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THE PHYSICAL STATE OF POTASSIUM ION IN THE LIVING CELL

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Abstract

This review summarizes more than 30 years of experimental testing (and confirmation) of a key postulate of the association-induction (AI) hypothesis: most K^+ ions in resting cells are adsorbed on β- and γ-carboxyl group of cell proteins in a close-contact one ion-one site manner. Failure of healthy, cytoplasm-free, squid axon-membrane sacs to selectively accumulate K^+ over Na^+ and success of muscle cells without a functional cell membrane (and postulated pumps) to achieve the same, point to the cytoplasm as the seat of selective K^+ accumulation. Four independent techniques unanimously confirmed the predicted localization in striated muscle cells, of the bulk of cell K^+ in the A-bands where 65% of the β- and γ-carboxyl groups are located.

Strict adherence to the Langmuir adsorption isotherm in the equilibrium distribution of K^+ in muscle cells in the absence and presence of competing ions, proved one ion-one site, close contact adsorption of cell K^+ on anionic groups. The "effectively membrane-pumpless open-ended cell" (EMOC) technique, further helped to establish close contact adsorption of K^+ to take place in the cytoplasm rather than the cell membrane. A pK_a of 3.9 obtained by titration of the cytoplasmic anions groups and a sensitivity to specific carboxyl group-reagent, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide HCl combine to establish that the cytoplasmic anionic sites adsorbing K^+ are indeed β- and γ-carboxyl groups.

Key Words: K^+, cell, living cell, adsorption, proteins, β- and γ-carboxyl groups, myosin, pK_a, water, carbodiimide

Introduction

Early concepts on the physical state of K^+ in living cells

As a rule, living cells are rich in K^+ and poor in Na^+, even though they may spend their entire lives bathed in a medium poor in K^+ and rich in Na^+. For some time in the past, this asymmetry in the distribution of this pair of otherwise highly similar ions had been ascribed to an absolutely impermeable cell membrane to both ions (Lucke' and McCutcheon, 1932) or to only one (Na^+) (Boyle and Conway, 1941). Radioactive tracer and other studies in the late 1930's and early 40's disproved this explanation (Heppel, 1940; Steinbach, 1940).

The Na pump hypothesis, a variant of the pump concept that had been considered off and on in the past (Lillie, 1923), gained popularity. In this hypothesis[1], the high level of cell K^+ and low level of cell Na^+ are the net result of a balance between continual outward pumping of Na+ on the one hand, and on the other hand, the presence within the cells of trapped anions (e.g., ATP, creatine phosphate) (Boyle and Conway, 1941), which dictate the presence of an equal number of cations within the cell (or cell organelle) in order to maintain its macroscopic electric neutrality. K^+ is elected to serve this role and becomes accumulated in the cell.

My own research in this area led to the discovery that the postulated sodium pump would consume far too much energy than the cell can afford (Ling, 1951, 1952, 1962). Within the confine of the law

[1] Ad hoc in nature, the Na pump hypothesis is not a part of a self-consistent, coherent general theory of solute distribution in living cells. For want of a better alternative known to me, the mechanism for K^+ accumulation given here is a somewhat arbitrary "marriage" of the relevant part of Boyle and Conway's theory of solute distribution (Boyle and Conway, 1941) and the Na pump concept (Dean, 1941).

of thermodynamics, the sodium pump hypothesis is thus untenable (see Discussion in Ling, 1988, p. 101). Forced to abandon a theory which had been the guideline and foundation for all my prior work including my Ph.D. thesis, I started to look for alternative interpretations for the outstanding physiological phenomenon of selective accumulation of K^+ in living cells. I began to find out that I was not alone in making this kind of effort.

Between 1910 and 1940, a number of investigators had suggested that the accumulation of K^+ in living cells is the result of the existence of this ion in the cell in an indiffusible or unionized form (Moore et al, 1912; Neuschloss, 1926; Ernst and Scheffer, 1928; Fischer and Suer, 1938; see also Ling, 1984, p. 46). The idea of Benjamin Moore and his coworkers deserves special emphasis.

Alone (Moore, 1906) and with his coworkers (Moore et al., 1912, p. 113), Moore argued that K^+ is retained in living cells because intracellular proteins have affinity for K^+. Na^+ is not retained in cells because intracellular proteins do not have affinity for Na^+. Moore and his coworkers also pointed out how K^+ accumulation may share a similar mechanism that concentrates oxygen in red blood cells and that selective accumulation of K^+ over Na^+ is not unique to living systems; soil does the same.

However, early efforts to demonstrate selective K^+ adsorption on isolated proteins in aqueous solutions had not been successful (see Lillie, 1923, p.120). Later attempts using more sophisticated methods only affirmed earlier impression that the uptake of K^+ by isolated proteins is in amount too low and lacking in the specificity necessary to account for the selective accumulation of this ion observed in living cells (Lewis and Saroff, 1957).

It should be pointed out that up to this point in time, all theories of indiffusible or non-ionized K^+ proposed are qualitative and descriptive in nature. <u>It was not until 1952 that for the first time, a quantitative, mechanistic theory of selective K^+ accumulation over Na^+ was presented</u> (Ling, 1952, p. 764). Primarily designed to explain the phenomenon as observed in living cells, it was explicitly stated that the proposed mechanism also can explain selective accumulation of K^+ over Na^+ in inanimate "model systems", including soil as already pointed out by Moore and his coworkers, permutits, and especially man-made (sulfonate type of) cation-exchange resins[2] (Ling, 1952, p. 772).

The key concepts introduced in my 1952 model can be briefly summarized as follows:

(1) Selective adsorption of K^+ over Na^+ in living cells and model systems results from the greatly enhanced degree of association of countercations like K^+ and Na^+ with the β- and γ-carboxyl groups of cell proteins and the more favorable electrostatic attractive energy between negatively charged β- and γ-carboxyl groups and the <u>smaller</u> positively charged (hydrated) K^+ than between the carboxyl groups and the <u>larger</u> (hydrated) Na^+.

(2) Native proteins are not expected to selectively adsorb K^+ over Na^+, because among other reasons, their β- and γ-carboxyl groups are locked in "salt-linkages" (Speakman and Hirst, 1931, 1932) with fixed ϵ-amino groups (of lysine residues), guanidyl groups (of arginine residues) etc. belonging to the same or adjacent proteins. As a result, these carboxyl groups are "masked". In contrast, similar β- and γ-carboxyl groups in resting cells are available for selective K^+ adsorption, because the proteins involved exist in a different steric and electronic conformation. To maintain that required conformation, the adsorption of ATP (and other protein(s) and <u>congruous anion(s)</u>, see below) on the protein is essential. How ATP and similar agents serve this role will be discussed in more detail below.

This early model of selective K^+ accumulation over Na^+ was expanded eventually into a full-fledged theory of the living cell called the association-induction (AI) hypothesis (Ling, 1962). Part of this theory will be described next.

<u>The physical state of K^+, (and of water and proteins) in living cells according to the association-induction (AI) hypothesis</u>

Water, proteins and K^+ are the three major components of all living cells. According to the AI hypothesis, these components within a resting cell exist together in a closely associated, high-(negative)energy--low-entropy state, called the <u>living state</u>.

In the living state, K^+ ions are selectively adsorbed singly (e.g., over Na^+) on the β -and γ-carboxyl groups as mentioned above, while water molecules are

[2] Apparently unaware of my earlier work, Harris and Rice (1956) published four years later a theory of selective cation accumulation in ion exchange resin by essentially the same mechanism.

adsorbed in multilayers on the exposed NH and CO groups of certain fully-extended protein chains. Like K^+ and water, most if not all cell proteins may themselves be directly or indirectly adsorbed and anchored onto a matrix of cytoskeletal proteins.

Since living cells as a rule possess a large quantity of proteins, and proteins as a rule carry a large percentage of β- and γ-carboxyl groups, selective adsorption as suggested for K^+ offers the cell the potential capability of accumulating solutes like K^+, to levels in the cell much higher than that found in the cells' environment. On the other hand, the reduced solubility in the bulk of cell water, which exists in the state of polarized multilayers, provides a mechanism for the cell to keep large solutes (e.g., hydrated Na^+) at a level below that found in the cell's environment. Together, in different combinations, these two types of mechanisms can account for the diverse levels of solute accumulation observed without the need of continuous energy expenditure (see also Troshin for his important contribution to this field, 1951a,b; 1958; 1966).

There are many solutes that are found only in the free state in the cell water (Ling and Hu, 1988). There is no solute in a living cell that is exclusively found in the adsorbed state. Nonetheless, due to the strong preferential adsorption of K^+ on the cell protein, only less than 2% of the K^+ in frog muscle cells is in the free state, the remainder being all adsorbed.

Why do K^+ and Na^+ associate with carboxyl groups of proteins in living cells when it is known that K^+ and Na^+ do not associate with carboxyl groups of, say, K^+ acetate or Na^+ isobutyrate in dilute solutions?

Unless there is close-contact association between the cations and the carboxyl groups, there is no way for the cells, or indeed any other systems, to tell K^+ apart from Na^+. These two ions differ from each other only in their short-range attributes (e.g., polarizability, Born repulsion constant); differences in short range attributes cannot be "perceived" and recognized unless the ion and (a part of) the cell (e.g., β- and γ-carboxyl groups) are in close-contact association.

Since Arrhenius, we have learned that in a relatively dilute solution, K^+, or Na^+, does not associate with its oppositely charged ions, like Cl^- or small organic anions carrying carboxyl groups (e.g., acetate ion). With this in mind, the key question is, "Why should virtually all K^+ in the cell associate with its oppositely charged protein carboxyl groups in resting cells?"

Table 1. The Activity of Na+ in Aqueous Solution of Na^+ Isobutyrate and Na^+ Polyacrylate.

Concentration of Na^+	Activity of Na^+	Activity Coefficient
Isobutyric acid, $CH_3CHCOOHCH_3$		
0.2	0.186	0.93
0.1	0.090	0.90
0.05	0.049	0.98
0.025	0.025	1.00
0.0125	0.0122	0.98
Polyacrylic acid $(-CH_2CHCOOHCH_2-)_n$		
0.2	0.060	0.30
0.1	0.0315	0.315
0.05	0.0146	0.292
0.025	0.0058	0.232
0.0125	0.0021	0.168

(From Kern, 1948, by permission of Makromolekulare Chemie)

For a long time, full dissociation of K^+ and Na^+ from anions in solution had been used as a major argument against the concept of K^+ adsorption in cells. But this situation has changed. There is now a good explanation for this apparent inconsistency (see Ling,1984). To facilitate its presentation, I shall introduce a familiar example.

On a very cold but clear day, frozen laundry dries in the open air. Yet at the same temperature, the laundry will not dry in a small enclosed space. This difference in drying or not drying arises from the vastly greater free volume available to evaporated water molecules in the wide open space, not enjoyed by their counterparts in a more confined one. In other words, the entropy of dissociation of the water molecules in the open air is large enough to offset the strong attraction of the ice surface for the water molecules. Frozen ice then sublimates. In a smaller enclosed space, the entropy gain is not large enough to offset the attraction. Water then remains frozen.

Consider a dilute solution of say, NaCl or K acetate. The dissociated ions have access to the entire volume of the container.The dissociation of these ion pairs is virtually complete as a result of the large entropy gain accompanying the process. The upper part of Table 1, taken from Kern (1948), shows that this is also true of the salt, Na^+ isobutyrate; the activity coefficient of Na^+ measured in the solutions are close to unity. Cl^-, acetate and isobutyrate are all univalent anions. When dissociated, each ion carries

a unit electronic charge. The entropy gain (and other factors) are enough or more than enough to overcome the attraction between the cation and the anion.

Now if we join two carboxyl groups closely together as in a dicarboxylic acid, the electric field created by the negative charges of the two dissociated carboxyl groups will overlap. As a result, the electrostatic attractive energy of the carboxyl groups for the second dissociating cation is enhanced, as shown by the 1000 fold smaller second dissociation constant (K_2) than the first one (K_1) of oxalic acid (HOOC-COOH) ($K_1=5.9x10^{-2}$; $K_2=6.4x10^{-5}$).

When a much larger number of carboxyl groups are joined into a polyanionic macromolecule, we have what in the AI hypothesis is called a semi-fixed-charge system. Here the same physical principle seen in the dissociation of dicarboxylic acid, operates at a more intense level and begins to approach the principle underlying the law of macroscopic electroneutrality, according to which the total number of cations and anions in a macroscopic object must be equal (Guggenheim, 1950, p. 330). Therefore no matter how large the volume of solution in which the polyanionic macromolecule is dissolved, the dissociated Na^+ or K^+ must remain confined within the volume containing the fixed carboxyl groups. This volume is usually quite small,and it restricts the entropy gained on K^+ dissociation. As a result, the association of K^+ with fixed carboxyl groups of the polyanionic macromolecules is very much increased (Ling, 1960).

There is a second reason for the enhanced association of K^+ with the anionic groups of a polyanionic macromolecule. As mentioned above, water molecules around a model semi-fixed-charge system (or in a bona fide fixed charge system such as a living cell) tend to exist in the state of polarized multilayers (Ling, 1984, 1990). The solubility of large solutes like hydrated K^+ in this polarized water is, as a rule, lower than that in a dilute salt solution. The reduction of solubility of the dissociated K^+ in the polarized water further reduces the entropy gain accompanying K^+ dissociation, and provides a second reason for the enhancement of association of K^+ with fixed carboxyl groups of the anionic macromolecules (Ling 1984, p. 150).

In support of these theoretical reasonings, it has been repeatedly demonstrated that the degree of association of the fixed ions with their countercations greatly increases when monomeric anions (e.g., isobutyrate) are joined into a linear polyanionic macromolecule (i.e., polyacrylic acid) (Table 1) (Kern, 1948; Ling and Zhang, 1983; Ling, 1984, p. 148).

Why do isolated proteins in an aqueous solution adsorb little or no K^+ ?

Even though most proteins contain many β- and γ-carboxyl groups and hence have the potential of adsorbing many K^+ ions, in reality most isolated proteins adsorb little K^+ or other alkali-metal ions (Carr, 1956). As mentioned earlier, one main reason for this scanty adsorption is that most proteins also contain many fixed cations (e.g., ϵ-amino groups, guanidyl groups). At near neutral pH, a great majority of these fixed cations and fixed β- and γ-carboxyl groups form salt linkages. Although this explanation was offered in 1952 for the scanty adsorption of alkali-metal ions on isolated native proteins (Ling, 1952), its experimental verification came much later. In 1984, Ling and Zhang (1984) demonstrated stoichiometric adsorption of alkali-metal ion on all "unmasked" fixed carboxyl groups in alkali-denatured bovine hemoglobin, in which the positive electric charges of the competing fixed cations has been neutralized by OH^- ions at high pH. As a result, the alkali-denatured protein has become a bona fide polyanionic macromolecule just like polyacrylic acid (Table 1). Like polyacrylic acid, the alkali-denatured hemoglobin adsorbs Na^+ or K^+ on all its unmasked carboxyl groups on a close-contact one-ion--one-site basis (Figure 1).

What makes intracellular proteins behave toward K^+ more (but not exactly) like alkali-denatured proteins than like isolated native proteins?

There is abundant evidence that, like alkali-denatured proteins but unlike isolated proteins, intracellular proteins can adsorb K^+ and Na^+ (see below).

The explanation offered by the AI hypothesis for this phenomenon is that the cell proteins are under the influence of ATP (and some other as yet unidentified proteinaceous component also present within the cells and congruous anions, Ling 1990, Chapter 8). The next section discusses theoretical mechanism for the action of ATP and other cardinal adsorbents.

How does ATP (and other cardinal adsorbent) control protein conformation and selective K^+ adsorption?

If a horse-shoe magnet is brought into contact with one terminal nail in a chain of loosely tethered nails, this terminal nail will become magnetically polarized. This polarized nail will polarize its nearest-neighboring nail, which in turn will polarize the nail further downstream and the process may repeat itself a number of times. If surrounding the nails are randomly distributed iron filings, the magnetized nails will attract and polarize them also, causing them to assume a more ordered distribution pattern dictated by the location of the nails.

