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THE STATE OF POTASSIUM IN SKELETAL MUSCLE AND IN NON-MUSCLE CELLS

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Abstract

The relationship between ions, water, and the electrical properties are fundamental to our understanding of cellular function. This paper is primarily directed at reviewing the theoretical explanations for the changes in cellular potential and ionic composition which are associated with early postnatal development of skeletal muscle. The findings are: (a) a two-fold reduction in tissue hydration and a significant reduction of the diffusive motion of cellular water; (b) ten-fold decrease in cellular sodium; (c) six-fold decrease in tissue chloride; (d) the concentrations of intracellular potassium, and of extracellular sodium, potassium and chloride were constant; and (e) the cellular potential changed by 55 mV. A review of the literature concerning the physical state of potassium and water is made. The theoretical explanations of these findings are evaluated in terms of the classical membrane theory and the association induction hypothesis.

KEY WORDS: State of potassium, skeletal muscle.

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Introduction

During the first 65 years of this century, cellular water was viewed as a passive medium in which constituents were dissolved with a structure indistinguishable from bulk water. The intra- and extracellular solutions, separated by a semi-permeable membrane, were assumed to obey the physical laws for dilute solutions. This view has served the biologist well, providing a physical basis for transient membrane permeability to sodium, cellular potential, solute distribution, and volume regulation [1,3,8,9,11,30,44,52,60, 63]. The specific hypothesis of Bernstein contained not only the assumptions of ideal solution theory, but also the assumption that the cell membrane of a resting cell was impermeable to sodium [3,30]. The assumptions contained in Bernstein's hypothesis appeared valid until 1939, when reports appeared posing serious challenges to that part of the theory which argued for the impermeability of the resting cell membrane to sodium [27] (see Ling in Discussion with reviewers for further details). Later, the hypothesis of Bernstein was extended to what is essentially the present concept of the excitable cell; and, is often referred to as the membrane-pump, or ionic theory [26,30]. The theory of dilute solutions, however, remained fundamental to the membrane-pump concept [26,30,63]. And, in specific, potassium was considered to be free and dissolved in the cellular water.

Numerous experiments have provided evidence consistent with the membrane-pump view [1,12,13, 30,52,59,60,63]. Less well known, however, are the reports that provide evidence challenging the validity of the assumptions basic to the membrane-pump view [4,6-10,14,17,19,20,28,35-40,42-50,54-56,61,62,64,65]. Additional studies will be presented which provide tests for these divergent views.

The Cellular Potential and Electrolyte Composition in Skeletal Muscles of Developing Rat: Statement of a Problem

Various laboratories throughout the world have reported dramatic changes in the electrical properties of skeletal muscle and in the muscle electrolyte concentrations in developing animals

[2,4,21-24,51,57,58,67,70]. The results from a series of studies coming primarily from our laboratory [21-25] will be summarized in order to demonstrate the overall changes in voltage, water (content and physical properties), and electrolytes associated with postnatal development of rat skeletal muscle.

Changes in cellular polarization occur between birth and maturity in skeletal muscle [4,22-24, 58]. Drawing from these studies it is concluded that, between birth and 60 days of postnatal life, the cellular potential changes from -25 mV to -80 mV (i.e., a change of 55 mV).

In the case of the gastrocnemius muscle of the rat, the percentage of water decreases from 90% at birth to 77% in the mature rats. On a dry weight basis, this means a change in hydration of approximately 9 gm/gm at birth to approximately 3.5 gm/gm at 65 days of age. Nuclear magnetic resonance (NMR) studies have been made on muscles taken from developing animals. The results from high resolution studies show that the line widths (for the proton NMR) increased from 5 Hz at 2 days of age to 13 Hz by 30 days of age [25]. Likewise, pulsed NMR studies of the water hydrogen nuclei relaxation times (T_1 and T_2) made during the same developmental period gave results consistent with the high resolution NMR studies [25]. These data were interpreted to mean that, during development, an increase in the order of the water molecules occurred within the cells of the muscle.

The changes in potassium, sodium, and chloride concentrations of the rat gastrocnemius between 2 and 65 days of age are given in Figure 1 [24].

