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Potassium Binding Sites in Muscle: Electron Microscopic Visualization of K, Rb, and Cs in Freeze-Dried Preparations and Autoradiography at Liquid Nitrogen Temperature Using ^{86}Rb and ^{134}Cs

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Summary. Normal frog sartorius muscles and muscles in which a major portion of the intracellular K^+ was reversibly replaced by Rb^+ or Cs^+ were frozen, freeze-dried and embedded without chemical fixation or staining. Dry-cut sections of these preparations reveal striation patterns with higher contrast than those of wet-cut sections of the same preparation. The results suggest that in the living state the alkali metal ions are mainly localized in the A bands and Z lines of myofibrils. This idea is confirmed by a new autoradiographic technique by means of which the distribution of Rb^+ and Cs^+ in frozen-hydrated single muscle fibers has been investigated. The findings support the association-induction hypothesis according to which most cell K^+ and other alkali-metal ions are not free in cell water but are adsorbed to beta- and gamma-carboxyl groups of cell proteins.

Introduction

The idea that the major cellular cation, K^+, is not distributed evenly within striated muscle dates back to the turn of the century (MaCallum 1905) and has been reviewed by Ernst (1963), and by Nesterov and Tigyi-Sebes (1965). Chemical precipitation and microincineration techniques suggested a localization of K^+ in the anisotropic bands of voluntary muscle. These techniques were questioned, however, and indirect evidence accumulated in support of the concept that K^+ is freely dissolved in cell water and evenly distributed in muscle and other cells (Fenn 1936; Gersh 1938). In the current version of the membrane-osmotic theory the high level of cell K^+ is maintained by a continuous "uphill" pumping which compensates for the continuous "downhill" diffusion of K^+ out of the cell. Free cell K^+ is responsible for a major part of cellular osmotic pressure, and the resting potential is due to the distribution of K^+ between the cellular and extracellular pools.

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There exist, on the other hand, strong reservations about the correctness of these ideas, and evidence of K^+ binding theories has been offered (Ling 1962; Ernst 1963; Troschin 1958). Among these the association-induction hypothesis (AIH) of Ling 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 electrical phenomena, in terms of explicit molecular mechanism (Ling and Peterson 1977; Ling 1978b, 1979). One prediction of the AIH concerns the localization of K^+ in striated muscle, and can now be tested experimentally (Ling 1962, 1977b). The AIH maintains that beta- and gamma-carboxyl groups of cell proteins are the primary sites of K^+ localization due to a physical adsorption (Ling 1952, 1962). Since myosin contributes more than 60% of the beta- and gamma-carboxyl side chains of 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. The experiments reported here take advantage of the fact that the alkali-metal ions K^+, Rb^+, and Cs^+, and Tl^+ ions are accumulated in muscle by the same mechanism, as they replace each other in a mole-for-mole fashion under physiological conditions (Ling 1977a). The subcellular localization of one species would therefore imply localization of adsorption sites for all four ions. In addition, Rb^+ with atomic weight of 85, and Cs^+ with atomic weight of 133, are heavy enough to be visualized by transmission electron microscopy (TEM) if they are unevenly distributed in the specimen (Thomson et al. 1969), and ^{86}Rb and ^{134}Cs are much more suitable for autoradiography than the short-lived ^{42}K.

I have previously shown by TEM that Cs^+ and Tl^+ are indeed localized in the A bands and Z lines of freeze-dried and embedded muscle (Edelmann 1977). Ling (1977b) has shown a similar pattern using ^{134}Cs and ^{204}Tl autoradiography of air-dried single muscle fibers. The purpose of the work reported here is first to determine if all the alkali-metal ions, K^+, Rb^+ and Cs^+, can be visualized in muscle sections, and second to confirm the alkali-metal ion localization by an independent method. The latter was accomplished using autoradiography at liquid nitrogen temperature, a technique that avoids all the preparative steps, such as cryosectioning, freeze-drying, embedding, or air-drying, which might cause dislocation of mobile ions.

