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State and Distribution of Potassium and Sodium Ions in Frog Skeletal Muscle

Chin Ok Lee* and William McD. Armstrong

Department of Physiology, Indiana University School of Medicine, Indianapolis, Indiana 46202

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Summary. A modified method for constructing cation-selective glass membrane microelectrodes is described. The method permits routine fabrication of electrodes with tip diameters less than 1 μ and exposed tip lengths of 2 to 5 μ. These electrodes had tip resistances in the range 10^7 to 10^9 ohms and gave stable and reproducible potentials in standard NaCl and KCl solutions. Potentials were unaffected by pH in the range 6 to 8 and satisfactory calibration curves were obtained over the temperature range 15 to 25 °C. Pairs of electrodes with different K-Na selectivity coefficients were used to measure K^+ and Na^+ activities in frog sartorius fibers. In fibers containing normal amounts of these ions, Na^+ activity was much less than would be predicted from chemical analysis of the muscles, assuming that all the apparent muscle Na^+ is present in an osmotically active form in the myoplasm. Possible origins of this discrepancy are discussed. Following a 48-hr soak at 5 °C in a K-free medium, the apparent Na^+ concentration and activity of the fibers both increased and the K^+ concentration and activity decreased. Comparison of the changes in fiber K^+ and Na^+ activities under these conditions with the corresponding changes in fiber concentrations strongly suggests the existence in normal fibers of at least two intrafiber K^+ compartments, both of which exchange with external Na^+ but only one of which is detectible by a microelectrode located in the myoplasm. Ca^{++} appears to exert a strong regulatory effect on Na^+-K^+ exchange between these compartments and the external medium and on the distribution of intrafiber Na^+ and K^+ between these compartments.

At present, much interest centers upon the state of Na^+ and K^+ in skeletal muscle fibers and other cells. Many investigations have indicated that the internal Na^+ of skeletal muscle is not uniformly distributed throughout the apparent fiber water. Indirect evidence for this conclusion was obtained in radiotracer studies of the kinetics of Na^+ and K^+ exchange in whole muscles (Carey & Conway, 1954; Simon, Shaw, Bennett & Muller,

* *Present address:* Departments of Medicine and Physiology, Pritzker School of Medicine, University of Chicago, Chicago, Illinois.

1957; Harris, 1963). These studies strongly suggested that a considerable fraction of apparent fiber Na^+ is not present in an osmotically active form in the cytoplasm but is sequestered, either in a special region or regions within the muscle fiber, or in an external region which is inaccessible to conventional extracellular markers such as inulin.

Recently, more direct evidence for inhomogeneous distribution of muscle Na^+ has been obtained. By comparing the steady-state nuclear magnetic resonance (NMR) spectrum of fresh frog skeletal muscle with that of extracts obtained by ashing at high temperature and HCl digestion, Cope (1967) found that about 65 to 70% of the Na^+ in fresh muscle is NMR-invisible and suggested that this fraction represents Na^+ which is complexed with macromolecules in the muscle fiber. Similar though quantitatively different results were reported by Martinez, Silvidi and Stokes (1969). Direct NMR evidence for the existence of complexed Na^+ in muscle was subsequently obtained by Czeisler, Fritz and Swift (1970) and by Cope (1970).

Measurements of intrafiber Na^+ activity with cation-selective glass microelectrodes have yielded essentially similar results. Hinke (1959) found that the intrafiber Na^+ activities in isolated crab and lobster muscles maintained in appropriate Ringer's solutions were only about one-third of the apparent fiber Na^+ concentrations, a discrepancy far too large to be accounted for by any reasonable estimate of the intrafiber Na^+ activity coefficient, assuming that all the apparent fiber Na^+ behaves as if it were in a homogeneous solution (Caldwell, 1968). Essentially similar findings were subsequently reported for frog sartorius muscle (Lev, 1964; Kostyuk, Sorokina & Kholodova, 1969; Armstrong & Lee, 1971) and giant muscle fibers of the barnacle *Balanus nubilus* (McLaughlin & Hinke, 1966).

On the other hand, direct measurements of K^+ activities in muscle fibers of various animal species have usually been considered to support the idea that most of the fiber K^+ behaves as if it were in homogeneous solution in the fiber water and that, contrary to divergent views (Ling, 1962; Ling & Cope, 1969), complexed or sequestered K^+ can be neglected in the interpretation of such phenomena as resting and action potentials, ionic movements, and the maintenance of steady-state levels of inorganic ions in muscle (Caldwell, 1968). This conclusion is based mainly on the fact that the measured fiber K^+ activity in fresh muscle is frequently close to the value which would be predicted from the fiber K^+ concentration and the known mean activity coefficients of KCl solutions with K^+ concentrations comparable to that of myoplasm (Hinke, 1959; Lev, 1964). However, such predictions are open to question on at least two grounds. First, in com-

puting the apparent fiber K^+ concentration it is usually assumed that all or virtually all the fiber water behaves as normal solvent water. There seems to be little doubt that this is an oversimplification (Hinke, 1970). Second, the assumption that univalent ion activity coefficients in complex media like myoplasm which contain significant amounts of polyelectrolytes are essentially the same as those in simple electrolyte solutions containing comparable concentrations of these ions is questionable (Kostyuk *et al.*, 1969). Thus, the correspondence between observed and calculated K^+ activities in muscle could be to some extent fortuitous and cannot by itself be taken as sufficient reason for excluding the possibility that a significant fraction of the fiber K^+ is complexed.

Aside from these objections, recent studies in our laboratory (Armstrong & Lee, 1971) have provided direct evidence for a significant fraction of complexed K^+ in frog skeletal muscle and have indicated that this fraction of the fiber K^+ plays an important role in net Na^+-K^+ exchange under certain conditions. This evidence may be summarized as follows. Na^+ and K^+ activities and concentrations in frog sartorius muscles which had been stored for 24 hr at 5 °C in a K^+-free medium containing 120 mM Na^+ were compared with the same parameters in their companion muscles which had been equilibrated for 2 hr at 25 °C in a Ringer's solution containing 105.4 mM Na^+ and 2.5 mM K^+. As anticipated (Desmedt, 1953; Carey, Conway & Kernan, 1959), the muscles gained a considerable quantity of Na^+ (27 ± 2 mM; mean $\pm$SE: $n=8$) and lost an equivalent amount of K^+ (26 ± 2 mM) during storage at 5 °C. However, the concomitant changes in Na^+ and K^+ activity (4.7 ± 0.4 and -8.4 ± 1.3 mM) were much smaller than would be predicted following replacement of 26 mM of osmotically active fiber K^+ by an equivalent amount of osmotically active Na^+. It was concluded, therefore, that the major fraction of fiber K^+ lost under these conditions was originally complexed or sequestered in some fashion and that the Na^+ which replaced it was similarly complexed or sequestered within the muscle fiber. Since the technique of storage at low temperature in K^+-free Ringer's solutions followed by a period of "recovery" at room temperature in solutions containing K^+ has been widely used in studies of the electrophysiological and ion transport properties of frog skeletal muscle and since most interpretations of these studies are based on models which assume a single intracellular compartment for Na^+ and K^+ (Caldwell, 1968; Kernan, 1972), it seemed appropriate to investigate further the changes that occur in intrafiber Na^+ and K^+ activities when the fiber concentration of these ions in isolated muscles is experimentally altered. The results of this investigation are reported herein.

Materials and Methods

Construction and Calibration of Cation-Selective Glass Microelectrodes

Open-tip micropipettes were drawn on an electrode puller (Alexander & Nastuk, 1953) from NAS 27-04[1] K^+-selective glass capillary tubes (Corning Glass Company, Medfield, Massachusetts; outside diameter 0.04 ± 0.003 inches, inside diameter 0.02 ± 0.003 inches). The micropipettes were about 5 cm long, had shank lengths of 12 to 15 mm and tip diameters less than 1 μ. After drawing, their tips were sealed under 200× magnification as described by Kleinzeller, Kostyuk, Kotyk and Lev (1969). Sealing was checked by immersing the electrode, tip downwards, in distilled water for about 30 min and then inspecting the tip region under 200 and 800× magnification. Water inside the tip or shank indicated a defective seal. Electrodes which passed inspection were filled successively with methanol, distilled water, and 3 M KCl by the method of Tasaki, Polley and Orrego (1954).

The microelectrodes were insulated as shown in Fig. 1. Insulating pipettes were drawn from Kimax glass capillaries (Kimble Products, Toledo, Ohio; outside diameter 1.5 mm, inside diameter 1.3 mm), had tip diameters of about 1.5 μ, were approximately 15 mm in total length and had shank lengths of about 7 mm. About 50 μ of the tip were filled with paraffin wax by momentary immersion in molten paraffin. The pipette was then attached to a plug of modelling clay in the open end of a glass tube (Fig. 1-1) which was in turn fixed to a micromanipulator. A sealed cation-selective micropipette was attached to the electrode holder of a second micromanipulator and mounted near the open end of the insulating pipette. Both micropipettes were mounted over a microscope stage and carefully aligned so that the tip of the sealed micropipette was opposite the center of the open end of the insulating pipette. The insulating pipette was then moved backwards over the sealed pipette until the tip of the latter was very close to the paraffin tip (Fig. 1-2). This movement was performed under 200× magnification and,

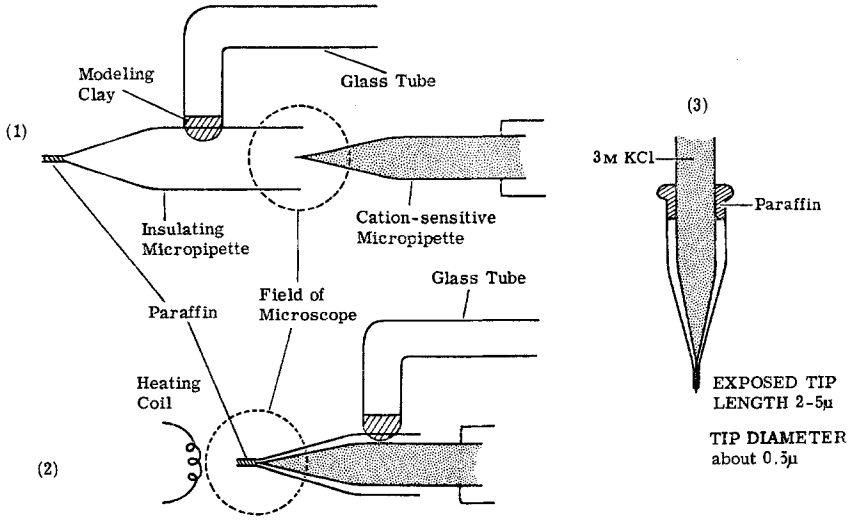

Fig. 1. Construction of cation-selective glass microelectrodes for measurement of intracellular Na^+ and K^+ activities

1 This terminology for glass composition follows that of Eisenman (1962).

during it, adjustments were made as necessary to keep the sealed micropipette aligned with the axis of the insulating pipette. When both micropipettes were positioned as indicated in Fig. 1-2, the heating coil (attached to the holder of a third micromanipulator) was brought close to the tip of the insulating pipette and the paraffin was carefully melted. At this point the insulating pipette was again moved back over the sealed micropipette until the tip of the latter was observed just outside the tip of the former. When the paraffin plug between the pipettes had solidified, the exposed tip of the sealed micropipette was inspected under 800× magnification and any paraffin adhering to it was removed by very careful heating with the platinum coil. Finally, the open end of the insulating pipette was fixed to the shaft of the sealed micropipette by careful application of 2 to 3 drops of molten paraffin (Fig. 1-3). In experienced hands this technique allowed the exposed tip length of cation-selective glass to be reduced to 2 μ or less.

