An Analysis of the Leakages of Sodium Ions into and Potassium Ions out of Striated Muscle Cells

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ABSTRACT Net sodium influx under K-free conditions was independent of the intracellular sodium ion concentration, $[Na]_i$, and was increased by ouabain. Unidirectional sodium influx was the sum of a component independent of $[Na]_i$ and a component that increased linearly with increasing $[Na]_i$. Net influx of sodium ions in K-free solutions varied with the external sodium ion concentration, $[Na]_o$, and a steady-state balance of the sodium ion fluxes occurred at $[Na]_o$ = 40 mM. When solutions were K-free and contained 10^{-4} M ouabain, net sodium influx varied linearly with $[Na]_o$ and a steady state for the intracellular sodium was observed at $[Na]_o$ = 13 mM. The steady state observed in the presence of ouabain was the result of a pump-leak balance as the external sodium ion concentration with which the muscle sodium would be in equilibrium, under these conditions, was 0.11 mM. The rate constant for total potassium loss to K-free Ringer solution was independent of $[Na]_i$ but dependent on $[Na]_o$. Replacing external NaCl with $MgCl_2$ brought about reductions in net potassium efflux. Ouabain was without effect on net potassium efflux in K-free Ringer solution with $[Na]_o$ = 120 mM, but increased potassium efflux in a medium with NaCl replaced by $MgCl_2$. When muscles were enriched with sodium ions, potassium efflux into K-free, Mg^{++}-substituted Ringer solution fell to around 0.1 pmol/cm^2·s and was increased 14-fold by addition of ouabain.

INTRODUCTION

The current concept of the sodium and potassium ion distributions in striated muscle cells is that passive leakage fluxes of these cations are balanced by metabolically driven pumped fluxes to maintain a physiological steady state for both cations. In the case of potassium ions, the passive net flux is usually low as the muscle fiber membrane potential normally approximates the potassium equilibrium potential (Adrian, 1956; Mullins and Noda, 1963). A

considerable inward leakage flux of sodium ions must, of course, be balanced by an outward flux due to active transport of sodium ions. The purpose of this work is to characterize the time and concentration dependencies of the sodium and potassium ion leakage fluxes in muscle and to separate these fluxes from pumped components as much as possible by experimental operations.

The ionic steady state achieved in muscle cells depends upon the external potassium ion concentration, $[K]_o$. Fenn and Cobb (1934) found that frog sartorius muscles neither gained nor lost potassium ions over a period of many hours if $[K]_o$ was maintained at a value of 4.9 mM. The unidirectional fluxes of potassium ions measured with ^{42}K are consistent with this finding (Sjodin and Henderson, 1964). The steady state achieved with respect to the internal sodium ion concentration also depends on $[K]_o$. Mullins and Noda (1963) observed sartorius muscles to exhibit a low rate of sodium ion gain over a period of several hours when $[K]_o$ in Ringer solution was maintained at a value of 2.5 mM. When $[K]_o$ is elevated to 5 mM, fresh sartorius fibers lose sodium ions in Ringer solution ($[Na]_o$ = 105 mM) to achieve a new steady-state internal sodium ion concentration of about one-half the normal value (Sjodin and Beaugé, 1968). It seems clear that at some intermediate value of $[K]_o$, the normal internal sodium ion concentration will be maintained in a steady state.

It is evident that for muscles stored in Ringer solution with $[K]_o$ in the range discussed, net leakage rates (true leakage flux – pumped flux) will be low and composite in that they will reflect slight imbalances in the passive and pumped components of flux. One can visualize some ways in which a study of leakage fluxes might be carried out. It would obviously be desirable to eliminate the pumped flux entirely, in which case the measured net leakage flux would equal the true passive leakage flux. The passive flux may be altered by the operation of removing the pumped flux, however, as the two components of flux need not be mutually independent. An attempt could be made, for example, to poison the pumped components of flux using various metabolic and transport inhibitors. A major difficulty with this method is the incomplete inhibition of active transport obtained with most and perhaps all inhibitors. Another difficulty is that the inhibitor may affect membrane permeabilities and so alter leakage rates.

A second possible method would be to measure active transport rates at various steady states and to equate the leakage flux to the appropriate active flux. There are at least three major difficulties with this method. First, it is difficult to measure the total active transport rate itself precisely unless one has a means to completely unfuel the transport system (Mullins and Brinley, 1967). It has not been possible to do this so far in muscle cells. As previously emphasized, inhibitors result only in partial inhibition. A second difficulty with this method is that of insuring that a true steady state has ensued at

some new desired condition. This means proving that concentrations have not changed over a long period of time, which requires a large number of observations for statistical significance. A third difficulty is that of correcting for possible interference due to exchange diffusional components. Even though such components do not cause departures from the steady state, being one-for-one exchanges of the same ion, it is sometimes difficult to separate them completely from active components. A portion of active sodium transport in muscle is activated by external sodium ions (Sjodin, 1971). In addition, this component appears to involve a one-for-one exchange of sodium ions under some conditions (Keynes and Steinhardt, 1968). Any component of active sodium transport that is of this sort will not contribute toward balancing the leakage flux.

A third method of approach offers less serious obstacles and was chosen as the main procedure for this work. When muscles are stored in Ringer solution from which potassium ions have either been omitted or reduced in concentration, the fibers lose potassium ions and gain sodium ions (Fenn and Cobb, 1934; Steinbach, 1940; Boyle and Conway, 1941; Edwards and Harris, 1957). The net gain of sodium ions is a consequence of the removal or reduction of the activating effect of external potassium ions on sodium pumping (Keynes, 1954; Sjodin and Beaugé, 1968: Sjodin, 1971). The net inward sodium flux under these conditions is down an electrochemical gradient and is likely to consist largely of a sodium leakage flux. The sodium inward net flux under these conditions can still be perturbed by sodium pumping as at least two sources of pump activation remain, namely that due to external sodium (Sjodin, 1971) and that due to potassium ions leaking out of the cells. The activation due to sodium is not likely to present a problem as such activation is likely to result in a one-for-one sodium exchange which will not contribute to sodium net flux (Keynes and Steinhardt, 1968). The possible activation due to accumulation of potassium ions leaking out of the fibers cannot be ignored, however.

The net outward leakage of potassium ions occurring from muscle fibers when $[K]_o$ is nominally equal to zero results because K^+ influx has been reduced nominally to zero and because the potassium ion distribution is no longer near electrochemical equilibrium. The muscle fiber membrane hyperpolarizes to values between -110 and -120 mV when $[K]_o = 0$. Mullins and Noda (1963) estimate the potassium equilibrium potential under these conditions to be -146 mV. The net efflux of potassium that occurs when $[K]_o = 0$ is thus down an electrochemical gradient and is, again, likely to consist largely of a passive leakage flux.

Because the latter procedure for measuring leakage rates offers the least number of difficulties, it was selected as the main method of procedure in the experiments to be reported. The ways in which leakage fluxes determined in

this manner might be influenced by residual ion pumping and by the presence of external potassium ions are also examined.

METHODS

Intracellular Ion Contents

Internal cellular concentrations of sodium and potassium ions were determined by the method of "back-extrapolation" to determine amounts residing in the intracellular compartment. Efflux of both cations was measured into Na- and K-free $MgCl_2$-substituted Ringer solution (Mg Ringer) for 2.5 h. To facilitate the unloading of noncellular compartments, a varying time schedule of sample collections was made as follows: 15 s, 1 min, 2 min, 2 min, 5 min, 10 min, 10 min, followed by four sample collections at 30-min intervals. Muscles were then blotted, weighed, and dried in the oven at 105°C for 1 h. Muscles were weighed again to obtain the dry weight and were then ashed in platinum. The ash was dissolved in a drop of 1 N HNO_3 and then brought to 10 cm^3 volume with deionized water. The final muscle ash and all efflux samples were assayed for sodium and potassium by flame photometry as previously described (Sjodin and Henderson, 1964; Sjodin and Beaugé, 1968). The fractions of total analytical sodium and potassium remaining in the muscle during efflux were plotted against time semilogarithmically and the straight lines obtained after 30 min were extrapolated to the axis of ordinates to determine the fraction of total quantities to be attributed to the intracellular compartment. Intracellular sodium and potassium contents were calculated from the intercepts and the total amounts of both cations in the muscles initially. During the 2.5 h exposure to Mg Ringer solution muscles underwent a small net loss in total cation content which was accompanied by an expected average weight loss of about 4% in the case of fresh muscles. Final cation contents were computed on the basis of corrected weights.

To measure net cation leakage rates, muscles were placed in K-free Ringer solution containing sodium ions at a concentration of 120 mM for varying time intervals. After exposure to K-free Ringer solution for the desired time interval, muscles were run through the same sequence of cation efflux measurement in K-free Mg Ringer solution described earlier. Cation contents of these muscles were determined in the same manner employed for fresh muscles. In such measurements, a paired control fresh muscle was always analyzed to provide initial cation contents.