Material and Methods

Frog sartorius muscles were isolated in a sterile fashion from Northern American leopard frogs (Rana pipiens pipiens, Schreber), and used either as they were containing a normal amount of K^+, or after a major portion of cell K^+ (at least 70%) was replaced with Rb^+ or Cs^+ (the latter a reversible process; Ling and Bohr 1971). For autoradiography the muscles were loaded with Rb^+ or Cs^+ labelled respectively with ^{86}Rb or ^{134}Cs.

Method I: Localization of Alkali-Metal Ions in Freeze-Dried, Embedded Muscles. Freeze-drying and embedding were carried out as described earlier (Edelmann 1977, 1978a, 1978b). Muscles were shock-frozen in quick-freezing pliers (Reichert, Vienna) at liquid nitrogen temperature, freeze-dried at $-80°$ C for 3 days and at $-60°$ C for 6 days. Freeze-drying was carried out in a newly-developed apparatus with the necessary reduction of water vapor pressure in the chamber provided by cryosorption pumping (Zeolite 13X, Leybold Heraeus, Köln) and cold traps. Frozen-dried pieces were

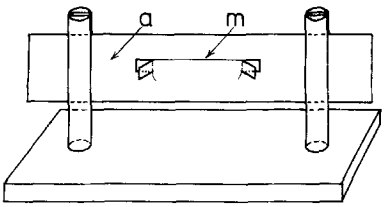

Fig. 1. Holder for aluminium foil to which a single muscle fiber is attached. *a* aluminium foil, *m* muscle fiber

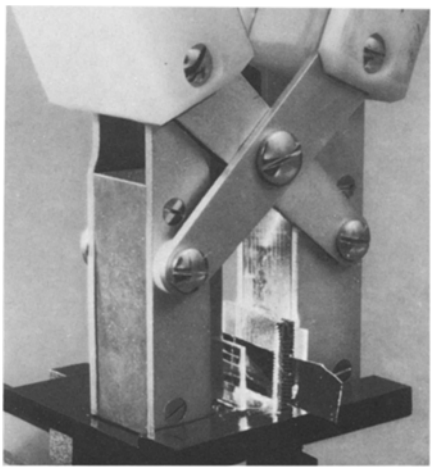

Fig. 2. Quick-freezing pliers. Two glass slides are glued on. Between the slides the aluminium foil (appears *dark*) can be seen with two attached muscle fibers (appear *light*)

directly embedded in ERL-4206 mixture (Spurr 1969) at −15° C. Dry- and wet-cut sections of thickness 0.3 µm were examined in a Siemens Elmiskop I at 80 kV acceleration voltage.

Method II: Localization of Alkali-Metal Ions in Frozen-Hydrated Muscles by Autoradiography at Liquid Nitrogen Temperature. About 70% of the K^+ of sartorius muscles was replaced by non-radioactive Rb^+ or Cs^+. The muscles were then placed into vials containing 1 ml of radiolabelled Rb^+ or Cs^+ and incubated at 4° C for several days to allow full equilibration of specific radioactivity between solution and muscle. Mean Rb^+ or Cs^+ concentration, expressed in terms of cell water, was at least 100 mM and the radioactivity was about 1 µCi/mg muscle. Very thin (about 20 µm) single muscle fibers were dissected and stretched to different degrees on aluminium foil as shown in Fig. 1, still in contact with labelled Ringer's solution. The aluminium foil with attached fibers was then rinsed in distilled water for a few seconds to remove adherent radioactive solution and fixed in the holder shown in Fig. 1. The holder was kept in a humidity chamber and a constant air stream saturated with water vapor was blown against the fibers to prevent them from drying.

