Scanning Microscopy, Vol. 2, No. 2, 1988 (Pages 851-865)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/88$3.00+.00

THE CELL WATER PROBLEM POSED BY ELECTRON MICROSCOPIC STUDIES OF ION BINDING IN MUSCLE

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(Received for publication April 03, 1987, and in revised form December 22, 1987)

Abstract

The question whether cell K^+ is free or bound in the striated frog muscle has been investigated during the last 10 years by different cryomethods and electron microscopy. The results support the view that most of cellular ions are osmotically inactive and that therefore the observed cell water activity must be explained by a model which assumes a specific cell water structure. According to the association-induction hypothesis, cell water is influenced by macromolecules and has low solubilities for Na^+ and other solutes which therefore are partly excluded from cellular water. Autoradiography of frozen hydrated Na^+ loaded muscles and microanalytical studies with freeze-dried cryosections of ouabain treated muscle support the view that cell water has the proposed Na^+ exclusion property. It is concluded that problems such as cell volume regulation and muscle contraction cannot be understood completely without taking into account cellular ion binding and a specific cell water structure; in addition, mainly due to these cell properties it seems to be impossible to avoid volume changes of cells and subcellular compartments during conventional chemical fixation and dehydration of biological specimens.

This paper is dedicated to Prof.Dr. Hellmuth Sitte on the occasion of his 60th birthday.

Key Words: Cell water, potassium binding; sodium exclusion; cell volume, muscle contraction, electron microscopic artifacts, freeze-substitution, low temperature embedding.

Introduction

In cell biology the physical state of ions and water in living cells is a controversial issue. The commonly taught membrane pump theory is based on the assumption that most cell water and ions exist in a physical state that is essentially a dilute aqueous solution. According to the alternative association induction hypothesis (AIH) of Ling (1962, 1984) the cell represents a cooperative protein-ion-water complex. Proteins, water and solutes exist in a physical state different from that of an aqueous protein salt solution. Let us consider the following example: it is an experimental fact that living frog muscle cells are isotonic with an 0.1 M aqueous NaCl solution. This demands that the water activity within the cell be equal to that of this solution. Since the total ionic concentration in the cell is approximately 0.1 M and K^+ ion constitutes the bulk of the cations it follows that the bulk of this K^+ as well as the intracellular anions must be in a free state - provided the intracellular water is in the same free state as in the outside solution (Hill and Kupalov, 1930; Hill, 1930). Alternatively, if it can be shown that the bulk of cellular K^+ is bound to macromolecules and not dissolved in the cell water, it must be concluded that the cell water is electrostatically influenced by cellular macromolecules in such a way that its activity is reduced to that of a 0.1 M NaCl solution.

The purpose of this paper is to show how several aspects of the cell water problem can be investigated by electron microscopy when using newly developed cryotechniques.It is shown firstly that electron microscopic studies of ion binding in muscle support the view that cell water has properties different from that of a dilute aqueous solution and secondly that microanalytical studies may help to answer the question to what extent Na^+ exclusion by living cells is caused by cellular water. Finally, the problems of cellular electrical potential, cell volume regulation, muscle contraction, and artifacts introduced during

specimen preparation for electron microscopy are briefly discussed; it is assumed that these phenomena cannot be understood completely without taking into account the cellular ion binding and the concept of a specific cell water structure.

Electron microscopic studies of K^+, Rb^+, Cs^+ and Tl^+ binding in muscle

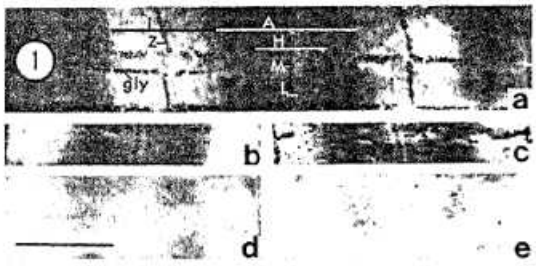
In a review article in 1975, Edzes and Berendsen concluded that the experimental results obtained with ion sensitive microelectrodes indicate little or no binding of alkali cations in muscle and other cells (Edzes and Berendsen, 1975). This conclusion was questioned by Ling (Ling et al. 1973, Ling,1984) who pointed out that ion sensitive microelectrodes damage the protein-ion-water complex and that therefore the liberation of weakly bound K^+ must be expected; Ling maintained that many other experimental results permit no other conclusion than that cellular K^+ is bound.

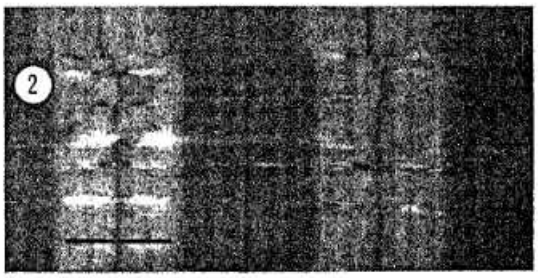
Stimulated by this controversy, we looked for experiments for testing the alternative views. The following established facts pointed to a promising test: 1) Water is not evenly distributed in striated muscle; the water content is slightly higher in the I band compared to the A band (Huxley and Niedergerke, 1958). 2) The putative binding sites for K^+ are β-and γ-carboxyl groups; myosin contributes more than 60% of the β- and γ-carboxyl chains of all the muscle proteins and is found primarily within the A bands of the striated muscle (Ling, 1977b). Hence, if K^+ is freely dissolved in cell water, K^+ follows the water distribution; we expect a slightly higher K^+ concentration in the I band than in the A band. On the other hand, if K^+ is bound to β- andγ-carboxyl groups we expect a preferential K^+ accumulation in the A band.

The following question now had to be answered: which method can be used to detect the "true" subcellular K^+ distribution in living muscle? It was evident that results of earlier trials of K^+ localization in striated muscle (e.g., Macallum, 1905; Nesterov and Tigyi-Sebes, 1965; Kallay and Tigyi-Sebes, 1969) had not been accepted because dislocation of the ions during preparatory steps could not be excluded. It was therefore necessary to develop new independent techniques for electron microscopy and to investigate whether ion redistribution artifacts can be avoided during preparative procedures. Associated with this goal was the problem of how the cellular K^+ distribution could be most effectively detected. Bearing the following considerations in mind, we decided to use the method of transmission electron microscopy: It is an established fact that living cells accumulate Rb^+, Cs^+, and Tl^+ according to the same mechanism as K^+, i.e., by a pumping mechanism or by adsorption to negative fixed charges (Numerous investigators have demonstrated the similarity of cellular uptake of K^+ and of the K^+ surrogates Rb^+, Cs^+, and Tl^+. See for example Epstein and Hagen, 1952; Lubin and Schneider, 1957; Mullins and Moore, 1960; Ling and Ochsenfeld, 1966; Bange and Van Iren, 1970; Van Iren and Van der Spiegel, 1975). Specifically with a living frog sartorius muscle it has been demonstrated that alkali-metal ions and Tl^+ replace each other reversibly in a mole-for-mole fashion under physiological conditions (Ling and Bohr, 1971b; Ling, 1977a). The subcellular localization of Rb^+, Cs^+ or Tl^+ would therefore imply detection of sites normally occupied by K^+. In particular Cs^+ and Tl^+, with the atomic weights of 133 and 204, respectively, should be directly visible under the transmission electron microscope if these electron dense ions are unevenly distributed in a muscle preparation. It must again be emphasized that we are dealing here with a basic problem which can be tackled not only with K^+ but also with Rb^+, Cs^+ or Tl^+ when using the methods of ion replacement introduced by Ling. For example, if Tl^+ rather than K^+ is the main cellular cation the water problem mentioned in the Introduction and the problem of cellular cation binding will remain the same.

