

Can We See Living Structure In A Cell?

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Abstract: Colloid chemistry (κολλᾶ: glue, or gelatin) was introduced in 1861 after the discovery of **protoplasm**, which exhibits gelatin-like properties. Some 80 years later, colloid chemistry (and with it, the concept of protoplasm) was largely abandoned. The membrane (pump) theory, according to which cell water and cell solute like K^+ are free as in a dilute KCl solution, became dominant. Later studies revealed that rejecting the protoplasmic approach to cell physiology was not justified. Evidence against the membrane (pump) theory, on the other hand, has stood the test of time. In a new theory of the living cell called the association-induction (AI) hypothesis, the three major components of the living cell (water, proteins and K^+) are closely associated; together they exist in a high- (negative)-energy-low entropy state called the **living state**. The bulk of cell water is adsorbed as polarized multilayers on some fully extended protein chains, and K^+ is adsorbed singly on β - and γ -carboxyl groups carried on aspartic and glutamic residues of cell proteins. Extensive evidence in support of the AI hypothesis is reviewed. From an extension of the basic concepts of the AI hypothesis and the new knowledge on primary structure of the proteins, one begins to understand at long last what distinguishes gelatin from other proteins; in this new light, new definitions of protoplasm and of colloid chemistry have been introduced. With the return of the concept of protoplasm, living structure takes on renewed significance, linking cell anatomy to cell physiology. Finally, evidence is presented showing that electron microscopists have come close to seeing cell structure in its **living state**.

Key Words: α -actinin, actin, adsorption, association- induction hypothesis, ATP, β - and γ -carboxyl groups, colloid, cryoprotection, gelatin, glycerol, K^+ ions, living state, living structure, membrane-pump theory, multilayer adsorption, protein conformation, protoplasm, q-value, water.

Historical Background

MANKIND IS now poised on the threshold of revealing the ultimate structure of matter, seeing the beginning of time, and documenting every gene that encodes a human being. Yet, we know little about what has made all these achievements possible: **life**. This lopsided understanding of Nature is not accidental. The path to knowledge has always been smoother in exploring the unexplored, than in rethinking the familiar.

From before Aristotle, there have been two divergent schools of thought on what **life** is: life as *organization* and life as *emergent action* (see Hall, 1969a). Both views were addressed to life at the organism level. That all living organisms are constructed of microscopic units of *cells* was not recognized until the middle of the 19th century.

Between 1835 and 1840, two landmark events took place: Felix Dujardin's description of "sarcode" (Dujardin, 1835); Theodor Schwann's announcement of the "cell theory" (Schwann, 1839). These events marked the beginning of another pair of divergent thoughts on the nature of life. These ideas were related to the two earlier schools of thoughts on life, though until now, not recognized as such.

Dujardin named the substance which he obtained from protozoa (Infusoria) **sarcode**, and described it as "pulpy, homogeneous, gelatinous, without visible structure and yet organized..." (Dujardin, 1838). Strongly emphasizing the gelatinous nature of sarcode, he also referred to it as the "living jelly". Sarcode was later renamed "protoplasm", after Max Schultze (1863) and others had demonstrated that Dujardin's sarcode isolated from protozoa and von Mohl's "protoplasm" (1846) isolated from higher plants and animals are similar.

Seeking to provide a physicochemical basis for inquiries into the nature of the "living jelly", Thomas Graham (1861) introduced the term, "colloid" from the Greek word *κόλλα*, meaning glue or gelatin. With the coining of this new word, Graham launched a new branch of chemistry, the colloid chemistry.

For well over half of a century after Dujardin's discovery, protoplasm continued to inspire splendid optimism on the future study of life. Thus in 1853, Thomas Huxley described protoplasm as the "*physical basis of life*" (Huxley, 1853). Half a century later, Locy (1908) hailed the recognition of the living substance, protoplasm, as the "beginning of modern biology". However, not long after Locy's pronouncement, history began to steer away from the course, which he, Huxley and others had so vividly foreseen. The colloidal approach to cell physiology continued to develop for a while in the 1920's. Then abruptly it receded into the background, as chronicled by the termination of the English-language Journal of Colloidal Chemistry.

The alternative theory of the living cell with emphasis on the cell membrane assumed dominance. Its first champion was no other than Theodor Schwann, even though the plant physiologist, Wilhelm Pfeffer (1877) was traditionally given this credit. Schwann regarded the inside of cells as essentially that of a homogeneous transparent liquid. He attached great importance to an enclosing cell membrane, an anatomical structure which, at that time, neither he nor anyone else could have seen. To this as yet invisible structure, Schwann imputed "metabolic power" to manipulate chemically the fluid substances inside (and outside) the cell (see Hall, 1969b, p. 194). Schwann's idea of the living cell with a metabolically powered cell membrane foreshadowed the concept of the living cell as taught in most textbooks today under the title of the membrane-pump theory. According

to this theory, the chemical composition of living cells reflects the continual, energy-consuming activities of postulated pumps located in the cell membrane. The three major components of the living cell — water, proteins and K^+ — are separate and independent from each other. That is, water in the cell is normal liquid water. Cell K^+ (and other ions and solutes) are also free like those found in dilute aqueous solutions. And cell proteins exist in the same conformation as that which they assume *in vitro* when purified, dissolved in a dilute buffer solution and referred to as native (for the origin and meaning in current usage of the term “native protein”, see Addendum at end of paper).

Having accepted these basic tenets of the membrane-pump theory, some biologists began to repudiate the existence of protoplasm.

If there is no “living substance” or protoplasm, then the best an electron microscopist can hope to see are mechanical parts that are never alive to begin with. It is amazing how close this seemingly new view is to the view of George E. Stahl (1659–1734) expressed two centuries ago: “God had created for man’s salvation a body and an ‘anima’ or soul” (Rothschuh, 1973, p. 121). The bodily machines are passive instruments of this anima.

Can the postulated membrane pumps be the anima or soul? I doubt that even the most ardent proponent of the membrane-pump theory would agree to that. If the membrane pump is not the anima, then “Is there anything alive at all in a cell?”, “If all its parts are not alive, then is the cell alive?” If the cells are not alive, and yet you and I are alive, then the essence of life must reside elsewhere than our bodily parts, all made of cells. This is precisely George E. Stahl’s position.

Was Stahl right after all? Are dead mechanical parts truly the best electron microscopist can hope to see? A more definitive answer will be given in the last section of this paper. For the moment, I may say that the answer depends on the validity of the membrane-pump theory. After all, it is this theory that underlies and gives credence to the belief that there is no protoplasm. Judging by what are at this very moment being taught to students at all levels, what researches are being supported, what subject matters appear regularly in the wide-circulating journals, an innocent observer would be hard put not to believe that the membrane-pump theory had been unequivocally established a long time ago. In fact, nothing could be further from the truth.

To substantiate my opinion on this serious issue — i.e., that the currently accepted and widely taught concept of the living cell is wrong and is misleading biomedical research — I will begin by asking and answering the question: “What has caused the majority of biologists in the 1930’s to abandon so precipitously the once highly cherished protoplasmic approach to cell physiology?”

Physiology is based on physics. The late 19th century and early 20th century saw rapid progress in the development of physics of dilute solutions. This progress augmented the standing as well as the membership of supporters of the membrane-(pump)-theory, which is in fact a dilute-solution theory of the living cell.

In contrast, the physics required to explain the more complex colloidal phenomena was either not yet in existence or otherwise beyond the reach of cell physiologists at that time. As a result, there were few colloid chemists. And, in spite of valiant efforts, they did not have the essential information and needed “tools” to uncover the hidden attributes of colloids. For an example, they had not clearly stated what distinguishes colloids from non-colloids. In hindsight, one sees that by overemphasizing the importance of the (larger) size of colloid materials, colloid chemists contributed to the loss of identity and the rejection

of colloidal chemistry, with the emergence of macromolecular chemistry, which really is a chemistry of large molecules but not colloid chemistry.

There was also a more immediate and more conspicuous cause for the rejection of the colloidal approach, i.e., a set of decisive experimental evidence against the colloidal theory of cell physiology but in favor of the membrane pump theory. Or so it seemed.

Colloidally-oriented cell physiologists like Benjamin Moore, Martin Fisher (1979–1962) and Ross A. Gortner (1885–1942) believed that a substantial portion of cell water and cell K^+ is not free but in some way bound. Gortner and his coworkers showed in model systems (especially gelatin gel) that part of the water does not dissolve sucrose and is thus *nonsolvent*. Others believed that bound water does not freeze at -20°C , and is thus *non-freezing* (for review see Gortner, 1938).

A.V. Hill (1886–1977) was a Nobel laureate and a towering, commanding figure in his time. Hill argued that if nonsolvent water exists in frog muscle cells, the equilibrium level of a solute like urea in the cell water should be lower than that in the external medium. Yet P. Eggleton and H. V. Horton provided Hill with the data showing equal distribution of urea between muscle-cell water and the external medium. Hill concluded that there is no nonsolvent water in frog muscle cells and all cell water is free (Hill, 1930). MacLeod and Ponder (1935) demonstrated equal partition of ethylene glycol between erythrocyte water and the external medium, affirming and extending Hill's conclusion that all cell water is normal liquid water.

Hill and Kupalov (1930) also showed that frog muscle is in vapor equilibrium with an isotonic NaCl solution. Since all water in frog muscle cells is free liquid water — as the urea distribution data had proved to Hill's satisfaction — nor was there any doubt that Na^+ and Cl^- as free ions in an isotonic solution, Hill and Kupalov further concluded that cell K^+ [and its anionic counterpart(s)] which alone can match the external Na^+ and Cl^- in concentrations, must also be free.

Complementing Hill's conclusion on cell water, Blanchard (1940) questioned the existence of "bound water" anywhere. He called attention to the fact that large volumes of pure water can be supercooled to -21°C . Therefore the presence of water in model systems that does not freeze at -20°C does not prove the existence of bound water. He also argued that the presence of high concentrations of proteins in biological materials mechanically blocks the formation and propagation of ice crystals. This mechanical blocking effect renders freezing-point lowering seen in living systems dubious evidence for the presence of bound water. However, Blanchard, for unexplained reasons, did not mention the important experimental observation of Moran (1926), who showed that water in 65% gelatin gel does not freeze at liquid air temperature (somewhere between -182.96°C and -195.8°C). (Yet Blanchard did cite this particular publication of Moran as Reference 39 in his list of references). No normal liquid water can stay unfrozen at liquid-air temperature (see also, Ling and Zhang, 1983; Ling, 1988b).

Ernst lived long enough to testify some 30 years later how it was Hill's findings that brought about the dramatic, and sweeping abandonment of the colloidal approach to cell physiology in favor of the membrane (pump) theory (Ernst, 1963, p. 112).

New experimental data to be described below in the Section on "Cell Water" show that the concept of water that has no solubility for *any* solutes (i.e., categorically "non-solvent") is faulty. And it was this faulty part of the idea of bound water that Hill, MacLeod and Ponder had truly disproved. As will be also made clear below, *their* findings did not prove that water in living cells is normal liquid water, even though Hill ap-

parently convinced himself and many of his contemporary biologists that they did. Without establishing that cell water is truly normal liquid water, Hill and Kupalov's argument that cell K^+ is free, falls apart.

Thus, in hindsight, the abandonment of the protoplasmic approach to cell physiology was not justified, even though it might have appeared so at the time. I now summarize the major evidence, which contradicts the membrane-pump theory and its postulates of free water and free K^+ .

Disproof of the Membrane-Pump Theory

Most living cells contain a high concentration of K^+ and a low concentration of Na^+ , even though these cells spend their lives in a medium rich in Na^+ and poor in K^+ . This asymmetrical distribution of a pair of highly similar solutes reflects a fundamental attribute of all living cell. Not surprisingly, all theories of the living cell thus far proposed have been formulated with this fact in mind.

As mentioned earlier, the founder of the Cell Theory, Theodor Schwann, had envisaged a key role of the cell membrane in determining the chemical composition of the cell. However, the membrane theory that had evolved after Schwann and that had culminated in the theory of Boyle and Conway (1941), did not invoke pumps. Instead, it relied on a postulated sieve-like membrane to explain the abundance of K^+ and scarcity of Na^+ in the cell. In this version of the membrane theory, solutes fall into two classes: Those that are too large to pass through postulated rigid membrane pores are impermeant and stay permanently outside the cell. Na^+ belongs to this category. Other solutes that are small enough to traverse the narrow pores are permeant and accumulate in the cell. K^+ belongs to this category. With the advent of the radioactive-tracer technology, Na^+ and a host of other supposedly impermeant solutes were shown to be permeant, invalidating the central theme of the Boyle-Conway theory (Cohn and Cohn, 1939; Heppel, 1939; Ling 1992, Sect. 9.1.2.). Gradually the Na-pump theory was adopted.

It should be pointed out that, then and now, the Na pump theory offered no molecular mechanism for its main function, i.e., Na pumping (Ling, 1992, Section 2.1); then and now, the Na pump theory is *ad hoc*. That is, the Na-pump theory is not part of a general theory explaining the distribution pattern of *all* solutes in the cells. Instead, the pumping of Na^+ has been treated as a separate and independent event. This "independent" approach, starting with the inauguration of the Na pump, has been followed by advocates of other pumps as well.

Is there enough energy to operate the Na pump and other needed pumps?

The answer is, No.

In 1962, I presented the results of a three-year-long inquiry into the feasibility of the postulated Na pump from a thermodynamic standpoint (Ling 1962, p. 189–212). In this study I compared the minimum energy need of the postulated Na pump in frog muscle cells for a recorded period of time, with the maximum energy available to these cells during the same period of time. Frog muscle's remarkable ability to withstand total interruption of its active metabolism made clear-cut answer within reach (see Appendix 1 at the end of this article for additional description of this study).

The last three sets of fully-completed experiments showed that *the minimum energy need of the postulated Na pump is from 1500% to 3000% of the maximum available energy* (Six other sets of fully completed experiments produced even larger discrepancies).

Disparities of this magnitude are decisive by any standard. Yet they do not come close to accurately representing the even greater true disparity. For in my calculations described above, I assumed that ATP, ADP and CrP all carry large amounts of utilizable free energy as it was once widely believed (ATP, -29.3 kcal/mole; ADP, -15.0 kcal/mole; CrP -12.8 kcal/mole). However, the work of Podolsky and Morales (1956) and of George and Rutman (1960) had left little doubt that there is no such readily available free energy trapped in these phosphate bonds. Since most of the maximum available energy I computed came from these sources, the actually available energy in the poisoned muscle was considerably smaller, i.e., *by another factor of no less than 500 times*. Yet this enormous energy imbalance comes from just one postulated pump.

A survey of the distribution of other solutes in frog muscle and other cells revealed that virtually all solutes found in the cells require pumps (Ling, 1992, Sect. 2.2.5). Nor are solutes requiring pumps limited to those found in the cells and in their natural environments. They must also include other solutes studied, that were for the first time synthesized by organic chemists. These man-created chemicals could not have been in contact with the cells' ancestral genomes; it would be difficult to envisage how new genes encoding the required pumps could have developed anticipating the future creation of the new chemicals.

Pumps are required for all these natural and artificial solutes not just at the plasma membrane. They are also required, as a rule, at the membranes of various subcellular particles. Just to mention one, the sarcoplasmic reticulum (SR) of voluntary muscle has been estimated to have a total surface (membrane) area 50 times bigger than that of the plasma membrane. Since under otherwise identical conditions, the energy need of a pump is directly proportional to the surface area of the membrane; the same pump located in a similar membrane and operating under the same condition, would require 50 times more energy at the SR membrane than at the plasma membrane (Ling, 1992, Sect. 2.2.6.).

Since the work described above was published 50 years ago, there has been no challenge in print against the experiments I performed and described, nor the conclusion I reached (however, see Ling, 1988a, discussion with I. Zs.-Nagy, on p. 911–913). In the meanwhile, the essence of my finding has been twice confirmed (Jones, 1965; Minkoff and Damadian, 1973).

Overwhelming as the evidence against the pump concept is from energy consideration, it is by no means the only evidence against the membrane-pump theory- and its specific postulates of free water, free ions and native proteins in living cells. Partly due to its relevance to subjects to be discussed below, one more set of evidence will be presented; this set of evidence shows that the (postulated) membrane pumping is not the mechanism living cells employ in maintaining their asymmetrical K^+/Na^+ distribution

Is a functional membrane pump needed for the selective accumulation of K^+ and exclusion of Na^+ ? The answer is also No.

A frog sartorius muscle consists of about 1000 elongated muscle cells, each running all the way from one end of the muscle to the other end. A razor-blade cut across the muscle away from its tapering tibial end exposes the cytoplasm of every cell and is not

followed by membrane regeneration as revealed by electron microscopy, persistent high permeability to sucrose through the cut end etc. (Ling, 1978; Cameron, 1988; Edelmann, 1989).

If the cut end of the muscle alone is exposed to a Ringer's solution containing labelled K^+ and Na^+ , while the remaining intact portion of the muscle is suspended in air or vaseline as shown in Figure 1, one obtains what is called an *effectively membrane-pump-less-open-ended cell* or EMOC preparation (Ling, 1978).

The loss of pump functions in an EMOC preparation follows from the fact that the (postulated) pumps in the intact part of the cells cannot function: the surrounding air (or vaseline) is not a source of K^+ for the inward K pump, nor can it function as a *sink* to receive Na^+ for the postulated outward Na pump.

Figure 2 shows that despite the absence of functional membrane pumps, the uptake of labelled K^+ and of labelled Na^+ in the part of the muscle cells away from their cut ends appeared quite normal. That is, K^+ and Na^+ distribution here follows a pattern essentially the same as that observed when entire *intact* and normal muscles were incubated for a shorter period of time in a Ringer's solution containing similar radioactively labelled isotopes: uptake of labelled K^+ to levels higher than in the surrounding medium and uptake of labelled Na^+ to levels consistently below that in the external medium.

It was shown that the drug ouabain, long accepted by proponents of the membrane-pump theory as a specific inhibitor of the postulated Na pump, functions quite normally in an EMOC preparation. Added to the Ringer's solution bathing the cut end, it depressed the uptake of labelled K^+ and it raised the uptake of labelled Na^+ , even though there is no functional membrane pump.

Precaution was taken against the possibility that Na^+ might be pumped from the cells into the extracellular space and returns thence via that same space to the Ringer's

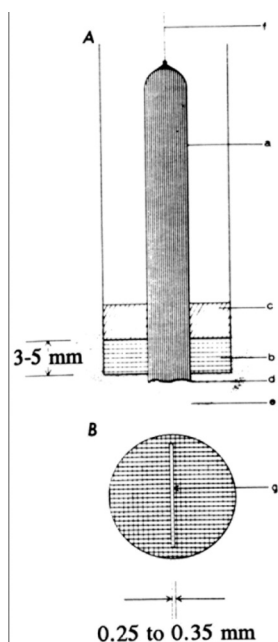


FIGURE 1. Diagram of the EMOC preparation. A. side view. B. bottom view. Only the cut end of the muscle is in direct contact with the labelled Ringer's solution, a. sartorius muscle; b, silicone rubber gasket; c, vaseline; d, cut end of muscle; e, bathing solution; f, anchoring string; g, slit in silicone rubber gasket. (From Ling, 1978).

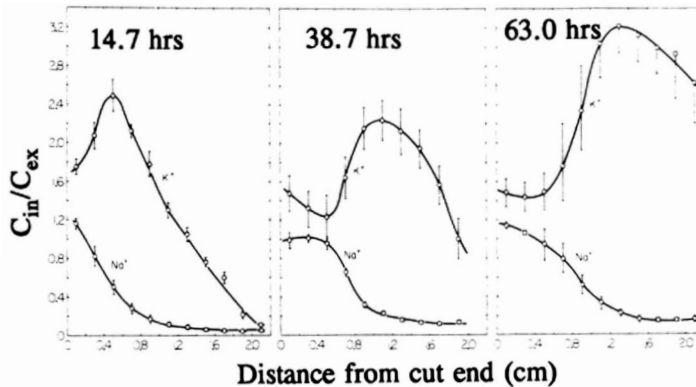


FIGURE 2. The simultaneous influx of labelled K^+ and labelled Na^+ into sartorius muscle through their cut ends. The three groups of frog sartorius EMOC preparations were exposed to normal Ringer's solutions labelled with both ^{42}K and ^{22}Na for 14.7, 38.7 and 63.0 hr, respectively. The abscissa represents the distance of the mid-point of each cut segment from the cut surface of the muscle fibers. The ordinate represents the ratio of the labelled ion concentrations in the water of each muscle segments (C_{in}) over the concentration of the same labelled ion in the solution bathing the cut end of the muscles at the conclusion of the experiment (C_{ex}). Each point was the average of 4 (14.7 hr), 10 (38.7 hr) and 4 (63.0 hr) experiments, respectively, the distance between the two longitudinal bars being twice the standard error (from Ling, 1978).

solution bathing the cut end of the muscle: the silicone-rubber gasket (b) shown in Figure 1 hugged the muscle so snugly that the total cross-sectional area of the extracellular space at that location was reduced from its normal size (9%) to nearly one-tenth that size (1%). However, I discovered later that this precaution was unnecessary.

There is no pump propelling Na^+ to move in a fixed direction within the extracellular space — from common sense and as witnessed by the fact that the cut could be applied to either the tibial or the pelvic end with similar result. Therefore, backward transport of labelled Na^+ into the Ringer's solution bathing the cut end must rely on the development of a diffusion *head*. A careful calculation shows that in order to move enough Na^+ via the extracellular space to explain the observed difference between K^+ and Na^+ accumulated, the concentration of labelled Na^+ in the extracellular space must be as high as 5 M in order to provide the necessary diffusion head. To test if such a high concentration of labelled Na^+ in fact existed, the fluid in the extracellular space of an EMOC preparation after 50 to 53 hours of incubation was collected and analyzed. The concentration of labelled Na^+ in the collected extracellular fluid was found to be 95.5 ± 0.3 mM, which was essentially the same as the concentration in the Ringer's solution (100 mM) bathing the cut end of the muscle. Therefore, the required diffusion head of Na^+ between the extracellular space and the Ringer's solution bathing the cut end did not really exist.

Indeed, there is no indication that these postulated pumps — if for argument's sake, one assumes they existed — had pumped any Na^+ into the extracellular space at all. Were it otherwise, the excess Na^+ (and accompanying anion(s)) thus accumulated in the extracellular space beyond its normal 100 mM level would have raised the osmotic activity in the extracellular space beyond that in the cell. As a result, water would have been drawn from the cells into the extracellular space, thereby increasing the weight percentage of the extracellular-space fluid. In fact, at the conclusion of 50-53 hours of incubation, the

percentage of the extracellular-space fluid measured $9.4\% \pm 0.72\%$, which is within the range of values obtained from normal frog sartorius muscles (8% to 10%) (Ling and Kromash, 1967; Ling and Walton, 1975).

Concluding the second set of evidence against the membrane-pump theory, I want to mention that a more complete set of the evidence can be obtained from my earlier volume (Ling, 1984) and especially from my book, “A Revolution in the Physiology of the Living Cell” (Ling, 1992), published by R. E. Krieger (Melbourne, FL, 32902-9542).

Two New Theories of Living Cells

I have shown above why the selective accumulation and exclusion of solutes from living cells is neither due to absolute membrane permeability (or impermeability), nor due to membrane pumps. The year 1951 saw the introduction of two new theories: the late A. S. Troshin’s *sorption theory* and the early version of my association-induction hypothesis then known as Ling’s *fixed charge hypothesis*. Though undeclared, and perhaps even unrecognized at the time, the introduction of both theories represented in fact a return to the protoplasm-oriented cell physiology of the 1920’s and 1930’s.

Troshin’s sorption theory

From his studies of the distribution in living cells and model systems, of alanine, creatine, galactose and other nonelectrolytes, Soviet biologist A. S. Troshin suggested that each solute in living cells may exist in two forms: dissolved in the cell water or adsorbed or otherwise complexed to macromolecules (Troshin, 1951, 1952; for review, see Troshin, 1958, 1966). The fraction in the cell water may be lower than that in the external medium because cell water resembles water in model “coacervates” — a complex colloid-rich material (containing as little as 50% water and as much as 85% water), which stays as a distinct phase separate from the surrounding colloid-poor aqueous solution. Troshin cited the earlier work of Hollemann *et al.* (1934) who showed that coacervates prepared from gelatin and resorcinol contain at equilibrium less solutes like Na_2SO_4 than in the surrounding medium. Why water in coacervates accommodates less Na_2SO_4 was not explained by Hollemann *et al.*, nor by Troshin.

