Ling, G.N. (2006) A convergence of experimental and theoretical breakthroughs affirms the PM theory of dynamically structured cell water at the theory's 40th birthday. In: Water and the Cell (Pollack, G.H., Cameron, I.L. and Wheatley, D.N., eds.), Springer Verlag, Berlin, New York, p. 1-52.

CHAPTER 1

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

GILBERT N. LING

Damadian Foundation for Basic and Cancer Research, Tim and Kim Ling Foundation for Basic and Cancer Research, c/o Fonar Corporation, 110 Marcus Drive, Melville, NY 11747, E-mail: gilbertling@dobar.org

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,

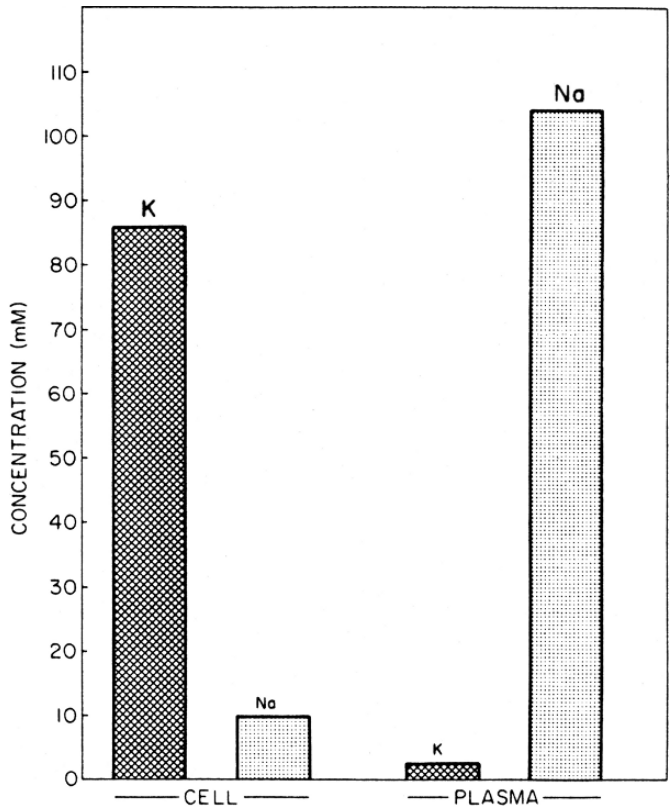

Figure 1. Potassium ion (K) and sodium (Na) concentration in frog muscle cells and in frog blood plasma. Concentrations in frog muscle cells and in frog blood plasma are given respectively in millimoles per liter of cell water or plasma water (from Ling 1984 by permission of Plenum Press)

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

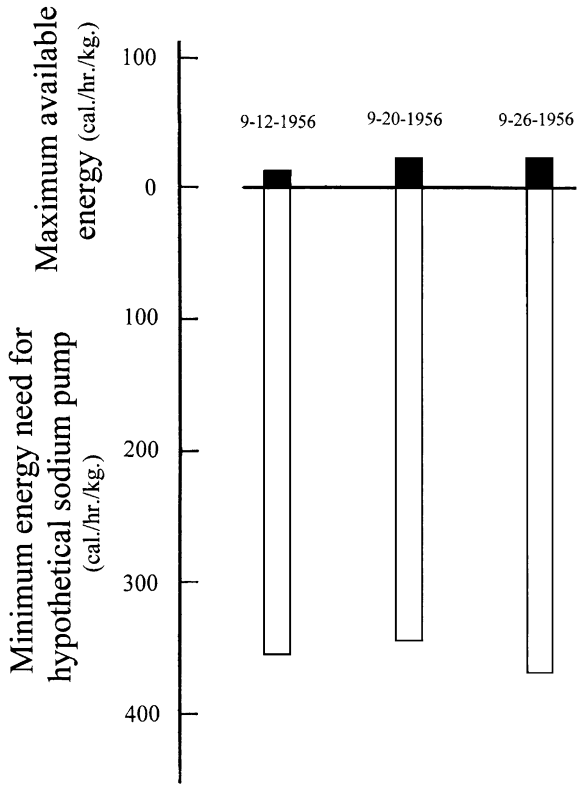

Figure 2. A comparison of the maximally available energy of (poisoned) frog sartorius muscle cells at 0 °C (upward black bars) and the minimum energy need to pump Na^+ against both (measured) electric potential gradient and a concentration gradient. Duration of the experimental observation for experiment (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).

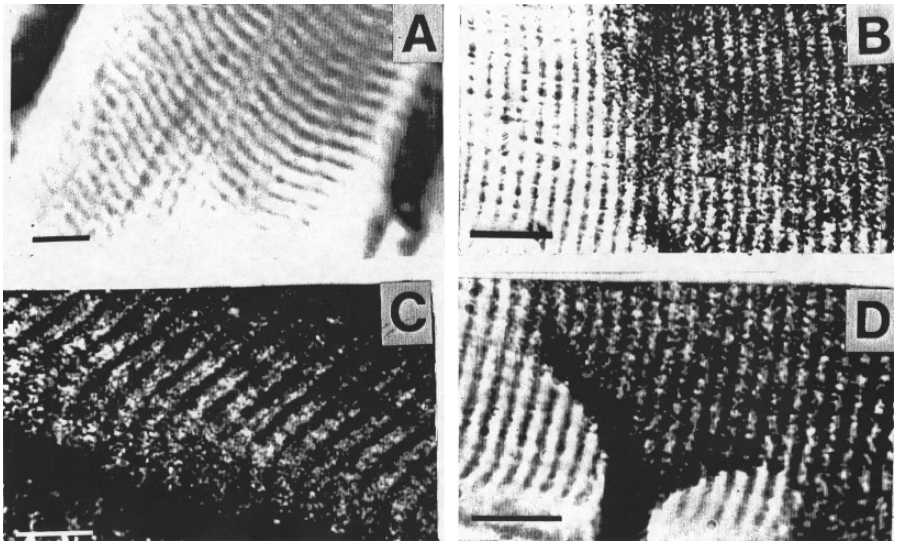

Figure 3. Auto-radiographs of dried single muscle fibers. (A) Portion of a single muscle fiber processed as in all the other auto-radiographs shown here but not loaded with radioisotope. (B), (C) and (D) were auto-radiographs of dried muscle fibers loaded with radioactive ^{134}Cs while living and before 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.

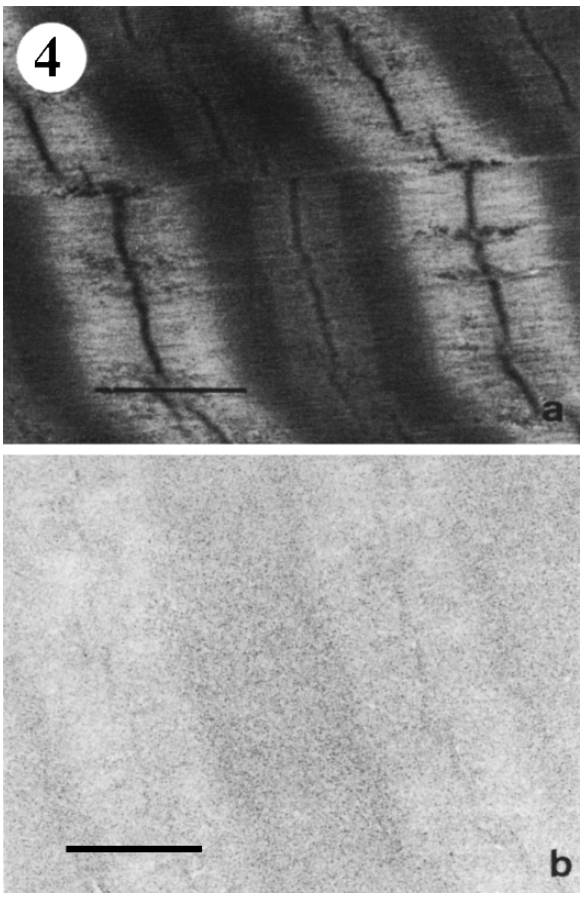

Figure 4. Cut sections of frog sartorius muscle 'stained' with a solution containing 100 mM LiCl and 10 mM CsCl by procedure described in Edelmann 1984. In a, the section was obtained by freeze-drying and embedded. In b, the muscle was fixed with glutaraldehyde and then embedded. Note that selective 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

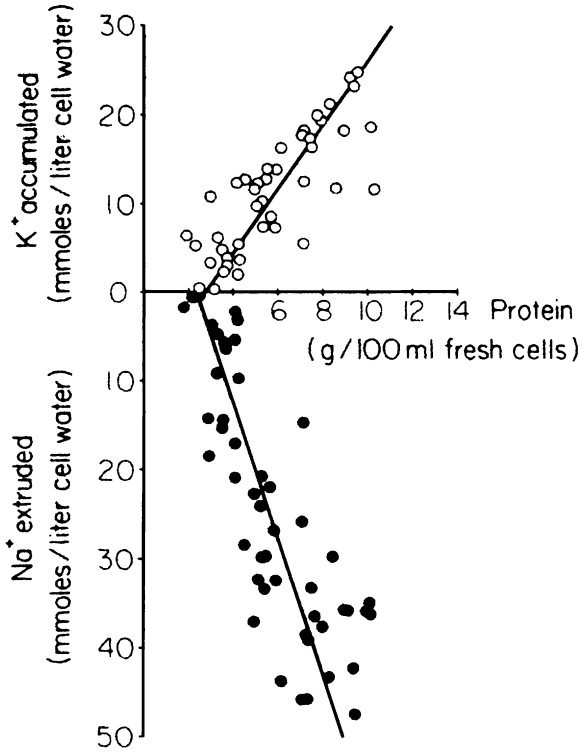

Figure 5. Re-uptake of K^+ and extrusion of Na^+ from red-blood-cell ghosts prepared from washed human red blood cells. The study followed rigorously a procedure described in (Freedman 1976). Freshly drawn blood was obtained (mostly) from different donors. When blood from the same donors was used, it 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,

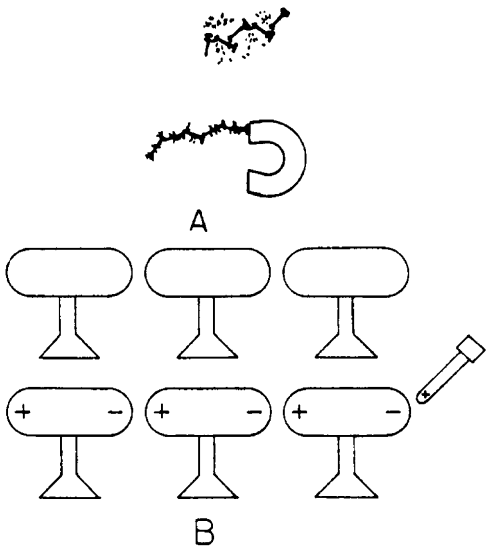

Figure 6. Two models demonstrating information and energy transfer over distances due to propagated short-range interactions. (A) A chain of loosely tethered soft-iron nails is randomly arrayed and does not interact with the surrounding iron filings. The approach of a magnet causes propagated alignment 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.

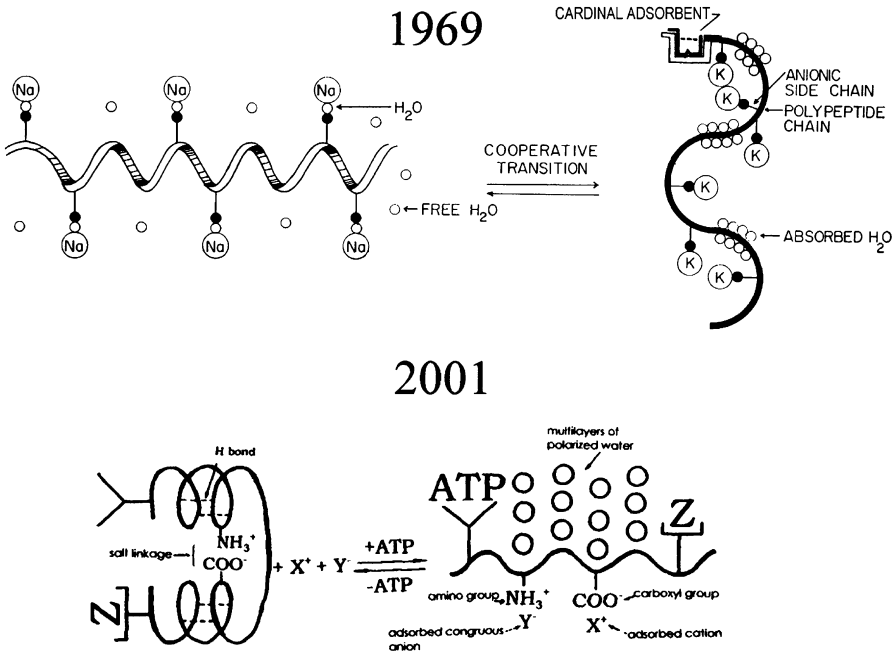

Figure 7. Two diagrammatic illustrations published respectively in 1969 and in 2001. The original legend of the 1969 presentation reads: 'Diagram of a portion of a protein molecule undergoing an auto-cooperative transformation. For simplicity, adsorbed water molecules in multilayers are shown as a single 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).