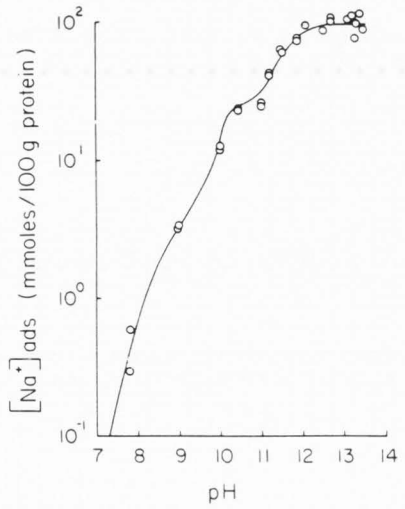

Figure 1. The adsorption of Na^+ by bovine hemoglobin at increasing pH. Data demonstrates the quantitative relation between fixed cations neutralized and Na^+ adsorbed. Points are experimentally measured concentrations of Na^+ adsorbed on 10% hemoglobin at different pH's. The solid line going through or near most of the experimental points indicates the number of fixed cations neutralized at different pH's and is calculated from the sum of the theoretical titration curves of all the α-amino groups, ε-amino groups, and guanidyl groups in 100 g of bovine hemoglobin [From Ling and Zhang, 1984, by permission of Physiol. Chem. Phys. & Med. NMR.].

In the AI hypothesis, ATP (as well as other cardinal adsorbents) behaves like the horse-shoe magnet; the cell proteins, like the chain of loosely tethered nails; and the K^+ (and water molecules) like the iron filings. Only here, instead of magnetic polarization, electrical polarization, also known as induction provides the key mechanism.

While the magnet-nail-iron filing analogy gives some insight into the theory, the theory is in fact more complicated. The interested reader is advised to consult other publications (Ling, 1984, p. 206; Ling 1990, Chapter 7 and 8). Suffice it to say that in the living system, the inductive effect serves a key role in an energy and information transfer mechanism that operates over long distances, a mechanism not unlike that underlying the propagation of a disturbance applied to one end of a chain of frictionless seesaws linked end-to-end with short pieces of string (Ling 1990, Chapter 7).

In another aspect, the cell model (according to the AI hypothesis) differs from the magnet-nail-iron filing model. A horse-shoe magnet can bring about only one type of action, while the effects of cardinal adsorbents fall into two categories: those that donate electrons to the system

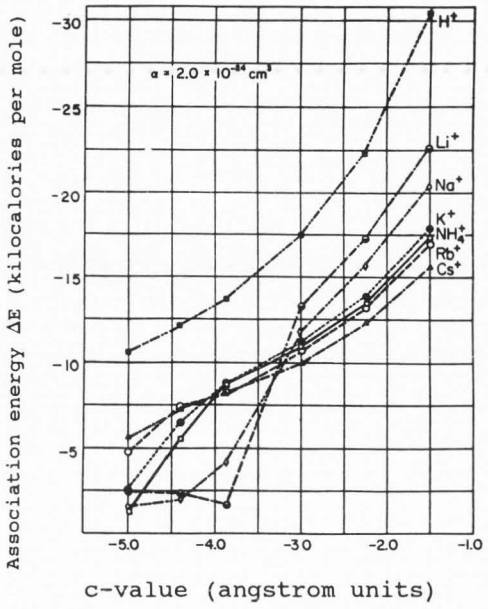

Figure 2. Relation between calculated association energy ΔE of various cations and c-value of the anionic oxyacid group. Polarizability of the anionic group is 2.0 x 10^{-24} cm^3 [From Ling, 1960, by permission of J. Gen. Physiol.].

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are called electron-donating cardinal adsorbent (EDC), and those that withdraw electrons from the system are called electron-withdrawing cardinal adsorbent (EWC). ATP, for example, is an EWC. Interaction with ATP tends to withdraw electrons away from β- and γ-carboxyl groups both near and far. How short-range inductive effect can offer the foundation for the propagated long-range effect is beyond the scope of this article to describe. Again the interested reader is recommended to read a larger document, especially Ling 1990, Chapter 6 and 7.

Theoretical computations led to results illustrated in Figure 2, which shows that an oxyacid group like a β- or γ-carboxyl group does not have a fixed preference for K^+ (over Na^+) as in my earlier model presented in 1952 and mentioned above. In that earlier model, each ion, K^+ or Na^+ is assumed to have a finite and unchanging hydrated ionic diameter. In the new model, the effective hydrated diameter for each ion is obtained by energy minimization, and it varies with (among other factors) the electron density of the anionic carboxyl groups with which it interacts. The electron density of anionic carboxyl groups is quantitatively described by a parameter called the c-value (shown as the abscissa in Figure 2) and is given in Ångstrom units.

As the c-value changes, the same carboxyl groups may prefer K^+ over Na^+ or vice versa. Even more important, is the fact that a third type of ubiquitous

cationic partner also exists in the form of fixed ϵ-amino groups and guanidyl groups. As mentioned earlier, if the carboxyl groups prefer these groups, salt linkages will be formed. This seems to be the case with most native globular proteins in a dilute aqueous solution, which adsorbs little K^+ or Na^+. Interaction with ATP [and a second proteinaceous auxiliary cardinal adsorbent and congruous anion(s) see Ling 1990, Chapter 8] in the living cell lowers the c-value of the carboxyl groups to such an extent that selective adsorption of K^+ over both Na^+ and fixed cations occur in consequence. This is the situation that prevails in normal resting cells according to the AI hypothesis.

In contrast, the removal of the competing fixed cations by alkaline pH leaves the carboxyl groups of a native protein, hemoglobin, at apparently a higher c-value. Hence the modest preference for Na^+ over K^+ as observed by Ling and Zhang (1984).

What was the major evidence that had led many at one time to the belief that cell K^+ is free in living cells? What further evidence have disproved them or made them equivocal?

There were three pieces of experimental evidence in support of the free-K^+ concept that played key roles in the broad acceptance of the free K^+ doctrine. These evidence along with their reevaluation in the light of more recent evidence are discussed next:

Evidence based on vapor-pressure measurement:

Urea was found to distribute equally between the cell water of frog muscle and extracellular water. This finding led A.V. Hill (1930) to conclude that water in living cells is simply normal liquid water. Frog muscle is in vapor pressure equilibrium with an isotonic NaCl solution. Since there is little question that the Na^+ and Cl^- in the NaCl solution are fully dissociated and since K^+ is the only solute within the cell at a high enough concentration to match that of the external free Na^+, Hill concluded that cell K^+ must also be free.

With the introduction and experimental verification of the polarized-multilayer theory of cell water and its subsidiary size-dependent, solute-exclusion theory, Hill's basic conceptual equation, that water which exhibits normal solubility for urea is simply normal liquid water, no longer holds. Thus solutions of (denatured) proteins and polymers, which like the living cells exhibit reduced solubilities for Na^+, sugars and other solutes, nonetheless exhibit normal solvency for urea (Ling et al, 1980; Ling and Hu, 1988).

Furthermore, free cell K^+ is no longer needed to balance the osmotic activity of external Na^+ because there are extensive evidence that cell water exists in the state of polarized multilayers (Ling 1988, 1990, Chapter 6), and polarized water itself exhibits strong osmotic activity (Ling, 1983).

Evidence based on K^+-mobility measurement:

The mobility of K^+ was measured in isolated squid axons, and found to be close to that of sea water, which contains primarily free alkali-metal ions (Hodgkin and Keynes, 1953). This finding was widely believed to have proven that cell K^+ is indeed free (Katz, 1966, p. 42). Unfortunately, though not in the least difficult to understand, Hodgkin and Keynes relied on the continued electrical excitability of the isolated axons as proof that the cytoplasm of the axons under study was healthy. Later work from Hodgkin's own (and other) laboratory showed that the health of the squid axon cytoplasm and the electrical excitability of the squid axon are not necessarily linked. Thus squid axons with all its cytoplasm removed (and perhaps flushed down the drain or otherwise destroyed) continue to conduct normal electrical impulses, when suitably stimulated.

Using muscle cells, Ling and Ochsenfeld (1973) were able to demonstrate comparably high mobility of K^+ in frog muscle cells (as Hodgkin and Keynes measured in isolated squid axons) but only when the muscle cells were deliberately killed. When the muscle cytoplasm remained healthy, the K^+ mobility measured was only 1/8 of that in a dilute KCl solution of similar ionic strength.

Evidence based on intracellular K^+-activity-coefficients measurements:

With the development of the K^+-selective intracellular microelectrode, the K^+ activity coefficients in the cytoplasm of squid axons and giant barnacle muscle fibers were measured and shown to be close to that a free KCl solution, providing another set of evidence in favor of the free-K^+ concept (Hinke, 1959, 1961; Lev, 1964).

However, with the intracellular K^+ activity of more and more varieties of cells measured, K^+ activity coefficients ranging from 0.27 (White 1976; Khuri et al 1974) to as high as 1.2 (Lee and Armstrong 1972; Palmer et al., 1978) have been reported, all by careful, experienced and skillful workers. This striking heterogeneity of the K^+ activity coefficients measured contradicts the theory of free cell K^+, which predicts essentially the same activity coefficient of K^+ in all types of cells. An in depth analyses revealed that such heterogeneity is precisely what one would have expected if

different types of cell cytoplasm possess different degrees of vulnerability to the intruding electrodes, which may be "microscopic" to the human eyes and called "microelectrodes", but are in fact, ramrods of enormous dimensions to the truly microscopic and delicate protoplasm being studied (Ling, 1984, p. 252 to 257).

Evidence in support of the theory that K^+ accumulation in living cells is due to its selective adsorption on cytoplasmic protein sites

A variety of experimental evidence exists in support of the theory that K^+ accumulation in living cells results from selective adsorption on cell proteins (see, for example, Ling, 1984, Chapter 8; Ling 1990, Chapter 4). Only some of these evidence will be discussed here in the section immediately following and other sections further beyond.

Selective K^+ accumulation against concentration gradients does not occur in squid axon membrane sacs with perfectly normal cell membrane, energy sources but no cytoplasm (Ling, 1965b, footnote on p. 95; Ling and Negendank. 1980, p. 222). In contrast, K^+ accumulation persists in frog muscle cells effectively without a functional cell membrane and postulated pumps but with intact cytoplasm (Figure 3) (see below for details).

Selective reaccumulation of K^+ can occur in human red blood cells after lysed cells have been resealed in the presence of ATP, if and only if the ghosts retain a significant amount of hemoglobin (and one or more additional as yet unidentified non-hemoglobin protein originally present in the cell) and therefore remain red or pink. White ghosts with intact cell membranes and normal membrane K,Na-activated ATPase (widely believed by supporters of the membrane pump theory to be a key part or the entirety of the postulated sodium pump) do not reaccumulate K^+. Indeed, pink and red ghosts reaccumulated K^+ in concentration, directly proportional to the concentration of residual hemoglobin in the ghost (Ling et al, 1984).

Since hemoglobin makes up 97% of the cytoplasmic proteins of red cells, it is hypothesized that hemoglobin provides most if not all the β- and γ-carboxyl groups adsorbing K^+. The observed rectilinear relationship between K^+ reaccumulated and the residual hemoglobin content of the ghosts offers direct confirmation.

Evidence of localized distribution of K^+ in voluntary muscle cells

In voluntary muscle cells, major cytoplasmic proteins occupy prominently different cytological locations. Thus myosin is found only in the A-bands, while

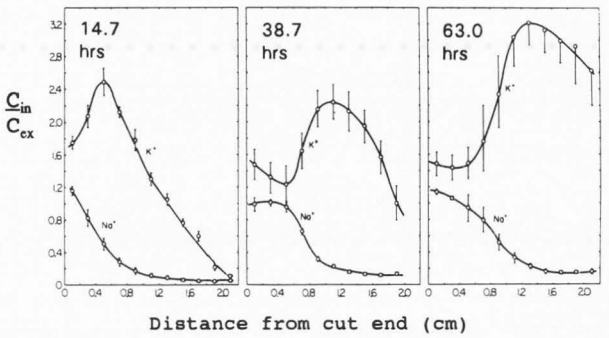

Figure 3. The simultaneous influx of labelled K^+ and labelled Na^+ into sartorius muscles through their cut ends. The three groups of frog sartorius EMOC preparations were exposed to normal Ringer solutions labelled with both ^{42}K sand ^{22}Na for 14.7, 38.7 and 63.0 hours, respectively. The abscissa represents the distance of the midpoint of each cut segment from the cut surface of the muscle fibres. The ordinate represents the ratio of the labelled ion concentrations in the water of each muscle segment (C_{in}) over the concentration of the same labelled ion in the solution bathing the cut end of the muscle at the conclusion of the experiment (C_{ex}). Each point was the average of 4 (14.7hr), 10 (38.7 hr) and 4 (63.0 hrs) experiments respectively, the distance between the two longitudinal bars being twice the standard error. [From Ling, 1978, by permission of J. Physiol.].

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actin is found mostly in the I-bands. Since myosin carries some 66% of the muscle cell's β- and γ-carboxyl groups (Ling and Ochsenfeld, 1966), the AI hypothesis predicts that K^+ adsorption should occur on myosin and hence in the A bands. The concentration of K^+ and its surrogates, Cs^+, Tl^+ etc., at the A bands has been confirmed by a variety of methods, including autoradiography (Ling, 1977b; Edelmann, 1980b), transmission electron microscopy (Edelmann, 1977, 1984, 1986), energy-dispersive X-ray microanalysis (Edelmann, 1978; Trombitas and Tigyi-Sebes, 1979; Edelmann, 1983), and laser mass-spectrometer microanalysis (Edelmann, 1980a, 1981). Unanimously they show that K^+ and other ions like K^+ are adsorbed mostly on specific proteins located in the A bands as predicted by the AI hypothesis (Figure 4 and 5).

Evidence that K^+ ions are associated with protein sites in a close contact, one ion-one site adsorption

The demonstrated localized distribution of K^+ in voluntary muscle cells may be interpreted in two ways: K^+ hovers around the anionic protein sites as a part

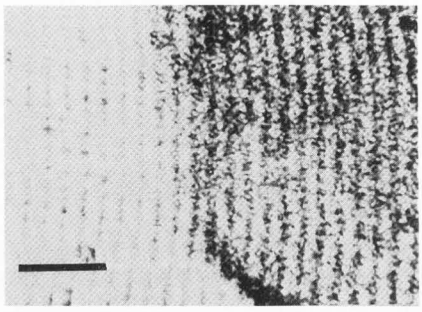

Figure 4. Autoradiograph of air-dried single frog muscle fiber, previously loaded with ^{14}Cs-labeled Cs^+ while the muscles were alive. Muscle fibers were partially covered with photoemulsion. Note: silver granules are seen primarily at loci corresponding to the dark or A bands [From Ling, 1977, by permission of Physiol. Chem. Phys.].

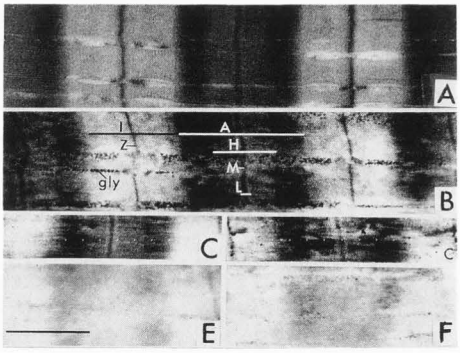

Figure 5. Electron micrographs of frog sartorius muscle. (A) Muscle fixed in glutaraldehyde only and stained only with uranium by conventional procedure. (B) EM of section of freeze-dried Cs^+ -loaded muscle, without chemical fixation or staining. (C) Tl^+ loaded muscle without chemical fixation or staining. (D) Same as (C) after exposure of section to moist air, which causes the hitherto even distribution of thallium to form granular deposits in the A band and Z-line. (E) Section of central portion of (B) after leaching in distilled water. (F) Normal "K^+-loaded" muscle. Scale bar: 1 μm [(A) from Edelmann, unpublished. (B to F) from Edelmann, 1977 by permission of Physiol. Chem. Phys.]

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of a countercation cloud; K^+ is adsorbed on the protein site in a <u>one-on-one, close-contact adsorption</u>.

This uncertainty was resolved by the demonstration (1) that the accumulation of radioactively labelled K^+ in muscle (and other) cells is quantitatively inhibited by the presence of other alkali-metal ions in strict obedience to the <u>Langmuir</u>

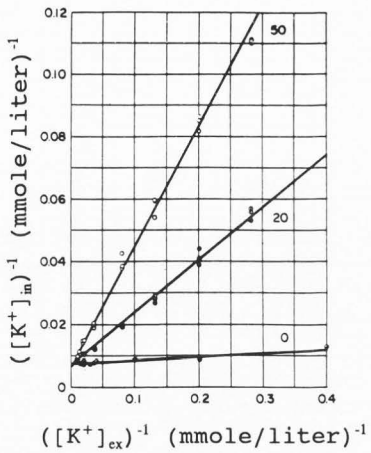

Figure 6. Equilibrium distribution of labelled K^+ in the presence of 0, 20, and 50 mM non-labelled K^+ as competing ion.

