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History of the Membrane (Pump) Theory of the Living Cell from Its Beginning in Mid-19th Century to Its Disproof 45 Years Ago — though Still Taught Worldwide Today as Established Truth

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Abstract: The concept that the basic unit of all life, the cell, is a membrane-enclosed soup of (free) water, (free) K^+ (and native) proteins is called the membrane theory. A careful examination of past records shows that this theory has no author in the true sense of the word. Rather, it grew mostly out of some mistaken ideas made by Theodor Schwann in his Cell Theory. (This is not to deny that there is a membrane theory with an authentic author but this authored membrane theory came later and is much more narrowly focussed and accordingly can at best be regarded as an offshoot of the broader and older membrane theory without an author.)

However, there is no ambiguity on the *demise* of the membrane theory, which occurred more than 60 years ago, when a flood of converging evidence showed that the asymmetrical distribution of K^+ and Na^+ observed in virtually all living cells is not the result of the presence of a membrane barrier that permits some solutes like water and K^+ to move in and out of the cell, while barring — absolutely and permanently — the passage of other solutes like Na^+.

To keep the membrane theory afloat, submicroscopic pumps were installed across the cell membrane to maintain, for example, the level of Na^+ in the cell low and the level of K^+ high by the ceaseless pumping activities at the expense of metabolic energy. Forty-five year ago this version of the membrane theory was also experimentally disproved. In spite of all these overwhelming evidence against the membrane-pump theory, it still is being taught as verified truth in all high-school and biology textbooks known to us today.

Meanwhile, almost unnoticed, a new unifying theory of the living cell, called the association-induction hypothesis came into being some 40 years ago. Also little noticed was the fact that it has

received extensive confirmation worldwide and has shown an ability to provide self-consistent interpretations of most if not all known experimental observations that are contradicting the membrane-pump theory as well as other observations that seem to support the membrane pump theory.

KEY WORDS: membrane theory, membrane-pump theory, cells, cell biology, cell membrane, membrane permeability, semi-permeability, osmosis, osmotic pressure, potassium ion, sodium ion, cell water, cell sap, association-induction hypothesis, plasmolysis, deplasmolysis, cell volume change, protozoa, foraminifera, protoplasm, sieve membrane theory, mosaic membrane theory, nucleus, nucleolus

THE DUTCH spectacle-maker, Zacharias Jansen invented the compound microscope in the year 1590 (Disney *et al* 1928.) Nineteen years later, Galileo Galilei of Italy independently did the same. For a long time afterward, the invention was more a toy than a scientific instrument. It was often used to peer at the outer anatomy of small living creatures including mites in cheese, ants, bees and, allegedly, also animals shaped like whales and dolphins in the human blood (Singer 1915.)

However, a new chapter in the use of the compound microscope opened with the arrival of the physicist-natural philosopher, Robert Hooke (1635–1703.) Though handicapped by a crippling illness in his childhood; he, nevertheless, earned the reputation as "a Person of a prodigious inventive Head, so of great Virtue and Goodness" (Hall 1969, Vol. 1, p. 295.) That is not to mention his equally prodigious skill in making and improving mechanical devices, including the compound microscope (Disney *et al* 1928, pp. 112–114.)

Armed with the improved compound microscope, Hooke began to explore with persistence and accuracy into the world of the small. One of the subjects of his early studies was a thin sheet of cork, which looked under his microscope like a bee comb of tiny walled cavities (Figure 1). For them, he gave — in his book, *Micrographia* (Hooke 1665) — the Latin name, *cellula*— the English equivalent of *cell*.

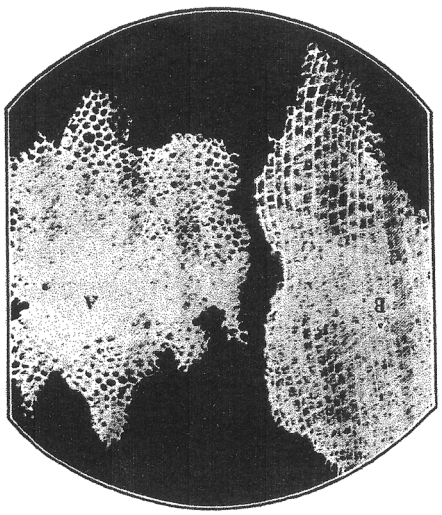


FIGURE 1. The earliest known picture of what Robert Hooke called cells in thin sections of cork from his *Micrographia.*

Subsequently, Hook also used the same name, *cellula*, to describe cross sections of the vascular bundles (of fresh Fennel, Carrats, Teasels, Fearn etc.,) which were often filled with fluid. Here too, Hooke correctly believed that they were cross sections of narrow longitudinal pipes that transport water and nutrients between different parts of the plant.

> It is truly to the regret of concerned Mankind that no likeness of Robert Hooke survived. In part at least this came from the mean-spiritedness of a rival, Sir Isaac Newton, who had instituted the removal of a portrait of Hooke at the Royal Society ("Robert Hooke" wikipedia; Manuel 1968, p. 136.)

Six years after the publication of Hooke's *Micrographi,* the Royal Society of London received two important manuscripts. One was from the British botanist, Nehemiah Grew (1641–1712) (Grew 1672, 1682); the other from the Italian anatomist, Marcello Malpighi (1628–1694) (Malpighi 1675, 1686.) Independently, both described the micro-anatomy of plants (and in the case of Malpighi, that of animals as well.) Both saw in the plants a mass of "bubbles"; both believed that these bubbles were filled with air. However, from here on, the two pioneer investigators saw things differently.

Following Hooke, Grew called the individual bubbles *cells*. Later, he compared the "mass of bubbles" with *lace,* the name of textile fabric of threads or fibers. Historian-biologist, John R. Baker pointed out that this misleading comparison brought in another widely-adopted misleading name, *tissue* — which means a woven fabric (Baker 1952, p. 158.) Furthermore, Grew thought that the holes in the fabric of the fibers comprise the cavities of the cells. Thus, in Grew's view, not only are all the fibers continuous with one another, the cavities among the fibers are also continuous with one another.

In contrast, Malpighi believed that each of these bubbles is a closed unit and referred to them as *utriculi (*utricles*) or saccula (*sacs*)* (Malpighi 1675, see also Baker 1952, p. 160.)

During the 30 years following, these two divergent views co-existed. That is, until the early 19th century, when the combined efforts of half a dozen micro-anatomists resolved the conflict in favor of (Malpighi's) *vesicular theory*. We shall return to this subject after a short visit to the world of animals cells.

The discovery of the animal cell and what was to be called the cell nucleus

The first animal cell discovered through the microscope is the red blood cell. On that, we are certain. However, by whom is a question that may never have an exact answer — though it must be one of the three: Jan Swammerdam (1637-1680), Antoni van Leeuwenhoek (1632–1763) and Marcello Malpighi, the last already mentioned above. The time of this discovery was 1673 or earlier (see Baker 1948, p. 110, also Harris 1999, p. 15.)

The first animal cell discovered also led to the discovery of the cell nucleus. Thus, in a letter dated March 3, 1682 to Robert Hooke, Leeuwenhoek showed a drawing of a red blood cell from a fish. This drawing contains what we would now unhesitatingly call a nucleus (see Harris 1999, p. 76) — even though the word, *nucleus*, was not to be introduced until 1831, when Robert Brown coined that name (Brown 1833.) Shortly before, the same Robert Brown also described the random motion of suspended particles that was to be named after him — the *Brownian motion* (Brown 1828.)

Four years after the introduction of the name, nucleus, Rudolf Wagner (1805–1864) gave an accurate description of a still smaller structure he saw inside the nucleus, the *nucleolus* (Wagner 1835.)

Leeuwenhoek's discovery of the cell nucleus was nowhere nearly as well known as his earlier discovery of bacteria, which he called *animacules* — first made public in 1676 in a letter to the Royal Society of London dated October 9 of that year (see Dobell 1932, Leeuwenhock letter No. 16.) Leeuwenhock made this discovery in a water infusion of hot pepper — when he was looking for the causal agent that made pepper hot to taste. However, the simple and yet powerful microscopes that enabled him to see these minute *animacules* call for a short comment.

As pointed out above, Robert Hooke improved and used what is known as a compound microscope, a tubular structure with two lenses, i.e., one objective lens and one ocular lens. In contrast, Leeuwenhoek's microscope is not a compound microscope at all. Rather, Leeuwenhoek's microscope was a single glass globule he made himself. According to John Harris, both Leeuwenhock and Schwammerdam learnt to grind these lens from a highly talented master, Johannes Huddle (1628–1704), who beside being an advanced mathematician, was the Mayor of Amsterdam. As if that was not an intriguing enough accomplishment for one single person, Huddle also taught lens grinding to the excommunicated great Jewish philosopher, Spinoza {Benedict (Baruch) de Spinosa} and thus, perhaps, giving him a means of paying his rents. Sadly, inhaling the glass powder might also have exacerbated his already poor health. He died of consumption at the age of 45 (1632–1677) (Scruton 1986.)

Leeuwenhoek bequeathed most of his one-lens microscopes to the Royal Microscopic Society. Of the 26 microscopes examined the best one had an astonishing magnifying power of 160 (Disney *et al* 1928, pp. 160–161.) We know that the best light microscope today has a magnifying power of about 1000 but only with an oil-immersion lens. Without oil immersion, the best magnifying power is usually about 400.

The collective evidence establishing that plant cells are independent entities

A strong and vigorous supporter of Grew's concept that both the plant cavities and fibers are continuous was the French botanist, Brissseau de Mirbel (1776–1854) (see Baker 1952, p. 160.) However, other investigators reached the opposite conclusion.

G. R. Treviranus demonstrated in thin sections of the buds of buttercup plants (Ranuculus species) that the separating walls are double (Treviranus 1805.) This finding strongly suggests that what are called cells are stand-apart units rather than cross sections of continuous channels as Grew and Brisseau de Mirbel believed.

Following G. R. Treviranus's footsteps, four other investigators, H.F. Link (Link 1807), L. C. Treviranus (Treviranus 1811), J.J.P. Moldenhawer (Moldenhawer 1812) and H. Dutrochet (1837) reached the same conclusion that cells are not continuous but independent of one another.

Charles François Brisseau-Mirbel
(1776–1854)

Finally, Brisseau de Mirbel publicly admitted his earlier error in these words. "*Today when I have obtained the most direct proof of the utricular composition of the tissue, I understand and I see the spaces, which I neither understood nor saw before, and I retract my objections to the fine discoveries of M. Tréviranus*." (Mirbel 1835.)

> Historian, John R. Baker described this passage as *magnanimous*. I completely agree. For Mirbel's honesty, courage and self-denial demonstrated in this public admission of his prior error and his generous and kindly compliments on his one-time opponent embodies the inner spirit of a scientist at his or her best.

With the exception of mature plant cells, the boundaries between most living cells are hard to see even with the finest of light microscopes. In contrast, the cell nucleus is much more visible. It was thus a brilliant postulation of botanist, Mathias Schleiden (1804–1881) that each plant cell possesses a nucleus for this postulation offered a basis for regarding the presence of a nucleus as evidence for the existence of a cell. Indeed, without this postulation, it would be much harder to make the case that all plants are made up of individual cells — as Schleiden did in the article he published in 1838 (Schleiden 1838.)

Mathias Schleiden
(1804–1881)

Although Schleiden did *not* mention or discuss a "Cell Theory" as such, nor did he study animal cells, he and Theodor Schwann have often been described as the co-founders of the Cell Theory for both plants and animals.

However, there are reasons to view this exclusive assignment of credit with reserve. Schwann published his Cell Theory in 1839 (Schwann 1839.) There were at least four other scientists who had made the same discovery *before* both Schleiden and Schwann: Lorenz Oken (1805, Singer 1959); Henri Dutrochet (1824); Purkinje (1834 or earlier, see Harris 1999, pp. 85–87, see also Studnička 1927); Valentin (1834, see Valentin 1836, 1839.) I shall return to the subject below.

Lastly, it should also not be overlooked that Schleiden's convenient one cell-one nucleus postulation is, strictly speaking, incorrect. Slime mold and inter-nodal cells of freshwater giant algae, Chara contain many nuclei (see Baker 1952, p. 177.) The mature mammalian red blood cell contains no nucleus at all (Lehmann and Huntsman 1961, see their Figure 29(a) on page 119.)

Johannes Müller's Institute of Physiology in the University of Berlin

It is true that Theodor Schwann (1810–1882) and Mathias Schleiden together are widely accredited as the authors of the Cell Theory. according to which all animals and plants are made of similar basic units, called the cells. However, Schleiden did not make this claim because he was a botanist and did not study animal cells. On the other hand, Schwann did study animal cells, wrote on the Cell Theory and is widely acclaimed as the originator of this theory. Yet, Schwann achieved all this in exactly five years between 1834, when he came to Berlin, to 1839, when he left Berlin (Harris 1999, p. 98.) Equally astonishing was

the historical fact: once he left Berlin, he never gave a backward glance at his Cell Theory or defended it against gathering evidence challenging its validity (see below.)

How could a single man gain such historic, landmark fame in such a short length of time and why did he abandon the Theory that gave him so much? In trying to answer these questions, we take a broader look at the extraordinary environment where Schwann did that five year's work.

Between 1830 and 1850, new physiological laboratories were springing up in universities all over Germany like mushrooms in a rain-permeated forest. This unusual undertaking was a part of a broad movement in the founding of state-financed semi-independent institutes in German universities in the 19th Century. Its overall purpose was nothing else than the promotion and nurturing of the searching for truth — labeled *Wiesenschaften* or *pure science* (McClelland 1980, Part III.)

The creation and continued support of these institutions of unshackled search for truth wrote an unparalleled chapter in the history of Mankind. And, to no small measure was this made possible by the insight, dedication and pervasive honesty of the Prussian and German bureaucrats (Kirchner 1958, p. 97, 164.)

One of the most famous of the German physiological institutes was the Institute of Physiology of Johannes Müller. It was one of the first of its kind (see p. 12 below, line 21 from bottom) and it was located in the University of Berlin, the *créme de la créme* of all German universities.

Johannes Müller was born in Koblenz in 1801. A brilliant student, good in both languages and mathematics, he completed his doctor's thesis when he was only 21. At the age of 32, he became the chairman of the physiological institute just mentioned. Hard working, well informed and strong in his convictions, Müller was also broad-minded and tolerant of different views. It was thus for good reasons — personally and statewide — that he had gathered around him a large number of the brightest among the young generation (Rothschuh 1973, p. 310.)

Nothing could demonstrate Müller's broadmindedness better than the variety of students he taught. Thus, he was at once the teacher of Herman von Helmholtz (1821–1894), Emil DuBois-Reymond (1818–1896) and Ernst von Brücke (1819–1892), three of the *Reductionist Four* — who believed that the laws governing the inanimate world govern the living as well — and the teacher of Theordor Schwann, who did not allow his Magnus Opus, *Mikroscopische Untersuchungen* to be published before obtaining the approval of (Archbishop of Malines of the) Catholic Church (Harris 1999, p. 101.)

Müller was himself a vitalist. That is, he believed that the basic mechanism of life, or *cause vitae*, couldn't be explained by the laws that govern the inanimate universe. Thus, Emil DuBois-Reymond recalled how he tried in vain to convince his teacher of the rigorous validity of physical laws in living organisms (Rothschuh 1973, p. 199.)

It was also not hard to imagine that Müller was closer to Schwann, with whom he not only shared a small room or Kabinet but also the only available advanced Plössl microscope (Rothschuh 1973, p. 201, Harris 1999, p.85.)

But in the exciting atmosphere that physiological studies enjoyed, many students nationwide have to be taught. For that, new textbooks and new journals, in which to publish are in high demand. To fill those needs, Müller had on the one hand, his famous *Handbuch der Physiologie* and for the other, his equally famous journal *Müller's Archiv für Anatomie und Physiologie und für wissenschftliche Medizin*. Armed with all these potent

weapons, Müller had no trouble in promoting Schwann's book *Mikroscopische Untersuchungen*, in which Schwann promulgated his Cell Theory to its astonishing popularity and unmitigated adoption by the German textbooks (see Harris 1999, p. 106.) However, that was what one sees on the surface. I shall return to what lay deeper below the unquestioning acceptance of what Schwann wrote in his *Mikroskopische Utersuchungen*.

As made clear in earlier passages, an important part of the membrane (pump) theory is the presence around each living cell of an enclosing membrane. True, this concept had already been given by Henri Dutrochet earlier (see below) but Durtrochet made no claim for having *seen* the cell membrane. To Dutrochet, it was just a theoretical concept, an extrapolation from familiar, macroscopic membranes like pig's bladder, intestinal loops etc.

Theodor Schwann
(1810–1882)

In contrast, the very title of Schwann's magnus opus, *Microscopical Researches into the Accordance in the Structure and Growth of Animal and Plants* (English translation of Henry Smith) implies that what he reported was from what he actually saw under the microscope. And, he said that he saw the cells and described them as *membrane-enclosed cavities.* Furthermore, this description was not only applied to the cell but also to its enclosure, the nucleus and the nucleus's enclosure, the nucleolus. So the three are like a set of Chinese boxes: the smallest nucleolar box sits inside the small nucleus box and the nucleus box sits inside the largest cell box. The spaces between these boxes are filled with fluid and this fluid constitutes the cell content (Schwann 1839, p. 177.) But that is not all. He had much more to add.

On page 175 of the original German text, Schwann wrote: "Nach Schleiden liegt er bei Pflanzen zuweilen in der dicke der Zellmembran, so dass er auch auf seiner inneren, gegen die Zellenhöhle gerichteten Fläche von einer Lamelle von Zellwande bedeckt ist."..." (On page 177 in Henry Smith's English translation: "According to Schleiden, in the plant cells, it (the cell nucleus, *GL's addition*) sometimes lies in the thickness of the cell membrane so that its (the nucleus's, *GL's addition*) internal surface, which is directed toward the cell cavity is covered by a lamella of the cell wall.")

Schwann's statement that the cell nuclei can be found *in der Dicke* (within the thickness) of the cell membrane is corroborated by what Schleiden wrote in his own article, "Contribution to Phytogenesis" (1838.) Thus on page 16 of this article in its English translation by Henry Smith — which was appended to the end of Smith's translation of Schwann' *Mikroskopische Untersuchungen* — one reads the following: "It is evident from the foregoing, that the cytoblast (Schleiden's name for the cell nucleus, Schleiden 1838, p.233) can never be free in the interior of the cell, but is always enclosed in the cell-wall, and (so far as we can learn from the observation of those cytoblasts which are large enough to allow this very difficult investigation) in such a manner that the wall of the cell splits into two laminae, one of which passes exterior, and other interior to the cytoblast. ..." (p. 241.)

Clearly, what Schleiden and Schwann called indiscriminately the *cell wall* or *cell membrane* is wide enough to accommodate a cell nucleus. Thus if we can find out the size of the cell nuclei they studied, we would be able to estimate the minimum width of what

Schleiden and Schwann called the cell membrane. And from that width, maybe we can figure out exactly what Schwann and Schleiden called the cell membrane (*alias* cell wall.)

It so happens that Schleiden in his 1838 article actually gave us measurements of the widths of the plant cell nuclei he observed. They ranged from the narrowest at 0.0009 Paris inch to the widest at 0.0022 Paris inch. Now, each Paris inch is equal to 2.7 cm. Converted into CGS units of length, these widths are respectively 24.3 microns or 243000 Å and 59.4 microns or 594,000 Å. Both are orders of magnitude wider than the 60 Å-wide affair we call cell membrane today. We now return to the question raised, *What did Schleiden and Schwann see and call the cell membrane or cell wall in 1838–1839?*

To find the right answer, we must not forget that the time was before 1839, when the technology of the light microscope was still in its infancy. Only very large mature plant cells could be recognized as individual units and they were the favorite materials for study.

Figure 2A is an illustration of such a large mature plant cell that I took from page 129 of the 1937 (3rd) Edition of *An Outline of General Physiology* by L. V. Heilbrunn, who in turn took it from Miller's *Plant Physiology.* Figure 2B, on the other hand, is taken from page 661 of the second edition of S. Glasstone's popular "Textbook of Physical Chemistry" (Glasstone 1946.)

In both Figure 2A and Figure 2B, the largest part of the cell is occupied by the central vacuole, filled with cell sap, a clear watery fluid. Surrounding the vacuole is a thin layer

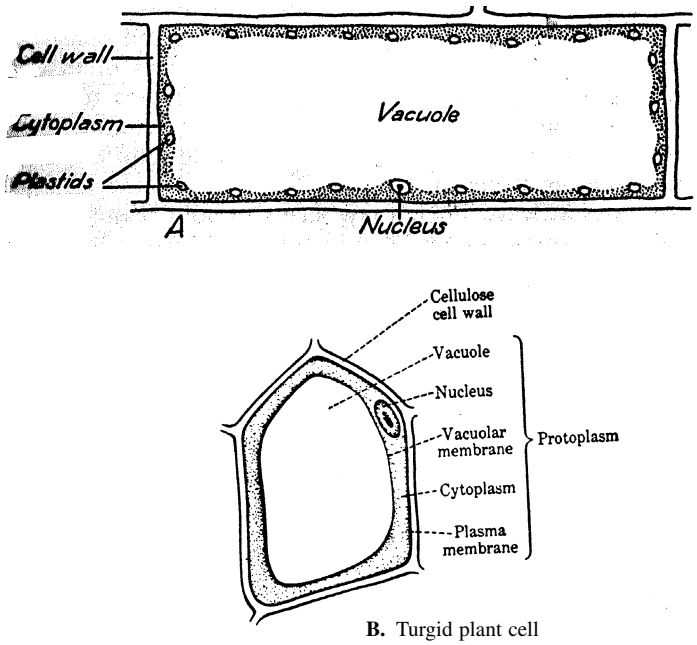


FIGURE 2. Two diagrammatic illustrations of mature plant cells. A. from Miller's *Plant Physiology* reproduced in Heilbrunn's "An *Outline of General Physiology*" 3rd ed., Saunders, Philadelphia, 1937. B. from Glasstone's *Textbook of Physical Chemistry*, van Nostrand, 1946.

of cytoplasm and inside this layer of cytoplasm lies one or more nuclei. That, is, of course, all we need to know — for it tells us exactly what Schleiden and Schwann saw and called the cell membrane (or cell wall) and the cell content.

What Schleiden and Schwann saw and called the "cell membrane" or "cell wall" was what Heilbrunn's picture and Glasstone's picture both labeled as the cell wall *plus* the layer of cytoplasm lying under the cell wall. Or in still greater detail, Schleiden and Schwann's cell membrane *alias* cell wall includes the true cellulose cell wall, and the adjoining layer of cytoplasm with its outer surface covered by what we now call the plasma or cell membrane and its inner surface covered by what is now called the vacuolar membrane or tonoplast. What Schwann saw filling his "cell membrane"-enclosed cavity is the entirety of the clear watery vacuolar sap. He then assumed that all living cells, plant and animal alike, young and old alike, all have a similar makeup of a *membrane enclosed puddle of clear watery sap.*

Out of this sequel of one mistaken identity built upon another mistaken identity emerged what appears to be the earliest rendition of what has been known as the *membrane theory*. Notwithstanding, neither Schleiden nor Schwann referred to the above-described picture of what the typical plant and animal cell looks like as the membrane theory. It was just a part of Schwann's *cell theory*.

However, Schwann did not stop there. He went on to suggest what would be the essence of the latest version of the membrane theory, known as the *membrane pump theory—taught universally worldwide as established truth today*. To achieve that, he first pointed out that the content of the cell is (as a rule, *added by GL*) different from the external fluid (which Schwann called *cytoblastema*) — yet, in fact, the fluid that fills the space between the cell membranes and the nuclear membrane gets there by the process of imbibition (a term, at Schwann's time, meaning simply swallowing) and as a result cannot be substantially different from that of the external fluid ("daher ein Zwichenraum zwichen ihr und der Zelle entstehen muss, der durch blosse Imbibition mit Flüsigkeit gefüllt wird. so kann diese Zelleninhalt nicht wesentlich vershieden sein von dem äussern Cytoblastem.) (Schwann 1839, p. 197.)

Schwann continued that "I think therefore that, in order to explain the distinction between the cell-contents and the external cytoblastoma, we must ascribe to the cell-membrane not only the power in general of chemically altering the substances ... but also of separating them such that certain substances appear on its inner and others on its outer surface. The secretion of substances already present in the blood, as, for instance, of urea, by the cells with which the urinary tubes are lined, cannot be explained without such a faculty of the cells." (p. 199, Smith's English translation.)

As an example, Schwann cited the galvanic pile, which is known to be able to separate chemical substances. He even suggested that the orientation of the axes of the atoms making up the cell membrane may play a role in the exercise of the metabolic power (pp. 196–197.) In different words to be sure, but if one compares what he sketched here with what we now call the membrane pumps, there is no uncertainty on what he was trying to say. Thus, Schwann had envisaged from mistaken identity what he thought was the cell membrane (but really not) and what he saw as the cell content as clear water fluid (but really not) and then postulated membrane-located submicroscopic pumps.

Major progress made in cell anatomy and cell physiology after Schwann left Berlin

As I have alluded to briefly above, of his fifty odd years of professional activity, Schwann spent only five years between 1834 and 1839 on subjects related to his hugely successful *Mikroskpische Untersuchungen*. In 1839, the year his book was published, he accepted a chair in Louvain, Belgium. Ten years later he moved to Liège, where he remained and stayed as a bachelor until the end of his life. In all these long years, he never published any work or interacted with other workers — as a rule brilliant and intelligent (see below) — in the field of micro-anatomy, which had, nevertheless, given him enduring fame. This is strange. Isn't it?

Historian Henry Harris offered an answer to Schwann's prolonged silence. "Schwann remained silent because he knew he was wrong." (Harris 1999, p. 195.) Harris made no specific reference on what specific subject was Schwann wrong. However, three pages before, Harris pointed out that the so-called "Gesetzen" ("laws") Schleiden proposed and Schwann adopted *that new cells always originate within old cells* — was wrong. The work of Belgian botanist, Barthélemy Dumortier (1797–1878) published first in 1832 (Dumortier 1832) and the later work of Hugo von Mohl ((1805–1872) published five years later in 1837 (von Mohl 1837) established that new plant cells do not originate from within old cells. Instead, they multiply by fission.

However, as I made clear above, this is not the only subject on which Schleiden and Schwann erred and erred in a big way. Thus, what Schwann regarded as the cell membrane and the cell content were grossly mistaken also. Cells are not membrane-enclosed puddles of clear liquid water. Evidence pointing to these errors were already on hand even before Schwann departed Berlin — notably in the historic work, to be described next, of the French zoologist, Felix Dujardin and the French botanist, Henri Dutrochet.

Discovery of sarcode, later (unfairly) replaced by a better-sounding name, protoplasm

Historian Thomas Hall published in 1969 a two volume treatise on *Ideas on Life and Matter: Studies in the History of General Physiology 600 B.C.–1900 A.D.* In the beginning of Chapter 39 on page 171 of Volume II, Hall wrote that the whole study so far has been a prelude to *sarcode,* which was the title of that chapter. And, it came from a very important French scientist, Felix Dujardin.

Felix Dujardin (1801–1860) was what we could now call a proto-zoologist. But at his time, he would be called a zoologist as the word protozoon had not yet been invented. Some of the protozoa were called *Infusoria* at that time.

> Protozoans are single-celled organisms that live in water. In modern taxonomy, protozoa are divided into four divisions: (1) ***Flagellates*** are usually oval in shape, usually carry chlorophyll and move about by whipping around a hairlike flagella. Englena is a flagellate. Volvox is a lasting aggregate of many flagellates, looking like a ball. (2) ***Sarcodena*** include amoeba and amoeba-like creatures. (3) ***Sporozoa*** reproduce by spores. It includes the malarial parasites. (4) ***Ciliates*** all have on their surface fine hairs called cilia, which help them to move around and capture food. Paramecium is a ciliate. Stentor, a trumpet like protozoa measures 1 or 2 millimeters in length; it is one of the largest single-celled animal. Another large cili-

> ate is Verticella. It is shaped like a funnel with a long stem. In the time of Dujardin, ciliates like these were known as Infusoria (Buchsbaum 1965.) Another kind of unicellular organism called Foraminifera may grow to 18 centimeters in diameter. They live in the oceans and we will have a chance to talk about their role in the history that was to follow.

Dujardin studied ciliates and larger multi-cellular animals including earthworms, insect larvae and so on (Dujardin 1835.) What fascinated him was the material that oozed out of the cells when they were crushed. He did not claim to be the first to have seen this substance but took great pains to cite and describe the earlier workers and what they found. They include Trembley's egg-white like materials (1744), K. F. Wolff's zellenförmiges Gewebe (1759), Karl Rudolphi's mucus material (1807), L.C. Treviranus's streaming Gallert (1811.)

> This good habit of referring to all prior work does not take much to do but, in my opinion, it is highly admirable. For it could mean so much for those scientists who had passed away — for this is the glue that makes scientific research a shared adventure of all humanity, living, dead and yet to come. And, small or big as it may be, the truth one helped to uncover has lasting value for all humanity.

Dujardin decided to give a name to this living jelly and he chose the name, *sarcode.* He then went on to describe what sarcode is like: "glutinous, transparent substance, insoluble in water, contracting into globular masses, attaching itself to dissecting-needles and allowing itself to be drawn out like mucus; lastly, occurring in all the lowest animals interposed between the other elements of structure" (Dujardin 1835.)

Meanwhile, investigation with the same general orientation on both animal and plant tissues continued with great vigor and increasingly better microscopes .

Thus, Meyen (1837) arrived at the conclusion similar to that of Dujardin: the cavity of true Infusoria is filled with a slimy, somewhat gelatinous substance. Jones (1841) claimed that the lowest form of animals consists of gelatinous parenchyma (p. 6.) In the same year, Kützing (1841) gave the name, Amylidzell to the material lining the inside of the cell wall of mature plant cells. One recalls that this is what Schwann wrongly regarded as an integral part of what he called cell wall or cell membrane.

Another three years later, the Swiss botanist, Karl Nägeli (1844), then a professor at the University of Zurich, described a slightly granular colorless "Schleimschicht" under the entire inner surface of the cell wall of mature green algae and some fungi. In young cells, on the other hand, the material that makes up "Schleimschicht" in mature cells, filled the entire cell. Nägeli acknowledged that his "Schleimschicht" and Kützing's Amylidzelle are the same thing. In the same year, Hugo von Mohl (1805-1872) introduced yet another name for what Kützing called Amylidzell and what Nägeli called Schleimschicht: *Primordialschlauch* or *utriculus primordialis* (von Mohl 1844, col 275.)

von Mohl pointed out that when a cell nucleus is present in the plant cell, it lies in the primordial utricle as shown in Figure 2A and Figure 2B above, further affirming the earlier conclusion that Schwann and Schleiden's cell membrane or cell wall includes the outer cellulose cell wall and this primordial utricle (von Mohl 1844, col. 276.) von Mohl also showed that what used to be called the cell wall or cell membrane — as Schleiden and Schwann did — have two components: the outer layer of true cell wall is mostly made of cellulose and it stains blue with iodine while the inner layer contains proteins and it stains yellowish-brown with iodine.

Two years later, von Mohl reaffirmed and extended what Nägeli pointed out earlier on the profound difference in the anatomical structure of the interior of young and old plant cells (von Mohl 1846.) Young plant cells do not possess a sap-filled central vacuole. But as the plant cell develops, sap-filled irregularly-distributed spaces began to appear. As a result, the kind of material that once fills young cells takes on the form of an irregular network as illustrated in von Mohl's drawing of what he called a typical plant cell (von Mohl 1851) reproduced here as Figure 3. As the cell grows older, the size of the sap-filled spaces increases and coalesces. Eventually, only one gigantic central vacuole remains.

Figure 4-1 taken from Bayliss (1927), who in turn took it from Kühne (1859) is that of a staminal cell of spiderwort (Tradescantia *virginica.)* Figure 4-2 shows a younger staminal cell after being subjected to moderate electric shocks (A) and stronger shocks (B.)

In 1846 — twelve years after Felix Dujardin gave the name, sarcode to the living jelly from lower animals — von Mohl gave the name, *protoplasm*, to the material that fills young plant cells, forms strands in middle-aged plant cells and eventually makes up the primordial utricle of old plant cells. von Mohl in all likelihood did not know that the word, *protoplasma*, had already been introduced by Purkinje to describe the ground substance of cells (Purkinje 1839, see Purkinje 1840.)

The next major step forward was the identification of what Dujardin called sarcode from animal cells with what von Mohl called protoplasma in plant cells. History shows that this effort began in the city of Breslau.

At the end of the World War I, the city Breslau was given to Poland and took on the Polish name Wroclaw. However, in the early 19th century, the city was under the rule of the Kingdom of Prussia. After the German unification, Breslau became the sixth largest city of the German Empire.

Jan E. Purkinje (1787–1869), was at the time professor of physiology and anatomy of the University of Breslau and also the founder of the first institute of physiology in Germany — founded a year before Müller's Institute in Berlin was founded. Equipped with a fine achromatic Plössl microscope, Purkinje and his students began to study both plant and animal tissues of diverse kinds.

Jan Evangelista Purkinje
(1787–1869)

Beginning in the early 1830's, Purkinje noted a fundamental similarity between the living substance of plant and animal tissues (Harris 1999, pp. 85–87.) He presented his findings in the year 1839 in a meeting of the Silesian Society for National Culture. In this meeting, he actually used the word "Protoplasma" in a scientific context for the first time to describe the living matter of living cells. A report of this address was published in the following year (Purkinje 1840.) In this article , he wrote: "...the correspondence is most clearly marked in the very earliest stages of development — in the plant in the cambium (in the wider sense) and in the animal in the Protoplasma of the embryo..."

In 1850, Purkinje left Breslau to accept the Professorship of Physiology and Anatomy at the University of Prague. Not long after, Ferdinand Cohn (1828–1898), a native of Breslau, became a member of the faculty of the same University. However, it took a long time before he could obtain some laboratory space. When that opportunity finally arrived, he turned it into the first Institute of Plant Physiology in Germany.