In 1958 Troshin applied his theory to the distribution of ions in living cells, including K^+ and Na^+ (Troshin, 1958, p. 158). He had not addressed himself to the question why K^+ is selectively adsorbed while the closely similar Na^+ is not, a question that had become increasingly my preoccupation from the late 1940’s on.

The early association-induction (AI) hypothesis, known as Ling’s fixed charge (LFC) hypothesis

In 1951 and 1952, I presented what was called Ling’s Fixed-charge (LFC) hypothesis, the primordial version of the AI hypothesis (Ling 1951, 1952). In the LFC hypothesis (as it is in the AI hypothesis), the abundance of K^+ in living cells is due to the more favorable electrostatic interaction of this positively-charged ion with negatively-charged fixed anions, and the preferable adsorption of K^+ on these fixed anions occurring in consequence. The most important fixed anions in the cells are β - and γ -carboxyl groups carried respectively on the side chains of aspartic-acid and glutamic-acid residues of cellular proteins.

Fixation of these anions (on the proteins) enhances the association of these fixed anions with monovalent cations like K^+ and Na^+ (Ling, 1990; 1992, Section 4.1). The resultant long-lasting, *close-contact*, one-ion — one-site adsorption holds the key to the preferential accumulation of K^+ over Na^+ — because K^+ and Na^+ differ from each other only in short-range attributes, which like say, the texture of a piece of fabric, can only be felt and recognized through *close contact*.

Figure 3 reproduces the theoretical model I presented in 1952. It was pointed out that K^+ and Na^+ exist as hydrated ions in water and hydrated K^+ is smaller than hydrated Na^+ .

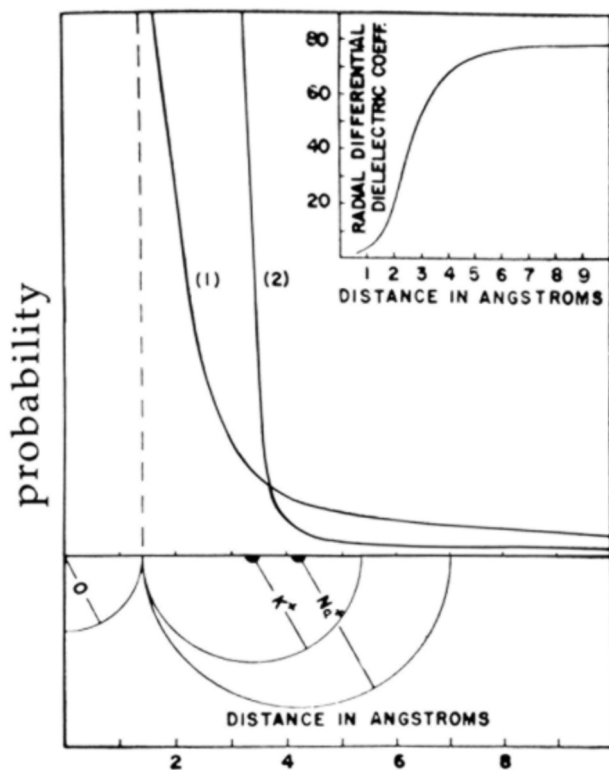


FIGURE 3. Theoretical model for the selective adsorption of K^+ over Na^+ on fixed anionic sites.

The main figure (2) shows how the difference in the smaller hydrated diameter of the K^+ than the larger hydrated diameter of the Na^+ illustrated in the bottom, and the phenomenon of rapidly decreasing dielectric constant in the immediate vicinity of ions, combine to provide a much higher statistical probability (ordinate) that the fixed anion is adsorbing the smaller hydrated K^+ than the larger hydrated Na^+ . This follows from the fact that only the smaller ion is capable of taking advantage of the favorable probability in the close vicinity of the fixed anion; the larger one is left out.

Abscissa represents the distance in Ångstrom units from the center of singly charged oxygen atom of the fixed oxyacid group.

Half circles in bottom figure illustrate the diameters of the hydrated K^+ and Na^+ ions. Top inset shows the sharp decline of the radial dielectric constant of water as one approaches closely the center of an ion (dielectric saturation). Curve (1) shows another probability curve if the phenomenon of dielectric saturation is ignored. (From Ling, 1952).

Furthermore, water molecules in the hydration shells of these ions are intensely polarized by the electric charges of the ions and are thus *dielectrically saturated* (i.e., its dielectric constant is close to unity rather than the much higher value for normal bulk phase liquid water, 81) (Debye and Pauling, 1925). Based on these facts, I computed a 10 times higher statistical probability of a fixed anion adsorbing a K^+ ion than a Na^+ ion. This higher probability of the fixed anion associating with K^+ predicts selective accumulation of K^+ (over Na^+) in living cells as observed.

Not long after the introduction of Ling's fixed charge hypothesis, it became clear that a selective mechanism that depends on an *unchanging* set of hydrated ionic diameters and hence an unvarying selectivity rank order, correctly describes the behavior of *some* fixed-charge-systems but not of others. Thus the sulfonate type of ion exchange resin, like many living cells, selectively accumulates K^+ over Na^+ (in agreement with the LFC hypothesis); the carboxylate type of exchange resins, on the other hand, actually selectively accumulates Na^+ over K^+ (in disagreement with the LFC hypothesis). Partly in response to the need to provide a model capable of explaining both sets of observations, I began to look for a more fundamental solution to the problem and this effort eventually led to the development of a general theory of the living cell, or more specifically, *a physical theory of the living state, called the association-induction hypothesis* (Ling, 1962).

Early in this endeavor, I realized that I must address myself to an old question that has, as pointed out earlier, interested philosophers and scientists from ancient times: "What is the meaning of being alive?"

The Concept of the "Living State" According to the Association-Induction Hypothesis

The raising of a human baby from an early embryo once kept in liquid nitrogen, made dramatic news. Though little noticed in this context, this event had perhaps a greater and more lasting significance for its impact on mankind's unending search for the meaning of life: *for this event has in fact provided a powerful evidence against the philosophical view of life-as-emergent-action*, including growth, metabolism, motion, reproduction (and membrane pumping). All these emergent actions must come to a stop at a near 0°K temperature. Since continued activity is life; discontinued activity at the near 0°K temperature could only mean death — a term, by definition, irreversible. That being the case, it seems far more reasonable to abandon this man-made emergentist theory, than to postulate that a *miraculous* resurrection occurs each time a frozen human embryo or a frozen red blood cell is warmed up to resume physiological activity again.

In contrast, the success in raising a human baby from a once-frozen embryo supports the concept of the *living state*. Introduced as a part of the association-induction (AI) hypothesis in 1962, the concept of the living state is entirely new. Nonetheless, it may be regarded as an offshoot of the philosophical school of thought of *life as organization*. Next, I will introduce the concept of the living state with a simple model and then discuss the fundamental difference between the life-as-organization and the living state.

Consider a number of soft iron nails, joined end-to-end with bits of string (Figure 4A). Their distribution is random and they do not interact with the iron filings scattered among them. If now a strong magnet is brought into contact with the free end of one of

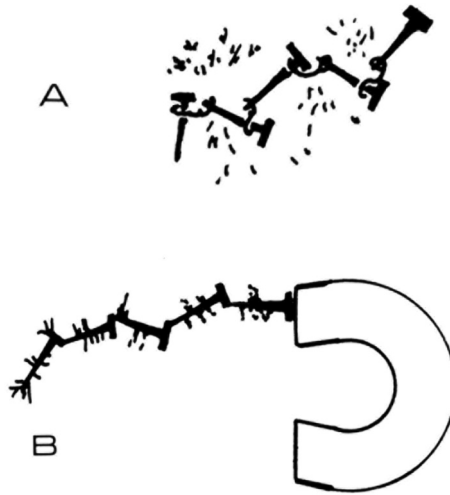


FIGURE 4. A chain of soft iron nails joined end to end with pieces of string is randomly arrayed and does not interact with the surrounding iron filings. The approach of magnet causes propagated alignment of the nails and interactions with the iron filings. (From Ling, 1969).

the terminal nails, the nail will be magnetically polarized. This magnetized nail will in turn polarize the nail next to it and the process may repeat a number of steps further. As a result, not only do the nails assume a less flexible and less random distribution, the iron filings are also magnetized, associate with the nails, and assume a more ordered pattern of distribution (Figure 4B).

Taken as a whole, the nail-chain-iron-filing system has shifted from its original low (negative) energy-high entropy state before the introduction of the big magnet to a high (negative) energy, and low entropy state after the interaction. (Entropy is, of course, a parameter measuring the randomness of a system, see Ling 1992, Chapter 3, Endnote 2 for a succinct discussion).

As mentioned above, the three major components of all living cells are water, proteins and K^+ . In the AI hypothesis, the cell proteins are like the tethered nails; water and K^+ are like the iron filings. Water, K^+ and proteins are all in close association, and thus diametrically opposite to the basic tenets of free K^+ , free water in the membrane-pump theory.

The equivalent of the big magnet is a class of small but powerful molecules called the *cardinal adsorbents*. Cardinal adsorbents include many drugs, hormones, Ca^{2+} , transmitters. Not all cardinal adsorbents are required in maintaining the living state, but of those that do, by far the most important is ATP. Whereas in the magnet-nail model, interaction is magnetic polarization; in their living counterpart, it is electrical polarization, or **induction**.

Being alive, according to the association-induction hypothesis, signifies a specific condition or state, called the living state. In this living state, the major components of the living cells (water, proteins and K^+) and other minor but critical components (e.g., ATP) are closely associated in a properly organized manner. The closely-associated and properly-

organized protein-water- K^+ -ATP systems in cells exist at a high- (negative)- energy-low-entropy condition. It is the maintenance of this high (negative)-energy — low entropy state that distinguishes the living state from the concept of life-as-organization (only). As an equilibrium phenomenon, the maintenance of the living state requires no energy expenditure and is compatible with ambient as well as absolute zero temperature.

Indeed, the title of my 1962 monograph, “A Physical Theory of the Living State: the *Association-induction Hypothesis*” (Ling, 1962) introduces the term “living state”, and sets it apart from its plebeian usage. The living state as defined in the AI hypothesis is uniquely an embodiment of this hypothesis because the living state originates from the full *association* of all the critical components of the cell (water, proteins, K^+ , ATP) and the *inductive* interaction among them.

Figure 5 shows a circular, submicroscopic portion of a living cell. Note that the bulk of cell water molecules are adsorbed in multilayers on the fully-extended polypeptide chains of some cell proteins. Virtually all the cell K^+ is adsorbed singly on β - and γ -carboxyl groups carried respectively on aspartic and glutamic acid residues. In the cell water, both Na^+ and K^+ exist at concentrations considerably lower than in the extracellular bathing medium.

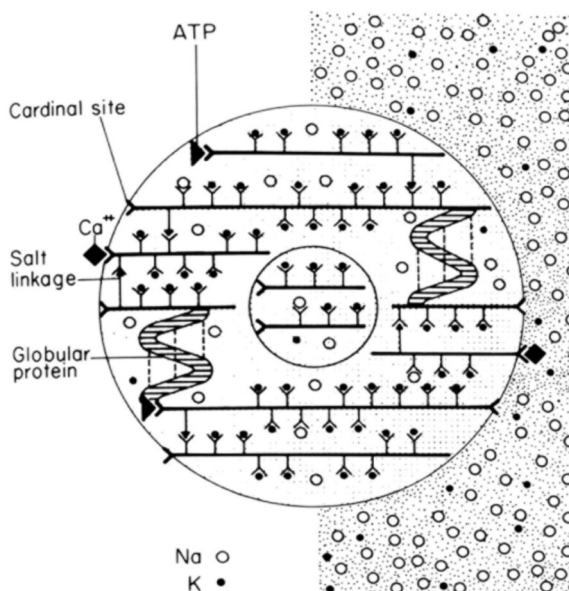


FIGURE 5. Diagrammatic illustration of a submicroscopic portion of the cell substance, showing the interaction among the three most abundant components of the living cell: water, proteins and K^+ and cardinal adsorbents, Ca^{2+} and ATP. Selective accumulation occurs as a result of the preferential adsorption on β - and γ -carboxyl groups of cell proteins. Na^+ exclusion results partially from the failure to compete against K^+ for the β - and γ -carboxyl groups and partially from the exclusion from the bulk-phase cell water which assumes the dynamic structure of polarized multilayers in consequence of interaction with fully-extended protein chains present pervasively throughout the cells. (From Ling, 1969).

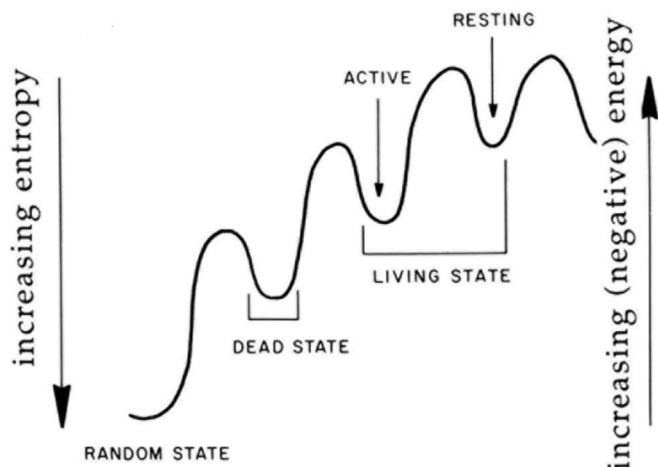


FIGURE 6. Diagrammatic illustration of the (negative) energy and entropy profile of the living state, the dead state and the ultimate random state. Diagram also indicates the existence of a resting living state and an active living state (From Ling, 1992).

Figure 6 illustrates the energy and entropy relationship between the living state and the death state as well as the ultimate random state. Of importance in interpreting functional activities of living cells is the *active living state*, which is a metastable state in between the *resting living state* and the death state.

With the living state defined, *protoplasm can be redefined as the generic name for the different types of closely associated system of protein-water- K^+ -ATP and other unspecified but essential minor elements maintained at the high (negative)-energy — low-entropy living state.*

Thus after a long estrangement, we are renewing our acquaintance with the concept of “protoplasm”, which had at one time inspired great vision and eloquence.

The living state pertains to the intact living cell, as well as to its constituent parts, including membranes, mitochondria as well as gelatinous material sometimes called cytosol. In the definition of protoplasm given by Dujardin, von Mohl and Max Schultze, protoplasm was limited to the gelatinous cytosol; in the present definition, protoplasm extends to other components and structures of the living cell as well. With this broader definition established, an old question reawakens: “How far downward life extends in the direction of increasing smallness.” (Lovejoy, 1936, p. 237). I will try to answer this question in the Section on “ATP functions as a cardinal absorbent” below.

Cell Water

Water is by far the largest component of protoplasm; yet in the context of the membrane pump theory it has been reduced to merely a simple solvent. I will in the five following subsections discuss how *association* and *induction* among water molecules contribute to the creation and maintenance of the living state in cells and their constituent parts. I begin

with the polarized multilayer theory of cell water introduced in 1965, completing the association-induction hypothesis (Ling, 1965a). This will be followed by summaries of the results of extensive testing of this theory. When all this is done, I will repeat once more the same sequence of theory first followed by results of experimental testing, but this time it will be on the second largest non-protein component of the cell, K^+ .

The polarized multilayer theory of cell water

According to the *polarized multilayers (PM) theory of cell water*, all or virtually all water molecules in a living cell participate in the dynamic structure of polarized multilayers for the following reasons.

The formula H_2O is a better representation than say HOH for water, because the two positively charged H atoms of the “real life” water molecule are not symmetrically located on each side of the oxygen atom but are located on the two corners of a tetrahedron with the oxygen atom occupying its center. Located at the other two corners of the tetrahedron are the oxygen atom’s two negatively charged “lone pairs” of electrons. This asymmetry provides the water molecule with a large permanent dipole moment (equal to 1.86 debyes).

The large permanent dipole moment, in conjunction with a sizable *polarizability*, (equal to $1.444 \times 10^{-24} \text{ cm}^3$) (i.e., the propensity to develop an additional induced dipole in an electric field) enables water molecules to interact strongly with, and adsorbs onto both positively charged (P) sites and negatively charged (N) sites on solid surfaces. Each water molecule thus polarized and adsorbed on an N or P site in turn polarizes and adsorbs another water molecule and the process repeats itself a number of steps further. Electrical polarization, also called *induction*, thus brings about the *association* of water molecules in the cells.

Now if alternating P and N sites are arranged at suitable distance apart like a checker-board (an NP system), or if two such NP surfaces are face-to-face in close juxtaposition (an NP-NP system) or if alternating N and P sites are carried on linear chains among a matrix of chains (an NP-NP-NP system), the interaction with and among the water molecules will be intensified. Here, individual water molecules in immediately neighboring rows of polarized water molecules would be oriented in opposite directions. Since oppositely oriented dipoles attract each other, this lateral attraction between adjacent adsorbed water molecules in neighboring rows further stabilizes the water-molecule — to — water-molecule interaction. Thus the interplay of induction and association can produce a stable and yet highly flexible, three-dimensional dynamic structure of polarized multilayers of water molecule (Figure 7). That molecules with permanent dipole moments like water form polarized multilayers on appropriate polar surfaces was not my invention. That was physics and it was history. Indeed, theoretical physicists like de Boer and Zwicker (1929) and Bradley (1936), had long ago derived rigorous equations describing multilayer adsorption of polar gases on appropriately charged surfaces. Bradley’s multilayer adsorption isotherm has since then received both theoretical (see Brunauer *et al.*, 1938) and repeated experimental confirmations (e.g., Hoover and Mellon, 1950; Ling and Negendank, 1970, Ling, 1984, pp. 288–289). On the other hand, that the bulk of *cell water exists in the dynamic structure of polarized multilayers* is entirely my suggestion (Ling, 1965a).

According to the PM theory, the bulk of cell water assumes the dynamic structure of polarized multilayers. Some cell proteins existing in the fully-extended conformation with their alternating positively-charged NH sites and negatively-charged CO sites function as

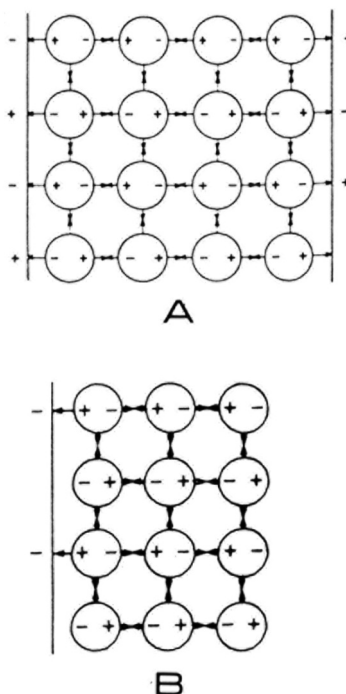


FIGURE 7. Diagrammatic illustration of how arrays of alternately positively and negatively charged fixed sites on a matrix of linear chains or juxtaposed surfaces (A), or of alternately negatively charged and neutral sites (B) can produce the dynamic structure of polarized multilayers of water molecules. A stable three-dimensional cooperatively linked assembly of water molecules follow from charge-dipole interaction with the fixed charged sites and from lateral interaction with oppositely oriented water molecules. (From Ling, 1989).

NP-NP-NP systems; and, as such, are directly exposed to the bulk-phase water, polarizing it in multilayers. Some years ago I gave reason why actin might be a major candidate for this role (Ling 1979a, p. 47; for very exciting new information on this issue, see answer to last question raised by Dr. Clegg in the Discussion with Reviewers at the end of this article).

Direct experimental confirmation that multilayers of water molecules are polarized and adsorbed on what I call a simple NP system was achieved by Harkins (1945). He demonstrated no less than five layers of water molecules are adsorbed on the surface of titanium dioxide crystals. Each additional layer of water molecules is adsorbed less strongly than the preceding one.

The study of water-vapor adsorption in model NP-NP-NP systems and living cells (Ling and Hu, 1988, pp. 267–268; Ling and Negendank, 1970; Ling and Ochsenfeld, 1989, pp. 39–40) have confirmed the essence of Harkins's findings. In gelatin gel, for example, no less than twelve layers of water is adsorbed and polarized between adjacent gelatin chains.

If water assumes the dynamic structure of polarized multilayers, its physicochemical properties may be expected to differ from normal liquid water. The first property of polarized water examined in some detail is the solvency for various solutes. This solvency study offers insight into why Na^+ , sugars and free amino acids are as a rule found in lower concentrations in cell water than in the surrounding medium.

Subsidiary theory of solute distribution in polarized water in living cells and model systems

From theoretical consideration of the energy (or more correctly, enthalpy) and entropy change in transferring a solute from normal water to polarized water (i.e., water assuming the dynamic structure of polarized multilayers), the “size rule” was deduced (Ling 1970, 1979a, 1979b, 1985; Ling and Sobel, 1975; Ling and Hu, 1988). Briefly, the (true) equilibrium distribution coefficient or q-value of solutes between polarized water and normal liquid water varies with the molecular size; and (for solutes having similar assortments of atoms) also with the molecular weight of the solute. The q-value is defined as follows:

$$q = [\text{S}]_{\text{in}} / [\text{S}]_{\text{ex}} \quad (1)$$

where $[\text{S}]_{\text{in}}$ and $[\text{S}]_{\text{ex}}$ are the equilibrium solute concentration in the cell or model and in the external bathing solution respectively. A plot of $[\text{S}]_{\text{in}}$ against $[\text{S}]_{\text{ex}}$ yields a straight line with a slope equal to q.

However, obedience to equation 1 depends on the existence of the solute S exclusively in the form of *dissolved* solute in the cell or model. Only then does the plot of $[\text{S}]_{\text{in}}$ against $[\text{S}]_{\text{ex}}$ assume the shape of a straight line. When one is not certain whether or not part of the solute in the cell or model may or may not be adsorbed on some macromolecules, the ratio of solute concentrations in the two phases will be more safely referred to as an *apparent equilibrium distribution coefficient* or p-value.

A corollary of the polarized multilayer (PM) theory is *that proteins can function as NP-NP-NP systems only if the polypeptide chains exist in the fully-extended-conformation with its backbone CO groups (N sites) and NH groups (P sites) directly exposed to the bulk-phase water*. In this case the bulk phase water will exhibit reduced solubility for salts like sodium sulfate, sugars like sucrose, free amino acids like glycine, each known to exist at lower concentration in cell water than in the surrounding media. On the other hand, *if the backbone CO and NH groups are locked in a-helical, b-pleated sheet and other inter- or intramacromolecular H-bonds as is the case in most native proteins* (for history and definition of the term native protein see Addendum at the end of the article), *the influence on water solvency for Na^+ salts, sucrose and glycine will be minimal or not observable*. Experimental confirmation of these theoretical predictions has been reported earlier (Ling *et al.*, 1980a).

It has been shown that in the 12 *native* proteins and one carbohydrate (chondroitin sulfate) studied, the p-values of Na_2SO_4 observed are indeed close to unity, indicating unaltered, or weakly-altered solvency of the water containing each of the twelve native proteins and one carbohydrate, when compared to normal liquid water (Ling *et al.*, 1980a).

In contrast, *the p-value of Na_2SO_4 in a solution of gelatin is considerably lower than unity. This is a matter of high significance*, even though this finding is not new.

I have mentioned earlier how gelatin and gelatin-like “living jelly” have intrigued biologists from the earliest days and that colloids are the namesake of gelatin. I have also mentioned that water in gelatin gel has reduced solubility for Na_2SO_4 as Holleman *et al.* and Troshin had demonstrated. But none of these investigators had offered a mechanism why gelatin behaves differently from most native proteins.

Not until 1978, did I put in print the first explanation for the unique effect of gelatin on water, and hence the distinctive feature of colloids (see Ling et al., 1978, 1980a,b). Long before, accurate knowledge on the primary structures of gelatin and the other 12 proteins has become readily available. When seen in the light of the PM theory, the different primary structures of gelatin and of the other native proteins provided the clue as to why gelatin behaves the way it does and in ways different from that of native proteins.