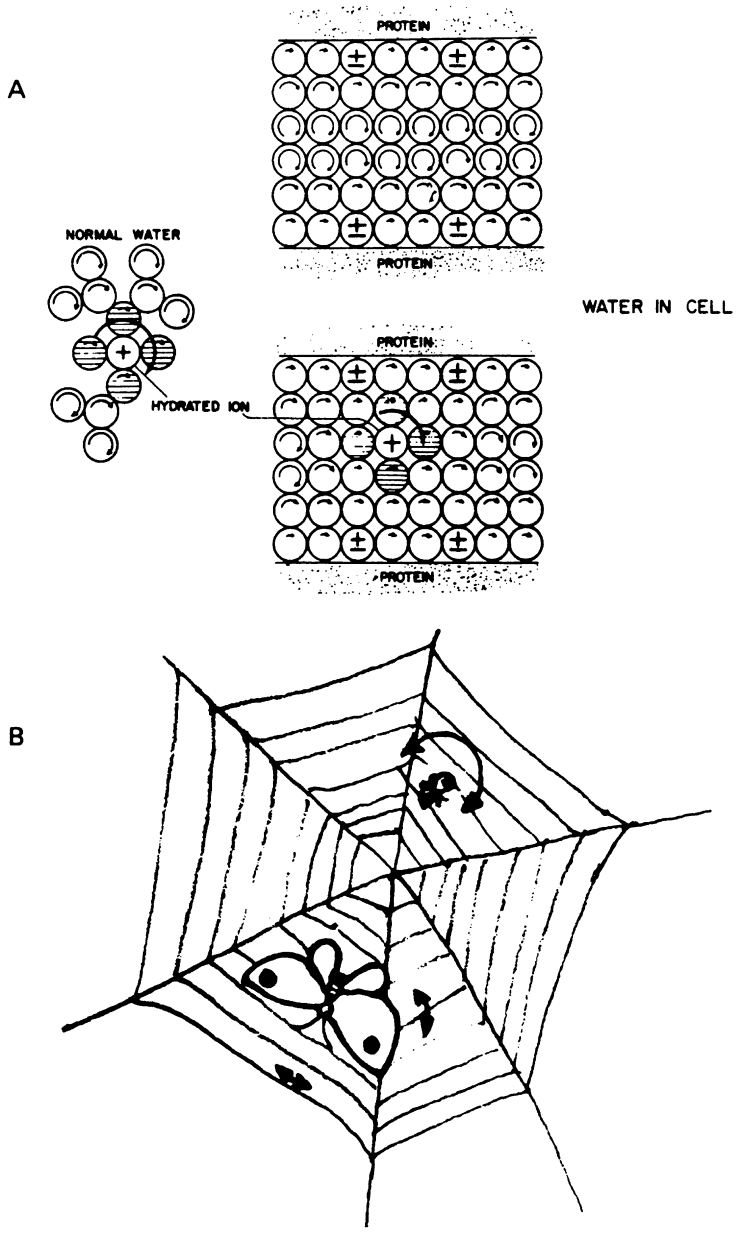

Figure 8. Motional reduction in polarized-oriented water. (A) Diagrammatic illustration of the reduction of rotational (and translational) motional freedom of a hydrated Na^+ ion in water assuming the dynamic structure of polarized multilayers. Size of the curved arrows indicates degree of rotational 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

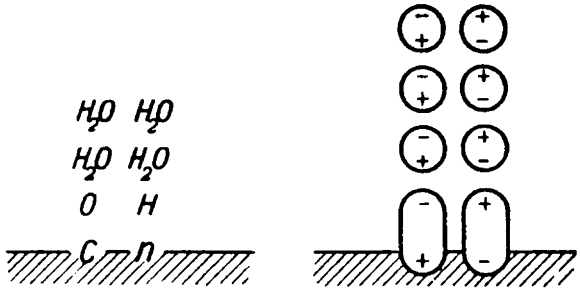

Figure 9. Theoretical model of de de Boer and Dippel showing how dipolar NH and CO groups of gelatin can polarize and orient multiple layers of water molecules (from de Boer and Dippel 1933)

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.)

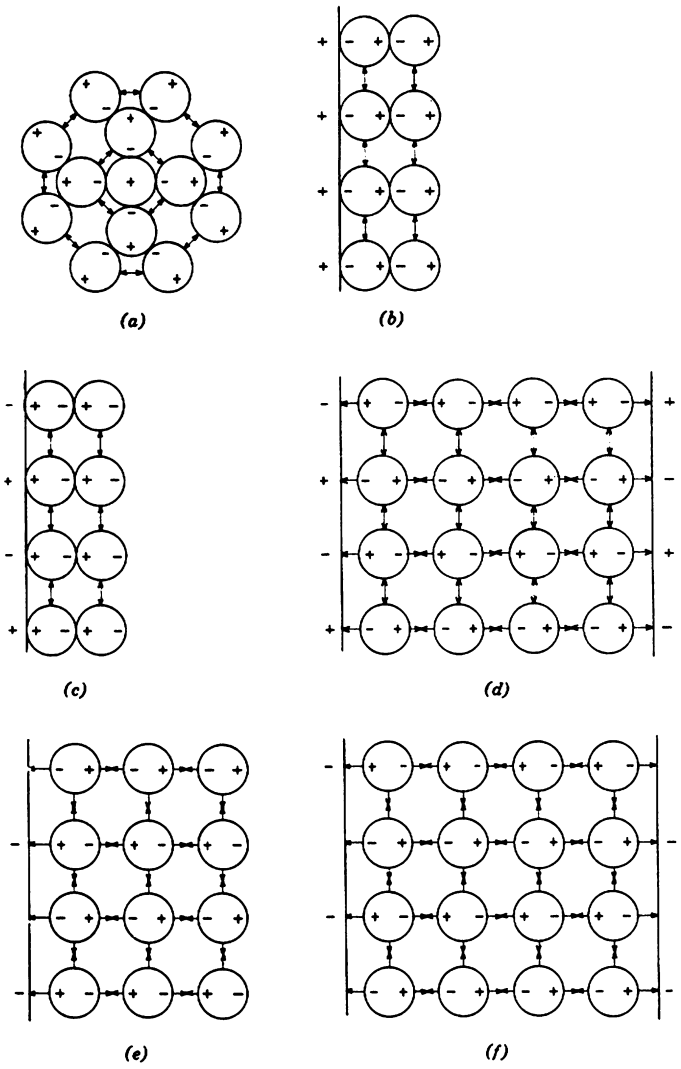

Figure 10. Diagrammatic illustration of the way that individual ions (a) and checkerboards of evenly distributed positively charged P sites alone (b) or negatively charged N sites alone (c) polarize and orient water molecules in immediate contact and farther away. Emphasis was, however, on uniformly 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.)

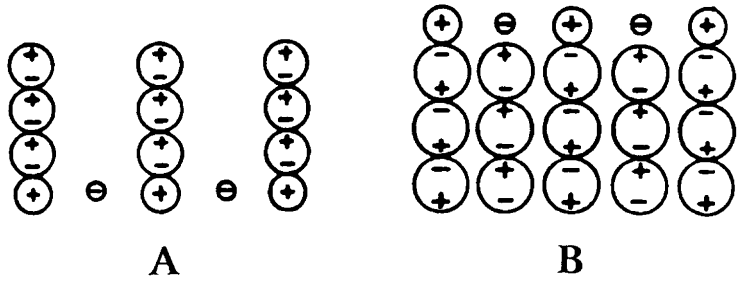

Figure 11. Reproduction of figures presented in the paper by de Boer and Zwikker in 1929 showing their vision of a checkerboard of alternating N and P sites and two different ways they saw how water molecules might be reacting to the charged sites. Although de Boer and Zwikker's Polarization Theory had encountered serious problems, their earlier explicit presentation of what I call the NP surface concept has been of great importance in the subsequenct development of the PM theory. Their reproduction reminds us of their contribution (A and B are respectively what de Boer and Zwikker labeled as Figure 3 and 4 respectively in their original paper)

values of a's can be plotted against the entire left hand side of Equation (2) to yield a straight line, one then regards the data as fitting the equation.

A decade later, Brunauer, Emmett and Teller (the Edward Teller of the Hydrogen bomb fame) demolished the de Boer and Zwikker polarization theory (Brunauer et al. 1938). They did this on the ground that electrical polarization alone – as it is the case in the polarization theory – cannot bring about adsorption of more than one layer of gas molecules even for a gas like argon with one of the largest *polarizability* among the noble gases. Brunauer, Emmett and Teller then offered their own theory, which was nicknamed BET theory after the first letters of their names.

Unfortunately, the BET theory is of no help to me either since virtually all the water that forms multiple layers in their model is simply normal liquid water. As such, it could not fulfill the need of polarized-oriented water demanded by the PM theory with altered physico-chemical properties. This leaves only Bradley's theory for polar gases with a permanent dipole moment like water. Again, despite the endorsement by Brunauer, Emmett and Teller (ibid, p 311) Bradley's theory for gases with permanent dipole moments also has serious problems. But until something better turns up (see below) that was all we had to work with.

4.3 Colloid, Its Birth, Its Unjustified Abandonment and Its Eventual Restoration – with a New Definition Based on the PM Theory

Thomas Graham (1805–1869) was an English chemist. He spent a good part of his life studying diffusion. He was not a cell physiologist and did not even use the word, protoplasm at all in his famous defining paper of 1861 (Graham 1861). Nonetheless, there is little question that he had initiated an important exploration into the nature of protoplasm by introducing the name and concept of colloid, the namesake of gelatin or glue (κολλή).

Under the heading of colloid chemistry and the leadership of a number of capable scientists like Dutch scientist, H.G. Bungenberg de Jong, a great deal of highly valuable information has been obtained. Nevertheless, colloid chemistry like the concept of protoplasm has lost its bearing and has become all but extinct from the cell physiology – until the emergence of the AI Hypothesis but especially its subsidiary, the PM theory of cell water.

A near-fatal mistake was a wrong-headed definition of colloid – in terms of the size of the colloidal particles. So when macromolecular chemistry came into being, colloid chemistry lost its identity.

It was in 1985, when I offered (my first) new definition of colloid (Ling 1985). This and a later more up-to-date definition quoted below were the offspring of two new developments:

(i) The full elucidation of the amino acid composition of gelatin (Estoe 1955), revealing large percentages of proline (13%) and hydroxyproline (10%), both lacking the positively H of the usual peptide NH group, cannot form an intra- or inter-macromolecular H-bonds; an even larger percentage of glycine (33%), which has a very low *α-helical potential* (see Table 4 in Ling 2001, p 145) and thus low propensity to form such intra- or inter-macromolecular H bonds.

(ii) The introduction of the PM theory, which suggested that exposed NHCO or NCO of polypeptide chains polarize and orient in multilayers of cell or model water. Combined, these new ideas and facts led eventually to the latest new definition of colloid as follows:

A colloid is a cooperative assembly of fully extended macromolecules (or large aggregates of smaller units) carrying properly-spaced oxygen, nitrogen or other polar atoms and a polar solvent like water, which at once dissolves and is polarized and oriented in multilayers by the macromolecules or equivalents. (See Ling 2001, p 84.)

In this new definition to colloid chemistry, the difference between macromolecular chemistry and colloid chemistry requires no further explanation. This new definition also affirms the insight and genius of Thomas Graham in being able to see the importance of gelatin as a *bona fide* colloid. Thus, most isolated proteins exist in the folded conformations with their backbone NHCO groups locked in H-bonds and are thus unavailable to polarize and orient the bulk-phase water. Gelatin, in contrast, is at least $(13\% + 10\% + 33\% =)$ 56% in the *fully extended conformation* in consequence of its inherent amino-acid makeup. The fact (to be presented below) that gelatin hydration matches those of PEO, PEG, PVP, each with its entire collection of NO sites fully exposed to bulk-phase water suggests that more than 56% of the NCO and NHCO groups of gelatin are fully exposed to the bulk-phase water.

4.4 Experimental Verification of the PM Theory of Cell Water and Model Systems

For a full account of the experimental verification of the PM theory, the reader is strongly advised to go to my 1992 and/or 2001 books. The limited presentation below emphasizes vapor sorption and solute exclusion studies with all the remaining subjects mentioned by names only in section 4.4.1 immediately following.

4.4.1 Overall triple experimental verification of the PM theory on eight sets of physico-chemical attributes of cell water and their models

An inanimate model is called a *positive model* or a *negative model,* depending on whether or not it can duplicate effectively a cell physiological phenomenon due to the presence or absence of a critical feature or property. The affirmation of the respective comparisons between the living cell and a positive model, between the living cell and a negative model and between the positive and negative inanimate models is called a *triple confirmation* (Ling 2003). While the term, positive and negative models apply to all models, more specific names were given to the specific models for cell water. Thus a positive model for polarized-oriented water is called an *extrovert* model; a negative model is called an *introvert* model.

Since the introduction of the PM theory in 1965, worldwide testing resulted in the triple confirmation of all eight sets of basic attributes of cell water so far studied in depth: (1) osmotic activity; (2) swelling and shrinkage; (3) freezing point depression; (4) vapor sorption at near saturation; (5) NMR rotational correlation time $\{\tau_r\}$; (6) Debye dielectric reorientation time $\{\tau_D\}$; (7) rotational diffusion coefficient from quasi-elastic neutron scattering; (8) solute exclusion. (For references of all these experimental studies, see Ling 1992, p 108; 2001, p 78.) In what follows, I shall discuss in more detail only two of these subjects: vapor sorption at near saturation and solute exclusion.

