Water and the Cell

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Cover design: The structural relationship of water molecules is shown in a cross-sectional view of a collagen fibril in a fully hydrated tendon; the tight hexagonal packing in a total cluster of 18 water molecules around each tropocollagen molecule forms a firm (tightly associated) monolayer. The design was taken with the kind permission of Professor Gary Fullerton from his paper (Fullerton GD and Amuroa MR. *Cell Biology International* **30**, 56–65, 2006 – Figure 2), in agreement with the publishers, Elsevier Press. It was selected and redrawn by Denys Wheatley, and finally placed against the same background image in white against the pale blue of the cover with the assistance of Springer-Verlag (Graphics).

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PREFACE

This edited volume deals with the state of water in the vicinity of biological interfaces, both intracellular and extracellular. This issue is of critical importance, for the cell is extremely crowded with interfaces, and as a result practically all cell water is interfacial. The character, or state, of this water may therefore be central to cell function.

What is meant by the 'state of water?' Few would question that water coming out of a household tap is a liquid, but water in an ice cube is something altogether different: it is a solid that floats on tap water (also known as bulk water). It is water in the solid state.

The fact that ice floats is an indication that it is less dense than water. Clearly, the physical properties are different. Water molecules below 0 °C form a crystal. In this crystal, the two positively charged hydrogen atoms of water bind to the double negative charges of oxygen atoms of two adjacent water molecules. The resulting crystal lattice is arranged in such a way as to be less dense than tap water, and constituent water molecules are also less mobile.

But what of water adjacent to surfaces, be they biological or inanimate? Does this represent yet another state of water, distinct from solid or liquid? Water's positively charged hydrogen atoms might be expected to bond to a negatively charged surface, whereas its negatively charged oxygen atom ought to bond to a positively charged surface. One might then ask if the dipolar orientation of the first bound water layer might provide an arrangement of charges that causes a second layer of dipolar water molecules to form. If so, how many layers might build? How might the physical properties of this water differ from those of bulk water?

These issues are dealt with in depth in the chapters of this volume. Several chapters imply that the ordered interfacial zone may extend considerably farther than generally envisioned.

There is appreciable background for such thinking, both theoretical and experimental. For example, consider the surface of a polished silver chloride crystal. Positive Ag^+ and negative Cl^- charges are spaced at a distance of about 3 Angstroms, or about the diameter of a water molecule. Thus, the surface charges form a checkerboard, whose spacing is equal to the size of a water molecule. In this case the positive charge of one water-hydrogen atom could bind to a negatively charged surface-Cl^-, while the negatively charge of oxygen of another water molecule could bind to a positively charged surface-Ag^+. Bound water molecules in this first layer could then hydrogen bond to one another to form a highly polarized monolayer that could serve as the nidus for formation of a second layer. In this

situation, it is predicted that numerous water layers could form (Ling 2003). In fact Hori (1956) has demonstrated that water between the surfaces of two quartz or polished glass plates spaced less than 100 μm apart does not freeze at temperatures as low as −90 °C. The inability to freeze is an indication of structuring. The span in question is 30,000 water-molecule diameters. Clearly, at these inanimate surfaces, multiple layers of water molecules are perturbed relative to water molecules in bulk.

What about the water in biological systems? Is the state of water in cells similar to bulk water, or is it organized? When a ciliated protozoan, such as *Tetrahymena*, growing in a dilute proteose peptone media is smashed between a microscope slide and a cover slip, the cortex of the cell ruptures, and a water-immiscible substance flows out and separates as a drop of cytoplasm (Cameron, unpublished). Further smashing of such extrusions can fractionate the drop into smaller immiscible droplets. This is a common observation, seen in various forms by many others. It indicates that protoplasm in the cell is immiscible in a dilute solution, and that retarding the flow of fluid/water from the cell protoplasm does not necessarily require an intact cell membrane; it is inherent in the physical features of the protoplasm itself.

These observations alone would appear to cast doubt on the tenet of the cell membrane as the critical water-diffusion barrier between the extracellular environment and the cytoplasm, where the intracellular water is assumed to be relatively free to exit the cell upon membrane disruption. Even in the absence of a membrane, the protoplasm does not dissipate.

The chapters in this monograph deal with water at the interfaces of both inanimate and biological systems. The biological systems include both filamentous and globular proteins, hydrophilic and hydrophobic surfaces, extracellular materials, and cells. What evolves is that water within cells is to a major extent ordered differently than bulk water, and functions not as an inert solvent, but as an active player. Most of intracellular water is adsorbed onto surfaces, which themselves are dynamic.

Understanding water order in biological systems is key to an understanding of life processes and to an understanding of diseases.

The material in the book should be of value to any person interested in the role of water inside the cell. This includes professionals in the area of cell biology, chemistry, and biochemistry. It also includes students interested in understanding the underlying basics of life.

The reader will be richly rewarded with insights difficult or impossible to obtain in current textbooks, which generally treat water merely as a background carrier with limited significance.

It was Albert Szent-Gyorgyi, Nobel Laureate, who stated that ‘Life is water dancing to the tune of macromolecules.’ Szent-Gyorgyi’s famous pronouncement is borne out in the contents of this volume. Water is definitely a major player in the biology of the cell.

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CHAPTER 1

A CONVERGENCE OF EXPERIMENTAL AND THEORETICAL BREAKTHROUGHS AFFIRMS THE PM THEORY OF DYNAMICALLY STRUCTURED CELL WATER ON THE THEORY'S 40TH BIRTHDAY

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Abstract: This review begins with a summary of the critical evidence disproving the traditional membrane theory and its modification, the membrane-pump theory – as well as their underlying postulations of (1) free cell water, (2) free cell K^+, and (3) 'native'-proteins being truly native.

Next, the essence of the unifying association-induction hypothesis is described, starting with the re-introduction of the concept of protoplasm (and of colloid) under a new definition. Protoplasms represent diverse cooperative assemblies of protein-water-ion – maintained with ATP and helpers – at a high-(negative)-energy-low-entropy state called *the resting living state*. Removal of ATP could trigger its auto-cooperative transition into the low-(negative)-energy-high-entropy *active living state* or *death state*.

As the largest component of protoplasm, cell water in the resting living state exists as polarized-oriented multilayers on arrays of some fully extended protein chains. Each of these fully extended protein chains carries at proper distance apart alternatingly negatively charged backbone carbonyl groups (as N sites) and positively charged backbone imino group (as P sites) in what is called a NP-NP-NP system of living protoplasm. In contrast, a checkerboard of alternating N and P sites on the surface of salt crystals is called a NP surface.

The review describes how eight physiological attributes of living protoplasm were duplicated by positive model (extroverts) systems but not duplicated or weakly duplicated by negative model (introverts) systems. The review then goes into more focused discussion on (1) water vapor sorption at near saturation vapor pressure and on (2) solute exclusion. Both offer model-independent quantitative data on polarized-oriented water.

Water-vapor sorption at physiological vapor pressure ($p/p_o = 0.996$) of living frog muscle cells was shown to match quantitatively vapor sorption of model systems containing exclusively or nearly exclusively fully extended polypeptide (e.g., polyglycine, polyglycine-D,L-alanine) or equivalent (e.g., PEO, PEG, PVP). The new Null-Point Method of Ling and Hu made studies at this extremely high vapor pressure easily feasible.

G. Pollack et al. (eds.), Water and the Cell, 1–52.

Solute exclusion in living cells and model systems is the next subject reviewed in some detail, centering around Ling's 1993 quantitative theory of solute distribution in polarized-oriented water. It is shown that the theory correctly predicts *size dependency* of the q-values of molecules as small as water to molecules as large as raffinose. But this is true only in cases where the excess water-to-water interaction energy is high enough as in living frog muscle (e.g., 126 cal/mole) and in water dominated by the more powerful extrovert models (e.g., gelatin, NaOH-denatured hemoglobin, PEO.) However, when the probe solute molecule is very large in size (e.g., PEG 4000), even water 'dominated' by the weaker introvert model (e.g., native hemoglobin) shows exclusion.

Zheng and Pollack recently demonstrated the exclusion of coated latex microspheres 0.1 μm in diameter from water 100 μm (and thus some 300,000 water molcules) away from the polarizing surface of a poly(vinylalcohol) (PVA) gel. This finding again affirms the PM theory in a spectacular fashion. Yet at the time of its publication, it had no clear-cut theoretical foundation based on known laws of physics that could explain such a remote action.

