Physiol. Chem. Phys. & Med. NMR (1994) 26:121–203

The New Cell Physiology:
An Outline, Presented Against its Full Historical Background, Beginning from the Beginning

Gilbert N. Ling

Damadian Foundation for Basic and Cancer Research.
c/o Fonar Corporation, 110 Marcus Drive, Melville, New York 11747

Abstract: The primary objective of this review is to present a brief outline of the new cell physiology. First introduced in 1962 under the title of the association-induction hypothesis, the theory is now confirmed in its essence, after more than thirty years of world-wide experimental testing. While minor, and even more than minor, improvements and revisions may yet be needed, the weight and scope of the confirmatory evidence have left little doubt that a comprehensive and coherent new theory of cell physiology is established. For the first time in history we have available a realistic cell physiology, awaiting to unite and to guide future biomedical research and teaching.

The review also demonstrates in passing how, as well as why, the old cell physiology has fizzled so miserably. Instead of providing the guiding light for all biomedical research, it has degenerated into a fairy tale of make-believe. This degradation began with a wrong theory. The situation has grown steadily worse with time by the impact of premature progressivefragmentation on something inherently indivisible; and by the universal adoption of thepeer-reviewsystem which gives to those with strong vested interests in the preservation of the status quo absolute power in determining who prospers and who is cast out (see Endnote 9).

It is thus hardly surprising that up to now, despite superlative chemical engineering know-how available and the lure of great financial awards, not a single drug has been designed from understanding, an understanding that will remain forever beyond reach as long as the guiding theory of the cell physiology is basically wrong.

In contrast, in its brief history, and despite its minuscule list of subscribers and their endless debilitating struggle merely to stay alive (see Endnote 9), the new cell physiology has already generated one mankind-enhancing medical technology of major proportions, magnetic resonance imaging or MRI (see Ling, 1984, p. vii; 1992, p. xxv).

To this day, most biomedical research scientists, teachers as well as students, still operate under the delusion that the old cell physiology, a little battered perhaps, is still basically sound. By far the greatest majority of them simply do not know enough; and it is hard for them to find a convenient

source of relevant information that will help in this regard. A second purpose of the present review is to fill this need.

To a few "true believers" of the old cell physiology the preservation of the ***status*** quo is of overriding importance. We owe largely to these few, and to the fear and opportunism they inspire all around, the unbelievable success of universal suppression and submission we witness: a whole generation of young biomedical scientists growing up totally "oblivious" of a major revolutionary development in the foundation science of their own interest--exploding right in front of their eyes.

Common sense tells us that there is a limit how long one can prop up a dead body and teach generation after generation of students that it is alive. That this has been happening for so long in regard to the disproved membrane-pump theory did not occur by accident. It can be traced at least partially to the fragmentation of biomedical science that has been going on for a long time. Fragmentation limits one's awareness. When the area of awareness is small enough, even the keenest observer cannot know if what he is looking at is a part of a corpse or a living patient.

With this thought in mind, I realized that it is not enough to enumerate all the merits of one theory and the lack of merits of another, no matter how lucidly and how faultlessly presented. As a remedy, I have provided the reader with, in broad strikes, a full history of cell physiology, so that he or she can see, so to speak, the "whole elephant"; and thus equipped, grasp the finality of the conclusions reached.

PHILOSOPHERS OF YORE classified knowledge into two categories: ***physics,*** describing the dead world; ***physiology,*** the living one. By the middle of the 20th century, physics had already scored many of its greatest achievements and reached maturity. Physiology, in contrast, remains to this day poorly understood at its most basic level. This is not to deny that major, indeed spectacular, progress had been made in some specific aspects of certain areas of the life sciences. An outstanding example is genetics, the expression of what German biologist, August Weissman (1834–1914), called ***germ plasm.*** Understanding of Weissman's ***soma,*** namely, ***cellphysiology proper,*** is in a bizarre position, where the theory being taught and learned continues to be the wrong one.

Yet, it is primarily the correct understanding of cell physiology proper, in conjunction with what we know of genetics and of other relevant areas of specialization, that holds the key to evolving the means to protect mankind from incurable diseases like cancer and deadly pathogens extant and others that are certain to evolve. Here the power of genuine knowledge, or lack of it, may be a matter of life or death on a grand scale. In the sobering words of Charles van Doren in his "A History of Knowledge" (1991, p. 355): "the human race might come to a tragic decision point: try to reproduce and die. Or simply die"; that is if we fail to bring under control just one of our true enemies, the AIDS virus.

In the middle of the 19th century, advances made in the construction of microscopes led to the twin discovery of the ***living cell*** as the basic unit of all life, and of the substance of living cells ***orprotoplasm,*** which Thomas Huxley (1825–1895) called "the physical basis of life". Almost immediately after these discoveries, scientists began to take sides on the two divergent schools of thought on cell physiology. One school believed that it is the thin membrane covering of the cell that plays the central role in cell physiology, culminating in the introduction of the ***membrane theory.*** The other school believed that it is the protoplasm that is the seat of life activities, leading to the ***protoplasmic*** (or colloidal) approach to cell physiology.

In the membrane theory the mystery of life resides in the submicroscopiccell membrane. The cell content is seen as essentially a dilute aqueous solution. Thus one may also see the membrane theory of the living cell as a dilute solution theory of the living cell.

The late 19th and the early 20th century saw rapid progress in the physics and physical chemistry of dilute solutions. Willard Gibbs, Peter Debye, Walther H. Nernst, Svante A. Arrhenius, Jacobus S. van't Hoff, and Wilhelm Ostwald were among the scientists developing dilute solution theories. Based on the new insights revealed, proponents of the membrane theory soon were able to offer plausible mechanisms for all four primary physiological manifestations of the living cell: solute distribution, water and solute permeability, cell volume shrinkage and swelling as well as cellular electrical potentials.

With its roots in the accomplishments of the highly respected physics, the proponents of the membrane theory had little difficulty in attracting into their ranks some of the brightest minds of the time, including Wilhelm Pfeffer, Ernst Overton, Julius Bernstein, Leonor Michaelis, Archibald Vivian Hill, Alan Hodgkin, Andrew Huxley, Peter Mitchell and others. Numerically superior, and occupying some of the most listened-to intellectual "pulpits" of the world (e.g., Cambridge University of England), the success of the proponents of the membrane theory can be gauged by the Nobel prizes they won one after another.

After an exciting beginning, the protoplasmic or colloidal approach had not moved much beyond the descriptive and the qualitative phase. At the time there was no simple physical theory to lean on. The physics that would one day come to the rescue of the colloid-oriented cell physiologists was yet to develop or, for other reasons, unavailable to those involved. Proteins, the pivotal component of living protoplasm, were poorly understood then. Though their adherents knew that protoplasm is gelatin-like (see below), they did not have the essential basic information to explain in what critical way gelatin is different from other proteins. Nor could they explain convincingly how a colloid is different from a non-colloid or crystalloid. Their increasing emphasis on the large particle size as the distinguishing feature of colloids was leading nowhere. Large molecules do not always make colloids; nor do colloids always contain large molecules.

Vastly outnumbered and outgunned, cell physiologists believing in the colloidal approach were overwhelmed by their opponents. A crucial experimental finding—as it was widely held to be at the time—was published in 1930 by the Nobel laureate, Archibald Vivian Hill against the protoplasmic view of the living cell. In its wake the colloid-oriented cell physiology, in the English-speaking countries at least, quietly receded into obscurity.

It was at about that time that the membrane theory reached what appeared at the time to be the zenith of its development. In 1941 Boyle and Conway published the leading article in the 100th volume of the (English) Journal of Physiology beginning at page one. The prominently displayed paper was in many ways the culmination of a number of long cherished ideas of the proponents of the membrane theory of the living cell.

While the focus of the Boyle-Conway paper is on the selective accumulation of K^+ over its closely similar Na^+ in living cells, it also reaches out to touch on other important subjects of cell physiology including cell volume changes in solutions containing a high concentration of K^+ and the influence of external K^+ concentration on the cellular resting potential. The central objective in this grand synthesis was to quantitate the by-then-familiar molecular sieve idea of cell membranes as brought forth previously by Traube, Nathanson, Ruhland, Collander, Bärland and others: cell membranes possess (rigid) pores of uniform dimensions which act as molecular sieves determining which ions are permeant (e.g., K^+) and which are not (e.g., Na^+). It might well be in the minds of the many enthusiastic readers

that, before celebrating the final victory of the membrane theory, we go through the formal steps of demonstrating unequivocally the obvious truth that the cell membrane is totally impermeable to the (hydrated) Na^+ ion and to other large hydrated ions which the theory predicted to be too large to pass through the narrow membrane pores.

Accordingly, extensive research was carried out in laboratories across the world (for details, see pg. 152 below). The results were not expected. Quite the contrary, they were cataclysmal. In contradiction to one of the oldest cherished beliefs, ***the cell membrane proves to be permeable to*** Na^+.

In this dumbfounding, anticlimactic situation, one guesses that the principles might have wanted to wash their hands of the whole thing. In any case, it is a fact that no deep soul-searching reexamination of the problems followed immediately. Instead, a "patch" (or pump) was applied to fix the leak of the membrane to the Na^+ ions. This is how the sodium-pump hypothesis came into existence.

A few years later, I came to the United States. One Monday afternoon in 1947 I gave a seminar in the Department of Physiology of the University of Chicago where I was a graduate student under the famous cell physiologist, Professor Ralph W. Gerard. The topic of my talk was "the Sodium Pump". I recall opening my talk with something like: "The most certain thing about the sodium pump is that no one knows what it is". High and low I searched, but I could not find that enthusiastic, single-minded advocate of the theory that had stood behind all other hypotheses of note that I knew. There was no mechanism proposed for the pump either, just a name.

After my talk, two of my professors (and highly cherished friends) took me aside and told me that I should leave the subject alone. It was a holy cow. There was nothing to be gained by making a martyr of oneself. I remember feeling very grateful for their kindness in going out of their way to make the suggestions. But I had difficulty convincing myself that I should follow them. Besides, my curiosity was aroused. So I began to do some simple experiments. One thing led to another. . .

However, not until 1951 did I publish my first short note presenting straightforward experimental evidence against the sodium pump hypothesis and offering a new molecular mechanism whereby most Na^+ is kept out of the living cells while its closely similar sister, alkali metal ion, K^+, is selectively accumulated within the cell. In this theoretical model, both K^+ and Na^+ are free to roam in and out of the cell without the need of a sieve-like membrane as in Boyle and Conway's membrane theory, nor a continuous pumping mechanism as it is in the case of the postulated Na pump (Ling, 1951).

In the following ten years, two major events took place. A definitive disproof of the sodium pump theory on the basis of energy consideration was completed; twice confirmed, the finding and conclusion have remained unchallenged ever since. My theory of selective accumulation of K^+ and exclusion of Na^+ grew and grew until it assumed the shape of a general theory of the living cell, called the ***association-induction hypothesis (AI hypothesis).*** The disproof of the membrane-pump hypothesis as well as the introduction of the AI hypothesis (and supportive evidence) was published in 1962 in a 680-page monograph entitled "A Physical Theory of the Living State: the Association-Induction Hypothesis" by Blaisdell. It was followed by my second volume, "In search of the Physical Basis of Life" published by Plenum in 1984. In this 791-page volume, I presented the results of extensive testing of the hypothesis in the preceding twenty-two years.

Another eight years passed before my third volume appeared in print, bearing the title:

"A Revolution in the Physiology of the Living Cell" (Krieger, 1992). A short 378 pages, this volume summarizes all the key evidence that has disproved the membrane-pump theory and established the association-induction hypothesis.

With due humility and full awareness that some part of the theory may yet need significant revision, I do feel confident that I now have a theory that fits most, if not all, existing relevant information known to me. It is in easy harmony with findings that had disproved the membrane-pump theory. It is fully able to explain all the four major attributes of the living cell, mentioned repeatedly above, but in ways that are solidly based on modem physics. And it is quantitative and given in equation form.

I believe that the new cell physiology based on the association-induction hypothesis has for the first time in history provided a realistic foundation toward the eventual construction of a rational drug therapy. When immunization alone is unable to cope, drugs designed on the basis of true understanding are without question the main weapons in mankind's defense against our ever-evolving deadly enemies, the viruses.

While a part of this presentation summarizes highlights of my 1992 monograph just mentioned—and it was mostly this part that was verbally communicated in the 1992 Society of Experimental Biology (SEB) symposium held in Lancaster, England and organized by Professor Michael Bingley—this presentation also offers another theme not found in the 1992 work: It provides a summary of the "entire" history of cell physiology, beginning with the invention of the compound microscope.

It is my belief that only those who know the history of long distance running, can fully understand and correctly evaluate the significance of Roger Bannister's first four-minute mile. So only those who know the full history of cell physiology, can fully understand and correctly evaluate what I call the New Cell Physiology.

Before embarking on my history of cell physiology I must point out a somewhat unusual strategy I choose to adopt in carrying out this enterprise. There is no need of any unusual strategy in dealing with history before I entered the picture in the later 1940's. I shall just tell it as it was in the best way I know how. However, for the history after my entry, I will make efforts to write in such a way as if I and most of my scientific work have never been known. Only after the history of cell physiology without my participation has been largely told will I present my own work to show how it has contributed to the complete disproof of the last version of the membrane theory, i.e., the membrane-pump theory; how cogently it meets the demands created by the demise of the membrane pump theory; and why the new cell physiology deserves attention so far denied. Not only because it is the only theory of cell physiology that has successfully stood worldwide experimental testing for well over a quarter of a century, but also because there is no alternative left.

The Discovery of the Living Cell

The road leading toward the discovery of the living cell began with the invention of the microscope. According to reviewers C. Singer (1915) and A.N. Disney (1928), the compound microscope was independently invented around the year 1609 by Zaccharias Jansen in Holland and by Galileo in Italy. However, with a single lens microscope Anton van Leeuwenhoek saw for the first time the teeming world of the little living creatures, which have been, from the very beginning of mankind's existence, everywhere around as well as

within each human body. Yet until that very moment, they had remained entirely outside of the human knowledge.

In the year 1676, van Leeuwenhoek described his discovery in a letter to the Royal Society of London. Leeuwenhoek's story of little living creatures, which he referred to as *animalcules* (see Dobell, 1932), was confirmed in 1678 by Robert Hooke (1635–1703) of England. However, long before that, Hooke had observed many other objects through his microscope and had published his observations in his book: "Micrographia; or, Some Physiological Descriptions of Minute Bodies Made by Magnifying Glasses with Observations and Inquired Thereupon" (London, 1665). Nearly a hundred years we're to pass before the little animalcules of Leeuwenhoek received the name *Infusoria* from Wrisberg. This christening was a significant step forward; it also inadvertently added to the confusion at that time. Infusoria included (what was to be recognized later to comprise) two large classes of very different living organisms: bacteria and protozoa.

In his Micrographia, Robert Hooke described what he saw in sections of cork through his microscope. Cork is the elastic outer bark of the cork oak. However, Hooke did not know this and thought that he was looking at some "Fungus or Mushrome". In this "Fungus" he saw many pores and called them *cells* (1665). He believed that these cells represented channels for the transport of fluids from one part of the plant to another. If Hooke could be revived three centuries later, he would be astonished to know what has befallen the name he had casually give to the holes in a piece of dead cork.

In his treatise, "The Cell", American cytologist, E.B. Wilson, pointed out in 1928 that calling the basic units of life "cells" was a mistake: the living cells are not hollow chambers as the name suggests but are solid bodies. However, the mistake was clearly not committed by Robert Hooke. Rather, it was the zigzagging course of acquiring new knowledge that had left stranded a name which at one time appropriately described the subject it intended to describe.

Jean Baptiste Lamarck (1744–1829) was an example of the kind of universal mind of the French Enlightenment (see Hall, 1969, vol. 2, pp. 133–148). As a starter, we owe to him the name, *Biology*. We owe to him also the theory of evolution, known later as Lamarckism: the transmission of acquired characters. Of more direct interest here, Lamarck laid great emphasis on the *cellularity* of living things. For this reason, some historians thought that Lamarck had anticipated Schwann in propounding a general cell theory. This is, strictly speaking, not true. Lamarck's cellularity was an extension of what Robert Hooke called cells, rather than what E.B. Wilson called cells. Thus, like Robert Hooke, Lamarck also regarded cells as a system of interconnecting open chambers. He made no mention that these cells were the fundamental discrete units of life.

A. R. Rich (1926) considered René J. H. Dutrochet (1776–1847), another French contemporary of Lamarck, to be the true founder of the cell theory. Rich had a much more solid foundation for his claim. Dutrochet did announce in 1824 and 1826 (and thus 13 years ahead of Schwann) that plants as well as animals are made up of living cells.

Most if not all textbooks being used today tell us that the founders of the cell theory were two German botanists, M.J. Schleiden and Theodor Schwann. Schwann announced his cell theory in 1839 in his famous monograph, "Mikroskopische Untersuchungen über die Übereinstimmung in der Struktur und dem Wachstum der Thiere und Pflanzen" (Microscopic Investigations on the Resemblance of the Structure and Growth of Animals and Plants). Schleiden's role is now recognized to be secondary and not on an equal footing with Schwann.

In Schwann's view of the living cell, the containing membrane--of both the cell and the nucleus—was prior in importance to their content which, according to Schwann, was a homogenous and transparent liquid. On the other hand, ***the cell membrane was postulated to have, for example, metabolic power, by means of which the cell membrane controls the chemical composition of the fluid outside as well as inside the cell.*** It is interesting to note that the living cells, as seen by Schwann at the publication of his monograph, were not completely unrelated to the earlier view of Lamarck on the cell, i.e., fluid-filled chambers.

Schwann (as well as Schleiden) was a botanist. His main subject of observation might well have been mature plant cells which are enclosed in a rigid wall—the cell wall. Mature plant cells also contain a large central vacuole filled with a simple watery liquid. Thus one could guess how Schwann came to the conclusion that living cells are bags of clear liquid enclosed in a membrane and on his emphasis on the cell membrane—which could not be visualized even today with the best light microscope in existence. What Schwann thought to be the cell membrane, therefore, might be primarily the thin layer of protoplasm surrounding the central vacuole; what he thought to be the cell content (or cytoplasm) might be the watery liquid filling the central vacuole. This surmise, if it had validity, will also make it easier to understand why in later years Schwann changed his mind about the relative importance of the cell membrane versus the cell substance. However, Schwann's change of heart was partly also in response to findings that came from another line of microscopic investigations with emphasis on the ***contents*** of living cells, this time animal cells.

The Discovery of Sarcode or Protoplasm

The microscope not only made it possible to see the outer appearance of living cells, it has also revealed what was inside living cells. In 1744 Abraham Trembly obtained from fresh water polyps a glairy, viscous substance. In 1786 Otto Fredrik Miiller described a transparent gelatinous substance that came from the inside of some protozoa. Between 1803 and 1811, Trevranus reported on the streaming movements of an intracellular "gallerte" (gelatin) in large plant cells. In 1810 Lorenz Oken described what he called an "Urschleim" (primordial slime) making up the substance of living cells (see Hall, 1969, vol. 2, pp. 172–174).

This prior work culminated in the announcement in the year 1835 by French microanatomist, Felix Dujardin, of a living substance he called ***"sarcode".*** That Dujardin had much interest in animals rather than plants and was thus a (proto)zoologist could be seen from zoological journals in which he published his critical work (Dujardin, 1835, 1841). Sarcode was isolated from protozoa (Infusoria)—which like all animal cells, do not possess a rigid cell wall nor a large water-filled central vacuole—and was described by Dujardin as "a pulpy, homogenous, gelatinous substance without visible organs, yet organized . . .". Dujardin was a scientist of admirable honor and integrity, scrupulously giving credit to all prior work that might conceivably be considered relevant to his discovery.

Six years after Dujardin's announcement of the sarcode, botanist Hugo von Mohl (1844) published his finding: in the center of a young plant cell, there was a nucleus, as Schwann and Schleiden described earlier, but surrounding the nucleus von Mohl found a viscous fluid which he called "*protoplasm*". Note in particular that von Mohl studied ***young*** plant cells which, like animal cells, do not have water-filled large central vacuoles found in mature plant cells.

It was largely the work of Ferdinand Cohn (1850) which established that plant protoplasm, and animal sarcode are identical and that this substance in cells is the primary seat of living activity. Franz Unger believed that protoplasm was a protein substance "consisting of the same nitrogenous compound that is present in every animal" (see Hall, 1969, vol. 2, p. 213). However, neither Cohn nor Unger attempted to replace the name sarcode, derived from the study of animal cells with the name protoplasm, derived from the study of plant cells. It is to Robert Remak (1852) we owe the redefinition of "protoplasm" to stand for the "living jelly" of both plant and animal cells.

With more and more attention devoted to the protoplasm, the relative importance of the cell membrane and the cell content underwent a dramatic shift. First Franz Leydig announced in 1857: "Cell contents are of a higher dignity than the membrane". It was followed by the famous ***"protoplasmic doctrine"*** by ***Max*** Schultze (1861; Hall, 1951, p. 449): "The protoplasm represents a spherical lump held together by its own inherent consistency. These cells do not possess a membrane chemically different from the protoplasm. The cells are membraneless little lumps of protoplasm with a nucleus."

At that time, it was already widely known that protoplasm emerging from living plant or animal cells does not mix with the surrounding aqueous medium (see Endnote 1) (for a visual demonstration of protoplasm, see Kuroda, 1964, or Figure 2.5 reproduced from Kuroda in Ling, 1984, for a photograph of isolated protoplasm suspended in an aqueous medium). Since no simple water solution can stay immiscible with another simple water solution, this immiscibility offers very convincing evidence that protoplasm has the innate quality to remain apart from the surrounding medium. This type of observation led Max Schultze to suggest that the surface of the cell protoplasm is covered with nothing other than a layer of protoplasm like that making up the substance of the living cell.

A pioneer in the field of protein chemistry, Willy Kiihne (1837–1900)—who isolated the contractile protein of muscle cells and named it ***myosin*** and who also introduced the name ***enzyme*** for the biological catalyst (see Rothschuh, 1973, p. 240)—suggested that the protoplasm of a living cell is covered with a very thin layer of coagulated protein (see Endnote 1).

It is noteworthy that in his later life Theodor Schwann apparently changed his mind on the relative importance of the cell membrane and the cell content. He reminded us that in the third part of his "Untersuchungen" he had spoken of "solid cells without membranes and without cavities" (Florkin, 1960).

Just as interesting was the changing of heart of English biologist, Thomas H. Huxley (1825–1895), whose famous and eloquent defense of the crucial role of protoplasm as the ***physical basis of life*** in 1853 came after an earlier period of time when he was an equally vigorous proponent of the primary importance of the cell membrane (Huxley, 1853; Hall, 1969, 2: 199).

I do not quote these opinion changes in a derogatory manner. On the contrary, I regard this willingness to make drastic changes in scientific opinions in the face of new scientific evidence as an expression of the highest form of dedication to the search for truth. On this, science and politics are different. Scientists are not obligated to keep "campaign promises". Scientific reasoning and experimental evidence alone should decide what future scientific position one ought to hold. Belief in this basic principle by many scientists as well as a broad subscription to honor, courage and clear thinking offer some of the reasons why Alfred Rus-

sell Wallace (1821–1913),—Charles Darwin's co-founder of the theory of evolution—called the 19th century, the *wonderful century.*

In summary, biologists from Jean Lamarck to Max Schultze —with the aid of the improving microscopic techniques in the span of about 100 years—gradually reached a general consensus acknowledging the existence of a common basic unit of all life: the living cell. They debated about the relative importance of the cell membrane and the protoplasm, and even the existence of one or the other of these two entities. A more definitive answer to these fundamental questions awaits a closer examination of not just how each component looks under a still better magnifying glass (e.g., the electron microscope), but also how the various components function. The early efforts in this direction inaugurated the new science of *cell physiology.*

The Beginning of Cell Physiological Investigation: The Invention of Models of Protoplasm and of Cell Membranes

The construction of *models* has played a very important role in the progress of science, especially in that critical early phase of a science still seeking foundation knowledge as in Michael Faraday's study of the interaction between electricity and magnetism; in Rudolf Hertz's study of electromagnetic waves; and in the still very early stage in the development of cell physiology from its beginning some 150 years ago until the present. In the construction of models we hope to capture the essence of a natural phenomenon, isolating it from the incidental and the irrelevant. The simplicity of the model permits quantitative verification or refutation of a hypothesis on one or another aspect of the natural phenomenon, while the natural phenomenon in its entirety would not tolerate. We owe the genius of the English chemist, Thomas Graham, for introducing a most cogent model of protoplasm almost immediately after protoplasm was discovered. Graham also played a key role in the invention of another important model, the model for the cell membrane.

Thomas Graham (1805–1869) was by profession a chemist. He held the office of the Master of the Mint, an office held by Isaac Newton about 160 years before. Graham spent most of his life studying diffusion and was well-known for his Law of Diffusion: the rate of diffusion of a gas is inversely proportional to the square root of its density. Graham's interest in diffusion brought him into contact with the newly discovered protoplasm and its widely recognized gelatin-like character.

In 1861, in a paper published in the Philosophical Transactions entitled "Liquid Diffusion applied to Analysis", Graham broadly separated chemical substances into two classes. One class called *crystalloid* includes salts, sugars, and alcohols, and these crystalloids diffuse rapidly. Substances belonging to the other class diffuse much more slowly but are also distinguished by the gelatin-like characteristics of their hydrates. "As gelatin appears to be its type, it is proposed to designate substance of the class as *colloids"* (from the Greek work for gelatin or glue, κολλα).

From Graham's definition *protoplasm is* a *colloid or a collection of colloids.* A branch of chemistry called *Colloid Chemistry* was thus launched. Colloid chemistry then offered the physicochemical basis for the continued investigation of protoplasm. This approach of cell physiology may be called the *protoplasmic approach to cellphysiology* and it grew out

of the view of cells as seen by Max Schultze whose microanatomical studies led him to conclude that it is the substance of the cell that mattered most and that the cell membrane is only a modified layer of the protoplasm found at the cell surface.

However, Graham also contributed to the alternative approach to cell physiology initiated by Theodor Schwann who believed in his early days that it was the cell membrane that counts, the cell interior being nothing more than a clear fluid. Graham's approach to what we may call the ***membrane approach to cell physiology*** lay in his study of the ability of certain special membranes that allowed the passage of water but not that of larger solutes—a phenomenons known as ***dialysis.***

Graham invented "dialysis". In this technique, the interpolation of a thin partition, or what he called ***septum,*** allows the separation of crystalloids, which pass through the septum, from colloids, which do not. The septum Graham used was (vegetable) parchment paper "sized" with gelatinous starch. Graham pointed out the similarity of these man-made septums and ***animal membranes*** used in earlier studies (see below).

Graham also mentioned that it was the colloidal starch hydrate introduced into the interstices of the septum that slowed down the passage of diffusing materials, stopping it altogether when the diffusing material was itself a colloid. Among those colloids that did not pass through his dialysis septums was copper-ferrocyanide gel.

Moritz Traube (1826–1894) was a German Jewish merchant who conducted scientific experiments in the basement of his house in Berlin. Traube invented a good and historically important artificial membrane: a thin layer of precipitated colloidal copper ferrocyanide (Traube, 1867).

