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Electron Microscopic Demonstration of Potassium Binding Sites in Muscle

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The Controversy Concerning the Physical State of K^+ in Living Cells

Modern textbooks present as fact the view that K^+ – the main cellular cation – is freely dissolved in free cellular water. Evidence for this view is based on at least 4 different kinds of experiments.

a) Living cells are isotonic with an 0.1 M aqueous NaCl solution. This demands that the osmotic activity within the cells be equal to that of this solution. Since the total ionic concentration in the cell is approximately 0.1 M and K^+ ion constitutes the bulk of the cations, if follows that the bulk of this K^+ as well as the intracellular anions must be in a free state (Hill 1930).

b) Improved histochemical methods refuted earlier findings and led to the claim that potassium is evenly distributed in muscle cells and probably not bound to specific proteins (Gersh 1938).

c) Studies of isolated actomyosin and of muscle homogenates failed to show any significant degree of selective K^+ binding (Szent Györgyi 1945; Erdös 1946; Lewis and Saroff 1957).

d) The theory of cellular electrical potential derived by Hodgkin and Katz (1949) predicts the correct magnitude of the resting potential. Since this theory is based on the assumption of complete dissociation of intracellular K^+, it follows that the bulk of the intracellular K^+ must be in the free state.

Taken together, these experiments appeared to provide proof for not only the concept of free cellular K^+, but also for the correctness of the following basic assumptions of the membrane theory: A very thin cell surface structure, called cell membrane, separates the extracellular from the intracellular pool and regulates the cell volume by its semipermeable properties; this membrane possesses special transport systems (pumps) to maintain asymmetric solute distributions, and is responsible for electrical potentials, called membrane potentials.

There exist, on the other hand, bulk phase theories which offered evidence for K^+ binding to intracellular macromolecules (Troschin 1958; Ling 1962; Ernst 1963). If cellular K^+ is bound the interpretations of osmotic and electrical phenomena cannot be those given by the membrane theory. In fact, the bulk phase theory that is most advanced theoretically and experimentally – the association-induction hypothesis (AIH) of Ling – provides explicit molecular mechanisms for cytoplasm-dependent selective K^+ accumulation, cytoplasm-dependent osmotic behaviour, and cell-surface-dependent electrical potentials (Ling 1962; Ling and Peterson 1977; Ling 1979 a).

The only way to resolve scientific controversies is to perform suitable tests. Since the state of cellular K^+ is one crucial issue for the competing theories it seemed promising to determine once more whether or not K^+ is evenly distributed throughout the striated muscle cell.

The proponents of the membrane theory believe that "the distribution of potassium in the cell is not essentially different from the distribution of water" (Fenn 1936). The AIH, on the other hand maintains (Ling 1952, 1962) that β- and γ-carboxyl groups are the primary sites of K^+ localization due to a physical adsorption. Since myosin contrib-

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utes more than 60% of the β- and γ-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. This prediction should also hold for Rb^+, Cs^+ and Tl^+ since it is generally accepted that Rb^+, Cs^+ and Tl^+ are accumulated in living cells by means of the same mechanism as K^+. Furthermore, Ling has shown that these ions replace each other in muscle in a mole-for-mole fashion under physiological conditions (Ling 1977).

Distribution of K^+, Rb^+, Cs^+ and Tl^+ in the Striated Muscle

Analysis of Sections of Freeze-dried, Embedded Muscle

A freeze-drying and embedding technique was used which provided preservation of ultrastructure of chemically unfixed muscle (Edelmann 1978, 1979, 1980 a). The main steps of this technique are (a) quenching of the tissue at a polished metal surface; (b) freeze-drying exclusively below -60 °C; (c) embedding in Spurr's low viscosity medium (Spurr 1969) at -15 °C; and polymerization of the Spurr medium at 40 °C.

Frog sartorius muscles were 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^+, Cs^+, or Tl^+. [The latter being a reversible process (Ling and Bohr 1971 b).]