It was therefore with great joy to announce at the June 2004 Gordon Conference on Interfacial Water, the most recent introduction of a new theoretical foundation for the long range water polarization-orientation. To wit, under ideal conditions an 'idealized NP surface' can polarize and orient water *ad infinitum.* Thus, a theory based on laws of physics can indeed explain long range water polarization and orientation like those shown by Zheng and Pollack.

Under near-ideal conditions, the new theory also predicts that water film between polished surfaces carrying a checkerboard of N and P sites at the correct distance apart would not freeze at any attainable temperature. In fact, Giguère and Harvey confirmed this too retroactively half a century ago

Keywords: water, cell water, polarized multilayers, association-induction hypothesis, AI Hypothesis, polarized multilayer theory, polarized oriented multilayer theory, PM theory, long-range water structure, water, vapor pressure, super-cooling, non-freezing water, silver chloride crystals, glass surface, BET theory

Symbols and Abbreviations: a, amount of water (or other gas) adsorbed per unit weight of adsorbent; α, polarizability; BET Theory, the theory of multilayer gas adsorption of Brunauer, Emmett and Teller (1938); d, distance between nearest neighboring sites on an NP surface; E^n, (negative) adsorption or interaction energy of water molecules polarized by, but far removed from an idealized NP surface (see Figure 27); μ, permanent dipole moment; NO surface or system, a checkerboard of alternatingly negatively charged and vacant sites; NO-NO-NO system, a matrix of arrays of properly-spaced negatively charged N sites and vacant O sites; NP surface, a checkerboard of alternatingly negatively charged N sites and positively charged P sites; NP-NP system, two juxtaposed NP surfaces; NP-NP-NP system, a matrix of more or less parallel arrays of linear chains of properly spaced N and P sites; p/p_o relative vapor presssure equal to existing vapor pressure; p, divided by the pressure at full saturation under the same condition; PEG, poly(ethylene glycol); PEO, poly(ethylene oxide); PVA, polyvinyl alcohol; PVP, polyvinylpyrrolidone; PM Theory, the Polarized-Oriented Multilayer Theory of Cell Water; PO surface, a checkerboard of alternatingly positive P sites and vacant O sites; PP surface, a checkerboard of uniformly positively charged sites; q-, or q-value, the (true) equilibrium distribution coefficient of an ith solute between water-containing phase of interest (e.g., cell water) and a contiguous water-containing phase such as the bathing medium; r, the distance between nearest neighboring water molecules; ρ-, or ρ-value, the apparent equilibrium distribution coefficient may include bound solute in addition to what a q-value represents

For not telling the whole truth, Martha Stewart went to jail. Many know that. In contrast, few are aware that many more than one scientist, teacher, textbook writer etc. have been engaged knowingly or unknowingly in telling half-truth and untruth. But they don't go to jail. Instead, they are blissfully honored and rewarded for passing half-truths and untruths as the whole truth and teaching them to generation after generation of young people now living and yet to come. Why does a civilized society built on the laws of equal justice, openly condone the opposite?

A moment of reflection would reveal an obvious cause: a rarely discussed 'Achilles heel' in even the finest forms of governments in existence. That is, the vast number of our species whose wellbeing and even survival hang on what we decide to do or not to do today have no say in making those decisions – since they are not born yet.

Martha Stewart went to jail because not telling the whole truth caused some monetary and related losses to people now living. And these living people *had* votes and voices. As a result, government officials took action. Yet those same government officials or their equivalents would probably only shrug – if that, – when told that many scientists and science teachers were doing what Martha Stewart did – only on a much grander scale.

For, as a rule, what a sound basic science can offer lies in the *future* – e.g., in practical applications built upon new knowledge that basic research brings to light. Those future applications would be the modern equivalents of the steam engines, the electric motor, the electric generator, and the wireless telegraphy. None of these was invented out of thin air. They grew out of the progress made in earlier basic science.

Only by now, our need for further progress in basic science, especially basic physiological science, has far surpassed that of the past. For Mankind will soon face problems it has not faced before: overpopulation, exhaustion of natural resources, increasingly more deadly diseases beyond what our make-believe understanding of living phenomena could cope with – to mention only three.

But seen from the viewpoints of the research-funding agencies, members of school boards and even the Nobel Prize committees, research and teaching based on the most up-to-date valid new knowledge or based on some popular, but erroneous idea might not seem to matter that much.

To begin with, they usually do not have the up-to-date expertise or adequate time to know and understand the difference. And the few who did find out are alone. The majority, who may see little gains but more headaches for themselves in rocking the boat, easily outvotes them.

Nonetheless, the condoned blurring of what is right and what is wrong cannot continue indefinitely. Look at Enron, the seventh largest US corporation before its downfall and A.B. Anderson, the once gold standard of accounting worldwide. For in a global capitalist economy, individual nations and even the world as a whole have become bigger versions of corporations like Enron and A.B. Anderson. They too cannot long endure if the line between what is truth and what is falsehood is being blatantly ignored.

At this juncture, I like to quote Andy Grove, one time CEO of Intel, who wrote the book: *Only the Paranoid Survive* (Grove 1996.) For what separates a paranoid from a normal counterpart is the preoccupation of the paranoid with the *future* (and the preoccupation of the normal with *now*.) As a self-diagnosed paranoid, Andrew Grove saved Intel by making drastic changes in the makeup of the company and in transforming the world's largest semiconductor maker to the premier manufacturer of microprocessors.

That is why in Andrew Grove and those who think and act like him lies the real hopes of the future. They live in the present but they keep their eyes open to what lies ahead. They are the alert bus drivers on a treacherous mountain road. In some ways, they are Plato's philosopher kings.

It is on this note of hope, that I write the following review on the basic science of life, or cell physiology, which had seen a profound (but artificially hidden) change that Andrew Grove would have called a *strategic inflection point*. Only this one occurred half a century ago.

1. THE FIRST UNIFYING THEORY OF CELL PHYSIOLOGY AND THE SUBSEQUENT VERIFICATION OF ITS ESSENCE

Fundamentally speaking, cell physiological research is like solving a gigantic crossword puzzle. Like the crossword puzzle, cell physiology also has just *one unique solution*. But to reach out to that unique solution, cell physiologists of the past faced an insurmountable obstacle.

That is, when the study of cell physiology began, the physico-chemical concepts needed to construct the correct unifying theory were not yet available. An *incorrect guiding theory* was doomed to be introduced and it was (see below.) And as time went on, this incorrect theory would either kill that branch of science, or worse: it would be taught as unqualified truth to younger generations living and yet to come.

Meanwhile, the study of cell physiology broke up into smaller and smaller fragments or specialties. In time each specialty spawned its own lingo, its own methodology and its own subspecialties; the contact of each specialty with other specialties become less and less frequent and more and more perfunctory. The cumulative result is as Durant described: 'We suffocated with uncoordinated facts, our minds are overwhelmed with science breeding and multiplying into speculative chaos for want of synthesis and a unifying philosophy.' (*The Story of Philosophy*, Durant 1926, reprinted repeatedly till at least 1961, p 91).

Now, Durant's complaint addressed the lack of a correct unifying philosophy or theory, which alone can bind together and make sense out of the senseless fragments. Then, often quietly and little by little, the obstacles to produce a correct unifying theory of cell physiology gradually melted away – when the most relevant aspects of physics and chemistry reached maturity in the late 19th and early 20th century.

Therefore, in broad terms it was not entirely surprising (although it has never ceased to be surprising to me) that some forty years ago a unifying theory of cell

physiology built upon mature physics and chemistry made its debut. It bears the name, the *association-induction* (AI) *hypothesis* (Ling 1962). Worldwide experimental testing and confirmation of its essence followed rapidly – as chronicled in three additional monographs published respectively in 1984, 1992 and 2001 (Ling 1984, Ling 1992, Ling 2001).

It would seem that the day would soon arrive when swift progress would light up another new age in science (of the living) like the one (of the dead) in the 17th – early 20th centuries. Unfortunately, forty years afterward, it has not happened yet.

As it stands today, few biomedical researchers, teachers or students here and abroad have ever heard of these books and what they tell, let alone understanding or teaching them. Instead, obsolete ancient ideas called the membrane theory, or its later version called the membrane-pump theory, are still universally taught as proven truth at all level of education – long after both have been thoroughly and resoundingly proven to be wrong.

