MILESTONE IN PHYSIOLOGY

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Evolution of our understanding of cell volume regulation by the pump-leak mechanism

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All animal cells are surrounded by a flexible plasma membrane that is permeable to water and to small ions. Cells thus face a fundamental problem: the considerable tension that their membranes would experience if the osmotic influx of water, driven by the presence of impermeant intracellular ions, was left unopposed. The pivotal study that described the cell's remedy for this impending osmotic catastrophe—the "pump-leak mechanism" (PLM)—was published in the *Journal of General Physiology* by Tosteson and Hoffman in 1960. Their work revealed how the sodium pump stabilizes cell volume by eliminating the osmotic gradient. Here we describe the mechanistic basis of the PLM, trace the history of its discovery, and place it into the context of our current understanding.

Introduction

The pump-leak mechanism (PLM) provides a mathematical demonstration of how the action of the Na^+ pump (Na^+/K^+ ATPase [NKA]) stabilizes cell volume against osmotic forces that drive water in. This mechanism is now part of the standard canon of physiology and is one of the principles that physiologists are urged to teach as part of the standard curriculum (Carroll et al., 2012). The history behind the PLM is a peculiar one that has suffered reversals, misattributions, and misunderstandings. This can be ascribed in part to the somewhat tortuous manner in which it made its way into the world. It is the vagaries of this history that we would like to trace because they provide an instructive case study of the origins and evolution of a central idea in physiology.

We also wanted to call attention to the mechanism itself because, unlike, for example, the Hodgkin and Huxley (1952) model of nerve conduction, the PLM is not often recognized as a formal mathematical model firmly grounded in physicochemical principles with few assumptions. Importantly, the PLM model can help make sense of a cell's response to changes in its ionic environment. Most biologists tend to think of the NKA as serving to establish the Na^+ and K^+ gradients, which are necessary for generating action potentials and for powering many types of coupled transport systems. Excitement about the then newfound ability to understand brain mechanisms in terms of ion conductances caused the spotlight to shine on the ion gradients. Other key roles of the Na^+ pump were ignored, including a function for the ouabain binding site in cell signaling that was only discovered decades later (Xie and Askari, 2002). Nevertheless, the primordial function of the NKA is likely that of cell volume stabilization (Stein, 1995). Most cell biology textbooks recount the essentials of the PLM, but few get to the root of its mechanism. The PLM has also, we believe, suffered from a rather uneuphonious name, which brings to mind plumbing problems rather than the intricate art of balancing ions. Nevertheless, the hydraulic analogy is helpful for didactic purposes.

Ion channels and pumps knit together much of the machinery that drives most physiological processes. Contemporary physiological research focuses, to a large extent, on what could be thought of as the nanoscale mechanics of proteins. Nevertheless, integrative models of physiology, à la Hodgkin–Huxley, still provide a framework and play an enormously important role in codifying our understanding of physiology. The PLM is one such integrative mechanism.

To appreciate fully the historical origins of the PLM, we believe that a clear understanding of the mechanism is essential. Indeed, frequently publications state that the NKA is involved in stabilizing cells, but there often seems to be some confusion about how it does so. Therefore, to set the stage for our historical discussion, we thought that it would be helpful to recap the essentials of the PLM as set forth in Tosteson and Hoffman (1960) (and see Armstrong, 2003). Readers familiar with the

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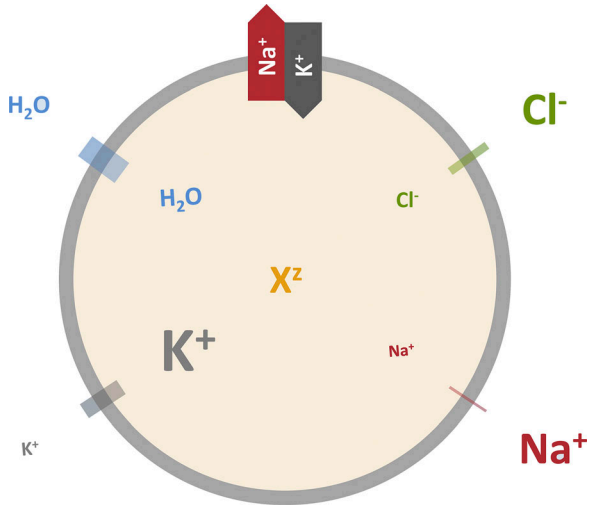

Figure 1. **The essentials of the PLM.** The asymmetry in ion distributions between the cytoplasm and extracellular space is established by an NKA (top). The flexible membrane is permeable to Na^+, K^+, Cl^-, and water. Impermeant molecules (X), mostly anions, with an average charge valence z, are trapped in the cytoplasm.

PLM can skip forward to the section entitled "A history of the PLM."

Mechanistic essentials of the PLM

To demonstrate how the PLM can stabilize a cell's volume, we consider a simple spherical cell with a flexible membrane that is permeable to Na^+, K^+, Cl^- (the primary contributors to extra- and intracellular tonicity), and water (Fig. 1). The cell also contains impermeant molecules, which we will refer to as X, that have an average charge valence, z. Throughout the paper, we will use capital X to refer to the impermeant molecules and their molarity, whereas lowercase x will denote the number of moles of X (see the Appendix for symbols used). The stability of the cell then hinges on the action of the NKA, which actively drives Na^+ out and K^+ in.

How the operation of the pump, together with the cellular permeability and impermeant molecules, stabilizes cell volume becomes apparent when one analyzes the set of equations that characterize the fluxes of ions and water in the cell.

