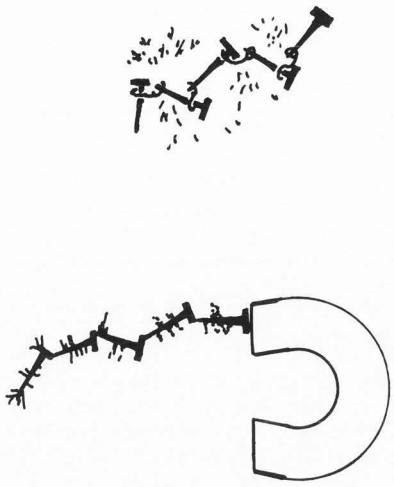

Fig. 1. A chain of soft iron nails joined end to end with pieces of string is randomly arrayed and does not interact with the surrounding iron filings. The approach of a magnet causes propagated alignment of the nails and interactions with the iron filing. [From Ling (1969), by permission of International Review of Cytology.]

include proteins, water, and K^+. Like the nail-chains and iron filings they may also exist in two (or more) discrete states. One state is a high energy state in which all these components are closely associated and together maintained at a high energy, called the living state. In the alternative death state, these cell components become disassociated and assume a high entropy, and low energy. Here the equivalent of the properly aligned sun and moon, or the big magnet, is a biologically controlling agent of fundamental importance, the ultimate product of all energy metabolism: ATP. Not as the source of a package of utilizable energy as once widely believed, but as an agent with great power electrically to polarize, to assemble, and to raise to a high energy-low entropy state the collection of proteins-water-ions which make up the living matter. This theory of a key role of ATP in the maintenance of the living state is based on the unequivocal facts that (i) though devoid of a package of energy in its phosphate bonds, it demonstrates strong power to polarize and interact with specific controlling sites, called the cardinal sites of key cell proteins, as evidenced by, for example, its -14 Kcal/mole of binding energy onto myosin (Wolcott and Boyer, 1974; Goody et al. 1977) - which strangely enough is in numerical value exactly equal to the erroneously determined free energy of hydrolysis of the two ATP phosphate bonds - and (ii) by the one striking feature of the unique component of all living matter - the partially resonating and thus highly polarizable polypeptide chain of the proteins, which is eminently suited to serve the role of the tethered chains of soft nails which propagate the polarization and in so doing, interact with the water molecules and ions just as the nails pick up singly or in multilayers the iron filings.

The Living Cell as a Complex System of Closely Associated Living Matter Existing at a High Energy Living State. As mentioned above, a living cell of a specific type maintains a constant quantitative relationship among all its components, most prominently displayed among the three largest components: water, proteins, and K^+. The membrane-pump theory has been primarily concerned with the question how K^+ (and Na^+) concentration are kept constant in cell water; it has never seriously dealt with the constancy of the protein-water relationship.

According to the AI hypothesis, the rule of constant proportionality among the cell's constituents is the natural consequence of their mutual interaction that led, for example, to the accumulation of both water and K^+ in the cell and the effective exclusion of other components found abundantly in the cell's environment, such as Na^+.

The constancy of the ratio of the cell protein and cell K^+ in the living state arises from a constant share of the total β- and γ-carboxyl groups (carried on aspartic and glutamic acid residues) of the cell's proteins in a specific electronic configuration, defined by the relatively low electron density measurable as low pK_a value or low c-value with high preference for K^+ adsorption (for details, see Ling, 1984, p. 155-163) and by the constancy of the effective concentrations of competing ions for the same β- and γ-carboxyl groups (e.g., Na^+, fixed cations).

The constancy of the ratio of cell proteins, cell water, and Na^+ is partly due to the inability of Na^+ to compete successfully against K^+ for the β- and γ-carboxyl groups and partly due to the low solubility of Na^+ (Cl^-) in water existing in the state of polarized multilayers as the entire cell water content does.

The constancy of the ratio of cell protein and cell water in the living state arises from the existence of a constant proportion of the cell proteins assuming such a conformation (the "fully extended conformation", see below for details) that has a strong tendency to adsorb in multilayers all the cell water (and more, if circumstances had allowed).

The equilibrium water content (per unit of cell protein) is thus determined by the equilibrium between the tendency of the "fully extended" cell proteins to polarize and to adsorb in multilayers still more water and the restraining forces of (1) "salt linkages" formed between some fixed anions (e.g., β- and γ-carboxyl groups) and fixed cations (e.g., ε-amino groups carried on lysine side chains) among different proteins (Ling, 1962, p. 244; Ling and Peterson, 1977); and (2) the opposite tendency to lose water in consequence of the lower osmotic activities created by the lower equilibration concentration of Na^+ ions in the cell water (due to its low solubility) than that in the external medium (Ling, 1980; Ling and Ochsenfeld, 1987).

In summary, in a living cell maintained at its high energy resting living state the constancy of cell water contents arise from their multilayer adsorption of all the cell water on a

fixed proportion of the cell proteins existing in the fully extended conformation. The constancy of cell K^+, also virtually all in the adsorbed state arises from the constancy of a fixed proportion of the cell's β- and γ-carboxyl groups existing in a low electron density state, with high preference for K^+ over its competitors as will be described next.

Cell K^+ is Virtually All Adsorbed on β- and γ-carboxyl Groups in Living Cells: Theory and Evidence. Most isolated proteins adsorb none or very little K^+ or Na^+. To explain this, I suggested that the β- and γ-carboxyl groups in isolated proteins are locked in salt linkages and thus not available for the binding of alkali-metal ions (Ling, 1952). Recently Ling and Zhang (1984) have completely confirmed this postulation. When the competing fixed cations (α-amino groups, ε-amino groups, and guanidyl groups) of the salt linkages are neutralized by titration with NaOH, for each mole of fixed cation neutralized, one mole of Na^+ becomes adsorbed onto the unmasked β- and γ-carboxyl groups in a stoichiometric manner.

Since 1977 on, the work of Ling (1977a), Trombitas and Tigyi-Sebes (1979), but especially Edelmann (1977, 1980, 1981, 1984) have left no question that in frog muscles, the bulk of cell K^+ is found primarily at locations where most of the β- and γ-carboxyl groups are found, i.e., at the A bands (and Z-lines). This was anticipated on the basis of the theory because more than 60% of the muscle cells' β- and γ-carboxyl groups are found in myosin and myosin is exclusively found in the A bands.

In frog muscle, various monovalent ions with identical long-range attributes (i.e., monovalent cations) but different in short-range attributes (e.g., polarizability, Born repulsion constant) which can only be told apart if in close contact, have entirely different effects in displacing cell K^+, an effect shown to be independent of the existence of a functional cell membrane (and postulated pumps) (Ling, 1977b, 1978). This finding left cytoplasmic sites as the seat of ionic selectivity by close contact adsorption.