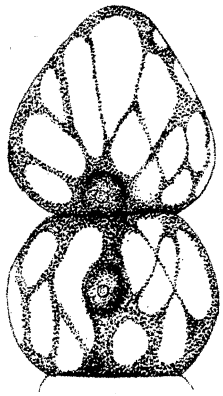


FIGURE 3. An illustration of a part of a typical plant cell. (from von Mohl, 1851, Table I, Figure 7)

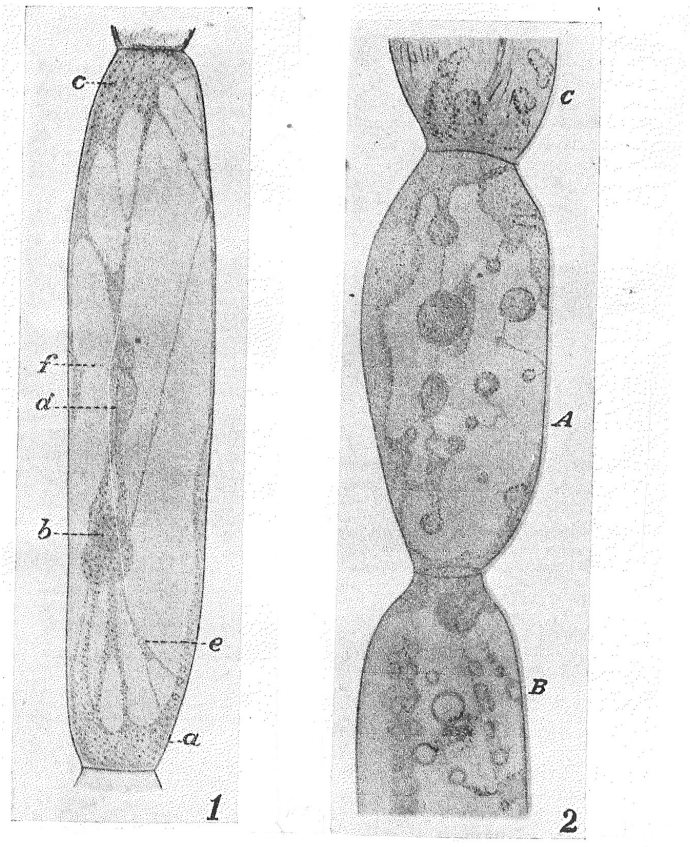


FIGURE 4. Cells of Staminal Hairs of Tradescantia *virginica*. (1) Normal resting cell: a, cell wall; b, nucelus; c, protoplasm; d, wave of contraction in protoplasm; e, web-like okate arising from the coalescence of two fine threads; f, moving bridge between two stronger protoplasmic currents. Length of cell, 0.3 mm. (2) Younger plant cell excited by electric shocks applied parallel to the long axis. A. shocks of moderate strength; B. shocks of stronger strength. In C, the protoplasm is coagulated by rupture of cell and entrance of water. Length of cell A, 0.145 mm. (from Kühne 1864, Pl. i, Figures 1 and 4 as reproduced in Bayliss's *Principles of General Physiology*, 4th ed., shown as Fig. 5 on p. 5, 1927.)

Cohn's best known work was in bacteriology. However, one can make a strong case that his contribution to cell anatomy and physiology was just as important if not more so. Thus, it was Cohn who had clearly demonstrated that what Dujardin described as transparent, water insoluble, contractile substance and named sarcode from animals are the same as what von Mohl names protoplasm of plant cells (Cohn 1847.) However, Cohn made no suggestion to call both by one name. That came later. It was Robert Remak (1815–1865) who suggested that both be named protoplasm (Remak 1852.)

The cell as a lump of membraneless protoplasm with a nucleus

In 1857, F. Leydig, professor of zoology at Göttingen, made an unambiguous statement that cell walls are not essential to a living cell — see Baker 1952, p. 164 for details — (Leydig 1857.) Anton de Bary (1831–1888), professor of botany at Freiburg, Halle and Strasburg worked on slime molds and declared in 1860 that the amoeba-like germinated spore cells called *swarmers* or swarm cells — had no cell membrane (de Bary 1860, 1864.). Soon afterward, de Bary's view was confirmed and extended by Max Schultze (1825–1874), the 36-year old young professor of botany at Bonn. Indeed, Max Schultze became the champion of the concept of membraneless living cell.

Schultze asked rhetorically the question, What is the most important kind of cell? (Schultze 1861, p. 8; Hall 1951, p. 451.) He answered that it had to be the embryonic cells, because they give rise to all the diversity of animal cells. An examination of the embryonic cells led him to conclude that they are not covered with a membrane chemically different from protoplasm. Hence, cells are membraneless little lumps of protoplasm with a nucleus....(..aber eine vom Protoplasma chemisch different Membran besitzen diese Zellen nicht. Sie sind hüllenlos Klümpchen Protoplasma mit Kern.) (Schultze 1861, p. 9.)

On the surface, one may find the bare statement that embryonic cells have no membrane anti-climatic. For in fact, it is hiding an important piece of relevant information. Namely, *with the best light microscope available then or now, you cannot see a membrane on these embryonic cells or any other animal cells*.

The readers will recall that on the alleged location of the plant cell nuclei, I have established that what Schwann (and Schleiden) called the cell wall or cell membrane is the true cellulose cell wall (which stains blue with iodine) plus the layer of material that stains yellowish-brown with iodine. The reader will also recall that this yellowish-brown staining layer has been given all kinds of names by a succession of investigators until von Mohl came along, and replaced all of them with a final name. And that final name, is nothing else than *protoplasm*.

With that, the only visible cell membrane is revealed as a mistake. Aside from the dead cell wall, the plant cell has no more cell membrane than the embryonic cell or any other animal cell.

In support of his notion that the surface of cells are chemically similar to that of the underlying protoplasm, Schultze cited Kühne's observation that small amoeba of fresh and salt water often fuse with one another (Kühne 1859.) Schultze also went to some length in his study of Polythalamia and Monothalamia — different forms of the Formaininifera — and Radiolaria. They are all single-celled amoeboid protists, usually less than 1 mm in size. However, as mentioned earlier, Foraminifera measuring as large as 18 cm in diame-

ter have been described. To show just how big that single cell is, one only needs to remember that a standard football (soccer ball) measures 25.4 cm in diameter (Figure 5.)

Each of these single-celled organisms carries with it an elaborate shell or *test*, which Schultze compares with the cell wall of plant cells. Only the membrane of plant cells have no holes, while the test of Foraminifera and Radiolaria have an abundance of perforations, through which the outer layer of protoplasm reaches out in the form of fine naked strands. These protoplasmic strands regularly fuse together when they touch. Moreover, the protoplasm in these strands is streaming constantly — just like the protoplasm in plant cells — especially as illustrated in the staminal cell of Tradescantia shown in Figure 4, taken from the work of Kühne (1864.)

In general, Schultze's 1861 paper was accepted and supported by a number of the most prominent biologists of the time, includeing Brücke, Hannstein and particulrly, Thomas Huxley, who gave his famous lecture in a Presbyterian church in Edinburgh on a Sunday evening in 1868.

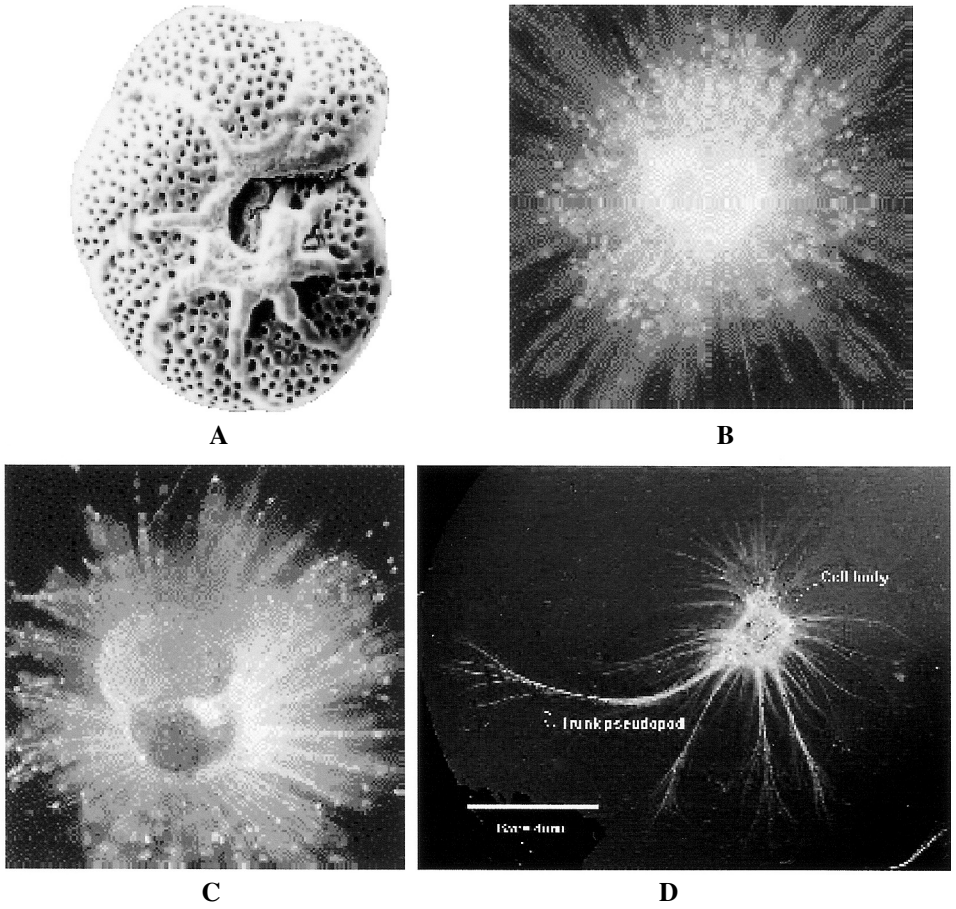


FIGURE 5. The shell or text (A) of Foraminifera. B and C show living spinose planktonic foraminifers. (from Liebes *et al*, 1998, by permission of John Wiley and Sons) D. a giant Antartic foraminifera, Astramina (Note bar = 4 mm length.)

There he told his largely lay audience that protoplasm is the physical basis of life, with such force and eloquence that he held his audience mesmerized. To give an indication of the depth of interest aroused, the issue of the Fortnight Revuew, in which the written version of his talk was printed, was reprinted an unprecedented seven times.

Henry L. Menken (1880–1956), the indomitable, straight-shooting American newspaper man of the Baltimore Sun praised Huxley as the greatest Englishman of the Nineteenth Century for "working that great change in human thought" which marked that Century. In agreement with Menken on his comment, I have something else to add.

Thomas Huxley not only was the eloquent advocate of the concept of protoplasm as the physical basis of life, he also became known at Darwin's Bulldog in fighting for Darwin's theory of Natural Selection and Evolution.

Yet in the beginning, Huxley was opposed to both the concept of evolution (as given by Lamark, for example) and the concept of protoplasm (Huxley 1853.) However, when he was face to face with new evidence uniformly pointing to a direction that contradicted his old beliefs, he lost no time making two 180-degree turns. *And henceforth Huxley became the strongest and ablest advocate of the view he once opposed. In this magnificent act of courage and self-denial, he and Brisseau de Mirbel mentioned earlier made true progress of science within reach.*

However, as the saying goes, One hand alone cannot clap. Thus to admire Huxley for what he had done, implies that there were others that inhabited the Victorian England of Huxley's time. For instead of slandering or ignoring him as he was in the habit of dosing out one bitter medicine after another, they offered him one honor after another, including the Presidency of the Royal Society for five long years.

How did the membrane theory of the living cell begin?

As illustrated in Figure 6, the cell membrane taught at both secondary and college level currently is in essence a phospholipid layer some 60 Ångstrom units (Å) thick. A structure so thin falls far below the resolving power of even the best light microscopes at 2

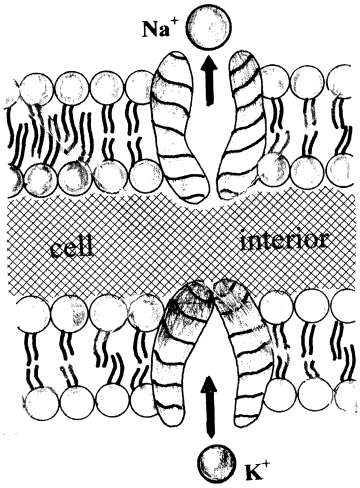


FIGURE 6. A diagrammatic illustration of what is given in most if not all current US high school and college biology textbooks, representing phospholipid bilayer cell membrane pierced by sodium (potassium) pumps.

microns or 2000 Å. Thus, it is safe to say that before the invention of electron microscopy in 1931 by Max Knoll and Ernst Ruska of Germany and its ancillary technology developed in the 1930's and 1940's, nobody had actually seen the cell membrane — even though some scientists including Schleiden and Schwann thought they did. Yet given time, the error was corrected and the new concept that living cells are membraneless lumps of protoplasm with a nucleus emerged and was broadly accepted — for at least 30 years (see Locy 1908, pp. 273–275; Kepner and Stadelmann 1985, p. viii.)

Yet, a look of any contemporary high-school and college biology textbooks shows that something has gone wrong, and seriously so. For who would believe that on this day in the 21st century, even the word, protoplasm, has disappeared.

The picture of the living cell in all the biology textbooks is very much like that in the mind of Theodor Schwann, before he discovered that he was wrong (Ling 2006.) For clues to an answer to this mystery, we return to the middle of the 18th Century.

Pig's bladder

Abbé Nollet (1700–1770) was the Preceptor in the Natural Philosophy to King Louis XV of France. Once the scientific opponent of Benjamin Franklin's one fluid theory of electricity, Nollet (1748) was also the first recorded investigator of what came to be known as the osmotic phenomenon.

Nollet filled a bottle with alcohol and covered the mouth of the bottle with a sheet of flattened pig's bladder, tied down securely with a piece of string. He then sank the bottle in a tub of water. Hours later, he noted that the bladder membrane bulged outward. In another trial, he immersed a water-filled bottle in alcohol. Now the bladder membrane bulged inward. Nollet concluded that the bladder membrane is more permeable to water than to alcohol.

Nothing much happened in the years immediately following Abbé Nollet's report until the arrival on the scene of a French scientist of commanding stature by the name of Henri Dutrochet (1770–1847.) Henceforth, the study of water and solutes movements across animal and man-made membranes became a quantitative science.

Abbé Nollet
(1700–1770)

Henri Dutrochet
(1776–1847)

Dutrochet, an impoverished but brilliant young Frenchman of noble descent, believed that life is movement. (For evidence that he had later changed his mind on this point, see p. 19 below, 19 lines from top.) And, that the major difference between organic and inorganic bodies is that the organic bodies were *vesicular* in nature, i.e., they are *hollow sacs or cavities filled with fluid.*

Dutrochet studied the movement of water and solutes in and out of both animal membranes (e.g., pig's bladder, loops of intestine) and plant tissues. He invented the word, *endosmosis* to describe movement into the membrane-enclosed fluid and *exosmosis*, to describe movement out of the membrane-enclosed fluid. To gain quantitative data, Dutrochet invented what he called an *endosmometer*. This simple instrument is in fact an inverted funnel, with the larger end securely covered with a membrane of pig's bladder.

When different fluids are added to either side of the membrane and the two-way movements take place, a pressure difference between two sides of the endo-osmometer was observed. The magnitude of the pressure difference is revealed by the rise or fall of the fluid level inside the endo-osmometer. This pressure difference was given the name, the *osmotic pressure.*

Dutrochet conducted his experiments of endo- and exosmosis on plant and animal tissues but visualized similar events going on at the microscopic cell level. Most of Dutrochet's later work was summarized in his (last) book published in 1837 (Dutrochet 1837) and thus two years ahead of Theodor Schwann's *Mikroskopische Untersuchungen*, which came in print in 1839.

Accordingly, Dutrochet was two years ahead of Theodor Schwann in introducing the concept that the living cell is a membrane-enclosed cavity containing a clear watery fluid. Schwann believed that he saw the cell membrane but, as made clear above, it was a mistake. Dutrochet did not claim to have seen the cell membrane. So his theory was based on indirect evidence revealed as endosmosis and ectosmosis.

Dutrochet believed that cells in general but animal cells in particular are in outer appearance highly similar to one another. Nonetheless, they are different from one another in the chemical makeup of the fluid inside the cell cavity. The diversity in its chemical makeup, in turn is due to the different *secretary activities* of the cell membranes (Dutrochet 1837, p. 470.)

By the 4th decade of the 19th century, the substance if not the name *semipermeable membranes* was well known. Only these were *macroscopic* membranes like pig bladder and intestinal loops. Cell membrane was also a familiar concept to Dutrochet but only in theory. Unfortunately, Dutrochet's original books in French are not easily accessible in English-speaking countries like the United States. For this reason, we have reasons to thank Professor A.S. Rich of the Johns Hopkins Medical School of Baltimore in the United States.

In 1926 Rich published in the Bulletin of Johns Hopkins Hospital an article entitled, *The Place of H. Dutrochet in the Development of the Cell Theory* (Rich 1926.) Rich pointed out in his book that Dutrochet had introduced the essence of the cell theory 13 years ahead of Theodor Schwann. Thus, translated in English, the relevant parts of Dutrochet's book reads: "All of the organic tissues of plants are made of cells." "Now the observation on animals which we have just described already confrims this view." (Rich 1926, p. 345) "The physiological connections which I have established between plants

and animals made it clear that there is but a single physiology.... I hope that some day, out of these first attempts, there will be born a new science — *general physiology*."(Rich 1926, p. 359)

Yet, Schwann in his *Mikroskopische Untersuchungen* made no mention of this, nor Dutrochet's discovery that "... the cell is the secreting organ *par excellence*. It secrets, inside itself, substances which are, in some cases, destined to be transported to the outside of the body by way of the secretary ducts, and, in other cases, destined to remain within the cell which has produced them, thus playing specific roles in the vital economy" (Dutrochet 1824, English translation of Rich 1926, p. 348.)

Indeed, Schwann made no mention of Dutrochet at all. Yet there are evidence that Schwann not only knew of Dutrochet but might have borrowed Dutrochet's ideas without giving him due credit. (For details, see page 364 of Rich 1926.)

All these pointed to the fact that Schwann was not in the habit of admitting his own error and in adopting the correct answer which new evidence brought to light. In sharp contrast, as Rich pointed out, Dutrochet was in the habit of consistently and readily admitting his earlier mistakes including what is contained in the following passage: "He, (Dutrochet) himself, however, with his customary self-honesty wrote some years later: 'I know that at first I went too far in considering endoosmosis as the fundamental phenomenon of life' — " (Dutrochet 1837, Rich 1926, p. 354.) Unfortunately, the majority of investigators paid little attention to this admission but continued in the direction that Dutrochet pursued in his early years.

New concept of colloids and of the colloidal state

An English chemist, Thomas Graham (1805-1869), primarily engaged in studying the phenomenon of diffusion, modified Dutrochet's endo-osmometer and gave it the simpler name of *osmometer*. Using a piece of sized parchment paper for its membrane barrier, he converted an osmometer into what he called a *dialyzer*.

Thomas Graham
(1805–1869)

Soon Graham discovered that the water-soluble substances he investigated could be sorted out into two groups. One group containing sugars and inorganic salts travel very fast through the dialyzer membrane and as a rule can assume crystalline form. Graham called this class, *crystalloids*. In contrast, substances like gelatin diffuse very slowly and do not pass through the dialyzer membrane. He called these substances *colloids*, based on the Greek word for glue or gelatin, *kolla.* As a rule, colloids do not form crystals (Graham 1861.) As examples, he showed that neither gelatin nor tannin can go through the sized parchment, nor could the brown gelatinous copper ferrocyanide formed when a solution of copper sulfate is mixed with a solution of potassium ferrocyanide.

These reported findings fell on the fertile mind of a tradesman in Berlin named Moritz Traube (1826–1894.) As a result, Traube produced two artificial membranes of historical

importance. Unlike Graham's sized parchment membrane, which is selectively impermeable to colloids but not to crystalloids, Traube's membranes were impermeable to (some) crystalloids as well (Traube 1867.)

One membrane was made on the end of a glass rod. The glass rod was first dipped in a solute of non-jelling gelatin and the gelatin coated glass rod dipped a second time into a solution of tannic acid. Hardened, the gelatin film on the end of the glass rod could be slipped off in the form of a little thimble with selective permeability properties to both colloids and crystalloids. This was a simple but remarkable achievement.

The second membrane Traube made was even more remarkable. Instead of a glass rod, he now started out with a narrow glass tube or what I call a pipette. When a solution of potassium ferrocyanide is drawn into this pipette and its fluid-filled tip gently lowered into a solution of copper sulfate, a thin film or membrane of reddish brown copper-ferrocyanide gel now covers the opening of the pipette. Traube found that once this membrane is formed, it would not allow additional formation of copper ferrocyanide precipitate either inside the pipette or outside.

This self-limiting precipitation shows that the copper ferrocyande gel membrane formed does not permit the passage of either one of the membrane forming crystalloids, positively-charged copper ion or negatively-charged ferrocyanide ion. The only thing it does allow to pass through appears to be water.

Trying to explain the behavior of the precipitation membrane, Traube suggested the *atomic sieve theory.* That is, the copper ferrocyanide membrane has pores of a critical size so that they would allow the small water molecules to go through but not the larger copper ion and the ferrocyande ion. That done, the ball passed onto the hands of Wilhelm Pfeffer (1845–1920.)

Pfeffer was born in Grebenstein, Germany. Though he received a Ph.D. degree in Chemistry in Göttingen, he became mostly a botanist. One of Pfeffer's major contributions was making highly accurate quantitative studies of osmotic pressure across the copper ferrocyanide membrane Traube invented.

Pfeffer did this by an invention of his own. He allowed the copper ferrocyanide membrane to form inside the wall of an unglazed porcelain pot. The sturdy wall of the porce-

Moritz Traube
(1826–1894)

Wilhelm F. Pfeffer
(1845–1920)

lain pot becomes the equivalent of Graham's sized parchment and Traube's ephemeral film at the tip of his glass pipette. This improvement has made possible precise measurements of osmotic pressure and Pfeffer took full advantage of the opportunity thus created.

As an example, Pfeffer filled the copper-ferrocyanide infiltrated porous pot with a concentrated solution of cane sugar (or sucrose) and placed the pot in turn in a container filled with pure water, Soon, water begins to move from the outside compartment to the inside compartment. On the other hand, if the top of the pot is connected to a manometer, a pressure will be registered. This is, (Dutrochet's) *osmotic pressure* produced by the concentrated sucrose solution. Here, the pressure is not the result of two opposing movements, one endosmotic and the other exosmotic as in the earlier studies of Dutrochet and others but it measures just one movement of water only. That simplification has made it possible to make reproducible results of the osmotic pressure (Pfeffer 1877; Hamburger 1904.)

Before long, Pfeffer was able to show that the osmotic pressure measured is directly proportional to the concentration of the sucrose solution inside the pot and inversely proportional to the absolute temperature. When Dutch botanist Hugo de Vries learned about these exciting findings, he brought it to the attention of another Dutrchman, Jacobus H. van't Hoff (1852–1911.)

Born in Rotterdam in 1852, Jacobus van't Hoff was still in his twenties, when he (and J. A. Le Bel) independently discovered what we now call *stereochemistry*. Within the next ten years, van't Hoff also introduced the concept of the *principle of mobile equilibrium* that is better known as the Le Chatelier principle. Awarded the first Nobel Prize of Chemistry in 1901, he was, with Wilhelm Ostwald, often regarded as the founders of the modern science of physical chemistry.

J. H. van't Hoff
(1852–1911)

Of particular interest in the history of the membrane theory was van't Hoff's formulation of his membrane theory of osmotic pressure. Based on the experimental findings of Pfeffer that osmotic pressure is related to absolute temperature and sucrose concentration, the theory took the shape entirely analogous to the ideal gas law: (van't Hoff 1885, 1885a, 1886, 1887, 1888)

$$\pi V = R' T, \tag{1}$$

where π is the osmotic pressure. V is the volume of solution containing one mole of sucrose and is therefore equal to 1/C, where C is the concentration of sucrose. T is the absolute temperature. R', a constant, can be computed from the equation and numerical data Pfeffer provided, to approach closely the gas constant, 1.987 cal.deg^{-1}. Equation 1 can be written in a different form:

$$\pi = CR'T. \tag{2}$$

Thus, Pfeffer's precise data on the osmotic pressure provided the foundation for van't Hoff's important work. But Pfeffer did more.

First, Pfeffer's success with his modification of Traube's model set in motion a saga of the creation of better and better copper-ferrocyanide membrane models. Thus, in the

version of Morse (1911), an osmotic pressure created by a sucrose solution stays unchanged for 60 days. This was a feat that has made the copper-ferrocyanide osmometer close to the ideal semipermeable membrane in van't Hoff's original definiton — *permeable to water but nothing dissolved in it* (van't Hoff 1885.). And, in general terms, the finding was in harmony with Traube's sieve theory, introduced in the year 1867 and mentioned above.

Subsequent investigations, however, did not support this theory. In general, the pores found in artificial membranes are far too large to act as meshes of a mechanical sieve that would bar the passage of sucrose with a molecular diameter of 9.9 Å. Indeed, Bigelow and Bartell (1909) showed that unglazed porcelain plates — with or without clogging precipitate of one kind or another — with pores as wide as 0.37 micron in diameter and thus 3700 Å wide demonstrate osmotic activity, thus effectively barring the passage of molecules two orders of magnitude smaller (Table 1.)

> At the time when A. Findlay wrote his monograph on Osmotic Pressure (Findlay 1919) the majority of investigators favored the so-called *Solution Theory*. That is, substances that can dissolve in the membrane will pass through, a substance that does not dissolve will not. Liebig, for example, favored this view (Liebig 1847.) However, only water could fill the 0.37 micron-wide pores in unglazed porcelain Bigelow and Bartell studied. At the center of the 3700Å wide, water-filled pores, the water molecules are thousands of water molecules away from the nearest solid wall. That these water molecules could, nevertheless, be profoundly modified was only given backing by the theoretical and experimental work done in the last years of the 20th and beginning of the 21st century (see Ling 2004.) That is a chapter of cell physiology beyond the time span covered by this review. But interested readers can access these new discoveries in Ling 2006, 2006a.

Second, Pfeffer suggested that the surface of the living cell exists as what he called *Plasmahaut* in agreement with what Meyen, Brown, Kützing, Nägeli and von Mohl found out earlier and described above. Additionally, *Plasmahaut* means *protoplasm skin,* which is different from a membrane — because the structure of any skin is more or less continuous with what lies under the skin and not sharply separated from what lies under the skin with a new interface as that found on the outer surface of a skin. That said, it must also point out that Pfeffer did not, indeed could not have seen the cell membrane at that time. So, it was just one more conjecture.

Pfeffer then went on further to conjecture that each time protoplasm comes into contact with water, a new Plasmahaut is formed at the new intersurface (Pfeffer 1877, p. 143.) The evidence he quoted for this conjecture was not new: namely, when protoplasm (or sarcode) comes out of broken animal or plant cell, it does not dissolve in the bathing water. This phenomenon has an earlier and simpler interpretation. Namely, protoplasm is *water-insoluble* — a characterization that came from Dujardin in 1835 for what he called sarcode but now more widely known as protoplasm.

By drawing upon a historic analogy, one may suggest that the view held by Dujardin is a *Pre-existence Theory* while the view held by Pfeffer is an *Alteration Theory*.

> The analogy is a familiar page in the history of physiology. E. DuBois-Reymond, believed that the electrical potential difference called *demarcation potential* or *injury potential* — measured across the cut surface of a muscle or nerve — exists before the cut is made, a view labeled *Preexistence Theory*. In contrast, his student, L. Hermann believed that the potential difference came only after the cut is made, a view known historically as the *Alteration Theory* (Hermann 1888; DuBois-Reymond 1848–49.) This controversy was resolved in favor of the pre-existence theory when minimizing the injury inflicted on the muscle cell in making

TABLE 1. The size of the pores in unglazed porcelain to produce an osmotic effect. According to Jurin's Law, $a^2 = hr$, where r is the radius of a capillary tube and h is the height to which a liquid ascends due to capillarity. At 19°C, a^2 for water equals 14.846 mm. Thus by measuring the pressure (P in mm of Hg) just high enough to force water out of a membrane saturated with water, one can estimate the radius or diameters of the pores of that porous membrane. Table A shows that the diameter (D) of fine glass capillaries estimated this way agree well with the diameters of the capillaries measured directly under a microscope (column 3.) Table B demonstrates the widest diameters of pores in samples of porcelain discs to produce an osmotic effect. In Table C, the pores of the porcelain discs were clogged with barium sulfate precipitates and in Table D, they were clogged with finely divided sulfur. Data indicate that the untreated porcelain discs continue to show osmotic effect when the pores are as wide as 0.37 micron in diameter. Clogging pores with barium sulfate and sulfur increased the maximum pore diameter demonstrating an osmotic effect to 0.59 and 0. 49 micron respectively. (from Bigelow and Bartell 1909)

TABLE A

P.	D, calculated.	D, observed.
22	0.099	0.114
33	0.066	0.064
48	0.046	0.047
65	0.034	0.033
258	0.0085	0.008

TABLE B

P.	D.	Osmotic effect
2.5	1.18	None.
2.6	1.14	None.
4.5	0.65	None.
5.0	0.59	None.
6.0	0.49	None.
7.0	0.42	Possibly a slight effect.
8.0	0.37	Surely some effect.
8.5	0.34	More effect.
15.0	0.19	Yet more effect.

TABLE C

P.	D.	Osmotic effect
3	0.98	None
4	0.74	Slight effect
5	0.59	Marked effect.
6	0.49	Marked effect.
12	0.24	Marked effect.

TABLE D

P.	D.	Osmotic effect
3.2	0.93	None.
4.0	0.74	None.
4.5	0.66	None.
5.0	0.59	Possibly a slight effect.
6.0	0.49	Surely some effect.
8.0	0.37	Marked effect.
12.0	0.25	Marked effect.
14.0	0.21	Marked effect.
18.0	0.16	Marked effect.

electric contact with the interior of the cell maximizes the magnitude of the electric potential difference measured — as that achieved with a Gerard-Graham-Ling (alias Ling-Gerard) microelectrode (Ling and Gerard 1949, also see below.)

We now consider some known facts and experimental findings that may help us to decide which of the two alternative theories is closer to the truth.

(1) It is in everybody's experience to witness the creation of a cloud of countless water droplets by blowing on an atomizer. Each water droplet formed stays separate from the other droplets and from the surrounding medium, air.

Now, water molecules at the droplet surface are attracted by intermolecular forces only from inside but not from the outside. The surface layer of water molecules may thus be in its spatial and energy configuration somewhat different from water molecules deep down in the droplet. So, it seems not uneasonable to consider the surface has developed a *skin*. That is, as long as we recognize that chemically speaking, whether it is at the surface or deep down under the surface, they are all water and not chemically different.

(2) By adding salts to an aqueous solution of gelatin at 30° C, Pauli and Rona saw the separation of the solution into two phases: a gelatin-rich phase below and a gelatin poor phase above (Pauli and Rona 1902.) This phenomenon was later given the name *coacervation* and the gelatin-rich phase, *coacervate* by Dutch colloid chemists, Bungenburg de Jong and Kruyt (Bungenberg de Jong and Kruyt 1929.)

Does the creation of this new interphase suggests the formation of a skin at the surface of the gelatin-rich phase?

The answer is a qualified yes. If such a skin is formed, its chemical composition cannot be different from what makes up the gelatin-rich phase. The reason is simple: nothing else beside salt, water and gelatin exist in the whole system. However, it is possible that their relative proportion might not be the same at the surface or down below. However, coacervation is not primarily an interfacial phenomenon. It involves the entire system.

Indeed, many investigators have expressed the view that living cells are themselves coacervates. They included Bungenburg de Jong (1893–1977) in his younger days (Bungenberg de Jong 1932) and the outstanding Soviet cell physiologist, A. S. Troshin (for review of ideas of still earlier workers, see Troshin 1966, pp. 58–73.) Both were able to demonstrate wide-ranging similarities between living cells and coacervates, including such bulk-phase properties like solubility for ions and sugars. Why Bungenberg de Jong should abandon his earlier position in his later days was not explained by himself (see Ling 2001, pp. 31–32) but not too difficult to make a guess. It could include the immense pressure to toe the line that cell water is just normal liquid water (see Ling 1997, 1997a) and that the critical mechanism that makes colloids colloids was still a thing of the future (Ling 2001, pp. 83–84.)

(3) W.W. Lepeschkin expressed the opinion that Pfeffer's hypothesis that a precipitation membrane — like that of copper-ferrocyanide — forms at the exposed surface of protoplasm is wrong (Lepeschkin 1930.) In support, Lepeschkin showed that he could collect protoplasm from the plant, *Bryopsis plumosa* and shake it vigorously in sea water to produce countless little protoplasmic droplets. The total surface area of these droplets could be 1000 times bigger than that of the original protoplasm collected.

If each one of these little droplet aquires a membrane made of a non-protoplasmic material, say lipoids (see below), two problems arise.

If the new lipoid membrane derives its lipoid by stretching the old one, that new membrane would be less than a single hydrogen atom thick. This was the argument given by Lepeschkin himself against Pfeffer's theory.

In my view, there is a second strong argument against the formation of new membrane or skin if the formation of the skin is what protects the protoplasm from dissolving away. This is the question of time — that is, the time needed to engineer such a new and effective skin. It is like trying to build a coop for a big flock of un-tethered chickens. Most of these chickens would have flown away or walked away before the coop is ready for business.

Finally, I want to correct a mistake of my own making. For many years, I believed and repeated that belief in many publications that Pfeffer introduced the membrane theory. When finally I got hold of a copy of Pfeffer's *Osmotische Untersuchungern* for the first time and read it from cover to cover, I realized to my horror, that he did not introduce the membrane theory.

Pfeffer summarized his life's work in this book. The first edition was published in 1877 (Pfeffer 1877). An unaltered second edition was issued in 1921. The term, *membrane theory* did not appear once in either edition. Nor did Pfeffer suggest another different publication where he had introduced a theory bearing that name. To correct this mistake that I learnt from hearsay and actually helped to spread was one of the motivations that set me on the course of writing this article on the history of the membrane theory.

