SCANNING ELECTRON MICROSCOPY/1984/II (Pages 875-888)
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0586-5581/84$1.00+.05

SUBCELLULAR DISTRIBUTION OF POTASSIUM IN STRIATED MUSCLES

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(Paper received January 17, 1984, Completed manuscript received May 30, 1984)

Abstract

Microanalytical experiments have been performed to answer the question whether the main cellular cation, K^+, follows the water distribution in the striated muscle cell or whether K^+ follows the distribution of negative fixed charges (β- and γ-carboxyl groups of aspartic and glutamic acid residues). Subcellular localization of K and/or of the K surrogates Rb, Cs, and Tl has been investigated by the following methods: 1) Chemical precipitation of K with tetraphenylborate. 2) Autoradiography of alkali-metals and Tl in air-dried and frozen-hydrated preparations. 3) TEM visualization of electron dense Cs and Tl in sections of freeze-dried and plastic embedded muscle. 4) X-ray microanalysis of air-dried myofibrils and muscle cryosections. The experiments consistently show that K, Rb, Cs, and Tl do not follow the water distribution but are mainly accumulated in the A band, especially in the marginal regions, and at Z lines. The same sites perferentially accumulate Cs or uranyl cations when sections of freeze-dried, embedded muscle are exposed to these electron microscopic stains. It is concluded that the detected uneven distribution of K, Rb, Cs, and Tl in muscle is neither a freeze-drying artifact nor an embedding artifact and may result from a weak ion binding to the β- and γ-carboxyl groups of cellular proteins.

KEY WORDS: Striated muscle, potassium, rubidium, cesium, thallium, ion localization, ion precipitation, air-drying, freeze-drying, artifacts, autoradiography, cryoultramicrotomy, X-ray microanalysis, ion binding.

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Introduction

The idea that the major cellular cation, K^+, is not evenly distributed within striated muscle cells dates back to the turn of the century (Macallum, 1905). Chemical precipitation and microincineration techniques suggested a preferred localization of K^+ in the anisotropic bands of voluntary muscle (reviewed by Ernst, 1963). These techniques were questioned, however (Gersh, 1938), and indirect evidence accumulated in support of the concept that K^+ is freely dissolved in free cellular water (Hill, 1930; Fenn, 1936; Edzes and Berendsen, 1975). This view is the basis of the membrane theory and of its theoretical interpretations of semipermeability, selective solute accumulation and exclusion, osmotic swelling and shrinkage, and cellular electrical potentials. Despite the great success of the membrane theory during the last 40 years a minority of scientists questioned the correctness of the basic idea of free cellular K^+ and tried to explain experimental findings in terms of K^+ binding theories. Among these the association- induction (AI) hypothesis of Ling (1962) is the most advanced from both a theoretical and experimental standpoint and this theory accounts for selective K^+ accumulation in living cells, as well as osmotic and electric phenomena, in terms of explicit molecular mechanisms (Ling and Peterson, 1977; Ling, 1982). Following the basic ideas of both the membrane theory and the AI hypothesis one can now deduce distinctly different predictions concerning the distribution of K^+ ions in the striated muscle: according to the membrane theory the K^+ in the muscle cell follows the water distribution. Since the water content in the I band is higher than in the A band (Huxley and Niedergerke, 1958) the membrane theory would expect a higher accumulation of K^+ in the I band than in the A band. The AI hypothesis, on the other hand, maintains that β-and γ-carboxyl groups are the primary sites of K^+ due to a physical adsorption. Since myosin contributes more than 60% of the β- and γ-carboxyl side chains of all the muscle proteins and is found primarily within the A bands of striated muscle, more K^+ should be localized in the A bands than in the I bands (Ling, 1977b). These predictions should also hold for Rb^+, Cs^+, and Tl^+ since it is generally accepted that these ions are accumulated in living cells by means of the same molecular mechanism as K^+ (Numerous investigators have demonstrated the similarity of cellular uptake of K^+ and of

the K^+ surrogates Rb^+, Cs^+, and Tl^+. See e.g. 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). Furthermore it has been shown with living frog sartorius muscle that alkali-metal ions and Tl^+ replace each other in a mole-for-mole fashion under physiological conditions (Ling, 1977a). The subcellular localization of Rb^+, Cs^+ or Tl^+ would therefore imply detection of sites normally occupied by K^+.

In this review microanalytical investigations are described which were stimulated by the conflict between the membrane theory and the K^+ binding theories and which were carried out to test qualitatively the above-mentioned alternative predictions.

Methods and Results

Chemical Precipitation of K^+

The most convincing results obtained with a precipitation technique were provided by Nesterov and Tigyi-Sebes (1965). They used single fibrils of the wing muscle of the honey-bee and treated them either with a $Na_3Co(NO_2)_6$ solution or with $NaB(C_6H_5)_4$.

In the first method the fibrils were investigated before and after treatment by means of an interference microscope which allows one to determine the dry mass distribution in a biological object (Hale, 1958). It was found that the mass increase in A bands was statistically significant only after treatment with $NaB(C_6H_5)_4$. From the measured data it was calculated that about 60% of total cellular K^+ was concentrated in the A bands while the total volume of A bands was about 35% of muscle volume. In addition, it was found that K^+ was also concentrated at Z lines.

In the second method the distribution of the dry mass in wet preparations was determined by photographic photometry. Fig. 1 shows interference microscopic pictures of a fibril in the native state (Fig. 1a) and after treatment with $NaB(C_6H_5)_4$ (Fig. 1b). The dark areas represent the A bands separated by the light I bands containing the dark Z lines. A contrast enhancement is clearly visible in Fig. 1b. Two recordings of the dry substance distribution in the same fibril before and after treatment with $NaB(C_6H_5)_4$ are given in Fig. 2 demonstrating the large mass increase in the A bands after treatment.

Air-drying

Autoradiography. Autoradiographic results concerning the localization of Cs^+ and Tl^+ in air-dried single muscle fibers have been published by Ling (1977b). Frog semitendinosus muscles were loaded with Cs^+ or Tl^+ respectively labeled with radioactive ^{134}Cs or ^{204}Tl. Single fibers were dissected on glass slides and allowed to dry thoroughly either at their natural length or stretched. The air-dried fibers were coated by the dip method with a layer of Ilford K5 emulsion which was rapidly dried. After exposure (1-2 weeks at -20°C) the emulsion was developed in a Kodak-19 developer. Typical results are given in Fig. 3. Fig. 3a shows an autoradiograph of a ^{134}Cs-loaded unstretched muscle in which the photographic emulsion covers only part of the muscle cell. The rows of dense granules corresponding to the localization of ^{134}Cs are aligned with the dark A bands that are seen in that part of the photograph not covered by the photographic emulsion. In Fig. 3b a stretched muscle fiber is seen, with sarcomere length of about 3.5µm. With this extension finer structures are revealed: The silver granules are not evenly distributed in the A bands but are accumulated at the two edges of the bands; in addition, narrow rows of silver grains are visible in the centers of the I bands and thus at the position of the Z lines. An autoradiograph of a ^{204}Tl-loaded muscle fiber is given in Fig. 3c, demonstrating again uneven distribution of the radioactive ion in the muscle preparation.

X-Ray Microanalysis. Trombitás and Tigyi-Sebes (1979) investigated air-dried isolated single myofibrils of the honey-bee. A transmission electron micrograph of a stretched fibril is reproduced in Fig. 4a. Paired X-ray analyses of an A band and an I band, shown in Fig. 4b, demonstrate a high K concentration in the A band and a low K concentration in the I band. Trombitás and Tigyi-Sebes pointed out that the K peaks stemming from the Z lines of a stretched preparation were as high as those of the A bands.