Isolated frog sartorius muscles were incubated in sterile modified Ringer solutions containing labelled ^{42}K until equilibrium which took less than 20 hours at 24°C. Intracellular labelled K^+ concentration plotted reciprocally against the external labelled K^+ concentration in a single frog sartorius muscle; line obtained by the method of least squares. The convergence of the 3 sets of straight lines on the same locus on the ordinates indicates full obedience to the Langmuir adsorption isotherm which was derived from the fundamental assumption that each adsorption sites is occupied by no more than one ion at a time [From Ling and Ochsenfeld, 1966 by permission of J. Gen. Physiol.]

<u>adsorption isotherm (Figure 6), which was derived on the basis that each site adsorbs only one ion at any one time</u>; and (2) that the ability of an equimolar concentration of <u>two</u> different alkali-metal ions (K^+ and Cs^+) to reduce the level of radioactively labelled K^+ in frog muscle cells are quite different (Figure 7). Since as part of a countercation cloud, the effects of two monovalent cations are identical, and since (as pointed out earlier in the case of K^+ and Na^+) the only difference between K^+ and Cs^+ lies in their short-range attributes (e.g., Born repulsion constant, polarizability) which cannot be "felt" unless the site interacting with the ions are in close contact, the different power of K^+ and Cs^+ to displace labelled K^+ established that the cell K^+ is adsorbed in a close-contact manner.

<u>Evidence that K^+ adsorption occurs on cytoplasmic sites rather than on membrane sites</u>

Having established the one-on-one,

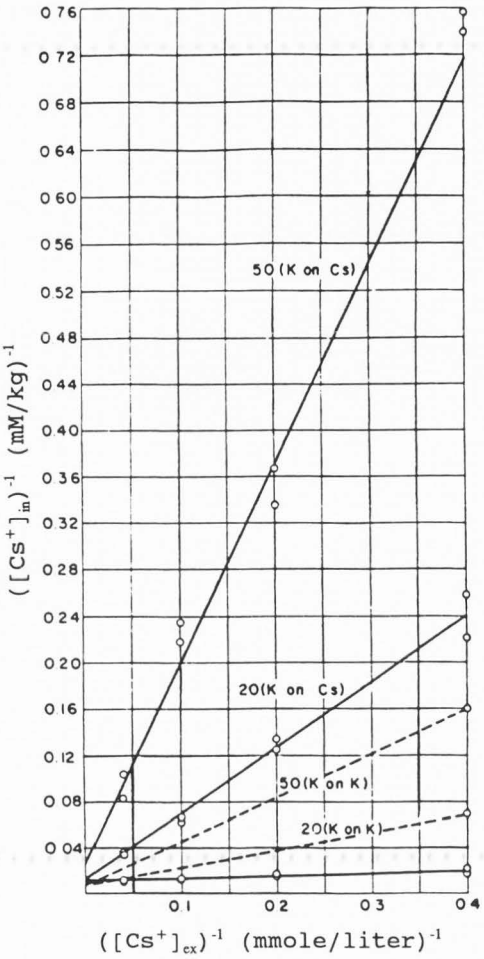

Figure 7. Equilibrium labelled Cs^+ concentration in muscle cells plotted reciprocally against the external labelled Cs^+ concentration, with which it is in equilibrium. Competing K^+ concentrations are 0, 20 and 50 mM respectively. Both Cs^+ and K^+ were in the form of acetate (24°C). Each point represents a single determination on one frog sartorius muscle; lines obtained by the method of least squares. The effect of K^+ ion on the accumulation of labelled K^+ ion (dashed) taken from Figure 6 for comparison. Note that the same concentration of K^+ exercises a much greater effect on suppressing the accumulation of labelled Cs^+ than on labelled K^+. [From Ling and Ochsenfeld, 1966, by permission of J. Gen. Physiol.]

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close-contact adsorption, I shall now demonstrate that the protein providing these adsorption sites are not on the cell membrane but are on the intracellular proteins. In this effort I rely on a technique already briefly mentioned above: the effectively membrane(pump)less open-ended cell (EMOC) muscle preparation. In this preparation, part of the cell membrane is

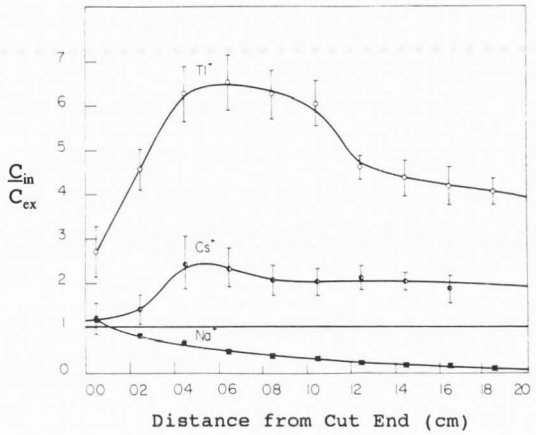

Figure 8. Simultaneous accumulation of labelled Na^+, Cs^+ and Tl^+ in the same frog sartorius muscle EMOC preparations with the open ends which alone were exposed to the Ringer solution containing these labelled ions.

Source solution contained 1 mM ^{204}Tl-labelled Tl^+. 1 mM ^{131}Cs-labelled Cs^+, and 100 mM ^{24}Na-labelled Na^+. Ordinate represent ionic concentration in cell water divided by the final concentration of the same ion in the source solution bathing the cut-end at the conclusion of the experiment. Abscissa represent distance form the cut ends. Incubation was for 3 days at 25°C. Data indicates selectivity for $Tl^+ > Cs^+ > Na^+$ quite similar to that seen in normal muscles. [From Ling, 1977a, by permission of Physiol. Chem. Phys.].

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amputated and only the bare cytoplasm at the cut end exposed to a Ringer solution containing the radioactively labelled ions. The remaining intact portion of the cell membrane (and postulated pumps) are made nonfunctional by being suspended in air or vaseline, which cannot serve as the "source" for the inward K^+ pump nor "sink" for the outward Na^+ pump. Yet in the EMOC preparation, the relative selectivity for different alkali-metal ions and Tl^+ remains the same as in normal cells (Figure 8). This finding shows that the seat of ionic selectivity lies in the cytoplasm and not in the cell membrane.

Evidence that the cytoplasmic protein sites that adsorb K^+ in a one-on-one close contact manner are mostly, if not all, β- and γ-carboxyl groups

I will discuss two types of experimental evidence that support the concept that it is the β- and γ-carboxyl groups of intracellular proteins that adsorb K^+ in a one-on-one close contact manner:

A frog sartorius muscle usually contains some one thousand muscle cells each measuring approximately 3 cm long and some

60 μm in diameter. Each muscle cell goes from one end of the muscle to the other without interruption. Razor-blade cuts of the muscle create a population of 2 mm long muscle segments with both ends open. The cut ends do not regenerate new cell membranes (Ling, 1978; Cameron, 1988).

In an ordinary frog's Ringer solution, these muscle segments deteriorate rather rapidly. After many years' searching, I finally discovered an additive (polyethylene glycol, PEG 8000), that partially preserves the cell's ability of maintaining selective ionic accumulation for at least 6 hours (Ling 1989). In work as yet unpublished, Ling and Ochsenfeld demonstrated that the amount of labelled alkali-metal ion taken up by the segments in the presence of the additive depends on the pH of the medium. The concentration of the labelled ion decreases sharply as the pH falls to around 4 (Figure 9). The pH of the inflection point of the titration curve is 3.9, which is characteristic of protein carboxyl groups.

The main component of the interaction between the negatively charged carboxyl groups and positively charged K^+ is electrostatic in nature. Removal of the negative charges of the carboxyl group is therefore expected to remove the ability of the carboxyl groups to adsorb K^+.

Chemical reagents that are known to alter or remove carboxyl groups from proteins have been used by protein chemists for a long time. It is only in recent times that reagents which combine a high degree of specificity with mildness of reaction have been discovered. Among them is the water soluble carbodiimides, which interacts with the protein carboxyl groups, producing a modified carboxyl group that no longer bears a negative electric charge (Hoare and Koshland, 1967). In work very recently finished, Ling and Ochsenfeld (1989) provided further evidence that the sites adsorbing K^+ and other alkali-metal ions are indeed carboxyl groups, when they demonstrated that exposure of the cut muscle segments to the water soluble carbodiimide, EDC [1-ethyl-3-(3-dimethylamino propyl) carbodiimide] greatly reduces the concentration of alkali-metal ion taken up by the cut muscle segments at pH above the pK_a of the carboxyl groups but not below it. This discrimination follows from the fact that only at pH above the pK_a of carboxyl groups do these groups adsorb alkali-metal ions. At pH below the pK_a of the carboxyl groups, alkali-metal ions are found only in the cell water and therefore indifferent to carboxyl reagents like EDC (Table 2).

There are 3 types of carboxyl groups carried on proteins: α-carboxyl groups on C-terminal amino acid residues; β-carboxyl groups carried on aspartic acid side chains and γ-carboxyl groups carried on glutamic acid residues. Neither the pK_a values determined, nor the carboxyl reagents like EDC can tell us precisely

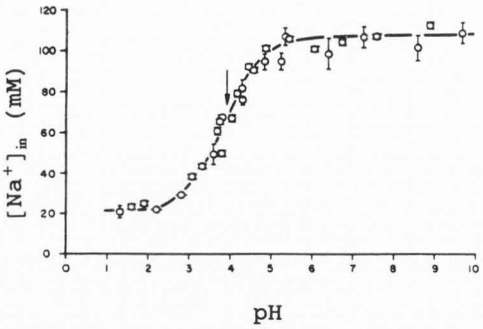

Figure 9. Effect of pH on the accumulation of labelled Na^+ in 2 mm long muscle cell segments with both ends open. Bathing solution contained 16.7 mM labelled Na^+ and 2.5 mM K^+. [From Ling and Ochsenfeld, to be published].

Table 2. The Effect of Carboxyl Reagent, EDC on the Uptake of Labelled Na^+ by 2 mm long, open-ended frog Muscle Segments

	pH	[Na*] (μmoles/g final wet weight)
control	2.4	12.5 ± 0.22 (n=4)
	8.0	55.6 ± 0.87 (n=4)
	11.0	57.2 ± 0.95 (n=4)
EDC	2.3	10.7 ± 0.14 (n=4)
	10.5	29.0 ± 0.58 (n=4)
	11.0	37.0 ± 0.90 (n=4)

Frog sartorius muscles were cut into 2 mm long segments with both ends open. Joined together by fascia along the edges of the muscle segments, each band of 5 or 6 segments were handled as a unit. The control segments were preincubated for 2 hours at 4°C in a medium containing 16.7% polyethylene glycol M.W. 8000 (PEG 8000) 2.5 mM KCl, 9.45 mM Na_2SO_4, while the experimental segments were incubated in a similar solution containing in addition 100 mM EDC (see text). Both media were kept at approximately pH 4 by the addition of H_2SO_4. Both control and experimental segments were rinsed and transferred to another PEG-Ringer solution containing radioactive ^{22}Na. After 135 minutes of incubation at 0°C, the segments were taken out, blotted dry, weighted and analyzed for the contents of labelled Na and water.

which is which. However, it is known that the protein carboxyl groups in muscle cell proteins are virtually all β- and γ-carboxyl groups (Bailey, 1948; Mihalyi, 1950; Kominz et al, 1954). The high concentrations of carboxyl groups revealed by acid titration (Figure 9) as well as by EDC (Table 2) leave no doubt that what these carboxyl groups are overwhelmingly β- and γ-carboxyl groups.

Conclusion

Scientific research of the past has enabled physicists to penetrate the deepest mystery of the inanimate world, engineers to land Man on the Moon and geneticists to successfully decipher the genetic code. The dazzling achievements in these and other fields of science might have blurred the perception of the very primitive condition of one area of science that holds great promise for the future welfare of mankind: the science of cell physiology.

To underscore the weakness of our understanding of cell physiology, one only needs be reminded of the fact that to this day there does not exist a single drug that was created from our understanding how it works or why it works--understanding that can only come with the maturity of the science of cell physiology.

The living cell is the basic unit of all life.. Cell physiology is therefore the scientific foundation for all biomedical investigations. The majority of cell physiological phenomena can be put under one of the four classical subjects of cell physiology: (1) solute distribution; (2) solute permeability; (3) cell volume regulation and (4) cellular electrical potentials. Three of these four subjects (1,3 and 4), as taught in textbooks, are built upon the now disproved assumption that cell K^+ is free[3].

Besides adding another major piece of experimental evidence for the AI hypothesis and against the membrane-pump theory, the demonstration that the bulk of cell K^+ is adsorbed is important in two other ways:

(1) In conjunction with the demonstration of the adsorbed state of the bulk of cell water (Ling, 1984, 1988), the finding completes the establishment of the existence of the three major components of the living cell in a closely linked, and functionally coherent association. The coherence of the major components of the living cell brings back to life an ancient concept: the existence of a living matter, the protoplasm.

Only protoplasm in the present light is not just a substance. It is more than just a substance. It is a substance (composed of protein-water and K^+), existing in a high-(negative)-energy, low-entropy living state. To maintain this state, the adsorption of the end product of cell metabolism, ATP [as well as a second as yet unidentified proteinaceous cardinal adsorbent and congruous anion(s)] is essential.

(2) The demonstration of the adsorbed state of cell K^+ endows the cell with a "base line" from where to launch into an active state and to return to afterwards. Such reversible functional activities are what many living cells are engaged in from the moment it is created until it dies. To stress the importance of adsorbed (and desorbed) K^+ in such functional activities, I have presented another review in this symposium on such a functional activity: active solute transport across epithelial membranes.

In summary, "free K^+ or adsorbed K^+" has far more significance than the specific knowledge about the physical state of this specific ion in the living cell; rather, the choice here is a critical one from a broader and longer perspective. It is as if it were, that a great deal of future real and effective progress in biomedical research hangs on the intactness of a short chain, one link of which is the correct and precise knowledge of the physical state of K^+ in the living cells.

[3] Disproof of the free K^+ doctrine is but one of the many decisive experimental evidence against this hypothesis (see below, and also Ling, 1984, 1988, 1990).

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Discussion with Reviewers

L. Edelmann: I have shown that proteins in sections of freeze-dried and embedded and even of freeze-substituted and low temperature embedded muscles can be "stained" by a solution containing 10 mM of the electron-dense Cs^+ ions indicating that the binding capacity of proteins can be captured in an embedded preparation (Edelmann 1989, "The Physical State of Potassium in Frog Skeletal Muscle Studied by Ion-sensitive microelectrodes and by electron microscopy: Interpretation of Seemingly Incompatible Results. Scanning Microscopy, 3, 1219). Preliminary unpublished experiments show that at pH 4(c_{H+} =0.1 mM) staining with Cs^+ and therefore Cs^+ binding (or adsorption) is greatly reduced. The result is in harmony with your finding shown in Fig. 9 and may be explained by a very high preferential adsorption of alkali-metal ions in proteins compared to that of H^+ ions. Is such a high preference in ion adsorption predicted by your model of ion association at fixed charges of proteins?

Author: The answer is "Yes". However, before elaborating on this answer, I want to call attention to the historical significance of Dr. Edelmann's success in capturing the living state in naked, isolated cytoplasm (as defined in the AI hypothesis, see Ling 1962, p.xxii; 1984, p. 147; 1990, Chapter 3). He achieved this in thin sections of frog muscle prepared with his new freeze-drying-embedding technique (Edelmann 1980a), as evidenced by the ability of these thin sections to selectively accumulate *in vitro* K^+ over Na^+ at a ratio comparable to that seem in living cells.

I myself have spent much efforts in attempting to demonstrate selective accumulation of K^+ over Na^+ in bare cell cytoplasm. Obviously, we were by no means alone in this effort.

Although eventually I discovered the preserving function of polyethylene glycol (PEG 8000) (see Figure 9 and Ling 1989), and that the preserved muscle cytoplasm retained its ability of accumulating alkali-metal ions, the selectivity ratio in the adsorption of K^+ over Na^+ is greatly diminished (Ling and Ochsenfeld, to be published).

