The Concentration Dependence of Sodium Efflux from Muscle

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ABSTRACT Frog sartorius muscles subjected to overnight loading with Na^+ in K-free Ringer in the cold were subsequently labeled with Na^{24} and then immersed in choline Ringer and the efflux of Na^{24} followed for 4 hours. The initial efflux of Na^+ appeared to be 17 pmole/cm^2 sec.; this value was maintained for 20 minutes and was followed by an abrupt decline to about 9 pmole/cm^2 sec. This latter rate was maintained for the next 20 minutes of efflux. The efflux then declined gradually with time and reached values of the order of 0.1 pmole/cm^2 sec. The back addition of counts lost from muscles enabled one to calculate the relationship between efflux and $[Na]_i$ for muscle. This roughly approximates an S-shaped curve with a value at half-saturation of about 17 mmole Na per liter of fiber water. The efflux-concentration curve is closely described by assuming that 3 Na^+ are transported per carrier cycle.

INTRODUCTION

In normal frog sartorius muscle the ion fluxes across the fiber membrane are approximately in balance and the flux ratio for potassium and chloride ions corresponds rather closely with that predicted for a membrane potential of −91 mv (Hodgkin and Horowicz, 1959 *a*, *b*). The Na^+ fluxes, although approximately in balance, do not correspond to the values demanded by the flux ratio relationship and it was proposed (Dean, 1941) that Na efflux is brought about largely by an energy-consuming mechanism or Na pump. A demonstration of such a pump was provided by Steinbach (1940), who subjected muscles to an experimental treatment that increased $[Na]_i$; he then showed that the muscles could extrude this Na against an electrochemical gradient.

A second mode of efflux for Na^+ was proposed by Ussing (1947) with the term "exchange diffusion" or an enforced exchange of Na for Na across the membrane. This sort of an effect has been recently demonstrated by Keynes and Swan (1959), who also found that exchange diffusion disappeared appar-

ently with an increase in $[Na]_i$. The experimental work to be described is an extension of Na efflux measurements to a wider range of $[Na]_i$.

Finally, a third mode of Na^+ efflux is the purely passive leak of Na^+ from the fibers. This efflux is very difficult to measure experimentally and the most reasonable way to have an estimate of its magnitude is to measure Na influx and apply the flux ratio relationship. As influx must be expected to include exchange diffusion, conclusions about efflux will be too high. A calculation shows, however, that Na leakage efflux is considerably less than 1 per cent of the total Na efflux for fresh muscle with a normal membrane potential, hence passive efflux can be ignored.

To determine the efflux of Na^+ when the fiber concentration is high requires that measurements be made very soon after the transfer of muscles from a loading solution to a Na-free medium and this, in turn, leads to difficulties in separating the fiber efflux from that of the extracellular space of muscle. Our experimental conditions have been designed to make the best possible separation of these two fluxes. Exchange diffusion has been eliminated as a factor in the measurements to be described because these necessarily require that efflux be into a Na-free medium. The leakage efflux of Na is difficult to estimate when $[Na]_i$ is high and membrane potential is depressed. It appears likely, however, that it is not more than a few per cent of the pump efflux.

METHODS

Frog sartorius muscles from *R. pipiens* were used exclusively in this work. In a typical experiment, three pairs of muscles were dissected and threads were tied to the tendons at each end of the muscle. The threads served to tie the muscles to small Teflon frames (shown in Fig. 1) with enough tension to maintain the muscle at body length. The subsequent transfer of muscles through the various experimental solutions could then be effected by moving the muscle and frame as a unit. Subsequent to their dissection muscles tied to frames were placed in 10 ml of K-free Ringer and held at 4°C for from 12 to 16 hours. At the end of this loading period, three muscles (designated *A* muscles) were placed in special Teflon cups made so that the entire muscle could be covered when 1.5 ml of Ringer solution was present in the cup. The solution in these cups was K-free Ringer containing Na^{24} and the muscles were loaded 2 to 3 hours at 4°C in this radioactive solution. Fifteen min. before the end of the exposure to Na^{24}, the muscles were transferred to a new solution at 4°C containing K-free Ringer, Na^{24}, and sucrose-C^{14}. The counts due to sucrose were adjusted to be roughly 1 per cent of the counts for Na^{24}. The pairs to the *A* muscles were not loaded with Na^{24} but were loaded with sucrose as outlined above. These muscles (designated *B* muscles) were washed 15 to 20 minutes at 4°C in two changes of tris or choline Ringer and were then analyzed for Na, K, C^{14}, and water. The *A* muscles were removed from the Na^{24} and sucrose C^{14} solution, clamped in the efflux apparatus in which all solutions were at 20°C, given a 10 sec. wash in either tris or choline Ringer, and the efflux apparatus was then started. The muscles were passed through tubes