Very recently, and in work yet to be published Ling and Ochsenfeld, relying on a new way of maintaining viability of frog muscle cells cut into 2 mm long segments (Ling, 1987), demonstrated that the Na^+ (and K^+) adsorbing sites are indeed β- and γ-carboxyl groups with a pK_a value of 3.9 typical of low c-value β- and γ-carboxyl groups.

With this fundamental problem resolved, the qualitative theory of selective cooperative adsorption of K^+, first demonstrated in 1966 (Ling, 1966) and subsequently widely confirmed in a large variety of living cells in my own and other laboratories (Ling, 1984, Chapter 11; Ling, 1988) has taken on new significance that mere quantitative agreement between theory and experimental data points were not able to achieve.

Multilayer Adsorption of Cell Water. In 1965, I presented the polarized multilayer theory of cell water as a subsidiary of, and hence completing the basic structure of the AI hypothesis (Ling, 1965; 1972; 1984).

In this theory, proteins can affect the property of water profoundly and in large quantity only when they exist in the " fully extended" conformation with the backbone NHCO groups directly exposed to the bulk phase water. The alternatingly positive (P) NH sites and negative (N) CO sites of a matrix of proteins chains with the NHCO directly exposed constitute an NP-NP-NP system. The bulk phase water in such a system is polarized in multilayers by these N and P sites.

Two points must be made very clear in order to avoid misleading impression. Firstly, the dynamic " structure" of water is much less defined and weaker than the static structure of ice or other crystals (Ling, 1979). Thus if water molecules in an ice crystal are compared to beads joined to their neighboring beads by rigid rods in a regular 3-dimensional lattice; then water molecules in the state of polarized multilayers may resemble similar beads joined to their nearest neighbors by weak rubber bands which in turn are fastened onto a springy framework under constant agitation. In fact, if a single x-ray picture is taken of the water for an exposure time many orders of magnitude longer than the rotational correlation time, no discernible structure is expected to be seen. Only if the protein chains are themselves entirely fixed and only if many x-ray photographs are taken repeatedly from a fixed position, can pattern of long-range spatial distribution of the water molecules be recognized (and in this aspect, different from normal liquid water, which has only short-range order). Secondly, the number of layers of polarized water molecules between adjacent fully extended protein chains in a living cell varies from cell type to cell type but is uniformly small: from a few to perhaps ten (Ling, 1983).

In the polarized multilayer theory of cell water, the cells are wet not because they are " soaked", nor because they contain a high concentration of osmotically active free K^+ (which is now established to be adsorbed and thus unable to serve this function) but because in the fully extended conformation the cell protein has a great propensity to sorb a great deal of water.

An experiment that can shed light on the validity of this theory is to find out if protein that for one reason or another does exist in the fully extended conformation will sorb enough water to match that of a living cell from a vapor phase containing water vapor at a relative pressure equal to that of a Ringer solution and if proteins which do not exist in the fully extended conformation sorb much less water.

Until very recently there had been great technical difficulty to conduct this study. Thus the vapor pressure of a Ringer solution is very close to saturation (p/p_o = 0.9966) and vapor equilibrium which takes only days to reach at vapor pressures usually studied (up to p/p_o =

0.95) takes months and even years to reach at pressures near saturation. However, recently Ling and Hu (1987) have developed a new method to study vapor pressure up to 0.99997. With this new technique we were able to confirm the prediction that only proteins (e.g., gelatin) and polymers (e.g., polyethylene oxide) that exist in the fully extended conformation sorb enough water to match that given by the total water content per unit cell protein in living cells. These new findings are in full harmony with the demonstration that about 5% of the water in frog muscle cells is tightly bound as monolayers and that the remaining 95% of cell water follows the predictions of Bradley's theory of polarized multilayers (Bradley, 1936; Ling and Negendank, 1970).

The Theory of Solute Exclusion from Cell Water and its Experimental Confirmation. That water associated with biological materials can have unusual properties was known since the earliest days of biology, often described as " Schwellungswasser" or bound water. Various attributes were at one time thought to distinguish such water including " non-solvency water", " osmotically inactive water", or " nonfreezing water". Many of these earliest investigators were impressed by the similarity between living protoplasm and gelatin gel or sol. and the term " colloids" (Greek: κολλα, meaning glue or gelatin) was coined by Thomas Graham (1861) to describe materials sharing their common characteristics. Unfortunately the basic criteria used to detect the bound water could not stand rigorous testing. In the 1940's the colloidal approach to living phenomena had all but vanished. Thus the demonstration that urea distributes itself equally between muscle cell water and its external aqueous medium led A. V. Hill to the conclusion that all muscle cell water is normal (Hill, 1930). The concept of nonsolvent bound water was thus dealt a fatal blow. Similarly the recognition that even pure water could be supercooled to -20° C, threw serious doubts that freezing point lowering observed in living matter was indicative of the unusual " bound water" and not simply due to the barrier effect of high concentration of proteins which blocks ice crystals propagation and formation (Blanchard, 1940).

These findings and interpretations which played key roles in the broad acceptance of the membrane theory and the rejection of the colloidal or protoplasmic approach to the interpretation of living phenomena, took on different meanings with the introduction of the polarized multilayer theory of cell water and its new concepts (Ling, 1965; 1972).

In the polarized multilayer theory, water existing in the state of polarized multilayers has reduced solvency for molecules and hydrated ions in a size-dependent manner. That is, small molecules and molecules like urea that can fit into the dynamic water structure of the polarized multilayers may not be excluded at all. Only large molecules and hydrated ions that cannot fit into the dynamic water structure are excluded. This is called the " size rule". Hydrated Na^+, sugars, and free amino acids which are as a rule excluded from the water of living cells are large and do not fit the dynamic polarized multilayered water structure and are therefore partially excluded.

A parameter was introduced to measure the degree of exclusion: the equilibrium distribution coefficient or q-value. A q-value of 0.5 means that the solute has a solubility half of that in normal liquid water. The q-value of Na^+, sugar, and free amino acids are usually in the range of 0.1 to 0.5. Another useful practical parameter is the ρ-value, or apparent equilibrium distribution coefficient when the solute in the cell or model system may not all be in the cell or model water but may be partly adsorbed.

Why should only large molecules and hydrated ions be excluded and have low q- (or ρ) values? There are two alternative reasons: one is entropic and the other enthalpic. The enthalpic reason, simply put is that it requires more energy to excavate a hole to accommodate the solute in polarized water than that gained in filling up the hole left behind in normal water when the solute is transferred from the normal water to the polarized water. The entropic reason is that the motional freedom, translational and especially rotational, are more severely hampered the larger and more complex the solute molecule is, for essentially the same reason that a larger butterfly will more easily become entangled in and become snared by a spider web than a small one (Ling and Sobel, 1975; Ling, 1984, p. 169-172).

Thus the basic theory of solute exclusion in the polarized multilayer theory of cell water already shows that Hill's demonstration that urea distributes equally between cell water and the external medium did disprove the (categorical) nonsolvent water concept but it did not prove the normalcy of cell water which at the time Hill and others believed. But this is only theory. It was the subsequent testing of the theory that made the new interpretation of Hill's history-making finding indisputable.