Typically a pump-leak model has five equations which comprise four differential flux equations and an algebraic equation for the voltage (see the Appendix), based on well-established physico-chemical principles. The voltage equation is simply determined by the sum of all charges and the total membrane capacitance (Eq. 1 in the Appendix). A water flux equation, in which water's passage is determined by the osmotic pressure difference and the water permeability of the membrane (Eq. 2 in the Appendix) governing osmotic balance across the membrane. The other three flux equations govern the movements of the permeable ions, Na^+, K^+, and Cl^-, which are determined by the transmembrane ion gradients and voltage, and active transport, if it is operative (Eqs. 3, 4, and 5 in the Appendix). The first two equations are sometimes referred to as "constraint equations" (Fraser and Huang, 2007). The voltage equation defines the forces that drive the intracellular compartment close to electroneutrality, while the water flux equation encapsulates the forces that nullify the osmotic gradient across the membrane.

With an appropriate choice of conductances and pump rate, which can be quite broad (see the Appendix), the cell will settle into a steady state, in which the voltage, volume, and ion concentrations remain constant (Keener and Sneyd, 2009; Mori, 2012). For each permeable ion, there are two ways for a steady state to occur: (1) If the ion is not actively transported, the equilibrium (Nernst) potential of the ion is equal to the membrane potential. (2) If the ion is actively transported, the passive flux is balanced exactly by its active transport, and the Nernst potential of the ion is not equal to the membrane potential.

Like the Hodgkin–Huxley equations, the PLM equations allow one to calculate how the voltage changes with time, but there are three important differences: (1) The cell volume can expand or shrink as water moves in or out. (2) Intracellular ion concentrations can change. (3) Impermeant ions are included in the model, and they exert both osmotic and electrical effects.

At the core of PLM are a heterogeneous collection of impermeant molecules, X (sometimes called nondiffusible solutes, impermeable anions, etc.) trapped within the cell. In the context of this model, these impermeant molecules exert an osmotic effect, which is a colligative property. Therefore, one need only take into account the total number of molecules and their average charge. It is the impermeant molecules that essentially provide the need for stabilizing the cell against inundation by water.

The impermeant molecules within a cell are a sizeable fraction of the contributors to the osmolarity within a cell. They represent about 100 milliosmoles (mOsm)/liter of the total osmolarity, which is ~290 mOsm/liter. Textbooks sometimes wrongly refer to X as proteins, whereas proteins represent <10 mM of the total osmolarity (Milo and Philips, 2015).

X is a mixture of macromolecules and numerous small organic metabolites, including many phosphate-containing compounds such as ATP. These metabolites represent ~90% of the impermeant constituents. A cell without impermeants, clearly an impossibility, would be stable without a pump, but subject to changes in the extracellular ionic conditions. Turning on the NKA in such a "cell" would drive the volume to zero.

The impermeant molecules set up a Donnan effect because, in addition to their osmotic effect, they are charged and attract counterions to preserve electroneutrality (see Eqs. 1 and 2 in the Appendix). However, and this is crucial, cells cannot be at a stable Donnan equilibrium unless they can develop and sustain a transmembrane pressure (Sperelakis, 2012). So, for example, in the model cell proposed above, if ions are not actively pumped, the volume will increase without limit, unless the membrane and/or cytoskeleton can oppose the pressure generated by the osmotic influx of water.

What the NKA does, essentially, is "null out" the effect of X, which has a net negative charge (z is in the range −2 to −0.7; Kay, 2017) By driving Na^+ out of the cell, it indirectly establishes a negative membrane potential that moves Cl^- out of the cell, in a sense making room for X. It cannot be stressed enough that a

cell, any cell, is not at a Donnan equilibrium. There are instances where Cl^- and K^+ can obey the Donnan relationship (in which the product of the external K^+ and Cl^- concentrations equals, approximately, the product of the internal K^+ and Cl^- concentrations; Hodgkin and Horowicz, 1959); however, in these cases, the cell is in a dynamic steady state, sustained by ATP hydrolysis, and it is *not* in thermodynamic equilibrium. Sometimes it is claimed that dead or dying cells are in a Donnan equilibrium (Dreier and Reiffurth, 2015), but such an equilibrium cannot actually be achieved, and it is probably best to view the volume increase as being driven by a Donnan effect.

The precise meaning and implications of the Donnan effect have sometimes been mischaracterized in textbooks. This has led to misconceptions about the Donnan effect that have bedeviled our understanding of how, for example, Cl^- distributes across membranes (Voipio et al., 2014).

It is possible to set up a hypothetical cell in which a so-called "double Donnan" equilibrium prevails, where no energy is needed to sustain the cell volume (Fraser and Huang, 2007). This requires, however, that the membrane be completely impermeable to Na^+. If there is even a minute permeability to Na^+, as is the case in all biological membranes, the cell will become unstable.

The temporal behavior of a pump-leak system, in its approach to a steady state or after a perturbation of the system (e.g., by a change in extracellular osmolarity), is driven by ion pumping. The precise course of the changes is constrained by osmotic and ionic (electroneutrality) balance. Mori (2012) has shown that the PLM equations can be characterized by a modified free energy equation, where for a broad range of parameters (i.e., conductances and extracellular ion concentrations), the system settles into a stable steady-state (Fig. 2). The system behaves much like a chemical equilibrium, governed by the law of mass-action that moves to minimize its energy and settle into equilibrium if perturbed, like a globally asymptotically stable point in a nonlinear dynamical system (Strogatz, 1994). The difference is that the PLM is a dynamic steady state rather than a thermodynamic equilibrium. Hence, what is minimized is not the thermodynamic free energy but a suitable modification thereof. The mechanism is very robust, which is just what one needs to operate in an environment where the extracellular osmolarity is likely to fluctuate and cellular parameters (e.g., ion conductance) may vary with time and conditions.