Two clean glass microscope slides were glued to the inner surfaces of the pair of prongs of the quick-freezing pliers (Fig. 2). One of the glass slides had been previously coated with Formvar. A layer of Ilford K-5 emulsion about 1 to 3 µm thick was placed on top of the Formvar layer by the dip method. (Rogers 1969). Pliers, with glass slides glued on, were cooled to liquid nitrogen temperature. The muscle fiber, stretched on its holder, was then quickly frozen as it was pressed between the rapidly-closing plier tongs. With this procedure the fibers were flattened, frozen and brought into contact with the emulsion simultaneously. The glass slides were clamped together, removed from the pliers, and stored in liquid nitrogen. Exposure lasted 1 to 3 weeks. The frozen muscle fibers were separated – still at low temperature – from the emulsion-coated glass slide, and the slide then warmed up to room temperature. The emulsion was developed in Kodak D19 developer for 3 min and fixed in the usual manner. The autoradiogram, still attached to the Formvar film on the slide could now be examined by light microscopy. The Formvar film carrying the

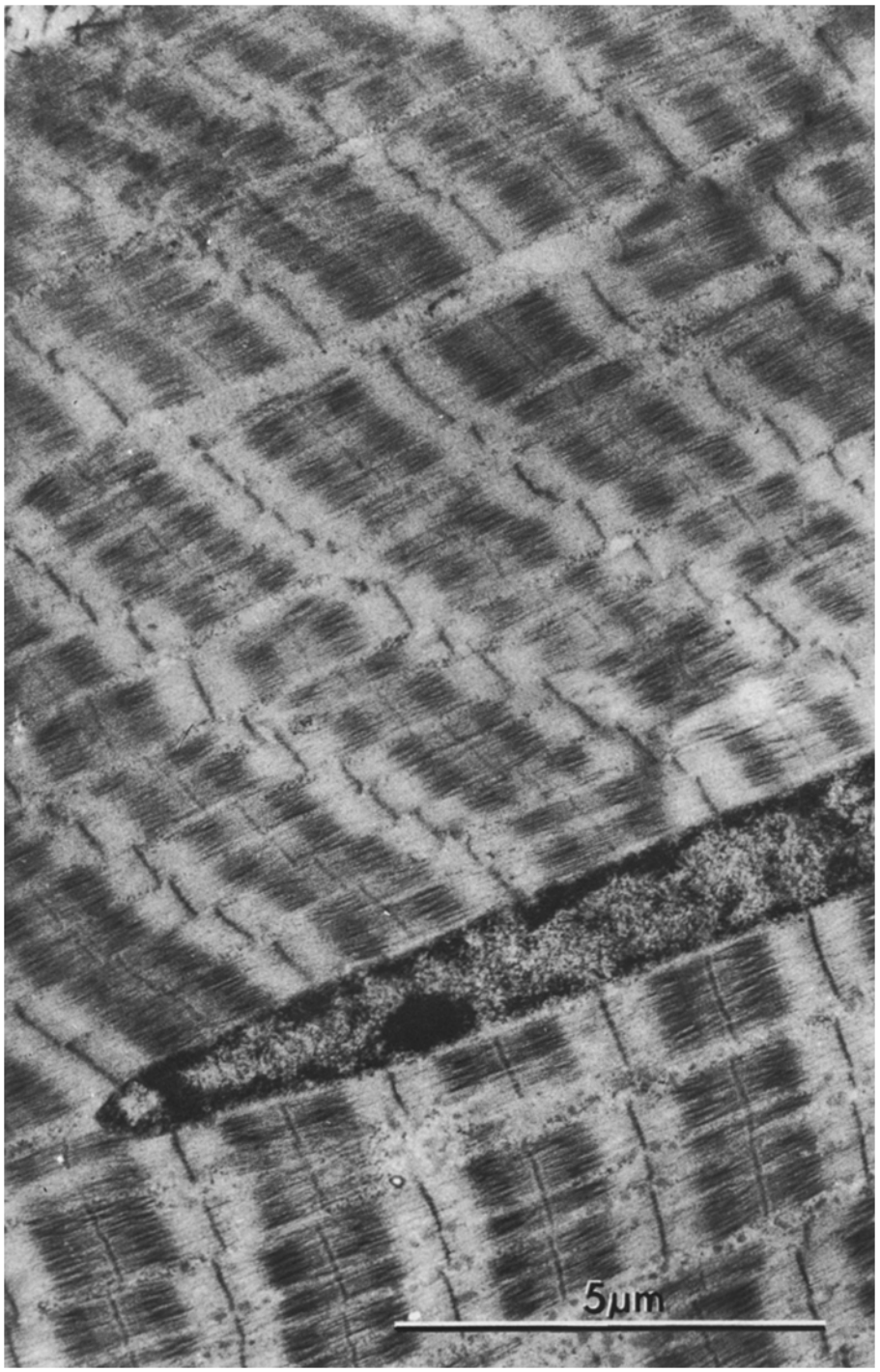

Fig. 3. Ultrathin section of a chemically unfixed frog sartorius muscle, freeze-dried, embedded in Spurr's resin, stained with 5% uranyl acetate (5 min) and lead citrate (5 min)

autoradiogram could also be cut into small areas by a razor blade, removed from the slide, and transferred to electron microscopic grids. This procedure was carried out in a Petri dish filled with distilled water.

Results

TEM Visualization of K, Rb and Cs in Sections of Freeze-Dried, Embedded Muscle

Electron micrographs of sections of freeze-dried and embedded muscles are shown in Figs. 3 and 4. Figure 3 was taken from a wet-cut ultrathin section of a Cs-loaded muscle stained with uranyl acetate and lead citrate in the usual manner. This micrograph demonstrates the usefulness of the new freeze-drying and embedding technique (Edelmann 1978a, 1979): Large areas can be sectioned (Sitte 1979), which reveal good preservation of ultrastructure of chemically-unfixed muscle. The sections shown in Fig. 4 were taken from zones with similar structure