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What Determines the Normal Water Content of a Living Cell?

Gilbert Ling

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

Abstract: Most living cells contain a large amount of water. To improve our understanding of this fundamental phenomenon of cell physiology, five theories are critically examined in the light of three sets of relevant experimental findings. These findings are: (1) the diversity and specificity of the percentage water content to tissue type; (2) the limitation imposed by the Law of the Conservation of Energy on postulating membrane pumps and (3) the non-extractability of cell water from the open ends of muscle cells whose membrane covering has been surgically removed. Two of the five theories examined are called respectively the accidental theory (Theory I) and the direct water pump-leak theory (Theory III); both are introduced for the first time here as working hypotheses. Three others theories examined were published; they comprise the Donnan membrane equilibrium theory (Theory II), the indirect pump-leak (Theory IV) and the polarized-oriented multilayer (PM) theory of cell water (Theory V.) The PM (Theory V) alone is in harmony with, and supported by all three sets of the experimental findings. The remaining theories are shown to be non-applicable to cell water by at least two of the findings

THIS COMMUNICATION begins with a brief description of five hypotheses or theories (designated Theory I to V respectively) on the basic physico-chemical mechanism by which living cells maintain their normal water content. Of the five theories considered, three were published. The remaining two are, to the best of my knowledge, published for the first time here (as working hypotheses) — even though it is very likely that these ideas have occurred to others.

An examination of each of these theories follows in the light of three sets of relevant experimental findings to be described.

I. Five theories on the underlying mechanism of the (high) water content of living cells

(1) **Theory I** (The Accidental Theory) **High water content of living cell as an accident**

According to this, the simplest of the five theories, water is found in large amount in the living cell because the cell lives in water — be it water in an ocean, water in a pond

or water in tissue fluid. Thus, in this theory, living cells are chockfull of water for the same reason that an old beer-can fished out of a river is chockfull of water.

(2) Theory II. (Donnan's Theory of Membrane Equilibrium — Donnan 1924) **High water content of living cells due to osmotic activity of entrapped impermeant and permeant ions in cells**

In the version introduced by Boyle and Conway (1941), muscle cell membrane is postulated to be impermeable to certain intracellular organic anions. These impermeant anions, a matching concentration of (permeant) K^+ cation as well as other permeant intracellular ions of both signs, draw water into the cell and maintains it at a high steady level.

(3) Theory III. (The Direct Water Pump-Leak Theory) **High water content in living cells as a result of an incessant inward pumping of water molecules balanced by its outward leakage**

In the membrane pump theory, each living cell is surrounded by a cell membrane so thin that if one stacks 10,000 of them in a pile, the pile would have a thickness of this piece of paper at which you are looking (Jain 1972, Table 4.4, p. 110.) For the most part, this membrane is postulated to exist in the form of a phospholipid bilayer, which has a permeability for water (Jain 1972 p. 117), for alkali metal ions (Miyamoto and Thompson 1967) and for D-glucose (Wood *et al*. 1968) orders of magnitude lower than real-life living cell membrane (for water, see Ling 1987, 2001 p. 127; for alkali metal ions, see Ling 1997 pp. 150–152; for D-glucose, see Ling 2001, p. 252.) As such, the substance of the postulated phospholipid bilayer membrane would act as a virtually impenetrable barrier to the intra-, extra-celllular traffic of water and substances dissolved in water. It is an assortment of specific channels (e.g., "potassium channels", "sodium channels", "water channels") as well as membrane-straddling devices called membrane pumps that have been postulated to permit the traffic of water and solutes to proceed. By their ceaseless activities 24 hours a day and 7 days a week, the membrane pumps maintain unchanging the chemical makeup of each type of living cell.

A by-no-means comprehensive survey made in 1968 shows that 18 membrane pumps had already been formally introduced by that time (Ling *et al.* 1973.) Some of these are not single pumps but categories of pumps like the sugar pumps or free amino acid pumps, each comprising a multitude of individual pumps.

In the context of this membrane pump philosophy, the large amount of water found in living cells can also be explained by a (direct) water pump, which I postulate here as a working hypothesis. That is, water accumulates inside living cells in large amount because water pumps located in the phospholipid bilayer membrane ceaselessly pump water into the cells against its constant outward diffusion or "leakage." Like all the other pumps already postulated, the energy needed to operate the water pump must also come from the cell's energy metabolism.

(4) Theory IV. (The Indirect Pump-Leak Theory) **High water content of living cells as an indirect consequence of the ceaseless activities of the sodium-potassium pump**

This indirect water pump model is an extension of the (non-water) membrane pump theories already formally postulated. Thus, the accumulation of a large and unchanging amount of water in living cells is seen as the indirect consequence of the net result of K^+ ion being pumped into the cell and Na^+ ion being pumped out of the cell by the sodium

pump (*alias* the sodium-potassium pump) (Dean 1941; Skou 1965.) The resultant rising osmotic activity inside the cell draws water into the cell and maintains it at the high level against a steady leak of water from the cell (Dowben 1969, Gutknecht *et al.* 1978.)

(5) Theory V: (The Polarized-Oriented Multilayer (PM) theory of cell water) **High water content of living cells in consequence of the polarization-orientation of multilayers of water molecules by cell proteins**

In 1965 I presented in the Symposium on *Forms of Water in Biological Systems* held in New York, the polarized multilayer (PM) theory of cell water as an integral part of the larger unifying theory of the living cell called the **association-induction hypothesis** (Ling 1962.) Recently the name has been amended to **polarized-oriented multilayer theory of cell water**, although the short name, PM theory, remains unchanged (Ling 2003.)

In this PM theory, "*all* or nearly all water molecules in a living cell exist as polarized multilayers oriented on the surface of cell proteins" (Ling 1965, p. 407.) Figure 1 is a reproduction of the original figure (Figure 5) used at that time to illustrate the theory. It emphasizes the graded motional restriction of (layers of) water molecules in consequence of direct or indirect interaction with cell proteins.