Flux Measurements Using ^{22}Na and ^{42}K

For comparison with the net sodium influx rates measured as previously described, sodium influx was also measured employing ^{22}Na ions as tracers. Muscles were soaked in K-free Ringer solution labeled with ^{22}Na at known specific activity for given time intervals. Efflux was then performed into unlabeled Ringer solution and the counts per minute of ^{22}Na remaining in the muscles were plotted against time semilogarithmically. The intracellular radioactivity was obtained by extrapolation of the straight line obtained for washout of cellular ^{22}Na to zero time. The value of intracellular radioactivity so obtained was corrected for curvature due to back flux.

The method applied using ^{42}K was the same as that previously discussed in detail

(Sjodin and Henderson, 1964). One modification was necessary, however, when muscles were desired which contained sodium ions at an elevated concentration and which were also labeled with ^{42}K. To obtain results with ^{42}K which agreed well with results obtained by measuring unlabeled K movement, it was necessary to obtain rather complete percentage equilibrations of the intracellular potassium with solution ^{42}K. To achieve high equilibration in a reasonably short period of time (3 h) muscles were placed initially in a 5 mM K Ringer solution labeled with ^{42}K. As muscles maintain a normal sodium content under such conditions, it was necessary to reduce $[K]_o$ and place muscles in the cold (4°C) to elevate the internal sodium concentration. To maintain high tracer equilibration during the period of sodium enrichment, the external potassium concentration was reduced 10-fold to 0.5 mM at constant specific activity. If this procedure was not followed and, instead, muscles were placed in a high specific activity solution of ^{42}K at a very low total K_o concentration, tracer equilibrations were low even after long periods in the cold.

Radioactive tracer experiments were carried out on separate muscles which were not used in the net flux experiments.

Membrane Potentials

Measurements were made with microelectrodes filled with 3 M KCl. Microelectrodes were connected to a DC amplifier with electrometer tube input and grid current of 10^{-12} A or less via a silver-silver chloride electrode. The output voltage of the DC amplifier was measured with a digital voltmeter after careful adjustment of the DC amplifier gain to 10.0. Membrane potential readings were made to the nearest millivolt. The output of the DC amplifier was also displayed on an oscilloscope to determine the rapidity with which the membrane potential developed at the microelectrode tip. Only membrane potentials which developed instantaneously and which remained stable were recorded. Tip potentials were in the range of 0 to −5 mV as measured with a Keithley 610 A electrometer (Keithley Instruments, Inc., Cleveland, Ohio) and microelectrode resistances were in the range of 6 to 10 MΩ. Tip potentials varied in Na-free solutions. In Mg^{++}-substituted media, tip potentials were reduced to about one-fourth their magnitude in Na Ringer solution. An uncertainty of a few millivolts thus exists in the membrane potentials recorded in Mg^{++} media. Membrane potentials were monitored for 10 to 20 min for each set of recorded values to make certain they were stable.

Muscles

Whole sartorius muscles from *Rana pipiens* were carefully dissected, mounted on platinum frames, and handled as previously described (Sjodin and Henderson, 1964). Mounted muscles were then used in flux experiments. Muscle weights were in the range of 35–50 mg. When only membrane potentials were to be measured, muscles were mounted horizontally in a lucite cell in which the microelectrode tip was positioned with a micromanipulator. The majority of the experiments were performed on freshly dissected sartorius muscles. Because of the nature of the measurements, many muscles were stored in Ringer solution for several hours during the course of the experiments. In cases in which only potassium efflux was to be measured

in muscles with elevated Na contents, such muscles were prepared in the usual way by storage in K-free Ringer solution at 4°C until the desired sodium concentration was attained. All measurements were carried out at a temperature of 20.0 ± 0.2°C.

Solutions

The standard K-free Ringer solution employed for the measurement of Na and K leakage rates was of the following composition (millimoles/liter): NaCl, 120; $CaCl_2$, 2; Tris buffer, 1. The pH was adjusted to 7.35 by neutralization with HCl. The K-free and Na-free $MgCl_2$-substituted Ringer solution was of the following composition (millimoles/liter): $MgCl_2$, 86.3; $CaCl_2$, 2; Tris buffer, 1; pH = 7.35. The osmotic pressure of both solutions was equal to 230 mosmol/liter. K-free solutions with variable sodium concentrations were prepared by mixing the above two solutions in the desired proportions. The 5 mM K, 120 mM Na Ringer solution was prepared by adding KCl to the K-free formula. The 3.5% increase in total osmotic pressure that resulted was tolerated in order not to change the sodium concentration. Solution osmotic pressures were monitored by the freezing point depression method.

RESULTS

The Intracellular Sodium Ion Concentration in Normal Fresh Sartorius Muscle

There are at least three problems encountered in arriving at accurate estimates of the true intrafibrillar sodium ion concentration. These problems may be briefly stated to be the extracellular sodium, a possible separation of internal sodium into two or more cellular compartments, and the loss of sodium ions from muscle according to an approximately cubic relation at low internal sodium concentrations rather than a simple exponential loss. These problems have been encountered and discussed by previous authors (Keynes and Swan, 1959; Mullins and Frumento, 1963; Keynes and Steinhardt, 1968). The method of careful extracellular space determination to allow subtraction of purely extracellular sodium is only a partial aid as one is still left with the problem of compartmentalization of cellular sodium. In this work, the method of determining the kinetics of the loss of total analytical sodium to a Na-free medium was employed with extrapolation of the slowest exponential loss observed to $t = 0$ to estimate the intrafibrillar sodium. This method would give the true intrafibrillar sodium content in a relatively straight forward manner were it not for the problem of a sodium loss according to a power law in the low internal concentration range. The normal sodium content of sartorius muscles appears to lie just above the concentration at which nonlinearity begins to appear in a semilogarithmic plot of sodium washout in an Na-free medium (Mullins and Frumento, 1963; Sjodin, 1971). A sodium washout that begins at the normal sodium content of fresh muscle will thus tend to show a flattening of the slow cellular sodium loss on a semilogarithmic plot versus time. The flattening or slowing effect due to operation of the power law on sodium efflux will cause the intracellular sodium content to be underesti-

mated. The lower the rate of sodium loss during the kinetic analysis, the smaller will be the error due to entering the cubic region of efflux. For this reason, washout curves were obtained in a K-free as well as an Na-free medium. Magnesium chloride was employed as a replacement for NaCl.[1] Further slowing of the sodium efflux in K-free, $MgCl_2$-substituted Ringer solution occurs upon addition of ouabain to the medium (Beaugé and Ortiz, 1972).

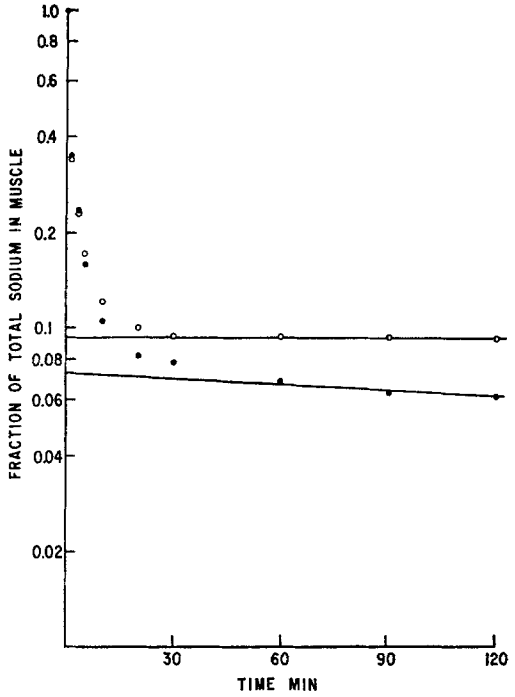

FIGURE 1. The fraction of the total muscle Na that remains in the muscle is plotted semilogarithmically against the time in contact with K-free and Na-free $MgCl_2$-substituted Ringer solution. Solid dots: absence of ouabain; open circles: efflux in presence of 10^{-4} M ouabain.

