

The Physical State of Potassium in Frog Skeletal Muscle Studied by Ion-Sensitive Microelectrodes and by Electron Microscopy: Interpretation of Seemingly Incompatible Results

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Abstract: According to the commonly accepted membrane pump theory most of cellular K^+ ions are freely dissolved in free cellular water; the alternative association-induction hypothesis postulates that the bulk of cellular K^+ is adsorbed (weakly bound) to cellular proteins that are maintained in a specific labile state in the cytoplasm of a living cell. K^+ activities measured with ion-sensitive microelectrodes in the cytoplasm of frog skeletal muscle seem to confirm the claim that most of cellular K^+ ions are free in cellular water. On the other hand, it is evident from electron microscopic ion binding studies that in frog skeletal muscle most of cellular K^+ ions are adsorbed to cellular proteins. The conflicting results can be explained with the assumption that a damage of the cytoplasm caused by the impaling microelectrode leads to a liberation of adsorbed ions. Using the light microscope tests the possibility that microelectrodes damage the muscle cytoplasm. It is found that microelectrodes produce visible traumas that increase with time. Electron microscopic ion binding studies with damaged muscle support the view that monovalent cations are liberated in the disturbed area of a muscle fiber. It is concluded that a K^+ -sensitive microelectrode is not suited to determine the concentration of free K^+ ions in intact frog skeletal muscle.

KEY WORDS: Membrane pump theory, association-induction hypothesis, potassium binding, ion localization, ion-sensitive microelectrodes, striated muscle, freeze-substitution, low temperature embedding.

Introduction

THE commonly taught membrane pump theory (MPT) is based on the assumption that most cellular water and ions exist in a physical state that is essentially a dilute aqueous solution. According to an alternative theory, the association-induction hypothesis (AIH) of Ling [31, 38] the cell represents a metastable cooperative protein-ion-water complex. Proteins, water and solutes exist in a physical state different from that of an aqueous protein salt solution. In particular, it is assumed that most of the cellular K^+ ions are adsorbed to β - and γ -carboxyl groups of cellular proteins and that the bulk of the cellular water is differently structured than extracellular free water. This controversial issue is addressed in a recently published book entitled, "The State of Water in the Cell" (W. Negendank, L. Edelman, (eds.), Scanning Microscopy Intl., AMF O'Hare, Chicago 1988, 1–113). In criticizing the predominance of the AIH throughout this book a reviewer wrote the following [49]: "It is amazing that, at a time where ion selective microelectrodes can directly measure free ion contents and find a majority of potassium to be in free form, Ling's hypothesis is endorsed and tacitly expanded to a generalized hypothesis by some of the contributors without major objections".

It remains, however, an open question as to what extent ion-sensitive microelectrodes are suited to detect free and adsorbed K^+ ions in living cells. According to the AIH the cytoplasm is a highly sensitive protein-ion-water system that is disturbed by an impaling microelectrode. As a consequence K^+ ions may be liberated from adsorption sites and the microelectrode is then detecting artefactual high K^+ ion concentrations [38, 47]. So far this argument is not widely recognized; and it is usually believed that a K-sensitive microelectrode impaled into the cytoplasm is detecting the same concentration of free K^+ ions as it exists in intact cells, provided the microelectrode is perfectly sealing the punctured cell membrane [17, 56].

It is evident that the above-mentioned critique is justified if it is proven that in first approximation the cytoplasm is an aqueous protein salt solution as described by the MPT. In the author's opinion such proof is missing. On the contrary, recent electron microscopic studies with frog skeletal muscle have led to the conclusion that most of cellular K ions are adsorbed to cellular proteins of the muscle — a conclusion opposite to the expectations of the MPT [11, 14] and also opposite to conclusions derived from studies with K-sensitive microelectrodes [26–30, 56]. Because of this conflict which eventually may be solved by interpretations provided by the AIH new experiments were devised to test the AIH and to answer the following questions: 1) Do microelectrodes disturb locally the cytoplasmic structure in skeletal muscle of the frog. 2) Is it possible to detect by electron microscopic methods a liberation of alkali-metal ions from adsorption sites of the sarcoplasm, which has been mechanically injured?

In order to enable the reader to understand why the AIH is chosen as a working hypothesis an Appendix is presented which contains a critical comparison between AIH and MPT.

Materials and Methods

Experiments with microelectrodes

Single intact skeletal muscle fibers were prepared at room temperature from semitendinosus muscles of Northern American leopard frogs (*Rana pipiens pipiens*, Schreber). The *caput posticum* of a semitendinosus muscle was fixed on a plastic frame kept in Ringer's solution in a Petri dish. Under the control of a stereo light microscope most of the fibers were cut and removed in such a way that only a few single fibers remained fixed at the tendons of the muscle. During the dissection the Ringer's solution was frequently changed and before and after the microelectrode experiment (see below) the single fibers were monitored for viability by electrical stimulation. The frame with the mounted single fibers was transferred (still in the Petri dish) into a small container the bottom of which was made of a glass slide normally used for light microscopy (Figure 1a). This container was then transferred to a light microscope (Zeiss, Standard 14) and the fibers were inspected with phase contrast optics (objective: Ph2, Neofluar 40/075). Glass microelectrodes with a tip diameter of about 0.3 μm were drawn on an electrode puller and mounted to a micromanipulator. Under visual control a microelectrode was inserted into a single muscle fiber (Figure 1b). Photographs were taken after different lengths of time. During the experiment the Ringer's solution bathing the muscle fibers was constantly renewed as shown in Figure 1b. Altogether 10 different single fibers were impaled with microelectrodes. In some experiments the tips of the microelectrodes were sealed with molten wax; these electrodes produced the same results as electrodes with open-ended tips.