(For the benefit of conscientious science historians of the future, I would like to point out that this was the first time that a member of the human race formally suggested that

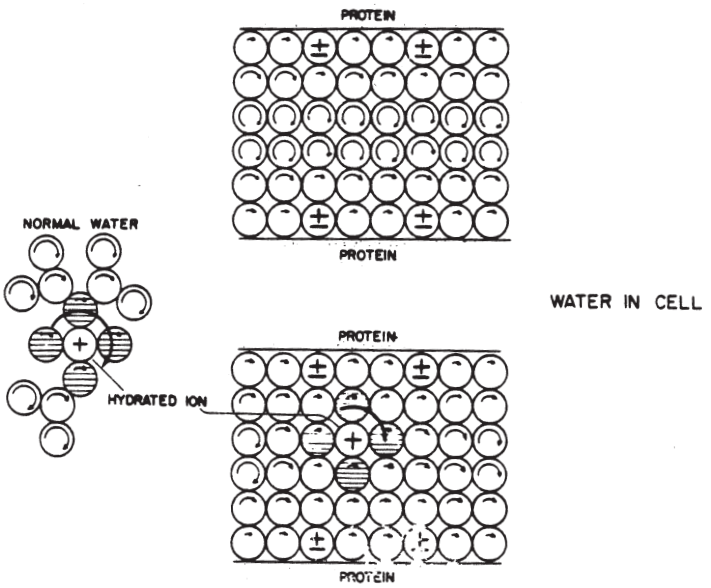

FIGURE 1. The diagrammatic illustration that launched the first polarized multilayer theory of cell water content as well as the first theory of solute exclusion in consequence of the dynamic structuring of cell water. The left figure shows a hypothetical diagram of a hydrated (e.g., sodium) ion in normal liquid water. The length of the arrows in the water molecules and elsewhere was intended to roughly illustrate the degree of rotational freedom of the molecule involved (from Ling1965, by permission of the New York Academy of Sciences, USA., copyright 1965.)

the large amount of water making up a human being and his/her non-human cousins is not normal liquid water.)

This PM theory presented in 1965 offers an explanation why a large amount of water exists in all living cells, clear and simple. However, the immediate aim of that presentation was to describe a new molecular mechanism for the maintained low level of Na^+ and other solutes found in living cells — *in lieu* of the postulated sodium pump (see below, for evidence of its excessive energy need.) As an equilibrium state, the unequal intra-, extra-cellular distribution of Na^+ and other solutes in this new theory does not demand continual energy expenditure.

II. Three sets of relevant experimental facts and what they tell us about the five theories listed above

(1) **Finding I. The similarity of the water contents of cells serving the same physiological function and divergence of the water content of cells serving different physiological functions.**

The water contents of living cells are, roughly speaking, specific and to varying degree predictable. As the data presented in Tables I and II demonstrate, the cell water content tends to be similar in tissues or cells serving the same physiological function even among widely different kind of living species (Table I.) In contrast, the water content tends to be different among tissues or cells serving different physiological functions even in the same species (Table II.)

Rough as it is, the specificity of water content shown in Tables I and II could not be accidental. So the accidental theory of cell water is out from these facts alone.

Nor can the Donnan theory (Theory II), the direct water pump-leak theory (Theory III), the indirect water pump-leak theory (Theory IV) predict the specificity of cell water content.

In contrast, the **PM theory** (Theory V) can explain readily the specificity of water content: a specific amount of water accumulates in a specific kind of cell because the free energy of water adsorption on the **specific proteins** in that type of cells is most favorable. In the PM theory, the quantity and kinds of proteins determine the total amount of water in each type of living cells. Accordingly, the PM theory anticipates that cells serving the same physiological function have similar assortments of proteins — even among different living organisms. On the other hand, cells serving different physiological functions even in the same organism would have different profiles of the kind and amount of proteins. In fact, Ling, Reid and Murphy (1986) have already confirmed this anticipation 18 years ago. Their figures are reproduced here as Figures. 2, 3 and 4..

Figure 2 shows the SDS-PAGE profiles of (all) the proteins of each of the three kinds of tissues (liver, lung and spleen) from four different strains of mice (A: Balb/c; B: $C_3D_{2-}F_i/J$; C:57BL; D: DBA/2.) The protein profiles of the same tissues in all four strains of mice are highly similar; the protein profiles of different tissues in the same or different strains of mice are consistently different. Figure 3 shows the SDS-PAGE runs of (all) the proteins in tissues of representative species of five classes of animals: A. Fish (gold fish); B. Amphibian (frog); C. Reptile (skink); D. Bird (chicken); E. Mammal (mouse.) Again, the total protein profiles are similar for the same tissue from representatives of all five

TABLE I. Water contents of red blood cells from different animals (from Ponder 1948.)

Man	63.9 to 66.1 ± 1.4*
Rabbit	63.3 to 66.8 ± 1.1*
Ox	58.6 to 71.2 ± 1.4*
Sheep	60.5 to 70.9 ± 1.1*
Pig	62.6
Horse	61.3
Dog	64.4
Cat	62.4
Goat	60.9

TABLE II. Water contents of eight types of living cells of North American leopard frogs.

	Cell Water Content (%)	Extracellular Space (%)	Tissue Water Content (%)
Egg	49.4 ± 1.94		
Heart	80.3 ± 0.32	15.7 ± 0.72	83.4 ± 0.35
Kidney	77.0 ± 0.33	17.6 ± 1.44	81.0 ± 0.50
Liver	68.3 ± 0.36	15.9 ± 1.38	73.3 ± 0.46
Muscle	77.4 ± 0.52	8.20 ± 0.32	79.3 ± 0.49
Oviduct	75.8 ± 0.46	10.8 ± 1.44	78.4 ± 0.54
Spleen	75.1 ± 0.40	7.94 ± 1.60	77.1 ± 2.48
Testis	82.1 ± 1.10	18.5 ± 3.64	85.4 ± 1.14

Tissues were isolated and immediately put into cold Ringer phosphate medium (4° C) in what is known as Medium 731 that permits long term preservations of isolated frog muscle for up to 8 days at room temperature, much longer at 4° C. (Other details and composition of the 731 medium are described by Ling and Bohr, 1969.) The water contents of the cells were computed from the total tissue water contents after correcting for the water trapped in the exracellular space (column 3.) The volume percentages of the extracellular fluid were determined by the centrifugation technique of Ling and Walton (1975) described in the text and in the legend of Figure 7. All weights were determined on a torsion balance kept in a cold room. A standardized blotting procedure was used in all cases to remove adhering fluids. After the last weighing in the wet state, the tissues were air-dried in a warm room for 3 days before transferring to an over maintained at 100° C and dried for another 24 hours before final weighing to determine the dry weight.

classes of animals. But the protein profiles are uniformly different from different tissues from the same or different classes of animals.

Of course, to perform different cell physiological functions *also* requires a specific assortment of proteins in each type of living cells. Why water, polarized and oriented to a different degree, may be a key component of the living mechanisms underlying physiological functions is a part of the central theme of the association-induction hypothesis, of which the PM theory is an integral part (Ling 1962, 1984, 1992, 2001.)