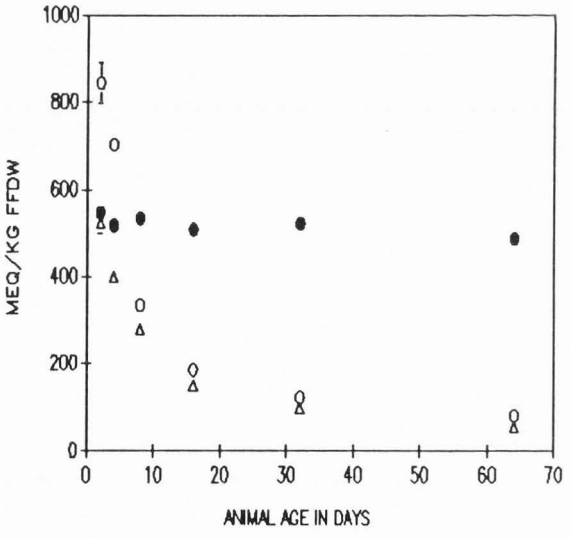

Figure 1. The data on the rat gastrocnemius muscle sodium (unfilled ovals), potassium (filled ovals), and chloride (unfilled triangles) are given in milliequivalents per kilogram of fat-free dry weight (FFDW). The abscissae is animal age in days.

The potassium concentrations, when related to the fat-free dry muscle mass, vary little with age. Sodium and chloride both decrease significantly. At four days of age and earlier, the sodium concentrations exceed those of potassium -- a remarkable finding. Direct intracellular measurements confirmed that these total tissue (dry weight) measurements reflect the intracellular concentrations of potassium and sodium [57].

In summary, during the first 64 days of postnatal life in rat skeletal muscle, there are changes in water (both in concentration and by physical properties), sodium, and chloride. Potassium, on the other hand, does not change significantly. The variations (or lack thereof) are: (**a**) a two-fold reduction in tissue hydration and three-fold, or more, change in the NMR line widths and relaxation times; (**b**) ten-fold decrease in cellular sodium; (**c**) six-fold decrease in tissue chloride; (**d**) the concentrations of intracellular potassium, and of extracellular sodium, potassium and chloride were constant; and (**e**) the cellular potential changed (in absolute value) by 55 mV.

Proposed Explanation of the Data

Classical Membrane-Pump Theory.

This explanation was rejected for four reasons.

Cellular potential. Classically, the voltage across the cell surface has been described as an equilibrium given by the equality between electrical and osmotic work. Thus, the resting cellular potential is defined by equation (1) -- the Nernst equation or the potassium equilibrium potential. Thus, the potential, **E**,

$$E = RT/F \ln\{\frac{[K]_i}{[K]_o}\} \qquad (1)$$

is an explicit function of the intra- and extracellular potassium concentrations ($[K]_i$ and $[K]_o$, respectively). In equation (1), **R**, **T**, and **F** are constant and, respectively the ideal gas term, the absolute temperature, and the Faraday. Considering the changes in cellular potential (**E** for the immature muscles and **E′** for mature muscles), we can evaluate equation (1) for the expected change in $[K]_i$ in a manner similar to that of Zierler [71]. Since $[K]_o$ did not differ between the two states, we have

$$e^{\Delta EF/RT} = \frac{[K]_i'}{[K]_i} \qquad (2)$$

where $\Delta E = |E - E'|$, and $[K]_i'$ represent the mature state. The resulting 55 mV change in cellular potential, requires an 830% increase in cellular potassium. And, as indicated above, no increase in cellular potassium was observed [24,57].

It remains possible that changes in membrane permeability might resolve this difficulty. Again, we used an analysis similar to that of Zierler [71] involving the Goldman-Hodgkin-Katz (GHK) equation which describes the cellular potential as a function of membrane permeabilities and ion concentrations, and is presented in equation (3).

$$E = RT/F \ Ln\{\frac{P_k[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o}{P_k[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i}\} \quad (3)$$

In equation (3) P_k, P_{Na}, and P_{Cl} are the membrane permeabilities to potassium, sodium, and chloride respectively. Our analysis differs from Zierler's in that we have used the modified GHK equation. According to Katz [ref. 30 pg 62], we may assume "...that the chloride ions are in equilibrium in resting muscle tissue (that is, $[Cl]_o/[Cl]_i = e^{EF/RT}$..." Such an assumption allows the deletion of the chloride term in equation (3) resulting in

$$E_r = RT/F \ Ln\{\frac{P_k[K]_i + P_{Na}[Na]_i}{P_k[K]_o + P_{Na}[Na]_o}\} \quad (4)$$

which is referred to as the modified GHK equation. Assuming that equation (4) describes the potential in both the mature and the immature states, and remembering that both $[K]_o$ and $[Na]_o$ do not change throughout the experiment, then applying the manipulations that gave equation (2) will give equation (5) -- the primes again defining the mature state.

$$\frac{[K]_i'}{[K]_i} = \frac{P_k}{P_k'}\{e^{\Delta EF/RT} + \frac{e^{\Delta EF/RT} P_{Na}[Na]_i}{P_k[K]_i} - \frac{P_{Na}'[Na]_i'}{P_k[K]_i}\} \quad (5)$$