In 1977 the first electron microscopic visualization of Cs^+ and Tl^+ distribution in striated frog muscle was published (Edelmann, 1977). Dry cut sections from freeze-dried and embedded Cs^+ or Tl^+ loaded muscle showed a preferential accumulation of the electron dense ions in the A band especially at the outer edges and at Z lines (Fig. 1). It was interesting to observe that the ions accumulate at the same sites which are stained by the uranyl cation in conventionally prepared muscle (Fig. 2). According to the AIH this finding was to be expected (Ling, 1977b); Hodge and Schmitt (1960) had shown that the uranyl ion binds to the β- and γ-carboxyl groups which are the putative binding sites of alkali cations. In 1980 alkali cation binding at cellular proteins was demonstrated <u>in vitro</u>: sections of freeze-dried embedded muscle exposed to alkali cation solutions are heavily stained by Cs^+ (Edelmann, 1980b,1984a, 1986b). Here too, the binding pattern is similar to that of the uranyl ion pattern in conventionally prepared muscle (Fig. 3a). However, sections of chemically fixed muscle do not bind (are not stained by) alkali cations (Fig. 3b) suggesting that the protein conformation maintained in the living cell (and at least partly in the freeze-dried embedded preparation) is essential for alkali cation binding.

The uneven distribution of Cs^+ and Tl^+ observed in freeze-dried embedded muscle, previously loaded with these ions, confirmed the AIH, and did not corroborate free cation dissolution in cellular water. This finding has been subsequently





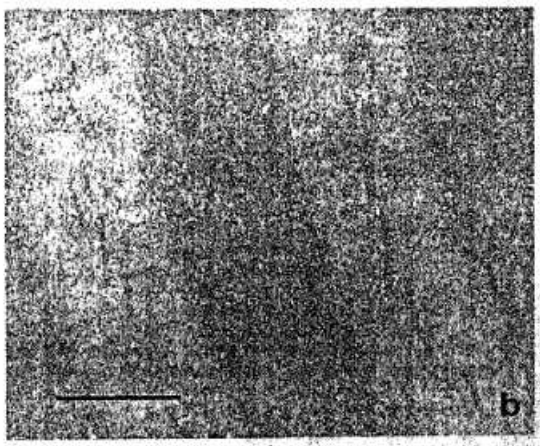


Fig. 1. 0.3 µm thick dry-cut sections of freeze-dried and embedded frog sartorius muscles. (a) cesium loaded muscle, (b, c) thallium loaded muscle; (b) was obtained immediately after sectioning, (c) after exposure of a section to humid atmosphere. (d) Central part of (a) after storage of the section in distilled water. (e) Normal potassium containing muscle. A, A band; I, I band; Z, Z line; H, H zone; M, M line; L, L zone gly, glycogen. Bar= 1 µm. (From Edelmann, 1977. Reprinted by permission).

Fig. 2. Frog sartorius muscle fixed in glutaraldehyde and conventionally embedded; 0.2 µm thick section, stained only with uranyl acetate. Bar=1µm.

Fig. 3. Muscle sections "stained" with a solution containing 100 mM LiCl and 10 mM CsCl as described by Edelmann (1984a). (a) Freeze-dried and embedded muscle, no chemical fixation. (b) Glutaraldehyde fixed and conventionally embedded muscle. Cs^+ binding sites are only visualized in the freeze-dried preparation. Bars= 1 µm. (From Edelmann, 1986a. Reprinted by permission).

confirmed in different laboratories by additional new independent methods (Ling, 1977a; Trombitas and Tigyi-Sebes, 1979; Edelmann, 1983, see review Edelmann, 1984a). However, no precise answer to the following questions has been given: Is it conceivable that during the freeze-drying process the alkali cations become bound selectively to A bands? Can the results be explained by freeze-drying and embedding artifacts? Can the results be explained by the assumption that a large amount of cations are still free?

In order to answer these questions in a straightforward manner, experiments with frozen hydrated preparations have been carried out. After revealing an uneven distribution of Rb^+ and Cs^+ in frozen hydrated single muscle fibers by autoradiography at liquid nitrogen temperature (Edelmann, 1980a), the most impressive demonstration of Tl^+ binding in muscle was possible by EM investigation of frozen hydrated cryosections (Edelmann, 1984b). EM pictures of normal K^+ containing frozen hydrated muscle sections and of Tl^+ loaded muscle are given in Fig. 4. While K^+ containing muscle sections only reveal very faint ultrastructural details, the Tl^+ loaded muscle sections show an intensely stained ultrastructure similar to that of a conventionally prepared and stained muscle section. The visualized Tl^+ accumulation pattern of the living muscle confirms the results obtained with the above mentioned freeze-drying and embedding technique as well as with other techniques, namely that K^+ and K^+ surrogates preferentially accumulate in the A bands and at Z lines. In addition to this, the intense protein staining caused by the electron dense Tl^+ implies that most of the cellular Tl^+ is bound to the proteins and not dissolved in the surrounding water: otherwise

a very poor contrasting or even a negative staining of the proteins would have to be expected.

Summing up, one can conclude that EM studies of ion binding in muscle strongly support the view that most of cellular cations are bound and hence osmotically inactive: thus the working hypothesis that cell water activity is mainly determined by the influence of cellular macromolecules appears justified.

Na^+ exclusion by cellular water: a problem for analytical electron microscopy

Cells living in an environment rich in Na^+ usually maintain a low intracellular Na^+ concentration, although the ion is constantly exchanged. This phenomenon is explained in the membrane pump theory by the operation of an energy consuming Na^+ pump localized in the cell membrane. In a detailed study, Ling demonstrated that the energy requirement of the hypothetical Na^+ pump of the frog muscle cell under conditions where the energy sources have been blocked were such that it would consume 15 to 30 times as much of the entire energy that the cell commands (Ling, 1962). This finding - which to date has not been invalidated experimentally - led Ling to conclude that there must be a non-energy consuming molecular mechanism responsible for the Na^+ exclusion. Ling postulated the following (Ling, 1965, 1984): In living cells certain proteins called the matrix proteins are present throughout the cell. These matrix proteins exist in an extended conformation with the NHCO groups exposed to, and polarizing in multilayers, all or virtually all of the bulk phase cell water dipoles. The water so polarized suffers both translational and rotational restriction and has reduced solubility for complex molecules including the large hydrated Na^+ ion.

Several aspects of this hypothesis can be and have been tested with success in the past including multilayer polarization (Ling and Negendank, 1970) and diffusion of water (Trantham et al., 1984; Rorschach, 1985). It has also been shown with model systems that water can be influenced by macromolecules in such a way that its Na^+ solubility is lower than that of pure water (Ling et al. 1980a, b; Ling and Ochsenfeld, 1983; Ling and Murphy, 1983; Ling 1983). The question we now ask is: how can we straightforwardly test the postulation that cell water keeps the cell Na^+ low without the help of a Na^+ pump?

Earlier physiological studies with striated frog muscle provided the following results: the cellular K^+ can be replaced stoichiometrically and reversibly by Na^+ either by lowering the external K^+ concentration or by ouabain (Fig. 5). The interpretation of this phenomenon is different in the alternative views of the cell. According to the membrane pump theory the Na^+ pump ceases to function at low external K^+ and ouabain poisons the pump. In both cases the intracellular concentration of free Na^+ increases to a high value and the cell loses the accumulated K^+. The poisoning effect of ouabain may be reduced by increasing the external K^+ concentration. According to the AlH the accumulation of K^+ or of Na^+ in a living cell is due to a physical adsorption to negatively charged sites of cell proteins. The ion adsorption can be described by the following equation (1) (Ling and Bohr, 1971b; Ling, 1984):