The effects of the osmotic and electrical constraints on the PLM can be shown graphically by plotting the steady-state intracellular concentrations as a function of the pump rate (Fig. 3). It is easiest to see this relationship when $z = -1$, but the constraints hold true for any value of z. The osmotic constraint is evident from the fact that the sum of the concentrations of all intracellular species is equal to extracellular osmolarity for all values of the pump rate. Similarly, the adherence to electroneutrality is apparent from the fact that the sum of the charges of the anions matches that of the cations. If the pump rate (p) is zero, the volume is not stable (see Eqs. 8 and 15 in the Appendix), showing that a stable passive Donnan equilibrium is not possible. In Fig. 3 with $p < 0.2$, the volume changes exponentially with p, so small fluctuations of p will translate into large fluctuations

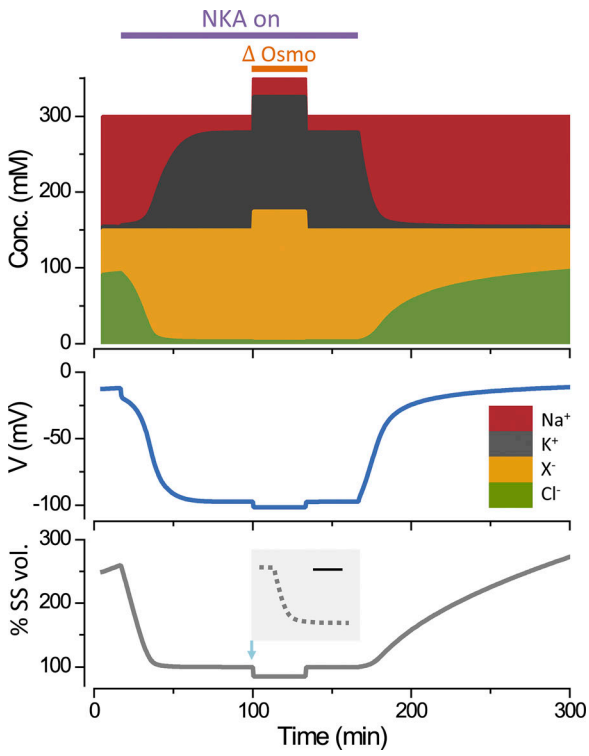

Figure 2. **The action of the NKA stabilizes cell volume.** The line labeled "NKA on" indicates when the pump is turned on and then off (p = 0.5 µC cm^{-2} s^{-1}). The composition of the extracellular solution is changed during the period indicated by "ΔOsmo," to 350 from 300 mOsm/liter, by adding an impermeant uncharged molecule. The intracellular ion concentrations ([Conc.], top panel) are plotted as a function of time, with the concentrations stacked on top of one another. The voltage (middle panel) and cell volume (lower panel) are plotted on the same timescale. The inset on the lower panel shows the change in volume, at a higher temporal resolution (bar, 0.5 s), when the osmolarity is increased. When the pump is turned off, the cell volume increases, slowly but without limit, showing that the system is unstable. The volume is expressed as a percentage of the steady-state volume (% SS vol.). The equations and parameters are given in the Appendix.

of the volume; for example, see Lew and Tiffert (2017) regarding the increase of red blood cell (RBC) volume as the pump rate declines with cell age. For $p > 0.2$, the volume asymptotes, thereby removing the system's susceptibility to p fluctuations. It seems likely that cells have a mechanism for ensuring that they operate in this latter range.

It may seem as if a feedback mechanism is needed to control a pump-leak system. However, the PLM requires no feedback to maintain volume stability, but by itself cannot ensure a return to the original volume after a sustained extracellular osmotic perturbation. The PLM can be considered as a kind of "engine" whose dynamics are encapsulated in the equations. Since cell size varies tremendously across different cell types (Ginzberg et al., 2015), it seems likely that cells have feedback mechanisms for setting cell size by controlling x and/or z (Fraser et al., 2005). This, in turn, implies that cells must have sensors for measuring their size, something likely to be of no small importance for cell biology.

Before moving on, it is worth making a few additional points about the PLM. (1) It can be shown that there is a direct

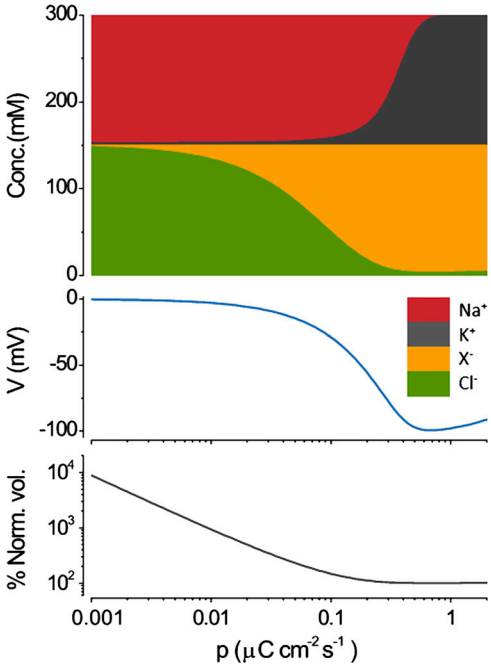

Figure 3. **Constraints on the PLM.** The steady-state intracellular ion concentrations ([Conc.], top), voltage (middle), and volume (bottom) are plotted as a function of the NKA pump rate (p). The concentrations are stacked as in Fig. 2. The steady-state solutions shown are the analytical Keener–Sneyd solution given in the Appendix. The hyperpolarization induced by the action of the NKA drives Cl^- out of the cell. Water moves out of the cell, preserving osmotic and charge balance, leading to a decrease in volume and an increase in the concentration of X^z. As p is increased further, K^+ progressively substitutes for Na^+. Osmotic balance is shown again by the fact that the sum of all intracellular ions at any p equals the extracellular osmotic strength. Electroneutrality is also preserved at all p; the sum of anions = sum of cations; $z = -1$. The volume is normalized by that at $p = 2$ (% Norm. vol.). The figure has been modified from Kay (2017), with a temperature of 37°C rather than 25°C.