For the details of the widely taught, but incorrect misguiding theory and alternatives, you must consult my most recent book, *Life at the Cell and Below-Cell Level*. It is the only book that takes you through the complete history of cell physiological research, beginning with the invention of microscopes and the first perception of the living cell as the basic unit of all life forms.

Here I offer a short cut to a part of the hidden scientific history as well as a list of references to the original sources of publication. But, above all, this article ends with an account of some important new discoveries that occurred *after* the publication of *Life* in the year 2001.

2. THE COMPLETE DISPROOF OF THE MEMBRANE (PUMP) THEORY AND ITS ANCILLARY POSTULATIONS

According to the membrane theory, each living cell is a small puddle of ordinary liquid water. In this ordinary liquid water is freely dissolved small salt ions of various kind, mostly potassium ions (K^+), and large molecules, mostly proteins (and some RNA and DNA).

Substances like K^+ and sodium ion (Na^+) are, as a rule, found in the cell at concentrations different from their counterparts in the surrounding medium (Figure 1). This type of asymmetrical solute distribution was seen as the consequences of a sieve-like cell membrane. With rigid pores of exactly the same and correct size, the cell membrane permits the intra-, extra-cellular traffic of ions and molecules smaller than the membrane pores but keeps out ions and molecules larger than the pores – absolutely and permanently.

When the sieve membrane idea failed to explain the asymmetrical distribution of K^+, Na^+ and other solutes, an *ad hoc* membrane pump theory was installed in its place. Then, it is a battery of submicroscopic pumps in the cell membrane that are installed to maintain the *status quo*. The sodium (potassium) pump, was a prominent example. Located in the cell membrane, this pump is postulated to push sodium ion (Na^+) out of the cell and to pull potassium ions (K^+) into the cell,

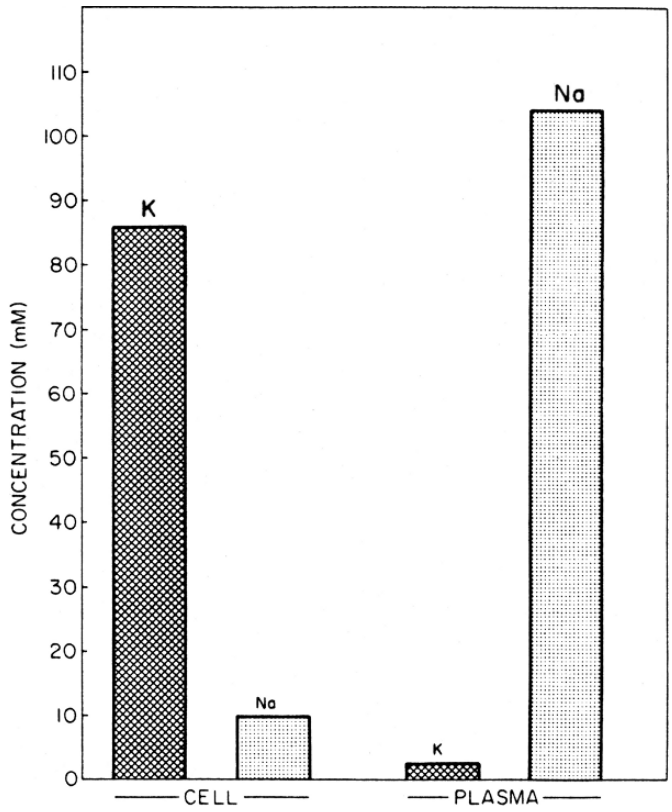

Figure 1. Potassium ion (K) and sodium (Na) concentration in frog muscle cells and in frog blood plasma. Concentrations in frog muscle cells and in frog blood plasma are given respectively in millimoles per liter of cell water or plasma water (from Ling 1984 by permission of Plenum Press)

24 hours a day, 7 days a week without stop. As mentioned above already and to be elaborated some more below, this membrane pump model did not fare better than the original sieve membrane theory.

The following is an itemized list of the decisive experimental findings. These findings have passed the final verdicts on the fate of both the original sieve membrane theory and the membrane-pump theory – as well as on the fate of the ancillary assumptions on which both the sieve membrane and the membrane pump theory were built.

2.1 Disproof of the Sieve-like Cell Membrane Concept

The sieve concept separates ions and molecules into two categories. Those that are able to pass through the membrane barrier and those that are (permanently and absolutely) unable to do so. This concept of all-or-none segregation reached the peak of its development with the publication of the famous paper by Boyle and Conway

on page 1(to page 63) of the 100th volume of the prestigious (English) Journal of Physiology (Boyle and Conway 1941.) However, even before the paper appeared in print, contradictory experimental evidence were rapidly collecting. Included were those from Conway's own laboratory (Conway and Creuss-Callaghan 1937) – showing that ions and molecules supposedly to be too large to traverse the postulated membrane pores, in fact, can enter and leave the cells with ease (Ling 1952, pp 761–763).

Table 1 taken from a more recent paper of Ling et al. (1993) shows that solutes from the small, like water, all the way to raffinose (molar volume, 499 cc) can all traverse the cell membrane without difficulty. Clearly, the asymmetrical distribution of solutes is not due to a sieve-like mechanism.

2.2 The Disproof of the Membrane Pump Theory

In 1952 I first presented results of my earlier study on the (would-be) energy requirement of the hypothetical sodium pump in metabolically inhibited frog muscle. To halt respiration, I used pure nitrogen (in addition to sodium cyanide). To halt glycolysis, the alternative route of energy metabolism, I used sodium iodoacetate.

Table 1. The time required for each of the 22 solutes investigated to reach diffusion equilibrium in isolated frog muscle cells. The data as a whole show that with the exception of three pentoses an incubation period of 24 hours at 0 °C is adequate for all the other solutes studied. The three pentoses took about twice as long or 45 hours to attain equilibrium (from Ling et al. 1993, by permission of the Pacific Press, Melville, NY)

Solute	Equilibration time (hours)
water	$\ll 1$
methanol	<20
ethanol	<20
acetamide	<10
urea	<24
ethylene glycol	<10
1,2-propanediol	24
DMSO	<1
1,2-butanediol	24
glycerol	<20
3-chloro-1,2-propanediol	24
erythritol	<20
D-arabinose	<45
L-arabinose	<45
L-xylose	<45
D-ribose	<24
xylitol	24
D-glucose	<15
D-sorbitol	<10
D-mannitol	<24
sucrose	<8
raffinose	10

The results showed that the minimum energy need of the sodium pump would be at least 400% of the maximum available energy.

In years following, the technique was steadily improved so that by 1956, I was able to achieve the highest accuracy in the last three sets of experiments, the results of which are shown graphically in Figure 2. Now, the minimum energy need of the sodium pump was shown to be no longer 400% as from early studies, but at least 1500% to 3000% times the maximum energy available (Ling 1962, 1997). Clearly, the asymmetrical distribution of solutes is not due to membrane pumps either.

2.3 The Disproof of the Free Cell Water Postulation

The free cell water postulation was disproved when Ling and Walton showed that centrifugation at 1000 g for 4 minutes quantitatively removes all free water found in the extracellular space of the isolated frog sartorius muscle. Yet the same centrifugation treatment failed to extract any water from within the cells (Ling and Walton 1976) – after (part of) the cell membrane has been surgically removed and electron microscopy revealed no membrane regeneration following surgery (Cameron 1988).

2.4 The Disproof of the Free Cell Potassium Postulation

The free cell potassium postulation was also fully disproved on at least four accounts.

First, in healthy cells, the diffusion coefficient of $K^+(D_K)$ was found to be only 1/8 of that in an isotonic solution, while in the same preparation the diffusion coefficient of labeled water was reduced only by a factor of 2. Killing the muscle by prior metabolic poisoning increased the K^+ diffusion coefficient to close to that in an isotonic KCl solution; injury produced a D_K in-between that of the healthy living cell and that of the dead cell (Ling and Ochsenfeld 1973).

Second, if the bulk of cell K^+ is free, an impaling intracellular K^+-sensitive microelectrode should register a uniform activity coefficient of cell K^+in all types of cells probed. And that uniform activity coefficient should match the activity coefficient of free K^+ in a KCl solution of similar ionic strength. In truth, the activity coefficients actually measured among different cell types varied from as low as 0.3 to as high as 1.2 (Table 8.2 in Ling 1984).