Plasmolysis and the so-called osmotic method for measuring membrane permeability

As illustrated in Figure 2A and 2B, each mature plant cell is imprisoned inside a rigid box of cellulose, the (true) cell wall. In 1855 Swiss-German botanist, Karl Nägeli (1817–1891) described how the part of the mature plant cell enclosed by the cell wall and later given the namer *protoplast* by Hannstein (Hannstein 1880), shrinks away from the cell wall when the cell is immersed in a concentrated salt solution as illustrated in Figure 7 (Nägeli 1844.)

Nägeli was not the first to describe this phenomenon. N. Pringsheim had described a similar observation a year before Nägeli (Pringsheim 1854.) However, it was Hugo de Vries (1848–1935), professor of botany in Amsterdam, that had turned this phenomenon into a widely adopted method for studying cell membrane permeability.

Hugo deVries
(1848–1935)

There are at least three notable undertakings of this remarkable scientist, Hugo de Vries, that are worth repeating. First, he was, as already mentioned, the one that had brought Pfeffer's accurate data on osmotic pressure to the attention of van't Hoff. Second, he was one of the trio that had resurrected Mendel's forgotten work on genetics (Watson 1977 p. 8; Ayala and Kiger 1980, p. 30.) Thirdly, he was the one that suggested mutation as the cause of evolution (Hall 1969, Vol, 2, pp. 346–348.) Now, we return to what de Vries contributed to the understanding of osmossis.

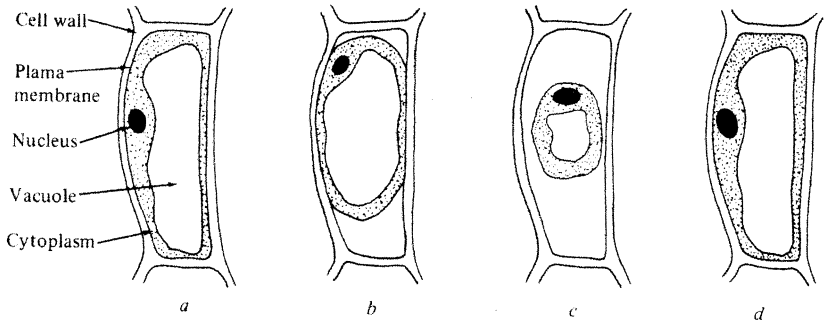
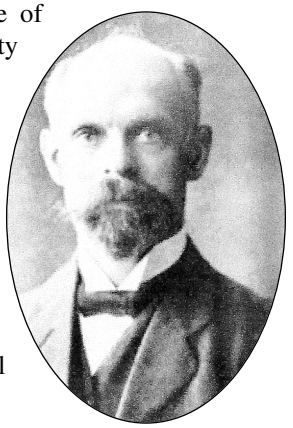


FIGURE 7. Diagrammatic illustrations of successive stages in the plasmolysis (b,c) of a mature plant cell (a) and deplasmolysis (d). (from Dowben 1969, by permission of author).

de Vries gave the name *plasmolysis* to the phenomenon of shrinking protoplast (de Vries 1884) and its reversal, *deplasmolysis* (de Vries 1888.) Under the general name, the "osmotic method", de Vries used it extensively in his study of cell membrane permeability. More specifically, the method determines the minimum concentration of a solute in the bathing medium that would cause a noticeable shrinkage of the protoplast i.e., the so-called *plasmolytic threshold solution* (*plasmolytische Grenzlösung)* (Overton 1895, p. 170.) The substance that shows the lowest threshold plasmolytic concentration is considered the most impermeant.

In a paper published in 1871, de Vries showed that in cells of the root of red beet immersed in a concentrated solution of table salt or sodium chloride, the protoplast stayed shrunken at the same size for 7 days (de Vries 1871, p. 123.) This led de Vries to conclude *that the cell membrane of red-beet root cells is impermeable to sodium chloride (NaCl.)*

Ernest Overton (1865–1933) was another scientist who used volume change of both plant and animal cells — under the name of "osmotic method" — to study the permeability to a wide variety of substances. That Overton was also a keen follower of Pfeffer could be seen from the vocabulary he adopted, including *Plasmahaut*, *Diosmotische Eigenschafte, isosmotische Konzentrationen* etc. But Overton also introduced his own views on what these terms represent.



Charles Ernest Overton (1865–1933)

Of particular interest is a figure of the mature plant cell that Overton presented in his 1895 paper as (his) Figure 1, reproduced here as Figure 8. (Note that he referred to what we now call cell wall as Cellulosemembran — reflecting yet once more the unending confusion of cell wall and cell membrane.)

A far more significant feature introduced by Overton shown in this figure is what Overton called *Aussere Grenzshicht alias*

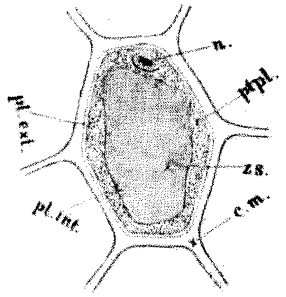


FIGURE 8. Schema eines Pflanzenzelle (Design of a plant cell). Note what Overton designated as Aussere Grenzschicht or Aussere Plasmahaut (pl.ext) and innere Grenzshicht or Vacuolenhaut (pl.int) are each represented as continuous lines of recognizable width and well delineated on both sides. c.m. cellulosemembran; ptpl, protoplasma; n, Nucleus; zs, Zellsaft. (from Overton, 1895, his Fig. 1 on page 160)

Aussere Plasmahaut. Note that in the diagram, the Plasmahaut is well-delineated by a similar sold line *on both sides*. This is not what Pfeffer said (and later translated into English by Kepner and Stadelmann (1985) that *the plasma membrane limits may be undefined on the inside.* (p. 139.) From this dichotomy, it is clear that the widely accepted concept of cell membrane being clearly defined on both inside and outside is at least in part due to Overton. Pfeffer's Plasmahaut is more aligned with Max Schultze's model of the living cell while Overton's cell membrane is more in line with Theodor Schwann's cell membrane alias cell wall.

Using de Vries's osmotic method, Overton determined the plasmolytic threshold for many inorganic and organic compounds on the filamentous fresh-water green algae, Spirogyra. The following is detailed account of one of his key studies (Overton 1895.)

First, Overton discovered that an 8% or 0.234 M sucrose solution would cause a very weak but perceptible shrinkage of the Spirogyra protoplast. He then used a solution containing beside 0.234 M sucrose another solute, grain alcohol or ethanol, but found the protoplast did not shrink further from that produced by 0.234 M sucrose alone. Overton concluded that the Plasmahaut of Spirogyra is fully permeable to ethanol. In contrast, addition of a similar concentration of ethylene glycol or glycerol on top of the sucrose caused an immediate shrinkage of the protoplast followed by a slow return to the original volume. This led Overton to the conclusion that the protoplast membrane is less permeable to ethylene glycol and glycerol than to ethanol. On the other hand, hexoses, mannitol or free amino acids, when applied alone, causes "permanent" plasmolysis. The shrunken protoplast stayed shrunken. Overton concluded that the protoplast membrane is totally impermeable to these solutes.

Some time later, Overton carried out a parallel study on a representative example of the animal cell, the frog muscle, including the gastrocnemius, the sartorius, the cutaneous pectoral muscle and the hind-leg muscle (Overton 1902.) Unlike Spirogyra, frog muscles are not imprisoned in a rigid cellulose box, and can therefore both shrink and swell, while Spirogyra protoplast can only shrink.

In his study on frog muscle, Overton started out with table salt or sodium chloride (NaCl) rather than sucrose. He determined the concentration of NaCl that would cause neither swelling nor shrinkage of the muscle cells. He then called this concentration of 0.7%, the *isotonic* concentration of NaCl.

Using this 0.7% NaCl as the starting point, he then repeated what he did with Spirogyra and obtained similar results. Methyl alcohol or methanol, when added to 0.7% NaCl

solution, caused no cell shrinkage. Glycerol when added to the same salt solution brought about an immediate shrinkage followed by a slow return to the initial volume. Sucrose, mannitol and free amino acids when added caused a "permanent" shrinkage. These findings led to the conclusion that the *frog muscle cell membrane is highly permeable to methanol, less permeable to ethylene glycol and glycerol but totally impermeable to sucrose, mannitol and free amino acids.*

These results and their interpretations raised questions. After all, all living cells need sugars and amino-acids for their survival and growth. Their impermeability to the cell membrane demands an answer. The one Overton offered was the same that Dutrochet first offered in 1824 and Schwann offered 1839: *secretion*. Or in more modern lingo, *active transport*.

In summary, Overton by studying the different patterns of shrinkage of plant and animal cells derived a model of the living cell that is basically similar to those offered by Dutrochet and by Schwann. In all three version, the cell is seen as a membrane-enclosed body of essentially normal liquid water, with the cell membrane engaged in the control of the chemical substances found or not found in the living cell. It does this either passively as through its "mechanical" permeability or by the process of energy-consuming *active transport* — then referred to respectively as "Metaboliche Kraft" (Schwann) or secretive activity (Dutrochet and Overton.) However, Overton went one step further by offering a specific chemical makeup of the cell membrane. That came in the form of his famous, "Lipoidal Theory" (Overton 1899.)

From the different permeability of some 500 chemical compounds that Overton tried out on plant and animal cells, he reached a set of conclusions known as Overton's rules. Thus, one rule says that substances that are soluble in oil or lipoids enter the living cells faster. In another rule, substances that are highly soluble in water enter the cell slowly. Based on these empirical rules, he suggested that all living cells are covered by a thin layer of lipoid materials — where the word lipoid is a loosely defined category of chemicals including phospholipids, lecithin etc.

Overton's lipoidal theory introduced in 1899 was a more specific example of the broader Solution Theories of membrane permeability like that first introduced by L'Hermit (1855) and of the more focussed suggestion of Quinke that a lipid layer covers living cells (Quinke 1898.)

To explain his theory, L'Hermit offered an elegant experimental model. If water is gently introduced into a glass cylinder containing some (heavy) carbon tetrachloride (CCl_4), that water will stay as a separate layer on top of the CCl_4 because water is lighter. Now, if we then add to the cylinder some of the still lighter ether, that ether will stay as a layer on top of the water layer. After that, we wait and will soon witness a demonstration of selective permeability of ether over CCl_4 through the water layer or membrane — in consequence of the different solubility of ether and CCl_4 in water. And, this is how it works.

Since ether is soluble in water but CCl_4 is not, ether would enter and permeate the water layer to reach and accumulate in the CCl_4 but CCl_4 , being water-insoluble, cannot enter or permeate the water layer to reach the ether layer. This, was, of course an elegant demonstration of the principle of the solution theory of membrane permeability. It works well in an experiment of short duration, say a few days.

However, the experiment will no longer work if the duration of the experiment is much longer. Indeed, given enough time, equilibrium would be reached among all three com-

ponents. At that distant time, the amount of ether in the CCl_4 layer as well as the amount of CCl_4 in the ether layer would be totally indifferent to the presence of the water layer. It may be mentioned that up to the end of the 19th century, cell physiologists had been thinking mostly in short time scales. That would soon change.

Overton's Lipoidal Theory received support many years later from the study of Collander (1954) in work summarized in Figure 9. The data on first look appears most impressive. However, a closer look reveals a serious weakness. In fact, the weakness revealed was only a part of growing evidence that not only Overton's Lipoidal Membrane theory but the basic assumption underlying the plasmolysis and deplasmolysis method are increasiangly in doubt. Three sets of these evidence will be considered next.

(1) The lipoidal membrane is not semipermeable

The data shown in Figure 9 clearly shows that the lipoidal membrane *is not* ***semipermeable***.

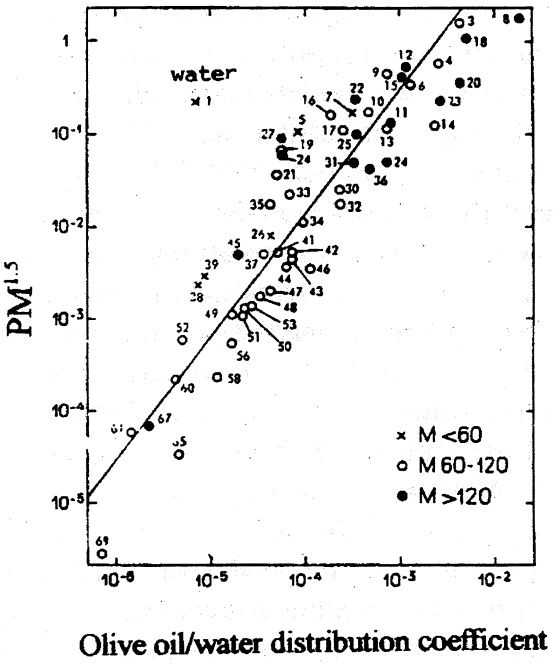


FIGURE 9. Correlation between the permeation power of various non-electrolytes into *Nitella mucronata* cell sap on the one hand and their respective oil solubility and molecular weights on the other. Ordinate represents $PM^{1.5}$ where P is the measured permeation rate of a solute studied and $M^{1.5}$ is its molecular weight raised to the power 1.5. To the best of my knowledge, this factor, 1.5 has no theoretical basis and is introduced to produce a straighter line. Abscissa is the solute's distribution coefficient between olive oil and water. The word, water, is added by the present review writer and not in the original graph. For the meanings of all the numbers in the graph, the reader must consult the original article. Only a selected few will be given here. 1, water; 3, methyl acetate; 7, ethanol; 8, paraldehyde; 52, ethylene glycol; 69, glycerol. (from Collander 1959, his Fig. 1 on his page 43, by permission of Elsevier (formerly Academic Press)

As mentioned earlier, it was van't Hoff who introduced the term, *semipermeability* (van't Hoff 1885.) It means a membrane, which allows the passage of water but none of the substances dissolved in water. Clearly, this is only a stipulation, never seen in real life. Notwithstanding, all cell membranes have been routinely referred to as semipermeable because they all demonstrate the highest permeability toward water. However, the oil/water distribution coefficient of water itself — shown as abscissa in Collander's figure shown here as Figure 9 — is not higher than that of ethanol but 200 times lower than that of ethanol. That being the case, it would predict a permeability of ethanol 200 times higher than water, contrary to facts including what Abbé Nollet first showed in pig's bladder.

(2) In plant cells, it is the tonoplast, and not the plasmahaut, that acts as a semipermeable barrier during plasmolysis

Höfler invented a method for measuring the size of the (irregularly-shaped) protoplast in a plant cell (Höfler 1918.) With its help, he demonstrated that the plant cells he studied acted like a perfect osmometer — as it was proudly announced by reviewers Lucké and McCutcheon in 1932 (Lucké and McCutcheon 1932, pp. 86–87.)

Later work, however, led Höfler to a different conclusion (Höfler 1926, 1931, 1932.) Namely, it is the tonoplast that immediately surrounds the central vacuole that acts like the semipermeable barrier. In contrast, the plasma membrane was quite permeable to sucrose — contradicting the conclusion he himself reached earlier as well as the long-held belief, in particular the belief derived from the extensive work of Overton described above, that the cell membrane, *alias* plasmahaut, is impermeable to sucrose. Subsequently, Chambers and Höfler confirmed Höfler's later conclusion by isolating the tonoplast-enclosed central vesicle and demonstrated sucrose-concentration dependent shrinkage of the assembly (Chambers and Höfler 1931.)

(3) Sucrose and galactose enter and accumulate in frog muscle cells at the same time causing their sustained shrinkage

This simple but highly important finding of Nasonov and Aitzenberg shows that the ability of a concentrated solution of a chemical compound to cause cell shrinkage does not depend on the impermeability of the cell membrane to this compound. Thus, they showed the supposedly impermeant sucrose (and galactose) not only penetrate muscle cells, they cause sustained shrinkage. of the muscle cells at the same time (Nasonov and Aitzenberg 1936; Kamnev 1938) (Figure 10.)

This finding and its later extensive confirmation and extension (see Ling 1992, pp. 249–272) have falsified much of the original conclusions on the nature of the cell membrane permeability and impermeability from plasmolysis, deplasmolysis and other cell-volume-change studies. The data also show that volume changes of the protoplast of plant cells and of animal cells as a rule do not reflect the physical characteristics of the cell membrane but reflect primarily the nature of the protoplast as a whole. New explanations of the phenomena, which fall beyond the time period of history which the present review covers, will not be described here. Interested readers can access to new facts and information from Ling 1992, pp. 249–272 and http://www.physiologicalchemistryandphysics.com/pdf/PCP19-159_ling.pdf> <http://www.physiologicalchemistryand physics.com/pdf/PCP19-177_ling_ochsenfeld.pdf>

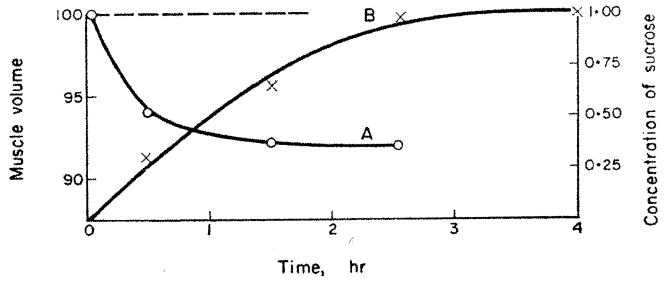


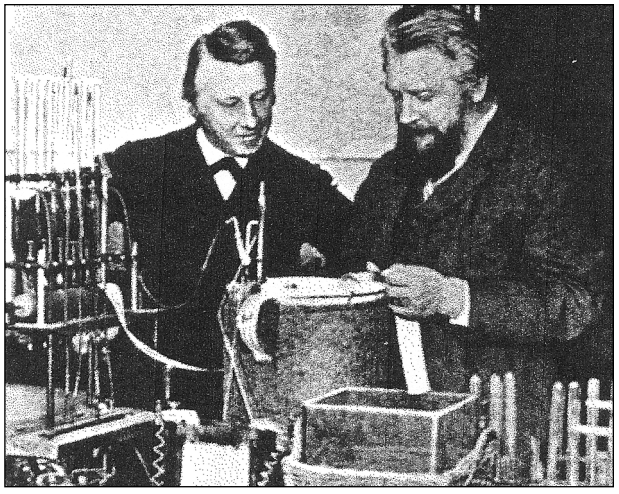
FIGURE 10. Time course of weight change of a frog muscle in a hypertonic (4%) sucrose solution (A) and of concomitant gain of sucrose by the muscle (B). Weight change given as percentage of control. Sucrose accumulation given in per cent per 100 grams of muscle cell water. (from Nasonov and Aizenberg 1937, Kamnev 1938)

A theory explicitly named the MembraneTheory finally arrived-but it was intended for a different subject matter

Thus far, we have gone to some length tracking down the real originator(s) of the membrane theory — to this day still widely taught as proven truth around the world. So far, we have found three: Dutrochet, Schwann and Overton — even though none of them claimed that authorship.

So it is somewhat anticlimactic that someone finally came forth with a theory bearing the name, the membrane theory — only to find, instead of the familiar one that deals with membrane permeability and related phenomena, a membrane theory of the electric potential instead.

Historically, this specific membrane theory began with the studies of the electric potential difference measured across two salt solutions separated by a copper-ferrocyanide-precipitation membrane. And, it was conducted by the outstanding Russian-German physical chemist already mentioned once, Wilhelm Ostwald (1853–1932.)



J. H. van't Hoff (1852–1911); Wilhelm Ostwald (1853–1932)

Toward the end of his paper, Ostwald suggested that the electrical potential of muscle and nerve, and indeed even that of the electric fish could originate from a similar mechanism (Ostwald 1890, p.80.)

However, in 1900, J. S. MacDonald — apparently unaware of Ostwald's suggestion — stole the show. For

it was MacDonald who first demonstrated that the so-called *injury potential* (or demarcation potential) measured between the intact surface of a nerve and its cut end could be shown to vary with the logarithm of the concentration of K^+ in the medium bathing the intact portion of the nerve (MacDonald 1900.)

Two years afterward, Julius Bernstein, a student of Ludwig von Helmholtz at the University of Berlin, took up the suggestion of Ostwald (Bernstein 1902, p. 541) and proposed the *membrane theory* for the electrical potential across the normal cell membrane of muscle and nerve cells (Bernstein 1902, p. 542.)

Based on an equation proposed earlier by W. Nernst (1864–1941) (Nernst 1889), Bernstein wrote an equation for this *membrane potential,* E, on the assumption that the cell membrane is permeable to K^+ but impermeable to both the intracellular anions and to Na^+:

$$E = RT/F \; ln \; (p_2 / p_1), \tag{3}$$

where R is the gas constant, T, the absolute temperature, F, the Faraday constant. p_2 and p_1 are respectively the osmotic pressure of the inside and outside of the cell. Since osmotic pressure is directly proportional to the concentration of the permeant ions as shown in Equation 2 above, the ratio, p_2 / p_1 is equal to the ratio of the two (permeant) K^+ concentrations. Accordingly, Equation 3 can be written in another form:

$$E = 2.303 \; (RT/F) \; \log \; ([K^+]_{in} / [K^+]_{ex}), \tag{4}$$

where $[K^+]_{in}$ and $[K^+]_{ex}$ are respectively the intra- and extra-cellular concentrations of what Bernstein recognized as the major permeant ion, K^+.

Bernstein's theory of cellular electric potential requires that the cell membrane is completely impermeable to anions. It also requires that all the intracellular K^+ are free and so is the bulk-phase cell water. Yet, a careful investigator would have no trouble even then locating evidence pointing to the opposite.

Julius Bernstein
(1839–1917)

Walther Hermana Nernst
(1864–1941)

Thus, Macallum (1905) and especially Menten (1908) showed microchemical evidence that cell K^+ is not free and evenly distributed in cell water but localized, on the two edges of the dark bands of striated muscle .

There were also repeatedly reported evidence that not all cell water is free liquid water. Thus, E. Overton noted that when frog muscle was placed in an artificial Ringer's solution of half osmotic strength, the muscle did not swell to twice its original weight but only to about a third higher than its original weight. Overton concluded that at least some of the water in frog muscle cells is what he called "swelling water" ("*Schwellungswasser*") (Overton 1902.) This was far from an original opinion. Wilhelm Pfeffer offered the same explanation for a similar phenomenon years before (Pfeffer 1881, 1897.) Similarly, Hofmeister (1891) and Rudolf Höber (1906, p. 61, 62 and 70) Rubner (1922) and Thoenes (1925) had made similar suggestions.

However, it was Gortner and his coworkers that strongly argued that a substantial part of the water in living tissues is "bound water" (Gortner 1930.) Since such bound water has been shown to have lost its normal solvency for solutes such as sucrose (Gortner 1938 pp. 279–280), this part of the water was sometime referred to as "non-solvent water". The implicit assumption was that water that has lost its solvency for sucrose has lost its solvency for all solutes that normal water dissolved. But there was no experimental proof for this hypothetical doctrine.

Then, a very powerful cell physiologist came on the scene. His name is Archibald Vivien Hill or A. V. Hill for short. Based on two simple sets of experiments, he scored a total victory for the free water and free K^+ doctrines of the membrane theory.

How A. V. Hill persuaded the opinion makers of the day that both cell water and cell K+ are free

Archibald Vivian Hill (1886–1977) was a remarkable English scientist. Tall, handsome, athletic, Cambridge-educated and married to Margaret Keynes, daughter of the famous economist, John Neville Keynes and sister of the equally famous economist, John Maynard Keynes. In 1922 A.V. Hill and Otto Meyerhof were conjointly awarded the Nobel Prize of Physiology and Medicine. All these Nature-made and Man-made admirable assets can be read in any biography of A. V. Hill explaining why he was so highly regarded and influential. But there was something else in Hill that had made him even more persuasive as a cell physiologist.

Archibald V. Hill
(1886–1977)

Sir William Maddox Bayliss was the author of the highly popular *Principles of General Physiology*. Bayliss believed that the greatness of a scientific investigator does not lie in his never making a mistake, rather it lies in being able to give up his once cherished idea when cogent evidence point to its fallacy. And this sentiment was printed in the Preface of all editions of the book including the 4th edition which Bayliss was too ill to complete. In his place, a good friend took over the task of getting the book published and it was. That friend was no other

than A.V. Hill who expressed his admiiration of Bayliss's book as "the greatest of its kind." And, Hill's own personal behavior pattern showed that he thoroughly shared Sir Bayliss's belief in what de Mirbel, Dutrochet, Thomas Huxley, Höfler and William Bayliss all believed and lived.

Hill studied muscle contraction and in time, he offered what was known as the "lactic acid theory" for muscle contraction. In this theory, muscle contraction was brought on by the production of lactic acid. However, a German biochemist, by the name of Gustav Embden (Embden *et al* 1926) argued that lactic acid production comes after and not before muscle contraction and therefore could not be the initiator of muscle contraction. And the two were engaged in a bitter battle back and forth, until a critical discovery was made and announced by a Danish scientist, Einar Lundsgaard.

Lundsgaard discovered that exposure to the chemical, iodoacetate (IAA) suppressed the production of lactic acid by muscle tissue without materially altering its ability to contract (Lundsgaard 1930.) Faced with this new cogent evidence, Hill admitted his earlier mistake. In an article he wrote for the *Physiological Review* under the title, "The Revolution in Muscle Physiology" Hill wrote, "He who laughs best who laughs last." Hill then admitted that he was not the last to laugh (Hill 1932.)

This event showed that Hill was a scientist who put the search for truth above his own ego — an ethical belief that makes scientific revolution possible as the title of his article so indicated. That said, we now return to the two pivotal sets of experiments Hill and his coworker, Kupalov, carried out.

Both are extremely simple. In one, he and Kupalov, measured the vapor pressure of normal living frog muscle and found that it equals that of an isotonic Ringer's solution — hardly surprising by itself (Hill and Kupalov 1930.) In the other experiment, Hill showed that the steady level of the probe molecule, urea, equals exactly that of urea in the bathing solution. This indicates that there is not "non-solvent" water in these frog muscle cells. That being the case, the solute within the cell that is high enough in concentration to produce a vapor pressure matching that of an isotonic NaCl solution of the bathing Ringer's solution can only be K^+ {and its companion anion(s)} in the cell. Ergo, both cell K^+ and cell water are completely free (for a complete review of this historical page, see Blanchard 1940.)

Hungarian physiologist, E. Ernst (1895–1981) who had witnessed all these, wrote later about the historical aftermath of this chapter. Ernst showed how in one stroke, Hill convinced opinion makers of the time, including W.O. Fenn, Rudolf Höber, F. Buchthal to abandon their earlier belief in bound water and bound K^+ and to adopt wholesale the free water-free K^+ doctrine of the membrane theory (Ernst 1963, p. 112.)

> Eventually, Hill's concept of free K^+ and free cell water were proven wrong. However, the subject falls outside the time span the present review covers and will not be discussed here. Interested readers can find detailed answers to Hill's powerful but mistaken conclusions on p. 100 in Ling 2001. Alternatively, the reader can access the key experimental basis for the refutation in <http://www.physiologicalchemistryandphysics.com/pdf/PCP21-19_ling_ochsenfeld.pdf>

Now, beside free water and free K^+, Bernstein's membrane theory of cellular electric potential also requires that the cell membrane be impermeable to anions like Cl^- (and Na^+.) Bernstein did not put this prediction to a test himself but Loeb and Beutner did.

In 1912, Loeb and Beutner introduced another membrane model, the apple skin (Loeb and Beutner 1912.) They demonstrated that the anions of the salt solutions placed on either side of the apple skin exercised no influence on the electric potential measured across it. The conjecture that this anion insensitivity reflects a low anion permeability of the apple skin was confirmed by Leonor Michaelis (1875–1949) and Fujita by direct measurement of ion passage through another membrane model, the dried collodion membranes (Michaelis and Fujita 1925.) In addition, Michaelis and Fujita showed that while the dried collodion membrane is virtually impermeable to anions, it is permeable to all mono-valent cations studied; their rates of permeation follow the rank order: $H^+ > Rb^+ > K^+ > Na^+ > Li^+$.

This is, of course, the same rank order of the mobilities of these cations in normal liquid water but the differences among them are greatly exaggerated here (Michaelis 1926, p. 39, Col. 1.) To explain, Michaelis pointed out that in aqueous solutions, these ions do not exist in their naked atomic form but exist in a hydrated form. And, as such they assume a rank order in their relative size exactly the opposite of the rank order of their respective atomic sizes, as suggested in the theories of Born and Fajans (ibid p. 42, Col 1.)

Michaelis went on to suggest that the narrow pores of the dried collodion membrane and apple skin adsorb and fix anions on their surfaces, thus endowing the pores with negative electric charges. And, in some way these negative electric charges put to a stop movement of free anions like Cl and slow down the movement of monovalent cations in a reverse order as that in their hydrated ionic diameters (Michaelis 1925, p. 36.)

We recall that it was Ostwald's suggestion that Bernstein took up with very satisfying consequences. Another scientist that took note of Ostwald's tip was chemist, F. Donnan.

Donnan's theory of membrane equilibrium

F. Donnan (1870–1956) introduced an equation describing the relationship between the electrical potential difference ($\pi_2 - \pi_1$), and the concentrations of permeant ions across two contiguous aqueous phases separated by a membrane that is impermeable to one ionic species found only in one phase (Donnan 1911, 1924):

Leonor Michaelis
(1875–1949)

Frederick Donnan
(1870–1956)

$$\pi_2 - \pi_1 = \{RT/nF\} \log (C_2 / C_1), \quad (5)$$

where π_2 and π_1 are respectively the electric potential in phase 2 and phase 1. R, T and F are as in Equation 3. C_2 and $C_{1,}$ are the concentrations of the permeant cation in phase 2 and phase 1 respectively. n is the valency of the permeant cation. $\pi_2 - \pi_1$, the electric potential difference between the two phases is equivalent to Bernstein's membrane potential though not identical. If the permeant ions present include monovalent cation K^+ (and Cl^-) and if phase 2 and 1 are respectively called intra-cellular and extra-cellular, designated by subscripts, in and ex, the concentrations of K^+ in the two phases are then represented by $[K^+]_{in}$ and $[K^+]_{ex}$ respectively, and Equation 5 then assumes a form similar to Equation 4 presented earlier.

The Donnan theory does not require the membrane to be impermeant to chloride ion as in Bernstein's Membrane Theory of the cellular membrane potential. Thus, in the Donnan theory (alone) can the potential difference also be written as

$$\pi_2 - \pi_1 = \{RT/F\} \log ([Cl^-]_{in} / [Cl^-]_{ex}), \quad (6)$$

where $[Cl^-]_{in}$ and $[Cl^-]_{ex}$ are the concentration of the (monovalent) chloride ion in the cell water and in the external bathing solution respectively. And,

$$[K^+]_{in} / [K^+]_{ex} = [Cl^-]_{in} / [Cl^-]_{ex} , \quad (7)$$

where these K^+ and Cl^- concentrations are ***equilibrium*** concentrations. Moreover, the Donnan theory predicts that in the same system, the equilibrium concentraton ratio (λ) of all permeant ions, positively charged as well as negatively charged, are predictable according to the following general equation:

$$\{C_2 / C_1\}^{1/n} = \{A_1 / A_2\}^{1/m} = \lambda , \quad (8)$$

where C_2 and C_1 are the equilibrium concentration of a cation of valency n in phase 2 and 1 respectively, while A_1 and A_2 are the equilibrium concentration of anions A of valency m.

Donnan's theory of membrane equilbrium gained attention when it became increasingly clear that, the highly asymmetrical distribution in living cells of the pair of chemically almost indistinguishable cations, K^+ and Na^+ was not an exception but a general rule.

A shared attribute among most if not all living cells: asymmetric distribution of K^+ and Na^+

In 1807, the great Swedish chemist, J. J. Berzelius (1779-1848) determined the mineral contents of muscle tissue (Berzelius 1840.) Half a century later, Julius Katz carried out an exhaustive analyses of eight elements — K, Na, Ca, Mg, Cl, S, P, Fe and water — in the muscle tissue of thirteen vertebrates, ranging from human to eel. Hidden amongst the large number of numbers is the striking and consistent asymmetry in the K and Na contents of muscle tissues — clearly demonstrated and recognized by the author of the work (Katz 1896.) Table 2 reproduces the K and Na data of Katz.

TABLE 2. H_2O, K^+ and Na^+ contents of the muscles of various vertebrates. Data given originally in weight percentage have been converted to millimolarity per kilogram of wet weight. (from J. Katz 1896)

	H_2O (%)	K^+ (mmoles / kg wet wt.)	Na^+ (mmoles / kg wet wt)
Human	72.53	81.94	34.72
Pig	75.89	64.92	67.83
Cattle	75.80	94.08	28.36
Calf	75.39	97.21	37.38
Deer	75.27	85.92	30.63
Rabbit	76.83	103.6	19.89
Dog	76.42	85.55	41.02
Cat	75.14	99.03	31.70
Chicken	68.38	94.62	41.36
Frog	81.61	78.76	24.02
Cod	80.63	85.57	43.10
Eel	84.92	25.26	5.33
Pike	79.88	103.8	9.85

Two years after the publication of Katz's monumental work, Emil Abderhalden (1877–1950), a student of the great German chemist, Emil Fischer who almost single-handedly worked out the structure of proteins, demonstrated that human red blood cells contain a high concentration of potassium ion (K^+) but no sodium ion (Na^+) at all, whereas the blood plasma in which the red blood cells spend their lives contains only 6.59 mM of K^+ but a whopping 193 mM of Na^+. Abderhalden's original table, is reproduced here as Table 3 but data are given in molarity rather than in weight percentage as they were given in the original publication (Abderhalden 1898.)

This report of zero Na^+ in rabbit red blood cells could have led others to suspect that a similar situation might obtain in other cells. As an example, the small concentration of Na^+ in vertebrate muscle tissue shown by Katz might originate from outside the muscle cells like the extracellular space and the connective tissues intermingled with the muscle cells. Thus in (the 4th edition of) the *Principle of General Physiology* mentioned above, its author, William Bayliss wrote "It is almost certain that there is no sodium ion in frog muscle cells" (Bayliss, 1927, p.121.)

TABLE 3. K^+ and Na^+ contents of rabbit blood plasma and rabbit red blood cells. Data given originally in weight percentage have been converted to millimolarity (plasma) and millimority per kilogram wet weight (red blood cells.) (from Abderhalden 1898)

	plasma (MM)	Red blood cells (mmoles/kg)
Potassium	6.59	133
Sodium	193	0

Historically, this perception marked a turning point in the history of the membrane theory. From here on, the membrane can no longer be seen as an instrument that determines merely the *rates* of passage in and out of the living cells of water and solutes. The cell membrane must also determine what can enter or leave as an *all-or-none* event. That is, impermeant solutes are not judged on the time scale of a few hours or even a few days but for all intent and purposes, as long as the cell lives.

