K^+ LOCALIZATION IN MUSCLE CELLS BY AUTORADIOGRAPHY, AND IDENTIFICATION OF K^+ ADSORBING SITES IN LIVING MUSCLE CELLS WITH URANIUM BINDING SITES IN ELECTRON MICROGRAPHS OF FIXED CELL PREPARATIONS

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• ***Localization of K+-adsorption sites on two peripheral regions of the A band and the Z line in frog voluntary muscle cells has been demonstrated with the aid of autoradiography and radioactive ^{134}Cs and ^{204}Tl. The findings confirm the association-induction hypothesis, according to which intracellular K^+ is adsorbed on the β- and γ-carboxyl groups of intracellular proteins.***

INTRODUCTION

Two mutually exclusive theories concerning ion concentration in the living cell are currently in competition. The membrane-pump theory maintains that intracellular K^+ is in the free state.[1–4] Alternatively, the association-induction hypothesis (which by virtue of development since first proposed has attained the status and rigor of a formal theory) maintains that the bulk of intracellular K^+ is adsorbed on the β- and γ-carboxyl groups of cell proteins.[5–10] In furthering understanding of the living cell, accurate knowledge of the physical state of intracellular K^+ is of a decisive importance that can be assessed from the following considerations:

(I) According to membrane-pump theory, the resting and action potentials of living cells depend on the existence of all or virtually all intracellular K^+ in the free state.[11–14] Adsorption of the bulk of intracellular K^+ would therefore contradict the membrane theory of cellular potentials. On the other hand, in the association-induction hypothesis cellular potentials are surface phenomena, their magnitudes not directly related to the macroscopic intracellular K^+ concentration.[6,10,15–18] The hypothesis, therefore, would be in full harmony with a finding of adsorption of the bulk of cell K^+.

(2) According to membrane-pump theory, half the osmotic activity of living cells is maintained by intracellular K^+, which is presumed to be free.[19,20] If the bulk of intracellular K^+ should prove not free, the intracellular osmotic pressure would, according to this theory, be reduced by a factor of 2 and thus would be too low to account for the water content normally maintained. On the other hand, according to the association-induction hypothesis, the osmotic activity of the

cell's interior is primarily due to long-range polarization of cell water by the NH and CO groups of certain protein chains in the extended conformation.[21–25] Demonstration of adsorption and consequent loss of osmotic activity of the bulk of K+, therefore, would offer strong support for the hypothesis.

Since 1952, when the β- and y-carboxyl groups (belonging respectively to the aspartic and glutamic acid residues) of cell proteins were first proposed as the sites of K^+ adsorption in living cells,[5–9] much has been learned about the nature of muscle cells. Recent conclusions have proved to be in accord with the basic concepts introduced in the association-induction hypothesis and have given rise to the following new predictions concerning ion distribution:

(A) Localization of K^+ in the A bands of voluntary muscle cells. Myosin, which contains more than 60 per cent of the β- and y-carboxyl side chains of intracellular proteins in voluntary muscle cells,[26,27] is now known to constitute the substance of the thick filaments and hence the **A** bands of striated muscles.[28–30] With this added information, the original theory of the β- and y-carboxyl groups as the sites of K^+ adsorption would demand that the bulk of intracellular K^+ be located in the **A** bands of the voluntary muscle cells.*

(B) Localization of cesium (Cs^+) and thallium (Tl^+) in A bands. In order to test the crucial issue of K^+ localization in the **A** band, autoradiography appeared to be theoretically appropriate. Unfortunately, radioactive K isotopes are not available at high enough specific activity to yield simple clear-cut answers. However, other long-lived isotopes with high specific activities are available, notable ^{134}Cs and ^{204}Tl. Both have been shown to accumulate in living cells in the same manner as K^+ does, and both compete stoichiometrically for the same adsorption sites in frog muscle cells.[26,27] When these factors are taken into account, the idea that K^+ is adsorbed on the β- and y-carboxyl groups suggests that Cs^{134} and Tl^{204} would behave similarly and would also be located in the *A* band.