Completed electrodes were stored in 3 M KCl. Usually they could be kept for about a week without significant changes in their properties but, normally, they were used as soon as possible after fabrication.

Calibration of sealed and insulated microelectrodes was performed just before the beginning of an experiment by measuring their potentials with respect to a grounded reference electrode (a polythene tube containing 3 M KCl in 3% (w/v) agar attached to a calomel half-cell) in solutions containing 0.005, 0.01, 0.05 and 0.1 M KCl or NaCl. These and all other solutions used in this investigation were made from reagent grade chemicals and distilled water which had been passed twice through a mixed-bed ion exchanger. The circuitry for the measurement of potentials was essentially similar to that described by White and Armstrong (1971) except that a high impedance amplifier (Keithley model 610B electrometer, input impedance $>10^{14}$ ohm) was used. Connection between this and the calomel half-cell which was in turn connected via a KCl/agar bridge to the microelectrode was made by means of a screened cable and the circuit was arranged to permit simultaneous display of data on a cathode ray oscilloscope, a Brush Mark 240 pen recorder and a digital voltmeter (White & Armstrong, 1971). All the numerical values for potentials reported herein were taken from the digital voltmeter.

Under conditions where K^+ and Na^+ are the only ions to which the microelectrode shows appreciable sensitivity, its behavior can, in theory, be expected to obey the following equation (Nicolsky, 1937; Eisenman, 1962):

$$E = E_0 + RT/F \ln (a_K + k_{KNa}\, a_{Na}). \tag{1}$$

E is the potential measured by the electrode in a solution containing K^+ and Na^+ at activities a_K and a_{Na}, respectively; E_0 is the potential observed in a solution in which the term in parenthesis is unity; R, T and F have their usual meanings; and k_{KNa} is the selectivity coefficient of the microelectrode given by the equation

$$k_{KNa} = \exp F/RT(E_{Na} - E_K) \tag{2}$$

where E_{Na} and E_K are the measured potentials in solutions containing Na^+ and K^+ ions, respectively, at the same activity.

Eq. (1) predicts that, in pure NaCl or KCl solutions, E should be a linear function of $\log a_{Na}$ or $\log a_K$, and that the slope of this line (2.303 RT/F) should be the same in both sets of solutions at the same temperature. Further, since k_{KNa} is primarily determined by the composition and structure of the glass (Eisenman, 1962) it might be anticipated that this parameter should remain constant for all electrodes derived from the same glass capillary. In practice, however, individual microelectrodes made from the same glass capillary can vary somewhat in their properties (Lev, 1964) probably because of slight changes in the structure and composition of the glass at the tip region which occur during drawing and resealing of the micropipette. Thus the empirical slope (S) of

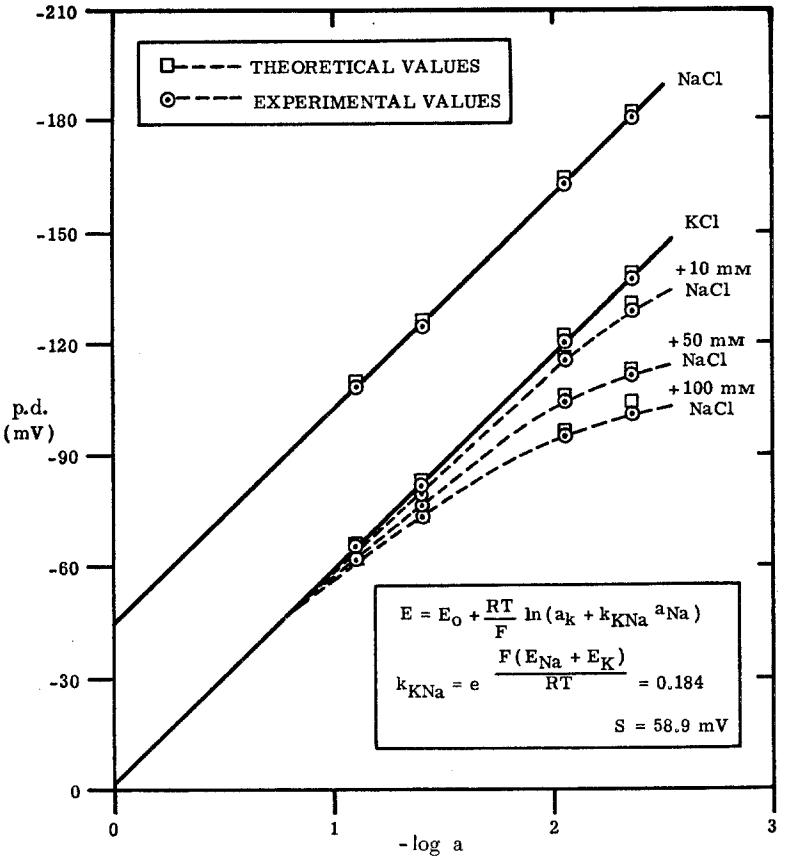

Fig. 2. Response of a typical potassium ion-selective glass microelectrode to Na^+ ions, K^+ ions, or mixtures of both

the potential *vs.* activity curve can deviate from its theoretical value and can be different in KCl and NaCl solutions, respectively. Also, k_{KNa} is usually different for each electrode. Hence, calibration of individual electrodes is an essential prerequisite to their use for determining ionic activities.

Fig. 2 shows a representative set of calibration data from this investigation. In this figure the circles indicate the potentials observed at 25 °C and pH 7.2 in pure KCl solutions, pure NaCl solutions, and mixtures of these. a is the mean activity of the calibrating solution calculated from its known concentration and mean activity coefficient, which, for a uni-univalent electrolyte, is assumed to be equal to the cationic activity coefficient (Robinson & Stokes, 1965). From the results obtained in pure KCl and pure NaCl solutions, the intercepts of both with the p.d. axis (E_0^K and E_0^{Na}) were determined. The equation $E = E_0^K + RT/F \ln a_K$ was then used to compute the potentials to be expected for the given values of a_K (square symbols in Fig. 2). Similarly, the equation $E = E_0^{Na} + RT/F \ln a_{Na}$ was used to predict the p.d. values for Na^+. The solid lines in Fig. 2 are drawn to fit these computed values. It is seen that the agreement between calculated and observed potentials is excellent as is that between the experimental slope in KCl (58.9 mV) and its theoretical value at this temperature (59.1 mV). As a further

test of the performance of the electrode, k_{KNa} was estimated from these data and Eq. (1) was used to predict the potentials in KCl solutions in which 10, 50 and 100 mM NaCl were incorporated (squares and dashed lines of Fig. 2). These were then compared with the observed potentials under similar conditions. As seen in Fig. 2, agreement between predicted and observed results was highly satisfactory.

Fig. 2 is an example of the rather rigorous evaluation to which the cation-selective microelectrodes were subjected early in this investigation. Because our glass had a different origin from that used by other workers (Lev, 1964; Kostyuk *et al.*, 1969) further studies of the electrochemical properties of the microelectrodes were also undertaken at this stage. Since some unexpected results emerged from these, they are described in this paper. Later it was considered sufficient to calibrate the electrodes in pure KCl and pure NaCl solutions only and to estimate E_0, k_{KNa}, and the slopes of the p.d. *vs.* $-\log a$ curves empirically from the results obtained. Electrodes were deemed acceptable if the slopes in KCl and NaCl did not deviate by more than ± 2 mV from 2.303 RT/F and did not differ by more than ± 1 mV from each other. k_{KNa} was usually estimated as $\exp \Delta E/S$ where ΔE was the difference in the potentials recorded in 100 mM KCl and 100 mM NaCl, and S was the average of the slopes obtained in KCl and NaCl solutions, respectively.

For the determination of a_K and a_{Na} in unknown mixtures of K^+ and Na^+, two acceptable electrodes with different k_{KNa} values are required. Within the limits discussed above, the mean slopes S' and S'' may also differ. Finally, for the measurement of intracellular Na^+ and K^+ activities it is necessary either to refer the intracellular potentials E' and E'' recorded with the cation-selective electrodes to an intracellular reference electrode or, if an external reference electrode is used, to measure the membrane potential E_m independently with a conventional microelectrode. For technical reasons, the latter expedient was adopted in this study. Open-tip electrodes drawn from Kimax glass, filled with 3 M KCl by the method of Tasaki *et al.* (1954), and with tip resistances of 20 to 50 MΩ and tip potentials not exceeding -5 mV were used. This method yields the following equations for a_K and a_{Na}:

$$(E' - E_M - E'_0)/S' = \log(a_K + k'_{KNa}\, a_{Na}) \tag{3}$$

$$(E'' - E_M - E''_0)/S'' = \log(a_K + k''_{KNa}\, a_{Na}). \tag{4}$$

Determination of Intrafiber Na^+ and K^+ Concentrations and Activities

These experiments fell into two general categories. Those in the first category were an extension of previously reported studies (Armstrong & Lee, 1971) in which Na^+ and K^+ concentration (C_{Na} and C_K) and activities in muscles following storage at 5 °C in a K^+-free medium were compared with those in their companion muscles which had been allowed to equilibrate at room temperature (25 °C) with a normal Ringer's solution. The second category of experiments involved the measurement of C_{Na}, a_{Na}, C_K and a_K in muscles which were first stored at 5 °C in a K^+-free medium and were subsequently re-immersed for 2 hr at room temperature in a medium containing K^+ ions.