In fact, the thinkers in this field began to do what one may say recast van't Hoff's idea of semipermeability in a different time scale. So instead of having in one category one item, water alone as being able to pass through or permeant, we will add to water also some specific solutes that are also considered permeant. However, all other solutes will be put *into the permanently and absolutely impermeant* category.

Molecular sieve theories again

Although the atomic sieve idea of Traube did not pan out for unglazed porcelain barrier with or without colloidal additives as shown in Table 1 (Bigelow and Bartell 1909), negative correlation of rate of permeation with molecular size continued to be reported by investigators including Bigelow and Bartell. Thus, Table 4 shows Fujita's data on the permeability of non-electrolytes ranging from methanol to glucose through two kinds of colloidion membranes, one more dried than the other (Fujita 1926.)

In a series of papers published between 1908 and 1912, Ruhland offered what he called the *Ultrafilter Theory*, which, on the surface, is just another name for Traube's atomic sieve theory. In 1925 Ruhland and Hoffmann published the data they obtained by the osmotic or plasmolysis method on sulfur bacteria (Table 5.)

Here, they demonstrated a close correlation between the permeability of the nonelectrolytes and their respective molecular volumes — given in the form of molar refraction MR_D (see Glasstone 1946, pp. 528–529, for derivation of the identity of MR_D and actual volume of molecules.) The last column of their table shows a lack of correlation between the rates of permeation and the ether/water distribution coefficients of the solutes studied, thus refuting Overton's lipoidal theory.

A few words need be added to give Ruhland's view of how pore size could produce a graded permeation rate rather than an all-or-none permeant-impermeant expectation. The difference lies in the uniformity or lack of uniformity in the pore size. In Ruhland's theory he did not propose a uniform pore size of a certain diameter. Rather, he visualized that the cell membrane has pores of different sizes. Since small solutes would be able to traverse through both small and larger pores, they enter more rapidly. In contrast, the larger solutes would have less pores available for them and hence enter more slowly.

Given what we found out about lipoidal membranes — which are permeable to lipoid soluble substances but practically impermeable to electrolytes — and living cell membrane — which show permeability to both nonelectrolytes and electrolytes — it seemed natural for someone to introduce a compromise. Indeed, that was what Nathanson did in what is known as *the mosaic theory* (Nathanson 1904a, 1904b.)

In this theoretical idea, the living cell membrane might represent a mosaic membrane containing both lipoidal areas and pores. Indeed, what later on was introduced by Davson and Daniellie as the so-called Paucimolecular membrane theory (Davson and Danielli

TABLE 4. Relative permeability (P) of two types of collodion membranes, one with wide pores (a) and the other with narrow pores (b). Molecular weight of solutes are shown under M. Portions of the original table giving the values of $PM^{1/2}$ of the solutes are not shown. (Fujita 1926)

Substances	MR_D	M^c	Relative permeability (a)	Relative permeability (b)	$PM^{1/2}$ (a)	$PM^{1/2}$ (b)
Methyl alcohol		32	1.22	9.24	6.9	52.4
Acetone		58	1.11	7 08	8.5	53.9
Formamide		45	1.06	4.11	7.1	27.6
Ethyl alcohol		46	1.15	2.98	7.8	20.2
Propyl alcohol		60	1.00	1.03	7.7	8.0
Urea	16.67	60	1.00	1.00	7.7	7.7
Butyl alcohol		74	0.85	0.82	7.3	7.1
Ethylene glycol	14.40	62	0.80	0.27	6.3	2.1
Glycerol	20.63	92	0.81	0.22	7.7	2.1
Chloral hydrate		165	0.81	0.11	10.4	1.4
α-Monochlorohydrin		110	0.70	0.07	7.3	0.7
Glucose	37.54	180	0.54	0.00	7.2	0.0

TABLE 5. The permeability of the sulfur bacteria, Beggiatoamirabilis to various nonelectrolytes obtained by the plasmolysis method. MR_D, the molecular extinction of the different solutes equal the molecular volumes of the respective nonelectrolytes. (from Rhuland and Hoffmann 1925)

Substance	Threshold plasmolytic concentration	MR_D	Distribution coefficient between: ether-water
Urea	0·35	16·67	0·0005
Ethylene glycol	0·09	14·40	0·0068
Methylurea	0·01	18·47	0·0012
Thiourea	0·075	19·59	0·0063
Glycerol	0·009	20·63	0·0011
Ethylurethane	0·015	21·01	0·6370
Lactamide	0·007	21·13	0·0018
Malonamide	0·007	22·92	0·0003
Dimethylurea	0·005	23·43	0·0116
Butyramide	0·00125	24·11	0·0580
Erythritol	0·001	26·77	0·0001
Succinamide	0·0015	27·54	0·0002
Arabinose	0·0008	31·40	0·0001
Diethylurea	0·003	32·66	0·0185
Glucose	0·00055	37·54	0·000l
Mannitol	0·00055	39·06	0·0001
Sucrose	0·00020	70·35	0·0001

1943) as well as the Singer-Nicolson model (Singer and Nicolson 1972) widely taught in all levels of education are variants of the Nathanson idea. As such, they all share a weakness. Namely, given enough time, the lipoidal part of the membrane would be no barrier to the passage of solutes big or small. As a result, all solutes would end up at the same concentration found in the surrounding media. And, since most living cells live a long time, the mosaic membrane in all its various forms cannot explain the sustained asymmetrical solute distribution patterns seen in all living cells.

To explain the sustained asymmetry of K^+ and Na^+ distribution in living cells, a return to the sieve membrane with rigid pores of a uniform size

From studies of perfused frog legs, Mond and Amson came to the concluson that the muscle cell membrane is totally impermable to anions and to cations like Na^+ and Li^+ but permeable to K^+ and Cs^+ (Mond and Amson 1928). To interpret their data, they adopted the model of Leonor Michaelis mentioned earlier. That is, the muscle cell membrane shares the basic characters of dried collodion membrane with narrow pores lined with negative charges. Since hydrated Na^+ and Li^+ are larger and suffer more collisions and resistance, they show slower mobility in the collodion membrane pores, while hydrated K^+ and Cs^+ are smaller and therefore suffer less resistance in their motion through the narrow membrane pores (Michaelis 1925, 1926.) However, this model was soon challenged by Boyle and Conway from the University of Dublin.

Boyle and Conway started out with a careful investigation of the movement of Cl^- when frog muscles were incubated in a Ringer's soluton containing a high concentration of KCl. They found a large gain of both K^+ and Cl^- by the muscle cells without impairing the cell membrane's normal ability to keep the high concentration of Na^+ in the external solution from entering the cell *en masse*. This study led Boyle and Conway to conclude that the normal frog muscle cell membrane is in fact quite permeable to Cl^-.

Edward J. Conway
(1894–1968)

That being the case, the installation of negative charges in the pores in order to explain the anion impermeability of the dried collodion membranes is no longer required. With this complication out of the way, Boyle and Conway then proposed once more a pure sieve membrane theory with uniform pore size (Boyle and Conway 1941.) What was more, they then took the model one step further and made it a quantitative theory for both anions and cations as shown in the historically highly important Table 6.

What the theoretical model tells us is that *all pores in the muscle cell membrane have exactly the same size*. And, this critical pore size is so uniform and the wall of the pores so rigid, that the pore size determines what can enter the cell and what cannot — all on a permanent and absolute basis. That their argument is not merely fantasy but based solidly on the known mobility data of all the anions and cations under consideration is shown in the top part of Table 6.

TABLE 6. Boyle and Conway's table describing their unifying and quantitative sieve membrane theory The mobility data given on the left-hand side of the table were from the International Critical Table and Chemicher Kalender. The relative ionic diameters were calculated on the basis of their relative mobilities and on the assumption that K^+ has a relative diameter of unity. (from Boyle and Conway, by permission of the Journal of Physiology)

Velocities of ions under gradient of I V./cm. or 0·5 V./cm. for divalent ions				Relative ion diameters (diameter of potassium ion = 1·00)			
Cations		Anions		Cations		Anions	
H	315·2	OH	173·8	H	0·20	OH	0·37
Rb	67·5	Br	67·3	Rb	0·96	Br	0·96
Ca	64·2	I	66·2	CS	1·00	I	0·97
NH_4	64·3	Cl	65·2	NH_4	1·00	Cl	0·98
K	64·2	NO_3	61·6	K	1·00	NO_3	1·04
Na	43·2	CH_3COO	35·0	Na	1·49	CH_3COO	1·84
Li	33·0	SO_4	34·0	Li	1·95	SO_4	1·89
Ca	25·5	HPO_4	28	Ca	2·51	HPO_4	2·29
Mg	22·5			Mg	2·84		

So as it was suggested by Netter twelve years before (Netter 1928), Boyle and Conway also suggested that the muscle cell behaves in their ionic distribution according to Donnan's theory of membrane equilibrium.

In the theory, certain unspecified organic anions in the cell are too large to move out of the cell through the narrow pores and stay inside permanently, thereby serving the role of Donnan's impermeant anion(s). The presence of these impermeant anions creates a large value of the Donnan ratio, λ, as defined in Equation 7. However, since *(hydrated) Na^+, the major external cation, is too large to penetrate the pores, it stays outside absolutely and permanently*. Meanwhile the smaller hydated K^+ can enter the cell via the membrane pores, until it reachs the concentration dictated by the value of λ, which is apparently equal to about 50 or so. Cl^-, in contrast, distributes at an intra-, extra-cellular ratio equal to the reciprocal of λ, which is quite low as also in agreement with known facts.

But that is not all. The theory simple as it is can not only explain selective permeability and selective ionic accumulation but also the cellular electric potential as a Donnan membrane potential and the swelling and shrinkage or volume control on account of the osmotic effect.

In other words, Boyle and Conway's sieve theory as presented in 1941 was a unifying theory able to explain all four basic physiological manifestations of the living cell. To complete the verification of this unifying membrane theory, one goes back to what A.V. Hill did in his two famous papers, one by himself and one in cooperation with Kupalov, which provided experimental proof of the free cell water and the free cell K^+ doctrine of the membrane theory (Hill 1930; Hill and Kupalov 1930.)

Thus, the 63-page long opening article of the 100th volume of the *Journal of Physiology* (London) from Boyle and Conway holds a historic position in presenting the most complete and finalized version of the membrane theory.

This theory was not introduced by Wilhelm Pfeffer as I had once wrongly believed and retracted above. Who then really introduced the membrane theory? The reader knows by now from the preceding pages that it received contributions all the way from the time Robert Hooke introduced the word cellulae or cell in 1665 until Boyle and Conway introduced their molecular sieve theory in 1941.

First, consider the word, cell. It is a walled cavity or a little box. A cell will remain a cell if it is kept in a vacuum. In this case, the only thing in existence is the wall, distinguished by its continuity and hence its acting as a barrier that separates a small space from the surrounding usually larger space. So when Theodor Schwann introduced his Cell Theory, he postulated that all animal and plant cells are walled cavities filled with a clear fluid. As such, the wall or membrane owns a clearly delineated surface on both the inside and outside faces. Whatever the cell does and is, it must be based on the property or activity of this membrane. This, then is the central theme of the membrane theory.

Now, most historians denied that Hooke had seen living cells but he introduced the word, cell. And, among the things he saw, there could be dead cells, dried up cells — but cells nonetheless. So Hooke in my view definitely contributed to the membrane theory since the cell is a continuous spherical membrane. But so did Abbé Nollet though he was even less involved consciously. Just the same, his work introduced the central theme of the membrane theory — semipermeability of a membrane barrier.

The next person on the time line was Henri Dutrochet. Conceptually, he transferred macroscopic endosmosis and exosmosis into the microscopic world of living cells. But his work was under-appreciated despite all the fine qualities, high intelligence, originality and honesty of this great scientist.

The most influential contributor to the membrane theory was without question Theodor Schwann. His magnus opus, *Mikroskopische Untersuchungen*, gained instant and unquestioning support by all around and was immediately built into the dominant German textbooks — despite the profusion of mistakes one piled upon another in his work and a pontific, self-seeking attitude through and through.

After Schwann, the membrane theory changed from an anatomical concept to a physiological concept. Traube, Pfeffer, van't Hoff all made significant contributions toward the membrane theory, So did de Vries, Overton, Michaelis, Mond, Netter and finally, Boyle and Conway.

Experimental testing of the Boyle-Conway's version of the membrane theory and its outcome

So there are many winning merits in this theory of Boyle and Conway. Its appearance on page 1 of the 100th volume of the prestigious *Journal of Physiology* (London) symbolizes the broad esteem in the minds of many workers at the time. However, though rarely if ever announced publicly, there is also a slippery side to the underlying postulation of a uniform pore size that sorts solutes into two sharply separated categories, permeant and impermeant. In fact, this separation of solutes into two sharply separated categories already began with van't Hoff's seemingly casual launching of his *concept of semipermeability*. That is, a membrane that is permeable to water but not to solutes dissolved in water.

Now, the word, semipermeability describes a physical attribute, presumably there as long as the membrane exists. So that means, as long as the membrane lasts — which in theory could be *forever*, it is absolutely impermeable to the solute dissolved in water. Of course, in reality, no such membrane has ever been found or created.

Nonetheless, as mentioned once already, in the hands of Morse, a copper-ferrocyanide membrane could sustain an osmotic pressure of 12 atmosphere for 60 days without showing sign of deterioration. A. Findlay who wrote about this admiringly could be forgiven for saying that "it appears to be truly semipermeable." However, my guess is that neither Morse nor Findlay would venture a suggestion that this pressure could be sustained for 60 years. Yet 60 years may very well be the life spans of many living cells in a human being, an elephant or a turtle.

Then we have the famous experiment of de Vries's red-beet root cells, that could sustain a shrunken plasmolyzed protoplast for seven days. One could hardly extrapolate from that to a life span of 60 year-old cell either.

Yet, there is no indication that as a human or an elephant ages, their cells would become filled with Na^+ rather than K^+. On the contrary, the asymmetric distribution of thus pair of ions is, generally speaking, age-independent. So the Boyle-Conway Sieve version of the membrane theory really stands on the thin ice of an idealized physical attribute that does not jibe with reality. With this in mind, it is not all too surprising that as soon as a clearly-defined theory like that of Boyle and Conway appeared in print in 1941 — indeed even before that, the theory was engulfed in a torrent of contrary evidence and totally destroyed. With it, died the sieve version of the membrane theory of the living cell as well as the different mosaic membrane models containing as an integral part the mechanical sieve or ultrafilter notion.

Unanimity in the conclusions of the earliest studies — the cell membrane is permeable to Na^+ and other large hydrated ions — theoretically predicted to be absolutely impermeable

The suddenness of the demise of the sieve version of the membrane theory owed to no small extent to the advent of a powerful technique, the radioactive tracer technology in the late 1930's. This technology is not only extremely accurate, *it is also the only technology that makes direct measurement of ion permeation and exchanges possible for the first time in history*.

However, not all the incisive experiments conducted to test the theory involved radioactivity. The following studies are listed according to the sequential order of their publication dates.

1912: P. Gérard found that in feeding dogs with an excess of K^+ salt, cells of the liver and kidney gained K^+ and lost Na^+ without a change in the concentrations of either ion in the blood plasma (Gérard 1912.)

1931: Wu and Yang injected NaCl solutions into the veins of dogs and found a rise in the concentration of Na^+ in the muscle cells. The authors concluded that the muscle cell membrane is permeable to Na^+ (Wu and Yang 1931.)

1933,1934: Kaplanskii and Boldyreva kept carps in a 1.5% NaCl solution for 70 days and found a less than 5% increase in the Na^+ concentration in the blood plasma, while the Na^+ concentration in the muscle tissues nearly tripled from 40 mg. per cent to 111.4 mg. per cent. These authors too concluded that the muscle cell membrane of carp muscle is permeable to Na^+ (Kaplanskii and Boldyreva 1933, 1934.)

1939: Cohn and Cohn injected radioactive isotope, ^{23}Na in isotonic saline into the veins of dogs and assayed the radioactivity of samples of blood plasma and red blood cells at different lengths of time after the initial injection. They showed that the ratio of the radioactivity in the red blood cells as a fraction of that in the blood plasma steadily rose until it reached above 65% after about a day. The authors concluded that the red blood cell membrane of the dog is permeable to Na^+ (Cohn and Cohn 1939.)

1939: Heppel fed rats on a low potassium diet for from 34 to 44 days before the animals were sacrificed and their muscle analyzed for its water, K, Na (Cl, P) contents. Heppel's data showed that the average K content of the muscle tissue had fallen from the normal tissue value of 109 mmoles per kg. fresh weight to 64.1 mmoles/kg., while the Na content rose from the normal value of 19 mmoles/kg to 54 mmoles/kg. Other studies show that the gain of Na in the muscle tissue was almost entirely inside the muscle cells. The author concluded that the muscle cell membrane of rats is permeable to Na^+ (Heppel 1939.)

In a later paper published in 1940, Heppel showed that the time it took for radioactive ^{24}Na in the muscle of K^+-depleted rats to reach the same ratio to the total muscle Na^+ as that found in the blood plasma was only 60 minutes (Heppel 1940.)

1940: B. Steinbach incubated isolated frog muscles in a modified Ringer's solution containing no K^+ for 17 hours and found the muscles lost a substantial amount of their intracellular K^+ in exchange for an equi-molar concentration of Na^+. When similar K^+-depleted muscles were subsequently incubated in a Ringer's solution containing 10 mM K^+, the muscles regained their lost K^+ simultaneously with extrusion of the extra Na^+ gained. These data indicate that the muscle cell membrane is fully permeable to both K^+ and Na^+. The author concluded that the exchange is fully reversible (Steinbach 1940.)

1940: S.C. Brooks studied the accumulation of Na^+ and other alkali metal ions in Spirogyra — the alga on which Overton conducted much of his famous plasmolysis studies —, Nitella and Amoeba proteus. In Spirogyra bathed in water containing 5 mM labelled Na^+, the time for the radioactive Na^+ in the cells to reach a level ten times higher than in the bathing medium was only 15 seconds. The much slower accumulation of radioactively labeled alkali metal ion in the cell sap inside the central vacuole than in the surrounding protoplasm further affirms the earlier finding of the much lower permeabiliity of the tonoplast than the plasma membrane by Höfler (and Chambers) mentioned earlier (Brooks 1939, 1940.)

In summary, the seven sets of independent studies unanimously demonstrated that the cell membrane of both animal and plant cells are permeable to Na^+. This result fully and

squarely contradicts Boyle and Conway's landmark paper on the sieve theory of the living cell before it even got published in the year 1941.

In theory, demonstration that cell membrane is permeable to Na^+ is by itself sufficient to disprove the theory of Boyle and Conway. However, other evidence show that virtually all the other cations and anions supposedly too big to traverse the pores of the sieve-like cell membrane and listed in Table 6 are able to permeate the cell membrane as well:

(1) **Li^+:** Fenn (1936)
(2) **Ca^{2+}:** Campbell & Greenberg (1940); Rothenberg & Field (1948)
(3) **Mg^{2+}:** Conway & Cruess-Callaghan (1937); Fenn & Haege (1942)
(4) **acetate ion:** no data on acetate *per s*e but carnosine, *alias* β-alanylhistidine, which contains in it an acetate moiety, is permeant (Eggleton & Eggleton 1933)
(5) **sulfate ion** (SO_4): (Ling 1962, Figure 11-31 on p. 333)
(6) **inorganic phosphate ion** (HPO_4): no data on membrane permeability *per se* but hexose monophosphate, which contains the phosphate moiety is cell membrane permeable (Roberts & Wolffe 1951)

Other solutes long considered impermeable to copper-ferrocyanide membranes of Traube, and Pfeffer and to living cell membranes of de Vries and Overton are sucrose and free amino acids. They too have also been shown to be membrane-permeable:

(7) **sucrose:** (Kolotilova & Engel'gardt 1937; Levine & Goldstein 1955)
(8) **(free) amino acids:** (Eggleton & Eggleton 1933)

Boyle and Conway's sieve theory was not put forth *de novo* for the first time. It was the culmination of a great deal of research beginning with Moritz Traube's Atomic Sieve Theory introduced in 1867. Therefore, the year 1940 could be taken as the date that the early (sieve) versions of the membrane theory came formally to an end. That was 67 years ago.

The membrane theory now began an internal change. Instead of sieves or ultrafilters, the sodium pump moved to center-stage, only to be disproved also in the course of another twenty years. Since I played a significant role in this part of the history, I will continue my narrative with a talk I gave in the world-famous Physiology Department of the University of Chicago on a Monday afternoon in the spring of 1948.

My first encounter with the sodium pump hypothesis

I was born in Nanking, China to a scholarly family of Confucian beliefs. The two countries in which I have spent most of my life are China and America. However, before I was born, these two countries were once at war — following what was known as the Boxer Rebellion. After the sacking of Peking, the Chinese government paid indemnities over the years to eight nations including the United States.

However, the United States decided not to use the indemnity money for her own benefits. Instead, she persuaded the Chinese government to build two new institutions with that money.

One institution was the Tsing Hua University built in the suburb of Peking. In time, this university has become what I may call a blend of MIT and Harvard.

> Parenthetically, a sizable number of the current political leaders in China were educated in that University. As an example, the current President of China, Hu Jin-tao was graduated from Tsing Hua with a degree in Hydraulic Engineering.

The second institution the US helped China to set up was the Boxer Scholarships. Its purpose was to provide full financial support for advanced education in the United States for twenty-some chosen scholars — one in each field of study, including physics, biology, mathematics, economy, medicine. These Boxer scholars were chosen on the basis of a nation-wide, competitive examination. Following traditional civil servant examinations, the examination lasted a whole week and the names of the participants were sealed at the outset and not disclosed to anyone until the result were announced.

Since its inauguration, five Boxer examinations had been held until 1938 when the Japanese invasion of China put an end to many hopes including that of winning the Boxer Scholarship. Then, for reasons unknown to me to this day, it was resumed once more in 1943 (for the last time) — and with it, came an unprecedented new rule: fresh college graduates with no working experience were allowed to participate — a small technical change of momentous importance to me, because I would be graduating from college in the summer of that year and would have no working experience to qualify for participation in that examination if the old rule had not been changed.

Then the best of the best breaks arrived. I won the biology (or more exactly, zoology) slot of the Boxer Scholarship. My roommate, Chen-ning Yang won the physics slot. Figure 11 is a copy of the list of chosen scholars announced by the Tsing Hua University administering this the Sixth Boxer Exam.

In 1945, Yang, I and most of the other 20 Boxer scholars flew over the Himalayan, visited Calcutta and Bombay before boarding the USS Liberty Boat, General Steward on her return to the US. The voyage lasted one month, taking us through the Suez Canal, the Mediterranean Sea and the Atlantic Ocean.

Then, on a cold November afternoon, we approached Manhattan as a light snow was falling on the Hudson River. The tall, dark and totally motionless skyline set against the sky of a darkening evening was a visual experience I could never erase from my mind nor duplicate in the long years to come.

By Christmas time, I was already in Chicago. Better still, I had the opportunity of meeting my much-admired professor, Ralph W. Gerard. Right away, he was enthusiastically talking about the topic of a Ph.D. thesis for me, something about a small piercing electrode.

Beside taking required courses, the first leg of my Ph.D. program was largely technical. A microelectrode technique initiated earlier by Professor Gerard and his student, Judith Graham (Gerard and Graham 1942; Graham and Gerard 1946) was not yet in working condition. A random set of membrane potentials measured across individual muscle cells of frog sartorius muscles ranged from 41.0 to 80.4 millivolts — far too scattered for the technique to be used to make quantitative measurements, which were badly needed.

Judith Graham was an intelligent and capable young woman, then trying valiantly to do her Ph.D. thesis while also raising two young children. Such a combined enterprise would

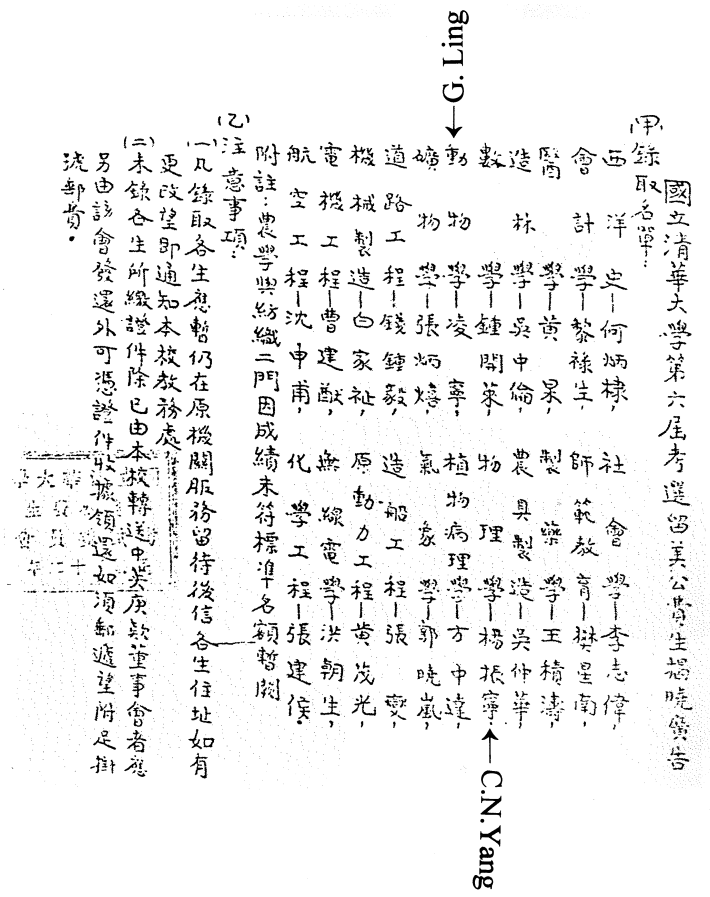


FIGURE 11. 1944 Tsing Hua University announcement of the list of chosen scholars, one in each of the twenty-two fields of study, on the basis of the 6th (Boxer) nationwide examination held in 1943 at different locations throughout China. Each chosen scholar would receive full financial support to complete advanced education in a University in the US chosen by the scholar involved.

be difficult at any time, but that was wartime. In my view, she simply did not have the time to do the nitty-gritty details. As my full scholarship took care of all my worldly needs, I had the time to try out different ways to make the electrode work better. Good luck once more favored me and I was able to get the microelectrode in working order before too long.

Two years later in 1948, I completed my Ph.D. thesis on the effects of metabolism, temperature and other factors on the membrane potential of single frog muscle fibers. The thesis was also published conjointly with Professir Gerard and, a visiting scientist from the West Coast, Walter Woodbury in four papers in the *Journal of Cellular and Comparative Physiology*. The findings by and large confirmed Bernstein's membrane theory of cellular resting potentials.

So, for a while, I enjoyed being quite popular among my peers — including the privilege of teaching Nobel laureate, Professor Alan Hodgkin of the Cambridge University of England how to prepare a usable microelectrode — then referred to not infrequently as the Ling-Gerard microelectrode. After that, the microelectrode technique spread rapidly worldwide.

To the best of my knowledge, there is no record on just how wide it has spread. However, there is a partial record made by the Swiss cardiac physiologist, Professor Silvio Weidemann, who wrote in 1971 a review on "The Microelectrode and the Heart; 1950-1970" (Weidemann 1971.) Under the section heading, "The cardiac descendents of Gilbert Ling", Weidmann showed how the art of pulling, filling and prodding was mostly handed on through personal contacts. He then provided detailed documentation on the names and locations of scientists engaged in heart physiology research with the microelectrodes.

A summary of this documentation was given in the form of three maps reproduced here in Figure 12. This reproduction is, in my view, important for the integrity of history. Since my subsequent challenge of the sodium pump hypothesis — to be described below — has drastically diminished my popularity among some of the most influential cell physiologists. As a symbol of displeasure, the term, Ling-Gerard microelectrode has become stripped of its marker and reduced to a nondescript "glass capillary microelectrode." This said, I return to the time I was actively using and improving that microelectrode to fathom what lies at the foundation of the electric potential of single frog muscle fibers.

Indeed, we were so excited about our success in affirming Bernstein's membrane theory of electric potential, we had little time for anything else. And that included the time needed to learn about and face the cataclysmic impact of the new radioactive-tracer technology that had by this time already played a key role in shattering the foundation of the (sieve version of the) membrane theory — with which, the reader of the prior pages of this review by now has been well acquainted.

As a result and suddenly, the sodium pump hypothesis was getting attention. Next thing you know, I was invited to do a library research and report what I found to my fellow graduate students and the faculty — in the departmental seminars held regularly on each Monday afternoon. The time was somewhere in the spring of 1948 and the title of my scheduled talk, *the Sodium Pump.*

Monday came. I began my talk with an apology. I told my audience that try as I did, I just could not find anything substantial about the sodium pump. Indeed, the only thing that I was sure of was that nobody seemed to know much about the sodium pump.

After that brief introduction, I went on to tell my audience my general dissatisfaction with the sodium pump hypothesis. I found on the library shelves an abundance of new evidence showing that it was not just the sodium ion that was in difficulty. A lot of other ions and non-electrolytes also exist in the cell at a concentration quite different from that in the bathing medium and yet fully membrane-permeable. It was the entire sieve version of the membrane theory that was in trouble. And, that included my own Ph.D. thesis, which was founded on the assumption that the membrane theory had full validity. And, the electric potential differences measured across the surface of individual muscle cells is truly a *membrane potential* — as described in the title of my Ph.D. Thesis, "Membrane Potential and Metabolism of Muscle" (Ling 1948.)

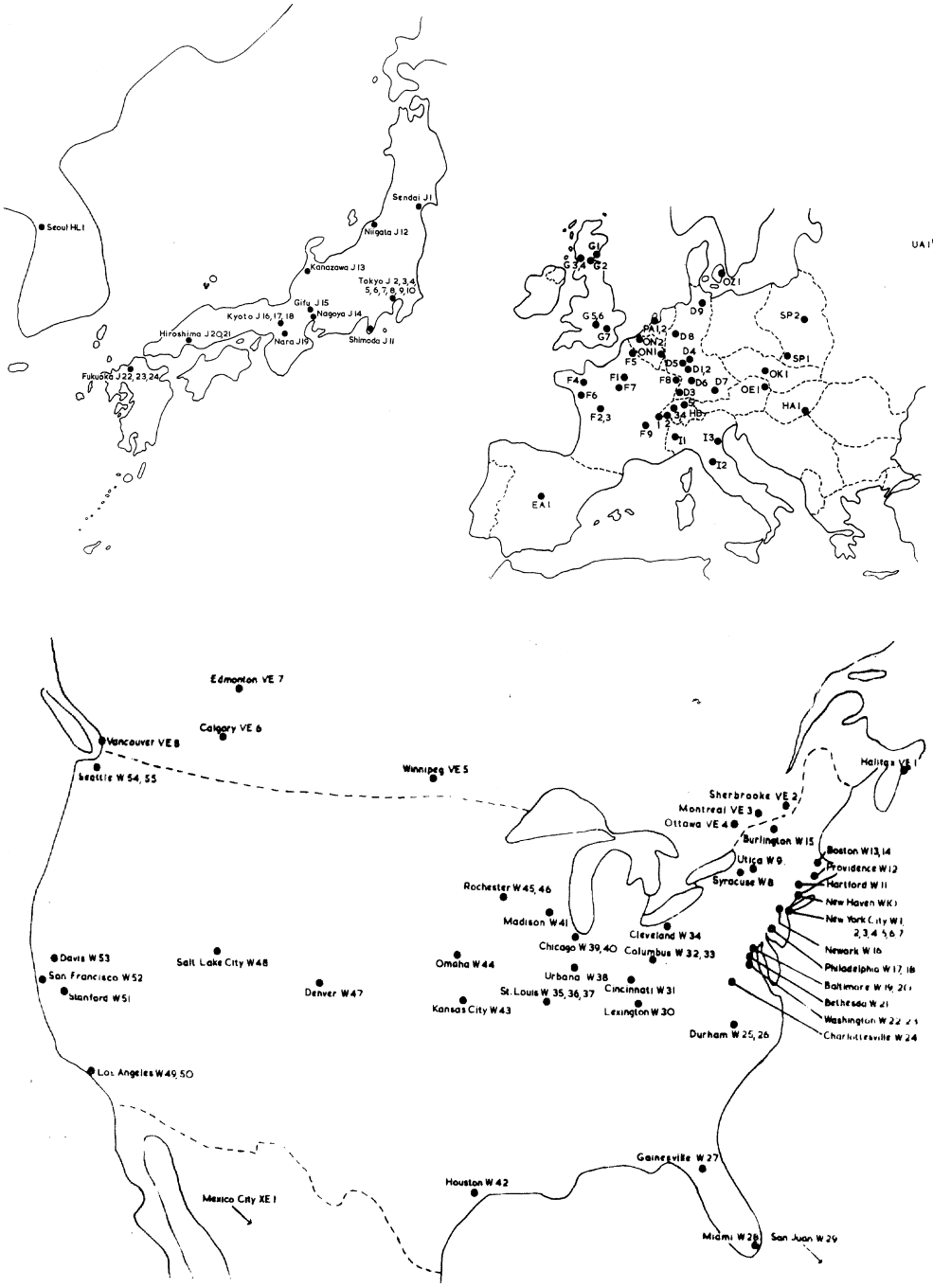


FIGURE 12. Geographic locations of research laboratories that have adopted the Gerard-Graham-Ling (alias Ling-Gerard) microelectrode to study the physiology and pathology of the heart. (from Weidemann's *The Microelectrode and the Heart 1950–1970*, Aulo Gaggi Publisher, Bologna)

So I asked myself and my audience what is the point of singling out one item and propose a theory specifically dealing with that one item alone and turn our back on everything else? That was more or less the question on which I ended my talk. I was entirely unprepared for what followed almost immediately after I stepped down from the podium.

For two of my most respected professors approached me in turn and using almost exactly the same words, gave me the same message: The sodium pump is a sacred cow. Leave it alone. There is no point making yourself a martyr.

I was at once startled and moved by what they told me. I thanked each most sincerely for their concern about my future. At that time, I thought that they were just overly worried. Who else beside myself could care about what one of the countless graduate students thinks about such a purely academic idea? Moreover, the central issue as I saw was a lack of solid information on the postulated sodium pump. It was just floating on hearsay.