(C) *Uranium binding sites relative to K^+ adsorption sites on electron micrographs.* In 1960 Hodge and Schmidt[41] reported the results of their electron microscopic investigation of uranium- and phosphotungstate-stained tropocollagen macromolecules. Whereas the phosphotungstate binds to the positively charged guanidyl groups of the arginine side chains, cationic uranium ions probably bind to the β-carboxyl groups of the aspartic acid residues and the y-carboxyl groups of the glutamic acid residues of the protein. If in the living state the β- and y-carboxyl groups indeed adsorb K+, one can deduct that sites in the living cell that adsorb K^+, Cs^+, and Tl^+ are the same sites that show uranium stain in an electron micrograph of the same type of cell. As it is well known from conventional .EM plates of uranium-stained voluntary muscle cell preparations, uranium binding sites are located pri-

* **A** careful survey of the literature reveals that a variety of evidence has already accumulated supporting the notion of K^+ localization in the A bands. Some of this evidence, however, is suggestive rather than conclusive, which may partly explain why it has not gained as much attention from cell physiologists as might be expected.[31–40]

marily in the two marginal regions of the A band (separated by a less densely staining middle portion, or the H band) and on the Z line situated in the center of the I band.[30,41] Thus identification of the β- and y-carboxyl groups as the site of uranium uptake and straining would extend localization of predicted K^+ adsorption sites to beyond those on myosin and to involvement of proteins of the Z lines.

It was to test the above predictions, and thereby falsify or further verify pertinent tenets of the association-induction hypothesis, that the experimentation reported here was undertaken.

MATERIALS AND METHODS

The autoradiographic technique used for this study was modified after similar techniques used in localizing soluble materials in tissues.[42–47] Sterile isolated frog semitendinosus muscles were incubated for 1 to 5 days at 25°C in a Ringer-GIB medium[48] containing (only tracer)** ^{134}Cs and ^{204}Tl at activities between 100 microcuries/ml and 1 millicurie/ml. To minimize the hazards of drying, all subsequent dissections were carried out in an enclosed humidified environment. First the loaded muscle was placed in a pool of the original incubation solution and was separated into small bundles of about 10 to 30 muscle fibers each. The fiber bundles were then rinsed in distilled water for a few seconds to remove adherent radioactive materials. Single fibers were dissected on glass slides and allowed to dry thoroughly, either at their natural lengths or stretched. The slides with the dried muscle fibers were then coated by the dip method with a layer of Ilford K5 emulsion approximately 5 μ thick. After further drying, the emulsion-coated slides were in some cases dipped for 4 sec in a scintillation fluid.[49] The scintillation fluid contained 7.0 g of PPO and 20 mg POPOP per liter. Exposure of the photographic emulsion in the presence of silica gel lasted from 1 to 2 weeks at —20°C, followed by development in a Kodak-19 developer.

It should be mentioned that localization of alkali metal ions in living cells has long been one of the more difficult problems in autoradiography. The reason is that these ions tend to dissolve and diffuse away when the sections are exposed to the warm emulsion. To overcome this basic difficulty I relied, on the one hand, on rapid drying of the emulsion and, on the other, on an unusual feature of our technique: the use of extremely thick (10 to 20 μ) and thoroughly dried whole muscle fibers. By that means when a small layer of localized isotopes becomes solubilized and washed away, the deeper layers of isotopes apparently remain unaltered and produce the autoradiographs desired. It is doubtful, however, that this method would apply readily to other cells because few other cells possess the great stability

** While the best results were obtained from muscles labeled with tracer alone, other muscles with a substantial portion of K^+ replaced by labeled and non-labeled Cs^+ gave similar though somewhat poorer results.

and regularity in cytological structures of voluntary muscle fibers in general and frog voluntary muscle fibers in particular.