Unlike most other proteins, a large proportion of the amino-acid residues of gelatin (denatured collagen) are in the form of proline (12%) and hydroxyproline (9%), each lacking a H atom on the peptide nitrogen atom and thus unable to form H-bonds. An even larger proportion of the amino-acid residues is glycine (33%), a well-known “helix breaker”, i.e., an amino acid residue, whose NH and CO groups as a rule do not engage in forming α -helixes (see the Section on “Inductive effect and protein conformation” below; also Chou and Fasman, 1978). When proline, hydroxyproline and glycine coexist in a protein like gelatin, *a major portion (at least equal to $12\% + 9\% + 33\% = 54\%$) of the gelatin molecule do not form α -helixes or other intra- or intermacromolecular H bonds and therefore remain permanently in the fully-extended conformation.* As a result, gelatin polarizes water in multilayers with reduced solubility for large solutes including hydrated Na^+ , and sulfate ions as observed.

(The new insight into the peculiarity of gelatin and the other supportive evidence to be described below permits a new definition of Thomas Graham’s **colloids** and **colloidal condition** as follows: colloids are materials that polarize and adsorb multiple layers of water, thereby causing the bulk-phase water (or other polar liquids) to assume the dynamic structure of polarized multilayers; colloidal condition describes the aggregate properties of the system of colloids and the surrounding water (or other polar liquids) assuming the dynamic structure of polarized multilayers).

In contrast, the polypeptide chains of the twelve other more normal native proteins do not contain large proportions of non-helix-forming amino-acid residues. As a result, their backbone NH and CO groups are largely engaged in α -helical and other intramolecular H-bonds and, as such, are unable to interact with the bulk phase water. A lack of significant influence on the solvency of the bulk phase water is predicted and observed by experiments.

Ling *et al.* (1980a) also showed the effects of synthetic polymers, polyethylene oxide (PEO), polyvinylpyrrolidone (PVP) and methylcellulose on water solvency. Like all proteins, these polymers also contain suitably-spaced oxygen atoms as N sites (with their negatively charged lone pairs of electrons) but in place of the positively charged P sites in an NP-NP-NP system, these polymers have only vacant or O sites. Accordingly they are referred to as NO-NO-NO systems. Like gelatin and unlike most native proteins, these polymers are also unable to form α -helical or other secondary structure due to the lack of NH groups. Accordingly, one may expect that all three polymers also have strong polarizing influence on the dynamic structure of water and its solvency. It was shown that they

are indeed as effective as, or more effective than gelatin in reducing the solvency of the bulk-phase water for Na^+ sulfate.

Native proteins which are without effect on the solvency for Na sulfate, sucrose and glycine became effective after exposure to concentrated solution of urea (or of guanidine HCl). Both denaturants are well known for their ability of opening up the secondary structure of native proteins, thereby transforming these folded native proteins into the fully-extended-conformation. With the backbone NH and CO groups directly exposed to the bulk phase water, full power in reducing the solvency for the same set of probe molecules studied was conferred onto the hitherto inactive native proteins (Ling and Ochsenfeld, 1989). (Data to be presented below demonstrates the alkali-denatured proteins behave similarly).

Ling and Ochsenfeld also demonstrated that while the bulk-phase water containing urea-denatured proteins have reduced solubility for sucrose (and glycine and Na sulfate), the same water has a p -value of 1.006 ± 0.008 for urea (from ten proteins studied).

On the one hand, this data agrees with the "size rule" (Ling, 1987), since urea is a much smaller molecule than hydrated Na^+ and sulfate ions, glycine and sucrose; on the other hand, *this data shows that the near-unity p -value of urea in water does not prove that the water is all normal liquid water.* A belief otherwise on the part of A.V. Hill had played a key role in the abandonment of the protoplasmic approach to cell physiology as mentioned above.

The development of two classes of models: extroverts and introverts

Solvency studies described above and elsewhere made it possible to separate various proteins and polymers into two groups:

"Introverts" including almost all native proteins, proteins denatured by sodium dodecyl sulfate (SDS) and by n-propanol. Both SDS and n-propanol unravel only the tertiary structure while leaving unchanged or even enhancing the secondary structure. The NH and CO groups of the polypeptide chains of the introverts are internally neutralized and as a result have minimal effects on the solvency of the bulk-phase water.

"Extroverts" including gelatin, oxygen-containing polymers like PVP, PEO, PEG (polyethylene glycol, $[\text{H}(\text{CHO})_n\text{OH}]$), PVME (polyvinyl methyl ether), and urea-, guanidine HCl-, as well as NaOH-denatured proteins. The NH and CO groups of the polypeptide chains of the extrovert proteins and the oxygen atoms of the linear polymers are directly exposed to the bulk-phase water, reducing its solvency for Na_2SO_4 , glycine and sucrose.

The recognition of these two distinctly different groups of models has provided a powerful set of tools to study and describe a spectrum of physicochemical properties beside solvency of extrovert-dominated polarized water in comparison with near-normal liquid water in the presence of inactive or less active introvert models. *The full gamut of distinctive characteristics of the polarized water thus recognized and delineated in turn provide the means of identifying its pervasive presence in living cells.*

Investigations on the different physicochemical properties of polarized water in comparison with normal or nearly-normal water

With the help of the two classes of model systems, five types of physicochemical properties of water in living cells and in the two groups of model systems have been investigated

by my laboratory and other laboratories across the world (for details, and references to original articles, see Ling, 1992, Chapter 5):

Motional freedom: (1) quasielastic neutron scattering studies of the translational and rotational diffusion coefficient of water in two kinds of living cells (brine-shrimp-cyst cells, frog muscle) (Trantham *et al.*, 1984; Heidorn *et al.*, 1986) and one extrovert model system (35% PEO solution) (Rorschach, 1985); (2) ultra-high-frequency dielectric studies of the rotational motional freedom (Debye dielectric relaxation time) on brine-shrimp-cyst cells and rabbit tissues on one hand, and PEO, PVP and PVME solutions on the other (Clegg *et al.*, 1984; Kaatze *et al.*, 1978); (3) nuclear magnetic resonance relaxation times (T_1 , T_2) and rotational correlation times on a variety of living cells and tissues in addition to solutions of PEO, PVP, gelatin (Damadian, 1971; Seitz *et al.*, 1980; Ling and Tucker, 1980; Ling and Murphy, 1983).

Water vapor adsorption: (1) obedience to Bradley's polarized multilayer adsorption isotherm of water vapor adsorption in frog muscle and on gelatin gel (Ling and Negen-dank, 1970; Ling, 1984, p. 288); (2) water vapor adsorption at physiological vapor pressure (near saturation, $p/p_0=0.9968$ for frogs) of frog muscle, extrovert models PEG, PEO, gelatin and introvert models of native proteins (Ling and Hu, 1987).

Osmotic activity: (1) osmotic activity (Ling, 1983); (2) sustained shrinkage (and swelling), in concentrated solutions of partially excluded **permeant** solutes (Na_2SO_4 , sorbitol), of extrovert models (PEO, PVME, gelatin, urea-denatured proteins) in dialysis sacs but not of introvert models (native proteins) (Ling and Ochsenfeld, 1987).

Freezing behavior: (1) microcalorimetric studies of the freezing point, freezing rate and the amount of water frozen in extrovert models including PEO, PVME, gelatin, urea-denatured proteins and in introverts including 6 native proteins and 1 SDS denatured protein (Ling and Zhang, 1983; for further discussion see Comments and Suggestions at the end of the present article); (2) forms of ice formed in living cells and model systems (Miller and Ling, 1970).

Solvency: (1) solute distribution in water under the domination of a wide variety of both extrovert and introvert models and in three types of living cells: frog muscle, frog ovarian eggs, and mouse Ehrlich-ascites-cancer cells (see below).

Space does not allow more than merely mentioning the list of subject titles here, but in order to achieve the specific purposes of this communication, described in the title of the review, I shall continue the discussion on *solventy of water in model systems and living cells*.

Solute distribution in water of frog muscle and model systems: Obedience to "size rule" and exceptions

Ling and Hu (1988) showed plots of the equilibrium concentration of various nonelectrolytes in dialysis sacs containing 39% native bovine hemoglobin against their concentrations in the external bathing solutions. In each case, the distribution curve is a straight line, and the slope of the straight lines, which equals the q -value of that solute in the water in the sac, does not deviate very much from one another. In a similar plot of the same set of nonelectrolytes in 18% NaOH denatured bovine hemoglobin, the distribution curves are straight lines also. However, the slopes, or q -values are widely different.

When the two sets of q -values are plotted against the molecular weights of the non-electrolytes, Figure 8 is obtained. Note that here the q -values of all the non-electrolytes in

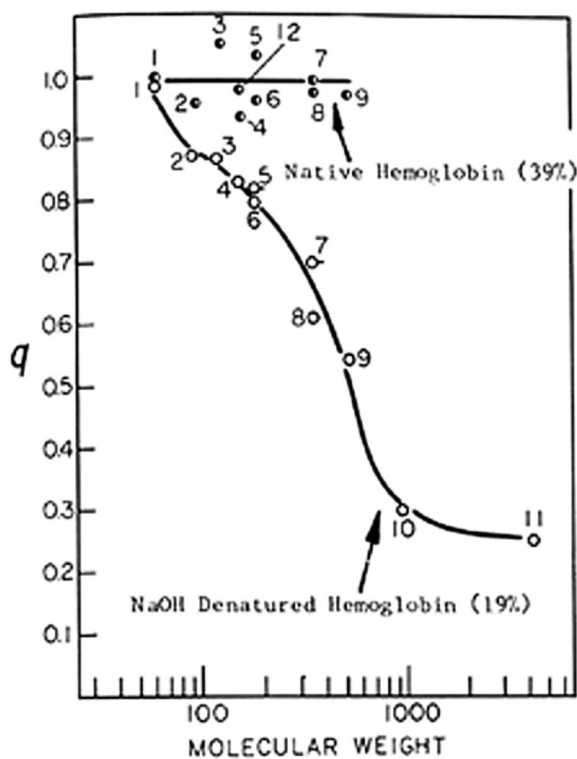


FIGURE 8. The relation between the true equilibrium distribution coefficients or q -value (ordinate) of the solutes, and the molecular weights of the solutes in 39 % of native hemoglobin at neutral pH (half-filled circles) and of 19% solution of NaOH-denatured hemoglobin at alkaline pH (empty circles): 1 ethylene glycol; 2 glycerol; 3 erythritol; 4 xylitol; 5 sorbitol; 6 D-mannitol; 7 sucrose; 8 trehalose; 9 raffinose; 10 inulin; 11 PEG 4000; 12 D-xylose. The relation between the equilibrium distribution coefficients or q -values of solutes in solutions of polyethylene glycol [PEG 8000, $\text{H}(\text{OCH}_2\text{CH}_2)\text{OH}$] at final PEG concentration of 15% and in gelatin gel at final concentration of 18% is not shown here. They bear resemblance to the plot of the q -values against the molecular weights of similar solutes in solutions of NaOH-denatured hemoglobin but little or no resemblance to native hemoglobin. (From Ling and Hu, 1988).

39% native hemoglobin are close to one, indicating that water in the 39% native hemoglobin solutions has solvency close to that of normal liquid water to all the solutes listed. In contrast, most of the q -values from the NaOH denatured hemoglobin solutions decrease steadily with increasing molecular weight.

The minimal effect of native hemoglobin on the solvency of all the nonelectrolytes studied confirms the prediction of the PM theory that introvert models with the backbone NH and CO groups locked in α -helical and other intramacromolecular H bonds do not react or react weakly with the bulk phase water. As a result the solvency of water in the 39% native hemoglobin solution is not different from that in the dilute salt solution outside the sacs, as revealed by the unchanging q -value close to unity.

In contrast, the extrovert model of NaOH denatured hemoglobin shows pronounced change of the solvency of the bulk-phase water. In the altered water, the distribution of most of the nonelectrolytes studied follow the “size rule”: low q-value for large molecules with high molecular weights; high q-values for small molecules with low molecular weights all essentially along a continuous line.

Also found are similar size-dependent q-values for similar solutes in two extrovert systems, PEO and gelatin (Ling and Hu, 1988). While the q-values of the PEO solutions were obtained from similar straight-line plots like those mentioned above, the data from gelatin were taken from Gary-Bobo and Lindenberg (1969) and were determined from single points.

Taken as a whole, these studies have confirmed the dramatic differences of the introvert native protein and the extrovert models including NaOH denatured protein, PEO and gelatin on the solvency of the bulk phase water. All three different types of extrovert models, existing in the fully-extended conformation but for different reasons, exercise similar effect on the solvency of the bulk phase water. Water so acted upon by the extrovert models demonstrates solvency alteration in accordance with the size-rule.

In parallel with these model studies my coworkers and I have also completed similar studies on the distribution of an even larger series of nonelectrolytes in three kinds of living cells: frog muscle, frog ovarian eggs and mouse Ehrlich ascites cells (Figure 5.9 in Ling, 1992).

These three set of data, including *the data from frog muscles, when seen side by side with the data of Figure 8, offer one of the simplest and most direct proof of the PM theory in particular and the AI hypothesis in general.*

This sense of confidence is inspired by the striking similarity between the q-value - vs -molecular-weight plots in these living cells and in all three extrovert models mentioned above and by the lack of resemblance at all to the q-value - vs - molecular-weight plot of introvert model of native hemoglobin.

Of sixteen of the twenty-three nonelectrolytes studied, the q-values commendably follow the “size rule”, i.e., the larger the molecular weight the lower the q-value. Yet seven other nonelectrolytes exhibit q-values considerably higher than expected. Note in particular that both urea and ethylene glycol belong to this category. They demonstrate q-values close to unity.

One recalls that it is the near-unity equilibrium distribution of these two solutes in frog muscle and human erythrocytes respectively that had at one time led Hill, Ponder and others to the belief that cell water is just normal liquid water, and the world-wide acceptance of the membrane-pump theory and the abandonment of the protoplasmic approach to cell physiology.

The data presented also confirmed Hill's reported finding on urea distribution in frog muscle and extended MacLeod and Ponder's finding on equal ethylene-glycol distribution in erythrocytes to frog muscle also. However, the gamut of size-dependent solvency demonstrated provides compelling evidence that water in frog muscle cells assumes the dynamic structure of polarized multilayers. Why do urea and ethylene glycol have higher q-values than their respective molecular weight suggest will be discussed in the next subsection.

From results of these and the other six collections of extensive and world-wide studies on different physicochemical properties briefly mentioned above, one concludes that

water in living cells studied without exception strongly resemble water in extrovert models and not at all, or very weakly resembles water in introvert models.

Taking also into consideration the disproof of both the sieve model and the pump model — the only two other known alternative mechanisms for asymmetrical solute distribution in two contiguous spaces (see Ling, 1992, Section 1.1), one concludes that *the bulk of cell water in living cells in their resting state assumes the dynamic structure of polarized multilayers due to interaction with fully-extended cell protein(s).*

A new theory of cryoprotectants according to the AI hypothesis (including an explanation for the high q-values of urea and ethylene glycol)

Urea and ethylene glycol are not the only solutes that disobey the “size rule”, having (near-unity) q-values higher than their respective molecular weights predict. Three other nonelectrolytes belonging to this group of seven exceptional solutes are glycerol, acetamide, and 1,2-propanediol. All three as well as ethylene glycol are all well-known cryoprotectants, i.e., their inclusion in the bathing medium of cells protects them from injury that may occur following freezing and thawing (Polge *et al.*, 1949; Lovelock and Bishop, 1959; Nash, 1962; Boutron and Kaufmann, 1979). I am not aware of prior studies on the cryoprotective activities of 1,2-butanediol and 3-chloro-1,2-propanediol. Their moderately excessive (but non-unity) q-values suggest some cryoprotective capability of these two compounds.

According to a new theory of cryoprotection based on the AI hypothesis, cryoprotectants may be divided into three types. In one, typified by ethylene glycol, glycerol, 1,2-propanediol (the *glycerol type*), the cryoprotective activity originates from their possession of one or more pairs of vicinal hydroxyl groups which, like water molecules themselves contain at once proton-donating and proton-accepting groups and which can readily anchor and polarize nearby water molecules, thereby stabilizing the dynamic structure of water in living cells (for additional discussion on cryoprotectants, see concluding section of Comments and Suggestions). Cryoprotectants of this group tend to have higher q-values than their respective molecular weight predict.

A second type of cryoprotectants owes its activity to the strong electron donating power of atoms like the oxygen atom in dimethyl sulfoxide (DMSO). The oxygen atom (with its lone pairs of electrons) of *DMSO* enhances the polarized water dynamic structure in a way like that exerted by the N sites of an NO-NO system, causing the enhancement of water-to-water interaction through *induction*. As a rule, the effectiveness of the DMSO type of cryoprotectants increases with the charge density of the lone pairs of electrons on the oxygen (or nitrogen) atom. T. Nash (1962) first pointed out the importance of strong “basicity” in cryoprotectants. Note that, in contrast to cryoprotectants of the glycerol type, DMSO does not have exceptionally high q-value. Its q-value of 0.72 is close to the expected value.

A third type of cryoprotectants (the *PEG type*) are exemplified by large oxygen-containing linear polymers like PEG-8000 which are members of the “extrovert” models. Their large molecular weights, their extremely low q-value — and hence virtual exclusion from the cell water — and their strong power in reducing water activity, as well as other aspects of their protective action to be mentioned below make them safe agents used to reduce the water contents of the cell. Since the least stable part of the polarized, multi-layered water is farthest from the NP-NP-NP chains, removal of water from the cells

reduces the least stable water and decreases the chances of ice formation (see Ling and Zhang, 1983; Ling, 1988b).

Earlier I have mentioned how human embryos have been successfully preserved in liquid nitrogen to develop later into a live human baby. The success of this enterprise relied on the use of cryoprotectants. One very effective formula (VS1) used for preserving embryos includes a combination of one or more members of each of the three types just described: glycerol and propylene glycol (alias 1,2-propanediol) (glycerol type); DMSO and acetamide (DMSO type) and polyethylene glycol 8000 (PEG type) (Rail, 1987). The use of such a combination of different types of cryoprotectants is in harmony with the view that each type enhances the dynamic structure of cell water in a different but synergistic way as suggested in theory. (For additional discussion on how cryoprotectants protect, see Comments and Suggestions at the end of the present article).

Cell K^+

Earlier, I have briefly described a theory of selective accumulation of K^+ over Na^+ , then called Ling's fixed charge (LFC) hypothesis. In this theory, preferential accumulation of K^+ in living cells follows in consequence of the more favorable electrostatic interaction of the smaller hydrated K^+ (than the larger hydrated Na^+) with fixed anions. In living cells these fixed anions exist largely in the form of β - and γ -carboxyl groups carried respectively on aspartic and glutamic acid residues of cell proteins. It was pointed out that myosin alone — which makes up 54% of the total muscle proteins — carries enough β - and γ -carboxyl groups to adsorb all the K^+ found in muscle cells (Ling, 1952, p.774). (For more recent evidence that myosin carries from 67% to 80% of the β - and γ -carboxyl groups adsorbing K^+ , see Ling and Ochsenfeld, 1991).

Confirmation of the predicted localization of K^+ and its surrogates, Cs^+ and Tl^+ in the A bands of striated muscle cells.

Myosin contains a major share of all the β - and γ - carboxyl groups in muscle cells (Ling and Ochsenfeld, 1966). In 1873, Engelmann already knew that myosin occurs only in the A bands of striated muscle, a perception repeatedly confirmed by later workers (see Ling, 1984, p. 227 for references). *When these two sets of facts are taken into account, the LFC hypothesis predicts that the bulk of cell K^+ must be located in the A bands.*

A survey of the literature brought to light quite a number of papers demonstrating the occurrence of cell K^+ in the A bands. Regrettably there were also serious criticisms for the various techniques used (for details, see Ling 1984, pp. 228–229). It seemed wise to devise new and hopefully better ways of testing the prediction. The method I chose was autoradiography. The two radioactive isotopes of K^+ are either too expensive (^{40}K), or too short-lived (^{42}K). Instead, I used two K^+ surrogates, thallium-204 (^{204}Tl) or cesium-134 (^{134}Cs). Both isotopes have long half lives, are relatively inexpensive and can physiologically replace the bulk of cell K^+ .

Figure 9 shows that in an EMOC preparation, both Tl^+ and Cs^+ are selectively accumulated like K^+ in muscle cells (over Na^+ , compare with Figure 2). Not surprisingly, the selective accumulation of Tl^+ and Cs^+ also does not require membrane pumps. It was also shown that inclusion of a higher concentration of K^+ (30 mM, rather than 0.5 mM in the

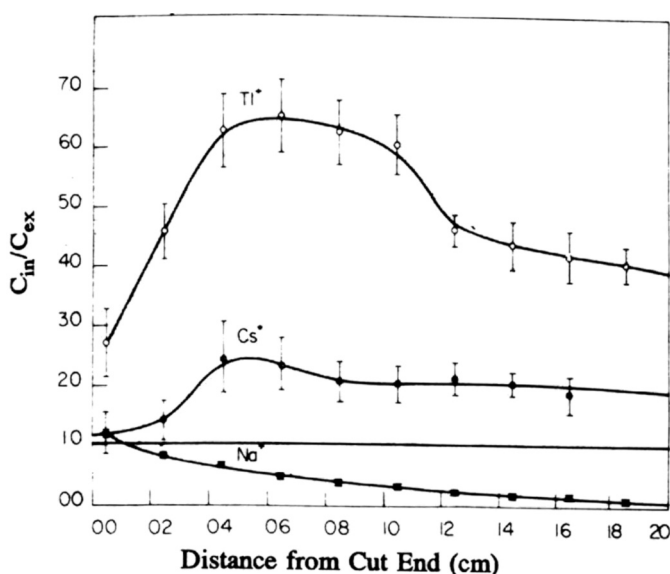


FIGURE 9. Demonstration that like K^+ , Tl^+ and Cs^+ are selectively accumulated over Na^+ in the absence of functional (postulated) membrane pumps (From Ling, 1977a).

control) in the Ringer's solution bathing the cut end of the muscle significantly reduced the level of Tl^+ accumulated, demonstrating competition of K^+ for the same anionic sites adsorbing Tl^+ . (For more rigorous proof of stoichiometric competition for a limited number of sites, see Ling and Ochsenfeld, 1966; Ling, 1977a).

Using the technique described by Ling and Bohr (1969), I incubated isolated frog semitendinosus muscles in a Ringer solution containing radioactive ^{134}Cs -labelled Cs^+ (or ^{204}Tl -labelled Tl^+) at $25^\circ C$ for 1 to 5 days. Single fibers were then isolated from the ^{134}Cs or ^{204}Tl loaded muscles, dried rapidly and coated with photoemulsion. Figure 10 shows an autoradiography made. The location of the silver granules indicate that labelled Cs^+ ions were not evenly distributed in the muscle cells but were mostly in the A bands, confirming the theoretical prediction (Ling, 1977b).

Other striking confirmation of the predicted localized distribution of K^+ surrogates, Tl^+ and Cs^+ came from Edelmann (1977). He took advantage of the high electron density of this pair of K^+ surrogates and was able directly to visualize their distribution after they had replaced most cell K^+ during prior incubation of isolated living muscles before freeze-drying (Figure 11).

Note in particular how granular deposits appeared at the A bands following exposure of the electron microscope sections of the Tl^+ -loaded sections to room temperature (and moisture) for 1 hour (Figure 11). These granules are most likely Tl phosphate crystals which have very low solubility in water. The appearance of these granules confirms that the darker area seen in Figure 11 are truly due to the electron dense Tl^+ ions, and not due to overdeveloped image of the somewhat higher A-band-protein density.

This earlier work was extensively confirmed and extended in years following by Edelmann mostly (1984, 1986, 1988, 1989, 1991) and by Trombitas and Tigyi-Sebes (1979).