I then asked myself, Why not do some simple bread-and-butter experiments testing the sodium pump hypothesis? After that, we will have some real thing to argue about. Furthermore, at that time I not only had all the needed facilities and frogs to carry out that kind of a study and the time needed to do the experiments. All I needed was to switch my attention from the membrane potential to the sodium ion content of the same tissues.

So I did go ahead with a simple bread-and-butter experiment. Its aim was to find out if cutting off the energy sources of the muscle cells would stop or at least slow down the postulated pumping and thus bring about a rise of cell Na^+ and a fall of cell K^+?

Now, the two major sources of energy are respiration, which converts glucose or glycogen to carbon dioxide and glycolysis, which converts glucose or glycogen to lactic acid. To block the energy production, I exposed isolated frog muscles and nerves to both pure nitrogen (N_2), which suppresses respiration, and iodoacetate (IAA), which suppresses glycolysis and to a 0°C temperature, which would decrease the rate of inward leakage of Na^+ into the muscle or nerve cells less (by diffusion with a low temperature coefficient) than it would decrease the postulated outward pumping (a chemical process with a higher temperature coefficient.) After five hours of incubation, the K^+ contents of frog muscle and nerve were analyzed; and the results, presented in Table 7 show that the concentration of K^+ does not change in either the muscles or the nerves.

Table 8 presents the result of a later study in which both the K^+ and Na^+ contents of five kinds of frog tissues (muscle, nerve, testis, kidney, and heart) were analyzed after they were exposed to IAA, 99.99% pure nitrogen for 7 hours and 45 minutes at 0°C. Neither the K^+ nor the Na^+ contents of all five kinds of tissues thus treated showed any significant departures from their control pairs.

This finding was at once surprising and not surprising. Surprising because metabolic poisons like those used have as a rule profound influence on the accumulation of inorganic salt ions in plant cells (Lundegårdh and Burström 1933; Machlis 1944) and so is low temperature (alone) (Ulrich 1941.)

However, it is also not surprising because frog muscle (and nerve) are known to contain a third source of energy in the forms of adenosinetriphosphate (ATP) and creatine phosphate (CrP.) Thus, according to the then widely-taught theory of Lipmann (1941), each molecule of ATP carries two *high energy phosphate bonds* represented as ~P and each creatine-phosphate bound contains one ~P and this (high) energy (supposedly to be stored in these high energy bonds) is supposed to be available for biological work performance. That being the case, the store of ATP and CrP in the muscle and nerve cells

TABLE 7. The combined effect of Na iodoacetate, pure nitrogen and 0° C temperature on the K^+ concentration in frog muscles and nerves. Incubation lasted 5 hours in Ringer's solution containing 0.5 mM Na iodoacetate (IAA) at 0° C in an atmosphere of pure nitrogen. The controls were in plain Ringer's solution in air. (from Ling 1952, by permission of the Johns Hopkins University Press, Baltimore)

Type of Tissue	Muscle No.		Weight (gms.)	mM. K+/l. of intracellular water
Sartorius	1	Control	0.0870	60.7
	2	Expt'l	0.0750	69.8
Semitendinosus	1	Control	0.0710	72.6
	2	Expt'l	0.0795	81.8
Tibialis anticus longus	1	Control	0.0938	71.1
	2	Expt'l	0.0900	79.2
N. ischiadicus +	1	Control	0.0300	38.1
N. tibialis + N. peroneus	2	Expt'l	0.0260	39.5
Sartorius	1	Control	0.0730	73.4
	2	Expt'l	0.0700	78.0
Semitendinosus	1	Control	0.0660	83.0
	2	Expt'l	0.0730	77.4
N. ischiadicus +	1	Control	0.0260	42.8
N. tibialis + N. peroneus	2	Expt'l	0.0242	40.0
			Muscles	Nerves
Average		Control	100.0%	100.0%
		Expt'l	105.2%	98.5%

could have explained why normal concentrations of cell Na^+ and K^+ were maintained for at least 5 hours in the N_2-IAA poisoned muscles and nerves kept at 0° C.

But there was still another theoretical possibility that should not be rejected offhand. Namely, the possibility that an as-yet undetected energy source exists beyond respiration, glycolysis and ~P-containing compounds, ATP and CrP. So I started to search for evidence of the existence or non-existence of a fourth energy source. Before long, I found the answer.

Based on the total heat output in similarly poisoned frog muscle measured by Hill and Parkinson (1931) and by Hukuda (1931) and some new data of my own (published eventually in 1973 by Ling *et al* 1973, pp. 11–12), I was able to conclude that there was no fourth energy source beyond respiration, glycolysis and ~P carrying compounds (Ling 1952, pp. 764–765; Ling *et al* 1973, pp. 11–12.)

With the possibility of a fourth energy source out of the way, I was ready to design an experiment, which had the potential of disproving the sodium pump hypothesis. The essence of this study lay in finding out if the minimum energy need of the postulated sodium pump maintained at 0°C would fall comfortably within the boundary set by the maximum energy available to the muscle cell for a chosen period of time at 0° C, during which the steady levels of both K^+ and Na^+ stay unchanged like those shown in Table 7.

TABLE 8. The combined effect of iodoacetate, pure nitrogen and low temperature on the K^+- and Na^+-contents of various frog tissues. Isolated tissues of North American leopard frogs (Rana pipiens pipiens, Schreber) were incubated at 0°C for 7 hours and 45 minutes. The modified Ringer's solution had been equilibrated with 99.99% pure nitrogen (Linde Corp.) before the tissues were introduced. At the conclusion of the experiment, the individual tissues were weighed and projected into 3 ml of distilled water to be heated in a 100°C water bath for 10 minutes or in 3 ml of 0.1 N HCl without heating. K^+ and Na^+ contents were analyzed by flame photometry on a Beckman DU spectrophotometer with a flame photometer attachment on aliquots of the tissue extracts containing a fixed amount of "radiation buffer" (i.e., 100 mM Na for K^+ assay; 100 mM K for Na^+ assay.) Muscle 2 and 3 represent respectively the semitendinosus and tibialis anticus longus muscles . "nerve" refers to sciatic nerve axons. (from Ling 1962)

Frog No.	Tissue	Control or experiment	Tissue wt, mg	K, µM/g	Na, µM/g
1	muscle 2[a]	control	96.8	78.6	25.8
	muscle 2	experiment	96.8	78.6	28.5
	muscle 3[a]	control	94.6	82.8	23.0
	muscle 3	experiment	93.6	81.1	24.6
2	muscle 2	control	109.6	70.2	18.0
	muscle 2	experiment	109.6	73.0	18.0
	muscle 3	control	105.6	67.6	20.8
	muscle 3	experiment	103.6	70.5	20.5
3	muscle 2	control	85.4	74.8	25.2
	muscle 2	experiment	84.6	74.4	30.9
	muscle 3	control	100.0	64.6	30.5
	muscle 3	experiment	101.4	75.6	23.2
4	muscle 2	control	86.4	55.5[b]	41.6[b]
	muscle 2	experiment	86.4	79.5	18.5
	muscle 3	control	88.8	45.1[b]	57.7[b]
	muscle 3	experiment	91.3	77.5	24.9
	nerve	control	33.0	34.8	73.0
	nerve	experiment	31.4	35.0	69.8
5	muscle 2	control	100.2	71.8	29.9
	muscle 2	experiment	101.0	71.0	29.9
	muscle 3	control	83.2	71.0	24.5
	muscle 3	experiment	84.3	62.0	34.4
	testis	control	26.8	56.0	35.8
	testis	experiment	23.0	56.7	41.3
	kidney	control	66.4	30.0	51.4
	kidney	experiment	64.0	35.2	51.9
	nerve	control	32.8	38.7	62.8
	nerve	experiment	28.0	35.8	51.1
6	muscle 2	control	100.0	70.5	36.7
	muscle 2	experiment	102.6	75.5	26.3
	muscle 3	control	97.0	71.4	31.0
	muscle 3	experiment	97.0	65.6	52.0
	testis	control	26.4	43.5	52.3
	testis	experiment	17.4	39.1	47.1
	kidney	control	74.2	40.4	60.7
	kidney	experiment	69.4	45.9	54.8
	nerve	control	30.2	37.1	79.5
	nerve	experiment	28.6	40.6	84.0

TABLE 8 (continued)

Frog No.	Tissue	Control or experiment	Tissue wt, mg	K, μM/g	Na, μM/g
7	muscle 2	control	102.2	75.2	38.6
	muscle 2	experiment	103.6	86.7	27.5
	muscle 3	control	97.0	83.8	29.5
	muscle 3	experiment	97.0	82.6	28.1
8	muscle 2	control	88.0	83.0	27.6
	muscle 2	experiment	82.0	86.2	31.7
	muscle 3	control	80.4	78.4	33.7
	muscle 3	experiment	83.4	84.4	27.6
9	muscle 2	control	98.2	80.0	28.6
	muscle 2	experiment	94.8	84.3	27.4
	muscle 3	control	92.6	78.9	30.4
	muscle 3	experiment	92.8	77.4	35.9
10	muscle 2	control	90.8	71.5	29.6
	muscle 2	experiment	90.8	72.7	32.8
	muscle 3	control	98.2	73.5	28.1
	muscle 3	experiment	100.3	72.8	26.1
9	heart	experiment	97.6	29.8	52.5
5	heart	experiment	104.0	21.4	38.7
6	heart	experiment	88.6	33.9	48.6
7	heart	experiment	90.4	22.2	63.0
8	heart	control	83.4	36.3	56.3
4	heart	control	80.0	45.0	49.0
10	heart	control	83.4	40.2	47.2

Before going into more details, I shall begin with a brief account of why energy is needed to pump the Na^+ out of the cell (and K^+ into the cell.) I shall then go into more details on how to measure both the minimum energy need of the sodium pump and the maximum available energy of the poisoned muscles all at 0°C.

Since it is vitally important to maintain a constant temperature of 0°C, as much as possible, all operations and the instruments used to carry out these operations were kept at 0°C by being kept at temperature equilibrium with a mixture of water and cracked ice mixture. The large container of this ice-water mixture as well as the essential measuring at just above freezing were all installed in a constant temperature room maintained at just above freezing.

Suppose you have a wet basement. To remedy the situation one can install and operate a sump pump to remove the unwanted water. This needs energy, because a weight of water has to be moved by the pump against a gravity gradient. That is, pumping water from a lower energy position on the floor of the basement to the higher energy position at the street level.

Pumping Na^+ out of muscle cells involves moving each Na^+ against, not one, but two unfavorable gradients. One is an electrical gradient. Because the sodium ion is positively charged, to move it from within the cell to outside it must be pushed against the 85 mV inside negative-outside positive membrane potential (better, resting potential.) The other gradient is a concentration gradient that arises from the much lower Na^+ concentration inside the muscle cell than that outside in the bathing medium.

With both the magnitudes of the two gradients determined, we already know the minimum amount of energy that must be spent to move one sodium ion out. That known, the last task would be to determine the rate of sodium ion pumping or the number of Na^+ that must be pushed out from within the cell in a unit time. Multiplying the amount of energy that must be spent to move one Na^+ out of the cell by the number of Na^+ that are moved out of the cell per unit time, say an hour and again by the duration of the experiment in hours yields the minimum energy need for the sodium pump to keep the level of Na^+ at the physiological low level for the duration of the experiment. With the design of the experiment made clear, I describe next the experimental details.

One begins with getting living tissue for the experiment. More specifically, one isolates a number of small muscle-fiber (or muscle-cell) bundles — with some 50 to 150 totally intact muscle fiber or cells in each bundle — from the pair of double-headed *semitendinosus* muscles from each thigh of one North American leopard frog (Rana pipiens pipiens, Schreber.)

Some of the isolated muscle fiber bundles would be used in Part 1 of the study with the purpose of determining the maximum energy available — under the assumption that the energy would be used for one and only one purpose: to pump Na^+. To achieve that goal, one determines the total contents of ATP and CrP of the poisoned muscle — by enzymatic methods, described in Ling 1997a, Appendix 1 — at the beginning of an experiment and at the conclusion of the experiment. Their differences would provide the data to compute the maximum energy available to the muscle fibers to keep the postulated sodium pump going for the duration of the experiment, which lasted as a rule from 4 to 10 hours.

Other muscle-fiber bundles isolated from the (same) frog would be used for Part 2 of the study. The overall purpose of Part 2 is to determine the minimum energy need of the sodium pump to keep the Na^+ and K^+ unchanged at their normal physiological levels through the duration of the experiment.

The muscle fiber bundles were exposed to the metabolic poisons in exactly the same manner but, in addition, also exposed to radioactive tracer, ^{22}Na-, or ^{24}Na. Each of the isotope-loaded and N_2-IAA-poisoned muscle fiber bundle was then tied to a long piece of surgical thread and mounted at the bottom of an U-tube, which in turn was placed inside the "well" of a well-type γ-scintillation counter shown in Figure 13. The radioactivity in the muscle fiber bundle is then continually monitored while a stream of non-radioactive, N_2-IAA Ringer's solution kept at 0°C flows through the U-tube at a steady rate. 99.99% pure nitrogen gas, further purified by passage through heated copper coils, bubbles through the reservoir of N_2-IAA Ringer's solution continually. The radioactivity counts collected yield data on the rate of pumping of Na^+ from the muscle cells.

Other similarly treated muscle fibers bundles were periodically pulled out of (and returned to) the U-tube and had their resting potential measured with a Gerard-Graham-Ling microelectrode (*alias* the Ling-Gerard microelectrode) in an electric potential measuring setup placed within the same cold room maintained at just above the freezing point.

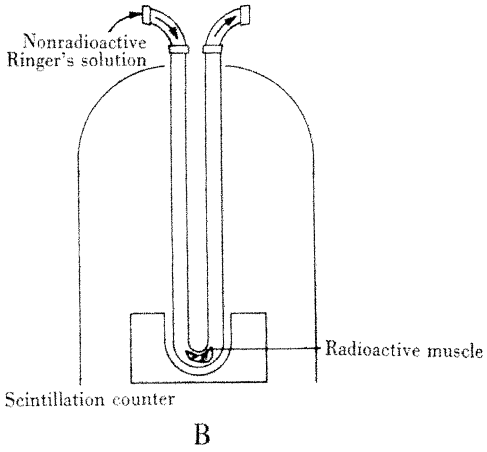


FIGURE 13. The U-tube, γ-scintillation counter assembly of Ling-Schmolinski for the study of ^{22}Na- .^{24}Na- (and other γ-emitting radio-isotope-) labelled Na^+ efflux rate studies of single and multiple frog muscle cells. (from Ling 1962, in his Figure 8-5 on page 198)

The experiments outlined above were carried out more or less steadily with technological improvements all along over a period of roughly six years between 1951 and 1956.

Over the six-year period many experiments were performed some complete, others incomplete (for more details, see legend of Figure 8.9 in Appendix I of Ling 1997a.) Without exception, all of them confirm and extend the earliest results reported in 1952: the minimum energy need was 400% of that maximally available.

However, the work first reported in 1952 was done with less sophisticated methods. Refined methods which evolved as time went along reached the peak of accuracy in the three sets of experiments performed in the year 1956. For their historic importance, the data are given in figure form (Figure 14) and also as a table as they were first presented in 1962 (Table 9.)

These three sets of data of September 1956 show that the minimum energy need of the sodium pump is from 15 to 30 times, or 1500% to 3000% of the maximally available energy. Based on these findings, I reached the conclusion that the sodium pump hypothesis is in conflict with one of the most fundamental laws of physics: The First Law of Thermodynamics also known as the Law of Conservation of Energy.

> It is of interest that this law was first enunciated by one of the greatest scientist in history, physiology-physicist, Ludwig von Helmholtz.

By any standard, a disparity of 1500% to 3000% is highly significant. However, even these figures are gross under-estimations for a variety of reasons including the following:

(1) **~P idea disproved.** Truly available energy is far below that assumed in the computation that led to the 1500% to 3000% figure. To obtain the 1500% −3000% value, I took what was then higher values of the free energy of the ~P bonds from the literature −14.3 kcal/mole (ATP → ADP + P), −15.0 kcal/mole (ADP → AMP + P) and −12.8 kcal/mole

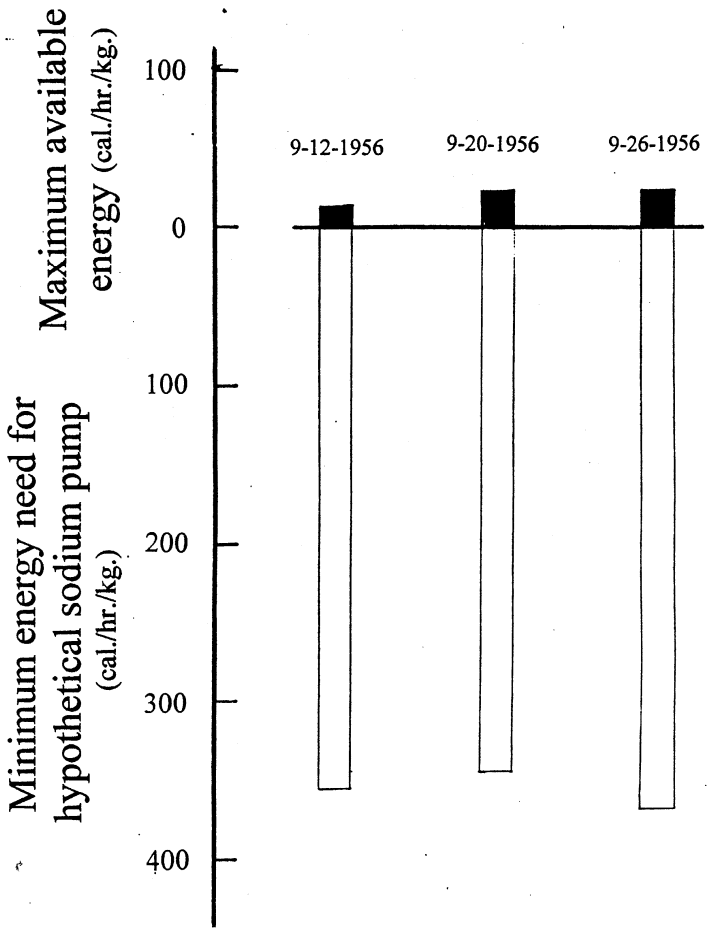


FIGURE 14. Graphical illustration of the energy balance sheet for the postulated Na pump in frog sartorius muscles at 0° C. This is the same set of data presented in Table 9 but in greater detail. The minimum rate of energy delivery required to operate a Na pump according to the membrane-pump theory was calculated from integrated values of the measured rates of Na^+ exchange shown in a table in the Ling 1962 monograph labeled Table 8.7 and the energy needed to pump each mole of Na^+ out against the measured electrical and concentration gradients (Table 8.5 and Figure 8.7.) The maximum energy-delivery rate was calculated from the measured hydrolysis of CrP, ATP and ADP, the only effective energy sources available to the muscles which were poisoned with IAA and N_2. Total inhibition of respiration and of glycolysis was assured by the simultaneous presence of 0.001 M NaCN (in addition to N_2) and verified by the actual measurement of residual lactate production (in addition to IAA.) There is no significant difference if the data on flux rate for series A in Table 8.7 are used rather than those for series B. Details of one of the three complete sets of data obtained in September of 1956 are given in Table 8.4 of 1962 monograph. It should be pointed out that six more sets of similar experiments were completed (3-20-53, 4-12-54, 4-13-55, 5-20-55, 5-30-55 and 8-9-55); the duration of soaking in the poison gave a mean maximum rate of energy delivery even lower than the data from the three sets used in these cacluations. Since the development of the final procedure for ATP-ADP determinations as described in Appendix D of 1962 monograph was not completed until the end of 1955, the earlier data have not been included. (from Ling 1962 in his Table 8.9 on page 211)

TABLE 9. The energy balance sheet for the (hypothetical) sodium pump in frog sartorius muscles at 0° C. The minimum rate of energy delivery required to operate a Na pump according to the membrane-pump theory was calculated from integrated values of the measured rates of Na^+-efflux (given in Table 8.7. This and other Tables and Figures referred to below are from Ling 1962 and can also be found in Appendix I of Ling 1997a.) and the minimum energy need to pump each mole of Na^+ out against the combined electrical and concentration gradients measured (Table 8.5 and Figure 8.7.) The maximum energy delivery rate was calculated from the measured hydrolysis of CrP, ATP and ADP , the only effective energy sources available to the muscle tissues which were poisoned with IAA and N_2. Total inhibition of respireation and of glycolysis was assured by the simultaneous presence of 0.001 M NaCN (in addition to N_2) and verified by the actual measurement of residual lactate production (in additon to IAA.) Details of one of the three complete sets of data obtained in September of 1956 cited in the Table are given in Table 8.4. (from Ling 1962)

Date	Duration, hr	Rate of Na exchange, integrated average, M/kg/hr	$\psi + E_{Na}/\tilde{\delta}$ integrated average, mv	Minimum rate of energy required for Na pump, cal/kg/hr	Maximum rate of energy delivery, cal/kg/hr	$\left[\frac{\text{Minimum required energy}}{\text{Maximum available energy}}\right]$
9-12-50	10	0.138	111	353	11.57 (highest value, 22.19)	3060%
9-20-56	4	0.121	123	343	22.25 (highest value, 33.71)	1542%
9-26-56	4.5	0.131	122	368	20.47 (highest value, 26.10)	1800%

(CrP → Cr + P). However, later work of Podolsky and Morales (1956) demonstrated that the original value of the enthalpy change involved in the ATP → ADP reaction has a substantial contribution from the heat of neutralization of the H^+ liberated during the reaction and not free to energy biological work performance. So the very concept of high-energy-phosphate bond has become untenable. Since virtually all the (maximally available) energy is that involved in the hydrolysis of ATP, ADP and CrP, the "downsizing" would further enhance the disparity between energy available and energy needed by large factors.

(2) **Many more pumps needed** The sodium pump is, as already pointed out above, only one of an ever-lengthening list of needed pumps to keep the cell afloat. Thus, it was not surprising that in a by no means comprehensive search, Ling *et al* (1973) had compiled a list of membrane pumps that have already been formally introduced and reproduced here as Table 10. Some of these newly added pumps are pumps for whole categories of solutes like sugars and free amino acids. Therefore, the real list in individual pumps required are actually much lengthier than the table appears to tell. Each one of these many pumps would require energy to keep on operating. Added together, that would truly dwarf the 1500% to 3000% figure cited above.

Table 11 shows another list of the diverse compounds. A considerable portion of them show below-unity intracelluar-extracellular concentration ratios — called the *q-values*. Yet

TABLE 10. A (partial) list of membrane pumps already formally postulated assembled in 1968. For detailed information on the source references, see Ling *et al* 1973. (by permission of Annals of New York Academy of Sciences, Blackwell Publishing)

Solute	Direction	System	Reference*
Na, K	coupled	many cells	169
Ca^{++}	outward	RBC, striated muscle	170, 171
Mg^{++}	outward	frog sartorius	172
$Choline^{+}$	inward	RBC	173
Amino acids	inward	RBC, muscle, tumor	174–176
D-xylose	inward	rat diaphragm	177
D-xylose	outward	rat diaphragm	178
Na^{+}	inward	frog sartorius	179, 180
Noradrenaline	inward	vascular smooth muscle	181
Prostaglandins	inward	mammalian liver	182
Curarine	inward	mouse diaphragm	183
Br^{-}, I^{-}, ReO_4^{-}, WO_4^{-}	outward	Ascites	184
CU^{+2}	inward	Ascites	185
Aminopterin	inward	Yoshida sarcoma	186
Cl^{-}	inward	squid axon, motor neurons	187, 188
Mn^{++}	inward	*E. coli*	189
Cl^{-}	outward	*E. coli*	189
Sugars	inward	*E. coli*	189
Amino acids	inward	*E. coli*	189
Tetracycline	inward	*E. coli*	190

they are all fully membrane permeable, reaching diffusion equilibrium in the time specified in the second column. Most of these compounds are not found in the natural environment of frog muscle cells, some like DMSO and chloro-propanediols came into existence on this planet in the laboratories of some organic chemists. Yet, they too are like Na^+ found with a below unity q-value and membrane-permeable, thus requiring an energy consuming pump. In theory, the number of organic compounds that future efforts of organic chemists could produce is without limits. So, the ultimate energy requirements of all of them together would be infinite. And the energy disparity would also become infinite.

(3) **No space in the cell membrane to accommodate an infinite number of pumps.** But even that is not the only argument against the membrane pump model. There is another question that arises from the simple physical fact that two subjects cannot at the same time occupy the same space. That is, the cell membranes which have been assumed to accommodate the diverse pumps have only a limited volume — an extremely small volume if one recalls the usual value assigned is about 60 Å thick. How can that limited space provided by the membrane of each living cell accommodate an infinity of pumps? It simply cannot.

TABLE 11. The time to reach full equilibrium in the distribution of all the listed (radioactively labeled) chemical compounds between the bathing Ringer's solution and the frog muscle cells and the *true equilibrium distribution coefficient* or *q-value* ot each of the chemicals listed. (data partly from Ling *et al* 1993)

Solute	Equilibration Time (hours)	q-value
water	<< 1	1.00
methanol	<20	0.91
ethanol	<20	0.81
acetamide	<10	1.00
urea	<24	1.05
ethylene glycol	<10	1.02
1,2-propanediol	24	0.83
DMSO	<1	0.72
1.2-butanediol	24	0.87
glycerol	<20	1.00
3-chloro- 1,2-proranediol	24	0.89
erythritol	<20	0.29
D-arabinose	<45	0.27
L-arabinose	<45	0.27
L-xylose	<45	0.26
D-ribose	<24	0.26
xylitol	24	0.22
D-glucose	<15	0.23
D-sorbitol	<10	0.23
D-mannitol	<24	0.22
sucrose	<8	0.13
raffinose	10	0.10

In years following, the essence of the energy-disparity finding were twice confirmed (Jones 1965; Minkoff and Damadian 1973.) Years later something truly bizarre happened.

A friend told me that he read an article written by a *Science* magazine reporter by the name of Gina Kolata. In this article, she claimed that new experiments of two of my former graduate students, Chris Miller and Jeffrey Friedman, had demonstrated that the energy need of the sodium pump I calculated was excessive and that the sodium pump really exists.

On further inquiry, I learnt that no such new experimental finding ever existed. The whole thing represents a sad and miserable page in the history of science, when innocent young students were coerced into activities of which they should be profoundly ashamed. However, this episode had one minor redeeming value. It propelled me to update extensively the earlier work in a 75-page article entitled: "Debunking the Alleged Resurrection of the Sodium Pump Hypothesis." Published in 1997, the article is also available online as a searchable pdf file (Ling 1997a.)

In summary, the sodium pump hypothesis in specific and the membrane pump hypothesis in general violate the most basic law of physics, the Law of the Conservation of Energy and thus are totally erroneous. Since the pump model was the last remedy to keep alive the disproved sieve version of the membrane theory, *the disproof of the pump model spells the end of the membrane theory*. The date of this disproof is 1962 and hence more than forty years ago.

Notwithstanding, the sodium pump is being taught as truth to all American students — and to students outside America also to this very day. This is admittedly a strange scenario. However, I am confident that eventually, it would all come out all right. Meanwhile, we must be very patient. Remember this. Nothing truly worthwhile comes easy.

Searching for the Physical Basis of Life — after the demise of the membrane theory

In the first edition of his book, *Biology and Its Makers*, first published in 1908, William A. Locy wrote: "Now for the first time physiologists began to have their attention directed to the actually living substance; now for the first time they saw clearly that all future progress was to be made studying this living substance—the seat of vital activity. This was the beginning of modern biology." (Locy 1908, p. 275.)

That was exactly a full century ago. Have we done what Locy suggested during this long period of time? Broadly speaking, the answer is Yes. However, most of the investigators no longer call themselves cell physiologists or even use the name, protoplasm. Instead, they call themselves biochemists, biophysicists, endocrinologists, embryologists, enzymologists, protein chemists, electro-physiologists, pharmacologists, toxicologists etc. etc.

To emphasize that within bounds this division of labor was not mistaken but rather, an unavoidable and necessary step in the progress toward a fuller and more coherent understanding. One can make this point more convincingly with the help of an analogy, an analogy of a gigantic and multi-dimensional cross-word puzzle. As such, it is beyond what anyone person could solve. So there is no better alternative beyond cutting up the cross-word puzzle into small pieces and work on them separately — first.

The overall result is, of course, fragmentation. This would be a difficult phase of the enterprise. A great deal of time, energy and resources would be wasted in efforts wrongly directed. However, in theory at least, one day some one will be lucky enough to sew them together into a whole again in the right way — if we know how to preserve and retrieve what are truly valuable information and what is simply junk. Fortunately, Google could be vastly helpful in this task (see below.)

However, for the physiologists, there is another hurdle to overcome. To explain we use the cross word puzzle model again but in a different context.

An ordinary cross word puzzle involves putting the right words in unique places. To do that successfully requires the command of a large and suitable vocabulary. A New York Times cross-word puzzle is thus beyond the reach of a third grader. Nonetheless, if he or she continues to learn more and more words, one day he or she would be able to erase his or her earlier wrong entries and replace them with the correct ones and solves the puzzle.

In solving the physiological cross-word puzzle, it is the relevant physical and chemical laws and concepts that are inserted at the right places. At the time when Locy called for the study of protoplasm in 1908, some key physics and chemistry were not yet available.

However, by the time I arrived at the United States in 1945, the essence of three major advances were already on hand. They are (1) the molecular structure of proteins, worked out mostly by the great German chemist, Emil Fischer (1852-1919); (2) the branch of physics called *Statistical Mechanics*, — which connects the properties and behaviors of atoms and molecules to the properties and behaviors of macroscopic subject made up of the atoms and molecules — invented by the great Austrian physicist, Ludwig Boltzmann (1844–1906) and (3) lastly, the *Induction Theory*, a theory first introduced by American chemist, G. N. Lewis (1875–1946) who showed that electronic polarization (or depolarization) emanating from atoms in one part of a molecule can affect the properties and behaviors of atoms in another part of the molecule.

Thus armed, I was able to introduce what became known as Ling's Fixed Charge Hypothesis in 1952. This turned out to be the embryonic version of a unifying theory of living phenomena at the cell and below-cell level and given the name, the association-induction (AI) hypothesis. It was published in 1962 by the Blaisdel Publishing Co. under the title: *A Physical Theory of the Living State: the Association Induction Hypothesis*. Three years later in 1965, an integral part of the AI Hypothesis, called the Polarized-(Oriented) Multilayer Theory of Cell Water was added (Ling 1965) thus completing the presentation of the AI Hypothesis.

Three other monographs have been added since then: *In Search of the Physical Basis of Life* published by Plenum Publ. Co., in 1984; *A Revolution in the Physiology of the Living Cell* published by Krieger Publ. Co. in 1992, and finally, *Life at the Cell and Below-Cell Level* published by the Pacific Press in 2001.

For those seriously interested, there is no substitute to reading these books, especially the last one. However, as a starter, I would recommend that you go to http://www.gilbertling.org/lp6c.htm for two abstracts of the association-induction hypothesis.

Let us now turn our attention away to the past and to those that are to inherit the future and how what we do or not do may shape their individual and collective destinies. As a start, let us think about the thousands upon thousands of biology teachers in America alone, who are now teaching year in and year out, generation after generation of young Americans a theory of what we all are at the most basic level — that is no more valid than

the flat-earth theory. Yet, this is done as part of public and private education in *science*, which has no other purpose beyond the search for the truth.

Partly in response to the (unspoken) need for help of both the teachers and their wards, I wrote and published a fourth book*, Life at the Cell and Below-Cell Level* (Ling 2001, pp. iv–v).

In order to bring this book to the attention of biology teachers, I paid $ 1200 for a full-page an ad in the popular *American Journal of Biology Teachers*. Then something happened just two weeks before its scheduled appearance in print. From the mailman, I received a note returning the $1200 I had paid for the ad and the message, that the top man of that organization believed that my ad is in conflict with the aim of the magazine. I wrote no less than six letters pleading with him that he was wrong but all to no avail. I did not get a single word in reply.

This seemingly incredible episode brought back to mind what once long ago my professors warned me — incredibly to me at the tiime — not to tangle with the sodium pump, which in their minds, had even then ceased to be a scientific hypothesis open to criticism and questioning by anyone, but has become a sacred cow; and as such, "exempt from criticism and questioning" (Webster Collegiate Dictionary.) The incredible episode has shown me most convincingly that this exempt from questioning is not just in theory but in reality — through the actions of the likes of the Executive Director of NABT.

That said, I want to reassure my readers that things are not all that hopeless as it might sound. There are ways to get around these petty tyranny. At the moment, it is a little ironic that it is the scientists at two noted Russian scientific institutions, Dr. Vladimir Matveev of the Leningrad Institute of Cytology and Dr. Alexander Maligin of the Pavlov Institute of Physiology and their crews who who apparently realized the great potential opportunity suddenly open to the high-school and college students to learn about what is happening to the "science of all sciences" (DuBoi-Reymond 1853,) (cell) physiology. As a first step, presently these cell physiologists are translating into Russian, *Life at the Cell and Below-Cell Level*.

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Regulation of 5-Aminolevulinic Acid-dependent Protoporphyrin IX Accumulations in Human Histiocytic Lymphoma U937 Cells

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Abstract: The aim of the present work is to clarify the mechanism(s) that regulates the accumulation of protoporphyrin IX (PpIX) in human histiocytic lymphoma cell line U937 incubated with 5-aminolevulinic acid (ALA). Biosynthesis and accumulation of PpIX in the cells was determined after incubation with 0.1 ~ 5 mM ALA using a flow cytometric technique. The synthesized endogenous PpIX was found to localize predominantly in the mitochondrial region of the cells. The ALA-enhanced PpIX synthesis was suppressed by the presence of either β-alanine, a competitive inhibitor of β-transporters on cell membranes, or carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone, an uncoupler of mitochondrial oxidative phosphorylation. In contrast, cellular accumulation of PpIX was enhanced by the presence of either deferoxamine (an iron chelater), $MnCl_2$ (a ferrochelatase inhibitor), or Sn-mesoporphyrin (heme oxygenase inhibitor). These results suggest that ALA-enhanced accumulation of PpIX in U937 cells was regulated by cellular uptake and conversion of ALA to PpIX and by degradation of Heme.