Experiments with freeze substituted and low temperature embedded muscles

Under sterile conditions frog sartorius muscles (*Rana pipiens pipiens*, Schreber) were incubated at room temperature for 4 days in K^+ -free Ringer-GIB medium [40] containing 2.5 mM Cs^+ as described by Ling and Bohr [41]. About 80% of cellular K^+ was then replaced with Cs^+ [6]. A muscle was transferred to a humidity chamber (100% humidity) in which adhering fluid was gently removed with a wet filter paper. The muscle was cut with

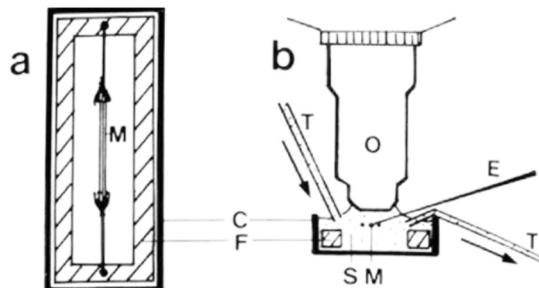


FIGURE 1. Muscle preparation for microelectrode experiments. (a) Top view of a plastic frame F with mounted single fibers M in a small container C. (b) Cross-section of container C with muscle preparation under the objective O of a light microscope; a microelectrode E is inserted into a muscle fiber. Arrows indicate a flow of Ringer's solution S through stainless steel tubes T into and out of container C.

a razor blade perpendicular to the direction of the muscle fibers. After 30 min the two pieces of the cut muscle were rapidly frozen as described elsewhere [15]. The 30 min wait between cutting and freezing was arbitrarily chosen to produce a visible damage of the muscle cytoplasm at the cut end (see Discussion). Frozen muscle pieces were freeze substituted in pure acetone at -80°C for one week and low temperature embedded at -60°C in Lowicryl K11M as described elsewhere [15]. Intact K^+ -containing and Cs-loaded muscles were cryofixed, freeze substituted and low temperature embedded in the same way. Diatome diamond knives were used for obtaining ultrathin, $0.1\ \mu\text{m}$ thick and $0.2\ \mu\text{m}$ thick wet-cut sections, glass knives for $0.3\ \mu\text{m}$ thick dry-cut sections. Ultrathin sections were stained with uranyl acetate and lead citrate. $0.1\ \mu\text{m}$ thick and $0.2\ \mu\text{m}$ thick sections were exposed to a staining solution containing 100 mM LiCl and 10 mM CsCl as described elsewhere [11].

Results

Typical results of the microelectrode experiments are shown in Figure 2. The impaled fibers show a spreading disturbance of the muscle structure. After 30 min (Figures 2f, h) the disturbed area has a diameter of several sarcomeres. Specific features of the artifacts produced by the microelectrode are:

- 1) The impaled muscle fibers show signs of local contraction (e.g., Figures 2d, e).
- 2) At the place of the impalement one observes a local swelling of the disturbed area (e.g., Figures 2e, f, and h).

Figure 3 shows a typical result of a $0.1\ \mu\text{m}$ thick section stained with a solution containing 100 mM LiCl and 10 mM CsCl. The electron-dense Cs ions preferentially stain proteins of the A band and of the Z line. The ultrastructure of the muscle cannot be seen in unstained sections (Figure 3b).

Results obtained with a cut sartorius muscle are shown in Figure 4. Muscle contraction can be observed near the cut end (Figure 4a) whereas most of the fiber appears in a perfect resting state (Figure 4b). In the undisturbed parts of the muscle the electron-dense Cs^+ ions are localized preferentially in the A bands and at Z lines as can be demonstrated with dry-cut sections (Figure 4c). For comparison see Figure 4d, which has been obtained from an intact K^+ -containing muscle and which shows a rather poor contrast between A band and I band regions. A dry-cut section of a contracted part of the cut sartorius muscle shows dark precipitates irregularly distributed over an almost homogeneous area, the periodicity of the contracted sarcomeres is barely visible (Figure 4e). Figures 4f and g show wet-cut $0.2\ \mu\text{m}$ thick sections "stained" with the LiCl-CsCl solution. The section obtained from the damaged part near the cut end (Figure 4f) appears almost unstained (no Cs^+ -binding) whereas the section of an intact area of the same muscle fiber (Figure 4g) is stained similar to the section shown in Figure 3a.

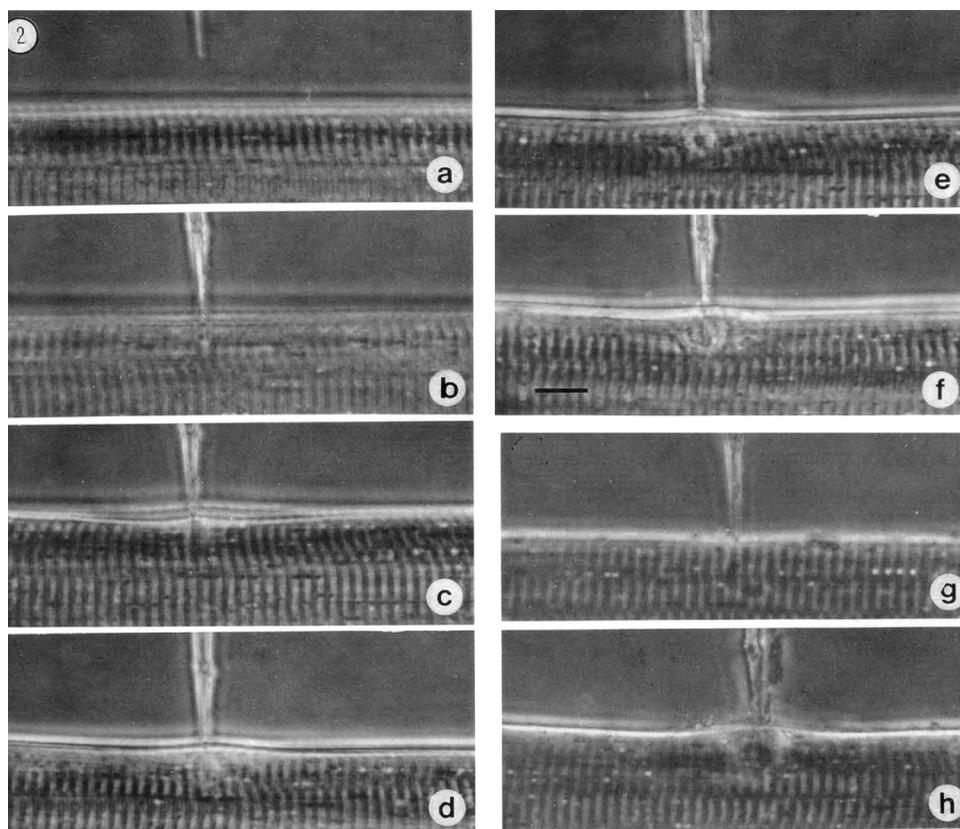


FIGURE 2. Light microscopic photographs of frog semitendinosus muscle fibers impaled with glass microelectrodes (**a**, **g**) controls, (**b**) - (**f**) after 1, 5, 11, 20, 30 min of insertion of the electrode into the muscle fiber shown in (**a**); (**h**) after 30 min of insertion of the electrode into the fiber shown in (**g**). Bar: 10 μ m.