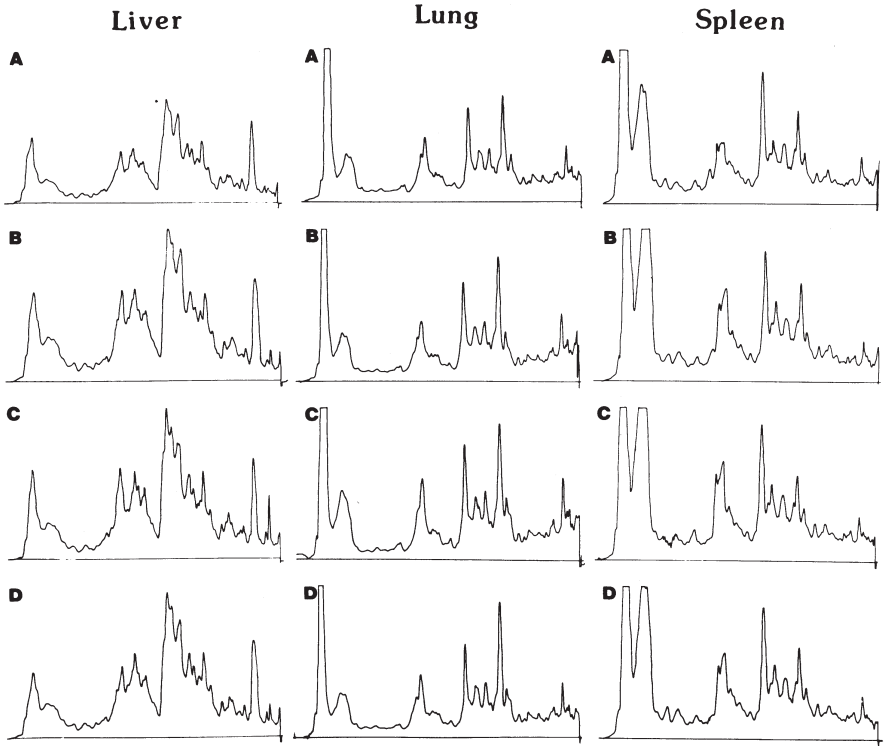

FIGURE. 2. Densitometer tracing of SDS-PAGE runs of normal liver, lung and spleen tissues from four strains of mice: A, Balb/c; B, C_3D_2Fi/J; C, C57BL; D, DBA/2. 20–40 mg of normal tissues were frozen in liquid nitrogen and ground in cold stainless tube and grinder. 20 times tissue weight of 50% glycerin was added to the suspension and homogenized. To 0.05 ml of the homogeneous suspension was added 0.2 ml of sample buffer containing 4.0 part 10% SDS, 2.5 part of 0.5 M Tris-HCl at pH 6.8, 1.0 part mercaptoethanol, 1.6 part 0.025% Bromophenol Blue and 6.9 parts water. The mixture was heated in a boiling water bath for 2.5 min. 65 µl of the cooled sample was placed in each well of the electrophoresis gel. For comparison, all samples and standards were run on the same gel and their densitometer tracing taken at a single setting (from Ling *et al*., 1986.)

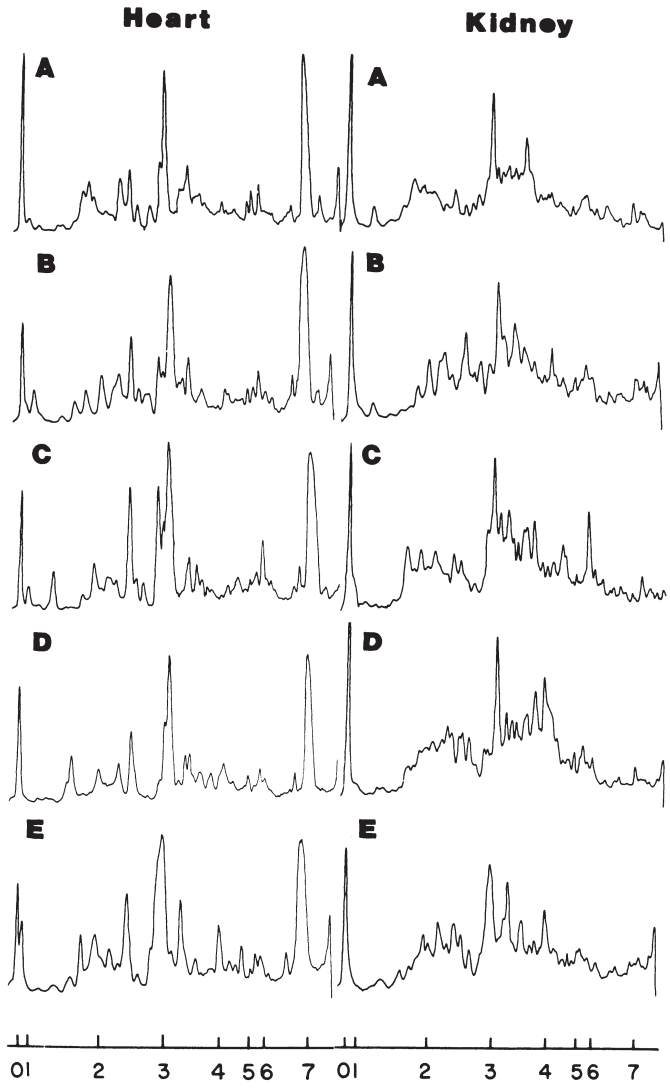

FIGURE 3. Densitometer tracings of SDS-PAGE runs of normal heart and kidney tissues from representative species of each of five classes of animals: gold fish, leopard frog, skink, chicken, mouse. Methods were same as described in legend of Figure 2. Numbered stubs on the abscissa correspond to positions of standards from Sigma Chemical Co., St. Louis, MO: 0, lysozyme (M.W. 14,400 daltons); 1, soybean trypsin inhibitor, 21,500; 2, carbonic anhydrase, 31,000; 3, ovalbumin, 45,000; 4, bovine serum albumin, 66,200; 5, phosphorylase B, 92,500; 6, β-galactosidase, 116,250; 7, myosin, 200,000. (from Ling *et al*., 1986.)