KEY WORDS: aminolevulinic acid, β-transporters, ferrochelatase, heme oxygenase-1, protoporphyrin IX

Abbreviations: ALA, aminolevulinic acid; FBS, fetal bovine serum; FC, ferrochelatase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; HO, heme oxygenase; NAO, 10-nonyl acridine orange; PpIX, protoporphyrin IX; PDT, photodynamic therapy; TMRE, tetramethylrhodamine-ethyl-ester.

Aminolevulinic acid (ALA)-induced synthesis of protoporphyrin IX (PpIX) is significantly high in a variety of tumor cells in various organs (1–5). In ALA-based photodynamic therapy (PDT), ALA taken up by tumor cells is metabolized to PpIX, which sensitizes cells to photo damage leading to apoptotic or necrotic cell death (6). Fluorescence-based diagnosis using ALA provides new method for the detection and/or treatment of various cancer cells (7). Since ALA is impermeable through membrane/lipid bilayers (8) and the biosynthesis of heme occurs both in cytosol and mitochondria, the efficacy of ALA-dependent PDT is restricted by cellular uptake of ALA and/or accumulation of photosensitizer PpIX (9). However, the mechanism of preferential accumulation of PpIX in malignant cells remains obscure.

To understand the mechanism for the preferential accumulation of PpIX in tumor cells, various factors have been studied, such as ALA uptake by cells (10, 11), mitochondrial properties (12) and molecules involved in PpIX metabolism including porphobilinogen deaminase (13), ferrochelatase (14), iron content (15) and transferrin receptor (16). Cellular activities to uptake ALA differ significantly with cell types. In adenocarcinoma cells and LM3 mammary adenocarcinoma cells, ALA has been shown to be taken up by β-transporter (17, 18). However, it has been reported that tumor-specific PpIX accumulation is generated by ALA conversion rather than by its initial uptake because no significant difference in the overall uptake of ALA was observed (5).

ALA is converted to porphobilinogen, uroporphyrinogen III and then to coproporphyrinogen III in cytoplasm. In some tumor cells, the preferential accumulation of PpIX is strongly affected by the activity of porphobilinogen deaminase that synthesize uroporphyrinogen III (5–20). Coproporphorphyrinogen III, a precursor of PpIX, is transported into mitochondria by an ATP-dependent mechanism (12). These observations suggest that the synthesis of endogenous PpIX from ALA is affected by cell and mitochondrial membranes.

The rate-limiting enzyme in heme biosynthesis is ALA synthase, which is the first step enzyme in heme biosynthetic pathway. The synthesized endogenous PpIX from ALA is converted to heme in mitochondria by ferrochelatase and cellular levels of Fe^{2+}. Recent study showed that not only low levels of ferrochelatase but also the augmented uptake of ALA contributed to the ALA-induced accumulation of PpIX in cancer cells (14). In differentiating B16 melanoma cells, however, the expression and activity of ferrochelatase did not correlate with accumulation of PpIX in cells (20). Furthermore, accumulation of PpIX has been affected by the activity of heme oxygenase (HO)-1, a key player for reducing cytotoxicity of heme (21). Thus, the mechanism of the increased accumulation of PpIX in cancer cells is not fully elucidated. The purpose of the present work is to clarify the mechanism of PpIX accumulation in U937 cells to induce efficient cell death by PDT.

Materials and Methods

Chemicals

ALA, β-alanine, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP), PpIX and deferoxamine were obtained from Sigma Chemical Co. (St. Louis, MO). Sn-mesoporphyrin and N-methyl protoporphyrin IX (Frontier Scientific) were obtained

from Funakosi (Tokyo). 10-nonyl acridine orange (NAO) and tetramethylrhodamine-ethyl-ester (TMRE) were obtained from Molecular Probes (Eugene, OR). All other chemicals were of analytical grade and obtained from Nacalai Tesque (Kyoto). NAO, TMRE and cyclosporine A were dissolved in DMSO and stored in aliquots at 4°C until use.

Cell culture

Histiocytic lymphoma U937, obtained from American Type Culture Collection (Rockville, MD, USA) were maintained in RPMI 1640 medium (Minimum Essential Medium Alpha, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) with 100U/ml penicillin and 100 μg/ml streptomycin. Cell cultures were established in 75cm^2 flasks and kept in humidified atmosphere with 5% CO_2 at 37°C as described in previous paper (22).

Assay for intracellular mitochondrial membrane potential in U937 cells

Cells (1 x 10^6 cells/ml) were washed twice with serum-free medium and incubated with various concentrations of ALA and 50 μM FCCP in serum free culture medium for 3 h. After incubation, the cells were washed twice with PBS, stained with 10 nM NAO or 100 nM TMRE for 15 min at room temperature in the dark. After washing with PBS twice, cells were resuspended in PBS and subjected to a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA) to determine the mitochondrial membrane potential in cells (23, 24).

Detection of PpIX in cells cultured in the presence of ALA under fluorescence microscopy

Cells were feeded in 6-well plates and cultured with serum free cultured medium containing various concentrations of ALA (0.5 ~ 5 mM) for 3 h. Then, the cells were stained with 10 nM NAO or 100 nM TMRE for 15 min at 37°C. The cells were washed with PBS and fluorescence of NAO, TMRE and PpIX was observed by fluorescence microscopy (Ziess, Axiovert 200) with a 100 W halogen lamp. Fluorescence images were made by a highly light-sensitive thermo-electrically cooled charge-coupled device (CCD) camera ($ORCA_{II}$-ER, Hamamatsu, Japan). The filter combinations used were composed of a 450 nm excitation filter, 510 nm beam splitter and a 515–565 nm emission filter for NAO; G365 nm excitation filter, a FT580 nm beam splitter and a up LP590 nm emission filter for PpIX; and a 488 nm excitation filter, 505 nm beam splitter and a 564 nm emission filter for TMRE (25).

Flow cytometry of cellular PpIX

U937 cells were grown on tissue culture plates and were incubated for 24 h. ALA was diluted in RPMI-1640 medium to a stock solution of 1 M and a final concentration of 0.1 – 5 mM was incubated with cells for 30 min – 3 h. After incubation with ALA, the cells were washed with PBS (without Ca^{2+} and Mg^{2+}) and scraped off with a rubber policeman. After 10 min of centrifugation at 1100 r.p.m., the medium was decanted and 0.5 ml of PBS (without Ca^{2+} and Mg^{2+}) was added. The suspension was filtered and measured using a Fluorescence-Activated Cell Sorter (Becton Dickinson FACS Calibur, Mountain View, CA, USA). In all, 20,000 cells were measured in each sample (ex. 488 nm, em. 650 nm) (21).

Excitation and emission spectra of PpIX in cells exposed to ALA

U937 cells were incubated with 1 mM ALA for 6 h in medium minus FBS. Samples were transferred to a quartz cuvette which was positioned in a spectrofluorometer (Hitachi 650–10S). Excitation for PpIX was 410 nm, and the fluorescence emission was scanned from 430 to 720 nm. Background autofluorescnce was determined in cells that had not been incubated with ALA. Emission for PpIX was 635 nm, and the fluorescence excitation was scanned from 400 to 600 nm.

Results

Excitation and emission spectra of ALA-mediated intracellular PpIX

The absorption spectra of extracted protoporphyrin showed a similar curve to that described by Calzavara-Pinton, Venturini, and Sala (21, 26) (data not shown). Excitation and emission spectra of fluorescence in U937 cells incubated with 1 mM ALA for 6 h in the absence of FBS were the same as those with authentic PpIX (Sigma Chemical Co.) in the presence of cultured cells (Figures 1A and 1B). Figures 1C and 1D showed excitation and emission spectra of the cells incubated for 6 h with 1 mM ALA (Figure 1).

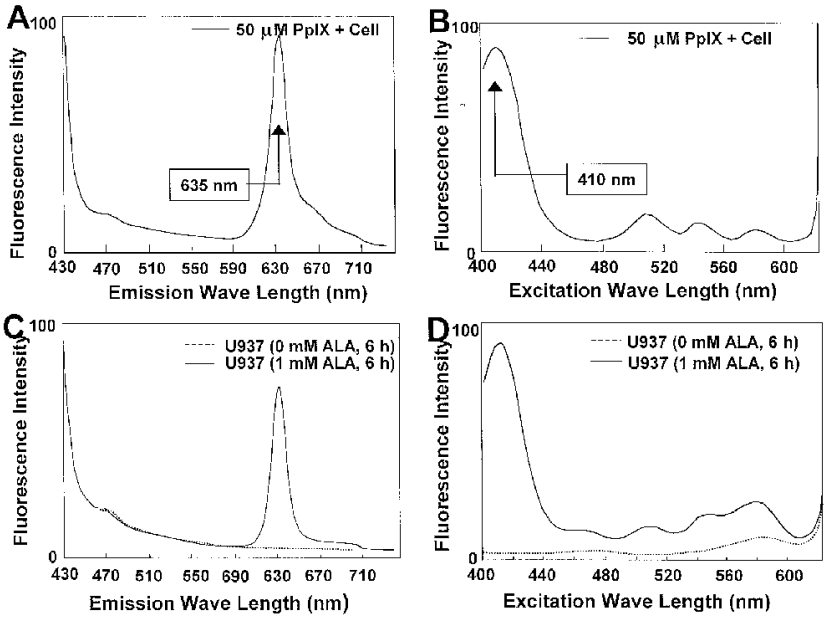

FIGURE 1. Excitation and emission spectra of U937 cells exposed to ALA. U937 cells (5 x 10^5 cells/ml) were incubated with 1 mM ALA for 6 h and the excitation and emission spectra were obtained using fluorescence spectrophotometer (Hitachi 650–10S). A) Fluorescence emission spectra of 50 μM PpIX in the presence of cells was obtained by excitation wavelength at 410 nm. B) Fluorescence excitation spectra of 50 μM PpIX in the presence of cells were obtained at 635 nm emission wavelength. C) Fluorescence emission spectra of accumulated PpIX in the cells mediated by ALA were obtained by excitation wavelength at 410 nm. D) Fluorescence excitation spectra of accumulated PpIX in the cells mediated by ALA were obtained at 635 nm emission wavelength.

The maximum excitation and emission wavelengths of cells cultured with ALA were 410 and 635 nm, respectively. The excitation wavelength was the same with that of ALA-mediated PpIX in WiDr cells (26). The levels of PpIX in cells not incubated with ALA were below the detection level.

Accumulation of PpIX in ALA-treated U937 cells

U937 cells were incubated with various concentration of ALA for 3 h. Figure 2 shows accumulation of cellular PpIX measured by cytometoric analysis using FACScan as a function of concentration of added ALA and time of incubation. Figure 2A shows an actual histogram of analyzed results and Figure 2B shows the ALA-concentration dependent increase in PpIX in U937 cells. Accumulation of cellular PpIX was linearly increased as a function of added ALA concentration for up to 1 mM. As shown in Figures 2C and 2D, the ALA-mediated PpIX accumulation was time dependent after incubation with ALA. The time dependent curves were different between 1 mM and 5 mM of added ALA (data not shown) and definite lag time was observed in the cells incubated with low concentration of ALA.

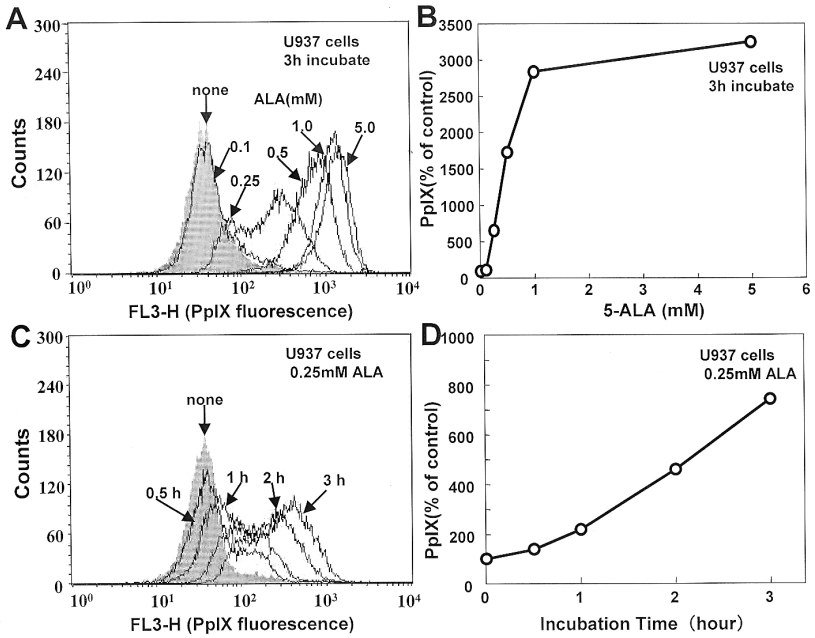

FIGURE 2. Accumulation of PpIX in U937 cells after incubation with various concentrations of ALA and various times of incubation. U937 cells were cultured in the standard medium in the presence of various concentrations of ALA for the indicated time. A) Cellular content of PpIX measured by flow cytometry using FACScan FL3-H. B) Concentration dependent curve of accumulated PpIX in U937 cells after incubation for 3 h using the program of Cell-Quest (Softwar version 3.1) of Becton Dickinson. C) Flow cytometric measurement of PpIX fluorescence after incubation of the cells with 0.25 mM ALA for various times. D) Time dependent curve of accumulated PpIX in U937 cells after incubation with 0.25 mM ALA for various times. Similar results were obtained in 3 separate experiments.

Subcellular localization of ALA-induced PpIX in U937 cells

The subcellular localization pattern of accumulated ALA-mediated PpIX and a mitochondria stained with cardiolipin specific probe, NAO, were studied by fluorescence microscopy. NAO and PpIX in Figure 3 show the fluorescence microscopic pictures of U937 cells after staining with NAO, or incubated with ALA, respectively. The granular patterns of the cells were initially similar for both PpIX and NAO, indicating that the distribution of PpIX primarily coincided with mitochondria. However, at later time points, PpIX fluorescence increased in the cytosol, especially in the peri-nuclear (data not shown). This is probably due to the diffusion of PpIX from mitochondria to cytoplasm.

Effect of β-alanine on the accumulation of ALA-mediated PpIX

ALA-induced PpIX accumulation is regulated by various factors including ALA transport system in cell membrane. 5-ALA, but not 5-ALA methyl ester, was transported by beta-amino acid and GABA carriers in human adenocarcinoma cell line, and the transport was attenuated by 85% in the presence of 10 mM β-alanine (17, 18). To elucidate the mechanism of ALA transport in U937 cells, the effect of β-alanine on the ALA-mediated accumulation of PpIX was examined. As shown in Figure 4, the ALA-mediated PpIX accumulation was attenuated by β-alanine in a concentration dependent manner. 1 mM β-alanine attenuated 60% ALA-induced PpIX accumulation (Figure 4). The suppressing activity of β-alanine was also confirmed by fluorescence microscopic picture of accumulated PpIX in the cells (Figure 5). The result indicated that the ALA transport in U937 cells also occurred through BETA transporters.

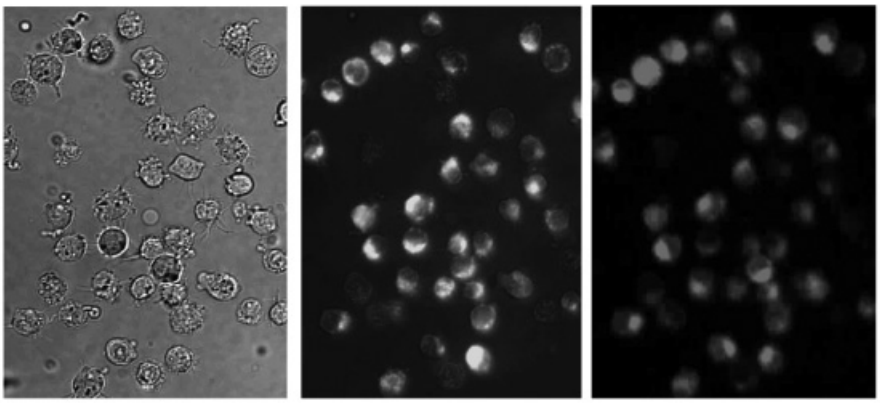

FIGURE 3. Intracellular localization of PpIX in U937 cells after incubation with ALA. U937 cells were cultured in the standard medium in the presence of 1 mM ALA for 3 h. Cells were washed with PBS and stained with 10 nM NAO for 15 min before observation under fluorescence microscopy. Bright, NAO and PpIX show the cells observed under bright light and fluorescence microscopy using filter of ex/dm/em = 450nm/510nm/515–565nm for NAO and ex/dm/em = 365nm/580nm/590nm for PpIX, respectively. Bright, bright light microscopic picture. NAO, NAO stained cells. PpIX, PpIX synthesized cells. Similar results were obtained in more than 3 separate experiments.

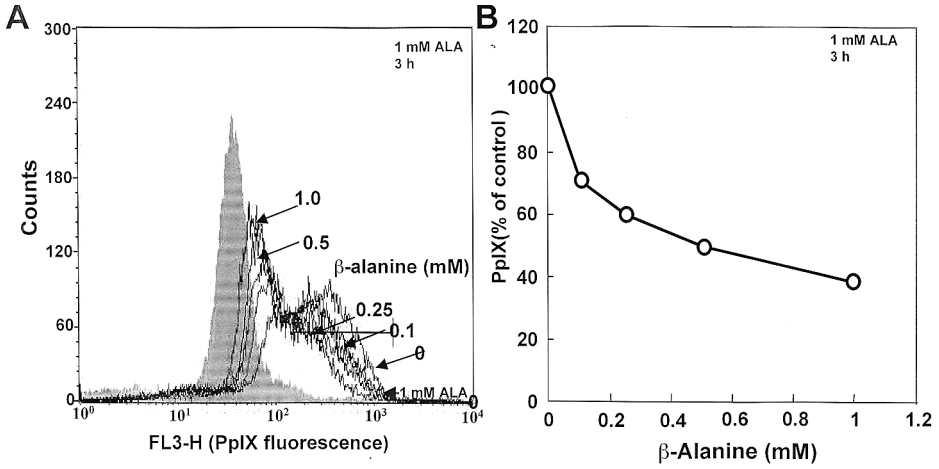

FIGURE 4. Concentration dependent inhibition of ALA-induced PpIX accumulation in U937 cells by β-alanine. Experimental conditions were the same as described in Figure 2. A) Accumulation of ALA-induced PpIX in the presence of various concentrations of β-alanine. B) Concentration dependent inhibition of PpIX accumulation in U937 cells after incubation with 1 mM ALA for 3 h in the presence of various concentrations of β-alanine. Similar results were obtained in 3 separate experiments.

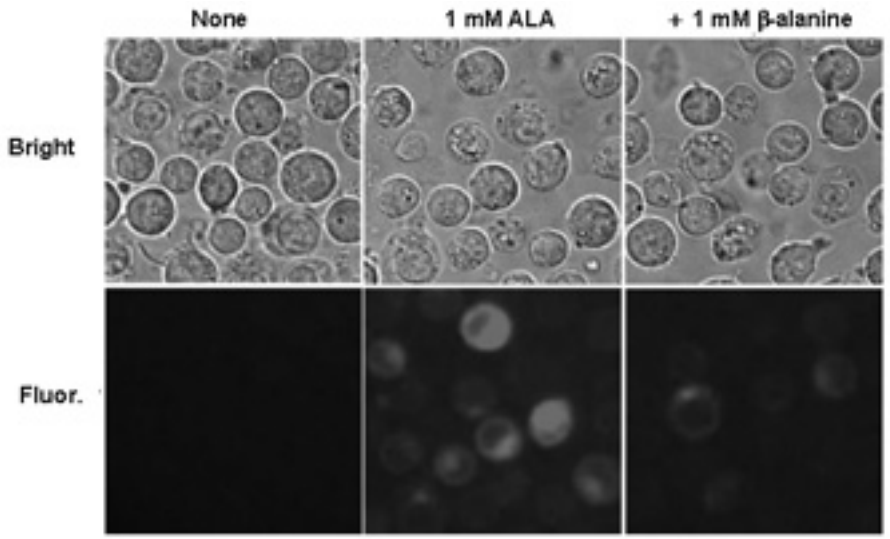

FIGURE 5. Inhibition of ALA-induced PpIX accumulation in U937 cells by β-alanine. Experimental conditions were the same as described in Figure 4. U937 cells were incubated for 3 h with 1 mM ALA in the presence or absence of 1 mM β-alanine. Bright, the cells as described in Figure 3. Fluor, fluorescence microscopic picture. Similar results were obtained in 3 separate experiments.

Effect of FCCP on the accumulation of ALA-mediated PpIX

It has been reported that biosynthesis of coproporphyrinogen III from ALA and transport of coproporphyrinogen III into mitochondria occurred via an ATP-dependent process in MLA cells and normal animal cells (12). Thus, the effect of FCCP, an uncoupler of oxidative phosphorylation in mitochondria, on the ALA-medicated PpIX accumulation was examined to confirm the involvement of the energy requirement reaction. Membrane potential of U937 cells, monitored by the fluorescence intensity of TMRE, was not affected by high concentration of ALA (5 mM). However, the membrane potential was decreased after treatment with 50 μM FCCP in the presence or absence of ALA. ALA-mediated PpIX accumulation in the cells was decreased in the presence of 50 μM FCCP (Figure 6). These results indicate that the ALA-mediated PpIX accumulation in U937 cells depend on the energy metabolism.

Effect of deferoxamine and $MnCl_2$ on the accumulation of ALA-mediated PpIX

Ferrochelatase is the terminal enzyme of the heme-biosynthetic pathway and is thought to be the rate-limiting step for heme production. This pathway required iron and then attenuated the heme synthesis by low concentration of iron chelator deferoxamine (27). Thus, the effect of deferoxamine on the accumulation of ALA-mediated PpIX was studied in U937 cells. 10~500 μM deferoxamine strongly increased the accumulation of ALA-mediated PpIX in U937 cells. After incubation with 0.1 mM deferoxmin for 3 h, fluorescence of ALA-mediated PpIX increased more than 15-fold of the control. The accumulation depended on the concentration and time of incubation with deferoxiamine (Figure 7).

The effect of $MnCl_2$ (28), an inhibitor of ferrochelatase, on the accumulation of ALA-mediated PpIX was examined. As shown in Figure 8, $MnCl_2$ also strongly increased accumulation of ALA-mediated PpIX. After 3 h incubation of U937 cells with 1 μM $MnCl_2$

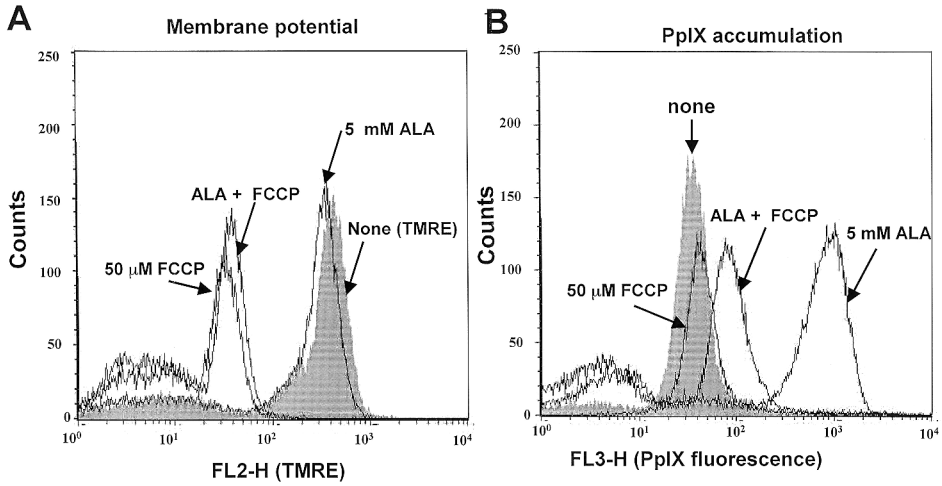

FIGURE 6. Inhibition of ALA-induced PpIX accumulation in U937 cells by uncoupler of oxidative phosphorylation. U937 cells were incubated with 5 mM ALA in the presence or absence of 50 μM FCCP. A) Depolarization of mitochondrial membrane potential by 50 μM FCCP in the presence or absence of 5 mM ALA. Membrane potential was monitored by flowcytometric analysis of TMRE fluorescence. B) Inhibition by 50 μM FCCP of PpIX accumulation in U937 cells incubated with 5 mM ALA for 3 h. Similar results were obtained in 3 separate experiments.

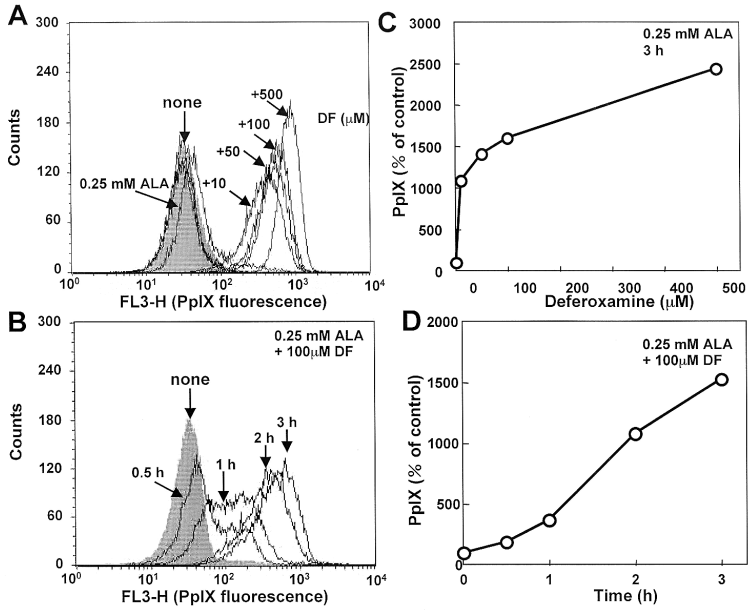

FIGURE 7. Increase in PpIX accumulation in U937 cells by deferoxamine. Experimental conditions were the same as described in Figure 2. A) and C) Increase of ALA-induced PpIX accumulation in U937 cells in the presence of various concentrations of deferoxamine (DF) monitored by flowcytometry. B) and D) Time dependent increase in ALA-induced PpIX by deferoxamine. Similar results were obtained in 3 separate experiments.

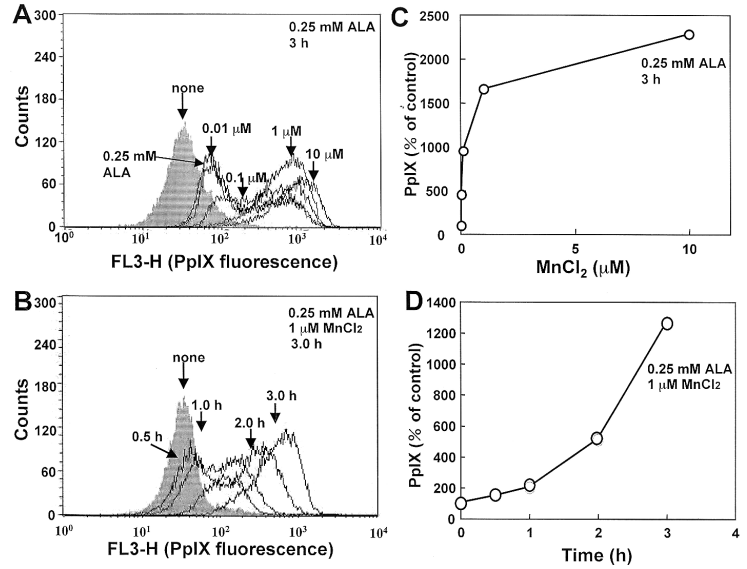

FIGURE 8. Increase in PpIX accumulation in U937 cells incubated with ALA in the presence of $MnCl_2$. Experimental conditions were the same as described in Figure 2. A) and C) Increase of 0.25 mM ALA-induced PpIX accumulation in U937 cells in the presence of various concentrations of $MnCl_2$ monitored by flowcytometry. B) and D) Concentration dependent increase in ALA-induced PpIX by $MnCl_2$. Similar results were obtained in 3 separate experiments.

in the presence of 0.25 mM ALA, more than a 12-fold increase in PpIX accumulation was observed (Figures 8C and 8D). These results indicate that metabolism of accumulated ALA-mediated PpIX is suppressed by inhibiting ferrochelatase and iron.

Effect of Sn-mesoporphyrin on the accumulation of ALA-mediated PpIX in U937 cells

Heme contents in cells are regulated by HO. It is well known that Sn-mesoporphyrin is a tipical inhibitor of HO (29). Thus, it was expected that accumulation of ALA-mediated PpIX was affected by the changes in HO activity. As expected, accumulation of ALA-mediated PpIX was increased by Sn-mesoporphyrin (Figure 9). The stimulating activity of Sn-mesoporphyrin depended on the concentration and time of incubation. The stimulating activity was very strong and more than 7.5-fold of accumulation was induced by the incubation of U937 cells with 0.1 μM Sn-mesoporphyrin in the presence of 0.25 mM ALA.

Discussion

The aim of the present work is to clarify the mechanism that regulates the accumulation of ALA-enhanced PpIX in U937 cells. Kinetic analysis using fluorescence technique revealed that the synthesized endogenous PpIX localized preferentially in mitochondria and

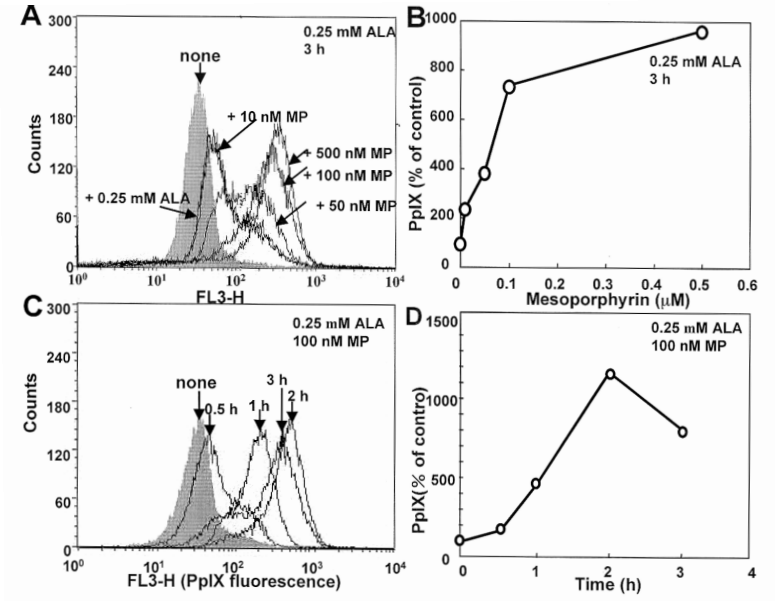

FIGURE 9. Increase in PpIX accumulation in U937 cells incubated with ALA in the presence of mesoporphyrin. Experimental conditions were the same as described in Figure 2. A) and B) Increase of ALA-induced PpIX accumulation in U937 cells in the presence of various concentrations of mesoporphyrin (μM) monitored by flowcytometry. C) and D) Time dependent increase in ALA-induced PpIX. Similar results were obtained in 3 separate experiments.

peri-nuclear regions in cells incubated with ALA. The cellular accumulation of PpIX was suppressed by β-alanine and FCCP but enhanced by deferoxamine, $MnCl_2$ and Sn-mesoporphyrin. These results suggest that ALA-enhanced accumulation of PpIX in U937 cells is determined by several steps including cellular uptake of ALA and *de novo* synthesis of PpIX and degradation of Heme (Figure 10).

Although exogenously administered ALA increased cellular levels of PpIX in most tissues, accumulation of PpIX occurred more markedly in tumor cells than in normal cells (1–5, 9). Thus, physicochemical properties of PpIX have been used for PDT of patients with tumors (30); photoactivation of tissues preferentially kills tumor cells without eliciting severe toxicity to progenitor cells and stem cells (31). Although the ALA-dependent PDT has been used successfully in the treatment of oncological and non-oncological diseases, the mechanism of this modality remains to be elucidated.

To study the mechanism of preferential accumulation of ALA-derived PpIX in malignant cells, several factors have been analyzed, such as ALA synthase, the rate-limiting enzyme in heme biosynthesis, cellular uptake of ALA (10, 11, 17, 18), mitochondrial properties (12), key molecules for PpIX metabolism (13, 21), ferrochelatase activity (14, 28), contents of iron (15, 23) and transferrin receptor (16, 34). The results of these analyses suggest that the efficacy of PDT likely depends on cellular activity of ferrochelatase, a rate-limiting enzyme in heme biosynthesis (28). However, Ohgari *et al.* (14) reported

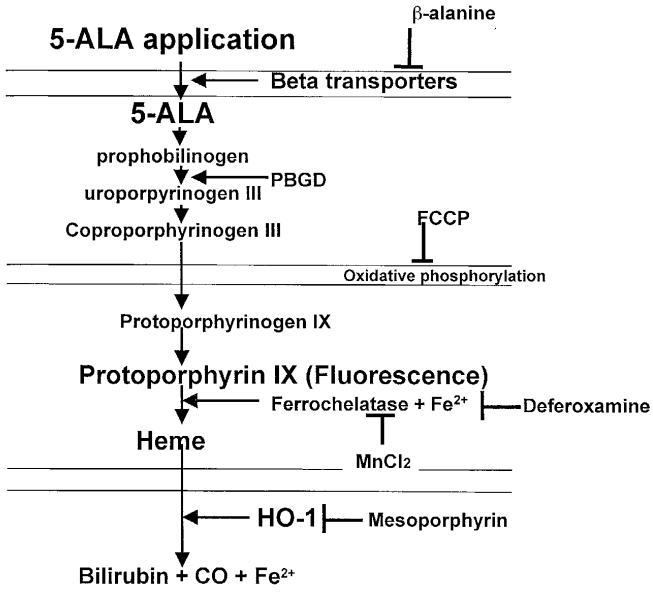

FIGURE 10. Schematic representation the regulatory mechanism of 5-ALA-mediated PpIX accumulation in U937 cells. The results of this experiment show that 5-ALA-mediated endogenous PpIX accumulation in U937 cells is suppressed by β-alanine and FCCP and is stimulated by $MnCl_2$, deferoxamine and mesoporphyrin. The results indicate that ALA-mediated PpIX accumulation in U937 cells is regulated by initial ALA uptake, ALA conversion to PpIX and metabolism of accumulated PpIX.

that not only the low level of ferrochelatase but also the augmented uptake of ALA contributed to the ALA-induced accumulation of PpIX in MethA cells, L929 cells and Balb/3T3 cells. Since no significant difference in the overall uptake of ALA was seen among different cells, preferential accumulation of PpIX in tumor cells seems to depend on the step of ALA conversion rather than its initial uptake (5). Furthermore, preferential accumulation of PpIX in tumor cells was strongly influenced by the difference between the activities of PpIX-generating porphobilinogen deaminase and PpIX-converting ferrochelatase (5). However, gene expression and activity of porphobilinogen deaminase in the differentiating B16 melanoma cells correlated with PpIX synthesis but not with ferrochelatase levels (21). It is apparent that the accumulation of PpIX is determined by several factors including uptake of ALA, porphobilinogen deaminase and ferrochelatase activities. Although the activity of ferrochelatase generally decreases in a variety of tumor cells, inhibition of the enzyme and/or elimination of iron by chelating agents further increased the accumulation of PpIX. Furthermore, preliminary experiments in this laboratory revealed that the accumulation of PpIX in primary cultured cells (Renal proximal tubule epithelial cells, RTEC, and Bladder smooth muscle cells, Bd-SMC) with normal ferrochelatase activity was not affected by a specific inhibitor of the enzyme. Thus, the mechanism of preferential accumulation of PpIX in tumor cells should be studied further.