The first category included three sets of experiments. In all three the general procedure was the same except for the time of immersion at 5 °C and the Ca^{++} content of the K^+-free medium. The composition of the immersion media used is given in Table 1. The experimental procedure has been described previously (Armstrong & Lee, 1971) and may be summarized as follows. Pairs of sartorius muscles from adult frogs (*Rana pipiens*) were used. One muscle was immersed for 2 hr in 20 ml normal Ringer's solution (Table 1) at room temperature (25 ± 1.0 °C) in a Lucite chamber (inside dimen-

Table 1. Composition of Ringer's solutions

Ringer's solution [a]	K^+	Cl^-	Na^+	Ca^{++}	HPO_4^{2-}	$H_2PO_4^-$	SO_4^{2-}	Mannitol	Ionic strength
Normal	2.5	96.1	105.4	1.8	2.3	0.8	5	30	0.1206
K-free	0	108.2	120.0	1.8	2.3	0.8	5	5	0.1327
K-free low Ca^{++}	0	104.8	120.0	0.09	2.3	0.8	5	10	0.1276
Recovery 1	10	103.6	105.4	1.8	2.3	0.8	5	15	0.1281
Recovery 2	10	100.2	105.4	0.09	2.3	0.8	5	20	0.1230

[a] Concentrations in mEquiv or mmoles/liter.

sions 80×20×16 mm). Two small pieces of cork, 22 mm apart, were fixed to the bottom of the chamber and the muscle was attached to these by fine pins driven through the tendon at each end. The companion muscle was immersed in 50 ml of a K^+-free medium containing 1.8 or 0.09 mM Ca^{++} (Table 1) and placed in a refrigerator at 5 °C for 24 or 48 hr.

Following 2 hr of equilibration, membrane potential measurements were begun on the control muscle and at least 10 measurements were recorded. Membrane potentials were accepted only if they remained constant within 1 mV for at least 1 min after reaching a stable value and if the tip potential of the electrode did not change by more than 1 mV between impalement and withdrawal. Further, if the membrane potentials of the control muscle were consistently less than 80 mV the experiment was abandoned.

Following this, the open-tip microelectrode was replaced by a cation-selective microelectrode and at least four or five potentials were recorded. This procedure was then repeated with a second cation-selective microelectrode with a different k_{KNa}. (Electrodes were paired so that the ratio of their k_{KNa} values was at least 3/2. Usually it was considerably greater than this.) Finally, a second series of measurements was made with an open-tip microelectrode as a check on the stability of the preparation. The whole sequence of potential measurements required about 2 hr to complete.

The cation-selective microelectrodes were recalibrated at the end of the above sequence of measurements. Results were accepted only if these calibration potentials were within 1 mV of those recorded previously and if the potential recorded by the electrode in the bathing medium had remained constant within this limit during the period of its experimental use. Other criteria for acceptability of cation-selective microelectrodes are discussed under Results.

Penetrations of the tissue were made under 120× magnification. To reduce electrical noise, the tissue chamber, half-cells, electrometer-amplifier, micromanipulators, etc. were housed in a well-grounded Faraday cage. In addition, all moving parts were grounded and the system and its supporting table were mounted on foam rubber to dampen mechanical vibrations.

At the end of the electrical measurements, the muscle was removed from the bath, lightly blotted, weighed, and dried to constant weight at 105 °C. Total Na^+ and K^+ and extracellular (^{35}S-labeled sulfate) volume were estimated as described elsewhere (Armstrong, Lurie, Burt & High, 1969) and C_{Na} and C_K were computed from the results.

After a 24- or 48-hr immersion at 5 °C in a K^+-free medium the companion muscle was placed in the tissue chamber which was then filled with the bathing medium. The chamber and its contents were allowed to warm to room temperature and the sequence

of electrical and chemical measurements described above for the control muscle was performed.

In the second category of experiments both sartorii from a single frog were immersed for 24 hr in a K^+-free solution containing 0.09 mM Ca^{++} (Table 1). Following this, one muscle was analyzed directly for total water, extracellular water and total Na^+ and K^+. Its companion was re-immersed for 2 hr at room temperature in recovery solution 1 or 2 (Table 1) after which it was subjected to the sequence of electrical and chemical manipulations already described.

Results

Electrochemical Properties of Cation-Selective Microelectrodes

Since cation-selective glasses invariably show some H^+ selectivity (Eisenman, 1962), it was necessary to ensure that, under the conditions of our experiments, the effect of H^+ on the electrode potential was negligible. A number of electrodes were therefore calibrated in NaCl and KCl solutions at pH 3.0, 4.0, 6.0, 7.2 and 8.0. The pH was adjusted by addition of small amounts of Tris or HCl. Fig. 3 shows the relationship between the electrode

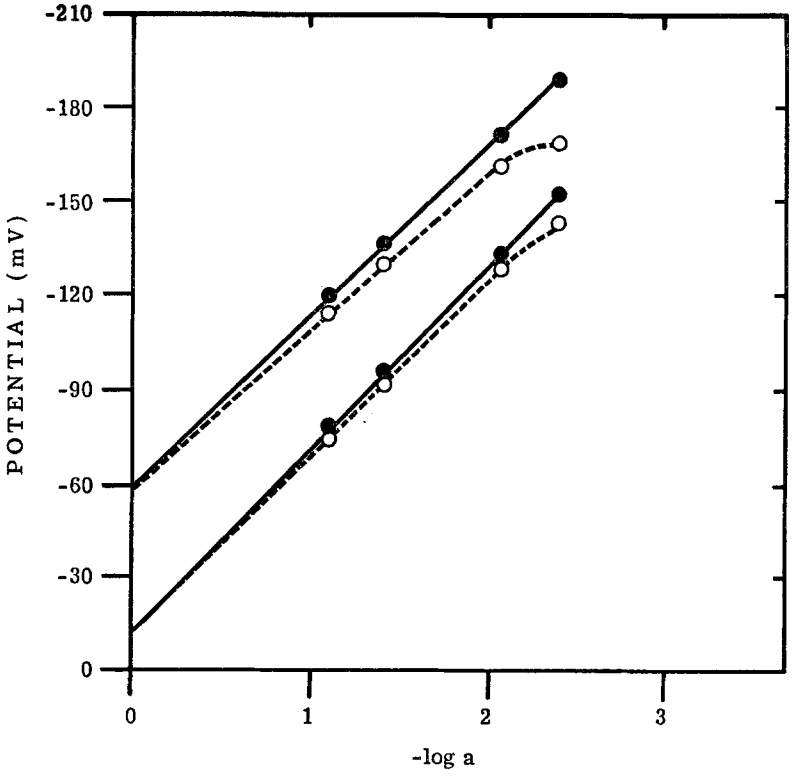

Fig. 3. Response of a typical potassium-selective glass electrode at pH 6.0 (•) and pH 3.0 (○) to Na^+ (upper two curves) and K^+ (lower two curves) ions

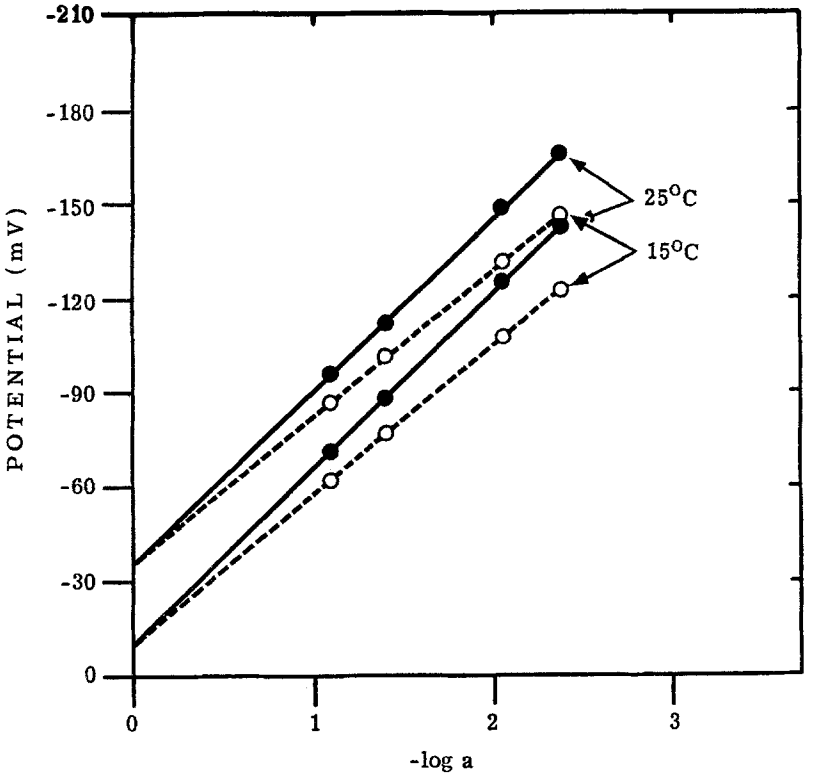

Fig. 4. Effect of temperature on response of a potassium-selective electrode to sodium (•) and potassium (○) ions

potential and ionic activities at pH 3.0 and 6.0 for one of these electrodes. It is evident that at pH 6.0 parallel straight lines were obtained in KCl or NaCl solutions. Similar results were obtained at pH 7.2 and 8.0. At pH 3.0 (Fig. 3) the relationship between electrode potential and $-\log a$ was not linear over the range tested. The deviation from linearity increased with decreasing KCl or NaCl concentration and was greater in NaCl than in corresponding KCl solutions. Evidently H^+ has an appreciable effect on the overall potential registered by these electrodes at this pH. Therefore, an unequivocal value for k_{KNa} cannot be derived under these conditions. Similar results were obtained at pH 4.0. It was concluded that, at pH 6 or above, H^+ had no measurable effect on the potentials registered by these electrodes in solutions containing 5 mEquiv/liter or more of Na^+ or K^+.