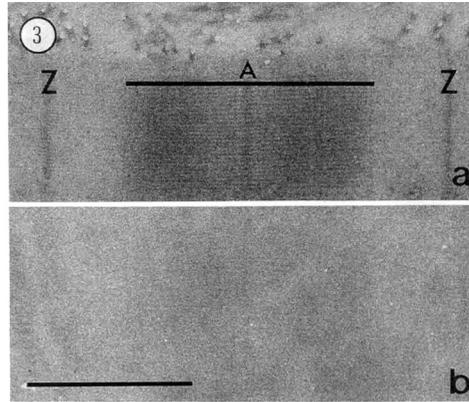


FIGURE 3. $\sim 0,1 \mu\text{m}$ thick *wet-cut* sections of frog sartorius muscles after freeze-substitution and low temperature embedding, (a) without any staining, (b) exposed to a solution containing 100 mM LiCl and 10 mM CsCl as described in [11] p. 884. Mainly myosin filaments of the A band (A) and proteins of the Z line (Z) bind the electron dense Cs⁺. Bar: 1 μm .

Discussion

Experimental determination of the physical state of K⁺ in living frog skeletal muscle

Results obtained with ion-sensitive microelectrodes. Experimental studies on frog skeletal muscle of Hill [22], Fenn [19] and Gersh [20] provided indirect evidence for the view that virtually all water and K⁺ ions exist in the free state in living cells. With the development of ion sensitive microelectrodes it was expected that free and bound cellular K⁺ could be determined unequivocally [18]. Experimental testing of nerve and muscle cells yielded the following results: the intracellular K⁺ activity agreed in first approximation with the intracellular K⁺ concentrations multiplied by an activity coefficient equal to that of K⁺ in an aqueous solution of ionic strength similar to that expected in living cells (e.g., [23, 28, 30]). The basic tenet of the MPT — *virtually all K⁺ is in free solution* — seemed to be confirmed. However, Ling criticized the results in the following way [33]: An ion-sensitive microelectrode can monitor only the ionic activity in a microscopically thin layer of fluid in immediate contact with the microelectrode tip. Thus even though the bulk of the cytoplasm may be in a perfectly good state of health and its K⁺ in a normal physiological state, the inserted ion-sensitive microelectrode cannot “see” that K⁺. It can only detect the activity of K⁺ in the microscopic portion of the cytoplasm that must have been forcibly torn apart to make room for the impaling ion-sensing electrode. The recorded activity is therefore that of a disturbed cytoplasm and not that of normal cytoplasm. In response to this argument Dick and McLaughlin [4] agreed that trauma could indeed liberate K⁺ ions but they pointed out that such liberated K⁺ would soon diffuse away while in actual measurements the K⁺ activity remained more or less the same for as long as 30 min. This view was not accepted because of the following two reasons [47]: 1) Experiments with K⁺ containing droplets injected into squid axons show that the diffusion of free K⁺ inside the cytoplasm is so slow that it can hardly be detected within 30 min. 2) The disintegration of the cytoplasm caused by the impaling electrode is spreading progressively to healthier regions during the experiment; thereby additional liberation of K is