In the polarized multilayer theory, all proteins have the innate ability to polarize in multilayers the bulk phase water but isolated native proteins normally do not exercise this ability because the NH and CO groups which are instrumental in polarizing water are inwardly directed by forming α-helical, β-pleated sheets and other intra- or intermacromolecular H bonds with other CO and NH groups. It is only when, for one reason or another, these CO and NH groups are prevented from being internally neutralized and become directly exposed to the bulk water that the inherent ability of polarizing the bulk phase water becomes unmasked.

From the early 1970's on, my laboratory has undertaken systematic studies of solutions of many proteins and found, in agreement with theory, that they have as a rule, very little ability to reduce the solvency of Na^+, sugar, and free amino acids (Ling, 1984).

A gelatin solution or gel, in contrast, behaves quite differently. It has strong influence in reducing the solvency of the bulk phase water for Na^+ salts and other probes. And it is

not difficult to see why. When collagen is denatured to form gelatin, most of the interchain H bonds are broken. The amino acid residues making up gelatin contain large proportions of proline and hydroxyproline (which due to the lack of a proton on its pyrole N cannot form H bonds) and glycine (which is one of the strongest helix-breakers), which make gelatin unusual in that they exist to a substantial degree in the fully extended conformation. As such,gelatin can polarize water in multilayers (Ling, 1984, p. 174).

This understanding is of historical importance because it shows that Thomas Graham had instinctively recognized something unique about gelatin solution or gel and abstracted the set of attributes and called them gelatin-like or colloidal.

It can now be said that in fact colloids are those systems that provide the NP-NP-NP system or equivalents to polarize the solvent (water) in multilayers. It is the characteristics of solvents in the state of polarized multilayers that we see among colloidal systems including many characteristics of living cells.

Other Model Systems and the Collective Traits of Water in the State of Polarized Multilayers. Proline and hydroxyproline residues of gelatin provide only negatively charged CO (N) sites. Without a proton on their pyrrolidine nitrogen atoms, they do not provide positively charged P sites. Since the formation of an H-bond (in an α-helical or β-pleated sheet conformation) requires a proton-accepting (N) site and a proton-donating (P) site this deficiency of P sites prevents formation of α-helix and other intramolecular H bonds, thereby offering one basic mechanism how a gelatin remains " fully extended" in a given solution. But how can one be certain that these alternatingly negative sites and neutral (O) sites also polarize water in multilayers? The answer was provided when we began to study a collection of oxygen-containing synthetic polymers in which as illustrated by polyethylene oxide $(-CH_2-CH_2-O-)_n$ oxygen atoms with their negatively charged lone-pair electrons are separated from their nearest neighboring oxygen atoms by the correct distances (Fig. 2). These NO-NO-NO systems, also including polyethylene glycol (PEG), polyvinylpyrrolidone (PVP), and polyvinylmethyl ether (PVME), turned out to be just as effective as gelatin in reducing the solvency for Na salts, sugar, and free amino acids.

Another class of models for the water polarizing matrix protein visualized in the living cells are proteins denatured by urea, guanidine HCl, alkali, and acids. That urea and guanidine HCl can convert the " self-satisfied" native proteins into " fully extended" water polarizing proteins is not difficult to understand because they are known to be strong H-bonding agents, and they unravel the α-helical, β-pleated sheet secondary conformations. Studies of the urea effect not only demonstrated that native proteins with no or little influence on water solvency can be converted by urea (or guanidine HCl) into NP-NP-NP systems polarizing and reducing the solvency of

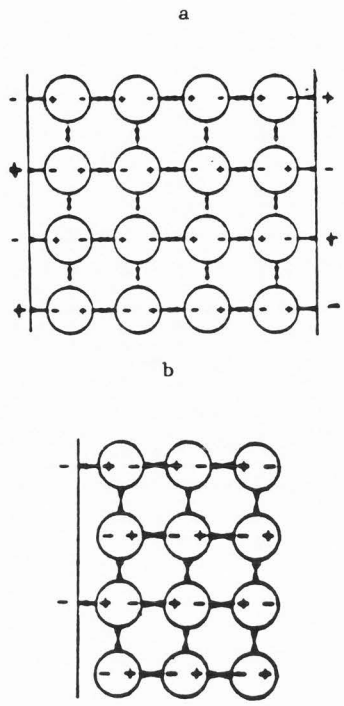

Fig. 2. Diagrammatic illustration of polarized water in an NP-NP system (a) and in an NO system (b)

the bulk phase water to Na salts, sugar, and amino acids as revealed by low ρ-values. They also clearly showed that urea, itself, is not excluded at all from the polarized water, exhibiting a ρ-value of unity (Ling, 1984, p. 177), thereby confirming experimentally our theoretical rebuttal to Hill's history-making conclusion that cell water is normal because the q-value for urea is unity.

For the moment, we may safely state that whether for structural reasons (e.g., gelatin) or in consequence of denaturation (e.g., by urea, NaOH), proteins or polymers interact with water, it is not just solvency for Na^+ salt, sugar, and free amino acids that are affected, but a whole gamut of distinctive properties revealed that are not shared by solutions of native proteins but are shared by living cells. Among these physico-electrical properties of water associated with fully extended protein chains and oxygen-containing polymers are, beside size-dependent exclusion of Na^+ salts, sugars, and free amino acids (Ling et al., 1980; Ling and Ochsenfeld, 1983): shortening of NMR relaxation times (T_1, T_2) and lengthening of NMR correlation time (Ling and Murphy, 1983); enhanced osmotic activity far beyond that predicted by molar concentration (Ling, 1983); freezing point lowering (Ling and Zhang, 1983); shrinkage in hypertonic solution (without a semipermeable membrane) (Ling and Ochsenfeld, 1987); extensive vapor sorption at partial vapor pressure of Ringer solution and higher (Ling and Hu, 1987).

Just as important was the successful confirmation of the reduced rotational (and translational)

freedom of water molecules by NMR methods both in model polymer-dominated water and in living cells (polymer: Ling and Murphy, 1983 - living cells: Ling and Tucker, 1980; Seitz et al., 1980); by high frequency dielectric dispersion methods both in polymer dominated water and in living cells (polymers: Kaatze et al.,1978 -living cells: Clegg et al.,1984); and by quasielastic neutron scattering methods both in polymer-dominated water and in living cells (polymers: Rorschach, 1985 - living cells: Trantham et al., 1984; Heidorn et al., 1986). In all cases,in both polymer-dominated water and in living cells there was demonstrated a rather modest reduction of rotational (and translational) motional freedom. The degree of motional restriction increases with decrease of water content and increase of polymer or protein contents - in full harmony with the polarized multilayer theory of cell water (Ling, 1965; 1972; 1979, p. 51; 1984).

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.

Bonting SL (1970) Sodium-potassium activated adenosinetriphosphatase and cation transport. In: Membranes and Ion Transport (Vol. 1) Bittar EE (ed), Wiley-Interscience, NY, 257-363.