Returning to your question, I want to point out that for the selective adsorption of five alkali-metal ions (Cs^+, Rb^+, K^+, Na^+ and Li^+) in addition to H^+ and NH_4^+, I introduced three theoretical models, differing from one another in the choice of the polarizability of the carboxyl groups. Being the first one computed, the model with the lowest polarizability of 0.876×10^{-24} cm^3 was also the first one displayed (see Figure 3 in Ling 1960) even before my 1962 monograph was published, where all three models were presented. Years later Dr. D. Reichenberg of the National Physical Laboratory of Middlessex, England during his visit to my laboratory in Philadelphia brought my attention to the fact that the polarizability of the carboxyl groups is considerably higher than 0.876×10^{-24} cm^3. As a result, I focussed my attention to the model I calculated with the much higher polarizability of 2.0×10^{-24} cm^3 and had used this model ever since whenever the details of the theoretical ionic selectivity values of carboxyl groups in living cells and carboxyl group-carrying models were discussed (see Ling and Bohr 1971, p.580; Ling and Fu, Physiol. Chem. Phys. & Med. NMR 20:61, 1988). (However, on a number of other occasions when the details of the computed values for carboxyl groups were not involved, the old figure with the lower polarizability rather than the correct one for carboxyl groups with the higher permeability has been cited).

As Figure 2 (with the higher polarizability) shows, the carboxyl groups have much higher preference for H^+ than for K^+, as well as the other alkali-metal ions and NH_4^+. Indeed, the preference ratio in the adsorption of H^+/K^+ has been estimated for the β- and γ-carboxyl groups at the muscle cell surface to be about 150, a value that is very close to the measured H^+/K^+ preference ratio of the carboxyl groups on the surface of oxidized collodion-coated glass electrodes (Ling 1990).

In summary, the AI hypothesis predicts theoretically a high H^+/K^+ selec-

tivity in the adsorption on carboxyl groups on proteins as well as carbxyl group-carrying model systems studied. Dr. Edelmann's new finding of the high sensitivity of Cs^+ adsorption to pH in his ultra-thin muscle section, maintained at the living state, further supports the notion that β- and γ-carboxyl groups belonging mostly to myosin (see Ling 1990) selectively adsorb K^+ over Na^+ when the muscle cells are maintained at its high-(negative)-energy-low-entropy living state.

I. Cameron: What is the concentration of EDC used as mentioned in Table 2?
Author: The concentration of EDC used in the experiment described in Table 2 is 100 mM. This is the standard concentration originally used by Hoare and Koshland (1967) and by many other investigators thereafter (e.g., Mozhayeva et al., Pfluegers Arch. 406: 31, 1986).

I. Cameron: What is the "additive" and the concentration of the additive used to partially preserve the muscle cell's ability to maintain selective ion accumulation for at least 6 hours? Have you tested this additive to see if it influences the pK_a of simpler and well known zwitterions?
Author: The "additive" is polyethylene glycol, M.W. 8000 (PEG 8000, Carbowax, Fisher Scientific.)(see Ling, Physiol. Chem. Phys. & Med. NMR, 21: 13, 1989). It was used at a concentration of 16.5% (w/v). At this concentration, an aqueous solution of PEG 8000 exhibits the same osmotic activity as that of an isotonic 0.118 M NaCl solution. We have not tested the effect of PEG 8000 on the pK_a of well-known simple Zwitterions. We have, however, tested on a well known simple carboxylic acid, acetic acid. The presence of 16.5% PEG 8000 increased the pK_a of acetic acid by about 0.1 pH unit (24°C).

I.Cameron: Is it not possible that K^+ within a cell might also be selectively adsorbed directly to: ATP, CrP or to fixed phosphate groups on proteins or nucleic acids?
Author:In the first theoretical quantitative model of selective K^+ adsorption (over Na^+) (Ling, 1952, p.770) as well as the later more complex model (Ling, 1960, 1962), the anionic groups adsorbing the monovalent ions considered have always been (monovalent) oxyacids. Since both carboxyl and phosphate groups are oxyacids, clearly there is no fundamental theoretical reason for rejecting offhand the phosphate groups as the sites of selective alkali-metal ion adsorption.

Direct selective binding of K^+ on free ATP and CrP ions (phosphocreatine), like the association of K^+ with other monomeric small anions in dilute aqueous solutions, is not expected to be extensive. My vague recollection is that K^+ and Na^+ binding on ATP were reported and the results were less than exciting. The extremely strong adsorption of ATP on myosin (see answer to your fourth question) and the localized distribution of CrP demonstrated in frog muscle (Hill, J. Physiol. 164: 31, 1962) suggest that neither ATP nor CrP exist as free ions in the cell. Therefore, the question of K^+ and Na^+ binding on free ATP and CrP may be only academic.

The situation becomes different if the ATP and CrP are adsorbed on a monovalent fixed cation of a cell protein. Under this condition, the degree of counterion association is greatly increased (see Section on "Why do K^+ and Na^+ associate with carboxyl groups ..." in the text). Indeed, in the 1952 article just mentioned a diagram (Figure 8) illustrating the selective adsorption of K^+ on ATP and other multivalent anions, which are themselves adsorbed on cell proteins. Several new experimental findings, to be described next, led me to reject this concept later.

Uniformity of K^+ Adsorption in One Cell Type: One of the new findings is the great uniformity in the quantitative aspects of the selective accumulation of K^+ in frog muscles. All the K^+ in frog muscles is described by a simple Langmuir adsorption isotherm with a single adsorption constant of 665 $(mole/liter)^{-1}$ on adsorption sites totalling 143 mmoles per kilogram of fresh weight (Ling and Ochsenfeld, J. Gen. Physiol. 49: 819, 1966). The great uniformity in the K^+-adsorbing properties of such a high concentration of anionic adsorption sites does not jibe with the diversity of a heterogeneous groups of K^+ adsorbing sites including the phosphate groups of ATP, and of CrP and the carboxyl groups of cell proteins.

Uniformity of K^+ Adsorption among Different Cell Types: In frog muscle, the total concentration of the phosphates roughly matches that of cell K^+ (see Ling, 1952, Table 3). In years following the publication of my 1952 paper, selective K^+ adsorption in various other cell types were reported. These cells, in contract to frog muscle, as a rule do not contain a high concentration of organic phosphates. Instead, the anionic counterpart of cell K^+ may be chloride (as in human red blood cells), glutamate (as in guinea pig brain and retina cells) and isethionic acid ($HOCH_2CH_2SO_3H$)(as in squid axons). Being monovalent, neither the chloride ion, nor the isethionate ion can adsorb onto the protein fixed cationic sites and still retain a net anionic charge for adsorbing K^+.

Finally the abundant phosphate groups in the nucleic acids were examined. In fact, the work of Negendank and Schaller (J. Cell. Physiol. 98: 95, 1979) shows that the adsorption of K^+ (and of Na^+) in nucleic acid-rich human lymphocytes are not significantly different from that in nucleic acid-poor cells like muscle cells. Thus one suspects that most if not all nucleic acid phosphate groups in the resting lymphocytes are locked in salt linkages with fixed cationic side chains of histones and other nuclear proteins and are unavailable for K^+ (and Na^+) adsorption.

Thus, as shown in Table 11.4 of Ling (1984), there is also a striking degree of uniformity in the adsorption of K^+ amongst different cell types, including those with high phosphate contents, and those with low phosphate contents.

In summary, only the β- and γ-carboxyl groups of cell proteins have the pK_a observed, are ubiquitous enough, are present in all cells at high enough concentration and possess the required degree of uniformity to function as the near-universal anionic sites for the adsorption of K^+ in nearly all living cells.

The one known exception are the halophilic bacteria which can selectively accumulate K^+ to a concentration as high as 5 M in an external medium containing 3.5 M NaCl. A K^+ concentration this high is far in excess of the concentration of β- and γ-carboxyl groups that are in the cells. Ginzburg et al. ruled out a pumping mechanism and suggested that the cell K^+ must be bound, but for reason not given, they made no reference to the AI hypothesis (J. Membr. Biol. 5: 78, 1971).

Later I suggested that under these specific circumstances the vastly more numerous backbone carbonyl groups might offer the additional needed sites for the selective K^+ adsorption in halophilic bacteria and had cited experimental evidence in support of this idea (Ling, Molec. Cell Biochem. 15: 159, 1977, p. 162). For a later, full discussion of this problem, see Ling (1990) endnote 8 of Chapter 4.

While the phosphates in muscle tissues are not believed to offer their anionic charge(s) for the adsorption of K^+, these phosphates groups like Cl in human erythrocytes, glutamate in guinea pig brain and retina, isethionic acid in squid axons, nonetheless play an indirect role in the adsorption of K^+ as congruous anions.

A congruous anion functions as the in vivo counterpart of the OH^- groups in the in vitro unmasking the β- and γ-carboxyl groups for the adsorption of alkali metal ions (see Figure 1). However, in stead of removing the positive charge of the fixed cations altogether as the OH^- ions do, the congruous anions liberate the β- and γ-carboxyl groups, (under the influence of the cardinal adsorbent ATP and a proteinaceous cardinal adsorbent called Protein X) by adsorbing onto and thus effectively neutralizing the fixed cations. Since each pair of salt linkage contains one carboxyl group and one fixed cationic group, the matching concentration of K^+ adsorbed and the number of congruous anions adsorbed are expected and observed.

I. Cameron: Is there direct in vivo or in vitro evidence to support your statement that adsorption of ATP controls protein conformation and selective ion adsorption? Author: It is known that hemoglobin can exist in two alternative conformations: a rigid T-sate corresponds to the deoxygenated state; a relaxed R-state corresponds to the oxygenated state (for an interpretation of the cause of the phenomenon, see Ling, 1984, footnote p. 222). Chanutin and Curnish (Arch. Biochem. Biophys., 106, 433, 1964; Arch. Biochem. Biophys. 121, 96, 1967) showed that the binding of ATP (or 2,3-diphosphoglycerate) shifts the hemoglobin conformation to the T state. Hemoglobin is not an ATPase, its effect on the hemoglobin conformation is entirely due to its adsorption on the hemoglobin molecule.

The contractile proteins of glycerinated muscle (e.g., the flight muscle of the inset Lethocerus maximus) can also exist in at least two conformation states: contracted and relaxed. Under suitable conditions, interaction with ATP drives the contractile proteins into the relaxed state. Goody and his coworkers (Biophys. J., 15: 687, 1975) showed that the maintenance of the relaxed state by ATP does not depend on its hydrolysis but depends only on its adsorption. This conclusion is supported by the fact that interaction of glycerinated Lethocerus muscle with ATP analogues -- which may not be hydrolyzed at all but have similar large binding constant on myosin as ATP -- also brings about the relaxed conformation.

The effect of ATP on the conformation of hemoglobin (the major cytoplasmic protein of erythrocytes) and the actomyosin system (the major cytoplasmic protein of muscle cells) provides in vitro evidence that the adsorption of ATP controls protein conformation.

So far the best in vivo evidence that ATP binding controls the adsorption of K^+ was the demonstration of a quantitative relationship between the level of ATP in frog muscle cells and the level of K^+ in the cells. To manipulate the levels of ATP in the cells, 11 toxicologically divergent poisons were used, each producing the same quantitative relationship between ATP concentration and K^+ concentration (Gulati et al., Biophys. J. 11, 973, 1971).

The true significance of this finding cannot be fully appreciated without taking into considerations the following:

a) ATP does not contain a package of "high energy" (Podolsky and Morales, J. Biol. Chem. 218, 945, 1956; George and Rutman, Prog. Biophys. Biophys. Chem. 10, 1, 1960). Therefore whatever ATP does physiologically, it cannot do so by delivering such energy through its hydrolysis.

b) It is now known that some of the proteins with high affinity for binding specific solutes (e.g., sugars) contain highly conserved region in the protein molecule which shares primary structure with other ATP-binding proteins, including myosin (Higgins et al., EMBO J. 4:1033, 1985).

c) Although ATP does not carry a package of "high energy", in its phosphate bonds, it does bind onto myosin with an enormous affinity. Indeed the binding constant is between 10^{10} to 10^{11} (Goody et al., Europ. J. Biochem. 78: 317, 1977). This binding constant is equivalent to a free energy of adsorption of -14 kCal/mole, which in magnitude matches the (once wrongly believed) utilizable energy stored in the two so-called "high energy phosphate bonds" of ATP (also equal to -14 kCal/ mole). The binding energy of ADP on myosin is only half of that of ATP. Indeed the most outstanding characteristic in the interaction of ATP and myosin is its extremely high binding energy. In the AI hypothesis, high binding energy is what distinguishes a "cardinal adsorbent", that controls the cooperative transition between alternative conformation states. So far, the AI hypothesis has offered the only theory, known to me, that makes use of this unique interaction between ATP and an intracellular protein like myosin.

I. Cameron: Have you completely ruled out molecular sieving of the larger hydrated Na^+ vs. the smaller hydrated K^+ ion within globular proteins as a contributing mechanism of selective K^+ accumulation within cells?

Author; Selective K^+ accumulation signifies the accumulation of K^+ to a level significantly higher than in the surrounding medium. Molecular sieving per se is therefore unsuited to explain selective K^+ accumulation.

Molecular sieving is a mechanism that has been considered favorably by other scientists (but not myself) for the size-dependent solute exclusion from a phase containing macromolecules: small solute molecules are found in concentration higher than those of larger solutes in the macromolecule-containing phase. But neither small nor large solutes exists in the macromolecule-containing phase at concentration higher than in the external medium (which does not contain the macromolecules) as in the case of selective K^+ accumulation.

While molecular sieving per se is unsuited to explain selective K^+ acuumulation, molecular sieving in conjunction with the presence of fixed anionic charges has been argued for the selective accumulation of K^+ over Na^+ in (sulfonate type of) ion exchange resins. However, this theory became no longer tenable when it was demonstrated the in carboxylate type of ion exchange resins, Na^+ is preferred over K^+. Molecular sieving allows only one rank order of selectivivity according to the size of the hydrated ions, giving rise of selective accumulation of K^+ over Na^+ but not the other way around as observed.

In concluding the answer to the question posed, I want to mentions that most isolated globular proteins do not bind and thus "accumulate" K^+ or Na^+ in vitro at physiological pH (7.5). Carr studied the binding of K^+ and of Na^+ on a total of 14 proteins (Arch. Biochem. Biophys. 62: 476, 1956). Only three, pepsin, β-lactoglobulin and zein show some binding (15 moles per 10^5 grams of proteins or less). Even in these proteins there is virtually no discrimination between K^+ and Na^+. Among the 11 other globular native proteins that show no demonstrable binding of either K^+ or Na^+at all, are human and bovine hemoglobin. This failure to demonstrate any K^+ or Na^+ binding on isolated globular hemoglobin was in total accord with other studies reported by Beatley and Klotz (Biol. Bull. 101: 215, 1951) and by Morris and Wright (Austral. J. Expt. Biol. Med. Sci. 32: 1486, 1954).

As pointed out repeatedly above, hemoglobin makes up 97% of the total intracellular proteins of erythrocytes. If hemoglobin truly exists in the living cells in its native globular state as studied by these workers, clearly the observed selective accumulation of K^+ over Na^+ in erythrocytes cannot be due to binding on this intracellular protein. Yet as I have shown in Figure 1, if hemoglobin is made to exist in a different conformation by exposure to alkali hydroxide, we have an altogether different ball game. Now every carboxyl group binds a Na^+ or K^+, the total binding capacity far exceeding that seen in living erythrocytes.

I. Cameron: Yours and other data convinced me that most of the intracellular K^+ is reduced in its movement and indeed adsorbed but would you have cell physiologists ignore the enormous amount of careful experimental work and numerous reports dealing with membrane-pumps? Surely there is valuable information in at least some of these reports.

Author: I am convinced that the associative aspect of the AI hypothesis (K^+ and H_2O adsorption in resting cells) is now

fully established and that the corresponding basic tenets of membrane pump theory are thoroughly disproved (see Ling, 1990). My ideas have been fully presented in the two books I already published (Ling 1962, 1984), in one soon to appear in print (Ling 1990) and in many shorter writings like the present one. This is all I can do. What other cell physiologists choose to do or not to do is entirely up to them.