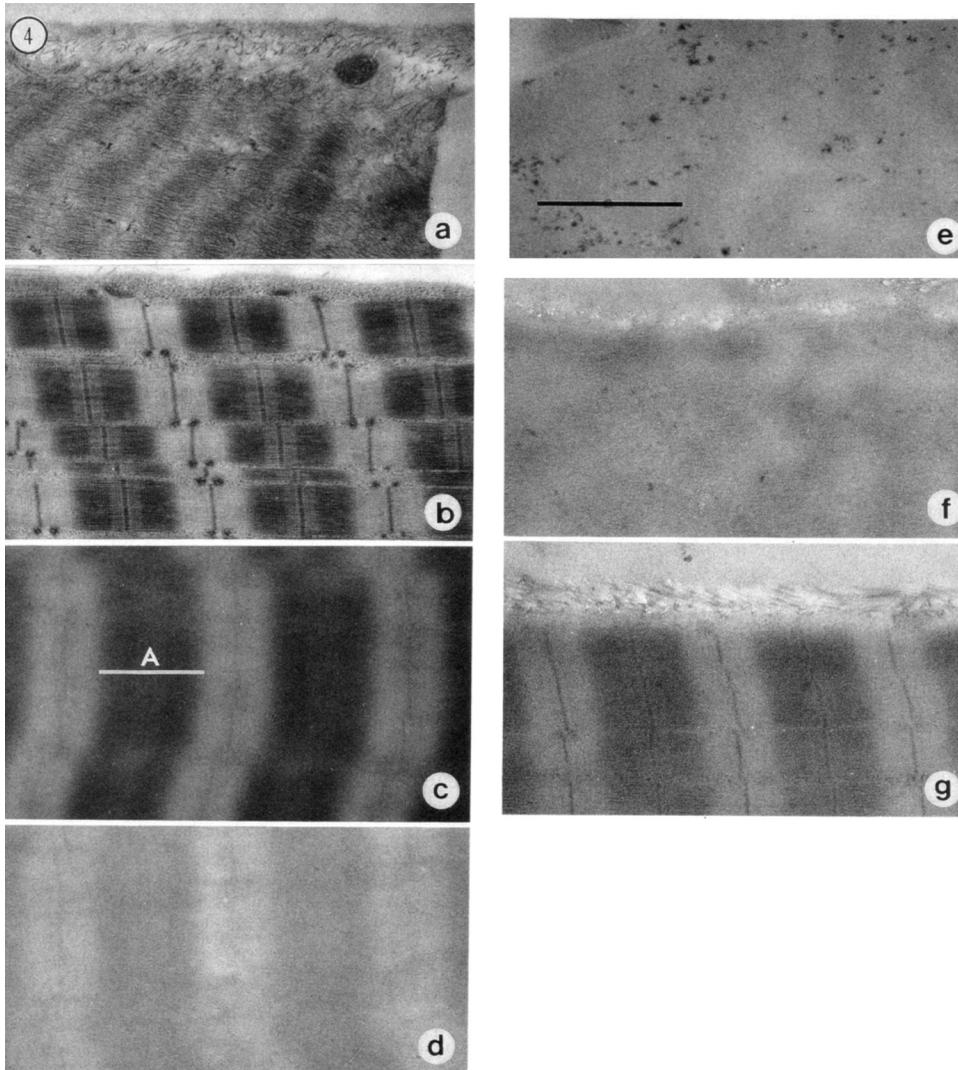


FIGURE 4. Cut frog sartorius muscles (loaded with the electron dense Cs^+ as described in the text) — after freeze-substitution and low temperature embedding.

(a) Ultrathin section stained with uranyl acetate and lead citrate showing the cut end of a muscle fiber (see text); (b) stained ultrathin section of the same cut fiber at a distance of about $0.4 \mu\text{m}$ from the cut end.

(c) $0.3 \mu\text{m}$ thick *dry-cut* section obtained from the same place as (b); the electron dense Cs^+ ions are mainly localized in the A bands (A); for comparison see (d) which shows a $0.3 \mu\text{m}$ thick *dry-cut* section of a normal K^+ containing muscle.

(e) $0.3 \mu\text{m}$ thick *dry-cut* section obtained from the same place as (a). Dark precipitates (most likely Cs^+ -containing precipitates) are irregularly distributed over an almost homogeneous area (f).

(f) + (g) $0.2 \mu\text{m}$ thick *wet-cut* sections exposed to a solution containing 100 mM LiCl and 10 mM CsCl as described in [11]; (f) obtained from an area near the cut end, (g) obtained from the same place as (b). The section shown in (f) is almost unstained, (g) shows a staining pattern similar to that shown in Figure 3a. Bar: $2 \mu\text{m}$.

to be expected. These arguments, however, were not further considered and Edzes and Berendsen wrote in 1975 [17]: “The experimental results point to little or no binding of the alkali cations and certainly not to a strong preferential K^+ binding”. This statement includes results obtained with K^+ -sensitive microelectrodes and frog skeletal muscle [26–30].

Results obtained with electron microscopic methods. Stimulated by the controversy between AIH and MPT electron microscopic experiments were designed to investigate the physical state of K^+ in living cells by methods that avoid mechanical disturbance of the cytoplasm. The idea was to test the following predictions of the MPT and the AIH concerning the localization of K^+ in the striated muscle: According to the MPT K^+ ions are freely dissolved in the free water of the striated muscle cells, and their localization follows the water distribution. Since the water content in the I band is higher than in the A band [25] the membrane theory would predict a higher amount of free K^+ ions in the I band than in the A band. The AIH, on the other hand, predicts a higher accumulation of K^+ in the A band compared to the I band because β - and γ -carboxyl groups are primarily found on myosin in the A bands [35]. These opposing predictions should also hold for the electron dense Rb^+ , Cs^+ , and Tl^+ as these ions accumulate in frog skeletal muscle by means of the same mechanism as K^+ ; they replace each other reversibly in a mole-for-mole fashion under physiological conditions [34, 41]. This implies that we can tackle the basic problem of cation binding in muscle with all four different cations. For example, if about 80% of cellular K^+ has been replaced by Cs^+ or Tl^+ , which are then found to be bound at cellular proteins, we must conclude that also K^+ is bound to the same proteins in a normal K^+ containing muscle.

Starting in 1976 several newly developed cryotechniques have been used to localize alkali-metal ions and Tl^+ in the striated muscle of the frog by electron microscopic methods. Either muscles with their normal K^+ content or muscles in which about 80% of the cellular K was replaced by Rb^+ , Cs^+ or Tl^+ have been investigated. The methods used include analysis of sections of freeze-dried [6] or freeze-substituted [14] and embedded muscle, autoradiography of frozen-hydrated single fibers using ^{86}Rb and ^{134}Cs [7], electron probe X-ray microanalysis of freeze-dried cryosections [10], and visualization of Tl^+ in frozen-hydrated cryosections [12], (for reviews see [11, 14]).

The main findings and conclusions of these studies are the following: In the normal K^+ containing frog skeletal muscle and in muscle loaded with the electron-dense surrogates Rb^+ , Cs^+ or Tl^+ the accumulated ions are preferentially localized within the A bands, especially at the 2 marginal regions and at the Z lines. These results fulfill the expectations of the AIH and are not in accordance with the predictions of the MPT. Of particular are the results obtained with frozen-hydrated preparations:

Autoradiographs visualizing the Cs^+ -distribution in a Cs^+ -loaded muscle cell (Figure 5a, b) show that the concentration of Cs^+ is low in the I bands and high in the A bands [7]. Since the concentrations of free Cs^+ in the cellular water must be equal in the A bands and in the I bands one can only conclude that the concentration of the cellular alkali-metal ion in the cellular water is low. 2). Micrographs of frozen hydrated cryosections of Tl^+ loaded muscle (Figure 6b) show that individual filaments (mainly myosin filaments in the A band, but also I band filaments) and Z line proteins are “stained” by Tl^+ [14]; this implies that most of the cellular Tl^+ ions are bound to proteins and not dissolved in the surrounding water: otherwise a very poor contrasting or even a negative staining of proteins