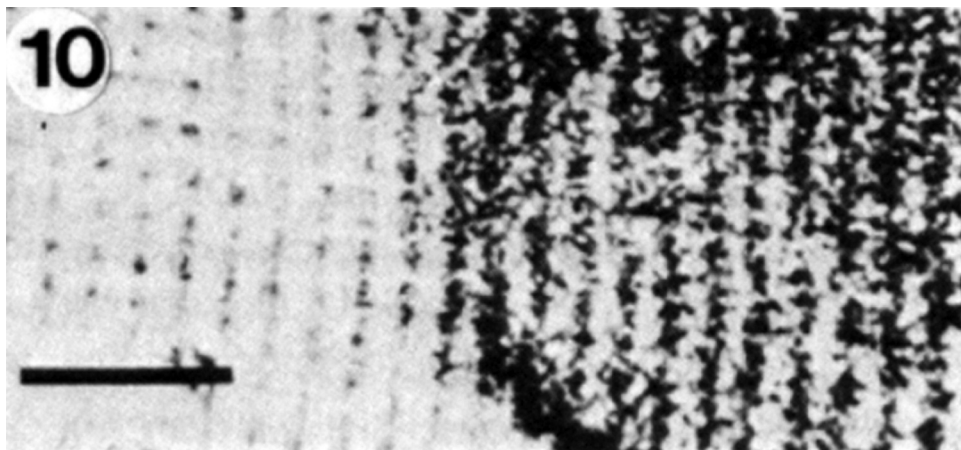


FIGURE 10. Autoradiograph of air-dried single frog muscle fiber, which was loaded before drying with ^{134}Cs while the muscle was perfectly normal. Muscle fiber was incompletely covered with the photoemulsion, permitting the recognition that the silver granules and hence labelled Cs^+ was primarily located in the A bands of the muscle cell (From Ling, 1977b).

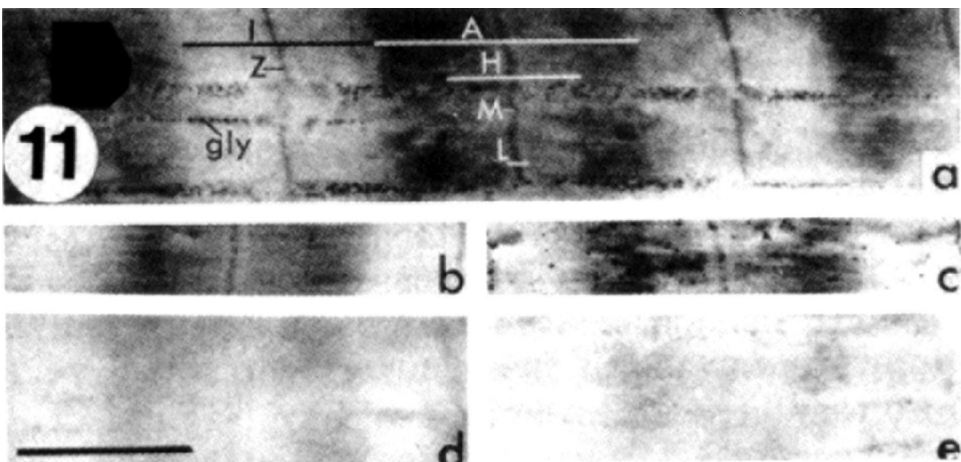


FIGURE 11. Electron micrographs of dry-cut, unstained sections of freeze-dried frog sartorius muscles. Living muscle had been loaded with Cs^+ (a) and Ti^+ (b,c) prior to freeze-fixation, freeze-drying and embedding. (b) was obtained immediately after sectioning, (c) after exposure of a section to room atmosphere for 1 hr. (d) central part of (a) after storage for 2 days in distilled water. (e) Normal " K^+ -loaded" muscle. Abbreviations used: A, A band; H, H zone; M, M-line; L, L zone; Z, Z line; gly, glycogen granules. Scale bar: 1 micrometer (From Edelmann, 1977).

A variety of techniques was used, including direct observation of the predicted localized distribution of K^+ *itself* by the method of energy-dispersive X-ray microanalysis (see also von Zglinicki, 1988; Gupta, 1989). Parallel observation on frozen fully-hydrated preparations in both autoradiography and transmission electron microscopy, eliminated the possibility that the observed distribution was an artifact due to drying (or freezing) (for review, see Ling 1992, Section 4.4.1.).

Stoichiometrical displacement of K^+ from their normal adsorption sites by Na^+ : by decreasing the K^+/Na^+ ratio in the bathing medium or by exposure to a cardinal adsorbent, ouabain.

The X-shaped pair of curves on the left of Figure 12 shows the equilibrium concentration of K^+ and Na^+ in frog muscles changed in a stoichiometric manner, when the ratio of extracellular K^+/Na^+ ratio was varied. These data indicate that under normal conditions, Na^+ is kept away from the adsorption sites because they cannot compete successfully against K^+ due to the weaker adsorption of Na^+ on these sites. When K^+ is absent or present at very low concentration, Na^+ takes its place.

The X-shaped pair of curves on the right shows that the intracellular concentration of the two ions also changed profoundly on exposure to a pharmacological concentration (10^{-7} M) of the cardiac glycoside, ouabain. Contrary to conventional belief, ouabain's ability to reduce the level of cell K^+ and increase that of cell Na^+ does not depend on its inhibition of the activity of pumps as pointed out earlier. All points in Figure 12 are experimental and the solid lines going through or near the points are theoretical according

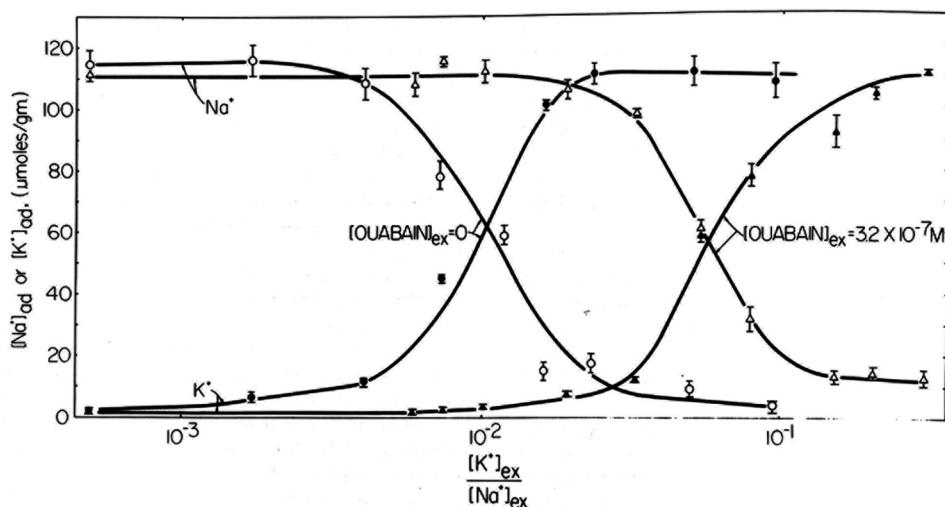


FIGURE 12. The equilibrium concentrations of K^+ and Na^+ in frog muscle in the presence of varying concentration ratios (abscissa) of these ions in the bathing medium. The left pair of X-shaped curves were derived from normal muscles while the right pair of X-shaped curves were obtained from muscles incubated in media also containing 3.2×10^{-7} M of ouabain. The reciprocal of the abscissa reading at the intersection of each pair of the X-shaped curves is equal to the intrinsic equilibrium constant (K^0), which changed from 100 to 21.7 in response to the treatment with ouabain. (From Ling and Bohr, 1971).

to the general equation for solute distribution in living cells (Ling 1965b, equation 8; 1984, equation 11.6; 1992, equation 16).

Figure 12 here demonstrates how interaction with the cardinal adsorbent, ouabain brings about an across-the-board, uniform decrease in the *intrinsic equilibrium constant* ($K^{\circ\circ}$) for the K^+/Na^+ exchange by a factor of about five (from 100 to 21.7).

Demonstration that K^+ (or its surrogates) in resting cells are adsorbed on β - and γ -carboxyl groups

I chose two ways to identify β - and γ -carboxyl groups as the sites adsorbing K^+ , Na^+ or their surrogates: by their characteristic pK_a (ca 4.0) through acid titration; by their specific sensitivity to carboxyl-specific reagents. Unfortunately, the cell membrane barrier prevents direct access to these intracellular sites. Cutting the muscle cells into 2 mm wide segments with both ends open effectively removes the membrane barrier. Unfortunately, deterioration soon sets in, with loss of the capability of the muscle segments to adsorb K^+ or any other alkali metal ions. After a number of unsuccessful attempts, I finally found an additive (polyethylene glycol-8000, PEG-8000) which can preserve the bulk of the K^+ -adsorbing sites with exposed cytoplasm (Ling, 1989). One recalls this agent is also used as a cryoprotectant.

Under the protective action of PEG-8000, Ling and Ochsenfeld were able to titrate and determine the pK_a of 3.85, which is characteristic of β - and γ -carboxyl groups. We were also successful in eliminating the alkali-metal ion adsorbing groups with the carboxyl specific reagent, 1-ethyl-3-(3-dimethylamino propyl)carbodiimide (for details, see Ling and Ochsenfeld, 1991; Ling 1990, 1992, Section 4.4.4.).

Without PEG-8000, neither set of experiments could have been accomplished. However, even in its presence, the β - and γ -carboxyl groups undergo significant changes. Thus the high selectivity of K^+ over Na^+ seen in normal cells and illustrated in Figure 12, for example, is greatly reduced. In addition, these carboxyl groups now acquire specific affinity for divalent ions, not seen in their normal physiological state. This new-found affinity for divalent ions suggests that as a result of the injury (and under the protective action of PEG 8000), part of the carboxyl-carrying proteins might have undergone a transition to the helical conformation, thereby bringing into close approximation normally isolated carboxyl groups, thereby endowing them with the ability of chelating divalent cations.

Acid titration of muscle segments in the absence of PEG-8000 revealed that most of the alkali-metal adsorbing sites have disappeared, in agreement with the well-known fact that dead muscle does not selectively adsorb K^+ or Na^+ ions. One asks, "What are the underlying causes of these subtle and yet profound changes?" The answer I offered is that the conformation of the involved proteins has changed (Ling, 1952). This answer in turn raises the question: What then keeps the protein in its normal resting conformation?

Earlier, I have demonstrated that normal cell water exists in the state of polarized multilayers and by implication, a major portion of the proteins in the cell must exist in the fully extended conformation, then one is also left with the pressing question: What makes these protein assume this fully-extended conformation?

We have shown how exposure to NaOH or urea can change introvert native proteins into extrovert fully-extended proteins. However, neither NaOH nor urea exists in the cells, certainly not at the concentration required. Nature must have provided a more subtle way of achieving this goal. To search for understanding, we turn to another page of the AI

hypothesis, which was addressed to the question what determines the secondary structure of proteins and what can alter them. According to the AI hypothesis, like that in water polarization, the key words are again electronic polarization, or *induction*.

At the outset, I point out that as we enter into the inductive aspect of the association-induction hypothesis, we find ourselves in an area, that is, relatively speaking, not as extensively tested and firmly established as the *associative* aspect of the theory. While I strongly believe that this inductive aspect is also in general correct, further improvements and alterations on specific points may well be necessary in time to come.

Inductive Effect and Protein Conformation

Acetic acid, CH_3COOH is a weak acid with a pK_a value of 4.76; its H^+ is held tightly by the anionic carboxyl group. Trichloroacetic acid is a strong acid with a pK_a less than unity; its H^+ is held loosely by a similar anionic carboxyl group. This is the classic example cited by G.N. Lewis when he argued for his Induction theory: the greater electronegativity of the chlorine atoms (compared with that of the H atoms they displace) causes a greater unequal sharing of paired electrons between the newly installed chlorine and the carbon atoms. This asymmetrical sharing of electrons in turn causes a similar displacement in the next carbon-to-carbon link, and the inductive effect propagates all the way down to the singly charged oxygen atom of the carboxyl group. As a result, the effective electron density of the carboxyl group is reduced. G.N. Lewis's inductive effect has long since become the bona fide part of the accepted knowledge in theoretical organic chemistry (Hammett, 1970; Exner, 1972; Chiang, 1987).

Since inductive effect determines the properties and behaviors of small organic molecules, it should exercise similar effect on the properties and behaviors of large organic molecules like proteins, which are only small organic molecules (i.e., amino acids) linked together. I have also shown that inductive effects are created not only by substitution involving the breaking and formation of covalent bonds, but also by substitution involving the breaking and formation of ionic or H-bonds as well (Ling, 1992, Section 6.1). On the basis of this reasoning and supportive evidence, I introduced in 1962 a key role of the inductive effects in the AI hypothesis in general and in a new theory of proteins in particular (Ling, 1962).

In the ensuing 52 years I have been collecting from the literature and my own research a growing body of evidence in support of my theory of proteins. Again the interested reader should consult my 1992 monograph for details. Here I shall begin with the specific question, By what mechanism does a protein "know" how to return to its native secondary structure (describing the pattern of folding of the amino acid residues in α -helical, β -pleated sheet and random coil conformations), after having lost it in consequence of interaction with denaturants, followed by subsequent removal of the denaturants (Anfinsen, 1967).

Studies on the empirical relationship between the primary structure and the secondary structure of proteins revealed by X-ray diffraction studies of protein crystals, led to the recognition of three types of interactions between the side chains and the backbone NH and CO groups involved in forming the secondary structure: long-range, intermediate-range and short-range interaction. *The least understood is the short-range interaction*

which is also the most important. Thus in the words of reviewer Scheraga: “The conformation of an amino acid residue in a polypeptide or protein is determined in very large measure (though not exclusively) by the short-range interactions between a side chain and atom of the backbone of the same amino acid residue...” (Scheraga, 1974). Yet to the best of my knowledge, my induction theory for the short-range interaction first introduced very briefly in 1964 (Ling, 1964) and elaborated later is the only mechanism for the short-range interaction offered so far.

In this inductive theory of short-range interaction, each amino acid side chain, due to its specific structure, has a *specific electron-donating strength*. Through this inductive effect, the electron-donating strength of each side chain determines the electron density of its own backbone carbonyl group.

The relative electron-donating strength of each of the 19 common amino acid residues was obtained from the pK_a 's of the corresponding carboxylic acids [e.g., formic acid, $HCOOH$, for glycine, $H_2C(NH_2)COOH$]. A positive linear correlation of +0.75 or better was obtained between the set of pK_a values and the “ α -helical potentials” of the 19 amino acid residues provided by Chou and Fasman (1978) and by two other groups of scientists (Ling, 1986).

An important secondary conclusion derived from these studies is the finding that *high electron density of the backbone carbonyl oxygen atoms favors the formation of the α -helical and other introverted structure*. In contrast, low electron density at the backbone carbonyl oxygen atoms favors the fully extended conformation and interaction of the backbone with the bulk phase water. Therefore, if the electron density of the backbone carbonyl oxygen can be made to change coherently, it would be possible to control the conformation of the protein and through its change, the physical state of the bulk-phase water.

The Control of Protein Conformation by Cardinal Adsorbents

The inductive model also helps to resolve what may on first sight appear to be an insoluble paradox. Each protein has one and only one uniquely defined primary structure. Since the primary structure determines its secondary structure, each protein should have only one uniquely determined secondary structure.

Yet it is also well-established that a protein can assume another different secondary structure by interacting with small molecules which I call cardinal adsorbents. Thus not only can the bona fide cardinal adsorbent ATP change the conformation of isolated actin or myosin, even ATP's non-hydrolyzable analogue, Mg-imido ATP can do the same (Marston *et al.*, 1979).

The resolution of this apparent paradox lies in that adsorption of the cardinal adsorbent alters the inductive effects asserted by the protein side chains. As a result, a new set of inductive effects created by the primary structure and the cardinal adsorbent together operates to bring about a new secondary structure (Ling, 1980).

In the AI hypothesis, proteins respond to the adsorption of a cardinal adsorbent by a chain reaction of self-propagating cycles of inductively mediated changes of the electron (and/or positive charge) density of the nearest-neighboring backbone CO and NH group respectively. As a result of their altered electron-or positive charge density, changes in the

preference for available alternative partners occur. Exchanges of the partners of the backbone NH and CO groups may then follow (e.g., from partners in the form of other protein CO and NH groups, to partners in the form of the O and OH end of water molecules as part of the bulk-phase water). The result is an all-or-none change of the conformation of the protein.

Effective cardinal adsorbents are divided into two classes: *electron-donating cardinal adsorbents (EDC)*; and *electron-withdrawing cardinal adsorbent (EWC)*. Interaction with an EDC produces an across-the-board increase in the electron density of both the backbone carbonyl groups and functional groups on short side chains including the β - and γ -carboxyl groups carried respectively on aspartic and glutamic acid residues. Conversely the interaction with an EWC produces an across the board decrease of electron density of the backbone CO groups and functional groups on short side chains.

To facilitate the understanding of the consequence of the across-the-board changes of electron density in the β - and γ -carboxyl groups, I introduce the c-value concept next.

The Electron Density of the β - and γ -Carboxyl Groups and its Representation by the c-Value

By replacing the three H atoms on the methyl groups of acetic acid (CH_3COOH) with three chlorine atoms (CCl_3COOH), a weak acid, with strong affinity of its carboxyl groups for H^+ is converted into a strong acid with weak affinity of its carboxyl group for H^+ . This part is familiar. H^+ is but only one of the monovalent cations the living cell encounters. K^+ and Na^+ are other examples. The question was raised: How would the relative affinities for K^+ and for Na^+ change with change in the carboxyl groups following the H to Cl substitution? In order to make a precise answer possible, one must be able to represent the change of the carboxyl group quantitatively. To do so, the concept of the c-value was introduced.

Briefly, the c-value is a measure of the electron density of the singly-charged oxygen atom in a carboxyl group. Thus acetic acid has a high acid dissociation constant, or pK_a value and a high c-value. Trichloroacetic acid has a low pK_a value and a low c-value. The following offers a more rigorous definition of the c-value:

The assumption is made that the difference in the pK_a of diverse carboxyl or other oxy-acid groups can be quantitatively simulated by moving a unit negative charge along a straight line going through the center of the carboxyl oxygen atom and the center of the cation interacting with the negatively charged oxygen atom. The displacement of this unit negative charge toward the cation in Ångstrom units would entail an enhancement of the interaction such as seen in acetic acid with strong affinity of the oxyacid group for the H^+ . Conversely, a displacement away from the cation, leading to a negative c-value, would correspond to a decreased affinity for the cation as in the case of trichloroacetic acid.

With the c-value defined I constructed what was called a linear model, in which a cylindrical cavity is carved out in a continuous dielectric. Four configurations 0, I, II and III were assigned corresponding to 0, 1, 2, and 3 water molecules being placed between the carboxyl oxygen atom and the interaction monovalent cations, including H^+ , Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ , or NH_4^+ . The statistical probability of each one of the configuration for a specific cation and at a specific c-value of the carboxyl oxygen atom was computed. Fi-

nally the adsorption or association energy of each of the cation at different c -values was computed by a Born charging method. The result obtained for a carboxyl group with a polarizability of $2.0 \times 10^{-24} \text{ cm}^3$ is shown in Figure 13.

Figure 13 illustrates that the relative affinity between a pair of ions is not a constant but varies with the c -value. Thus at one low c -value, K^+ is highly preferred over Na^+ . As the c -value rises this preference declines until a certain high c -value is reached, where from the selectivity order is reversed and the Na^+/K^+ ratio continues to increase some more.

Almost all proteins contain trifunctional amino acid residues. When incorporated into the protein chain, it is these trifunctional amino acids that provide the protein with reactive functional groups. Prominent among these functional groups are the fixed anions and fixed cations. So far we have discussed the fixed anions in the form of β - and γ -carboxyl groups carried respectively on aspartic and glutamic acid residues. With few exceptions, all proteins also carry fixed cations in the form of α -amino groups carried on N-terminal amino acids, ϵ -amino groups and guanidyl groups carried respectively on lysine and arginine residues. All three fixed cations are various modification of the ammonium ion, NH_4^+ . However, these fixed cations are bulkier and thus more electrically polarizable; in consequence of their being attached, they have less motional freedom.

When fixed anions join fixed cations on the protein they form what is called *salt linkages*. Salt linkages are important components of the tertiary structure of a protein. Recently Ling and Zhang (1984) have fully confirmed the contention made first in 1952 that

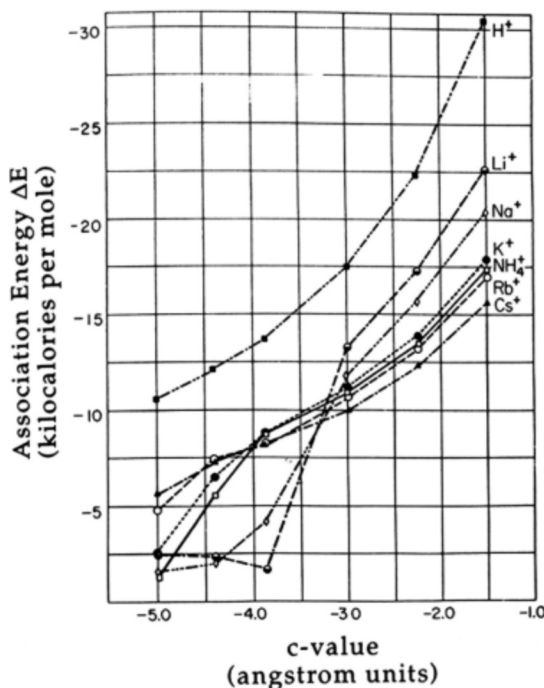


FIGURE 13. The theoretically computed dissociation energies of H^+ , Li^+ , Na^+ , K^+ , Rb^+ , Cs^+ and NH_4^+ from a singly charged anionic site with a polarizability equal to $2.0 \times 10^{-24} \text{ cm}^3$. (From Ling, 1962).

virtually all the β - and γ -carboxyl groups of native proteins do not adsorb significant amounts of K^+ or Na^+ because they are locked in salt linkages (Ling, 1952). Neutralization of the cationic charge by raising the pH liberates the salt-linkage-bound β - and γ -carboxyl groups and a stoichiometric adsorption of K^+ (or Na^+) follows in consequence (Ling and Zhang, 1984)

In summary, at relatively low c -value, the preference of the carboxyl groups for the fixed cation is similar to that for K^+ (since the calculated K^+ and NH_4^+ adsorption curves in Figure 13 are close to each other). However, as the c -value rises to higher values, the preference for the fixed cations increases more steeply than that for K^+ . Eventually the preference for the fixed cation overtakes even that for Na^+ , which as mentioned earlier, is more preferred at higher c -values. Therefore, as the c -value proceeds from low to very high, the preference of the carboxyl groups goes through the sequence: K^+ to Na^+ to fixed cations. That native proteins do not adsorb either K^+ or Na^+ and their β - and γ -carboxyl groups are entirely locked in salt linkages are in harmony with the theoretical deduction that native proteins are proteins in which the β - and γ -carboxyl groups have very high c -values. To maintain the living state in which K^+ is adsorbed preferentially over both Na^+ and much fixed cations, the c -value of the β - and γ -carboxyl groups must be lowered in some physiological way.

ATP (and its “Helpers”) Functions as an Electron Withdrawing Cardinal Adsorbent or EWC in Maintaining the Living State

In describing the living state we relied on the soft nails-iron-filing-big magnet analogy. I then pointed out that the maintenance of the living state depends on an analogous electronic mechanism, where ATP, the ultimate product of metabolism serves its critical role not by its hydrolytic delivery of a package of energy stored in a special phosphate bond. (As mentioned earlier, this once highly cherished idea turns out to be wrong). Rather ATP serves its function by virtue of its extremely strong electronic interaction with the appropriate *cardinal site*. The resultant electronic polarization of the protein molecules may then bring about a change of its conformation. An in-depth examination of the consequence of interaction of ATP with protein models led to the conclusion that *ATP functions as an electron withdrawing cardinal adsorbent, or EWC*.

In the preceding pages and on two different occasions I have pointed out the following: (1) low electron density of the backbone carbonyl groups favors the fully extended conformation and the multilayer polarization of the bulk-phase water, and that water assuming this dynamic structure has reduced solvency for large hydrated ions like Na^+ (and K^+); (2) low electron density or c -value favors the selective adsorption of K^+ on β - and γ -carboxyl groups; (3) interaction with a suitable EWC creates an across-the-board lowering of the electron density of the backbone carbonyl groups and the c -value of β - and γ -carboxyl groups; (4) ATP functions as such an EWC.