Equation (5) may be evaluated for various reasonable limits. For example, in the neonatal state, it seems likely that, at most, $P_k \approx P_{Na}$ and that $[Na]_i \approx [K]_i$. It also seems reasonable that $P_{Na}'[Na]_i' << P_k[K]_i$. These limits represents a minimum for equation (5) and reduces to an approximate form given in equation (6).

$$\frac{[K]_i'}{[K]_i} \approx \frac{P_k}{P_k'} \ 2e^{\Delta EF/RT} \quad (6)$$

This analysis reveals that, when membrane permeability changes are included, an increase in $[K]_i$ is required which is greater than that expected from equation (2) which does not include permeability. In fact, for equation (6) to equal equation (2) (i.e., for $[K]_i'/[K]_i$ to be equal to one), P_k' would have to be 1660% greater than P_k.

The observation of a lack of a relationship between $[K]_i$ and the cellular potential has been reported by several investigators [ref. 48 pp 465-467 for review, 71]. Our findings reported here for muscles of developing animals are, in general, consistent with those reports.

Changes in cellular sodium. Because of the original assumptions that the ions are in solution, the changes in intracellular sodium (i.e., the ten-fold decrease during the first 64 days of postnatal life) require an active transport system. As early as 1962, Ling reported a quantitative analysis of the energy requirements for membrane transport in skeletal muscle; and found the energy required to pump sodium exceeded the energy available by 1500% - 3060%. See Ling [ref. 42 Table 8.9 pg 211, 45] and Hazlewood [20] for two detailed accounts of the calculations. To the best of our knowledge, no refutation of the energy demands of the pump have been reported which involves the use of quantitative data.

Tissue hydration. According to the classical theory, cell volume is proportional to the inverse of external osmotic pressure. In early postnatal development, however, changes in intracellular volume (hydration) occur in the absence of changes in external osmotic pressure. Also, the physical properties (i.e., the relaxation times) of water in skeletal muscle changed during the first 64 days of postnatal life. In the conventional membrane-pump view, the water within cells is considered to behave physically like a dilute solution. Thus, the discovery that the physical properties of cellular water are different from ordinary bulk water was not predicted. Explanations of this observation, therefore, require that *ad hoc* additions be made to the classical theory.

The physical state of solutes in cells. The evidence is now overwhelming that significant amounts of intracellular potassium, and other solutes including metabolites are adsorbed [8,9,15,20,36,54,69]. These findings are contrary to the fundamental assumptions of the classical theory that the cellular solutes are in solution -- adsorption of a significant fraction of the solutes was not a part of the conceptualization. Other theories do, however, incorporate the ideas that adsorptive processes are needed to describe the data at hand.

Association-Induction Hypothesis (AIH)

Cellular solute and water distribution.

In this theory, the intracellular concentration of any ion will be determined by its electrostatic association to macromolecular fixed charged sites, and its solubility in cellular water. For example, cellular potassium is selectively accumulated over that of sodium primarily by two mechanisms: a) potassium is preferentially adsorbed to fixed charges on proteins, nucleic acids, etc. within the cell [14,15,28,48,54,55]; and b) sodium is entropically excluded from the organized cytoplasmic water [15,49,55,56].

Equation (7) provides a simplified formalization of the words in the previous paragraph, assuming only one type of fixed charged site.

$$[S]_{in} = a\,[S]_{int} + [S]_{ad} \quad (7)$$

where $[S]_{in}$, a, $[S]_{int}$, and $[S]_{ad}$ are intracellular concentrations of any solute S, a is the concentration of cytoplasmic water, $[S]_{int}$ is the concentration of solute S in the cytoplasmic water, and $[S]_{ad}$ is the concentration of the adsorbed solute S. Thus, a $[S]_{int}$ is the fraction of $[S]_{in}$ that is dissolved in the aqueous phase of the cell and $[S]_{ad}$ is that fraction of $[S]_{in}$ which is adsorbed. If one now defines q_s as the equilibrium distribution coefficient for any solute S, we have equation (8):

$$q_s = [S]_{ex}/[S]_{int} \quad (8)$$

where $[S]_{ex}$ is the concentration of solute **S** in the extracellular fluid, and $[S]_{int}$ is the same as in equation **(7)**. (Please note: q_s is not to be confused with ρ_s which is the ratio of $[S]_{ex}$ to $[S]_{int}$.) Substituting equation **(8)** into equation **(7)** we have equation **(9)**.

$$[S]_{in} = a\, q_s\, [S]_{ex} + [S]_{ad} \quad (9)$$

Since there will be a finite number of fixed charges in a cell, $[S]_{ad}$ is a saturable fraction (i.e., $[S]_{ad}$ will become constant as $[S]_{ex}$ approaches physiological limits). On the other hand, q_s (or $[S]_{int}$) is non-saturable (i.e., its value is determined by the physical properties [solubility] of the cellular water).