preservation. In this figure 0.3 µm thick dry-cut sections (Fig. 4A, C, D) are compared to 0.3 µm thick wet-cut sections (Fig. 4B, E). All photographs were prepared under the same conditions. The staining intensities shown in these micrographs result only from the dry material present in the freeze-dried, embedded muscle. It is apparent that sections of Cs-loaded (Fig. 4A) and of Rb-loaded (Fig. 4C) muscles show good contrast due to the uneven distribution of the electron-dense Cs or Rb. The alkali-metal ions are preferably accumulated at those sites which are stained by lead and uranium after the conventional chemical fixation, embedding and staining procedures (Edelmann 1978b). When Cs- or Rb-loaded muscles are wet-cut, then the sections show poor contrast (Fig. 4B), indicating that much of the alkali-metal ions leach out into the distilled water. Dry-cut sections of normal (K^+-containing) muscle (Fig. 4D) also yield micrographs with a slightly improved contrast when compared to wet-cut sections of normal muscle (Fig. 4E). The difference in contrast was confirmed by means of an intensity meter adapted to the electron microscope: The intensity of the electron beam penetrating the section was about 10% higher under the I band than under the A band in wet-cut sections of normal muscle and 20% higher in dry-cut sections of normal muscle. The differences were up to 40% in dry-cut sections of Rb- or Cs-loaded muscles.

Autoradiography at Liquid Nitrogen Temperature of Muscle Fibers Loaded with Radiolabelled Rb or Cs

Best results were obtained with stretched muscle fibers. Light and electron microscopic pictures of the same Cs-loaded stretched muscle fiber are shown in Fig. 5A and 5B. These autoradiographs show that the radioactive ion which is in contact with or near the emulsion during exposure is unevenly distributed within the muscle fiber. In Fig. 5B the periodicity of the striation pattern is about 4.4 µm, indicating that the sarcomeres had been stretched to this length. The dark deposits are concentrated in bands with a width of about 1.5 µm. In some cases a faint line of silver grains can be seen between two dark bands (arrow). An autoradiograph of a Rb-loaded fiber in which the sarcomeres had