Third, if cell K^+ is free, its location in frog muscle should be higher in the I bands where the water content is higher than in the adjacent A bands. Instead, the great majority of K^+ is located at the edges of the A bands and at the Z line. (For in depth, definitive work, see Edelmann 1977, 1984, 1986; for earlier and less-than exhaustive work, see Macallum 1905; Menten 1908; Ling 1977; Tigyi et al. 1980–81; von Zglinicke 1988.) (Figures 3, 4).

Fourth, this regionally-accumulated, radioactively-labeled K^+ could be ‘chased away’ by adding competing alkali metal ions like Rb^+ or Cs^+ to the external

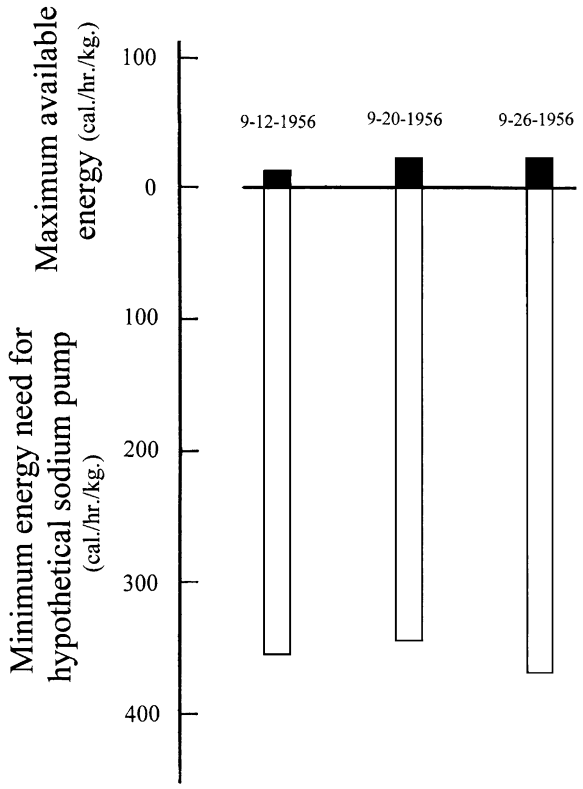

Figure 2. A comparison of the maximally available energy of (poisoned) frog sartorius muscle cells at 0 °C (upward black bars) and the minimum energy need to pump Na^+ against both (measured) electric potential gradient and a concentration gradient. Duration of the experimental observation for experiment (9-12-1956) lasted 10 hrs; Experiment 9-20-1956, 4 hrs; Experiment 9-26-1956, 4.5 hrs. Active oxidative metabolism was suppressed by exposure to pure nitrogen (99.99%, in addition to 0.001 M NaCN); glycolytic metabolism, by sodium iodoacetate and doubly insured by actual lactate analysis before and after the experiment. Other detailed studies reported in 1952 (Ling 1952, Table 5 on page 765) and in 1962 (Ling 1962, Table 8.4) showed respectively that under similar conditions of 0 °C temperature and virtually complete inhibition of active energy metabolism the K^+ and Na^+ concentrations in frog muscle, nerves and other tissues remain essentially unchanged for as long as the experiments lasted (5 hrs. for the 1952 reported experiment, and 7 hrs 45 min in the 1962 reported findings). (For additional details, see Ling 1962, Chapter 8 and Ling 1997. Since the book referred to here as Ling 1962 has been out of print, its entire Chapter 8 has been reproduced as an Appendix in the article, Ling 1997 bearing the title: *Debunking the Alleged Resurrection of the Sodium Pump Hypothesis*.) In the computations, it was assumed that the frog muscle cell does not use its metabolic energy for any other purpose(s) than pumping sodium ion and that all energy transformation and utilization are 100% efficient (from Ling 2004, by permission of the Pacific Press, Melville, New York)

medium. The extent of displacement varied with the *short-range attributes* (e.g., size) of the displacing ions – indicating that the K^+ ions are engaged in close-contact adsorption and not free in the cell water (Ling and Ochsenfeld 1966).

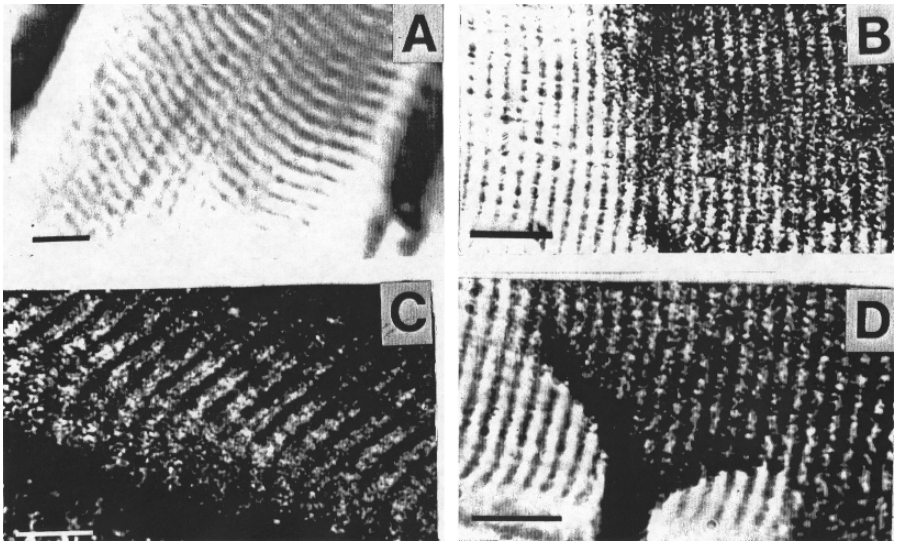

Figure 3. Auto-radiographs of dried single muscle fibers. (A) Portion of a single muscle fiber processed as in all the other auto-radiographs shown here but not loaded with radioisotope. (B), (C) and (D) were auto-radiographs of dried muscle fibers loaded with radioactive ^{134}Cs while living and before drying. (B) and (D) were partially covered with photo-emulsion. Muscle in (C) was stretched before drying. Bars represent 10 micrometers. Incomplete coverage with photo-emulsion in B and D permits ready recognition of the location of the silver grains produced by the underlying radioactive ions to be in the A bands. Careful examination suggests that the silver grains over the A bands are sometimes double. A faint line of silver grains also can be seen sometimes in the middle of the I bands, corresponding to the position of the Z line (from Ling 1977, by permission of the Pacific Press, Melville, NY)

(For additional confirmatory work from X-ray adsorption fine structure of cell K^+, see Huang et al. 1979; for first order quadrupole broadening of Na^{23} in K^+-depleted frog muscle of Cope and Ling, see Ling 2001, p 187–190.)

2.5 The Disproof of the Postulation of the Existence of All Intracellular Proteins in the Conformation Conventionally called 'Native'

From section 2.4 above, we know now that K^+ in living cells is not free. That is just another way of saying that virtually all cell K^+ is in some way bound. In mature human red blood cells, which have no nucleus, nor significant amount of DNA or RNA, the only macromolecular component large enough in size and amount to provide enough binding sites for cell K^+ is proteins. And of the proteins in mature mammalian red blood cells, fully 97% is hemoglobin. This leaves hemoglobin as the only bearer of binding sites in mature mammalian red blood cells for cell K^+ as well as the bulk of cell water – also shown to be not free in section 2.3 above.