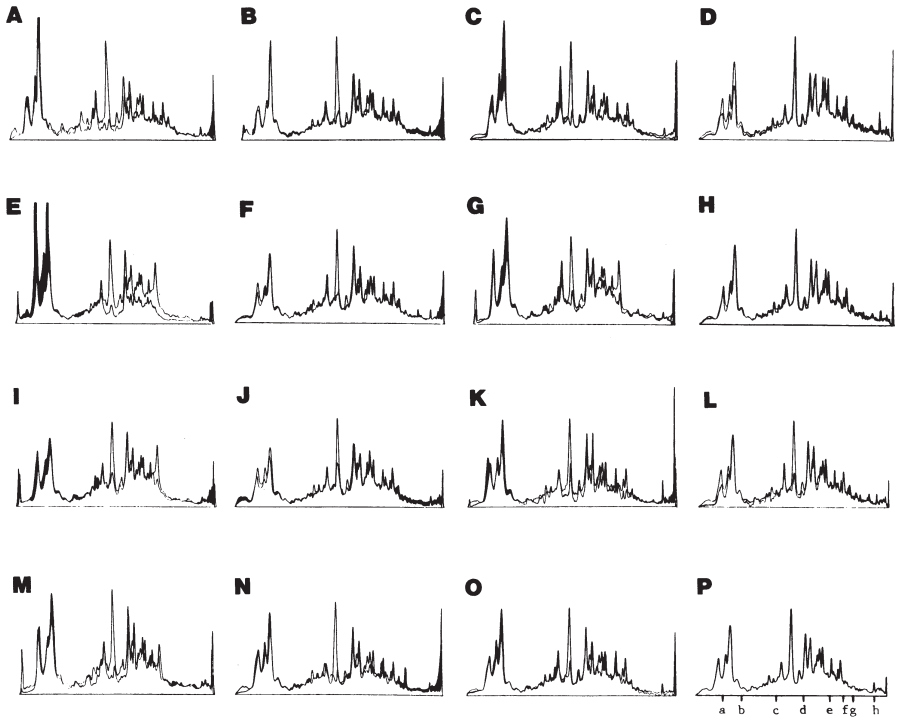

FIGURE 4. Densitometer tracings of the SDS-PAGE separated protein bands of mouse ascites cancers. On each tracing from one kind of cancer cell is superposed a similar tracing from Ehrlich ascites cancer cells, which serves as an internal standard to fine-tune the similarity (and differences) of the the tracings. Darkened (shaded) areas represent lesser abundance of the protein of that molecular weight in the Ehrlich tracing. Unshaded areas represent greater abundance of the protein of the molecular weight in the Ehrlich standard tracing. Downward stubs on abscissa on the abscissa of P shows positions of molecular weight standards: a, lysozyme (M.W., 14.4 kilodaltons or kd); b, soybean trypsin inhibitor (21.5 kd) c, carbonic anhydrase, (31.0 kd); d, ovalbumin (45.0 kd); e, bovine serum albumin, (66.2 kd) f, phosphorylase B (92.5 kd); g, galactosidase, (116.3 kd) h, myosin (200.0 kd.) (from Ling *et al.*, 1986.)

Finally, Figure 4 shows highly similar protein profiles from 15 kinds of mouse (ascites) cancer cells — even though the tissues from which the cancer cells were derived are widely different. This similarity of protein profiles among cancer cells of different tissue origins is in harmony with the fact that cancer cells as a class tend to have high water contents (Ling and Tucker 1980, Table 2; Beall *et al.* 1984, Tables 5-8, 5-9 and 9-10.) And it also supports the idea expressed many years ago by Greenstein, Knox and others (Greenstein 1947; Knox 1967) that cancer cells represent a reversion to a common, embryonic form of cells.

In summary, the specificity of the water content to the type of living cells cannot be explained by the accident theory; the Donnan membrane equilibrium theory, the direct pump-leak theory nor the indirect pump- theory. On the other hand, the water-content specificity is in full accord with the PM theory, according to which it is the kind and

amount of different proteins in a cell that determine its total water content. The experimental demonstration that the protein profiles indeed reflect the physiological function of the cell adds more weight to the PM theory.

(2) **Finding 2. Limitation of the postulation of membrane pumps set by the Law of Conservation of Energy**

One of the most fundamental laws of physics in our Universe is the law of the conservation of energy — also known as the First Law of Thermodynamics. In the simplest of terms, it says that without energy, work cannot be done.

In 1952 I presented in the Symposium on Phosphorus Metabolism held at the Johns Hopkins University in Baltimore, results of my early study of the energy need of the hypothetical sodium pump. Based on the rather limited data then on hand, I reached the tentative conclusion that if the muscle cells spend all their energy in pumping sodium, the minimum energy need of the sodium pump would still require 400% of the maximally available energy (Ling 1952, p.767.)

In the succeeding years I continued to improve the methods used in the earlier study and the accuracy in this line of investigations. Eventually, I counted a total of 78 sets of complete and incomplete experiments. Their results uniformly point in the same general direction my 1952 report showed, namely, frog muscle under rigorously controlled conditions, does not have enough energy to operate the postulated sodium pump.

The last three sets of completed studies carried out in 1956, considered to be the most accurate of all my studies, are graphically represented in Figure 5. They show that the minimum energy need to operate just one of the postulated pumps, the sodium pump (*alias* the sodium-potassium pump) is from 15 to 30 times the maximum available energy (Ling 1962 Chapter 8; data reproduced and expanded in Ling 1997.) These energy balance studies have left no doubt that both Theory III, the (direct) water pump-leak theory and Theory IV, the indirect pump-leak theory are also not tenable.

Theory V, the PM theory, is once again in accord with the results of the energy balance study. As multilayer adsorption, the accumulation of a large amount of water in living cells is an expression of an equilibrium phenomenon. As such, it does not entail a steady expenditure of energy.

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(3) **Finding 3. High speed centrifugation, which quantitatively removes all water in the extracellular space of a frog sartorius muscle fails to extract any detectable amount of water from the cut ends of muscle cells after their covering cell membrane has been surgically removed.**

According to the membrane theory or its variant, the membrane-pump theory, cell water is essentially normal liquid water (for history, see Ling 2001, Chapt. 5.) The Donnan membrane equilibrium theory (Theory II), the direct water pump-leak theory (Theory III) and the indirect pump-leak theory (Theory IV) are all extensions of the membrane (pump) theory. As such, these three theories as well as the accidental theory (Theory I) all see water in living cells as normal liquid water. Theory V, the PM theory, differs.

A familiar property of normal liquid water is its propensity to flow from a high place to a low place. This kind of water flow is caused by the force (or weight, w) acting on an object of mass m, by the gravitational acceleration g of the Earth, where the force or weight is equal to the product of the mass and the gravitational acceleration, g. A man-

