PREFERENTIAL LOCALIZED UPTAKE OF K^+ AND Cs^+ OVER Na^+ IN THE A-BAND OF FREEZE-DRIED EMBEDDED MUSCLE SECTION: DETECTION BY X-RAY MICROANALYSIS AND LASER MICROPROBE MASS ANALYSIS

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• *Sections of freeze-dried embedded frog muscle were exposed to aqueous solutions containing various combinations of the salts LiCl, NaCl, KCl, and CsCl. Energy dispersive X-ray microanalysis and laser microprobe mass analysis of these sections showed selective and preferential accumulation of K^+ (and Cs^+) over Na^+ on specific protein sites in the A-bands. The selectivity coefficient exceeded 10 and involved a total K^+ accumulation of about 40 mmoles/kg (in comparison with a value of about 80 mmoles/kg in normal living frog muscle). These findings support the view that selective K^+ adsorption on intracellular proteins is the primary cause of selective accumulation of K^+ in living cells.*

INTRODUCTION

The debate concerning the physical state of potassium (K^+) in living cells has been going on for more than half a century.[1-8] The conventional membrane theory assumes most K^+ to be dissolved freely in cell water and the high cell K^+ level to be dependent upon a membrane pump, a Donnan equilibrium, or both. The bulk-phase theories, on the other hand, assume cell K^+ to be somehow "bound" to intracellular macromolecules. The association-induction hypothesis in particular attributes the high cell K^+ level to specific physical adsorption onto anionic β- and γ-carboxyl groups of cell proteins.[9]

Inability to demonstrate selective K^+ adsorption by proteins *in vitro* played a decisive role in the early rejection of the bulk-phase theories and acceptance of the membrane-osmotic-pump theory.[10] Studies of isolated actomyosin and of muscle homogenates failed to show any significant degree of selective K^+ adsorption.[11-13]

Another approach to the issue of the physical state of cell K^+ is to determine whether potassium is distributed evenly throughout the cell or is localized to regions rich in protein carboxyl groups. Striated muscle provides a unique preparation with which to study this question, as its major proteins are segregated at locations in the cell and the myosin-rich A-bands contain most of the cell's β- and γ-carboxyl side chains.[14] Furthermore, the alkali-metal ions K^+, Rb^+, Cs^+, and Tl^+ replace each other, in frog muscle under physiological conditions, in a mole-for-mole fashion.[15,16]

Indeed, three recently developed and independent methods have shown that potassium and other alkali-metal ions accumulate primarily in the A-bands of frog sartorius muscle. These methods are (1) transmission electron microscopy (TEM) of Cs^+ and Tl^+ in freeze-dried and embedded but unfixed muscle,[17,18] (2) autoradiography of Rb^+ and Cs^+ in both air-dried muscle[19] and frozen-hydrated muscle,[18] and (3) electron microprobe analysis of freeze-dried muscle.[20]

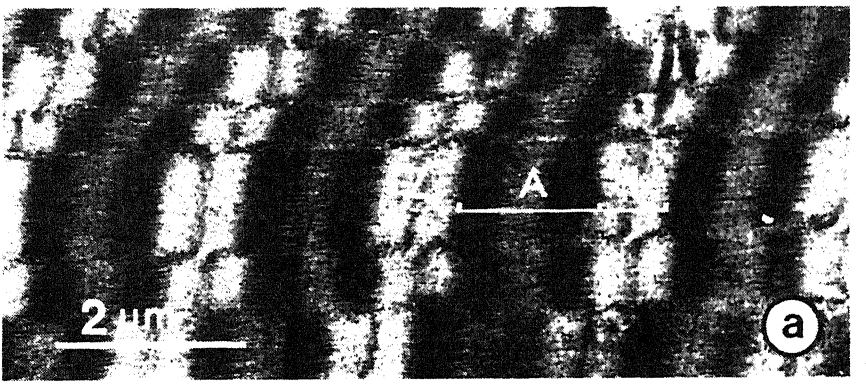
The study reported here used freeze-

dried embedded muscle to re-investigate the fundamental question of whether it is possible for an *in vitro*, non-living preparation to adsorb alkali-metal ions in a selective fashion. To detect the ions, both the electron microprobe and a new technique—the laser-activated microprobe mass analysis (LAMMA)—were used.