The response of several electrodes to Na^+ or K^+ at pH 7.2 was measured at 15 ± 1 °C and 20 ± 1 °C as well as at 25 ± 1 °C. Fig. 4 shows calibration curves at 15 and 25 °C for the same electrode. These and additional results

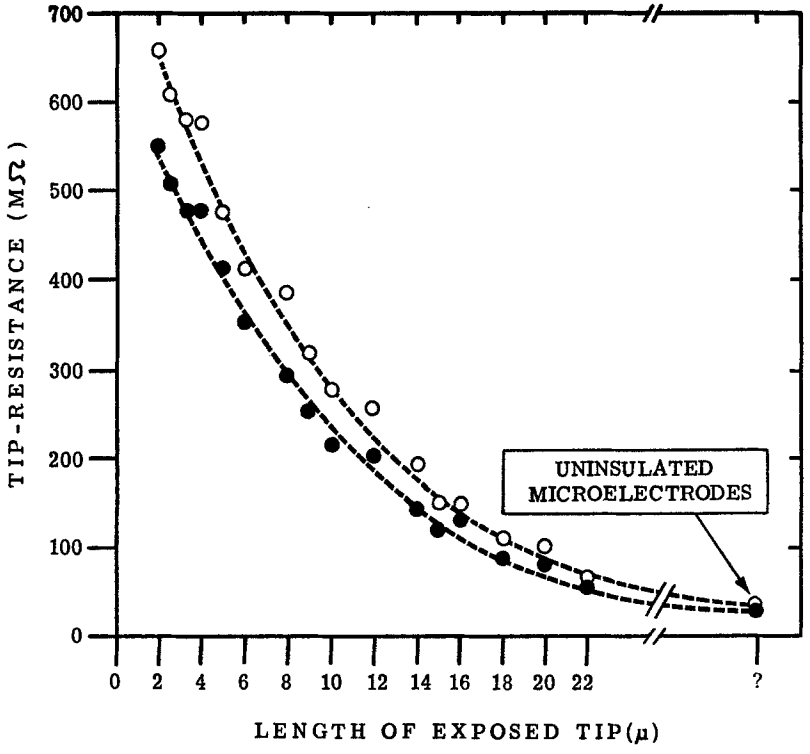

Fig. 5. Relationship between tip resistance and exposed tip length of a potassium-selective glass microelectrode in 100 mM NaCl (○) and 100 mM KCl (●)

obtained can be summarized as follows. Electrodes which give linear and virtually parallel responses to NaCl and KCl at 25 °C gave similar responses at 20 °C. When the slope at 25 °C was close to the theoretical value (2.303 RT/F), the slope at 20 °C was also close to this value. At 15 °C the slopes were usually lower than would be predicted from the decrease in T (Fig. 4). We have no explanation for this phenomenon at present. However, it should be pointed out that electrodes prepared by the present technique are perfectly usable at 15 °C providing that calibration is done at this temperature since results such as those illustrated in Fig. 4 permit a single value of k_{KNa} to be used over a wide range of activities.

Fig. 5 shows the relationship between tip resistance and exposed tip length for microelectrodes immersed in 100 mM NaCl and 100 mM KCl. To measure tip resistances, a resistance box containing five resistors (10, 50, 100, 1000, 10,000 MΩ) and an open circuit was connected in parallel with a cation-selective electrode and the Keithley electrometer. The resistors were connected in parallel with each other, were well shielded, and could be

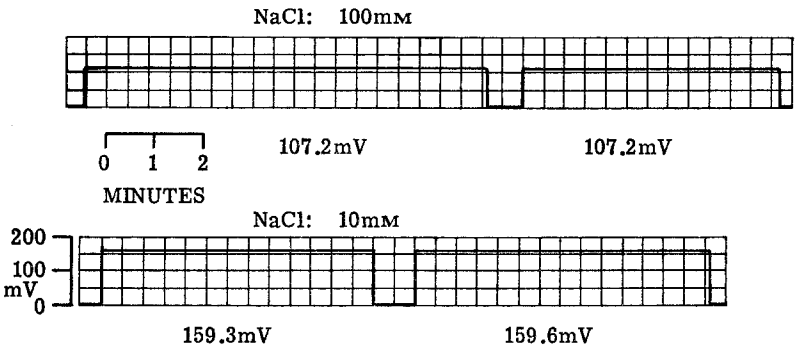

Fig. 6. Two successive recordings of potentials registered by a potassium-selective glass microelectrode in 100 mM and 10 mM NaCl solutions

switched individually into the measuring circuit. If the microelectrode is considered to have its own voltage source E_S together with a resistance R_S and a capacitative element, E_S can be measured with the resistance box at open circuit. When the resistance box is switched to a resistance R, R_S can be obtained from the measured voltage V by the following equation

$$R_S = R(E_S - V)/V. \tag{5}$$

Several notable results emerged from these resistance measurements (Fig. 5). First, depending on the exposed tip length, resistances ranged from about 10^7 to about 10^9 ohm. These values, as pointed out by Lev (1969) are considerably less than one would predict from the resistivity of dry NAS 27-04 glass and the probable dimensions of the tip (about 10^{11} ohm for electrodes with exposed tip lengths of approximately 5 μ). However, they are what one would expect from Eisenman's (1968) conclusion that ionic diffusion coefficients in hydrated glass of this composition are about 1,000 times greater than in the dry glass. Second, tip resistance decreased with increasing length of exposed tip (which could be adjusted during construction of the microelectrodes). This effect was observed in both NaCl and KCl solutions. Resistances were higher in 100 mM NaCl than in 100 mM KCl.

Finally, since electrode stability is important in the determination of ionic activities, and since Lev (1969) reported somewhat unstable potential readings when microelectrodes made from K^+-selective glass were immersed in NaCl solutions, we examined the stability of our electrodes on 0.01 and 0.1 M NaCl solutions at pH 7.2. Fig. 6 shows some typical recordings. It is evident from this figure that, following immersion in either solution, the electrode potential repidly attained a stable value which remained steady thereafter for relatively long periods.

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Figs. 7 and 8 show examples of recordings obtained during these experiments. The numerical values given for potentials in both these figures were taken from the digital voltmeter. In Fig. 7 calibration data for a cation-selective microelectrode (upper tracing), four successive intrafiber potentials obtained with this electrode in a control muscle (middle tracing), and ten recordings with an open-tip electrode of membrane potentials in the same muscle (lower tracing) are shown.

Three features of the recording shown in Fig. 7 are particularly noteworthy. First, it is apparent that, following immersion in an electrolyte

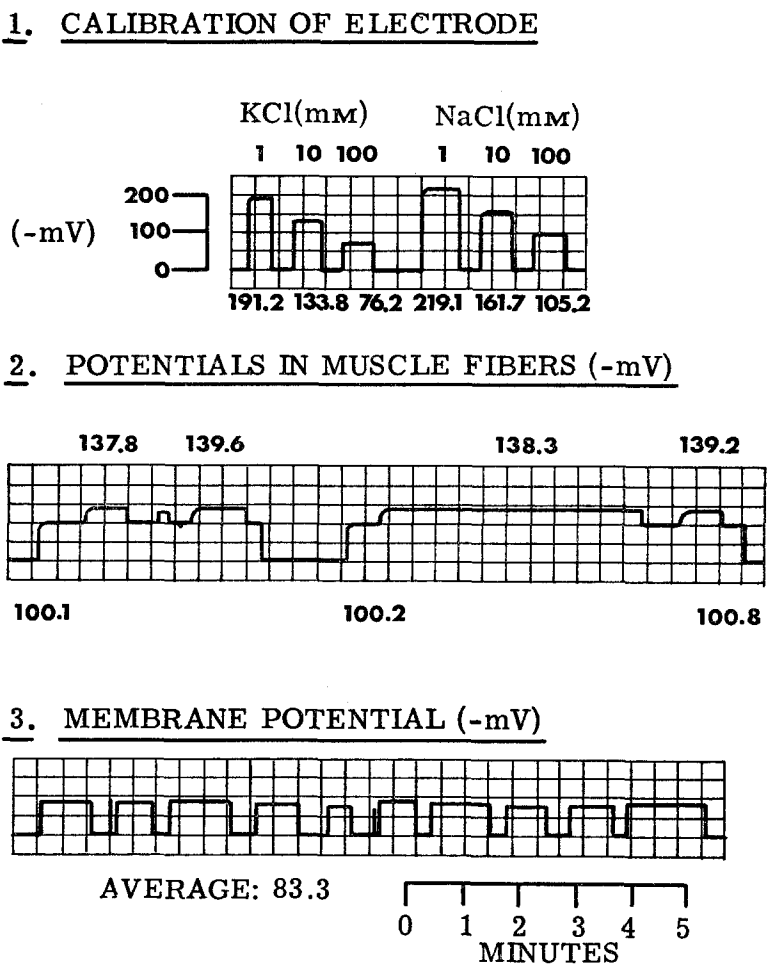

Fig. 7. Recordings of potentials for determination of intrafiber Na^+ and K^+ activities. Upper tracing: calibration of microelectrode. Middle tracing: four successive measurements of potentials in muscle fibers with the calibrated microelectrode. Lower tracing: 10 measurements of membrane potentials with an open-tip microelectrode