Freeze-Drying and Embedding

The following results were obtained with freeze-dried and embedded frog sartorius muscle either containing the normal amount of K^+, or after a major portion of cell K^+ had been replaced with Cs^+ or Tl^+. (Ling and Bohr, 1969, have developed a method for the in vitro preservation of frog muscle tissues at 25°C for up to 8 days, as judged by the criteria of resting potential, the K^+ and Na^+ contents, and mechanical contractility. Using this method it is possible to replace reversibly most of cellular K^+ with Rb^+, Cs^+, or Tl^+ in the living frog muscle. Ling and Bohr, 1971; Ling, 1977a). The muscles were incubated for 4 days in K^+-free medium containing 2.5mM Cs^+ or for 8 h in a medium containing 4mM Tl^+ and 2.5 mM K^+. At least 2/3 of cell K^+ were then replaced with Cs^+ or Tl^+. The muscles were frozen using a modified method of Handley et al. (1981). In a humidity chamber a muscle - freed of adhering fluid by light blotting on wet filter paper - was put on a 20µm thick copper foil (Fig. 5a,

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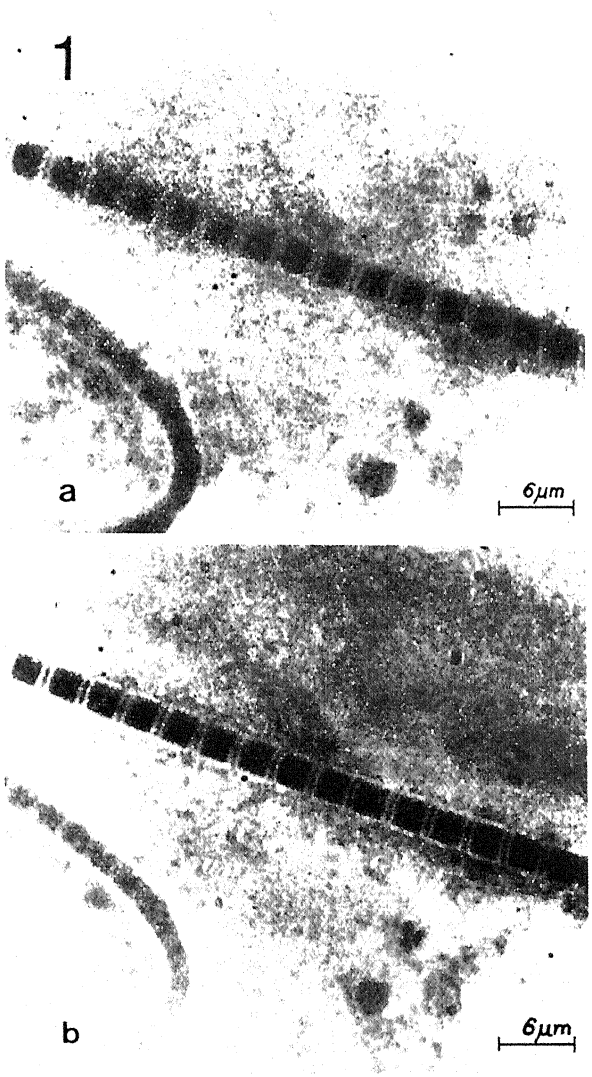
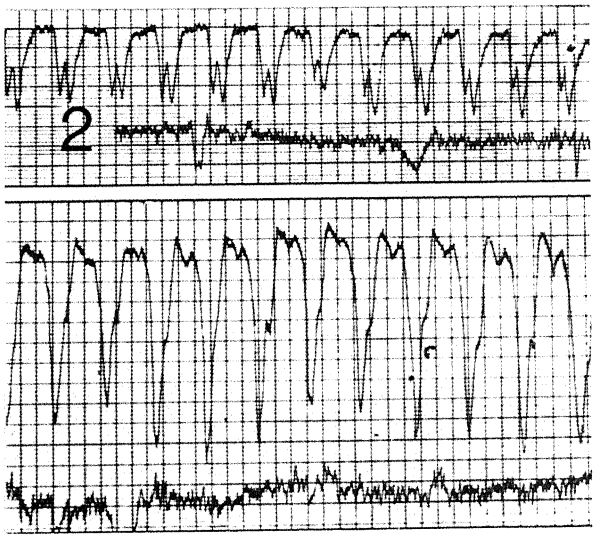
Fig. 1: Interference microscopic picture of a fibril of the wing muscle of the honey-bee.
(a) Native fibril before treatment.
(b) Fibril after $NaB(C_6H_5)_4$ treatment.
(From Nesterov and Tigyi-Sebes, 1965, Reprinted by permission)

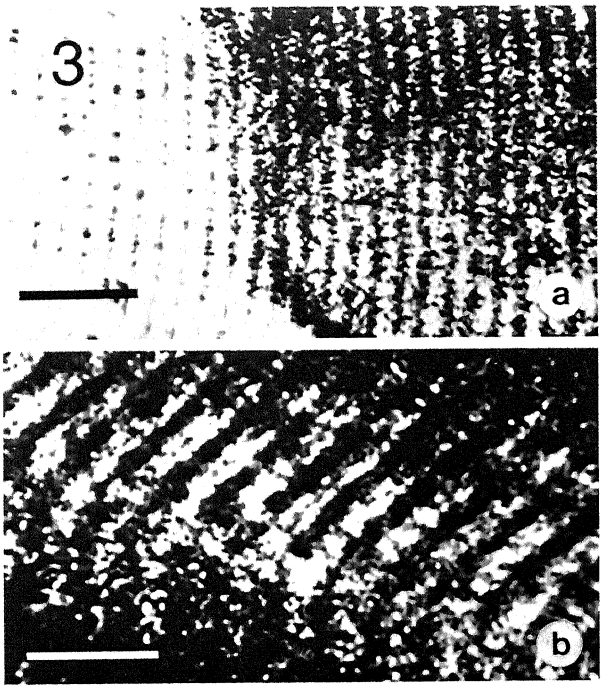
Fig. 2: Dry substance distribution in fibril before (top) and after treatment with $NaB(C_6H_5)_4$ (bottom).
(From Nesterov and Tigyi-Sebes, 1965, Reprinted by permission)

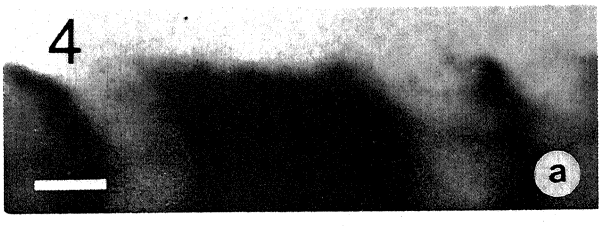
Fig. 3: Autoradiographs of frog muscle fibers.
(a) Single ^{134}Cs-loaded fiber partially covered with photographic emulsion.
(b) Single ^{134}Cs+-loaded fiber that had been stretched before drying.
(c) Single ^{204}Tl+-loaded fiber. Bars=10µm
(From Ling, 1977b, Reprinted by permission)

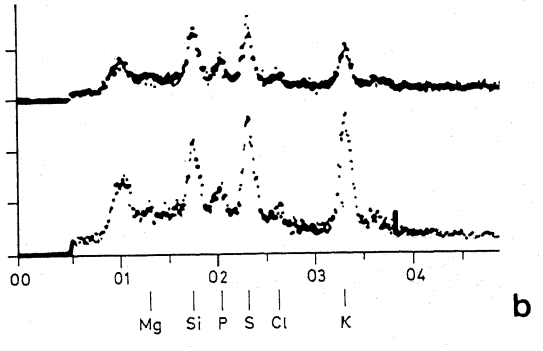
Fig. 4: Isolated myofibril of honey-bee flight muscle.
(a) Transmission micrograph of a stretched fibril. Bar=1µm
(b) X-ray spectra from I band without Z line (top) and from A band (bottom).
(From Trombitás and Tigyi-Sebes, 1979, Reprinted by permission)











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top view and cross section). The foil was folded (Fig. 5b) and fixed with a pair of forceps (Fig. 5c), cross section and side view). By means of an air pistol the forceps with the covered muscle was then plunged into liquid propane (-190°C) at a speed of 10 m/s (Edelmann, 1983). Freeze-drying was carried out as described earlier (Edelmann, 1978a) with the necessary reduction of water vapour pressure in the freeze-drying chamber provided by cryosorption pumping and cold traps. After freeze-drying at -80°C for 3 days and then at -60°C for 6 more days, the muscles were directly embedded in Spurr's resin (Spurr, 1969) at -20°C and kept at this temperature for several hours. After slowly warming the embedded tissue (10°C/h) polymerisation was carried out at 40°C for at least 8d. Sections were examined in a SIEMENS Elmiskop IA at 80kV acceleration voltage.