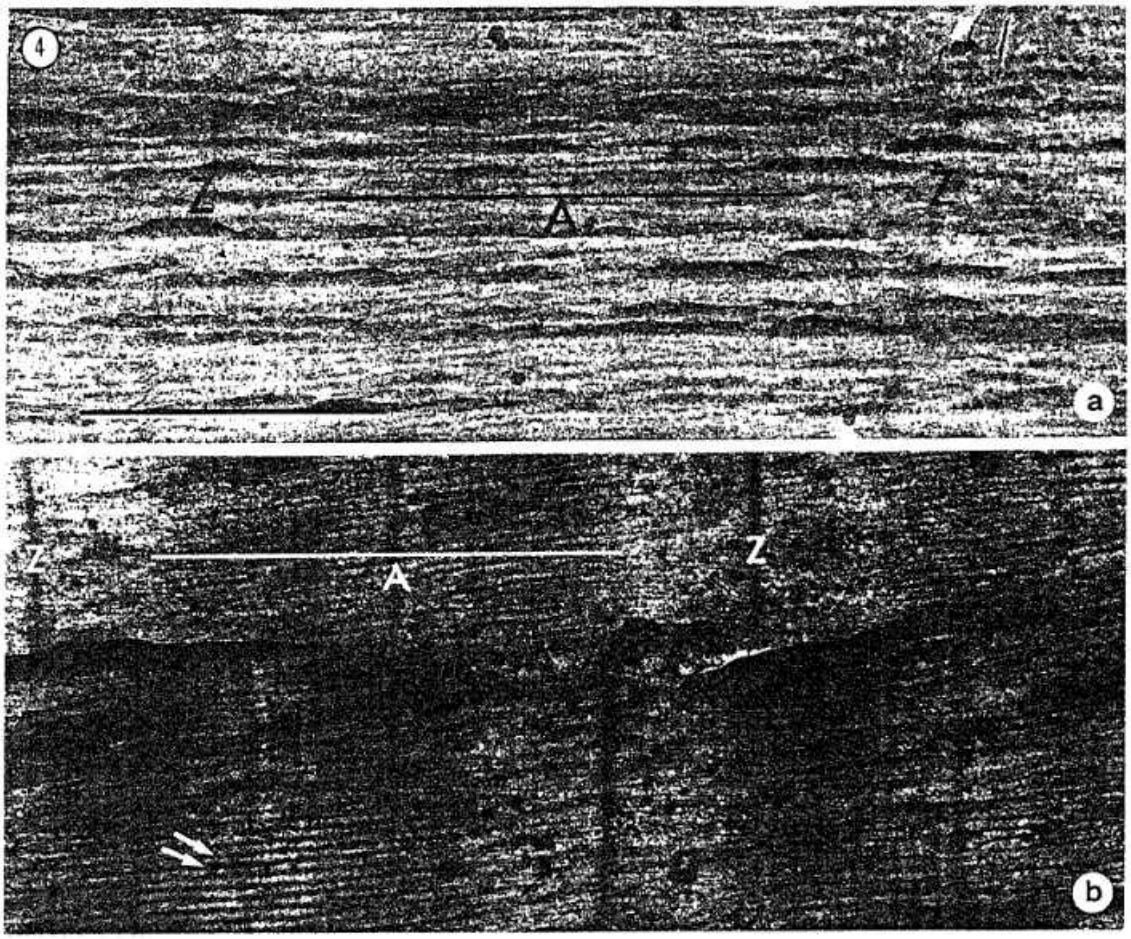
$$[p_i]_{ad} = \frac{[f]}{2}\left\{1 + \frac{Y - 1}{[Y^2 + 4 \cdot Y \cdot \exp(\gamma^{ij}/RT)]^{0.5}}\right\} \quad (1)$$

with $Y = [p_i]_{ex} \cdot K^{\infty}_{j \to i} / [p_j]_{ex}$, where
$[p_i]_{ad}$=concentration of ith adsorbed ion(e.g.,K^+),
$[p_i]_{ex}$=extracellular concentration of ith ion,
$[p_j]_{ex}$=extracellular concentration of jth ion,
$[f]$ =concentration of adsorption sites,
$K^{\infty}_{j \to i}$ =equilibrium constant of exchange of j for i
$-\gamma^{ij}/2$=energy of nearest-neighbour interaction between adsorption sites.

According to this equation the adsorption of Na^+ or K^+ in the cell is described by the three parameters $[f]$, $K^{\infty}_{j \to i}$ and $-\gamma^{ij}/2$. The interpretation of cellular K^+ and Na^+ accumulation according to Fig. 5 and equation (1) is as follows: the cellular binding sites are either occupied by Na^+ or K^+; at a low external K^+ concentration (e.g., $[K^+]_{ex}$=0.1mM, $[Na^+]_{ex}$=100 mM; $[K^+]_{ex}/[Na^+]_{ex}=10^{-3}$) the same protein sites are occupied by Na^+ which bind K^+ at higher external K^+ concentrations (e.g., $[K^+]_{ex}$=2.5 mM, $[Na^+]_{ex}$=100 mM, $[K^+]_{ex}/[Na^+]_{ex}=2.5\times10^{-2}$).

Fig. 4. 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. The Tl^+ accumulation pattern is similar to the Cs^+ patterns shown in Figs. 1 and 3. Bar = 1 µm.

Fig. 5. Effect of ouabain (3.2×10^{-7}M) on the equilibrium distribution of K^+ and Na^+ ion in frog sartorius muscle. Curves with open (Na^+) and filled (K^+) circles were equilibrium distribution data from muscles not treated with ouabain. $[Na^+]_{ad}$ and $[K^+]_{ad}$ (ordinate) are intracellular, $[Na^+]_{ex}$ and $[K^+]_{ex}$ (see abscissa) extracellular concentrations. (From Ling and Bohr, 1971a. Reprinted by permission).


4
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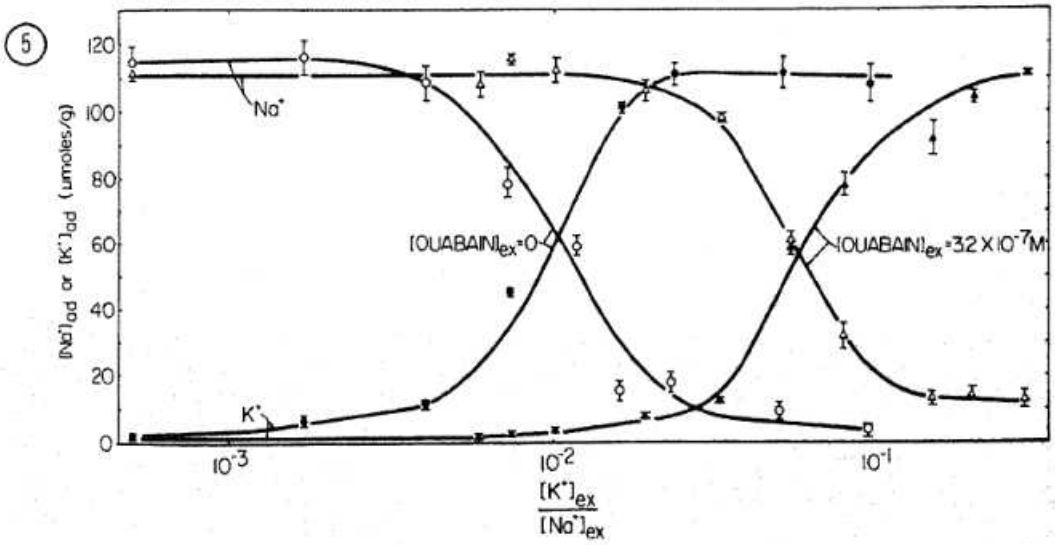

5
120
100
80
60
40
20
0
[Na+]ad or [K+]ad (umoles/g)
Na+
K+
[OUABAIN]ex=0
[OUABAIN]ex=3.2 X 10-7M
10-3
10-2
10-1
[K+]ex / [Na+]ex

Addition of 3.2×10^{-7}M ouabain shifts the K^+ and Na^+ adsorption isotherms to the right indicating that only one parameter namely $K^{\infty}_{Na \to K}$ changes from 100 to about 20. With $[K^+]_{ex}$=2.5 mM and $[Na^+]_{ex}$=100 mM Na^+ is then still preferentially adsorbed at the cellular proteins.

According to the considerations described earlier concerning the cellular K^+ accumulation, the alternative interpretations of the cellular Na^+ uptake may be tested: muscles are prepared in which the cellular Na^+ and K^+ concentrations are similar to that of the outside solution either by lowering the external K^+ or by addition of 3.2×10^{-7}M ouabain in the outside solution. Micro-analytical detection of the cellular Na^+ distribution should then reveal whether the Na^+ follows the water distribution (higher in the I band than in the A band) or whether Na^+ is preferentially accumulated at the same sites (A band and Z line) which have been shown to accumulate K^+ or the K^+ surrogates Rb^+, $Cs^+$and Tl^+.