Traube's discovery was based on a simple observation. When a drop of copper sulfate was brought into contact with another drop of potassium ferrocyanide, a thin layer of reddish brown copper ferrocyanide precipitate formed at the boundary of contact. Once such a layer was formed, no further formation of copper ferrocyanide precipitate occurred. Traube soon realized the significance of this arrest of further precipitation: The thin layer of copper ferrocyanide prevented the further passage of copper and of ferrocyanide ions to the opposite side. In other words, he had discovered a superior membrane model that is not only impermeable to colloids as was the case of Graham's starch-sized parchment paper but impermeable to crystalloid copper and ferrocyanide ions as well. Traube published his observation in 1867. It soon came to the attention of plant physiologist, Wilhelm Pfeffer.

Introduction of the Membrane Theory

Wilhelm Pfeffer (1845–1920) made a further improvement of Traube's artificial membrane by depositing the copper ferrocyanide gel within the porous wall of an unglazed porcelain pot. The new membrane now has the strength to withstand handling and applied pressure. Pfeffer then showed that across such a fortified copper ferrocyanide membrane there was movement of water from the compartment containing a lower concentrated sucrose solution to the other compartment containing a higher concentration of sucrose. In contrast, no significant amount of sucrose passed through the membrane (Pfeffer, 1877).

One recalls that Rent Dutrochet introduced his version of cell theory before Schwann. In earlier times Dutrochet also studied the movement of water in and out of solutions enclosed in an animal membrane sac (e.g., dried pig bladder). He called these movements of water in and out of the sac ***endosmosis*** and ***exosmosis*** respectively (Dutrochet, 1827). From these

words, the concept and terminology of ***osmosis*** and related phenomena emerged. Thus the pressure that must be applied to the side containing a higher concentration of sucrose to prevent movement of water into this compartment from the compartment containing a more dilute solution of sucrose is called ***osmotic pressure.*** Pfeffer was able to show that the osmotic pressure is directly proportional to the concentration of the sucrose in the sucrose solution and to the absolute temperature. Two significant events soon followed the acquisition of these accurate data.

In the year 1877, Pfeffer published his work in a monograph entitled, "Osmotische Untersuchungen". On the basis of Pfeffer's findings, the Dutch physicochemist J.H. van't Hoff (1852–1911) formulated and announced what was to be known as the ***van't Hoff Law of Osmosis*** (1887): The product of the volume of the sucrose-containing compartment and the osmotic pressure is equal to the product of the molar concentration of sucrose, the gas constant and the absolute temperature. From this Law one could make the prediction that the product of the volume of the sucrose-containing solution and the concentration of sucrose is a constant. An osmotic system that demonstrates such a relationship was later referred to as behaving like a *perfect* ***osmometer*** (Lucké and McCutcheon, 1932).

In his "Osmotische Untersuchungen", Pfeffer also postulated that living cells are covered with a thin covering having the characteristics of the copper ferrocyanide membrane. He called this theory the ***membrane theory.*** In introducing the membrane theory Pfeffer had, in fact if not in intention, returned to the earlier view of Theodor Schwann emphasizing the primary importance of the cell membrane in a living cell over its content.

In conjunction with van't Hoff's Law of Osmosis, Pfeffer's membrane theory of the living cell would predict a quantitative relationship between cell volume and the concentration of impermeant solute in the bathing medium. When living cells are exposed to solutions of different concentration of a solute like sucrose, the product of the sucrose concentration and the equilibrium volume of the cell should be a constant. Experiments designed to test this prediction of the membrane theory were soon undertaken with strikingly affirmative results.

Experimental Testing of Pfeffer's Membrane Theory of Osmotic Behaviors of Living Cells

In 1855 the influential German botanist, Carl von Nageli, described the phenomenon of *plasmolysis* as the shrinkage of the protoplasmic mass of an adult plant cell from its rigid encasing cell wall when the cell is exposed to a concentrated solution, say of sucrose. Hugo de Vries (1871, 1885) demonstrated that the protoplast of the root cells of common beet (Beta vulgaris) plasmolyzed in a strong solution of NaCl and the entire cell substance inside its enclosing rigid cell wall, called ***protoplast,*** remained shrunken for as long as several weeks. This observation led de Vries to the conclusion that the root ***cell membrane is completely impenetrable to NaCl.*** Similar experiments on this and other cell types led to the broad belief that NaCl cannot penetrate the cell membrane. In 1902, E. Overton showed that frog muscle retains its natural volume when immersed in a 0.7% NaCl solution. Thus a 0.7% NaCl solution is ***isotonic*** to the frog muscle. When a high concentration of methyl alcohol is included in the 0.7% NaCl solution and a frog muscle immersed in this solution of NaCl and methyl alcohol, the muscle volume remained unchanged; while a muscle immersed in

an isotonic NaCl solution containing also a high concentration of ethylene glycol first shrank and then expanded. These facts led Overton to conclude that the frog muscle cell membrane is highly permeable to methyl alcohol and only slowly permeable to ethylene glycol. While all of these findings were qualitatively in accord with Pfeffer's membrane theory of osmotic behaviors of the living cell, it was the work of K. Hofler that offered ***quantitative*** support.

Hofler found a way of accurately measuring the volume of the shrunken protoplast when the cell was immersed in a concentrated solution of sucrose (Hofler, 1918). Using this method, and choosing as his experimental subject the parenchyma cells of the plant Tradcescantia elongata, he verified the predicted constancy of the product of the external sucrose concentration (varying between 0.3 M and 0.6 M) and the volume of the shrunken protoplast (see Lucké and McCutcheon, 1932). Thus the parenchyma cells did indeed behave like perfect osmometers as the theory of Pfeffer (and van't Hoff) had predicted.

Unfortunately the state of optimism thus created lasted only a short time. Later work of Hofler compelled him to reverse his earlier conclusion.

As mentioned above, mature plant cells are different from young plant cells and from "all" animal cells in that only in mature plant cells is there a large central vacuole filled with clear watery liquid. The membrane covering this central vacuole is known as the ***tonoplast.*** Between the tonoplast and the cell membrane or plasma membrane is the cell cytoplasm or protoplasm. The iconoclastic later work of Hofler revealed that only a part of the mature plant cell functions like a perfect osmometer; that part is the tonoplast-enclosed central vacuole.

In cooperation with American scientist, Robert Chambers, who was most skilled in microsurgical work, Hofler isolated the tonoplast-enclosed central vacuoles from mature plant cells and observed their volume change when immersed in solutions containing different concentrations of sucrose. The theoretically predicted constancy of the product of external sucrose concentration and vacuolar volume was confirmed on these isolated central vacuoles. They, rather than the whole cells, behaved like perfect osmometers (Chambers and Hofler, 1931). Nor was this entirely surprising since the content of this "vesicle" is definitely a simple watery liquid.

In other experiments on whole mature plant cells, other scientists showed that in concentrated salt solutions, the central vacuole does shrink as expected. However, the cytoplasm surrounding the central vacuoles actually swelled (Plowe, 1931). Our next questions is: "Do animal cells which do not possess a large central vacuole behave like perfect osmometers?"

Hamburger (1904) found that when red blood cells were transferred from a 0.9% to a 1.5% NaCl solution, the volume shrank but only by 17.5%. If the cells behave like perfect osmometers, then the product of the concentration and volume should be constant. In that case, the volume shrinkage should be 40%. However, Hamburger showed that the agreement between theory and observation would be greatly improved if the volume is reduced by 50–55%. This volume was referred to as Nichtloessender Raum or nonsolvent volume.

Historically, it was believed that nonsolvent is equal to the volume of the cell solids. In the light of later, more accurate and complete knowledge, the solid volume is too small to account for the nonsolvent volume. Thus the percentage solid weight of human red blood cells is 35%; the density of whole human red cells is 1.096 or approximately 1.10 (see Ponder, 1948, pp. 119–120). Since roughly 65% of the cell weight is water with a density

of unity, the cell solid density is equal to (1.10 − 0.65)/0.35 = 1.29 and the cell solid volume is thus 0.35/1.29 = 0.27 or 27%, which accounts for only about half of the nonsolvent volume.

Ernst Overton, whose studies of the volume changes of frog muscles in concentrated solutions of methyl alcohol and of ethylene glycol I have mentioned above, reported in the same article also on his study of the swelling of frog muscle in NaCl solutions below isotonic (0.7%), or ***hypotonic*** NaCl solutions (Overton, 1902).

If muscle cells behave like perfect osmometers as Pfeffer's membrane theory predicts, then a reduction of the concentrationsof the NaCl concentration in the bathing solution from isotonic (0.7%) to one half of that strength (0.35%) should lead to a two-fold or 100% increase of the volume of the frog muscle. In fact, Overton showed that the volume increase was only a third of the original weight. Since the total solid weight in frog muscle (20%) is even less than in red blood cells (35%), the volume of the cell solid volume is even less able to account for the discrepancy. Overton then suggested that at least a part of the water in muscle does not exist as normal liquid water but in the form he calls ***Quellungswasser*** or ***swelling water***. Indeed to make Overton's observed volume conform to the prediction of the membrane theory, more than 50% of the nicht loessender Raum must exist in the form of Quellungswasser.

Nobel laureate, A.V. Hill, whose work published in 1930 was briefly referred to above and will be again in greater detail below, reported in the same article (Hill, 1930) that he had repeated and confirmed Overton's observation. However, Hill offered a different explanation, i.e., the muscles he himself as well as Overton used were injured to such an extent that 25% of the muscle cells have lost their normal semipermeability.

First, it is not clear to me why Hill chose to perform most of his studies on the much thicker cylindrical gastrocnemius muscles (diameter: ca. 5 mm) rather than the much thinner sartorius muscles (thickness: ca. 0.6 mm), on which E. Overton carried out his experiments. This is all the more puzzling when one reads the specific reasons Overton gave for not using the gastrocnemius muscle and for using the sartorius muscles, e.g., too slow for the gastrocnemius muscle to reach equilibrium.

In hind sight, and after more than 45 years of more or less continually studying the frog sartorius muscle, I and my colleagues have learned how to isolate frog sartorius muscle without injury and to keep it alive ***in vitro*** at 25°C for more than a week, at the end of which it was shown that the muscle did not lose a significant amount of its K^+ content, its normal resting potential or its mechanical contractility (see Ling and Bohr, 1969). Using the technique learned, we have also repeated Overton's work on swelling frog sartorius muscle in half-isotonic solution and found that if Overton had included in his hypotonic solution, half of the normal K^+ and Ca^{++} content in a normal Ringer solution (Ringer, 1882–83), the increment of muscle volume in a half-isotonic solution would be 50% rather than 33% (as reported by Overton) (see Figure 9.16 on page 247 of Ling, 1962). Such a difference between a 33% increment and a 50% increment does not detract us from the judgment that the essence of Overton's conclusion was correct: frog muscles do not behave like perfect osmometers.

That injury did not materially affect the volume change in hypotonic solutions can be seen from the fact that the swelling in hypotonic solution after a 4-hour incubation was not significantly different in similarly treated intact sartorius muscles and sartorius muscles

which had been cut into 2 mm and 4 mm wide segments with both ends of each segment open (for evidence that no membrane regeneration takes place at the cut ends, see below) (Ling and Walton, 1976, Fig. 1).

In summary, in mature plant cells only the tonoplast-enclosed central vacuoles behave like a perfect osmometer as predicted by Pfeffer's membrane theory of osmotic behaviors of living cells. Animal cells do not have central vacuoles and, as exemplified by human red blood cells and frog sartorius muscle, do not behave like perfect osmometers either.

The Colloidal or Protoplasmic Approach to Cell Physiology

Martin H. Fischer and Gertrude Moore (1907) demonstrated that the swelling behaviors of frog muscle in acids, alkalis and salt solutions bear strong resemblance to those of swollen beef fibrin and of gelatin (Fischer, 1909). Fischer and Moore pointed out that in muscle cells as well as fibrin (and gelatin) the affinity of the colloids for water is not constant but variable. In both, the affinity increased sharply on the addition of acids as well as of alkalis. This variability of ***swelling water*** in living cells formed the core of Fischer's concept of oedema and nephritis (Fischer, 1909). In 1938, Martin Fischer and Werner J. Suer wrote: "it (protoplasm) is held to be a union of protein, salt and water in a giant molecule". (The most important salt ion in the cell is K^+. The question whether the cell K^+ is free or in some way united to the proteins will be discussed below under the subject of "solute distribution"). (see Endnote 2 for more about Martin Fischer).

As time went by, the term "bound water" was more often used in replacement of the more cumbersome "swelling water" or "imbibition water". Efforts were made to characterize and measure quantitatively bound water in models of living cells. ***The cryoscopic method*** was based on the assumption that bound water does not act as a solvent for dissolved solutes. This was how the concept of "nonsolvent water" came from Newton and Gortner (1922). ***The calorimetric method*** depended on the assumption that bound water could not freeze at the temperature at which normal liquid water freezes (i.e., –20° to –30°C). This was then the beginning of what was known as "nonfreezing water" (Rubner, 1922; Robinson, 1931).

Using the cryoscopic method, Newton and Martin (1930) demonstrated a good correlation between draught resistance of various species of grasses and what they measured in the expressed cell sap as "nonsolvent water". They also showed that the percentage of "nonsolvent water" was considerably higher in solutions or gel of gelatin and agar than in solutions containing an equal concentration of vegetable albumin. This conclusion is in agreement with parallel studies of Thoenes (1925) on "nonfreezing water" in gelatin and agar by calorimetric method. This author also showed that dog and guinea pig muscle contained between 19% to 26% of bound water (see also Gortner, 1938, pp. 283–287).

In 1926 T. Moran demonstrated that even at the low temperature of liquid air (ca. –190°C), no ice formed in a 65% gelatin gel. Since 65% gelatin contains 35% water, this gelatin gel retained 0.54 grams of non-freezing water per gram of dry gelatin.

Moran also showed that the percentage of "nonfreezing water" at –3°C increased sharply with decreasing pH brought about by the addition of more and more HCl. Moran's findings were in harmony with Fischer and Moore's demonstration of steeply increasing percentage of ***swelling water*** in fibrin and in gelatin with the addition of an increasing amount of HCl (see above). Assisting Moran in the work described, was a Mr. H.P. Hale whose help

Moran acknowledged at the end of the article published in the Proceedings of the Royal Society of London.

In 1932 Robert Chambers who had cooperated with Höfler in isolating the tonoplast-enclosed central vacuole of adult plant calls cooperated with the same Mr. H.P. Hale in their freezing experiments of isolated frog muscle and other cells (Chambers and Hale, 1932). They observed that in isolated single muscle fibers, ice formed only in the form of elongated spikes without any branches and thus was quite different from the normal ice crystals with feather-like hexagonal dendrites. When the muscle fiber was twisted, the ice spikes also assumed a twisted pretzel-like shape (Chambers and Hale, 1932). Chambers and Hale's observations were confirmed by Rapatz and Luyet in 1959 and by Miller and Ling in 1970.

In 1934 Dorothy Jordan-Lloyd and T. Moran published in the Proceedings of the Royal Society of London their beautiful work on the water retained in gelatin gels when subjected to high pressure. They showed that with increasing pressure, up to 7000 lbs per square inch, there was rapid loss of water from a 66% gelatin gel to about 33%. However, with the pressure increasing from 7,000 to 38,000 Ibs per square inch, the water content remained essentially constant at about 0.4 to 0.5 gram of water per gram of dry gelatin. This figure agrees well with the nonfreezing water at –190°C reported by Moran alone in 1926.

In the above I have taken special pains to document the extensive mutually supportive evidence that gelatin—which typifies colloids—contains a sizable amount of water which resists freezing even at liquid air temperature, and could not be squeezed out by pressure as high as 38,000 Ibs per square inch. Still other studies showed that this water also does not dissolve sucrose.

All in all, these findings offer evidence for a remarkable progress toward the understanding of protoplasm, the physical basis of life, as Thomas Huxley so eloquently described. And there seemed little reason to doubt that more important progress would continue to be made in this protoplasmic approach to cell physiology.

Could one believe then that the future of cell physiology would be at that very time about to make a violent turn so that the whole protoplasmic approach would be more or less abandoned, and the major English-speaking journal of colloid chemistry, the ***Journal of Colloid Chemistry,*** terminated? But history shows that this indeed happened. Moreover, it came from the work of one man in the form of one paper communicating, in essence, a single set of experimental data which would take a competent experimentalist no more than a day to accomplish. It is often said that a single falling apple changed the course of the progress of physics. Did something of this kind happen in cell physiology?

The Man and His Single Experimental Finding that Derailed the Protoplasmic Approach to Cell Physiology

Archibald Vivian Hill (1886–1977) was tall in stature, athletic in build and all in all a most commanding type of a person. He received his education at the Trinity College of Cambridge University but from 1923 on worked at the University College of London. In 1922, Hill and Otto Meyerhof were conjointly awarded the Nobel prize for their study of muscle physiology (see below).

The experimental finding that was to change the future course of cell physiology appeared in 1930 in the 106th volume of the Proceedings of the Royal Society of London,

Series B. In this paper Hill presented the experimental work of Mr. P. Eggleton and Mr. H.V. Horton who demonstrated that ***urea distributed equally between the cell water of isolated frog sartorius muscles and water of the external bathing solution.***

Based on this set of findings Hill concluded that frog sartorius muscle does not contain any significant amount of bound water. Were it otherwise, the average equilibrium concentration of urea in the muscle cell water would be correspondingly lower than that in the external medium.

Confirmations of Hill's contention that no bound water exists in living cells came in rapid succession. MacLeod and Ponder (1936) found that ethylene glycol also distributes itself equally between water in red blood cells and in the external medium. Hunter and Parpart (1938) showed equal distribution of ethylene glycol between water in frog abdominal muscle and the external medium. The overall impact of Hill's announcement and its sequels was electrifying.

E. Ernst who was an eyewitness to all these events, recalled in 1963 how, in response to A. H. Hill's work, the opinion makers of the day, including Rudolf Hober, W.O Fenn and F. Buchthal, all renounced the concept of bound water (and bound K^+) and began to subscribe in total to the idea that living cells represent a simple aqueous solution obeying the osmotic laws of van't Hoff etc. (Ernst, 1963, p. 112).

It must be emphasized that the derailment of the protoplasmic approach to cell physiology was largely a matter of opinions. Hill poked a hole in an idea that was then popular among the protoplasm-oriented cell physiologists and they were caught without a defense and, as a result, a historical turnabout took place. Hill did not even deal with the important findings of Moran and of Jordan-Lloyd mentioned above, for example. He and his supporters were most probably not aware of the important work of the Soviet scientists, Dmitri Nasonov and his colleague, E.I. Aizenberg, published in Russian.

In 1937, the Soviet biologists, D.N. Nasonov and E.I. Aizenberg, exposed isolated frog muscles to concentrated solutions of various solutes including 20% urea, 1.69% alanine, and 1.8% (and 4%) sucrose (Nasonov and Aizenberg, 1937; see also Troshin, 1966, pp. 37–52). They then followed the change of weights of the muscles as well as the intracellular concentrations of each of the solutes to which the muscles were exposed. In all cases, while the concentration of each of the solutes was steadily increasing in the cell—thereby establishing that they were all able to penetrate the cell membrane—the muscles were steadily losing weight until a specific steady (shrunken) state was reached and ***maintained.*** (Nasonov and Aizenberg's results were confirmed and extended first by Troshin and by themselves individually, see Troshin, 1966, p. 47; see also Nasonov, 1938; Aizenberg, 1939), and much later still by G. N. Ling (Ling, 1980, 1987a).

These studies showed that cells as a rule do gain or lose water in a sustained manner in solutions containing low or high concentrations of solutes respectively. However, unlike that predicted by the membrane theory, the effectiveness of these solutes in causing sustained cell-volume changes ***are not causally related to the impermeability of the cell membranes to these solutes. Permeant solutes do the same.***

This generality applied to neutral solutes, as those mentioned above, as well as to charged electrolytes like NaCl. In history, sucrose and NaCl had long been regarded as impermeant solutes to the living cell on the basis of a circuitous argument: sucrose and NaCl cause sustained shrinkage because they are impermeant to the cell membrane; they are impermeant to the cell membrane because they caused stained cell shrinkage.

Nasonov and Aizenberg's finding showed, to the contrary, that there is no causal relationship between impermeability through the cell membrane and the ability of an electrically neutral solute to cause cell shrinkage. The advent of radioactive tracer technology facilitated the demonstration of a similar lack of causal relationship between membrane impermeability and the ability of an electrically charged solute like NaCl to cause sustained cell shrinkage. In response, the sodium pump hypothesis was introduced as I have already mentioned.

In the 1950s, T.H. Wilson (1954) and A. Leaf (1956) presented the view that the continual activity of the postulated sodium pump makes the Na^+-permeable cell membrane ***effectively impermeable*** to the Na^+ ion. Undoubtedly it was the language barrier that prevented Wilson, Leaf and many others from familiarity with the Soviet scientists' important work. If they did know Nasonov and Aizenberg's work, they would be forced to postulate pumps for urea, alanine, galactose, and sucrose as well, because these solutes behave just like the Na^+ ion, being permeable and yet able to cause sustained shrinkage at high concentration. In 1966, an English translation of Troshin's book was published by Pergamon Press, making available the Russian work described above. However, I have not been able to detect any major response to the work now in English from the supporters of the membrane-pump theory. And it is not difficult to see why.

In summary, the osmotic behaviors of living cells do not follow the predictions of the membrane theory with or without an Na pump. It is here that the long history of explaining cellular volume changes in terms of the membrane theory came to an end.

The Membrane Theory of Cellular Electrical Potential

In 1757 Leopoldo M.A. Caldani elicited contraction of isolated muscle by sparks from a discharging Leyden jar (see Rothschuh, 1973, p. 140). In 1786 Alosius (alias Luigi) Galvani again elicited the contraction of frog muscles, this time inadvertently when a metal hook inserted into the frog's spinal cord accidentally touched the metal plate on which the frogs' legs were lying. Galvani believed that the electricity was originally stored in the brain of the frog and this "animal electricity" was sent down to, and stored on, the surface of the muscle much as in a Leyden jar. Galvani's claim of animal electricity was strongly disputed by physicist, Alexandro Volta, who believed the electricity was entirely due to the bimetallic contacts.

The ultimate outcome of this controversy was strangely divergent. For those writing about physics and physicists, Volta's position was upheld: Volta was the one who spoke the last word (see Morgan, 1954, p. 42). Just after the French Academy of Sciences had repudiated Anton Mesmer's claim of ***animal magnetism,*** it is not surprising that skepticism was entertained by some physicists against Galvani's claim of ***animal electricity.*** However, for those writing about biology, Galvani's position was upheld (see below). In any case, investigation of the electrical activities of muscle continued in Italy.

In 1841 C. Matteucci discovered that the intact surface of the isolated frog muscle was electrically positive in reference to the cut surface of the muscle. In the next year, Matteucci reported to the Academy of Science in Paris what he called "Secondary Twitch" (Matteucci, 1842): Two frog muscles were isolated with their sciatic nerves' connection to each muscle kept intact. He then placed the nerve of one muscle (muscle A) onto the surface of

the second muscle (muscle B) and with a pair of metal electrodes stimulated the nerve of the muscle B. In consequence, not only did muscle B respond with contractions, muscle A also responded with contractions. Thus Matteucci learned that activity of muscle B had excited the nerve of muscle A.

According to historian, Karl E. Rothschuh, Galvani had carried out an experiment quite similar to the one described above (and thus years ahead of Matteucci). The trouble was that Galvani published this work anonymously in 1794—parenthetically one asks, Why anonymously? Was he very afraid of being dealt with as was Anton Mesmer? In any case, Galvani was entirely different from Mesmer. Thus in the words of Rothschuh: "Galvani placed the nerve of a muscle-nerve preparation on the fresh section of another muscle, eliciting contractions when the nerve and muscle established contact. For him, this was the decisive proof of the existence of an "animal electricity". Rothschuh further pointed out that this particular experiment was further confirmed by the German naturalist, Alexander von Humboldt, who published his work in the two volumes entitled "Versuche über die gereizten Muskel—und Nerven Fasern" (Rothschuh, 1973, pp. 141–143).

Electrical potential difference was also found between the intact and cut surface of nerves. Later a steady electric current was observed between the intact and cut surface of an isolated nerve by Emil duBois-Reymond. He called this electrical current ***demarcation current*** or ***injury current*** and the potential difference between the intact and cut surface in muscle and nerve ***"demarcation potential"*** or ***"injury potential"*** (du Bois-Reymond, 1848–1849).

Du Bois-Reymond also discovered that if a brief electric stimulus was applied to a nerve, the magnitude of the injury current decreased. This change was attributed to what he called a "negative Schwankung" (negative variation). Later du Bois-Reymond was able to "buck out" the standing injury potential and isolated the negative Schwankung in pure form, known later as the ***action potential.*** The demarcation potential was seen as the foundation of the action potential and as an imperfect expression of a lasting and standing electrical potential difference across the cell surface called the ***resting potential.*** Still later, the resting potential was referred to with increasing frequency as the ***membrane potential*** (for comments on replacing the noncommittal name, resting potential, with the name prescribed by an unproven theory and for similar renaming of the whole or part of cell physiology, membrane physiology, see Endnote 3).

In 1890 Wilhelm Ostwald (not to be confused with Wolfgang Ostwald mentioned earlier) measured the electric potential differences between two salt solutions separated by a copper ferrocyanide membrane. Based on these observations, he ventured the suggestion that the muscle and nerve potential as well as the electric current delivered by an electric eel may have a similar origin.

In 1900 J. S. MacDonald published a paper in the Proceedings of the Royal Society of London entitled "The Demarcation Current Considered as a Concentration Cell", in which he expressed the view that the inside of the nerve is a more concentrated electrolyte solution than the external medium in which the nerve was bathed. He then demonstrated a linear relation between the demarcation potential measured and the logarithm of the concentration of potassium salt in the external medium. MacDonald probably did not know about Ostwald's suggestion and made no reference to it. This is not the case with another investigator, Julius Bernstein, who was once a student of the great physicist-physiologist Hermann von Helmholtz.

Julius Bernstein (1902) acknowledged the suggestion of Ostwald and proceeded to present his *"membrane theory"* of cellular electric potentials. By making certain simplifying assumptions [e.g., the cell membrane is impermeable to anions and to the cation, sodium (Na^+) but permeable to the cation, potassium (K^+)], Bernstein arrived at an equation which predicts that the resting potential should be inversely proportional to logarithm of the concentration of K^+ outside the cell—as MacDonald had already demonstrated. In addition, the potential should also be directly proportional to the absolute temperature.

In years following, the technique of recording the resting potential has markedly improved. The success in isolating the giant axons of squids and other aquatic animals offered one new approach. The employment of the Gerard-Graham-Ling glass capillary microelectrode offered another (Graham and Gerard, 1946; Ling and Gerard, 1949). With these improved techniques, the resting (and action) potential can be measured with accuracy. And soon it was shown that the predicted relationship between the resting potential of a variety of living cells and the logarithm of the external K^+ concentration was universally confirmed. So was the linear relationship between the absolute temperature and the resting potential.

However, the predicted relationship between the logarithm of the ***intracellular K^+ concentration*** and the resting potential proved troublesome. Of the 14 laboratories that had examined this relationship, four found confirmation. Ten others could not (see Ling, 1992, pp. 276–277).

Another kind of serious discord concerns the critical assumption of Bemstein that the cell membrane is impermeable to sodium ion (Na^+). As mentioned above, since the time of de Vries, the concept of membrane impermeability to the sodium ion has been widely accepted. Yet as also mentioned repeatedly above, studies with radioactively labelled Na^+, as well as nonlabelled Na^+ have unequivocally disproved the concept of impermeability of the cell membrane to Na^+ ions, a concept on which the membrane theories were built.