T. von Zglinicki: How is the c-value defined?
Author: Acetic acid has a pK_a value of 4.76. Trichloroacetic acid has a pK_a of lower than 1. This is a classical example of the inductive effect. It demonstrates how distant substitution of 3 H atoms by the more electronegative Cl atoms reduces the electron density of the carboxyl groups and hence the free energy of interaction between the ionized carboxyl group of the acid with H^+. It is also known that the interaction energy between the ionized carboxyl group and H^+ is primarily electrostatic (Kossiakoff and Harker, J. Amer. Chem. Soc. 60: 2047, 1938).

The c-value is a parameter introduced to represent the underlying cause of the change of the interaction energy between acetic acid and H^+. Unlike the pK_a value which represents an interaction and is therefore not an independent parameter, the c-value is an independent parameter describing mainly electron density of the ionized carboxyl group (or of any other oxy-acid group). Acetic acid has a high pK_a because the carboxyl group of acetic acid has a higher c-value. Trichloroacetic acid has a lower pK_a value because the carboxyl group of trichloroacetic acid has a lower c-value.

A rigorous definition of the c-value begins with a prototype oxygen atom bearing a single negative electronic charge located at the center of the atom. To this singly charged oxygen atom is then added through covalent (and other linkages), more and more additional atoms so that in the end an acetic acid or a trichloroacetic acid is built up. As a result, the electron density of the anionic singly charged oxygen atom and other nearby atoms are changed. This change may be a decrease, an increase or no change from the prototype singly charged oxygen atom with H^+. Each of these changes can be matched exactly by moving the single electronic charge at the center of the prototype oxygen atom either closer to or farther away or not at all respectively from the monovalent cation along a line joining the centers of the prototype oxygen atom and the monovalent cation. The distance traversed by the single electronic charge in Angstrom units, is called the c-value.

The c-value is positive if the single negative charge moves toward the monovalent cation. It is negative if its moves away from the monovalent cation.

T. von Zglinski: How can the counterion condensation theory of G. S. Manning (G.S. Manning, Quart. Rev. Biophys. 11 (1978) 179-246) which also rests on the equilibrium of dissociation entropy and (electrostatic) binding to a fixed set of charges, be related to the AI hypothesis?
Author: Manning's "Counterion Condensation Theory" was first published in 1969 (Manning, J. Chem. Phys.51:924, 1969). The "Theory of Enhanced Counterion Association in Fixed Charge Systems" was first presented nine years earlier (Ling, J. Gen. Physiol., 43: 149, 1960). I know of Prof G. S. Manning's work and have in fact cited in my 1984 monograph the very same review you mentioned. Both the "Counterion Condensation Theory" (CC theory) of Manning and my "Theory of Enhanced Counterion Association in Fixed Charge Systems" (ECA theory) (as part of the much broader Association-Induction hypothesis) deal with the increased degree of counterion association with fixed ions, and are thus departing from the conventional belief that ionic dissociation in aqueous solutions is as a rule, near completion. Both theories regard the overlapping of the electric field of neighboring fixed charges as the cause, or one of the causes (ECA theory) of the enhanced counterion association.

There are also many profound differences. Only two will be mentioned:

The ECA theory deals primarily with (localized) one-ion one-site, close contact association of monovalent cations on protein carboxyl groups in living cells and in models including proteins and polyelectrolytes. The CC theory, on the other hand, is more involved with delocalized condensation of ions on polyelectrolytes in solution. The CC theory does not present a quantitative theory of localized adsorption on discrete groups, as indicated by Dr. Manning's statement " The extent to which condensed counterions are dehydrated and localized near discrete groups, that is, the 'tightness' of binding, may be expected to vary both with the nature of the charged groups and the counterion species...." (Manning, 1978, cited in the question p. 185). The ECA theory has provided a quantitative theory answering these problems (see Figure 2 and Ling, 1962, p.61 to 84). In this theory, the c-value concept plays a key role and the amount of hydration between a certain cation and a fixed anion at a specified c-value was obtained by energy minimization. The theoretical results indicate that a Na^+ ion interacting with an oxyacid group with a low c-value retains to varying

degree its its hydration shell. The same Na^+ interacting with a similar oxyacid group with a high c-value becomes dehydrated and is more tightly adsorbed.

Reviewer IV: The paper is completely out of time: the problem of the state of K^+ ions was treated in a much more complex way already 12 years ago by Pieri et al., J. Ultrastruct. Res. 59, 320-331 (1977): see the text on page 322 there! The main trouble is that if Ling is right, he is not new and has to give credit to the previous literature maintaining the "immobility" concept of K^+. If Ling is wrong, then the paper is not worth publishing at all. If Ling wants to reopen the discussion on the state of K^+, he has to discuss the NMR evidence and add new facts. However, he fails to do so.

Author: I think what the reviewer was trying to say is that my paper is "out of date" in the sense that my ideas are not original and that I have not given credit to the earlier true originator(s) of the idea. Let us examine just how he has reached such a conclusion (before examining if the conclusion has validity). To begin, I reproduce verbatim page 322 of the Pieri et al. paper he cited:

" I. The Mobility of Light Elements in the Cell. Several approaches exist to this problem. Nuclear magnetic resonance studies revealed that a considerable fraction of Na^+ of the tissues is "invisible" for NMR, and the conclusion of several authors was that this indicates a firmly bound portion of the intracellular Na^+ [see for refs. (Monoi, Biophysical J. 11: 645, 1974)].It has also been described using NMR that the K^+ ions are completely associated with the fixed charges of macromolecules in muscle and brain, i.e., no free K^+ exists (Cope and Damadian, Physiol. Chem. Phys. 6: 17, 1974). Others, however, were of the opinion that the NMR data obtained are to be interpreted in the basis of the theory for the quadrupole relaxation in systems with a slow correlation time (Berendsen and Edzes, Ann N. Y. Acad. Sci. 204: 459, 1973; Edzes and Berendsen, Ann. Rev. Biophys. Bioeng. 4: 265, 1975; Monoi, op cit.; Monoi, Biophys. J. 14, 653, 1974), offering a rationale for the existence of only one species, i.e., free Na^+, K^+ and Cl^- in the tissues. (Author's note: I think what the authors were trying to say is that each of these ions exists in the cell in one state, the free state). This interpretation agrees quite well with the conclusions obtained by another approach. Measurements of the activity coefficients using different methods in KCl solution, liver tissues homogenates or solutions containing biological macromolecules showed with little doubt that the freedom of motion of K^+, Na^+ and Cl^- ions in biological systems (except some bacteria (Ginzburg et al., Biochem. Biophys. Acta 173: 370, 1969; Ginzburg et al., J. Gen. Physiol. 55: 187, 1970; Ginzburg et al., J. Membr. Biol. 5: 78, 1971; Ginzburg et al., J. Membr. Biol. 6: 259, 1971) closely resembles that of the liquid state, however, with the addition of an electrostatic influence by the fixed charges on the intracellular macromolecules (Edzes and Berendsen, 1975, op. cit.; Pauly, Biophysik 10: 7, 1973).

Therefore, it seems to be reasonable to consider the monovalents mentioned above as free ions in the cell water ..."

From this writing, one finds out that Piere et al. were not the true originators of the "immobilized"-K^+ concept. Rather, they only quoted some earlier reports supporting the notion that cell K^+ and Na^+ are bound. Next they quoted other reports supporting the notion that K^+ and Na^+ are not bound. Pieri et al. then declared--less convincingly--that it was reasonable to consider these monovalent ions as free ions in the cell water.

Reviewer IV took me to task for not giving credit to "previous literature" on "immobilized" K^+, citing the Pieri et al. paper quoted verbatim above. Since Pieri et al. were definitely not the originator(s) of the "immobilized" K^+ concept, who then are the authors of this "previous literature" that the reviewer had in mind?

Pieri et al. first quoted a 1974 publication of Monoi and then a publication of Cope and Damadian. Are Monoi, Cope and/or Damadian the true originators of the "immobilized" K+ concept? To find out, let us read the papers of Monoi, Cope and Damadian cited.

The first sentence of the Cope and Damadian paper cited--which was published in 1970 and not in 1974 (Nature, 228: 76, 1970)-- states "Although there is recent evidence that cell potassium is complexed-....". In support of this statement, Cope and Damadian cited 4 earlier publications on the subject, including the following two:

Ling GN, Cope F, Science 163: 1336, 1969;

Ling GN, Ochsenfeld MM, Biophys. J. 5: 777, 1965

Let us next look at the 1974 Monoi's paper the Reviewer also mentioned. The first (earlier) publication Monoi cited here is that of Cope (Biophys. J. 10: 843, 1970), who stated on its first page the following:

> " Na^+ and K^+ are believed mostly to be complexed to cell macromolecules...A basis for comprehensive theoretical analysis ... has been developed by Ling (Ling, G.N. 1969. Intern. Rev. Cytology. 26: 1; Ling, G.N. 1962. A Physical Theory of the Living State, Blaisdell Publishing Co., New York.) ..under the name of

the "association-induction" hypothesis..." .

Thus, in a roundabout way, one finds that G. N. Ling, namely myself, was the originator of the "immobilized" K^+ idea after all.

Reviewer IV also admonished me that if I want to reopen the discussion of K^+, I have to discuss NMR evidence.

In fact, the paper of Ling and Cope cited above did just that. And that was published 20 years ago. Furthermore, in years following I have provided new theoretical reasoning as well new experimental data showing that in regard to the significance of the "NMR-invisible Na^+", the original conclusion of Cope, (rather than that of Berendson and Edzes) was essentially correct (Ling, 1984, p. 263-268; Ling and Zhang, Physiol. Chem. Phys. & Med. NMR 15: 251, 1983): approximately half of muscle cell Na^+ is adsorbed.

The reviewer also cautioned me that if I want to discuss the physical state of K^+ in living cells, I must add new facts, implying that I have not done so. In fact, two sets of key experiments establishing that the K^+ adsorbing sites are β- and γ-carboxyl groups and shown in Figure 9 and Table 2 of the present paper were entirely new then and now. Indeed these data have not been published even at this moment. Can there be anything newer than these?

Reviewer IV: The terminology of Ling is wrong: adsorption means something else in the chemistry as compared to that he wants to suggest.

Author: I am thankful that Reviewer IV brought this issue up. His brief commentary will be answered fully, because it tells more than what appears on the surface.

The general phenomena of the taking up of gases by liquids and solids were desingnated "absorption" more than a century and half ago (see J. S. T. Gehler, Phys. Wörterbuch, Leipzig, 1825, 1: 40). Later M.L. Frankenheim (Die Lehre von der Cohäsion, Breslau, 1835, p. 158) introduced the term adsorption for uptake of gases in pores or upon surfaces. However, Frankenheim's introduction of the term, adsorption, became forgotten; it was reintroduced by H. Kayser 46 years later at the suggestion of the noted physiologist, E. du Bois-Reymond (Kayser, Ann. Phys. 14: 451, 1881).

Henceforth adsorption has been used exclusively to describe the occurrence of a higher concentration of any component at the surface of a solid or liquid, while the more or less uniform penetration into solids is called absorption.

In years following, one direction in the investigation of adsorption deals with its occurrence at surfaces or interfaces. It was along this line of approach that R. A. Gortner in his "Outlines of Biochemistry" (John Wiley and Sons, 2nd edition,1938, p. 211) stated "Those substances which decrease surface energy tends to concentrate at a liquid-vapor interface, and those substances that decrease interfacial energy tend to concentration at a liquid-solid or liquid-liquid interface. This phenomenon of concentrating at the interface is called adsorption". More recent development in this general direction can be found as part of the studies of interfacial phenomena (see J.T.Davies and E. K. Rideal "Interfacial Phenomena" Acad. Press, New York, 1963, 2nd edition, Chapter 4). Possibly Reviewer IV--along with Gortner of long ago-- believes (erroneously) that this kind of adsorption is the only kind of adsorption. Interfacial adsorption has become less and less relevant as the precision of our knowledge of cell physiology increases and our approaches become more and more "focussed".

An important landmark in the progress of our broadening understanding of adsorption in general and what I shall refer to as "localized adsorption" in particular was the introduction of a theory on adsorption by the American chemist, Irving Langmuir (1881-1957). Langmuir visualized the adsorption of gases to occur not at a uniform solid surface but at a finite number of localized centers of attraction or "active spots" on the surface. The equation he introduced for the adsorption of gases at a fixed temperature bears his name, i.e., Langmuir adsorption isotherm (Langmuir, J. Amer. Chem. Soc. 40: 1361, 1918). Accordingly, the amount of gas adsorbed does not increase steadily with increasing concentration (or pressure) of the gas, but asymptotically approaches a limiting value corresponding to the density of "active spots" or adsorption sites on the surface.

As more and more knowledge was gained,it has become increasingly clear that adsorption of gases and other substances are not limited to the surfaces and interfaces of solids and liquids as one time thought, but may occur within what one would recognize as a solid. A clear example is the sorption of water vapor within crystalline and non-crystalline proteins. While inert gases like nitrogen, argon etc. do adsorb primarily on the exposed surfaces of these proteins, water adsorption, vastly larger in quantity, is on specific adsorption sites (polar side chains and in particular the keto and imido groups of the polypeptide chain) mostly in the interior of the proteins (Benson et al., J. Amer. Chem. Soc. 72: 2095, 1950; ibid, 72: 2102, 1950; for a more complete review see Alexander and Hudson, "Wool: Its Chemistry and Physics", Reinhold Publ. Co., New York, 1954, p.

115; Ling and Hu, Physiol. Chem. Phys. & Med. NMR 19: 251, 1987, p. 264-269).

In an article entitled "Adsorption als Folge von Polarization. Die Adsorptionsisotherme" de Boer and Zwikker presented an adsorption isotherm describing adsorption of (polarized) multilayers of gas molecules on solid surfaces (Z. Physik. Chem. B3: 407, 1929). In 1936, S. Bradley derived a formally similar polarized multilayer adsorption isotherm with the main difference that gaseous molecules with large permanent dipole moments (e.g. H_2O with dipole moment = 1.86 debyes) were considered. Later theoretical considerations led Brunauer et al to the conclusion that only molecules with permanent dipole moments can form deep polarized multilayers.

An implicit assumption in deriving the Langmuir adsorption isotherm was the absence of site-to-site interaction. In this model each adsorption is independent of other absorptions. In 1964, Ling and Yang, using one-dimensional Ising method, derived a cooperative adsorption isotherm for one(solute)-on-one(site) adsorption with interaction between nearest neighboring adsorption sites (Ling, J. Biopolymers 1: 91, 1964).

What then is localized adsorption? Localized adsorption involves the attraction of molecules (or ions) to, and momentary capture by discrete sites on a much larger, and more or less immobilized macromolecule or polar solid surface. After a relatively long "residence time" on the site, each adsorbed molecule eventually returns to the surrounding medium without being chemically altered. In other words,localized adsorption represents a reversible equilibrium phenomenon. Underlying the favorable free energy for the adsorption is the attractive force (between the adsorbed molecule and the site) which is fundamentally electrostatic in nature (e.g., Coulombic, ion-dipole, dipole-dipole, van der Waal). The three adsorption isotherms (Langmuir, Bradley, Yang and Ling) offer part of the theoretical framework of the theory of the living cell called the association-induction hypothesis.

Within the last 30 years, one of my most exciting experience has been the experimental demonstration that the adsorption of ions and nonelectrolytes in living cells follow rigorously either the Langmuir adsorption isotherms or the cooperative Yang-Ling adsorption isotherms. In addition, the uptake of water by living cells and model systems also rigorously follow the Bradley adsorption isotherms. The interested reader can find details of these confirmations of the theory in my 1984 book, "In Search of the Physical Basis of Life" already in print and "A Revolution in the Physiology of the Living Cell" (Krieger Publ. Co., Melbourne, FL) soon to be.

To shed more light on the reasons why so many otherwise well-informed biomedical scientists know so little about key fundamental subject as adsorption, I shall try to introduce the reader to some other "forces" that, though largely unseen, often decide on what one knows and what one does not know.

In 1953, Irving Klotz, Professor of Chemistry at the Northwestern University contributed a chapter on the subject of what he called "Protein Interaction" to the monograph series, "The Proteins: Chemistry, Biological Activity and Methods" Academic Press, New York, Volume I, Part B, Chapter 8). A key equation he introduced as Equation 16, is the Langmuir adsorption isotherm. In the remainder of the chapter this Equation was shown to describe adsorption of small molecules on a variety of isolated proteins. Yet, curiously enough, in stead of acknowledging the confirmation of Langmuir's theory of molecular adsorption in the proteins studied, this is what Professor Klotz said:

"Equation (16)... is essentially the same as that derived by Langmuir (loc. cit.) to describe certain adsorption isotherms. It seems appropriate to emphasize, therefore, that the adherence of a set of binding data to Eq. (16) does not justify the assertion that the process is adsorption. Equation (16) is also an expression of the law of mass action". In support, Prof, Klotz cited Linderstrom-Lang (Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim., 15, No.7, 1924...) (p. 749).