Visualization of K, Rb, Cs, and Tl by Transmission Electron Microscopy (TEM)

Electron micrographs of sections of freeze-dried and embedded muscles are shown in Fig. 1 (Edelmann 1980 a). 0.3 µm thick dry-cut sections (Fig. 1 A, C, D) are compared to 0.3 µm thick wet-cut sections. 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. 1 A) and of Rb-loaded muscles (Fig. 1 C) show good contrast due to the uneven distribution of the electron-dense Rb or Cs. Similar results were obtained with Tl-loaded muscle (Edelmann 1977). When Cs or Rb-loaded muscles (or Tl-loaded muscles) are wet-cut, then the sections show poor contrast (Fig. 1 B) indicating that much of the ions leach into the distilled water. Dry-cut sections of normal (K^+ containing) muscle (Fig. 1 D) also yield micrographs with a slightly improved contrast when compared to wet-cut sections of normal muscle (Fig. 1 E).

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 20% higher under the I band than under the A band in dry-cut sections of normal muscle and only 10% higher in wet-cut sections of normal muscle. The differences were up to 40% in dry-cut sections of Rb- or Cs-loaded muscles.

Electron Probe X-ray Microanalysis

Dry-cut sections of normal, of Cs- and of Tl-loaded muscles prepared as described above, were analysed in a Siemens Elmiskop ST 100 F (Edelmann 1978 b). The results can be summarized as follows:

a) The concentrations of alkali-metal and Tl^+ ions are about three times higher in the A band compared to the I band including the Z-line.

b) The cation concentration is much higher in the narrow Z-line than in the surrounding I band.

c) The two marginal regions of the A band accumulate considerably more alkali-metal ions than the middle portions of the A band.

These investigations confirm the results described on p. 942 and visualized most clearly in Fig. 1 A.

Autoradiography of Air-dried Muscle Fibers Using ^{134}Cs and ^{204}Tl

Frog semitendinosus muscles were loaded with Cs or Tl labeled with radioactive ^{134}Cs