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.

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.

Conway EJ, Kernan RP, Zadunasky JA (1961) The sodium pump in skeletal muscle in relation to energy barriers. J. Physiol. 155: 263-279.

Edelmann L (1977) Potassium adsorption sites in frog muscle visualized by cesium and thallium under the transmission electron microscope. Physiol. Chem. Phys. 9: 313-317.

Edelmann L (1980) Potassium binding sites in muscle. Electron microscopic visualization of K, Rb, and Cs in freeze-dried preparations and autoradiography at liquid nitrogen temperature using ^{86}Rb and ^{134}Cs. Histochemistry 67: 233-242.

Edelmann L (1981) Selective accumulation of Li^+, Na^+, K^+, Rb^+, and Cs^+ at protein sites of freeze-dried embedded muscle detected by LAMMA. Fresenius Z. Anal. Chem. 308: 218-220.

Edelmann L (1984) Subcellular distribution of potassium in striated muscles. Scanning Electron Microsc. 1984; II: 875-888.

George P, Rutman RJ (1960) The high energy phosphate bond concept. Prog. Biophys. Biophys. Chem. 10: 1-53.

Goody RS, Hofmann W, Mannherz HG (1977) The binding constant of ATP to myosin S_1 fragments. Europ. J. Biochem. 78: 317-324.

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

Heidorn DB, Rorschach HE, Hazlewood CF, Ling GN, Nicklow RM (1986) Neutron scattering studies of water in frog muscle. Biophys. J. 49: 92A.

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.

Jones AW (1965) The water and electrolyte metabolism of the arterial wall. Ph.D. Thesis, University of Pennsylvania, Phila., PA.

Kaatze U, Gottmann O, Podbielski R, Pottel R, Terveer U (1978) Dielectric relaxation in aqueous solutions of some oxygen containing linear hydrocarbon polymers. J. Phys. Chem. 82: 112-120.

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

Lehninger AL (1964) The Mitochondrion, Benjamin, Menlo Park, CA. Chapter 2.

Ling GN (1952) The role of phosphate in the maintenance of the resting potential and selective ionic accumulation in frog muscle cells. In: Phosphorous Metabolism (Vol. II) Mc Elroy WD, Glass B (eds), The John Hopkins University Press, Baltimore, MD, 748-795.

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 (1966) All-or-none adsorption by living cells and model protein-water systems: Discussion of the problem of permease-induction and determination of secondary and tertiary structures of proteins. Fed. Proc. 25: 958-970.

Ling GN (1969) A new model for the living cell: A summary of the theory and recent experimental evidence in its support. Int'l. Rev. of Cytol. 26: 1-61.

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 (1977a) K^+ localization in muscle cells by autoradiography, and identification of K^+ adsorbing sites in living muscle cells with uranium binding sites in electron micrographs of fixed cell preparations. Physiol. Chem. Phys. 9: 319-328.

Ling GN (1977b) Thallium and cesium in muscle cells compete for the adsorption sites normally occupied by K^+. Physiol. Chem. Phys. 9: 217-225.

Ling GN (1978) Maintenance of low sodium and high potassium levels in resting muscle cells. J. Physiol. 280: 105-123.

Ling GN (1979) The polarized multilayer theory of cell water and other facets of the association-induction hypothesis concerning the distribution of ions and other solutes in living cells. In: The Aqueous Cytoplasm, Keith AD (ed) Marcel Dekker, Inc., NY, 23-60.

Ling GN (1980) The role of multilayer polarization of cell water in the swelling and shrinkage of living cells. Physiol. Chem. Phys. 12:383-384.

Ling GN (1983) Studies on the physical state of water in living cells and model systems: III. The high osmotic activities of aqueous solutions of gelatin, polyvinylpyrrolidone and poly (ethylene oxide) and their relation to the reduced solubility for Na^+, sugars, and free amino acids. Physiol. Chem. Phys. and Med. NMR 15: 155-165.

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

Ling GN (1987) A new method for prolonging the survival of cut frog muscle segments. Physiol. Chem. Phys. and Med. NMR 29: xxx.

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

Ling GN, Hu W (1987) Studies on the physical state of water in living cells and model systems. VIII. Water vapor sorption on proteins and oxygen-containing polymers at physiological vapor pressures: Presenting a new method for the study of vapor sorption at close to and including saturation. Physiol. Chem. Phys. and Med. NMR 19: 251-269.

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 solutions of native and denatured protein, gelatin, and oxygen-containing polymers immersed in solutions of Na salt and of sugar and sugar alcohols. Physiol. Chem. Phys. and Med. NMR 19: 177-192.

Ling GN, Peterson K (1977) A theory of cell swelling in high concentrations of KCl and other chloride salts. Bull. of Math. Biol. 39: 721-741.

Ling GN, Sobel AM (1975) The mechanism for the exclusion of sugars from the water in a model of the living cell: The ion-exchange resin: Pore size or water structure? Physiol. Chem. Phys. and Med. NMR 7: 415-421.

Ling GN, Tucker M (1980) Nuclear Magnetic Resonance Relaxation and Water Contents in Normal Mouse and Rat Tissues and in Cancer Cells. J. Nat. Cancer Inst. 64: 1199-1207.

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.

Ling GN, Zhang ZL (1984) A study of selective adsorption of Na^+ and other alkali-metal ions on isolated proteins: A test of the salt-linkage hypothesis. Physiol. Chem. Phys. and Med. NMR 16: 221-235.

Ling, GN, Miller C, Ochsenfeld MM (1973) The physical state of solutes and water in living cells according to the association-induction hypothesis. Ann. NY Acad. Sci. 204: 6-50.

Ling, GN, Walton C, Ling MR (1979) Mg^{++} and K^+ distribution in frog muscle and egg: A disproof of the Donnan theory of membrane equilibrium applied to the living cells. J. Cell. Physiol. 101: 261-278.

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

Minkoff L, Damadian R (1973) Caloric Catastrophe. Biophys. J. 13: 167-178.

Peachey L (1965) The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. J. Cell Biol. 25: 209-231.

Podolsky RJ, Morales MF (1956) The enthalpy change of adenosinetriphosphate hydrolysis. J. Biol. Chem. 218: 945-959.

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.

Schoenheimer R (1942) The Dynamic State of Body Constituents, Harvard University Press, Cambridge, MA. p. 62-65.

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, Persoone G, Sorgeloos P, Rolls O, Jaspers E (eds), Universa, Wetteren, Belgium, 2: 545-555.

Skou JC (1960) Further investigations on a (Mg^{++} and Na^+)-activated adenosinetriphosphatase possibly related to the active, linked transport of Na^+ and K^+ across nerve membrane. Biophys. Biochim. Acta 42: 6-23.

Spector WS (1956) Handbook of Biological Data, Saunders, Phila., PA, Table 55.

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.