Translocation of synthesized endogenous PpIX from mitochondrial matrix to cytosol also plays an important role in the accumulation of PpIX. A recent report describes the localization of ATP-binding caset transporter (ABC) in mitochondrial membranes that transports synthesized endogenous PpIX into cytosol (32). Furthermore, Krishnemurthy *et al* (33) reported that inhibition of the ABC-transporter suppressed the accumulation of PpIX in cells. Thus, mitochondrial ABC transporter may also determine the accumulation of PpIX in tumor cells. This possibility should be studied further.

The present work demonstrates that synthesized endogenous PpIX accumulated in U937 cells after incubation with 5-ALA. The synthesis of PpIX was suppressed by β-alanine and FCCP, suggesting that ALA uptake by β-transporter (17, 18) and mitochondrial ATP play important roles in the synthesis and accumulation of PpIX (17, 27). In contrast, deferoxamine, $MnCl_2$ and Sn-mesoporphyrin enhanced the ALA-dependent accumulation of PpIX (27, 28). These observations suggest that ALA-dependent accumulation of PpIX was enhanced by inhibiting synthesis and/or degradation of heme (29). Thus, the efficacy of PDT of malignant tumors could be improved by selectively modulating endogenous synthesis and accumulation of PpIX in tumor .

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Exposure to an Additional Alternating Magnetic Field Affects Comb Building by Worker Hornets

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Abstract: Oriental hornet workers, kept in an Artificial Breeding Box (ABB) without a queen, construct within a few days brood combs of hexagonal cells with apertures facing down. These combs possess stems that fasten the former to the roof of the ABB. In an ABB with adult workers (more than 24 h after eclosion), exposed to an AC (50 Hz) magnetic field of a magnitude of B = 50–70 mGauss, the combs and cells are built differently from those of a control ABB, subjected only to the natural terrestrial magnetic field. The effects of the additional magnetic field consist of (a) 35–55% smaller number of cells and fewer eggs in each comb, (b) disrupted symmetry of building, with many deformed and imperfectly hexagonal cells, and (c) more delicate and slender comb stems.

KEY WORDS: magnetic field, Oriental hornet, comb building, Hymenoptera, gravity-oriented comb cells.

OUR LABORATORY has long been engaged in investigating the biology of the Oriental hornet *Vespa orientalis* (Hymenoptera, Vespinae). This is an annual insect whose fertilized queen hibernates and in spring founds a new nest in the subterranean hollow. This nascent nest grows and thrives throughout the summer till the end of the season in autumn (Ishay *et al*., 1967; Spradbery, 1973). Shortly after foundation of the nest, the first worker hornets eclode, their number steadily increasing thereafter, amounting in this species to several hundreds or more in the month of August. When such worker hornets are removed from their natural nest and transported to the laboratory where they are assorted in groups of 5–20 individuals into Artificial Breeding Boxes (ABBs), they commence building one

or more brood combs out of soil, paper or any other available, electrically-insulating material. The combs are built suspending down from the roof of the ABB and affixed to it by one or more (as needed) interconnecting stems. The number of cells within each comb ranges between 10–25, with their apertures facing down in the direction of the gravitational force. In the absence of a queen the workers will oviposit a single, unfertilized egg, in each of the built cells, which will give rise to male offspring (Ishay & Sadeh, 1975). To construct such a comb in the absence of a queen takes the workers about two weeks (Ishay *et al.*, 1995). In a normal nest the building of cells and combs is continuous throughout the whole season. In our long-range searches in the field for hornet nests we have occasionally come across subterranean nests that were founded at the bases of high-tension electric poles (i.e., up to 1 meter from the base). Interestingly, the brood combs extracted from such nests differed from the combs in ordinary nests in several respects, to wit: a) they were less 'fertile', i.e., there were many empty cells in them which, although oviposited into, did not give rise to any living brood; b) the number of combs was smaller than customary; and c) there was no sexual brood in them because the built cells were not large enough to house males or queens.

Aware of the fact that high-tension electric poles also create around them a magnetic field ranging from 10 to 25mGauss (measured one to two meters from the base of the pole), we deemed it worthwhile to create an artificial magnetic field in the laboratory and attempt to assess its effect on the comb-building activity of laboratory-grown hornets. First, however, we compared the combs built in vicinity to high-tension poles with those built in any other location in the field. Such a comparison is presented in Figure 1.

Figure 1A shows the underside of a 'normal' comb, revealing cells housing larvae (l) and beneath them, cells housing the silk coated pupae (s). Note also several worker hornets (w) walking upon the comb and probably nursing the brood. Note further that the larvae and pupae are densely packed, leaving no empty cells between them (such as would indicate death and expulsion of the contained brood). Figure 1B presents a top view of 'normal' combs, showing the spaced stems (see arrows) interconnecting between the tiered combs and those connecting the combs to the roof of the ABB. Figure 1C, on the other hand, shows a comb extracted from a nest located near the base of a high-tension electric pole. Here, we note the paucity of larvae that have succeeded to pupate, with the majority of the brood dying and expelled from (or dropping out of) their cells. We can reasonably assume that the longer the exposure of the brood to the magnetic field conditions, the more damage the brood sustains. Figure 1D also pertains to the 'exposed' combs, showing the stems (see arrows) in their upper part to be more delicate or slender than in the 'normal' combs (Figure 1B).

In the wake of these preliminary observations, we proceeded to set up in our laboratory a system whereby we could alter the normal magnetic field of the earth and thus enable us to assess the effect of the altered conditions on the longevity and also the building activity of worker hornets. Initially (Kisliuk & Ishay, 1978) we found that an additional horizontal magnetic filed is lethal for adult worker hornets (more than 24 hours after eclosion) and their larvae, but juvenile hornets (0–24 hours after eclosion) are capable of adapting to the additional magnetic field, and build combs commencing at the sites with high field intensity and proceeding towards sites of lower field intensity. Subsequently (Kisliuk & Ishay, 1979), we learned that the introduction of a magnetic field that counteracts the vertical component of the earth's magnetic field and thereby creates a total 'zero field', is lethal also to juvenile worker hornets and completely disrupts the comb-

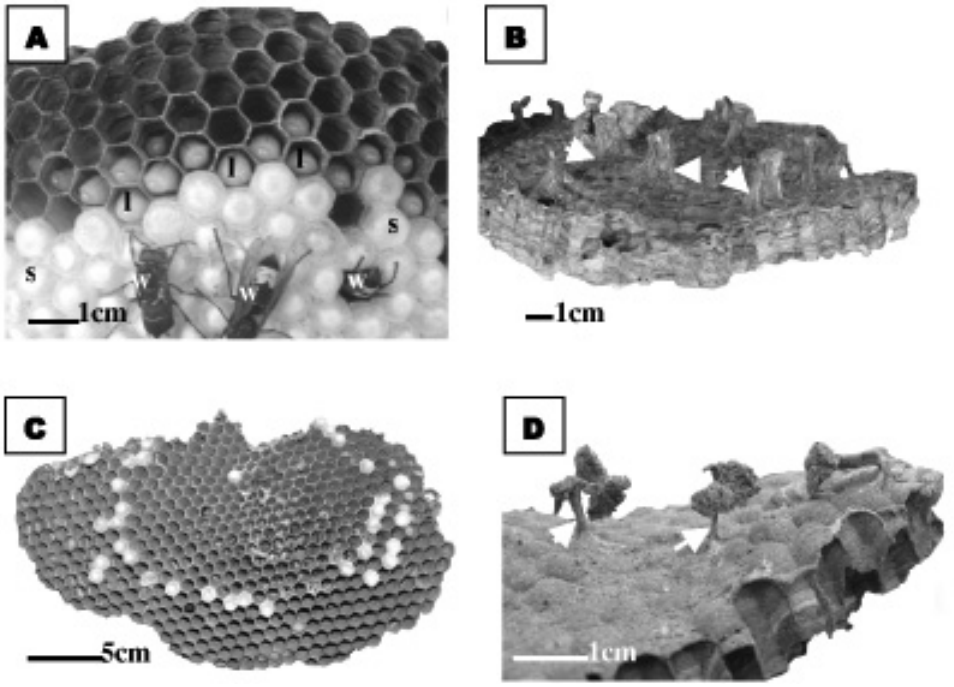

FIGURE 1. Top and bottom views of a brood comb from a *V. orientalis* nest in its natural subterranean location vs. one from a nest built beneath a high-tension electric pole.
A- Picture showing the underside of a comb from a 'natural' nest. One can see developing larvae (l), silk-coated pupae (s) and three adult workers (w) walking on the pupal silk caps. Note the continuity of the brood deposition, without empty cells in between. Bar=1cm.
B- Picture showing the top side of a 'natural' comb. Arrows indicate the stems projecting from the comb, which either connect one comb to another or else connect it to the roof of the nest (or the ABB). Bar=1cm.
C- Picture showing the underside of a comb in a nest built near the base of a high-tension electric pole. Here there is paucity of occupied cells, with an abundance of vacant cells (whose brood died and fell out), and a few silk caps indicating pupal stages that have ecloded. Bar= 5cm.
D- Top view of a comb in a nest built near the base of a high-tension electric pole. Again as in Figure 1B, arrows mark the interconnecting comb stems, but here they are much more delicate and slender. Bar=1cm.

building orientation of adult worker hornets, which suggested that the terrestrial magnetic field is the main guideline for vespan building orientation. In the face of these findings, we deemed it worthwhile to use the accumulated data to devise further experiments along these lines but with greater in-depth programming. The results of these later experiments are described and discussed herein.

Materials and Methods

Worker hornets were obtained from combs extracted from nests in the field during the summers of 2005 and 2006. The method of collection was as previously described (Ishay,

1975). The collected worker hornets were placed in groups of 10–20 individuals in artificial breeding boxes (ABBs), where they received: a) clumps of soil mainly red loam, as building material; b) cubes of sugar; c) a vial containing tap water; and d) the whites of hard-boiled eggs and morsels of tuna fish (as protein source). The ABBs were dispersed randomly, ensuring, however, that the test ABB was within the magnetic field while the control ABB was at some site remote from the test ABB. In all, we ran 8 cycles of test and control, using AC current, at a frequency of 50Hz, and a magnitude of 50–70mGauss. The latter was applied in a direction perpendicular to the gravitational force. The ABBs were all kept in the dark.

Results

The overall experiment lasted two weeks, during which time test worker hornets were exposed to and remained within a magnetic field while control worker hornets were at varying distances from a magnetic field. Upon termination of the experiment, the series of tests run yielded results that were not uniform but rather variable in each test, yet showed common trends as follows: 1) Fewer cells were built by the test groups than by the control groups, as witness Figure 2A *vs*. Figure 2B. The total average numbers of cells built by the test groups were about 35–55% smaller than the control groups. It is worth to mention that it was almost impossible to compare the building parameters along the season as initially many cells are built and later only few cells are built etc. Same differences were observed in groups of hornets derived from various families. 2) Insofar as fertility, the control groups yielded the maximal number of eggs, i.e., each comb cell housed an egg or larva. Contrariwise the test combs contained only few eggs and no larvae. 3) Insofar as cell symmetry, the cells in combs of the control groups were perfectly hexagonal and uniform throughout dimension-wise, whereas the cells in combs of the test groups were rather asymmetrical, i.e., their sides were non-uniform in length, which lent the cells a deformed shape (compare Figures 2A,C *vs*. Figures 2B,D).

Discussion

In this study we assess how nest construction by hornets is affected by exposure to a magnetic radiation at a frequency of 50Hz and a magnitude of B=50-70mGauss which is directed perpendicular to the gravitational force. Non-ionizing magnetic radiation is ubiquitous and has many sources, both man-made and environmental, such as power lines, the static magnetic field of the Earth, solar activity, etc. Behavioral studies have shown that the capacity to sense the Earth magnetic field, and possibly other magnetic fields of extremely low frequency and magnitude, is widely developed in a variety of species, which use this capacity for navigation and guidance over long and short distances (Ritz *et al*., 2004; Johnsen & Lohmann, 2005), and this by extracting needed information for estimating the north-south direction. This natural 'GPS' (Global Positioning System) ability assists animals in navigation. However, little is known about the mechanisms of interactions between magnetic fields and biophysical systems. In this regard, systems and several hypotheses have been proposed: electromagnetic induction, modulation of bio-

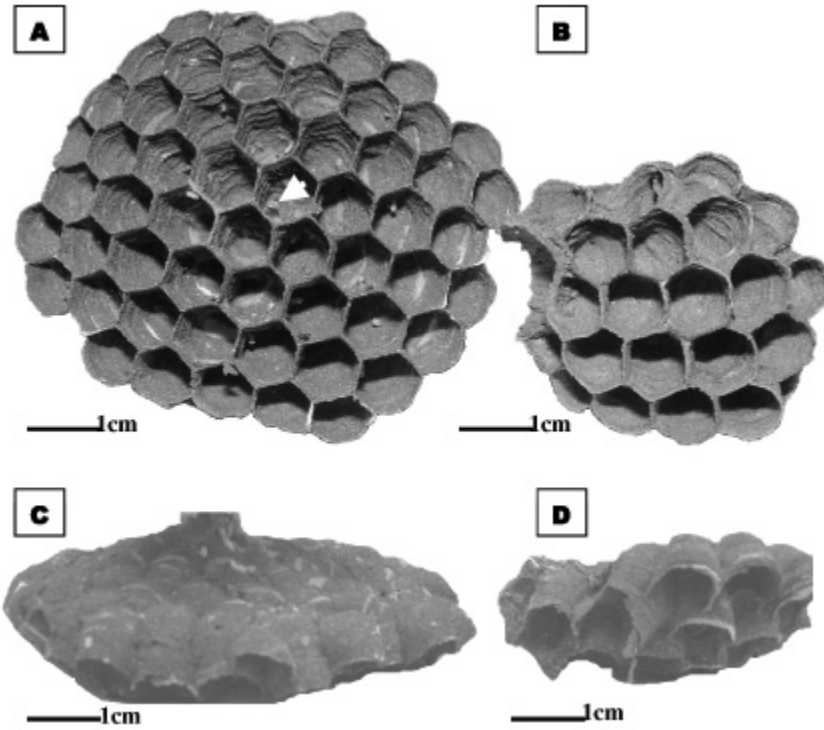

FIGURE 2. Morphological and structural comparisons between control combs built in a 'natural' environment and test combs built under additional magnetic field.
A) Control comb build of red loam soil. All the completed cells are perfectly hexagonal at their lower end. Arrow marks an egg-containing cell. Bar=1cm.
B)- Test comb built within an additional magnetic field. Note: a) the smaller number of cells; b) the distorted shape of the cells, which are not perfectly hexagonal; and c) the absence of any eggs. Bar=1cm.
C) Top view of the control comb, showing a fairly level roof surface. Bar=1cm.
D) Top view of test comb built within a magnetic field, showing the rather terraced and bumpy appearance of the roof. Bar=1cm.

genic magnetite (Kirschvink, 1992), chemical reactions sensitive to weak magnetic fields (Weaver *et al*., 2000), or cyclotron and stochastic resonances at the cellular, ion-channel, and molecular levels, (Gailey, 1999; Lin, 2003).

Kirschvink (1992) posits in insects, including honeybees, interaction between weak magnetic fields and magnetite (Fe_2O_3), suggesting that the magnetic field reacts directly with magnetite crystals (50nm in diameter), which, if connected through cyto-skeletal filaments to gated channels, may cause the latter to open (see also Davila *et al*., 2005). Iron biominerals were found to be deposited in the common hornet (*Vespa affinis*) within the cytoplasm of trophocytes (Hsu, 2004). Weaver *et al*., (2000) envision chemical reactions in which the magnetic field affects the orientation of reactants and thus changes reaction rates. The influence of magnetic fields on the orientation of comb building by honeybees has also been studied. In bees there is some interaction of orientation with the magnetic field and vision. It was found that honey-bees build a new comb in the same magnetic

direction as the parent hive (in the absence of other external cues) (De Jong, 1982). Hornets may also react to the magnetic field and vision for orientation but in the actual experiment we tested only (or mainly) the building abilities. Hornets always prefer to build their combs in darkness so there is probably no interaction with vision in this respect. In our case, we assume that the introduction of an alternating magnetic field induces or initiates currents in the body of the worker hornet which cause it to build its comb in an abnormal way.

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An Unanswered 2003 Letter Appealing on Behalf of all Mankind to Nobel Laureate Roderick McKinnon to Use His Newfound Fame and Visibility to Begin Restoring Honesty and Integrity to Basic Biomedical Science by Rebutting or Correcting Suspected Plagiarism in His Nobel-Prize-Winning Work

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Abstract: The centerpiece of this document is an unanswered letter of appeal from the author to Professor Roderick MacKinnon of the Rockefeller University dated November 17, 2003. The aim of the appeal is summarized in the title of this communication. In addition to the 2003 letter, there are also two follow-up letters in this communication, each containing a copy of the 2003 letter and each repeating the appeal. The follow-up letters, dated February 22, 2008 and April 2, 2008 respectively, were also unanswered. To make sure that these letters reached their destination, each was certified with delivery time and date affirmed. Thus the February 22 letter was delivered on the February 24 by the US Postal Service. Two copies of the April 2 follow-up letter were sent. The first copy was delivered by Federal Express on April 4. The second copy of the April 2 letter was delivered by the US Postal Service on the same day. Thus all told three additional copies of the 2003 letters were delivered to, and must be in the hand of Professor MacKinnnon. All these efforts were made to make certain that Professor MacKinnon's refusal to answer my registered 2003 letter was not due to his not having received a copy of that letter.

November 17, 2003

Dear Dr. MacKinnon:

Cell physiological research, on which both you and I have spent much of our lives, is like solving a gigantic crossword puzzle. Like the crossword puzzle, cell physiology also has just one unique solution. But to reach out to that unique solution, cell physiologists of the past faced an insurmountable obstacle.

That is, the physico-chemical concepts needed to construct the correct unifying theory were not yet available when the study of cell physiology began. An incorrect guiding theory was introduced (see below) and as time wore on, taught more and more as unqualified truth. Meanwhile, the study of cell physiology broke up into smaller and smaller fragments or specialties. In time each specialty spawned its own lingo, its own methodology and its own subspecialties; the contact of each specialty with other specialties become less and less frequent and more and more perfunctory. The cumulative result is as Durant described: "We suffocated with uncoordinated facts, our minds are overwhelmed with science breeding and multiplying into speculative chaos for want of synthesis and a unifying philosophy." ("The Story of Philosophy", Durant.)

Now, Durant's complaint addressed the lack of a valid unifying theory, which alone can bind together the scattered fragments and offer pathways out of this chaos toward deeper understanding. However, the obstacle to produce a valid unifying theory gradually evaporated when physics and chemistry matured in the late 19th and early 20th century. Therefore, it was not entirely surprising that some forty years ago a unifying theory of cell physiology built upon mature physics and chemistry made its debut. It bears the name, the *association-induction* (AI) *hypothesis*. (See Book #1 listed under "Books" in enclosed Document #1.) Worldwide experimental testing and confirmation of its essence followed rapidly — as chronicled in three additional full-sized monographs published respectively in 1984, 1992 and 2001 (Books #2, #3 and #4 in Document #1) as well as many scientific papers, including those listed under "Articles" in Document #1.

It would seem that the day would soon arrive when swift progress would light up another new age in science (of the living) like the one (of the dead) in the 17th–early 20th century.

The sad truth is forty years later, that day is yet to come. Long after the verification of the essence of the AI Hypothesis, few biomedical researchers, teachers or students here and abroad have ever heard of all these, let alone understanding or teaching them. But why? After all, we live in an age of unprecedented personal freedom and enjoy means of virtually instant communication far and near. What has gone wrong to reproduce this gargantuan backward movement toward the Dark Ages? Who will be the ultimate victims? Ultimately the victim is the entire Mankind. But some are affected more immediately.

To demonstrate that the harm is being inflicted right now on countless innocent scientists, teachers and students across the world by this global information embargo, I focus on your own outstanding scientific work — for which you are awarded the 2003 Nobel Prize. I begin by asking you two questions: First question: do you know that your work is built on the foundation of the membrane pump theory (which was disproved forty years ago) and thus doomed to collapse sooner or later? Second question: do you know that years ago I introduced for the first time a variety of the basic concepts now found (unac-

knowledged) in your Nobel-Prize winning work on potassium channel? Put differently, do you know that you might be at risk of committing plagiarism? I fully expect that you would answer no to both questions; you really did not know. And accordingly, I shall begin briefing you on some of the critical information that has been withheld from you, beginning with the subject matter of my first question.

If you did not know that the membrane pump theory has been disproved some forty years ago, one legitimate reason could be that this disproof itself has been disproved. If true, one should find that out by consulting the Citation Index or another abstracting publication. However, a thorough search through these media revealed no disproof of the disproof.

In other words, then (or now), no *bona fide* scientific rebuttal, let alone disproof of my disproof of the membrane-pump hypothesis existed. Instead, two bits of gossips have been circulating around on the grapevine. Possibly, they might have created the impression on you that the membrane pump hypothesis is still alive and kicking.

One of these grapevine messages was the offspring of the union of a fact and a popular belief. The *fact* is that Peter Mitchell and Jens Skou have each been awarded a Nobel Prizes for their work on (the postulated) membrane pumps (see below.) And the *popular belief* is that Nobel Prizes are never given to scientific works that have not been proven beyond doubt and are in veracity and originality on equal footing with History's greatest like those of Planck and of Einstein, for example

The other gossip centers on a scientist by the name of Christopher Miller. He, along with several of my other former graduate and postgraduate students, left my laboratory *en masse* in the seventies. The grapevine story reads something like this. When young and trusting, Miller and the others made the mistake of volunteering to study under me, and to participate in research for varying number of years — until they suddenly saw the light and courageously returned to their new-found faith in the membrane pump hypothesis. Since nobody could have known my work better than my students who shared their day-to-day lives with me for years, their *en masse* departure suggests that there must be something seriously wrong with what I taught: the AI Hypothesis or my disproof of the membrane pump theory or both.

In the following I will examine with you the truth behind the gossip. My first contention is that Nobel Prize Committee members and their favorite advisors were not Gods; as human mortals, they make mistakes. And from a careful analysis of the proven mistakes they have made, I found that they were a part of the network of victims-(unknowing) perpetrators of the information embargo. My second contention is that the real cause of the mass exodus of my students was that in a state of panic, they felt that a clean break from my scientific work and me was the only way they could secure jobs after graduation. Details follow.

Peter Mitchell received the Nobel Prize of Chemistry (1978) for his Chemiosmotic Hypothesis. This was astonishing because I have never heard of anyone else being thus honored for merely introducing a hypothesis — a hypothesis that has not been experimentally confirmed then or later. To save space here, I enclose as Document #2, the first 15 pages of a critical review of Mitchell's Chemiosmotic Hypothesis that I wrote in 1981. It shows that the hypothesis itself and its supportive arguments are seriously contradicted by facts.

Thus according to the Chemiosmotic Hypothesis, the energy used in the synthesis of ATP in mitochondria comes from dissipating what he calls a "Protomotive Force," a

composite of a H^+-ion gradient and an electric potential gradient across the inner membrane of mitochondria. However, it turned out that the H^+ ion gradient is negligible if in existence at all. And the electric potential gradient, instead of being maintained at the theoretically required inside-negative voltage of 200–300 mV, turns out to be only 10–20 mV and in the wrong direction (for a simple account of this disproof, see p. 510 of Book #2 in Document #1.) It is hard to believe that Mitchell did not know these incisive experimental contradictions. Yet to the best of my knowledge, he did not publicly abandon this theory or make drastic changes in it or subscribe to the association-induction hypothesis, which has no trouble explaining most if not all known facts with few additional ad hoc assumptions. This inactivity seems to confirm a saying attributed to a Nobel Prize winner in the biomedical field that once you have won a Nobel Prize you are not allowed to correct your mistakes. True or not, you will soon find out.

Jens C. Skou of the University of Aarhus of Denmark also won the Nobel Prize for Chemistry (1997) — specifically for his work on the hypothetical **sodium pump**. His work is, therefore, not just resting on the foundation of the membrane pump theory but is in fact the centerpiece of that membrane pump hypothesis. To gain a better understanding of Skou's work, I read all his publications I could lay my hands on, including the following.

In 1990 Skou gave the Fourth Datta Lecture. Its printed version bears the title: "The Energy Coupled Exchange of Na^+ for K^+ across the Cell Membrane: The Na^+, K^+ -pump" (FEBS 268, 314, 1990.) In the opening section of this paper, he wrote "that the energy from metabolism of the muscle was not high enough to account for the sodium flux.... The answer to the problem was given by (Hans) Ussing (of the University of Copenhagen) namely, that beside the active transport (or pumping) of sodium, there is a sodium-for-sodium exchange, an exchange diffusion, which energetically is neutral." (p. 314)

To the best of my knowledge, this statement is the first and the last Skou wrote on the problem of energy shortage. What is puzzling is that he made no mention of an (expected subsequent successful) verification of this exchange diffusion hypothesis. Yet as he himself made clear, the validity of his life's work on the sodium pump depends on the validity of this hypothesis.

Thus, unbelievable as it is, we find Nobel Laureate Skou also in the role of victim-(unknowing) perpetrator of this global information embargo. For the truth is that not only is there not a single piece of supportive evidence for the exchange diffusion hypothesis, those who studied it in depth arrived at the opposite conclusion.

Thus, between 1955 and 1970, four independent laboratories have tested this hypothesis on four different kinds of living cells. They unanimously reached the same verdict: Ussing's exchanged diffusion hypothesis has no validity (Hodgkin and Keynes, J. Physiol. 128: 61, 1955; Hoffman and Kregenow, Ann. NY Acad. Sci. 137: 566, 1966; Buck and Goodford, J. Physiol. 83:551, 1966; Ling and Ferguson, Physiol. Chem. Phys. 2: 516, 1970).

Thus, Skou continued to believe that the energy shortage problem had been successfully resolved by Ussing's exchange diffusion hypothesis — long after that hypothesis had been thoroughly disproved. Without the help of the hypothetical exchange diffusion mechanism, the energy shortage persists and as such invalidates the sodium pump hypothesis as well as the broader membrane pump hypothesis. Nonetheless, the Nobel Prize Committee for Chemistry of 1997 saw fit to award the Nobel Prize for his work on the sodium pump anyway. This mindless decision on something given so much public trust,

is one instance that suggests what I mentioned earlier: the Nobel Committee members and their favorite advisors are themselves part of the victim-(unknowing) perpetrator network.

However, other than verifying my contention that Nobel Committees are not infallible, Skou's failure to deal with the excessive energy need of the postulated sodium pump was really no more than a minor footnote, if that, in history. To prove or disprove the sodium pump (and the larger membrane pump hypothesis) requires something far weightier. And in essence that was what I tried to achieve — a long time ago.

From 1951 till the middle 1956, I carried out all told some seventy (70) sets of complete and incomplete experiments all giving essential similar results but with increasing reproducibility. The last three sets of complete experiments conducted in 1956 were what I believe to be the most accurate. I then made two simplifying assumptions. First, frog muscle cells use all their available energy exclusively for just one purpose, i.e., pumping Na^+. Second, that all involved processes operate at 100% efficiency. Based on these assumptions, I showed that the minimum energy need for the sodium pump would still be at least 1500% to 3000% of the maximum energy available under the condition.

Within the following ten years, none have disputed my conclusion or the methods used to reach that conclusion. In contrast, the essence of my finding has been twice confirmed. (However, for a bizzare later event involving my former students Jeff Friedman and Chris Miller, see below.)

My conclusion, that the sodium pump hypothesis in specific and the membrane pump hypothesis in general are disproved, should also be viewed from the perspective of the total picture. That is, the sodium pump is but only one of an ever-lengthening list of more and more pumps. Each one of these postulated pumps must derive its energy need from the same source, now shown to be inadequate to cope with just one (sodium) pump alone. For an admittedly incomplete list of the names of pumps already proposed by 1968, see Table 2 (in Document #3 enclosed), — which was collected by Chris Miller from the literature in 1968.

The details of my work on the energy balance of the hypothetical sodium pump were presented in 1962 as a chapter (8) in my first book, "A Physical Theory of the Living State." (Book #1 listed in Document #1) But since this book is no longer in print, I have reproduced *verbatim* this entire chapter as Appendix 1 in enclosed Document #4.

However, other than Chapter 8 and its reprinted version in Document #4, there are other "contacts" which can lead you to the original work. Thus, under the heading "Articles" in the enclosed Document #1 are also the journal names, volume and page numbers of 18 reviews and original articles published in (mostly) easily accessible journals spanning a period of forty years between 1952 and 1992. In the same Document #1, there is also a list of the ISBN, titles, names of publishers etc. of four monographs published respectively in 1962, 1984, 1992 and 2001. In each of these publications, the disproof of the sodium pump hypothesis (and the membrane pump hypothesis in consequence) was discussed at different levels of details.

Beside its primary purpose of providing the information they contain, I have put together this list also to demonstrate that the absence of public awareness of the disproof of the sodium pump (and the membrane pump hypothesis) had nothing to do with difficulty in locating the original publication.

Next I fill in the historical details of the *en masse* exodus of my students. The story really began at a much more honest time in the history of biomedical sciences.

From the late fifties on I had gradually gathered around me a small band of bright and idealistic young students. To a person, each has made substantial contributions to real science.

Then the lion's teeth and claws were suddenly upon us and upon all those who have come to share my scientific view. A coordinated siege began. As an example, NIH program director, Dr. Paul Bowman told me that our NIH support might be terminated *permanently*. As I scrambled to save my laboratory, panic seized my graduate students.

Four of them including Chris Miller went back to Swarthmore College and asked Professor Savage to stop introducing the AI Hypothesis to new students. Bill Negendank, who went along with the group, told me later that the reason given for their request was a concern about not being able to get jobs on account of their prior association with me. Negendank, however, chose to remain with my laboratory. Holding a MD degree, he saw no danger of being unable to earn a living. However, I was not to find out how much more some of my terrified former student(s) had to degrade themselves beyond severing their ties to my scientific work and me to achieve the comfort and security … until another 20 year later.

In 1976 and thus 14 years after my publication of the disproof of the sodium pump hypothesis, a fledgling science reporter for the ***Science*** magazine published an article in that prominent journal entitled "Water Structure and Ion Binding: A Role in Cell Physiology?" (Science: 192: 1220.) In this article she announced that two scientists, Jeffrey Friedman and Chris Miller had produced "crucial experiments and calculations … that provide strong evidence for the existence of pumps." (p. 1220)

I was entirely flabbergasted when a friend told me about this publication and its main message — twenty years later. One reason for my surprise is that twenty years before, the same Gina Kolata had sent to me and several other scientists a manuscript she wrote with the same, or similar title and asked for our comments. We each thanked her for her courtesy and our comments were eventually all published in a later issue of Science. Totally unknown to us, she did not publish the manuscript sent to us, but a new version containing the above-quoted claim of my former graduate students, Jeffry Friedman and Chris Miller. Nor did Kolata tell us of this manuscript switch, nor send us a reprint of the altered manuscript when published.

When questioned twenty years later, she refused to give me a Yes or No answer to this (obvious) switch. In response to my other request for a copy of the report describing the alleged new crucial experiments and calculations, she apologized, claiming that she was so young and inexperienced that she included the statements (apparently from a nameless but influential scientist) without even checking with Friedman or Miller.

When I asked Friedman and Miller for the document presenting the alleged crucial experiments and calculations, Friedman never answered. Miller did answer but claimed that he had never published the material and had in fact destroyed it after circulating it among friends and therefore it no longer exists.

In fact, the circulated material was not completely destroyed. I was able to collect most if not all of it from Miller's Ph.D. thesis, which up to that time I had not seen. Immediately it became clear why he would not want me to discuss it with him. First, *his alleged crucial experiment* — apparently concocted by the unnamed scientist providing the gossip to Gina Kolata — *never existed*. The alleged crucial calculation refers to Miller's claim that the sodium ion efflux rate used in my energy calculation is ten times faster than

the values determined by other cell physiologists. On the surface, this seemed like a reasonable cause for questioning the value used. In fact, it was something for which he ought to feel thoroughly ashamed.

For to arrive at his conclusion, he had turned upside down the sequence of time. Thus, what he did was like claiming that Galileo (1564-1842 AD) was wrong in believing that the earth revolves around the sun — because Aristotle (384–322 BC) and Claudius Ptolemy (2nd C. AD.) have shown that the sun revolves around the earth.

To make this clear, I have included the first 21 pages of an attached 1973 review (Document #3,) which gives a summary of our then ***new*** experimental findings on the sodium ion flux rates of frog muscle cells. The data on these pages present what the subsection title says: "Evidence for a Major Error in Assessing the Intra-Extracellular Exchange Rate of Na^+ Ion." In other words, the published Na^+ efflux rate of all cell physiologists up to that point (myself excepted) were too slow by a factor of ten at least and therefore grossly mistaken.