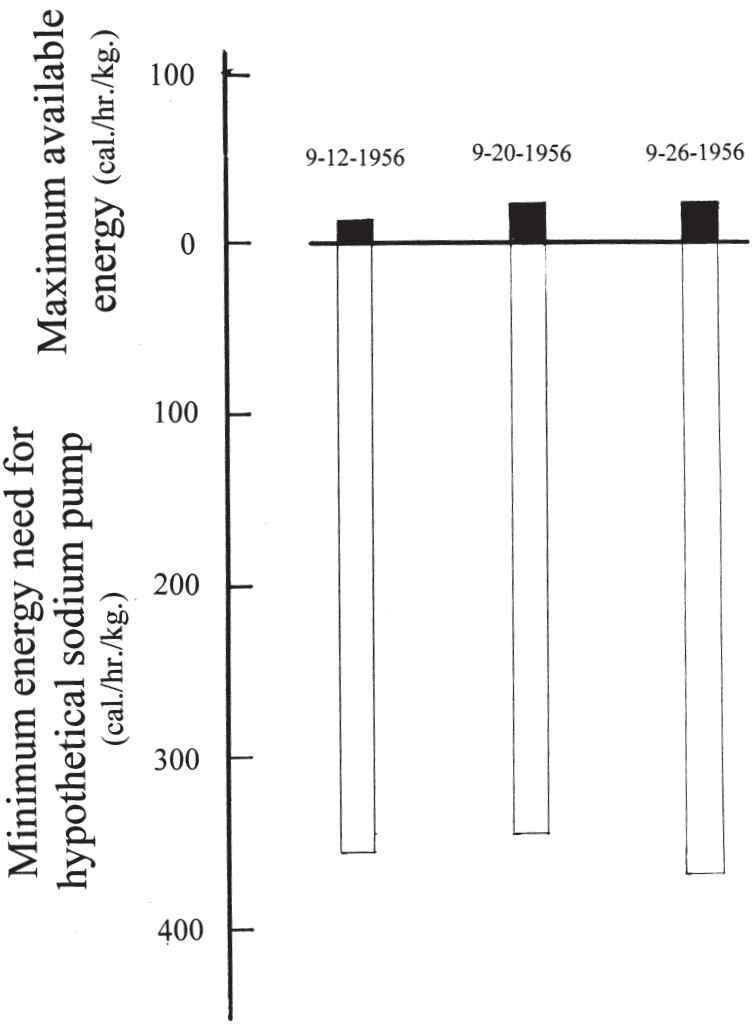

FIGURE 5. A comparison of the maximally available energy of (poisoned) frog sartorius muscle cells at 0°C (upward black bars) and the minimum energy need to pump Na^+ against both (measured) electric potential gradient and a concentration gradient. Duration of the experimental observation for experiment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

made centrifuge, by spinning an object at high speed, can exert a much stronger pulling force than gravitation.

Thus, in theory at least, spinning a broken living cell in a centrifuge may produce a decisive experiment that could tell us if water in living cells is indeed mostly normal liquid water (as predicted in Theory I, II, III and IV) or polarized-oriented — and thus less free to flow — as predicted in Theory V, the PM theory (Ling 1965, 2003.) However, to achieve that goal, we must first establish that such centrifugation at high speed can indeed extract all or virtually all authentic normal liquid water found in a cell preparation. That in turn requires a population of highly similar living cells that can be handled easily as a unit.

It turned out that Nature has provided us with just what we need in the form of a frog sartorius muscle. As illustrated in the top picture of Figure 6, this is a thin sheet of a muscle on the ventral side of a frog's thighs, about 3-cm long, a third to half of a centimeter wide. It also comes with a predictable amount of indisputably normal liquid water in the extracellular space of each sartorius muscle — that past studies have established quantitatively. This gives us, so to speak, a built-in marker that would tell us if the centrifugation technique to be described immediately below can indeed remove all the free water in a muscle and nothing beyond that.

A freshly isolated frog sartorius muscle is blotted dry on moist filter paper to remove adhering fluid by a standardized procedure described by Ling, Neville *et al.* (1969, pp. 87–88) and weighed on a torsion balance kept in a humidity-controlled glove box. The

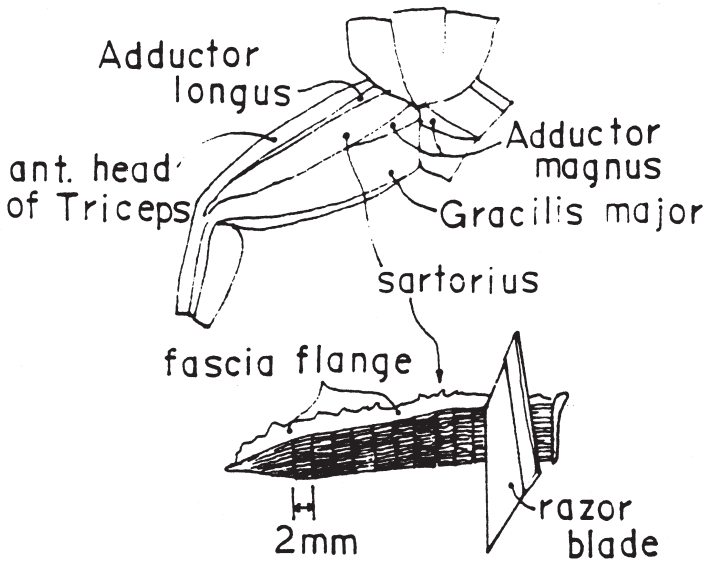

FIGURE 6. The top figure shows the ventral surface of the skinned thigh of a leopard frog, demonstrating the location and shape of the sartorius muscle. The bottom figure illustrates how razor blade cuts can make frog muscle cell segments with open ends. But since the illustration was from a different project, the product is not the accordion-like assembly of 2 mm- and 4mm-wide segments but fully isolated assemblies of segments containing uniformly 2 mm wide muscle cell segments. (From Ling and Ochsenfeld 1991.)

muscle is then placed upon a stack of properly-wetted filter paper (for details, see Materials and Methods of Ling and Walton 1975) and the assembly of muscle and moist filter paper wrapped in a sheet of paraffin film (Parafilm) to form a hermetically-sealed packet. Each packet is then placed at the bottom of an empty 250-ml centrifuge-tube-shield in a refrigerated centrifuge and spun at from 50 to 2000 g for varying duration. Following that, the muscle is removed from the packet and weighed again to obtain the amount of liquid water lost to the muscle as a result of the centrifugation.

As shown in Figures 7 and 8, the weight loss of the muscle is constant at a centrifugal force between 400 g and 1400 g (all for 4 minutes) and averages 9.3%. At 1000 g, spinning from 2 to 16 minutes extracted on the average 9.4% of the weight of the muscle..

Statistically speaking, these values of 9.3% to 9.4% are not different from the average weight percentage of water, 9.1% ± 0.76 (S.D.), found in the extracellular space of the sartorius muscle earlier with the aid of four new and different independent methods: the low-inulin probe method, 10.3%; poly-L-glutamate method, 8.9%; single muscle fiber sucrose space method (9%); ^{86}Br method, 8.2%. (For sources of publications, see Ling and Walton 1975, p. 217.) The good accord of the centrifugation extractable weight and the weight percentage of water in the extracellular space established that centrifugation for 4 minutes at 1000 g quantitatively removes all the normal liquid water from the extracellular space of a frog sartorius muscle but leaves water and other materials inside the cell unchanged.

Our next chore was to find a way of destroying the membrane barrier to the free flow of water in and out of the cell; that is, if cell water truly exists as (free-flowing) normal liquid water as prescribed by Theory I, II, III and IV. Again the anatomical makeup of the sartorius muscle makes this task easy to achieve and to achieve effectively.