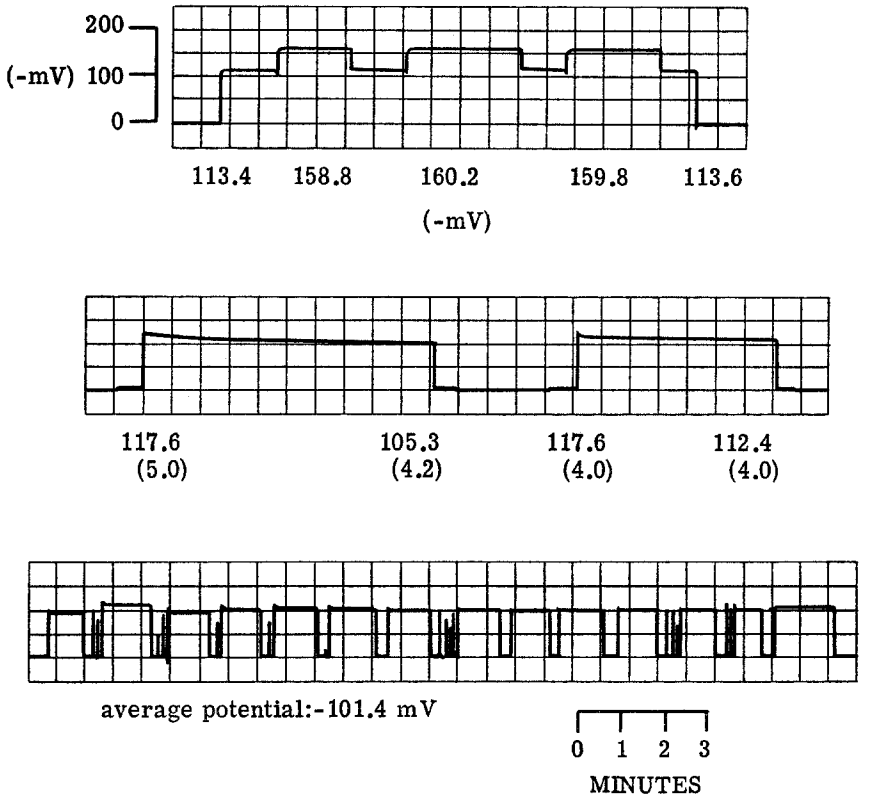

Fig. 8. Recordings of potentials measured in a muscle immersed for 48 hr in a K^+-free Ringer's solution containing 1.8 mM Ca^{++}. Upper tracing: potentials measured with a potassium-selective glass microelectrode. Lower two tracings: membrane potentials (for details *see text*)

solution (upper and middle tracings) the cation-selective microelectrode rapidly reaches a stable potential, indicating that the response time of these electrodes is quite small. Second, intracellular potentials registered by this electrode have similar characteristics (middle tracing). Third, the potential registered by the cation-selective microelectrode in the bathing solution remained virtually constant throughout the experiment (middle tracing). We found during this investigation that, as long as this is so, the characteristics of the electrode remain the same and recalibration is not necessary after each series of potential measurements.

Fig. 8 shows recordings from a muscle which was first stored at 5 °C for 48 hr in a K^+-free Ringer's solution containing 1.8 mM Ca^{++} and then allowed to warm up to room temperature in the same solution. The values in parentheses are tip potentials. The top tracing in this figure shows three

Table 2. Sodium and potassium activities (a), concentrations (C), and membrane potentials (E_m) in frog sartorius muscle fibers [a]

Group	n	Fiber H_2O (g/g dry wt)	C_{Na} (mM)	a_{Na} (mM)	C_K (mM)	a_K (mM)	E_m (mV)
I	6	2.85±0.09	26±1	6.3±0.4	140±2	93.9±0.7	84.7±0.5
II	6	2.81±0.11	56±1	19.4±0.8	110±4	83.4±2.2	100.2±0.7
III	6	2.68±0.05	29±1	7.7±0.2	142±1	95.4±0.3	84.7±0.3
IV	6	2.73±0.06	71±3	38.5±2.0	95±5	66.4±2.7	42.9±0.6
V	7	2.59±0.04	27±1	7.5±0.2	141±1	97.3±0.9	84.7±0.3
VI	7	2.87±0.05	103±2	55.3±1.5	56±2	47.8±1.3	42.1±1.6

[a] Mean value ± standard error given for each parameter listed. Groups I, III and V are control muscles immersed for 2 hr at 25 °C in normal Ringer's solution. Group II, companion muscles to Group I immersed for 48 hr at 5 °C in K^+-free Ringer's solution. Group IV, companions to Group III immersed for 24 hr at 5 °C in K^+-free, low Ca^{++} Ringer's solution. Group VI, companions to Group V; immersion as for Group IV but for 48 hr (Ringer's solutions designated as in Table 1).

successive impalements of the muscle with a cation-selective microelectrode. The lower two tracings are recordings of open-tip membrane potentials in the same muscle. Those in the middle tracing were recorded while the muscle was warming to room temperature. The bottom tracing was taken with the muscle at room temperature and immediately before impalement with the cation-selective microelectrode. It is evident from these two tracings that membrane potentials recorded during successive impalements in the warm-up phase were significantly higher immediately after impalement than those recorded after the muscle bath had attained temperature equilibrium with its surroundings. Also, potentials recorded during warm-up tended to decline significantly during impalement. Following warm-up to room temperature, stable membrane potentials were recorded. We do not at present have any satisfactory explanation for the instability of the potentials recorded during the warm-up period. It may be an artifact depending on the properties of the open-tip microelectrodes.

Table 2 summarizes the results obtained with muscles which were immersed for 24 or 48 hr in K^+-free Ringer's solutions containing 1.8 or 0.09 mM Ca^{++}. Groups I and II of this table show the data obtained with muscles immersed for 48 hr at 5 °C in a K^+-free Ringer's solution containing 1.8 mM Ca^{++}. These results are very similar to those previously reported (Armstrong & Lee, 1971) for muscles which had been immersed for 24 hr under the same conditions. In the control group (Group I) a_{Na} was, as reported earlier by Lev (1964) and by us (Armstrong & Lee, 1971),

much lower than would be predicted on the assumption that all the apparent intracellular Na (C_{Na}) is in homogeneous solution. This was also the case for the other two groups of control muscles (Groups III and V) in Table 2. By contrast, in all three control groups, a_K is, at first glance, consistent with the idea that all, or virtually all the myoplasmic K^+ is in an osmotically active state.

Following immersion in K^+-free Ringer's solution (Group II, Table 2) C_{Na} increased and C_K decreased as expected (Desmedt, 1953; Carey *et al.*, 1959). The average increase in C_{Na} (30 mM) was the same as the decrease in C_K indicating a one-for-one exchange between these ions during immersion. At the same time a_{Na} also increased significantly ($p < 0.001$) and a_K decreased ($p < 0.005$). There was no significant difference ($p > 0.05$) between these activity changes. Further, although the increase in a_{Na} and the concomitant decrease in a_K under these conditions were somewhat larger than the corresponding changes previously found in muscles immersed for 24 hr (4.7 ± 0.4 and 8.4 ± 1.3 mM, respectively, Armstrong & Lee, 1971), they were not sufficiently great to account for the changes observed in C_{Na} and C_K, assuming that the extra Na^+ taken up during immersion remained as osmotically active Na^+ within the fibers and replaced an equivalent amount of osmotically active myoplasmic K^+. Thus, these results appear to lend further support to our suggestion (Armstrong & Lee, 1971) that an appreciable fraction of the fiber K^+ in normal muscle is "bound" or sequestered in at least one state or compartment which does not contribute to the activity measured by the cation-selective microelectrode.

It is also worthy of note that the average membrane potential of the muscles in Group II of Table 2 was considerably higher than that of the corresponding control muscles. A similar hyperpolarization (average E_m 100.3 ± 2.2 mV) was previously observed with muscles immersed for 24 hr under similar conditions (Armstrong & Lee, 1971). Also, as previously found with muscles immersed for 24 hr, there was no significant change in fiber water during the 48-hr immersion procedure.

It appeared to be of interest to examine changes in a_{Na} and a_K under conditions where more extensive replacement of fiber K^+ by Na^+ could be achieved. Since the increase in C_{Na} and the decrease in C_K (30 mM in each case) observed with the Group II muscles of Table 2 were not much greater than the corresponding changes in these parameters (27 ± 2 and 26 ± 2 mM, respectively) previously found after 24-hr immersion under similar conditions (Armstrong & Lee, 1971), it did not seem profitable to pursue this objective by further increases in immersion time. Rather, advantage was

taken of the observations of Dee and Kernan (1963) and of Cross, Keynes and Rybova (1965) that frog sartorius muscles, when immersed overnight in the cold in K^+-free Ringer's solution with a lowered Ca^{++} content gain considerably more Na^+ and lose more K^+ than muscles immersed under similar conditions in K^+-free solutions containing the normal amount of Ca^{++}. Table 2 shows the results of two sets of experiments in which one member of a pair of companion muscles was immersed at 5 °C for 24 hr (Group IV) or 48 hr (Group VI) in a K^+-free solution containing 0.09 mM Ca^{++} (Table 1). It is evident from Table 2 that the results obtained with the control groups of muscles (Groups III and V) in these experiments were essentially similar to those found with the muscles of Group I.

Following a 24-hr immersion at 5 °C in this low Ca^{++} medium, the changes in C_{Na} (42 ± 3 mM) and a_{Na} (30.8 ± 2.0) were considerably larger than those found after 24-hr (Armstrong & Lee, 1971) and 48-hr immersions (Group II, Table 2) in a medium containing 1.8 mM Ca^{++}. Also, as found for the latter two sets of conditions, there was no significant difference, at the 0.05 confidence level, between the increases in C_{Na} and a_{Na} and the corresponding decreases in C_K and a_K (51 ± 3 and 29.1 ± 2.9). It is also noteworthy that, unlike muscles which were immersed in media containing 1.8 mM Ca^{++}, the muscles of Group IV, Table 2 were markedly depolarized following immersion.

Even more striking changes in sodium and potassium concentrations and activities, particularly the latter, were observed in muscles which were immersed for 48 hr in a solution containing 0.09 mM Ca^{++} (Group VI, Table 2). As noted with the sets of immersed muscles already discussed (Groups II and IV of Table 2; *see also* Armstrong & Lee, 1971) there was no significant difference at the 0.05 confidence level between the increase in a_{Na} (46.9 ± 1.7 mM) and the corresponding decrease in a_K (49.5 ± 1.1 mM). However, unlike these other instances, the increase in C_{Na} (73 ± 4 mM) for the muscles of Group VI, Table 2 was significantly smaller at this level than the corresponding decrease in C_K (83 ± 3 mM) though rather marginally so ($0.05 > p > 0.025$). In addition, there was a significant increase ($p < 0.05$) in the amount of fiber water during immersion under these conditions. As was found with the muscles of Group IV, Group VI muscles were strongly depolarized compared to their controls (Table 2).