To obtain as slow a rate of sodium loss as possible, 10^{-4} M ouabain was also added to the K-free and Na-free washout medium. The results of such a kinetic analysis performed both in the absence and presence of 10^{-4} M ouabain are shown in Fig. 1. A fast component of sodium washout predomi-

[1] Tris chloride and magnesium chloride have previously been employed as sodium chloride substitutes to provide Na-free and low-Na media for muscles. Both NaCl substitutes have similar properties in increasing the potassium-activated sodium efflux in muscle and in reducing the sodium efflux in K-free solutions (Beaugé and Ortiz, 1970, 1972; Sjodin, 1971). In muscles with elevated sodium contents, the two NaCl substitutes have been shown to be quantitatively similar, the average rate constants for Na loss to Na-free, K-free media being 0.25 h^{-1}, Tris (Sjodin, 1971) and 0.27 h^{-1}, $MgCl_2$ (Beaugé and Ortiz, 1972). In the present work $MgCl_2$ was selected as the NaCl substitute because, for incubation periods lasting several hours, muscles maintained higher potassium ion contents in $MgCl_2$-substituted solutions than in Tris-Cl-substituted solutions. In several experiments performed for a comparison, average sodium contents determined by the same method using Tris-Cl or $MgCl_2$ did not differ significantly.

nates in the kinetics during the first 30 min both in the presence and absence of 10^{-4} M ouabain. The fast fraction contains extracellular sodium and probably contains rapidly moving sodium from fiber depots in close proximity to extracellular fluid. It could be argued that the fast component does not represent a different compartment, but results because the sodium concentration declines such that the cubic or nonlinear region is reached shortly after beginning washout. This explanation is rendered implausible by the fact that the muscle in the ouabain-containing solution gives an extrapolated intrafibrillar sodium concentration of 11.8 mM/kg fiber water which agrees with the final concentration in this muscle at the end of 2 h of washout. From work previously cited, the sodium content of this muscle has not entered the nonlinear or cubic region during the entire experiment and the extrapolated concentration should represent the true intrafibrillar concentration. The muscle in the solution not containing ouabain, however, gives an extrapolated sodium concentration of 9.2 mM/kg fiber water. At the end of 2 h of washout, this muscle had a sodium concentration of 7.8 mM/kg fiber water. On the basis of the previous work referred to, the muscle not exposed to ouabain has just entered the cubic region of sodium efflux. The lower estimate for intrafibrillar sodium concentration in this muscle is consistent and expected. The error introduced in this particular experiment amounts to a 22% underestimate of sodium concentration.

The average values for both sodium and potassium ion concentration obtained in a large number of such experiments appear in Table I. In many experiments, the rate of loss of the slowly moving sodium in the presence of 10^{-4} M ouabain was greater than the rate observed in the muscle giving the data for Fig. 1. The rate was always significantly and considerably lower than in the absence of ouabain, however. One cannot be absolutely certain that some muscles did not begin to enter the cubic region of efflux even in the presence of 10^{-4} M ouabain. It should be realized that the average concentration of 10.6 mM/kg fiber H_2O could still represent somewhat of an underestimate. The underestimate of sodium concentration that occurs if ouabain is not used, however, amounts to about 30% on the average. For comparison, concentrations obtained by soaking muscles for 10 min in K-free and Na-free Tris Ringer solution to remove extracellular sodium and potassium are included in Table I. In this method, muscles remained for the entire 10 min interval in the same wash solution and were not transferred from tube to tube. The potassium concentrations obtained by all methods are essentially equivalent. The latter method, however, overestimates the intrafibrillar sodium concentration by 60%. Obviously, a 10 min wash in the same Na-free solution does not suffice to remove all of the extracellular sodium. Transferring muscles from tube to tube according to the schedule in Methods removes the extracellular sodium more efficiently. Fig. 1 indicates that the total muscle sodium is only about one-third greater than the extrapolated value after 10 min of washout involving solution transfers. The difference between the extrapolated

TABLE I

FRESH MUSCLE CATION CONTENTS

Method	No. of observations	A. Sodium contents $[Na]_i$					
			SD	SE		SD	SE
		mmol/kg wet wt			*mmol/kg fiber H_2O* *		
Extrapolation, K and Na-free + ouabain	31	6.55	±1.80	±0.32	10.6	±2.9	±0.5
Extrapolation, K and Na-free Ringer solution	17	4.47	±1.53	±0.37	7.2	±2.5	±0.6
10 min K-free Tris Ringer solution soak	33	10.51	±2.00	±0.35	16.9	±3.2	±0.6

Method	No. of observations	B. Potassium contents $[K]_i$					
			SD	SE		SD	SE
		mmol/kg wet wt			*mmol/kg fiber H_2O*		
Extrapolation, K and Na-free Ringer Solution	48	86.8	±7.2	±1.0	140.0	±11.6	±1.6
10 min K-free Tris Ringer solution soak	33	86.3	±5.1	±0.9	139.2	±8.2	±1.5

* To express ionic concentrations in units of millimoles per kilogram of fiber water requires a knowledge of the dry weight of the muscles, the wet weight of the muscles, and the percentage of the muscle weight due to the extracellular fluid. If W_W = wet weight, W_D = dry weight, and W_S = weight of extracellular fluid, then the factor by which ion contents in millimoles per kilogram wet weight must be divided to obtain fiber water concentrations is:

$$1-\left[\frac{W_D}{W_W}+\frac{W_S}{W_W}\right].$$

The ratio W_D/W_W was measured for all muscles used in this investigation by drying muscles in an oven at 105°C for 1 h, after obtaining wet weights, and then reweighing. The average value of the ratio W_D/W_W was 0.204 ± 0.004 (SE), n = 81. The extracellular space was not measured but Johnson (1956) reports an average inulin space for sartorii from *Rana pipiens* of 18 ± 0.5 (SE) percent of muscle weight, n = 56. These numbers were used to calculate fiber water concentrations in the present work.

value and the 10 min value amounts to about 2 μmol Na per g wet weight of muscle. As this is equivalent to about one-tenth the extracellular sodium in blotted muscles, it is probable that it represents some rapidly moving fiber sodium in addition to sodium that has not yet left the extracellular space.[2] An

[2] An uncertainty in assigning sodium contents to different cellular compartments lies in the amount of sodium which washes out of the extracellular space in these experiments. Mullins and Frumento (1963) measured the washout of extracellular sodium from sartorius muscles as an exponential process with a time constant of 2.4 min. On this basis, extracellular washout would be essentially complete in 10 min. However, Mullins and Frumento also found that about 5% of the sucrose

additional uncertainty in the distribution of the muscle sodium is that due to the presence of a fraction that is either inexchangeable or exchanges very slowly with labeled sodium. There is evidence that this fraction of the total muscle sodium is situated in the connective tissue and amounts to about 2 mmol/kg of muscle (Harris and Steinbach, 1956 *a,b*). Sodium contents in Table I should be corrected by this amount if this interpretation of the very slowly exchanging sodium fraction is correct. As this fraction was not measured in this work, the corrections have not been made.

The extrapolation method in the presence of 10^{-4} M ouabain appears to have the least number of possible errors of all methods available in this laboratory for determining intrafibrillar sodium concentration. The method gives reasonable and very reproducible results. As differences in sodium concentration are the quantities sought in this investigation, any small errors that may remain are of even less importance. Accordingly, in this work, the intrafibrillar sodium concentration is operationally defined as that determined by extrapolating sodium washout curves in K-free and Na-free Mg-substituted Ringer solution containing 10^{-4} M ouabain.

The Kinetics of Sodium Net Gain in a K-free Medium

One of a pair of muscles from the same frog was subjected to the procedure for determining the intrafibrillar sodium ion concentration immediately after dissection. The other muscle of the pair was placed in K-free Ringer solution for a given interval of time. At the end of this period, the muscle was subjected to the same procedure for determining intrafibrillar sodium ion concentration. The difference in sodium ion concentration between the paired muscles was taken as the net sodium gain in the given interval of time. Results are plotted in Fig. 2 (dots, lower curve). Similar experiments were performed in the presence of 10^{-4} M ouabain. Results are plotted in Fig. 2 (triangles, upper curve). Ouabain increased sodium net influx by about 40%. In all experiments performed, initial and final potassium ion contents of muscles were also determined. Potassium ion losses from muscles are plotted against time in Fig. 2 both in the absence of ouabain (open circles) and in the presence of 10^{-4} M ouabain (open squares). Potassium losses occurring with or without the presence of ouabain fall along the same line describing sodium ion gain in the presence of ouabain.

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emerging from muscles was lost with a time constant that was very long compared to that for a rapid initial washout. It is not possible, at present, to decide whether such components represent diffusion from the innermost interspaces or are due to unloading of superficial fiber compartments associated with tubular elements. It appears, however, that the uncertainty in the total intrafiber sodium in muscles exposed to 10 min of wash involving solution transfers in Na-free media must be about 5% of the extracellular sodium. This amounts to approximately 1 μmol of Na per g of muscle which is not enough to account for all of the extra sodium remaining in the muscle after only 10 min of washout.