METHODS AND RESULTS

Freeze-drying and embedding of sartorius muscle of North American leopard frogs was carried out as described in earlier papers.[21,22] Sections 0.2 μm thick were wet-cut with a diamond knife and collected on Formvar-coated grids. The sections were exposed to aqueous solutions containing different concentrations of LiCl, NaCl, KCl, and CsCl (pH adjusted to 7.0 with tris buffer) in the following manner: Grids bearing the sections were floated with section side down on drops of the solution for 5 min. The grids were picked up with a pair of forceps and vigorously moved through the air to remove the adhering fluid by centrifugal force. This method yielded sections showing large areas free of salt crystal artifacts when observed by TEM. The sections were stored in a dry chamber until further examination. The exposure of sections to solutions containing CsCl and other alkali-metal ions always yielded an improved contrast under TEM compared to unexposed sections, the best staining occurring with CsCl-LiCl solutions as shown in Fig. 1a. A-band and Z-lines are heavily stained showing that large amounts of alkali-metal ions are accumulated at these sites.

Energy dispersive X-ray microanalysis[23] of exposed sections showed that each detectable species of alkali-metal ion (Na, K, Cs)



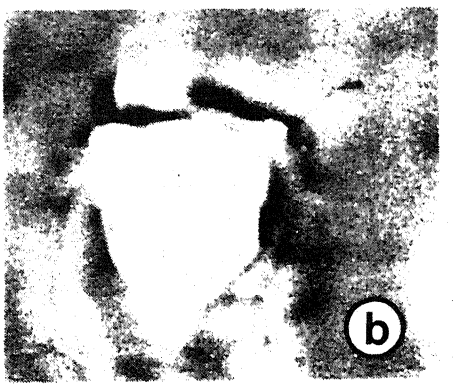


FIGURE 1. 0.2 μm-thick sections of chemically unfixed frog sartorius muscle, freeze-dried and embedded. The sections were exposed for 5 min to aqueous solutions containing (a) 100 mM LiCl and 10 mM CsCl and (b) 100 mM NaCl and 10 mM KCl. The latter section was perforated at roughly the location of the A-band with a laser beam from a LAMMA instrument. A = A-band. I = I-band. Z = Z-line.

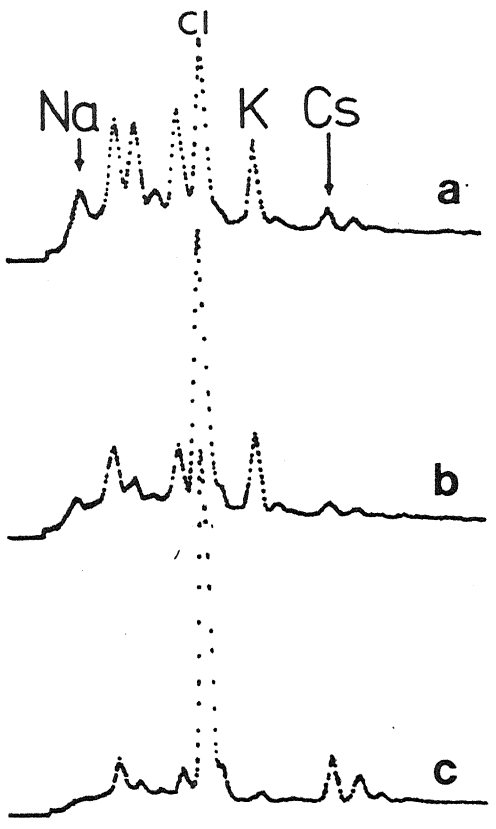


FIGURE 2: Energy dispersive spectra (a) from a gelatine standard containing 50 mM NaCl, 50 mM KCl, 10 mM CsCl; (b) from an A-band region of muscle sections exposed to solutions containing 50 mM NaCl, 50 mM KCl, 10 mM CsCl and (c) containing 50 mM LiCl, in addition to 50 mM NaCl, 50 mM KCl, and 10 mM CsCl.

was accumulated locally within the muscle section. As a rule, peak intensities of the ions over the A-bands were 3 to 5 times higher than over the I-bands (Z-line excluded). The areas that contained only embedding medium and looked clean under the TEM were free of alkali-metal ions on X-ray microanalysis.