Recovery Experiments

It is well established (Keynes & Maisel, 1954; Steinbach, 1954; Carey *et al.*, 1959; Cross *et al.*, 1965) that the increase in fiber sodium and the

Table 3. More sodium and potassium activities (a), concentrations (C), and membrane potentials (E_m) in frog sartorius muscle fibers [a]

Group	Fiber H_2O (g/g dry wt)	C_{Na} (mM)	a_{Na} (mM)	C_K (mM)	a_K (mM)	E_m (−mV)
I	2.85±0.05	30±2	14.6±0.8	136±2	80.5±1.5	57.4±1.1
II	2.93±0.05	27±1	7.7±0.4	139±1	107.7±0.8	54.3±0.3

[a] These fibers were immersed for 24 hr at 5 °C in K^+-free Ringer's solution containing 0.09 mM Ca^{++}. Group I re-immersed for 2 hr at 25 °C in "Recovery solution 1" (Table 1). Group II similarly re-immersed in "Recovery solution 2" (Table 1). Mean value ± standard error (n=4) shown for each parameter listed.

concomitant decrease in fiber potassium induced by soaking frog sartorius muscles at low temperature in K^+-free Ringer's solutions are readily reversed by subsequently transferring the muscles at room temperature to Ringer's solutions containing potassium. This "recovery" process has been shown to involve active extrusion of sodium ions in exchange for external potassium ions (Caldwell, 1968). It seemed of interest in this study to examine changes in intrafiber sodium and potassium activity during recovery and the effect on such changes of the Ca^{++} concentration in the recovery medium. Two sets of experiments were performed. In each set both sartorii from a single frog were immersed for 24 hr at 5 °C in a K^+-free Ringer's solution containing 0.09 mM Ca^{++}. Following this, one member of each pair was analyzed for Na^+ and K^+ to ensure that the expected uptake of Na^+ and loss of K^+ had occurred. The companion muscles were re-immersed for 2 hr at 25 °C in a solution (Table 1) containing 10 mM K^+ and either 1.8 (Group I of Table 3) or 0.09 mM Ca^{++} (Group II, Table 3).

The results obtained following immersion in K^+-free Ringer's solution can be summarized as follows. C_{Na} (73±3 and 74±3 mM) and C_K (94±3 and 93±3 mM) in muscles analyzed after the first immersion did not differ significantly between the two sets of experiments and were not significantly different from the corresponding values previously found with muscles immersed under the same conditions (Group IV, Table 2). Presumably, therefore, the changes in a_{Na} and a_K that occurred in these muscles were also similar to those previously observed (Group IV, Table 2).

Several points emerge from a consideration of the muscles which were allowed to "recover" for 2 hr at 25 °C in Ringer's solutions containing 10 mM K^+ (Groups I and II, Table 4). First, C_{Na} and C_K did not differ significantly between the two groups and were essentially the same as the

values found for "normal" muscles (Table 2). Thus, one might conclude that all or virtually all the sodium taken up during the first immersion was exchanged for external K^+ ions during the recovery period and that this exchange was unaffected by the Ca^{++} concentration of the recovery medium. Similarly, there was no significant difference between their water contents at the end of the recovery period. Further, there was a partial but highly significant repolarization of the fibers in both groups during recovery (compare Groups IV and VI of Table 2).

Closer inspection of the data in Table 2 does, however, reveal one interesting difference between muscles allowed to recover in a solution containing 1.8 mM Ca^{++} and those re-immersed in a solution containing 0.09 mM Ca^{++}. a_{Na} was significantly higher and a_K was significantly lower ($p < 0.001$) in the former than in the latter suggesting that external Ca^{++} does in fact influence Na^+-K^+ exchange between different cellular compartments and the external medium.

Discussion

Origin of Low Tip Resistance in K^+-Selective Glass Membrane Microelectrodes

As already pointed out, the tip resistances of the K^+-selective electrodes used in this study were much lower than would be predicted from the bulk resistivity of NAS 27-04 glass and the calculated dimensions of the exposed tip. Also, tip resistance was inversely proportional to exposed tip length with most of the observed decrease occurring in the range from 2 to about 25 μ (Fig. 5). Lev (1969) had previously observed comparably low tip resistances in a number of his electrodes. These were made from an essentially similar glass and were not much different from ours in their general dimensions. Lev (1969) concluded that the low resistance exhibited by these electrodes was due to "incomplete sealing" (i.e., the existence of narrow pores in the sealed tip of the glass capillary). This interpretation was strengthened by the occasional observation that microelectrodes made from an Na^+-selective glass (NAS 20-10) showed K^+ selectivity and, when this occurred, their resistances were anomalously low. Thus, Lev (1969) concluded that, in such electrodes, cation-selectivity was determined by the specificity characteristics of the pores rather than those of the glass as a whole. A full discussion of the theory of incompletely sealed electrodes is given in his paper.

However, the results reported herein, particularly the electrode resistance data shown in Fig. 5, suggest a different interpretation for the relatively

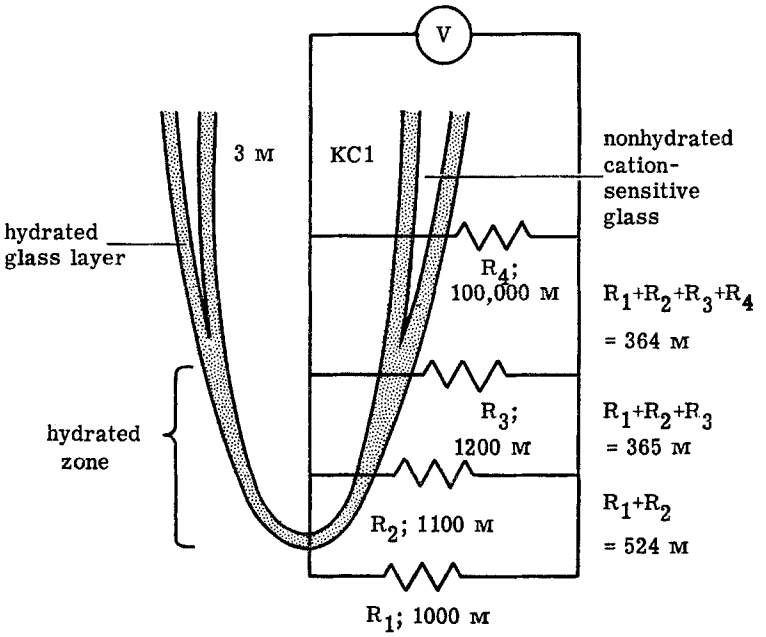

Fig. 9. Schematic representation of the tip of a potassium-selective glass microelectrode. R_1, R_2, R_3: resistances of hydrated glass membrane. R_4: resistance of nonhydrated glass membrane

low resistance of our electrodes. In this interpretation, which is illustrated schematically in Fig. 9, it is proposed that the resistance of the microelectrode is determined mainly by the mobility of cations in an area of the glass tip which is fully hydrated. Eisenman (1967, 1968) has suggested that the thickness of the hydrated glass layer in K^+-selective glass is of the order of 10 μ. Assuming Lev's (1969) estimate of about 0.2 μ for the minimum thickness of the glass wall at the tip it is evident that the zone of complete hydration could, as indicated in Fig. 9, extend for a considerable distance back along the shank of the electrode.

For illustrative purposes we have inserted a number of plausible resistance values (assuming that the resistance at any point of the glass wall depends on its thickness at that point, and that the hydrated glass wall has a much lower resistivity than the nonhydrated wall) in Fig. 9 and have, as shown therein, calculated the resultant electrode resistances for various exposed tip lengths. It is apparent that the values obtained are similar, and show a similar dependence on tip length, to the experimental values shown in Fig. 9, bearing in mind the fact that the resistance of a real electrode is a continuous function of tip length and not a step function as implied by

the simplified scheme shown in Fig. 9. For NAS 27-04 glass this relationship cannot readily be extracted from Lev's (1969) model in which the total resistance of the microelectrode is the resultant of two parallel ionic permeability pathways, a direct high resistance pathway through the glass and a relatively low resistance pathway through a pore or pores in the immediate vicinity of the tip. However, it is consistent with the existence of a completely hydrated tip having a resistivity some 1,000 times lower than that of dry glass, as expected from Eisenman's (1968) finding of an increase in the diffusion coefficients of ions in hydrated, compared to dry glass.

The model illustrated in Fig. 9 also enables the length of the fully hydrated zone at the tip of the electrode to be calculated from the equation

$$\lambda' = \sqrt{R/R'\lambda^2} \tag{6}$$

where λ' is the length of the hydrated zone, R is the tip resistance of the uninsulated electrode (exposed tip length $\to \infty$), and R' is the resistance at an exposed tip length λ. The average calculated value of λ' for 12 electrodes used in this study was 34 ± 3 (SE) μ.

State and Distribution of Fiber Sodium

In this connection, two major questions arise from the results reported in this paper. The first of these concerns the state and distribution of Na^+ in muscles containing normal amounts of this ion. The second relates to the state and location within the muscle fiber of the extra Na^+ accumulated, in exchange for K^+, during immersion at low temperature in a K^+-free medium.

Concerning the first question, two points should be noted. First, total fiber Na^+, estimated by chemical analysis, of the muscles belonging to Groups I, II and III of Table 2 is in good agreement with other estimates of the total fiber Na^+ content of frog sartorius muscles under similar conditions (Carey & Conway, 1954; Lev, 1964) and the state and distribution of Na^+ in these muscles may be assumed to reflect, to a first approximation at least, the *in vivo* status of Na^+ in this tissue. Second, the marked discrepancy found between C_{Na} and a_{Na} in these muscles (Table 2) agrees well with the results of earlier studies with cation-selective microelectrodes on skeletal muscles of the frog (Lev, 1964; Kostyuk *et al.*, 1969; Armstrong & Lee, 1971) and several other species (Hinke, 1959; McLaughlin & Hinke, 1966).