Cellular water. Applications of quasielastic neutron scattering (QNS) techniques have led to new insights into the physical properties of water in biological systems [61,65]. From the QNS studies we have learned that the rotational diffusive motion is reduced in *Artemia* cysts [65] and in skeletal muscle [61]. These new findings are consistent with other studies of diffusion [ref. 20 pp 229-241 for review, 50,62] and indicate that cellular water is in a physical state different from that of ordinary bulk water -- a condition consistent with that proposed by the association-induction hypothesis. In theory, such changes would be reflected in the q_s value in equation **(9)** above; and, the expected physical consequence is the exclusion of solute from the aqueous phase [49]. Under normal physiological conditions, the equilibrium distribution coefficient all solutes (q_s) is a constant and, for most, less than one. Changes in the physiological state of the tissue alter the physical properties of water and hence the q_s. Thus, we are proposing that during early postnatal development, q_{na} in equation **(9)** decreases. On the other hand, the macromolecular species adsorbing potassium [substitute **K** for **S** in equation **(9)**] does not appear to change.

Cellular potential. The cellular potential has been shown to be relatively insensitive to changes in the intracellular concentration of potassium [ref. 48 pp 465-467 for review], as in the case reported here. Because of this important short coming, alternative interpretations were sought. The cellular potential (**V**) as described by the surface adsorption model of the AIH in its simplest form, is offered for consideration.

$$V = \text{constant} - (RT/F)\mathrm{Ln}\{X_k[K]_{ex} + X_{na}[Na]_{ex}\} \quad (10)$$

where X_k and X_{na} are the surface adsorption energy constants for potassium and sodium, and $[K]_{ex}$, $[Na]_{ex}$, **R**, **T**, and **F** have their usual meaning [ref. 48 Chapter 14 pp 463-500]. This formulation offers an explanation for the postnatal changes in cellular potential referred to in this paper [18,22-24,58]. In the context of the association-induction hypothesis, emphasis is placed on the surface charge of cells and the external concentration of monovalent ions. Direct and indirect tests of this theory have been reported [14,23].

The State of Potassium in Non-Muscle Cells

Here, we are going to give a brief survey of our previous experimental findings indicating several-fold decrease of the chemical activity of potassium in different cells and also in the nucleus of cells. In essence, these experiments can be divided into two groups: **a)** observations on cell nuclei; **b)** studies of release kinetics of potassium from detergent "opened" cells.

Observations on Cell Nuclei

Stained Polarization Microscope Studies. Isolated DNA filaments show intensive birefringence in the polarization microscope. However, the DNA inside the nucleus of an intact interphase cell is optically isotropic [32,34]. In isolated nuclei the DNA will become gradually birefringent if the concentration of free K^+ and/or Na^+ in the solution the nuclei are exposed to exceeds 70 mM [31-33]. Furthermore, we found that DNA in the nucleus of an intact living interphase cell cannot be exposed to a free electrolyte (free K^+ solution) exceeding a concentration value of 70 mM [32,33]. If one determines the potassium content of isolated nuclei and divides that number by the water content one obtains a "concentration" 150 mM or higher. Recent findings utilizing the X-ray microprobe technique indicate that if all the nuclear potassium were in solution, it would exceed 70 mM [6,7]. Consequently, a certain mechanism, or mechanisms must exist in the nucleus of a living cell which decreases the free K^+ concentration to a level below 70 mM in the close vicinity of DNA [31,33]. We propose that such an ion-regulatory mechanism must include nuclear proteins.

Previously, we hypothesized that loosely bound nuclear proteins are the essential constituents of the intranuclear ion-regulatory machinery [31,33]. Supporting these ideas is the observation that most of the loosely bound nuclear proteins are removed from the nucleus during the isolation procedure and also during the treatment with different solutions. In other words, in the isolated nuclei where the reversible ion-induced DNA birefringence was observed at or above 70 mM free ion concentration, the loosely bound nuclear proteins were not present. In addition, the findings that the DNA of chromosomes of dividing cells is birefringent provides support for the ion-regulatory role of loosely bound nuclear proteins [32,34]. It is also known that when the chromosomes are formed and the nuclear membranes are disintegrated, the majority of the loosely bound nuclear proteins are released from the nucleus to the cytoplasm. At this stage larger regions of DNA might be transiently exposed to free electrolytes exceeding 70 mM, which is the critical DNA-birefringence-inducing ion concentration found in our studies.