Since the cell membrane is permeable to the Na^+ ion, Bernstein's equation of the cellular resting potential as such is no longer valid, because the key justification for disregarding Na^+ in Bernstein's equation of resting potential disappears.

Under this condition, one could only deal with the sum of intercellular K^+ and Na^+ and the sum of extracellular K^+ and Na^+, rather than the intracellular and extracellular K^+ concentration alone as Bernstein did. Since the sum of intracellular Na^+ and intracellular K^+ is roughly equal to the sum of the extracellular Na^+ and K^+, the ratio of the intracellular and extracellular sums are about equal. But the logarithm of unity is zero. The now correctly written version of the Bernstein equation would not predict the existence of a resting potential at all. Yet an electrical potential difference of considerable magnitude definitely exists across the frog muscle and many other cell surfaces. The need of a new quantitative theory thus arose. In response, two different theories were suggested. Only one of these will be briefly discussed here. Following the strategy mentioned at the outset of this historical review, I will describe the second theory introduced by myself toward the end of the paper.

The first theory was proposed by A.L. Hodgkin and B. Katz under the name of ***ionic theory*** (Hodgkin, 1951). The ionic theory was proposed by Hodgkin—in the wake of Hodgkin and Katz's brilliant discovery that the magnitude of the action potential depends on the concentration of Na^+ in the external medium (Hodgkin and Katz, 1949). It has been known for some time that when the external Na^+ is removed and replaced by sucrose, the muscle or nerve becomes inexcitable—as was first reported by E. Overton in 1902 (Over-

ton, 1902). Hodgkin and Katz gave an elegant interpretation for this phenomena when they showed that the height of the action potential, or more precisely what is called the "overshoot" —the excess voltage shift beyond the annulment of the inside-negative outside-positive resting potential —is linearly dependent on the logarithm of the external Na^+ concentration. Based on these and other observations, Hodgkin and Katz (1949a) introduced what has been known as the Hodgkin-Katz-Goldman(HKG) equation.

In form, the HKG equation resembles the original Bemstein equation, with the exception that instead of intra- and extracellular K^+ concentrations alone, the equation includes also the intra- and extracellular concentrations of two other ions: Na^+ and the negatively charged chloride ion (Cl^-). So the key term of the equation represents the logarithm of the ratio of not just K^+ or even K^+ and Na^+ but the sum of the intracellular K^+, intracellular Na^+ and extracellular Cl^- divided by the sum of the extracellular K^+, extracellular Na^+ and intracellular Cl^-. Moreover, each of the K^+ concentration terms is multiplied by a constant P_K representing the permeability of the cell membrane to this ion. Similar permeability constants P_{Na} and P_{Cl} are attached to the Na^+ and Cl^- terms respectively.

This ionic theory overcomes the difficulty which confronts the Bemstein equation in regard to the intrusion of the Na^+ concentrations after the demonstration that this major external cation is also permeant to the cell membrane. Here by giving higher values to P_K than to P_{Na}, the concentration of both ions can be represented but with different impact on the magnitude of the resting potential. The high external Na^+ concentration was made far less effective in bringing down the magnitude of the resting potential by employing a very small value of h a , so that the product of the external Na^+ concentration and its permeability constant P_{Na} remains small.

What was even more exciting was that by assigning a drastically higher value to P_{Na}, Hodgkin and Katz could quantitatively predict the value as well as the electric sign of the action potential. This was no mean accomplishment and accordingly they and another of their coworkers, Andrew Huxley, received the Nobel Prizes for the year 1963.

The ionic theory described by the Hodgkin-Katz-Goldman equations suffers from two major shortcomings. Worse, the authors of these equations were unable to, or for some other reason(s) would not, defend their theory against serious published criticisms concerning these shortcomings (Ling, 1962, 1984, 1992). Nor did they accept an official invitation to enter into a televised debate with myself and others on the subject of cellular electric potential and related subjects at Atlantic City scheduled for 1968, claiming prior engagement in a letter dated the year before.

The first difficulty encountered by the ionic theory is the same that unsettles the original Bemstein's membrane theory: namely, of the 14 laboratories studying the problem, only a minority of four laboratories found that the resting potential depended on the intracellular concentration of K^+. Ten others could not confirm this prediction. Their difficulties could not be due to their lack of technical expertise, because none of them had any trouble confirming the predicted relationship between the resting potential and ***external*** K^+ concentrations. To the best of my best knowledge, neither Hodgkin, nor Katz nor anyone else came forth with an explanation of this major discrepancy.

A second discrepancy between experimental facts and the Ionic theory is even more serious. This concerns the participation of the Cl^- concentration terms. In the rigorous derivation of the Hodgkin-Katz-Goldman equation (due largely to David Goldman) the three terms included in the equations for K^+, Na^+ and Cl^- are of roughly equal importance.

Of course, each term is weighted according to its permeability constant. However, studies of R.H. Adrian reported in 1956, and by A.L. Hodgkin himself in conjunction with P. Horowicz reported in 1959, conclusively demonstrated that profound alterations in the concentration of external Cl^- ion produced no noticeable change of the resting potential of frog muscle.

A legitimate explanation of this indifference to external Cl^- concentration would be a very low permeability of the frog muscle to this ion. In fact, just the opposite is the case. Hunter and Padsha showed in 1959 that the permeability of frog muscle membrane to the Cl^- ion is twice as high as that of K^+. Lorkovic and Tomanek (1977) reached a similar conclusion from their studies of mammalian muscles.

Nonetheless, the authors of the HKG chose to delete the Cl^- terms from the equation (Katz, 1966). Repeatedly protesting this action, I pointed out that it is not within the rule of rigorous science to chop off a part of a derived equation when the predicted relation involving that part is not experimentally confirmed. I also pointed out that the claim by Katz (1966, p. 62) that the Cl^- term can be deleted because this ion has reached diffusion equilibrium is not valid. The reasons are given by Ling in 1978 (Ling, 1978, pp. 416–417) and in 1984 (Ling, 1984, pp. 463–465). Thus most electrical potentials measured in the inanimate world, including those measured by Ostwald across copper ferrocyanide membranes—which started Bemstein on his formulation of the Bernstein equation—are equilibrium potentials.

Finally let us consider a third serious contradiction from the study of inanimate models. This concerns the failure over a period of some seventy years to produce a single inanimate model that confirms the existence anywhere —xcept in the minds of theoretical scientists— of "membranes potential" arising from the different permeabilities of electrically charged particles or ions through a membrane.

The story has an almost unbelievable quality: How could a failure of this fundamental nature, unanimously confirmed again and again over such a long span of time, be kept entirely out of the reach of cell physiologists researching on what they call so confidently, the membrane potential? (Ling, 1984, pp. 470–473; 1992, pp. 282–285).

From the 1920s on, there have been serious efforts aimed at confirming the membrane theory of cellular electrical potential. To do so, various simple inanimate membrane models were chosen. When such a membrane partitions two electrolyte solutions, electric potential differences like those measured across the surface of living cells like muscle and nerve were often observed. As mentioned earlier just before the work of Thomas Graham was introduced, confirmation of a theory on inanimate model is the very first step that a theory-maker ought to try before waxing too eloquent on how well his theory can explain a comparable but more complicated living phenomenon.

Technically there is little difficulty in testing the Bemstein or the Hodgkin-Katz-Goldman equation on some simple inanimate membrane systems separating K^+, Na^+, Cl– and other ions of interest. Indeed, to conduct such an experiment one needs nothing much more than a potentiometer (e.g., a pH meter). It is no surprise that no less than four different kinds of membrane had been thoroughly investigated over a period of some 70 years. Finding these publications proved far more difficult; it took more than thirty years to get the four sets of data together and compare their main conclusions.

The four membrane models thoroughly studied were the glass membrane, the collodion (nitrocellulose) membrane, the oil-layer membrane, and the phospholipid membranes. Note

that the copper-ferrocyanide gel membrane which inspired Wilhelm Ostwald to suggest a common mechanism underlying the potential difference across such a membrane and the living cell surface was not thoroughly studied. The unsupported gel membrane is obviously too fragile; a gel membrane deposited in the interstices of a porous unglazed porcelain candle does not satisfy the basic requirement of a ***simple*** model.

At the beginning, all four sets of investigators were trying to test the membrane potential theory. As mentioned above, in this theory, a potential difference (the membrane potential) is created between two aqueous phases separated by an artificial or living semipermeable membrane. The potential difference arises from the ***unequal permeabilities*** of the diffusing ions.

When their respective investigations were concluded, the investigators all reached the conclusion that the membrane potential they were looking for was not what they found. In all cases, the ionic permeability of the membrane proved irrelevant to the creation of the measured potential difference.

On what they did find, they were in complete agreement, even though they were often unaware of each other's work—being in some cases separated by long periods of time. To describe the essence of what they all found I present next, in some detail, the work of Colacicco, published in 1965 in the journal, ***Nature*** (Colacicco, 1965).

Colacicco measured the electrical potential difference between two aqueous KC1 solutions of different strengths separated by a thin layer of oil. If the KCl solution is 100 mM on one side and 1 mM or the other side, their ratio would be 100. The common logarithm of 100 is 2. Now, if the model follows the theory proposed by the proponent of the membrane theory, one may expect to measure a potential difference of close to 100 millivolts. In fact, none was measured.

One may argue that this failure to observe an expected potential arose from the impermeability of the oil layer to both K^+ and Cl^- ions.

Colacicco then introduced into one compartment a very small amount of the anionic detergent, sodium dodecylsulfate or SDS. SDS is a strong electrolyte; the Na^+ component is fully dissociated as a free cation. The dodecylsulfate anion has a long saturated hydrocarbon chain ending on a negatively charged sulfate group. The saturated hydrocarbon has a high solubility in the oil layer and poor solubility in water; in contrast, the charged sulfate end has a very low solubility in oil but high solubility in water. The introduction of SDS (to one side of the oil layer) leads to a compromise: the formation of a molecular layer of SDS with the hydrocarbon end of the molecule imbedded in the surface of the oil layer while the polar sulfate end remains in the contiguous water phase.

With the introduction of this minute amount of SDS, a dramatic change took place. Now instead of dead "silence", Colacicco observed a solid potential difference. Furthermore, this potential difference measured across the oil layer is sensitive to the concentration of K^+ ion (but not that of the Cl^- ion) in the SDS-containing compartment (only). This SDS-containing compartment becomes electrically positive with respect to the other compartment. The potential difference measures +39 mV with a KCl concentration of 1.0 M, increasing steadily with decreasing KCl concentration. When the solution contains the least concentration of K^+ (i.e., in "pure" water), the potential was +158 mV. In contrast, the potential difference is indifferent to the concentration of either K^+ or Cl^- in the compartment (containing no SDS) on the other side of the oil layer.

Colacicco's finding shows that a potential difference is created at the oil surface when it is exposed to **SDS.**

A membrane separating two aqueous solutions like the simple, unadulterated oil layer creates no potential difference as long as neither one of its two interfaces carries fixed electric charges. When fixed charges are introduced to one interface, an electric potential difference is created that is sensitive to the concentration of the free ion bearing the opposite charge as that of the fixed charge. Thus the fixed charge provided by the negatively charged sulfate ion of the **SDS** molecule creates a potential difference, sensitive to the~'concentration in the bathing fluid, but no sensitivity to the free Cl^- anion.

The question might be raised: "Could the effect of **SDS** in producing the K^+ sensitive potential be due to an ion-carrier or "ionophore" effect?" That is, could **SDS** make the oil layer selectively permeable to K^+? this hypothesis of **SDS** serving as a K^+ carrier is contradicted by the indifferenceof the system to the K^+ concentration in the other compartment containing no **SDS.** For if **SDS** acts by enhancing K^+ permeability, the potential would be a membrane potential. As such, it must be sensitive to the K^+ concentration on both sides of the oil layer. This is contrary to what Colacicco observed.

Colacicco conducted additional experiments. In these companion experiments, Colacicco employed a cationic detergent, cetyltrimethylammonium bromide (CTAB) instead of anionic detergent **SDS.** CTAB also has a long aliphatic tail but instead of a negatively charged group at the end of the molecule, CTAB has a positively charged quaternary ammonium ion at its end. The introduction of CTAB therefore also creates an electric double layer with, however, the layer of fixed *cations* anchored onto the oil surface. As a result, the system acquires a new sensitivity to the Cl^- concentration in the phase containing the CTAB but not in the other compartment. The electrical polarity is just the opposite of the SDS-induced potential difference. At the lowest Cl^- concentration in "pure" water, the potential difference measured was –220 mM. The potential is in this case indifferent to the K^+ concentration in both compartments. This finding by itself might also be explained on the basis of the membrane potential hypothesis: That is, if one postulates that CTAB makes the oil layer selectively permeable to CL^-. The fact that the potential is indifferent to the concentration of Cl^- in the opposite compartment containing no CTAB, however, refutes this interpretation.

Colacicco then combined these two experiments. He introduced **SDS** to one compartment and CTAB to the other compartment, and measured the electrical potential difference across the oil layer.

Now, if **SDS** truly makes the oil layer selectively permeable to K^+ and the CTAB truly makes the oil layer selectively permeable to Cl^-, the simultaneous addition of **SDS** to one compartment an CTAB to the other compartment would have created an oil layer permeable to both K^+ and Cl^- ions. In other words, one would now have a membrane fully satisfying the definition of the semipermeable membrane for the generation of a membrane potential. And if both compartments contain the same (lowest) concentration of KCl provided by "pure" water, one would predict a potential difference of zero mV from either Bernstein's equation or that of the HKG equation.

What Colacicco actually observed was the opposite. He found that the measured potential is the *sum* of the **SDS** and CTAB potentials. Thus with the "pure" water on both sides, the potential difference measured was 370 mV.

The essence of what Colacicco observed agrees with the results of the study of the three other sets of inanimate models mentioned above: glass membrane, collodion membrane, and phospholipid membranes (for review see Ling, 1992, pp. 282–285).

In summary, the currently widely taught ionic theory is a modified membrane potential theory where the electrical potential difference is believed to originate from the different permeability of various ions through the cell membrane. The ionic theory cannot explain the indifference of the resting potential to the concentration of external Cl^-, which in muscle cell traverses the cell membrane faster than even K^+, nor to the indifference to the intracellular concentration of both K^+ and of Na^+ as observed by ten of the 14 laboratories that have investigated the phenomenon. But the greatest difficulty facing the ionic theory is the failure to find a single experimental model that demonstrates a membrane potential which is created by the different membrane permeabilities to different ions. Unable to resolve these difficulties, the proponents of the membrane theory of cellular electric potential are stuck.

Life's Source of Energy: Fire, Lactic Acid, ATP

Ancient Greek philosophers saw a close relationship between life and fire. Heraclitus regarded fire as the most important component of the human body. Democritus considered the soul and mind comprising the same sort of small, round, fiery atoms. These thoughts lay buried during the Middle Ages and were to re-emerge after the Renaissance.

In the 16th century, Paracelsus and Francis Bacon likened life to a fire. Paracelsus further proposed that air contains something essential for both the ordinary flame and the *flamma vitalis*. Anton Lavoisier in 1780 quite clearly demonstrated that the substance Paracelsus suspected in air is oxygen and that animal respiration is a form of combustion in which oxygen molecules are "burnt" with the same products as if they had burned in a fire.

René Descartes (1595–1650) equated life with motion and especially with *feux sans lumière* (fire without luminance). Now fire produces both light and heat. If Descartes is right and life is indeed "feux sans lumière", clearly it must be heat produced by the living fire that drives the living engine. In harmony with this idea, William Harvey (1578–1657) believed that the central importance of blood circulation was the distribution of life-giving heat.

In the late 19th century, more specific theories appeared relating heat on the one hand, and the most prominent among life's manifestations: muscle contraction. An example was the theory of Engelmann. In 1873, Engelmann suggested that heat provided by metabolic reactions causes the "dark bands" of the voluntary muscle to imbibe water from the "light bands" (see Figure 8 below for a photograph of these bands) and assume a shorter spherical shape, hence muscle shortening. To confirm his theory he demonstrated heat-induced reversible contractions of an artificial muscle in the form of a catgut string. Unfortunately, Engelmann's model was shown to be untenable on theoretical grounds by physiologist Adolf Fick (better known for his Laws of Diffusion).

Fick showed that in order to achieve the heat-activated contraction Engelmann suggested and at the efficiency known for muscle contraction, the muscle must be heated to a temperature well above the boiling point of water (i.e., 114°C). No muscle could survive such a high temperature, even if such a superheated condition could be obtained.

The conclusion from the Engelmann-Fick encounter is historically far more important than recognized. *Fick's disproof had abruptly put an end to one of the most venerable ideas of biology, that heat is the source of energy for biological workperformance.*

Not only is heat produced by the proposed feux sans lumière, proven to be unsuitable to drive the biological machine, some real life machines can also get by without oxygen at all. This is also contrary to the ancient belief that life is related to fire since ordinary fire invariably is put out by the removal of this gas.

Thus J. B. van Helmont (1577–1644) believed that fermentation rather than fire produced the vital heat. In his early years, Thomas Willis (1621–1675) voiced a similar belief. However, it was the investigation of Louis Pasteur that clearly established two kinds of life, one in the presence of oxygen and which he named "aerobic" and another in the absence of oxygen, which he called "anaerobic". Pasteur showed that the microorganism responsible for the butyric fermentation and putrefaction of meat, eggs or other protein-containing materials are examples of anaerobic life.

However, life of cells belonging to the "higher" form of life also can continue in the absence of oxygen. Muscle cells offer an example of this type. The first clear demonstration of this phenomenon was due to Fletcher and Hopkins (1907). They showed that when frog muscles were isolated and deprived of the normal oxygen supply, lactic acid was formed steadily as long as the muscles remained excitable. This lactic acid production ceased upon the admission of oxygen.

In muscle it is the breakdown of glycogen that gives rise to the lactic acid and the process is known as ***glycolysis.*** In 1930, Otto Warburg offered a broader definition for glycolysis as "splitting of carbohydrates into lactic acid" (Warburg, 1930).

In the 1920s a heated controversy developed between Gustav Embden and A.V. Hill—Hill was a strong champion of the "lactic acid theory" of muscle contraction in the 1920s. Hill and Otto Meyerhof were awarded the 1922 Nobel Prize "for the discovery of the fixed relation between the consumption of oxygen and the metabolism of lactic acid in muscle".

Embden objected to Hill's lactic acid theory of muscle contraction. He believed that the production of lactic acid followed rather than preceded muscle contraction—in contradiction to Hill's idea that lactic acid produced gave rise to contraction. The Hill-Embden controversy came to an abrupt end when Einar Lundsgaard entered the scene with new evidence. Lundsgaard published his finding of muscle contraction without lactic acid, based on his discovery that the drug, iodoacetic acid (IAA), arrests the production of lactic acid in muscle. Yet the IAA-poisoned muscle continued to contract, for a limited number of times, in a manner not significantly different from normal muscle. The elimination of lactic acid—the major product of glycolysis—as the causal agent of muscle contraction shifted the attention to a newly discovered phosphorus-containing compound, phosphagen.

Fiske and Subbarow (1925) in the United States and Eggleton and Eggleton (1927) in England independently reported the discovery of a labile compound, which for a while was called ***phosphagen,*** but later was accepted as ***phosphocreatine.*** It was soon demonstrated that contraction of muscles exposed simultaneously to oxygen-free pure nitrogen and IAA depends on the presence of phosphocreatine. With successive contractions the content of phosphocreatine decreased until it became completely exhausted. At about this time, the muscle stopped contracting and has indeed already entered the state of rigor. These findings suggested that phosphocreatine is more intimately tied to the mechanism of muscle contraction. But then another important discovery was announced by K. Lohmann.

In 1935 Lohmann announced the discovery of another phosphorus-containing compound from muscle: ***adenosinetriphosphate*** or ATP. In addition he also discovered a very important reaction, known now as the Lohmann reaction. In this reversible reaction catalyzed by

the enzyme, creatine kinase, a molecule of creatine reacts with a molecule of ATP to produce a molecule of phosphocreatine and a molecule of adenosinediphosphate or ADP.

When simultaneously poisoned with pure nitrogen and IAA, both oxidation and glycolysis of the muscle cells are inhibited. When such a poisoned muscle is made to contract, its ATP content remains unchanged while its store of phosphocreatine declines steadily. This insensitivity of ATP content to contraction threw doubts on the contention that ATP is a more immediate "energy source" for the contraction than phosphocreatine. Many years later, a specific drug, fluorodinitrobenzene(FDNB) was discovered by Infante and Davies (1962). This drug inhibits the Lohmann reaction. When muscles exposed to pure nitrogen, IAA and FDNB are stimulated, the expected fall of ATP concentration in a contracting muscle was at long last revealed. The reason that in the absence of FDNB no ATP concentration fall could be detected is due to its extremely rapid replenishment at the expense of prosphocreatine through the activity of the creatine kinase.

When normal muscle contracts very vigorously, its phosphocreatine content also declines but only momentarily. Soon after the cessation of contraction, the phosphocreatine concentration returns to normal. Since in living muscle phosphocreatine cannot decompose or be resynthesized without the intervention of creatine kinase, clearly the restoration of the phosphocreatine level is via the resynthesis of ATP. This resynthesis of ATP occurs during the metabolic steps of glycolysis and of oxidative respiration.

As mentioned above, the splitting of carbohydrates into lactic acid is called glycolysis. Glycolysis occurs largely in animal cells. In plant cells, the equivalent is fermentation which splits carbohydrates not into lactic acid but into ethyl alcohol and carbon dioxide. Yeast fermentation was discovered long before glycolysis of muscle.

Indeed, in the middle of the 19th century a great controversy raged between Louis Pasteur, who believed that alcoholic fermentation could only occur in the presence of intact living cells according to his Vitalistic Theory and, on the other hand, Jons Berzelius, Justus von Liebig and others who believed that fermentation is the result of "catalysts" (later given the name "enzymes" by Willy Kiihne). In 1897 E. Buchner succeeded in demonstrating alcoholic fermentation by a cell-free extract of yeasts, thus finally settling the dispute in favor of Liebig, Berzelius and the proponents of catalysis theory. Later glycolysis was also found to occur in muscle juice free from intact cells. That fermentation as well as glycolysis can occur in cell-free extracts greatly facilitated the study of the intermediate steps leading to the breakdown of glycogen or sugar. in both fermentation and glycolysis for each molecule of glucose consumed, two molecules of ATP are generated.

Fermentation and glycolysis are the means of plant and animal cells to manufacture ATP in the absence of oxygen. In the presence of oxygen, the compounds that are converted to lactic acid or ethanol anaerobically enter into a different route of reaction; these reactions take place in the form of a cycle of steps, known as the tricarboxylic acid cycle.

For each turn of the cycle, acetate (two-carbon fragment derived from either glycolysis or fermentation of carbohydrates or from amino acids or fatty acids) yields two molecules of carbon dioxide and four pairs of (bound) hydrogen atoms. These hydrogen atoms are then fed into the respiratory chain--comprising a series of electron carriers—eventually to react with molecular oxygen to form water. Ultimately, a large number of ATP molecules are formed from ADP in the process known as oxidative phosphorylation. The total number of ATP molecules formed from the complete aerobic oxidation of each glucose molecule is 36.

After the disproof by Adolph Fick that heat energizes motion, we have come a long way

to the clear-cut understanding of what fermentation, or feux sans lumibre eventually produces from food materials they work on, namely, ATP. But then the key question became: "How does ATP energize motion and life?" In response the "High Energy Phosphate Bond Concept" was introduced.

The introduction of this concept was founded on the calorimetric studies of the ***heat*** or ***enthalpy of hydrolysis*** of ATP conducted in the laboratory of Otto Meyerhof in the 1920s and 1930s. The data indicate that unlike ordinary phosphate bonds, the splitting of each of the two terminal phosphate bonds of ATP entails the production of –12 Kcal/mole of heat. Assuming that the entropy change with the hydrolysis of the phosphate groups to be relatively trivial, Meyerhof and his coworkers concluded that the two terminal phosphate bonds are "high-energy" with a free energy of hydrolysis equal to about –12 Kcal/mole per bond. Other findings and reasoning led to the conclusion that the two terminal phosphate groups of ATP, the terminal phosphate group of ADP, and the phosphate bond of phosphocreatine all belong to what were called "high-energy phosphate" bonds.

In 1941, Fritz Lipmann elaborated on this theme and included a whole variety of biological work performance in the same category as muscle contraction that is being energized by the high energy contained in the high energy phosphate bonds of ATP. Looking back on the history of mankind's view on life, one cannot help feeling that this high-energy-phosphate-bond theory, if proven correct, may be one of the most important in the history of mankind's attempt to understand itself and life in general. Unfortunately, serious problems soon arose.

Podolsky and Kitzinger (1955) and Podolsky and Morales (1956) in very carefully conducted calorimetric measurement with the most sophisticated instruments available, showed that the earlier values of –12 Kcal/mole for the hydrolysis of the terminal phosphate group of ATPs is wrong. The correct value determined is only -4.75 Kcal/mole. At this value, there is no "high-energy" to speak of.

In a comprehensive review, George and Rutman (1960) showed that high "standard free energy" of hydrolysis of ATP obtained from the measurements of the equilibrium of ATP hydrolysis was largely the result of an error of omission. That is, in the hydrolysis of ATP, a hydrogen ion is produced. As a result, a correct estimation of the ***standard free energy*** of hydrolysis should be measured in a medium having a hydrogen ion concentration of 1 molar, equivalent to a pH of 0. In fact, the reaction was carried out at a pH of 7. It is the Le Chatelier principle rather than a high free energy content that drives the ATP to its hydrolysis. The two terminal phosphate groups of ATP do not contain a larger amount of energy per group than that of the "low energy" phosphate bond of AMP in full harmony with the conclusion of Podolsky and his coworkers.

Over 30 years have gone by and, to the best of my knowledge, no one has openly disputed the conclusions of Podolsky, Kitzinger, Morales, George and Rutman. It is thus most distressing that one continues to read in all kinds of scientific textbooks about how the high-energy contained in the high-energy phosphate bonds of ATP continues to energize muscle contraction as well as other energy-requiring biological activities, among which may be mentioned the so-called sodium pump postulated to regulate the level of sodium ion in living cells (see below).

Thus, from all we know, neither heat, nor the production of lactic acid, nor the special high energy contained in the terminal phosphate groups of ATP energizes biological motion and life.

This is where the energy problem stands as of this date. Some textbooks continue to teach

the high-energy-phosphate bond concept as if the work of Podolsky, Kitzinger, Morales, George and Rutman never existed. Others managed to hint that the idea is still valid in some inexplicable way.

The Membrane Theory of Water, Ion and Nonelectrolyte Permeability

The *sine que non* of the membrane theory is the selective permeability of the cell membrane. In particular the permeability of a membrane to water but not to dissolved solutes—a set of phenomena described as *semipermeable* by van't Hoff-demanded a mechanism. Two broad answers were offered: the *molecular sieve theory* of Traube and the *solution theory* of M. L'Hermite (1855). To the solution theory may be placed the *negative adsorption theory* of Justus von Liebig (1862) and the *lipoidal theory* of *E*. Overton (1899).

(1) The Molecular Sieve Theory of Traube

Traube suggested that the cause for the semipermeability of his copper ferrocyanide gel membrane is the presence in the gel membranes of small pores, which are large enough to permit the passage of small water molecules but too small to allow the passage of copper ions, ferrocyanide ions or sucrose. This molecular sieve theory was soon disproved by electron diffractions and other studies of the copper ferrocyanide gel. The interstices measured are far too large to bar the passage of molecules like sucrose (see Glasstone, 1946, pp. 656–657). Despite the disproof of Traube's molecular sieve interpretation of the semipermeability of his model membrane, the idea was resurrected again and again to explain behaviors of the living cells.