The rate of sodium ion gain is independent of the internal sodium ion concentration up to a concentration of 70 mM/kg fiber water which represents about one-half replacement of intracellular potassium by sodium. This result would be expected if the measured gain in sodium content is due to an inward

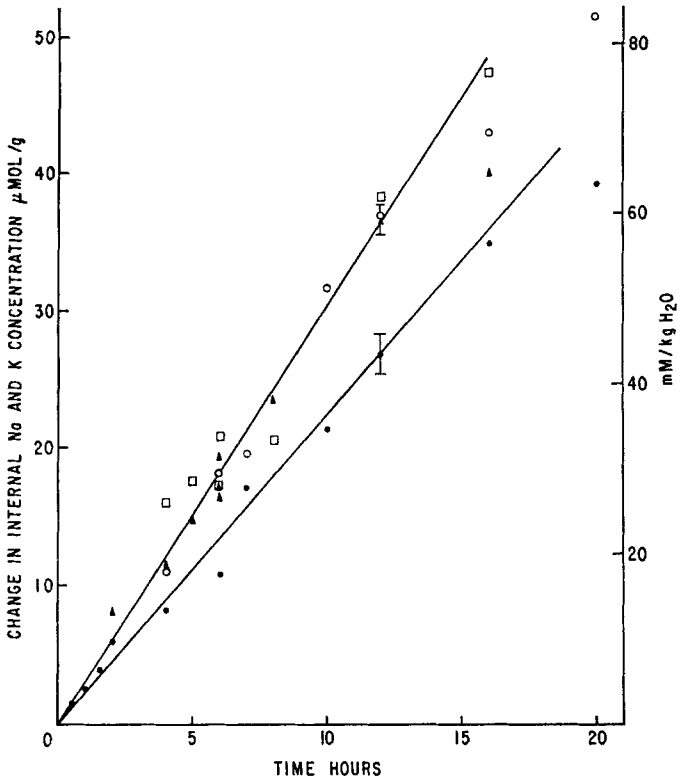

FIGURE 2. The gain in intracellular Na and loss of K occurring when muscles are placed in K-free, 120 mM Na Ringer solution at 20°C are plotted against the time of incubation. Points with vertical lines represent average values ±1 SE of seven or eight experiments each. Solid dots: Na gain; solid triangles: Na gain in the presence of 10^{-4} M ouabain; open circles: K loss; open squares: K loss in the presence of 10^{-4} M ouabain. The K loss points at 12 h refer to average values of seven and eight experiments each. For clarity in the graph, the standard errors have been omitted for these data points. The magnitudes of the standard errors for K loss are approximately twice those for the Na data points at 12 h. All other points refer to single experiments. The units for the left hand axis are micromoles per gram wet weight.

passive net flux of sodium ions, ϕ_{Na}, that obeys the constant-field flux equation:

$$\phi_{Na} = P_{Na} \frac{E_m F}{RT} \left(\frac{[Na]_o - [Na]_i e^{E_m F/RT}}{e^{E_m F/RT} - 1} \right), \quad (1)$$

where E_m is the membrane potential, P_{Na} the sodium permeability coefficient, and other symbols have their usual significance. The muscle membrane poten-

tials during such experiments are reported in another section and were more negative than $E_m = -100$ mV. Under these conditions, the term containing $[Na]_i$ has a negligible numerical value throughout the experiment and sodium net influx is proportional to $[Na]_o$.

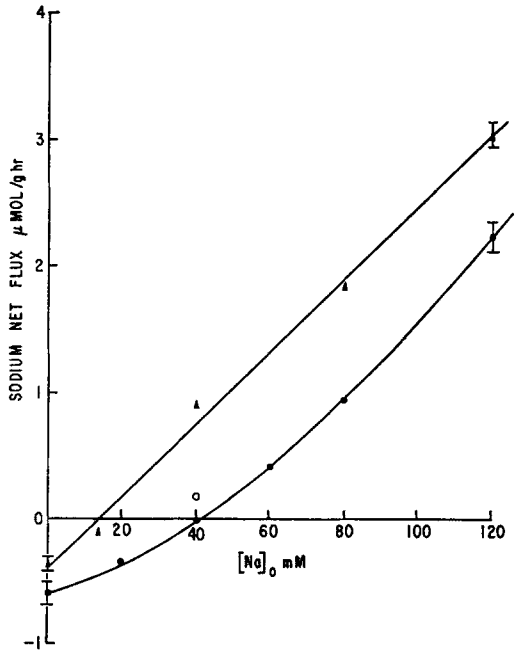

FIGURE 3. Na net flux measured in K-free media at 20°C is plotted against the Na ion concentration in the medium. NaCl in the medium was replaced with an osmotic equivalent of $MgCl_2$ to achieve different sodium ion concentrations. Positive values denote net influx; negative values denote net efflux. Points with vertical lines denote average values of eight or more experiments ±1 SE. Other points are averages of two experiments each. Solid dots: normal muscle; solid triangles: presence of 10^{-4} M ouabain. Net fluxes were determined after 12-h incubation periods. The open circle refers to a single experiment performed using Tris as a sodium substitute in the absence of ouabain. In the unit μmol/g·h, g refers to muscle wet weight.

The Dependence of Net Sodium Gain on the External Sodium Ion Concentration

Eq. (1) predicts a linear dependence of passive net sodium influx on the external sodium ion concentration under the conditions of these experiments. Net sodium gain experiments were performed as before but at different external sodium ion concentrations. The results appear in Fig. 3 (dots, bottom curve). A significant departure from linearity occurs, differing from the predictions of Eq. (1). Moreover, the flux changes sign at an external sodium ion concentration of 40 mM. At the membrane potentials of the muscle fibers in these experiments, Eq. (1) does not predict a change in the sign of the flux

until $[Na]_o$ falls to values of a few tenths of a millimole per liter. It seems evident that the net gains of sodium measured represent combinations of inward leakage and outward pumping of sodium. In a K-free Ringer solution, these components of flux balance one another at $[Na]_o$ = 40 mM. As $[Na]_i$ = 10 mM and the membrane potentials are more negative than −100 mV, such a flux balance requires a net outward active transport of sodium ions equal to the leakage rate at $[Na]_o$ = 40 mM. The possible sources of pump activation are potassium ions leaking out of the cells or external sodium ions themselves.[3]

The same type of experiment was performed in the presence of 10^{-4} M ouabain. Results are plotted in Fig. 3 (triangles, upper curve). The net flux in ouabain follows a linear dependence on $[Na]_o$ in agreement with predictions of Eq. (1). In the presence of ouabain, a balance of the sodium fluxes occurred at an external sodium ion concentration of about 15 mM in K-free solutions. Had all active sodium extrusion been abolished by 10^{-4} M ouabain and K-free conditions, one can calculate that a purely passive flux balance would have been expected at $[Na]_o$ = 0.11 mM. As the observed value of $[Na]_o$ at flux balance was over 100 times this concentration, muscle fiber membranes are apparently able to perform active transport against a considerable electrochemical gradient with potassium ions removed from the medium and in the presence of ouabain at a concentration known to completely inhibit potassium-activated sodium extrusion.[4]

The Action of External Potassium Ions on Net Sodium and Potassium Fluxes in the Presence of 10^{-4} M ouabain

In the work previously reported, experiments were performed in a K-free medium so that a net sodium influx would be induced in the absence of transport inhibitors. It is clear that the net sodium influx so induced must include a significant component of passive leakage of sodium ions down an electro-

[3] As some of the characteristics of the curves in Fig. 3 are probably determined by potassium ions leaking out of the fibers, at least in the absence of ouabain, different Na substitutes may result in somewhat different curves.

[4] An alternative explanation of these results might be that the observed total flux balances are due to fluxes occurring across different membranes bounding different compartments. In this event, a pseudo-steady state might occur with one compartment gaining sodium and another compartment losing sodium at an equivalent rate. This explanation is rendered implausible because sodium exchanges in muscle occur with time constants of the order of 1 h. The steady states observed occurred over a 12 h period. Each individual compartment involved has thus had a period of several time constants' duration to come to a steady state at the altered external sodium concentration. A redistribution of cellular sodium in different compartments is not excluded, however. If much of the cellular sodium in low Na media moved from a region of high membrane potential to a region of low or zero membrane potential with no change in total sodium content, the results might be explained on the basis that ouabain inhibits the sodium pump much more than it seems to. As such an explanation remains entirely *ad hoc*, the one offered is preferred in the absence of further evidence concerning the distribution of cellular sodium.

chemical gradient. It is of interest to examine the influence of external potassium ions on the rate of inward sodium leakage. If potassium ions are added to the medium in the absence of ouabain, stimulation of the sodium pump will reduce the total net inward movement of sodium ions. At an external potassium ion concentration of 5 mM, the net inward sodium movement is reduced to zero or actually becomes an outward movement depending upon the value of $[Na]_o$, (Sjodin and Beaugé, 1968). As ouabain is known to completely abolish potassium-activated sodium extrusion, experiments were performed in the presence of ouabain at a value of $[K]_o$ = 5 mM. Results are reported in Table II where a comparison is made between net flux in the presence of external potassium and the previously determined values of net flux in the absence of external potassium ions. The action of 5 mM K^+ is to reduce the net fluxes occurring in a K-free ouabain-containing medium by approximately

TABLE II

Na AND K NET FLUXES AND MEMBRANE POTENTIALS UNDER DIFFERENT CONDITIONS

Conditions					Membrane potential	
$[K]_o$	$[Na]_o$	Ouabain, 10^{-4} M	Net Na influx ±SE	Net K efflux ±SE	Initial	Final
mM			*μmol/g·h*		*mV*	
0	120	−	2.24±0.12 (8)	3.08±0.23 (8)	−113.6±1.3	−125.4±1.4
0	120	+	3.05±0.09 (7)	3.18±0.23 (7)		−123.4±1.4
5	120	+	2.55±0.13 (6)	2.55±0.16 (6)	−79.4±0.8	−71.9±0.7

Number of observations indicated in parentheses.