These results lead to estimates of the apparent fiber activity coefficient (a_{Na}/C_{Na}) in the range 0.2 to 0.3, a value which is inconsistent with the

presence in an osmotically active form within the muscle fiber of all the apparent fiber Na^+. Thus, one is led to conclude that a substantial fraction of the apparent fiber Na^+ is in a region which is inaccessible to a microelectrode inserted into the fiber and is also not included in the extracellular Na^+ measured by conventional means (i.e., the equilibrium volume of distribution of a chemical marker which is presumed to equilibrate with all the extracellular water but not to penetrate the fiber membrane). Two general explanations have been advanced to account for these findings. The first (McLaughlin & Hinke, 1966; Kostyuk *et al.*, 1969) postulates an internal location for the fiber Na^+ which is not detected by intracellular microelectrodes and considers this Na^+ fraction to be either attached to specific intracellular binding sites (e.g. on sarcoplasmic macromolecules such as myosin or on intrafiber organelles such as the sarcoplasmic reticulum) or sequestered within intracellular organelles (e.g. sarcoplasmic reticulum or nuclei). The second (Caldwell, 1968) proposes that the low apparent activity coefficient for Na^+ in muscle is most probably due to localization of much of the apparent fiber Na^+ in extrasarcoplasmic structures, specifically the central elements of the triad system, which are in direct communication with the exterior of the fiber (Huxley, 1964).

Supporting evidence for both these points of view can be found in the literature. For example, thermal denaturation (Hinke & McLaughlin, 1967) and light-scattering studies (McLaughlin & Hinke, 1968) on muscle fibers from the giant barnacle *Balanus nubilus* have been interpreted as suggesting that considerable amounts of Na^+ may be bound to myosin in this tissue and selective binding of Na^+, with respect to K^+, by myosin has been demonstrated (Lewis & Saroff, 1957). In addition, Hinke, Caillé and Gayton (1973) have shown that glycerol-treated fibers from *Balanus* selectively bind Na^+ ($k_{Na}/k_K \sim 3$) when exposed to mixed KCl/NaCl solutions in which the K^+/Na^+ ratio is between 24 and 29. Although the Na^+/K^+ selectivity ratios found in these studies are not startlingly high they are consistent with a preferential binding of Na^+ by sarcoplasmic macromolecules in intact muscle fibers. Furthermore, NMR studies of Na^+ in muscle have been widely interpreted as providing evidence for Na^+ binding (Cope, 1967, 1970; Martinez *et al.*, 1969; Czeisler *et al.*, 1970) although an alternative interpretation, involving a nuclear quadrupolar interaction rather than the division of cellular Na^+ ions into two discrete populations, has been proposed for the ^{23}Na NMR spectrum of biological tissues (Shporer & Civan, 1972). Finally, it would seem that the low Na^+ activity coefficients observed by Kostyuk *et al.* (1969) in polyacrylamide solutions could also be interpreted as indicative of binding or complexing of Na^+ ions by

macromolecular systems. Similarly, evidence for compartmentation of Na^+ in subcellular organelles such as the nucleus and mitochondria in frog skeletal muscle (Sorokina & Kholodova, 1970) and other tissues (Dick, Fry, John & Rogers, 1970) has been reported.

On the other hand, plausible arguments can be advanced for the presence of a significant fraction of fiber Na^+ in extracellular regions such as the transverse tubules of the sarcoplasmic reticulum which may not be included in estimates of the extracellular space obtained by conventional methods. For example, in *Carcinus* muscle, Shaw (1958) found that exchange of fiber Na^+ with external ^{24}Na ions involved two kinetic components of which the faster one accounted for about 70% of the total. The same author found that when *Carcinus* muscles were immersed in a medium containing 27 mM Na^+ their Na^+ content fell from 56–83 mM to 18–23 mM after 10 min. The latter figure is close to the sarcoplasmic Na^+ concentration which one would predict from Hinke's (1959) estimate of the average sarcoplasmic Na^+ activity in this tissue (13.5 mM). Similarly, Carey and Conway (1954) obtained evidence that, in frog sartorius muscles, approximately 8 mEquiv fiber Na^+ per kg muscle is located in a "special region" which is freely permeable to Na^+ as well as to K^+ and which is presumably extracellular, though it should be noted that Hodgkin and Horowicz (1959) working with single sartorius fibers did not find any clear kinetic evidence for the existence of such a region.

The results of Allen and Hinke (1971) on net Na^+-Li^+ exchange in single fibers of barnacle muscle are also of interest in this context. These authors found that when fiber Na^+ is replaced by external Li^+, intrafiber Na^+ activity falls much more slowly than the apparent intrafiber Na^+ concentration indicating the existence of at least two Na^+-Li^+ exchange rates, the slower of which appears to be associated with "free" myoplasmic Na^+. These results could be interpreted in favor of an extracellular location for the "bound" Na^+ previously found in these fibers (McLaughlin & Hinke, 1966). It is of interest that Beaugé and Sjodin (1968) and Keynes and Steinhardt (1968) obtained kinetic evidence for at least two Na^+-Li^+ exchange pathways in frog sartorius muscles.

It is evident from the above discussion that, in muscles containing normal amounts of Na^+ (Groups I, III and V of Table 2) the excess fiber Na^+ (over that estimated from measurements of a_{Na}) could be located intracellularly or extracellularly or both. Our experiments do not permit an unequivocal decision on this point, although the results obtained with muscles previously soaked in K-free Ringer's (Group II of Table 2, and Armstrong & Lee, 1971) do provide strong evidence for the existence of

intracellular sites in muscle which are capable of binding sodium ions and lead one to infer that some of the "excess" Na^+ in normal muscles is intracellular, as proposed by McLaughlin and Hinke (1966). It is, however, clear that if, in fact, a substantial amount of this sodium is extracellular, the method used in this investigation (the determination of the steady-state volume of distribution of sulfate ions) for the estimation of extracellular Na^+, and methods using other chemical markers, e.g. inulin, which give essentially similar estimates for this parameter (Johnson, 1956; Kernan, 1972) are seriously in error and that the true intrafiber Na^+ concentration is significantly less than values based on such estimates.

Estimation of the Na^+ concentration of sartorius fibers from Na^+ efflux kinetics (Sjodin & Beaugé, 1973) yields values which are in good agreement with that predicted from our results if a_{Na} is taken as reflecting the true intrafiber Na^+ concentration. According to Sjodin and Beaugé (1973) their most reliable estimates of intrafiber Na^+ were obtained by extrapolation to zero time of the slowest exponential component of Na efflux observed in a K^+- and Na^+-free medium containing 10^{-4} M ouabain. Thirty-one experiments of this kind gave a mean fiber Na^+ concentration of 10.6 ± 2.9 (SD) mmole/kg fiber water. Analysis, in terms of Debye-Hückel theory (Robinson & Stokes, 1965) of the ionic composition of myoplasm in frog sartorius muscle (Kernan, 1972) gives a myoplasmic Na^+ activity coefficient of 0.77. This combined with the overall average for a_{Na} found in this investigation (Groups I–III of Table 2) gives a value of 9.4 ± 0.7 (mean $\pm$ SD: $n = 20$) mEquiv/kg fiber water for the intrafiber Na^+ concentration. These two values do not differ significantly at the 0.05 confidence level.[2]

More definite inferences can be drawn concerning the location of that fraction of the extra Na^+ accumulated during immersion in K^+-free media containing 1.8 mM Ca^{++} which is not accounted for in terms of an increase

2 This agreement between two estimates of intrafiber Na^+ concentrations obtained by completely different methods is gratifying. However, there is a major uncertainty in both calculations. This concerns the value assigned to the fiber water. In deriving this from the wet and dry weights of their muscles, Sjodin and Beaugé (1973) used Johnson's (1956) figure of 18% for the inulin space in frog sartorii. This is not too different from the mean sulfate space found in our experiments (about 20%) but both these estimates of intrafiber Na^+ concentration may be complicated by the existence of nonsolvent water with the fibers (as discussed subsequently in this paper) and, perhaps more seriously in the present context, by the fact that, if external solutes such as inulin and sulfate ions lead to significant over-estimates of intrafiber Na^+, they may also cause the volume of intrafiber water to be similarly overestimated. This need not necessarily affect the agreement between the values obtained but raises doubts about their absolute accuracy.

in a_{Na} (Group II, Table 2). It is difficult to explain this accumulation in terms of a localization of the extra Na^+ in regions such as the transverse tubules which are in direct communication with the external medium, even though these elements have been reported to swell considerably under certain conditions (Dydynska & Wilkie, 1963). As shown in Table 2 (Groups I and II) and as previously reported (Armstrong & Lee, 1971), this process of Na^+ accumulation involves a one-for-one exchange for fiber K^+. The relatively large amounts of K^+ involved in this exchange could not originate from regions having virtually the same ionic composition as frog plasma, and, in any case, there is abundant evidence in the literature that the K^+ ions which participate in this exchange are intracellular. It seems reasonable, therefore, to conclude that a substantial fraction of the extra Na^+ accumulated in K^+-free media is adsorbed or sequestered in one or more intracellular compartments which are inaccessible to cation-selective microelectrodes.

State and Distribution of Fiber Potassium

The results with muscles containing normal amounts of K^+ (Groups I, III and V, Table 2), together with those found previously under similar conditions (Armstrong & Lee, 1971) support the generally accepted idea (Caldwell, 1968; Kernan, 1972) that the greater part of the fiber K^+ is osmotically active. Thus, the overall average K^+ activity for Groups I, III and V of Table 2 (95.8 mM) when divided by an assumed myoplasmic K^+ activity coefficient of 0.77 gives a value of 124 mEquiv/kg fiber water for the intrafiber K^+ concentration. Considering the probable uncertainties in the estimates of both the myoplasmic activity coefficient and the total fiber water (and particularly the possibility that, as already mentioned, the latter may be an overestimate) this is sufficiently close to the overall average observed C_K for these three groups (141 mM) to suggest that all, or virtually all of the fiber K^+ behaves as if it were in homogeneous solution. However, the existence of a significant amount of complexed or sequestered K^+ within the fibers which is preferentially exchanged for external Na^+ during immersion at 5 °C in a K^+-free medium was strongly indicated by previous experiments in this laboratory (Armstrong & Lee, 1971) and appears to be confirmed by the results obtained for the muscles in Group II of Table 3. This is perhaps best illustrated by considering the apparent K^+ activity coefficients (calculated as a_K/C_K for the muscles in Groups I and II of Table 2). Assuming that C_K (Table 2) represents a true myoplasmic concentration and that replacement of some 30 mM osmotically active K^+ by

an equivalent amount of Na^+ during immersion at 5 °C does not substantially alter the mean ionic strength of myoplasm, current theories of the behavior of strong electrolytes in aqueous solution (Robinson & Stokes, 1965) would not lead one to predict any significant change in the intrafiber K^+ activity coefficient under these conditions. However, the increase in the ratio a_K/C_K calculated from the data for Groups I and II in Table 2 (0.672 ± 0.003 to 0.762 ± 0.017; mean values $\pm$ SEM) is highly significant ($p < 0.01$) indicating a significant increase in the ratio of "free" to "bound" K^+ during immersion at 5 °C in a K^+-free medium.