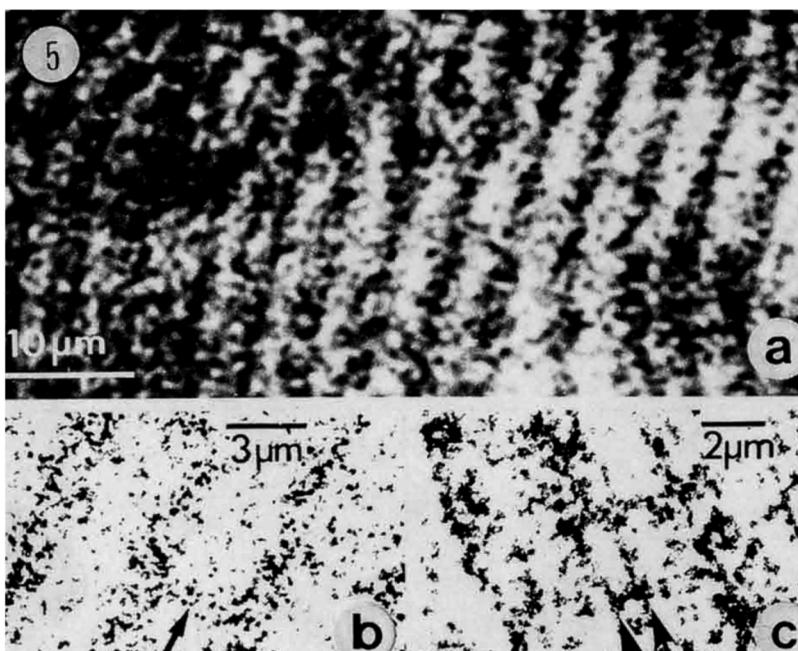


FIGURE 5. Autoradiographs of *frozen hydrated* frog muscle fibers, (a) Light microscopic ^{134}Cs autoradiogram of a stretched Cs^+ -loaded fiber, (b) Electron microscopic ^{134}Cs autoradiogram of a stretched Cs^+ -loaded fiber. The sarcomere length is about $4.4\ \mu\text{m}$. A line of silver grains between two dark bands (A bands) indicates the Z line (arrow). (c) Electron microscopic ^{86}Rb autoradiogram of a stretched Rb^+ -loaded fiber. The sarcomere length is about $3.3\ \mu\text{m}$. Arrows indicate dark lines at the outer edges of an A band. From [7], reprinted by permission.

would have to be expected. *Of utmost importance is the fact that these results have been obtained with fully hydrated cryosections.* The sections have been kept at about $-170\ \text{C}$ and photographed in a Zeiss EM 10CR at a magnification of 5000 (electron exposure below $1000\ \text{e}^-/\text{nm}^2$). No mass loss of the area irradiated during photographing could be detected. From these electron microscopic studies it was concluded that most of cellular cations are bound to cellular proteins. A recent quantitative X-ray microanalysis concerning the localization of K^+ in rat heart muscle cells led to a similar conclusion [55].

Explanation of seemingly incompatible results. The experimental findings presented in Figure 2 give an explanation for the conflicting results obtained with the described different techniques. This explanation is essentially that predicted by Ling *et al.* [47]: A microelectrode cannot be inserted into a muscle cell without disturbing the cytoplasmic ultrastructure in the vicinity of the microelectrode. Furthermore, the trauma does not have a stationary localization around the microelectrode but spreads with time. For instance, after 11 min the disturbed area seen in Figure 2d will exceed the area shown in Figure 6b. A reasonable explanation for the high activity of K^+ seen by a K^+ -sensitive microelectrode is that K^+ ions are released from disturbed cytoplasmic proteins and freely dissolved in the free water of the disturbed area. The observed local swelling at the place of deterio-

ration may be caused by the increasing concentration of liberated ions in the disturbed area with the result of water movement towards this place of reduced water activity.

The observed progressive deterioration of the cytoplasm around the microelectrode may explain why K^+ -sensitive microelectrodes did not detect large variations in K^+ -activities; such variations would be expected if a small microelectrode could detect the K^+ ions liberated only from an A band region (high value) or from an I band region (low value). One may speculate that low K^+ -activities have been detected at the beginning of microelectrode experiments (when the damage was still small) and that such findings were neglected because they were attributed to improper sealing of the cell membrane around the microelectrodes. It could be worthwhile to repeat K^+ activity measurements with a simultaneous control of the produced damage to clarify this issue. The possibility however that the high stable K^+ activities reported in past microelectrode work were obtained with microelectrodes which produced no structural artifacts is very unlikely for the following reasons: 1) The microelectrodes used for the present investigations were smaller (tip diameter about $0.3\ \mu\text{m}$) than those used for K^+ activity measurements in frog skeletal muscle (e.g., [30], tip diameter up to $1.5\ \mu\text{m}$). With no exception even these small microelectrodes produced structural artifacts, which were visible under the light microscope. 2) The center of deterioration produced by the microelectrode is near the electrode inside the cell and not at the place of the membrane where the microelectrode is inserted (see e.g., Figure 2e). This means that the damage is not starting from an improperly sealed cell membrane.

Ion adsorption in intact and damaged muscle cytoplasm studied by electron microscopy

Ion adsorption in intact skeletal muscle cells has been established by the above discussed electron microscopic methods. Independent evidence for the view that cytoplasmic proteins do weakly bind or adsorb alkali-metal ions has been obtained with the following *in vitro* experiments: Sections of freeze-dried embedded muscle exposed to alkali cation solutions show a selective binding of the different ions (e.g., K^+ , Rb^+ , Cs^+) by the same proteins which accumulate the ions in the living cell [8, 9, 11]. Figure 3b shows that freeze substituted and low temperature embedded muscle can also be used to demonstrate this phenomenon: The electron-dense Cs^+ ions stain the ultrathin section because they are adsorbed preferentially to filaments of the A band and to Z line proteins. The ion adsorption sites are the same as those visualized in frozen hydrated cryosections of Tl^+ -loaded intact muscles (Figure 6b). It is noteworthy that proteins of a glutaraldehyde fixed muscle do not bind alkali-metal ions [13]. These results confirm the postulation of the AIH that cellular proteins are able to adsorb alkali-metal ions but only if they are maintained in certain conformations similar to those which they assume in the living cell. Apparently freeze-substitution and embedding carried out exclusively at rather low temperatures can be used to capture the capability of proteins to adsorb alkali-metal ions.