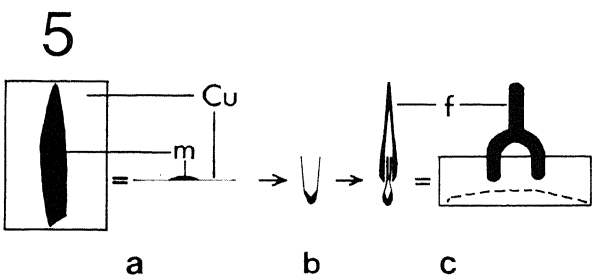


Fig. 5: Preparation of frog sartorius muscle for cryofixation. Cu, Cu foil; m, muscle; f, forceps. For details see text. (From Edelmann 1983, Reprinted by permission)

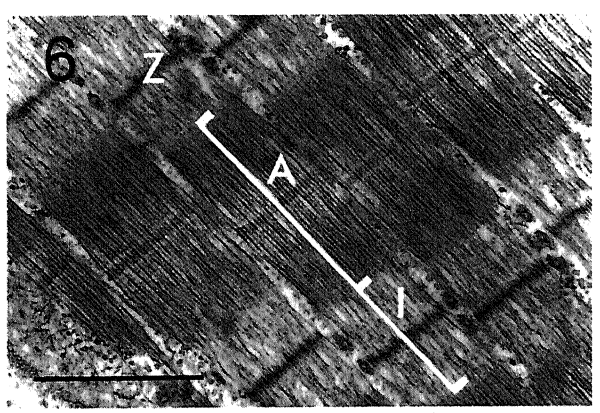


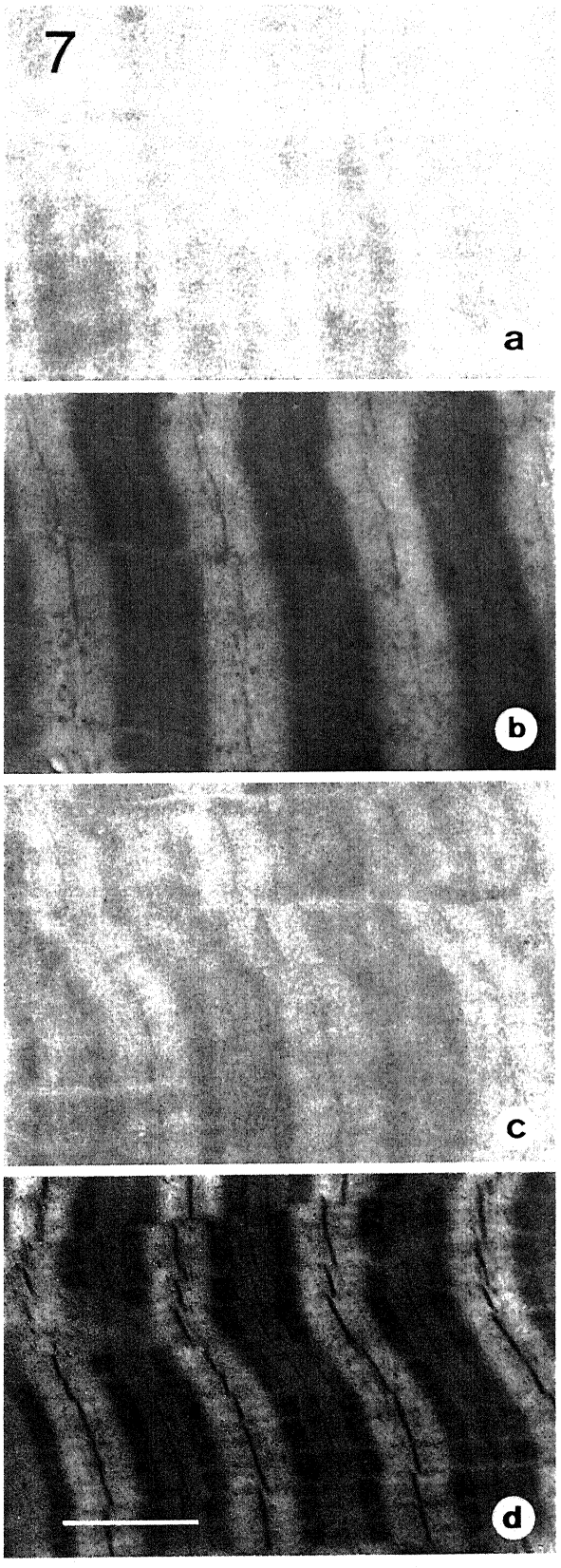
Fig. 6: Ultrathin section of a chemically unfixed frog sartorius muscle, freeze-dried, embedded in Spurr's resin, stained with 5% uranyl acetate and lead citrate. A, A band; I, I band; Z, Z line. Bar=1µm

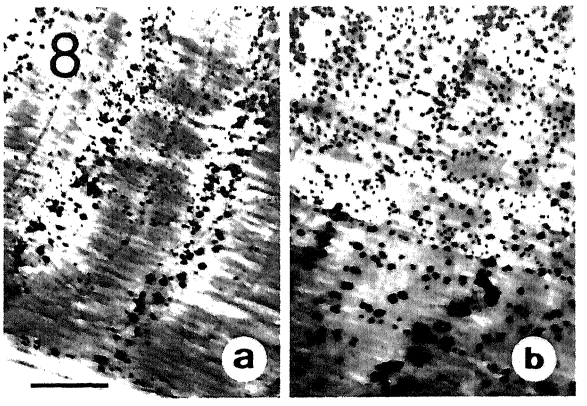
A conventionally stained ultrathin section (Fig. 6) demonstrates the quality of structure preservation. The sections shown in Fig. 7 were taken from zones with similar structure preservation. Here the section thickness was about 0.3 µm. The figure shows dry cut sections of normal K^+ containing muscle (Fig. 7a) and Cs^+-loaded (Fig. 7b), or Tl^+-loaded muscle (Fig. 7d). A wet cut section of a Cs^+-loaded muscle is given in Fig. 7c. All photographs were prepared under the same conditions. The staining intensities of the sections result only from the dry material present in the freeze-dried embedded muscle. By comparing the section of the normal K^+-loaded muscle with muscle sections loaded with Cs^+ or Tl^+ one observes a distinct qualitative increase in contrast in the latter sections. It is evident that the electron dense Cs^+ and Tl^+ are mainly localized in the A bands, especially at the outer edges, and at Z lines. The wet cut Cs^+-loaded muscle shows poor contrast, indicating that Cs^+ ions leach out into the distilled water in the knife trough (see also Edelmann, 1977, 1980a). The difference in contrast as demonstrated here was confirmed by means of an intensity meter adapted to the electron microscope (Edelmann, 1980a).

In a preliminary X-ray microanalytical study (Edelmann, 1978b) carried out in collaboration with G. Hubert (SIEMENS, Karlsruhe) the uneven distribution of Cs and Tl visualized in sarcomeres of freeze-dried embedded muscle could be confirmed; an uneven distribution of K was also established. We observed, however, that the characteristic peak intensities were only stable at very low probe currents(10^{-10}A/0.2µm^2). Without a liquid N_2-cooled specimen holder (which was not available) the ion distribution was very sensitive to electron irradiation. Redistribution of ions due to the energy from the electron beam has been reported by Hodson and Marshall (1971), and a further example will be given next.

In the following section some possible artifacts are visualized. Dislocation and precipitation of Tl^+ is observed in incompletely freeze-dried preparations (Fig. 8). These muscles had been freeze-dried for 2 d at -80°C and for 3 d at -60°C. In Fig. 8a and in the lower part of Fig. 8b the Tl^+ precipitates are mainly localized in the I bands whereas the upper part of Fig. 8b shows Tl^+ precipitates in the marginal regions of the A bands and in the I bands. Fig. 9 demonstrates dislocation and precipitation of Cs^+ (Fig. 9a) and Tl^+ (Fig. 9b) after exposure of the section to room atmosphere for several days (Cs^+) or for 12 h (Tl^+).