Professor Klotz's reluctance to use the term adsorption is, in my view, unjustified, even though it is shared by influential figures like Linderstrom-Lang of Stockholm. (Linderstrom-Lang had treated proteins as a sphere with its electric charge uniformly smeared over its surface. His model does not jibe with molecular adsorption of the kind the Langmuir adsorption isotherm describes, Linderstrom-Lang, 1924 loc. cit., see also Ling, 1962, p.xxviii).

Mass action law describes chemical equilibrium. As such it does not automatically produce a relationship between the reacting species seen in the Langmuir adsorption isotherm (see Glasstone, Textbook of Physical Chemistry, 2nd Ed., van Nostrand-Reinhold, New York, 1946, p. 816). To produce the relationship in the Langmuir adsorption isotherm, one has to make specific assumptions etc. But then that was what Langmuir did. Can one do it again and not call it a Langmuir adsorption isotherm? The basic ethical rule of Science forbids that kind of practices.

Adsorption is not the only highly relevant protein behavior that has become a "No No" in protein chemistry--a develop-

ment which could trace at least part of its origin to Linderstrom-Lang. Another important concept in understanding proteins became a "No No", also as a result of the (strong but in my view, mistaken) idea of Linderstrom-Lang (and possibly also of his coworker, Jacobsen; Jacobsen and Linderstrom-Lang, Nature 164: 411, 1949): the concept of salt-linkages. As already mentioned in the text, salt linkages are electrostatic bonds formed between anionic protein carboxyl groups and cationic protein amino and guanidyl groups first suggested by Speakman and Hirst (1931). Long after Perutz and coworkers have unequivocally demonstrated the existence of the salt-linkages in hemoglobin in their monumental X-ray analysis of its crystalline structure (Perutz, Nature 228: 726, 1970), many protein chemists are still very reluctant to use this term.

Ultimately, the prejudice against "adsorption" and "salt-linkage" concepts can be traced to the incorrect extension of the discovery (Arrhenius; Debye and Hueckel) of the limiting law of total ionic dissociation (in infinitely dilute solutions). As a result, it has become "verboten" to consider "adsorption on proteins" and "salt-linkages" --both representing association phenomena-- in realms the limiting laws are not intended to cover: the behaviors of macromolecules and living cells (Ling, 1962).

I describe adsorption (and salt-linkages) in some detail because both phenomena are highly relevant to the subject matter of the paper under discussion, and because their unnatural neglect, in my opinion, has much to do with the poor knowledge of the major roles "adsorption" and "salt-linkages" play in cell physiology.

Reviewer IV: Ling is fighting against a phantom-concept: "...the close-contact selective adsorption of K^+ takes place in the cytoplasm and not on the cell membrane." To best of my knowledge, nobody has ever claimed that the selective intracellular K^+ accumulation is directly taking place in the cell membrane.

Author: To understand Nature, a scientist must consider all other conceivable alternatives hypotheses--that can also more or less predict the experimental finding under consideration--in addition to his or her own hypothesis, regardless whether or not other scientists have already suggested these alternative concepts.

Having said this, I want to add that one does not need to go far to find a model of selective K^+ accumulation in cytoplasm that, in my opinion, is in need of discriminatory mechanism at the cell membrane like the one I considered (but rejected, see text).

In one of the following questions (see below), Reviewer IV describes a 50-100 greater permeability of the cell membrane for K^+ than for Na^+, and uses this observation to construct a theory of selective K^+ accumulation in the cell cytoplasm without, however, explaining how this selective permeability could be achieved.

One recalls that the use of radioactive isotopes and other studies have long ago disproved the sieve idea of Mond and Amson (which was also adopted without acknowledgement by) Boyle and Conway (Ling, 1984, p.32), where small membrane pores were postulated to allow the permeation of the smaller hydrated K^+ but not that of the larger hydrated Na^+. After the discovery that antibiotics like valinomycin and similar compounds could selectively ferry K^+ (but not Na^+) across artificial lipid bilayers, extensive efforts had been made to find natural ion-specific ionophores in cell membranes. As ruefully pointed out by a participant in these extensive efforts, Paul Mueller pointed out that these efforts had all ended in failure (see Mueller, Ann. N.Y. Acad. Sci. 264: 97, 1975; Ling, 1984, p. 380). Thus the membrane pump theory has run out of molecular mechanism to explain selectivity between K^+ and Na^+.

As mentioned repeatedly in the text, the only differences in the attributes of K^+ and Na^+ are their short-range attributes and that without lasting close-contact association with a cell component, these ions cannot be told apart. Therefore a molecular mechanism must be found, they would be hard put not to invoke close contact association with cell membrane β- and γ-carboxyl groups.

While in the association-induction hypothesis, selective accumulation of K^+ over Na^+ in living cell is primarily an expression of the properties of the cytoplasmic proteins and water--and experimental evidence discussed in the text amply support this concept-- selective adsorption on the β- and γ-carboxyl groups at the cell surface does play key roles in explaining selective ionic permeability in all types of living cells as well as in explaining active ion transport across bifacial cells described in the companion paper I wrote for this volume.

Thus in 1952, I first briefly suggested that the same discriminatory mechanism proposed for the selective accumulation of K^+ over Na^+ in the cytoplasm may also operate at the cell surface,i.e., selective adsorption on β- and γ-carboxyl groups. This prediction was confirmed 13 years later by Ling and Ochsenfeld (Biophys. J., 5: 777, 1965). Here the data affirm the predictions derived from the hypothesis that the great majority of entrant K^+ into frog muscle cells do so by

first associating with, followed by libration around the cell surface fixed carboxyl groups and then desorption and entry into the cells. The adsorption energy of K^+ on the cell surface carboxyl groups was found to be --like that on cytoplasmic carboxyl groups--at least 40 times stronger than that for Na^+. As a result, much of the (adsorption-desorption) routes on the cell surface mediating K^+ entry is denied to Na^+. Like the anionic groups that adsorb alkali-metal ions in the cytoplasm (see text, and Figure 9), the anionic groups associate (momentarily) with entrant alkali metal ions at the cell surface also exhibit pK_a values characteristic of β- and γ-carboxyl groups.

Reviewer IV: The membrane theory of cell physiology explains the intracellular accumulation of K^+ on the basis of the following well-established facts:

(i) The presence of so-called "fixed negative charges" in the cytoplasm (evidently beta- and gamma-carboxyl groups on proteins, and also phosphate-groups of nucleic acids), the macromolecular carriers of which are practically unable to leave the cells, because the membrane is not permitting their free movement, attract a great number of positive charges from outside of the cell. (The paper of Ling agrees with this concept but this is not creating a reason for publishing it).

(ii) Due to the fact that in its resting state the membrane is highly permeable for K^+ and is 50-100 times less so for Na^+, the main ion which will be accumulated is K^+ and not Na^+, in spite of the fact that this latter is present in considerably higher concentration in the extracellular space.

Author: The presence of high concentrations of free (and masked) fixed negative charges (β- and γ-carboxyl groups) in living cells was first suggested in 1952 by myself as the basis for the selective accumulation of K^+ over Na^+ in living cells (Ling, 1952). The lack of correct factual information on the part of an established scientist like Reviewer IV shows how timely it is to convene this symposium and to engage in questions and answers like the present. My paper is a review (even though it also presents important new findings for the first time, see below and I (had to) mention some of my earlier but still valid ideas.

In my theory, the high concentration of β- and γ-carboxyl groups in the cell is important because they offer the sites for the selective adsorption of K^+ over Na^+.

In (ii) Reviewer IV suggested that in the presence of an abundance of fixed β- and γ-carboxyl groups, a much higher inward membrane permeability to K^+ than to Na^+ would lead to the selective accumulation of K^+ over Na^+ in the cell. This is an impracticable (but by no means new) idea.

Robert Dean who is often credited for introducing the sodium pump hypothesis, described 48 years ago a similar model as the one Reviewer IV just mentioned: "a differentially permeable membrane will produce a concentration difference at the expense of kinetic energy of the diffusing molecules" (Dean, Biol. Symp. 3: 331, 1941, p. 346). He then continued: "This violates the second law of thermodynamics and is in fact a Maxwellian demon...".

In general principle I agree with Dean's rejecting differential membrane permeability as the mechanism for a non-energy consuming selective accumulation of K^+ over Na^+. In my opinion, such a mechanism, considered by Dean in 1941 and by Reviewer IV now, violates the First Law of Thermodynamics (law of conservation of energy) rather than the Second Law of Thermodynamics (heat of itself, without the intervention of any external agency, cannot pass from a colder to a hotter place) as Dean contended. (For a discussion of the cause of this difference, see Ling 1990, Endnote 1 in Chapter 8).

Reviewer IV: Whenever the cell membrane becomes depolarized by electric or chemical stimuli, its Na^+ permeability increases for a short time, i.e., a measurable Na^+-influx and K^+-efflux appears, and the consequences of this phenomenon are eliminated by the energy-dependent Na^+-pump. On the other hand a sustained depolarization of the cell membrane causes a permanent shift of the intracellular Na^+/K^+ ration toward very high values, i.e., the K^+-accumulation is shifted to Na^+-accumulation, like in the case of cancer cells.

If Ling wishes to question these basic interpretations of cell physiology, he has to make a much better job, otherwise the declarations he makes against the membrane-theory are insufficient.

Author: In 1962 I presented evidence that the postulated Na^+ pump would consume far more energy than the total energy the cell commands (see text). In other words, the hypothesis violates the First Law of Thermodynamics.

What the reviewer has done in (ii) of the previous question is to devise a non-energy consuming mechanism for the selective accumulation of K^+ over Na^+ in living cells. In the present question he tries to delegate the postulated Na^+ pump to deal only with net transport of Na^+. However, as I have pointed out above, his theory of non-energy consuming K^+ accumulation, like the postulated Na pump, also violates the First Law of Thermodynamics and therefore equally untenable.

As I pointed out in the text, selective accumulation of K^+ and exclusion of Na^+ are but two examples of the cell's

capability of selectively accumulating and/or excluding an endless array of solutes. So what is needed is a general theory that can cope with the asymmetrical solute distribution phenomenon as a whole, rather than endlessly and hopelessly playing a game of make-belief that there is only one problem, i.e., the K^+ and Na^+ problem--such as the membrane pump theory supporters have been doing for the better part of the 20th Century.

The association-induction hypothesis, already more than a quarter of a century old, offers just such a general, comprehensive theory. The association-induction hypothesis can explain the equilibrium distribution of K^+,Na^+ as well as the endless array of other solutes without violating any basic laws of physics, and in a quantitative manner (see Ling, 1984, Chapter 11, Ling, 1990, Chapter 8).

The dissatisfaction of Reviewer IV with my presentation is to be expected. While there is little doubt that my writing can be improved (One wonders whose cannot), there is limitation to how much a short paper like mine can tell about a comprehensive theory like the association-induction hypothesis after 30 years of extensive experimental testing. There is too much unfamiliar information that cannot be digested and mastered in a short period of time. Furthermore, the information, once fully understood, will inevitably force painful rethinking of what one might have long accepted as indisputable truth. None of this is easy; some can be painful (see also: Ling in "The State of Water in the Cell" Scanning Microscopy International, Chicago, p. 102, 2nd paragraph; Current Contents: Clinical Medicine, Vol. 17, No.44, 1989, p.22, or alternatively Current Contents: Life Sciences, Vol. 32, No. 44, p.23).

B.L. Gupta: All proteins found inside a living cell in vivo are "native" to the cell by definition. If such proteins are not expected to selectively adsorb K over Na, then how does one explain >100 mM of K in almost all the cell-types so far analyzed in situ in cryosections with the electron probe microanalysis? Your argument also conflicts with the statement that isolated proteins have to be denatured by unrealistically harsh (for the cell) conditions before they would bind K or other alkali metal ions? Please clarify the expression "native" for binding K in a living cell.

Author: According to the Webster dictionary, the word native, means "found in nature esp. in an unadulterated form" (Webster New Collegiate Dictionary, 1977, p.765). Therefore, Dr. Gupta's statement "All proteins found inside a living cell in vivo are 'native' to the cell by definition" is logically impeccable. But it is also unrealistic. Because the term "native protein" as used in the literature today has a different meaning --even though the pioneer users of this term might have thought that their "native" proteins were in agreement with Dr. Gupta's definition.

Studying isolated proteins in vitro, early protein chemists soon discovered that these proteins could exist in more than one state (without changing their primary structure). Thus gentle heating may convert a water-soluble protein into a water-insoluble one. Wu (Chinese J. Physiol. 5: 321, 1931) then offered the first definition of protein denaturation: "Denaturation is a change in the natural protein molecule whereby it becomes insoluble in solvents in which it was previously soluble." Wu's "natural protein" is the equivalent of the term "native protein" as used by the majority of workers in this field today, and it in this context that I used the term "native protein".

If we could do it all over again, we might consider reserving the term native protein to protein assuming the conformation in the living cell and introduce a new name for what it is now commonly used to designate.

Knowing how difficult it is to change convention, one might as well use the term "native " as it is being used today and introduce a new term for the state of protein existing in the living cells. Indeed, this is what I have done. It is called the living state (Ling, 1984, p. 147; Ling, 1990, Sect.3.2).

An implicit assumption underlying the introduction of the term living state is that the living state and the native state are as a rule not the same for many important intracellular proteins. A major manifestation of this difference between the two states is underscored by the strong experimental evidence demonstrating that myosin selectively adsorbs K^+ in the living state but it does not in the native state.

The reasons why the purest and best (native) hemoglobin--the premier cytoplasmic protein of red blood cells--that one can obtain do not selectively adsorb K^+ over Na^+ at a scale comparable to that believed to exist in the living cells is given in the paper (see sections on pages 739-740 of the text). One prediction of the AI hypothesis is that if the competing fixed cations (for the β- and γ-carboxyl groups) are removed, the hemoglobin will regain its ability of selectively adsorbing K^+ and Na^+. One way to get rid of the competing fixed cations is by exposure of the protein to high pH and our experiment (Figure 1) has fully confirmed this prediction.

The success of this experiment does not imply that the intracellular pH is high. Quite the contrary, all existing

evidence point to a neutral intracellular pH. The success of the high pH experiment only points out that the β- and γ-carboxyl groups have the inherent capability of adsorbing alkali metal ions on a large scale. The maintenance of hemoglobin in the living K^+-adsorbing state depends on other mechanism(s) than exposure to an alkaline environment. One possible mechanism is presented on page 240 of the paper.

B. L. Gupta: Your argument that a Na-pump would consume far too much energy in maintaining high K and low Na in a living cell is incomplete. It does not allow for the fact that most of this energy invested in the "primary pump" for electrochemically polarizing the cell membrane (and other organelle membranes) is recovered through a range of secondary active transport processes as well as other work. All modern text books of cell biology, physiology and biochemistry include this concept.
Author: "Active transport" by definition is not passive and therefore must require energy expenditure, whatever their given names, primary or secondary. Similarly the performance of work also requires energy expenditure. One cannot **recover** the energy loss from one energy-spending operation from another energy-losing operation. For this reason I cannot see how: "most of this energy invested in the"primary" pumps ...is recovered through a range of secondary active transport precesses as well as other work".

The concentration of K^+ in most living cells is many times higher than that in the bathing medium, while the concentration of Na^+ is many times lower than in the bathing medium. At one time, this asymmetrical distribution of these two chemically highly similar ions was attributed to the absolute impermeability of the cell membrane to both or (later) only one ion (Na^+).

In 1939 and 1940, radioactive tracer and other studies proved beyond doubt that the cell membrane is permeable to both ions. Why do these ions fail to diffuse through the cell membranes and reach the same concentration inside the cell water as those in the external water? In answer, two types of mechanisms were presented. In one, this sustained asymmetrical ion distribution is the result of the unceasing activities of energy-consuming metabolic pumps located in the cell membrane; in the other, the asymmetrical distribution of K^+ and Na^+ reflects the different physicochemical environment for these ions within the cells than in the external medium. Troshin's Sorption theory ("The Problem of Cell Permeability", Pergamon Press, 1966) and my Association-Induction (AI) hypothesis belong to the second type.