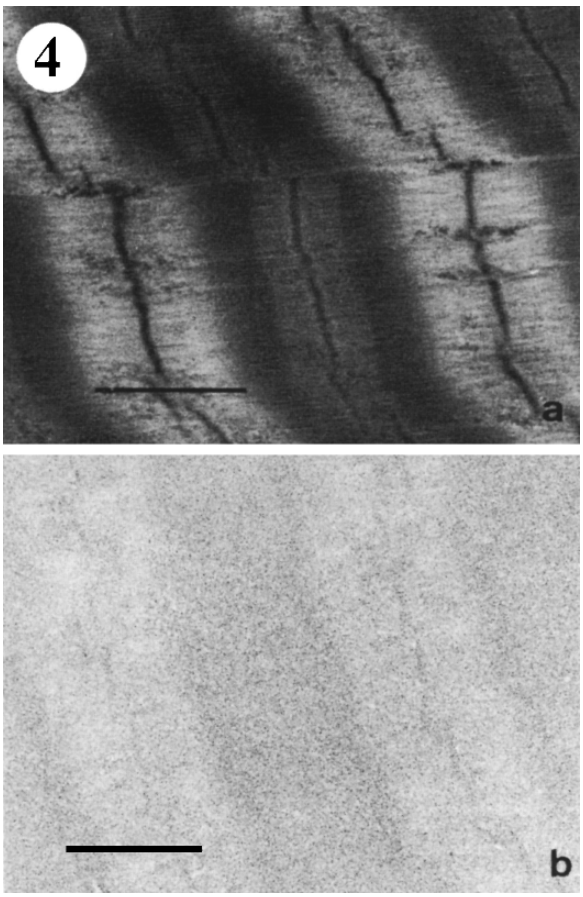

Figure 4. Cut sections of frog sartorius muscle 'stained' with a solution containing 100 mM LiCl and 10 mM CsCl by procedure described in Edelmann 1984. In a, the section was obtained by freeze-drying and embedded. In b, the muscle was fixed with glutaraldehyde and then embedded. Note that selective uptake was only observed in the freeze-dried preparation. Taken together, this type of studies has demonstrated the successful capturing of the *resting living state* of the muscle cells by the adsorption staining procedure introduced by Edelmann (from Edelmann 1986, by permission of Scanning Electron Microscopy International)

The conclusion that both cell K^+ and cell water must be bound to hemoglobin in mature human blood cells, offers an unusual opportunity. That is, an opportunity to put to test the widely-accepted idea that intracellular proteins exist in what is conventionally called native state and as such can be obtained from any biochemical supply house in a bottle – often in crystalline forms. However, there is so far no evidence that what we call native hemoglobin really means what it is supposed to mean, i.e., as it exists in living red blood cells.

For if this popular but unproved idea is correct, a water solution of mammalian hemoglobin at the concentration that it occurs in red blood cells (35%) should selectively bind K^+. In addition, the bulk of surrounding 65% water should have low solvency for Na^+ sulfate.

To put this prediction to a test, a 35% hemoglobin solution was enclosed in a dialysis sac, and allowed to reach diffusion equilibrium with K^+ and Na^+ in the solution bathing the sac. Analysis of the ionic concentration in the bathing solution revealed no or virtually no accumulation of K^+ by the hemoglobin in the sac (Beatley and Klotz 1951; Table 1 in Ling and Zhang 1984). Nor does that 65% water in the sac show reduced solvency for Na^+ (as sulfate) (Table 2A, also Table IX in Ling and Ochsenfeld 1989). In other words, store-bought native protein is not native in the true sense of the word.

Now, if we expose human red cells to a hypotonic lysing solution containing ATP, the red cells hemolyze, losing varying amounts of its hemoglobin as well as most of its K^+ and gained Na^+. If we now 'reseal' the *hemolyzed red cell*s or '*ghosts*' by adding sucrose to make the lysing solution isotonic, they would regain more or less their original volume and their lost K^+. In addition, they would extrude the extra Na^+ gained. Most significant was that the amount of K^+ gained as well as the Na^+ extruded are directly proportional to the hemoglobin retained and/or recaptured in the ghosts. In ghosts with no hemoglobin, neither was K^+ regained nor Na^+ extruded (Figure 5).

In summary, cell K^+ and cell water are not free but are 'bound'. In mature mammalian red blood cells, the only major cell component that could bind these small molecules and ions is hemoglobin. Yet store-bought hemoglobin called native does not work. In contrast, hemoglobin in healthy living red blood cells as well as in 'resealed' ghosts – in the presence of ATP – does work. So there is a profound difference between what is conventionally called 'native' and what is truly native – that is, as it occurs in normal living cells. The following simple experimental finding does offer a clue as to the cause of this difference.

In this simple experiment, we titrated the native hemoglobin with NaOH (Ling and Zhang 1984). As the added OH^- neutralizes the positive charges of the ϵ-amino groups of the lysine side chains and the guanidyl groups of the arginine side chains, a profound change takes place in the hemoglobin.

As a result of this change, the up-to-now impotent hemoglobin not only can now adsorb selectively large amount of K^+ (or other alkali metal ions), but also profoundly alters the solvency of the bulk phase water. In the end, the mix of NaOH-titrated hemoglobin and its adsorbed K^+ and water begin to look like what it might be like inside normal red blood cells. But that is not all that make them look similar.

In addition, the NaOH-treated hemoglobin solution is no longer the free-flowing liquid the simple hemoglobin solution once was. The viscosity of the solution has gone up so much that it now takes on the form of a solid gel.

With proper micro-dissecting tools, one can cut up a red blood cell into small fragments without losing its hemoglobin. This retention indicates that hemoglobin is not free but attached to the

red stroma proteins (Best and Taylor 1945, p 7.) Certainly there is no question that fresh meat (muscle cells) is in the form of a fairly rigid gel and so is axoplasm of a squid axon (Hodgkin 1971, p 21).

For those used to preparing protein solutions, pure crystalline store-bought native hemoglobin is remarkable in that even at a concentration of 40% (w/v), a hemoglobin solution still flows freely like water. Of course, this is in keeping with the well-known fact that the 'native' hemoglobin molecules are tightly folded and more or less spherical structures (Perutz et al., 1968).

Table 2. The apparent equilibrium distribution coefficient or ρ-value of Na^+ (as sulfate) in water containing native proteins (A), gelatin (B) and PVP (C, E.) and PEO (D.). The ρ-value differs from the (true) equilibrium distribution coefficient or q-value in that the solute in the cell or model water may not all exit in cell water as it is the case with the q-value. However, the ρ-values shown here are all at, or below unity. This means that if some of the solute is adsorbed on the protein or polymer, its quantity was minimal. a, $NaSO_4$ medium; b, Na citrate medium (from Ling et al. 1980 by permission of the Pacific Press, Melville, NY)

Group	Polymer		Concentration of medium (M)		Number of assays	Water content (%) (mean ± SE)	ρ-Value (mean ± SE)
(A)	Albumin (bovine serum)		1.5	a	4	81.9 ± 0.063	0.973 ± 0.005
	Albumin (egg)		1.5	a	4	82.1 ± 0.058	1.000 ± 0.016
	Chondroitin sulfate		1.5	a	4	84.2 ± 0.061	1.009 ± 0.003
	α-Chymotrypsinogen		1.5	a	4	82.7 ± 0.089	1.004 ± 0.009
	Fibrinogen		1.5	a	4	82.8 ± 0.12	1.004 ± 0.002
	γ-Globulin (bovine)		1.5	a	4	82.0 ± 0.16	1.004 ± 0.004
	γ-Globulin (human)		1.5	a	4	83.5 ± 0.16	1.016 ± 0.005
	Hemoglobin		1.5	a	4	73.7 ± 0.073	0.923 ± 0.006
	β-Lactoglobulin		1.5	a	4	82.6 ± 0.029	0.991 ± 0.005
	Lysozyme		1.5	a	4	82.0 ± 0.085	1.009 ± 0.005
	Pepsin		1.5	a	4	83.4 ± 0.11	1.031 ± 0.006
	Protamine		1.5	a	4	83.9 ± 0.10	0.990 ± 0.020
	Ribonuclease		1.5	a	4	79.9 ± 0.19	0.984 ± 0.006
(B)	Gelatin		1.5	a	37	57.0 ± 1.1	0.537 ± 0.013
(C)	PVP		1.5	a	8	61.0 ± 0.30	0.239 ± 0.005
(D)	Poly(ethylene oxide)		0.75	a	5	81.1 ± 0.34	0.475 ± 0.009
			0.5	a	5	89.2 ± 0.06	0.623 ± 0.011
			0.1	a	5	91.1 ± 0.162	0.754 ± 0.015
(E)	PVP	Q	0.2	b	4	89.9 ± 0.06	0.955 ± 0.004
		S*	0.2	b	4	87.2 ± 0.05	0.865 ± 0.004
		Q	0.5	b	3	83.3 ± 0.09	0.768 ± 0.012
		S	0.5	b	3	81.8 ± 0.07	0.685 ± 0.007
		Q	1.0	b	3	67.0 ± 0.26	0.448 ± 0.012
		S	1.0	b	3	66.6 ± 0.006	0.294 ± 0.008
		Q	1.5	b	3	56.3 ± 0.87	0.313 ± 0.025
		S	1.5	b	3	55.0 ± 1.00	0.220 = 0.021