RESULTS

In Fig. 1A, a section of single muscle fiber that was not exposed to radioactivity but was otherwise processed exactly as the other specimens shows the usual striations of light and dark bands. The distinct dark A band and light I band in this figure clearly show that drying and other procedures did not obscure the periodic structures normally observed in the living state. Figure 1B shows an autoradiograph of a ^{204}Tl-loaded muscle cell. In this picture, the silver granules are mostly seen in rows, an average 2.7 microns apart. By themselves, the data of Fig. 1B indicate ^{204}Tl localization either in the A band or in the I band.

Figures 1C and 1D are autoradiographs of ^{134}Cs-loaded muscles in which the photographic emulsion covered only part of the muscle cells. The rows of dense silver granules corresponding to the location of ^{134}Cs are aligned with the dark A bands in that part of the photographs not covered by the photographic emulsion. These data show that ^{134}Cs and by inference Tl^+ and K^+ are indeed located primarily in the A bands of living frog muscle cells.

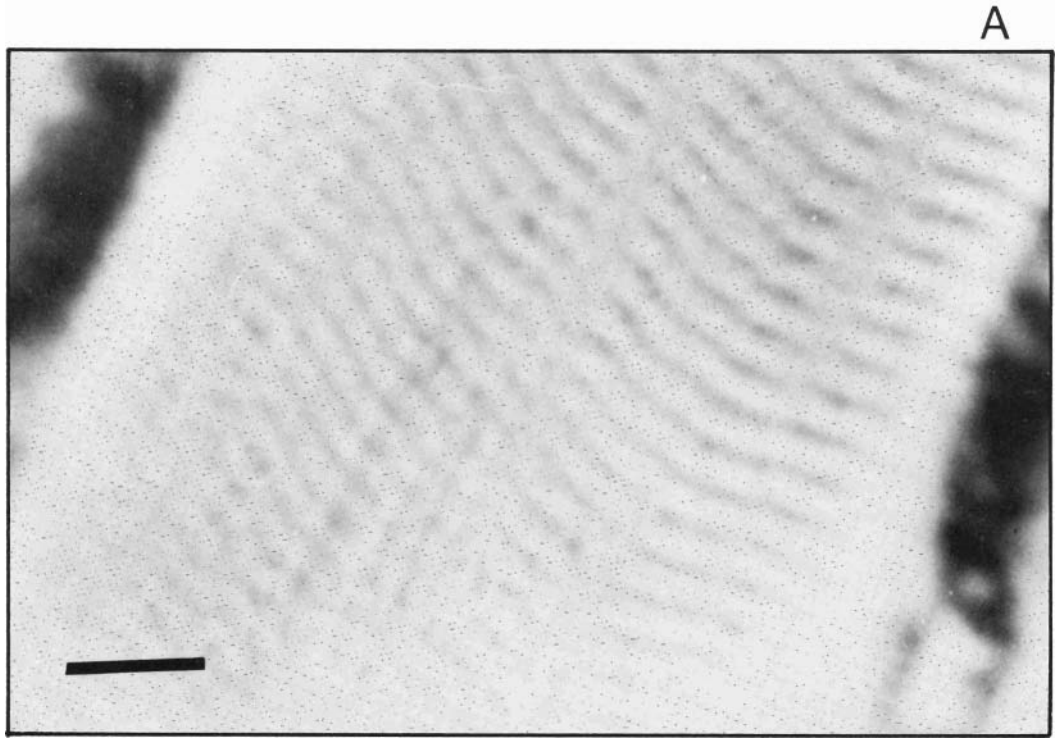
In Figs. 1E and 1F the muscle fibers are seen stretched, with sarcomere lengths of approximately 3.5 and 4.4 μ respectively. In the upper halves of each figure the silver granules are in sharper focus, revealing that the ^{134}Cs adsorbing sites are not uniformly distributed in each A band but tend to be distributed in two rows separated by a space of lesser silver grain density. Furthermore, another narrow row of silver granules can be visualized in the center of the I band and thus at the position of the Z line.