The AI hypothesis was first presented in full in a monograph entitled "A Physical Theory of the Living State" (Blaisdell Publ. Co., Waltham, Mass. 1962). In Chapter 8 of this volume, I summarized the results of a major effort I made in the preceding 3 years, demonstrating that under strictly controlled conditions, the minimal energy need of the Na^+ pump is from 15 to 30 times the total energy the cells command. In the 27 years following, neither the experiments conducted nor the conclusion drawn from them have been challenged in print; the essence of the finding has been twice confirmed (A.W. Jones, Ph. D. thesis, Univ. Pennsylvania, Philadelphia 1965; Minkoff and Damadian, Biophys. J. 13: 167, 1973) (see Nagy in Ling, 1988, p.101).

The decisive experiments rests on the fact that frog muscle can sustain its normal K^+ and Na^+ distribution pattern for as long as 7.74 hours (Ling, 1984, Table 4.4) when all its "active metabolism" had been arrested by a combination of low temperature (0°C, which slowed down outward pumping of Na^+ more than it slows down its inward diffusion), pure nitrogen (which arrested respiration) and iodoacetate (IAA) (which arrested glycolysis). Under these conditions the only remaining energy source of the muscle cells resided in the store of ATP, ADP and phosphocreatine (CrP) within the muscle (for evidence that there was no additional unknown source of energy, see Ling, 1952, p.764-766; Ling et al., 1973, p.11). Thus the decrease in concentration of these compounds during a period of time of maintained asymmetrical K^+ and Na^+ distribution in the absence of active metabolic activity offers an exact estimate of the maximum energy available to the cells during this period of time. The minimum energy need of the postulated Na^+ pump for the same period of time can also be calculated as follows:

Na^+ is positively charged and it occurs at an intracellular concentration ten times lower than in the external bathing solution. To pump such a positively charged ion from the inside of the cell to the outside it must overcome an electrical potential gradient i.e., the resting potential which is electrically more positive outside than the inside the cell. To pump Na^+ from the cell interior to the outside, it must also overcome a 10:1 Na^+ concentration gradient since the Na^+ concentration is 10 times higher outside the cell than inside the cell. A simple calculation shows that only a very small fraction (i.e., 0.2%) of the Na^+ leaving the normal resting muscle cells does so by simple passive diffusion. Virtually all the Na^+ leaving the normal resting muscle cells (according to the basic assumption of the membrane pump theory) does so via the postulated Na^+ pump (Ling, 1965, footnote on p. S105).

In summary, one can calculate the minimum energy need of the Na^+ pump in frog muscle under the rigorously defined conditions described (0°C, pure nitrogen and IAA) from (1) the rate of Na^+ efflux measured (hence the rate of pumping), (2) the resting potential recorded and (3) the Na^+ concentration gradient (i.e., the ratio of intracellular Na^+ concentration over the extracellular Na^+ concentration) also monitored.

From the last three sets of fully completed experiments I carried out in 1956 and presented in detail in the monograph "A Physical Theory of the Living State" Blaisdell, Waltham, Mass. (Ling, 1962 p. 211; see also Ling 1984, p. 214, 1988 p. 90) I arrived at the conclusion that it requires at least 15 to 30 times of energy to operate the Na^+ pump (under the experimental condition described) than the energy the cell commands. Six other sets of fully completed experiments performed earlier yielded a similar conclusion with average disparity even bigger than cited here.

In order to save the Na Pump concept, three types of remedial postulations were presented and discussed next. I suspect that it is the third remedial hypothesis (the non-energy-consuming Na pump concept) that Dr. Gupta was referring to:

(1) Ussing's Exchange Diffusion: in this postulation there is a mandatory Na^+ for Na^+ exchange at the cell membrane which does not change the chemical composition of the cell, and therefore requires no energy expenditure. The purpose of this postulation was to reduce the pumping rate derived from the measured Na^+ efflux rate to a lower, and perhaps more feasible one. This postulation was disproved by the demonstration of continued rapid Na^+ efflux in Na^+ free media in a variety of living cells studied (for ref. see Ling, 1984 p.63, 126).

(2) Sequestration of Na^+ in the sarcoplasmic reticulum in muscle cells was also suggested as a means to reduce the energy need of the Na^+ pump, since the energy need is proportional to the Na^+ concentration in the cytoplasm. It too was disproved by the demonstration of even distribution of cell Na^+ throughout a normal frog muscle with no evidence of such sequestered distribution (Ling and Walton, Physiol. Chem. Phys. 1975; Somlyo et al., J. Cell Biol. 74: 828, 1977).

(3) Non-energy consuming Na Pump (Glynn, Trends Biochem Soc. 2: N225, 1977). In this remedial postulation, the inward diffusing Na^+ is postulated not to enter the cells by free diffusion but channeled into the postulated Na^+ pump and in that process turns the pump "engine" backward, generating ATP. The ATP thus generated is then used to pump the Na^+ out of the cell again. As a result, a steady low level of Na^+ ion in the cell is maintained indefinitely without energy consumption at all. (Knowingly or otherwise, the authors of this idea were in fact engaged in yet another effort of designing a perpetual motion machine which can perform work without spending energy. Since no such perpetual machine exists in the inanimate world, the introduction of the non-energy-consuming Na pump idea represents a return to Vitalism once flourishing in the 17th and 18th century and long since abandoned).

The foundation for this non-energy consuming Na^+ pump theory was the belief once held by some biochemists: energy released in dissipating an ion concentration gradient could be used to regenerate ATP from ADP. This concept has since then been unequivocally disproved by Kanazawa (Seikagaku 44: 323, 1972), Boyer et al. (in Biochemistry and Biophysics of Mitochondrial Membranes, Azzone et al., eds. Academic Press, N.Y., 1972, p. 343), Masuda and deMeis (Biochem. 12: 4581, 1973), Taniguchi and Post (J. Biol. Chem. 250: 3010, 1975) and Knowles and Racker (J. Biol. Chem., 250: 1949, 1975). Unanimously they had shown that the dissipation of an ion gradient does not generates ATP (for further details, see Ling, 1984, p. 514). Nonetheless, the idea built on this disproved idea lives on.

In fact before my first monograph was in print in 1962, it became obvious that the above-described estimates of the energy need of the Na^+ pump have grossly underestimated the disparity between the minimum energy need of the Na^+ pump and the maximum energy available.

First Podolsky and Morales (J. Biol. Chem. 218: 9845, 1956) and then George and Rutman (Prog. Biophs. Biophys. Chem 10: 1, 1960) separately but quite conclusively showed that ATP, ADP and CrP do not carry large packages of utilizable free energy for biological work performance in their phosphate bonds. Yet virtually all the available energy I counted on in my calculation was based on the assumption that there were large stores of utilizable free energy in these phosphate bonds. When these erroneous assumptions are corrected, the calculated ratio of the maximum available energy over that of the minimum nervy needed would dwarf the earlier 1500% to 3000% figures.

Furthermore, so far we have only considered pumps located at the plasma membranes. Solute distribution across the membranes of subcellular particles also tends to be asymmetrical and requires the installment of additional pumps and they were. Furthermore, the energy need of a pump varies with the membrane surface area and the surface area of the subcellular particles are as a rule larger than the plasma membrane. Thus the surface area of

the sarcoplasmic reticulum in muscle has been estimated at 50 times that of the plasma membrane (Peachey, J. Cell Biol. 25: 209, 1965). Thus it is not unlikely that the energy need for each subcellular particle-membrane pump may exceed the corresponding pump at the plasm membrane by a large factor.

Above all, the reader must realize that the Na^+ pump is but only one of the many pumps already postulated (see Ling et al., Ann. N.Y. Acad. Sci., 204: 6, 1973, p. 9 for a list compiled in 1968) and the vastly greater number of pumps that must be, but not yet postulated. Recent investigation of the cellular distribution of many "exotic" water-soluble organic compounds (Ling, 1990, Figure 5.8B) showed that even though it is unlikely that the genome of the animal has ever been exposed to these exotic chemicals, they as a rule also maintain intracellular concentrations different from that in the external medium and therefore require the presence of membrane pumps. The list of new pumps to be postulated is therefore an open-ended one, since more and more new water soluble compounds are going to be synthesized with no end in sight.

B.L. Gupta: Furthermore, your own hypothesis now also required vast quantities of ATP. How do you reconcile these apparently conflicting views?
Author: In the AI hypothesis, ATP functions as a cardinal adsorbent. In that role ATP resembles other cardinal adsorbent such as Ca^{++} or acetylcholine. The primary function of ATP is controlling the electron distribution in cell protein molecules and maintaining the cell proteins at the high (negative) energy-low entropy resting living state. Serving this role does not require the (continual) hydrolysis of ATP. Therefore in my hypothesis, no "vast quantities of ATP" has ever been required to maintain the cell at the living state. Only a few millimoles of ATP are needed to serve its vital function by its adsorption as such, without breaking down and resynthesized continually. Hydrolysis of ATP occurs only at the time when it benefits the cell by (temporarily and reversibly) removing the ATP as during certain functional activities of the cell.

B. L. Gupta: Other workers who still believe in membrane phenomena have also noted anomalies in the original Hodgkin and Keynes argument ... but their interpretations include the per-axonal structures ... Similarly you do not refer to the work of Richard Adrian ... on the sartorius muscle of frog. Please comment.
Author: Hodgkin and Keynes in their original 1953 work believed that they had demonstrated the rate of diffusion of K^+ in isolated squid axons to approximate that of K^+ diffusion in sea water. As I pointed out in the text, their criterion for insuring the health of the axoplasm was faulty. In well preserved muscle cytoplasm, the K^+ mobility Ling and Ochsenfeld measured is 8 times slower than in an isotonic KCl solution. This slow rate of diffusion in normal frog muscle can be made to approach the faster diffusion in a dilute KCl solution if the muscle cell is injured or killed by metabolic poisons. Frequent consultations of the Citation Index (and other similar media) have reassured me that except one short query by Kushmerick which was promptly answered (Ling, Trends Biochem. Sci.Lett.Ed. 4: N134, 1979), none challenged our conclusions.

I was not aware of the others' criticism of the work of Hodgkin and Keynes, nor of the work of Richard Adrian you referred to.

B. L. Gupta: Heterogeneous injury to different cells by intracellular microelectrodes ... is not the only explanation for a large range in previously reported K-activity coefficient inside living cells. Apart from the technical imperfections in microelectrodes in a lot of this early work, there was the primary problem of obtaining reliable estimates for the total K (and other ions) inside the cells. The electron probe microanalysis of cryosections have remedied this saturation. Where ever both sets of data are available, the intracellular activity coefficients for the monovalent ions appear to be around 0.5. Why not consider this more recent evidence from a number of labs?
Author: As mentioned above, the K^+ mobility measured in a region of a frog muscle cell near a razor-blade cut is faster than in a region farther away from the cut end. In fact, under proper illumination the slowly spreading deterioration of the muscle cytoplasm created by the amputation is visible as a spreading cloudiness of the normally clear cytoplasm. That a cut inflicted on the cell by a microelectrode in not fundamentally different from the injury inflicted by a razor blade cut has been also demonstrated by Edelmann (Scanning Microsc. 3, 1219, 1989).

With this fact established, one must recognize that the concept of measuring normal K^+ activity in a living cell is faulty in its very inception. The sensing surface of the electrode never "sees" the K^+ activity in the normal cytoplasm away form the electrode. In stead, it only "sees" the K^+ concentration in the thin fluid film gathering around the electrode surface, being an exudate from the cytoplasm torn and damaged by the very same intruding electrode.

Dispersive microprobe analysis can indeed make more accurate measurement of

the total K^+ concentration in isolated, small and inaccessible cells, especially in tissues containing a mingled conglomeration of different types of cells and non-cellular tissues. But it has its own special problems (e.g., difficulty in assessing the contribution of extracellular fluid contribution); and offer no better accuracy for tissues containing a uniform population of similar cells. But even in the case where dispersive X-ray microanalysis is superior, that advantage cannot compensate for the inherent faultiness of the K^+ activity measured with the intracellular microelectrode. Reinforcing one or more strong links in a chain containing one fatally weak link adds nothing the strength of the whole chain.

B. L. Gupta: You say that hemoglobin-free (white) RBC ghosts (properly sealed) do not accumulate K in the presence of ATP etc. However, the process of reconstitution with excessive washing can a) damage the membrane skeleton and b) may lead to inside-out ghosts which would not accumulate K anyway. Furthermore, isolated primary secretive pumps (H^+, K^+/H^+, Na^+/K^+, Ca^{2+}) have been reconstituted in pure lipid bilayers vesicles ("liposomes") which have no enclosed proteins. These vesicles do accumulate respective ions as expected, leading to the saturation of electromotive force of the pump, unless a parallel leak is provided. Please comment.

Author: The criticism of possible damage in preparing white ghosts by exhaustive washing does not apply to the data I mentioned in the text. All the ghosts under discussion were prepared by exactly the same mild procedure with no excessive washing involved. Yet the amount of hemoglobin left in the resealed ghosts prepared from blood of different healthy donors varied widely. And there was a quantitative relationship between the K^+ reaccumulated and Na^+ extruded on the one hand, and the amount of hemoglobin retained in the resealed ghosts on the other (for details, see Ling et al, 1984, Figure 5). In resealed ghosts containing no or nearly no hemoglobin, reaccumulation of K^+ was not observed.

Claim had been made that ATP energized transport of Na^+ in inside-out synthetic lipid vesicles containing isolated Na, K-activated ATPase, an enzyme widely believed to be either a part of, or the entirety of the postulated Na pump. This claim was not justified as pointed out by Ling and Negendank in a comprehensive critical review on this and other claims of similar nature, entitled: "Do Isolated Membranes and Purified Vesicles Pump Sodium? A Critical Review and Reinterpretation" (Persp. Biol. Med. 23: 215,1980). The claim turned out to be based on results arising largely from an artifact. Briefly, the apparent difference seen in the concentrations of labelled Na^+ found in the ATP-treated vesicles in comparison with those found in control vesicles not treated with ATP was due to the different amounts of labelled Na^+ that had leaked out from the vesicles during the passage of the vesicles through the Sephadex column--a step needed to separate the fragile labelled Na^+-loaded vesicles from the incubation solution providing the source of the labelled Na^+. (For full detail, see Ling and Negendank, 1980, p.226-236).

For a full critical review of Mitchell's Chemiosmotic hypothesis, which Dr. Gupta seemed to have accepted, and serious errors in the key concept of protomotive force etc., see Ling (Physiol Chem. Phys., 13: 29, 1981, p.33-44.)

B. L. Gupta: The evidence for a non-uniform distribution of Na, K, Cl in the cytoplasm does not come only from your and Edelmann's work on muscle. The earliest microprobe work on frozen-hydrated sections in Cambridge Biological Microprobe Laboratory shows this in insect epithelia (Gupta et al., 1976, Nature 264: 284; Gupta et al., 1977, in "Transport of Ions and Water in Animals" [eds. Gupta et al.], pp. 83-143, Academic Press; Gupta and Hall 1979, Fed. Proc. 38: 144-153) and in rabbit ileum enterocytes (Gupta et al, 1978, Microscopica Acta, Suppl. 2, p 46-64). Additionally, the microprobe work also shows the selective adsorption of K and Ca over Na (and Cl) outside the cells in the pericellular mucoids such as glycocalyx, basement membranes etc. (Gupta 1989 in "Electron Probe Microanalysis" [Eds. Zierold and Hagler], Springer Verlag, Berlin pp. 199-212). You do not discuss any of this and other similar work. Please comment.

Author: I am delighted to learn that you have also discovered localized distribution of K^+ in the cytoplasm of living cells and truly regret that I did not know about it until now. However, neither Dr. Edelmann nor I myself claimed that we were the first to have made such demonstrations. Indeed, in my first publication alone (Ling, Physiol. Chem. & Phys., 9: 319, 1977) I cited no less than 10 earlier references of earlier demonstration of localized distribution of K^+ in living cells including the work of Macallum dated 1905.

Additionally, the specific purpose of the work of myself and of Dr. Edelmann was more than just demonstrating localized distribution of cell K^+. Our work was designed to test a specific localized distribution that was predicted by the AI hypothesis: most of the cell K^+ or its surrogate Cs^+ and Tl^+ ions should be found in the A bands of the striated muscle.