Figure 14 is presented for three purposes:

It diagrammatically illustrates how all these factors contribute to the creation and maintenance of the living state, a key feature of which is the preferential adsorption of K^+ over Na^+ on the β - and γ -carboxyl groups and the reduced solvency for free Na^+ (and K^+) in the cell water existing in the state of polarized multilayers. Note also that

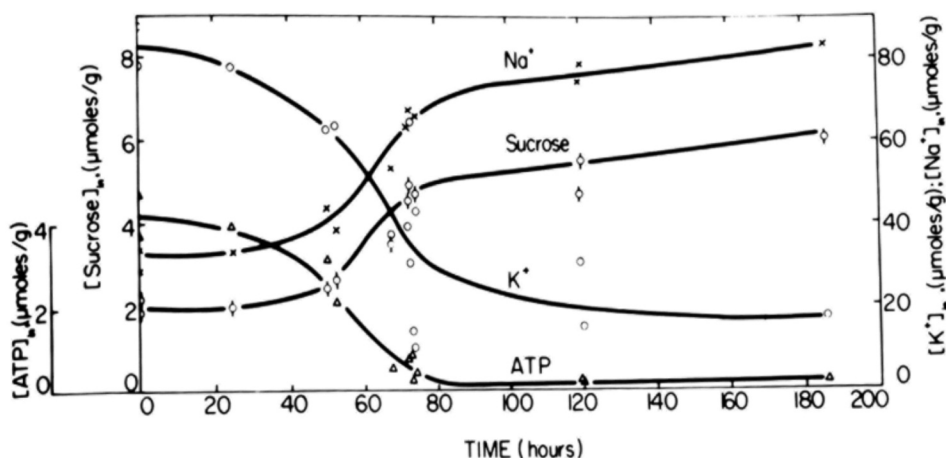


FIGURE 15. The equilibrium concentrations of K^+ , Na^+ , sucrose and ATP in frog muscles exposed to a low concentration of sodium iodoacetate (0.2 mM) for different length of time (abscissa) at $0^\circ C$. (Ling and Ochsenfeld).

concentration and Na^+ concentration clearly indicate that the Na^+ taken up is dissolved in the depolarizing cell water and not stoichiometrically replacing adsorbed K . Were it otherwise, and the cell water remains unchanging, then sucrose which cannot replace cationic K^+ , would have remained unchanged in concentration. Therefore the antipodal changes observed belong to Type B.

In brief, as ATP concentration declines, K^+ is desorbed. While a small fraction of Na^+ might have taken up the β - and γ -carboxyl groups, most of these groups become locked in salt linkages with fixed cationic groups. In the meanwhile, water is depolarized, and with it, there is a parallel increase of the q -value for large hydrated ions, Na^+ , as well the large hydrated nonelectrolyte, sucrose.

Can We See the Living Structure? Studies of Edelman Suggested that He and Other Electron Microscopists May Have Seen the Living Structure

A good and widely employed method of recognizing and counting the number of dead cells is the *dye method*. By dispersing the mixed population of live and dead cells in a Ringer's solution containing trypan blue, rhodamine B or nigrosin, dead cells stand out deeply stained while living ones are not. This distinction does not originate from an increase of membrane permeability to the dyes upon cell death. Both live and dead cells are permeable to these vital dyes. From Troshin we learnt that even though normal living frog muscle was only lightly stained by 0.02% rhodamine B, the concentration of this dye in healthy muscle cells at equilibrium was found to be 9.5 times higher than in the external bathing solution. When the muscles cells were dead, the dye concentration rose to 54.9 times higher than in the external medium. Only then did the muscle cells look brightly red (Troshin, 1966, p. 206).

Now the molecular weight of rhodamine B is 479. From the molecular weight vs. q-value plots (Figure 5.9 in Ling, 1992), one estimates that the q-value of this dye in normal frog muscle cell water could not be much higher than 0.1. When the muscle dies, the q-value of rhodamine should sharply increase to unity. However, this increase in rhodamine B concentration in the cell water could not be the main cause for the observed increase in coloration. The bulk of rhodamine B in both living and dead muscle cells belongs to the adsorbed fraction, according to another rule (the surplus-adsorption rule) introduced in the AI hypothesis: *whenever a solute is found in the cell water at equilibrium concentration exceeding considerably the concentration of the same solute in the external medium, that excess is probably adsorbed in the cell.*

Following this rule, one concludes that both living and dead cells adsorb rhodamine B but the dead cells adsorbed a great more rhodamine B than the living ones. The question is, "Why?"

The AI hypothesis offers a possible answer. In cell death, depletion of the EWC, ATP occurs. As a result, the cell proteins undergo an auto-cooperative conformation change. In the resulting conformation, the proteins have enhanced adsorption for the dyes and the adsorbed dye also exhibits a more intense red color.

This interpretation is in accord with the well-known fact that dyes adsorbed on proteins often undergo profound color changes when the protein undergoes drastic conformation changes. Thus, as is well known, cooking turns lobsters red. The carotinoid pigment, astacin is green when adsorbed onto the lobster carapace protein in its natural state. When astacin is adsorbed onto the cooked carapace protein, the pigment becomes bright red.

As mentioned repeatedly above, the ability of taking up some solutes at high levels while keeping others at much lower level is seen in all living cells. The selective accumulation of K^+ over Na^+ offers striking examples of both phenomena. In live muscles, the K^+ concentration is some 40 times *higher*, while Na^+ is 10 times *lower* than the respective concentrations in the bathing medium. Yet when the cells die, the K^+ concentration falls while the Na^+ concentrations rises, each to approach and eventually to attain its respective concentration in the surrounding medium. The distribution of K^+/Na^+ , under certain circumstances, may serve as even better monitor than the artificial vital dyes in informing us about the life/death of cells.

I have described earlier how Edelman had succeeded in demonstrating the selective accumulation of Cs^+ , Tl^+ in the A bands of frog voluntary muscle. Frog muscle physiologically loaded with these electron-dense ions was freeze-dried, embedded in plastic and the adsorbed electron-dense Cs^+ and Tl^+ in dry-cut section directly visualized under an electron microscope (Figure 11).

In 1980, Edelman introduced a new method of *staining* freeze-dried, embedded frog muscle sections *after* the sections have been cut. 0.2 mm-sections were wet-cut and momentarily exposed to a solution containing different alkali-metal ions. The fluid adhering to the section was removed by a centrifugation procedure before the section was examined for its ionic uptake. (This method is described by Dr. Edelman in a paper also presented in this conference).

In Figure 16, I have reproduced Dr. Edelman's LAMMA spectra of a gelatin film containing 50 mM each of LiCl, NaCl, and KCl but only 10 mM CsCl (a). This standard spectrum shows that at the same concentration, each ion gives a spectrum of different intensity. Figure 16d shows the LAMMA spectra of a muscle section, which had been exposed to a solution containing 100 mM of NaCl but only 10 mM of KCl. Yet the ion

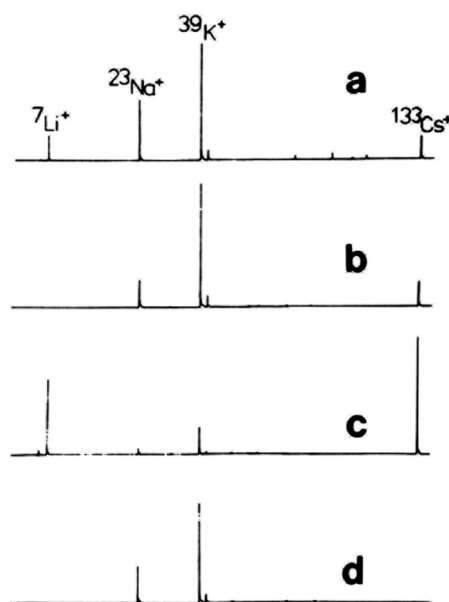


FIGURE 16. Laser Microprobe Mass-spectrometer Analysis (LAMMA)-spectra: (a) gelatin standard containing 50 mM LiCl, 50 mM NaCl, 50 mM KCl, and 10 mM CsCl — gelatin used to keep ions evenly distributed. LAMMA spectra b, c, and d are from A-band region of frog muscle sections following exposure to a solution containing respectively the specific composition: (b) 50 mM NaCl, 50 mM KCl, 10 mM CsCl; (c) 50 mM NaCl, 50 mM KCl, 10 mM CsCl and 50 mM LiCl; (d) 100 mM NaCl, 10 mM KCl. (From Edelmann, 1980).

contents of the muscle vaporized by the laser beam shows a K^+ concentration considerably higher than that of Na^+ . Thus a 10-to-1 selectivity of K^+ over Na^+ is demonstrated. The amount of K^+ adsorbed was estimated to be about 40 mmoles per kilogram of fresh muscle cells, in comparison with about twice that concentration in fresh living muscle cells. In other words, *the cut section appears to be able to accumulate K^+ over Na^+ at a selectivity ratio and in the amount approaching that seen in **living** frog muscle cells. If this data can be confirmed and reproduced at will, then Dr. Edelmann had in fact succeeded in demonstrating the maintenance of the living state of thin frog-muscle-section by his freeze-drying, and embedding technique.*

Although Dr. Edelmann has not elaborated further on this particular experiment, he has approached the problem in another way. He demonstrated in the LAMMA spectrum c in Figure 16 that the inclusion of 50 mM of LiCl in addition to 50 mM of both NaCl and KCl and also 10 mM of CsCl, greatly enhanced uptake of Cs^+ (and of Li^+). While it requires (the less accessible) LAMMA instruments to measure Li^+ , Na^+ and K^+ , transmission electron microscopy permits easy visualization of Cs^+ as he has earlier demonstrated in muscle physiologically-loaded with Cs^+ and shown in Figure 11. One reasons that if the freeze-dried, embedded section of Figure 16d could selectively accumulate K^+/Na^+ at a favorable ratio of 10 to 1, the freeze-drying and embedding technique had in fact captured the frog muscle cell in its living state, or at least not far away from its living state. Since the section giving rise to Figure 16c was prepared by an entirely similar procedure,

it would seem reasonable to assume that section had also retained at least partially the living state. If so, the demonstrated intensive uptake of Cs^+ in the company of LiCl may be regarded as a substitute method for revealing a partially preserved living state. Fortunately, Dr. Edelmann had conducted some additional experiments which, when viewed side by side with some unpublished work of my own laboratory, lend independent support for this stipulation as follows:

I have pointed out earlier that after a razor-blade cut, the exposed cytoplasm of frog muscle does not regenerate a cell membrane. Instead, the cytoplasm rapidly deteriorates as can be seen from the electron microscopy plate of Dr. Cameron. Other evidence of spreading deteriorating protoplasm can be seen in the gradual loss of the ability of selectively taking up labeled K^+ and of excluding labeled Na^+ when the time of incubation of the EMOC preparation increased (Figure 2).

Elsewhere I presented experimental data similar to those shown in Figure 2. Only here, beside the uptake of *labelled* K^+ and *labelled* Na^+ , the *total* cell K^+ along the length of the muscle is also presented. The labeled K^+ uptake observed in each section closely follows the level of total K^+ in that section. That is, low uptake of labeled K^+ and high uptake of labeled Na^+ in sections, which have lost their adsorbed K^+ ; high labeled K^+ uptake and low Na^+ uptake in section still retaining a high total K^+ content. In summary, the muscle substance near the cut is no longer maintaining its living state; yet, in the same muscle fiber 1.2 to 1.6 cm away from the cut, the living state is well preserved. Could a difference in ATP concentration be the cause of this striking difference?

Figure 17 shows a similar K^+ - and Na^+ -distribution profile like those shown in Figure 2, except that the ATP concentrations in the cut sections are also presented. Recalling

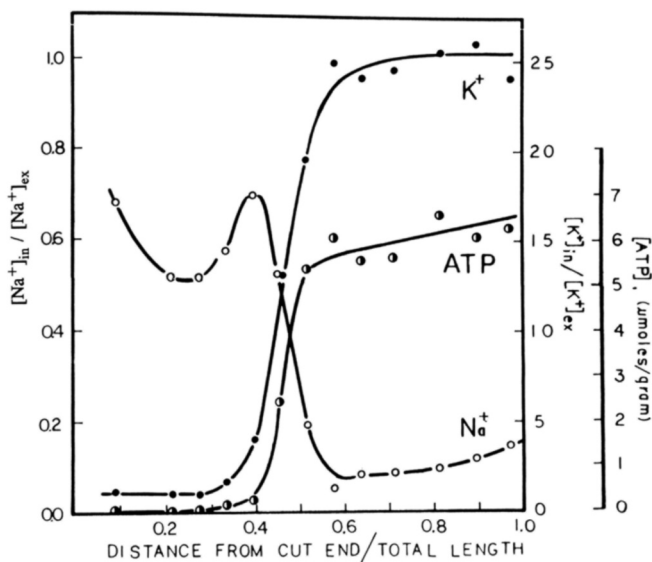


FIGURE 17. The distribution profiles of total K^+ and ATP concentration as well as the $[\text{Na}^+]_{\text{in}}/[\text{Na}^+]_{\text{ex}}$ ratio of labelled Na^+ in a frog sartorius muscle after the cut end of the muscle had been exposed for about 50 hours at 25°C in an EMOC preparation. The Ringer's solution bathing the cut end of the muscle contained 100 mM of labelled Na^+ and 2.5 mM of (nonlabelled) K^+ . (Ling and Blackmann).

what the data of Figure 15 have shown, one may say that the ability of selectively accumulating K^+ and of excluding Na^+ was lost when the ATP concentration fell to zero or near zero in response to the spreading injury from the cut. Near-perfect preservation of the living state only 5 millimeters away is matched by an equally well-preserved ATP content. These data once more confirm the theory that ATP plays a key role in the maintenance of the living state. Thus if *in vitro* super-adsorption of Cs^+ in the A band truly offers another way of monitoring the living state, then only sections prepared from muscle right at the cut end will not take up Cs^+ intensely in the presence of Li^+ ; however, a little distance away from the cut, there should be intense Li^+ -promoted super-adsorption of Cs^+ . In fact, an observation made by Edelmann and published in 1989 had already confirmed this prediction.

Edelmann cut a frog sartorius muscle with a razor blade and allowed the cut muscle to stay in a humidity chamber for 30 minutes before freezing. The frozen pieces were freeze-substituted and low temperature embedded in Lowicryl K11M. Thin sections were prepared from the vicinity of the cut end of the muscle and exposed to a solution containing 100 mM LiCl and 10 mM CsCl. As shown in Figure 18c, little observable structure could be seen in section near the cut end. Yet in another section prepared from the same cut muscle a short distance away from the cut edge, a well-stained picture was obtained as shown in Figure 18d. In Figure 2, I have demonstrated how after the razor-blade cut, deterioration begins at the cut end spreading slowly toward the intact end. Comparing Figure 18 with Figure 17, and keeping in mind the much shorter interval between time the

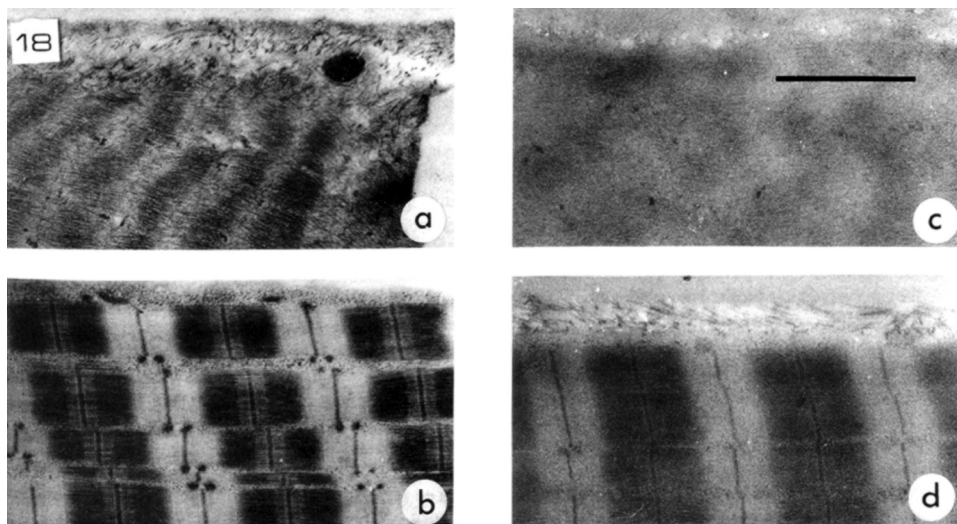


FIGURE 18. Electron micrographs of a portion of a transected frog muscle cell at or near the cut end. Frozen muscle pieces were freeze-substituted in pure acetone at -80°C for one week, and low temperature embedded at -60°C in Lowicryl K11M. (a) An ultrathin section stained with uranyl acetate and lead citrate showing the cut end of the muscle fiber, (b) Similarly stained ultrathin section of the same cut muscle fiber at a distance of about 0.4 mm from the cut end, (c) and (d) 0.2 μm thick wet-cut sections which were exposed to a solution containing 100 mM LiCl and 10 mM CsCl, (c) section obtained at the cut end, (d) section obtained at a location 0.4 mm from the cut end and thus similar to (b). The section in (c) is almost unstained. Section in (d), in contrast shows a staining pattern similar to that shown in (b). (From Edelmann, 1989).

muscle was cut with a razor blade and freezing in the thin muscle section shown in Figure 18 (i.e., 30 min.) than in the much wider sections in Figure 17 (50 hrs), one tentatively concludes that Li^+ -promoted super-adsorption of Cs^+ at the A bands and Z lines following exposure of the cut section to a solution containing 100 mM LiCl and 10 mM CsCl offers another way of monitoring and demonstrating the maintained living state of the frog muscle in the freeze-dried, or freeze-substituted and embedded sections.

This tentative conclusion, if fully confirmed in the future, would have established that the freeze-drying or freeze-substitution and embedding in plastic — and most probably the other even more sophisticated methods of preparing specimens for electron microscopy beginning with rapid cooling (e.g., cryoelectron microscopy of vitrified specimen and of high-pressure cooling) — has already made it possible to preserve and to see the living structure.

Concluding Remarks

Living structure, the central subject in the title of this review, is a term not widely used. Yet *living structure* and *lifeless structure* are as different as New York City is different from the ruin of Pompeii. Disuse of the term could be traced to its position in a no-man's land between physiology (which describes *living*) and anatomy (which describes *structure*). However, the primary reason for the disuse is the incorrect, but widely taught theory of cell physiology (the membrane pump theory), according to which, there is no protoplasm; ergo no living structure.

The EMOC preparation, the PEG-preserved 2 mm muscle sections, and the freeze-dried, or freeze-substituted muscle sections embedded for electron microscopy demonstrate how one can, in the absence of a functional cell membrane (and membrane pumps), preserve to varying degree the functional and anatomical intactness of the living structure, as monitored by the persistent accumulation of K^+ over Na^+ . With these basic concepts set straight, a new opportunity of close cooperation between electron microscopists and cell physiologists looms large and bright. After all, anatomy and physiology are like the right and left leg of a human being. Neither can progress soberly by itself without a balanced progress of the other.

Some Comments and Suggestions to Electron Microscopists

As I pointed out in this paper and elsewhere, many of the major attributes of the living cell that have been ascribed to cell membrane, pumps etc. actually reflect the unusual properties of the cell water. These unusual properties of the cell water (according to the AI hypothesis) in turn, reflect the existence of cell water as polarized multilayers.

Once one accepts the key role of cell water in living phenomena, it is no longer surprising that the very modest efforts spent in investigating the nature of cell water has already generated one mankind-enhancing diagnostic instrument of great power, magnetic resonance imaging (MRI) (see Ling, 1984, footnote on p. vii; Ling 1992, endnote 1 in Chapter 1).

Similarly, the little-noticed study of freezing of water by Father Luyet and his coworkers at his American Foundation for Biological Research, published almost exclusively in

the obscure, by-now-long-defunct “Biodynamica”, had given rise to diverse practical exploitations of benefits to mankind.

In 1937 Luyet first suggested the use of vitrification as a means of cryopreservation. Four years later, Luyet and Hartung (1941) demonstrated that treatment with ethylene glycol enhanced the survival of vinegar eels following rapid freezing in liquid nitrogen and rewarming. In 1946 Rostand successfully preserved the motility of frog spermatozoa for 6 to 22 days at -4° to -6° C in media containing 10 to 20% glycerol. However, at the time of their publication, these findings were largely ignored. It was the accidental discovery of a beneficial effect of glycerol in preserving frozen fowl spermatozoa by Polge, Smith and Parkes (1949) that caught worldwide attention. Cryopreservation with the aid of glycerol and other agents has since then already revolutionized live-stock breeding, food-technology, surgery, and even child-bearing. However, most appropriate for the subject matter of this Symposium on better ways of preparing specimens for electron microscopy, is *the possibility that vitrification has actually provided the means of preserving and seeing the living structure*.

In response to the suggestion of Dr. Ludwig Edelman (see Discussion with Reviewers), I have expanded and rewritten the final passages of my original manuscripts. Much of the comments and suggestions is based on earlier studies of myself and my former graduate student, Dr. Z. L. Zhang on the freezing behaviors of cell water and model systems. The comments and suggestions will be described under four subheadings: (1) the structure of vitrified cell water; (2) how does a cryoprotectant protect? (3) the life and death of cell structure; (4) quantification of the transition of cell structure from living to dead.

(1) *The structure of vitrified cell water*. Investigators in cryopreservation have often expressed the view that vitrified water in living cells is *amorphous*. However, recently others have pointed out that vitrified water is *polymorphous* (see Mayer and Bruggeller, 1983; Dubochet *et al.*, 1988, p. 143).

This newer view agrees with my own: vitrification by rapid cooling is like taking a rapid snapshot; it does not first undergo a homogenization step during which an amorphous structure of water is created before solidifying. Rather, vitrification by rapid cooling catches the water in its momentary configuration whatever that may be.

According to the AI hypothesis, the bulk of water in a living cell exists in the state of polarized multilayers. The extensive evidence in support of this view has been briefly reviewed in this paper and in detail elsewhere (Ling, 1992). Therefore, *vitrification must have preserved the polarized-multilayer structure in living cells* (and in extrovert model systems).

(2) *The mechanism of cryoprotectant action and the beneficial effect of rapid cooling and thawing*. As already mentioned in the text above, there are three (related) types of cryoprotectants, according to the AI hypothesis: the glycerol type, the DMSO type and the large-molecular-weight PEG type. Though different in their specific mode of action, each enhances the stability of the polarized-multilayer state of cell water (as part of the resting living state of the cell).

Our prior investigation of slow freezing of water in aqueous solutions of introvert and extrovert models offer support for this idea. Ling and Zhang have shown that the freezing points of water in solutions of 6 introvert native proteins (for definition of “native proteins” see Addendum) determined by differential microcalorimetry cooling at the rate of 10°C per min (Ling and Zhang 1983). The observed freezing points of the protein solutions were independent of the protein concentration between 5% and 50% (w/v).

Now a 50%–5% = 45% protein solution contains about 4 M amino-acid residues. Each amino acid residue has the potential of offering one CO and one NH group. Yet an increase of 4 M of amino acid residues had no observable influence on the freezing point of water in the solution. In contrast, under similar conditions even a 3 M solution of urea lowers the freezing point of water by 20°C. Clearly not all solutes at equimolar concentration depresses the freezing temperature to the same degree. The interpretation for this difference in terms of the AI hypothesis is that the bulk of backbone CO and NH groups of the *native proteins* are locked in intra-macromolecular H bonds and no longer available for interaction with the bulk-phase water whereas the polar CO and NH₂ groups of urea are free to do so. This interpretation is supported by the totally different responses of the same proteins after urea denaturation discussed next.

If the same native bovine hemoglobin is added to a 9 M urea solution to a final concentration also of 50%, the freezing point no longer stayed put at that of a 9 M urea solution (233°K); instead, the freezing exothermic peak disappeared altogether (at least at or above the lowest temperature observed, 212°K). Here the protein becomes denatured by the urea; in consequence, its backbone NH and CO groups are no longer locked in intramacromolecular H bonds but become exposed to the bulk phase water. Water polarization and freezing-point depression follow. The freezing point lowering described here is shared by all other extrovert models we studied (Ling and Zhang, 1983).