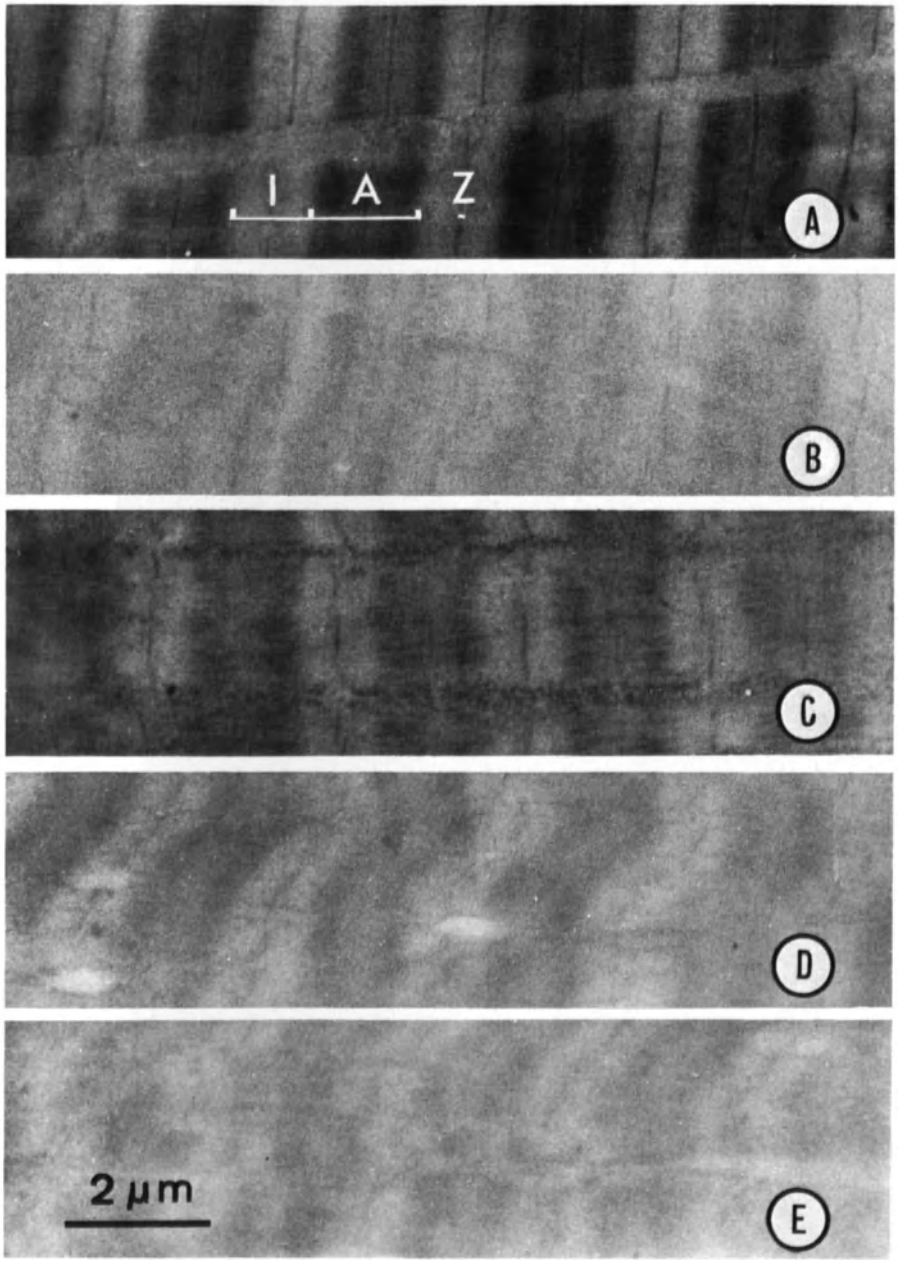

Fig 1 A–E. Transmission electron micrographs of 0.3 µm thick sections of freeze-dried frog sartorius muscle. **A** Dry-cut section of a Cs-loaded muscle. **B** Wet-cut section of a Cs-loaded muscle. **C** Dry-cut section of a Rb-loaded muscle. **D** Dry-cut section of a normal K-containing muscle. **E** Wet-cut section of a normal K-containing muscle. *A–A* band. *I–I* band. *Z–Z* line. (From Edelmann 1980 a. Reprinted by permission)

or ^{204}Tl (Ling 1977). 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. 2. Figure 3 μm 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

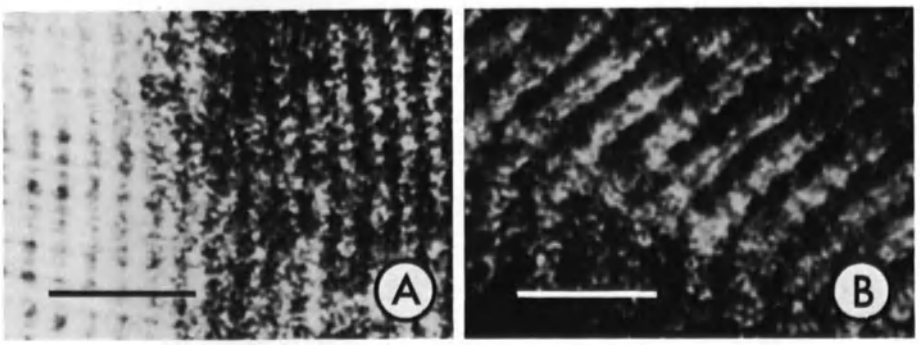

Fig. 2 A, B. Autoradiographs of frog muscle fibers. **A** Single ^{134}Cs-loaded fiber partially covered with photographic emulsion. **B** Single ^{134}Cs-loaded fiber that had been stretched before drying. Bars represent a length of 10 μm. (From Ling 1977 b. Reprinted by permission)

2 A 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 location of ^{134}Cs are aligned with the dark A bands in that part of the photograph not covered by the photographic emulsion. In Fig. 2 B a stretched muscle fiber is seen, with sarcomere length of about 3.5 μm. In this case narrow rows of silver grains occur in the centers of the I bands and thus at the position of the Z-lines.