That different scientists should fall into the same pit over and over again throughout history arose from teaching increasingly focussed on currently popular issues and from less and less attention paid to what had led to the present state of knowledge. Repeated observations in somewhat different settings of the same fact—the rate of solute permeation is size-dependent—triggered each time the same old response.

(2) The Lipoidal Membrane Theory of Overton

In support of his solution theory, L'Hermite poured into a cylinder three liquids: chloroform, water and ether—in that order. In time, ether—which is soluble in water—moved through the water layer to enter the chloroform layer, expanding its volume. Chloroform did not enter the top ether layer because it had no solubility in water. Here the middle layer of water functions as a "semipermeable membrane". Solutes that dissolve in the "membrane", traverse the membrane; solutes that have no solubility in the "membrane" cannot.

Following L'Hermite's reasoning, E. Overton—whose work on osmotic swelling of frog muscle we have already discussed—suggested that living cells are covered with a thin layer of oil or lipoid and that it is this lipid layer that functions like the water layer of L'Hermite. Overton's lipoidal theory was supported by his finding that those substances which have high solubility in lipids appear to have higher permeability rate into living cells (Overton, 1895). However, Overton overlooked a major exception to his rule: water, which is relatively insoluble in oil but has the highest permeability through the living cell surface.

In years following, Overton's lipoidal membrane theory and Traube's atomic sieve theory were spliced together in similar or different ways. Thus pores were introduced into the lipid layer to account for the "abnormally" high permeability to small molecules in

Nathansohn's ***mosaic theory,*** (Nathansohn, 1904) and in Ruhland's ***ultrafilter theory*** (Ruhland, 1908). Later, layers of globular proteins were added onto the two surfaces of the lipoid layer to account for the much lower surface tension of living cells measured than the pure lipid-water interface demands (Harvey and Danielli's ***Paucimolecular Membrane Theory,*** 1936). Yet another version of the atomic sieve idea was launched in the theory of Boyle and Conway (1941). Even more elaborate variations of the mechanical atomic sieve came still later. In this case, small pores, called "potassium channels", were postulated for the exclusive passage of small K^+ ions, and larger pores, called "sodium channels", for the exclusive passage of the larger Na^+ ions (Katz, 1966).

The visualization of a trilaminar membrane ("unit membrane") in cells stained with specific electron microscopic stains was seen as strong evidence in favor of the paucimolecular membrane model (Robertson, 1960). The persistence of trilaminar structure after prior removal of 95% or more of membrane lipids (Fleischer ***et al.,*** 1967; Morowitz and Terry, 1969) was apparently little heeded by most main-stream "membrane physiologists". However, this observation led "non-main-streamers" including myself, to the conclusion that lipids or phospholipids could not be in the form of a continuous all-covering layer. They also reasoned that the failure of lipid removal to influence the microanatomy of the cell surface could be better understood if the phospholipids exist as isolated patches. In that case, phospholipid removal could then have avoided detection in an electron microscopic picture (see also Sjostrand and Berhnard, 1976). This interpretation is also in harmony with results of studies on K^+ permeability in the absence and presence of antibiotic "ionophores" to be described next.

Phospholipid bilayers as such have virtually no permeability to ions like K^+, Na^+ and Cl^- (Miyamoto and Thompson, 1967). In contrast, all living cells studied have high permeability toward these ions as mentioned before (see below also).

Valinomycin (and other "ionophores") could increase ***one thousand-fold*** the K^+ permeability of bilayers of phospholipids isolated from sheep red cells (Andreoli ***et al.,*** 1967). Yet contrary to the prediction from the lipoidal membrane theory, these ionophores had ***no effect*** on the K^+ permeability of the inner mitochondria1 membrane of rat liver (Maloff ***et al.,*** 1978), nor of the plasma membrane of squid axon (Stillman ***et al.*** 1970), nor of frog muscle (Ling and Ochsenfeld, 1986) nor of frog ovarian eggs (Ling and Ochsenfeld, 1986).

Other studies revealed that the apparent high permeability of artificial lipid bilayers to water, quantitatively matching the water permeability of living cells, was the result of a major error in estimating the permeability of water through living cell membranes. Correctly measured, the permeability of living cell membrane to water has been shown to be ***several orders of magnitude higher than reported for the (correctly measured) permeability of artificial phospholipid bilayers to water*** (Ling, 1987, 1992, p. 225).

Taken together, these and other investigations on the permeability of cell membranes to water, ions and nonelectrolytes lend support to the following conclusion: phospholipids either do not exist in significant amount or, in cases where they do, they exist in the form of isolated patches. As such, these patches do not significantly contribute to the permeability characteristics of the cell membranes in physiologically active cell membranes like the inner membrane of rat liver mitochondria, and the plasma membrane of squid axon, frog muscle and frog eggs (for a lone exception, see Endnote 4).

As a historical landmark I would like to mention the work of my former colleagues at the Eastern Pennsylvania Psychiatric Institute in Philadelphia. It was the announcement in 1962

of the success in the preparation of lipid bilayer "black" membrane by Paul Muller, Donald Rudin and their colleagues that started the large-scale investigation on this membrane model (Muller ***et al.,*** 1962). However, as mentioned above, the phospholipid bilayer membrane has virtually no permeability to ions like K^+, Na^+ and Cl^-. Yet as shown in the preceding sections, the permeability of the cell membrane to these ions is a well established fact and absolutely vital to cellular physiology. The creation of cellular electrical potentials offers one example.

Thus if the lipid bilayer theory has any validity at all, ways must be found to convert these ion-impermeable membranes into ion-permeable ones. It was under these circumstances that the discovery that antibiotics like valinomycin (which had failed the tests as safe bacteriocidal agents) which had specific ion transporting ability and thus functioned as an "ionophore" was greeted with great enthusiasm. It seemed only a matter of time, before one could find out the real-world equivalent(s) of these ionophores and the ionic permeability dilemma would be resolved. It is not surprising that both the National Institute of Health and the National Science Foundation provided much money to facilitate this search.

Thirteen years later, Paul Muller provided this sad bottom line: "A lot of us have spent a wasted ten years or so trying to get these various materials into bilayers . . ." (Muller, 1975).

Paul Müller and I were miles apart in our views on living cells. His arrival at the Eastern Pennsylvania Psychiatric Institute more or less marked the beginning of my departure from the Institute that one time I loved so much. Nonetheless, I admire Müller's candidness in saying publicly and honestly what obviously was painful to him. Any scientist knows how to crow about his or her success. It takes genuine courage to admit mistakes publicly. In my view, it is one of the most important traits of a really good professional scientist.

Solute Distribution in Living Cells

According to the membrane theory, membrane permeability (or impermeability) offers the physical foundation for all major cell physiological manifestations. Thus far we have reviewed three of these: cell volume control, cellular electrical potentials and cell permeability itself. In all three cases I have already shown why these original interpretations on the basis of the membrane permeability have failed. I now turn to the fourth major physiological manifestation of the living cell: the maintenance of the unique chemical composition of both small and large molecules in the living cell. In earlier pages, I have also mentioned very briefly how its interpretation of solute distribution on the basis of membrane permeability (and impermeability) had also run aground, forcing the postulation of the sodium pump. The history of our investigations on solute distribution will now be examined in greater detail.

I would like to point out that ***without this attribute of maintaining a more or less constant chemical composition of the cell content different from its environment, the living cells would be unable to gather the building blocks of their vital structures. Under that condition, the living cells would not have existed at all.*** It is not surprising that all major theories of living cells were introduced with a focus on this subject.

Among the most prominent differences between the chemical composition of the living cells and their aqueous environments are those of the pair of alkali metal ions, K^+ and Na^+.

In 1896, Julius Katz published the results of the first exhaustive analyses of the contents of these two and six other kinds of minerals (K, Na, Fe, Ca, Mg, P, Cl and S) in the volun-

tary muscle tissues from six mammals (man, pig, cattle, rabbit, dog, cat), one bird (chicken), one amphibian (frog), and three fish (cod, eel, pike).

Two years later, Abderhalden published the water contents and the contents of six minerals (K, Na, Ca, Mg, Cl, PO_4) of both the erythrocytes and serums of three mammals (rabbit, bull, dog). No Na could be detected in the erythrocytes of the rabbit, while high concentrations of Na were found in the erythrocytes of both bull and dog. In contrast, the K content of the rabbit erythrocytes is very high but much lower in those of bull and dog (Abderhalden, 1898).

The K and Na contents of the serums are more or less similar in all three animals Abderhalden studied, being very high in Na and quite low in K. Like rabbit erythrocytes, the human erythrocytes also have a high K content, but low Na content.

All the muscle tissues reported by Katz have a much higher concentration of K than Na.

(1) The Original Membrane Theories Including the Molecular Sieve Theory of Boyle and Conway

From osmotic studies on erythrocyte of different kinds, Hamburger (1891) came to the conclusion that erythrocyte membrane is completely impermeable to all cations. However, Benjamin Moore and Herbert Roaf from the University of Liverpool objected (Moore and Roaf, 1908), pointing out that according to the then accepted view, "the whole exchange (of K^+ and Na^+ ions) is supposed to be regulated for the cell by an inert membrane by which it is enclosed, and which, like a prison wall, keeps the potassium and phosphate ions within while it equally prevents sodium ions from entering. The membrane theory takes no trouble to explain how prisoners are introduced within the prison yet introduced in some way that must be, for as growth proceeds, cells multiply, and there is no diminution in the number of imprisoned ions in each." Moore and Roaf suggested that "the varying concentrations of sodium, potassium, chlorine and phosphatic ions within and without the cell are an expression of specific affinities of the definite colloids of each particular cell-type for these ions, and do not mean that there is a membrane acting as a closed gate to these ions". In support, they pointed out that soil also preferentially takes up potassium over sodium even though no "membrane retention hypothesis" had been put forward for this case (Moore and Roaf, 1912). Unfortunately, very few heeded Moore and Roaf's impeccable reasoning or accepted their explanation for selective solute distribution.

Nonetheless, from the initial position that the cell membrane is completely impermeable to cations (Hamburger, 1904), the position had shifted in the 1930s to one in which it was agreed that the cell membrane is permeable to K^+ but not to Na^+ and to all anions.

As mentioned earlier in Volume 100 of the Journal of Physiology (London), an historically important paper was published by P. J. Boyle and E. J. Conway (Boyle and Conway, 1941). Their article presented what was the final grand synthesis of the original membrane model of the living cell. It was also its swan song.

Boyle and Conway postulated that the frog muscle cell membrane contains rigid pores that are small in a highly uniform manner. On the basis of an estimated list of the ionic sizes (i.e., the ion itself plus its coat of more or less permanently attached layer of water molecules), the smaller hydrated K^+ ions enjoy the privilege of permeating the cell membrane pores and staying within the cells, while the larger hydrated Na^+ ions are too large to traverse the small membrane pores and, as a result, stay outside the cell as long as the cells live. With this critical pore size determined, Boyle and Conway were able to ex-

plain also why small Cl^- can enter and leave the cells—supported by new evidence they provided—while the much larger Mg^{++} could not--or at least as the theory predicts (see below).

This is scientifically speaking a good model, because it is a ***general*** *theory* contending with all solutes in spirit at least. It is also good because it lends itself to decisive experimental testing. Thus the theory would be invalidated if proof can be produced that shows larger ions like Na^+ and Mg^{++} can in fact traverse the cell membranes. Surprisingly, evidence of this kind was gathering long before Boyle and Conway's paper appeared in print in 1941.

(1) In 1931, Wu and Yang demonstrated entry of Na^+ into rabbit muscle following intravenous injection of NaCl solution into the animal.

(2) In 1934, Kaplanski and Boldyreva demonstrated an accumulation of a high concentration of Na^+ in fish muscle when carp were kept in water containing 1.5% NaCl. The plasma Na^+ concentration in the fish remained normal.

(3) In 1937, E.J. Conway and *G.* Cruess-Callaghan demonstrated entry of Mg^{++} into surviving frog muscles.

(4) In 1939, Heppel demonstrated a gain of Na^+ in muscles of rats fed a low K^+ diet.

(5) In 1940, Steinbach demonstrated a (reversible) gain of muscle cell Na^+ when isolated frog muscles were incubated in a solution containing a low K^+ but the normal high concentration of Na^+.

From 1939 on, the availability of radioactive Na^+ isotopes made it easy to carry out experiments further proving that the plasma membrane is unquestionably permeable to Na^+ as well as other large hydrated ions (for references, see Ling, 1992, pp. 207–208). The disproof of Boyle and Conway's molecular sieve theory of ion distribution was complete.

(2) The Sodium-Pump Hypothesis

The failure of the Boyle-Conway molecular sieve theory of the living cell thus repeats a similar failure of the Traube molecular sieve theory for his copper ferrocyanide gel membrane. The sodium pump hypothesis, already familiar to the reader by this time, was adopted.

According to the sodium pump hypothesis, Na^+ can also traverse the cell membrane and yet stays at a steady low intracellular concentration due to the ceaseless operation of a postulated sodium pump. Located in the cell membrane, the pump throws out Na^+ ions at the same rate as they enter the cell.

The patch-work nature of this sodium pump hypothesis was also briefly mentioned earlier. It was proposed without a molecular mechanism for the main article, the pump; and it left in limbo the distribution of other ions and solutes. The focussed attention on Na^+ ion alone gave the false impression that only this particular ion needed "fixing", while in truth the entire membrane theory has fallen apart.

Is There Enough Energy to Operate the Sodium and Other Pumps? The Answer is No.

More than forty years ago, I presented early evidence against the Na pump hypothesis (Ling, 1951, 1952). Ten years later in 1962, I presented in full a detailed account of an inquiry into the feasibility of the postulated Na pump from a thermodynamic standpoint (Ling, 1962, pp. 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 a clear-cut answer within reach.

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 under the assumption that the cell does not require energy for anything else besides pumping Na.*

Disparity of this magnitude is decisive by itself. Yet it belies the even greater true disparity. For in my calculations presented in 1962, 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) mentioned above left little doubt that there is no utilizable "high energy" trapped in these phosphate bonds. Since most of the (maximum) available energy in my computation 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. And we need many more than one 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 for the same reasons that led to the postulation of the sodium pump: permeability through the cell membrane and presence within cells at concentrations quite different from what the membrane theory would have predicted (Ling, 1955, p. 90; 1992, Sect. 2.2.5). Nor are solutes requiring pumps limited to those found in the cells and their natural environments. They must also include other solutes studied, numbering among those which were for the first time created by 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 in anticipation of 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 (Peachey, 1965). 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 some thirty years ago, there has been no challenge in print against the experiments I performed and described, nor the conclusions I reached. (However, for written questions of recognized and anonymous scientists and my answers, see Ling, 1988a, discussion with I. Z. Nagy, on pp. 911–913; Ling, 1990a, discussion with Reviewer IV on pp. 755–760; Ling, 1992, discussion with Reviewer I on pp. 439–445). In the meantime, the essence of my finding has been twice confirmed (Jones, 1965; Minkoff and Damadian, 1973).

Overwhelming and incontrovertible as the evidence against the pump concept is from the energy consideration, it is far from being 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 next.

Are There Sodium Pumps in the Muscle Cell Membrane? The Answer Is Also No

A frog sartorius muscle comprises about 1000 fiber-like elongated muscle cells. Each muscle cell runs all the way from its pelvic origin to its other end, some three centimeters away at the muscle's tibial insertion (Ling, 1973, p. 299). A razor-blade cut across the muscle away from its tapering tibial end exposes the cytoplasm of every one of the 1000 cells. This cut is not followed by regeneration of a new membrane as revealed by electron microscopy (Cameron, 1988; Edelmann, 1989) and the persistent high permeability to sucrose through the cut end etc. (Ling, 1978).

If the cut end of the sartorius muscle 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, 1973, 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 can no longer 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 in an EMOC preparation, the uptake of labelled K^+ and of labelled Na^+ in the part of the muscle cells away from their (injured) cut ends appeared quite normal (Ling, 1978). That is, K^+ and Na^+ concentrations found here follow a pattern not significantly different from 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 ions: 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 (Schatzmann, 1953; Glynn and Karlish, 1975), functions in an EMOC preparation much as it does in intact muscle cells. 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.***

Careful precaution was taken against the possibility that Na^+ might be pumped from the intact portion of the muscle cell in an EMOC preparation into the extracellular space, and returns thence via that same space to the Ringer's 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, it was found later that this precaution was unnecessary.

As witnessed by the fact that the cut could be applied to either the tibial or the pelvic end with similar result, there is no pump propelling Na^+ to move along the length of the sartorius muscle in a fixed direction within the extracellular space. Backward transport of labelled Na^+ into the Ringer's solution bathing the cut end, if it exists, must rely on the development of a diffusion ***head.*** That is, the labelled Na^+ concentration in the intact end of the muscle must be substantially higher than in the Ringer's solution bathing the cut end. The question is, Exactly how much higher?

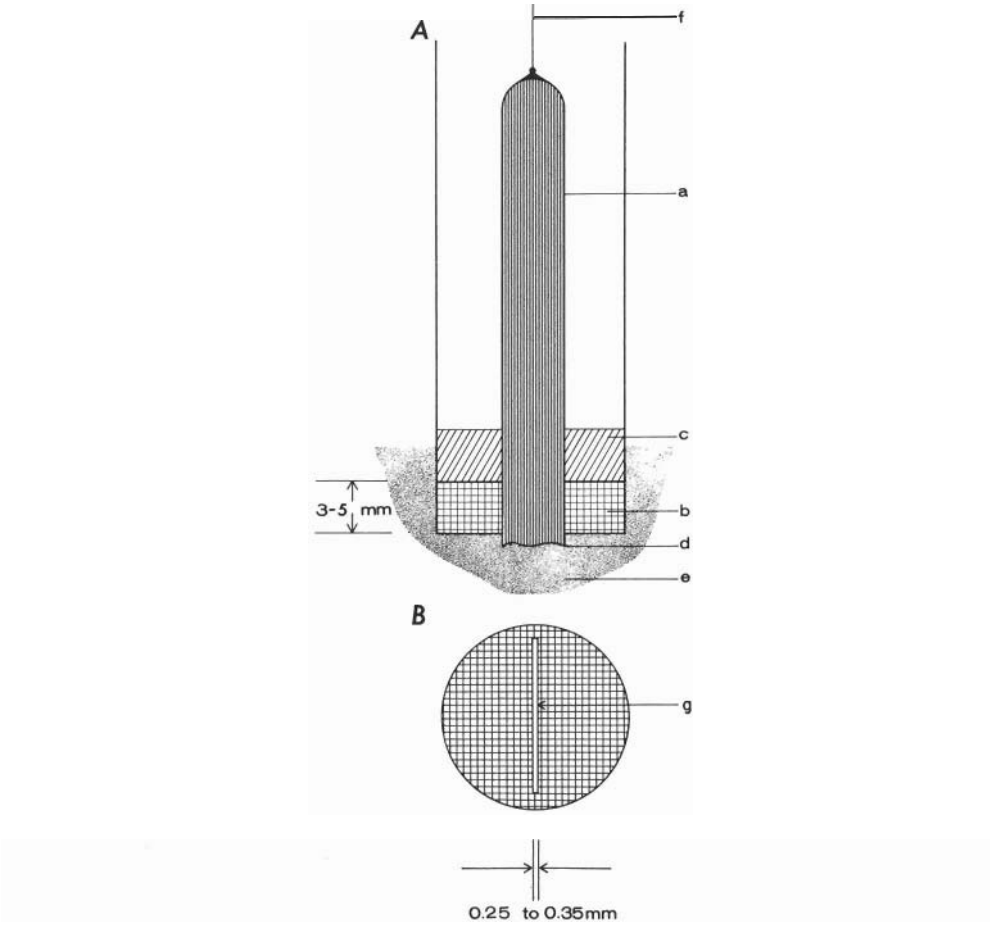

FIGURE 1. Diagram of the EMOC preparation. A, side view. B, bottom view. Only the cut end of the muscle is in direct contact with the labelled Ringer's solution. a, sartorius muscle; b, silicone rubber gasket; c, vaseline; d, cut end of muscle; e, bathing solution; f, anchoring string; g, slit in silicone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

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 not be lower than 5 Molar in order to provide the necessary diffusion head. To find out if such a high concentration of labelled Na^+ in fact

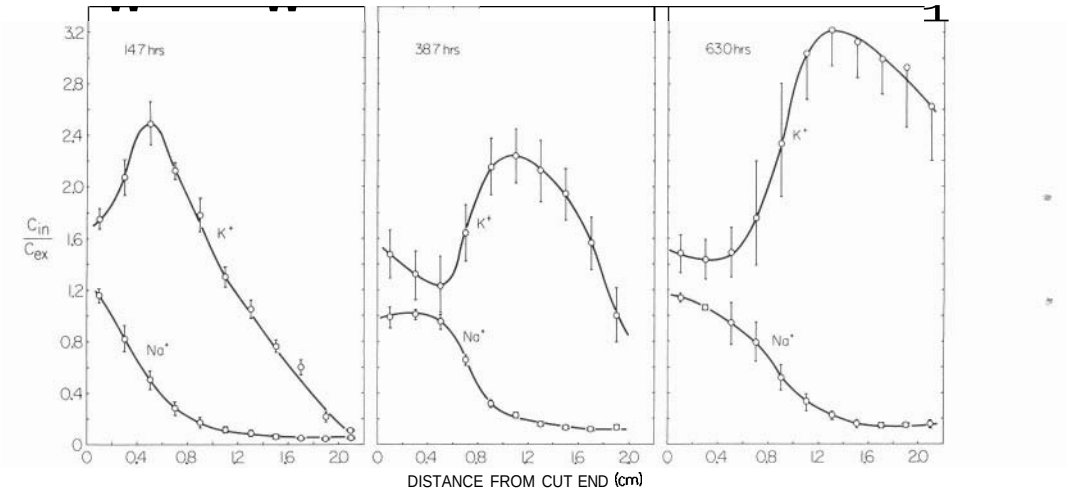

FIGURE 2. The simultaneous influx of labelled K^+ and labelled Na^+ into sartorius muscle through their cut ends. The three groups of frog sartorius EMOC preparations were exposed to normal Ringer's solutions labelled with both ^{42}K and ^{22}Na for 14.7, 38.7 and 63.0 hr, respectively. The abscissa 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 segment (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. By permission of J. Physiol.)

existed, the fluid in the extracellular space of an EMOC preparation after 50 to 53 hours of incubation was collected by a centrifugation technique earlier described (Ling and Walton, 1975) and analyzed.

The concentration of labelled Na^+ in the collected extracellular fluid was found to be 95.5 ± 0.3 mM, which was not higher than the concentration in the Ringer's solution bathing the cut end of the muscle, i.e., 100 mM. Therefore, the required diffusion head of Na^+ between the extracellular space and the Ringer's solution bathing the cut end did not exist. Accordingly, the low labelled Na^+ concentration in the intact end of the muscle in an EMOC preparation could not be due to a steady return of labelled Na^+ to its source solution bathing the cut end of the muscle.

Indeed, there is strong evidence that the postulated pump—if for argument's sake, one assumes it existed—had not moved a significant amount of Na^+ into the extracellular space at all. Were it otherwise, the excess of Na^+ (and accompanying anion(s)) added to the 100 mM originally present in the extracellular space 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% ± 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).

The EMOC studies led to the conclusion that an intact and functional membrane pump is not necessary for the maintenance of a low concentration of Na^+ and a high concentration of K^+ in living cells. Having said that, I now ask a deeper question, What can I make out of this statement beyond what was said?

When one says that there is no need of another television set in the house, the statement does not impugn the existence of television sets elsewhere. It is thus different from saying that there is no need of a functional membrane pump in the cell membrane to explain the low level of Na^+ and high level of K^+ in living cells, because the only justification for the existence of the postulated pump is the need of such a pump to maintain the low Na^+ and high K^+ in living cells. Removing this need, as the EMOC experiment did, one eliminates the Na pump altogether. Thus put in simpler language, ***the*** EMOC ***experiment proved that there is no Na pump in the muscle cell membranes, and by inference in any living cell membrane.***

While the EMOC experiment offers conclusive evidence against the existence of the Na pump in the cell membrane, one can also, by the method of exclusion, conclude that the ***selective accumulation of K^+ and exclusion of Na^+, as well as the controlling influence of the cardiac glycoside, ouabain, on the K^+/Na^+ distribution must reflect the property of the protoplasm.*** The compelling reason for this deduction is that there is nothing else beyond the membrane and the substance of the cell, the protoplasm.

In concluding the second set of evidence against the membrane-pump theory, proving that there is no Na pump in cell membranes, I want to add two more sets of relevant information. The first concerns the (true) active transport of ions and other solutes across frog skin, kidney epithelium, intestinal epithelium; the second concerns the hypothesized active transport of ions across squid axon membranes.

(1) ***True Active Transport:*** While there is now extensive unequivocal evidence against the sodium pump hypothesis for the distribution of Na^+ and K^+ in what I call ***unifacial*** cells-cells with one kind of membrane like muscle, nerve, red blood cells, this evidence does ***not*** apply to the altogether different kind of phenomenon, i.e., active transport of Na^+, K^+ and other solutes ***across*** what I call ***bifacial*** cells-cells with two different kinds of membranes facing respectively the two solutions which the layer of bifacial cells separate. Examples are the frog skin, kidney and intestinal epithelium. A theory of active transport in these bifacial systems based on the AI hypothesis was first presented in 1981 (Ling, 1981), then in 1984 (Ling, 1984, Chapter 17) and in its most up-to-date form in 1990 (Ling, 1990).

(2) Hypothesized Active Transport: The giant axons from the North Atlantic squids often measure 400 to 800 micra in diameter. The technique of removing all or virtually all the protoplasm or axoplasm from the inside of an isolated giant axon without harming the physiological integrity and activities of the axon membrane were perfected by Baker, Hodgkin and Shaw (1961) in England and by Oikawa, Spyroupolos and Tasaki (1961) in the United States. The stage was set in 1961 for another set of crucial experiments testing the sodium pump hypothesis.

After removing the axoplasm, one could fill the remaining sac with sea water containing the needed energy sources (e.g., glucose, ATP, phosphoarginine—the squid's counterpart of mammalian phosphocreatine) and tie up its open ends. By this simple procedure, one obtains an ***idealpreparation*** for testing of the validity of the sodium pump theory.

That is, if membrane pumps truly maintain the low Na^+ and high K^+ in normal squid axons, one expects to detect continued pumping of Na^+ out of the axonal membrane sac and continued pumping of K^+ into the axonal membrane sac, both against a concentration gradient. After a suitable period of incubation, one should find a higher K^+ and lower Na^+ concentration within the artificial axoplasm in the axoplasm-free membrane sac than their respective concentrations in the bathing sea water. Such a demonstration would go a long way in verifying the sodium pump hypothesis.

Indeed, extensive efforts were made in this direction by some of the most skilled and capable workers in this field. However, all efforts to demonstrate a net transfer of Na^+ and K^+ against concentration gradients failed.

On a spring day in 1963, I was privileged to be invited to attend a lecture given at the Johnson Research Foundation at the University of Pennsylvania. On that occasion Professor Richard Keynes of The Physiological Laboratory of Cambridge University was originally scheduled—as I believed—to report on the ***success*** of such an effort, but I was bewildered to hear from Professor Keynes that he was to talk about something entirely different, which he then did. But in response to a query from Professor George Karreman of the University of Pennsylvania, also invited to attend the lecture, Professor Jobsis (the chairman) and Professor Keynes simultaneously announced that "the experiment did not work".