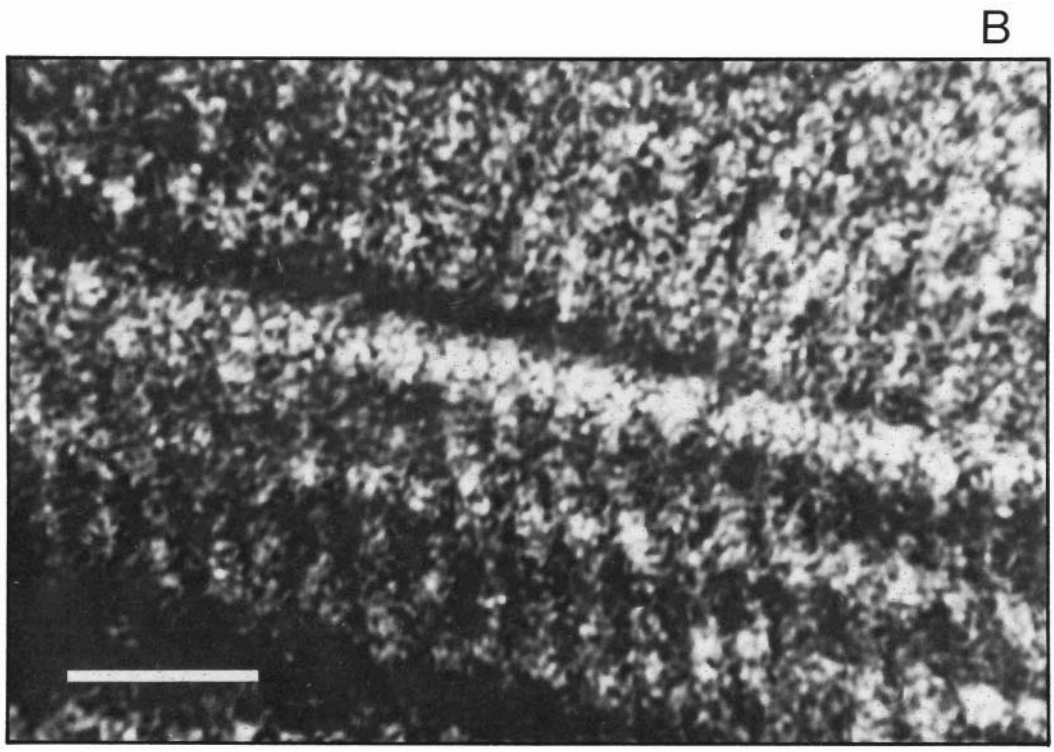
DISCUSSION

Before reaching a conclusion, let us examine in greater depth what the localization of ^{134}Cs and ^{204}Tl, and indirectly of K^+, in the A bands (and Z lines) tells us about the alternative theories concerning K^+ concentration in the living cell.