relationship between the number of moles of X, z, and cell size. Indeed, if x is zero, the cell size goes to zero (Eqs. 8 and 15 in the Appendix). (2) The stabilizing mechanism does not have to be a NKA, but can be some other active mechanism for driving Na^+ out of the cell (see below). (3) Pumping Na^+ alone is sufficient to stabilize a cell, but actively driving K^+ in or out, alone, will destabilize the cell. (4) In the Keener–Sneyd equations, the NKA is assumed to run at a constant rate, independent of ion concentrations. Using a more realistic version of the NKA dynamics, which includes a dependence on ion concentrations and nonlinear terms, does not change the overall behavior of the mechanism (Kay, 2017). This can be seen by comparing the dependence of the steady-state ion concentrations, volume, and voltage on pump rate, with constant pumping and the realistic pump model (compare Fig. 3 to Fig. A2 in Kay, 2017). For the NKA to stabilize a cell, the Na^+:K^+ stoichiometry can be as low as 0.1:1, for both the constant pump rate and a more realistic model.

A history of the PLM

As the preceding section indicates, the PLM is a fairly straightforward, yet elegant, concept. But where did it come from? This was not the result of an epiphany. Rather, it was the culmination of an evolving understanding of the link between ionic and osmotic balance, and was made possible by the concurrent discovery of the NKA, which is such an integral part of the PLM.

There are a number of excellent historical accounts of the discovery of the NKA (Glynn, 1993; Robinson, 1997; Clarke and Fan, 2011), which we will not reiterate. Rather, we will focus on the parallel story of the origins of the PLM. Moreover, we will concentrate primarily on the role of ion pumping as it pertains to single cells, rather than to epithelia, because in epithelia the emphasis is on the role of the NKA in powering transepithelial transport of solutes (many via Na^+-coupled co- and counter-transport mechanisms) and water. Thus, the pump's essential role in cell volume stabilization is usually overlooked (see below). It is, however, worth remarking that the influential model of Koefoed-Johnsen and Ussing (1958), which is still a powerful explanatory paradigm in epithelial transport (Palmer and Andersen, 2008), also includes some aspects of the PLM. In its original formulation, however, the Koefoed-Johnson and Ussing model did not include impermeant ions and hence could not be used to understand cellular volume stability. We will also omit discussion of the regulatory volume changes that occur when cells are subjected to changes in extracellular solution osmolarity (Hoffmann et al., 2009).

Origins and some early pioneers

Knowledge of the asymmetry of sodium and potassium concentrations between the inside and outside of cells is one with a considerable vintage, having been discovered by Carl Schmidt (1850). The unearthing of this signature feature of almost all cells came less than 50 yr after the discovery of potassium and sodium in 1807 by Humphrey Davy. The 23-yr-old Davy was employed by the Royal Institution in London in 1801 to provide public lectures with demonstrations as part of its mission to promote science. He wrote to his friend, the poet Coleridge, about his experiments using batteries, which had only recently been developed by Volta (1800): "I have made some important galvanic discoveries which seem to lead to the door of the temple of life" (Holmes, 2010). Coming from a young man with little formal scientific training, this seems frankly megalomaniacal; in retrospect, however, he was spot on, since so many physiological processes are driven by the fluxes of these two ions.

The osmotic flux of water across the plasma membrane provides a constant challenge to the stability of cells. It is noteworthy that the first Nobel Prize in chemistry (1901) was awarded to Jacobus van't Hoff in recognition of his work on osmosis. This provided perhaps the first clear link between molecules and a macroscopically evident physical phenomenon. Remarkably, the precise molecular basis of osmosis remains unclear (Weiss, 1996).

The physical chemist Frederick Donnan, who did a postdoctoral stint with van't Hoff, provided a key part of the PLM story. The theory that he developed in his 1911 paper showed how impermeant molecules trapped within a membrane could generate a membrane potential (English translation in Donnan, 1995). Indeed, for the first half of the 20th century, it seemed as if the Donnan potential could account for the negative membrane potential of cells. Donnan himself pooh-poohed the

notion of ion pumps and felt that his mechanism was able to explain the membrane potential (Donnan, 1942). But it turned out to be something of a red herring. In fact, cells counteract the Donnan effect by actively driving an asymmetric ion distribution which, in the steady state, equalizes the water potential across the membrane (Blaustein et al., 2019).

The redoubtable Jacques Loeb, founder of the *Journal of General Physiology*, was a great enthusiast of Donnan's theory, so much so that 14 of his 61 papers published in *JGP* mention the Donnan effect. He went so far as to say, "If Donnan's theory of membrane equilibria furnishes the mathematical and quantitative basis for a theory of colloidal behavior of the proteins, as the writer believes it does, it may be predicted that this theory will become one of the foundations on which modern physiology will have to rest" (Loeb, 1922). It is worth recalling that because of his discovery that stimulating oocytes with ions could trigger the development of unfertilized oocytes, Loeb became a scientific celebrity in the United States. Such was his fame that Sinclair Lewis based the character Max Gottlieb in his novel *Arrowsmith* in part on Loeb (Fangerau, 2006).