Trombitas C, Tigyi-Sebes A (1979) X-ray microanalytical studies on native myofibrils and mitochondria isolated from honey bee flight muscle. Acta Physiol. Acad. Sci. Hung. 14: 271-277.

Wolcott RG, Boyer PD (1974) The reversal of the myosin and actomyosin ATPase reactions and the free energy of ATP binding to myosin. Biochem. Biophys. Res. Comm. 57: 709-716.

Discussion with Reviewers

H.E. Rorschach: " the continuation of life... [during cryopreservation]... does not necessarily depend on pumping or other ceaseless chemical

activities." This is an interesting point that raises a question: are there studies of the temperature-dependence of $[Na^+]/[K^+]$ ratios in the cell? Is there a temperature below which the " pump" ceases, but Na^+ and K^+ diffusion can still occur? Or is the $[Na^+]$ exclusion maintained at all temperatures?

Author: Yes. In some types of mammalian cells, there is a critical temperature at which the adsorbed K^+ is replaced largely by adsorbed Na^+. However this is more in keeping with the notion that K^+ and Na^+ are adsorbed on cell proteins in a cooperative manner and the critical temperature is simply the Curie point in a cooperative transition rather than a sudden cessation of Na^+ pumping at the critical temperature. Two sets of evidence may be cited in favor of this view: (1) the extensive evidence that the bulk of cell K^+ is adsorbed on localized sites in the cells; (2) in frog muscle, no such transition was observed between 0^oC and above ambient temperature. Since frog muscle exhibits typical K^+ and Na^+ distribution patterns seen in almost all cells and are therefore likely to follow similar basic mechanisms, the indifference of the $[Na^+]_{in}/[K^+]_{in}$ in frog muscle argues against the temperature dependent pump interpretation.

H.E. Rorschach: Ling tries to present a picture of the structure of the water in a " polarized multilayer". I believe he goes considerably beyond what we know at this point. On the one hand, he pictures the water as forming layers of aligned dipoles (with definite spatial and orientational order). On the other hand, he cautions against a structural picture. It would be helpful if he would tell us what to expect from x-ray or neutron diffraction studies on these layers in, say, protein crystals.

Author: Dr. Rorschach communicated to me over the telephone his concern that a number of scientists have rejected the polarized multilayer theory after one look at Fig.1 which shows an ice-like structure. As I have emphasized again and again, Fig. 1 is only a static idealized diagram to illustrate a constantly changing dynamic structure. Since it is not possible for me to present the reader with a movie of a three-dimensional model, Fig. 1, with explanatory notes is the best I can do.

That a theory is not entirely proven yet and thus ahead of what we truly know is entirely in keeping with the time sequence of the Scientific Method where understanding follows full experimental confirmation of the predictions of the theory.

Having said this, I must correct Dr. Rorschach's misunderstanding that there is " no evidence for this form". On the contrary there is already considerable evidence.

Firstly that oppositely oriented water dipoles attract each other while similarly oriented water dipoles repel each other is the consequence of the law of electrostatics. The question is, when there is a checkerboard of negatively charged N and positively charged P sites separated from the nearest neighboring sites, by roughly the diameter of a water molecule, would multilayers of water molecules be polarized and oriented? This question was carefully examined for water and other gaseous molecules by Brunnauer, Emmett, and Teller (1938), who concluded that only where the gas molecules have a large permanent dipole moment would such a polarized multilayer be expected and in that case the adsorption should follow the polarized multilayer adsorption isotherm derived by Bradley " text reference". Since water does have a large permanent dipole moment (1.86 debyes), the answer is clearly affirmative from theoretical consideration. There are also experimental confirmations:

(1) Bradley isotherm: In agreement with the polarized multilayer theory of cell water, 95% of the water in frog muscle cells follows the Bradley polarized multilayer adsorption isotherm (Ling and Negendank,1970 "text reference"). In the agreement with theory, water sorption on gelatin (which is a protein that exists in the fully extended form) also follows the Bradley polarized multilayer adsorption isotherm (Ling, 1984 " text reference").

(2) Enhanced adsorption energy of multilayers of adsorbed water: Prof. W. Harkins measured the molar energy of desorption of water layers from the surface of an NP system (titanium dioxide powder) minus the energy of vaporization ($E_a - E_1$). $E_a - E_1$ was 6,550 cal/mole for the first layer of adsorbed water molecules, 1380 for the second layer, etc. From these data, Harkins concluded that " While the effect of the attraction of the solid dies off rapidly, it extends to somewhat more than 5 molecular layers" (Harkins, 1945, p. 294). Simple electrostatic calculations reveal that if two titanium dioxide surfaces are closely juxtaposed to each other (constituting what is called an NP-NP system), the total number of water layers that have E_a energy significantly in excess of E_1 will exceed 10 layers and furthermore, the steepness of the "dying off" of attraction will be reduced (Ling and Ochsenfeld,1983 "text reference"). It has been shown that even if no more than 5% of the cell's proteins exist in the fully extended conformation it is sufficient to polarize all the cell water in layers (between nearest neighboring chains) no deeper than 10 (Ling, 1983 " text reference"). As mentioned above 95% of this water in frog muscle has also been shown to follow the Bradley polarized multilayer adsorption isotherm.

(3) Cooperativity in the multilayer sorption of cell water: Cooperativity, in the statistical mechanical sense, denotes the existence of significant near neighbor interaction among units of an assembly. The juxtaposition of N and P sites produces a cooperative type of interaction that the adsorption of one water molecule directly or indirectly on these sites enhances the adsorption energy of the immediately neighboring water molecules. The polarized adsorption is therefore (auto)cooperative with a positive nearest neighbor interaction energy (Ling, 1984). An important characteristic of a cooperative assembly is the existence of a critical temperature or Curie point. Indeed this has been demonstrated in frog muscle as far back as 1967; the q-value of sucrose in frog muscle abruptly changes from 0.2 to 1.0 between 38^o C and 40^o C (Ling, 1967; see also Ling, 1984, p. 358).

(4) The rotational (and other) motional restriction of the bulk of cell water and water in model systems: Another prediction of the polarized multilayer theory of cell water is that all or virtually all of the cell water suffers rotational (and other) motional restriction. This predicted rotational motional restriction has been verified by quasielastic neutron scattering (QENS) of brine shrimp cysts and frog muscle by physicists from the Physics Dept. of Rice Univ. led by Prof. Rorschach in collaboration with Hazlewood, Clegg and others. Conclusions from QENS studies were also at least qualitatively supported by ultra high frequency dielectric (UHFD) studies of water in brine shrimp cysts by Clegg and coworkers (Clegg et al, 1984 " text reference")and by parallel QENS as well as UHFD studies of model systems of oxygen-containing polymers that satisfy the criteria of NO-NO-NO systems, i.e., a matrix of chains containing N sites separated by distances of approximately two water molecules, from the nearest neighboring N sites.