Fig. 7: Transmission electron micrographs of 0.3µm thick sections of freeze-dried and embedded frog sartorius muscle.
(a) Dry-cut section of a normal K+-containing muscle.
(b) Dry-cut section of a Cs+-loaded muscle.
(c) Wet-cut section of a Cs+-loaded muscle.
(d) Dry-cut section of a Tl+-loaded muscle.
Bar=2µm





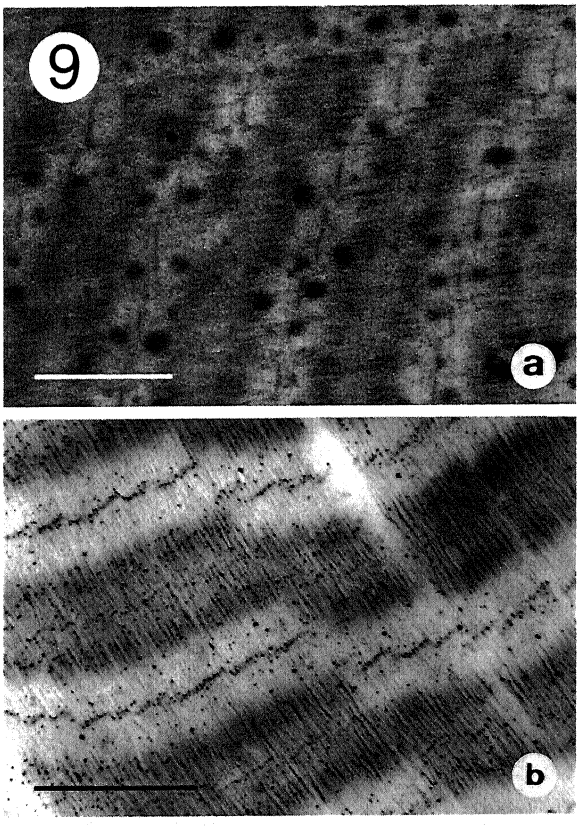


Fig. 8: Dry-cut sections of shortly freeze-dried (see text) and embedded Tl^+-loaded muscle. In (a) the Tl^+ precipitates are mainly localized in the I band, in (b) precipitates are mainly found near Z lines and in the marginal regions of the A bands (top). Bar=1µm

Fig. 9: Dry-cut sections of Cs^+-loaded (a) and Tl^+-loaded (b) freeze-dried embedded muscle exposed to room atmosphere for several days (a) and 12 hours (b). Bars=2µm

In this connection, results of Roomans and Sevéus (1976) should be mentioned. They observed extensive Rb^+ precipitation in dry-cut cryosections of Rb^+-loaded yeast cells after section exposure to room atmosphere. Another kind of artifact is shown in Fig. 10. After photographing a section of a Tl^+-loaded muscle (Fig. 10a) an electron beam of high intensity was focused in the upper region of the picture. Fig. 10b demonstrates the weakening of the contrast between A bands and I bands in the surroundings of the burned area, indicating that dislocation of the electron dense ions has taken place.

Autoradiography of Frozen-hydrated Muscle Fibers

Selected single fibers of frog sartorius muscles were used. In these fibers a major portion of the intracellular K^+ was reversibly replaced by Rb^+ or Cs^+. The replacing ions were labeled with ^{86}Rb or ^{134}Cs. A glass slide coated with a Formvar film and then with a 3µm layer of Ilford K5 emulsion was chilled to -196°C by exposure to liquid nitrogen. The fully hydrated fibers, maintained at resting length or stretched, were then quickly pressed against the chilled emulsion-coated glass slide. Thus flattened, the frozen fibers remained in contact with the emulsion at liquid nitrogen temperature until exposure ended. The emulsion was then separated from the muscle. After development and fixation of the emulsion in the usual manner, the autoradiographs were examined by light or electron microscopy. The latter examination was done after transferring small pieces of the Formvar film carrying the autoradiogram from the glass slide to an electron microscopic grid (Edelmann, 1980a). Results are given in Fig. 11. This figure demonstrates again the uneven distribution of alkali-metal ions in muscle fibers. Comparison of the results from fibers stretched to different degrees made evident that most of the alkali-metal ions were accumulated in the A bands of the muscle cells. (The A band width of a resting muscle is about 1.5µm and remains constant during stretching. In Fig. 11b the dark deposits are concentrated in bands with a width of about 1.5µm whereas the periodicity of the striation pattern is about 4.4µm. One may therefore conclude that a major portion of the ^{134}Cs is localized in the A bands). Cation accumulation at the Z lines could also be observed (see arrow in Fig. 11b).

Cryoultramicrotomy and X-Ray Microanalysis

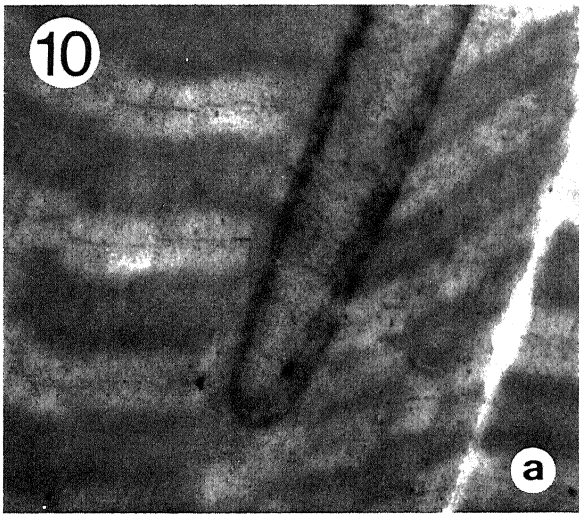
X-ray microanalysis on frozen-hydrated muscle sections was carried out by Bacaner et al. (1973). They found intracellular heterogeneity of monovalent ions (K^+, Cl^-) and concluded that there is perhaps ion binding within the cell. Sjöström and Thornell (1975) examined freeze- dried cryosections of frog toe extensor muscles. By means of X-ray microanalysis they found about equal concentrations of K in the middle of A bands and at Z lines. Somlyo et al. (1981) performed a quantitative electron probe study with freeze-dried cryosections of frog semitendinosus muscle. By comparing the middle parts of A bands with the central parts of I bands including the Z lines they found somewhat higher K concentrations in the latter regions. The authors concluded that the distribution of K followed the water distribution. These results seemed to be in conflict with the result obtained with the above mentioned different methods. In order to find possible explanations for these apparently conflicting results, I also carried out studies of frog sartorius muscles either containing K^+ or loaded with Rb^+, Cs^+ or Tl^+, with the cryosectioning technique.

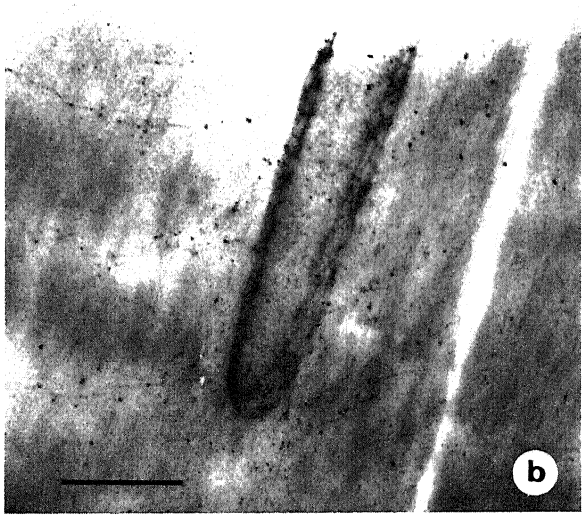
Electron microscopic examination of freeze-dried cryosections. The muscles were frozen as described above. Cryosectioning was done on an FC 4 REICHERT cryoultramicrotome (REICHERT, Wien). Small pieces of the frozen muscles were mechanically fixed in a vise type holder (Sitte and Neumann, 1983) and mounted in the microtome in such a way that a smooth surface of the muscle faced the edge of the glass knife. The smoothness of this surface indicated that this part had been covered with the copper foil during freezing and hence had been chilled with the highest speed. In addition, the muscles were oriented in such a way that sectioning could be done perpendicular to the filament direction. Under such conditions the inevitable section compression (Dubochet et al., 1983) does not diminish the original A band and I band widths. Sections about 0.2µm thick (purple to blue interference colours) were cut at specimen and glass-knife temperatures at or below -120°C. The sections were placed on Cu grids coated with Pioloform film and carbon. Freeze-drying of the sections was carried out at -80°C and $2 \cdot 10^{-6}$ Torr for 2 h, then the temperature was increased at 20°C/h to room temperature. The sections were examined in a SIEMENS Elmiskop IA at 80 kV acceleration voltage. Results are given in Fig. 12. The three photographs were prepared under the same conditions. The different staining intensities shown in Fig. 12a and Fig. 12b result from the different dry material present in the freeze-dried section: Fig. 12a shows a normal K^+-containing muscle and Fig. 12b a Cs^+-loaded muscle. Again, darkening of the A bands, especially at the marginal regions, and of Z lines is visible. Fig. 12c demonstrates section compression during sectioning. The sections shown in Fig. 12b and Fig. 12c were taken from the same preparation. In