20%. An equivalence in the magnitudes of the sodium and potassium net fluxes in the presence of ouabain is again observed.

As external potassium ions depolarize muscle fiber membranes and sodium passive leakage flux should be a function of the membrane potential more or less in accordance with Eq. (1), it was of interest to measure fiber membrane potentials under the different conditions employed. Average membrane potentials obtained are included in Table II. Figures reported are the averages of the means of 10–12 penetrations on several muscles (usually four muscles). Membrane potential values were monitored at 30-min intervals and are reported as initial and final values due to a modest change in potential during the course of a 6 h flux experiment. In the absence of external potassium, potentials increased (hyperpolarized) with time. In the presence of 5 mM K, membranes depolarized somewhat with time. Membrane potential changes followed an approximately exponential time-course with a half-time of about 1.5 h for K-free conditions and a half-time of about 3 h in the presence of 10^{-4} M ouabain and 5 mM K^+. The reduction in net sodium influx brought about

by the presence of 5 mM K in the medium in the presence of 10^{-4} M ouabain can be explained on the basis of a reduced driving force for inward sodium ion leakage. The force driving sodium ions inward is reduced in the presence of external potassium ions due to the decreased negativity within the fibers. Quantitatively, the reduction is less than that predicted by Eq. (1) suggesting that potassium ions may have other effects on sodium passive flux. In view of the antagonistic actions of K^+ and ouabain on sodium transport (Post and Albright, 1961), part of the action of 5 mM K^+ may be to reduce the effectiveness of ouabain. It should be noted that the change in net influx of sodium ions under K-free conditions that is induced by 10^{-4} M ouabain cannot be attributed to changes in fiber membrane potential.

The change in potassium ion net efflux brought about by addition of 5 mM K^+ to K-free ouabain-containing Ringer solution is accompanied by a large depolarization and the introduction of a large component of unidirectional influx. The changes are difficult to analyze. The consequences of adding ouabain to 5 mM K^+ Ringer solution bathing muscles can, however, be examined. The potassium ion fluxes in 5 mM K^+Ringer solution are in balance (Sjodin and Henderson, 1964) and the fiber membrane potentials are close to the potassium equilibrium potential, E_K (Adrian, 1956). When ouabain is added to the medium, a net loss of potassium ions from the cells is induced which causes the potassium equilibrium potential to become less negative inside the fibers. The membrane potential becomes less negative faster than the equilibrium potential for potassium ions, however, so that a driving force becomes available to move potassium ions out of the cells. The difference between E_m and E_K is from 5 to 7 mV over most of the time interval during which such experiments were conducted. The mechanism for the observed depolarization is unknown. An increased permeability to sodium ions could be involved. An electrogenic component to the membrane potential which becomes abolished in the presence of ouabain seems to offer the best possibilities for an explanation.

The Distinction Between Total Unidirectional Sodium Influx and Net Sodium Influx

The total unidirectional sodium influx may consist of a passive leakage flux down an electrochemical gradient and additional components with different properties. To measure total unidirectional sodium influx, ^{22}Na ions were employed as tracers. Sodium unidirectional influx was measured at varying internal sodium ion concentrations. Muscles with increasing intracellular sodium ion concentrations were prepared by the previously applied technique. Influx was subsequently measured over time intervals from 30 min to 1 h. Under the K-free conditions employed, $[Na]_i$ did not change appreciably

during the measurements. Results are plotted in Fig. 4. In marked contrast to sodium net influx, unidirectional sodium influx shows a strong dependence on the internal sodium ion concentration. The unidirectional sodium influx increases linearly with increasing internal sodium ion concentration. The solid line through the data points represents the best fit of a straight line to the points giving increased weighting to data representing the mean of several observations. It is of significance that the line fitting the data does not extrapolate to the origin at $[Na]_i = 0$ but meets the axis of ordinates at an influx value of about 4 μmol/g·h. This result suggests that the total unidirectional sodium in-

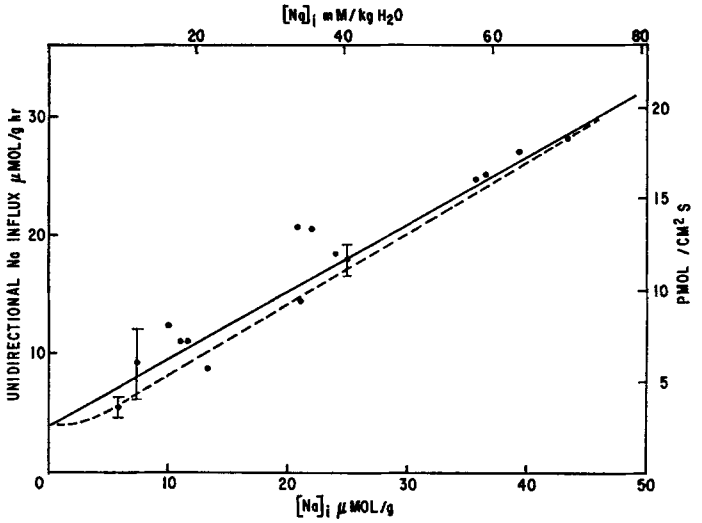

FIGURE 4. Unidirectional total Na influx measured using ^{22}Na ions as tracers is plotted against the Na ion concentration in the muscle. The solid line was drawn to fit the data. The dotted line was drawn from theoretical considerations explained in the text. Points with vertical lines are averages of four to six experiments ±1 SE. In the unit μmol/g·h, g refers to muscle wet weight.

flux is the sum of two components, one showing no dependence on $[Na]_i$ and one rising linearly with increasing $[Na]_i$.

The Residual Sodium Efflux

In the absence of both external potassium and sodium ions, a sodium efflux occurs that has the properties of an active transport. Even though this efflux is not against an electrochemical gradient, it is at least an order of magnitude greater than the value of efflux expected for a passive outward leakage of sodium ions. The residual sodium efflux has been reported to be inhibitable by cardioactive steroids but to differing degrees depending upon the sodium substitute employed. A variable strophanthidin sensitivity of the residual so-

dium efflux measured in K-free Tris-substituted media has been observed (Sjodin, 1971). The percentage of inhibition due to 10^{-5} M strophanthidin varied between 0 and 32% with an average inhibition of 13%. A very large inhibition due to 10^{-5} M strophanthidin in a K-free Tris medium was observed by Henderson (1971), but the control rate constant in the absence of strophanthidin was about 3 times larger than the average value reported by Sjodin (1971) so that it is difficult to compare the results. Beaugé and Ortiz (1972) report a 40% inhibition (one experiment) due to 10^{-4} M ouabain in a K-free, Tris-substituted medium. This is near the highest degree of inhibition observed by Sjodin (1971). A significant reduction in sodium efflux measured in K-free, $MgCl_2$-substituted solutions was always observed during application of 10^{-4} M ouabain both in previous work (Beaugé and Ortiz, 1970, 1972) and in the present work. The reason for the variable results in

TABLE III

RATE CONSTANTS FOR SODIUM ION LOSS TO K- AND Na-FREE, Mg-SUBSTITUTED MEDIA

Internal Na concentration	Control (±SE)	Presence of 10^{-4} M ouabain (±SE)	Ouabain-sensitive
		h^{-1}	
Normal	0.133±0.022	0.084±0.010	0.049
Elevated*	0.241±0.051	0.124±0.004	0.117

* $[Na]_i$ was elevated by storage of muscles in K-free Ringer solution for 6–12 h. The range of elevated sodium contents was 25–40 μmol/g.

Tris-Cl solutions is not known. It seems likely that the ouabain-sensitive activation of the sodium pump in K-free, Na-free media is due to potassium ions leaking out of the fibers. Variable results may be due to variable potassium accumulation in a region near the external activation sites. Experiments to be reported in the next section suggest that potassium ions leaking out of the fibers into K-free, $MgCl_2$-substituted media can, under some conditions, be pumped back into the cells.

In the course of this investigation, it was necessary to measure sodium net efflux on numerous muscles when the medium was both K- and Na-free. In addition to being reduced by ouabain, the rate constant for this component of sodium outward movement proved to be dependent on the internal sodium ion concentration. When the internal sodium ion concentration was elevated by as little as 40%, the rate constant for residual sodium loss was significantly elevated over that measured on fresh muscles with a normal sodium content. Further elevation of the internal sodium ion concentration resulted in no further increase in the rate constant observed. The results are summarized in Table III. Both ouabain-sensitive and ouabain-insensitive rate constants are

observed to be increased upon elevation of the internal sodium ion concentration. The activation of these components of sodium ion transport is maximal at internal sodium ion concentrations of approximately 15 mM and above. The normal internal sodium ion concentration, 10.5 mM on the average, produces less than maximal activation. Activation of this type can be described satisfactorily by a model which assumes that sodium ions are transported outwardly when two to three sites at the inner membrane surface are occupied by sodium ions.