Up to the present time the following tests have been carried out:

a) Autoradiography of Na^+-loaded single fibers at -196°C: muscles with a high Na^+ and a low K^+ content were obtained by reducing the extracellular K^+/Na^+ ratio to 5×10^{-3} (see Fig. 5). The Na^+ was labelled with radioactive ^{22}Na and the Na^+ distribution was visualized by autoradiography carried out at liquid nitrogen temperature (for details of the method see Edelmann, 1980a). A ^{22}Na autoradiogram is given in Fig. 6d. Figs. 6a-c show autoradiograms of frozen hydrated single fibers loaded with Cs^+ or Rb^+ labelled with ^{86}Rb or ^{134}Cs, respectively. From Rb^+ and Cs^+ we know that they are preferentially accumulated at the A bands. The similarity of the different autoradiograms showing periodical arrangements of silver grains suggests that Na^+ is also preferentially accumulated at the A bands. The Na^+ autoradiogram as it is does not of course prove that the Na^+ accumulation occurs at the A bands. However, the most interesting information provided by this autoradiogram is that a cell with about the same intra- and extracellular Na^+ concentration does exclude Na^+ from certain intracellular areas while accumulating it at other periodically arranged places. This supports the view that cellular water is able to exclude Na^+.

b) Frog semitendinosus muscles have been treated with 3.2×10^{-7} M ouabain for 2 days as described by Ling and Bohr (1971a) ($[Na^+]_{ex}$=100mM, $[K^+]_{ex}$=2.5 mM). The Na^+ and K^+ content of 4 muscles were found to be Na^+_{muscle}=93 ± 11 mmoles/kg tissue; K^+_{muscle}=13±3 mmoles/kg tissue. Similar ouabain treated muscles have been cryofixed by rapid contact with a LN_2 cooled copper block. (A detailed description of the cryofixation method will be described elsewhere; briefly, the muscle is placed on wet felt and covered with wet filter paper, immediately after removing the filter paper the muscle and the attached felt are frozen by a falling LN_2 cooled copper block). The frozen preparation is mounted in a vise type holder of a REICHERT-JUNG FC 4 cryoultramicrotome and sectioned. Frozen hydrated muscle sections are then transferred into a ZEISS EM 10 CR (collaboration with E. Zellmann, CARL ZEISS, Oberkochen). After freeze-drying of the sections in the

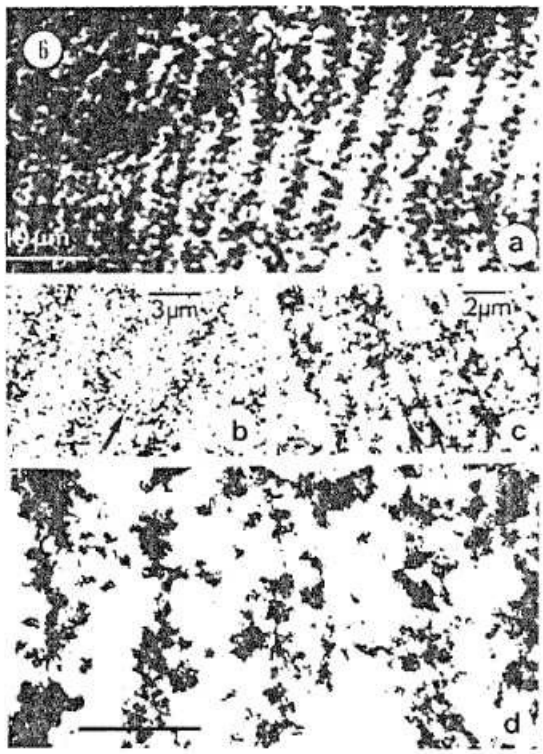


Fig. 6. Autoradiographs of frog muscle fibers. (a) Light microscopic ^{134}Cs autoradiogram of a stretched Cs^+-loaded fiber. (b) Electron microscopic ^{134}Cs autoradiogram of a stretched Cs^+-loaded fiber. The sarcomere length is about 4.4µm. Between two dark bands (A bands) a line of silver grains indicates the Z line (arrow). (c) Electron microscopic ^{86}Rb autoradiogram of a stretched Rb^+-loaded fiber. The sarcomere length is about 3.3µm. Arrows indicate dark lines at the outer edges of an A band. (Figs. 4a - c from Edelmann, 1980a. Reprinted by permission). (d) Electron microscopic ^{22}Na autoradiogram of a stretched frog sartorius muscle fiber which contained much Na^+ (labelled with ^{22}Na) and few K^+ (see text). The periodically arranged rows of silver grains indicate sarcomere lengths of about 3µm. Bar= 3µm. (From Edelmann, 1986a. Reprinted by permission).

electron microscope at -80°C for 1h the specimen holder was cooled to -163°C and several areas of the muscle sections have been analysed at an accelerating voltage of 100 kV and at a probe current of about 0.1 nA. Typical results of 2 neighbouring A band and I band areas are shown in Fig. 7 demonstrating that the Na^+ concentration is much higher in the A band than in the neighboring I band. This result corresponds with the expectation described above. It must be mentioned, however, that these "typical results" were obtained only in section areas which incidentally were not placed on the carbon coated Formvar film. We found that section areas which were in close contact with the film usually contained much less Na^+ than shown in Fig. 7; in addition, large differences of Na^+ concentrations could not be found in neighbouring A and I bands. (Similar results were obtained in collaboration with K. Zierold, Dortmund: Freeze-dried cryosections of ouabain treated muscle were analysed in a SIEMENS ST 100 F. Results obtained with a Link Quantem FLS program including background and film corrections were compared with results from dextran standards (Zierold, 1986); in most cases we found very small Na^+ concentrations in A band and I band areas). A tentative explanation of these results is the following: Complete freeze-drying of cryosections of Na^+ loaded muscle is more difficult than freeze-drying of normal K^+ containing muscle because it is more difficult to remove the hydration water from Na^+ ions than from K^+ ions; particularly, if some water remains captured after freeze-drying between the cryosection and the supporting film the Na^+ mobility may be such that ions are redistributed within the cryosection; even a dislocation of the Na^+ ions from the cryosection towards adsorption sites of the film are conceivable. This possibility must be investigated in future studies.

Implications

Experiments carried out to test two alternative theories of the cell support the view that most of cell cations are bound and that therefore cell water must be organized differently than free extracellular water. Methods are now available with which it should be possible to determine quantitatively the amount of bound cellular cations (e.g., microdensitometry of electron microscopic pictures obtained from frozen hydrated sections of Tl^+ loaded muscle) and to determine the distribution coefficient of Na^+ (and other solutes) between cellular water and free extracellular water (e.g., microanalysis of cryosections of ouabain treated cells). An exact determination of the Na^+ distribution coefficient should be possible if cellular areas can be found which do not contain Na^+ binding proteins