(5) Spacing requirement of oxygen-containing polymers: Striking similarity has been observed among water in living cells, in gelatin gel, in urea-denatured proteins, and in oxygen-containing NO-NO-NO polymer systems. Of those polymers studied, polyethylene oxide (PEO) is strikingly simple being linear chains of repeating units of $(-CH_2-CH_2-O-)_n$. PEO which has high solubility in water, sorbs an amount of water from an environment of physiological vapor pressure (p/p = 0.9966), in quantity similar to those of frog muscle - a relatively highly hydrated cell type (Ling and Hu " text reference"). Yet neither polyformaldehyde $(-CH_2-O-)_n$ with one less CH_2 group between a neighboring pair of oxygen atoms, nor trimethylene oxide $(-CH_2-CH_2-CH_2O-)_n$ with one more CH_2 group between a neighboring pair of oxygen atoms are even soluble in water (Stone and Stratta, 1967), demonstrating how important is the distance between neighboring N sites in the polymer in dissolving in and in sorbing large amounts of water, confirming the theory.

What can one expect from x-ray studies of water existing in the state of polarized multilayers? Theoretically, one would expect more sharply defined peaks occurring over large distances in x-ray radial distribution functions. Practically, one confronts the difficulty from the lack of pure polarized water and that to create the polarized water large concentration of polymer or proteins must be present generating problems not found in studies of pure liquid water or ice. Neutron scattering studies, on the other hand, have already proven their usefulness in demonstrating the 14-fold reduction of the rotational diffusion coefficient and 3-fold reduction of the translational diffusion coefficient of the less-hydrated brine shrimp cysts (Trantham et al. " text reference") and the 2-fold reduction of the rotational diffusion coefficient of the much more hydrated frog muscle (Heidorn et al. " text reference"). But as pointed out by Heidorn (1985) for more accurate and correct understanding of the QENS data, more refinement in the theory and underlying assumption of the theory are required.

As a rule only native proteins form crystals. Protein existing in the '' fully extended conformation" is usually referred to as " denatured". Denatured proteins as a rule do not form crystals. However, very recent work from our laboratory suggests that water associated with very high concentrations of native proteins also exhibits characteristics of polarized multilayers but of a much weaker sort. However, here the water polarizing N and P sites might be part of exposed surface in the " random coil" conformation or the polar groups on the protein surface. If polarized multilayers of water can indeed be created in highly concentrated native protein crystals their influence on water polarization may be more easily discernible by x-ray and other methods because the N and P sites themselves are not in constant motion as in, say, PEO solutions.

H.E. Rorschach: The sorption of water by cell proteins is an interesting question. I believe some reference to centrifuge studies, which show the strong retention of water by gels (but with little change in NMR parameters) would be of interest.

Author: Frog muscle cells, without intact cell membrane, can sustain prolonged centrifugation at from 400 to 1500 g. for 4 minutes without losing any of their cell water. The same procedure quantitatively removes all water in the extracellular space (Ling and Walton, 1976). This different response to centrifugation of extracellular normal liquid water and intracellular water adds further evidence that water in living cells is more tightly held than in free water in agreement with the polarized multilayer theory of cell water.

G. Roomans: The author states that K and Na are the most prominent solutes. One can of course have different ideas about the meaning of the word " prominent", but in most cells, the Cl concentration is higher than the Na concentration, and this ion, though not mentioned at all, is at least as " prominent".

Author: Here the word " prominent" is used in the historical context of being outstanding rather than merely abundant. Indeed both the abundance of K^+ and the scarcity of Na^+ in cells have played key roles in the formation of the major theories of living cells. Changes of K^+ and Na^+ concentration have profound influence on the resting and action potential of muscle and nerve while alteration of Cl concentration usually has no such effect on these widely studied cells.

G. Roomans: The author discusses the necessity of pumps for solutes to which the cell could not have been exposed in its evolutionary history. The view of special pumps for " unusual solute" is not even held by proponents of the " membrane pump theory". Accumulation or exclusion of such compound is generally explained by their affinity for pumps for " naturally occurring" solutes.

Author: The reviewer is right that the proponents of the membrane-pump theory deal with solute distribution problem in a one-pump-at-a-time

approach, and usually limited to familiar solutes. The AI hypothesis offered a general theory of solute distribution. To test this general theory, the distribution patterns of many solutes have been under investigation including many solutes that are not experienced by the cell's genome in the course of evolutionary history.

A very large number of these solutes are found at lower equilibrium concentration in the cell water ($[S]_{in}$) than in the external environment $[S]_{ex}$. In addition, there is very often observed rectilinear relationship between $[S]_{in}$ and $[S]_{ex}$ such that $[S]_{in}/[S]_{ex} < 1$ (see Ling, 1984, Fig. 11.17). The slope of this curve equal to the equilibrium distribution coefficient of the solute (q or q_s) are constant.

The rectilinear distribution with $q < 1$ has profound implications: Firstly the maintenance of the low level of S in the cell water could not depend on a process involving the combination with a limited number of sites, as it is in the case of membrane pumps. Were it otherwise, the curve should bend upward as $[S]_{ex}$ increases as the pumps sites become " saturated". The rectilinear distribution pattern widely observed among these foreign solutes, thus precludes the operation of pumps whether specifically designed for that foreign solute or originally intended for some other " naturally occurring solutes" as the cause of the observed low level in the cell water.

This inability of the pump model to explain the rectilinear distribution so often observed is probably why, despite the enormous effort spent in studying the Na^+ pump, Ca pump, etc., there is no quantitative equation proposed for the pump theory. Yet all one needs is to transpose a term of the equation that was proposed (e.g., Cohen and Monod, 1960) for solutes that distribute themselves at concentration higher inside the cell water, to obtain an equation for the case when $[S]_{in} < [S]_{ex}$. (For additional evidence against foreign solutes being transported by pumps intended for " naturally occurring" solutes, see Ling, 1988 " text reference").

G. Roomans: The author discusses the possible implications of preserving living cells at very low temperature. I do not follow the author in this mainly semantical exercise. A parked car is not necessarily broken down; some of them may be, but the majority will run happily as soon as you start the engine. The fact that one can " park" a cell at low temperature and then revive it may be a wonder of nature, but it proves or disproves nothing about pumping or not pumping. Actually, the best ways to preserve cells at low temperature involve treatment with glycerol or DMSO, which changes the ion content of the cell drastically (and, if I interpret the AI theory correctly, therefore must also change the energy state of the cellular proteins).
Author: If life is truly flame-like, then quenching of this " flame" can only mean death; lighting this " candle" again means the equivalent of the creation of new life. Similarly, if a car with its engine running is considered as equivalent to life; then turning off the engine must mean death. Turning on the engine again must mean the creation of new life. Since even parents don't create new life, they only give part of their lives to the next generation, I think most scientists thawing frozen embryos, or red blood cells would feel embarrassed, and rightly so, if told that they are in fact creating new life. It seems more logical to think the source of this embarrassment is the faulty flame theory.