of tris or choline Ringer. The usual program for time changes was ten 2 min. effluxes, followed by five efflux periods of 5 min. and fifteen periods of 10 minutes, and ending with three or four 20 min. efflux periods. In the early part of the efflux the muscles were sometimes changed every minute for 10 min. followed by ten 2 min. efflux periods. Efflux time programs were varied to avoid having critical parts of the efflux curve always fall after the same number of solution changes.

Preliminary experiments in which muscles were moved by hand from tube to tube during efflux indicated that some mechanical arrangement would have to be developed if relatively short time effluxes were to be measured with any degree of ac-

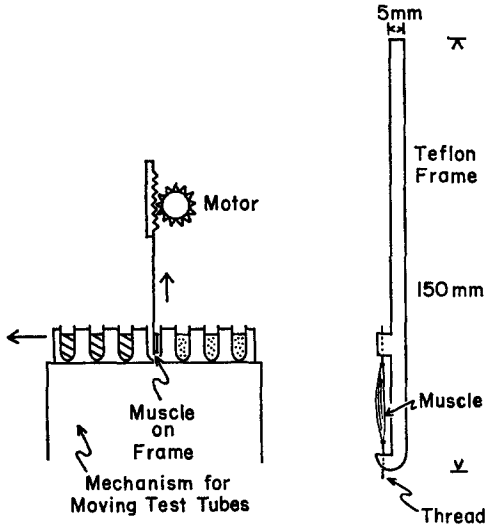

FIGURE 1. On the left the apparatus for moving muscles through efflux solutions is shown. The elevator motor is at the top with a muscle frame clamped to it. The hatched test tubes have already collected an Na^{24} efflux, the dotted test tubes are to receive efflux. On the right is a drawing of the Teflon mounting frame used to hold muscles during the experimental manipulations.

curacy. If only a single muscle were to be measured at a time, a continuous flow system with a motor-driven syringe appeared to be a feasible arrangement. For the simultaneous measurement of a number of muscles, however, such a system appeared to be more inconvenient than one in which the muscles were moved mechanically from tube to tube. The arrangement we used is shown in Fig. 1 and consists of two parts, a base carrying 225 test tubes and containing a mechanism such that the base moved in a boustrophedon pattern (*i.e.* activation of the base movement mechanism caused the test tubes to advance in unit steps along the x direction until the last test tube was reached; the next advance was one step in the y direction and subsequent advances were in a $-x$ direction). The second part of the apparatus was an elevator bar that carried the muscle frames. In operation, a timing signal from a clock activated the elevator motor and all the muscles were raised from their efflux tubes, the base mechanism then advanced, and the elevator motor reversed and lowered the

muscles into the next tube. The transit time between tubes was 2.6 sec. Our original designs included a channel in the Teflon frame to carry a tube for the aeration of the efflux solutions. While this stream of gas bubbles did not come in contact with the muscles on the frame, the stirring action of the stream caused the muscles to undergo torsional movements during efflux. A measurement of efflux from muscles under these conditions showed that while the usual pattern of curve shape was observed, occasionally, during a particular 5 min. efflux period the efflux might rise to ten times the value in the previous sample and then promptly return to a value consistent with previous measurements. The most likely interpretation of this effect is that the membrane of a single muscle fiber collapsed during the efflux period so that the entire radioactivity of the fiber was released into the efflux solution. Such effects disappeared when bubbling in the efflux solutions was discontinued; our opinion is that muscles are very sensitive to mechanical deformations when they have been isolated for the periods of time (up to 20 hours) necessary for carrying out the experiments described in this paper. Accordingly, great care was used throughout to prevent any deformation of the muscles.