Space limitation does not allow more details. Nonetheless, it gives me both pleasure and honor to cite investigators whose scientific insights, skill and dedicated efforts have made all these possible: Freeman Cope, Carlton Hazlewood, Raymond Damadian, Jim Clegg, Miklos Kellermayer, Bud Rorschach, E. Ernst, A.S. Troshin, Dimitri Nasonov and many others. Then there are still others whose work would be discussed in greater detail below.

4.4.2 Vapor sorption of living cells and model systems at relative humidity ranging from near zero to 0.99 and higher

Throughout history, many studies of the adsorption of water by proteins and other biomaterials have been reported. Almost all of these studies do not go beyond 95% saturation on the high end. This is unfortunate because the *physiological vapor pressure*, that is the vapor pressure of a typical Ringer's solution or plasma is way beyond 95%. For example, the relative vapor pressure of (frog) Ringer's solution is 0.996.

However, there is one notable exception. Namely, the comprehensive study by J.R. Katz (1919) of water vapor sorption on a wide variety of chemical substances and biomaterials at relative humidity as high as 100% saturation.

(1) *The earliest theory known to me of a common origin of water sorption in protoplasm and in gelatin by Heinrich Walter based on J.R. Katz's vapor sorption data*

J.R. Katz was not that certain about the accuracy of his data obtained at what he labeled as 100% saturation. So he gave these numerical figures behind $\pm$ sign. Nonetheless, the difference between the water vapor uptake of (denatured collagen

or) gelatin at 100% relative humidity (±4.6 grams of water per gram of dry protein) and so-called native proteins like hemoglobin (0.796 grams of water per gram of dry protein) could not be more striking. This subject will be brought up again below.

However, at the next highest relative vapor pressure Katz studied (96.5%), the uptake of water vapor by gelatin was only 0.64 grams per gram. Thus fully 86% of the water uptake of gelatin occurs above 96.5%, which is above the usual upper limit at 95%.

Four years after Katz's paper was published, Heinrich Walter (1923) at the Botanical Institute at Heidelberg reported that the volume of (vacuole-free) protoplasm from various algae, when immersed in sucrose solutions of different strengths, exhibit a certain quantitative pattern of variation. Walter then suggested that this pattern of swelling or shrinkage bears resemblance to the pattern of water vapor uptake by gelatin, starch etc., which Katz had demonstrated earlier. Walter's illustration is relabeled and shown here as Figure 12.

Alas, this perfectly reasonable idea of Walter was also ignored and so rarely cited that once again I was totally unaware of its existence until very recently. Thus to

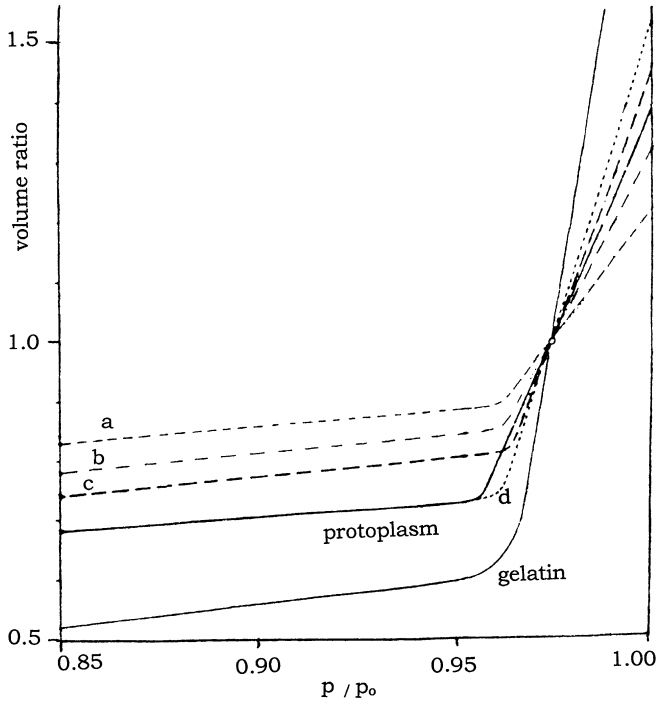

Figure 12. Heinrich Walter's demonstration of parallelism between the swelling and shrinkage of algae protoplasm in sucrose solutions of different strengths and water vapor sorption of gelatin, starch (a), nucleic acids (b) and casein (c) at different relative vapor pressure from J.R. Katz (1919) (from Walter 1923)

the best of my knowledge on this day (February 29, 2005), Walter was the first to suggest in 1923 that water in protoplasm and in gelatin share a common origin.

But Walter offered no idea on what that common origin is. When de Boer and Dippel did suggest ten years later that the polypeptide NHCO groups of gelatin could provide the seat of multilayer adsorption of water, they made no connection between their idea and Walter's. Fortunately, other facts known were able to lead me to make the several connections that spelled out as the PM theory.

(2) *Ling and Negendank's study of water sorption of surviving frog muscle cell strips at relative humidity from near zero to 0.996*

Having given due credit for Walter's idea, I must now point out that there is a gap of experimental knowledge. This gap lay between J.R. Katz's water vapor sorption data of gelatin (casein, starch and nucleic acids) and Walter's data, which he claimed to be water sorption on plant protoplasm obtained by soaking the plant cells containing the protoplasm in sucrose solutions of various strength.

There is no question that the cell membrane involved like all other cell membranes is quite permeable to sucrose (Table 1). So his data of the water uptake was not that of the protoplasm but that of protoplasm *plus* varying amount of sucrose. That was the best he could do at the time but it was in need of improvement. In fact such an improvement was made (in ignorance of Walter's earlier idea) and reported by Ling and Negendank in 1970 (Ling and Negendank 1970). And it consisted of making a similar vapor sorption study as J.R. Katz had done on gelatin and other materials in 1919, but now directly on living protoplasm under sterile conditions.

First, by dissecting frog muscle into narrow strips and exposing them to a vapor phase kept at different relative vapor pressure – all under sterile conditions – Ling and Negendank found that the time of water vapor sorption equilibrium at 25 °C was reached in about 5 days. Based on this knowledge, Ling and Negendank obtained the vapor sorption isotherm of living frog muscle cells at relative humidity ranging from 0.043 to 0.996 as shown in Figure 13.

The data can be sorted out into two parts. A small fraction making up about 5% of the total water uptake is taken up strongly at the lowest vapor pressure range. The interpretation we offered, that the 5% strongly bound water was taken up by polar side chains of cell proteins is in agreement with conclusions of the later work of Leeder, Watt and others (Leeder and Watt 1974).

The remaining 95% of the water adsorbed could fit the Bradley isotherm (shown for example in Figure 21 in Ling 2001) – much as Hoover and Mellon (1950) had demonstrated similar fitting of the Bradley isotherm of their data on water sorption of casein, cotton and especially polyglycine.

(3) *Hoover and Mellon's study of vapor sorption of polyglycine, proteins and other polymers*

I was not able to find out the molecular weight of the polyglycine Hoover and Mellon used. Nonetheless, it is not difficult to arrive at the conclusion that the

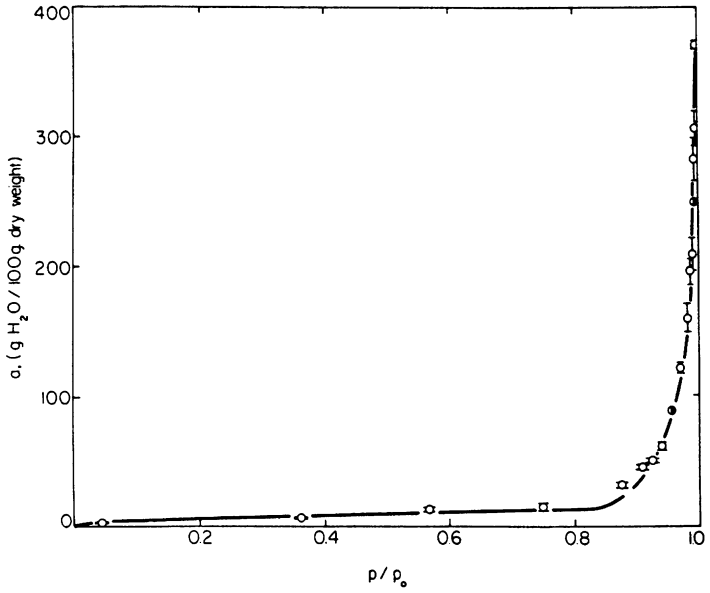

Figure 13. Water vapor sorption of surviving frog muscle cell strips at relative vapor pressure from 0.043 to 0.996. Small muscle cell strips were isolated under sterile conditions and incubated at 25° for 5 days to reach equilibrium. To prevent condensation of water on the wall of the wide glass tubes in which the muscle strips ware hanging, the entire tubes were immersed in a constant temperature bath maintained at 25 °C ± 0.05 °C. Different relative vapor pressures were provided by different concentrations of solutions of H_2SO_4 or NaCl (from Ling and Negendank 1970, by permission of the Pacific Press, Melville, NY)

polyglycine they used could not have been a small polypeptide. Or else these authors would not have referred to the polymer studied as high polymer in the title of the article.

On the assumption that they studied a high molecular weight polyglycine, one can reason that the terminal carboxyl and amino groups are trivial in number and that virtually all its water-sorbing sites were in the form of backbone NHCO groups. Whether this interpretation is completely right or only partly right, Hoover and Mellon themselves had inferred from their data that the backbone NHCO groups offer important sites of water vapor sorption. In addition, they also showed that the vapor uptake of polyglycine exhibits a sigmoid-shaped water sorption curve. This in turn has a two-fold significance.

First, it shows that the backbone NHCO groups are indeed important water sorption sites as pointed out earlier by Lloyd, Sponsler and others (Lloyd 1933; Lloyed and Phillips 1933; Sponsler et al., 1940).

Second, since there were no polar side chains in polyglycine, the abundant ‘extra’ uptake of water at very high humidity in this case at least was not built upon monolayers of water adsorbed on polar side chains as Leeder and Watt once

suggested for proteins (1974, p 344). *Rather, the entire sigmoid-shaped uptake begins and ends as polarized oriented multilayers on the backbone NHCO groups*.

To gain more insight on the role of backbone NHCO groups, we move to the water sorption data of Katchman and McLaren (1951) on a closely similar polypeptide, polyglycine-DL-alanine.

Like polyglycine, polyglycine-DL-alanine also has virtually all its potentially water-sorbing sites in the form of backbone NHCO groups. And its strongly accelerating sorption of water at relative vapor pressure approaching saturation once more reminds us of a similar pattern seen in the water sorption of surviving frog muscle cells shown in Figure 13. This close quantitative similarity is made even clearer in Figure 14, taken from Ling 2003, which in turn was a modification of a still earlier illustration first published in 1972 (Ling 1972).

This quantitative matching of water sorption of intact living frog muscle and of polyglycine-DL-alanine (1:1) on the upper end of vapor sorption is highly significant. It confirms the PM theory of cell water as being polarized and oriented primarily by the exposed NHCO groups of the fully-extended proteins in living cells.

(4) *Ling and Hu's introduction of a new fast technique and their study of vapor sorption of PEO, PEG, PVP, gelatin and several native proteins at physiological, and still higher vapor pressure*

A skeptic critique may ask, 'How do you known that the NHCO groups of either polyglycine or polyglycine-DL-alanine are not engaged in α-helical or in other intra-, or inter-macromolecular H bonds and thus not free to adsorb water?' The answers are as follows.

First, there is no problem with polyglycine. It is well known to exist in the fully extended form in water (Bamford et al. 1956, p 310.) In contrast, a block polymer of poly-L-alanine (of 130 residues) is known to be entiely water insoluble. Indeed, so tight is its α-helical folding that it would not unfold even in 8 M urea (Doty and Gratzer 1962, pp 116–117). The fact that the co-polymer, polyglycine-DL-alanine containing one part glycine and one part D,L-alanine is *fully water soluble* as well as its strong water sorption make it a good bet that the polyglycine-DL-alanine investigated by Katchman & McLaren does not contain large block of alanine residues but contain more or less randomly distributed mixed polymer and as such, fully extended.

However, to leave no doubt about the fully extended nature of these poly-amino acids, Ling and Hu undertook the studies of some other *extrovert models* of the NO-NO-NO type, which being without P sites cannot engage in α-helical of other intra-, or inter-macromolecular H-bonds as polyamino acids can.

Furthermore, in these newer studies there was yet another improvement over the Katchman-McLaren data, which did not go beyond 95% relative vapor pressure at its high end. As already pointed out above, when we talk cell physiology, it refers to its normal physiological environment. As mentioned above, the physiological vapor pressure for frogs is 0.996.