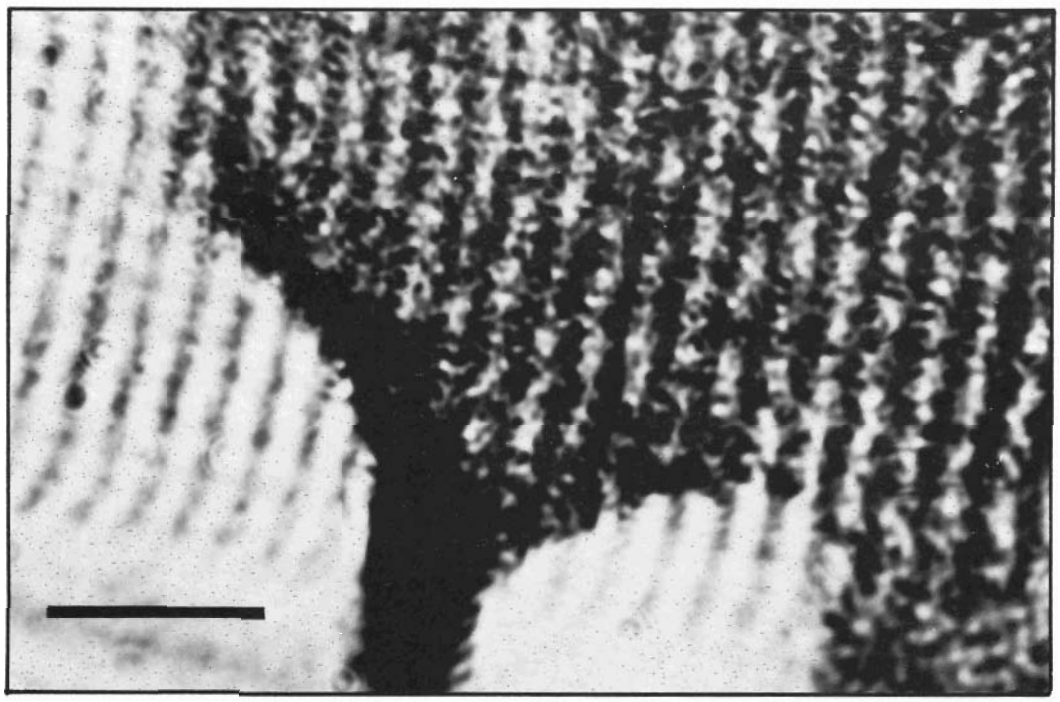
To account for the magnitude of the resting potential, membrane-pump theory requires that all intracellular K^+ not only be free but also be evenly distributed within the cell surface. The autoradiographs clearly show that this is not the case. To account for the visible localization of K^+, proponents of the membrane theory must postulate more membranes with pumps lining the surfaces of all the A bands and Z lines. But such a revised membrane model can be considered only if we disregard the fact that it violates the law of energy conservation.[5–10] In addition, it would conflict anew with other known facts. For example, such a model would predict wide fluctuation of electrical potentials along the length of each muscle fiber, with sharp peaks in the region of the A bands and Z line where K^+ concentration

FIGURE 1. Autoradiographs of frog muscle fibers. (A) Single fiber processed as those in the accompanying autoradiographs but not loaded with radioisotopes. (B) Single ^{204}Tl-loaded fiber. (*C*, D) Single ^{134}Cs-loaded fibers partially covered with photographic emulsions. (*E*, F) Single ^{134}Cs-loaded fibers that had been stretched before drying. (Bars represent a length of 10 micrometers.)