Electron microprobe spectra are shown in Fig. 2. Figure 2a shows the elemental peaks of cryosectioned and freeze-dried gelatine standard[24] in which 50 mM NaCl, 50 mM KCl, and 10 mM CsCl were present. The thickness of the standard was about 0.4 μm. Figure 2b shows a spectrum of an A-band region of a 0.2 μm-thick muscle section exposed to a solution containing the same ion concentration as the standard. There was a selective accumulation of the alkali-metal ions. The ratio of concentrations of two or more ions detected in an A-band differed markedly from the concentration ratio of these ions in the bathing solution. Comparing Fig. 2a with 2b, one obtains the selectivity rank order K > Cs > Na, which is the same rank order of selective accumulation in living frog muscle cells.[9] Figure 2c was taken from a section which was exposed to a solution containing 50 mM LiCl in addition to 50 mM NaCl, 50 mM KCl, and 10 mM CsCl. Here the rank order is Cs >> K > Na. Sections exposed in such a way were well stained, similar to the section shown in Fig. 1a. As a rule it was observed that sections exposed to the same solution yielded reproducible selectivity ratios, provided the structure preservation of the detected areas was similar.

In order to detect also Li^+ ion in muscle sections, elemental analysis was carried out

with a laser microprobe mass analyzer (LAMMA).[25] A small spot (diameter between 1.5 μm and 2.0 μm) of the section was evaporated under the control of a phase contrast light microscope and ionized by a focused UV power-pulse laser. The ions were collected by ion optics and analyzed in a time-of-flight mass spectrometer. The area of the muscle section destroyed by the laser beam and analyzed for ions was from an A-band region (Fig. 1b). Results are given in Fig. 3. Figure 3a shows a standard with 50 mM LiCl, 50 mM NaCl, 50 mM KCl, and 10 mM CsCl. Figures 3b and 3c are spectra from A-bands of sections treated as those from which Fig. 2b and 2c were taken. The selectivity order shown by Fig. 3b is $K^+ > Cs^+ > Na^+$ while that of Fig. 3c is $Cs^+ > Li^+ >> K^+ > Na^+$ in full agreement with the X-ray microprobe. Figure 3c illustrates that exposure of sections to solutions with LiCl yielded considerable accumulation of Li^+ in the muscle preparation and reaffirms the earlier observation that Li brought about a marked change in the selectivity order of Na, K, and Cs.

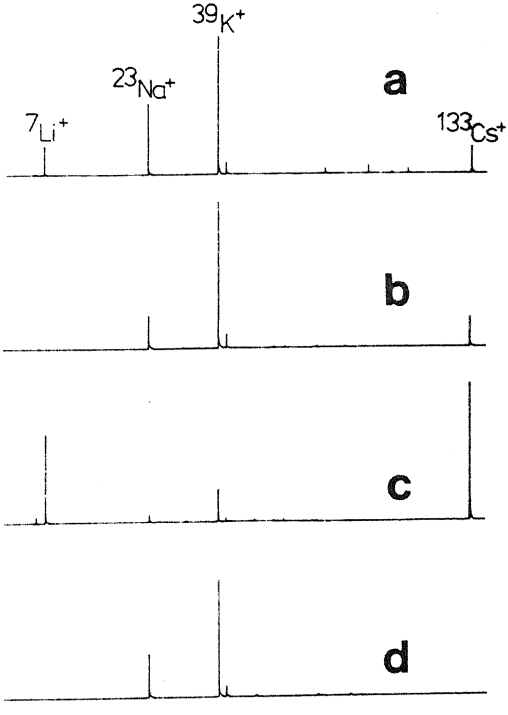


FIGURE 3. LAMMA-spectra (a) from a gelatine standard containing 50 mM LiCl, 50 mM NaCl, 50 mM KCl, 10 mM CsCl from A-band regions of muscle sections exposed to a solution containing (b) 50 mM NaCl, 50 mM KCl, 10 mM CsCl; (c) 50 mM NaCl, 50 mM KCl, 10 mM CsCl in addition to 50 mM LiCl; (d) only 100 mM NaCl, 10 mM KCl.