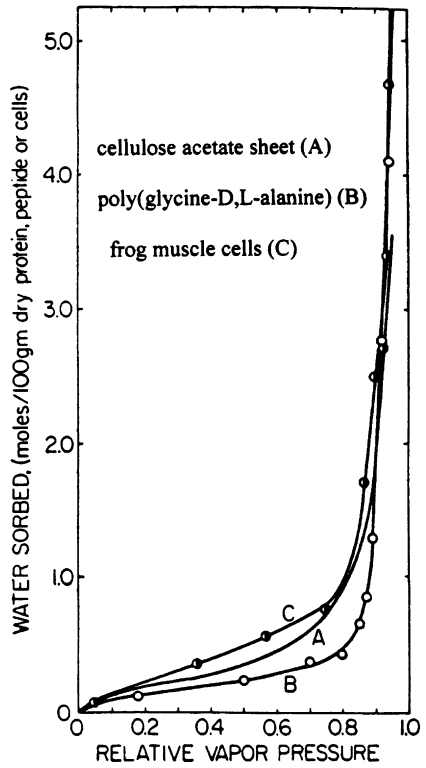

Figure 14. The strikingly similar steep uptake of water molecules at relative vapor pressure close to saturation of surviving frog muscle cells (marked C) and of the synthetic polypeptide, poly(glycine-D,L-alanine) (marked B), shows evidence that the backbone NH and CO groups (which are virtually all the functional groups that can interact with water molecules in the synthetic polypeptide) are the major seats of (multilayer) water adsorption in living cells as suggested in the polarized-oriented multilayer theory of cell water. The third curve from unpublished work of Palmer and Ling shows that water taken up by commercial cellulose acetate sheets is similarly adsorbed on dipolar sites – a matter of great significance because later work of Ling (1973) shows that the permeability of this membrane strikingly resembles the permeability of a live membrane (inverted frog skin) (figure reproduced from Ling 1972, by permission of John Wiley and Sons, Inc.)

To introduce our next subject, I ask the question, 'Why was J.R. Katz able to study water vapor sorption on biomaterials up to 100% relative vapor pressure and yet later work on vapor sorption, including that of Katchman and McLaren just quoted, shied away from relative vapor pressure higher than 95%?'

First, the continued dominance of the membrane (pump) theory with its free water and free K^+ assumptions has given a (false) reason to consider irrelevant the study of water sorption at physiological vapor pressure. But there is a second more legitimate reason.

At saturation or near saturation vapor pressure, the attainment of adsorption equilibrium is extremely slow and this slowness not only has made studies difficult

but also uncertain in accuracy. Thus in Katz's work, it took several months to reach what he considered as equilibrium but behind ± signs. Ling and Hu showed that in the case of water sorption of the polymer, polyvinylpyrrolidone (PVP), at a near saturation vapor pressure, the equilibrium level was still not reached after three hundred and twenty (320) days of incubation.

It was to overcome this forbidding difficulty that Ling and Hu introduced their new method, called *nullpoint-method*. This new method shortened the equilibrium time to only five days and with this gain, there was also a gain in full reliability. To save space, Figure 15 with a detailed legend will give the reader some idea on how it is done.

Using this new null-point method, Ling and Hu studied the vapor sorption of gelatin, three oxygen-containing polymers that belong to what we call NO-NO-NO extrovert systems and several 'native' proteins including hemoglobin. Among the NONONO extrovert models studied are polyvinylpyrrolidone (PVP), poly(ethelene oxide) (PEO) and polyethylene glycol (PEG). The vapor pressures studied were immediately below, at, and above the physiological vapor pressure for frog tissues, 0.9969. Also included in Figure 16 is the upper end of the water sorption of surviving frog muscle data of Ling and Negendank presented in full in Figure 13.

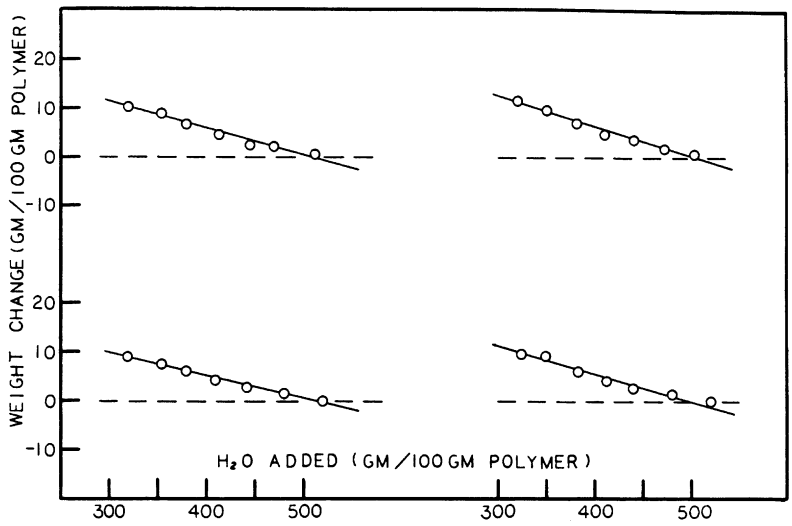

Figure 15. Quadruplicate determinations of the equilibrium water sorption in an atmosphere containing water vapor at a relative vapor pressure equal to 0.99775, of polyethylene glycol (PEG-8000) by the Null Point Method. The ordinates represent the percentage gains (or losses) of water in the different PEG samples after 5 days of incubation at 25 °C following the addition of different amounts of water to each sample (in grams of water added per 100 grams of dry sample weight) shown as abscissa. The zero gain (or loss) intercepts allows one to obtain from the quadruplicate set of data: 513.7, 505.0, 510.8 and 508.9 averaging 509.6 ± 3.65 (S.D.) (from Ling and Hu 1987, by permission of the Pacific Press, Melville, NY)

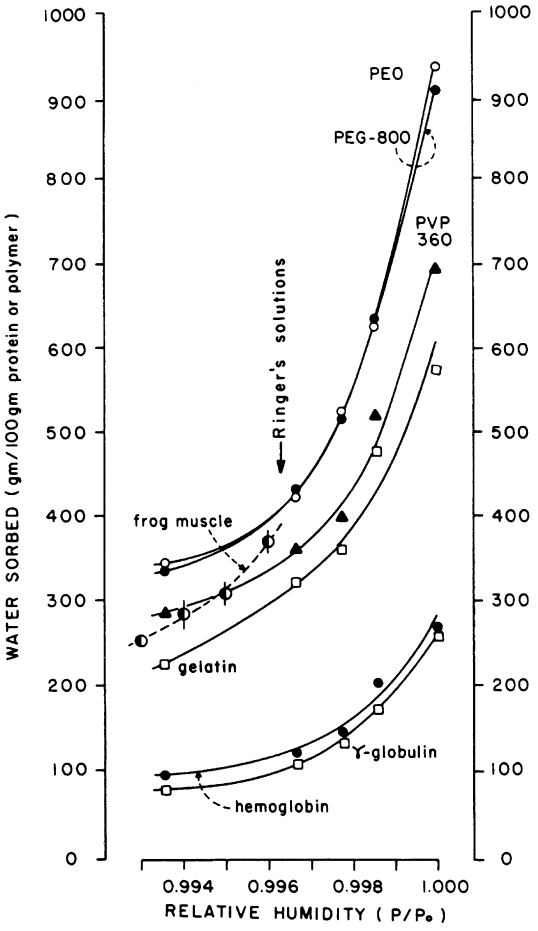

Figure 16. Equilibrium water vapor sorption obtained with the aid of the Null Point Method at relative vapor pressures from 0.99354 to 0.99997 of polyethylene oxide (PEO), polyethylene glycol (PEG-8000) polyvinylpyrrolidone (PVP-360), gelatin, γ-globulin, hemoglobin at 25 °C. (from Ling and Hu 1987, by permission of the Pacific Press, Melville, NY)

Note that at this extremely high physiological vapor pressure range, the uptake of water of these extrovert models has gone far above the water uptake at the upper limit of relative vapor pressure (95%) in the poly-glycine-DL-alanine study. As such, they match or even exceed the water uptake of living frog muscle cells. Since these extrovert NONONO models carry negatively charged oxygen atoms as N sites, but no positively charged P site in-between the N sites, they cannot form intra-, or inter-macromolecular H bonds. Accordingly, each of these oxygen atoms (together with its two negatively charged lone pair electrons and its adjacent vacant or O site) is free to polarize-orient multilayers of water as predicted in the PM theory.

The quantitative accord between the amount of water adsorbed of living frog muscle and these three NONONO polymers as shown in Figure 16 confirm one of the main postulates of the PM theory of cell water. That it is the exposed NHCO groups that polarize and orient by far the greater majority of water molecules in living cells.

(5) *Conflict with traditionally accepted low water binding data and a possible reconciliation*

Parenthetically, the demonstration that the so-called 'native hemoglobin' (and other 'native' proteins) do(es) not polarize and orient enough water to match that found in the living cells – even though these folded 'native' proteins do polarize and orient far more water than conventionally accepted by many first rate protein chemists (Table 3). The question may be raised: 'Which is right?'

The answer is probably that both are right. Thus, different investigators could be focussed on different portions of the affected water. The data obtained by equilibrium vapor sorption is direct and not model dependent. In contrast the methods used to obtain the data presented in Table 3 are almost all model-dependent and the values determined usually relied on certain simplifying assumptions.

In some of these cases at least, there is the possibility that what was measured was the more tightly held water bound to polar side chains of proteins mentioned earlier. Thus, you may recall, tightly bound water emerges as a separate fraction in the water vapor taken up by frog muscle cell strips already completed at very low vapor pressure. Its total amount was estimated to be about 5% of the total water content of the muscle cells. At a total water content of about 80%, that 5% amounts to 4 grams of sorbed water per 100 grams of fresh muscle. Taking a value of 20% total cell proteins, this fraction would be equivalent to 0.2 grams of water per gram of protein. This is a figure not far from the average water content listed in Table 3.

In the next section, I shall examine another physical property of cell water, its solvency for solutes of different sizes. This too is model independent. If at equilibrium a specimen of water accommodates only half of what that solute can dissolve in normal liquid water, at least 50% of the water in this specimen must be different from normal liquid water.

4.4.3 Solute exclusion from polarized-oriented water in living cells and in model systems

(1) *Theory of solute exclusion based on the PM theory*

Now, we are in a position to look at Figure 8A once more. You recall that that figure shows how proteins can polarize and orient multilayers of water. The figure to the left in Figure 8A also shows how restricted motional freedom might offer an (entropic) mechanism for the exclusion of solutes like the hydrated Na^+ – as illustrated in the analogy of a butterfly caught in a spider web shown in Figure 8B.

Table 3. Hydration of proteins

Protein	Reference listed at end of table	Technique identified at end of table	Hydration (g H_2O/g protein)
Serum albumin	5	A	0.2
	6	B	0.30–0.42
	10	C	0.19–0.26
	11	D	0.31
	7	E	1.07[a]
	7	F	0.75[a]
	8	G	0.43
	12	F	0.40
	12	H	0.48
	13	A	0.18–0.64
	14	A	0.15
	9	A	0.23
Avg.			0.32
Ovalbumin	2	I	0.18
	5	A	0.1
	6	B	0–0.15
	8	G	0.31
	7	E	0.45[a]
	9	A	0.18
Avg.			0.17
Hemoglobin	6	B	0.20–0.28
	11	D	0.10
	8	G	0.45
	7	E	0.36[a]
	7	F	0.69[a]
	9	A	0.14
	10	A	0.2
Avg.			0.25
β-Lactoglobulin	6	B	0–0.20
	7	E	0.72[a]
	7	F	0.61[a]
	5	A	0.4
	9	A	0.24
Avg.			0.25

A Dielectric dispersion; B X-ray scattering; C Sedimentation velocity; D Sedimentation equilibrium; E Diffusion coefficient; F Intrinsic viscosity; G NMR; H Frictional coefficient; I ^{18}O diffusion.

1. Fisher 1965; 2. Wang 1954; 3. Adair and Adair 1963; 4. Adair and Adair 1947; 5. Oncley 1943; 6. Ritland et al., 1961; 7. Tanford 1961; 8. Kuntz et al., 1969; 9. Buchanan et al., 1952; 10. Cox and Schumaker 1961a; 11. Cox and Schumaker 1961b; 12. Anderegg et al., 1955; 13. Grant et al., 1968; 14. Haggis et al., 1951; 15. Miller and Price 1946. (For details of sources of the publications, see Reference at end of Ling 1972) (from Ling 1972, by permission of John Wiley and Sons, Inc.).

However, to facilitate a more quantitative approach, I must first introduce a parameter called the q-value.

The q-value stands for the (true) *equilibrium distribution coefficient* of a solute between cell (or model) water and the usually normal liquid water in the surrounding

medium. The q-value refers exclusively to a solute (like sucrose) in the bulk-phase water of a living cell. As a rule, the q-value is at or below unity. Since according to the PM theory, solute distribution in living cell water and in model systems follows the Berthelot-Nernst distribution law (see Glasstone 1946, p 735), a plot of the equilibrium concentration of the solute against its concentration in the bathing medium should yield a straight line. And the slope of the straight line is equal to the q-value of that solute in the cell water or model water as the case may be.

In 1993, a full quantitative PM theory of solute exclusion involving both enthalpic and entropic mechanisms was published (Ling 1993; Ling et al. 1993). Working together, each mechanism provide its own size-dependent solute exclusion. Under the name, the 'size rule', the theory predicts that the larger the solute molecule, the lower is the q-value of that solute. Equation (A3), which summarizes the theory, is reproduced here as Appendix 1 given toward the end of this communication.