Studies of the Movement of the T-Antigen. Additional supportive evidence that loosely bound nucleoproteins may be regulated by the free monovalent ion concentrations comes from a systematic study of the large T-antigen of SV40 virus induced tumor cells. In this study, the majority of the T-antigen could be extracted from isolated nuclei of SV40 virus induced tumor cells in solutions containing monovalent cations between 70 mM and 150 mM concentrations [39]. The T-antigen is known to be released from the nucleus to the cytoplasm at mitosis. In H-50 chicken erythrocyte heterokaryons, the T-antigen migrates into the chicken erythrocyte nuclei just prior to gene activation and initiates nuclear swelling [35]. We interpret these findings to indicate an essential role of T-antigen in the volume-regulation of the nucleus through the condensation-decondensation of chromatin. The large T-antigen is in many respects similar to the nucleoplasmin -- the first protein named as a "molecular chaperon" [41]. (The proteins of the "molecular chaperon" family are considered to interact with newly synthesized proteins and maintain their extended state in an ATP-driven manner [16,5]. Ling, as early as 1970, hypothesized that the extended state of some proteins could be responsible for ion selectivity and for the polarization of water. He further postulated that the fully extended state was induced by ATP and possibly other bioregulants. We propose the following questions: (a) Do the proteins of the "molecular chaperon" family support Ling's hypothesis? (b) Is the large T-antigen a member of the "molecular chaperon" family? (c) Do the loosely bound proteins play a role in selective K^+ accumulation and in the organization of cellular water?)

Findings on Thymus Lymphocyte Nuclei Exposed to Different Electrolytes. Isolated thymus lymphocyte nuclei were incubated in buffers containing 3 mM each of Ca^{++} and Mg^{++} plus varying concentrations of K^+ and Na^{++} [37]. Nuclei isolated in buffers containing 15 mM K^+ plus 15 mM Na^+ had a smaller diameter, and the interchromatin spaces were reduced when compared to nuclei incubated in the absence of these two ions. Nevertheless their water-holding capacity, i.e. their water content after centrifugation at 100,000 g was higher. When the isolated nuclei were exposed to the calcium and magnesium buffers containing 75 mM K^+ and 75 mM Na^+ (i.e., monovalent ionic concentration of 150 mM), the chromatin became disrupted and their water-holding capacity elevated. Thus, exposure of nuclei to buffers containing total monovalent ionic concentrations (K^+ and/or Na^+) of 150 mM leads to destabilization of the chromatin structures of the nucleus. Also, the hydration of chromatin and hence, the volume of (membraneless) nuclei were sensitive to the monovalent ionic concentrations. Consequently, a mechanism within the nucleus of a living cell which could regulate the free K^+ and Na^+ concentration seems not only tenable but essential to cell homeostasis. We are proposing that the major constituents for this ion regulatory mechanism are the extractable proteins which, in the intact nucleus, are loosely bound to the nuclear matrix and to the chromatin structures.

Kinetics of Release of Potassium From Detergent "Opened" Cells

Three cell types (thymus lymphocytes, H-50, and chicken erythrocytes) were studied [7,36,38]. Thymus lymphocytes and chicken erythrocytes in suspension were exposed to Triton X-100 and Brij 58 non-ionic detergents. Above their critical micelle concentration both detergents disrupted or completely removed membrane lipids within 2 to 5 minutes. The release of potassium from Triton X-100 treated cells was immediate, following the removal of membrane lipids. (It should be noted that the removal of membrane lipids is concurrent with the disappearance of identifiable membrane structure as revealed by electron microscopy [38].) However, in case of Brij 58, the release of potassium did not follow the removal of membrane lipids. Rather it correlated with the mobilization of intracellular proteins and ATP [36,40]. The delayed release of potassium from Brij 58 "opened" cells was observed in all three cell types we examined. Considering the diffusion coefficient of K^+ in solution at room temperature to be of the order of 10^{-5} cm^2/sec, and the diameter of the cells to be 16 μM, then equilibration of potassium with external media should occur within 100 msec [9]. Thus, if cellular potassium were indeed free, its equilibration should have occurred within a second or so, and not requiring the several minutes we observed. Our findings of the delayed release of K^+ from detergent "opened" cells are consistent with others, indicating that K^+ ions are not freely dissolved in the water inside the living cell [15,20,48,54]; rather, they are in intimate contact with "energized" proteins. The concept of "energized" protein needs further justification, but in our view it indicates ATP induced high electron density sites on extended proteins associated to each other in a dynamic three dimensional matrix.