The efflux tubes from an experimental run (usually thirty to thirty-five from each of three muscles or a total of about one hundred in all) were sampled and aliquots of the solution were evaporated in planchets. These were subsequently counted in an automatic sample changer with an anticoincidence guard. Background was 2 CPM, and at least 1000 counts were measured on a given sample. After counting, the planchets were stored for 3 weeks and recounted to measure the C^{14} from sucrose used as a marker for extracellular space. At the conclusion of the experiment all muscles were weighed, dried at 105°C to determine water content, and then ashed in platinum. The ash was dissolved in water containing HCl and estimations of final K^+ and Na^+ were made by flame photometry. Another sample of the ash solution was counted to have a value for final radioactivity.

The easiest way to present the efflux data is to plot efflux in CPM/min. against total radioactivity in the fibers in CPM. As the muscles are in Na-free solution, the specific activity does not change with time and one measures therefore Na efflux as a function of fiber Na concentration. We have chosen to measure efflux and fiber Na concentration in absolute units and the following protocol was used to transform efflux in CPM/min. measured as a function of time into efflux in pmoles/cm² sec. as a function of $[Na]_i$. This transformation in no way affects the shape of the efflux-concentration curve.

(*a*) Obtain final radioactivity (decay and background corrected) of an *A* muscle ash (final CPM).

(*b*) Add to (*a*) the CPM of the last efflux sample, keep a running total, add the next to last efflux CPM, and continue to add the counts lost in backward order with respect to time until the first efflux sample is reached (initial CPM).

(*c*) Subtract from (*b*) the total sucrose C^{14} CPM independently measured (initial Na^{24} CPM).

(*d*) From the measured extracellular space and initial radioactivity of soak solution obtain CPM in extracellular space. Substract this value from (*c*) (initial fiber Na^{24} CPM).

(*e*) From the analytical Na content of the paired *B* muscle and (*d*) compute muscle specific activity (CPM/mol Na).

(*f*) Use the value obtained in (*e*) as a divisor for each value in the running total of counts in muscle from the end up to $t = 20$ min. (in *b*) and obtain moles Na in muscle as a function of time. From the measured water content of the muscle, concentration can be obtained as mmole/liter fiber water (mmole/l.f.w.).

(*g*) By dividing each efflux value in (*b*) by (*e*) efflux in moles Na/efflux period muscle is obtained as a function of time. Efflux as a function of concentration can be obtained from concentrations (*f*) corresponding in time to a particular efflux. Using 405 cm^2/gm muscle as a conversion factor, efflux in pmoles/cm^2 sec. can be obtained.

(*h*) For times earlier than 20 min. the counts in the extracellular space must be considered—they do not emerge instantaneously but contribute considerably to the efflux at 15 min. For a complete curve the extracellular counts must be added back to the total fiber counts and Equation 1 used to calculate fiber efflux from total efflux and space efflux. Such a procedure is unlikely to give useful results at times earlier than 10 min.

The Ringer solution used contained NaCl 110 mM, KCl 2.5 mM, and $CaCl_2$ 1.8 mM. Quantities of tris or choline chloride to give a concentration of 110 mM were used to prepare Na-free solutions, and the first seven efflux tubes of choline Ringer contained 50 μM *d*-tubocurarine to prevent activation of fiber junctions by choline. This reagent was omitted in a few experiments without any detectable change in the efflux curve.