Take a large molecule like sucrose (or hydrated Na^+) for example. In order to transfer a molecule of sucrose from an external bathing solution made up of normal liquid water into a living cell, a hole must be excavated in the cell water to accommodate the sucrose. Since the average water-to-water interaction in the polarized-oriented cell water is stronger than that in the normal liquid water outside, more energy would be spent in excavating the hole in cell water than the energy recovered in filling the hole left behind in the normal water of the bathing solution.

This enthalpy or energy difference per molecule is the enthalpic (or energy) component of the size-dependent solute exclusion. Figure 17 shows the theoretical q-value for solutes of different size in cell water or model water with different levels of water-to-water interaction energy (alone). This is the size-dependent *bulk phase* enthalpy component of the free energy difference that determines the q-value of a solute.

Next to discuss is the entropic component. Here too, the larger the solute molecule the greater is its structural complexity The greater the structural complexity, the larger is its rotational entropy. The greater its rotational entropy, the greater is its propensity to lose a significant part of it due to the more 'sticky' polarized-oriented cell water with stronger water-to-water interaction than in normal liquid water of the surrounding medium. This is illustrated in the spider web analogy of Figure 8B. It is the larger butterfly that suffers proportionally the more motional restriction, while a smaller butterfly may not lose that much motional freedom to allow it to fly away.

Then, there is a third component in the makeup of the full expression of exclusion given in Equation (A3) mentioned above. This third component is a measure of how well the surface of the solute molecule fits the water structure surrounding the solute molecule. For solute that can fit into the water structure, that favorable component of (negative) energy gained would be added to the q-value, making it higher than that due to size alone. On the other hand, if the surface component is unfavorable, it would make the q-value still lower.

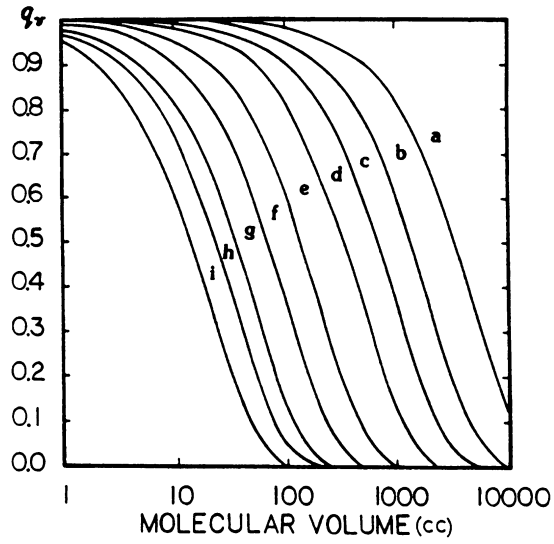

Figure 17. The theoretical volume (or solvent) component of the equilibrium distribution coefficient (q_v) for solutes of different molecular volume in water polarized at different intensity (See Equation A3 in Appendix 1). The intensity of water polarization due to the volume component of the polarization energy is given as the specific solvent polarization energy, ΔE_v. The specific value of ΔE_v in units of RT per cm^3 is indicated by the letter near each curve, where a represents 0.0002; b, 0.0005; c, 0.001; d, 0.002; e, 0.005; f, 0.01; g, 0.02; h, 0.03; i, 0.05. R is the gas constant and T the absolute temperature. At room temperature (25 °C), RT is equal to 592 cal./mole (from Ling 1993, by permission of the Pacific Press, Melville, NY)

(2) *Experimental testing of solute exclusion theory based on the study of small probe molecules with molar volume of 500 cc or less*

Based on what has been explained above, the PM theory could make certain predictions. Thus in normal resting living cells, the q-value of solutes should decrease with increasing size of the solute's molar volume, Figure 18 shows that this is true for frog muscle cells. Note that the slopes of the straight lines decrease with the increasing size of the solute. That slope, as mentioned earlier, is equal to the q-value of that solute.

According to the PM theory, water in extrovert models should demonstrate similar size-dependent q-values. Figure 19 and Figure 20 respectively show that this is true with two extrovert models studied in some detail, poly(ethylene oxide) or PEO and NaOH denatured hemoglobin.

On the other hand, water in a solution of so-called native hemoglobin – a well-established *introvert* model, should according to the PM theory, demonstrate a q-value close to unity for the same solutes of varying size that are excluded to varying degrees by normal living cells and by the extrovert models. Figure 21 shows that this expectation too is realized for solutes 500 cc or less in molar volume.

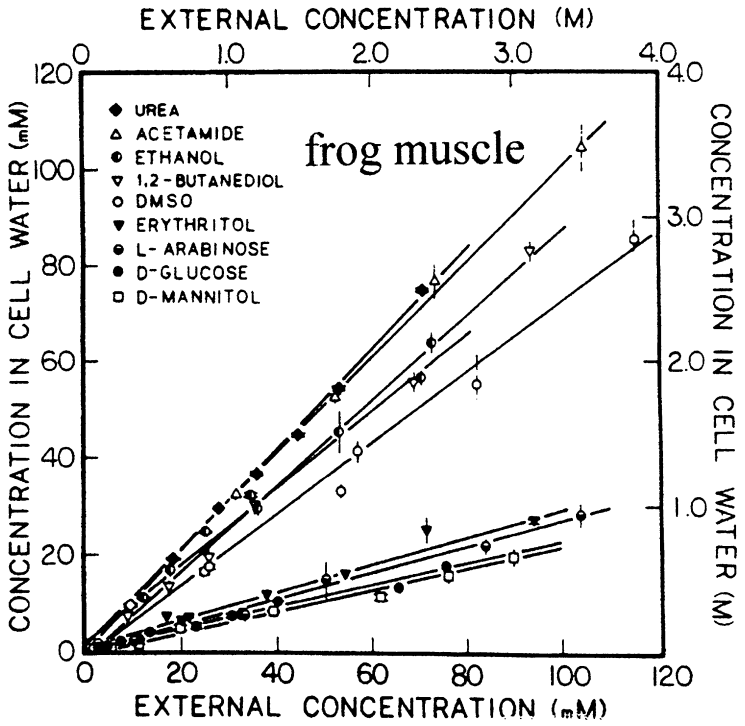

Figure 18. The equilibrium distribution of nine non-electrolytes in frog muscle cell water at 0 °C. Each point represents the mean ± S.E. of at least four samples. Incubation time (enough or more than enough to insure diffusion equilibrium in all cases) was 6 days for L-arabinose and L-xylose but 24 hours only for all others. Each of the straight lines going through or near the experimental points is obtained by the method of least squares, its slopes yielding the respective (true) *equilibrium distribution coefficient* or q-value of that solute (from Ling et al. 1993, by permission of the Pacific Press, Melville, NY)

The slopes of these straight line plots in Figures 18 to 21 yield respectively the q-values of the different solutes in muscle cell water, in the water containing NaOH-denatured hemoglobin, PEO and native bovine hemoglobin. These q-values are in turn plotted against the molecule weight of each solute in Figure 22 and its insets. Included in Inset B is also a set of data of gelatin, which were not our own but taken from Gary-Bobo and Lindenberg (1969). Unlike our data, their degree of exclusion was not obtained from linear plots from many experimental points at different concentrations. Rather, each point was from a single set of determinations at a specific concentration. Thus, this set of data is not rigorously obtained as are q-values. But judging from the general conformity, the deviations if any could not be too large.

The lines going through or near the experimental data points of the main figure and figures in the insets of Figure 22 were all obtained by visual inspection. All told, two kinds of curves were obtained. The curve in the form of a straight flat

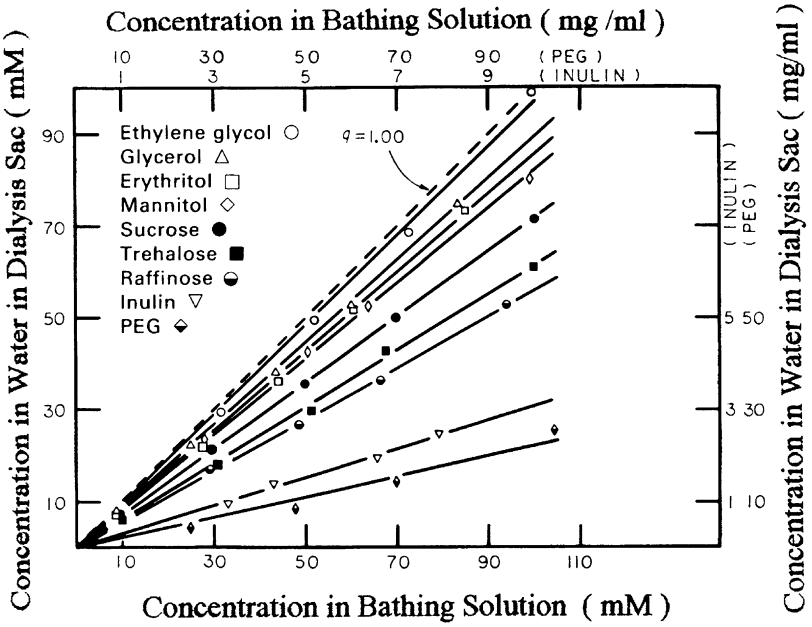

Figure 19. Equilibrium distribution of nine non-electrolytes in dialysis sacs containing bovine hemoglobin (18%) dissolved and incubated in, and denatured by 0.4 M NaOH. Incubation lasted 5 days at 25 °C. Symbol for each nonelectrolyte as indicated in figure (from Ling and Hu 1988, by permission of the Pacific Press, Melville, NY)

line was from the introvert model, native hemoglobin. The remaining four sets of data one from living frog muscle and the others from the three extrovert models all show a Z-shaped curve depicting decreasing q-values with increasing molecular weight of the solute. Note also that seven of the 21 experimental points from frog muscle do not fit in with the rest of the points, which provides the basis for the main Z-shaped line.

Next, we attempt to obtain quantitative data on the underlying mechanism of the solute exclusion in the living frog muscle cells. To achieve that, we need to put our theoretically derived equation (Equation A3) to work. That is, the frog muscle data points are plotted a second time in Figure 23. Here, instead of molecule weights of each solute studied, the q-values are plotted against their respective molecular volumes in cc as it is in Equation (A3).

More important, the lines going near and through the data points are not drawn by visual inspection. Instead, they are theoretical based on Equation (A3) cited in the Appendix. There are now two theoretical curves instead of one with seven apparently aberrant points.

One theoretical curve fits data points lower down and the other fitting the seemingly aberrant higher points in Figure 22. Only we now know that they are not aberrant at all. Indeed, the two theoretical curves were based on the same bulk-phase

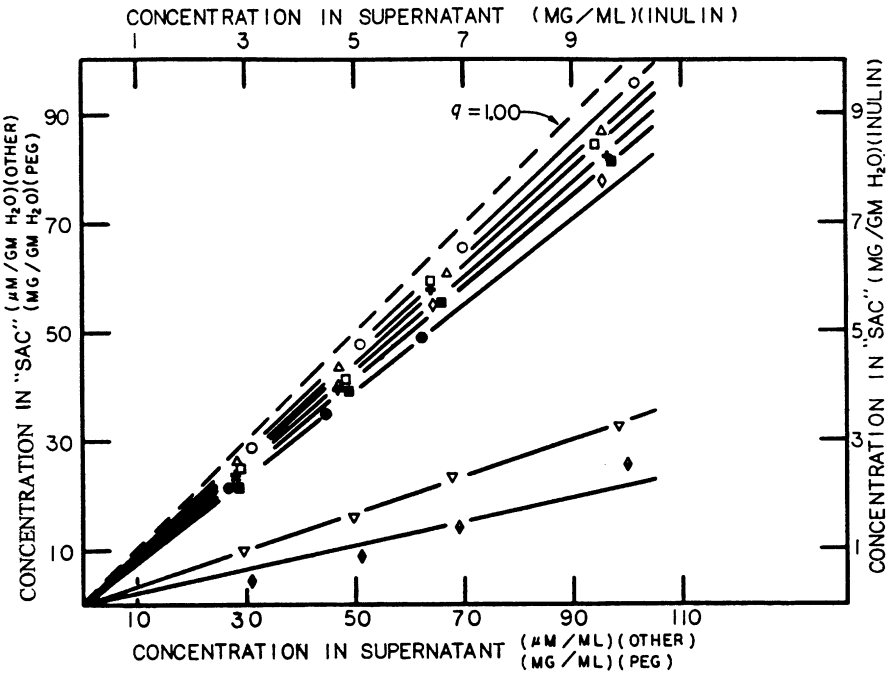

Figure 20. Equilibrium distribution of nine non-electrolytes in a solution of polyethylene oxide. Final concentration of the polymer was 15%. In addition, the solution also contained 0.4 M NaCl. The symbols used for all the non-electrolytes are the same as in Figure 19. D-xylose, which was absent in Figure 19 but present here, is represented by + (from Ling and Hu 1988, by permission of the Pacific Press, Melville, NY)

exclusion intensty, $\mathcal{U}_{vp}$, equal to 126 cal per mole. But the surface polarization energy $\mathcal{U}_s$ are not the same. It is 119 cal/mole for the lower curve but a good deal higher at 156 cal/mole for the upper curve.

This much higher surface polarization energy, $\mathcal{U}_s$, of the curve that better fit the seven 'aberrant' points is exciting because five of these seven solutes are chemicals known as *cryoprotectants*.