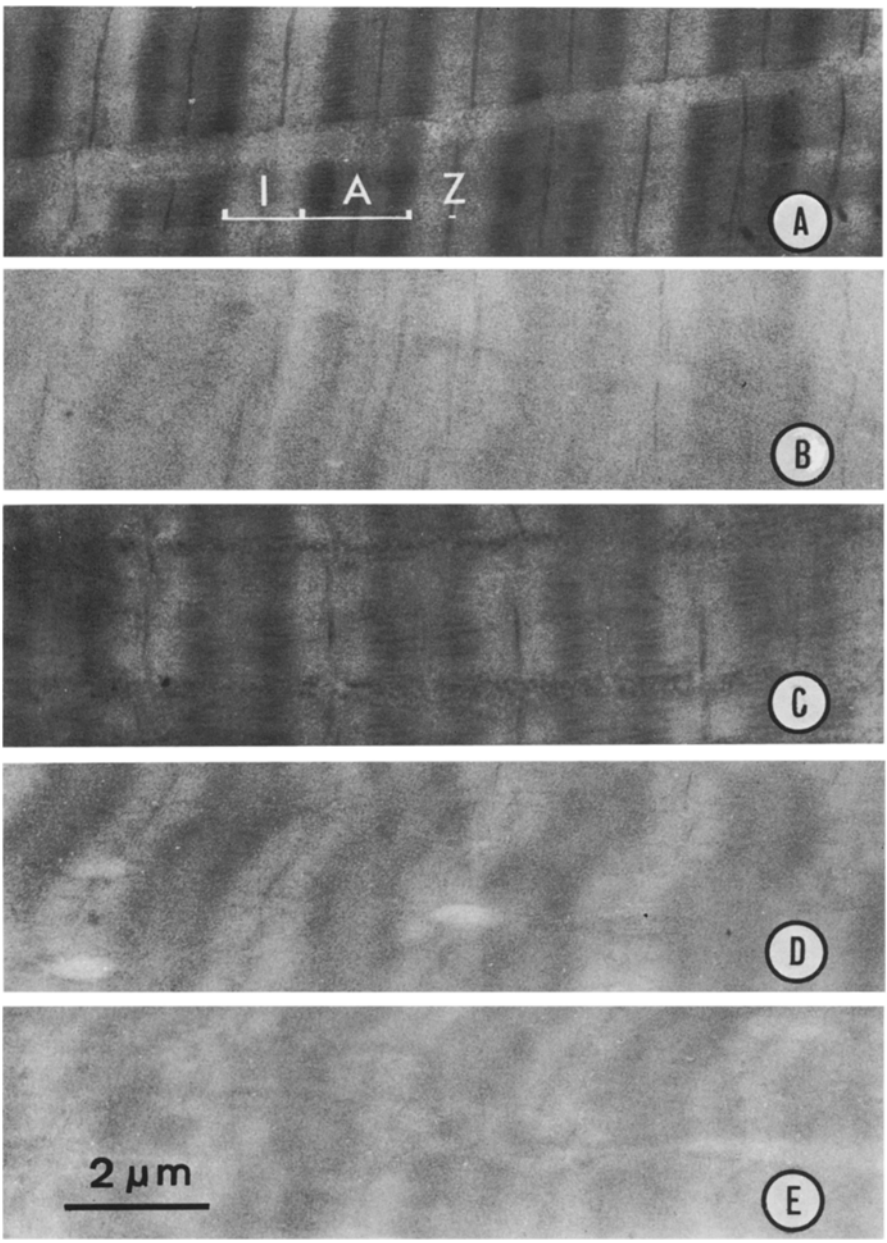

Fig. 4A–E. Transmission electron micrographs of 0.3 µm thick sections of freeze-dried frog sartorius muscle. **A** Dry-cut section of a Cs-loaded muscle. **B** Wet-cut section of a Cs-loaded muscle. **C** Dry-cut section of a Rb-loaded muscle. **D** Dry-cut section of a normal K-containing muscle. **E** Wet-cut section of a normal K-containing muscle. *A* – A band, *I* – I band, *Z* – Z line

been stretched to about 3.3 µm is shown in Fig. 5C. The dark bands which have approximately the same width as those of Fig. 5B are closer together than in Fig. 5B. Since the A band width of a resting muscle is about 1.5 µm (Fig. 4A) and remains constant during stretching whereas the I band changes