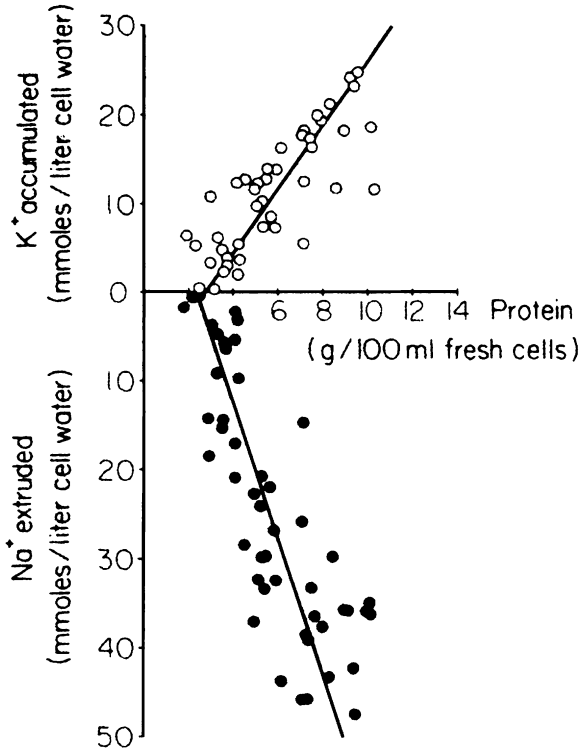

Figure 5. Re-uptake of K^+ and extrusion of Na^+ from red-blood-cell ghosts prepared from washed human red blood cells. The study followed rigorously a procedure described in (Freedman 1976). Freshly drawn blood was obtained (mostly) from different donors. When blood from the same donors was used, it was drawn at least 6 weeks apart. Each data point represents the difference of K^+ or Na^+ concentration in samples of the ghosts at the beginning of incubations and after 18 hours of incubation in the presence of ATP (37 °C). Straight lines shown in the graph were obtained by the method of least squares. Total protein content was obtained by subtracting the sum of the weights of lipids, phospholipids, salt ions, and sucrose from the dry weights of the ghosts (from Ling, Zodda and Sellers 1984, by permission of the Pacific Press, Melville, NY)

From this starting point, the observation that titration with NaOH should bring about a drastic increase in viscosity, there is only one reasonable explanation: *the tightly-folded 'native' hemoglobin molecules have dramatically unfolded in consequence*. Such an unfolded protein assumes the conformation know as *fully extended conformation*. And it is in this conformation, often also called denatured conformation, that it can adsorb K^+ and reduce the solvency of bulk-phase water for Na^+.

Ironically, this finding shows that we have the thing largely turned up side down. Not only is truly native protein not what we have been calling 'native'; what we commonly referred as 'denatured' is, at least in the case of hemoglobin, in fact closer to being truly native.

Indeed, a vast amount of experimental data has collected in the last forty years in support of this conclusion. I shall discuss them in a section below under the title of the Polarized-Oriented Multilayer Theory of Cell Water.

3. A BRIEF OUTLINE OF THE UNIFYING THEORY OF CELL AND SUB-CELLULAR PHYSIOLOGY: THE ASSOCIATION-INDUCTION HYPOTHESIS

As its title clearly indicates, the association-induction hypothesis is built upon the fundamental concepts of close-contact *association* among its constituent parts in the form of ions and molecules so that electrical polarization and depolarization (or *induction*) could link them into a coherent whole. To see how association-induction works, we begin by invoking an old concept, the concept of protoplasm.

3.1 The Restoration of the Concept of Protoplasm

In 1835 Felix Dujardin (1801–1860) described what he saw under the microscope: a gelatinous substance oozing out of the broken end of a protozoon (then called Infusoria.) Dujardin described this 'living jelly' as a 'pulpy, homogeneous, gelatinous substance without visible organs and yet organized. . .' (Dujardin 1835). Though he gave this gelatinous substance the name, *sarcode*, the name *protoplasm* was broadly adopted in the end.

Thirty-three years later, in his famous Sunday evening lecture in Edinburgh on November 8, 1868, Thomas Huxley called protoplasm 'the physical basis of life.'

The discovery of protoplasm inspired the introduction of the idea of colloid and colloid chemistry. Unfortunately, the understanding of both protoplasm and of colloid were handicapped by the lack of depth in our understanding of (relevant) physics and chemistry at that time – as I have already alluded to in broader terms at the beginning of this communication. This is one reason how the simpler membrane theory gained dominance. Indeed, by the beginning of the 21st century, even the word, protoplasm has become all but forgotten.

Nonetheless in my opinion, protoplasm has been there since life began. So it is a great honor for me to re-introduce this most basic knowledge of biology to the world again.

Given the substantial progress made in the revelant parts of physics and chemistry in the late 19th and early 20th centuries, the AI Hypothesis came into existence and with it, a new definition of protoplasm was born.

3.2 A New Definition of Protoplasm

According to the association-induction hypothesis, protoplasm remains the physical basis of life as Thomas Huxley first and rightly pointed out.

Only protoplasm is no longer defined by its appearance. True, protoplasm may exist in the form Dujardin described as 'pulpy, homogeneous, structureless and yet

structured . . .', but it may also assume a wide variety of other forms as well. What it looks like is only a superficial facet of its existence. What underlies protoplasm to make life possible defines protoplasm.

Except 'ergastic' matter such as the watery solution in the central vacuole of mature plant cells and the inclusions inside food vacuoles of protozoa, etc., all the living part of cells and their living appendages are made of protoplasm. An example of the makeup of automobiles may make the definition easier to understand.

The precise composition, properties and functions of different steel vary. They vary because each kind of steel must serve its specific function in an automobile. For the same reason, the precise composition, properties and functions of different protoplasm vary – in order to serve the specific function of that part of the protoplasm.

Nonetheless, all kinds of steel are steel. That is, they all contain as its major constituents, iron, carbon, other metals and nonmetals. Protoplasm is primarily a system of proteins, water, ions and other small and big molecules functioning as controlling *cardinal adsorbents*. As the *principle cardinal adsorbent*, ATP plays a critically important role in making the living alive.

A correct though variable chemical composition is only one common feature shared by all living protoplasm. Just as vital is how all these constituents are linked together electronically in what physicists called ferromagnetic cooperativity or more precisely what I call auto-cooperativity (Ling 1980). Thus the protein-water-ion-cardinal adsorbent system exists together at a low energy-low entropy state, or what I prefer to describe as high (negative) energy-low entropy state called the *living state*.

3.3 The Living State

Consider a chain of soft-iron nails joined end-to-end with bits of string (Figure 6A). Bring a strong horseshoe magnet close to the end of one of the terminal nails, a chain reaction follows. As a result, the loosely tethered chain of soft-iron nails assumes a more rigid configuration. And with that change, they also pick up the randomly scattered iron filings in the vicinity. Take away the magnet, the system more or less returns to its earlier more random configuration. (Similarly, an electronic rather than magnetic model can be constructed as shown in Figure 6B.)

What the magnet does in this model is to transform the system from a low (negative) energy-high entropy state to a high (negative) energy-low entropy state. According to the AI Hypothesis, protoplasm may also exist in these two alternative states.

However, instead of the tethered chain of soft-iron nails, we have the proteins with their partially resonating and highly polarizable polypeptide chains. And instead of iron filings, we have K^+ and water molecules. And instead of the horseshoe magnet,

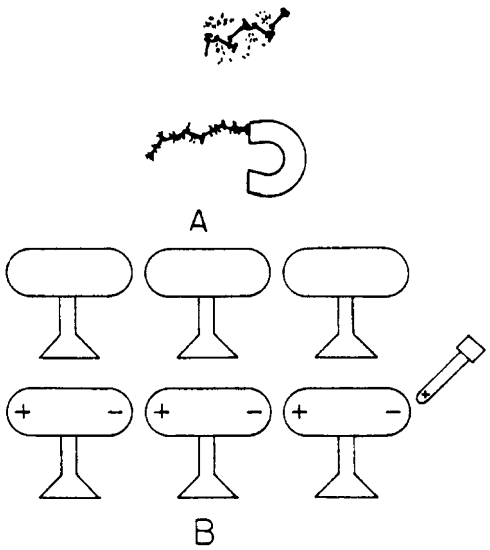

Figure 6. Two models demonstrating information and energy transfer over distances due to propagated short-range interactions. (A) A chain of loosely tethered soft-iron nails is randomly arrayed and does not interact with the surrounding iron filings. The approach of a magnet causes propagated alignment of the nails and interaction with the iron filings. (B) Electrons in a series of insulators are uniformly distributed before the approach of the electrified rod, R. Approach of the rod relocates the electrons by induction such that the insulator becomes polarized with regions of low electron density and regions of high electron density (from Ling 1969, by permission of Intern Rev Cytol)

we have the *principle cardinal adsorbent*, ATP (and its helper the protein Z). (See Figure 7 below.) Only here, the high (negative) energy, low entropy-state with ATP adsorption on the appropriate *cardinal site* constitutes what is known as the *resting living state*. The alternative low-(negative)-energy, high-entropy state is either the *active living state* (as in all reversible transitions) or *dead state* (in an irreversible transition). (Figure 7).