Now, cryoprotectants are chemicals, which when added to the culture medium protects the cells or embryos from freezing damages during storage in liquid nitrogen. One suspects that a sixth 'aberrant' chemical (urea) would have been yet another cryoprotectant if it were not for the fact that urea is also a protein denaturant, which might harm the cells.

(3) *Experimental demonstration of the exclusion of large macromolecules with a molar volume of 4000 cc by weakly polarized-oriented water*

At this juncture, it is timely to mention some new results of a study that we have just published (Ling and Hu 2004). It demonstrated that the water in a solution of 35% native bovine hemoglobin that shows no exclusion at all for solutes as small

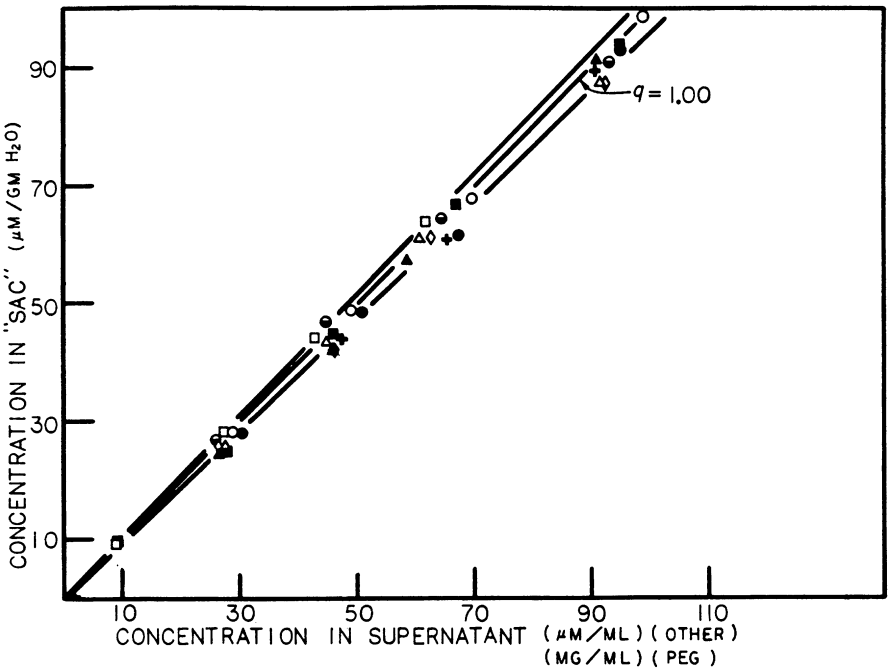

Figure 21. Equilibrium distribution of various non-electrolytes in a neutral solution of bovine hemoglobin (final concentration, 39% ± 1%) after 5 days of incubation at 25 °C. Solution also contains 0.4 M NaCl. Symbols used are the same as shown in Figure 19, except D-xylose, which is not in Figure 19 but present here. It is represented by + (from Ling and Hu 1988, by permission of the Pacific Press, Melville, NY)

as water to solutes as large as raffinose, is not normal liquid water either. Indeed, when the probe molecule used was poly (ethylene glycol) with a molecular volume of 4500 cc, it shows a q-value of only about 0.2.

The surprisingly low q-value for very large probes like PEG-4000 reminds us that the introvert models we studied so far are rarely, if ever the ideal introvert models (with the water-to-water interaction energy of perfectly normal liquid water). Rather, they are merely models with much lower "excess water-to-water interaction energy" than that of the extrovert models. Nonetheless, it is not normal liquid water. After all, some of the NHCO groups of *bona fide* globular native proteins are not all engaged in forming intra- or inter-macromolecular H bonds. Those NHCO groups existing as *free coils* or even *turns* as well as polar side chains (associated with the right kind of cations) may have some impact on water structuring as their counterparts in fully extended polypeptide chains.

Nonetheless, how large molecular size of the probe molecules can compensate for the weakness of the degree of polarization-orientation of the bulk phase water is clearly demonstrated here. This fact also puts us in a position better to assess the even more spectacular exclusion of really large probe molecules as reported by Gery Pollack's groups from Seattle, which will be discussed next.

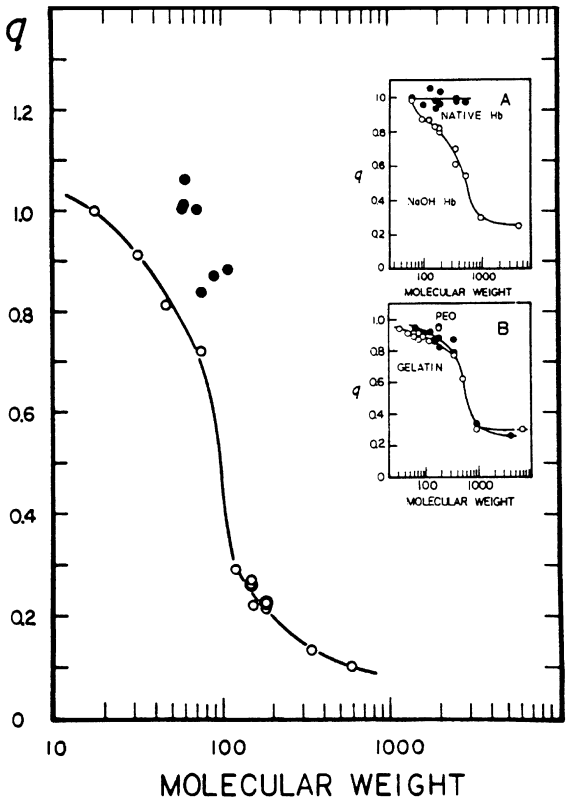

Figure 22. The (true) equilibrium distribution coefficients (or q-values) of 21 non-electrolytes in muscle cell water shown on the ordinate plotted against the molecular weights of the respective non-electrolytes. For comparison, q-value *vs* molecular weight plots of similar non-electrolytes in solutions of NaOH-denatured bovine hemoglobin (18%), gelatin gel (18%), poly (ethylene oxide) or PEO (15%) and native bovine hemoglobin (39%) are shown in the insets. Data on gelatin gel represent ρ- rather than q-values (see text) and were taken from Gary-Bobo and Lindenberg 1969 (from Ling et al. 1993, by permission of the Pacific Press, Melville, NY)

(4) *The exclusion of giant probe, latax-coated microspheres 1 μm in diameter*

The exclusion of latex-coated microspheres 1 μm in diameter by water dominated by the NONONO (or NPNPNP) surface of a polyvinyl alcohol (PVA) gel as revealed in the time sequence photography of Zheng and Pollack (2003) offered spectacular confirmation of the PM theory (Figure 24.) Because, like the saying goes, seeing is believing. There is no question here that the power of the NONONO surface could extend as far out as 100 μm or some 300,000 water molecules away from the PVA gel surface.

Polyvinyl alcohol, or PVA was a very good choice. As shown in Figure 25 taken from McLaren and Rowen's review. PVA has a strong affinity for water molecules.

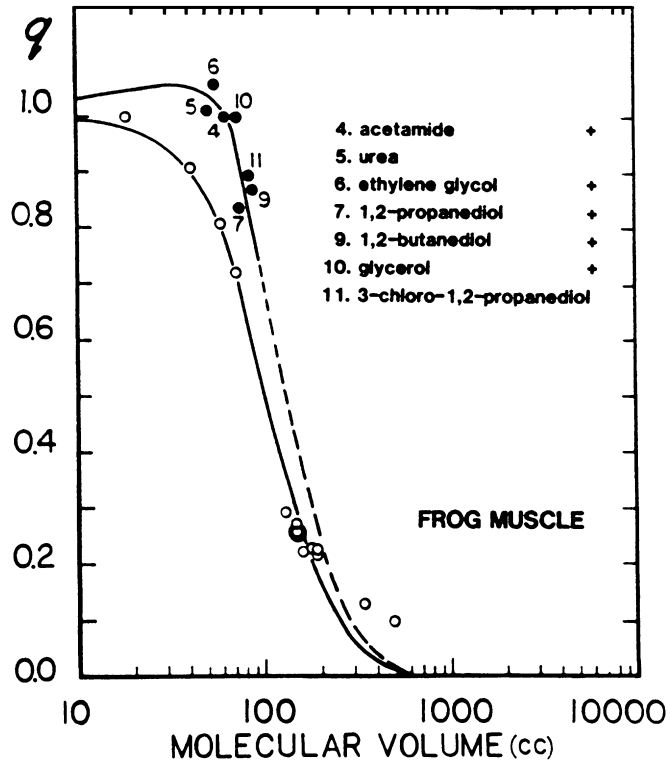

Figure 23. The equilibrium distribution coefficients (or q-values) of 21 non-electrolytes in muscle cell water plotted against the *molecular volumes* of the respective non-electrolytes. The points are experimental and are the same as shown in Figure 22. The lines going through or near the experimental points are theoretical according to Equation A3 in Appendix 1. For both the lower and upper theoretical curves, $\mathcal{U}_{vp}$, the 'exclusion intensity' for one mole of water, is the same at 126 cal/mole. $\mathcal{U}_s$, the surface polarization energy, on the other hand, is 119 cal/ mole of water for the lower curve and 156 cal/mole for the upper curve. Chemicals marked with the + signs are established cryoprotective agents (from Ling et al. 1993, by permission of the Pacific Press, Melville, NY)

From its structure, $\{\text{-CH}_2\ \text{CH(OH)-}\}_n$ but in fact an NPNPNP system because the OH groups can function both as a proton acceptor and proton donator. There are two interesting features that this astonishing finding has demonstrated.

First, the Zheng and Pollack figure reproduced as Figure 24 shows a clear-cut boundary between a zone of exclusion and a zone of admittance.

The most important reason could be what the PM theory has long maintained that the water polarization-orientation is strongly *auto-cooperative* (Ling 1980, p 39). The polarization-orientation of one water molecule greatly enhances the neighboring water molecules to undergo the same change. In other words, there is a strongly positive *nearest neighbor interaction energy*. This confirmation of the theory is the most important.

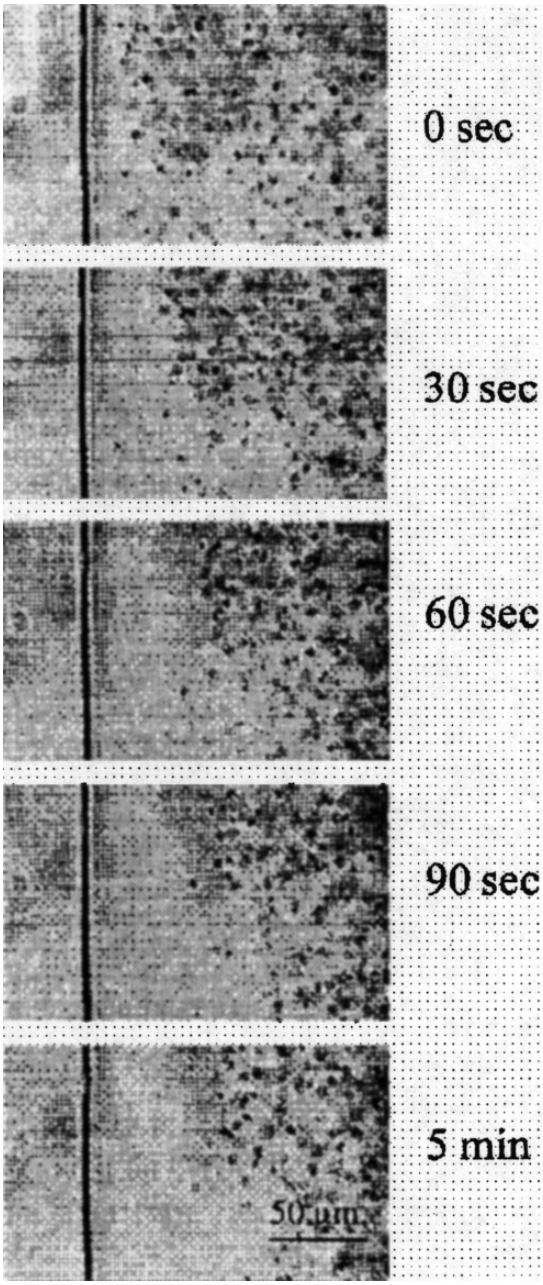

Figure 24. The emergence and progressive widening of an exclusion zone containing no microspheres as a function of time in a cylindrical channel of a polyvinyl alcohol (PVA) gel. Carboxylate groups-covered latex microspheres measured 2 μm in diameter (Zheng and Pollack 2003, by permission of Physical Review)

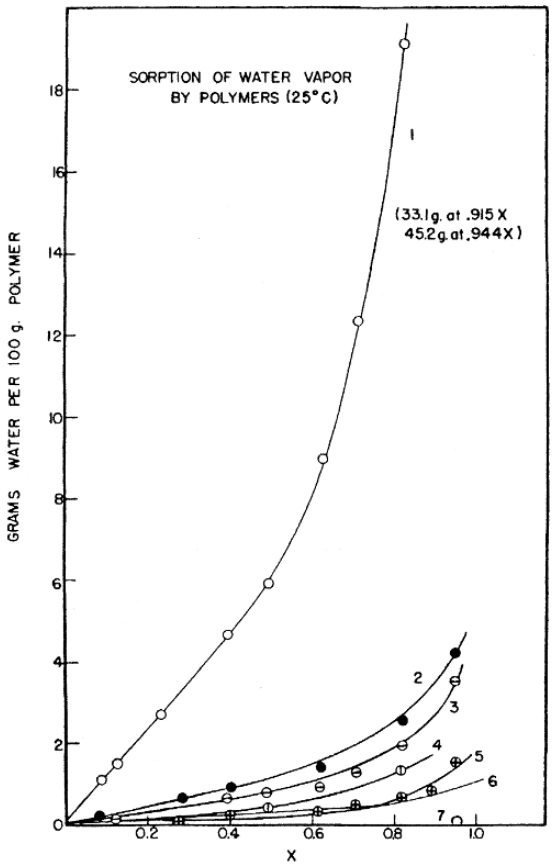

Figure 25. Sorption of water vapor by various polymers at 25°C. Polyvinyl alcohol (1); polyvinyl butyral (2); ethylene vinyl alcohol (3); rubber hydrochloride (4); vinylidine chloride-acrylonitrile (5); chlorinated polyethylene (points not shown) (6); polyethylene (7) (from McLaren and Rowen 1951, by permission of J. Polymer Science)

But there might be another contributing factor, which is trivial in nature but nonetheless contributes to what we see. When the molecular volume of the probe is very large as it certainly is the case here, the visual image might not look significantly different when the q-value of the microspheres varied say between 0.01 to 0.0001. This effect tends to enhance the all-or-none appearance.