A 50% PVME solution remains a highly viscous liquid in an ethanol-dry-ice mixture. When a glob of the solution was held between a pair of glass rods and plunged into this cooling mixture, the glob stays fluid. However, if one of the rods is metallic, the translucent glob abruptly turns white with ice-crystal formation, when heat from the fingers reaches the PVME solution via the conducting rod. On the other hand, if the PVME solution is cooled directly to –190°C, the translucent viscous fluid turns into a transparent solid glass. It seems reasonable to conclude that at –70 °C water in this extrovert-water system remains in the state of polarized multilayers. After further cooling and vitrification, the water exists as *vitrified polarized multilayers* as described in section (1) above.

The more-or-less random distribution of the PVME molecules in such a vitrified system and the more ordered but still-far-from-crystalline distribution of water-polarizing proteins in vitrified living cells, as well as the “dynamic” nature of the water structure in both, offer reasons against the prospect of success in employing conventional X-ray diffraction methods to detect the structure of vitrified polarized multilayers of water. Attempts to demonstrate *enhanced and farther-reaching short-range order* of vitrified polarized water with more sophisticated X-ray diffraction (and other) methods are altogether different and very worthy of consideration.

Another unusual behavior seen only in the chilled *extrovert* models (but not the *introvert* models) is the *warming exothermic peak* when the extrovert model is slowly warmed up again (also at the rate of 10°C/min) (Zhang and Ling, 1983). However, long before Luyet and his coworkers had reported a similar phenomenon.

Luyet *et al.* (1966) showed that when an aqueous solution of glycerol, ethylene glycol or polyvinylpyrrolidone (PVP) was rapidly cooled and then warmed, heat was given off during the warming step. These authors interpreted this exothermic response as due to devitrification. Since there is now overwhelming evidence that water in the presence of high concentration of PVP and other extrovert models exist as polarized multilayers, that aqueous solutions of glycerol and ethylene glycol also demonstrates *warming exothermic peak* suggests that water in concentrated solution of glycerol and ethylene glycol exists

at least partly as polarized multilayers and that their cryoprotective action, mentioned above, lies in their reinforcing the polarized-multilayer state of water in living cells, as suggested earlier.

Another interesting aspect of the warming-exothermic response is that even at the slow rate of warming (10°C/min), devitrification of the concentrated extrovert models does not occur until the temperature has risen to -35 to -30°C, which is a far cry from the devitrification temperature of vitrified pure liquid water (about -120°C) (Mayer, 1985). Clearly with rapid-enough warming, the vitrified polarized multilayers of vitrified water can return to the state of liquid polarized multilayers without going through a transitional stage of cubic or hexagonal ice formation. Thus the beneficial effect of rapid thawing of cryopreserved living cells shares a similar reason for the rapid cooling in preparing specimens for electron-microscopy: prevention of ice-crystal formation of water in the state of polarized multilayers (for further discussion on the subject, see last question of Dr. Clegg and answer below).

(3) *Life-and-death of cell structure.* Gross anatomists began their career dissecting and describing the bodily parts of cadavers. Obviously they had no other choice. With the introduction of microscopes, new ways of revealing the fine structures of tissues and cells were discovered involving the killing and fixing the cell structures with cross-linking agents, cutting the imbedded tissue into thin sections and staining them with a variety of vividly colored dyes before observing the tissue section under the light microscope. When the electron microscope was invented, the basic procedures used to prepare tissues and cell specimens did not materially change. Only the stains have changed. Instead of colored dyes, electron-dense stains are introduced. The tradition of killing the living specimens first continued in conventional electron microscopy.

Thus historical past might have played a part in this tolerance of the study of tissues and cells, beginning with their killing. Another possible factor for this tradition may be traced to the dominance of the Emergentists' view of life, i.e., only functional activities of the cells represent life, the cell substance are merely mechanical parts and are not living. I have presented many reasons why this view belongs to the past. With the disproof of the membrane-pump theory and the introduction of a new paradigm under the banner of the association-induction hypothesis, a gamut of new challenges to all biologists has arrived.

The new challenge to electron microscopists is special, exciting and demanding high-level skill: to test the hypothesis that beautifully preserved cell structures seen under the electron microscope may be only those of a beautifully preserved corpse and that a less beautifully preserved electron microscopical picture might have caught the essence or "soul" of the living structure much as painters and sculptors have strived to achieve throughout history. There is, however, one profound difference between the two enterprises. Even the untrained eyes can tell at an instance a living person from a dead corpse. To know what is living structure and what are well-preserved artifacts is itself an illusive challenge. In learning what is a living structure and what is a dead one, one must begin with the selection of a standardized, easy procedure for producing a well-defined dead cell. To the best of my knowledge, the easiest way is by using metabolic poisons to shut off the supply of energy of sturdy cells of North American frogs.

In the main text of this paper, I have gone into some depth presenting the reasons for my belief that Dr. Ludwig Edelman in particular and other advanced electron microscopist developing and using various rapid-freezing vitrification techniques (see Dubochet *et al.*, 1987, for example) might have, knowingly or quite "innocently", been seeing the

cell structure preserved at its living state. To put this hypothesis to a test, another comparison needs to be made between electron microscopy pictures obtained by the *in vitro* staining technique with Cs^+ in the presence of Li^+ which Edelmann presented at this symposium (Edelmann, 1991) and pictures of tissues which have been physiologically loaded with Cs^+ but processed by the various sophisticated vitrification methods. And these comparisons cannot be made without a high level of reproducibility in creating each type of these electron microscopical pictures.

While doing the autoradiography studies described in the text I came face-to-face with the much greater degree of hit-and-miss in these studies than my routine studies of cell physiology. My own limited experience in electron microscopy also suggested a similar low reproducibility. However, there is no question that this uncertainty varies with the researcher's skill and experience. Just as it was the accumulated "tricks" that created the Stradivarius reproducibly, so it seems to me worth while, as a cell physiologist attending an electron microscopists' meeting, to document in Appendix 2 below, a few hints at ways of obtaining healthy frog muscle cells, of physiologically loading these cells with Ti^+ or Cs^+ , and of producing killed frog cells in a standardized manner.

(4) *Quantification of the transition from living to dead structure by manipulating the level of ATP and other cardinal adsorbents known to sustain or to control the living state.*

ATP: I suggest that the relationship between ATP concentration in the freeze dried (or freeze substituted muscle sections and the ability of the sections to adsorb *in vitro* K^+ over Na^+ and/or to demonstrate Li-promoted super Cs^+ adsorption (according to Edelmann, 1991) is well worth the efforts to work out: The data of Figures 17 and 18 suggest that uptake of high concentration of Cs^+ depends on a normal ATP concentration. A systematic study of the super Cs^+ uptake in electron microscopy sections prepared from muscles containing a *wide range* of ATP concentration — following exposure of the muscles prior to freezing to 0.2 mM Na iodoacetate containing Ringer's solution maintained at 0°C over a long period of time (see Figure 15) — could set new guidelines toward the further investigations of the living structure in all kinds of living cells and in further perfecting the techniques of specimen preparation for electron microscopy. In addition, one would also like to know if the inclusion of ATP in the section-soaking solution affects the ionic uptake? If not, why not?

Drugs: To determine if prior exposure of the frog muscle to drugs like ouabain (see Figure 12) before freezing and embedding influences the Li^+ -promoted Cs^+ uptake by the Edelmann procedure: If the answer is positive, one could then ask: Would the presence of ouabain in the section-soaking CsCl-LiCl solution exercise a similar effect? If not, why not? If yes, the Edelmann procedure may offer an important new way to test the theory of drug actions according to the AI hypothesis (see above; Ling 1992, Section 7.3.2.). I may add that to understand drug action at the basic level is probably one of the most important and urgent tasks for a biologist at this point in history.

Addendum

According to the Webster dictionary, the word *native* means "found in nature esp. in an unadulterated form" (Webster New Collegiate Dictionary, 1977, p. 765). Therefore, one may say that "All proteins found inside a living cell *in vivo* are "native" to the cell by de-

finition” (see Ling, 1990, question of B. Gupta) is logically impeccable. But it is also unrealistic. Because the term “native protein” as used in the literature today has a different meaning — even though the pioneer users of this term might have thought that their “native” protein were in agreement with the reviewer’s definition.

Studying isolated proteins *in vitro*, early protein chemists soon discovered that these proteins could exist in more than one state (without changing their primary structure). Thus gentle heating may convert a water-soluble protein into a water-insoluble one. H. Wu (Chinese J. Physiology, 5: 321, 1931) then offered the first definition of *protein denaturation*: “Denaturation is a change in the natural protein molecule whereby it becomes insoluble in solvents in which it was previously soluble.” Wu’s “natural protein” is the equivalent of the term “native protein” as used by the majority of workers in this field today, and it is in this context that I used the term “native protein” here and elsewhere.

If we could do it all over again, we might consider reserving the term native protein to protein assuming the conformation in the living cell and introduce a new name for what is now commonly used to designate.

Knowing how difficult it is to change convention, one might as well use the term “native” as it is being used today and introduce a new term for the state of protein existing in the living cells. Indeed, this is what I have done. It is called the living state (Ling, 1984, p. 147; Ling, 1990, Sect. 3.2).

An implicit assumption underlying the introduction of the term living state is that the living state and the native state are as a rule not the same for many important intracellular proteins. A major manifestation of this difference between the two states is underscored by the strong experimental evidence demonstrating that myosin selectively adsorbs K^+ in the living state (Ling and Ochsenfeld, 1991) but it does not do so to the same level and with the same degree of high specificity in the native state.

The reasons why the purest and best (native) hemoglobin — the premier cytoplasmic protein of red blood cells — that one can obtain do not selectively adsorb K^+ over Na^+ at a scale comparable to that believed to exist in the living cells is given in the text (see Figure 14). One prediction of the AI hypothesis is that if the competing fixed cations (for the β - and γ - carboxyl groups) are removed, the hemoglobin will regain its ability of selectively adsorbing K^+ and Na^+ . One way to get rid of the competing fixed cations is by exposure of the protein to high pH and the experiment of Ling and Zhang (1984) has fully confirmed this prediction.

The success of this experiment does not imply that the intracellular pH is high. Quite the contrary, all existing evidence point to a neutral intracellular pH. The success of the high pH experiment only points out that the β - and γ - carboxyl groups have the *inherent capability* of adsorbing alkali metal ions on a large scale. The maintenance of hemoglobin in the living K^+ -adsorbing state depends on *other* mechanism(s) than exposure to an alkaline environment. (This addendum is a reproduction of one of the answers to a question raised by a reviewer in Ling, 1990).

Appendix 1

To obtain an accurate and precise comparison of available and needed energy, it would be desirable that all active metabolism of the muscle cells be brought to a halt. To do so, the

muscles were poisoned simultaneously with pure nitrogen (plus NaCN) (which stopped respiration), and sodium iodoacetate (which stopped glycolysis), at the temperature of 0°C (which should have slowed down the postulated outward pumping of Na⁺ more than its inward diffusion). Despite these severe treatments, the frog muscle maintained for many hours its normal high K⁺ concentration and its low Na⁺ concentration, its normal rate of Na⁺ efflux (virtually all due to pumping according to the basic postulate of the membrane pump theory and the law of physics) as well as its normal resting potential.

On first sight, the total indifference of the K⁺ and Na⁺ levels and other functional activities in frog muscle to the gross interference of its metabolism seemed incompatible with the metabolic pump viewpoint. However, one might argue that frog muscle contains large energy reserve in its high ATP, ADP and creatine phosphate (CrP) contents and it was this energy reserve that was being tapped to energize the Na pump. Indeed, I adopted this idea as my working hypothesis.

Thus, the energy need of the postulated pumping in the muscle — deprived of all its active metabolism — could only have come from the ATP, ADP and CrP in the muscle at the start of the experiment. By analyzing the contents of these compounds at the beginning of the experiment in one set of muscles (but after the poisons have taken their full effects on the target enzymes) and again in their paired muscles at the conclusion of the experiment several hours later, one could determine just how much of the ATP, ADP and CrP had been “used up”. The amounts of energy from each of the “used up” compounds were calculated and tallied to yield the maximum amount of energy that was available to the muscle during this period of time.

Throughout the whole experiment, the Na⁺ concentration remained essentially unchanged at levels much lower than in the external medium. At the same time, individual Na⁺ ions constantly moved in and out of cells. According to Na-pump theory, this unchanging level of Na⁺ is maintained by the continual outward pumping of this ion. Now each mole of (positively charged) Na⁺ ion pumped out must overcome both the (outside-positive) electrical potential gradient equal in magnitude to the resting potential of the muscle fibers measured, and the (outside high) Na⁺-concentration gradient, which is proportional to the logarithm of the ratio of extra- and intracellular Na⁺ concentration. The pumping rate of Na⁺ in moles of Na⁺ per square centimeter of membrane surface per hour could be determined from the labeled-Na⁺-ion efflux rate. From the data gathered, I determined the minimum energy need of the postulated Na pump.

Appendix 2

The animal of choice is small-to medium-sized North American leopard frogs (*Rana pipiens pipiens*, Schreber), preferably from the State of Vermont (e.g., from J. M. Hazen, Alburg, Vermont, USA 05440)(most of Dr. Edelmann’s work was carried out on this variety of frog). After arrival, the frogs are kept for a week in a tank filled to a height of about half-inch of tap water containing tetracycline (about 1 gram per 200 ml; veterinarian’s tetracycline is cheap), which is renewed as needed. Afterward, the frogs are kept preferably in running water or at least in water frequently changed. The aquarium must provide dry areas (e.g., a stone), as frogs do not like sitting in the water all the time. They are force-fed twice a week canned cat food with high protein content, preferably of the seafood variety, fortified with a powdered multivitamins (e.g., Centrum, Lederle, at the

rate of about 1.5 g per 8 ounce can). Artificial light is provided during the day hours. After some time on this regimen, the frogs become healthy and strong. You can tell that this stage has been reached, when the larger specimen will take some effort to hold in one hand. This standardized procedure was developed after the recognition much of the so-called winter frogs were simply starved frogs. Other commercially available animals are often both starved and sick. It was even later did I finally found out that even a good dog food does not contain enough vitamins to sustain captured frogs.

Sterilely isolated frog muscles can be kept in a modified Ringer solution for at least 8 days at 25°C without significant impairment of normal function. The formula for the Ringer solution (Solution 731) is given by Ling and Bohr (1969). It takes about 3 days incubation at 25°C in a modified 731 solution in which the normal 2.5 mM K^+ is largely replaced by Cs^+ or Tl^+ to produce what we called Cs^+ - or Tl^+ -loaded muscles. Presumably the same procedures can be applied to other frog tissues.

For a standardized procedure to produce a killed frog muscle or other frog tissues, I suggest the adoption of the dosage as well as route of administration introduced by E. Lundsgaard when he first discovered the pharmacological action of iodoacetate: inject into the dorsal lymph sac of a frog 0.022 ml of a 0.1 M solution of sodium iodoacetate per gram of fresh frog weight. The injected frog is kept in a darkened moistened container maintained at room temperature (about 25°C). After 5 hours (longer in colder room) the frog is in full rigor, with the rigid muscles held at more or less their normal "relaxed" length. The heart of the injected frog continues to beat long after the drug has reached all parts of the body. As a result, there is a degree of uniformity in the killing of all the tissues difficult to achieve without resorting to elaborate perfusing procedures.

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Discussion with Reviewers

J. Dubochet: The French scientist Jean-Marc Levy-Leblond proposed as an exercise for his physics students to criticize unusual theories as, for example, the one in which gravitation is explained by the fact that the universe is full of particles, rapidly moving in all directions and producing a uniform pressure on any object. The “shadow” of one object on the other produces an unbalanced pressure resulting in an apparent attraction forces. I cannot think of a better way to develop a true understanding -as opposed to the learning by heart of accepted ideas- than by finding out how much such a theory could be correct or why it is wrong. Regardless if association-induction hypothesis of Ling is correct or wrong, it has, at least, the huge advantage to raise good and fundamental question. I have presented it to my students with the same goal as Levy-Leblond. They were strongly challenged and liked it. I have also proposed it to a number of specialists of the cell potential and of the Na-K-ATPase. Most of them had hardly heard of it and they did not like it. The miserable level of reasons invoked by most of them to refuse the theory was the obvious proof that it should not too rapidly be disregarded-at least for didactic reasons.

Author: Prof. Dubochet’s comments concerning the divergent responses of established and young scientists to a new theory echoes similar observations made repeatedly by great scientists of the past (for direct quotations of Lavoisier, Darwin and Planck see Ling 1992, p.vii). I appreciate and agree with Prof. Dubochet’s comment. It is my hope that the freedom of testing and of adopting new ideas as well as the will of young scientists to fight for this freedom will survive in the future, despite the vastly increased power of the established scientists of today than in the past to dictate the future of other scientists both young and not so young. (For another important contribution of Prof. Dubochet see answer to Prof. Clegg’s comment following).

Reviewer I: Although I am a proponent of macromolecules to have hydration shells and of many K not being free but bound to proteins or as a salt, I cannot follow this fundamentalistic separation into two opposed theories; AIH against MPT. Having worked rather intensively on the pumps of *E. coli*, having made numerous experiments on the hydration of phage T4, I simply cannot accept this polarized view. Both theories are true and coexist; although I admit that many cell biologists do not consider bound water sufficiently.

Author: “Science is absolute and truth is everlasting” (Hegel). For every phenomenon in this Universe, no matter how vast or how small, there is a singular immutable solution.

To clarify, let us consider a small part of the truth, represented by the value of π . According to our scientific handbooks, π is correctly determined to be 3.1415926536... (In fact, it is known to far more than the number of decimal points cited here).

Now if from outer space, a delegation of scientists arrived bringing with them the notion that the value of π is not the one we believe in but 3.1415926546...

Hearing of this, should our own scientists for the sake of cosmic harmony, negotiate with these foreign scientists to settle for a value of somewhere between the two extremes, say, 3.1415926541...? I should say no.

Rather the two groups of scientists should carefully examine the foundation research that gave rise to each figure and find out which one is faulted and, if after a long period of time, our figure has successfully stood the test of time, our figure without any negotiated change should be accepted the truth, at this stated level of precision and until new evidence prove otherwise. Truth versus untruth is always black and white.

Reviewer I believes that the membrane-pump (MP) theory and the association-induction (AI) hypothesis are both right, and that the two theories can coexist. In answer I now reiterate the reasons why both theories cannot be right.

First, the basic tenets of the two theories are diametrically opposed. Thus the great majority of K^+ cannot be at once adsorbed and not adsorbed. The bulk of cell water cannot be both free and not free. Second, one of the two theories has been proven wrong a long time ago. As examples, my present article quotes two sets of critical studies (energy balance sheet; Effectively-Membrane-pump-less-Open-ended-Cell preparation (EMOC) studies), each of which alone, provides unequivocal evidence against the pump theory. Neither has been challenged in print in the long period of time since their full presentation in 1962 and 1978 respectively. (However, these two are only a part of the massive (undisputed) evidence against the membrane-pump theory. For a complete presentation, the reader may want to consult my book, “A Revolution in the Physiology of the Living Cell” (Ling, 1992).

Of course, Reviewer I could disagree. In that case, he should publicly explain why he disagrees. However, a careful search through the Citation Index shows that neither Reviewer I nor anyone else has done that.

In a certain qualified way, I feel apologetic to single out Reviewer I for the failure to respond to fundamental scientific challenges. He is vulnerable to my criticism, only because he chose to accept the invitation of reviewing my paper; after all, the failure to respond to these and other challenges was not that of Reviewer I alone (see Prof. Dubochet’s comments above; also, Ling 1988, p. 911-912).

It should also be pointed out that the disproof of the pump model applies only to solute distribution *within* “unifacial cells” like muscle, nerve, erythrocytes, *E. coli*, each in possession of a single uniform cell membrane. Active transport *across* “bifacial cell” systems

(e.g., frog skin, intestinal epithelium layer with two different membranes facing the source and sink of the transport) is a different matter. (For a new theory of active transport across bifacial cell layer based on the AI hypothesis, see Ling 1984, Chapter 17; Ling, 1990a).

Overwhelming evidence against the membrane pump theory is but a part of the reasons for rejecting the coexistence idea Reviewer I believes in. A second reason is that the AI hypothesis can explain known observations that the membrane pump theory can as well as those it cannot. And the AI hypothesis is extensively supported by the result of world-wide testing over a period of more than a quarter of a century.

Not only can the AI hypothesis explain these basic physiological manifestations that have interested biologists from its very early days, but it can do so in a quantitative manner. As pointed out, for example, in Chapter 8 of my recent book (Ling, 1992), the *general* equation for solute distribution in living cells, first briefly presented in 1965 (Ling, 1965, Persp Biol Med 9: 87, Equation 8; Ling, 1984, Equation, 11.5; Ling, 1992, Equation 16), and its various simplified versions (e.g., equation 1 of the present article) can quantitatively describe all known relevant and accurate data on solute distribution in living cells.

Therefore pumps not only violate the basic laws of physics, they are superfluous. Its unnatural preservation not only goes against the basic purpose of the Scientific Method, it also violate another important law of science, i.e., the law of parsimony: “Neither more, nor more onerous causes are to be assigned than are necessary to account for the phenomenon” (William Hamilton).

Reviewer I: I admit that many cell biologists do not consider bound water sufficiently. But not every water is bound! Therefore, also not every ion is bound.

Author: If I am not mistaken, what Reviewer I intended to say is that only part of the cell water and ions are bound. Before commenting on this statement, I would like first to recommend less use of the word *bound* especially in regard to water. This is the terminology of the past, and on many occasions no longer fits what we know now with much greater clarity.

A more appropriate term is “*adsorbed*” (For a full discussion of the meaning of “*adsorption*” see Ling, 1990, p. 756, response to Reviewer IV in middle of the left column). Thus according to the AI hypothesis, water is adsorbed in multilayers on the exposed backbone NH and CO sites of proteins and K^+ is adsorbed singly on β - and γ -carboxyl groups of aspartic and glutamic acid side chains. Water molecules in the state of polarized multilayers lose a substantial part of its rotational freedom (for theory: Ling, 1964, 1969; for experimental evidence: Trantham *et al.*, 1984; Heidorn *et al.*, 1986), but water molecules and K^+ ions, though adsorbed, are continually desorbing and moving away to adsorb on the same or different sites. To say that they are bound — and hence tied down to specific sites — exaggerates the degree of immobilization. On the other hand, there is extensive experimental support for the *adsorption* of K^+ and of the bulk-phase water (for definition of “*adsorption*”, see Ling 1990, p. 756, column 1, or Ling 1992, endnote 5 of Chapter 3; see also *ibid.*, Chapters 4 and 5; Ling 1988, 1988a, 1990).

I already pointed out that as a rule, each ion, nonelectrolyte or other solute can exist either exclusively as free solute dissolved in the cell water, or it may exist in two states: freely dissolved in the cell water and adsorbed on some macromolecules, mostly proteins.

For solutes that exist in the cell water, its concentration in the cell water demonstrates linear distribution pattern, according to equation 1. This is the case for many nonelectrolytes; the distribution curve is a straight line with a slope equal to the q -value of the solute. And as shown in both model systems and living cells, the q -value tends to follow the “size rule” (Figure 8).

For solutes that exist both as free solute and adsorbed — which are the case with both Na^+ and K^+ , then to the rectilinear fraction is added another fraction that is described by either a (hyperbolic) Langmuir adsorption isotherm or the more general Yang-Ling cooperative adsorption isotherm, often S-shaped. The concentration of each fraction of a solute in a living cell depends on the concentration of the adsorption sites for the solute, the concentration of the solute in the external medium, the concentration(s) (if any) of competing species, and other factors. I shall illustrate with the monovalent cation, Na^+ .

Na^+ is usually present at very high concentration in the external medium (e.g., 100 mM). There is also an abundance of potential adsorption sites for this ion. However, it is the presence of K^+ with its much stronger adsorption constant on these sites, that keeps the concentration of cell Na^+ under most conditions rather low (e.g., about 10 $\mu\text{moles/g}$ fresh cells) by reducing the concentration of adsorbed Na^+ . Due to the large size of the hydrated ionic size, and in obedience to the size rule, Na^+ has a rather low q -value. As a result, free Na^+ in the cell water occurs only at about one-tenth the concentration in the external medium, i.e., 10 mM. So roughly speaking, half of the cell Na^+ is free and half adsorbed.