Conclusions

Although most biophysicists, biochemists, cell biologists, and cell physiologists seem to agree that the cytoplasm is not comprised of a simple solution of electrolytes, proteins, enzymes, and various metabolites, it is hard to find otherwise in current textbooks and even in journal articles. In print we continue to find reports viewing the cellular organelles as membranous sacs with soluble contents and that outside the organelles, crowded solutions of enzymes and their substrates exist that are thought to be interacting through random collisions as in free diffusion [52,60,68]. The fact that all reactions within the living cell proceed in a well defined order in time and space, requires, we believe, a revision of the current model of the cell. It is suggested that any new model of cell function must take into account the spatio-temporal order in general, and, in particular, the intimate association of all, or most of the cellular proteins.

There is now compelling evidence that proteins of the living cell are bound together in a three dimensional framework within the cell [9,48]. The observations of the *in vivo* coordination of multienzyme systems is one indication of the extent of such protein-protein associations. The extensive interconnecting three dimensional skeleton of the cyto- and nuclear matrices extending even into organelles and through the cell surface into the extracellular protein matrix can only be considered as evidence of far-reaching protein-protein interactions. The time delayed, but simultaneous, release of potassium, proteins, and ATP from permeabilized (detergent opened) cells provides circumstantial evidence for co-compartmentation of these ions and molecules.

We hypothesize that, in the three dimensional "continuous" protein phase, the individual proteins are in a higher energy state and their associations to each other dynamically "stabilize" the energy in the individual proteins and the free energy in the whole complex. We further propose that the higher energy state of the proteins is an unfolded or extended state; and, that cellular potassium is dynamically adsorbed to the proteinaceous matrix of cells in the resting state. Continuing with the hypothesis, we are proposing for non-muscle cells that the loosely associated proteins of the nucleus and the cytoplasm exist in an extended (or unfolded) state and provide the sites for selective adsorption. It is thought that the process of selectivity is energized by ATP. In the case of skeletal muscle, the site of the adsorption seems primarily to be the Z-line and the A-band (myosin) [15].

Evidence from quasi-elastic neutron scattering studies and the use of other physical techniques shows that, when compared to bulk water, the diffusive motion of cellular water is reduced. These findings, combined with those experiments which demonstrate solute exclusion in protein-water solutions [49], lend support to the idea of the orderly exclusion of solutes from the aqueous cytoplasm. This orderly solute exclusion, dependent on physical properties of each solute, along with selective electrostatic adsorption of ions determine the total cellular solute concentration in mature cells [43-49]. We are proposing further that during early postnatal development the water-macromolecular interaction induces less order and high concentrations of sodium can occur [25]. Finally, our proposed view of cell water may have been in Albert Szent-Györgyi's mind when he stated in 1972 that: "Sixty years of research has taught me to look upon water as part and parcel of the living machinery, if not the hub of life. Water is the most extraordinary substance! Practically all its properties are anomalous, which enabled life to use it as building material for its machinery. **Life is water dancing to the tune of solids**" [ref. 64, p 9].

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Discussion with Reviewers

J.S. Clegg: Have you attempted to explain the postnatal changes in the cellular potential on the basis of the electrogenic pump concept?
Authors: This subject has been given considerable thought and, we do not favor this possibility for the following reasons: (1) it has been amply demonstrated that the cellular potential is independent of the intracellular concentration of most ions [see last paragraph of section on Cellular potential]; (2) the preponderance of evidence is supportive of the interpretation that the bulk on the intracellular ions are adsorbed rather than freely dissolved in the cytoplasmic water; (3) if the *ad hoc* postulate of an electrogenic pump is plausible, then the energy problem referred to in the section on Changes in cellular sodium must be resolved; and, (4) the extra energy requirements of an electrogenic pump -- in full operation for 30 days -- will also require explanation.