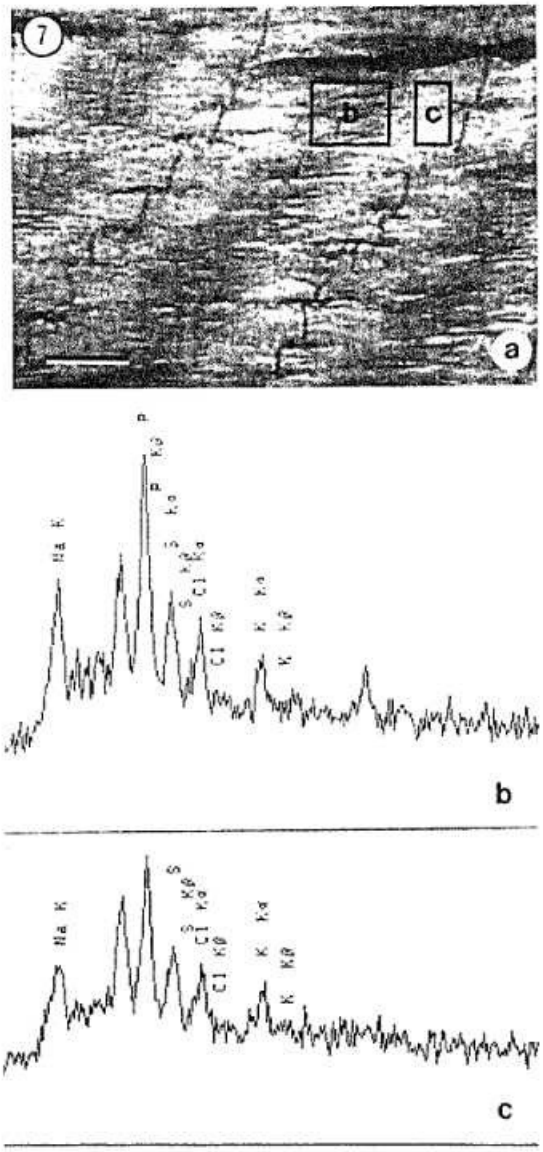


Fig. 7. Microanalysis of a freeze-dried cryosection of an ouabain treated frog muscle (see text). The rectangular boxes in (a) indicate the areas which have been analysed: (b) spectrum from the A band, (c) spectrum from the neighbouring I band. Spectrum (b) shows a much higher Na-peak than spectrum (c). Bar = 1µm.

The spectra have been obtained in collaboration with E. Zellmann, CARL ZEISS, Oberkochen, using a ZEISS EM 10 CR (STEM mode) equipped with an Ortec System 5000.

and if Na^+ redistribution artifacts can be excluded unequivocally. The results obtained so far imply that several cellular phenomena must be reinvestigated by taking account of cellular ion binding and water ordering by cellular macromolecules. A few examples should be mentioned:

Cellular electrical potential

If cell K^+ is bound, the cellular electrical potential cannot be that described in virtually all textbooks. The resting potential cannot be in first approximation a K^+ diffusion potential as described by the Hodgkin-Katz theory (Hodgkin and Katz, 1949). An alternative theory of the cellular electrical potential has been proposed by Ling (Ling, 1962, 1982, 1984). This theory is based on the assumption that the potential is determined by the density and nature of the anionic groups on the proteins of the cell surface and is thus independent on the cellular K^+ concentration (see also Edelmann, 1973).

Cell volume regulation

According to the membrane pump theory free solutes in the cell - including K^+ which is supposed to be free - help to balance the osmotic pressure of the cell interior to that of the outside solution; a Na^+ pump is required to explain why cells do not swell under normal conditions despite the colloido-osmotic pressure of intracellular macromolecules (Tosteson and Hoffman, 1960). The overwhelming difficulties with this concept have been reviewed by several authors (Ling, 1984, chapter 13; Negendank, 1984, 1986; Lechene, 1985). Lechene (1985) proposed that the cytoplasmic gel may be the main determinant of cell volume regulation. According to Ling, (Ling 1984; Ling and Walton 1976; Ling and Peterson 1977) the cell volume is determined by 3 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 concentration of particles (mainly ions) dissolved in the external solution and the lower concentration of particles dissolved in the multilayer water of the cytoplasm (which must not necessarily be a gel). At a stable cell volume the water activity (or osmolarity) of the external solution must be equal to that of the cytoplasmic water which is determined by the degree of multilayer structure and by the (usually) rather low concentration of freely dissolved particles. A most interesting finding is that the swelling and shrinking behaviour of dialysing bags filled with polymer solutions (no gels) is similar to that of living cells when changing the external ionic composition; it is important to note that these solutions also show sodium exclusion much as living cells do (Ling and Ochsenfeld 1983; Ling 1984).

Muscle contraction

In today's accepted models of muscle contraction cell water plays no specific role. However, as Ling (1984, p.566) pointed out shortening of a muscle is primarily an event of water movement. In a tentative model of muscle contraction based on principles of the AIH (cellular ion binding and cell water organization) Ling proposed that local liberation of bound K^+ and local differences of water activities may be the cause of water shifts within sarcomeres of the contracting muscle. This concept may be tested by microanalytical studies. Preliminary experiments with contracting muscles frozen during shortening reveal A band shortening in freeze-substituted and Lowicryl K 11M embedded muscle (Fig. 8). We have shown that alkali-metal and Tl^+ ions are preferentially found in the A bands of the resting muscle. Now we may ask whether a redistribution of K^+ or of K^+ surrogates can be observed in the contracting muscle which shows A band shortening. First results have been obtained with the freeze-substitution technique:

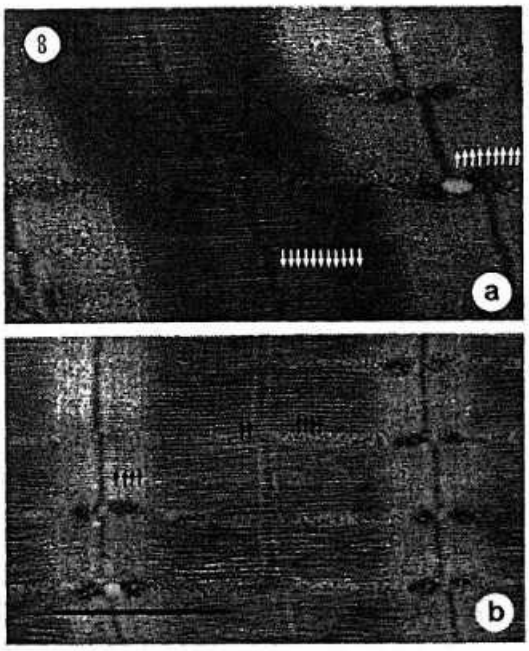
1) Freeze-substitution of resting frog muscle for 8d at -80°C and subsequent embedding in Lowicryl K11M at -60°C leaves most of cellular K^+ in the muscle preparation (unpublished).
2) A similar treatment of resting Tl^+ loaded or Cs^+ loaded muscle (Fig. 9a,c) reveals uneven distributions of the electron dense ions in the muscle similar to those obtained after freeze-drying and embedding (Fig. 1a, b) or after cryofixation and cryosectioning only (Fig. 4b).
3) Sections of freeze-substituted and K 11 M embedded contracted muscle loaded with Cs^+ before cryofixation reveal a rather poor contrast between I bands and slightly shortened A bands (Fig. 9d). By comparing Fig. 9d with Fig. 9c it appears that the electron dense Cs^+ ions have been redistributed during contraction.