RESULTS

Before it is possible to analyze the efflux from muscle at short times after transfer from radioactive solutions, it is necessary to consider the Na^{24} in the extracellular space. Our experimental arrangement actually contributed two spaces to the measurements made, one from the muscle mounting frame and the thread used to tie the muscle and a second from the muscle itself. Measurements were first made by mounting on the muscle frame the same quantity of thread used to tie the muscle and equilibrating the frame in Na^{24} for 2.5 hours. The frame was then mounted in the efflux apparatus, given a 10 sec. wash, and changed from tube to tube once each minute. Two such experiments are shown in Fig. 2A. The time constant for such an efflux is 0.6 ± 0.1 min. and the absolute value of the space averaged 10 μl. For a measurement of muscle extracellular space a muscle mounted on a frame was equilibrated with Na^{24} for 15 min. and the washout was followed by changing the muscle from tube to tube each minute. The results of this experiment are shown in Fig. 2B (upper curve) where the time constant for the last few points on the efflux curve is 2.4 min. (range 2.0 to 3.1 min., mean 2.5 ± 0.4). In the lower part of Fig. 2B is shown a washout of sucrose-C^{14} from a muscle. The sucrose washout data, obtained on most muscles used, suggest that about 5 per cent

of the sucrose counts emerge with a time constant that is very long compared with the extracellular space time constants. A similar finding that 3 per cent of the SO_4 and sucrose was lost slowly was made by Johnson (1955). The sucrose and Na^{24} space measurements agree in indicating a space for muscle of

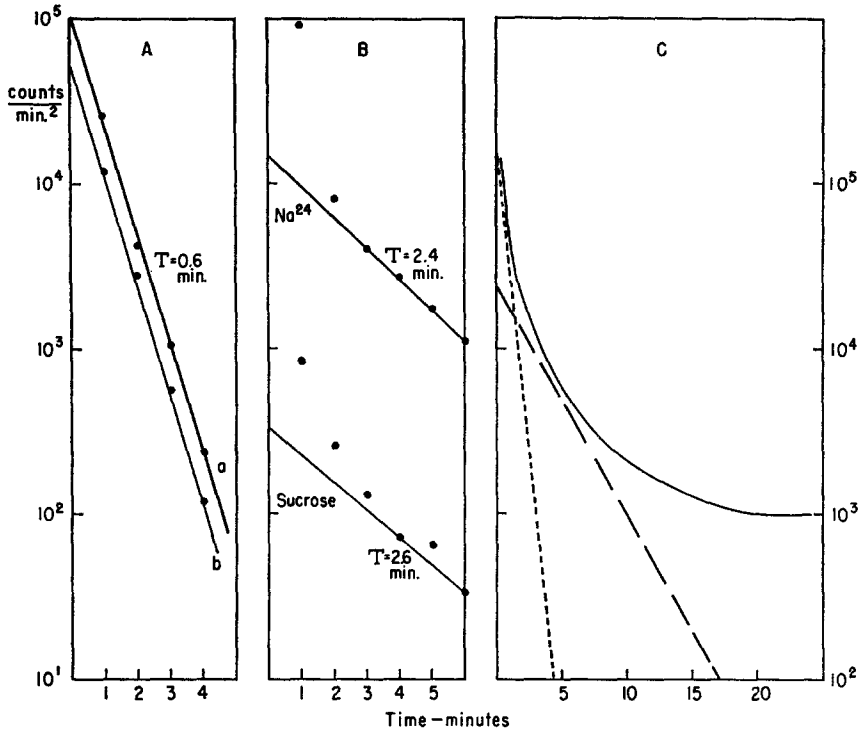

FIGURE 2. (A) The efflux of Na^{24} from two threads (*a* and *b*) of a length equivalent to that used to tie and mount muscles is plotted against time in minutes. Both threads showed a time constant for Na^{24} loss of 0.6 min. (B) The top curve is a plot of the loss of Na^{24} from a muscle loaded with Na^{24} for 15 min. prior to the efflux. The lower curve shows the efflux of sucrose-C^{14} from an *A* muscle loaded with both Na^+ and Na^{24}. The time constants for loss are shown on the curves. (C) The dotted line is a solution for $m_o = A \exp\text{-}t/\tau_1$ with $\tau_1 = 0.6$ min. and *A*, the counts contained in 10 μl of soak solution; the dashed line is a solution to $m_o = B \exp\text{-}t/\tau_2$ with $\tau_2 = 2.6$ min. and *B* equal to 20 per cent of 20 mg or 4 μl of soak solution. The solid line is a solution for equation (1) in the text.