J.S. Clegg: How much (i.e., relative %) of the total is free to diffuse? Are we talking all K^+ being adsorbed? Is there good evidence on this point? I think we need to know the Authors' position on this matter.
Authors: The first of your questions is difficult to answer because K^+ may jump from site to site and thereby diffuse [ref. 48 pg 243]. Likewise, K^+ may diffuse in the aqueous phase. You well know, because of exchange, in a given increment of time, the majority of the K^+ may be in solution or adsorbed. The practical side of this problem, how much is adsorbed, we think can be addressed. When one considers the work of Edelmann [ref. 15 and references therein] it seems very significant that the spatial distribution of potassium is neither homogeneous, nor in the aqueous phase, but localized to specific macromolecular structures within the sarcomere. Now, it seems to us, that if the majority of the K^+ were in solution we should see significant amounts in the interfilamentous spaces. Although this work is outstanding and to us quite compelling, the argument is qualitative and, by itself not convincing. Let us then consider our work [36] where as much as 70% of the K^+ is delayed in its release from permeabilized cells. As you have so aptly pointed out in your excellent review [9], equilibration of the cell interior is expected to occur in less than one second -- a 2-5 minute delay in K^+ release is consistent with the idea that the K^+ is adsorbed. Next, let us consider equations **(7)**, **(8)**, and **(9)** with K^+ substituted for S. If $[K^+]_{ex}$ (2.5 mM/L in the frog) is in equilibrium with the aqueous phase of the cell ($[K^+]_{int}$ in equation **(8)**) then 2.5 mM out of a total $\approx$ 100 mM would be present in the aqueous phase of the cell. Finally, from the studies in which the cooperative isotherms for potassium have been determined [ref. 48 pp 345-367] -- here $\approx$ 98% of the K^+ follows the isotherm for cooperative uptake. Now, these findings on frog sartorius muscle, coupled with Edelmanns' observations (which were made on the same muscle type)

become compelling to us that ≈ 98% of the cellular K^+ is adsorbed to proteinaceous structures within the cell.

J.S.Clegg: The idea of "extended" proteins is a central and critical matter for the association induction hypothesis, and the authors discussion in this paper, Thus, I believe it is reasonable for the authors to be a bit more specific about what "extended" means: thus, must the groups of all the peptide bonds be fully exposed to the surrounding environment? Must this happen for all peptide bonds simultaneously?

Authors: From our perspective, the data presented in this review are best explained by selective K^+ adsorption and a reduced solvency of the cellular water to most solutes in general and K^+ in particular. We know that globular proteins, in solution, do not exclude solutes to any degree [ref. 47 pg 174 Table 6.4]. These native globular proteins posses a high degree of α helical and β pleated sheet (partially extended) conformations which leaves primarily the amino acid side chains available to interact with the solutes and water. If the backbone of the amino acids were exposed to the solvent, then abundant charges would be available to interact with the solutes and water. One way to accomplish the latter is to denature proteins; and, when this is done, we find evidence for solute exclusion [see example for gelatin in ref. 47 pg 174 Table 6.4]. Furthermore, when polymers (and proteins) which cannot form a significant number of intra- or intermacromolecular H bonds, exclusion should occur. This expectation has been realized [see ref. 47 pg 174; 48].

Now, as to "how extended the proteins must be"; we do not know exactly! We postulate that they are in the fully extended state. Since, in frog muscle, the q-value for Na^+ is of the order of 0.1 (i.e., ≈ 90% of the water is not available for solvation), we would naturally expect a high degree of extension. This is expected because so much "backbone exposure" to the cellular water must be involved in the process of exclusion [for further discussion of this topic see ref. 47 pp 167-180; and, *Physiol. Chem. & Phys. & Med. NMR* 20: 293-317, 1988; 21: 19-44, 1989]. Also, the selective uptake of Na^+ by denatured (i.e., extended) hemoglobin has been demonstrated [*Physiol. Chem. & Phys. & Med. NMR* 16: 221-235, 1984]. Granted, direct evidence for protein extension is lacking; however, the indirect evidence for its occurrence is certainly sufficient to warrant further consideration. One might address the following question: if extended proteins are not the cause of the solute exclusion from the aqueous cytoplasm -- what is?

H.G. Hempling: The authors raise some interesting questions about energetics. Evidence from Ling's studies with gelatin and with native proteins indicates the necessity that the proteins be extended in order to organize water in multilayers. Is it not true however that much of the protein in non-muscle cells is not in that state? The authors would assign the energy of ATP to unfold these proteins, particularly those proteins loosely bound to DNA. How much energy is required? Is there enough from metabolism? I much prefer taking advantage of hydrogen-bonding and electrostatic forces to organize the water in the cell not so much to exclude ions as to limit the water's escaping tendency.

Having demonstrated many years ago that there was plenty of metabolic energy for the transport of potassium and extrusion of sodium against their electrochemical gradients in leucocytes, lymphocytes, and ascites tumor cells, I do not have difficulty explaining the asymmetric distribution of potassium and sodium. I am also ready to accept the existence of specific binding sites for potassium and sodium which the cells may use to regulate cell volume in conjunction with the regulation of ion channels within the membrane. It is the either/or, take it or leave it mentality to which I object. Fortunately, though they may not admit it, the authors, I sense, are equally ready to consider all possibilities.