Having said that, I must return to the most important feature of the observation: its extreme reach of the influence of the PVA surface. To the best of my knowledge, this spectacular demonstration of a distance of 100 μm-wide clear zone was not predicted by any of the theory of gas adsorption known at the time when Zheng and Pollack published this work in 2003.

4.5 To the Rescue, a New Theoretical Foundation of Truly Long Range Water Polarization and Orientation

In an earlier section, I have shown how two of the theories of multilayer gas adsorption, de Boer-Zwikker's polarization theory and Bradley's theory for multilayer adsorption of non-polar gases fell on the roadside. As pointed out by Brunauer, Emmett and Teller in 1938, polarization alone cannot do what it is supposed to do: polarize and immobilize multiple layers of gas molecules. Even for the most polar gases, the mechanism proposed could produce much more than a single layer of adsorbed gas.

This trio then proposed their own theory often known by its nickname, the BET theory. Unfortunately, the BET theory has its own weaknesses. First, it is hard to see why the allegedly normal liquid water – free from long-range influence from the solid surface – would nonetheless stay on as multilayers on the solid surface rather than evaporate at any relative vapor pressure less than 100% saturation (see Cassie 1945). Second, it cannot explain the intense 'extra' uptake of polar molecules like water at near saturation vapor pressure. Thus, the BET theory could adequately explain adsorption only at below 50% vapor saturation. Higher than that, theoretical predictions and experimental data sharply diverge (see Figure 26). Third, condensed as *normal liquid water*, it cannot explain the extensive experimental demonstrations of altered physico-chemical attributes of the deep layers of water collecting on suitable solid surfaces (Henniker 1944).

That leaves only Bradley's general theory for gas molecules with permanent dipole moments. Yet, despite an endorsement by Brunauer, Emmett and Teller (1938, p 311), it is also full of holes. The fact that it shares a formal equation (Equation 1) with two other theories no longer tenable is already bad. By Bradley's own admission, that data fitting his equation does not prove that his theory is confirmed weakens it further.

Perhaps the most damaging to the Bradley isotherm for gas molecules with permanent dipole moments is this. It gives not an inkling as to how deep a layer of water a water-polarizing surface can produce. And as you notice above, it is the lacking of the theory that can give us a definitive answer to that question that would leave the legions of exciting and wonderful experimental data like so many orphans.

Indeed, without the support of a sound theoretical foundation even these spectacular observations could run the risk of sharing the same fate that had overtaken the already long list of truly exciting observations of long range interaction (see Henniker 1944, also Figures 29 and 30) in being treated like curios and relics rather than sound scientific knowledge. When these clear-cut scientific facts are looked upon with uncertainty, the PM theory of cell water, which has its foundation on nothing better than the Bradley isotherm, also suffers.

For all these reasons, I felt jubilant when I discovered a short cut and through it, developed a new theoretical foundation for the long-range dynamic structuring of water molecules and other suitable polar gases.

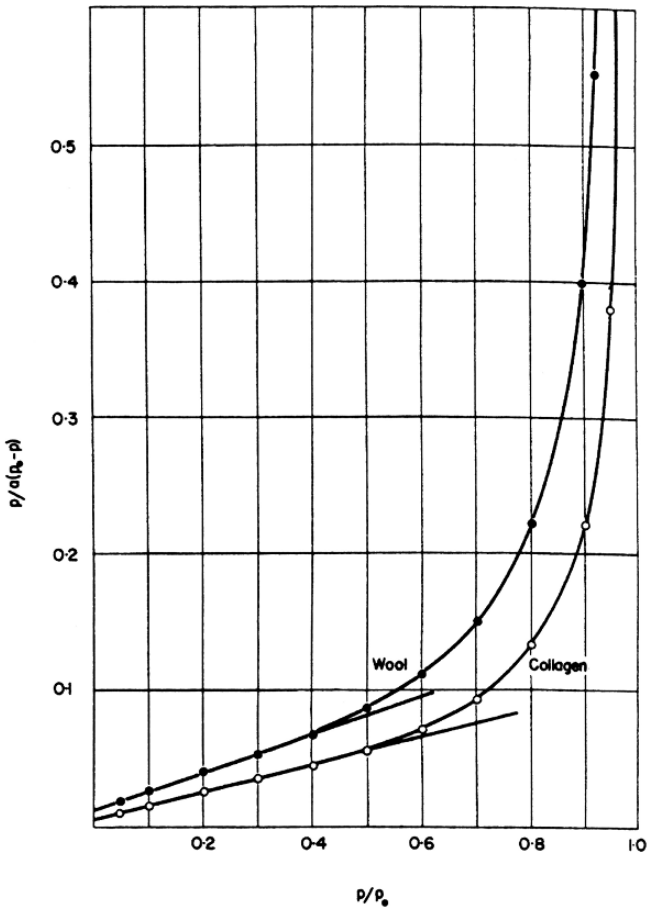

Figure 26. Water vapor sorption data from Bull (1944) on collagen and on wool were plotted according to the BET isotherm. Data fit the isotherm well up to about 40% relative vapor pressure. From this point and higher, the data and isotherm are far, far apart. In other words, the BET theory cannot explain the high uptake of water by these proteins at the high end of the vapor pressure values (from Ling 1965, by permission of the Annals of NY Academy of Sciences)

For details, the reader must consult my recent paper (Ling 2003). Suffice it to say here that even the best of human minds have limitations. Thus, when dealing with highly complex physiological problems, methods that had proved so powerful in dealing with simple systems may not yield equally rewarding results.

More specifically, the central problem in dealing with long range dynamic structuring of water lies in coping with the thermally agitated *permanent dipole moment* of the gas molecules near the polar surface. To overcome this difficulty, de Boer and Zwikker simply ignored the permanent dipole moment of the gas molecules. In his general theory for all gases on salt crystal surfaces, Bradley did not ignore the

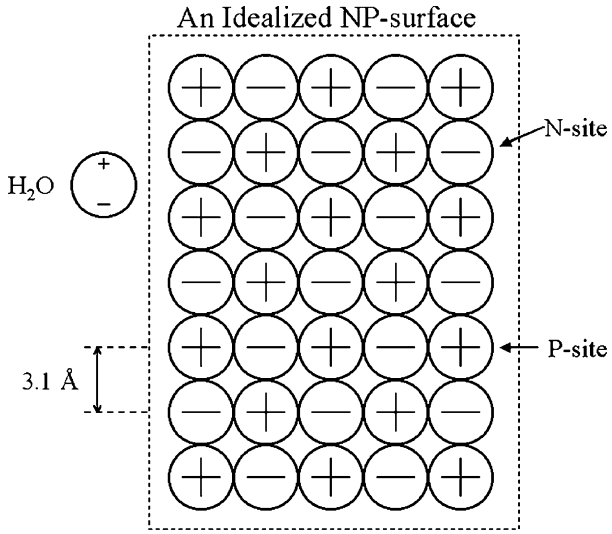

Figure 27. An idealized NP surface. The distance between a pair of the nearest-neighboring N and P site is equal to the distance, r, between neighboring water molecules in the normal liquid state and approximately 3.1 Å (from Ling 2003, by permission of the Pacific Press, Melville, NY)

permanent dipole moment. Rather, he ignored the fixed negatively charged sites or N sites of the salt crystal surface. So, instead of a study of what I would call a NP system or even an OP system, he in fact studied what I would call a PP system. As pointed out in section 4.1, such a uniformly charged surface cannot polarize-orient deep layers of polar gas molecules like water.

The short cut that I took was to get around the thermal agitation of the permanent dipole moments by bringing the temperature to just a little above absolute zero. I then introduced what I call an *idealized NP surface* (Figure 27) and added conditions that would eliminate extraneous factors like gravitation, border effects etc. The net result was that I have greatly simplified the problem by making electrostatics the sole determining factor in the polarization and orientation of water dipoles. Under this condition, each water molecule is fully characterized by three parameters: its diameter (r), its permanent dipole moment (μ) and its polarizability (α). Note also that the distance between the nearest neighboring N and P site is made to equal that of the water diameter, r, chosen.

The result of the computation is illustrated in Figure 28. The most striking feature is that the (negative) energy of water-to-water interaction, E^n, at the nth layer of water molecules, does not taper off with increasing distance from the idealized NP surface. Instead, as the distance increases toward infinity, E^n assumes a constant value described by the following equation:

$$(3) \qquad E^n = (4\mu^2 r^3)/(r^3 - 8\alpha)^2$$

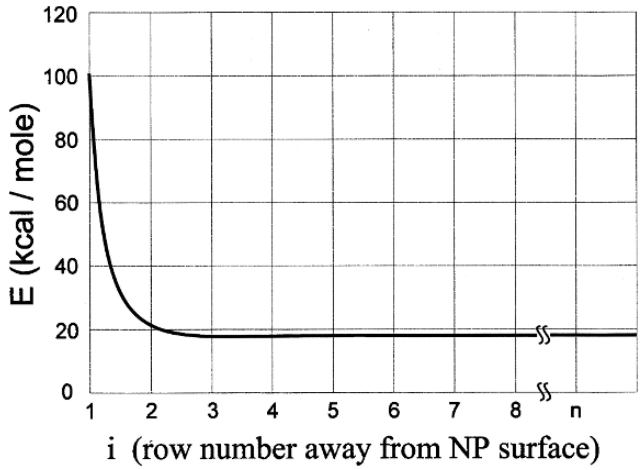

Figure 28. The adsorption energy of a water molecule in successive layers from an idealized NP surface at a temperature very near absolute zero. The theoretically computed adsorption energy per water molecule (E) at successive rows of water molecules away from an idealized NP surface. Note that as the distance between the water molecule and idealized NP surface increases, the adsorption energy does not taper off to zero. Rather, it continues at a constant value described by Equation 3. For detailed on the idealized NP surface, see Figure 27 (from Ling 2003, by permission of the Pacific Press, Melville, NY)

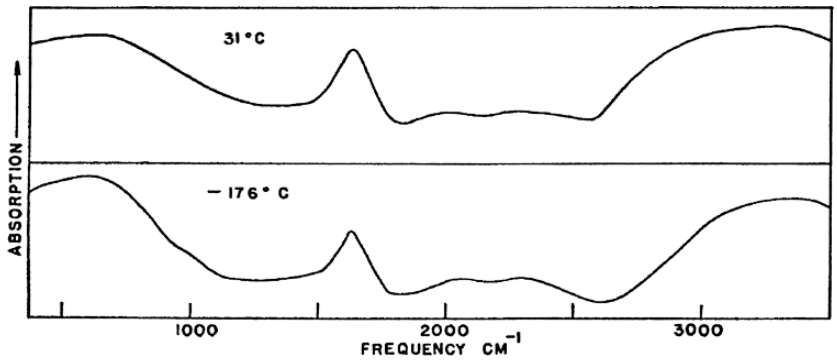

Figure 29. Infrared absorption spectrum of 10-micron-thick water film held between polished AgCl crystal plates. (For the near-ideal geometry of the AgCl NP surface, see Figure 9 in Ling 2003). The two (indistinguishable) spectra were observed respectively at ambiant temperature (top, at 31 °C) and at liquid air temperature (bottom, at −176 °). I am indebted to Dr. Rod Sovoie, the former associate of Dr. Giguère for the information on the thickness of the water film Prof. Giguère and Harvey studied (from Giguère and Harvey 1956, by permission of Canad. J. Chemistry)