In the foregoing section we explained the high K^+ activity measured by microelectrodes as being due to ions, which were liberated from proteins of the disturbed cytoplasm. In other words, we expect that proteins of a disturbed muscle cytoplasm change their conformation in such a way that alkali-metal ions like K^+ or Cs are no longer preferentially adsorbed. The best way of testing this possibility would be to freeze very rapidly a muscle cell together with an inserted microelectrode, to analyze the K distribution around the electrode in a frozen hydrated preparation and to determine the ion binding capacity of

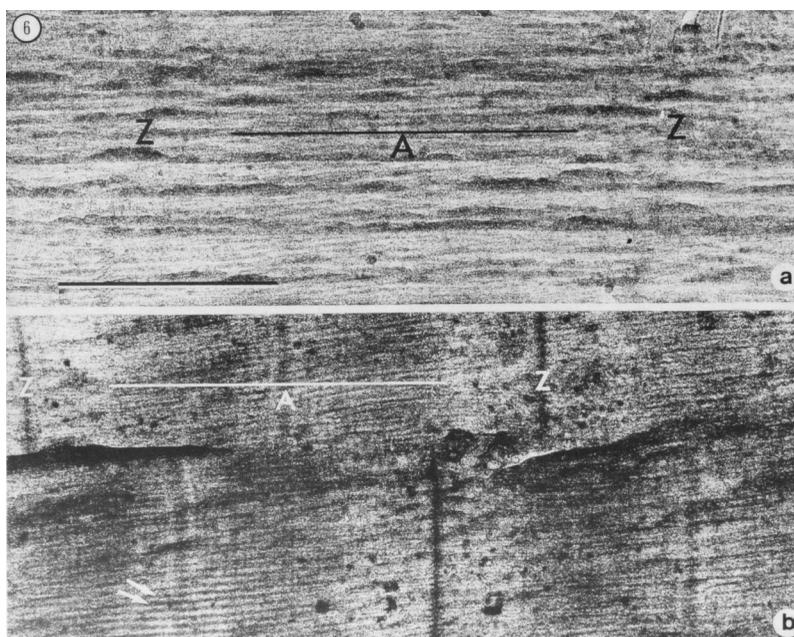


FIGURE 6. *Frozen hydrated* cryosections of frog sartorius muscle, (a) Normal K^+ containing muscle. Only very faint ultrastructural details can be seen. **A**, A band; **Z**, Z line; (b) Tl^+ -containing muscle. Dark myosin filaments (arrows) in the A bands (**A**) and dark Z lines (**Z**) indicate sites of preferential Tl^+ accumulation in the living cell. Bar: 1 μm . From [14], reprinted by permission.

proteins around the electrode after freeze substitution and low temperature embedding. Because of technical difficulties we have chosen to start with a simpler method. From EMOC (effectively membrane-less open-ended cell) studies it is known that after cutting of a skeletal muscle cell by a razor blade the cytoplasm at the cut end is deteriorating progressively, thereby changing its physicochemical properties [37, 43]. With the electron microscopic cryo-methods now available it is possible to investigate the events occurring during this deterioration under different conditions. First experimental findings obtained from a cut Cs^+ -loaded muscle are given in Figure 4 and described above. The following observations are relevant to the problem of ion adsorption in damaged muscle cytoplasm:

1) The subcellular distribution of electron- dense Cs^+ ions is completely different in the disturbed area near the cut end compared to an area where the muscle appears in a normal resting state (compare Figure 4e with Figure 4c). The disturbed area shows a poor contrast and some dark precipitates which most likely represent Cs - precipitates. Whether the poor contrast is caused by an even distribution of Cs^+ ions or whether the Cs^+ concentration is very low in the disturbed area of the freeze substituted and low temperature embedded preparation remains to be determined by future X-ray microanalytical studies. (It has been speculated that ions, which are freely dissolved in cellular water, cannot be

retained as easily as weakly bound ions in the biological specimen during freeze substitution and low temperature embedding [16]). In any case it can be concluded that a redistribution of the electron-dense ions must have occurred. Since the disturbed area shows muscle contraction (Figure 4a) a finding observed in earlier studies with intact contracting muscles is noteworthy [15, 16]. These muscles loaded either with Cs or Tl⁺ before freezing during contraction showed also ion redistribution; this phenomenon confirmed a postulate of a new model for the contraction of living muscle namely that alkali-metal ions (e.g., K⁺ ions in a normal K⁺-containing muscle) are liberated from their original adsorption sites during contraction ([38], chap. 16). With these findings and the observation that muscles impaled with microelectrodes show a local contraction (see Figure 4e) we have an experimental confirmation for the view that an impaling microelectrode causes a local liberation of adsorbed ions.

2) The disturbed area cannot be stained with the electron-dense Cs⁺ ions (Figure 4f) as is the case with intact muscle fibers (Figure 3a) or with intact areas of a cut muscle fiber (Figure 4g). This suggests that proteins of the damaged cytoplasm have lost their ability to adsorb (or bind) alkali-metal ions. If the ion adsorption capacity of proteins is greatly reduced during damaging of the cytoplasm an increase of free ions in the cellular water is inevitable.

Conclusion

Theoretical considerations and experimental findings obtained with electron microscopic cryotechniques support the view that the bulk of muscle K⁺ is adsorbed (weakly bound) to cellular proteins and that this binding can only be detected by methods, which do not disturb the cytoplasm. Ion sensitive microelectrodes, which impale the cell, produce artifacts in the cytoplasm and cannot be used to evaluate free and adsorbed ions in intact skeletal muscle.

Appendix

Association-induction hypothesis versus membrane pump theory

According to the classical membrane theory the cell interior is seen as a protein containing solution of free ions in free water separated from the external environment by a very thin cell membrane. In the early 1940's the membrane theory gained a high degree of public acceptance when it was able to explain with a few basic postulates the following four main physiological properties of living cells [1]: selective solute accumulation and exclusion, selective permeability, volume changes, and cellular electrical potentials. However, a basic assumption of the classical membrane theory was found to be incorrect when the cell membrane was shown to be permeable to Na⁺ ions [53]. In order to explain the observed low cellular Na⁺ ion concentrations it was necessary to postulate an energy consuming Na⁺ pump situated in the cell membrane [3]. Soon it became evident that more and more pumps had to be postulated in order to understand the observed asymmetric distribution between the inside and outside of living cells of many other substances (for review see [47]).