K^+ exists at a much lower concentration in the external medium, i.e., 2.5 mM. However, it has a much stronger adsorption constant than its chief competitor Na^+ . As a result virtually all the cell K^+ is adsorbed on β - and γ -carboxyl groups in the cell. The q -value of K^+ is somewhat higher than that of Na^+ . However, since the external K^+ concentration is so low (2.5 mM in frog plasma), less than 1 mM of free K^+ exists in (muscle) cell water. About 98% of the cell K^+ is adsorbed.

As a rule, virtually all the cell water exists in the state of polarized multilayers. Again there are all kinds of supportive evidence for this belief. As an example, ultrahigh frequency dielectric measurements of Clegg and coworkers (1984) showed that in brine shrimp cyst cells, there is no indication of the existence of normal liquid water.

Reviewer I: When you know all the work of Pollard and other cell biologists on the gel-sol conversions of the protoplasm, you can simply not write an article as if everybody would deny the protoplasm being a colloid. And when one knows now that the cytoplasm consists of fibrous and globular macromolecule then the old-fashioned concepts are completely revitalized!

Author: I had not said that *everybody* denies that the protoplasm being a colloid. I mentioned that the once prosperous English-language Journal of Colloid Chemistry went out of business and the peripheral events associated with this demise. After all, scientists do not frequently give up a long-established branch of science without a good reason. And I was trying to tell the reader what I think to be the reason(s) that looked good at the time (but no longer now).

I do not and have not denied that some scientists like Dr. Pollard mentioned may continue to use terms introduced by colloid chemists long ago. But there is no denial that the great majority of scientists involved with the study of living cells use such colloidal terms

sparingly, if at all. Indeed, one may ask, "Why should they do otherwise?" After all, the best definition of *colloids* is a solution or suspension containing large molecules of certain size. For such a system, one might just as well call it a macromolecular system. There is not much cause for joy, if classical colloid chemistry *as such* is indeed being revitalized as Reviewer I claims, and if being colloidal only means the presence of globular and fibrous macromolecules. Under that condition, I suspect that those who have been getting along without classical colloid chemistry may continue their disregard of colloidal chemistry without really missing anything.

One might argue that if classical colloid chemists of the 1920's and 1930's, like Drs. Ross Gortner, Martin Fisher were given more support and acceptance, they might have discovered much earlier what I was to discover years later: a new and more realistic definition of colloids (see text) and with it, further and deeper understanding of the living cell might have materialized than that achieved under the uncertain guidance of the membrane-pump theory.

Reviewer I: Now, to the bacteria: When the respiration of *E. coli* is arrested by KCN or O₂ starvation K leaks rapidly out and is replaced by Na of the medium. *The cell is still alive* and not a single macromolecule is lost. As soon as respiration starts again, energy dependent *pumping* expulses Na and pumps K in. The same is true for Mg, but as a slower rate. There is suggestive evidence that Mg is entirely bound to DNA; the dissociation is obviously not instantaneous. It is interesting to note here that all chemical fixatives commonly used *also* induce rapid leakage of K and Mg.

Author: It is well known that the strength of a chain does not depend on the strength of its strong links; it depends only on the strength (or lack of it) of its weakest link. Similarly the validity of a scientific hypothesis depends less critically on the strength of evidence that appears to support the hypothesis, it critically depends on other available and unexplained evidence that contradicts the hypothesis. (I shall call this the Rule of the Weakest Link).

The Rule of Weakest Link does not denigrate the importance of positive supportive evidence. On the contrary, positive supportive evidence is vital in affirming a theory; but only *after* it has been shown that a theory is not contradicted by major insurmountable negative evidence.

Positive supportive evidence varies tremendously in significance. Some positive evidence are very important because they confirm a unique prediction of a hypothesis. Thus the evidence that by sailing west one can arrive at the same location that one can reach by sailing east offers strong positive evidence for the hypothesis that the earth is round. This evidence cannot be explained by the only known alternative theory i.e., flat-earth theory.

In contrast, the kind of positive evidence Reviewer I offers in support of the membrane-pump theory is weak evidence. It can be explained just as well by the association-induction hypothesis (only in a more rigorous and quantitative manner). Indeed, the literature from the 1930's to the 1950's abounds in reports like those described by Reviewer I (for a list of references to some of these publications, see Ling, 1984, p. 61). Not only interference with metabolism by anoxia, cyanide (which suppress respiration), iodoacetate (which arrests glycolysis) but cooling to 0°C all produce loss of K⁺ and gain of Na⁺ reversibly in some, irreversibly in others in a variety of plant, animal and microbial cells.

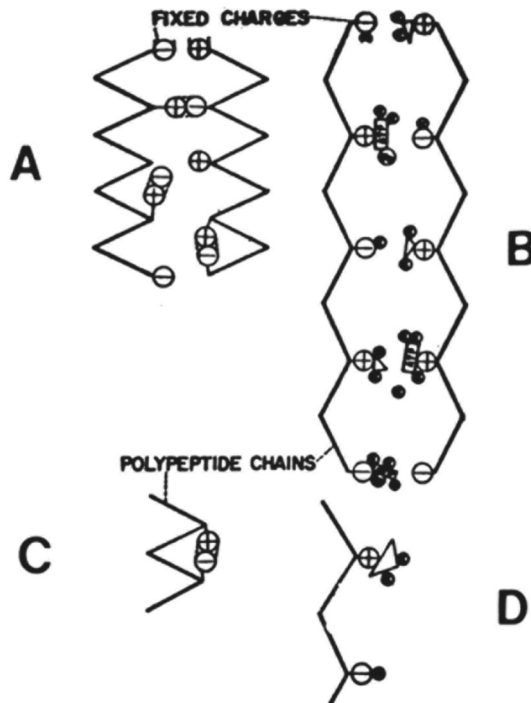


FIGURE A. Diagrammatic illustration of K^+ and anions (ATP, creatine phosphate represented as triangles) act together to split up salt-linkages formed between fixed (β - and γ -carboxyl groups and cationic ϵ -amino and guanidyl groups by adsorption onto the respective “liberated” sites. In this specific model multivalent anions offer extra sites for more K^+ adsorption; this concept was later abandoned for reasons given in Ling 1990, answer to question of Dr. Cameron (From Ling, 1952).

However, there are also experimental results of a different kind. They *contradict* the membrane-pump theory. These contradictory experimental data include the demonstration that prolonged exposure to anoxia, iodoacetate in addition to chilling (0°C), failed to produce any significant loss of K^+ in either frog muscle or frog nerve (Table 5 on p. 765 in Ling, 1952).

However, if one allows the experiment to last 50 instead of 5 hours, very slow changes occur as illustrated in Figure 15 at 0°C . More rapid changes of the K^+ and Na^+ concentrations occur in frog muscles if kept at room temperature.

If Reviewer I is right that this loss of K^+ and gain of Na^+ truly reflects the dysfunction of the Na pump, there is a good way of testing this hypothesis: Given the polarity of the resting potential, and the 10 times lower concentration of Na^+ in resting frog muscle cells, the laws of physics require that virtually all the outward fluxing Na^+ to be by pumping (see Ling, Fed Proc Symp 24: S103, footnote on p. 107). As a result, the membrane pump theory predicts a sharp decline in the rate of Na^+ efflux in response to metabolic interference. Indeed, such Na^+ -efflux studies had been performed in two different ways: at 0°C (in which there is no appreciable change of the total cell K^+ and Na^+ concentrations) and

at 25°C (at which there is rapid change of the K^+ and Na^+ concentration). In either case, there was no detectable change of the rate of Na^+ efflux.

Let me add further that this indifference of the Na^+ efflux rate to metabolic inhibition had already been reported by me as far back as 1952 (Ling 1952, p. 766). My finding was later fully confirmed by R. D. Keynes and G. W. Maisel using IAA and cyanide (Proc Roy Soc B142: 383, 1954) and again later by E. J. Conway and coworkers using IAA alone (J Physiol (London) 155: 263, 1961). Thus as far as experimental data is concerned, there is unanimity.

Reviewer I: Leirimo *et al.* (Biochemistry 26: 2095–2101 (1987)) have shown that the charge equilibrium of K^+ is largely achieved by glutamate⁻, certainly not Cl^- , as we believed for a long time. Overall, the proteins are approximately neutral, what means that about equal amounts of both anions and cations are bound. Some of the charged groups of macromolecules interact with each other (acidic groups of DNA with basic groups of histones and in the binding of proteins into oligo- and polymers which mostly involve several side chains; charged, hydrophobic and H-bonding).

Author: In Figure 14 of my paper being reviewed, I have diagrammatically illustrated that for a mole of K^+ to be successfully adsorbed onto a mole of β - and γ -carboxyl groups, there is the need of an equivalent amount of “congruous anions” (in addition to ATP and the Protein-X) to be adsorbed onto the fixed cation(s). The nature of congruous anions varies from cell type to cell type. In muscle, it is mostly organic phosphates, in human erythrocytes, it is Cl^- ; in mammalian brain cortex and retina, it is primarily glutamate (Ling, 1962, p. 251; Ling, 1990, p. 751, response to I. Cameron). Thus if Reviewer I cited Leirimo *et al.*'s work and pointing out how it supports my thesis illustrated in Figure 14, I would have understood why he cited Leirimo *et al.*'s recent work. However, Reviewer I did not make this connection. On the contrary, this case was cited to affirm his coexistence theory.

Yet there is little question that Leirimo *et al.*'s explanation that both K^+ and glutamate adsorb onto the cell proteins confirms precisely what I described in Figure 14. But the key concept of equal molarity (or more correctly for a more general case with multivalent ions, equal normality) of K^+ and congruous anions are adsorbed on the protein involved was first published by me many years before Leirimo *et al.* (1987) (see Ling, 1952), and a diagrammatic illustration of my earlier view published in 1952 is reproduced here as Figure A. (Only here the congruous anions are the organic phosphates e.g., ATP and creatine phosphate since I was then dealing primarily with muscle).

Reviewer I's quotation of Leirimo *et al.*'s 1987 publication reminds me of the same kind of accusation that I received once for failing to give credit to a third author's idea. However, after a little more careful literature search, it turned out that at the bottom of the confusion was the third author's failure to cite the same idea I had introduced many years before the third author (see Ling, 1990, Comments of and answer to Reviewer IV, p. 755).

Reviewer I: The whole pH homeostasis is also achieved at ATPase dependent proton pumping. These facts can simply not be ignored. Use of dead bacteria, as ion-exchange has been successful also, clearly allowing binding of ions (Damadian R (1971) Biophys J 11: 739–761). Pumps and binding coexist and are not exclusive.

Author: Here the reviewer reiterates his assertion that pumps and binding coexist. My answer is the same as before: they cannot and for the same reasons given. In fact, Mitchell's Chemiosmotic hypothesis is no more valid than the garden varieties of membrane pump, in violating a most inviolable law of physics: the Law of Conservation of Energy. However, I did more than just pointing out this violation. Thirty-three years ago, I published a 67-page article entitled "Oxidative Phosphorylation and Mitochondrial Physiology: A Critical Review of Chemiosmotic Theory and Reinterpretation by the Association- Induction Hypothesis", detailing why the Chemiosmotic hypothesis was not tenable and more (Physiol. Chem. Phys. 13: 29–96, 1981).

I have no argument with the finding of Raymond Damadian, which Reviewer I quoted. Dr. Damadian is a life-long friend who no more believes in the membrane pumps than I do. He and his student investigated the energy balance of *E. coli* and published their results in an article entitled "Caloric Catastrophe" (see Minkoff and Damadian, 1973).

Reviewer I: Obviously the work with cut muscle cells as presented by Ling is of interest; but, facing all other evidences, it cannot be used to dismiss pumps. The results are interesting for showing the slow release of bound ion. But who is doubting that acid and basic side chains of proteins are not neutralized? Every biochemist knows that his DNA is not in the form of acid but as a sodium salt. I admit that some biochemists forget this and make interesting mistakes. Still, one cannot generalize and insinuate by writing that they assume DNA to be a free acid.

Author: If there is one piece of decisive, unequivocal evidence that has proved beyond doubt that a certain man has committed murder, he is a murderer regardless of how many other pieces of evidence exist showing that he has been (otherwise) a law-abiding citizen. Similarly if there is one piece of decisive, unequivocal evidence that has proved beyond doubt that a certain scientific hypothesis is invalid, the hypothesis is not valid, regardless of how many other pieces of evidence exist which support this hypothesis. Of course, I am only reiterating what I call the Rule of Weakest Link in response to a similar question the Reviewer had raised earlier.

The EMOC experiment Reviewer I refers to is the second decisive, unequivocal evidence I chose to present in this review, disproving the membrane-pump hypothesis. As mentioned this evidence and conclusion drawn from it has been in the literature for more than thirty-five years. None has publicly disputed it.

My answer to the reviewer's rhetorical question "Who is doubting that acid and basic side chains of protein are not neutralized?" is that I really don't know. The really relevant question is not whether or not the polar side chains are neutralized; the really relevant question is why most isolated native proteins including those isolated from living cells (e.g., hemoglobin which makes up 97% of the intracellular proteins of red blood cells) do not adsorb K^+ or Na^+ at all (see Carr, 1956, Arch Biochem Biophys 40: 286). This was the fact that led me to propose (what I later referred to as) the Salt-linkage hypothesis in 1952 (Ling, 1952) and it was not until 32 years later that Ling and Zhang (1984) confirmed this hypothesis, according to which, the reason that hemoglobin (and most isolated native proteins (for definition, see Ling, 1990, p. 760, column 1, answer to B.L. Gupta, or alternatively, Ling, 1991, endnote 4, Chapter 3) do not adsorb K^+ nor Na^+ is not because the β - and γ -carboxyl groups are not neutralized. They are, but by the wrong cations, i.e., the fixed anions belonging to the same hemoglobin molecules forming salt linkages.

When these salt linkages are broken up as by high pH which neutralized the electric charge of the fixed cations, the β - and γ -carboxyl groups thus liberated then precisely and stoichiometrically adsorb K^+ and Na^+ . However the high selectivity of K^+ over Na^+ observed in living cells is not seen. In fact, there is a slight preference for Na^+ over K^+ . To explain that, one has to go to another chapter of the association-induction hypothesis that falls out of the confine of the present paper.

I am not sure that I agree with the reviewer that "Every biochemist knows that his DNA is not in the form of acid but as a sodium salt." I agree even less with his statement "Still, one cannot generalize and insinuate by writing that they assume DNA to be a free acid". Why Reviewer I makes a statement like this is obscure to me. It seems that whether or not DNA exists as a Na^+ salt or as an acid depends on the past experience of the DNA. Fresh out of the bottle from a commercial supplier, the DNA one purchases is often in the sodium form. However, if the dissolved DNA is passed through a cation resin column in the acid form, he will get DNA in the acid form.

Even worse, nowhere in my manuscript did I even mention DNA, much less that it is in the free acid form. I did discuss DNA at length in my monograph "In Search of the Physical Basis of Life" (Ling, 1984), in Chapter 18 on "The Control of Protein Synthesis" and in Chapter 19 on "Growth and Differentiation". *In vivo*, DNA can be coupled to nuclear proteins or ions. Even there I made no comment on what other biochemists believe or do not believe that DNA is in the free acid form.

Reviewer I: I can only recommend the author to complement his work on cut muscle cells with facts from well-defined cells, as are bacteria. They are amenable to significant experiments and provide unambiguous fact.

Author: I and my immediate associates have been working with a wide range of other living cell types including frog nerve, frog kidney, frog ovarian eggs, rat diaphragm muscles, ox retina, human red blood cells, amphibian red blood cells, hamster and rat intestinal epithelium, fourteen types of mouse cancer cells, five type of rat cancer cells, yeast cells, *Chlorella* cells, *E. coli* (see Ling 1962, p. 255) and so on. My favorite has been the frog muscle.

It is hard to understand just what does the reviewer mean when he refers to "well-defined cells such as bacteria". Well-defined in regard to what? Genetics, yes. But my primary interest is not genetics. Cell physiology is my main subject. As an investigator on the subject of cell physiology, I have not come upon any tissue or cell type that matches the frog muscle for studying cell physiology in the tremendous advantages it offers both in what we already know about the cell and what it offers for future investigation on the important subjects of the living phenomenon.

The claim that only "well defined cells like bacteria can provide unambiguous facts" reflects a tunnel-vision of the association-induction hypothesis: Cellular action and resting potential can be easily and directly studied in frog muscle cells; it cannot be easily and directly measured in tiny cells like bacteria. K^+ localization studies represent another subject that the striated nature of the muscle cell structure makes it uniquely suitable. Again bacterial cells are too small and too undifferentiated to serve this purpose. EMOC preparation can only be made on long cells like frog muscle cells. Again, there is no way to make EMOC preparations out of tiny *E. coli*.

Reviewer I: Instead of trying to refute pumps, why not give the experimental evidence for a large proportion of bound ions? Why must all ions be bound such that the pumps cannot work? After all, even the rather densely packed bacteria have 80% water. As mentioned already, not all of it can be bound and by that structured; solutions of DNA of 10 mg/ml can be made very easily *in vitro*. We have made equilibrium dialysis experiments with such DNA solutions. They are easily feasible and here the ion sensitive electrodes facilitate the work! As to DNA, the same could be done with other macromolecules, like proteins.

Author: A large amount of our foundation work was done with the equilibrium dialysis method. As an example, the data of Figure 8 may be mentioned.

The reviewer seems to feel a strong protective feeling toward the pump-concept as if it were a close friend or relative. It is only with this sentiment behind can one understand the statement "Why must all ions be bound such that the pumps cannot work?"

But the pump is only a man-made hypothesis. There is no reason to protect it or keep it after it has been clearly shown to be incorrect and superfluous. We have exhaustively demonstrated that the bulk of cell ions is adsorbed and most of the work has long been published and reviewed in my books "In Search of the Physical Basis of Life" and "A Revolution in the Physiology of the Living Cell" mentioned above (Ling, 1992).

As to the question how much of a solute in the cell is adsorbed and how much is free, I have already answered the question above and will not repeat again.

H.E. Rorschach: Ling refers in the text and in Figure 6 to a low (negative) energy state; I would guess that this means a more positive, or higher energy state. I believe it would be clearer to refer to the low energy as the more negative energy and the high energy as the less negative or more positive energy. This would change the way in which Figure 8 is plotted.

Author: I do appreciate this comment as I am aware of the awkwardness in using the term higher (negative) energy, lower (negative) energy etc. But after much deliberation, I decided to use this clumsy system rather than the alternative of more negative energy and more positive energy. The basis for this choice is as follows: I am primarily addressing non-physicists. For a non-physicist, that *more negative energy* denotes *stronger attraction* may not be self-evident. Thus, it is in the nature of common sense that two people strongly attracted to each other have *higher* (something) for each other (rather than lower (something) for each other). Following this instinctive feeling, when one says that for a pair of oppositely charged ions with *strong* affinity for each other to have more negative energy is not as clear as to say that they have high (something) between them. Once I accept this notion, that (something) can only be "(negative) energy".

L. Edelmann: I propose to change the title because over 90% of the article deals with the living structure as it emerges from physiological experiments: furthermore, the cited freeze-drying and freeze-substitution methods yield preparations in which the main component of a cell (namely water) is missing; hence the structure which can be visualized in these preparations is most likely not identical with the living structure even if some properties of proteins or another macromolecule are captured. An adequate title could be "The Living Structure of a Cell." In my opinion this title would be as effective as the old one

to stimulate others to investigate living structure or properties of living structure by electron microscopic methods.

Author: In view of the key role Dr. Edelman's work has played in the thesis I presented here, his opinion is never taken lightly. Yet after much deliberation, I still prefer to retain the old title. The main reason for this decision is that to switch to the shorter title suggested something important may be lost.

"Can We See the Living Structure of a Cell?" is more than just a technical question asked. It also poses a philosophical question. It is this philosophical component that will be lost in switching to the more technical title: "The Living Structure of a Cell".

It is true that 90% of the text is devoted to explaining the meaning of the living structure and its historical background. While a title is often intended to represent accurately what are between the covers, a title may also be something different, e.g., an invitation to a new adventure (the living structure) via something the potential reader already has an interest in: seeing.

According to the AI hypothesis, being alive means the maintenance of the complex protein-ion-water system in a specific high(negative)-energy-low-entropy state, called the living state. Therefore the living structure may be arbitrarily resolved into two components: the structure and the specific energy state. The living energy state is all or none; the structure is not. A person who has lost a great proportion of his bodily parts can still be alive. For the same reason a cell may be kept at the living state even after its major component part, water has been replaced by D₂O or whatsoever without toppling the energy state into its dead state.

I also understand why Dr. Edelman is conservative in regard to the question whether or not his own work has led to the visualization of the living structure of a cell. On the other hand, I, as a theorist, need not be equally conservative, as long as I do not misrepresent anything. Remember that I am primarily asking a question and giving the reasoning why I think electron microscopists using vitrification methods in general and Dr. Edelman in particular have come close to seeing the living structure. Whether I am right or not awaits future investigations.

L. Edelman: Your discussion of life is very interesting, in particular the idea of organization (supported by embryo experiments). However one may argue against your opinion that only dead mechanical parts can be visualized if the membrane-pump theory has some validity. One may argue that it is the organization of all the cellular membranes which keeps a cell alive and that this organization (living structure) may eventually be visualized and completely understood. It is apparently this belief (paradigm) that has lead to the dominance of membrane research in biological sciences. The revolutionary difference between this belief and your theory is that not only a tiny fraction of a cell but the whole living cell consists of living structure.

Author: "Life is organization" is a concept that could trace its origin to Lamarck (life as "état de choses") or even Aristotle (equating life-as-soul with form) (see Hall, 1969, p. 19). I think life is more than just organization; it is organization maintained at the living state. Indeed, as I mentioned in the text, a perfectly preserved dead cell may have organization but not the living state. In my view, Dr. Edelman's thin section has both organization and the living state because it can demonstrate a specific attribute of the living state, i.e, selective accumulation of K⁺ and its surrogate ions Cs⁺.

To study living structure of any kind, including the living cell membrane, one cannot separate physiological “living” from anatomical “structure”. Therefore if one wants to entertain the idea that only the cell membranes are alive and the rest is dead mechanical parts, one must be able to demonstrate that *isolated* membrane can *do* what the *whole* living cells can *do*.

Attempts in this direction were made again and again but these efforts had failed (see Ling, 1992, Section 2.3 and Section 2.4.4.). As an example, I may mention that a squid axon can be freed of its cytoplasm without impairing its physiological functions of the cell membrane, as witnessed by the maintained capability of conducting perfectly normal action potentials. When filled with sea water containing energy sources and tied at both ends, a membrane sac of the squid axon membrane offered an ideal preparation to test the prediction of the membrane-alive-everything-else-dead hypothesis: *net* outward transport of Na^+ and net inward transport of K^+ should follow incubation. Efforts in this direction by some of the most skilled workers failed.

In contrast, muscle cell deprived of a functional cell membrane (the EMOC preparation), or thin sections of freeze-dried or freeze-substituted muscle cells containing no intact membrane covering, selective K^+ accumulation over Na^+ persists.

Having said this, I want to point out that the pump concept is truly a part of the *emergentist view of life*. It is the continued *activity* — and not the mechanical parts that are engaged in — locomotion, metabolism, growth and membrane-pumping that represents life. From this viewpoint discussed in the text, even the membranes and their postulated pumps are in theory not alive.

G.M. Roomans: I have commented about the discussion on “life” and “death” with regard to frozen embryos as reviewer of a previous paper. I still feel that this discussion does not add anything to the scientific concept of the paper. That does not mean that the point has no philosophical interest (so has the question of the existence of an immortal soul). However, it cannot serve as a discriminator between scientific theories.

Author: I regret that I must express a different opinion again from that of Dr. Roomans on this subject — just as I did in response to the comments he made in his review of my earlier paper (Ling 1988, p. 100) on the same issue, i.e., the appropriateness of citing the survival of frozen embryo at near absolute zero temperature as evidence in choosing between the two alternative theories of life and death. I used this fact to stress that life cannot be defined as *continued functional activity* (which comes to a halt at such a low temperature) but can be defined as representing a state, the living state.

Electron microscopists of the past might not have been much interested in the question of life and death, because the specimen they used to prepare began with cell-killing.