Another towering figure, August Krogh, never published in *JGP*, but his influence looms large in physiology (Schmidt-Nielsen, 2013). He pioneered the rigorous linking of physics and chemistry to the explication of physiological questions. Krogh appealed to physiologists to reach out into the natural world for tractable models: "For a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied" (Krogh, 1929).

Krogh thought long and hard about the osmotic challenge to animals and, perhaps more than any of his contemporaries, recognized what was required to maintain osmotic balance. He wrote that osmotic homeostasis "can be done only by the steady expenditure of energy in special mechanisms adopted for the purpose. In such a case there is no equilibrium between the internal and external medium, but we use the word 'steady state' to characterize the situation" (Krogh, 1939).

The first use of the term ion "pump" has been attributed to Robert Dean, who spent some time in Krogh's laboratory while doing his thesis at the University of Cambridge, United Kingdom, with James Gray (Dean, 1987). On the basis of experiments showing that muscle reversibly loses potassium and gains sodium, Dean postulated that "…there must be some sort of a pump, possibly located in the fiber membrane, which can pump out the sodium, or, what is equivalent, pump in the potassium" (Dean, 1941). Dean's measurement of the permeability of RBCs to K^+ was published in *JGP* (Dean et al., 1941).

E.J. Conway and his colleagues contributed an important part of the PLM story: they showed that the imposition of two constraints (namely, balanced osmotic strength across the membrane and electroneutrality within and outside the cell) led to an equation that linked cell voltage with the number of impermeant molecules within the cell (Boyle and Conway, 1941). However, this alone did not solve the problem of how to stabilize a cell. He reached for a solution that was rather unnatural—and postulated that a cell could be stabilized by making itself impermeable to Na^+. This was essentially the "double Donnan" mentioned above. Nevertheless, at the end of his 1957 review, he commented that "it would appear necessary for the cell to possess an active mechanism for Na^+ extrusion as Na^+ ions could not be indefinitely excluded" (Conway, 1957).

Alexander Leaf and his associates also played a key part in the lead-up to the PLM. He coined the term "double Donnan," but it is clear from his writing that he did not mean it in the literal sense of complete impermeablity to Na^+, but that the action of the NKA is to make the cell effectively impermeable to Na^+ (Leaf, 1956). Maffly and Leaf (1959) demonstrated in a *JGP* paper that there is no sustained difference in osmolarity between the intracellular and extracellular compartment on the basis of melting point depression experiments. Later on, Macknight and Leaf (1977) published a meticulous review of the literature on cell volume regulation.

Hodgkin, in his Croonian lecture of 1957, states, "If a nerve or muscle fibre is to remain in a steady state there must be a balance between the rates at which Na and K leak through the membrane…and the rate at which these ions are pumped by the metabolic system." The published lecture includes the equations for osmotic balance and for electroneutrality, and concludes that an electroneutral ($1Na^+$:$1K^+$) pump could stabilize a cell with impermeant ions (Hodgkin, 1958). He did not, however, provide a formal proof of this.

Following on the work of Dean (1941), by the mid-1950s, J.E. Harris, I.M. Glynn, R.L. Post, T.I. Shaw, and others had elucidated key aspects of Na^+ pump function (see Glynn, 1993); this culminated with the serendipitous discovery of the NKA by Skou (1957). The concurrent studies on cell volume regulation (see above) led to a convergence of ideas; thus, by the late 1950s, it became apparent to a number of investigators that an active ion pump is required to stabilize cell volume. Ultimately, however, a mathematical model of all the forces at play in cells was required to comprehend the mechanism. To this end, in an appendix to their 1957 paper that established the correct stoichiometry for the NKA ($3Na^+$:$2K^+$), Post and Jolly (1957) provided a pared-down version of the PLM that shows how the mechanism works. They considered a model cell that was permeable to a molecule H and contained a fixed amount of an impermeant molecule C. They showed that in the absence of active transport the cell was unstable, whereas if C was pumped out of the cell, this would stabilize the cell. Although the Post and Jolly (1957) model vastly simplifies the situation by omitting charge entirely, it is useful didactically in that it makes evident, mathematically, how pumping molecules can serve to stabilize a cell under an osmotic threat.

Tosteson and Hoffman's revelation: A model that fits

The preceding pioneers set the stage for the landmark report by Tosteson and Hoffman (1960); see Fig. 4) that first clearly laid out the quantitative essentials of the PLM. The model, which is similar in form to that outlined above (see Summary of the PLM and the Appendix), was developed by Tosteson with some input from Hoffman when they were both research fellows "in Cambridge (UK) on those cold, dark mornings in the winter and spring of 1957" (Brobeck et al., 2013). Tosteson was working in Hodgkin's laboratory, trying (unsuccessfully) to measure the human RBC membrane potential with microelectrodes

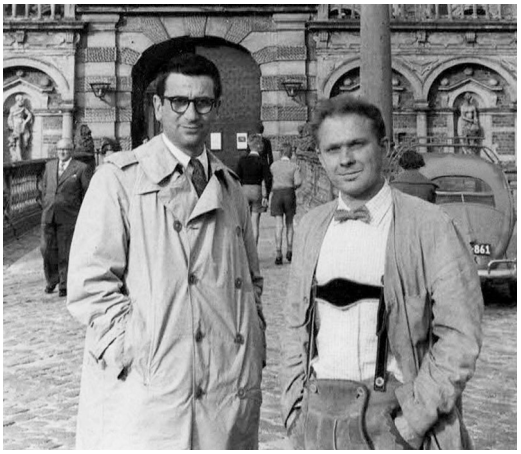

Figure 4. **Joseph Hoffman (left) and Daniel Tosteson (right).** Photograph from 1955 or 1956 at the Museum of National History, Frederiksborg Castle, Hillerød, Denmark, taken when Tosteson was a postdoctoral fellow in Hans Ussing's laboratory in Copenhagen before he moved to join Alan Hodgkin's laboratory at the University of Cambridge, UK. Photo courtesy of Dr. Magdalena Tosteson.