The Influence of the Internal Sodium Ion Concentration on Potassium Efflux into K-free and Na-free Media

The results in Fig. 2 indicate that the net rate of loss of potassium ions from muscles to K-free Ringer solution is independent of the internal sodium ion concentration. When the external sodium ion concentration was lowered by replacement with magnesium ions, potassium efflux declined and became reduced by about one-half in $MgCl_2$ Ringer solution with $[Na]_o = 0$. Also, the rate of potassium loss to K-free, Mg^{++}-substituted Ringer solution was found to depend markedly on the internal sodium ion concentration. If muscles were first enriched with sodium by placing them for several hours in K-free Ringer solution with $[Na]_o = 120$ mM, the rate of potassium loss subsequently measured in K-free, Mg^{++}-substituted Ringer solution fell to very low values. The rate constant for potassium loss in K-free, Mg^{++} Ringer solution with $[Na]_o = 0$ was about 10 times less for muscles with an elevated sodium content than for fresh muscles with a normal sodium concentration. The reduced net potassium efflux under these conditions was very sensitive to ouabain. Application of 10^{-4} M ouabain brought about an average increase in potassium efflux of 14-fold in such cases. The results are presented in Fig. 5 and in Table IV.

It has been stated that ouabain reduced the sodium efflux that occurs in K-free, Mg^{++} Ringer solution with $[Na]_o = 0$. It is of interest to compare the reductions in sodium efflux and the increases in potassium efflux brought about by the presence of ouabain. The comparison is made in Table IV. Muscles which served as controls and muscles exposed to ouabain were paired from the same animal. Both sodium and potassium ion effluxes were measured for each muscle. The increases in potassium efflux induced by ouabain were observed to be comparable to the decreases in sodium efflux brought about in the same muscle. The table column showing the ratio of the decrease in sodium efflux to the increase in potassium efflux shows that these quantities are essentially equal to within experimental errors.

The most reasonable interpretation of the results in Table IV is that the sodium pump in muscles with an elevated sodium content reaccumulates potassium ions leaking out of the cell and returns them to the cell interior when

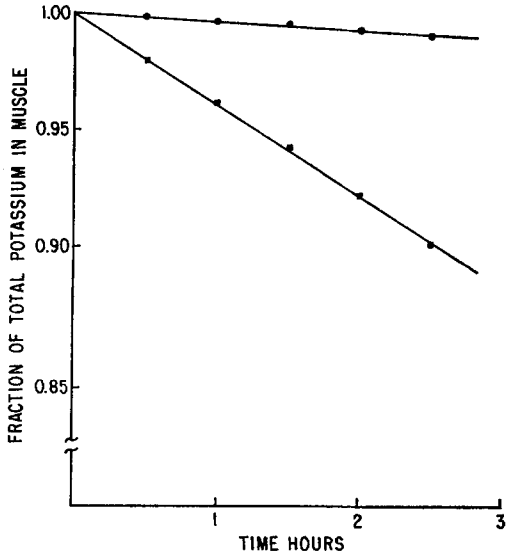

FIGURE 5. The fraction of the total muscle K remaining in the muscle is plotted semi-logarithmically against the time in contact with K-free and Na-free $MgCl_2$-substituted Ringer solution. Muscles were enriched with Na by storage in K-free, 120 mM Na Ringer solution before the measurement of K efflux. Solid dots: absence of ouabain; solid squares: presence of 10^{-4} M ouabain.

TABLE IV

THE EFFECT OF OUABAIN ON K AND Na EFFLUX IN HIGH Na MUSCLES IN Mg^{++} RINGER SOLUTION

	Rate constant		Efflux		Efflux change		
Ouabain*	K	Na	K	Na	K	Na	$\frac{\Delta Na}{\Delta K}$
	h^{-1}		$\mu mol/g \cdot h$				
−	0.0018	0.168	0.12	4.84			
+	0.0266	0.112	1.82	3.09	1.70	1.75	1.03
−	0.0022	0.133	0.16	3.59			
+	0.0194	0.075	1.49	2.17	1.33	1.42	1.07
−	0.0026	0.166	0.17	4.72			
+	0.0307	0.133	2.13	3.37	1.96	1.35	0.69
−	0.0038	0.195	0.23	5.09			
+	0.0405	0.120	2.32	3.31	2.09	1.78	0.85

* One of a pair of muscles was exposed to 10^{-4} M ouabain while the other muscle of the pair served as a control.

the external medium is nominally free of sodium ions. The mechanism which enables this effect to take place is the removal of the inhibiting action of external sodium ions on pump activation by external potassium ions. Removal of external sodium ions causes the sodium pump in muscle membrane to become sensitive to the activating effect of small external concentrations of potassium

ions (Sjodin, 1971). "Back pumping" of potassium ions leaking out of the cells might be expected to take place when $[Na]_i$ is elevated and $[Na]_o$ is zero. The action of ouabain is then readily explainable on the basis of the inhibitory effect of ouabain on the Na pump. Ouabain thus serves to elevate potassium efflux to the normal value one would expect if no "back pumping" of K^+ occurred.

Some potassium efflux measurements were made using ^{42}K ions as tracers. Comparisons with rate constants measured for the net loss of analytical potassium are made in Table V. This table also includes a summary of rate con-

TABLE V

SUMMARY OF RATE CONSTANTS FOR K LOSS FROM MUSCLES TO K-FREE SOLUTIONS

Muscle Na	$[Na]_o$	Ouabain	Rate constant ±SE*		E_m
			K loss	^{42}K loss	
	mM		h^{-1}		*mV*
Normal	120	−	0.0425±0.0028	0.0601±0.0021	−113.6±1.3
Normal	120	+	0.0443±0.0026	0.0509±0.0019	
Normal	0	−	0.0231±0.0038	0.0245±0.0035	−116.4±1.7
Normal	0	+	0.0306±0.0013		
Elevated	0	−	0.0026±0.0003	0.0033±0.0008	−113.8±2.5
Elevated	0	+	0.0373±0.0014		−112.0±2.2

* Results are presented as rate constants because all ^{42}K experiments and all net K loss experiments in Na-free media gave straight lines when plotted semilogarithmically. The net K-loss data in sodium Ringer solution plotted in Fig. 2 is included in the table by considering K loss to be an exponential process. The K lost from individual muscles to K-free, 120 mM Na Ringer solution was followed kinetically for 6 h by direct measurement of the potassium concentration in successive samples. Such data gave a straight line when plotted semilogarithmically. The data obtained by the "difference" method is not capable of resolving the exact kinetics as each experiment gives only one point on a curve. These data plot linearly in Fig. 2. The same data, however, expressed as rate constants for an exponential K loss, show a relatively constant rate coefficient as can be seen from the standard errors in this table.

stants obtained under various experimental conditions together with average muscle fiber membrane potentials measured during such experiments.

In measuring potassium efflux into K-free Ringer solution by the ^{42}K method, no special precaution was taken to highly equilibrate the muscles with ^{42}K Ringer solution initially. The muscles were about 20% equilibrated with tracer before beginning efflux measurements in these cases. The rate constant for ^{42}K loss is significantly higher than the rate constant for the loss of total potassium in these experiments. Previous experiments (Sjodin and Henderson, 1964) showed that the specific activity of the ^{42}K lost from lightly equilibrated muscles to K-free Ringer solution remained higher than the specific activity of ^{42}K remaining in the muscles for a period of about 2 h. A

compartment in the muscle fibers that is more highly equilibrated with ^{42}K than the intracellular compartment could, therefore, account for the present finding. In the experiments involving muscles with an elevated sodium content, special care was taken to highly equilibrate the muscles with ^{42}K Ringer solution before the beginning of efflux measurement as described in Methods. Unless this was done, rate constants for ^{42}K loss did not agree well with the rate constants for the loss of total K. The equilibrations with ^{42}K Ringer solution for the remainder of the rate constants reported in Table V were around 70% complete. Under these conditions, rate constants for tracer and nontracer potassium agreed well.

An Analysis of Measured Sodium Fluxes in Terms of the Pump-Leak Hypothesis

The sodium net influx measured in the absence of external potassium ions and in the presence of 10^{-4} M ouabain has some of the characteristics expected of a passive leakage of sodium ions. Under these conditions, net influx of Na^+ is independent of $[Na]_i$ (Fig. 2) and linearly dependent on $[Na]_o$ (Fig. 3). It is evident from Fig. 3, however, that even this component of sodium flux contains pumped components. In this section, an attempt will be made to separate pumped and leakage components of Na^+ flux by analysis. It should be clear that no further experimental separation of these components is possible using the experimental techniques employed in this investigation.

The net influx of sodium ions measured in this work will be denoted by J_{Na} and it will be assumed that this flux is the difference between a passive inward leakage flux, ϕ_{Na}, and an externally directed pumped flux, $\bar{m}_{Na}$. The fundamental flux equation is thus assumed to be:

$$J_{Na} = \phi_{Na} - \bar{m}_{Na}, \tag{2}$$

where ϕ_{Na} is given by Eq. (1). For reasons previously stated, the term involving $[Na]_i$ in Eq. (1) makes a negligible contribution under the conditions holding in these experiments. Thus one can rewrite Eq. (1) as:

$$\phi_{Na} = P_{Na} f(E) [Na]_o \tag{3}$$

where $f(E)$ is a function of the membrane potential deducible from Eq. (1).