In the AI hypothesis, K^+, Cs^+ and Tl^+ are adsorbed on protein carboxyl groups and prior examination of the concentra-

tions of glutamic and aspartic acid residues of all the muscle cell proteins by Ling and Ochsenfeld (J. Gen. Physiol., 49: 819, 1966, p. 841) showed that 65% of the β- and γ-carboxyl groups carried on the side chains of these amino-acid residue are found in myosin. Now, myosin has long been known to be found exclusively in the A bands (Englemann, Pfluegers Arch. 7: 155, 1873). Hence the predicted concentration of muscle cell K^+ in the A bands of striated muscle.

B.L. Gupta: Your argument on the voluntary muscle is faulted. I assume that the membrane around the intact part of the muscle was healthy (not dried in air) and the external glycosaminoglycan material was intact. If not this preparation is as moribund (and therefore irrelevant) as the squid axon that you deride. If intact and healthy, then the "sink" and "source" of Na and K respectively could be the extracellular matrix, with ion recycling across the cell membrane through the ion-pump and leak channels. How can you discount that? Was the EM structure of this muscle preparation examined?

Author: You have perhaps unintentionally misrepresented me by stating that I have derided Hodgkin and Keynes on their work on the squid axon. To deride means to laugh at contemptuously. I do not knowingly deride anyone.

The air phase in contact with the intact portion of the sartorius muscle in the Effectively Membrane-pump-less Open Ended or EMOC preparation was fully humidified. The muscle did not lose weight and were otherwise in fine condition.

In frog muscle, the only intracellular space that is directly linked to the outside is the T-tubules which occupies a very minute part of the cell volume (0.2% to 0.4%) (Huxley, Nature 202: 1067, 1964; Hill, J.Physiol. 175: 275, 1964; Peachey, J. Cell Biol. 25: 275, 1965).

Figure 3 shows the progressive accumulation of labelled K^+--to concentration exceeding that in the source solution--in the part of the muscle suspended in air and away from the cut end. In the meanwhile, radioactively labelled Na^+ in the same region remains far below the concentration of that ion in the same source solution.

Part of this difference seen in the amount of labelled K^+ and labelled Na^+ accumulated in the intact region of the muscle could be attributed to the different rates of inward diffusion. The diffusion coefficient of K^+ in normal frog muscle cytoplasm has been found to be 1.27 times higher than that of Na^+. This difference, however, could be exactly annulled by increasing the incubation time for Na^+ to 1.27 times longer than that for K^+.

Now the 3 sets of data shown in Figure 3 from left to right correspond to incubation time of 14.7, 38.7 and 63.0 hours respectively. Since 63.0/38.7 = 1.63 and 38.7/14.7 = 2.63, one would have more than compensated for the diffusion-rate bias by comparing the K^+ profile of the 14.7 hour experiment with the Na^+ profile of the 38.7 hour experiment or by comparing the 38.7 hour K^+ profile with the 63 hour Na^+ profile. In either case, the much greater gain of K^+ than Na^+ remain essentially unchanged.

Theoretically it is conceivable that Na^+ pumped out of the cells in the region where the muscle is suspended in air to find its way back to the Ringer solution bathing the cut end of the muscle. For the sake of argument, let us assume that this was actually what happened. Then by comparing the 14.7 hour K^+ profile and the 38.7 hour Na^+ profile we can calculate just how much labelled Na^+ has been pumped out of the normal membrane in the intact region of the muscle and found its way back to the source Ringer solution bathing the cut end. This turned out to be 4.12 micromoles in 38.7 hours.

If one assumes that much labelled Na^+ had indeed been pumped out of the muscle cells in the intact region, by what mechanism(s) did the labelled Na^+ delivered to the extracellular space find its way back to the Ringer solation bathing the cut end of the muscle?

Since EMOC preparation produced the same results regardless of whether one cuts off the tibial end and suspend the intact pelvic end of the muscle in the air or vice versa, there is obviously no Na^+ pump propelling Na^+ ion in the extracellular space from the pelvic to the tibial end or from the tibial end to the pelvic end.

Without a propelling pump, the only way that the labelled Na^+ that had been pumped into the extracellular space could find its way back to where it came from would be by diffusion. However, there cannot be a net movement of Na^+ by diffusion if there is no diffusion head. In fact one could easily calculate just how high the concentration of labelled Na^+ must be in the extracellular space of the intact end of the muscle, if it is to deliver 4.12 micromoles of labelled Na^+ to the source solution in 38.7 hours. This turned out to be 5.75 M!

At that time, I have evolved a simple and accurate method for determining both the volume of the fluid in the extracellular space of the EMOC muscle preparation and the concentration of labelled Na^+ in that fluid. With this method I proceeded to analyze the labelled Na+ concentration after an even longer period of incubation (55 hours). From 16 experiment performed, we obtained an extracellular space volume

equal to 9.4% ± 0.72% of the total cell volume, which is not significantly different from the extracellular space volume of normal frog sartorius method, i.e., 9%. I also found a labelled Na^+ concentration in the extracellular fluid collected from the extracellular space of the EMOC muscle equal to 95.9 ± 5.3 mM, which is slightly less than the labelled Na^+ concentration in the Ringer solution used.

In other word, there was no diffusion head or concentration gradient to have propelled any significant amount of labelled Na^+ from the extracellular space in the intact part of the muscle back to the source Ringer solution. Indeed, there was strong evidence that no extra Na^+ was pumped into the extracellular space at all. Were it otherwise, the extra Na^+ thus accumulated in the extracellular space would have made the fluid in the extracellular space fluid hypertonic. This hypertonicity would in turn draw water from the cells into the extracellular space, increasing its relative percentage volume. In fact, the extracellular space percentage volume remained quite normal. I conclude that we were wrong in assuming that labelled Na^+ were pumped from the cell to the extracellular space here, and by inference in normal muscle and non-muscle cells.

B.L. Gupta: You say that preparation of frog sartorius muscle with cut ends deteriorates rapidly in normal Ringers (original Ringers was for the intact frog's heart). But this Ringers is for the extracellular fluid and has high Na and Cl (about 100 mM each). Both will now have access to the cell-cytoplasm through the cut ends and both will influence the native conformation of proteins. Gibbons et al (1985, J. Cell Biol. 101, 1281) have shown that the ATPase activity of the protein dynein is activated some 10X in the presence of 150 mM of Cl as compared with the normal 30 mM Cl found; inside the cell. So what is the relevance of this pH 4 experiment in an otherwise totally unphysiological ionic conditions for the cytoplasm of a living cell?

Author: It is true that in a normal frog Ringer solution, 2 mm and 4 mm wide cut frog muscle segments rapidly deteriorate and lose their ability of accumulating alkali metal ions. But the experiments on the cut muscle segments we studied (described in Figure 9) were not conducted in a normal frog Ringer solution.

In stead, they were kept in a highly special incubation solution in which the 100 mM of Na^+ and Cl^- were replaced by an osmotically equivalent amount of the ingredient referred to as in the text as the additive. The additive is polyethylene glycol (molecular weight 8000, PEG 8000), used at a concentration of 16.4 %, which produces an osmotic pressure equal to that of an isotonic NaCl solution. In a Ringer solution, containing as its main osmotic ingredient PEG 8000, the muscle retains a major part of its ability of accumulating alkali-metal ions (see Ling, Physiol Chem. Phys. & Med. NMR 21: 13, 1989).

Dr. Gupta asked "So what is the relevance of this pH 4 experiment in an otherwise totally unphysiological ionic condition for the cytoplasm of a living cell?"

To answer, I quote a passage from the classical monograph, "Enzymes" by Malcolm Dixon and Edwin C. Webb (Academic Press, 1958, p. 3): "The early work on enzyme purification met with a good deal of criticism on the ground that it was 'unphysiological' to separate enzymes from cells and that only work on undamaged cells was valuable".

Would Dr. Gupta make similar objections to someone studying isolated enzyme proteins today? Or even determine the pH optimum of the activity of the isolated enzyme and in the process expose the isolated enzyme proteins to pH 4 or even lower? I don't think that he would. If this guess is correct and Dr. Gupta indeed sees nothing wrong in someone studying an isolated enzyme protein at a pH of 4, may I then ask, Why should Dr. Gupta then object to our studying the muscle cytoplasmic proteins at a pH of 4, when I did not even take these muscle proteins out of the muscle cells as the enzyme chemists routinely do?

A great wealth of information has been obtained from the in vitro studies of isolated enzymes. A great deal of more information will one day come from "permeabilized cells" of which the cut muscle segment nurtured in a solution containing PEG 8000 is a highly successful example.

B. L. Gupta: You have made a categorical statement implying that most if not all the cell-K is adsorbed on the cell-proteins in all cells. Your own arguments are essentially based on the study of striated voluntary muscle.

Author: Dr. Gupta is right in pointing out that most of my published work was on the study of frog voluntary muscle. However, I did not study only frog muscle. My other favorable cells include the human erythrocytes, frog ovarian eggs, 16 strains of mouse and rat cancer cells, E. coli, mouse and rat liver cells, frog nerves, frog brain, frog erythrocytes, ox retina, guinea pig smooth muscle (taenia coli), frog oviduct (gravid and non-gravid), frog kidney etc. From the study of these different tissues one finds that basically their general physiological properties are remarkably alike (see Ling 1984, Table 11.4, for example).

However, I would venture to say that even if I had studied only one cell type, and if I was lucky enough to have made a

correct set of observations and from it, drawn a valid conclusion. I still stand a good chance of being right in generalizing to the extent I did in this presentation. Witness the following:

Gregor Mendel discovered what is now known as the Mendelian Laws of Inherence. His discovery was based on experiments conducted on one life form, the garden peas. If Mendel had considered that his finding was of broad and general significance, we now know that he would be not too far from being totally right.

The spectacular further progress in what is now known as the molecular biology (of the gene) was based also on studies on one living form, the bacteria E. coli. Twenty eight years ago, Nobel laureate Jaques Monod and his coworker F. Jacob had, you may say, anticipated the question put to me today: To wit, Can you generalize about fundamental physiological mechanisms on the basis of the study of one living form only? Monod and Jacob's answer, with which I agree, was: "...anything found to be true of E. coli must also be true of Elephants." (Monod and Jacob, Cold Spring Harb. Symp. Quant. Biol., 26: 389, 1961, p.393).

B.L.Gupta: These (muscle) cells are generously endowed with a high concentration of closely packed proteins filling up almost all the cell volume. Most other cell-types are not so "fortunate". Even by your own reasoning, how can these less-well endowed majority have enough concentration of β- and γ-carboxyl groups in their cytosol for adsorbing all the K? The vast majority also have the task of allowing large fluxes of Na^+ and Cl^- (and some cases K^+) through them for supporting the essential function of secretion and excretion in the body so that the muscle can function. Are you not falling into the same pit as the membrane wallas who too generalized the evidence from atypical cell-type such as the squid axons and the frog muscle?

Author: I disagree with Dr. Gupta's opinion that frog muscle is "generously endowed with a high concentration of closely packed proteins filling almost all the cell volume". On the contrary, frog muscle along with brain tissue, and (maximally deviated) ascites cancer cells are close to being in the middle in as far as its protein (18-20%) and water content (80%) are concerned (Ling and Ochsenfeld, 1966, op cit p. 841). Some normal tissues have water content as high as 85% and protein content as low as 15% (e.g., frog testes, frog brain). Other tissues like the human erythrocytes contain only 65% water and about 35% proteins; mature frog ovarian eggs contain only 51% to 52% water and some 40% proteins.

Frog muscle contains twice as many β- and γ-carboxyl groups (260 to 288 mmoles/kg fresh weight) than the maximum concentration of K^+ the muscle cells adsorb (140 mmoles/kg) (Ling and Ochsenfeld 1966, loc cit). In terms of the number of carboxyl groups alone, other tissues with as little as 15% protein could still be able to adsorb the same concentration of K^+ per kg of fresh weight as the frog muscle. Nor is there evidence that all living cells must contain the same concentration of cell K^+. Some other cells with less cell proteins may simply have less cell K^+.

There is no conflict between the large fluxes of Na^+ and Cl^- through the (bifacial) epithelial cells or in and out of (unifacial) cells and the high concentration of β- and γ-carboxyl groups both needed and are present in the cells to adsorb K^+. These two events (K^+ adsorption and Na^+ and Cl^- fluxes) are by and large separate. K^+ occupy β- and γ-carboxyl groups. Intracellular Na^+ and Cl^- traverse through and occupy space largely in the cell water.

I do not think that it was the choice of squid axons and frog muscle as their experimental materials, that led what you call the "membrane-walla(h)s" to fall into their self-made pit. Their failure came largely from an incapability or unwillingness to follow the Scientific Method including the unspoken rule: think in terms of all known hypothesis and not just one currently popular, and when the existing evidence argue against the popular hypothesis, to turn to alternative hypotheses regardless of their current popularity or lack of it. After having said this, I must add, not without sympathy, that sometimes it may be extremely difficult to follow these simple paths.

B. L. Gupta: Was the concept of intracellular "fixed" anions and the structured water ever completely abandoned by all biologists? JD Bernal strongly argued in support of both (Symp Soc. Exp. Biol. 19: 17-32, 1965). The problem essentially was of producing the evidence. The measurements with the radioisotope fluxes etc, including the ones in your experiments, are always open to alternative explanations. Please comment.

Author: There are two comments I will make: one directed at what the words of the question seem to indicate; the other directed at the first sentence specifically.

The words of this question seem to say that (1) that the concept of intracellular "fixed" anions and the structured water seems completely abandoned by all biologists, and (2) that this global rejection of the concept followed from the lack of truly supportive evidence, since existing supportive evidence from my and

other laboratories came from the studies of radioisotope fluxes; and (3) evidence from the studies of radioisotope fluxes are always equivocal.

There is substance in what is said in (1) and this will be addressed below. On the other hand, (2) is simply incorrect. Decisive evidence for the intracellular fixed anions and structured water concepts do not involve studies of radioisotope fluxes, as this paper and others published on this topic and on "The State of Water in the Cell" (published in Scanning Microscopy by the Scanning Microscopy International) testify to.

Nor are studies with radioisotopes always equivocal as in (3). As an example, it was the study of radioisotope flux into dog erythrocytes which proved that the cell membrane is permeable to Na^+ (Cohn and Cohn, Proc. Soc. Exp. Biol. Med. 41: 445, 1939). How can this history-making, repeatedly confirmed, important experimental result be considered equivocal?

On the other hand, Dr. Gupta aptly raised a serious question in (1). To comment on them meaningfully I have to go back to history.

In 1951 and 1952 I first introduced the hypothesis (known as Ling's fixed charge hypothesis) that intracellular fixed anions in the form of protein carboxyl groups selectively adsorb K^+ over Na^+. Ten years later this nuclear concept evolved into the association-induction (AI) hypothesis. Along with the decisive experiments against the membrane pump theory, the AI hypothesis was published in my first monograph, "A Physical Theory of the Living State: the Association-Induction Hypothesis" (Ling, 1962). In 1965, the polarized multilayer theory of cell water, in which all cell water is "structured", was introduced, completing the AI hypothesis.

Unlike the ad hoc piecemeal membrane pump theory, the AI hypothesis has been a self-consistent coherent theory from its very beginning. It is largely built on concepts and laws of modern physics and its major postulates have been verified in studies on inanimate models. The AI hypothesis offers a unified theory interpreting all major aspects of cell physiology proper.

In 1984, I published a second book, "In Search of the Physical Basis of Life" describing the extensive relevant experimental evidence accumulated world-wide during the preceding quarter of a century. The results of these efforts were overwhelmingly against the conventional membrane-pump theory and in support of the AI hypothesis.

After another six years of intensive studies, I completed the writing of yet another (smaller) book, "A Revolution in the Physiology of the Living Cell". This volume will be published by Robert Krieger Publ. Co., Melbourne, FL, early in 1990, presenting formally and in detail the disproof of the membrane-pump theory and the establishment of a new paradigm.

In contrast, in the course of its 150 year long history, there has never been published in a single volume presenting the entire membrane pump theory. I believe that the lack of internal coherence and enduring confirmational evidence for the membrane-pump theory and the abundance of evidence against it do not permit the publication of such a volume.

Clearly, the lack of broad acceptance of the "intracellular fixed anions and structured water" concepts introduced in the AI hypothesis (and the continued teaching and subscription to the membrane pump theory) have nothing at all to do with a shortage of supportive experimental evidence for the AI hypothesis.