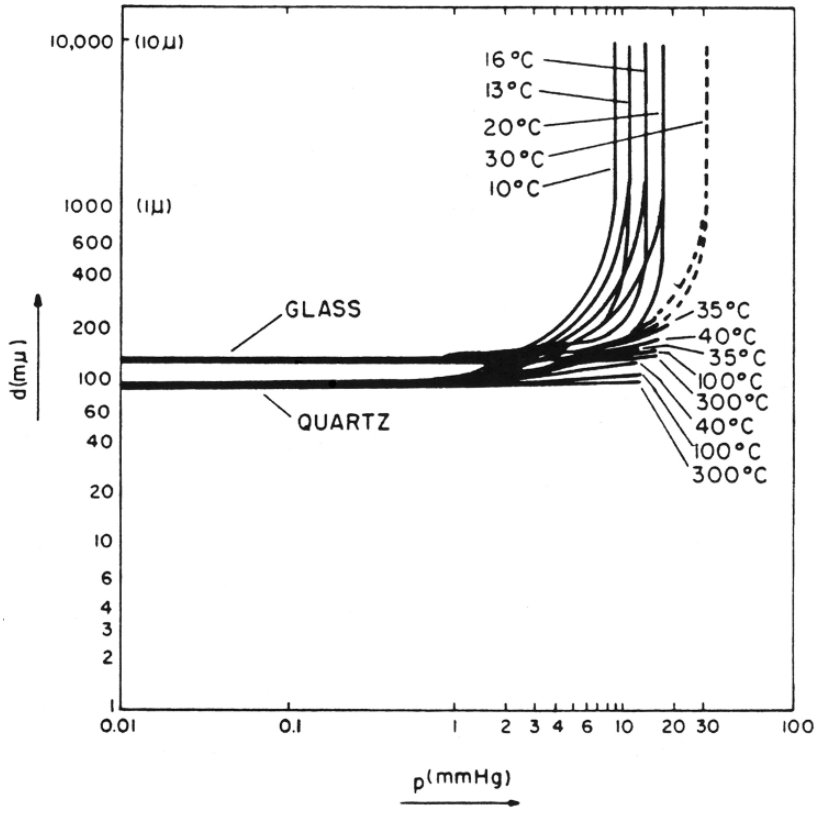

Figure 30. The vapor pressure (abscissa in mm of Hg) of water film at different thickness in mμ (or 10^{-7} cm or 10 Å) (ordinate). Water film of varying thickness was produced by placing water between one flat glass (or quartz) plate and one curved plate with radius of curvature of 35 m. The thickness mentioned above refers to the water film at the periphery of doughnut-like ring. When the thickness is 1 μ or thicker, the vapor pressure is not different from normal liquid water. However, when the thickness falls to 90 mμ (or 900 Å or some 300 water molecules thick), the vapor pressure becomes zero even at temperature as high as 300 °C (from Ling 1972, redrawn after Hori 1956)

Set μ equal to zero, E^n vanishes. This is why, as pointed out by Brunauer, Emmett and Teller that without the participation of the permanent dipole moment, polarization alone cannot produce multilayer adsorption. Also important is the value of r chosen. The value of 3.1 Å adopted was obtained by dividing the molar volume of water (18.016 cc) by the Avogadro number and taking its cube root as equal to r. For values of r shorter than 3.1 Å, the calculated E^n would be even stronger. For values much lower than 3.1 Å, the *ad infinitum* propagation of polarization-orientation would no longer be predicted. Ice crystals of different phases (e.g., ice I, ice X) may then emerge.

The most important contribution of this work is that a good theoretical foundation for the PM theory of cell water and for the long-range dynamic structuring of

water and other polar molecules is now on hand. And this includes the spectacular recent finding of Zheng and Pollack just described. A more detailed analysis of their findings will be forthcoming based at once upon the PM theory of long-range dynamic water structuring (Ling 2003) and the PM theory of solute exclusion (Ling 1993).

Two additional predictions from what is summarized in Equation 3 are: (1) that under proper conditions, water layers held between two surfaces with charge distribution approaching that of an idealized NP surface, would not freeze under any attainable low temperature; (2) that water under similar conditions would not boil at temperature as high as 400 °C. Both predictions have been confirmed retroactively by observations of Giguère and Harvey (1956) and of Hori (1956) reported almost half a century ago. Their key figures are respectively reproduced here as Figures 29 and 30.

ACKNOWLEDGEMENTS

I thank Dr. Raymond Damadian, and his Fonar Corporation and its many friendly and helpful members for their continued support, Margaret Ochsenfeld and Dr. Zhen-dong Chen for their dedicated and skillful cooperation, and librarian Tony Colella and Michael Guarino, director of Media Services and Internet Services for their patience and tireless assistance.

APPENDIX 1

$$\text{(A3)} \qquad q = \exp\left\{\frac{1.23v\Delta E_s\left[1-(1-b)\frac{(kv)^n}{1+(kv)^n}\right]-(\Delta E_v+1.23\Delta e^*)v}{RT}\right\},$$

where q is the equilibrium distribution coefficient of the solute in question. v is the molecular volume (molar volume) of the solute and it is in cm^3. b is a small fractional number describing the probability of (very large) molecules in finding adsorbing sites on the water lattice. k and n are parameters describing the steepness of the declining probability of finding adsorbing sites with increase of molecular volume. $\triangle E_s$ is the *specific surface (or solute) polarization energy* per cm^2 in units of $cal.mol^{-1}$ $(cm^2)^{-1}$, when the solute is moved from normal liquid water to the polarized cell water. $\triangle E_v$ is the *specific solvent polarization energy*, equal to the difference between the energy spent in excavating a hole 1 cm^3 in size in the polarized (cell) water and the energy recovered in filling up a 1 cm^3 hole left behind in the surrounding normal liquid water; it is in units of $cal.mole^{-1}(cm^3)^{-1}$. $\triangle e^*$ is the *increment of the activation energy* for overcoming the greater rotational restriction per unit surface area in units of $cal.mole^{-1}(cm^2)^{-1}$, when a solute is transferred from normal liquid water phase to the polarized water phase. R and T are the gas constant and absolute temperature respectively.

REFERENCES

Bamford CH, Elliott A, Hanby WC (1956) Synthetic Polypeptides: Preparation, Structure and Properties. New York: Academic Press, p 310
Beatley EH, Klotz IM (1951) Biol Bull 101:215
Benson SW, Ellis DA, Zwanzig RW (1950) J Amer Chem Soc 72:2102
Best CH, Tatlor NB (1945) The Physical Basis of Medical Practice. 4th edn., Baltimore: The Williams & Wilkens Co., p 7, column 2
de Boer JH, Zwikker C (1929) Zeitschr Phyisk Chem B3:407
de Boer JH, Dippel CJ (1933) Rec Trav Chem Pays-Bas 52:214
Boyle PJ, Conway EJ (1941) J Physiol 100:1
Bradley RS (1936a) J Chem Soc:1467–1474
Bradley RS (1936b) J Chem Soc:1799–1804
Brunauer S, Emmett PH, Teller E (1938) J Amer Chem Soc 60:369
Bull H (1944) J Amer Chem Soc 66:1499
Cameron IL (1988) Physiol Chem Phys & Med NMR 20:221
Cassie ABD (1945) Trans Farad Soc 41:450
Conway EJ, Creuss-Callaghan G (1937) Biochem J 31:828
Doty P, Gratzer WB (1962) Polyamino Acids, Polypeptides and Proteins. Stahmann MA (ed) Madison: Univ. Wisconsin Press, pp 116–117
Dujardin F (1835) Annales des sciences naturelles; partie zoologique, 2d sér. 4:364
Durant W (1926) The Story of Philosophy. New York: Pocket Books, A Division of Simon and Schuster, reprinted all the way to at least 1961
Eastoe JE (1955) Biochem J 61:58.9
Edelmann L (1977) Physiol Chem Phys 9:313
Edelmann L (1984) Scanning Electron Microscopy. II:875
Edelmann L (1986) Science of Biological Specimen Preparation. Müller M, Becker RP, Boyde A, Wolosewick JJ (eds) Chicago: SEM Inc, AMF O'Hare, p 33
Freedman JC (1976) Biochem Biophys Acta 455:989
Gary-Bobo CM, Lindenberg AB (1969) J Coll Interf Sci 29:702
Giguére PA, Harvey KB (1956) Canad J Chemistry 34:798
Glasstone S (1946) Textbook of Physical Chemistry. 2nd edn., New York: D.van Nostrand
Graham T (1861) Phil Trans R Soc. London, vol 151, p 183
Grove A (1996) Only the Paranoid Survive. New York: Doubleday Dell Publ
Hall TS (1969) Ideas of Life and Matter. Chicago: Univ Chicago Press, p 310
Henniker JC (1949) Rev Modern Phys 21:322
Hodgkin AL (1971) The Conduction of the Nervous Impulse. Liverpool: Liverpool Univ. Press, p 21
Hoover SR, Mellon EF (1950) J Amer Chem Soc 72:2562
Hori T (1956) Low Temperature Science. A15:34 (Teion Kagaku, Butsuri Hen) (English translation: No. 62, US Army Snow, Ice and Permafrost Res. Establishment, Corps of Engineers, Wilmette, Ill. USA)
Huang HW, Hunter SH, Warburton VK, Mos SC (1979) Science 204:191
Katchman BJ, McLaren AD (1951) J Amer Chem Soc 73:2124
Katz JR (1919) Koloidchem Beihefte 9:1
Leeder JD, Watt IC (1974) J Coll Interf Sci 48:339
Ling GN (1952) Phosphorous Metabolism (Vol II). McElroy WD, Glass B (eds) Baltimore: The Johns Hopkins Univ. Press, p 748
Ling GN (1962) A Physical Theory of the Living State: The Association-Induction Hypothesis. Waltham MA: Blaisdell
Ling GN (1965) Ann NY Acad Sci 125:401
Ling GN (1969) Intern Rev Cytol 26:1
Ling GN (1970) Intern J Neurosci 1:129
Ling GN (1972) Water and Aqueous Solutions, Structure, Thermodynamics, and Transport Properties. Horne A (ed) New York: Wiley-Interscience, pp 663–699

Ling GN (1973) Physiol Chem Phys 5:295
Ling GN (1977) Physiol Chem Phys 9:319
Ling GN (1980) Cooperative Phenomena in Biology. Karreman G. (ed) NewYork: Pergamon Press, p 39
Ling GN (1984) In Search of the Physical Basis of Life. New York: Plenum Publ Corp
Ling GN (1992) A Revolution in the Physiology of the Living Cell. Malabar FL: Krieger Publ Co
Ling GN (1993) Physiol Chem Phys & Med NMR 25:145
Ling GN (1997) Physiol Chem Phys & Med NMR 29:123 Available via http://www.physiological-chemistryandphysics.com/pdf/PCP29-2_ling.pdf
Ling GN (2001) Life at the Cell and Below-Cell Level: the Hidden History of a Fundamental Revolution in Biology. New York: Pacific Press
Ling GN (2003) Physiol Chem Phys & Med NMR 35:91 Available via http://www.physiological-chemistryandphysics.com/pdf/PCP35-2_ling.pdf
Ling GN (2004) Physiol Chem Phys & Med NMR 36:1 Available via http://www.physiological-chemistryandphysics.com/pdf/PCP36-1_ling.pdf
Ling GN, Hu WX (2004) Physiol Chem Phys & Med NMR 36:143 Available via http://www.physiologicalchemistryandphysics.com/pdf/PCP36-2_ling_hu.pdf
Ling GN, Ochsenfeld MM (1966) J Gen Physiol 49:819
Ling GN, Negendank W (1970) Physiol Chem Phys 2:15
Ling GN, Ochsenfeld MM (1973) Science 181:78
Ling GN, Walton C (1976) Science 191:293
Ling GN, Zhang ZL (1984) Physiol Chem Phys & Med NMR 16:221
Ling GN, Hu WX (1987) Physiol Chem. Phys. & Med. NMR 19:251
Ling GN, Hu WX (1988) Physiol Chem Phys & Med NMR 20:293
Ling GN, Ochsenfeld MM (1989) Physiol Chem Phys & Med NMR 21:19
Ling GN, Ochsenfeld MM, Walton C, Bersinger TJ (1980) Physiol Chem Phys 12:3
Ling GN, Zodda D, Sellers M (1984) Physiol Chem Phys & Med NMR 16:381
Ling GN, Niu Z, Ochsenfeld MM (1993) Physiol Chem Phys & Med NMR 25:177
Lloyd DJ (1933) Biol Rev Cambridge Phil Soc 8:463
Lloyd DJ, Phillips H (1933) Trans Farad Soc, vol 29, p 132
Macalum AB (1905) J Physiol (London) 32:95
McLaren AD, Rowen JW (1951) J Polymer Sci 7:289
Mellon EF, Korn AH, Hoover SR (1948) J Amer Chem Soc 70:3040
Mellon EF, Korn AH, Hoover SR (1949) J Amer Chem Soc 71:2761
Menten ML (1908) Trans Can Inst, vol 8, p 403
Perutz MF, Muirhead H, Cox JM, Goaman LCG, Mathews FS, McGandy EL, Webb LE (1968) Nature 219:29–32
Sponsler OL, Bath JD, Ellis JW (1940) J Phys Chem 44:996
Stirling AH (1912) James Hutchinson Stirling: His Life and Work. London, p 221
Tigyi J, Kallay N, Tigyi-Sebes A, Trombitas K (1980–81) International Cell Biology. Schweiger HG. (ed) Berlin: Springer, p 925
Walter H (1923) Jahrschr Wiss Bot 62:145
von Zglinicki T (1988) Gen Physiol Biophys 7:495
Zheng J, Pollack GH (2003) Phys Rev E68:031408