In the next section, I shall point out that according to the AI Hypothesis, protoplasm is basically an electronic machine. Surprising as it may seem, that recognition made in 1962 was also one of history's firsts.

The theory is that diverse variety of protoplasm all existing in the resting living state makes up the entire living cells. This in turn implies that *all cell water must also exist in a physical state different from that of normal liquid water.*

Of course, I have already shown in section 2.5 decisive evidence that no free water exists in typical cells like frog muscle. In the next section I shall go into a little more detail in reviewing the polarized-oriented multilayer (PM) theory of cell water and of inanimate systems demonstrating long-range dynamic water structuring.

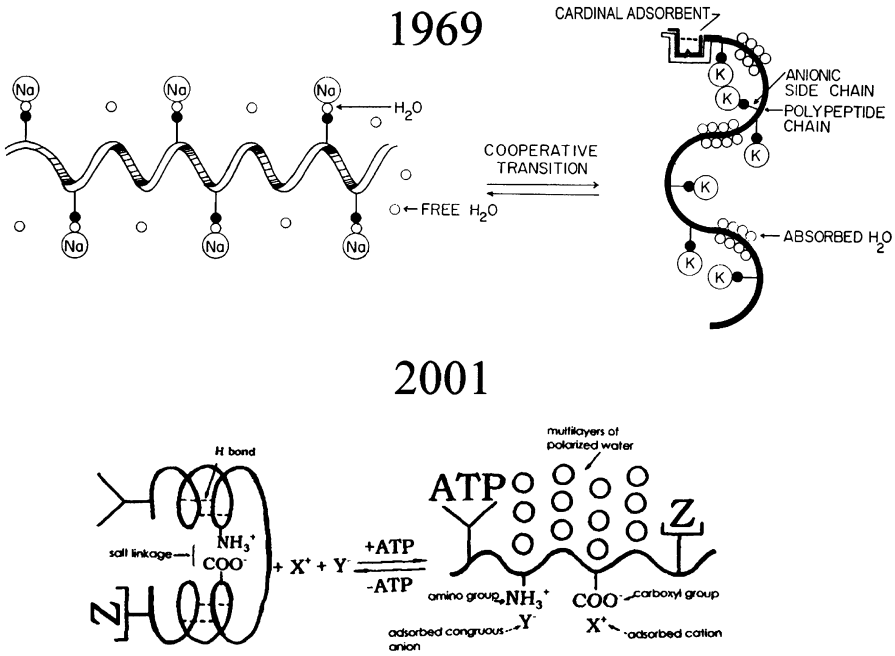

Figure 7. Two diagrammatic illustrations published respectively in 1969 and in 2001. The original legend of the 1969 presentation reads: 'Diagram of a portion of a protein molecule undergoing an auto-cooperative transformation. For simplicity, adsorbed water molecules in multilayers are shown as a single layer. W-shaped symbol represent a (principle) cardinal adsorbent like ATP.' (The 1969 figure shown above is a slightly modified version of the original to correct an illustration error). (from Ling 1969 by permission of the Intern Rev Cytol). The original legend to the 2001 version reads: 'Diagrammatic illustration of how adsorption of the cardinal adsorbent ATP on the ATP-binding *cardinal site* and of 'helpers' including the *congruous anions* (shown here as 'adsorbed congruous anion' and Protein-X (shown as Z) unravels the *introverted* (folded) secondary structure shown on the left-hand side of the figure. As a result, selective K^+ adsorption can now take place on the liberated β-, and γ-carboxyl groups and multilayer water polarization and orientation can now occur on the exposed backbone NHCO groups. The resting living state is thus achieved and maintained' (from Ling 2001 by permission of the Pacific Press, Melville, NY)

4. THE POLARIZED-ORIENTED MULTILAYER THEORY OF CELL WATER AND MODEL SYSTEMS

4.1 A Brief Sketch of the Theory

In 1965, three years after the publication of the association-induction hypothesis proper, the polarized multilayer theory – recently modified to read *polarized-oriented multilayer (PM) theory* of cell water and model systems was introduced. Figure 8A reproduces the key figure in my first public presentation at the New York Academy of Sciences symposium on the 'Forms of Water in Biological Systems' (Ling 1965).

What Figure 8A represents is twofold.

First, it suggests that all the water in all living cells is not normal liquid water but water assuming the dynamic structure of polarized-oriented multilayers.

Second, this picture diagram – again for the first time in history – presents a molecular mechanism by which solutes like Na^+ are kept at a low concentration in living cells on account of an unfavorable free energy of distribution. Note that this theory would not have been possible without the first part of the theory, i.e., *all the cell water* is altered water.

The language used in the 1965 presentation already hinted to those backbone NHCO groups as the primary sites of multilayer polarization and orientation of cell water. But it was not until 1970 and still later (Ling 1970, 1972) that the idea became firmly established in my thinking.

However, long before 1965, J. H. de Boer and C. J. Dippel (1933) had described their idea that multiple layers of water molecules could be adsorbed on the backbone NHCO groups of gelatin. Their original illustration is reproduced here as Figure 9. I did not know about the existence of this paper until last year.

Figure 10 is a reproduction of a figure published in 1972 in an article bearing the title 'Hydration of Macromolecules' in the monograph, *Water and Aqueous Solutions* (Ling 1972). As indicated by the small arrows, Figures 10a, 10b and 10c emphasize that nearest neighboring sites bearing electric charges of the same polarity, orient water dipoles in the same direction. Since water molecules oriented in the same direction repulse one another, multilayer water polarization would not occur on this type of surfaces.

It is only when nearest-neighboring sites bear alternatingly positive (P) and negative (N) electric charges that multiple layers of water molecules can be polarized and oriented in consequence of the attractions among all nearest neighboring water molecules. And to the best of my knowledge, it was the same deBoer mentioned above – with co-author, C. Zwikker – who first pointed out in print this idea (see below).

A checkerboard of alternating N and P sites are what I later designated as an NP system while two juxtaposed NP surfaces are called an NP-NP system (Figure 10d). When either the N or P sites is replaced by a vacant O site, we have what are called an NO-NO system (Figure 10f) or PO-PO system. Not shown in this illustration is what I call a NP-NP-NP system or NO-NO-NO system, which are *parallel arrays of linear chains carrying alternating N and P sites or alternating N and O sites respectively*. They are of central importance in water polarization in living cells because within living protoplasm, there are no *bona fide* flat surfaces like those on salt crystals.

4.2 Four Pre-existing Theories on Multilayer Gas Adsorption and their Respective Shortcomings

At the time when the PM theory was first introduced in 1965, there were four quantitative theories known to me for the multilayer adsorption of gaseous molecules.

C.P. de Boer and C. Zwikker offered the first quantitative theory of multiple layer adsorption of gases on the surface of salt crystals (de Boer and Zwikker 1929).