Sections exposed to a solution with 100 mM NaCl and 10 mM KCl accumulate more K^+ than Na^+ in the A-band of the muscle (Fig. 3d). Comparing Fig. 3d with Fig. 3a, one finds that the selectivity coefficient of K^+/Na^+ exceeds 10. The concentration of accumulated K^+ expressed in terms of cell wet weight is about 40 mM in the muscle section. Even higher concentrations of accumulated alkali-metal ions are detected if the sections are exposed to solutions with LiCl and CsCl.

Control experiments were also carried out with sections of glutaraldehyde-fixed and conventionally-embedded muscle preparations. Sections exposed to alkali-metal ion solutions did not show an improved contrast under the TEM when compared to unexposed sections, and only minute, non-reproducible amounts of alkali-metal ions could be detected by elemental analysis.

DISCUSSION

The results presented here show that K^+ and Cs^+ were *selectively* accumulated over Na^+ by freeze-dried, embedded muscle at those sites which accumulate K^+, Rb^+, and Cs^+ in living muscle.[17-20] These are the same sites that are stained by uranium and lead in the conventional chemical fixation and staining techniques. It seems reasonable to conclude that in all these cases electrostatic interaction between anionic groups of the proteins and the cations is responsible for the observed accumulation.[26] The idea of

electrostatic alkali-metal ion binding in sections of freeze-dried and embedded muscle is supported by the findings, first, that all ion species can be accumulated to a high degree, and second, when in competition with one another, ions accumulated in the A-band showed a high degree of specificity. From the first observation it is clear that the selectivity is not due to an exclusion of some ions from small water channels within the section. If only free counter-ions were to hover near the fixed charges, either in water channels within the section or at the outside of the section, no ion-specific selectivity would have occurred. It seems most likely that in the freeze-dried muscle preparation the short-range attributes of the alkali-metal ions (e.g., polarizability, Born repulsion constant) contribute to the electrostatic interaction with fixed anions. A detailed calculation of long-range and short-range forces between fixed anions and alkali-metal ions reveals that in a certain range of weak ion binding or adsorption high selectivity ratios are expected.[9]

Findings which deserve further investigation are the effects of Li^+ on the selective accumulation of the other alkali-metal ions: (a) Accumulation of Li^+ is accompanied by an *increase* of the total amount of accumulated alkali-metal ions. (b) Accumulation of Li^+ alters the rank order of selectivity among Na^+, K^+, and Cs^+. Conceivable interpretations would be that adsorption of Li^+ causes dissociation of salt linkages between fixed anion and fixed cation groups of the proteins thereby generating new alkali-metal ion binding sites;[7,9] the dissociation is accompanied by an additional accumulation of alkali-metal ions and Cl^- ions at the newly generated free sites, a prediction noticeable in the size of the Cl^- peak on Li^+ treated sections (Fig. 2c); at the same time the selectivity rank order of the alkali-metal ions changes. This sort of phenomenon has been discussed by Ling and attributed to inductive effects.[9]

In conclusion, the following are worth notice:

(1) Alkali-metal ions, especially Cs in Cs-Li solutions, alone provide good contrast for "staining" freeze-dried, embedded preparations for electron microscopy.

(2) Cellular proteins may be freeze dried, without chemical fixation, and thus maintained in an electronic, as well as steric configuration that is able to display *selective* adsorption of K^+ and other alkali-metal ions. This agrees with concepts introduced by the association-induction hypothesis: selective K^+ adsorption over Na^+ on anionic β- and γ-carboxyl sites of protein, primarily in the A-band, but only when the protein-ion-water system is maintained in its resting, metastable state. With this in mind it is not surprising that earlier experiments using isolated proteins or muscle homogenates failed to show significant selective K^+ binding.

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