The fundamental correctness of the new membrane pump theory (MPT) was doubted when Ling calculated the energy needed for the Na^+ pump to maintain the observed low cellular Na^+ concentration in frog skeletal muscle cells; Ling found the energy requirements of the Na^+ pump under conditions where the energy sources had been blocked were such that the Na^+ pump alone would consume 15 to 30 times as much energy as the entire amount that the cell commands ([31], chap. 8). This finding led Ling to develop a theory for a molecular mechanism for the selective accumulation of K^+ over Na^+ in living cells, which is not based on hypothetical ion pumps (for review see [31]). Later Ling presented a generalized theory of the living cell called the association-induction hypothesis (AIH) based on the following 3 concepts ([38], p. 375):

(C1) "The bulk of cellular water exists in a state of polarized multilayers; in this state water tends to exclude solutes and does so to variable degrees depending on the size and complexity of the solute. This provides the mechanism for the normal exclusion of Na from most cells."

(C2) "Solute are accumulated by the cell if they are adsorbed onto macromolecules within the cell; for example, cations are adsorbed onto fixed carboxyl groups and sugars onto hydrogen bonding groups of proteins. This provides the mechanism for the normal accumulation of K^+ by most cells."

(C3) "The polypeptide chain is especially well suited for the induction of electron distribution changes from one side chain to another. This underlies the interaction between sites that adsorb solutes, permitting them to function in a cooperative manner, and it underlies the ability of cardinal adsorbents (e.g., ATP, hormones, drugs) to affect a large number of sites in an allosteric manner".

According to the current version of the MPT the above mentioned four basic phenomena of living cells are interpreted as follows: Selective accumulation and exclusion are the result of energy consuming active and passive transport mechanisms situated in the cell membrane. Selective permeability is explained as follows: The cell membrane is seen as an envelope made of lipids in which complex charged and uncharged pores are incorporated as well as carriers or other active and passive transport mechanisms that are responsible for the translocation of ions, sugars, amino acids, and other substances across the membrane. Cell volume regulation is determined primarily by the osmotic pressure exerted by solutes that are freely dissolved within free cellular water. K^+ - the main cellular cation - is thought to be the main solute that helps to balance the osmotic pressure of the cell interior and of the extracellular medium. According to the MPT an intact cell membrane is essential for the maintenance of cell volume. The electrical potentials of living cells are membrane diffusion potentials, which can be described by the extra- and intracellular concentrations and the membrane permeabilities of certain ions. For instance, the resting potential of nerve and muscle cells is in first approximation a K^+ -diffusion potential as described by the Hodgkin-Katz theory [24].

According to the AIH the four cellular phenomena are quantitatively described by four sets of equations [39] and interpreted as follows [38, 39]: Solute exclusion is the result of cellular water properties (see C1) and solute accumulation is due to binding onto cellular macromolecules (see C2). In most cases this binding is very weak (adsorption) and dependent on the very labile state of the cellular protein-ion-water complex (fixed charge system). Energy is necessary to maintain the metastable state of the cytoplasm but energy is not required to move solutes into and out of the cell. Selective permeabilities of ions,

sugars, and amino acids are due to cell surface (or membrane) properties. The cell surface is seen as a fixed charge system (organized differently than the cytoplasm), which contains interstices filled with multilayers of polarized water and proteins with potential adsorption sites for ions, sugars, amino acids, and other substances. The movement of solutes into and out of the cell may occur via diffusion through the water and/or via adsorption and desorption from proteins or other macromolecules (for different views on cell membrane properties according to MPT and AIH see [36]). Cell volume regulation is determined by three factors: 1) the tendency of certain macromolecules to build up several layers of water dipoles (multilayer expansion), 2) the restrictive forces provided by salt linkages between fixed cationic and anionic groups of cellular proteins (salt linkage restraint), and 3) the disparity between the particles dissolved in the external solution and the lower concentration of particles dissolved in the multilayer water of the cytoplasm. (It is important to realize that according to the AIH the concentration of free K^+ and other ions in the cytoplasm is very low and that the decrease in the cell water activity to the value that is found in the extracellular fluid is mainly caused by the electrostatic influence of certain proteins on the cellular water). Cellular electrical potentials are seen as phase boundary potentials between the cell surface and the extracellular phase and have no direct relation to ionic permeabilities. The electrical potential is determined by the density and nature of the ionic groups on the macromolecules of the cell surface.

Taken together the AIH is a general theory based on a few postulations, which is able to explain the four basic phenomena of living cells in a consistent manner. One may ask why the theory is not generally accepted or at least generally discussed. The main reason is probably the strong conviction of most scientists that ion pumps have been proven unequivocally and that therefore neither the energy argument of Ling nor the logical consequence - a model without energy consuming membrane pumps - can be correct.

However, are membrane situated pumps and in particular ion pumps responsible for K^+ accumulation in and Na^+ exclusion from living cells really proven? Many scientists believe that the following observations prove that the Na,K -ATPase - first isolated by Skou in 1957 [52] - is the postulated ion pump (for review see [38], pp. 118–119): 1) Both systems are present in the cell membrane (the ATPase is in the membrane, the postulated pump must be in the membrane). 2) Both systems utilize ATP but not inosine triphosphate. 3) Both systems require the presence of Na^+ and K^+ . 4) Both systems require the same concentration of cations for half-maximal activity; ATPase activity and cation flux are significantly correlated. 5) Both systems are inhibited by cardiac glycosides.

As a consequence of these findings most scientists accepted ouabain inhibition and ATP induced increase in ion flux as an identification for active transport. In Best and Taylor's textbook of physiology we read: "An active transport process is defined, not by demonstrating that flux is thermodynamically uphill, but only by demonstrating that flux is coupled to metabolism" [51]. This definition, by statement, however is useless for scientific methods because it is not clear what fundamental assumptions are basic to the definition, hence it cannot be tested [21].