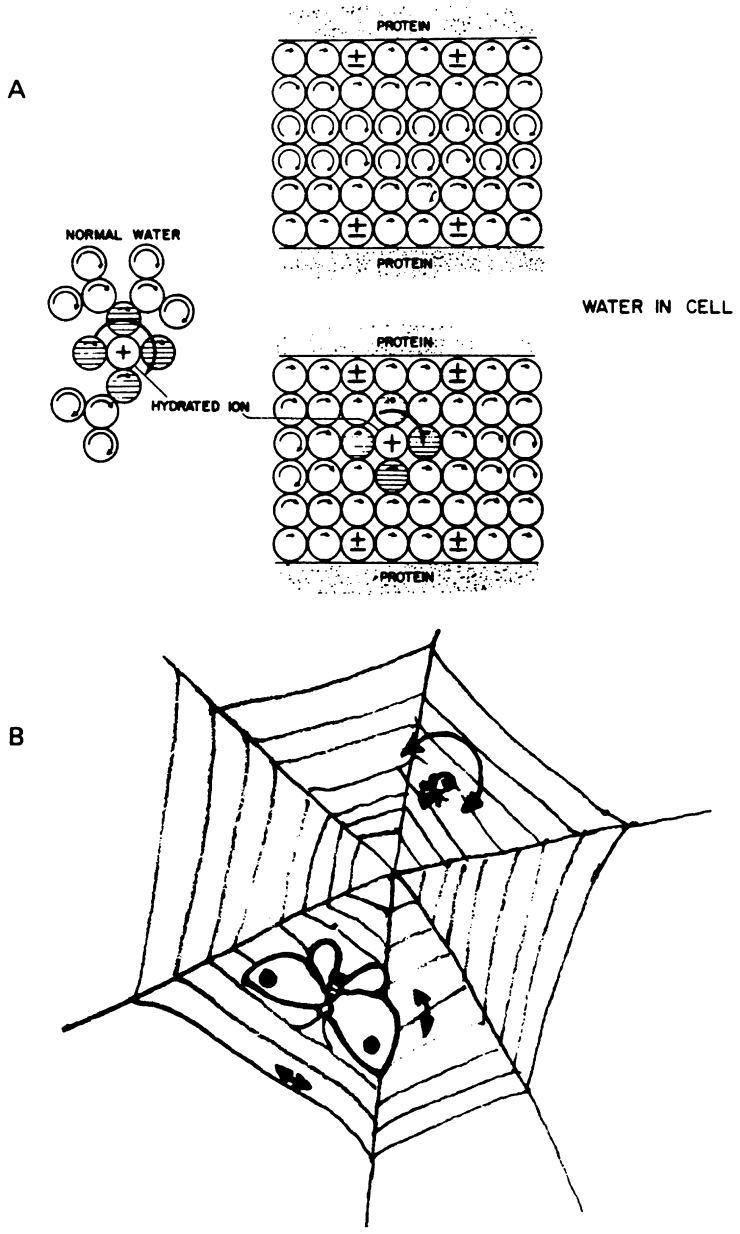

Figure 8. Motional reduction in polarized-oriented water. (A) Diagrammatic illustration of the reduction of rotational (and translational) motional freedom of a hydrated Na^+ ion in water assuming the dynamic structure of polarized multilayers. Size of the curved arrows indicates degree of rotational freedom of both the water melodies (empty circle) and hydrated cations. Reduced motional freedom is indicated by the smaller sizes of the arrows. (This part of the figure was taken from an early paper, Ling 1965). Now we know that one aspect of this diagram is less applicable to living cells. Thus, the degrees of

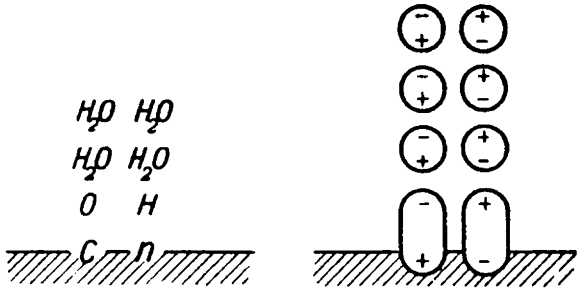

Figure 9. Theoretical model of de de Boer and Dippel showing how dipolar NH and CO groups of gelatin can polarize and orient multiple layers of water molecules (from de Boer and Dippel 1933)

They suggested, as mentioned above, that the presence of alternatingly positive-, and negative electrically charged sites allow the formation of deep layers of gas molecules on the surface of salt crystals as illustrated in their figures reproduced here as Figure 11.

de Boer and Zwikker's *polarization theory* was intended to describe the multilayer adsorption of all types of gas molecules, some without a *permanent dipole moment* like non-polar nitrogen, others with a permanent dipole moment like water vapor.

Within a decade or so after the publication of the de Boer-Zwicker theory, Bradley added two more theories of his own, one specifically for the multilayer adsorption of non-polar gas molecules without a permanent dipole moment (Bradley 1936a) and the other for polar molecules with permanent dipole moments (Bradley 1936b). Each of these three theories can be expressed by an equation of the same form:

$$(1) \qquad \mathrm{Log}_{10}(p_o/p) = K_1 K_3{}^{a} + K_4$$

where a is the amount of gas adsorbed by a unit weight of the adsorbent. (p_o/p) is the reciprocal of the relative vapor pressure. K_1, K_3 and K_4 are all constants at the same temperature. The meanings of each of the three constants vary from one theory to the other but are all too complicated to provide quantitative insights into the adsorption process. Equation (1) can be written in the double log form:

$$(2) \qquad \log_{10}[\log_{10}(p_o/p) - K_4] = a \ \log_{10} K_3 + \log_{10} K_1$$

If the data on the gas adsorbed (a) at different relative vapor pressures (p/p_o) are such that rational numbers can be found for each of the three constants so that the

Figure 8. polarization of water molecules far and near tend to be more uniform than that indicated in the diagram. (from Ling 1965, by permission of the Annals of New York Academy of Sciences). (B) Illustration of the greater degree of motional restriction of larger butterflies snared in a spider web. (Larger) size of arrow represents (greater) degrees of motional freedom (from Ling 1992, by permission of Krieger Publ. Co.)

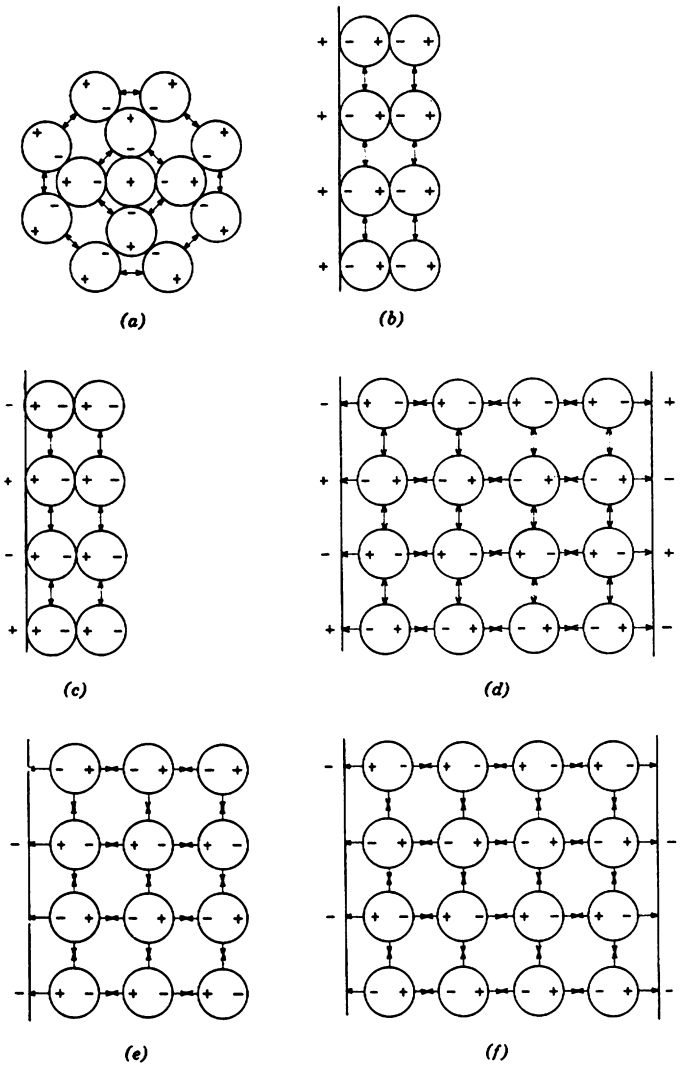

Figure 10. Diagrammatic illustration of the way that individual ions (a) and checkerboards of evenly distributed positively charged P sites alone (b) or negatively charged N sites alone (c) polarize and orient water molecules in immediate contact and farther away. Emphasis was, however, on uniformly distanced bipolar surfaces containing alternatingly positive (P) and negative (N) sites called an NP surface (d). When two juxtaposed NP surfaces are facing one another, the system is called an NP-NP system. If one type of charged sites is replaced with vacant sites, the system would be referred to as PO or NO surface (e). Juxtaposed NO or PO surfaces constitute respectively a PO-PO system or NO-NO system. Not shown here is the NP-NP-NP system comprising parallel arrays of linear chains carrying properly distanced alternating N and P sites (modified after Ling 1972, reproduced with permission of John Wiley and Sons., Inc.)