Authors: Our evaluation of the usefulness of the Goldman-Hodgkin-Katz (GHK) equation is presented according to the procedure introduced by Zierler in 1959. In our attempt to use the GHK equation to explain the postnatal changes in cellular potentials and in electrolyte composition, we do not find the **clarity** and **exactness** to which you refer.

Even though we did not interpret our findings in terms of the Bradley adsorption isotherm, your dissatisfaction with our inability to provide the reader with quantitative equations for the organization of cellular water is well appreciated -- a frustration we all share. On the other hand, the "lumping" of the so called "osmotically inactive water" into one term we also find somewhat disconcerting. Using one parameter leads one to think of cellular water as "bound" or "free" -- thus the q-value for each solute should be identical: an expectation lacking experimental confirmation. To be more specific, the size rule discovered by Ling cannot easily be explained by the Boyle-Van't Hoff relation [*Physiol. Chem. & Phys. & Med. NMR* 20:293-307, 1988].

Concerning extended proteins, please see our response to Dr. Cleggs' third question above.

The ATP, in our conceptualization, serves to induce a conformational change in certain proteins such that the selective adsorption of K^+ will be favored. Thus, we expect that the ATP content of a cell to be correlated with the K^+ content of the cell -- such an observation has been reported [ref. 47 pp 363-366]. It would seem that the energy requirements (adsorption in nature) are met. Nevertheless, more studies are needed to further examine the relationship between ATP and K^+.

We respect your statement about the adequacy of the energy; however, without details it is impossible for us to evaluate. Certainly, your statement offers no nullification of the detailed studies of the energy available versus that needed made by Ling [42,45] for frog muscle nor those of Minkoff and Damadian [see "Caloric Catastrophe" *Biophys. J.* 13: 167-178, 1973] for

bacteria.

As we all know, a theory evolves from a set of assumptions and/or hypotheses. When the assumptions and hypotheses are called to question by careful experimentation, it seems prudent to look for alternative explanations. In our opinion, all of the assumptions and hypotheses that gave rise to the classical membrane theory are called into question. As always, however, we remain open to all possibilities.

G.N. Ling: **(1)** Another scientist who had played a key role in the formulation of the Membrane Theory (besides Bernstein) was W. Pfeiffer, who in his monograph, "Osmotische Untersuchungen: Studien zur Zell-Mechanik" introduced in 1877 the Membrane theory. But even then, the idea was not new. Indeed, Theodore Schwann had expressed the essence of the Membrane Theory in his "Microscopical Researches" in 1839 (see Thomas S. Hall, *Ideas of Life and Matter*. Vol. 2 pg 194, University of Chicago Press, Chicago, 1969). Bernstein's Membrane Theory came much later and seems confined to the specific aspect of the cellular resting and action potential. As far as I know he did not refer to either the work of Schwann nor of Pfeiffer. I believe that the first true synthesis of the various aspects of the Membrane theory was that of Boyle and Conway (*J. Physiol.* Vol 100 pg 1, 1940).

Having said what seemed to be historical facts we must also be more understanding. Pfeiffer was a plant physiologist and Bernstein was primarily a muscle physiologist. A great gulf had already developed between the two denominations.

(2) The initial proofs of the membrane permeability to Na^+ were due to Cohn and Cohn, [*Proc. Soc. Exp. Biol. Med.* 41: 445, 1939] and to Heppel [*Amer. J. Physiol.* 127: 385, 1939]. Both sets of experiments were conducted directly on the permeation of Na^+. Hodgkin and Huxley's work was on action potential, their theory of action potential was based on indirect stipulation that if the cell membrane is permeable to Na^+ then ...

Authors: We basically agree and thank you for clarifying these historical facts. We only add two comments: **(1)** We (particularly CFH) view Bernsteins' hypothesis as the first unified theory of cell function which offered an explanation of cellular potential and electrolyte distribution and a role for the membrane in these processes; and , **(2)** within the context of the Bernstein hypothesis the resting cellular potential was, upon stimulation, thought to "collapse" to zero -- Hodgkin and Huxley observed the reversal potential experimentally.

G.N. Ling: A more detailed perusal of the earlier literature will show that Bernstein took Ostwald's idea and developed it into the Bernstein hypothesis [ref. 48 pp 21-22 and references therein]. Furthermore, it is important to point out that MacDonald [*Proc. R. Soc. London* 67: 310-, 1900] had already demonstrated that in nerve, the demarcation current was sensitive to the concentration of potassium in the external media.

Authors: We are convinced that it is important to have the history of an idea illuminated. It helps us to see better how the "parts relate to the whole".