22 ± 2 per cent (mean and SD). This is rather different from the value given by Desmedt (1953), who worked with European frogs but is in agreement with recent values by Steinbach (1961) working with *R. pipiens*.

As the efflux of Na^+ from muscle fibers initially appears to be constant with time (see discussion below) the efflux of Na from muscles can be represented as in equation (1)

$$m_o = A \exp\text{-}t/\tau_1 + B \exp\text{-}t/\tau_2 + C \quad (1)$$

where m_o is Na efflux, A is a constant, t is time, τ_1 is the time constant for efflux from the frame, B is a constant, τ_2 is the time constant for efflux from the muscle extracellular space, and C is a constant representing efflux from muscle fibers. Using the values for time constant given previously for frame and extracellular spaces and considering a 20 mg muscle with a 20 per cent

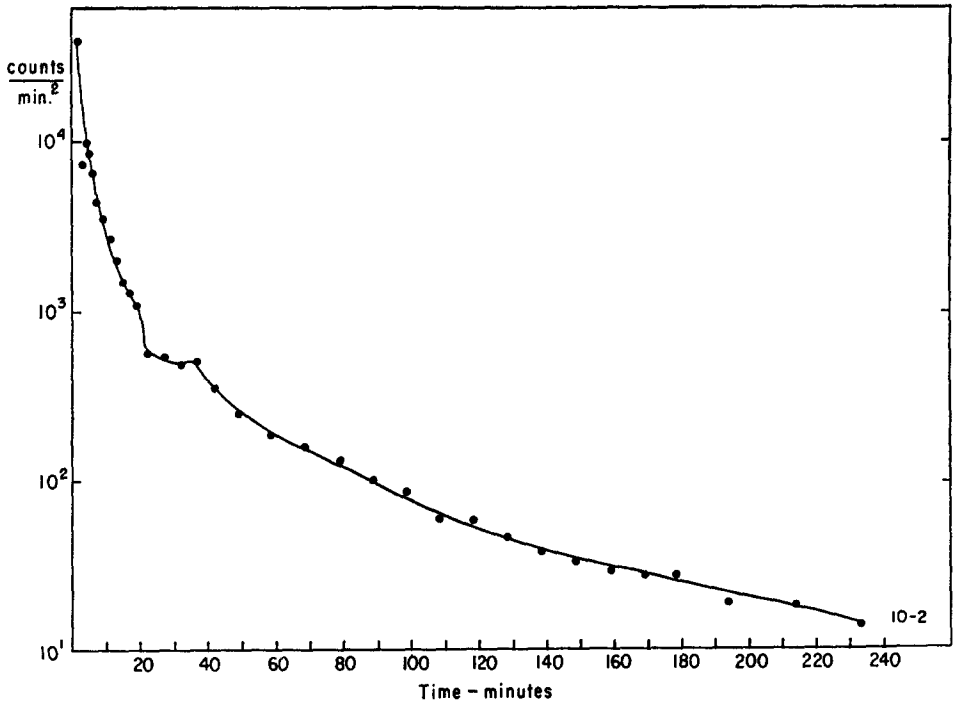

FIGURE 3. Efflux in cpm² (on a semilog scale) is plotted against time for muscle 10-2, in choline Ringer solution.

space, and a fiber efflux of 1000 CPM², Equation 1 yields the solid line shown in Fig. 2C. The dotted line is the washout from the frame and the dashed line is space washout. It is to be noted that at $t = 10$ min. the total efflux is only about twice the fiber efflux so that analysis of fiber efflux is still possible. At $t = 5$ min., the total efflux is so many times larger than fiber efflux that analysis is impossible.