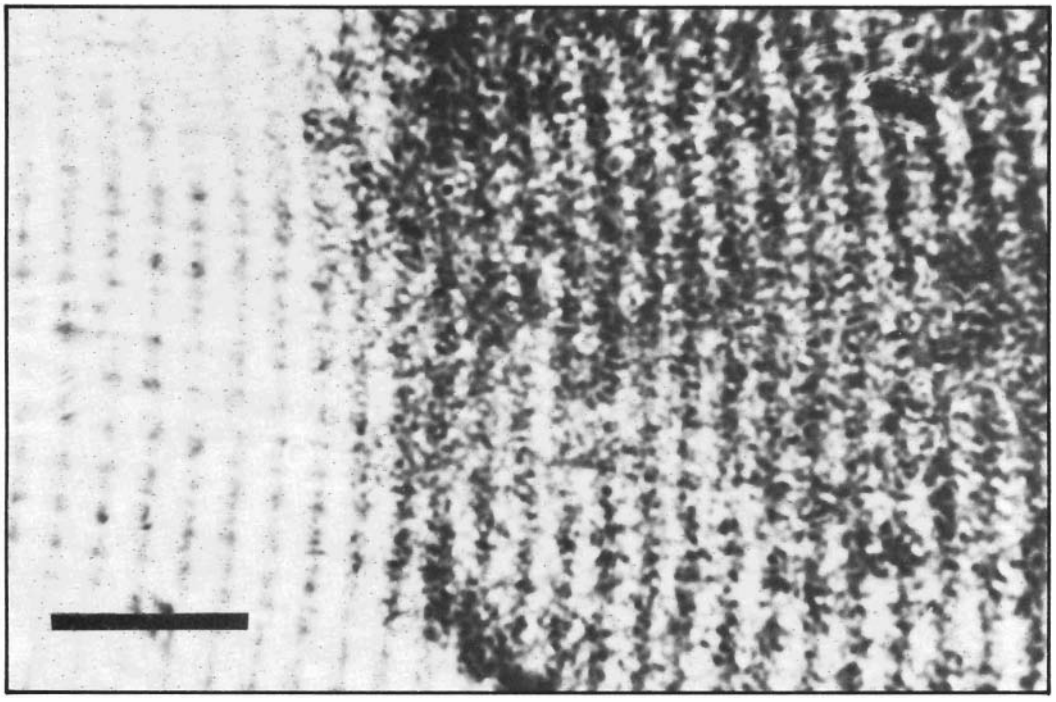

A


B

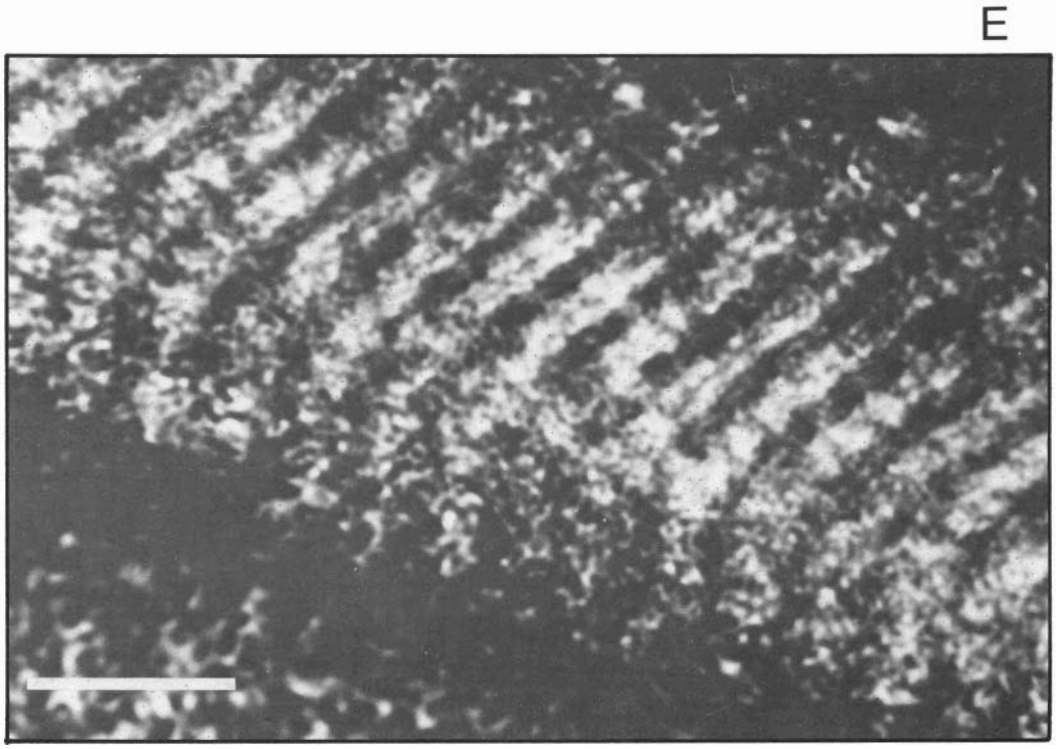
C

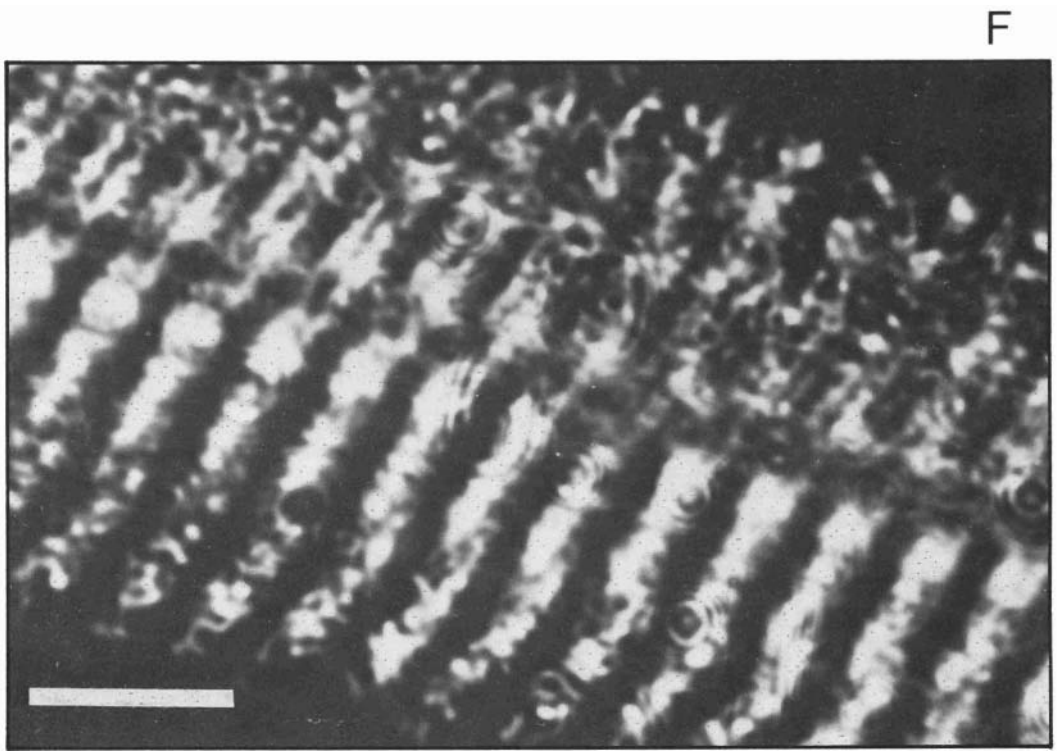


D



(Autoradiographs)





is high and with low electrical potentials in the I bands where K^+ concentration is low. The width of these regions is such that large fluctuations of potential could easily be picked up by a microelectrode, especially in stretched muscle-cell preparations. However, no such wide fluctuation has ever been observed in either relaxed or stretched muscle cells in thirty years of extensive experimentation.[50–53]

Localization of the bulk of cellular K^+ in the A bands and Z lines also conflicts with the membrane-theory interpretation of the osmotic equilibrium of living cells. The need to maintain macroscopic electroneutrality demands that the intracellular anions (largely creatine phosphate and ATP in muscle cells) must also be located within the regions where the bulk of K^+ is found; indeed there is independent evidence showing that this is the case for ATP.[53] With the bulk of both intracellular (free) cations and (free) anions confined to the A band and Z line, a gross disproportion of osmotic pressures would exist between these different regions and the I band. As a result, more and more water should migrate from the I bands into the A bands and Z lines. Since this does not actually occur, the tenet of free ion and free water basic to membrane-pump theory is clearly questionable.

In contrast, the association-induction hypothesis offers a comprehensive interpretation of the observations. K^+ as well as the bulk of intracellular anions are in the adsorbed state, and as adsorbed ions contribute little to the osmotic activity of the cell. That activity, as mentioned above, is provided primarily by the long-range effects of the intracellular proteins, probably with myosin and actin playing major roles.

While the localization of K^+ itself raises serious doubts about the membrane-pump theory, K^+ *localization* in regions where most β- and y-carboxyl groups are located cannot be directly equated with K^+ *adsorption* on those groups, since other interpretations exist.