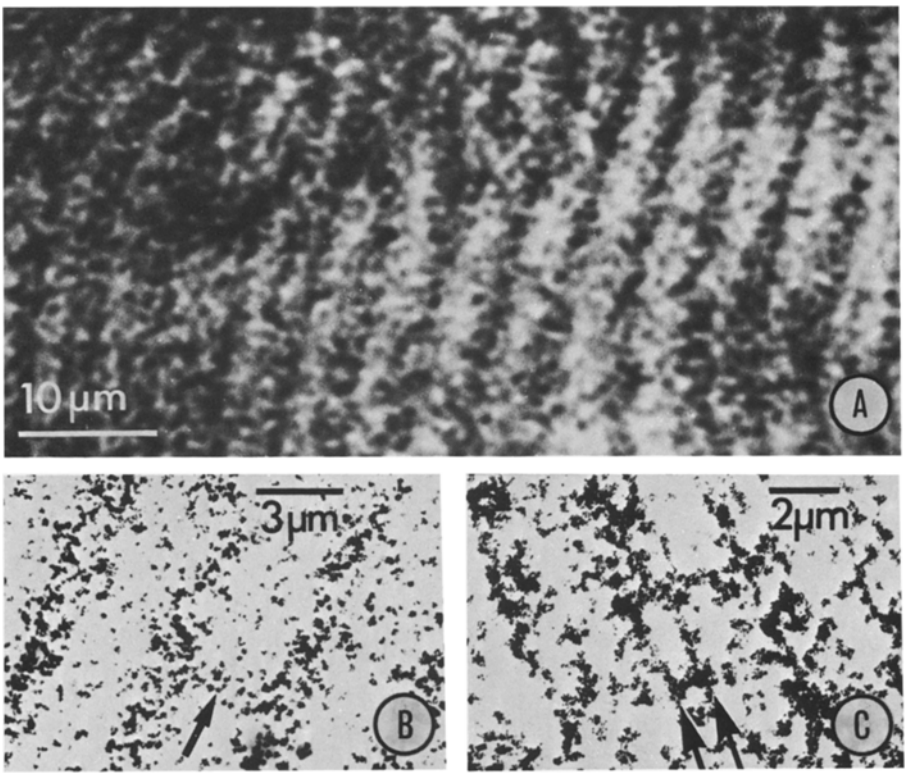

Fig. 5A–C. Autoradiographs of frog muscle fibers. **A** Light microscopic autoradiogram of a stretched Cs-loaded fiber. **B** Electron microscopic autoradiogram of a stretched Cs-loaded fiber. The sarcomere length is about 4.4 µm. Between two dark bands (A bands) a line of silver grains indicates the Z line (*arrow*). **C** Electron microscopic autoradiogram of a stretched Rb-loaded fiber. The sarcomere length is about 3.3 µm. *Arrows* indicate dark lines at the outer edges of an A band

in width, it can be concluded that the major portion of the ^{134}Cs or ^{86}Rb is localized in the A bands and that the arrow in Fig. 5B indicates Cs-accumulation at the Z line.

The dark bands in Fig. 5C show a high silver grain density at their outer edges (arrows). This finding suggests that the highest Rb concentration was present at the outer edges of the A bands as it was shown to be the case in freeze-dried, embedded preparations (Fig. 4C). A preferential accumulation of silver grains at the outer edges of the dark bands cannot be seen in autoradiographs of highly-stretched fibers (Fig. 5B). Locker and Leet (1975) have shown that at this degree of stretching the A bands are uniformly stained after conventional chemical fixation and staining procedures. Considering the above mentioned similarity between staining patterns of conventionally prepared muscle sections and accumulation patterns of alkali-metal ions it seems to be likely that the alkali-metal ions are uniformly distributed in the A bands of highly-stretched sarcomeres.

Control muscle fibers loaded with non-radioactive Rb or Cs did not yield autoradiographs with striation patterns.