Autoradiography of Frozen-hydrated Muscle Fibers Using ^{86}Rb and ^{134}Cs

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 from the muscle. After development and fixation of the emulsion in the usual manner, the autoradiographs were examined by light or electron microscopy (Edelmann 1980 a). Results are given in Fig. 3. 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 band of the muscle cells. Cation accumulation at the Z-lines could also be observed (see arrow in Fig. 3 B)

The high degree of consistency in the results using widely differing techniques is in itself persuasive evidence that the ion localization is not due to artifact. (Possible artifacts are discussed in the original publications.) Most important is the observation that the ions are localized at those sites which are stained in conventionally prepared (chemical fixation, dehydration, embedding) sections by uranium and lead.

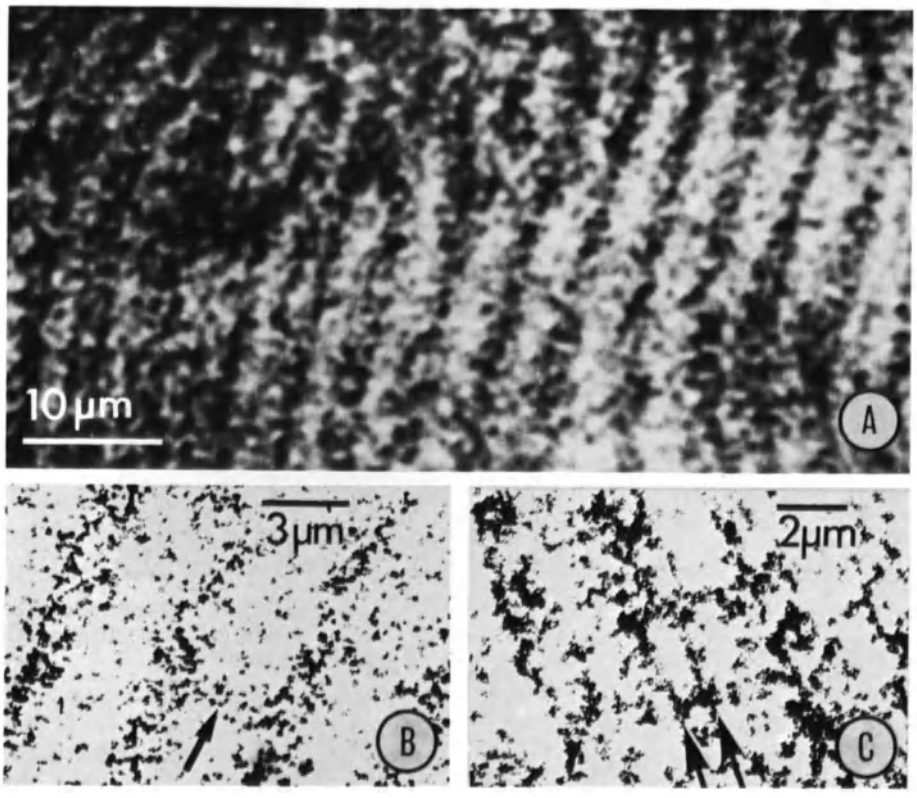

Fig. 3 A–C. Autoradiographs of frog muscle fibers. **A** Light microscopic autoradiogram of a stretched Cs-loaded fiber. **B** Electron microscopic autoradiogram of a stretched Cs-loaded fiber. The sarcomere length is about 4.4 µm. Between two dark bands (A bands) a line of silver grains indicates the 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. (From Edelmann 1980, Reprinted by permission)