As indicated above, the sartorius muscle contains some 1000 highly similar hair-like muscle fibers or cells, each running all the way from its distal tibial end to its proximal pelvic end without interruption (Ling 1973, p.299.). Nor is there any internal barriers across the width of the muscle cell that would prevent the water molecules from moving smoothly from the inside of one end of the cell to the other end (see Ling and Ochsenfeld 1973, Figure 1, A,B,C for evidence of unhindered (radioactively-labeled) water diffusion in the longitudinal direction along the length of the sartorius muscle cells.)

Accordingly, a simple razor blade cut across the muscle (at a point a few millimeters proximal to the tapering tibial end of the muscle) would destroy at once the intactness of the membrane of all the 1000-some muscle cells in the sartorius. The next task is to prove that the cut end of the muscle cell thus laid bare does not regenerate a new covering cell membrane. In fact, that too has been established from two different approaches, one with the aid of electron-microscopy; the other using chemical probes.

Figure 9 reproduced from Cameron (1988), shows that the exposed ends of the cut muscle cells do not regenerate new cell membranes. Table III from Ling (1973) shows that the strongly enhanced permeability of the cut end of muscle cells to radioactively labeled sodium ion and sucrose remained unchanged 24 hours later, again indicating no membrane regeneration.

Having found that spinning 4 minutes at 1000 g provides us with the means of removing exclusively all the normal liquid water from a frog sartorius muscle and that a razor blade cut destroys lastingly the integrity of the cell membrane as a diffusion barrier, we were in a position to perform the incisive experiment aimed at determining if the the bulk-

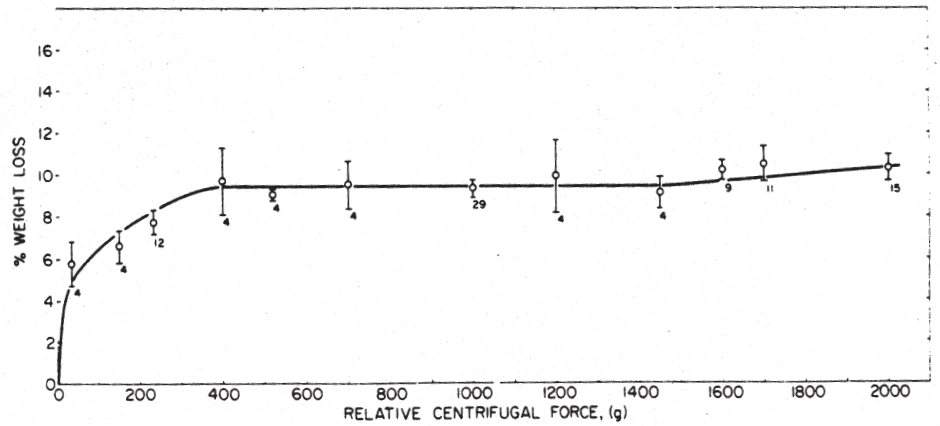

FIGURE 7. Weight loss as a percentage of initial weight (ordinate) following spinning in a hermetically sealed packet at different relative centrifugal force. Centrifugal force represented as multiples of g. Time of spinning was uniformly 4 minutes. Number under each experimental data point indicates number of independent experiments performed. The distance between the horizontal bars represents twice the standard errors (from Ling and Walton 1975.)

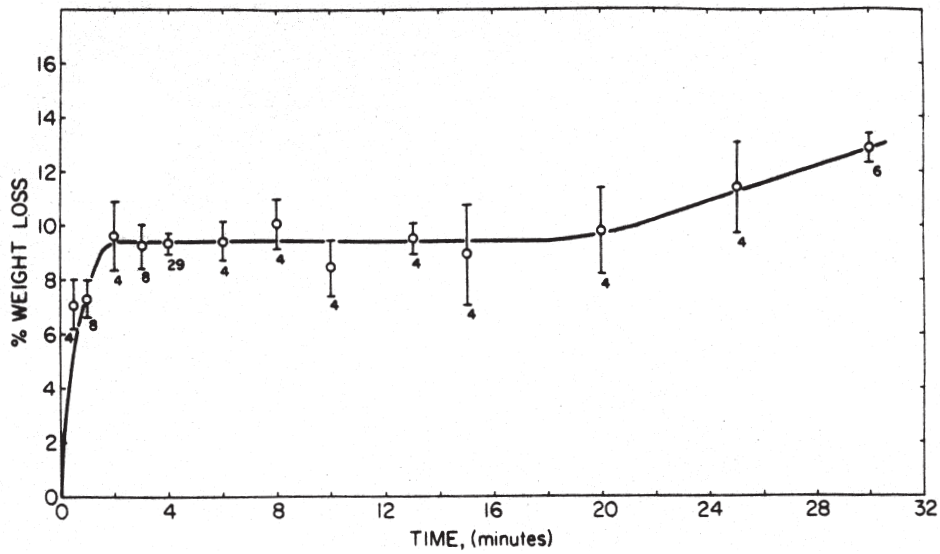

FIGURE 8. Percentage weight loss of frog sartorius muscle after centrifugation at 1000 g for varying lengths of time. Symbols have same meaning as described under Figure 7 (from Ling and Walton, 1975.)