This important negative experiment against the prediction of the theory of sodium pump was, to the best of my knowledge, never published. The only printed record of it was in the shape of short footnotes as well as brief reference in reviews I published describing what I heard at the lecture (Ling, 1965; 1984, p. 127; Ling and Negendank, 1980, p. 222). However, a similar failure to demonstrate ***net*** transport of Na^+ across a perfused squid-axon-membrane preparation still retaining its axoplasm was also observed by Brinley and Mullins in 1968; but they did publish what they found (Brinley and Mullins, 1968; Mullins and Brinley, 1969).

In the nearly thirty years following, no further report revising these earlier conclusions was published. In the past, it would be very difficult to establish that some paper was ***not*** published. The availability of periodicals like the Citation Index has overcome the great difficulty of locating unknown publications and has made a search for this type of unknown publication both easy and reliable.

Since the genuine Nature-made membrane itself does not pump Na^+ against a concentration gradient, it is hardly surprising that a claim of active transport of Na^+ in an "inside-out" synthetic vesicle containing the hypothesized sodium pump (i.e., Na, K-activated ATPase) turned out to be an experimental error (for detailed discussion, see Ling and Negendank, 1980; Ling, 1992, pp. 22–25).

These failures to demonstrate active transport in the ideal pure membrane preparation without protoplasm (as well as the less ideal man-made vesicles) provided the counterpart of the success in demonstrating selective uptake of K^+ over Na^+ in the Effectively Membrane-pump-less Open-ended Cell (EMOC) preparation. Together they had firmly established that ***Na (and other) pumps do not exist in living cell membranes. And that it is the cell protoplasm that provides the seat for selective ionic accumulation and exclusion.***

Before introducing the association-induction (AI) hypothesis, I present the early embryonic version of the AI hypothesis known as Ling's fixed charge hypothesis as well as the sorption theory of my friend, the late A.S. Troshin from what was then in Leningrad.

Two New Theories of the Living Cells

The year 1951 saw the introduction of the beginning of two new theories: the late A.S. Troshin's ***sorption theory*** and the early version of my association-induction hypothesis, Ling's ***fixed charge hypothesis.*** Though undeclared, and perhaps even unrecognized at the time, the introduction of both theories represents in fact a return to the protoplasm-oriented cell physiology of the 1920s and 1930s, which were largely abandoned by cell physiologist (in the English-speaking world at least), to no small extent in response to the experimental finding of Nobel laureate, A.V. Hill.

(1) Troshin's sorption theory

From his studies of the distribution in living cells and model systems of alanine, creatine, galactose and other nonelectrolytes, 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). Troshin also introduced a two term equation (which I have suggested on several occasions to be named the Troshin equation, see Ling, 1992, p. 203, Endnote 2).

The fraction of solute 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 and named "coacervate" by H. Bungenberg de Jong (1932). In support of his theory, Troshin cited the earlier work of Hollemann ***et al.*** (1934) who showed that the simple coacervate of gelatin contained 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. Nevertheless, Troshin's theory is a very important theory in the history of cell physiology.

In 1958 Troshin extended 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 physical mechanism whereby K^+ is selectively adsorbed while the closely similar Na^+ is not, a question that has become increasingly my preoccupation from the late 1940s.

(2) The early embryonic version of the association-induction (AI) hypothesis, known as Ling's fixed charge (LFC) hypothesis

In 1951 and 1952, I presented what was then called Ling's fixed-charge (LFC) hypothesis (Ling, 1951, 1952), the primordial version of the AI hypothesis to follow ten years later (Ling, 1962). 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, 1990a, 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 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^+. 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^+ provides the molecular mechanism for the selective accumulation of K^+ (over Na^+) in living cells as observed.

A selective mechanism that depends on an *unchanging* set of hydrated ionic diameters and hence an unvarying selectivity rank order has a serious drawback. This theoretical mechanism correctly describes the behavior of *some* fixed-charge-systems but not of others. Thus soil—as pointed out first by Moore and Roaf cited earlier—and the sulfonate type of ion exchange resin, selectively accumulating K^+ over Na^+ like many types of living cells; the carboxylate type of exchange resins, on the other hand, actually selectively accumulates Na^+ over K^+ (see Bregman, 1953).

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 our earlier, interested natural philosophers 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 provided 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.

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. Or even better, Lamarck's definition of life as: *état de choses*.

Consider a number of soft iron nails, joined end-to-end with bits of soft string (Figure 4A). Their distribution is random and they do not interact with the iron filings scattered

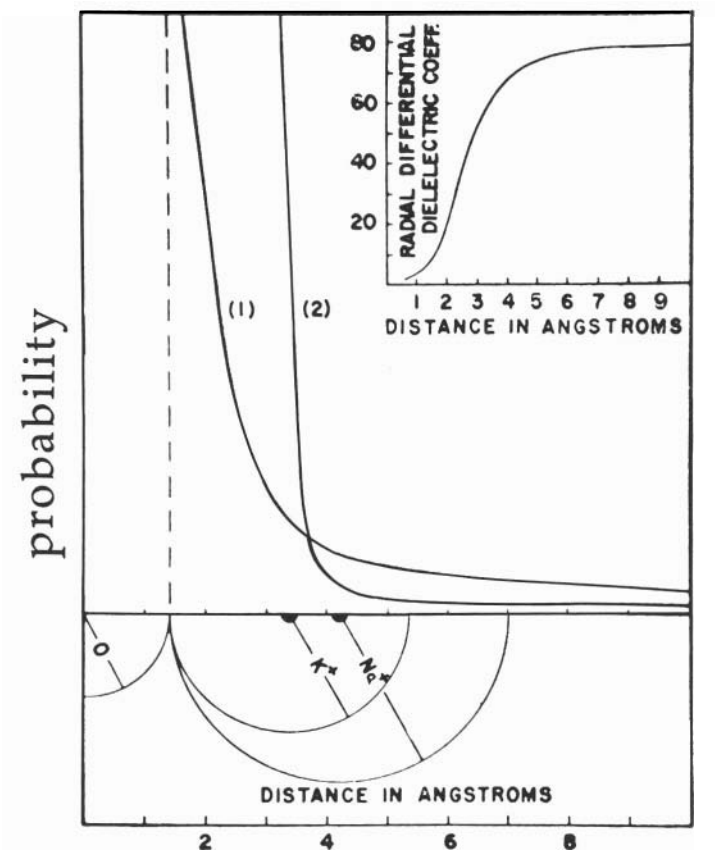

FIGURE 3 Theoretical model for the sellective adsorption of K^+ over Na^+ on fixed anionic sites.

The smaller diameter of the (hydrated) K^+ than that of the (hydrated) Na^+ (shown at the bottom of the figure) and the sharply decreasing dielectric constant as one approaches the center of an ion (shown in the inset) combine to provide a much higher statistical probability (curve 2) that the fixed anion adsorbs the smaller hydrated K^+ than the larger hydrated Na^+. This follows from the fact that only the smaller K^+ ion is capable of entering and occupying the spherical shell of "high-probability space" in the closest immediate vicinity of the fixed anion, represented here as a singly-charged oxygen atom.

Abscissa represents the distance in Angstrom units from the center of the singly charged oxygen atom of the fixed oxyacid oxygen atom. Curve (1) shows another probability curve if the phenomenon of dielectric saturation is ignored. (From Ling, 1952. By permission of the Johns Hopkins University Press).

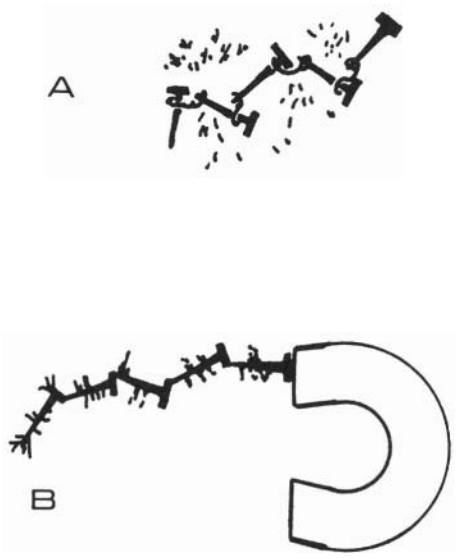

FIGURE 4. A chain of soft iron nails joined end to end with pieces of string is randomly arrayed and does not interact with the surrounding iron filings. The approach of the magnet causes propagated alignment of the nails and interactions with the iron filings. (Adapted from Ling, 1969.)

among them. If now a strong magnet is brought into contact with the free end of one of 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).

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 theory or its modified version, 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^{++}, transmitters. Not all cardinal adsorbents are required in maintaining the living state, but of those that

are, 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 his high-(negative)-energy, low-entropy state that distinguishes the living state of the AI hypothesis from the concept of life-as-organization (only) or the "life-is-life" ambiguity of Lamarck's "état de choses". As an equilibrium phenomenon, the maintenance of the living state requires no continual 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. (Evidence for these basic contentions will be presented in some detail below.)

With the living state defined, ***protoplasm can now be given a new definition 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.***

The living state pertains to the intact living cell, as well as to its constituent parts, including membranes, mitochondria as well as the 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.

Cell **Water**

Water is by far the largest component of protoplasm. Common sense tells us that without water, thee is no life. Yet in the context of the membrane pump theory it has been reduced to merely a solvent. I will in the five following subsections (1 through 5) 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 (PM) 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 in the following section the same sequence: theory first, followed by results of experimental testing. Only this time it will be on the second largest, non-protein component of the cell, the K^+ ion.

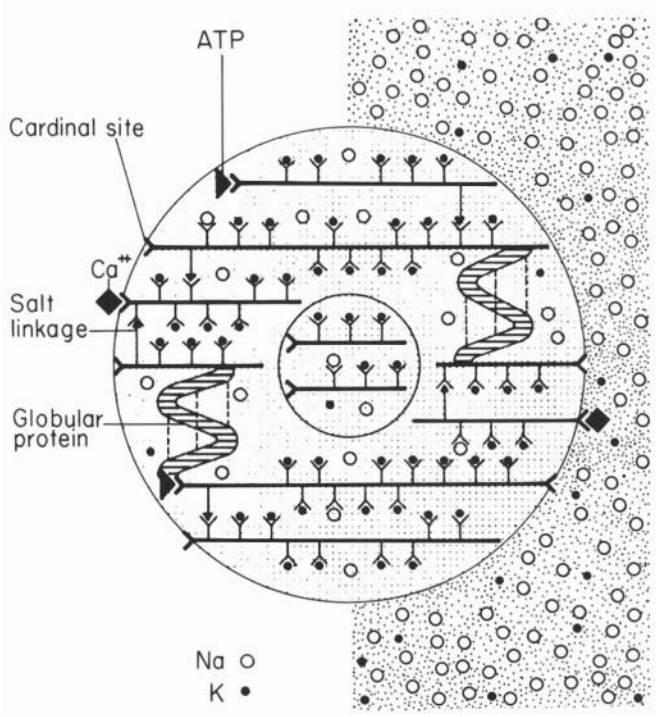

FIGURE 5. Diagrammatic illustration of a submicroscopic portion of the cell substance, showing the interaction among the three most abundant components of the living cell: water, proteins and K^+ and cardinal adsorbents, Ca^{++} and ATP. Selective K^+ accumulation occurs as a result of the preferential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

(1) 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.

The formula H_2O is a better representation than 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 comers of a tetrahedron with the oxygen atom occupying its center. Located at the other two comers 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). That is, for certain quantitative treatments, each water molecule may be visualized as a little electrically charged linear entity, one end positive and the other end negative.

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

Now if alternating positively charged P sites and negatively charged N sites are arranged in two dimensions at suitable distance apart like a checkerboard (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 similar 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 molecules (Figure 6).

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 old physics. Indeed, theoretical physicists like de Boer and Zwikker (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, Emmett and Teller, 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 my original suggestion (Ling, 1965a). (For R.A. Gortner's consideration of the evidence existing in 1930 that water might exist in the state of polymolecular layers and the reason for his backing away from suggesting that biologist's bound water is polymolecular, see Endnote 5).

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 functioning as NP-NP-NP systems; and, as such, these N and P sites are directly exposed to the bulk-phase water, polarizing and orienting it in multilayers. Some years ago I gave a reason why actin, an ubiquitous protein found in many if not all cells, might be a major candidate for this water-polarizing role in living cells (Ling, 1979, p. 47).

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 that 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.

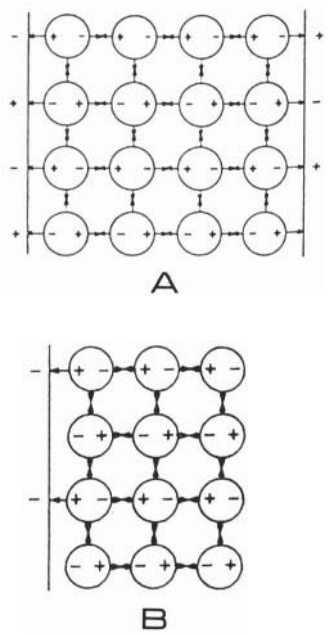

FIGURE 6. Diagrammatic illustration of how arrays of altematingly positively and negatively charged fixed sites on a matrix of linear chains or juxtaposed surfaces (A), or of altematingly negatively charged and neutral sites (B) can produce the dynamic structure of polarized multilayers of water molecules. 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.)

The study of water-vapor adsorption in model NP-NP-NP systems and living cells (Ling and Hu, 1987, 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. More rigorous proof of the *multilayer* water adsorption in living cells and model systems have been recently published by Ling (1993) and Ling, Niu and Ochsenfeld (1993).

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.

(2) 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 (for definitive treatment, see Ling, 1993; for earlier views of historic interests see Ling, 1970; 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*** of a solute is defined as the ratio of the concentration of this solute in the cell water at equilibrium represented as $[S]_{in}$ over the concentration of the same solute in the external bathing medium represented as $[S]_{ex}$, A plot of $[S]_{in}$ against $[S]_{ex}$ yields a straight line with a slope equal to the ***equilibrium distribution coefficient*** of solute S or ***q-value.***

However, obedience to the expectation of a straight line relationship 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 $[S]_{in}$ against $[S]_{ex}$ 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 water.

On the other hand, ***if the backbone CO and NH groups are locked in a-helical, β-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 Ling, 1992, endnote 4 on p. 37), 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 earlier reported (Ling, ***et al.,*** 1980; Ling and Ochsenfeld, 1989).

Thus in solutions of 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.

In contrast, ***the p-value of Na_2SO_4 in a solution of gelatin is considerably lower than unity.*** This is a matter of 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 Hollemann ***et al.***

and Troshin have demonstrated. But none of these investigators have offered a mechanism why gelatin behaves differently from most native proteins.

The polarized multilayer theory of cell water soon led to an understanding of the unusual and striking effect of gelatin on water, and hence the distinctive feature of colloids (see Ling, 1972, p. 691; 1992, pp. 81–84; Ling et al., 1978, 1980). Accurate knowledge on the amino acid composition of gelatin (denatured collagen) and the other 12 proteins has become available in the sixties (Piez *et al.* 1960; Tristram and Smith, 1963). 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 (Ling, 1984, p. 175).

Unlike most other proteins (which contain 5% or less proline and no hydroxyproline) a large proportion of the amino-acid residues of gelatin (denatured collagen) is in the form of proline (12%) and hydroxyproline (9%), each lacking an 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 in a protein molecule as a rule do not engage in forming a-helixes (see below; also Chou and Fasman, 1978). (Most other non-collagen proteins contain 10% or less glycine). 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 a-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 *colloid condition* as follows: *a colloid is the collective name given to the material and the bulk-phase water (or other polar solvent) which material polarizes and orients, thereby causing the bulk-phase water (or other polar solvent) to assume the dynamic structure of polarized multilayers; colloidal condition is one in which the material in question polarizes and orients the bulk-phase water (or polar solvent) to assume the dynamic structure of polarized multilayers.* (This, the latest definition of colloids and colloidal condition is a somewhat improved version of earlier similar definitions I suggested.)

In contrast to gelatin, 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 a–helical and other intramacromolecular 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 (for reasons that one can compare gelatin, a denatured protein with normal native proteins, see Endnote 6).

Ling *et al.* (1978) also studied 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 a-helical or other secondary structure due to the lack of NH groups. Accordingly, one may expect that all three polymers also have a 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) (Ling and Ochsenfeld, 1989). 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. (Data to be presented below demonstrates the alkali-denatured proteins behave similarly).

Ling and Ochsenfeld (1989) 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, 1987a), 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.*** In other words, the colloidal chemists like Newton, Gortner and others were mistaken in describing water that excluded sucrose as water that has lost its solvency for ***all*** dissolved substances, hence the name ***nonsolvent water.*** It is this mistake that had led A. V. Hill to conclude that the water in frog muscle cells is simply free water. This too was wrong, as will be made clear in a following section.

(3) 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 and 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" include gelatin, oxygen-containing polymers like PVP, PEO, PEG (polyethylene glycol, $[H(CHO)_nOH]$), 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-dominatedpolarized 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.

(4) Investigations on the different physicochemical properties of polarized water of extrovert and introvert model systems and of living cells

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, 1984); (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. (Darnadian, 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 Negendank, 1970; Ling, 1984, p. 288); (2) water vapor adsorption at physiological vapor pressure (near saturation, $p/p_o = 0.9968$ 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 six native proteins and one SDS denatured protein (Ling and Zhang, 1983); (2) different shapes of ice crystals 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 ***solvency of water in model systems and living cells.***

(5) 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 one from 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 nonelecrolytes, Inset A and B of Figure 7 are obtained. Note that here the q-values of all the nonelectrolytes in 39% native hemoglobin are close to one, indicting that water in the 39% native hemoglobin solutions has solvency close to that of normal liquid water for 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 nonelecrolytes studied confirms the prediction of the PM theory that introvert models with the backbone NH and CO groups locked in a-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 very different from that in the dilute salt solution outside the sacs, as revealed by the unchanging q-value close to unity (for further details, see Ling, 1993).

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 the 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 I and my coworker have. also completed but published one set of the data on frog muscles (see Ling ***et al.,*** 1993). Similar studies on the distribution of nonelectrolytes in two other kinds of living cells have been completed but not yet published: frog ovarian eggs and mouse Ehrlich ascites carcinoma cells.

These three sets of data, including ***the data from frog muscles (Figure 7), when seen side by side with the data from model studies shown in the insets of the same figure offer one of the simplest and most direct proofs 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 or more rigorously between the q-value-vs-molecular-volume plots in these living cells (see Ling, 1993; Ling, Niu and Ochsenfeld, 1993) and in all three extrovert models mentioned above and by the total lack of resemblance to the q-value-vs-molecular-weight plot of the introvert model of native hemoglobin.

In the frog muscle data, fourteen of the twenty-one nonelectrolytes studied, the q-values

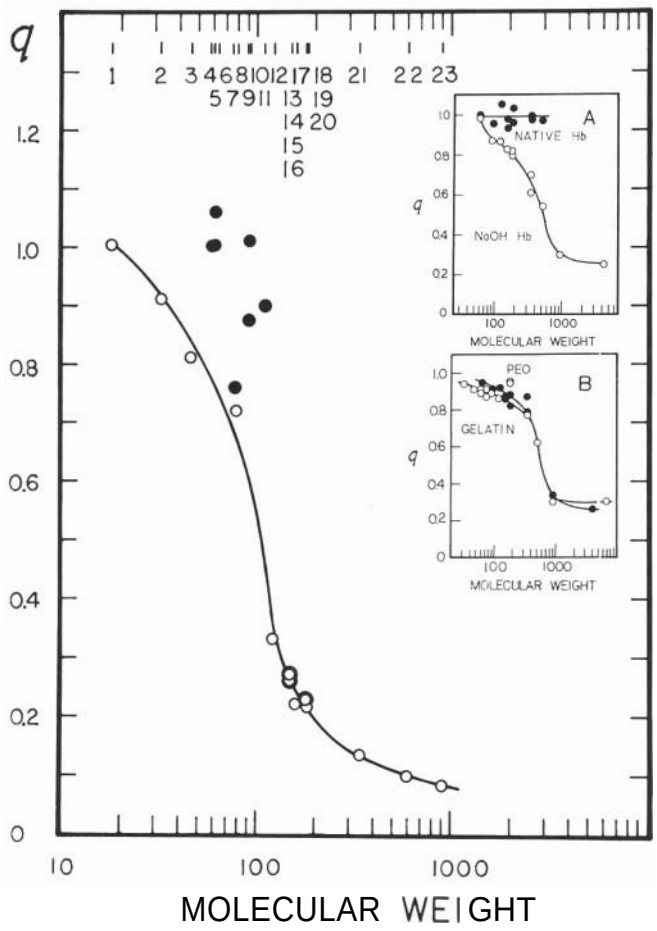

FIGURE 7. The equilibrium distribution coefficients or q-values of twenty-one nonelectrolytes in living frog muscle cells are shown on the ordinate and plotted against their respective molecular weights shown on the abscissa. For comparison, plots of q-values against molecular weights (q-w plots) of similar nonelectrolytes in solutions of native bovine hemoglobin (39%), NaOH-denatured bovine hemoglobin (20%), gelatin gel (18%) and the oxygen-containing polymer, PEO (15%). q-values of nonelectrolytes in both frog muscles and the inanimate models were obtained from rectilinear plots like those shown in Ling and Hu (1988) and in Ling, Niu and Ochsenfeld (1993). For the native hemoglobin data, the q-value of another large nonelectrolyte, poly(ethyene glycol) 4000 or PEG 4000, was added to a later version of a somewhat modified curve in Ling (1993) but not included here.

commendably follow the "size rule", i.e., the larger the molecular weight the lower the q-value. Yet seven nonelectrolytes exhibit q-values considerably higher than expected.

These exceptional solutes include urea and ethylene glycol. 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 A. V. Hill, MacLeod and Ponder, Hunter and Parpart and others to the belief that cell water is just normal liquid water. This belief in turn played a critical role in the world-wide acceptance of the membrane-pump theory and the dramatic abandonment of the protoplasmic approach to cell physiology in America and Western Europe. The data presented in Figure 7 confirm all of their findings but not their conclusion that cell water is normal free liquid water.

On the contrary, the ability of muscle cell water *not to exclude urea and ethylene glycol* side by side with the *ability of excludingpartially other solutes according to their molecular volumes* (and molecular weights), when seen side by side with the data from model studies shown in the inset A and B of Figure 7, demonstrates that water in frog muscles is by and large similar to water in the presence of extroverts. In both, the behaviors of water follow those predicted by the polarized multilayer theory (Ling, 1993).

Why urea and ethylene glycol have higher q-values than their respective molecular weight suggests is presented in detail in the more quantitative theory of solute exclusion that has recently appeared in print (Ling, 1993). Suffice it to say that these molecules and others like them fit and interact strongly with the dynamic water structure, so that in displacing water molecules they enhance rather than weaken the polarized multilayer dynamic structure.

From results of these and other 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 resembles 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).*

Cell K^+

In preceding pages, I have briefly described a theory of selective accumulation of K^+ over Na^+, earlier 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 y-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—arries enough β- and y-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 y-carboxyl groups adsorbing K^+, see Ling and Ochsenfeld, 1991).

(a) Confirmation of the predicted localization of K^+ and its surrogates, Cs^+ and Tl^+ in the A bands of striated muscles cells

Myosin, the muscle protein discovered and named by Willy Kiihne, contains a major share of all the β- and γ-carboxyl groups in muscle cells (Ling and Ochsenfeld, 1966). In 1873, T.W. Englemann, whose theory of heat-energized muscle contraction was discussed earlier, 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 seen together, the LFC hypothesis (as well as the association-induction 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 predictions. 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^+.

In an EMOC preparation, both Tl^+ and Cs^+, like K^+, are selectively accumulated in muscle cells over Na^+, indicating that 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 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's solution containing radioactive ^{134}Cs-labelled Cs^+ (or ^{204}Tl-labelled Tl^+) at 25°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 8 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).

Ludwig Edelmann (1977) provided additional striking confirmation of the predicted localized distribution of K^+ surrogates, Tl^+ and Cs^+. 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 9).

Note in particular how granular deposits appeared at the A bands following exposure of the EM sections of the Tl^+-loaded sections to room temperature (and moisture) for 1 hour (Figure 9–3). 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 9–2 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 (Trombitas and Tigyi-Sebes, 1979). A variety of techniques was used, including direct observation of

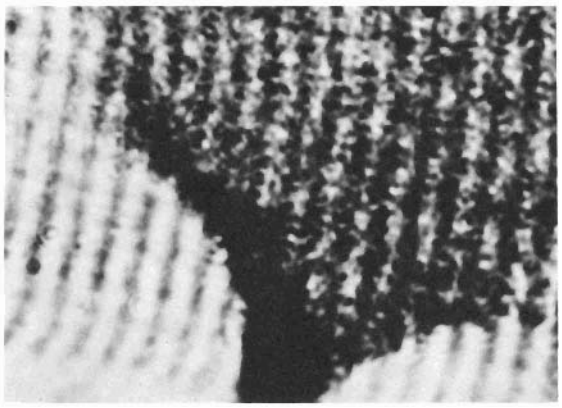

FIGURE 8. Autoradiograph of an air-dried single frog muscle fiber, which was loaded with ^{134}Cs while the muscle was perfectly normal and before drying. The muscle fiber was incompletely covered with the photoemulsion, permitting the recognition that the silver granules and hence labeled Cs^+ was primarily located in the A- or dark-bands of the muscle cell (From Ling, 1977b.)

the predicted localized distribution of K^+ itself by the method of 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).

(b) 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 10 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 and the ion distribution reached new equilibrium levels after sterile incubation at 25°C for 72 hours (Ling and Bohr, 1971). 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 (see Figure 3). 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 an extremely low or what one calls 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 in the description of the EMOC preparation studies. All points in Figure 10 are experimental and the solid lines going through or near the points are theoretical according to the general equation for solute distribution in living cells (Ling, 1965, equation 8; 1984, equation 11.6; 1992, equation 16).