Furthermore, it is assumed that the outward pumped flux of Na^+, $\bar{m}_{Na}$, can be separated into outward and inward components m_o and m_i such that:

$$\bar{m}_{Na} = m_o - m_i. \tag{4}$$

To effect an analysis of the fluxes it is necessary to know the value of ϕ_{Na}. Unfortunately, there does not appear to be an unambiguous method for determining the purely passive component of the sodium influx. The value of the

net sodium influx measured in the presence of ouabain cannot be used to estimate ϕ_{Na} with certainty as ouabain does not seem to completely inhibit the sodium pump. In the limit when $[Na]_i = 0$, all pumped and exchange components of sodium flux vanish leaving only the passive sodium influx. Hence, the most reasonable method for estimating ϕ_{Na} appears to be that of extrapolation to $[Na]_i = 0$ of the data in Fig. 4. The value for ϕ_{Na} obtained by this method is 4.2 μmol/g·h and this magnitude is used in further calculations. The disadvantage of this approach is that ϕ_{Na} may not be known very accurately. It should be noted that shifting the value of ϕ_{Na}, within certain limits, would alter the quantitative conclusions, but would not change the general qualitative conclusions.

From Fig. 2 and Table II, the net influx of sodium when $[Na]_o = 120$ mM is equal to 2.24 μmol/g·h. Using Eq. (2), $\bar{m}_{Na}$ under these conditions is 2.0 μmol/g·h. Thus under K-free conditions in the medium, the pumping rate is about one-half the true rate of inward passive leakage and this suffices to reduce the net sodium influx to a value about one-half of the true passive leakage influx.

The efflux component of $\bar{m}_{Na}$, m_o, has been previously studied in some detail (Sjodin, 1971). Under K-free conditions, sodium efflux is activated by external sodium ions and is given by $m_o = k_{Na}[Na]_i$, where k_{Na} is the rate constant which depends on the external sodium ion concentration but not the internal sodium ion concentration, provided that $[Na]_i$ exceeds a minimum value of about 10 mM. To carry the analysis further, use may be made of the observed fact that the measured net sodium influx, J_{Na}, is independent of $[Na]_i$. As J_{Na} and m_o have properties already experimentally determined, m_i must satisfy a functional relation consistent with these properties. To determine the functional relation, Eqs. (2) and (4) can be solved for m_i subject to the conditions described. The following equation is obtained for m_i :

$$m_i = k_{Na}[Na]_i - 2.0, \tag{5}$$

where k_{Na} is the rate constant previously described and where units of flux are in micromoles per gram·hour. The required relation to satisfy the data is, therefore, a linear dependence of m_i on $[Na]_i$. Total unidirectional sodium influx, J_i, must be equal to the sum of the true passive influx and m_i. One thus obtains the relation $J_i = \phi_{Na} + m_i$ which for $[Na]_o = 120$ mM becomes:

$$J_i = k_{Na}[Na]_i + 2.2, \tag{6}$$

in flux units of micromoles per gram·hour. For K-free conditions in the medium and with $[Na]_o = 120$ mM, $k_{Na} = 0.6$ h^{-1} (Sjodin, 1971). A limiting condition is evident from Eq. (5). The flux m_i may not become negative, i.e., a unidirectional influx may not become an efflux. The lowest value of m_i is

thus zero and this occurs at $[Na]_i$ = 3.3 mmol/g or 5 mM/kg fiber water. Eq. (6) may thus be expected to hold at $[Na]_i$ = 3.3 μmol/g and above. The physical interpretation of these details is that m_i, being an active component, requires a certain internal sodium ion concentration for activation. An internal sodium ion concentration of 5 mM is well within the cubic region and activation may be expected to fall rapidly to zero at lower internal sodium ion concentrations.

The total unidirectional sodium influx, J_i, in Eq. (6) is just the component of flux that was measured using ^{22}Na ions and plotted against $[Na]_i$ in Fig. 4. The dotted line in Fig. 4 is a plot of Eq. (6) above $[Na]_i$ = 5 mM. Below $[Na]_i$ = 5 mM, lack of activation reduces m_i rapidly to zero and the dotted line is, correspondingly, altered to approach the true inward passive leakage flux at $[Na]_i$ = 0. With this modification, Eq. (6) is seen to give a quite satisfactory fit to the data.

The action of 10^{-4} M ouabain on the measured net sodium influx, J, can be interpreted either as an increase in the sodium ion permeability, P_{Na}, or as a decrease in the active component, $\bar{m}$, or as a combination of these effects. The action of ouabain on active components of sodium flux is known from previous work. Keynes and Steinhardt (1968) observed that ouabain decreases both sodium efflux and sodium influx when potassium ions are nominally absent from the external medium. Sodium ion efflux and influx were reduced by approximately the same percentage. If flux components m_o and m_i are both reduced by the same fraction, it is clear that the difference between these components, $\bar{m}$, will also be reduced by this fraction. It has been observed that ouabain decreases sodium efflux by about 25% in the absence of external potassium ions (Sjodin, 1971). In the present work, assuming that ouabain reduces the flux component $\bar{m}$ to 75% of its normal value, an estimated value of 1.5 μmol/g·h results when the external sodium ion concentration is 120 mM. On the basis that only $\bar{m}$ is affected by ouabain, one thus computes an expected value of net sodium influx in ouabain of (4.2 − 1.5) = 2.7 μmol/g·h. As the value actually measured in ouabain was 3.05 μmol/g·h, it appears that ouabain may also increase the sodium ion permeability. To fit the data exactly, an increase in P_{Na} of 8% due to ouabain would be required. To obtain an exact fit with no action of ouabain on P_{Na} would require an estimated sodium leakage flux, ϕ, some 25% greater than the estimate presently used. The best conclusion seems to be that most of the effect of ouabain on the measured net sodium influx can be attributed to a reduction in the active flux components.

It is of interest to examine the behavior of the sodium flux components that have been postulated as the external sodium ion concentration is varied. Values of fluxes calculated from measured values of sodium net influx, J, and measured values of unidirectional sodium efflux, m_o, are presented in Table

VI. The column giving computed values of the flux component m_i shows the dependence of m_i on $[Na]_o$. It is evident that the sodium flux component, m_i, must be a linear function of $[Na]_i$ in accordance with Eq. (5) and must be a

TABLE VI

AN ANALYSIS OF Na FLUX COMPONENTS DEDUCED FROM MEASUREMENTS OF NET INFLUX AND UNIDIRECTIONAL FLUXES

$[Na]_o$	J	ϕ	$\bar{m}$	m_o	m_i
mM			*μmol/g·h*		
0	−0.60	0	0.6	0.6	0
20	−0.38	0.7	1.1	2.0	0.9
40	0	1.4	1.4	3.1	1.7
60	0.44	2.1	1.7	3.3	1.6
80	0.97	2.8	1.8	3.6	1.8
120	2.24	4.2	2.0	3.9	1.9

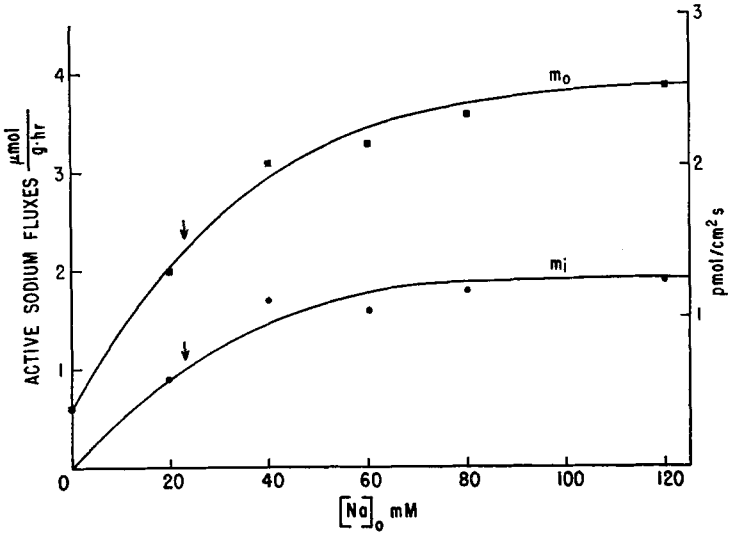

FIGURE 6. Active components of Na ion flux in frog muscle are plotted against the external Na ion concentration. The component m_o (squares) refers to efflux calculated from data of Sjodin (1971). The component m_i (dots) refers to influx and is calculated as described in the text. The arrows denote half-maximal fluxes. The units for flux on the left hand axis are micromoles per gram wet weight of muscle per hour.

Michaelis-type saturating function of $[Na]_o$. Both active components, m_o and m_i, are plotted against $[Na]_o$ in Fig. 6. Half-maximal values of flux are denoted by the arrows. The Michaelis constant is the same for both components of flux and has a value of approximately 23 mM.