(Hoffman J.F., personal communication). Hoffman was a postdoctoral fellow in F.J.W. Roughton's Department of Colloid Science, but was also working on RBC ghosts with Ronald Whittam in the Cambridge Physiological Laboratory (Hoffman et al., 1960).

One of us (Blaustein) was a student in Tosteson's laboratory at Washington University, St. Louis, MO, from 1959 to 1961, and was enthralled by his fascinating stories of those "miserable" mornings in Cambridge (echoed by Paul Horowicz, another Hodgkin laboratory veteran who had also joined the Washington University faculty). This led Blaustein to spend two memorable years in Hodgkin's laboratory less than a decade later (Blaustein, 2016).

In the autumn of 1957, Tosteson and Hoffman both joined Robert Berliner's Laboratory of Kidney and Electrolyte Metabolism at the National Institutes of Health (Bethesda, MD). There they used their new model to estimate transport parameters from experiments they performed on sheep RBCs. This was fortuitous, if not prescient; mammalian RBCs contain no nuclei or mitochondria and are relatively simple cells that behave like micro-osmometers. Based on Berliner's recommendation, they used cells from two different strains of sheep, one that had "normal" (high) levels of intracellular K^+ (HK) and another that had low levels of K^+ (LK). To follow the fluxes of ions, they employed radioactive Na^+ and K^+. Their PLM model was then used to estimate the parameters of the PLM. Their data were consistent with the PLM, and they could account for the difference between HK and LK RBCs in terms of the differences in pump rates and ionic permeabilities. To test their model, Tosteson and Hoffman showed that when the NKA was blocked by strophanthidin, a ouabain analogue, cell volume increased in proportion to the increase in cell cation content. Because the cation permeabilities were different in the two cell types, the rates of increase in cation content and volume were also different in the LK and HK cells, especially when the external K^+ concentrations were altered. This provided strong support for the model. The particular relevance of the HK/LK results is that even if the LK cells normally have relatively high Na^+ (137 mM) and low K^+ (17 mM) concentrations, they must still pump Na^+ out to maintain a stable cell volume (Tosteson and Hoffman, 1960).

In summary, Tosteson and Hoffman proposed a mechanism that could account for the stability of cells harboring impermeant molecules. The essential components of the model are the following: (1) a membrane that is flexible and permeable to water, Na^+, K^+, and Cl^-; (2) impermeant molecules in the cytoplasm, with a net negative charge; and (3) a pump for driving Na^+ out of the cell. These assumptions can be translated into a set of four differential equations, which, together with an expression for the intracellular voltage, constitutes the pump-leak equations (Eqs. 1, 2, 3, 4, and 5 in the Appendix).

Although Tosteson and Hoffman listed seven assumptions in their model, some are not necessary. Indeed, a PLM system like theirs, for a wide range of conductances and pump rates, will settle into a steady state in terms of volume, voltage, and intracellular ion concentrations (Mori, 2012). There is no need to assume that the system is at a steady state. The physics of the components takes care of that.

Tosteson and Hoffman recognized that permeable anions that are not pumped must be at equilibrium with the membrane potential (V). Further, they reported that the mean $[Cl^-]_i/[Cl^-]_o$ ratio was 0.67 in the HK and 0.70 in the LK cells. These values correspond (at 37°C) to $V = E_{Cl}$, the Cl^- equilibrium potential, of −10.7 and −9.5 mV, respectively. These values are very similar, despite the very different cation distributions in the two cell types, and they are comparable to the value, −9 mV, calculated from the Cl^- ratio in human RBCs (Hoffman and Laris, 1974). Given Tosteson's interest in the RBC membrane potential (noted above), it seems surprising that the sheep RBC membrane potentials were not reported (and apparently not calculated) in the 1960 article.

It is worth mentioning that bicarbonate anions, unlike protons (<1 μM), make a contribution to the extra- and intracellular tonicity. But little is known about how the complex interplay of the passive and active transport of H^+ and HCO_3^- (Occhipinti and Boron, 2015), a topic beyond the purview of this article, influences cell volume stability. In RBCs and many other cell types, however, without active anion transport, the $[HCO_3^-]_i/[HCO_3^-]_o$ ratio is identical to that of Cl^- because of a prevalent plasma membrane Cl^-/HCO_3^- exchanger. Therefore, as in Tosteson and Hoffman's PLM (Fig. 1), for convenience, Cl^- and HCO_3^- can be lumped together in the mathematical treatment.

Critics (cited in Post et al., 1967) questioned the pump-leak concept as well as the 3 Na^+:2 K^+ stoichiometry of the NKA by Post and Jolly (1957). To address these issues and provide independent support for the PLM, Post et al. (1967) employed the Tosteson–Hoffman model to measure the pump and leak Na^+ and K^+ fluxes in human RBCs. They confirmed that the pump Na^+:K^+ coupling ratio is 3:2, which is now widely accepted.