Now, in what Miller circulated around, he turned the time sequence upside down, claiming that my figure was too fast because other cell physiologists have shown figures that were ten times slower. Miller knew perfectly well what happened first and what happened last and what happened in-between. After all, he was a co-author of that very same paper (Document #3), containing the above quoted subsection with its clarifying title.

To make sure that all the misleading innuendoes and half truths circulating around were made known and straightened out, I wrote and published in 1997 a full review of the subject under the title: "Debunking the Alleged Resurrection of the Sodium Pump." A copy is enclosed and labeled Document #4. As already mentioned above, I attached as Appendix 1 to this Document #4, a reproduction of Chapter 8 of my now out-of-print book, "A Physical Theory of the Living State." You recall that it is in this Chapter 8 that the full original report on the energy balance study was presented. And as such, it documents the disproof of the sodium pump hypothesis in specific and the membrane pump theory in general.

But the harm was already done. During the 20 years, when I was not aware of Gena Kolata's manuscript switching and therefore could not have rebutted its falseness, she, Friedman and Miller as well as Science magazine have all become a part of the victim-(knowing or unknowing) perpetrator of the network of global deception.

However, before leaving this subject, let me turn you attention to page 161 of the "Debunking" article (attached Document #4). There I said on page 161: "T(t)here is little doubt in my mind that Miller and all my other graduate and postdoctoral students would have behaved altogether differently if they did not see a total hopelessness in front of them following what they once started to do: to lead the life of a real scientist…" I still have some hope for him. Now a Howard Hughes professor, the security of himself and his family is no longer a question. It is high time for Miller to make amends to avert the everlasting fate of being judged very harshly in human history. Next, I share with you what I dug out further: a pair of upstart "big-time" players in the global information embargo.

As you know too well, every scientist is overwhelmed by the plethora of publications coming off the press everyday. There is no way for anyone to read every publication every day and yet a scientist can rarely afford not to keep up with the literature. In response to this need, some review writers, especially those from highly respected scientific and

educational institutions, appoint themselves the arbiters of what the scientific community ought to know and, what not to know.

In the early 1970's, two youngish cell physiologists, I. M. Glynn and S.D.J. Karlish, found themselves in what one may call the Mecca of Cell Physiology, the Physiological Laboratory of the Cambridge University in England. Apparently, they were asked by the editor of the *Annual Review of Physiology* to write a review on the sodium pump and they did.

Here is what Professor H. R. Catchpole of the University of Illinois wrote about Glynn and Karlish's review, which appeared in volume 37, pp. 13–55 of the Annual Review of Physiology. ***"The first comprehensive review which mentioned the sodium pump in its title was that of Glynn and Karlish of 1975. Glynn and Karlish listed 245 articles in support of the sodium pump and none opposed. Yet Ling's idea has been around for 25 years, so had ours, so had Troshin's…"*** (Persp. Biol. Med. 24: 164, 1980.)

Among the "opposing" evidence against the sodium pump hypothesis systematically left out are all the experimental evidence against the sodium pump hypothesis as given in Chapter 8 of the 1962 monograph: "A Physical Theory of the Living State: the Association-Induction Hypothesis" and in the embryonic version of the association-induction (AI) hypothesis called Ling's Fixed Charge Hypothesis published in 1952 (Document #5), review articles like Document #3, as well as the supportive evidence for the AI Hypothesis both collected between 1952 and the year of publication of Glynn and Karlish's review, 1975, including 11 of the articles and reviews among the 18 listed under "Articles" in Document #1 and many others.

Glynn and Karlich were not alone. In 1988, I counted no less than five additional reviews and published symposia edited or written by Glynn and other scientists on a similar subject. Each followed unwaveringly the same tactic initiated by Glynn and Karlish in 1975, that is, citing only findings in support of the sodium pump hypothesis and none opposed. Still more of the same kind came in years after 1988.

The latest review dated 2002 is another review written by I. M. Glynn (alone) for the same *Annual Review of Physiology* under the title:"A Hundred Years of Sodium Pumping." Again the review cited ***only references in support of the sodium pump hypothesis and treated opposing evidence as if it had never existed.***

The brief summary of the reviews written in cell physiology shows that for nearly one half of a century, the dishonest style of writing reviews initiated by Glynn and Karlish has been adopted almost universally. By this unethical maneuvering, the reviewers have created a falsified history of science, which glorify the reviewers' own work and those sharing their view and cause not only the names but the work of all those who hold different scientific views to disappear. The key question is has cell physiology been always like this? The answer is a decidedly No. The deception began not much longer than half of a century ago, when a few misguided individuals took over the helm. Soon the absolute power they wielded corrupted them.

And to give you an idea what was once like to be a scientist — I mean, a real scientist, I cite what was seen as the behavioral guideline from Sir William Bayliss's "Principles of General Physiology" (4th edition, 1927) described by Professor A. V. Hill, Nobel Laureate, as " the greatest book of its kind."

"Shake your counter as boldly every whit,
Venture as warily, use the same skill,
Do your best, whether winning or losing it" (Browning)

"But at the same time, there must never be the least hesitation in giving up a position the moment it is shown to be untenable. It is not going too far to say that the greatness of a scientific investigator does not rest on the fact of his having never made a mistake, but rather on his readiness to admit that he has done so, whenever the contrary evidence is cogent enough."

Only five years after writing the Preface for the 4th edition of Sir Bayliss's book, Professor A.V. Hill was to show how true he was to Bayliss's guideline. Having been awarded the Nobel Prize did not prevent Hill from admitting and correcting a mistake he had once made and vigorously defended when the contrary evidence became cogent. Thus in an article he wrote for the Physiological Review under the title: "The Revolution in Muscle Physiology in which he made this final comment: "He laughs best, who laughs last" only it was Hill's scientific opponent, Gustav Embden, who did the last laughing. (PR 12: 56, 1932.)

Now, A.V. Hill was not only a key player in the field of muscle physiology, he was also a strong proponent of the precursor of the membrane pump theory, called simply the "membrane theory." Indeed, he almost single-handedly put to rout the protoplasmic-oriented cell physiologists in the early 1930's (See Chapter 7 of Book #4 in Document #1.).

Thus, the membrane theory was the only theory I was taught when I arrived in the United States and began my Ph.D. study in late 1945. My sponsor was Professor R. W. Gerard at the Department of Physiology in the University of Chicago. Aided by what I call the Gerard-Graham-Ling microelectrode technique, my early study of the electrical potential difference or "membrane potential" cross the surface of single frog muscle cells apparently offered support for the membrane theory.

It was in the fall of 1948, Professor Alan C. Hodgkin of the famous Physiological Laboratory of Cambridge University in Cambridge, England visited out department in Chicago. I had the pleasure of showing Hodgkin how to make and fill the microelectrodes (See Document #6.) He in turn suggested a little later to the editor of the Physiological Review to invite me to write a review. My review on the membrane potential was to appear at the same time as another review he was writing for the Biological Review. This was a high honor I greatly cherished. After all, I had not even gotten my Ph.D. degree.

But as I was gathering materials to write this review, I was increasingly alarmed by the virtual absence of substantial experimental support for the sodium pump hypothesis. Yet this sodium pump hypothesis is the foundation of my Ph.D. thesis in which the electric potential difference I routinely measured across the surface of muscle cell with the microelectrode were seen as a "membrane potential" — a name that came straight out of Bernstein's Membrane Theory. Eventually I decided to do some simple experiments of my own. I expected that the muscle cell should lose its K^+ on (simultaneous) exposure to pure nitrogen (which blocks respiration), sodium iodoacetate (IAA, which blocks glycolysis) and 0° temperature, [which slows down outward pumping of Na^+ (a process with a higher temperature coefficient) more than it slows down inward diffusion (with a lower temperature coefficient.)]

To my astonishment, the K^+ concentrations remained unchanged after 5 hours of incubation in N_2, IAA and 0°C. (See Table 5 of enclosed Document #5.)

Even though this kind of experiment did not by itself disprove the sodium pump hypothesis (and I was beginning to think of more incisive ones), I began to suspect that the claim I made in my Ph. D. thesis as well as my first four full-length papers (co-authored with Professor Gerard) that cellular electric potential is a membrane potential might be incorrect. After six requests for postponement I decided to give up my dream of glory and declined the invitation to write the review.

I also began to suspect that it was not a pump in the cell membrane that keep the cell Na^+ concentration low and the cell K^+ concentration high. But I had no better mechanism to offer for what caused the asymmetric distribution of this pair of chemically highly similar ions.

So when I left Chicago for my first job as an instructor in the Wilmer Institute at the Johns Hopkins Medical School in Baltimore, I was obsessed with the wish to find a new explanation for the asymmetric K^+/Na^+ distribution. Nonetheless, months and months went by, I got absolutely nowhere.

Then suddenly while sitting in cubicle in the basement of Welsh Library, an idea dawned on me that was to change the direction of my future cell physiological research altogether. It was a new mechanism for the selectivity of K^+ over Na^+ in living as well as non-living systems.

The essence of this idea has two components. The first component is what I later call the "principle of enhanced association through site fixation" (For up-to-date details, see p. 769 of Document #5 and p. 48 of Book #4 in Document #1.) Thus while very little K^+ associates with the negatively charged carboxyl groups of an acetate anion in solution, the association is intense when the carboxyl groups are fixed on the end of side chains of a protein molecule. The second component is fundamental statistical mechanics. That is, the probability of a β- or γ-carboxyl groups associating with the smaller hydrated K^+ is much higher than associating with the larger hydrated Na^+. If one takes into account the phenomenon of dielectric saturation, a K^+/ Na^+ selectivity ratio of about 10 was achieved. The main fixed anionic sites suggested for this role are the β- and γ-carboxyl groups of intracellular proteins, myosin for example.

I don't recall exactly what day or month that was when the new idea came into existence. But it could not be later than 1950, for the first publication I put out on the subject appeared in an abstract that appeared in print in 1951 (see Document #7.) A longer version was sent to Hodgkin, Hill, Katz, Harris and many others. All answered with encouraging comments. It was at about this time that Bill McElroy and Bentley Glass were organizing the second Symposium on Phosphorus Metabolism and I was invited to give a paper.

It was in this paper enclosed as Document #5 that a fuller exposition of my new idea on K^+ selectivity over Na^+ was presented (pp. 767–772.) Elated by this discovery I must have talked to some friends at the Hospital. To support my belief that the kind of global swindling perpetrated by Glynn and Karlish began at a much later date, I tell you a heartwarming story that took place in the big lecture hall of the Johns Hopkins Hospital. One day not too long after I found my new mechanism of selective accumulation, I was heading for the Welsh Library via the board-walk. There was a big overflowing crowd at the entrance of the lecture hall. I poked my head in to find out what was going on. Just as I

found out that it was Professor A.B. Hastings from Yale lecturing on his expertise subject, K^+ in living systems, someone yelled from the audience, "Is Dr. Ling in the audience?"

Not sure of what I heard, I hesitated but was eventually hustled all the way down until I was scribbling on the blackboard on the podium, describing my new hypothesis. After I finished, Professor Hastings, the honored guest speaker of the occasion, came to me and shook my hand, saying that all his life he suspected that the K^+/Na^+ selectivity has something to do with the different hydrated diameters of the two ions. Now you got it.

Long after this totally unexpected encounter and when things looked really bad, I always thought back with gratitude and admiration for having met Professor A.B. Hastings. Like Professor A. V. Hill, he too was a personification of what Sir Bayliss envisioned as a great cell physiologist.

While the mechanism was originally introduced to explain selective accumulation of K^+ over Na^+ in living cells, the mechanism suggested was in fact far more general and easily lends itself to other applications. Thus in 1953, it was extended to account for the s*elective K^+ permeability* of living cells (Document #8.) In 1956 my report at Atlantic City that the cellular resting potentials as well as glass electrode potentials are not membrane potentials but adsorption potentials at the cell or electrode surface was also based on the same basic mechanims of selective adsorption of K^+ over Na^{+}. That report also brought me into contact with George Eisenman and his coworkers in Philadelphia.

An invited lecture followed in which I apparently convinced Eisenman and others in my audience of the general validity of my theoretical model of selective K^+ adsorption over Na^+. Accepting my model, Eisenman and coworkers further extended it with the new idea that the selectivity for the 5 alkali metal ions could vary with what they described as a change in the field strength of the anionic site. This important new idea, as well as some relevant old idea from colloid chemist Bungenberg de Jong gave me both the impetus and some additional building blocks for a new adventure. That is, to develop my original simple model of selective K^+ accumulation (called Ling's Fixed Charge Hypothesis, see Book #4, Chapter 10) into the unifying theory, the AI Hypothesis (mentioned at the opening of this letter.) That was of course forty years ago when the AI Hypothesis became published in full. Three years later, the Polarized Multilayer Theory of Cell Water was added, completing the AI Hypothesis.

Recently a physicist friend who had some familiarity with my work sent me an email. In this, he told me that in his opinion I should be happy about your ion channel work being awarded the Nobel Prize for chemistry — because it is "very close" to my work. After spending some time in the library I realized that he was not wrong.

Indeed, it seems that the more I read of your work, the more I realized how correct my friend was. All except his opinion that I should be happy about all these. I am not. No one else would. Thus, would you jump with joy when someone else got the Nobel Prize for ideas that you introduced for the first time many years ago but was not acknowledged? Would you not cry "plagiarism" loud and clear so everyone would hear it and force the offender to restore to you what is rightly yours? To be more specific, I shall make one direct comparison and a few loose pointers on ideas you might have presented without the knowledge that I had introduced them earlier.

(1) In enclosed Document #9, Figure 1 shows carboxyl groups carried on a protein(s) lining the wall of a cell membrane pore. Here the carboxyl group serves as a selective

device (what you would call a "K^+ filter") for achieving selective K^+ permeability in and out of living cells. This figure was presented in 1965 in an article entitled: "Physiology and Anatomy of the Cell Membrane…" (Fed. Proc. Symposium 24: S-103, on page S-110.; see also enclosed Document #9 for a later exposition of the AI Hypothesis of ionic permeability; see also Chapter 13 of Book #4 for reason that the lipid bilayer part of this figure is no longer valid.) The pencil-encircled part of this figure is compared with a similar figure (Fig 10) reproduced from an article you published with Chris Miller in the J. Gen. Physiol. 23 years later in 1988. Your reference list does not include my name or my prior publication. I am inclined to think that you are an innocent victim here. But since Miller knew my work well (See pp. 39–40 in Document #3), there was no justification for his not acknowledging me as the original author — without committing plagiarism.

(2) The concept of cooperativity in the adsorpion of K^+ ions means that the adsorption of a K^+ on one carboxyl group increases the change of the nearest neighboring carboxyl group also adsorbing K^+. This is shown in the illustrations on both page 45 and page 47 in the enclosed Document #11. See also Documents #10 and 12 on cooperativity.

(3) The idea that a linear array of carboxyl groups can provide a mechanism for diffusion of K^+ faster than in free water is first shown in 1962 in Documents #13, p. 336. And again in enclosed Document #11, Fig. 22 on page 34. For evidence of C. Miller's familiarity of this accelerated diffusion, see <http://www.gilbertling.org/lp18.htm> and also p. 33 in Document #3.

(4) Adsorption or desorption of Ca^{++} on a nearby *cardinal site* (receptor site) controls the sodium current and a molecular mechanism to explain it. See Document #14, from Ling in "Die Zelle, Structur und Funktion (H. Metzner ed.) 3rd Ed. Wissenschftlich Verlag, Stuttgart, 1981; Section 15-6 in Book #4.

So you see, you are already a very active member of this global information embargo network. You have already done me a great deal of harm in taking as yours a good part of my life's work. And your being awarded the Nobel Prize would make many people of the world listen to and believe you rather than me. But wouldn't you like to be awarded for what are truly yours and not somebody else's? I am sure of that.

By recording only publications in favor of the membrane pump hypothesis and ignoring all opposed, Glynn and followers have done away with the search for truth as the goal of science and have installed in its place the perpetration and glorification of the *status quo* right or wrong. And over the long run, the sin/crime of the deception is going to be paid in the lives and suffering of countless innocent men, women and children. Just take one incurable disease, cancer as an example

In America alone, 1990 innocent men, women and children died of cancer everyday in the year 2000. Put differently, cancer kills more innocent Americans on any two ordinary days (3980) than on that single calamitous day, 9–11, 2001 at the World Trade Center (2801.)

As a cell physiologist yourself, I do not need to tell you that the chance of curing cancer (and of many other incurable and even more threatening disease(s) would be greatly improved if the theory of the living cell is heading in the right direction. From all the above, it is obvious that the membrane pump theory is not heading in the right direction, nor does it provide the barest minimum of a molecular mechanism for the control of the living machines by drugs and other "cardinal adsorbents". In contrast, the AI Hypothesis is heading in the right direction. And the AI Hypothesis does offer the foundation of drug

control (See Chapter 14 of Book #4.) Yet since 1988, all support from government agencies like NIH, NSF, ONR and private foundation like the Howard Hughes Medical Foundation have been lavishly and exclusively supporting workers subscribing to the membrane pump theory. Meanwhile all governmental financial support has been taken away from me and all others who have been pursuing science by the rule — abandoning the theory that has been unequivocally disproved (MPT) and following the affirmed one (AIH). But what has this unilateral support produced? For answer, let us return to Glynn and Karlish.

In their 1975 review, Glynn and Karlish cited 245 papers, exclusively favorable to the sodium pump hypothesis. In contrast, their 2002 review counts only 95 papers (also exclusively favorable). Of these 95, 64 are repeats of what was already reviewed in the 1975 review. This leaves only a total of 31 (favorable) papers that have accumulated worldwide in the 27 years since 1975. Thus despite lavish support by public institutions like NIH, NSF, ONR and private foundations like the Howard Hughes Medical Institution in the US alone, the average world-wide productivity is only *1.15* papers per year.

In contrast, during the same 27 year period, guided by the AI Hypothesis, my laboratory alone has produced ninety-five (95) original papers in addition to three major books (Books #2, #3 and #4 in Document #1). All came after the exodus of my former students and during the last 15 years entirely without governmental or private foundation support. It was Raymond Damadian and his tiny struggling Fonar Corporation that has permitted my little group to live on scientifically — in the form of salaries, shelter and facilities. For the first ten years, most of our miniscule laboratory operating expenses of about a thousand dollars per year came from my own pocket (i.e., my salary.) In the last few years, we also received a few thousand dollars from my son Tim and daughter-in-law, Kimberly to pay for additional expenses publishing my new book, "Life at the Cell and Below-Cell Level."

It is self-evident that so far humanity as a whole has failed to find a cure for cancer (and for many other incurable and even more threatening diseases.) One reason for this man-made tardiness is unquestionably that the verified and productive guiding theory has been blacklisted. As a result, promising young scientists no longer have the freedom to follow their conscience, their best judgements, the results of their experiments and the one and only ethical guideline eloquently expressed by Sir William Bayliss. Meanwhile, government science supporting agencies like NIH and NSF, and major priate funding agencies like the Howard Hughes Medical Foundation, have been exclusively supporting in the last fifteen years work based explicitly or implicitly on the long-defunct membrane pump theory.

In closing, I ask you another question. At this very moment, 19 year olds are asked to give up their most precious possession, their lives to protect American citizens. Shouldn't you and other intelligent and caring scientists like you, who have now the visibility and public trusts that come with the Nobel Prize, join me in righting the wrongs in basic cell physiological science, wherever they are?

As a token of good will, I am sending you (by separate mail) a gift. It takes the form of my latest book, "Life at the Cell and Below-Cell Level" listed as Book #4 in Document #1. You will find that it contains the first and only account of the history of cell physiology from its very inception to 2001. It tells not only the story of the AIH; it tells also the full story of the MPT as well. The over 500 single and multiple references in the book will lead to most of the information that might have been withheld from you in your past.

But it would be wrong to say that the job ahead for you would be easy. It is going to be a very challenging one. But that was the way, perhaps the only way of keeping alive and growing what the West has discovered in the 17th to 19th century that we call Science.

Sincerely yours,

Gilbert Ling
c/o Fonar Corporation
110 Marcus Drive
Melville, NY 11747

Document #1

ARTICLES

1. Ling, G.N. (1952) The role of phosphate in the maintenance of the resting potential and selective ionic accumulation in frog muscle cells. *Phosphorus Metabolism,* Vol. II, pp. 748–795 (W.D. McElroy and B. Glass eds.) The Johns Hopkins University Press, Baltimore.
2. Ling, G.N. (1955) Muscle electrolytes. *Amer. J. Phys. Med.* 24: 89–101.
3. Ling, G.N. (1965) The membrane theory and other views for solute pemeability, distribution and transport in living cells. *Pers. Biol. Med.* 9: 87–106.
4. Ling, G.N. and Ochsenfeld, M.M. (1965) Studies on the ionic permeability of muscle cells and teir models. *Biophys. J.* 5: 777–807.
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7. Ling, G.N. (1969) A new model for the living cell: a summary of the theory and recent experimenta evidence for its support. *Intern. Rev. Cytology* 26: 1–61.
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15. Ling, G.N. (1988) A physical theory of the living state: application to water and solute distribution. *Scanning Microscopy Intern.* 2: 899–913.
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18. Ling, G.N. (1997) Debunking the alleged resurrectrion of the sodium pump hypothesis. *Physiol. Chem. Phys. & Med. NMR* 29: 123–198.

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1. Ling, G.N. (1962) *A Physical Theory of the Living State: the Association-Inducuton Hypothesis.* Blaisdell Publ. Co., Waltham, Mass. (Book out of print but can be obtained at Amazon. com
2. Ling, G.N. (1984) *In Search of the Physical Basis of Life*. Plenum-Kluwer Publ. ISBN 0-306-41409-0
3. Ling, G.N. (1992) *A Revolution in the Physiology of the Living Cell*. Krieger Publ. Co., Malabar, Fl. ISBN 0-89464-309-3.
4. Ling, G.N. (2001) *Life at the Cell and Below-Cell Level: the Hidden History of a Fundamental Revolution in Biology.* Pacific Press, 110 Marcus Drive, Melville, NY 11747. ISBN 0-970-7322-0-1.

Document #2

Oxidative phosphorylation and mitochondrial physiology: A critical review of chemiosmotic theory and reinterpretation by the association-induction hypothesis. *Physiol. Chem. Phys.* 13: 29 (1981) or go to www.gilbertling.com, choose volume (and article) from drop-down list.

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The physical state of solutes and water in living cells according to the association-induction hypothesis. *Ann. NY Acad. Sci.* 204: 6 (1973)

Document #4

Debunking the alleged resurrection of the sodium pump hypothesis**.** *Physiol. Chem. Phys. & Med. NMR* 29:123 (1997) or go to www.gilbertling.com, choose volume (and article) from drop-down list.

Document #5

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Document #6

The microelectrode and the heart. by S. Weidemann in *Research in Physiology* (F. F. Kao, K. Kozumi and M. Vassalle. Eds.) Auto Gaggi Publishers, Bologna, pp. 3–25 (1971) p. 3

Document #7

Tentative hypothesis for selective ionic accumulation in muscle cells. *Amer. J. Physiol.* 167: 806

Document #8

Selective cellular permeability according to the fixed-charge hypothesis. Proc. 19th Intern. Pysiol. Congress, Montreal, (1953) pp. 566–567

Document #9

Physiology and anatomy of the cell membrane: the physical state of water in the living cell. *Fed. Proc. (Symposium)* 24: S-103, (1965) Figure 1 on p. S110

Document #10

The theory of the allosteric control of cooperative adsorption and conformation changes. In *"Co-operative Phenomena in Biology"* (1980) (George Karreman, ed.) Pergamon Press, New York pp. 39–69

Document #11

A new model for the living cell: a summary of the theory and recent experimental evidence in its support. *Intern. Rev. Cytology* 26: 1 (1969.)

Document #12

The role of inductive effect in cooperative phenomena of proteins. *Biopolymers Symposium No. 1,* pp. 91–116 (1964)

Document #13

A Physical Theory of the Living State: the association-induction hypothesis. Cited as Book #1 under Document #1.

Document #14

Elektrische Potentiale lebender Zellen. *Die Zelle: Stuktur und Funktion* (H. Metzner) Wissenschaft. Verlag, Stuttgart (1981) p. 385

February 22, 2008

Professor Roderick MacKinnon
Laboratory of Molecular Neurobiology and Biophysics
The Rockefeller University
1230 York Ave, New York, NY 10021

Dear Professor MacKinnon:

Five years have passed since I sent you a registered 17-page letter with various documents and a book — not long after you were awarded one half of the Nobel Prize for Chemistry of 2003.

I wrote the letter because I was truly flabbergasted by what you have (apparently) done. Thus, four major components of your Nobel-Prize winning work on ion channels — as summarized on pages 14-15 of my letter — were first introduced by me as the enclosed documents make irrefutable. Yet, I could not find a shred of evidence that you had acknowledged my priority and given me due credit. For this reason, I mentioned to you that you were at risk of having committed plagiarism. I then invited you to correct my mistake —if any — by telling me where you had in fact given me credit earlier.

Anyone would agree with me that those five full years offer more than enough time to write a short answer to my letter.The fact that you have not done so suggests that you are unable to rebut my accusation but also would not make the effort to set straight the serious harm done to me by plagiarizing a substantial part of my life's work as your own. If that is indeed your position, I would be forced to repeat what I did in 1986 to Prof. Bertil

Hille of the Department of Physiology in the Washington University in Seattle. You can read all the letters exchanged as well as the final verdict at www.gilbertling.org/lp16a.htm. I close this letter with a warning. If you do not respond to this last appeal within the next three weeks, I will make public in print and online the (unanswered) letter I wrote you on November 17, 2003 along with the present one.

Sincerely yours,

Gilbert Ling

Damadian Foundation of Basic and Cancer Research
Fonar Corporation
110 Marcus Drive
Melville, NY 11747

PS This letter will also be sent registered with a return slip so that I will know exactly if and when you get it.

PPS I also enclose a copy of the letter I sent you on November 17, 2003.

April 2, 2008

Professor Roderick MacKinnon
Laboratory of Molecular Neurobiology and Biophysics
The Rockefeller University
1230 York Ave, New York, NY 10021

Dear Professor MacKinnon:

More than a month have gone by since I sent you on February 22 of this year a follow-up letter of another unanswered one I sent you five years ago on Nov. 17, 2003. According to the Post Office, this second letter was delivered to you at 3:13 pm on February 26. Although the Post Office has the signature of the person who accepted the letter, the return slip apparently got lost and never reached me.

Because of the seriousness of the next step I would be forced to take, I decided to offer you one more chance to either refuting or admitting/correcting the suspected plagiarism described in detail on pages 14–15 of my 2003 letter.

All you have to do is to write a short note. In this note, you can either rebut (with indisputable supportive documented evidence) or correct the wrong done to me by your prior failure to give me due credit for ideas I first introduced but have been incorporated into your Nobel-Prize-winning work as your own original work. I will then help you publish it in Physiological Chemistry Physics and Medical NMR, of which I am the current Editor-in-Chief.

Enclosed are copies of both my original Nov 17, 2003 letter and the more recent one dated February 22 of this years

Sincerely yours,

Gilbert Ling

Damadian Foundation of Basic and Cancer Research
Fonar Corporation
110 Marcus Drive
Melville, NY 11747

PS. This letter, like its predecessors, will be sent registered with return slip so that I will know exactly when it is delivered. Only this time it will go by two separate routes: one by next-day registered mail and the other by Fed Ex.

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Identical Twins, Asymmetrical Mitosis and Bilateral Symmetry of Organisms

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Part I: Identical twins and bilateral symmetry of organisms

Identical (monozygotic/isogenic) twins in humans and other metazoa result when the first pair of daughter cells of the zygote separate and act as zygotes themselves.

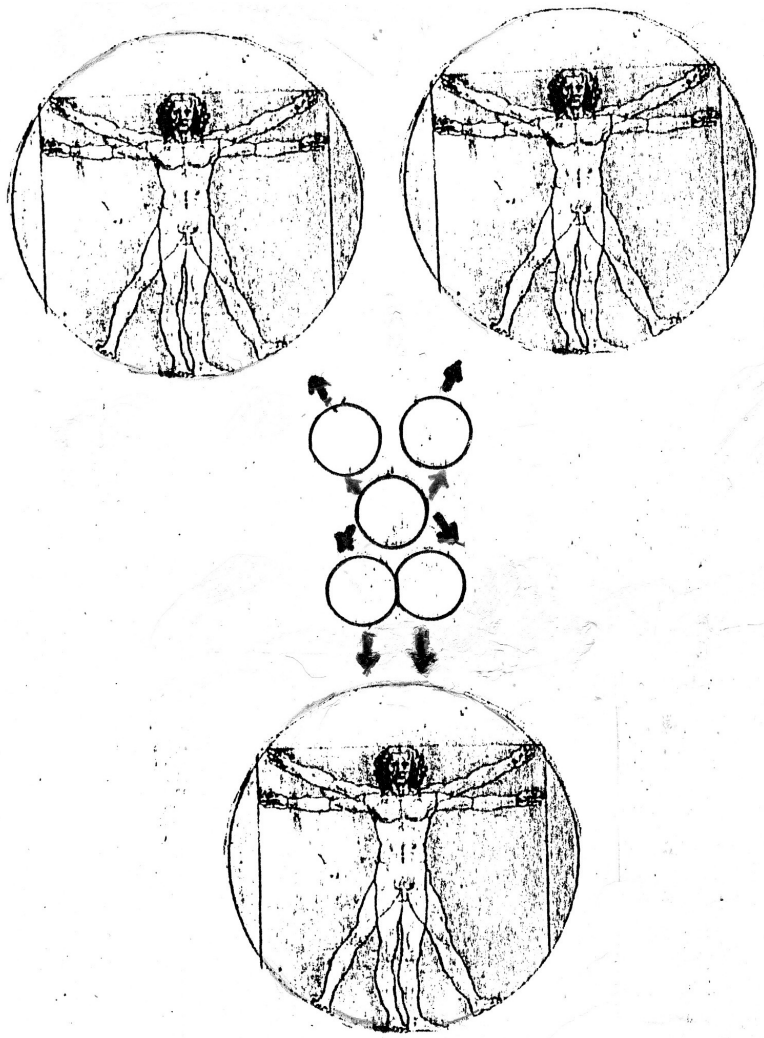
Does this phenomenon relate to the normal development of the individual when these daughter cells do not separate?

We believe the phenomenon of identical twins does give insight into the development of the individual in metazoa with bilateral (reflective) symmetry. It is proposed that the pair of daughter cells of the zygote that remain together, generate progeny that express only the lateral component of their bilateral potential. The medial component is suppressed by the apposition of the pair of daughter cells and their progeny, with a resulting individual with identical, medially fused, halves, differing by their dextro and levo orientation.

The reason for the exceptions to the bilateral symmetry pattern (e.g., heart, intestinal tract in humans) remains to be clarified. In these instances, one of a pair of identical stem cells may be suppressed, possibly by positioning. The existence of dextrocardia and *situs inversus* in humans, where organ orientation is opposite to normal, supports this hypothesis.

Support for the proposed relationship between the development of identical twins and bilateral symmetry in metazoa are the variations and permutations of conjoined twins. Here there may be reversion to bilateral potential of the subordinate (hierarchic) stem cells for the areas affected. Or there may be the converse, where there is partial expression of the bilateral potential of the stem cells involved, with reversion to the normal dextro and levo identical halves, with fusion. Midline separation of mirror image halves as in *spina bifida* (failure of fusion) also supports the proposed coherent, simultaneous generation of mirror-image halves by the lineage of the first two blastomeres.

Can it be otherwise? A contrarian perception is difficult; why would the generative potential of the identical daughter cells of the zygote be completely changed when in apposition? What mechanism other than simultaneous, coherent generation of complexly-differentiated, mirror-image halves by the paired daughter cells of the zygote can account for bilateral symmetry of organisms?



Part II: Reciprocal fallacies of studies of C. elegans development: If claims of asymmetrical mitosis are true, its use as a model for human development is flawed and vice versa

The question of differentiation of metazoa is of critical interest. The prevailing belief is that cells in metazoa proliferate by mitosis-only. But classical mitosis as an exact duplicating mechanism cannot explain differentiation.

Over the years, the theory of asymmetrical mitosis (somatic cell division, not the asymmetrical cell division that occurs in meiosis) has evolved as an approach for explaining differentiation in metazoa. Asymmetrical mitosis holds that daughter cells of this process differ in size, cytoplasmic contents, genetic programming and differentiated destiny (1).

The hermaphroditic nematode, C. elegans (1) has been a model for the study of asymmetrical mitosis, with implications for human development. Key evidence for this process has been from the study of the initial cell divisions of this nematode, especially the first division of its zygote.

It is claimed that the first two daughter cells of the zygote generate totally different organ systems, and that the initial divisions generate cells, each of which is dedicated to form one of the three principal axes of the C. elegans body (1). We could not find any references to studies following the development of the separated first two daughter cells of C. elegans. We did receive conflicting personal communications from C. elegans laboratories, one indicating that these daughter cells, when separated do form individuals (identical twins), the other indicating that they do not. The large number of fertilized eggs of C. elegans would make detection of spontaneous identical twins difficult; experimental separation of conjoined daughter cells of the zygote might obscure the issue because of cell damage. Another C. elegans researcher indicated that such experimental separation resulted in two arrested embryos, in favor of totipotentiality of the zygote's daughter cells. Experimental production of identical twins has been difficult. Tarkowski and Roblewska (2) generated an adult mouse from one blastomere of a two-cell-stage embryo. Mullen *et al.* (3) generated identical twin mice from separated blastomeres of a two-cell-stage embryo.

Inability of separated daughter cells of C. elegans to form identical twins would be discordant with the relationship between identical twin potential and bilateral symmetry (4), a characteristic of C. elegans (5). Further, since human daughter cells of the zygote have identical twin potential, the claim that each daughter cell of the C. elegans zygote generates progeny forming different organ systems would be a fundamental difference, discordant with the use of C. elegans development as a model for human development.

But even aside from the question of the developmental potential of the first two daughter cells of the C. elegans zygote, there are other reasons to question the asymmetrical mitosis claims of C. elegans developmental studies (1).

In reference to claims of asymmetrical mitosis later in C. elegans development, this is discordant with the complex interrelationships of differentiated body components.

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4. See part I of this paper.
5. Wikipedia

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