DISCUSSION

The net influx of sodium ions into muscle cells was found to be independent of the intracellular sodium ion concentration in the absence of external potassium ions. This independency was observed from the normal concentration up to about half replacement of the intracellular potassium with sodium. A possible interpretation of this result is that, in the absence of external potassium ions, the sodium pump produces no net transport of sodium ions and little or none of the sodium influx is balanced by the pump. In this case, all of the measured net influx of sodium ions would be a passive leakage flux which should obey Eq. (1). A difficulty with this interpretation is that measurements of sodium net influx as a function of the external sodium ion concentration did not result in the linear dependence on $[Na]_o$ predicted by Eq. (1). The deviation cannot be explained as a variation in P_{Na} with $[Na]_o$ as Fig. 3 indicates that the net flux of sodium changes sign at $[Na]_o = 40$ mM. This concentration is higher than $[Na]_i$ and the cell interiors are negative in electrical potential. Active transport must be performed, therefore, in order to maintain a flux balance at $[Na]_o = 40$ mM. This active sodium extrusion occurs in a solution nominally free of potassium ions. Previous work has studied the potassium requirement for sodium extrusion at different external sodium ion concentrations (Sjodin, 1971). At $[Na]_o = 40$ mM, it can be inferred that an external potassium concentration of around 0.5 mM would be required to activate the sodium pump sufficiently to achieve a flux balance for sodium ions. It is quite likely that potassium ions leaking out of the cells can produce local concentrations of this order at the external activation sites.

The action of ouabain on sodium net influx would also be understandable on the same basis. Ouabain produces a partial inhibition of the pump which thus elevates the measured net influx of sodium to values closer to the true passive inward leakage rate. Though the sodium net flux measured in ouabain appears to vary linearly with $[Na]_o$ (Fig. 3), there are two reasons for believing that a pumped component of sodium flux is still involved: (*a*) the values of net influx in ouabain are significantly lower than the most reasonable estimate of the passive inward leakage flux, and (*b*) the magnitude of net influx in ouabain plotted against $[Na]_o$ yields a line which does not extrapolate to the origin. The line crosses the abscissa at $[Na]_o = 13$ mM, which is greater than the magnitude of the intracellular sodium ion concentration. In view of the large negative electrical potential within the cells, active transport must be performed to achieve the flux balance observed at $[Na]_o = 13$ mM in the presence of 10^{-4} M ouabain. It is clear that the technique of defining passive fluxes as those that remain in the presence of ouabain cannot be applied to sodium ion movements in frog striated muscle fibers. Ouabain apparently decreases the efficiency of the sodium pump in muscle cells so that active trans-

port can only be performed against a very much reduced electrochemical gradient. In the present experiments, an approximately 10-fold reduction in the sodium ion gradient was required.

The mechanism for active transport under these conditions is not clear. Ouabain interferes with dephosphorylation of membrane-derived ATPases (Sen, Tobin, and Post, 1969). This action would be expected to seriously affect the utilization of ATP in energy transfer for active transport. Brinley and Mullins (1968), however, observed that application of the cardioactive steroid strophanthidin did not reduce sodium efflux in squid giant axons to the levels obtained in the absence of internal ATP. Until the exact mode of action of cardioactive steroids on ion transport is known, it is difficult to interpret the effects of ouabain and strophanthidin on sodium fluxes in muscle and in giant axons.

In contrast to the results obtained on net sodium influx, unidirectional sodium influx as measured with tracer ^{22}Na ions showed a strong linear dependence on the intracellular sodium ion concentration. At the normal sodium ion concentration within muscle fibers and for a considerable concentration interval above the normal concentration, sodium efflux also shows a linear dependence on $[Na]_i$ in the presence or absence of external potassium ions (Sjodin, 1971). The results are, therefore, suggestive of a sodium exchange process operating across the muscle cell membrane. Evidence for such an exchange has previously been presented on the basis of observed sodium-free effects (Keynes and Swan, 1959; Horowicz, 1965; Keynes, 1966; Sjodin and Beaugé, 1968; Beaugé and Sjodin, 1968; Sjodin, 1971). Though such an "exchange diffusion" of sodium ions is suggested by these observations, the mechanism and stoichiometry of the exchange are subject to various interpretations.

The interpretation that seems most satisfactory on the basis of the present results is to regard a portion of sodium influx as having some properties of an active transport as in squid giant axons (Brinley and Mullins, 1968). This component of sodium influx is denoted m_i in this work. The analysis applied indicates that this flux component requires a minimal intracellular sodium ion concentration for activation in much the same manner as for activation of outwardly pumped sodium by internal sodium ions. In addition, Keynes and Steinhardt (1968) have observed that ouabain inhibits this component of sodium influx. The inhibition of both sodium influx and efflux that occurs is consistent with the effect of ouabain on net sodium influx observed presently. Ouabain apparently inhibits both the flux components m_o and m_i to the same degree in the absence of external potassium ions. The resulting inhibition of the net outwardly pumped sodium flux, $\bar{m}$, causes the measured net influx to rise as observed. It should be emphasized that these effects apply only to K-free solutions.

A reduction in sodium efflux upon removal of external sodium ions occurs in the presence of strophanthidin (Sjodin, 1971). It is possible that this "glycoside insensitive" exchange occurs by a different mechanism. The evidence favoring this view is weak, however, as ouabain and strophanthidin have incomplete inhibitory effects on the sodium pump. An incomplete inhibitory effect of cardioactive steroids on a sodium-for-sodium exchange process is easy to visualize.

The sodium flux components m_o and m_i approach one another in magnitude only at elevated intracellular sodium ion concentrations. At the normal muscle sodium concentration, there is some evidence for a one-for-one sodium ion exchange. For example, from Table VI, the normal value of m_o is 3.9 μmol/g·h and that for m_i is 1.9 μmol/g·h. Removal of external sodium ions reduces sodium efflux by one-half so that m_o is reduced to1.95 μmol/g·h. It is apparent that, under normal conditions, Na-free conditions reduce sodium efflux by an amount equivalent to the influx contribution due to the component m_i occurring in the presence of external sodium ions. It is possible that this is a consequence of a one-for-one sodium ion exchange.

It is interesting to consider the values of these components as $[Na]_i$ rises: m_o and m_i approach the same value, but the Na-free effect fails to indicate a one-for-one stoichiometry. When $[Na]_i$ is increased fivefold, for example, m_o rises to 20 and m_i rises to 18 in the same flux units. As removal of external sodium ions reduces m_o by about 50%, the reduction in m_o due to sodium-free conditions is now only about one-half of the magnitude of the influx contributed by m_i in the presence of sodium ions. These considerations indicate the possibility of a change in the Na:Na exchange stoichiometry or a flexibility in the coupling as $[Na]_i$ rises.

Whatever the membrane mechanisms are which underlie these experimental observations, it is apparent that there is difficulty in determining the permeability of the muscle cell membrane to sodium ions by techniques involving the measurement of sodium ion fluxes. No directly measurable component of sodium ion flux appears to give an unambiguous determination of P_{Na}. The measurement of net sodium influx in the presence of ouabain and in the absence of external potassium ions gives the best estimate of P_{Na}. It seems impossible, under any conditions, to measure P_{Na} by means of a radioactive tracer for determining sodium ion flux, unless results are extrapolated to $[Na]_i = 0$.

The potassium efflux that occurs in K-free Ringer solution has some characteristics which suggest that it is a passive leakage of potassium ions. Potassium efflux under these conditions is insensitive to ouabain. Also, it is similar in magnitude to the estimate of the passive component of sodium influx obtained by extrapolating measurements of unidirectional sodium influx versus

$[Na]_i$ to the intercept at $[Na]_i = 0$. When $[Na]_i = 0$, it is assumed that no active components of sodium ion flux are present.

Some other properties of potassium efflux as observed in this work deserve mention. Significant changes in potassium efflux occurred in cases in which only minor changes in the resting membrane potential occurred (Table V). Also, replacement of external sodium with magnesium resulted in a decline in potassium efflux. Potassium efflux was approximately halved in Na-free, $MgCl_2$ media. It is not possible to state whether this effect is due to the absence of sodium, to the presence of magnesium, or to both. Further investigation of this problem is in progress.

Ouabain increased potassium efflux in K-free, $MgCl_2$ media. An effect of ouabain on potassium ion permeability does not seem likely as ouabain was without significant effect on potassium net loss in the presence of normal external sodium ion concentrations. It is possible that some potassium ions lost from fibers are pumped back into the cells at low external sodium ion concentrations. Support for this hypothesis is obtained from results observed when muscles are first enriched with sodium ions. Potassium efflux from such muscles is reduced to very low values in an Na-free medium (Table V). This would be expected if increased "back-pumping" of potassium ions occurred as a consequence of pump activation by the increased internal sodium ion concentration. Consistent with this interpretation is the observation that application of ouabain now causes a 14-fold increase in potassium efflux which serves to raise its magnitude to near the normal value. It thus seems that the ouabain effects in these cases are more likely to involve active fluxes than potassium permeability.

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