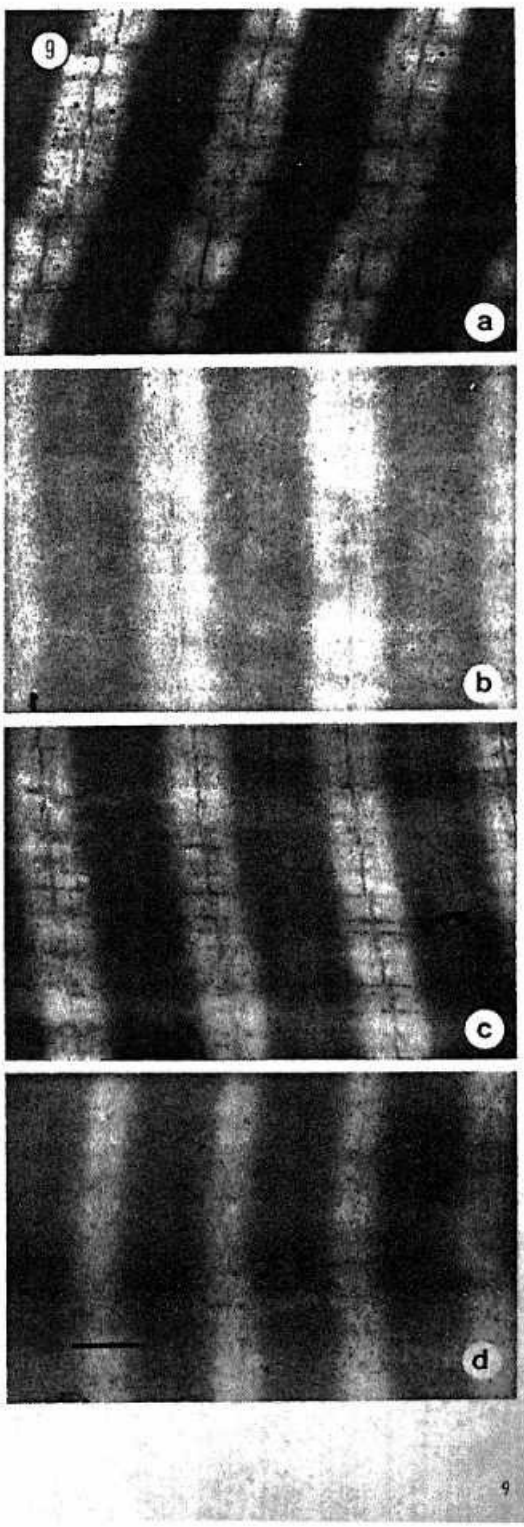
Artifacts during specimen preparation

One of the major problems of preparing biological specimens for electron microscopy is to maintain the volume of cells and subcellular compartments during the preparative procedures. So far, the events leading to swelling or shrinking of cells during chemical fixation and dehydration are far from being understood. It is interesting to observe that in many cases the osmolarity of the liquid containing the chemical fixative does not determine the degree of swelling or shrinking as anticipated (e.g., Bahr et al., 1957; see also review by Lee, 1984). In my opinion, one of the main reasons for the failure

Fig. 8. Freeze substituted frog sartorius muscles, uranyl acetate and lead citrate staining. The muscles have been cryofixed by contact with a LN_2 cooled copper block, freeze substituted for 7 days in pure acetone at - 80°C, embedded at - 60°C in Lowicryl K11M and polymerized at the same temperature. Freeze-substitution, embedding and polymerization has been carried out in a REICHERT-JUNG CS auto (Sitte et al., 1986). Bar = 1 µm.
(a) Slightly stretched muscle; a spring kept the muscle at a tension of 1 g. (b) Contracting muscle. The muscle, kept at a tension of 1 g, has been frozen after five stimuli at a rate of 50/sec (100 msec). The muscle shows A band shortening. Arrows in (a) and (b) point to faintly visible axial periodicities of about 40 nm.

Fig.9. Transmission electron micrographs of 0.3 µm thick sections of freeze-substituted and K11M embedded frog muscle (procedure as described in the legend of Fig. 8). (a) Dry-cut section of a Tl^+ loaded muscle. (b) Wet-cut section of a Cs^+ loaded muscle. (c) Dry-cut section of a Cs^+ loaded muscle. (d) Dry-cut section of a Cs^+ loaded contracting muscle. The muscle, kept at a tension of 1g, has been frozen after stimulation for 1 sec at a rate of 50/sec. The poor contrast of (b) indicates loss of electron-dense Cs^+ ions during wet-cutting. The rather poor contrast of the contracting muscle (compare d with c) suggests liberation and redistribution of the electron-dense Cs^+ ions during contraction. Bar = 1 µm.





to obtain an artifact-free structure preservation with conventional chemical fixation and dehydration methods is the transition of the living cell, a protein-ion-water complex, to a "dead" structured network which for example has lost the capability of K^+ binding and whose matrix proteins have lost their capability of ordering cell water. Liberation of ions and the change of water structure must lead to considerable water and ion shifts thereby producing inevitable cellular and subcellular volume changes. Only cryomethods appear to be suited to preserve certain structures which cannot be captured by conventional chemical fixation and dehydration. If, for example, the freeze substitution method is used, the water molecules of a fast frozen piece of tissue can be removed and the immobilized structure can be stabilized at low temperatures. An example is given in Fig. 10. Fig. 10a-d shows normal and swollen muscles which have been chemically fixed by glutaraldehyde (2%), dehydrated in acetone and prepared for electron microscopy in the conventional manner. The swollen muscles have been obtained by incubation for 2 days in sterile Ringer's solution containing 93 mM KCl as described by Ling and Peterson (1977). Weighing the muscles before and after incubation yielded a weight increase of 50%. The cross-sections of normal and swollen muscle fixed at the same resting length show similar distances between neighbouring myosin filaments. The swollen muscle shows large areas containing no cellular material. This appearance of the swollen muscle is not expected if one assumes that cell swelling induced by KCl is due to a dissociation of salt linkages which permits the incorporation of more water layers into the protein-ion-water complex due to the reduction of restrictive forces (Ling and Peterson, 1977). In order to evaluate the artifacts introduced by the chemical fixative and/or the dehydration agent normal and swollen muscles have been cryofixed on a polished LN_2 cooled copper block and freeze-substituted in a REICHERT-JUNG CS auto (Fig. 10e-h): it is evident that the distance between myosin filaments of normal freeze substituted muscle is much larger than that of normal chemically fixed muscle and that the myosin filaments of swollen muscles are still further apart from each other. In addition, large areas without cellular material could not be observed in KCl-swollen freeze substituted muscle.

Summing up, one can conclude that the realization of a specific cell water structure may open new ways for investigating unsolved problems in cell biology. The strategy proposed for solving these problems is to test an already existing cell water theory until verification or falsification.

Fig. 10. Cross-sections (a, c, e, magnification same as g) and longitudinal sections (b, d, f, magnification same as h) of frog sartorius muscle. (a) and (b) were obtained from a muscle fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h (4°C), dehydrated with acetone and embedded in Spurr's resin. (c) and (d) show a KCl-swollen muscle (see text) after fixation with 2% glutaraldehyde in 0.01 M cacodylate buffer for 2 h (4°C), dehydrated with acetone and embedded in Spurr's resin. (e) and (f) Freeze-substituted control muscle. (g) and (h) Freeze-substituted KCl-swollen muscle (see text). Freeze-substitution was carried out with 2% OsO_4 in acetone at -80°C for 2 days. Then the temperature was increased at a rate of 4°C/h to room temperature. The substitution solution was replaced by acetone and after 1h the acetone was gradually replaced by Spurr's resin. Freeze-substitution was carried out in a REICHERT-JUNG CS auto (Sitte et al., 1986). Each of the hexagons shown in (a, c, e, g) include 7 cross-sectioned myosin filaments; they are drawn to facilitate the comparison of the different photographs. Bars = 0.5 µm. (From Edelmann 1986a. Reprinted by permission)

Acknowledgements

This work was supported by Zentrale Forschungskommission der Universität des Saarlandes and by a grant from the city of Homburg/Saar. I thank E. Zellmann, CARL ZEISS, Oberkochen and K. Zierold and Sabine Dongard, Max-Planck-Institut für Systemphysiologie, Dortmund, for much help. I also would like to thank Mrs. E. Frank for her assistance in preparing the manuscript.