Selective Accumulation of Li^+, Na^+, K^+ and Cs^+ in Sections of Freeze-dried, Embedded Muscle

In this section some experiments are presented which were designed in order to re-investigate the fundamental question whether it is possible for an in-vitro, non-living preparation to adsorb alkali-metal ions in a selective fashion. The basic idea was: If cellular proteins are in fact able to accumulate selectively alkali-metal ions by electrostatic interaction the in vitro experiment should be carried out in such a way that the proteins have little chance of changing their physical and chemical properties. Freeze- dried and embedded material seemed to be suitable for such an experiment. Sections 0.2 µm thick of freeze-dried embedded frog sartorius muscle were exposed to aqueous solutions containing different concentrations of LiCl, NaCl, KCl, and CsCl. Energy dispersive X-ray microanalysis (Siemens, ST 100 F) and laser microprobe mass analysis (Leybold Heraeus, LAMMA 500) of these sections yielded the following consistent results (Edelmann 1980 b):

a) The sections accumulate each species of alkali-metal ions to a high degree; the ions are preferably localized in the A bands.

b) If the sections are exposed to two or more ion species the alkali-metal ions are accumulated with a high degree of specificity

c) If the sections are exposed to Na^+, K^+ and Cs^+ the rank order of selectivity is K^+

$>Cs^+>Na^+$ (The same rank order as in living cells). If the sections are exposed to Li^+, Na^+, K^+ and Cs^+ the rank order of selectivity is $Cs^+>Li^+ \gg K^+>Na^+$. In the latter case the sections are well stained due to the high Cs^+ accumulation (Fig. 4).

portion 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

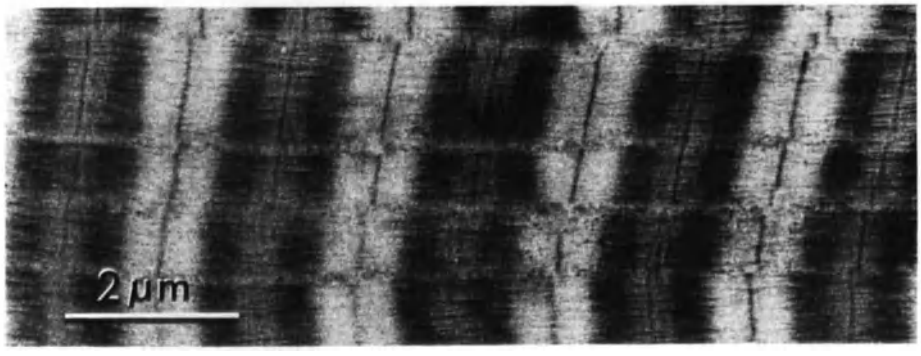

Fig. 4. 0.2 µm thick section of chemically unfixed frog sartorius muscle, freeze-dried and embedded. The section was exposed for 5 min to an aqueous solution containing 50 mM LiCl, 50 mM NaCl, 10 mM KCl and 10 mM CsCl

Conclusion: Cellular Potassium is Bound to Macromolecules

A comparison of the results of pp. 942–945 with those of pp. 945–946 shows that K^+ and other alkali-metal ions were selectively accumulated by freeze-dried, embedded muscle at those sites which accumulate K^+, Rb^+, Cs^+, and Tl^+ in living muscle. These sites – primarily located in the A bands – are the same that are stained by uranium and lead in the conventional chemical fixation and staining techniques. Since myosin contains a higher density of negatively charged β- and γ-carboxyl groups than, for example, actin, it seems reasonable to conclude that in all these cases electrostatic binding between anionic groups of the proteins and the cations is responsible for the observed accumulation.

The possibility that in living muscle the alkali-metal ions were only near the fixed charges as free counter ions 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 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.

Most important seems to be the finding that 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 occurs at anionic sites of cell

proteins, 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.