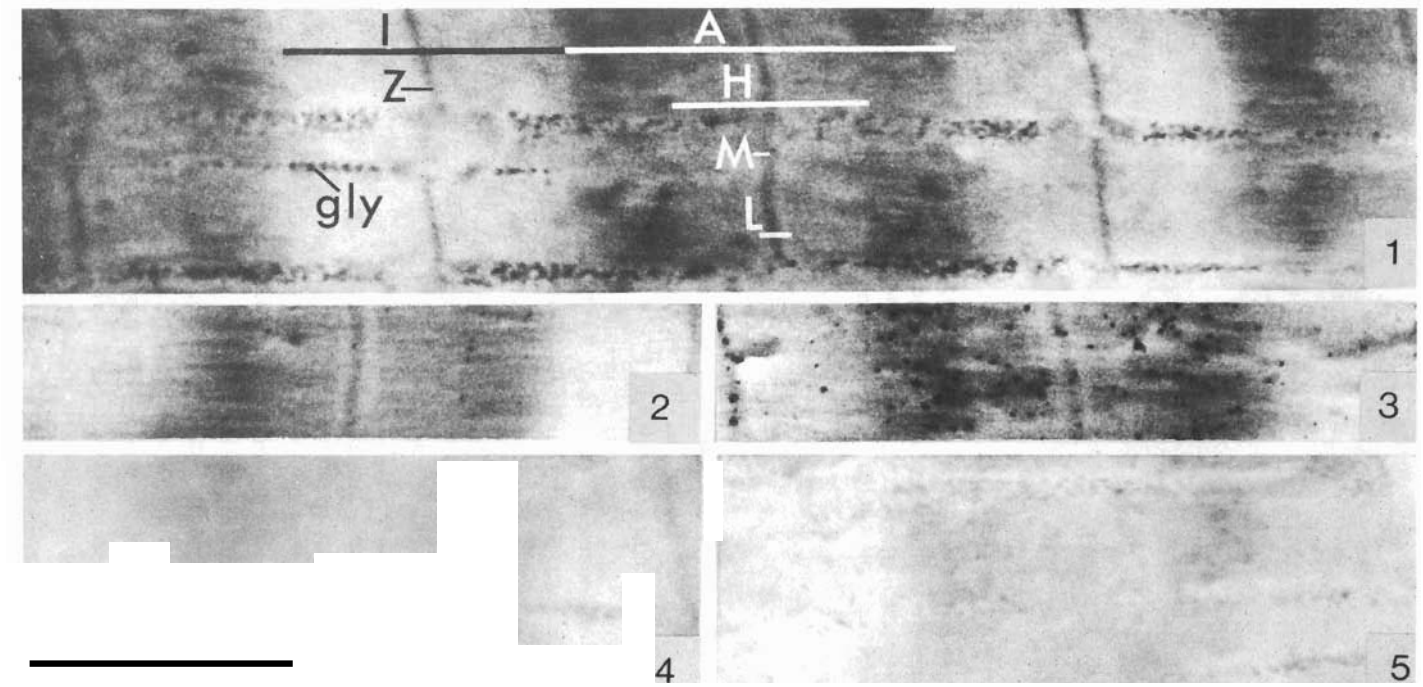

FIGURE 9 Electron micrographs of dry cut, unstained sections of freeze-dried frog sartorius muscles. Living muscle had been loaded with Cs^+ (1) and Tl^+ (2, 3) prior to freeze-fixation, freeze-drying and embedding. (2) was obtained immediately after sectioning, (3) after exposure of a section to room atmosphere for 1 hr. (4) central part of (1) after storage for two days in distilled water. (5) 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).

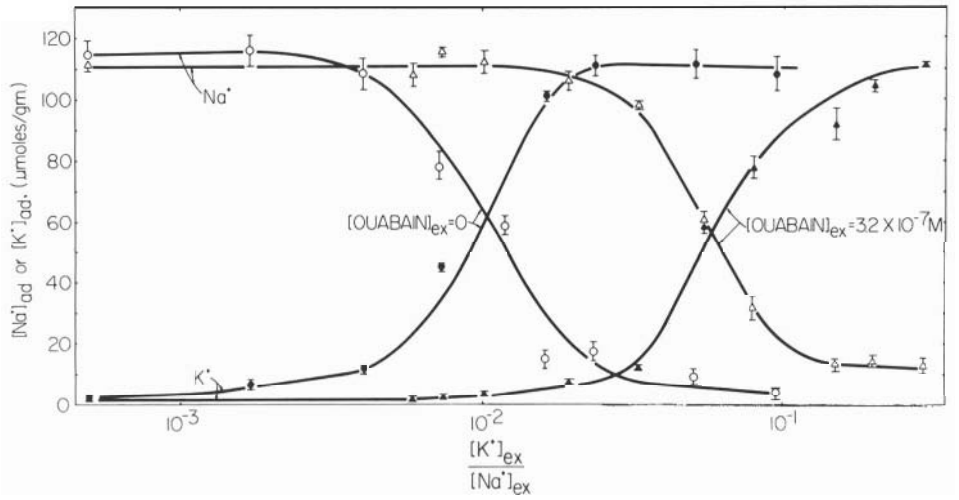

FIGURE 10. The equilibrium concentrations of K^+ and Na^+ in frog muscle (ordinate) in the presence of varying concentration ratios of K^+ over Na^+ of these ions in the bathing medium (abscissa). The left pair of X-shaped curves were derived from normal muscles while the right pair of X-shaped curves 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^{oo}), which changed from **100** to 21.7 in response to ouabain. (From Ling and Bohr, **1971**.)

Figure 10 here demonstrates how interaction with the cardinal adsorbent, ouabain, brings about an across-the-board, uniform decrease in the ***intrinsic equilibrium constant*** (K^{oo}) for the K^+/Na^+ exchange on the β- and y-carboxyl groups by a factor of about five (from 100 to 21.7). Evidence that the sites adsorbing K^+ or Na^+ were indeed β- and y-carboxyl groups are discussed next.

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

I chose two ways to identify β- and y-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. An early obstacle came from the cell membrane barrier which 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 (polyethyleneglycol-8000, PEG-8000) which can preserve the bulk of the K^+-adsorbing sites with exposed cytoplasm (Ling, 1989) well enough to carry out studies. (This agent is also used as a cryoprotectant by scientists engaged in preserving living cells at liquid nitrogen temperature.)

Under the protective action of PEG-8000 at O°C, 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 substantially reducing the alkali-metal ion adsorbing groups with the carboxyl specific reagent, 1-ethyl-3-(3-dimethylamino propyl)cabodiimide (for details, see Ling and Ochsenfeld, 1991; Ling, 1990; 1992 Section 4.4.4).

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. The question arises: What makes these proteins assume this fully-extended conformation? After all, most native proteins do not exist in the fully-extended conformation.

It is true that exposure to NaOH or urea can change introvert native proteins into extrovert fully-extended proteins. However, neither NaOH nor urea exist in the cells, certainly not at the high concentration required. Nature must have invented a more subtle way of achieving this goal. To search for understanding, we turn to another page of the AI hypothesis, which addressed the question as to what determines the way a protein folds (i.e., secondary structure) and what can alter it. 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 which is, relatively speaking not as thoroughly tested and firmly established as the *associative* aspect of the AI hypothesis. 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 the future.

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. Trichloroaceticacid 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 the outstanding American chemist, G.N. Lewis (1923) 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 a bona fide part of the accepted knowledge in theoretical organic chemistry (Derick, 1911; Lewis, 1923; Branch and Calvin, 1941; Ingold, 1953; Hammett, 1970; Chapman and Shorter, 1972; Chiang, 1987).

Since inductive effect determines the properties and behaviors of small organic molecules, it should exercise a 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 31 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 a-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, as demonstrated by Anfinson (1967)?

Studies on the empirical relationship between the primary structure (i.e., amino-acid residue sequence) 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 was the short-range interaction; it 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 atoms 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, though rarely if ever cited by those who should have a keen interest (see Endnote 7).

In this inductive theory of short-range interaction in proteins, 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 carboxyl group.

The relative electron-donating strength of each of the 19 common amino acid residues was obtained from the pK_a 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 "a-helical potentials" of the 19 amino acid residues provided by Chou and Fasman (1978) and by two other groups of scientists (Gamier ***et al.,*** 1978; and Tanaka and Sheraga, 1976; see Ling, 1986, 1992, pp. 118–121).

The primary conclusion of this study is that the short-range influence of the side chain in determining the secondary structure lies in the different induction effect each side chain exercises on (primarily) the electron density of the residue's own backbone CO group. Side chain that donates electrons enhances the electron density of its own backbone carbonyl oxygen, which in turn raises the probability of the formation of a-helical conformation of the peptide linkage. Conversely, a side chain that withdraws electrons from its own carbonyl oxygen atom reduces the probability in the formation of a-helical conformation. The success of the inductive interpretation of the hitherto unexplained mechanism, how primary structure determines secondary structure, declares its own importance—Anfinson was awarded the Nobel prize for establishing this relationship between primary and secondary structure but no mechanism for the relationship (Anfinson, 1967).

An important secondary conclusion derived from these studies is the general finding that ***high electron density of the backbone carbonyl oxygen atoms favors the formation of the α-helical and other introverted structures. In contrast, low electron density at the backbone carbonyl oxygen atoms favors the fully extended conformation and interaction of the back-***

bone 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 as well.

Resolving a Paradox: 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 but powerful 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 non-hydrolyzable Mg-imido ATP can do the same (Marston *et al.*, 1979).

The resolution of this apparent paradox lies in that *the adsorption of the cardinal adsorbent alters the inductive effects asserted by the protein side chains*. As a result, a new set of inductive effects is created by the primary structure and the cardinal adsorbent operating together to bring about a new secondary structure (Ling, 1986).

In the AI hypothesis, proteins respond to the adsorption of a cardinal adsorbent by an inductively mediated, self-propagating change of the electron (and/or positive charge) density of the nearest-neighboring backbone CO and NH group respectively. As a result, changes in the preference for alternative H-bonding partners occur. Exchanges of the partners of the backbone NH and CO groups may than 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 (Ling, 1992, pp. 142–149). (For discussion of oscillatory conformation changes in the transmission of inductive effect, see Endnote 8).

Effective cardinal adsorbents are divided into two classes: *electron-donating cardinal adsorbents (EDC) or electron-withdrawing cardinal adsorbents (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 y-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 (Ling, 1981; Ling, 1992, pp. 144–145).

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 groups for H^+. This part is familiar. H^+ is only one of the monovalent cations the living cell encounters. K^+ and Na^+ are

other examples. The question was raised: Would the relative affinities of the carboxyl groups for K^+ and Na^+ change following the H to Cl substitution? In order to make a precise answer possible, one must be abe to represent the change of the carboxyl group quantitative. To do so, the concept of the c-value was introduced (Ling, 1962, pp. 57–60; 1992, pp. 126–127).

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 (pK_a value) and a high c-value. Trichloroacetic acid (CCl_3COOH) 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 oxyacid 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 Angstrom units would entail an enhancement of the interaction such as that seen in acetic acid with strong affinity of the oxyacid group of 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 cawed out in a continuous dielectric. Four Configurations 0, I, II and III were assigned corresponding to zero, one, two, and three water molecules being placed between the carboxyl oxygen atom and the interaction monovalent cations, including H^+, Li^+, Na^+, K^+, Rb^+, Cs^+, or NH_4^+ (Ling, 1962, p. 61, Figure 4.3; 1984, pp. 155–157). 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. Finally the adsorption or association energy of each of the cations at different c-values was computed by a Born charging method. The result obtained for a carboxyl group with a polarizability of 2.0×10^{-24} cm^3 is shown in Figure 11.

As illustrated in Figure 11, 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 the selectivity order is reversed and the Na^+/K^+ ratio continues to increase.

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 y-carboxyl groups carried respectively on aspartic and glutamic acid residues. With few exceptions, all proteins also carry fixed cations in the form of a-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 modifications 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 anions on the protein they form what are called ***salt linkages*** (Speakman and Hirst, 1931). Salt linkages are major structural components of the tertiary structure of a protein (see Perutz, 1970). Recently Ling and Zhang (1984) have fully confirmed the contention made first in 1952 that virtually all the β- and y-carboxyl groups of native proteins do not adsorb significant amounts of K^+ and Na^+ because they are locked in salt linkages (Ling, 1952). Neutralization of the cationic charge by raising the pH liberates

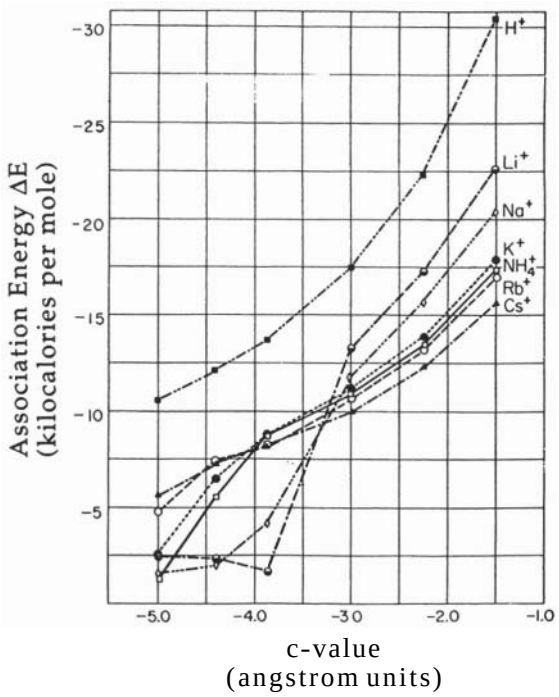

FIGURE 11. The theoretically computed dissociation energies of H^+, Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+ from a singly charged anionic site with a polarizability equal to 2.0×10^{-24} cm^3. (From Ling, 1962, by permission.)

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 of K^+ (since the calculated K^+ and NH_4^+ adsorption curves in Figure 11 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^+ as a result of (i) the much smaller entropy gain on dissociation of the fixed cation when compared to the free K^+ or Na^+, and (ii) the much greater polarizability of the bulky fixed cation than that of the NH_4^+ ion. 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 (for a full discussion, see Ling, 1992, pp. 127–128). That native proteins do not adsorb either K^+ or Na^+ and that their β- and y-carboxyl groups are mostly locked in salt linkages are in harmony with the theoretical deduction that native proteins are proteins in which the β- and y-carboxyl groups have very high c-values. To maintain the living state in which K^+ is adsorbed preferentially over both Na^+ and many fixed cations, the c-value of the β- and y-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—magnet analogy. I then pointed out that the maintenance of the living state depends on analogous electronic mechanisms, where ATP, the ultimate product of metabolism, serves its critical role, not on 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 on the protein involved. The resultant electronic polarization of the protein molecules may then bring about a change of its unfolding.***

As mentioned above, it was once widely believed that two of the terminal phosphate bonds are high energy phosphate bonds which were estimated to be –12 Kcal/mole. Subsequent investigations showed that this was incorrect. The final value estimated was 4.7 Kcal/mole; at this level, there is no high energy of which to speak. However, it remains a fact that ATP serves many important functional roles that ADP cannot. ATP must have some other attributes genuinely different from ADP. What can this be? The answer to this crucial question came gradually. But by now, there is no doubting what it is. ***It is the adsorption energy of ATP on the protein it affects.*** In the contractile muscle, the protein ATP acts on is myosin.

The binding constant of ATP on myosin is enormous, equal to 10^{10}–10^{11} (Mole–') (Goody ***et al.,*** 1977; Cardon and Boyer, 1978). This is equivalent to an average standard free energy of adsorption of –14.3 Kcal/mole (see Ling, 1992, p. 180). Note that this (genuine) adsorption energy is even higher than the once-widely believed but erroneous –12 Kcal/mole value miscalculated for the so-called high-energy-phosphate bonds. Just as reassuring is the now established fact that the biding constant of ADP on myosin—the hydrolytic product of ATP—is only 10^5 and thus 100,000 times weaker than the binding of its parent compound, ATP (Lowey and Luck, 1969; Marsh ***et al.,*** 1977). This great disparity between the binding energy of ATP and of ADP is vital if ATP acts as a switching-controlling mechanism (see below).

An in-depth examination of the consequence of the interaction of ATP with protein models led to the conclusion that ATP functions as a electron withdrawing cardinal adsorbent, or EWC (Ling, 1992, pp. 180–182).

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 y-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 y-carboxyl groups; (4) ATP functions as such an EWC.

Figure 12 is presented for two 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 y-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 to carry out its func-

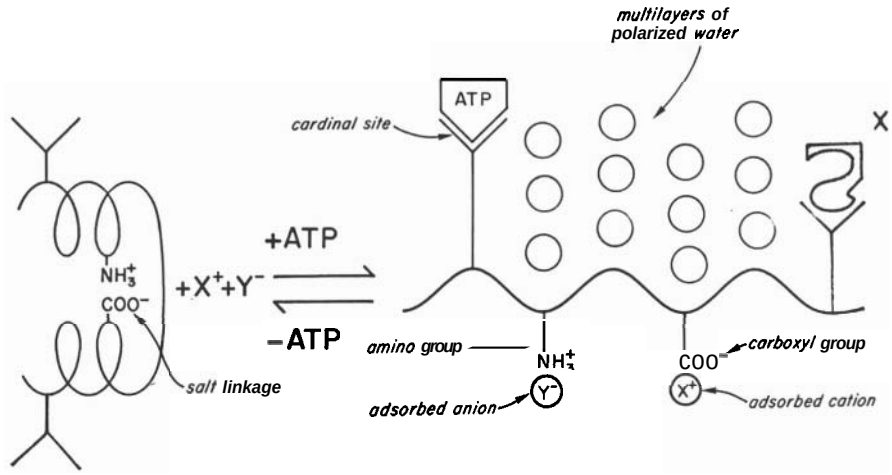

FIGURE 12. Diagrammatic illustration of how ATP and its "helpers", including the congruous anion and the protein X, control protein conformation. Note that the key events brought about by ATP and its "helpers" is the unravelling of the secondary structure, releasing the backbone NH and CO groups to interact with the bulk-phase water and the breaking up of salt-linkages so that the freed fixed cations can now interact with the "congruous anions" and the freed fixed β- and y-carboxyl groups can selectively adsorb K^+. (From Ling, 1992. By permission of Kreiger Publishing Co.)

tion, ATP requires at least two "helpers": "congruous anions" and an as-yet-unidentified helper protein component, referred to as protein X (Ling, 1992, pp. 182–186).

It also tells us that for each ATP molecule properly adsorbed on its cardinal site on the minimal protoplasmic unit, there would be a finite number of K^+ adsorbed. Similarly for each ATP adsorbed, there would be a finite number of water molecules engaged in the dynamic structure of polarized multilayers and, as a result, a proportional reduction in the concentration of Na^+ accommodated by the water. Since ATP increases the uptake of K^+ while it reduces the concentration of Na^+, one expects antipodal changes in the concentration of K^+, Na^+, when the ATP concentration is allowed to decrease very slowly to permit diffusion equilibrium of K^+, Na^+ (and sucrose) to be reached at all levels of the falling ATP concentration. This type of antipodal change of K^+ and Na^+ concentrations (to be referred to as Type B) is different from the type of antipodal changes involving the stoichiometric displacement of one ion by the other as illustrated in Figure 10 and to be referred to as Type A.

Figure 13 shows that as ATP concentration falls in a specific experiment at 0°C where 0.2 mM iodoacetate is used, the changes of K^+ and Na^+ concentration are like mirror-images as expected. A parallel course of change of sucrose concentration and Na^+ concentration clearly indicates 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 harmony with the findings demonstrated in Figure 13 were the data of Gulati ***et al.*** (1971) also on frog sartorius muscles, shown in Figure 14. Here the equilibrium concentration of K^+ and of ATP were assayed at different times after the poisons were introduced to

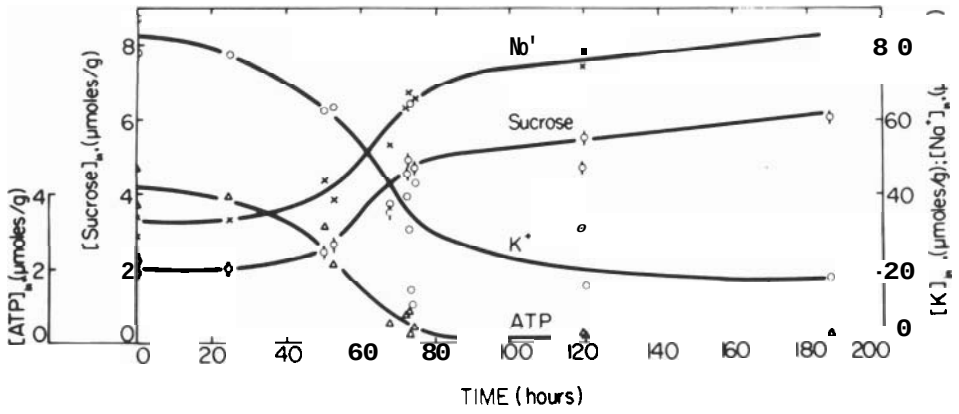

FIGURE 13. The equilibrium concentrations of K^+, Na^+, sucrose and ATP in frog muscles exposed to a low concentration of sodium iodoacetate (0.2 mM) for different length of time (abscissa) at 0°C. (From Ling and Ochsenfeld. By permission of Krieger Publishing Co.)

the medium bathing the isolated muscles. The ten poisons studied had widely diverse targets of their deleterious actions. Nonetheless, essentially the same linear relationship was found between residual ATP concentration in the muscle tissues and its remaining K^+ contents, regardless of what poison was used to lower the ATP concentration. This indifference to the way ATP depletion was produced underscored that it is the concentration of ATP alone that determines the level of K^+ in the muscle.

Summarizing the results of both Figure 13 and Figure 14, one may state that as ATP concentration declines, K^+ is desorbed and lost to the outside medium. While a small fraction of Na^+ in the cells might have taken up the β- and γ-carboxyl groups, most of these groups become locked in salt linkages formed with fixed cationic groups. In the meantime, water is gradually depolarized, and with it, there is a parallel increase of the q-value for large hydrated ions, Na^+, as well as the large hydrated nonelectrolyte, sucrose, until their intracellular concentrations approached those in the outside medium.

The New Cell Physiology: Its Role in Reviving a Dying Major Human Intellectual Enterprise

The first scientist that emphasized the cell membrane over the cell substance was Hermann Schwann, the founder of the Cell Theory. Since even using the best light microscope today, one cannot see a distinctive membrane structure at the cell surface, I suggested above that Schwann's (early) emphasis on the cell membrane was based on a mistaken identity. Whether my surmise is correct or not, there is no doubt that Schwann in his later years subscribed to an altogether different view of the living cell with emphasis on the protoplasm, such as enunciated by Max Schultze in his well-known Protoplasmic Doctrine.

Traube's discovery of, and Pfeffer's experimentation on, the copper ferrocyanide gel

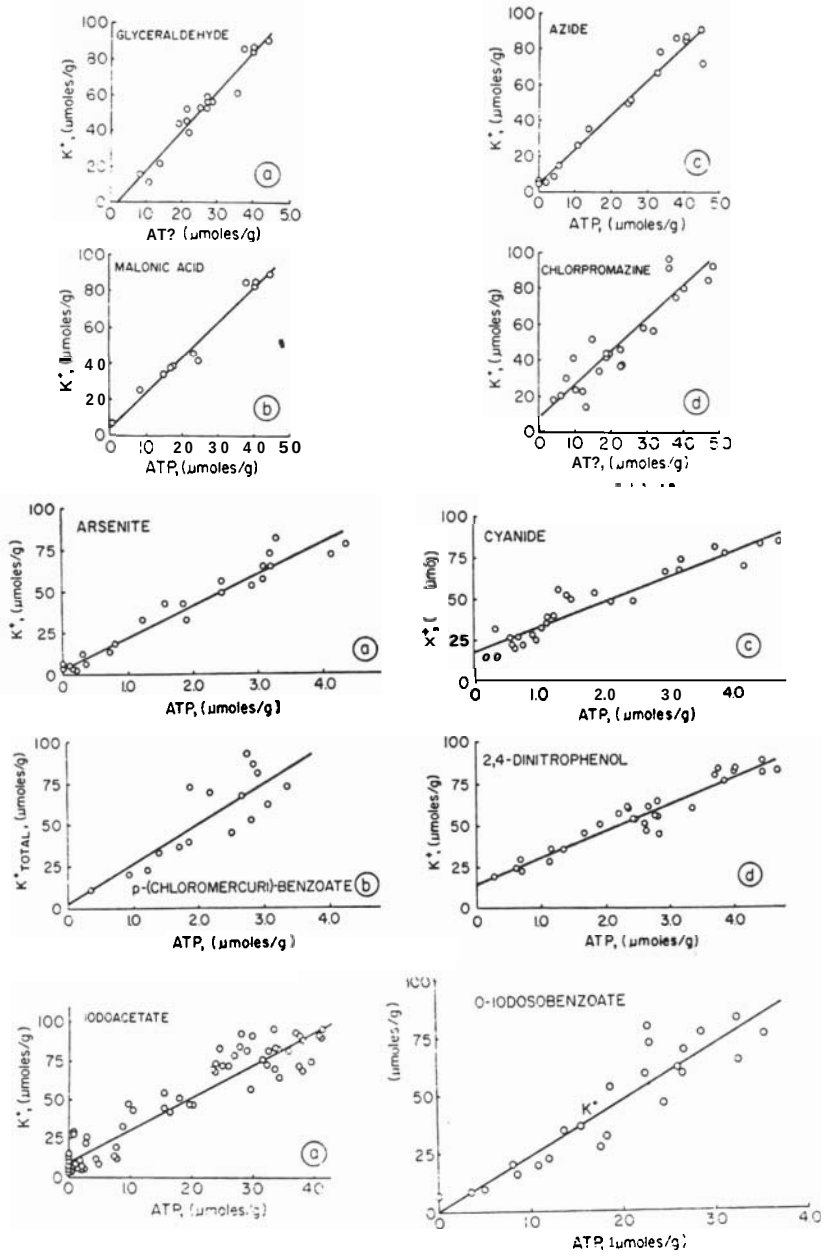

FIGURE 14. The relation between the equilibrium concentration of K^+ and the concentration of ATP in frog muscles exposed to 10 different poisons (as indicated) for different lengths of time at 25°C with shaking, followed by an equilibrium period of 2 to 4 hours at 1°C also with shaking to allow diffusion equilibrium for K^+ to be reached. (Gulati et al., 1971. Reproduced from the Biophysical Journal, 1971, Vol. 11, by copyright permission of the *Biophysical* Society.)

membrane officially launched the membrane theory. van't Hoff's introduced and defined the term 'semipermeability' as permeability to water but not to (any) solutes. de Vries' study of shrinkage of beet root cells in concentrated solution of NaCl and Overton's work on the volume changes of frog muscle in concentrated methyl alcohol, ethylene glycol convinced cell physiologists of the time that the semipermeability of the living cell membrane is different from the definition given by van't Hoff: solutes themselves appeared to fall into two classes, those that are permeable to the cell membrane (e.g., methyl alcohol, ethylene glycol) and those that are not (e.g., sucrose, NaCl).

It was based on the idea that cell membrane can be permeable to some solutes and ***absolutely impermeable*** to others, and drawing heavily upon the rapidly developing dilute solution physics that early subscribers to the membrane theory offered plausible mechanisms for all four classic manifestations of the living cells: asymmetrically solute distribution, selective permeability, volume control and the cellular electric potentials.

At the very moment when proponents of the membrane theory seemed to have won over their arch opponents, the subscribers to the protoplasmic theory of living cells; the foundation concept of their own membrane theory collapsed. The prototype impermeant solute, NaCl was found to be permeant. Hastily, the sodium pump hypothesis was introduced.

I have already presented a bird's eye view of what happened to each of the four major subjects of cell physiology from its inception to the present day, taking care to keep my own part out of the picture as long as possible until I reached the subject of the selective solute distribution. It is here that I allowed my own work to be brought back into the picture after the introduction of the sodium pump. As the reader knows by now, this work had unequivocally disproved the Na pump hypothesis: the energy consideration shows that the postulated pump is against the law of conservation of energy and thus not feasible. The EMOC preparation showed that the cell membrane does not contain membrane pumps. The failure of the ideal squid axon sac to demonstrate transport of K^+ and Na^+ against concentration gradients lends further support to the conclusion that the cell membrane does not contain ion pumps.

The disproof of the sodium pump hypothesis has for the second time disproved the membrane theory. The successful EMOC experiments and failed squid axon membrane sac experiments also unanimously pointed to the cell substance, the protoplasm as the seat of the selective solute distribution, thereby bringing to life once more the protoplasmic approach to cell physiology.

The key initial findings of the sorption theory of Troshin and of my association-induction hypothesis both appeared in print in the year 1951 and each was addressed to the basic problem of solute distribution in living cells and they are in harmony with each other. Indeed, the general equation for solute distribution introduced first in 1965 (Ling, 1965)—of which the Troshin equation is a special case—has been shown to describe all types of equilibrium solute distribution known to us in 1991 (Ling, 1965; Ling, 1992, Chapter 8). There is no need to say more on this subject here.