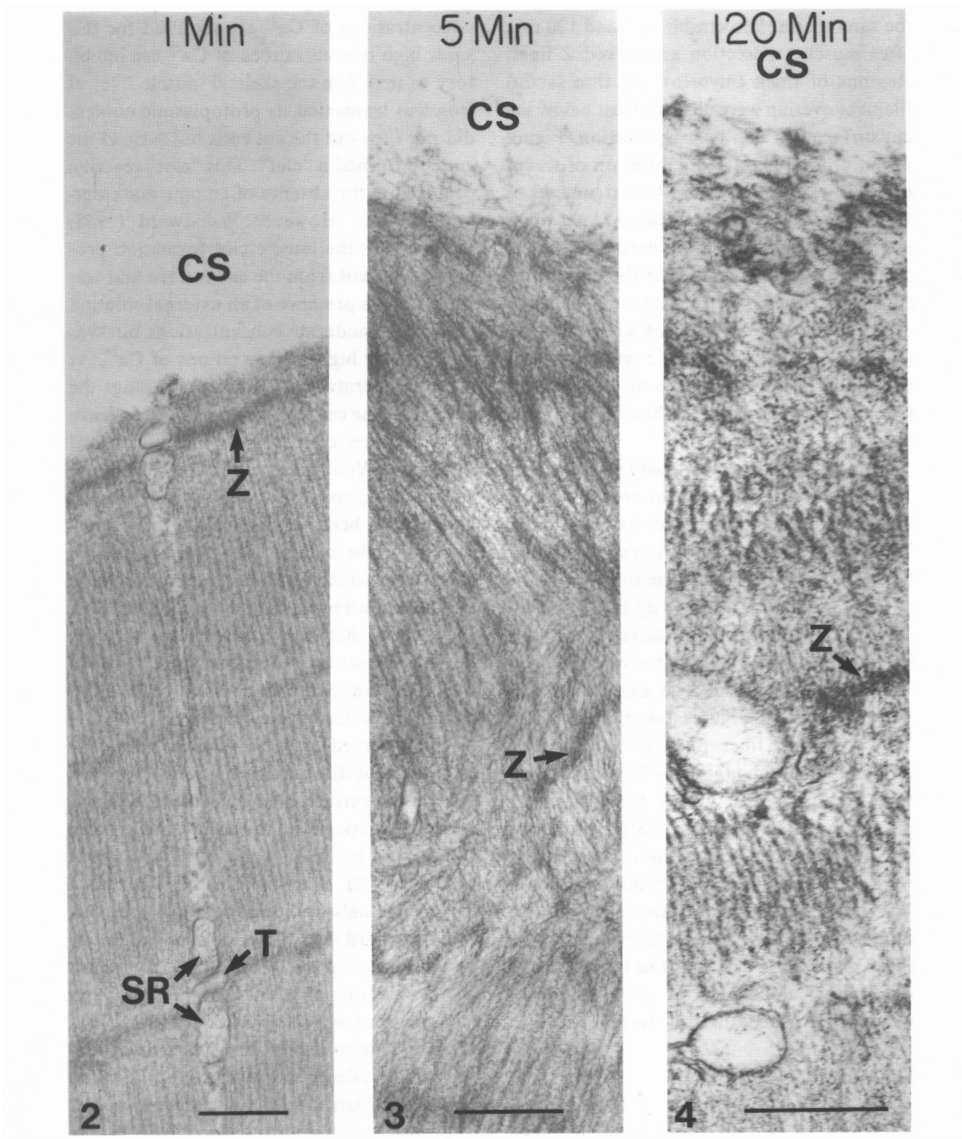

FIGURE 9. Electron-micrographs of the cut ends of 1 mm-long frog muscle fiber (cell) fixed at 1minute (Plate 2), 5 minutes (Plate 3) and 120 minutes (Plate 4) after trans-section of the muscle fibers. Plate 2 shows contraction of the sarcomere immediately next to the cut surface (CS). Plate 3 and 4 show progressive disorganization of the surface sarcomere and failure of the surface to regenerate a new plasma membrane. A one micron marker bar is at the bottom of each Plate. Z, T and SR represent respectively the Z-line, T-tubule and sarcoplasmic reticulum. (from Cameron 1988.)

Table III. Chemical evidence that the exposed surface of frog muscle cells after a (razor-blade) cut does not regenerate a new cell membrane.

	Rate before Cutting (μmoles/g)	Rate After Cutting (μmoles/g)		
		immediately after cutting	incubated after cutting	
sucrose	0.073 ± 0.013	0.174 ± 0.031	0.168 ± 0.016	(24 hrs)
Na^+		22.7 ± 2.5	22.3 ± 5.1	(51 hrs)

A frog sartorius muscle was cut crosswise (once) with a razor blade at a location proximal to the tapering tibial end of the muscle (to ensure the exposure of the inside of all the 1000-some muscle fibers to the labeled probes.) The cut muscle was then mounted in what is known as *Effectively Membrane-pump-less Open-ended Cell* (EMOC) preparation, which holds the bulk of the sartorius muscle in moistened air and exposes only the open end of the muscle to the Ringer's solution containing radioactively-labeled Na^+ and radioactively-labeled sucrose. Last column gives duration of exposure in hours to sucrose and labelled Na^+, respectively. Illustrations of the EMOC setup can be found in Ling 2001 p. 53 (from Ling 1973 reproduced in Ling 2001.)

phase water in frog muscle cells is truly normal liquid water. And here are the additional details on how the incisive experiment was actually carried out.

By making razor blade cut half way across the muscle on alternate sides, one or more accordion-like preparation(s) of muscle cell fragments can be made from a single sartorius muscle. (For more complete razor blade sectioning, see bottom illustration of Figure 6.) Each of these individual preparations contains a population of some 1000 muscle cell fragments, either 2 mm or 4 mm long and with both ends open. This accordion-like assembly of open-ended muscle was then carefully blotted on moist filter paper by the standard procedure mentioned above. Freed of extra surface-adhering fluid, the preparation was weighed, wrapped in Parafilm and spun for 4 minutes at 1000 g, before weighing again on the torsion balance in the humidity chamber — in exactly the same way as described for extracting normal liquid water from intact sartorius muscles. In 1976, Ling and Walton published their centrifugation study in Volume 191 of the Science magazine under the title, "What Retains Water in Living Cells?"

In Figure 10 reproduced from that 1976 publication, the bar-graphs, $a_1\ a_2$, $b_1\ b_2$, $c_1\ c_2$ show that cutting frog sartorius muscle or rat diaphragm muscle into 2- and 4-mm wide segments with two open ends did not significantly increase the percentage of *centrifugation extractable fluid* (CEF) shown in the top figures. The same percentages of CEF were obtained from the cut muscle segments as from their paired muscle that were kept intact. In other words, no outflow of (normal) liquid water from cells with open ends occurred. Since the same treatment (4 minutes of spinning at 1000 g) does remove quantitatively all the normal liquid water found in between the muscle fibers, this set of findings has clearly shown that there is no detectable amount of normal liquid water within frog sartorius muscle cells.

Furthermore, intact sartorius muscles or cut muscle segments, when leached exhaustively in large volumes of pure distilled water loses virtually all its K^+ and other intracellular solutes (for details, see Endnote 1 located at the end of this article.) Nonetheless, these leached muscles retained the same percentages of water content as their normal counterparts after centrifugation for 4 minutes at 1000 g. Indeed, the only major components left in these leached and centrifuged muscles segments are water and proteins.