For example, it has been argued that the localization could conceivably be an artifact produced during drying as a result of Cs^+ and $T1^+$ being crowded out by macromolecules from regions of high protein density into regions of low protein density. This cannot be the explanation, however, because the A band, where the bulk of K^+ is localized, has a higher protein concentration than that of the I band, where K^+ is not present or is present in low concentrations.[26,27] †

Another alternative explanation is that the localization may be due merely to the presence of fixed anionic charges in the A bands and Z lines and that K^+, Cs^+, and $T1^+$ are confined to regions not as adsorbed cations but merely as free counter-ions. However, this possibility was ruled out more than ten years ago, when the

† Myosin, which makes up more than 50% of the total muscle protein, is concentrated in the A band. Actin, which makes up the bulk of the protein in the I band, contributes only 15% of the total protein. Certainly in a stretched muscle (such as shown in Figs. 1E and 1F) the total protein of the I band (excluding the Z line) must be considerably lower than that of the A band, since the I band region is then either equal to or greater than the A band region in total volume.

critical difference between the attributes of ions within the ion cloud which balances fixed opposite charges and the attributes of ions adsorbed on discriminating sites were examined and tested experimentally.[26] It was shown that the concentration of the accumulated cations as part of a counter-cation cloud to balance the fixed negative charges, can be reduced by the presence of other cations in the system; in this respect, the behavior of free counter-cations resembles that of an adsorbed cation. But this competitive effect in the case of free counter-ions is only valance-specific and not ion-specific. That is to say, a cation—e.g., K^+—would be as effective in displacing accumulated free counter-cations Cs^+ as it is in displacing accumulated free counter-cation K^+, because as part of a counter-cation cloud, only the long-range attributes of these ions can be "felt" by the system; the long-range attributes of K^+ and Cs^+, being those of univalent cations, are the same. It is when the ions in question are in close contact with the fixed anions that the differences in the short-range attributes of the two univalent ions are felt, and the only significant differences between K^+ and Cs^+ are their short-range attributes (e.g., polarizability: $K^+ = 0.87 \times 10^{-24}$ cm; $Cs^+ = 2.79 \times 10^{-24}$ cm. *Born repulsion constant:* $K^+ = 26.5 \times 10^{-82}$ erg/cm^8; $Cs^+ = 82.5 \times 10^{-82}$ erg/cm^9). Experimental studies of Cs^+ and K^+ accumulation in frog muscles have revealed that ^{39}K is three times more effective in displacing accumulated ^{134}Cs-labeled Cs^+ than in displacing ^{42}K-labeled K^+, showing that the differences in the short-range attributes between the two ions are indeed perceived and play a major role in selective accumulation of the counter-cations.‡

One can only conclude that K^+ ions accumulated in frog muscle cells are not part of a diffuse counter-cation cloud and that they must be in close association with the fixed anions. In other words, K^+ in frog voluntary muscles is selectively adsorbed on the β- and γ-caryboxyl groups of myosin molecules located in the peripheral regions of the A band and on the β- and y-carboxyl groups of proteins located in the Z line. The autoradiographic work reported here fully confirms the recent finding of Edelmann who, using transmission electron microscopy, demonstrated with great lucidity the localization of K^+ in the A bands and Z line in frog voluntary muscle cells.[54]

‡ The question may be raised whether the sites of this discrimination are truly at the adsorption sites or in the cell membrane where carriers have been postulated to combine selectively with either K^+ or Ca^+. It was to settle this and other questions that the EMOC (effectively membraneless open-ended cell) technique was introduced. With that technique I have shown that the selectivity in the accumulation of cations followed the rank order $Tl^+ > K^+ > Cs^+ > Na^+$ in an EMOC preparation of frog muscle as had been shown to be the case in intact muscles. In the EMOC preparation the postulated pumps are rendered nonfunctional in part by amputation and in part by suspension in air, thereby being deprived of the "sinks" or "source" indispensible for the functioning of the postulated pumps."

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