G. Roomans: Since a virus does not have a membrane, wouldn't the study of ionic relationships of viruses give useful information with regard to the AI-hypothesis?
Author: This is a very good idea and I suspect further pursuit would yield valuable information. Indeed, already we know that the studies of Vaccinin virus by Mc Farlane et al. (1939) showed that water in amount equal to 5 to 7 times that of viral dry weight is inaccessible to sucrose - offering support that in this virus large quantities of cell water exists in a state similar to those of living cells with plasma membranes.

G. Roomans: Could you discuss cellular chloride content in terms of the AI hypothesis?
Author: Chloride is a major component of the external milieu of many living cells. Like various other solutes found in living cells, intracellular chloride tends to fall into two categories: freely desorbed in the cell water and adsorbed. The concentration of free Cl^- in a living cell varies with the " intensity of the degree of polarization (i.e., the " IP" factor) of the cell water. Thus in cells like frog muscle where the " IP" is high, q_{Cl} tends to be low; while in malignant cancer cells where the " IP" is low, q_{Cl} tends to be high. However IP factor is not the only deciding factor. Another factor is the nature of companion cations. In most Ringer solution, the comparison cation force is Na^+. The nature of companion cation is important because to preserve macroscopic electric neutrality, Cl^-, being an anion, cannot enter the cell and exist as free Cl^- without an accompanying cation or in exchange for another anion. If the accompanying cation is also exclusively found in the cell water as free cation, then its q-value will influence the q-value of the free Cl^- in the cell and they will share the same q_{Na^+,Cl^-} in the cell. A cation with a high q-value will raise the q-value of the free Cl^- in the cell water and vice versa. If the accompanying cation is preferentially adsorbed, the q-value for the Cl^- will increase. For these reasons, the q-value of Cl^- and other ions are as a rule much more complex than say that of an uncharged nonelectrolyte, in which case, the q-value is simply determined by the nature of the nonelectrolyte, usually following the " size rule".

Much greater variations occur among the size of the adsorbed fraction of Cl^- in different types of living cells. Major types of sites that adsorb Cl^- are the ϵ-amino groups and guanidyl groups carried on lysine and arginine side chains of cellular proteins. In 1962, Ling had presented evidence that Cl^- and other anion

binding follows a surprisingly unchanging rank order of preference, well known as the lysotropic or Hofmeister series, and that this constancy in the rank order according to Ling, reflects the strengths in anion binding on cationic amino groups' or its modification, guanidyl groups and the long saturated alephatic side chains which insulate the cationic charges density of these groups changes occurring in other parts of the protein molecules (in contrast to β- and γ-carboxyl groups with short side chains and widely varying affinities for the counterions, K^+, Na^+, etc.). Besides Cl^-'s intrinsic affinity for the fixed cations, another decisive factor is the nature of its companion cation. When the companion cation is strongly adsorbed (e.g., K^+) more Cl^- will be adsorbed on ε-amino groups and guanidyl groups potentially capable of adsorbing Cl^- (at the expense of " salt linkages" formed between β- and γ-carboxyl groups potentially capable of adsorbing K^+).

In summary, Cl^- distribution in living cells is complex, highly interesting and deserves far more studies than so far achieved.

I.Z. Nagy: The first part of the paper deals with the criticism of the membrane Na-pumps: as a matter of fact it declares that "...there has rarely been a theoretical concept that has been as thoroughly disproven as the membrane-pump hypothesis." Unfortunately, however, the reader cannot be convinced by the manuscript about the validity of this claim. The arguments cited by the author in favor of his claim are based on experiments on hibernated frog sartorius muscle, performed more than 30 years ago, and published in 1962. Moreover, the author claims that his findings have been " twice confirmed (Jones, 1965; Minkoff and Damadian, 1973)." However, as we learn from the reference list, Jones (1965) is a never published " Ph.D. Thesis Appendix". This cannot be found practically by anyone except the single University where the Thesis had been done, and anyway cannot be considered as a " confirmation" of the findings.

Author: Whether Dr. Jones' confirmatory work, published only as part of his Ph.D. thesis, constitutes a confirmation, I think it is best left to the reader of the paper to decide. All I need to point out is that Dr. Jones had carried out similar studies on a different kind of tissue reaching conclusions similar to mine and his Ph.D. thesis is undoubtedly available through interlibrary loan.

A valid theory as a rule has broad applicability to an observed phenomenon the theory is introduced to explain. The association-induction hypothesis, for example, can explain the distribution pattern of Na^+, K^+, and any other solutes in existence and yet to be created, at 0° C or at whatever temperature at which the living cell survives. If the Na pump theory can explain sustained low level of Na^+ and high level of K^+ only at say 25° C but not at 0° C, does it still deserve to be considered a theory? To test a theory, it must be done in as rigorous and as demanding situations as possible.

I.Z. Nagy: It is a very serious issue to claim that practically all the actual cell physiology is completely wrong when attributing a function to the sodium-pump in the membrane. If the author wants to gain attention to his ideas, he has to offer much more and much better established data than those in Table 1. First of all, an experiment performed at 0° C may completely be meaningless as regards the energetic calculations, especially because the system used is a tissue of a poikylothermic animal which regularly undergoes a hibernation during which all the physiological processes are extremely slowed down. It seems to be a conceptual error to measure " energy delivery" in a tissue poisoned by IAA and nitrogen at 0° C. On the other hand, the energetic calculations made by the author should have been compared by those made by the people on the opposite side: it is well known that numerous calculations have been performed according to which a portion of 20-50% of the biological energy yield is used for the maintenance of the sodium gradient between the intra- and extracellular space. Dr. Ling should have shown in detail why his calculations are different from those of the others, and where is the error (if there is any) in the calculations of the opposite side. In the present form his claims about the energetic balance of the sodium transport in the frog muscle remain only declarations without any support, and what is still worse, due to the missing explanations, one cannot even make any attempt to control them. It is not acceptable to make reference to a work written 25 years ago, and blame all the existing cell physiological knowledge in this respect without a careful analysis and comparison of the more recent experimental data. The publication of the present manuscript of Dr. Ling can only create further confusion, but it will not promote any open-minded discussion on the topics, which would be really necessary.

Author: Scientific truth is also ageless. Results of a crucial experiment valid 30 or even 100 years ago is valid today. So Dr. Nagy's unwillingness to accept my finding because it was published 25 years ago has no legitimate foundation. At any time, Dr. Nagy could have gotten a copy of the book (Ling, 1962 "text reference") from a library or through the interlibrary loan service or to use the Citation Index to reach the many review articles I have written on the subject. Among the more recent ones is my new monograph (Ling, 1984 " text reference") where the original finding first presented in 1962 was again reviewed in some detail.