In a recent *JGP* Perspective article, Sachs and Sivaselvan (2015) raised objections to the PLM. Their paper focuses on a few studies that show that some cells do not rapidly swell and

lyse when subjected to extreme osmotic stress by bathing in distilled water. They attribute this ability to withstand osmotic stress to the poroelastic properties of the cytoskeleton, and give the PLM rather short shrift. The PLM can stabilize cells, albeit at an altered size, even after a change in extracellular osmolarity (see Fig. 2). In the absence of osmotic stress, when metabolism is inhibited or the NKA (or other active ion pump that implements the PLM; see below) is blocked, most types of cells with flexible membranes swell as they accumulate Na^+, Cl^-, and water isosmotically (e.g., Leaf, 1956). In this respect, the RBC, which rapidly lyses in distilled water, serves as an excellent model. This behavior cannot be explained by cell mechanics, and demonstrates that cells require a PLM to maintain cell volume.

Evolution of the mathematics of the PLM

Tosteson and Hoffman wrote down the equations that encapsulate the PLM and provided key experimental support, but their exploration of the implications was limited. The first two more comprehensive theoretical explorations of the PLM were performed by Mackey (1975) and Jakobsson (1980); both acknowledged the influence of J. Walter Woodbury (1965). Keener and Sneyd (2009); first edition, 1998) developed a simplified model of the PLM, which captured all of its essential aspects and provided a very convenient closed form solution (see Appendix).

Other ways of implementing the PLM

Thus far we have only discussed how the NKA can be used to stabilize cells via a PLM; however, there are other combinations of transporters that can also provide volume stability, thereby helping validate the basic concept. Dog RBCs are an interesting case because they have a high intracellular Na^+ concentration, similar to the concentration in plasma, but not at electrochemical equilibrium, and they lack an NKA (Parker, 1973b). As Parker has shown, however, they do have an energy-dependent mechanism for extruding Na^+. This mechanism involves an ATP-driven Ca^{2+} extrusion pump and a Na^+/Ca^{2+} exchanger (NCX) that extrudes Na^+ in exchange for Ca^{2+} and normally stabilizes intracellular osmotic pressure and cell volume (Parker, 1973a; Parker et al., 1975). For a PLM to work, Na^+ has to be pumped out of the cell, but it does not have to be via an NKA.

Another variation on this theme is seen in cardiac muscle (Baumgarten, 2006). Takeuchi et al. (2006) observed that guinea pig cardiomyocyte volume regulation is preserved after ouabain blockade of the NKA that is expressed in these cells, which would seem to undermine the PLM. The large Na^+ electrochemical gradient in these cells, however, serves as a storage battery, and the prevalent NCX in the myocytes then helps to extrude Na^+ from these cells in exchange for entering Ca^{2+}, which is quickly extruded (by a plasma membrane Ca^{2+} pump) or sequestered and thus does not contribute to the osmotic load. This mechanism was substantiated by showing that ouabain itself did not increase cell volume, but subsequent addition of NCX blockers did increase volume. Regrettably, the authors apparently did not perform the converse experiment: if NCX was blocked first, we would not have expected cell volume to increase, but then blocking NKA with ouabain should have initiated a volume increase. This would have verified that the two transport systems, NKA and NCX, are independent but functionally coupled, and that the ATP-driven pump is required to power the system.

An analogous coupled system was reported to stabilize cell volume in mycoplasma, a form of bacteria without a cell wall; an outwardly directed proton pump operating in parallel with a Na^+/H^+ exchanger, that can drive Na^+ out of the cell (Linker and Wilson, 1985a,b). Subsequent studies, however, revealed that mycoplasma express a ouabain-insensitive primary (ATP-driven) Na^+ pump that serves to stabilize cell volume (Shirvan et al., 1989).

The lesson from all of these cases is that all cells face inundation by Na^+ and water and need to achieve volume stability. Thus, they all require some energy-dependent mechanism to drive Na^+ out of the cell. This can be accomplished directly by a Na^+ pump (e.g., the NKA) or by another active transport process coupled to an exchanger that can then extrude Na^+. One might think that actively pumping K^+ in might be an effective mechanism for stabilizing cell volume, but it depolarizes the cell and prevents Cl^- from moving out. Indeed, simulations show pumping K^+ alone destabilizes the cell and hence would be ineffective (Kay, 2017).

Conclusion

JGP had its origins in part in Loeb's desire for a physiology journal rooted in the "physico-chemical methods of analyzing life" (Andersen, 2005). Loeb's commitment to developing a mechanistic and quantitative physiology is nicely captured by Osterhout (1928): "[Loeb's] notion of biological research was simple: all the observed phenomena should be expressed in the form of equations containing no arbitrary constants. Anything short of this is to be regarded as merely preliminary."

Mathematical theories have often received something of a cold shoulder in biology (Goldstein, 2018), a sharp contrast with the fields of physics and chemistry, where mathematics is an indispensable part of the enterprises. Theory, however, provides a framework for making evident our understanding of a biological system; it also allows us to make predictions that go far beyond what one can do using logic and natural language. Imagine, for example, Hodgkin–Huxley's theory without mathematics; it simply becomes a narrative account of one thing following after another. Remarks by Heinrich Hertz (1889) on James Clerk Maxwell's theory of electromagnetic radiation aptly summed up the peculiar power of mathematics: "It is impossible to study this remarkable theory without experiencing at times the strange feeling that the equations and formulae somehow have a proper life, that they are smarter than we, smarter than the author himself." Of course, mathematical modeling, unless it can be experimentally verified, is usually a futile exercise. We believe that the PLM provides a wonderful example of the powerful coupling between theory and experiment.