The Question of Osmotic Balance and Cell Potential

The experimental findings reported here show that the experiments described under (b) and (c), p. 941 can no longer be used as evidence against K^+ binding in cells. Furthermore, the uneven distribution of K^+ in muscle invalidates the osmotic argument (see a, p. 941). Since the water activity inside the cell must be equal in regions of high and of low K^+ concentrations, and equal to the extracellular activity, then there remains only one reasonable conclusion: The low water activity inside the cell must be due to an interaction between water molecules and proteins. Direct experimental evidence for this view is now available (see Ling, this volume).

On p. 941 a fourth argument was mentioned which seemed to support the idea of free cellular K^+ ions: the argument derived from the Hodgkin-Katz theory of cellular electrical potential. According to this theory the resting potential of living cells is in first approximation a K^+ diffusion potential, dependent on extra- and intracellular concentrations of free K^+. The surface adsorption theory of Ling, on the other hand is based on the assumption that the potential is determined by the density and nature of the anionic groups on the proteins of the cell surface and thus independent on the cellular K^+ concentration. Experiments, designed to test both theories and carried out during the last 30 years support the surface theory of Ling (Edelmann 1973; Ling 1978, 1979a).

Other Evidence for K^+ Binding

Intact, uninjured muscle cells have a K^+ mobility 1/8 that of a dilute salt solution (Ling and Ochsenfeld 1973), that is not due to membrane barriers or to electrical potential gradients (Ling 1973, 1978, 1979b).

Ion-sensitive microelectrodes indicate a wide range of measured K^+ activities, from very low in intestinal mucosa cells (White 1976), to near-normal in nerve and muscle (Hinke 1959,1961), to very high (higher than the actual concentration) in oocytes (Palmer et al. 1978). How can these differences be explained?

The AIH views the cell as a cooperative protein-water-ion complex, and assumes (1) that the solvent properties of polarized cell water differ from those of extracellular water, tending to exclude solutes; and (2) that the alkali-metal ions are selectively adsorbed to sites on cell proteins. The ion-sensitive microelectrode measures ion activity within a microscopic film of fluid immediately surrounding the electrode tip, and hence within a region of the cytoplasm that has been damaged by the electrode (Ling et al. 1973). The AIH would predict that perturbation of the metastable protein-ion-water complex would lead to a local shift in cation affinity of fixed anionic sites from that for K^+ to, e.g., that for Na^+ and to a local increase of the water activity which has to be lowered by accumulation of free, e.g. injury-liberated, ions. Thus, (1) the wide variation in measured K^+ activities reflects the varied susceptibility of cells to such perturbation; and (2) the measured Na^+ activities are expected to be lower, as is in fact observed in a wide variety of cells (Walker and Brown 1977).[1]

[1] A similar explanation can be applied to the experimental findings of Horowitz et al. (1979), who injected globules of gelatin into amphibian oocytes to serve as a "reference phase". They found higher K^+ and lower Na^+ concentrations in the gelatin than in the external solution bathing the oocytes and concluded that these findings proved active transport. However, in contrast to the rapid Na^+ exchange, the intracellular-extracellular K^+ exchange in the gelatin-loaded oocytes was very slow (Horowitz and Paine 1979). Therefore within the duration of the experiment the bulk of injury-liberated K^+ ions could not have escaped to the outside solution and must remain accumulated in the reference phase. For electrostatic reasons the presence of a high concentration of K^+ in the gelatin then prevented therein an accumulation of Na^+ ions. Postulation of active transport in resting oocytes is not warranted

Strong direct evidence for K^+ binding has been provided by the use of the near-edge X-ray absorption spectrum in red cells (Huang et al. 1979).

Taken together, there is now extensive evidence for the view that the bulk of cellular K^+ is adsorbed. Further investigation of K^+ binding may be facilitated by applying the results obtained here with freeze-dried embedded muscle to other tissue. One may suppose that, as a rule, K^+ binding sites are in fact the ones that are visualized by uranium and lead staining of ultrathin sections of conventionally-prepared biological material.

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