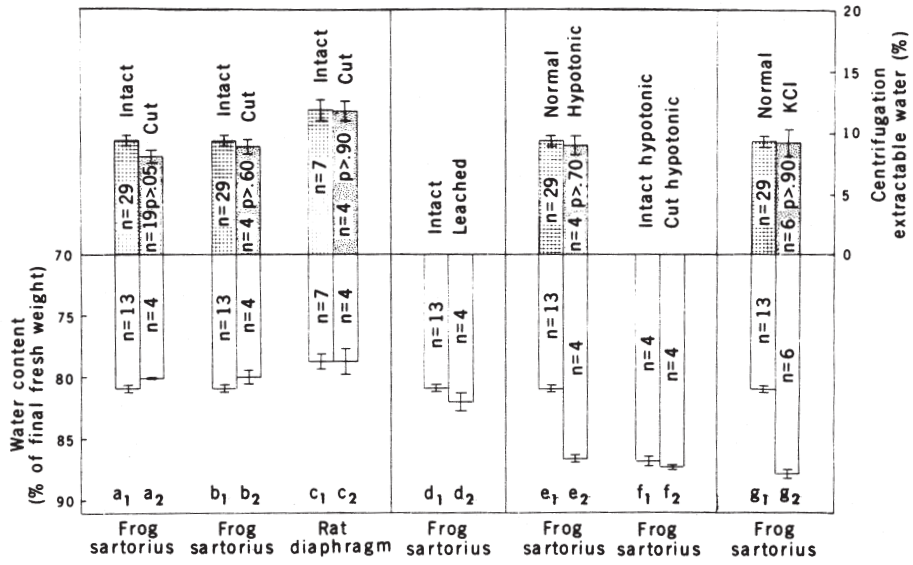

FIGURE 10. Data show that cutting frog muscle cells into 2 mm- and 4 mm-segments with both ends open, does not significantly increase the centrifugation-extractable water (a_1 a_2 , b_1 b_2 , c_1 c_2.) Nor does leaching (intact muscle cells) in large volumes of distilled water, which removes most of the solutes in the cells (see Endnote 1 for detailed data) significantly reduce the percentage of water retained in the leached muscle after centrifugation for 4 min. at 1000 g. (d_1, d_2.) Upper bars represent CEF; lower ones, water content at conclusion of the described treatments (from Ling and Walton 1976, by permission of Science.)

In summary, the centrifugation experiment has produced three sets of facts of far-reaching significance. (1) there is no free normal liquid water in frog muscle cells; (2) the retention of a normal amount of water in living cells does not depend on the presence of an intact, enclosing cell membrane and (3) nor does the retention of the cell water depend on the presence of a high concentration of intracellular ions like K^+.

III. Conclusions

It is not difficult to see that each of the three iconoclastic findings from the centrifugation experiment (no free water in cell; intact enclosing cell membrane unnecessary; high concentration in intracellular ions unnecessary) is enough to disprove four of the five theories of cell water considered. They are the accidental theory (Theory I), the Donnan equilibrium theory (Theory II), the direct water pump-leak theory (Theory III) and the indirect pump-leak theory (Theory IV.)

That the sodium pump demands far more energy than available has disproved yet another time the direct, and indirect pump-leak theories (Theory III, IV.).

The demonstration of function specificity of cell water content has disproved the accidental theory (Theory I) yet another time. Nor do the Donnan membrane equilibrium

theory (Theory II), the direct- and indirect pump-leak theories (Theory III and IV) predict the functional specificity of cell water content.

One theory alone, the polarized-oriented multilayer theory of cell water (Theory V) survived all three sets of experimental findings. In fact, like hand and glove, they are in full harmony with and eloquently support the theory.

Having said that, I must point out that it is important to recognize that the resistance to centrifugal extraction of intracellular water can only be expected from cells like the frog muscle, in which as a stiff gel, the intracellular proteins (and associated water-ion assemblies) are immobilized.

In contrast, even gravitational force alone (at 1 g) is able to cause the outpouring of normal liquid water from the large central vacuoles of mature plant cells,—which in the case of giant algal cells can be substantial in quantity. Furthermore, the protoplasm surrounding the central vacuole of these giant algal cells are also fluid in nature and can flow out of the cut end of the giant cell as shown in Figure 11, taken from Kuroda (1964.) Indeed, it was this type of fluid protoplasm emerging from broken protozoan cells that had led to the discovery of what was later named protoplasm by Felix DuJardin more than a century and half ago (Ling 2001, p.6.) However, what flows out of the cut end of the

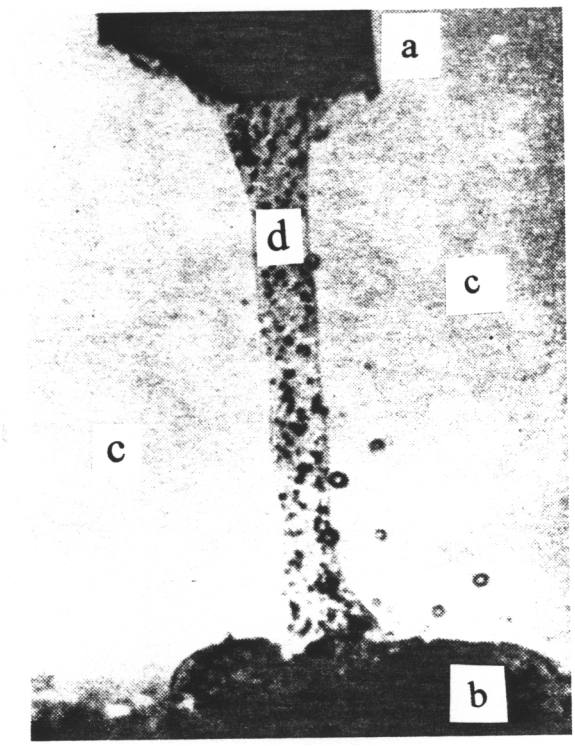

FIGURE 11. Outflow of protoplasm (endoplasm) (d) from the cut end of a Nitella cell (a) into a culture medium (c) The protoplasm collected as a flattened round drop (b) on the bottom of the cuvette. Note air bubbles in the culture medium. The picture was taken 5 minutes after the cut was made (from Kuroda, 1964 reprinted with permission of the Academic Press.)

Nitella cell is not normal liquid water but a fluid form of living protoplasm, of which water is an integral and indispensable part. Indeed, in a balanced salt solution, the isolated protoplasm could be kept alive for 3 days after its isolation (Kamiya and Kuroda 1957 ; Kuroda 1964, pp.32–33.)

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Endnotes

1. The concentrations of several cations found in frog sartorius muscles after extensive leaching in repeated changes of large volume of cold distilled water at 4° C: K^+, 0.242 ± 0.242 μmoles/gram fresh weight (in normal muscle, 85.8 μmoles/gram); Na^+, 1.34 ± 0.68 (normal 24.9); Mg^{++}, 1.90 ± 0.30 (normal, 10.8); Ca^{++}, 0.225 ± 0.093 (normal, 4.08.). Assuming that all the (remaining) ions estimated above are free and that an equivalent amount of anions (in the form of monovalent Cl^-) also exists in the leached muscle cells, the maximum osmolarity would be about 9.5 milli-osmolar. This is to be compared with the total osmolarity of 125.6 milli-osmolar from the above-mentioned intracellular cations alone in normal frog muscle. That is, if one assumes that these cations are all free as according to the membrane pump theory but not as according to the AI Hypothesis and its extensive supporting evidence as summarized in 2001 (Ling 2001, Chapter 10.)

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