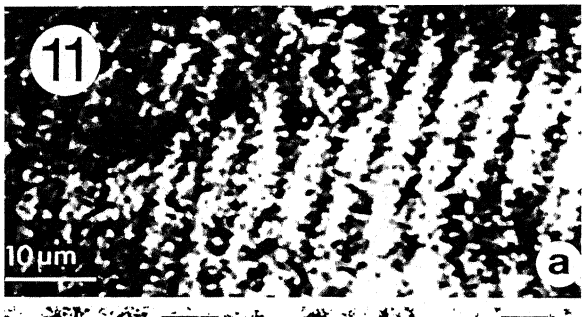
Fig. 10: Dry-cut section of a Tl^+-loaded freeze-dried and embedded muscle. After photographing (a) an electron beam of high intensity was focused at the top of (b). Bar=2µm

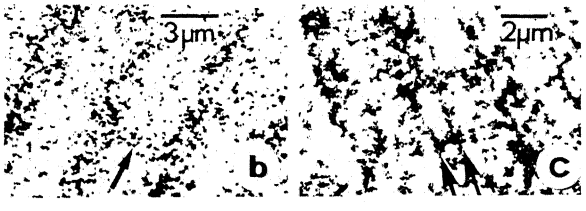
Fig. 11: Autoradiographs of frog muscle fibers.
(a) Light microscopic autoradiogram of a stretched Cs^+-loaded fiber.
(b) Electron microscopic autoradiogram of a stretched Cs^+-loaded fiber. The sarcomere length is about 4.4µm. Between two dark bands (A bands) a line of silver grains indicates the Z line (arrow).
(c) Electron microscopic 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.
(From Edelmann, 1980a, Reprinted by permission)

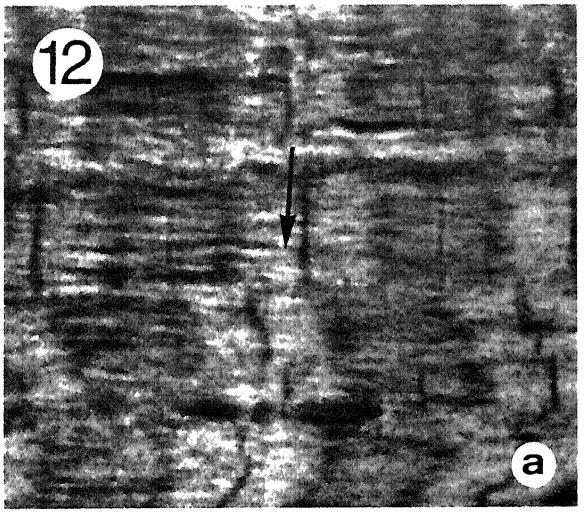
Fig. 12: 0.2µm thick cryosections of frog sartorius muscle. Arrows indicate sectioning direction.
(a) Normal K^+-containing muscle.
(b) Cs^+-loaded muscle
(c) Same preparation as in (b) with changed sectioning direction. Bar=2µm











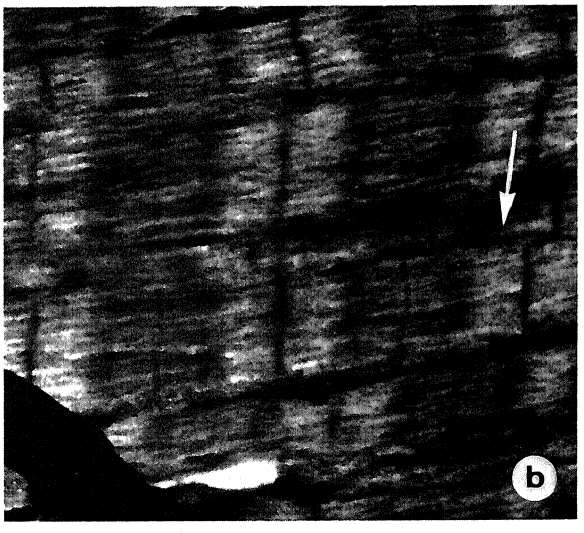


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Fig. 12b (and Fig. 12a) the sectioning direction was perpendicular to the longitudinal filament direction as described above whereas the sectioning direction was changed in Fig. 12c (see arrows). By comparing A band widths and lengths of sarcomeres of Fig. 12b and Fig. 12c, one finds that section compression is between 30% and 50% and that the section compression is uneven in the visualized area, being more pronounced in the I band than in the A band.

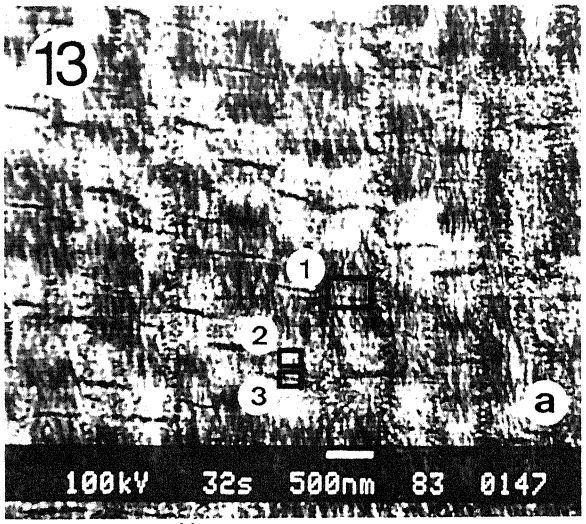
X-ray microanalysis of freeze-dried cryosections. The frozen-hydrated sections were transferred into a SIEMENS scanning transmission electron microscope (STEM) ST 100 F using the cooling chain described by Zierold et al. (1981). Freeze-drying was carried out in the instrument below -60°C. The STEM was operated at an acceleration voltage of 100 kV and a probe current of about 10nA. All analyses were done with a liquid N_2-cooled holder at -165°C to minimize contamination and radiation damage. For X-ray microanalysis the specimen was tilted 30° towards the Si (Li) energy dispersive X-ray detector (USC nuclear semiconductor) 15 mm distant from the grid. The sections were visualized on the screen of the ST 100 F and the exact positions of the analysed areas were recorded by tracing them from the screen. Spectra obtained after an analyzing time of 100 s were photographed from the screen of a multichannel analyser (Link systems, England).

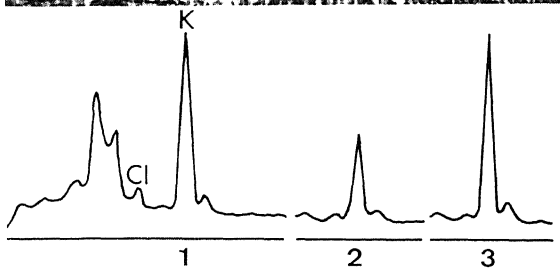
Fig. 13 shows STEM and X-ray microanalytical results (Edelmann, 1983). As a rule only small energy intervals of the spectra are given in order to facilitate the comparison of the K, Rb, Cs, or Tl peaks stemming from the numbered analysed areas of the sections marked out in small rectangular boxes. Fig. 13a shows results from a normal K^+-containing muscle. The figure demonstrates that the ratio between the K peaks (P) and the background (B) values (e.g. obtained from the right ends of the shown spectra) is lowest in the I band without Z line and that much higher P/B ratios are obtained from the A band and from the center of the I band which includes the Z line. Since the elemental concentration in an analysed volume of a thin section is in first approximation proportional to the P/B ratio (Hall et al. 1973) it is evident that much higher K concentrations are detected in the A band and at the Z line than in the I band without Z line. Fig. 13b shows results from a Rb^+-loaded muscle. Again the alkali-metal concentration is lowest in the I band excluding the Z line, highest at the outer edge of the A band and about equal in the center of the A band and in the center of the I band including the Z line. The Cs distribution shown in Fig. 13c is again similar to the distribution of K and of Rb. In Fig. 13d results of a Tl^+-loaded muscle are shown. In this case not all the K^+ had been replaced by Tl^+ but a considerable amount of K^+ remained in the muscle. (An estimate based on the actual Tl^+ loading procedure and results published by Ling (1977a) yields about 25 mmoles K^+/kg wet weight in the Tl^+-loaded muscle). Therefore both K and Tl peaks are shown in Fig. 13 d. In addition, gold (Au) peaks originating from the specimen holder in the electron microscope are recorded. K and Tl are distributed in the same uneven manner as was shown for K in normal muscle or for Rb and Cs in muscles containing these alkali-metals. (One may criticise that no quantitative data are given here. The main reason for showing only spectra is due to the fact that no computer facilities were available to determine correct concentrations of elements of the analysed areas. However, from the spectra shown here and in the original publication (Edelmann, 1983) one can estimate the following: 1. The peaks of K, Rb, Cs and Tl are about two times higher at the edges of the A bands (regions in which myosin and actin overlap) compared to the I bands without Z line (actin regions). 2. The peaks are consistently smaller in the middle of the A bands (myosin regions) than at their edges. 3. In the I bands the peaks are at least two times higher at the Z lines compared to the I bands without Z lines. 4. The background values obtained from the right ends of the different spectra of the same cryosection differ only slightly. Hence, the observed large differences of K, Rb, Cs, or Tl peaks cannot be due to variations of the section thickness).