In presenting the theory and evidence for the AI hypothesis, I have also gone into some details concerning how ATP serves its role in cell physiology now that we know it does not carry a package of high energy, as was widely believed at one time. The extremely high adsorption energy of ATP on the cell protein myosin in contrast to the 5000 fold weaker adsorption energy of its hydrolytic product ADP—all discovered after the AI hypothesis for ATP function had been proposed—fits the theory like hands fit gloves. The quantitative

relationship demonstrated between ATP concentration and the steady level of K^+ and Na^+ further confirm the theory and point to new avenues of research on the mode of actions of cardinal adsorbents in general and drugs in particular.

However, to show how well the new cell physiology has succeeded where the membrane theory with its sodium pump corollary has failed, I now return to the further development of the three other subjects of cell physiology (cell volume control, cell permeability and cellular electrical potentials) that I have described earlier with the assumption that the sodium pump hypothesis was still viable and in order to keep out of consideration anything in which the AI hypothesis had played a part. Now that the sodium pump hypothsis is thoroughly disproved and a full sketch of the AI hypothesis has been presented, I can tell the full story as it stands today.

(1) Cell volume regulations

The original membrane theory of cell volume regulation was squarely built on the concept that only impermeant solutes can cause sustained cell shrinkage. The work of Nasonov and Aizenberg published in 1937 disproved that. After the introduction of the sodium pump, Wilson and Leaf suggested that in the presence of the pump, the leaky membrane acts effectively as if it were impermeable to Na^+. The disproof of the sodium pump has also eliminated that corollary to the membrane theory. As far as I know, this is the end of the road for the membrane approach to cell volume regulation.

Where is the new interpretation of cell volume regulation? Before answering that I must point out that neither Nasonov and Aizenberg nor Troshin offered a molecular mechanism for the demonstrated sustained shrinkage of muscle cell in the presence of high concentrations of permeant solutes. In contrast, the new cell physiology based on the AI hypothesis does offer an explanation in a rigorous equation form, and it has been confirmed by experiments.

When a solute like NaCl is dissolved in water, the activity of water is reduced. Quantitatively, the water activity is equal to what is called ***partial vapor pressure.*** A more concentrated solution of NaCl has a lower vapor pressure and hence water activity and vice versa. In two adjoining phases, water will always move from a phase of higher activity to a phase of lower one. When water equilibrium is reached, it usually means that the water activities in the two phases are equal.

Normal frog muscle is in water equilibrium with a 0.7%--or more exactly, 0.118 M—NaCl solution as Overton first showed a long time ago. Frog muscle gains water from a 0.35% NaCl solution because the activity of water in the more dilute solution is higher.

From the summary of the AI hypothesis discussed above, we know that most solutes have a specific equilibrium distribution coefficient or q-values in cell water. The q-value of NaCl in frog muscle is close to 0.2. That means in a 0.7% NaCl solution the intracellular Na(Cl) concentration is only some 20% of that of the outside solution. Thus if no other balancing force exists, the cell will lose water and shrink until its intracellular concentration matches that outside. This of course does not happen. The cell volume stays constant because there is an opposing force trying to hold onto the water inside the cell. This opposing force is the force adsorbing multiple layers of water on the fully-extended protein chains in the cell. At exactly what point where the cell ceases gaining or losing water depends on the q-value of the solute and on the partial vapor pressure of the surrounding medium as formulated in an equation I introduced in 1987 (Ling, 1987a) and is listed as Equation 34 in my 1992

monograph. This equation correctly describes the type of data first reported by Nasonov and Aizenberg in 1937.

(2) Cell permeability

In 1953, as part of the embryonic version of the AI hypothesis, known as Ling's fixed charge hypothesis, I suggested that the presence at the cell surface of fixed β- and γ-carboxyl groups belonging respectively to the aspartic and glutamic acid residues of cell surface proteins could provide a mechanism for the preferential membrane permeability of many types of living cells to K^+ over Na^+ (Ling, 1953). This idea was extensively tested and confirmed in general principle from the studies of different types of living cells and a variety of inanimate fixed charge systems including ion exchange resins, sheep's wool, myosin gel (for review, see Ling, 1960; Ling and Ochsenfeld, 1965; Ling, 1984, pp. 396–403; 1992, pp. 230–233).

After the AI hypothesis in its fully-fledged form appeared in 1962 (Ling, 1962), the polarized multilayer (PM) theory of cell water was added in 1965, completing the theory (Ling, 1965a). Believing that qualitatively the cell membrane is not fundamentally different from the bulk-phase protoplasm-(unknowingly at that time to me) echoing the ideas of Max Schultze and of Willy Kiihne—I then suggested that it is water existing in the state of (intensely) polarized multilayers (rather than lipid bilayers) that provides the *continuous barrier* to diffusion of nonelectrolytes into living cells in lieu of the (by-now disproved) phospholipid bilayer hypothesis (Ling, 1973).

This idea of polarized water serving as the continuous diffusion barrier was then tested extensively in living cell membranes side by side with an artificial activated (hydrated) cellulose acetate membrane. The agreement found between the model and the living cells was better than yielding a good correlation (a linear correlation coefficient = +0.961); actually there was a 1-to-1 correspondence. In both the living cell membrane and the experimental model, the polarized water *functions* as a molecular sieve even though it is definitely not a *mechanical* one. Thus small molecules like water go through both the living membrane and the cellulose acetate membrane very rapidly. In contrast, larger molecules like sucrose go through the membrane several orders of magnitude more slowly and are thus to all intent and purposes impermeant. Yet the average pore diameter of the activated cellular acetate membrane was estimated at 44 Angstrom units. The diameter of sucrose, to which the membrane is virtually impermeable, is only nine Angstrom units. The physical mechanism underlying this strong exclusion of sucrose and other larger molecules from polarized water will be mentioned below.

At this junction, it would be appropriate to address the question often raised: Is there such a thing as the cell membrane? Schwann could not have seen a cell membrane, nor could Pfeffer, nor could Bemstein. Max Schultze did not believe that a cell membrane existed; he believed that in substance the cell surface is just another version of the protoplasm making up the whole cell. What I have found are in closer agreement with Schultze's than with Pfeffer's idea of cell membrane. The critical difference lies not in what makes up the surface layer. Rather it lies in what lies beneath the surface layer. There is overwhelming evidence that both the cell surface and cell substance share the basic attributes of protoplasm—a closely associated system of proteins-water-ions and cardinal adsorbents including ATP. However, cell physiologists have used the name cell membrane so long, I think that we should continue to use it only if one understands that the inside face of the layer does not separate it from another dilute solution as the outer surface undoubtedly does.

In some way, the cell membrane is like the "membrane" of a free-floating water droplet. Different from the bulk substance and yet not that different.

(3) Cellular resting potential

According to the membrane theory of Bernstein or the Ionic theory of Hodgkin, the cellular resting potential is a membrane potential or a modified membrane potential. The magnitude and polarity of the potential depends on the relative permeability of different ions present in the cell and in the surrounding medium. These theories are made doubtful after great efforts made over a period of some 70 odd years failed to turn up a single inanimate model membrane portraying such a membrane potential.

The ionic theory also cannot explain why the resting potential in frog muscle is indifferent to the (highly permeable) Cl^- ions, nor according to the majority of investigators, why the resting potential is indifferent to the concentration of intracellular K^+ concentration.

The ionic theory is contradicted by the demonstration that no sodium pump exists in the cell membranes. And it is the postulated sodium pump that maintains the low free K^+ ion in the cell required in the ionic theory.

I suggested briefly in 1955 and in greater detail in 1960 and 1962, that the resting potential is not a membrane potential (as I once believed, see Endnote 3) but a ***surface adsorption potential*** (Ling, 1955, 1960, 1962, 1992). The surface adsorption potential is created by the presence of fixed anions on the cell surface in the form of β- and y-carboxyl groups belonging respectively to the aspartic and glutamic acid residues of the cell surface protein(s). Like those in the cell interior, these β- and y-carboxyl groups also selectively adsorb K^+ and Na^+ and it is this selectivity in adsorption that gives rise to the greater sensitivity of the resting potential to external K^+ and less sensitivity to external Na^+. Supportive experimental evidence includes the following:

First, the surface adsorption potential theory of cellular potentials is in full accord with results of studies on all four types of model membranes, each of which demonstrates either a surface adsorption potential or something basically similar.

Second, being anionic in nature, the surface β- and y-carboxyl groups do not interact with similarly-chargedanions like Cl^-. It is therefore in full accord with the fact that frog muscle resting potential has no sensitivity to the concentration of external Cl^- ion.

Third, the entire cell constitutes a single phase (see also Nasonov, 1959, p. 171). The surface as well as the cell interior shares the same characteristics of a closely associated protein-K^+-water assembly maintained at a high (negative) energy, low entropy state. Therefore in each cell there is only one interface with normal liquid water and that interface is the one at the cell surface. This is why under normal conditions the potential is indifferent to the intracellular ionic concentration.

When the cytoplasm is removed and replaced with Ringer's solution, the β- and γ-carboxyl groups on the newly exposed protoplasmic surface may act like that normally present on the outside only, and as a result exhibit ion sensitivity. But this is a man-made artifact and as a result only a small percentage of investigators found sensitivity of the potential to the intracellular ion concentration (for details, see Ling, 1984, pp. 475-477; 1992, p. 315, Endnote 5).

Fourthly, I want to mention that a general equation for the cellular resting (and action) potential was introduced in 1979 (see Ling, 1992, pp. 289, Equation 48), which is capable

of explaining all the observations that can be explained by the HKG equation as well as those that cannot.

Indeed, the simpler version of the equation introduced earlier resembles a portion of the HKG equation formally. The only variables in the Ling equation (the temperature, the extracellular K^+ and Na^+ concentrations)are the same variables in the HKG equation that have been experimentally verified. The other variables in the HKG equation (intracellular concentrations of Cl^-, K^+ and Na^+ and extracellular concentration of Cl^-), which were not experimentally verified, are absent in the Ling equation (see Ling, 1992, pp. 280–287).

While the three-variable Ling equation is the entirety of a rigorously derived equation, the three-variable abridged version of the HKG equation is, of course, no equation at all. Nonetheless, each of the concentration terms in both the Ling equation and the (incomplete) HKG equation is multiplied by a constant. The meanings of the two sets of constants are, however, quite different. In the HKG equation, as mentioned earlier, the constants are permeability constants of each of the ions involved; the corresponding constants in the Ling equation are adsorption constant of the ions on the surface β- and γ-carboxyl groups.

A young German biophysicist by the name of Ludwig Edelmann designed and carried out an experiment on isolated Guinea pig heart muscle in order to find out what these constants really stand for. His result, the publication of which was to cost him not only his job but a career he had been trained for, confirmed that these constants had nothing to do with permeability but are adsorption constants (Edelmann, 1973). Edelmann then became an electron microscopist—for which he had no prior training—once more lending his courage and his talents in search for the truth wherever it lies (see Figure 9).

Concluding Remarks

The patchwork sodium pump hypothesis came into being after the disproof of the original membrane theory when its key assumption that sustained shrinkage of living cells could only be caused by concentrated solutions of impermeant solutes, proved wrong. In time, the sodium pump hypothesis was also disproved (1) by the demonstration that the hypothetical pump would consume energy many thousand times higher than available, (2) by the failure to demonstrate active transport of Na^+ and K^+ against concentration gradients in axoplasm-free, squid axon membrane sacs, and (3) by the demonstration of normal selective K^+ accumulation in, and Na^+ exclusion from muscle cells without a functional cell membrane and postulated pumps.

Old cell physiology, which was built upon these theories, reached the end of its legitimate life a long time ago. It is still being taught as truth to this very day by dint of the power of the vested interests (see Endnote 9).

In the meantime, a new cell physiology has grown into maturity. Started a little over forty years ago as Ling's fixed charged hypothesis, it evolved into the full-fledged theory of the living cell, the association induction hypothesis in 1962. The essence of this new theory has been confirmed extensively in the ensuing thirty years and summarized above.

Perhaps one of the most important characteristics of the new cell physiology is its extreme simplicity. For this reason, the validity of the new cell physiology can be argued in detail with rigor as it was in my 1992 volume (Ling, 1992). It can also be argued succinct-

ly on the basis of elementary logic and a few pieces of the key evidence alone. Firm in the belief that what is learned by experience lasts longer than what is learned by rote, I believe it worthwhile to give a summary of the new cell physiology—with emphasis on the new physiology of cell water—by taking you, the reader, through the experience of arriving at the new cell physiology by a short cut. Having done that, we will examine one by one just what the new cell physiology offers in disentangling some of the historic Gordian knots.

In this conducted tour, I shall begin with salt (NaCl) and sugar (sucrose). Both can traverse the cell membrane but are found at steady levels some 10–20% of those in the surrounding medium. Since, as shown earlier, this could not be due to pumps, their maintained low levels could only be due to their reduced solvency in the cell.

The cell is as a rule 20% proteins and 80% water. Of these two, only water can dissolve salt and sugar. We conclude that cell water must have reduced solvency for them.

As everyone knows both sugar and salt dissolve easily in normal liquid water. To account for the 10–20% levels of these solutes found in the cell water, at last 80% to 90% of cell water must be so profoundly different from normal liquid water that it either has no or little solvency for salt and sugar at all. Since the cell is primarily water and proteins, one has little choice but to conclude that proteins somehow bring about the profound change on so much water.

When a cell dies, it loses its ability partially to exclude salt and sugar and their concentrations in the cell water approach those in the surrounding media. Thus the protein(s) which act upon the water to reduce its solvency must also be able to withhold this effect.

Cell death can occur very quickly. This speed does not allow enough time for the disintegration of the cell proteins in the dying process. Therefore the change that does take place on cell death must involve a different kind of profound change of the proteins. Indeed, proteins are well-known for making such profound changes without disintegration: i.e., a change of the way a protein molecule folds upon itself. Historically connected with the process of protein denaturation, it involves primarily transitions between the tightly coiled a-helix conformation and what I call the fully-extended formation.

Water makes up some 80% of the cell weight. The molecular weight of water is 18.02. 90% of cell water amounts to (0.9 x 0.8)/18.02 = 40 moles of water molecules per kilogram of cells. Water molecules are polar and thus prefer to react with other polar groups. The most numerous polar groups carried by proteins are the NH and CO groups of the backbone. But these NH and CO groups are locked up and unable to react with water molecules if the protein exists in the a-helical form. Only when the protein exists in the random-coil or what I call fully-extended form can the maximum number of backbone NHCO sites be exposed and available.

Now if all the 20% cell proteins exist in the fully-extended form, there will be all told, 2001112 = 1.79 moles of NHCO groups per kilogram of cells, where 112 is the average weight of the amino acid residues in proteins. Dividing the total number of affected water molecules by the maximum number of NHCO groups one obtains 40/1.79 = 22.3. Thus each pair of NHCO groups must be able to polarize at least 22.3 water molecules. Since each NH of an NHCO group can form an H-bond with one water molecule while each CO groups with two, together the NHCO group directly coordinates and polarizes only three water molecules. Since each of these three water molecules can in turn polarize and orient three other water molecules, no less than three layers of water molecules must be polarized and oriented to add up to a total of 22.3 molecules. In fact, only a fraction of the cell protein

could be involved, and the average number of water molecules polarized and oriented must be even more than three layers. Since by definition, anything more than one is multiple, water must be polarized, and oriented in the form of *polarized multilayers* (for exact experimental proof, see Ling, 1993, and Ling *et al.*, 1993).

Since the fully-extended conformation brings about the profound change of the properties of water including its solvency (and other attributes mentioned above) in the living cell, death of the cell must involve eventually the change of the relevant proteins to the α-helical state. Extensive studies of the influence of metabolic arrests led to the conclusion that only the level of ATP in the cell is directly relevant. In other words, the presence and hence adsorption of ATP per se keep site cell proteins in the water polarizing (and K^+ adsorbing) living state.

Let us next take an inventory of what the experimental confirmation of the polarized multilayer theory of cell water offers in resolving the unresolved and seemingly unresolvable issues of the past:

(1) Sugar, salt as well as exotic man-invented molecules can be kept at low concentrations in the cell and within subcellular compartments without continual energy expenditure at all.

(2) Studies of extrovert models and living cells have shown that while polarized water excludes partially NaCl and sucrose, it does not exclude urea and ethylene glycol. This finding shows that A.V. Hill, MacLeod, Ponder, and others made a mistake in concluding from the equal partition of urea (and ethylene glycol) between cell water and extemal medium proves that cell water is normal liquid water. This mistake had serious adverse consequences including the erroneous and unjustified world-wide rejection of the colloidal approach to cell physiology.

(3) The polarized multilayer theory of cell water soon led to, for the first time in history, the introduction of a new and realistic definition of the word, colloids and colloidal condition.

(4) The polarized multilayer theory has also helped in offering for the first time a physico-chemical definition of protoplasm.

(5) The polarized multilayer theory also offers a logical way out of mistakes made again and again in relation to the size-dependency of solute permeation through living and inanimate membranes. Observation of this kind has seduced one cell physiologist after another into believing and introducing the erroneous mechanical sieve-like model of cell membranes. Instead, large domains of the cell surface filled with intensely polarized water *functions as* a molecular sieve allowing rapid passage of small molecules like water but is practically impermeable to larger molecules like sucrose.

(6) With the exception of mature plant cell, all living cell structures are made of protoplasm. Thus each living cell is a solid body of protoplasm—in harmony with, and support Max Schultze's *protoplasmic doctrine* introduced more than 130 years ago. As a continuous phase, each cell only makes contact with the extemal medium at its outer cell surface. It is at this interface that the (only one) surface adsorption potential (i.e., the resting potential) arises from the presence of fixed anions (as in muscle and nerve) and its preferred counteraction, K^+. The basic mechanism is shared with all four inanimate models mentioned above and illustrated by Collacicco's oil/water interface to which has been introduced the anionic detergent, SDS.

(7) The β- and y-carboxyl groups of the protoplasm of the cell substance normally selec-

tively adsorbs K^+ and Na^+, thereby giving rise to the bulk-phase selective accumulation of K^+ over Na^+. Similar β- and y-carboxyl groups at the cell surface not only give rise to the K^+-sensitive resting potential as just mentioned, it also creates a mechanism for the selective permeability to K^+ over Na^+. Since the majority of entering cations must first associate with the surface fixed anions, followed eventually by their dissociation into the cell interior, and since these surface carboxyl groups also preferentially take up K^+ and Na^+, the preferential K^+ permeability observed is explained.

(8) The new cell physiology resolves the difficulty created when ATP, the main product of the cell's energy metabolism, was found to contain no high energy in its phosphate bonds at all. ATP, like the horseshoe magnet in the nail-iron filing model, serves its function by polarizing the protein molecules it adsorbs on. The enormous adsorption energy measured, taken side by side with the 5000 times weaker adsorption energy of its hydrolytic product, ADP—findings made long after the introduction of the AI hypothesis—fits the theory of ATP function like hand in glove.

(9) It was to test the validity of the polarized multilayer theory of cell water that Dr. Freeman Cope, Dr. Carlton Hazlewood and others introduced nuclear magnetic resonance to the study cell water. And it was this beginning that led to Dr. Raymond Damadian's invention of the medical technology of great potency, now known as magnetic resonance imaging of MRI.

(10) Erwin Schrõdinger's introduction of what is known as the Schrodinger equation was claimed to be the greatest achievement of the human mind. Schrodinger wrote a little book, entitled "What is Life?" (Schrodinger, 1945), in which he argues that life represents a system that decreases entropy. In other word, life creates order out of randomness.

The living cell is the basic unit of all life. If life decreases entropy, living cells have to be the instrument in doing it. If cell living decreases entropy, cell death must increase entropy.

Now a living cell contains primarily proteins, water and K^+ ions. In the membrane theory or the membrane-pump theory, water and K^+ are both free. Since they cannot be freer than free, the only thing in living cells that in theory may gain entropy on cell death are the proteins. However, we know when cells like muscle die they undergo rigor mortis. In other word, the protein instead of gaining freedom of motion, loses freedom of motion and hence entropy on death. Is Schrodinger wrong?

Schrodinger was not wrong. It is the membrane or membrane pump theory on which the old cell physiology is built that is wrong.

In the new cell physiology, both water and K^+ are in the adsorbed and lower entropy state. It is this reduction of entropy that physicist Schrodinger has forecast and which we now confirm.

Concluding Remarks

The old cell physiology is dead; the new cell physiology is fully fledged and ready to soar. For this, I feel very privileged and grateful. Not many scientists are given the opportunity I have had in playing a significant role in laying a new foundation for future progress in cell physiology and in the healing science. But this is not the time to say, "I have done my part" and "Good bye". Thanks to the "true believers" and a bad system which they have learned to exploit, there are very few of us left to tell the story, wonderful and exciting beyond belief as it is.

To begin, we must get others who can appreciate the new cell physiology to join our cause. For that, I urge you to bring this review to the attention of others including your students. I promise to send them reprints if they will drop me a card. If there is enough interest I may even consider making the article into a booklet and selling it at an affordable price. This could be a good beginning.

However, the main thrust of our future efforts must be directed at informing lay people. After all, in the long run it is their lives and happiness that are at risk. For myself, I intend to write another book addressed to the public.

What kind of book is this going to be? First it must contain part of the materials presented in this review but also draw upon the histories of other sciences as well. The book will alert the public to the fact that one of the most precious "magical" gifts which it has inherited from the historical past, and which holds the key to protecting mankind from suffering and painful deaths from cancer, AIDS and other dreaded diseases is being trashed for lamentable reasons. And the people of the United States and elsewhere have both the obligation and the power to reverse this situation and restore integrity, enthusiasm and true productivity to biomedical research and teaching in the near future.

I anticipate positive responses. After all, only 31 years ago, Rachel Carson, then an unknown biologist, wrote "The Silent Spring". With this little book, she has miraculously turned the world around from its once suicidal course of mindless environmental destruction, to an increasingly more thoughtful approach to protecting the only habitat we have—for the benefit of our own and future generations of humanity and fellow living beings.

Endnotes

1. Carl von Nageli (1864, p. 1–158) described how protoplasm escaping from the crushed root cells of the water plant ***Hydrocharis*** did not disperse in the surrounding water but assumed the shape of little round balls which are impermeable to the dye aniline blue just like the intact root cells (see also Kite, 1913, 1913a). This and other similar observations are documented in Pfeffer's monograph (1877, p. 127 etc.), Pfeffer's paper (1890, p. 193) as well as the publication of Robert Chambers (1917). Wily Kühne (1864, p. 39) described similar protoplasmic balls from the ciliate protozoan, Stentor. Lepeschkin (1930) showed that protoplasm isolated from cells of ***Bryopsis plumosa*** could be broken up into many little water-immiscible droplets, estimated to have a total surface area 1000 times larger than that of the original intact cell. The combined surface area of the droplets being so much bigger, Lepeschkin reasoned that these numerous droplets could only be covered with a surface layer derived from the cell protoplasm.
2. Martin H. Fischer was an imaginative and pioneering colloid chemist, physiologist and physician and, above all, a courageous and wonderful person. After the first World War many German scientists were in dire condition. Fischer made great personal sacrifices in sending packages of household and scientific supplies to many of them, including his worst scientific opponents. In celebration of Fischer's 60th birthday, colloidal chemist, Wolfgang Ostwald quoted Schopenhauer: "As torches and fireworks pale and become invisible in the sunlight, so the mind of even genius and beauty are outshone and overshadowed by goodness of heart" (Ostwald, 1940).

3. This assignment of a specific name, the membrane potential, on the basis of a theory far from being proven was a serious violation of the basic rule of science. I say this with feeling because, as a graduate student, I myself had written four papers bearing "membrane potential" on their titles. I found out much later that this was wrong (see below) and that I had thus contributed my share to this mistake. Although later I spent much time trying to correct this mistake, it is a very difficult task, because by that time, it was written into the textbooks as a scientific truth—and thus implicitly sharing the same command for acceptance as, say, the Pythagorean theorem.
4. An exception appears to be the case of human red blood cells, which is known to have one of the highest membrane phospholipid contents. Of all the five types of cell membranes studied, that of the human red blood cells alone demonstrates a two-fold increase in its K^+ permeability in response to 10^{-7} M valinomycin, nonactin or monactin (Ling and Ochsenfeld, 1986). Since the same concentration of valinomycin increases by one thousand fold the K^+ permeability of bilayers of phospholipids isolated sheep red cells, the moderate twofold increase seen in living red blood cells shows that phospholipids, even in this unusual cell membrane, are very limited in coverage (for details see Ling and Ochsenfeld, 1986; Ling, 1992, p. 216–218).
5. The following is a verbatim quotation from Gortner (1930, pp. 684–685): "... there are nevertheless sufficient data on record to indicate that in adsorption from a solution we must consider that the adsorbed layer may be polymolecular." Thus Keyes and Marshall (1927) very definitely state that, "the first and succeeding layers, because of their special state, constitute new adsorptive surfaces which may adsorb molecules of the same species as the first layers or molecules of a different species. Each succeeding layer (the adsorbing molecules all of the same species) partakes of the special state of the first layers in lessened degree until finally a layer is reached which in the molecular state differs little from what may be imagined as a molecular "contact" arrangement". And Nutting (1927) reaches similar conclusions in dealing with the thickness of the water film at a silica-water interface, and concludes that this water film represents a layer of water from 100 to 120 molecular diameters deep and that the energy of adsorption is by no means confined to the first molecular layer nor is there any apparent break in the curves after the first layer has been formed. Unfortunately the properties of water in oriented adsorption films have not been sufficiently characterized to enable us to state whether or not this may be the type of water which the biologist is coming to call bound water."
6. Comparing (non-collagen) native proteins with gelatin is not entirely unlike comparing apples and oranges. These non-collagen native proteins are ***native*** proteins. Gelatin is not a native protein; it is collagen heat-denatured in water, acid or alkali. Collagen, itself an insoluble protein, becomes soluble gelatin as a result heat-denaturation. In contrast, non-collagen native proteins are as a rule soluble; but after heat-denaturation they become insoluble—as well exemplified by the cooking of egg white. Indeed, the first definition of protein denaturation characterizes the process by a loss of solubility (Wu, 1931). From this consideration, it is obvious that the true counterparts of gelatin are ***heat-denatured*** non-collagen proteins. But since heat denaturation ***reduces*** the interaction with water, to compare collagen with its native counterparts offers extra assurance that the peculiar amino acid composition of gelatin mentioned above is what underlies the enhanced interaction of gelatin with bulk-phase water.