What I have tried to demonstrate here is that with increasingly more sophisticated methods of vitrification through rapid freezing, freeze-drying and freeze-substitution as well as cryosectioning, electron microscopists have now attained a new level of achievement, i.e., seeing the *living* structure. In this effort, I must be able to recognize what is living and what is dead. Therefore I disagree with Dr. Roomans that talking about life and death in my paper is irrelevant. But I do agree with him that talking about “immortal soul” might be considered irrelevant if it is not a reference taken out of context.

G.M. Roomans: One might argue, that thermodynamics teach us that the energy required to maintain a K^+ gradient and a Na^+ gradient over the cell membrane does not depend on the way these gradients are created. This would imply that the same energy that the membrane-pump theory has to spend on keeping the membrane pumps going, in the AI hypothesis has to be spent on keeping the cellular macromolecules in the “living state”. How can one then discriminate between the two theories on the basis of energy requirement?

Author: The two theories are built on entirely different basic concepts and as a result, their respective needs of energy are disparate. And it is for this reason, that energy requirement constitute one of the key discriminating issues between the two theories.

In the membrane pump theory, the bulk of cell water and ions are free as in the surrounding extracellular fluid. The chemical potentials of say, Na^+ in the cell water and the external solution are respectively:

$$u_{iNa} = u_i^0 + RT \ln a_{iNa} \quad (A)$$

and

$$u_{oNa} = u_o^0 + RT \ln a_{oNa} \quad (B)$$

where a_{iNa} and a_{oNa} are the intra- and extracellular Na^+ activity, respectively; u_i^0 and u_o^0 are the standard chemical potential for Na^+ in the intracellular and extracellular phases respectively and they are equal. The chemical potential gradient Δu between the two phases is thus described by the following equation:

$$\Delta u = u_{iNa} - u_{oNa} = RT \ln (a_{iNa} / a_{oNa}) \quad (C)$$

The condition for equilibrium is that u_{iNa} and u_{oNa} are equal. Thus at equilibrium, the intracellular and extracellular activities of Na^+ must be equal. Yet in living cells the intracellular activity of Na^+ is only a fraction of that in the external solution. Therefore, energy must be spent continually to maintain this non-equilibrium distribution (by pumping), much as pumping is necessary to keep the water level in a leaky boat afloat. When gasoline used to pump the water is gone, the boat sinks, with equalization of the levels of water in and out of the boat.

An altogether different situation exists with AI hypothesis. For simplicity, let us only consider the Na^+ in the cell water (and disregard here the fraction of Na^+ that is adsorbed on protein β - and γ -carboxyl groups). Since cell water exists in the state of polarized multilayers, the standard chemical potential of Na^+ in the cell water is quite different from that in the external free water solution. The result is that at equilibrium when u_{iNa} and u_{oNa} are equal,

$$u_{iNa}^0 - u_{oNa}^0 = RT \ln (a_{iNa} / a_{oNa}) \quad (D)$$

Since the standard chemical potentials are not equal, the activities (or roughly, the concentrations) of Na^+ in the cell water and external solution are not equal. Indeed, it can be shown that

$$\exp \{ (u_{iNa}^o - u_{oNa}^o) / RT \} = q \quad (E)$$

where q , the equilibrium distribution coefficient was described in the text in Section (b). Combining equations D and E, one obtains equation 1 of the text:

$$q = a_{iNa} / a_{oNa} \quad (I)$$

Thus in the AI Hypothesis, no energy expenditure is needed to maintain the observed concentration gradient because the asymmetrical distribution of this and other solutes is an expression of an equilibrium phenomenon and as such requires no continual energy expenditure (in sharp contrast from the case of the membrane pump theory). In simpler language, the solubility of Na^+ in the cell water is lower than in the external free aqueous solution; to maintain such a difference in the solubility of a substance in two contiguous liquid phases does not require continued energy expenditure.

Indeed one may go one step further and ask why is the solubility of Na^+ lower in cell water than in normal free liquid water. For this, thermodynamics cannot offer us a mechanism. However, statistical mechanics can and does.

The key question here is, What does the standard chemical potential really represent? In the simplest case one can devise, the standard chemical potential is determined by the following equation:

$$u^o = -RT \ln (p.f.) \quad (F)$$

where R and T are respectively the gas constant and absolute temperature respectively; (p.f.) represents the statistical mechanic's "partition function", which is defined the sum of a series of exponential terms:

$$(p.f.) = \sum \exp (-\epsilon_r/kT) \quad (G)$$

where ϵ_r is the energy of a specific r th quantized state, k the Boltzmann constant and T , once more the absolute temperature.

From equations E and F, one derives:

$$q = (p.f.)_o / (p.f.)_i \exp(-E/RT) \quad (H)$$

where $(p.f.)_o$ and $(p.f.)_i$ are the partition functions of the (hydrated) Na^+ ion in the external medium and in the cell water respectively and E is energy difference in the ground states of the quantum-mechanical energy levels in the two media. Now each partition function is a product of several different partition functions, each representing the energy states corresponding to most importantly, the translational, vibrational and rotational motions of the hydrated ion.

Due to its large size and hence many rotational axes of symmetry, the dominant partition function of the hydrated ions is the rotational partition function, and it is this partition function that suffers the greatest proportional loss in the polarized water since each water molecule is polarized and thus anchored to its neighboring water molecules. This is one of the reasons for the size-dependent solute exclusion predicted and observed (see Figure 8).

Another cause for the size-dependency in solute exclusion is due to term E of equation H. A larger hole must be dug in the polarized water to accommodate a large solute in its inward transfer from the external medium. However the energy holding the water together in the polarized water is higher than in the external medium of free water. As a result more energy is spent in excavating the hole than gained in filling up the hole left behind. Again, the larger the solute the larger the energy difference and the smaller the q-value. However, this is what has been referred to as the bulk-phase enthalpy term.

There is in addition a surface enthalpy term. Thus if the surface structure of the solute is such that it can fit better than water itself into the structure of polarized multilayers of the surrounding water, then this component will tend to favor the transfer of the solute into the polarized cell water. Under certain condition, this favorable surface energy component can counterbalance the unfavorable bulk phase energy and entropy components and as a result, we find solutes with q-values higher than their respective molecular weights predict. As mentioned in the text, I feel that this might be one of the reasons for the high q-values of glycerol and its water-structure-stabilizing effect contributes to its cryoprotective efficacy.

G.M. Roomans: Diseases such as cystic fibrosis and diarrhoea would be highly interesting to study from the AI-hypothesis point of view, since they involve a defect in the regulation of normal ion transport. The question then becomes: where in the cell is this defect situated. Recently, the gene of which a mutation causes cystic fibrosis has been identified. The gene codes a protein called the cystic fibrosis transmembrane regulator (CFTR) protein, that is believed to regulate a chloride channel in the apical membrane of epithelial cell. In this view, the CFTR protein could be closely associated with the (apical) cell membrane. However, according to my understanding of the AI-hypothesis transepithelial transport would be described by an adsorption of chloride ions to cellular macromolecules followed by a unidirectional release following a conformation change of these macromolecules. If the membrane is not important in regulating ion transport, the localization of CFTR should be in the cytoplasm rather than in the membrane. Would this be a valid test of the AI hypothesis? In addition, the site of action of commonly used diuretic drugs would be interesting to study from this point of view.

Author: These are interesting suggestions. Apropos of the cause of diarrheal diseases, I may mention that in 1965 I took part in a Cholera Research Symposium and published my talk later in the Proceeding of the Cholera Research Symposium, entitled "Thoughts on the Molecular Mechanism of Normal Intestinal Mucosa as a Barrier to Sodium Ion Movement and Massive Fluid Loss in Cholera." I suggested then that normal mucosal cells (and intestinal blood vessels) offer effective resistance to the massive loss of water and electrolytes not just due to its cell membrane but to a large extent due to the resistance of the cytoplasm containing water in the state of polarized multilayers. A prediction of this hypothesis is that thicker mucosa offers greater resistance toward diarrhoeal diseases. In support of this idea was the remarkable finding that well-nourished people are highly resistant to cholera and they characteristically have very thick intestinal mucosa. In contrast, cholera patients as a rule have very thin intestinal mucosa not in consequence of the infection (as once believed) but a precondition leading to the high susceptibility. It would be interesting to see if this relationship between mucosal thickness and susceptibility to diarrhoeal diseases extend to other types of infections and if it is related to the greater susceptibility of children to diarrhoea.

In my model of active transport across frog skin, intestinal and kidney epithelia and other bifacial cell layers, the cytoplasmic protein nicknamed the “sponge protein” plays the central role. However, both the serosal and mucosal surface membrane also plays key roles in providing the basic mechanism of the unidirectionality. Thus if a drug turns out to act at either one of these membranes, rather than the cytoplasmic proteins it does not contraindicate this model. On the other hand, if it can be demonstrated that the locations of the key protein or drug action is in the cytoplasm, it would support the AI model.

At this junction, it may be appropriate to mention that we already know of instances where a protein supposedly to be located in the cell membrane turned out to be otherwise. This concerns what was called β -galactoside permease from *E. coli*. In 1966, Kolber and Stein published in *Nature* (209: 691) results of their success in isolating from *E. coli* this famous protein, specified by the Y gene. To their astonishment, the permease could not be found in the membrane fraction. Instead it all came from the cytoplasm. It seems that the procedure Kolber and Stein used might well be adaptable to studies aimed at pinpointing the location of CFTR protein.

J.S. Clegg: Although your hypothesis is based heavily on results from the study of striated vertebrate muscle (including the architecture of its myofilament system) if it is to be a general one, applicable to all animal cell, then result of non-muscle cell must also be included which are not referred to in the paper. Thus Keith Porter and his associates have more than the last decade produced a large body of high voltage electron microscopy (EM) observations on the cytoplasmic organization of animal (non-muscle) cells, which he refers to as the microtrabecular lattice (MTL); see Porter KR (1966) Structural organization of the cytomatrix. In: *Organization of Cell Metabolism* (GR Welch and JS Clegg, eds) pp. 9–26. Plenum Press, New York. These “fixed” images of cytoplasmic organization have been supplemented by studies on intact cells using fluorescence methods which confirm the existence of a highly cross-linked network of cytoplasmic fibrils (see Luby-Phelps K, Lanni K and Taylor DL (1988): The submicroscopic properties of cytoplasm as a determinant of cellular function. *Ann Rev Biophys Chem* 17: 369–396). These aspects and others on this issue have been reviewed frequently (see Clegg JS and Barrios MB (1989) The Cytosol”: a neglected and poorly understood compartment of eukaryotic cells, In: *Cell Function and Disease* (L Canedo and L Packer, eds.) pp. 159–170. Plenum Press, NY.) These citations are only a small sampling of the information available on “cytoplasmic organization” Thus, if Dr. Ling is to consider the hypothesis a general one (for animal cells), why doesn’t he consider this massive body of literature?

Author: I am sure that every writer of a scientific review paper confronts the same eternal problem: How to give credit and recognition to every other publication that deserves to be? The problem is not in collecting every paper that has bearing on the title but a matter of the limitation of the human mind. That is, at any one time, it can only absorb a very limited amount of information. Therefore merely listing every paper which a computer search can gather only clutter up the paper and make it difficult to understand, and the names and journal citations would be ignored.

Therefore, the next best choice is to cite only those papers that are directly relevant to the subject matter of the paper. As the reader of the present article knows by now, it is my opinion that biomedical teaching and research in the last half of a century has been under the strong domination of membrane pump theory. Since the early fifties, A.S. Troshin and

I have devoted our lives to return the lead of biological research abandoned since the later thirties and early forties.

The profoundly different emphasis of the two theories (membrane-pump vs AI hypothesis) mostly regrettably makes a huge amount of the experimental findings published by many scientists of limited use to the further investigation of the living phenomenon under the guidance of the AI hypothesis.

Having said all this, I must add that I am glad Dr. Clegg had taken the trouble to provide the detailed references of the multitude of papers which I did not cite in the present paper (but may do so in the future).

However, I am not unaware of Dr. Porter's work, having listened on a number of occasions to the 3-D presentation of high-powered EM pictures. My main comment on this particular approach is that with the use of regular glutaraldehyde fixation, the trabecular network seen in the EM plates might not accurately represent how these fibrous network really look like as part of the living structure, even though their pervasive presence in the cell is highly suggestive for a role like that envisaged for certain water-polarizing proteins in the cell (see below). Since I have only a limited space, I naturally prefer to discuss at length the kind of EM work like that of Dr. Edelmänn which from its beginning has attempted to preserve the living structure. It is my hope that the present paper and the work of Dr. Edelmänn might stimulate such highly capable and skilled workers to make use of the cryofixation, freeze-substitution and other advanced techniques the participants of this conference have so eloquently demonstrated.

Finally, Dr. Clegg raised the same question that was raised by another reviewer of another earlier publication in Scanning Microscopy (see Ling, 1990, p. 766 question of Dr. Gupta and answer): Can you develop a general theory for all living cells from the investigation of one cell type, frog muscle.

First a correction: I did not derive my major conclusion from the study of one cell type. I studied a variety of cell types, including frog muscle, frog eggs, human erythrocytes, many types of cancer cells etc. etc.

The study of phylogeny and of ontogeny teaches us that all cells have a common origin. That is, different cell types are like different models of the same design. Therefore basic properties like those discussed in this paper are most likely very similar in all cell types — and here I am *not* limiting myself to animal cells but include all living cells. As in my answer to Dr. Gupta's similar question raised earlier, I answer: the laws of inheritance that Gregor Mendel discovered was applicable to all inheritance even though Mendel worked on only one life form, the garden pea. Similarly, I see eye-to-eye with Monod and Jacob's truism: "anything found to be true of *E. coli* must also be true of elephants" (Cold Spring Harbor Symp Quant Biol 26: 389, 1961, p. 393).

J.S. Clegg: Another matter that I have raised with Dr. Ling several times in the past, and raise here again, concerns the specific nature of the proteins which are proposed to reversibly unfold (extend) tenable the cell water to be polarized into multilayers. To my knowledge these have never been identified. As I understand it, it is not easy to *reversibly* denature (i.e., "extend" globular proteins, notable to the extent required by the AI Hypothesis. Does Dr. Ling have any recent evidence on the identity of these intracellular proteins? Is there any evidence that globular proteins actually fully extend into "linear" polypeptide chains, in a fully reversible way, under intracellular conditions? (I note that

the ribonuclease studies of Anfinsen were done under highly non-physiological conditions.) My comments should be viewed as constructive: evidence for these matters could go a long way toward proving compelling evidence for Dr. Ling's hypothesis.

Author: Shortly after the polarized multilayer theory of cell water was introduced, I need to choose a name for the protein conformation with all its polypeptide NH and CO groups directly exposed to the cell water. The word "random coil" is technically correct for a solution of protein in this configuration, but may impose attributes on proteins in the cell which may be exposed but not random. The word "extended" conformation also could not be used in the same limited sense, but again cannot be used because it has already been used to designate the β -pleated sheet conformation. Nonetheless, both "random coil" and "extended" had been used in my earlier publications. It was only from 1980 on that I switched to the term "fully extended conformation". I made very careful description on what I mean by this term: It does not necessarily have to be extended like a rigid rod. The chief requirement is the exposure of the NH and CO groups to the bulk-phase water and not internally locked in α -helical and β -pleated sheet conformation. That these chains should also be arranged in parallel was also suggested, but not absolutely essential.

In theory each protein has the inherent capability of existing in at least two conformations: the helical conformation and the random-coil conformation. However, each protein is different in terms of the ease of the transition between the two states. Thus oxidized ribonuclease exists entirely in the random-coil state in the absence of denaturant like urea (Harrington and Schellman (Compt Rend Trav Lab Carlsberg, Ser Chim, 30, No 6, 21, 1956); it takes 1 M urea to denature β -lactoglobulin; 5 M urea is needed to denature serum albumin, even saturated urea solution (>10 M) cannot denature pepsin (see Ling, 1962, p. 176–178; 1964). Why different proteins have different stability in their secondary structure is the concern of the AI hypothesis but too lengthy to enter into a detailed discussion here (see Ling, 1991, Section 6.2).

The main question here is whether or not the fundamental helix-random coil transition — which underlies most conformation changes fast as well as slow — is fast enough to cope with rapid physiological changes under otherwise favorable conditions. The answer is a definitive "Yes". It takes 10^{-8} second or less to achieve this cooperative transition (Eigen and Hammes, 1963, Adv Enzymol 25:1; Ullman, 1970, Biopolym 9:471).

As I mentioned above, "fully extended polypeptide chain" means non-random random coil, or non-pleated-sheet extended conformation. It can involve a part or an entire protein molecule. Our current method of identifying the secondary structure of proteins cannot be extended to specific proteins inside a living cell yet. However, the ability to visualize living structure in cells would be certainly a major step in this direction.

Finally, the question of the as-yet-unidentified protein in the cell responsible for the multilayer polarization of the bulk-phase water. This is what I wrote on the subject years ago: "...all living cells have a common origin. It seems reasonable, therefore, that the same or very similar protein or proteins may serve the same purpose in all living cells. At this time there is no concrete evidence to substantiate the idea; nevertheless, I would like to suggest that such a "universally" present protein does indeed exist and that it probably includes actin as a major if not the chief, component.... One reason for this speculation is that (like gelatin) with its high proline content, actin has a natural tendency to exist in an extended state. Another reason is that actin has already been detected in a wide variety of

living cell, and it is not unlikely that it is present in all living cells” (Ling, 1979, p. 47). In 1984 I further suggested that within living cells actin might exist in what Tilney called “profilamentous” and/or what Oosawa and Kasai called G* actin and in this form the protein might offer its CO and NH groups for bulk-phase water polarization (Ling, 1984, p. 568).

During the Santa Cruz conference I had the great pleasure of meeting and learning from Prof. Jacques Dubochet some very exciting as-yet unpublished work which he and his coworkers G. Pruliere, E. Nguyen, P. Douzou and others had just accomplished. This work provides new evidence that actin may indeed play a role in polarizing cell water and that α -actinin, an actin-binding protein found in the Z line of striated muscle may act as the postulated protein-X of Figure 14.

G. Pruliere and P. Douzou (Biophys Chem 1989, 34: 311–315) showed that a 15 μ l drop of an aqueous solution of 1,2-propanediol (15%) and glycerol (8%) when plunged into liquid nitrogen turned milky, indicating the formation of ice crystals. However, if minute amount of F-actin (0.5%) and of α -actinin (0.1%) are added to the 1,2-propanediol-glycerol solution, the solution remains transparent after cooling to liquid nitrogen temperature. When frozen hydrated section of the frozen droplet was examined by cryo-electron microscopy and by electron diffraction, they revealed that the droplet was in a vitrified state without any ice crystals big or small, and that the proteins formed a microporous network containing much finer filaments than those of F-actin. Their new studies also showed that initially actin needs not be in the F-form. G-actin acts just as well. The authors concluded that “the proteins produce a global change in the properties of the liquid aqueous solution rather than acting locally and specifically on the growing ice crystal”. In terms of the AI hypothesis, α -actinin may act as the protein X of Figure 14, which aided by the water-polarizing influence of 1,2-propanediol and glycerol (both of which reveal higher q-value in frog muscle than their respective molecular weights dictate and both are well known cryoprotectants), convert actin into a more finely dispersed filamentous form apparently with its CO and NH groups directly exposed to and polarizing the bulk phase water, making it to assume the vitrified polarized multilayer state. The more finely dispersed actin filament produced by interaction with α -actinin seems to have some of the required attributes of the water-polarizing extrovert matrix protein(s) postulated by the AI hypothesis.

There are two more comments which I would like to make: (1) the extremely low concentration of actin needed to produce the global change of the bulk-phase water-glycerol-1,2-propanediol system is very encouraging for the AI model; (2) just as α -actinin appears to function as the Protein-X of Figure 14, it is possible that the α -actinin-actin system may in turn function as a higher-order Protein X in controlling the conformation change of other (major) proteins of the cell such as myosin in muscle and hemoglobin in erythrocytes.

Editor's Note: Paper presented at the 9th Pfefferkorn Conference, Santa Cruz, CA, August 1990; other papers presented at this Conference were published in Scanning Microscopy Supplement 5, 1991.

In 1999, the scientific journal, Scanning Microscopy went out of business. "CAN WE SEE LIVING STRUCTURE IN A CELL", which was published in Volume 6, (1992) of that journal lost its owner. And by law, the copyright of this and other articles return to their respective authors. The central importance of the article, including its long sections of debate between the author and reviewers, led me to the decision to give the article a new life by publishing it a second time in the journal, *Physiol. Chem, Phys. & Med. NMR*. But there is one problem. Major advances have been made repeatedly since 1992. To update the article, I shall present two short summaries of the most prominent advances as follows:

(I) The smallest units of life. There are two ways to look at the world we live in: a macroscopic view and a microscopic view. From its beginning, the association induction (AI) hypothesis has attempted to explain conventional macroscopic concepts of living phenomena like cells, membranes, pumps, sieves, semi-permeability etc in terms of the properties and activities of assemblies of microscopic molecules, atoms, ions and electrons.

According to the conventional view, the smallest unit of life is the cell. In the AI Hypothesis, the smallest unit of life is much smaller and has been in the past referred to as *biological fixed charge system* (Ling 1962, p. 530), *minimum unit of life* (the present article 1992, p. 34), *elementary living machine* (Ling 2001, p. 152). In the year 2008, I have replaced all of these names with a new name, *nanoprotoplasm unit* (NPU) (Ling 2006, p. 111; Ling 1989, Article #14, #15 listed on front page of Website; Ling, 2013 pp. 97–98).

As a typical example, the nano-protoplasmic unit (NPU) of the cytoplasm of mature human red blood cell (rbc) can be described by the formula $(\text{Hb})_1(\text{H}_2\text{O})_{7000}(\text{K}^+)_{20}(\text{ATP})_1$. In this formula, each component is joined to all the others directly or indirectly by primarily ionic bonds. As a rule, each NPU contains one protein molecule specific to that NPU, which is hemoglobin in mature human rbc cytoplasm and represented as $(\text{Hb})_1$. The subscript under each item refers to the number of that item in one NPU. To show how small each NPU is, I may mention that each mature human red blood cell contains on the average 300,000,000 NPU's. Also to be noted is that in weight each NPU contains mostly polarized-oriented dynamically structured water molecules beside potassium ion (K^+) and the most powerful electron-withdrawing cardinal adsorbent (EWC) ATP (Ling, 1989, Article #14 and #15 listed on the front page of the Website; Ling, 2013 pp. 97–98.)

(II) Revision of polarized multilayer (PM) theory of cell water to polarized oriented (POM) theory of cell water.

I introduced in 1965 the concept that all or virtually all the water molecules in living cells (and models) exists as dynamically structured water molecules for the first time in history. It was presented under the heading of polarized multilayer (PM) theory of cell water. In the diagrammatic illustration printed, the degree of motional restriction was shown to taper from the polarizing protein sites. For theoretical support, I relied on the work of de Boer and Zwicker (1929) and Bradley (1936). However, subsequent careful

study of their respective work revealed that both had difficulties dealing with the impact of thermal bombardment of the permanent dipole moment of polar molecules like water and were forced to make simplifying assumptions that did not do any good.

It was therefore with great joy I announced in 2003 the discovery of a theoretical short-cut, which enabled me to reach the astonishing conclusion that an ***Idealized*** (checker-board of) ***NP Surface*** can polarize and orient deep layer of water molecules *ad infinitum* (Ling, 2003 pp. 106–114). By analogy, I also reached the conclusion that the polarization mechanism can cover only a single layer of water molecules at the exposed backbone NH and CO groups of the protein backbone. In contrast, it was primarily the orientation mechanism that produces the dynamic structuring of the bulk phase cell water.

Thus strictly speaking, the original title of polarized multilayer theory is not quite correct. The more up-to-date title should be polarization-orientation multilayer (POM) theory of cell water.

A corollary of the POM theory is the prediction that water (polarized) oriented by the Idealized NP surface will not freeze at any achievable low temperature. Nor will it boil at temperature as high as or higher than 450° C. The non-freezing prediction was confirmed (retroactively) by the Canadian chemists, Giguère and Harvey (1956). The non-boiling prediction was retroactively confirmed by the Japanese scientist, T. Hori of Hokkaido University (1956).