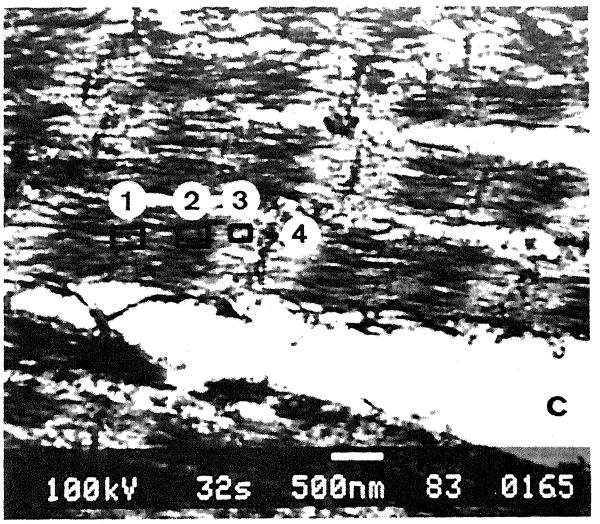
Although the detected uneven distribution of alkali-metals and Tl does not follow the water distribution, the results of Sjöström and Thornell (1975) and Somlyo et al. (1981) could be confirmed: by comparing suitable middle parts of A bands with central parts of I bands including the Z lines one finds about equal concentrations of K, Rb, Cs, or Tl in these regions or slightly higher values in the latter regions (see e.g. areas 1 and 3 of Fig. 13a, areas 1 and 4 of Fig. 13b, areas 1 and 4 of Fig. 13c, areas 4 and 6 of Fig. 13d).

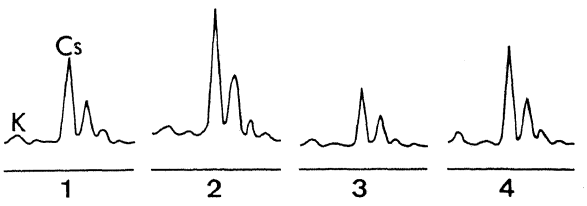
One possible source of error during the preparation of freeze-dried cryosections should be mentioned. Freeze-drying of the sections as described above and transfer through the air yielded sections with a more smooth appearance compared to that shown in Fig. 13. As a rule it was found that with cryosections that had been exposed to the air during transfer the differences in P/B ratios from different analysed areas (e.g. A band and I band without Z line) observed were smaller than those shown in Fig. 13 where no exposure to air occurred. Since freeze-dried tissue is highly hygroscopic (Neumann, 1958) it seems to be absolutely necessary to avoid any exposure of freeze-dried cryosections to the air. The humidity in the air appears to cause structure change and redistribution of ions inevitably (Roomans and Sevéus, 1976; Hodson and Williams, 1976).

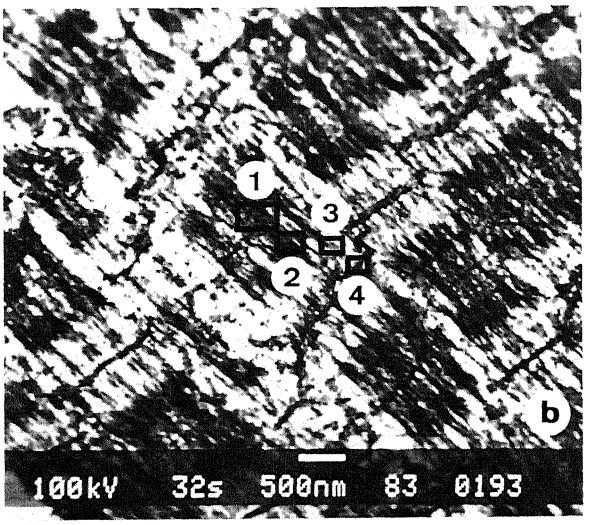
Fig. 13: Cryosections of frog sartorius muscle and X-ray spectra from numbered areas.
(a) normal K^+-containing muscle. (1) A band; (2) I band without Z line; (3) I band with Z line. Cl: K_α=2.621 keV; K: K_α=3.312 keV.
(b) Rb^+-loaded muscle. (1) middle region of an A band; (2) marginal region of an A band; (3) I band without Z line; (4) I band with Z line. Rb: L_α =1.694 keV.
(c) Cs^+-loaded muscle. (1), (2) A band regions; (3), (4) I band regions. K: K_α=3.312 keV; Cs: L_α=4.286 keV.
(d) Tl^+-loaded muscle (1) 2 sarcomeres; (2), (5), (6) A band regions; (3), (4) I band regions. K: K_α=3.312 keV; Au: L_α=9.712 keV; Tl: L_α=10.267 keV.
(From Edelmann, 1983, Reprinted by permission)

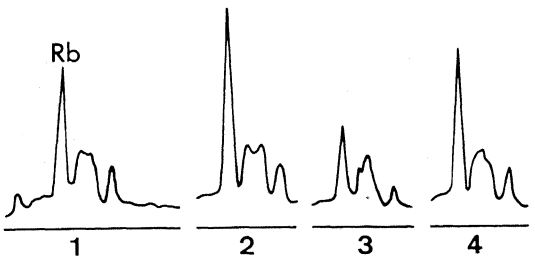


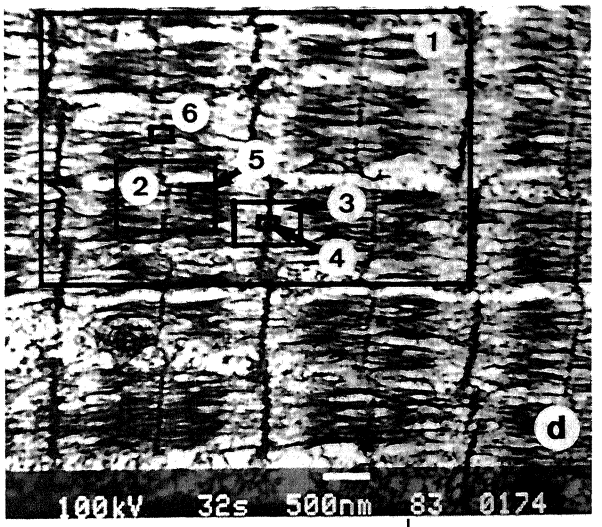












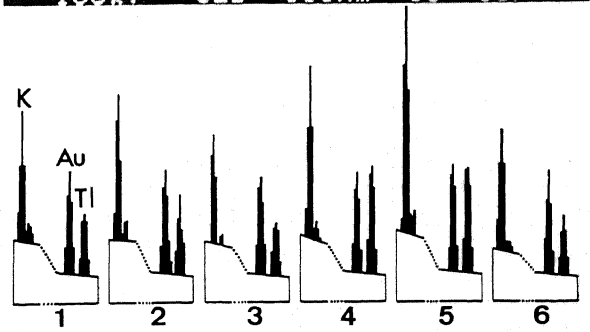


Figure Captions on Facing Page

Staining of Sections of Freeze-dried Embedded Muscle with Cs^+

Experiments were designed in order to re-investigate the fundamental question whether or not it is possible for an in-vitro, non-living preparation to bind or adsorb alkali-metal ions. The basic idea was: If cellular proteins are in fact able to accumulate selectively alkali-metal ions by electrostatic interaction, an in-vitro experiment should be carried out in such a way that the proteins have little chance of changing their physical conformations or their chemical properties. Freeze-dried and embedded material seemed to be suitable for such an experiment. Sections 0.2 µm thick of frog sartorius muscle were exposed to aqueous solutions containing different concentrations of alkali-metal ions. X-ray microanalysis and laser microprobe mass analysis (LAMMA) of these sections yielded the following consistent results (Edelmann, 1980b, 1981b): The sections accumulate each species of alkali-metal ions to a high degree. If the sections are exposed to two or more ion species the alkali-metal ions are selectively accumulated. Specifically, sections exposed to solutions containing LiCl and CsCl are well stained for electron microscopy due to the accumulation of a sufficient amount of the electron dense Cs^+ at specific protein sites. The methods finally adopted to obtain satisfactory staining results are the following (a detailed description of these methods will be published elsewhere): a) Sectioning is carried out with the salt solution in the knife trough. After 5 min the sections are picked up with a Formvar-coated grid. The grid with the drop of salt solution on the sections is transferred to a special grid holder in a small centrifuge, in which the grid is centrifuged and freed from the adhering fluid. b) The tissue is sectioned with a 0.2 M glucose solution essentially free of ions in the knife trough. The sections are collected with a grid and fixed in the centrifuge. The drop of glucose solution on the section-bearing grid is then replaced by a drop of a salt solution. After 5 min the solution is removed by centrifuging. With this method partial drying of the drop of salt solution on the grid during the necessary manipulations and hence increase of ion concentration can be avoided.