7. Though published in a journal widely read by protein chemists, my theory of a role of induction in the determination of secondary structure was rarely, if ever, cited, despite its great relevance. I cite this here to demonstrate the progressive fragmentation mentioned earlier and what it does to the progress of science: prevention of meaningful communication across artificial barriers between specialties.
8. Proteins dissolved in water undergo continual back-and-forth conformation changes at extremely high speed (e.g., 10^{-8} sec. for the random-coil ——-helix transitions) (see Ling, 1992, p. 146). If this rapid transition has general validity, the inductively transmitted electronic and conformation changes depicted in the AI hypothesis may then be superimposed on these oscillatory changes—much as radio signals transmitted are superimposed on oscillatory changes or carrier waves at fixed frequencies. The advantage offered by the oscillatory changes in sensitivity, in efficiency and in fidelity of transmitted messages was first understood and introduced by Alexander Graham Bell in his "far speaker", now known as the telephone. The same principle underlies modem distance transmission through wires, air etc. of all sorts. However, if this speculation is not mistaken, then Nature has been making use of the same principle since long long ago in its greatest invention, life.
9. As an illustration of the power of the true believer, the following may be cited. In 1983 the Academic Press published a 765-page volume under the title "Structure, Mechanism and Function of the Na/K Pump". Of the 253 contributors, not one presented evidence challenging or questioning the validity of the Na pump theory, or defending the sodium pump theory against massive published evidence against it. One hundred thirty seven papers were from the United States. Forty-four (44) National Institute Health (NIH) grants, running into millions upon millions of dollars, continued to support the study of the long-defunct Na pump theory. The majority of the 44 grants were approved and funded by the Physiology Study Section of NIH. The same Study Section in 1973, recommended (against the NIH rule) to the NIH that further support for my work should be stopped, citing among other trivial, incompetent and/or self-serving reasons, that my theory was not accepted (at that time) by a majority of scientists. From that time on, the Physiology Study Section and its successor sections had not approved and funded a single research grant from me or from any other scientists whose work had involved the new cell physiology. It was largely due to the courageous intervention of Dr. Stephen Schiaffino, Associate Director of the Division of Research Grants (DRG) of NIH, and of Dr. Arthur Callahan, Program Director of the Office of Naval Research, that I was able to continue my work until they both retired. In 1988, the new DRG director, Dr. Jerome Green, on the basis of a recommendation from a panel of scientists—which he and his subordinates chose against my desperate protest and which was dominated by my scientific opponents (all of whom in their lifetimes never challenged my science publicly)—terminated all my support, forcing the immediate closing of my laboratory at the Pennsylvania Hospital in October, 1988, at the height of its productivity. I was saved from instant scientific demise by my friend, Dr. Raymond Damadian and his MRI manufacturing company, Fonar, so that I have the chance to write this review as well as continue my research today.

References

Abderhalden, E. *(1898) Zeitschr. Physiol. Chem. 25:*65.
Aizenberg, E.I. *(1939) Arkh. anat., gistol. i embriol. 22:*86.
Andreoli, T.E., Tiffenberg, M. and Tosteson, D.C. *(1967) J. Gen. Physiol. 50:*2527.
Anfinson, C.B. *(1967) Harvey Lect. 61:*95.
Adrian, *R.H. (1956) J. Physiol. (London) 133:*631.
Baker, P.E., Hodgkin, A.L. and Shaw, T.I. (1961) *Nature 190:*885.
Bemstein, J. *(1902) Pflugers Arch. ges. Physiol. 92:*521.
Boyle, P.J. and Conway, E.J. *(1941) J. Physiol. (London) 100:*1.
Bradley, R.S. *(1936) J. Chem. Soc. 1936:*1799.
Branch, G.E.K. and Calvin, M. *(1941) The Theory of Organic Chemistry, An Advanced Course,* Prentice Hall, Englewood Cliff, N.J..
Bregman, J.I. *(1953) Ann. N.Y. Acad. Sci. 57:*125.
Brinley, F.J. and Mullins, L.J *(1968) J. Gen. Physiol. 52:*181.
Brunauer, S., Emmett, E.P.H. and Teller, E. *(1938) J. Amer. Chem. Soc. 60:*309.
Buchner, F. *(1897) Ber. Deut. Chem. Ges. 30:*117.
Bungenberg de Jong, H. *(1932) Protoplasma 15:*110.
Cameron, I.L. *(1988) Physiol. Chem. Phys. & Med NMR 20:*221.
Cardon, J.W. and Boyer, P.D. *(1978) Europ. J. Biochem. 92:*443.
Chambers, R. *(1917) J. Exp. Zool. 23:*483.
Chambers, R. and Hale, H.P. *(1932) Proc. Roy. Soc. (London) Ser. B. 110:*336.
Chambers, R. and Hofler, K. *(1931) Protoplasma 12:*338.
Chapman, N.B. and Shorter, J. *(1972) Advances in Linear Free Energy Relationships.* Plenum, New York.
Chiang, M.C. *(1987) The Role of Homologous Linearity of Organic Compounds.* Science Press, Beijing.
Chou, P.Y. and Fasman, G.S. *(1978) Adv. Enzymol. 47:*45.
Clegg, J.S., McClean, V.E.R., Szwarnowski, S. and Sheppard, R.J. *(1984) Phys. Med. Biol. 29:*1409.
Cohn, F. *(1850) Academiae Caesareae Leopoldino-Carolinae [of Halle] 22:*605.
Colacicco, *G. (1965) Nature 207:*936.
Conway, E.J. and Cruess-Callaghan, G. *(1937) Biochem. J. 31:*828.
Damadian, R. *(1971) Science 171:1151.*
deBoer, J.H. and Zwikker, C. *(1929)* Z. *Physik. Chem. B3:*407.
Debye, P. and Pauling, L. *(1925) J. Amer. Chem. Soc. 47:*2129.
Derick, C.G. *(1911) J. Amer. Chem. Soc. 33:*1152.
de Vries, *H. (1871) Arch. Neerl. Sci. 6:*117.
de Vries, H. *(1885) Jahrb. Wiss. Bot. 16:*465.
Disney, A.N. *(1928) Origin and Development of the Microscope,* London.
Dobell, C. *(1932) Antony van Leeuwenhoek and his "Little Animals".* New York, Harcourt, Brace and Co.
du Bois-Reymond, E. *(1848–1849) Untersuchungen uber Thierische Electricitat.* Reimer, Berlin Vol. I and II.
Dujardin, F. *(1835) Ann. d. sciences naturaelles, partie zoologiqw,* 2nd ser. *4.*
Dujardin, F. *(1841) Histoire naturelle des zoophytes: Infusoria,* Paris, *pp. 35–42.*
Dutrochet, R. *(1827) Ann. chim. et physique 35:*393.
Edelmann, L. *(1973) Ann. N.Y. Acad. Sci. 204:*534.
Edelmann, L. *(1977) Physiol. Chem. Phys. 9:*313.
Edelmann, L. *(1984) Scanning Electron Microscopy 11:*875.
Edelmann, L. *(1986)* In: *Science of Biological Specimen Preparation,* (M. Muller, R.P. Becker, A. Boyde, and J.J. Wolosewick, eds). SEM Inc., Chicago, Ill., p. *33.*

Edelmann, L. (1988) *Scanning Microscopy* 2: 1.
Edelmann, L. (1989) *Scanning Microscopy 3:*1219.
Edelmann, *L.* (1991) *Scanning Microscopy Suppl. 5:*S75.
Eggleton, M. and Eggleton, P. (1927) J. *Biol. Chem. 21:*190.
Englemann, T.W. (1873) *Pflugers Arch. Ges. Physiol. 7:*155.
Ernst, E. (1963) *Biophysics of the Striated Muscle.* Publishing House of the Hungarian Academy of Sciences, Budapest.
Findlay, A. (1919) *Osmotic Pressure.* Longman and Green, London, 2nd ed.
Fiske, C.H. and Subbarow, Y. (1925) J. *Biol. Chem. 66:*375.
Fletcher, W. M. and Hopkins, F.G. (1907) J. *Physiol. (London)35:*247.
Fischer, M.H. (1909) *Oedema,* Trans. College Physicians, Philadelphia 3rd ser. 31:457.
Fischer M.H. and Moore, G. (1907) *Amer.* J. *Physiol. 20:*330.
Fischer, M.M. and Suer, W. J. (1938) *Arch. Pathol. 26:*51.
Fleischer, S., Fleischer, B. and Stoekenius, W. (1967) J. *Cell Biol. 32:*193.
Florkin, M. (1960) *Naissance et deviation de la theorie cellulaire dan l'oeuvre de Theodore Schwann.* Paris, pp. 55-65.
Gary-Bobo, C.M. and Lindenberg, A.B. (1969) J. *Coll. Interf. Sci. 29:*702.
Galvani, Luigi (1953) *Commentary on the Effects of Electricity on Muscular Motion.* (M.G. Foley, trans.), Norwalk, Connecticut.
Gamier, J., Osguthrope, D.J. and Robson, B. (1978) J. *Mol. Biol. 120:*97.
George, P. and Rutman, R.J. (1960) *Prog. Biophys. Chem. 10:*1.
Glasstone, S. (1946) *Textbook of Physical Chemistry.* 2nd *Ed.,* Van Nostrand, New York.
Glynn, LM. and Karlish, S.J.D. (1975) *Ann. Rev. Physiol. 37:*13.
Goody, R.S., Hofmann, W. and Mannerz, H.G. (1975) *Europ.* J. *Biochem. 78:*317.
Gortner, R.A. (1930) *Trans. Faraday Soc. 26:*678.
Gortner, R.A. (1938) *Outlines of Biochemistry.* John Wiley & Sons, New York.
Graham, J. and Gerard, R.W. (1946) J. *Cell. Comp. Physiol. 28:*99.
Graham, T. (1861) *Philos. Trans. Roy. Soc. (London)*151:183.
Gulati, J., Ochsenfeld, M.M. and Ling, G.N. (1971) *Biophys.* J. *11:*973.
Gupta, B.L. (1989) *Symp. Soc. Exp. Biol. 43:*81.
Hall, T.S. (1951) *A Source Book in Animal Biology.* New York.
Hall, T.S. (1969) *Ideas of Life and Matter.* Univ. of Chicago Press, Chicago.
Hamburger, H.J. (1887) *Archiv. Anatom. und Physiol.* S41.
Hamburger, H.J. (1891) Z. *Biol. 28:*405.
Hamburger, H. (1904) *Osmotische Druck und lonenlehre.* Bergmann, Wiesbaden, Vol. 3.
Hammett, L.P. (1940) *Physical Organic Chemistry.* McGraw-Hill, New York.
Harkins, W.D. (1945) *Science 102:*292.
Harvey, E.N. and Danielli, J.F. (1939) *Usp. Souvrem. Biol. 10:*471.
Heppel, L.A. (1939) *Amer.* J. *Physiol. 127:*385.
Heidorn, D.B., Rorschach, H.E., Hazlewood, C.F., Ling, G.N. and Nicklow, R.M. (1986) *Biophys. J. 49:*92A.
Hill, A.V. (1930) *Proc. Roy. Soc. (London) Ser. B. 106:*477.
Hill, A.V. (1932) *Physiol. Rev. 12:*56.
Hodgkin, A.L. (1951) *Biol. Rev. 26:*239.
Hodgkin, A.L. and Horowicz, P. (1959) J. *Physiol. (London)148:*127.
Hodgkin, A.L. and Huxley, A.F. (1945) J. *Physiol. (London)104:*176.
Hodgkin, A.L. and Katz, B. (1949) J. *Physiol. (London)108:*37.
Hodgkin, A.L. and Katz, B. (1949a) J. *Physiol. (London)109:*240.
Hofler, K. (1918) *Ber. Dtsch. Bot. Ges. 36:*414.
Hollemann, L., Bungenberg de Jong, H.G. and Moddermann, R.T. (1934) *Koll. Beih. 39:*334.
Hoover, S.R. and Mellon, E.F. (1950) *J. Amer. Chem. Soc. 72:*2562.
Hunter, O.F. and Padsha, S.M. (1959) J. *Physiol. (London)146:*117.

Hunter, F.R. and Parpart, A.K. (1938) J. *Cell. Comp. Physiol. 12:*309.
Huxley, T.H. (1853) *The British and Foreign Medico-Chirurgical Review. 12:*285.
Infante, A.A. and Davies, R.C. (1962) *Biochem. Biophys. Research Commun. 9:*410.
Ingold, C.K. (1953) *Structure and Mechanisms in Organic Chemistry.* Cornell Univ. Press, Ithaca, N.Y.
Jones, A.W. (1965) *The Water and Electrolytes Metabolism of the Arterial Wall,* Ph.D. Thesis, University of Pennsylvania, Philadelphia.
Jordan-Lloyd, D. and Moran, T. (1934) *Proc. Roy. Soc. Ser. A 147:*382.
Kaatze, A., Goettmann, O., Rodbielski, R., Pottel, R. and Terveer, U. (1978) J. *Phys. Chem. 82:*112.
Kaplanskii, S. Ya. and Boldyreva, N. (1934) *Fiziol. Zh. 17:*96.
Katz, *B.* (1966) *Nerve, Muscle and Synapse.* McGraw-Hill, New York.
Katz, J. (1896) *Pflugers Arch. ges. Physiol. 63:*1.
Kite, G.L. (1913) *Amer.* J. *Physiol. 32:*146.
Kuhne, W. (1864) *Untersuchungen uber das Protoplasm und die Contractilitat.* Leipzig, pp. 1–158.
Leaf, A. (1956) *Biochem. J. 62:*241.
Lepeschkin, W.W. (1930) *Protoplasma 9:*269.
Lepeschkin, W.W. (1939) *Protoplasma 33:*1.
Lewis, G.N. (1923) *Valence and the Structure of Atoms and Molecules.* Chemical Catalogue Co., New York.
Leydig, F. (1857) *Lehrbuch der Histologie des Menschen and der Tiere.* Frankfurt, pp. 14–15.
L'Hermite, M. (1855) *Annales chim. phys. 43:*420.
Liebig, *J.* (1862) (see von Liebig, J.).
Ling, G.N. (1951) *Amer.* J. *Physiol. 167:*806.
Ling, G.N. (1952) *Phosphorus Metabolism.* (W.D. McElroy and B. Glass, eds). Johns Hopkins University Press, Baltimore, MD.
Ling, G.N. (1953) *Proc. 19th Intern. Physiol. Congr.,* Montreal, Canada, p. 566.
Ling, G.N. (1955) *Amer. J. Phys. Med. 34:*89.
Ling, G.N. (1960) J. *Gen. Physiol. 43:*149.
Ling, G.N. (1962) *A Physical Theory of the Living State:* the Association-Induction Hypothesis, Blaisdell, Waltham, Mass.
Ling, G.N. (1964) J. *Biopolymers 1:*91.
Ling, G.N. (1965) *Persp. Biol. Med. 9:*87, footnote on p. 95.
Ling, G.N. (1965a) *Ann. N.Y. Acad. Sci. 125:*401.
Ling, G.N. (1970) *Intern.* J. *Neurosci. 1:*129.
Ling, G.N. (1972) In: *Water and Aqueous Solutions, Structure, Thermodynamics and Transport Processes* (A. Home, ed.). Wiley-Interscience, New York, pp. 663–699.
Ling, G.N. (1973) *Physiol. Chem. Phys. 5:*295.
Ling, G.N. (1977a) *Physiol. Chem. Phys. 9:*217.
Ling, G.N. (1977b) *Physiol. Chem. Phys. 9:*319.
Ling, G.N. (1978) *Bioelectric. and Bioengineer. 5:*411.
Ling, G.N. (1980) In: *Cooperative Phenomena in Biology* (G. Karreman, ed.). Pergamon Press, New York, pp. 39-69.
Ling, G.N. (1980a) *Physiol. Chem. Phys. 12:*383.
Ling, G.N. (1981) *Physiol. Chem. Phys. 13:*29.
Ling, G.N. (1983) *Physiol. Chem. Phys. & Med NMR 15:*155.
Ling, G.N. (1984) In: *Search of the Physical Basis of Life.* Plenum, New York.
Ling, G.N. (1986) *Physiol. Chem. Phys. & Med. NMR 18:*3.
Ling, G.N. (1987) *Physiol. Chem. Phys. & Med. NMR 19:*199.
Ling, G.N. (1987a) *Physiol. Chem. Phys. & Med. NMR 19:*159.
Ling, G.N. (1988) *Scanning Microscopy 2:*899.

Ling, G.N. (1989) *Physiol. Chem. Phys. & Med. NMR 21:*13.
Ling, G.N. (1990) *Scanning Microscopy 4:*723.
Ling, G.N. (1990a) *Scanning Microscopy 4:*737.
Ling, G.N. (1992) *A Revolution in the Physiology of the Living Cell.* Kreiger, Melbourne, Fl.
Ling, G.N. (1992a) *Scanning Microscopy 6:*405.
Ling, G.N. (1993) *Physiol. Chem. Phys. & Med. NMR 25:*145.
Ling, G.N. and Bohr, G. (1969) *Physiol. Chem. Phys. 1:*591.
Ling, G.N. and Bohr, G. (1971) *Physiol. Chem. Phys. & Med. NMR 3:*431.
Ling, G.N. and Gerard, R.W. (1949) J. *Cell. Comp. Physiol. 34:*383.
Ling, G.N. and Hu, W. (1987) *Physiol. Chem. Phys. & Med. NMR 19:*251.
Ling, G.N. and Hu, W. (1988) *Physiol. Chem. Phys. & Med. NMR 15:*293.
Ling, G.N. and Kromash, M.H. (1967) J. *Gen. Physiol. 50:*677.
Ling, G.N. and Murphy, R. (1983) *Physiol. Chem. Phys. & Med. NMR 15:*137.
Ling, G.N. and Negendank, W. (1970) *Physiol. Chem. Phys. 2:*15.
Ling, G.N. and Negendank, W. (1980) *Persp. Biol. Med. 23:*215.
Ling, G.N. and Ochsenfeld, M.M. (1965) *Biophys.* J. *5:*777.
Ling, G.N. and Ochsenfeld, M.M. (1966) J. *Gen. Physiol. 49:*819.
Ling, G.N. and Ochsenfeld, M.M. (1973) *Ann. N.Y. Acad. Sci. 204:*325.
Ling, G.N. and Ochsenfeld, M.M. (1986) *Physiol. Chem. Phys. & Med. NMR 18:*109.
Ling, G.N. and Ochsenfled, M.M. (1987) *Physiol. Chem. Phys. & Med. NMR 19:*177.
Ling, G.N. and Ochsenfeld, M.M. (1989) *Physiol. Chem. Phys. & Med. NMR 21:*19.
Ling, G.N. and Ochsenfeld, M.M. (1991) *Physiol. Chem. Phys. & Med. NMR 23:*133.
Ling, G.N. and Sobel, A.M. (1975) *Physiol. Chem. Phys. 7:*415.
Ling, G.N. and Tucker, M. (1980) J. *Nat. Cancer Inst. 64:*1199.
Ling, G.N. and Walton, C.L. (1975) *Physiol. Chem. Phys. 7:*215.
Ling, G.N. and Walton, C.L. (1976) *Science 191:*293.
Ling, G.N. and Zhang, Z.L. (1983) *Physiol. Chem. Phys. & Med. NMR 15:*391.
Ling, G.N. and Zhang, Z.L. (1984) *Physiol. Chem. Phys. & Med. NMR 16:*221.
Ling, G.N., Ochsenfeld, M.M., Walton, C. and Bersinger, T.J. (1978) *Physiol Chem. Phys. 10:*87.
Ling, G.N., Ochsenfeld, M.M., Walton, C. and Bersinger, T.J. (1980) *Physiol. Chem. Phys. 12:*3.
Ling, G.N., Niu, Z.L. and Ochsenfeld, M.M. (1993) *Physiol. Chem. Phys. & Med. NMR 25:*177.
Lipmann, *F.* (1941) *Adv. Enzymol. 1:*99.
Lohmann, *K.* (1935) *Biochem.* Z. *282:*120.
Lorkovic, H. and Tomnek, R.J. (1977) *Amer.* J. *Physiol. 232:*C109.
Lowey, S. and Luck, S.M. (1969) *Biochem. 8:*3195.
Lucke, B. and McCutcheon, M. (1932) *Physiol. Rev. 12:*68.
MacDonald, J.S. (1900) *Proc. Roy. Soc. London 67:*310.
MacLeod, J. and Ponder, E. (1935) J. *Physiol. (London) 86:*147.
Maloff, B.L., Scordillis, S.P., Reynolds, C. and Tedeschi, H. (1978) J. *Cell. Biol. 78:*199.
Marsh, D.J., De Bruin, S.H. and Gratzer, W.B. (1977) *Biochemistry 16:*1738.
Marston, S.D., Tregear, R.T., Rodger, C.D. and Clarke, M.L. (1979) J. *Mol. Biol.* 128:111.
Matteucci, C. (1842) *C.R. Acad. Sci. (Paris) 4:*797.
McMahon, B.C., Hartung, E.J. and Walban, W.J. (1940) *Trans. Farad. Soc. 36:*515.
Minkoff, L. and Darnadian, R. (1973) *Biophys. J. 13:*167.
Miller, C. and Ling, G.N. (1970) *Physiol. Chem. Phys. 2:*495.
Miyarnoto, *V.K.* and Thompson, T.E. (1967) J. *Coll. Interf. Sci. 25:*16.
Moore, B. and Roaf, H.E. (1908) *Biochem.* J. *3:*55.
Moore, B., Roaf, H.E. and Webster, I. (1912) *Biochem.* J *6:*110.
Moran, T. (1926) *Proc. Roy. Soc. London, Ser. A. 112:*30.
Morgan, A. (1954) *A First Electrical Book for Boys.* Charles Scribner's Sons, New York.
Morowitz, H.J. and Terry, T.M. (1969) *Biochim. Biophys. Acta 183:*276.
Mullins, L.J. and Brinley, F.J. (1969) J. *Gen. Physiol. 53:*704.

Muller, P. **(1975)** *Ann.* N.Y. *Acad. Sci. 264:*97.
Muller, P., Rudin, D.O., Tien, T.M. and Wescott, W.C. **(1962)** *Nature 194:*979.
Nasonov, D.N. **(1938)** *Arkh. anat. gistol. i embriol. 19:*116.
Nasonov, D.N. **(1959)** *"Local reaction of protoplasm and gradual excitation".* Transl. by Y.S. Halpern. Available from the Office of Technical Services. U.S. Department of Commerce, Washington **25,** D.C.
Nasonov, D.N. and Aizenberg, E.I. **(1937)** *Biol. Zh. 6:*165.
Nathansohn, A. **(1904)** *Jahrb. Wiss. Bot. 39:*607.
Newton, R. and Gortner, R.A. **(1922)** *Bot. Gaz. 74:*442.
Newton, R. and Martin, W. McK. **(1930)** *Canad. J. Research 3:*336.
Oikawa, *T.*, Spyropoulos, C.S., Tasaki, I. and Teorell, I. (1961) *Acta Physiol. Scand. 52:*195.
Ostwald, Wilhelm **(1890)** *Z. Physik. Chem. 6:*71.
Ostwald, Wolfgang **(1940)** J. *Med. 20:*436.
Overton, E. (1895) *Vieterljahrschr. Naturf. Ges. (Zurich)40:*159.
Peachey, L.D. **(1965)** *J. Cell Biol. 25:*209.
Perutz, M. **(1970)** *Nature 228:*726.
Pfeffer, W. **(1877)** *Osmotische Untersuchungen,* Engelmann, Leipzig, p. **236.**
Piez, K.A., Weiss, E. and Lewis, M.S. **(1960)** *J. Biol. Chem. 235:*1987.
Podolsky, R.J. and Kitzinger, C. **(1955)** *Fed. Proc. 14:*115.
Podolsky, R.J. and Morales, M.F. **(1956)** *J. Biol. Chem. 218:*945.
Ponder, E. **(1948)** *Hemolysis and Related Phenomena.* Grune and Stratton, New York.
Rapatz, G. and Luyet, B. **(1959)** *Biodynamics 8:*121.
Remak, R. **(1852)** *Muller's Archiv f. Anat. Physiol. u. Wiss. Med. (Berlin),*p. **49.**
Rich, H.R. **(1926)** *Bull. Johns Hopkins Hospital 39:*330
Robertson, J.D. **(1960)** *Prog. Biophys. Biophys. Chem. 10:*343.
Robinson, *W.* **(1931)** *J. Biol. Chem. 92:*699.
Rorschach, H.E. **(1984)** In: *Water and Ions in Biological Systems.* (V. Vasilescu, ed). Plenum, New York.
Rothschuh, K.E. **(1973)** *"History of Physiology".* Kreiger, Melbourne, Fl.
Rubner, M. **(1922)** *Abh. preuss Akad. Wiss.,* Phys.-Math. Klasse, p. **3.**
Ruhland, W. **(1908)** *Ber. Dtsch. Bot. Ges. 26:*772.
Schatzmann, H.J. **(1953)** *Helv. Physiol. Pharmacol. Acta 11:*346.
Scheraga, H. **(1974)** *Current Topics Biochemistry 1973:*1.
Schrodinger, E. **(1945)** *"What is Life?"* Macmillan, New York.
Schultze, Max **(1861)** *Miiller's Archiv f. Anat. u. Physiol. u. Wiss. Med. (Berlin),*p. *1.*
Switz, P.K., Hazlewood, C.F. and Clegg, J.S. **(1980)** *The Brine Shrimp, Artemia. (G.* Persoone, P. Surgeloos, O. Roels, and E. Jaspers, eds). Universa Press, Wettem, Belgium, Vol. **2,** p. **545.**
Singer, C. **(1915)** The Dawn of Microscopic Discovery. *J. Roy. Microsc. Soc.,* pp. **317–340.**
Sjöstrand, F.S. and Bernhard, W. **(1976)** *J. Ultrastruc. Res. 64:*217.
Speakman, J.B. and Hirst, M.C. **(1931)** *Nature 127:*665.
Steinbach, B. **(1940)** *J. Biol. Chem. 133:*695.
Stillman, I.M., Gilbert, D.L. and Robbins, M. **(1970)** *Biochim. Biophys. Acta 203:*338.
Tanaka, S. and Scheraga, H.A. **(1976)** *Macromol. 9:*168.
Thoenes, F. **(1925)** *Biochem.* Z. *1657:*175.
Tinker, F. **(1916)** *Proc: Roy. Soc. 92A:*357, p. **362.**
Trantham, E.C., Rorschach, H.E., Clegg, J.C., Hazlewood, C.F., Nicklow, R.M. and Wakabayashi, N. **(1984)** *Biophys.* J. *45:*927.
Traube, M. **(1867)** *Arch. (Anat. u) Physiol.* **87–165.**
Tristram, G.R. and Smith, R.H. **(1963)** *Adv. Protein Chem. 18:*227.
Trombitas, C. and Tigyi-Sebes, A. **(1979)** *Acta Physiol. Scad. Sci. Hung. 14:*271.
Troshin, A.S. **(1951)** *Byull. Eksp. Biol. i Med. 31:*285.
Troshin, A.S. **(1952)** *Byull. Eksp. Biol. i Med. 34:*59.

Troshin, A.S. (1958) *Das Problem der Zellpermeabilitat*. Gustav Fischer, Jena
Troshin, A.S. (1966) ***Problem of Cell Permeability.*** Pergamon, New York.
van't Hoff, **J.H.** (1887) Z. ***Physik. Chem. 1:***481.
von Liebig, J. (1862) ***Annalen*** 121:78.
von Mohl, H. (1844) ***Botan. Zeit***. April 12, **19, 26;** May 3, 10.
von Nageli, C. (1885) ***"Primordial Schlauch" in "Pflanzen Studien",*** Heft *I*, pp. 1–20, Zurich.
von Zglinicki, T. (1988) ***Gen. Physiol. Biophys. 7:***495.
Warburg, ***O.*** (1930) ***The Metabolism of Tumors.*** (F. Dickens, trans.). Constable, London.
Wilson, E.B. (1928) ***The Cell in Development and Heredity.*** McMillan, New York, 3rd ed.
Wilson, **T.H.** (1954) ***Science 120:***104.
Wu, H. (1931) ***Chinese J. Physiol. 5:***321.
Wu, H. and Yang, E.F. (1931) ***Proc. Soc. Exp. Biol. Med. 29:***248.