Discussion

The results show that the alkali-metal ions K^+, Rb^+ and Cs^+ are unevenly distributed within muscle cells and that they are preferentially accumulated within the A band and, to a lesser extent, the Z line. These ions are accumulated at the same sites that are stained by lead or uranium in the conventional chemical fixation and staining techniques. This finding is now confirmed by three independent methods:

(1) Analysis of sections of freeze-dried, embedded muscle

(a) Visualization of K^+, Rb^+ and Cs^+ by TEM (reported here and by Edelmann 1977)

(b) Electron probe x-ray microanalysis (Edelmann 1978b)

(2) Autoradiography of air-dried muscle fibers (Ling 1977b)

(3) Low-temperature autoradiography of shock-frozen muscle fibers not dried or embedded (reported here).

Assessment of Artifact and Use of Low-Temperature Autoradiography

The high degree of consistency between the results using the above widely-differing techniques is in itself persuasive evidence that the ion localization is not due to artifact. There are, however, several independent observations that, in addition to the results of the autoradiography at liquid nitrogen temperature, argue against a freeze-drying or embedding artifact as the cause of the results obtained with TEM. (a) MacKenzie has shown that freeze-drying of KCl solutions leaves KCl unchanged in the pattern in which it was deposited during freezing (MacKenzie 1965). Since ice crystal artifacts are minimized in the well-preserved zone of muscle, which most clearly show the uneven ion distribution, the possibility of a major dislocation during freeze-drying may be ruled out.

(b) Measurements using radioactive tracers show that less than 7% of cellular alkali-metal ions leach out into the embedding medium.

(c) The very regular and highly reproducible distribution pattern speaks against the possibility of a major dislocation of ions due to plastic embedding.

However, the main argument against possible artifacts in freeze-dried, embedded, or air-dried muscle is provided by the low-temperature autoradiography reported here. Conditions required in order to see unequivocal periodic striation in the autoradiograph are first, that the fibers be well-flattened onto the emulsion, and second that they be frozen fast enough that they do not contract and shorten until after their removal from the emulsion and subsequent thawing. In spite of these potential problems, in spite of the thickness (several μm) of even the flat fibers, and in spite of the fact that ^{86}Rb and ^{134}Cs are high-energy beta emitters and not particularly well-suited for high-resolution autoradiography, the striated pattern is clearly evident (Fig. 5). Furthermore, the quickly-frozen, flat muscle fibers provide greater resolution of ^{134}Cs than in the prior studies of Cs tracers in sections of formol-fixed muscle (Giese and Rekowsky 1970) and in cryostat sections of chemically non-fixed muscle (Szentkuti and Giese 1973). Nevertheless, even these studies were able to show an uneven distribution of the ions. The technique of autoradiography at low temperature may be useful if developed further and applied to other biological phenomena.

After exposure of shock-frozen material, it could be removed from the emulsion, freeze-dried, prepared for TEM, and the autoradiograph correlated with the ultrastructure.

Distribution Patterns of Alkali-Metal Ions

Since myosin is concentrated in the A bands, and since myosin contains a higher density of negatively-charged beta- and gamma-carboxyl groups than, for example, actin, the obvious interpretation of the results of our studies is that the ions are adsorbed to these carboxyl groups. The alternate explanation, that the cations were actually free in solution in cell water and were only near the fixed negative charges as counterions seems unlikely for several reasons. First, if a large disproportion of free ions would exist between the A bands and the I bands then a gross disproportion of osmotic pressures would exist between these two regions. As a result water should migrate from the I bands to the A bands and cause their progressive swelling. Since this does not occur it must be concluded that the concentration of alkali-metal ions that are free in cell water is not higher in the A bands than in the I bands. Second, the notion of free cellular alkali-metal ions conflicts with recent studies of frog muscle by the "EMOC" technique (Ling 1973, 1978a). In those experiments the end of the muscle was cut off, and, since all cells run the length of the muscle, they were all cut open. The preparation was suspended in humid air and allowed to equilibrate with Ringer's solution in contact with the muscle only at the cut end. Not only was an asymmetric distribution of ions maintained between the muscle and the Ringer's in the absence of functioning surface membrane "pumps", but the rank order of selectivity ($K^+ > Cs^+ > Na^+$) was also maintained. In conclusion, the TEM and autoradiographic data reported here provide strong direct support for the sort of molecular adsorption mechanism provided by the association-induction hypothesis.

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