Results are given in Fig. 14 demonstrating preferred accumulation of electron dense alkali-metal ions in the A bands and at Z lines. The methods and ion concentrations of the staining solutions used are given in the figure legends. Fig. 14b shows artifacts which occur after storage of the stained sections for some days in grid boxes. These artifacts demonstrate changes in the ion binding capacity of the freeze-dried embedded material. Fig. 14c was obtained with a staining solution which yields excellent contrast (100 mM LiCl, 10 mM CsCl). Fig. 14d shows an 0.1 µm thick section exposed to alkali-metal ions in which ultrastructural details are visible. Especially, glycogen is heavily stained in this preparation. This is in harmony with earlier findings according to which Cs^+ accumulation was observed in glycogen in freeze-dried embedded Cs^+-loaded muscle (Edelmann, 1977).

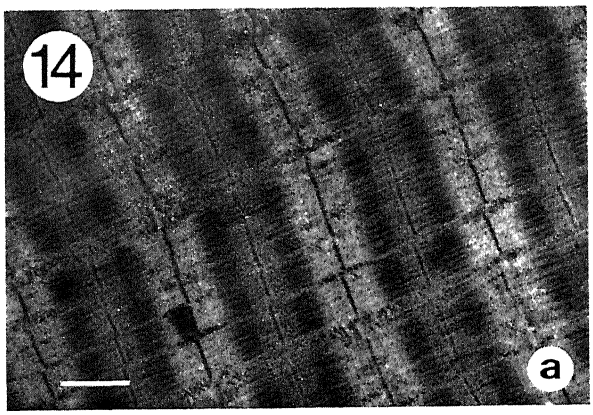
Since it was expected that chemical fixation of muscle cancels the alkali-metal ion binding capacity of cellular proteins, I carried out experiments with sections of glutaraldehyde-fixed and embedded muscle. Such sections exposed to alkali-metal ion solutions did not show an improved contrast under the electron microscope (Fig. 15), when compared to unexposed sections, and only minute, nonreproducible amounts of alkali-metal ions could be detected by elemental analysis.

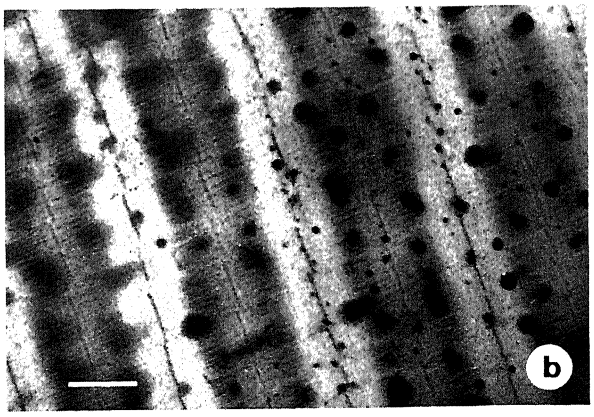
Discussion

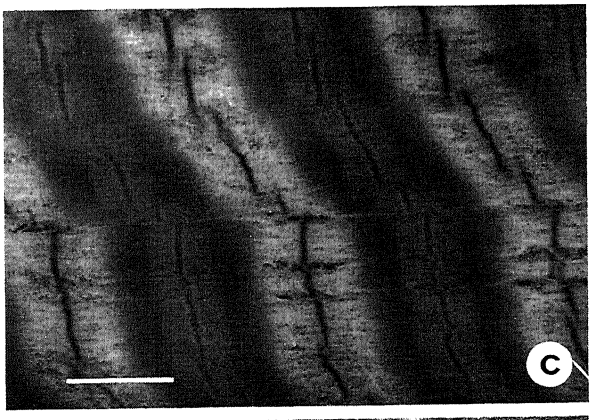
The results obtained with widely differing techniques consistently show the following: The alkali-metal ions K^+, Rb^+, Cs^+ and also Tl^+ are preferentially accumulated within the A band, especially at the two marginal regions, and at the Z line. The fact that frozen-hydrated preparations, freeze-dried cryosections and freeze-dried embedded preparations all yield qualitatively the same results supports the idea that the uneven ion distribution - visualized most clearly in freeze-dried embedded preparations - is neither a freeze-drying artifact nor an embedding artifact. In addition, although there is no doubt that precipitation (Van Steveninck et al., 1981; Van Steveninck and Van Steveninck, 1981) and air-drying techniques (Gullasch and Kaufmann, 1974) usually cause unacceptable elemental redistribution one may note that under favourable conditions even these techniques may yield meaningful results.

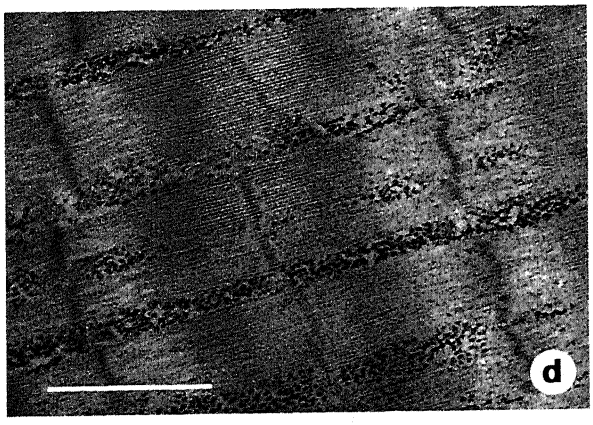
Let us now discuss the physico-chemical conditions which may be responsible for the observed uneven ion distribution in sarcomeres of the striated muscle. Most striking is the observation that the ion accumulation is highest at those locations which are stained by uranium and lead in sections of chemically fixed and embedded muscle. Although the detailed nature of uranium and lead staining mechanisms is not known it is generally assumed that uranyl and lead cations may bind to anionic sites of proteins (Hayat, 1981). Two studies dealing with uranium binding to proteins should be mentioned. Hodge and Schmitt (1960) investigated the binding of the uranyl cation to tropocollagen macromolecules by using 0.002 M uranyl nitrate. (Under these conditions the uranium in solution is cationic.) They concluded that the uranyl cations most likely bind to aspartic and glutamic acid residues of the protein. This conclusion is supported by a detailed study of Dounce and Lan (1949) dealing with uranium binding to several proteins. Fig. 16 shows a section of a freeze-dried embedded muscle stained with 0.002 M uranyl nitrate in the conventional manner. It is evident that the uranyl cation is accumulated at the same sites as alkali-metal ions and Tl^+. The similarity of ionic uptake suggests binding of K^+ and K^+ surrogates at negative fixed charges of the proteins. In addition, the results of the staining experiments with alkali-metal ions provide strong direct support for the idea of alkali-metal ion binding to cytoplasmic proteins. However, it is important to note that this binding must be very weak and may be altered or changed profoundly during preparative procedures in such a way that dislocation of the ions follows as a result. The electron microscopic observation of heavy, electron dense K^+ surrogates like Cs^+ and Tl^+ helped to visualize possible artifacts.