The apparent paradox implicit in the above paragraph is readily resolved if one assumes a finite fraction of nonsolvent water within the fibers. Evidence for osmotically inactive fiber water in muscle has existed for many years.[3] Recently, the question of structured, nonsolvent, or osmotically inactive fiber water has been intensively investigated by a number of techniques including osmotic (Dydynska & Wilkie, 1963; Reuben, Lopez, Brandt & Grundfest, 1963; Bozler, 1965; Hinke, 1970), microelectrode (Hinke, 1970) and NMR measurements (Cope, 1967, 1972; Hazelwood, Nichols & Chamberlain, 1969; Cooke & Wien, 1971; Chang, Hazelwood, Nichols & Rorschach, 1972; Civan & Shporer, 1972; Outhred & George, 1973). A full discussion of these results is outside the scope of this paper. However, there appears to be virtually universal agreement that intrafiber water is inhomogeneous although there is considerable diversity of opinion about the fraction of fiber water which is highly ordered. The exact relationship between "structured", "osmotically inactive", and "nonsolvent" water also remains to be clarified, although Hinke (1970) in a careful analysis of the fiber water in barnacle muscle has presented evidence that, in this tissue, the latter two terms may be synonymous.

Na^+-K^+ Exchange in Muscle; The Role of Ca^{++}

Table 4 (B, C and D) shows the concentration changes (ΔC_{Na} and $-\Delta C_K$) observed during the experiments summarized in Table 2. Also shown in Table 4 are the corresponding concentration changes ($\Delta C'_{Na}$ and $-\Delta C'_K$) calculated from the observed activity changes, assuming a mean ionic activity coefficient of 0.77 for myoplasm. For comparison, Table 4 (A) includes a similar analysis of earlier results obtained with muscles immersed for 24 hr at 5 °C in a K^+-free medium containing 1.8 mM Ca^{++} (Armstrong & Lee, 1971).

3 *See* McLaughlin and Hinke (1966) for a useful summary and discussion of the early literature.

Table 4. Observed (ΔC) and calculated ($\Delta C'$) changes in intrafiber sodium and potassium concentrations, together with observed intrafiber activity changes (Δa)[a]

Condition	n	ΔC_{Na}	Δa_{Na}	$\Delta C'_{Na}$	$-\Delta C_K$	$-\Delta a_K$	$-\Delta C'_K$
A	8	27±2	4.7±0.4	6±1	26±2	8.4±1.3	11±2
B	6	30±1	13.1±1.0	17±1	30±3	10.5±1.6	14±2
C	6	42±3	30.8±2.0	40±3	51±3	29.1±2.9	38±4
D	7	76±2	47.8±1.6	63±2	85±2	49.5±1.3	64±2

[a] Observations and calculations followed immersion of frog sartorius muscles at 5 °C in K^+-free Ringer's solutions (for details *see text*). Mean value (mEquiv/kg fiber water) ± standard error shown for each parameter listed.

From rows A and B of Table 4 it is evident that with muscles immersed in media containing 1.8 mM Ca^{++}, ΔC_{Na} and $-\Delta C_K$ are significantly greater than $\Delta C'_{Na}$ and $-\Delta C'_K$, respectively, indicating that the K^+ lost during immersion originates from at least two cellular compartments, one of which is not detected by activity measurements with cation-selective microelectrodes. Similarly, the Na^+ which replaces the K^+ lost during immersion appears to enter at least two regions of the muscle fiber, only one of which is accessible to these electrodes. The amounts of K^+ lost from and of Na^+ taken up by the "inaccessible" fiber compartment can be estimated from Table 4 as $(\Delta C_K - \Delta C'_K)$ and $(\Delta C_{Na} - \Delta C'_{Na})$, respectively. With muscles immersed for 24 hr (row A of Table 4) the values (mean ± SEM) obtained for these parameters are 15±1 and 21±2 mEquiv/kg fiber water. These do not differ significantly at the 0.05 confidence level. The value for Na^+ is, however, much greater than the concomitant increase in C'_{Na} (6 mEquiv/kg fiber water) indicating that the greater part of the Na^+ taken up by the fibers under these conditions is not detectable as osmotically active Na^+ by the electrode technique. Although not as clear-cut statistically, the corresponding data for K^+ are consistent with the idea that the greater part of the K^+ lost under these conditions also originates from a cellular fraction or fractions which are not accessible to a K^+-selective microelectrode.

Following a 48-hr immersion in a medium containing 1.8 mM Ca^{++} the values obtained for the corresponding changes in composition are 13±1 mEquiv/kg fiber water for Na^+ and 16±3 mEquiv/kg fiber water for K^+. Once again these values were statistically identical at the 0.05 level. Furthermore they did not differ significantly in magnitude from the corresponding values for $\Delta C'_{Na}$ and $-\Delta C'_K$, suggesting an approximately equal involvement of active and "inactive" cellular fractions in Na^+-K^+ exchange under these conditions.

Quite different results emerge from the data given in Table 4 for muscles immersed at 5 °C in K^+-free media containing 0.09 mM Ca^{++}. Following a 24-hr immersion ΔC_{Na} and $\Delta C'_{Na}$ were statistically identical indicating that all or virtually all the Na^+ taken up under these conditions entered the fibers as osmotically active Na^+. On the other hand, $-\Delta C_K$ under these conditions was significantly greater than $-\Delta C'_K$ ($p < 0.05$) suggesting a loss of osmotically inactive K^+ which was not replaced by external Na^+. Following a 48-hr immersion when the net loss of K^+ and net gain of Na^+ were much more extensive, both ΔC_{Na} and $-\Delta C_K$ were significantly greater than $\Delta C'_{Na}$ and $-\Delta C'_K$, respectively. However, the difference in magnitude between $-\Delta C_K$ and $-\Delta C'_K$ (21 ± 2 mEquiv/kg fiber water) was significantly greater than that between ΔC_{Na} and $\Delta C'_{Na}$ indicating that while some of the osmotically inactive K^+ lost under these conditions was replaced by external Na^+, this replacement was not complete.

In conclusion we would like to propose a model for net Na^+-K^+ exchange in muscle under the conditions of our experiments which, though admittedly tentative, is consistent with the findings presented in this paper and offers a basis for further study. In this model Na^+ and K^+ are assumed to be distributed between at least two intracellular compartments. One of these represents free or osmotically active Na^+ and K^+ and can be estimated from intracellular activity measurements. The other contains the bound or sequestered moiety of the fiber Na^+ and K^+. Direct exchange of K^+ for Na^+ and *vice versa* between each of these compartments and the extracellular medium together with internal exchange between the compartments is assumed.

In terms of this model, when muscles are exposed at low temperature to a K^+-free medium containing a concentration of Ca^{++} within the normal physiological range (Conway, 1957), uptake of Na^+ from the external medium during the first 24 hr occurs predominantly through a one-for-one exchange for bound or sequestered K^+ ions. At the same time a smaller amount of Na^+ exchanges on a one-for-one basis for osmotically active fiber K^+ (Table 4). On more prolonged exposure (Tables 2 and 4) there is an additional entry of Na^+ ions (again by a one-for-one exchange for K^+ ions) into the compartment containing free or osmotically active Na^+ and K^+.

The model suggests two effects of Ca^{++} on Na^+-K^+ exchange. First, the virtually complete restriction of Na^+-K^+ exchange to the osmotically active fiber compartment during a 24-hr immersion at 5 °C in a K^+-free medium containing a 0.09 mM Ca^{++} indicates that Ca^{++} plays an important regulatory role in controlling the permeability barrier between this com-

partment and the external medium. In media containing normal concentrations of Ca^{++} a relatively low permeability of this barrier to Na^+ could be the rate-limiting factor in Na^+-K^+ exchange across it. An increased Na^+ permeability in media low in Ca^{++} could increase the rate of such exchange. If the properties of this barrier are the principal determinants of the membrane potential the marked depolarization in fibers exposed to low Ca^{++} media (Table 2) is also readily understood in terms of an increase in Na^+ permeability. The data shown in Table 3 are also consistent with this view. During recovery in a low Ca^{++} medium of muscles which were previously immersed in a K^+-free solution containing 0.09 mM Ca^{++}, the decrease in a_{Na} and the increase in a_K were greater than in similarly treated muscles which were allowed to recover in a medium containing Ca^{++} at a concentration within the normal physiological range, again indicating that net exchange of osmotically active Na^+ and K^+ is facilitated by lowering the external Ca^{++} concentration.

A second effect of prolonged immersion at low temperature in low Ca^{++} media is suggested by the data of Table 4. Following a 24-hr immersion under these conditions there appeared to be a net loss of bound or sequestered K^+ which was not balanced by an equivalent gain of Na^+. Following a 48-hr immersion there was a significant increase in the "bound" Na^+ content of the fibers which was, however, significantly less than the total amount of K^+ lost from this compartment. This could be interpreted as arising from a change in the cation binding affinities of a population of intracellular binding sites which could in turn reflect conformational changes in intracellular macromolecules induced by depletion of myoplasmic Ca^{++} under these conditions.

Finally, it is evident that the results presented herein may have important implications for the overall regulation of the Na^+ and K^+ content of muscle fibers and the detailed mechanism of the Na^+ pump in this tissue. Riordan, Manery, Dryden and Stile (1972) have shown that there are at least two components of net Na^+-K^+ exchange in frog muscle, one of which is inhibited by ouabain and the other by ethacrynic acid. It is tempting to suggest that these two components might be associated with the two intracellular compartments discussed herein but, pending further investigation, this suggestion must be regarded as extremely tenuous.

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