The figure of 20% to 50% of the resting energy output of frog muscle is spent in operating the postulated Na pump were obtained by Levi and Ussing (1948), Harris and Burn (1949) and by Keynes and Maisel (1954) " text reference". These conclusions were drawn years before my work was published in 1962 from experiments performed under conditions different from mine. Nor were their experiments designed to test rigorously if the Na pump hypothesis was tenable - they merely compared the energy need at 25°C with the energy available from the resting oxygen consumption of the tissues. My experiment was specifically designed to test rigorously if the Na

pump hypothesis was tenable - therefore I selected the most stringent conditions in which the energy source was reduced to a minimum while the low level of Na^+, high level of K^+ were maintained for many hours (e.g., 7.4 hrs.). This stringent condition imposed on frog muscles was brought about by lowering the temperature to 0^oC and by full arrest of both respiration and glycolysis (see Ling, 1984, p. 90). 7.4 hours is many times longer than the Na^+ renewal, the time for 90% exchange of cell Na^+ at this temperature was only 80 minutes. Thus in terms of the membrane-pump theory, the cell Na^+ would have long before reached the concentration in the external medium (contrary to facts) if the poisons and chilling had truly stopped the (hypothetical) Na pump. These stringent experimental conditions provided the basis for a comparison to be made between the minimal energy need of the Na pump and the maximal energy available as shown in Table 1.

While I disagree to varying degree with almost all the statements Dr. Nagy expressed here, I nevertheless have sympathy for Dr. Nagy and other fundamentally innocent scientists who have been seriously let down by the leaders whom Dr. Nagy and others have trusted implicitly. After all, the subject matter was not exactly Dr. Nagy's specialty. Yet my findings of 1962 and subsequent discoveries might very well have direct bearings on Dr. Nagy's and other's work and interpretations. As an example of the type of information manipulation and suppression done again and again, I refer to the " first of its kind" review on the Na pump bearing " Na Pump" as its title, by I. M. Glynn and S. J. D. Karlish (1975) from the world renowned physiological laboratory of Cambridge University. Of a total of 245 references cited covering a period of more than twenty years, all in favor of the Na pump, not a single paper from my laboratory and any other laboratories which have experimentally refuted the Na pump concept was cited. This practice of citing only favorable evidence and not citing unfavorable ones is against the ethical rule of science and strikes at the very root of science which has no other purpose than the seeking of the truth.

I.Z. Nagy: Some further points must be mentioned too: Dr. Ling claims that," Three remedial postulations to keep down the energy have all been experimentally disproven". The reader of his paper cannot understand, why some more possibilities have not been considered in this aspect: For example, the rigidization of the cell membrane lipid layer at 0^o C (and the concomitant slowing down of the lateral diffusion of proteins) may well cause a very serious decrease of the resting membrane permeability for sodium, and this may explain a great part of the lower energy need for maintaining the homeostasis at such a low temperature. This is only one example, but it cannot be the task of the reviewer to list all the possible mechanisms and explanations which are just omitted by the author.

I am of the opinion that the defects of the manuscript listed above, and similar ones in other publications of the same author might have contributed to a great extent to the actual situation, mentioned also by Dr. Ling, namely, "... my findings and conclusions have not been challenged in print;" People, even if they would like to do so, are not able to judge the real content of the author's ideas because of the way he treats his own and the existing data.

Author: I cannot answer the question, " Why some more possibilities have not been considered (besides the three remedial postulations)?" Perhaps the answer is that it is not as easy as Dr. Nagy believes. Take for example, his idea of membrane " rigidization" - which I take to mean what is expressed by the English word, rigidification. One really does not need to make this hypothesis at all. We already know cooling to 0^o C does slow down Na^+ exchange, and according to the pump theory, lowers the energy need of the pump. Yet despite the slowing down of Na^+ " pumping" rate and it was shown to be 1500% to 3000% of the maximally available energy. So Dr. Nagy's remedial hypothesis does nothing at all to alleviate the energy crisis of the Na pump and is indeed rather pointless.

I must, however, protest Dr. Nagy's accusation that I have omitted some possible mechanisms and explanations. I have done no such thing. The three remedial postulations were the only ones published and my conclusion against the Na pump hypothesis has remained unchallenged in the last 25 years. A thorough search with the aid of Citation Index and Source Index bears me out on both accounts. However, if Dr. Nagy has some new challenges beyond these three remedial postulations, he should have it published. However, I would not recommend rushing too fast into publication, if these additional remedial hypotheses are as pointless as the " rigidization" hypothesis given above.

I.Z. Nagy: The second part of the paper deals with the " High Energy Living State" theory, proposed by Dr. Ling. The reader encounters again great difficulties when reading this part of the manuscript. Namely, the author claims in various places of the manuscript that there is a constancy of the ratio of cell protein, cell water, K^+ and Na^+. Unfortunately, this assumption is simply not true. A great number of data show that during ontogenesis and aging there is a continuous dehydration of the tissues (and the total body) meanwhile the protein (and total dry mass) content of the intracellular space increases considerably. Dr. Ling attributes an important role to the " constancy" of these ratios, but the facts strongly contradict the validity of his assumption. This compromises even the valid elements of his hypothesis on the living state, and renders again impossible an open, serious discussion on his proposals. I want to stress that the problem of interaction of water and ions with the macromolecules is one of the most important questions of the actual cell biology, and in this respect I am really open for any new proposal or idea or explanation. However, such new ideas must be based on solid facts and not on fantasies which are far from the biological reality.

Author: The meaning of the word constancy used is that of common sense and common usage. This constancy refers to the underlying fact that a biological handbook can list, say, the water content of human red blood cells as 63%, that of its K^+ content, 8.9×10^{-12} meq/cell and that of its Na^+ content 15×10^{-12} meq/cell (see Ponder, 1948, Table XII iii), just as one can normally describe human red blood cells as representing a biconcave disc without a nucleus (without going into a lengthy discussion how it looks before maturation, after senility, etc., etc.) all based on the underlying constancy in cells that are implicitly normal and mature. Indeed if no constancy at all exists there would have to be a table for each single red blood cell and the world of science would become a world of chaos.

Additional References

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

Cohen GN, Monod J (1960) Bacterial Permeases. Bacteriol. Rev. 21: 169-194.

Glynn IM, Karlish SJD (1975) The Sodium Pump. Ann. Rev. Physiol. 37: 13-55.

Harkins WD (1945) Surfaces of solids in science and industry. Science 102: 292-295.

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

Heidorn D (1985) A quasielastic neutron scattering study of water diffusion in frog muscle. Ph.D. Thesis, Rice University, Houston.

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.

Ling GN (1967) Effects of temperature on the state of water in the living cell. In: Thermobiology, Rose A (ed) Academic Press, NY, 5-24.

Ling GN, Walton CL (1976) What retains water in living cells? Science 191: 293-295.

Mc Farlane AS, Mc Farlane MG, Anies CR, Eagles GH (1939) Physical and chemical examination of vaccinia virus. Brit. J. Exper. Path. 20: 485-501.

Ponder E (1948) Hemolysis and Related Phenomena. Grune and Stratton, NY, Chap. 4.

Stone FW, Stratta JJ (1967) Ethylene oxide polymers. In: Encyclopedia of Polymer Science and Technology, Vol. 6, John Wiley and Sons, NY, 103-145.