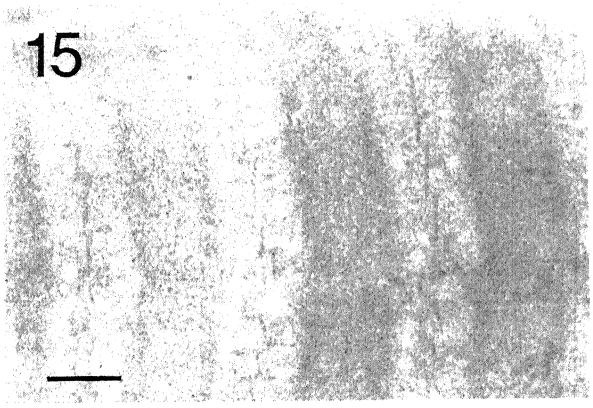
One remaining question concerns the amount of bound K^+ or K^+ surrogates. The present results do not allow an exact quantitative evaluation of that amount. However, by assuming that the concentration of free alkali-metal ions or Tl^+ is not higher











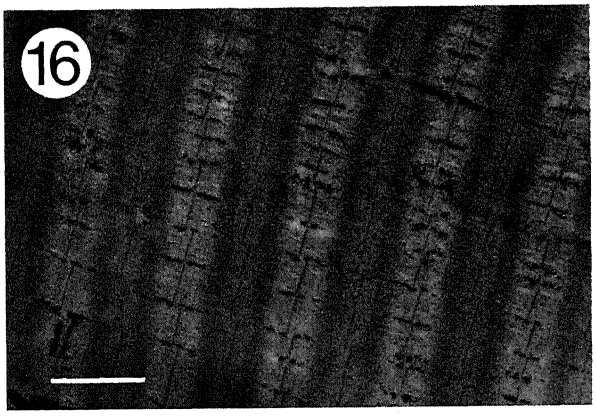


Fig. 14: Sections of freeze-dried and embedded muscle exposed to alkali-metal ion solutions (see text) according to "staining method" b): (a, b, c) and method a): (d). (a), (b), and (d) were exposed to solutions containing 50 mM LiCl, 50 mM NaCl, 10 mM KCl, 10 mM RbCl, and 10 mM CsCl. (c) was obtained with 100 mM LiCl and 10 mM CsCl. The section thickness is 0.2 µm (a, b, c) and 0.1 µm (d). (b) shows a section after storage for 2 days in a grid box. The precipitates at the right side appear more condensed; this side had been examined in the electron microscope (electron irradiated) immediately after staining the section. Bars=1µm

Fig. 15: Section of a glutaraldehyde fixed muscle prepared as the section shown in Fig. 14c. Bar=1µm

Fig. 16: 0.2 µm thick section of a freeze-dried and embedded muscle conventionally stained with 0.002 M uranyl nitrate. Bar=2µm

than the lowest detected ion concentration (e.g. in the I band without Z line) one may conclude that a major portion of the cellular ions must be bound. The assumption is reasonable because it is highly unlikely that a large disproportion of free ions (i.e. ions freely dissolved in free cell water) can exist between the A band and the I band; in this case a gross disproportion of osmotic pressure would exist between these two regions. In addition, taking into account the fact that actin contains about 14% free side chains (Kominz et al., 1954) which may also bind K^+, one may conclude that the concentration of free K^+ or K^+surrogates is probably still much smaller than the detected ion concentration in the I band without Z line. In order to evaluate precisely the amount of bound K^+ by microanalytical methods it will be necessary to determine quantitatively the ion distribution in freeze-dried cryosections or perhaps in frozen-hydrated cryosections. It is expected that highly stretched muscle fibers (Locker and Leet, 1975) would be most promising. In this case one may analyse muscle areas containing neither myosin nor actin nor Z line proteins.

In the past, objections to the idea of K^+ binding to cellular proteins were derived primarily from physiological experiments. Followers of the membrane theory have repeatedly cited experimental evidence against the AI hypothesis including ion selective electrode studies of free K^+ concentration and diffusion coefficient measurements (e.g. Edzes and Berendsen, 1975). The interpretation of these experimental findings however, have been explained explicity in terms of the AI hypothesis (Ling, 1984; see also Edelmann, 1981a). In addition, the widely cited claim of experimental proof of the Na pump in isolated membranes and purified vesicles - which would falsify an important part of the AI hypothesis - has been rebutted by Ling and Negendank (1980) by showing that the experimental findings are consistent with the AI hypothesis and may be explained without postulating a Na pump. According to Kernan (1980), active transport of ions into plant cell vacuoles invalidates the AI hypothesis. However, at no time did this hypothesis assert that active transport per se does not occur. Cooperative adsorption-desorption pumps derived from principles of the AI hypothesis were proposed to explain active transport of ions in plant cells with vacuoles (Ling, 1965) and across bifacial systems like frog skin and intestinal epithelium (Ling, 1981). To the best of my knowledge, the question whether the AI hypothesis may be accepted or rejected as a working hypothesis to explain cellular functions remains open in the literature now available. The findings reported here, however, clearly support the idea of K^+ binding to cellular proteins as predicted by the AI hypothesis.

Conclusion

The striated muscle is an ideal preparation to perform microanalytical work. The main cellular cation, K^+, can be reversibly replaced in the living muscle by the heavy electron dense Cs^+ or Tl^+. Localization of these ions is possible with optimal local resolution in the transmission electron microscope. By means of the electron dense ions artifacts during preparative steps can be visualized and avoided. Hence Cs^+- and Tl^+-loaded muscles may be used to test the quality of the preparation and of the analytical method used. The periodical arrangement of different muscle proteins facilitates detection of their different ion binding capacities.

Acknowledgements

This work was supported by Zentrale Forschungskommission der Universität des Saarlandes and by Vereinigung der Freunde der Universität des Saarlandes. I would like to thank Miss D. Jung for her assistance in preparing the photographs and Mrs. E. Frank for typing the manuscript.

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Discussion with Reviewers

M.F. Wendt-Gallitelli: I wonder, if it were not reasonable to look into the dependence of K distributions in skeletal muscles, frozen under different physiological conditions?
Author: According to the AI hypothesis K^+ desorption and adsorption are involved in contractile phenomena (Ling, 1984, In search of the physical basis of life, Plenum Publ. Corp. New York, 539-584). In my opinion it would be worthwhile to focus in future studies on a conceivable K^+ redistribution in muscle during contraction.

M.F. Wendt-Gallitelli: Could you comment upon the finding of high K concentration over the Z lines?
Author: According to the general interpretation this finding reflects a high density of β- and γ-carboxyl groups of the Z line proteins. Future studies should reveal if and how negative fixed charges are concentrated at Z lines.

M.F. Wendt-Gallitelli: In my opinion the two spectra of Fig. 6b only show, that the lower spectrum was taken in a denser region than was the upper spectrum, and that all elements in the lower spectrum show higher peaks.
Author: In a highly stretched hydrated fibril the protein concentration is higher and the water content is lower in the A band than in the I band. Therefore S and P peaks and background values are expected to be higher in the A band than in the I band of the air-dried fibril. On the other hand, one would expect a higher K peak in the I band if all K was formerly dissolved in the water of the fibril. Fig. 6b shows that the K peak is much smaller in the I band than in the A band and that the ratios between the K peak and the S or P peaks are smaller in the I band than in the A band.

M.F. Wendt-Gallitelli: In my opinion the loss of contrast in Fig. 10b must not necessarily occur by means of element redistribution. It can also be a result of contamination with C or Si during irradiation.
Author: This has to be checked in future studies. However, two arguments speak against this assumption: 1) A darkening of the area showing less contrast was not observed in the electron microscope. 2) The contrast is qualitatively different after irradiation of the section. This should not be the case if only contamination would weaken the former contrast.

R. Wróblewski: Have you found any characteristic localization of other elements detectable by means of X-ray microanalysis (sodium, magnesium, chlorine, sulphur)?
Author: I did not yet investigate the intracellular distributions of other elements. However, sodium and chlorine (see also Somlyo et al., 1981) seem to be preferentially localized in the A bands.

H.K. Hagler: What effects would warming the freeze-dried cryosections to room temperature have on K redistribution?
Author: Warming the sections to room temperature seems not to cause redistribution of K provided the sections are completely dehydrated and not exposed to water vapour.

H.K. Hagler: How do you exclude the possibility that during the drying process K^+ becomes bound selectively to A bands?
Author: In order to exclude this possibility I made the autoradiographic experiments with frozen-hydrated muscle fibers. Further studies with frozen-hydrated sections are planned.

Note added in Proofs:

After this review was written, frozen hydrated cryosections of a thallium (Tl) loaded frog sartorius muscle have been examined in a Zeiss EM 10 (Oberkochen, FRG), equipped with a cold stage. The section revealed Tl localization mainly at filaments of the A-bands and at Z-line proteins.