Despite this misleading definition of active transport it should have been possible to test the claim that the Na,K -ATPase is able to transport ions against electrochemical gradients. Indeed, several attempts were undertaken to prove such an active transport with pure membrane preparations. Unfortunately these attempts either failed or the results were at best equivocal. For instance, the perfused squid axon contains functioning Na,K -ATPase and is able to increase efflux by addition of ATP, and the efflux is sensitive to ouabain;

however, a net Na^+ efflux against an electrochemical gradient could not be observed (for review see [38], p. 127). This “negative” experiment shows that the good correlation between ATPase activity and ion fluxes does not reflect the postulated pumping activity of this enzyme but may reflect the hypothesis of Ling that configuration changes of the ATPase change its interactions with ions and water with the result of modified ion permeability rates. Similar results were obtained with nonleaky “white” ghosts obtained from red blood cells. These ghosts are not able to accumulate K^+ or extrude Na^+ despite the fact that they contain normally functioning Na,K-ATPase [45]. The claim that reconstituted purified phospholipid-ATPase vesicles pump Na^+ has been analyzed in a detailed study by Ling and Negendank [44]; they came to the conclusion that ATP did not actually cause a net gain of Na^+ by these vesicles and that the results can be better explained by the AIH. The criticism of Ling and Negendank has not been refuted in print.

These negative results either require one to refute Ling’s energy argument and to carry out new test experiments, which unequivocally prove the existence of pumps as postulated by the MPT, or to adopt an alternative working hypothesis for designing new test experiments. In the author’s opinion the AIH is the most advanced alternative model from both a theoretical and experimental standpoint which has proven its usefulness as a working hypothesis by many correct predictions a few of which should be mentioned here (for a complete list of successful predictions provided by the AIH see [39]):

Since 1965 it has been postulated that the bulk of cellular water is polarized in multilayers [32]. Experimental testing [42] shows that 95% of frog muscle water follow the Bradley multilayer adsorption isotherm [2]. According to the AIH cell volume regulation is primarily not due to membrane properties but is due to interactions between cellular proteins, ions and water. Experimental testing shows that the maintenance of normal muscle cell volume, its swelling in hypotonic solutions as well as in concentrated KC1 solutions are indifferent to the presence of an intact cell membrane [46]. A logical consequence of the multilayer theory of cell water and of water in solutions of certain polymers is that the water molecules suffer motional restriction, in particular rotational motional restriction. This prediction has been confirmed by quasi-elastic neutron scattering [50, 54] (see also [48]). Within the context of the AIH it is postulated that K^+ accumulation and Na^+ exclusion by living cells is due to cytoplasmic properties. Both phenomena have been verified by direct exposure of muscle cytoplasm to Ringer’s solution [37]. A prediction of the AIH is that K accumulation in muscle follows the distribution of β - and γ -carboxyl groups fixed to cellular proteins. This prediction has been confirmed by electron microscopic studies (see Discussion of this paper). The AIH predicts that the electrical potential of cells is dependent on ion adsorption at the cell surface and not on ion permeabilities. This prediction has been confirmed with guinea pig heart muscle cells by using K^+ , Rb^+ and Cs^+ ions [5].

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Discussion with Reviewers

C.F. Hazlewood: In studies of many non-muscle cells, the activity of potassium (measured by the potassium sensitive electrode) has been shown to be significantly reduced. Can you explain how the ion sensitive electrodes can give these opposite results?

Author: This problem has been discussed at length by Ling (text reference [38], pp. 252-257). He came to the following conclusion (p. 253): “There are three possible sources of artifacts in the intracellular microelectrode recording of intracellular K^+ -activity coefficient: (1) liberation of adsorbed K^+ , (2) localized depolarization of water with rise in its solubility for K^+ , and (3) interference by charged amino groups on proteins. Since all three of these sources of artifacts favor the recording of a spuriously high inactivity coefficient, it is remarkable that one finds so many reports of a low K^+ -activity coefficient (in epithelial cells, see Table on p. 255). A likely cause for this is a greater stability of the cytoplasm of these epithelial cells when compared to that of, for example, nerve and muscle.”

C.F. Hazlewood: It has been reported that cell nuclei swell (*in situ* and *in vitro*) when exposed to monovalent ionic concentrations of the order of 150 mM/l (see Kellermayer M. (1981). Soluble and “loosely bound” nuclear proteins in regulation of the ionic environment in living cell nuclei. In: Intl. Cell Biology. H.G. Schweiger (ed.) Springer Verlag, Heidelberg. 915-924; Hazlewood C.F., Kellermayer M. (1988). Ion and water retention by permeabilized cells. *Scanning Microsc.* 2, 267-273). These time dependent changes in nuclei came to mind when I saw your slide of the changes in the sarcoplasm at the tip of the microelectrode. Do you think these observations are related in any way to what is going on at the tip of your microelectrode?

Author: It is possible that the local swelling of the muscle observed at the place of the electrode impalement is not only due to movement of free water towards the place where liberated K^+ ions accumulate but also to an additional mechanism; cytoplasm exposed to high concentrations of alkali-metal ions may swell because specific salt linkages between

neighbored proteins may be dissociated leading to an expanded but still coherent cytoplasm with water polarized in multilayers [38, p. 445; 46]. A similar mechanism may be responsible for the swelling of nuclei exposed to high concentrations of monovalent ions.

Th. von Zglinicki: Micrographs like your Figure 6 contain an enormous amount of quantitative information which could easily be obtained by microdensitometry. I would like to encourage you to do so and to compare the results with the expected distribution of putative binding sites.

Author: Such studies are planned. In order to obtain reproducible quantitative information it is, however, necessary to produce first reproducible cryosections with the lowest possible compression during sectioning. We are currently trying to achieve this precondition.

Like the preceding article by Dr. Gilbert N. Ling, this article was also once published in *Scanning Microscopy*, which is now defunct. Because the paper contains important information that must be preserved, like Dr. Ling I also am grateful for the opportunity to reprint the article in *Physiol. Chem. Phys. and Med. NMR*.

Dr. Ludwig Edelmann