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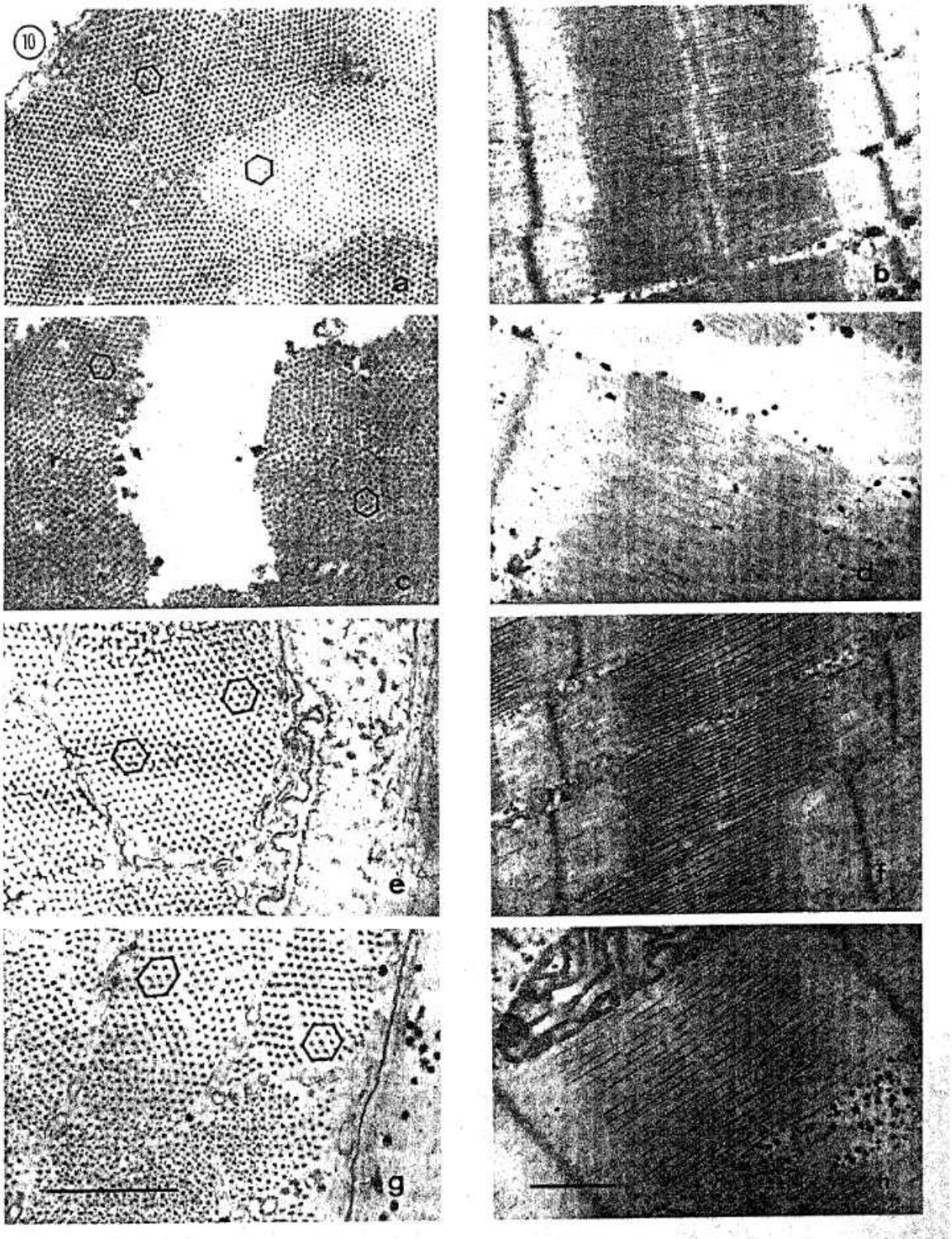
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Discussion with Reviewers

G.H. Pollack: Particularly intriguing are the author's observations of thick filament shortening during contraction. Although there have been numerous modern reports of this phenomenon (for review, cf. Pollack, Physiological Reviews 63(3):1049-1113, 1983) thick filament shortening is widely ignored in the muscle literature. The author's approach of quick-freezing contracting fibers and freeze-substituting would seem to avert some of the artifacts attendant with chemical fixation, particularly shrinkage. One question that arises is whether the author is able to resolve the axial periodicity in the A band well enough to ascertain whether shortening is uniform throughout the A band, or whether it occurs locally, within a restricted region of that band.

Another question regards I band periodicity. Often, an axial periodicity in the order of 40nm can be picked up in the I band. This periodicity remains constant during shortening. The constancy of this periodicity might be one way of checking for differential shrinkage artifacts: i.e., for possible shrinkage in the activated specimen, and none in the unactivated one. This could be one potential explanation of your result, though, the numerous reports of A band / thick filament shortening in the literature based on other methods including optical microscopy might militate against such an interpretation.

Author: I have not yet investigated systematically the axial periodicity in the A band and in the I band. The arrows in Fig. 8 point to very faintly visible periodicities in the resting and in the contracting muscle; in both cases the periodicity (A and I band) is in the order of 40nm. So far I have not seen a local shortening of thick A band.

filaments. A tentative explanation of the visualized A band shortening is as follows: The electron microscopic pictures mainly reflect the uneven binding of the electron microscopic stains to cellular proteins. It is conceivable that the myosin filaments are differently stained in the resting and in the contracting muscle (no stain at the outer edges of the A band) because certain binding sites are tightly linked (salt linkage) to other sites of the same or other proteins during contraction. This interpretation is supported by the finding that during contraction a considerable amount of electron dense Cs^+ ions appears liberated from sites which are occupied in the resting muscle (Fig. 9d).

M.B. Engel: Theoretical and experimental considerations favor the notion that intracellular water is organized, i.e., the solvent properties, dielectric constant, colligative properties and molecular arrangements differ from those of ordinary water (cited references of Ling, but also Joseph NR, Engel MB, Catchpole HR. (1961). Distribution of sodium and potassium in certain cells and tissues. Nature 191, 1175-1178; and others). The author's results are consistent with the foregoing statement. However, I do not believe that these experiments can be cited as establishing the case for structured water.
Author: I agree that many scientists have questioned the correctness of the basic assumption of the membrane pump theory namely that most of cell water and ions exist in a physical state that is essentially a dilute aqueous solution. It is my opinion, however, that the AIH of Ling is the most advanced concept from both a theoretical and experimental standpoint and perhaps most important it makes precise predictions which can be tested experimentally. The ion binding studies described in this paper were aimed to test one main postulation of the AIH. These studies give no information on the real structure of the cellular water. However, with the finding that the bulk of the main cellular cation K^+ is not freely dissolved in the cellular water one must conclude that it is (not may be) differently structured than extracellular free water.

M.B.Engel: When frog muscle is bathed in the media containing Rb^+, Cs^+, or Tl^+ "Surrogates" for K^+, or altered Na^+/K^+ ratios do the solutions affect the intracellular macromolecules? Is their state of aggregation and conformation altered?
Author: Electron microscopic pictures of healthy frog muscles which have been reversibly loaded with Rb^+, Cs^+ or Tl^+ look very similar to those obtained from normal K^+ containing muscle. Small differences of the ultrastructure of cellular macromolecules of differently loaded muscles are conceivable. Of particular interest would be to investigate structure changes of muscles exposed to solutions with high KCl, RbCl or CsCl concentrations; it has been reported (Ling and Peterson, 1977) that such muscles show a typical ion specific swelling and shrinking behaviour which is explained by the different interactions of the ions with cellular macromolecules.

M.B.Engel: Huxley and Niedergerke (1958) interpret their optical results as pointing to a higher concentration of soluble protein and other solutes in the I band. They do not specifically mention its relative water concentration. The author's results reflect a selective interaction of structural macromolecules with the cations. Inferences with respect to water structure in A and I bands can only be tenuous (although probably correct). The equilibrium distribution of Na^+ and K^+ ultimately depends on differences in the standard free energy of these ions with respect to the extracellular and intracellular phases.
Author: Huxley and Niedergerke found that the concentration of solids is only about 7g/100ml higher in the A band than in the I band. Since the difference of the solid myofibrillar proteins between A and I bands should give a much higher value they concluded that most likely soluble proteins are more concentrated in the I band. By taking the values of water and solid content of the muscle used by Huxley and Niedergerke the concentration difference of solids in the A and the I bands corresponds roughly to a 10% higher water content in the I band. Now, if the cellular cations do not follow the water distribution but are adsorbed at cellular proteins of A and I bands one must conclude that the water structure in A and I bands is different from that of the extracellular water which contains known amounts of osmotically active particles.

M.B. Engel: Putative binding sites are attributed to β- and γ-carboxyl groups - especially of myosin. What about the phosphate groups of nucleic acids?
Author: Putative binding sites are β- and γ- carboxyl groups of all proteins; it remains to be examined whether the alkali-metal ions are adsorbed with different selectivities to the β- and γ- carboxyl groups of different proteins. A selective weak binding of alkali-metal ions and Tl^+ to phosphate groups of nucleic acids is also conceivable; Cs^+ or Tl^+ may be used to visualize eventually such a binding.