One of the problems that we have noticed in reviewing the history of the PLM is that this theory is often taken for granted or seems not to be known. For example, one often finds all the elements of the PLM deployed in papers with no mention of the PLM or reference to Tosteson and Hoffman's *JGP* paper. Hodgkin–Huxley-like simulations are now widely used in

attempts to understand neural networks; however, simulations that incorporate water fluxes and flexible membranes are rare (Kager et al., 2007). It is perhaps worth asking if such simulations ought to be more widespread, since not only should models account for electrical phenomena but also for the stability of cells.

Most epithelia couple the action of the NKA to transport ions and other solutes and water, but they also require the pump to achieve cellular stability. This key role of the NKA is seldom mentioned in epithelial physiology papers (Lew et al., 1979), even though simulations of epithelia often include the NKA, impermeant ions, and flexible membranes: a PLM, in all but name (Larsen et al., 2000).

In the interpretation of experiments, even if a model is not made explicit, one interprets the responses in terms of an implicit model—frankly, even if one does not recognize it. It seems that if one is trying to make sense of ionic and osmotic phenomena, it would be prudent to adopt the PLM as the baseline assumption. Although the PLM considered here does not incorporate the mechanics of the cell and cytoskeleton, they can easily be included. Such models will be of value in determining the relative contributions of cell mechanics (Sachs and Sivaselvan, 2015) and ion transport to cellular stability.

A clear index of the importance of a cellular process is the amount of energy that cells spend on it. It has been estimated that cells in all animal phyla expend ∼20% of their energy budget on driving the NKA (Rolfe and Brown, 1997). It seems likely that the evolution of the PLM liberated cells from the straightjacket of the rigid cell wall and, in the words of Tosteson (1964), "It would appear reasonable to conclude that volume regulation is the most fundamental and primitive cellular function of active Na and K transport. Without it, we would all be, at best, mute dryads of the trees imprisoned in cellular walls."

Appendix

The pump-leak equations

We list here the pump-leak equations of Keener and Sneyd (2009) and the steady-state solution that they derived.

Capitalized solutes represent concentrations, and extracellular concentrations are denoted by a subscript *o* and are assumed to be fixed, while intracellular concentrations have no subscripts. Symbols are defined below.

The five pump leak equations are as follows:

$$V = \frac{Q}{C} = \frac{Fw}{AC_m}(Na + K - Cl + zX), \tag{1}$$

$$\frac{dw}{dt} = \nu_w P_f A(\Pi - \Pi_o), \tag{2}$$

$$\frac{dNa}{dt} = -\frac{A}{wF}\left(g_{Na}\left[V - \frac{RT}{F}ln\left(\frac{Na_o}{Na}\right)\right] + np\right), \tag{3}$$

$$\frac{dK}{dt} = -\frac{A}{wF}\left(g_K\left[V - \frac{RT}{F}ln\left(\frac{K_o}{K}\right)\right] - qp\right), \tag{4}$$

$$\frac{dCl}{dt} = \frac{A}{wF}\left(g_{Cl}\left[V + \frac{RT}{F}ln\left(\frac{Cl_o}{Cl}\right)\right]\right). \tag{5}$$

We introduce the following nondimensional variables:

$$\nu = \frac{FV}{RT}, \tag{6}$$

$$P = \frac{pF}{RTg_{Na}}, \tag{7}$$

$$\mu = \frac{w}{x}Cl_o. \tag{8}$$

We define:

$$\gamma = e^{-\nu}, \tag{9}$$

$$\alpha = \frac{Na_o e^{-nP} + K_o e^{qP\gamma}}{Na_o + K_o}, \tag{10}$$

$$\Upsilon = g_{na}/g_K, \tag{11}$$

Keener and Sneyd (2009) demonstrated that for

$$n\frac{Na_o}{g_{Na}} > q\frac{K_o}{g_K},$$

there is a range of *p* for which the system has a finite positive cell volume, with the following solutions:

$$Na = Na_o\gamma e^{-nP}, \tag{12}$$

$$K = K_o\gamma e^{qP\gamma}, \tag{13}$$

$$Cl = Cl_o/\gamma, \tag{14}$$

$$\mu = \frac{1 + \sqrt{1 - (1-\alpha)(1-z^2)}}{2(1-\alpha)}, \tag{15}$$

$$\gamma = \frac{-z + \sqrt{z^2 + 4\alpha\mu^2}}{2\alpha\mu}, \tag{16}$$

Symbols

A, membrane area; *C*, membrane capacitance; *F*, Faraday's constant; g_i, conductance of ion *i*; *n*, number of Na^+ pumped per NKA cycle; *p*, pump rate; *q*, number of K^+ ions pumped per NKA cycle; *Q*, net intracellular charge; *R*, universal gas constant; *T*, absolute temperature; *w*, cell volume; *x*, number of moles of the impermeant intracellular molecules; *z*, average charge valence of impermeant intracellular molecules; *V*, membrane potential; ν_w, partial molar volume of water; Π, the intracellular osmolarity; Π_o, the extracellular osmolarity; P_f, osmotic water permeability coefficient.

Default values of parameters

Cell radius = 5 µm; Cl_o = 150 mM; C_m = unit membrane capacitance = 1 µF cm^{-2}; g_{Cl} = 0.2 mS cm^{-2}; g_K = 0.3 mS cm^{-2}; g_{Na} = 0.01 mS cm^{-2}; K_o = 3 mM; *n* = 3; Na_o = 147 mM; $P_f = 7 \times 10^{-3}$ cm s^{-1}; *q* = 2; *T* = 37°C; *x*= 2.6×10^{-14} mol; *z* = −1.

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