Reviewer III: The discussion between supporters of membrane pump theory and AIH has reached somewhat polar "religious" dimensions which are finally contraproductive. Some "tolerant" concluding sentences might contribute towards a more fruitful common discussion.

Author: My "tolerant" concluding sentences are the following: History shows that major steps in the advancement of science cannot occur without development of new ideas. New ideas are usually proposed and defended by a minority and are oftentimes not compatible with concepts accepted by the majority; a polar discussion of revolutionary new ideas is in most cases inevitable. Scientists should appreciate a polar scientific discussion because it indicates that an unsolved problem exists. Why do many scientists try to avoid such discussions? The answer is very simple: Our peer review system strongly supports accepted concepts. A career is usually not possible without publishing in established journals and without obtaining money through grant proposals. In both cases it is necessary to convince established peers. As a consequence open debate that smacks of heresy is avoided.

The present case is a good example for the foregoing statements:The AIH has been published a quarter of a century ago. Although many exciting new findings of the last years have been obtained by experimental testing of this theory and although no theoretical or experimental disproof of the basic assumptions of the theory have been published there are still many peers who refuse to consider the AIH as a working hypothesis.

One may ask how we may come to a more fruitful common discussion of the problems indicated by the controversy between the membrane pump theory and the AIH. One suggestion is to present only results and not to mention the controversy at all. This suggestion is not acceptable particularly if the experiments leading to new results were specifically designed in order to test the two opposing theories. Another suggestion is to mix features of both theories together and to propose that the correct model is a combination of both theories. This behaviour appears clever but it could be wrong as can be seen from the outcome of past scientific controversies. At the present state of our knowledge a faster progress is expected if the proponents of both theories continue to verify or falsify the basic assumptions of their favourite model. A fair competition however appears only possible if more and more editors of journals as well as sponsors of grant proposals can be convinced that scientific controversies must be supported rather than suppressed. Our review system eventually needs "advocati diaboli" who must defend the minorities. We must change the present system which appears to be unable to give a fair treatment to a minority because usually the opinion of the majority to a dispute is used to judge the validity of the opinion of the minority.

C.A. Pasternak: The authors' results on retention of K^+ by detergent-treated lymphocytes or H-50 cells are interesting, but not too surprising. First, lymphocytes are known to contain very little cytoplasm (unless they are stimulated or transformed), the nucleus occupying an exceptionally large proportion of total cell volume. Second, it has been shown by others (e.g. Paine et al. (1981) Nature 291, 258; Tluczek et al. (1984) Dev. Biol. 104, 97) that certain cells (in this instance amphibian oocytes) contain surprisingly large amounts of bound K^+; (that work did, however, show that for other cells, K^+ content is approximately equal to K^+ activity, i.e. little bound K^+). In any case, authors' own results (Table 1) show very extensive loss of K^+ (125 to < 20 mMol/kg water) with only slight protein leakage (3mg/ml over controls) in Triton-treated cells.

Authors: We agree that our results demonstrating that the release of K^+ does not follow promptly the removal of the cell membrane are not surprising for those who have long been aware that K^+ is not freely dissolved inside the living cell. These findings may be new, however, to those who only know about the conventional view of the living cell or to those who continue to use equations derived from dilute solution theory to explain cellular potentials and ion distribution. Indeed the textbooks continue to present the cell as a membranous bag surrounding what may be taken as a dilute solution. Also our results should be surprising to the majority of the cell physiologists who consider the cell membrane to be filled with hypothetical pumps and channel proteins that control all that comes and goes from the cell.

The protein loss which occurs together with the loss of K^+ is not small. In fact the protein loss is quite substantial if we consider the large volume of the extracelluar liquid into which the protein is released. From other studies we know that the amount of proteins released by detergents can exceed 50% of the total cellular proteins.

C.A. Pasternak: Authors refer extensively (references 4,5,7,11,17,19,23-31,39) to results from laboratories that challenge the conventional view of a permeable membrane, containing ion pumps, that regulates cation content in cells. Apart from some general reviews (1,3,6,8,12,20-22,37) to the latter, experiments showing extensive leakage of K^+ out of, and Na^+ into, cells permeabilized by a whole host of different agents, are scarcely mentioned. This reviewer's work (Bashford et al. (1983) Biosci. Rep. 3, 631), showing an equilibration between intracellular and extracellular K^+ in permeabilized cells, is only one of hundreds of such reports; note that in most of these, where detergents are used to permeabilize cells, the concentrations are often less than those used here. Equally, there are many experiments in which K^+ activity is measured by intracellular electrodes and is found to be close to K^+ concentration (total K^+ divided by water content). In short, the authors' results with lymphocytes (the H50 cells do show K^+ leakage when permeabilized with Triton; hence the Brij results may reflect merely the lesser potency of Brij as a solubilizing agent) are the exception, not the rule.

Authors: We also are well aware of the hundreds of papers - in fact we have chosen references for the conventional membrane-pump view which would lead the reader to a good sampling of that general view point. Likewise, we have chosen only a few papers which offer alternative interpretations. It is correct that Bashford, et al. (2) have demonstrated, using various permeabilizing agents, an equilibration between intracellular and extracellular K. These findings are comparable to our studies with Brij 58; the equilibration, however, occurs over several minutes. If K^+ activity were close to K^+ concentrations as purported for the intracellular K^+ electrode studies, then the equilibration would occur over a period of a second or less with the loss of membrane integrity. Problems inherent in the K^+ selective electrode studies are well addressed in reference 28 (pp. 250-257). The evidence now available is quite substantial favoring the adsorption of K^+ within cells (reference 33 and pp. 227-269 of reference 28).

It is quite evident that Brij 58 has lesser potency than Triton X-100; however, the potency of Brij 58 to disrupt membrane integrity rapidly (often less than one minute) and to completely dissolve membrane structure within 5 minutes is well documented.

C.A. Pasternak: Why should not the detergent, having solubilized the plasma membrane and released its proteins, proceed to solubilize intracellular membranes and release additional membrane proteins, as well as cytoplasmic proteins?

Authors: It has been well proved that detergents are directly involved in both solubilization of lipids and solubilization of protein from the cell surface and also from the interior of the cell. Electron micrographs clearly document the disruption of membrane integrity, even the complete loss of membrane structure in specific studies.

I.L. Cameron: What is the basis for your proposal that fibrillar proteins in the intact cell selectively absorb K^+ over Na^+ and that these proteins selectively desorb K^+ upon transformation back to the globular shape with detergent treatment?

Authors: Fibrillar proteins like F-actin do not accumulate K^+ selectively in the test tube. A globular protein like G-actin also does not accumulate K^+ selectively in vitro. We are hypothesizing that both the cytoskeletal proteins and proteins associated to the skeleton form a three dimensional protein system where the individual polypeptides must be in their extended configuration, and this state is what we call high energy state with selective K^+ accumulating capability.

I.L. Cameron: What is the rationale for use of the symbol ρ instead of a descriptive title such as Ratio of concentration in the pellet over concentration in the supernatant?

Authors: The symbol ρ is a shorthand notation for the ratio of the "concentration" of the pellet over the concentration in the supernatant. It should be realized that the "concentration" in the pellet includes both adsorbed and aqueous phase solutes.

I.L. Cameron: In Table 2 the concentration of Na^+ in the supernatant is listed as 23.48 mMol/l while the concentration of Na+ in the lymphocyte "control" is listed as 39.80 mMol/l in Table 1. How is the relatively high concentration of Na^+ in the lymphocyte compared to the extracellular environment explained?

Authors: The value for the lymphocyte control (39.80 mMol/l) in Table 1 is comparable to the values for human lymphocytes (see reference 33). Unfortunately, we do not know the fractional distribution of intracellular sodium between the nucleus and the cytoplasm. The low extracellular (supernatant) sodium concentration results from the fact that the cells have been washed with TSCM solution (sodium free); therefore, most of the supernatant sodium comes from that which was trapped between cells and the small amount which may have leaked from the cells during the procedure.