A muscle loaded with sodium by an overnight soak in K-free Ringer at 4°C followed by a 2.5 hour equilibration with Na^{24} at the same temperature shows, upon its subsequent transfer to a choline Ringer at 20°C an efflux of Na^{24} such as that shown in Fig. 3. The features of special interest in this curve are: (*a*) a plateau where efflux is relatively constant between the 20th and 40th min.,

(*b*) a curved efflux-time relationship (on a semilog plot) extending from 40 min. to the end of the experimental period, and (*c*) in most muscles examined a plateau (the early plateau) between the 10th and 20th min. of efflux with a value in CPM[2] about twice that of the plateau (*a*). This early plateau is partly obscured by the washout of the extracellular space but is clearly present in three fourths of the muscles examined. The term early plateau is used to denote efflux

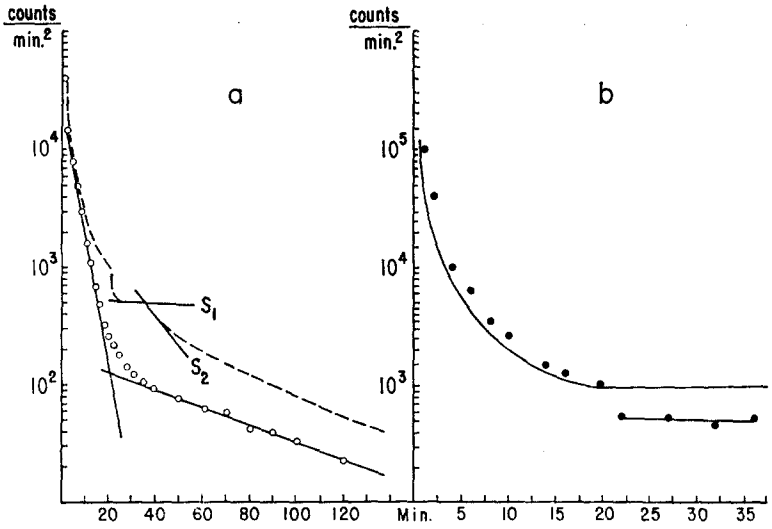

FIGURE 4. In (a) the upper dashed line is redrawn from Fig. 3 and shows the efflux of Na^{24} from muscle as a function of time. At t = 30 min. a tangent to this curve has been drawn to include the best fit by eye to two points on either side of this reference time point, this is S_1. At t = 40 min. another tangent to the curve has been drawn, and this is S_2. The solid line and experimental points represent the efflux from a muscle not loaded with Na and where $[Na]_i$ is constant during the experiment because efflux is into Na Ringer solution. Under these conditions, a simple exponential relationship between efflux and time is obtained. In (b) the solid line of Fig. 2C is redrawn; this shows the expected efflux resulting from the summation of two space effluxes and one intracellular compartment with a constant efflux. The experimental points from Fig. 3 are shown on this plot; they fit the calculated curve well until t = 20 min. but then decline to a value one-half that of the assumed constant efflux.

at 10 to 20 min. without any implication that efflux is constant during this time. The data are consistent with a constant efflux but the experimental error is too great. At times earlier than 10 min. after transfer of the muscles to choline Ringer, the analysis of the efflux curve becomes impossible because of the very small contribution of fiber efflux to the total.

In order to have some quantitative measure of the plateau between the 20th and the 40th min. of the efflux curve shown in Fig. 3, the slope of such a curve was taken at 30 min. and at 40 min. of efflux as shown in Fig. 4a. Such slopes,

on a semilog plot, were drawn to give the best fit to a straight line for two experimental points on either side of the 30 min. reference time and for two experimental points after 40 min. While the complexity of the efflux curve prevents one employing the conventional time constant, such slopes can be characterized as the time required for efflux to fall to $1/e$ of its initial value. The slopes S_1 and S_2 have been measured for all muscles studied with the result that the mean values are $S_1 = 110$ min., $S_2 = 32$ min., $S_1/S_2 = 3.4$. The range in values for the ratio S_1/S_2 was from 2 to 5 with a SD $= 1.2$. It is clear therefore that a highly significant change in slope occurs in all muscles studied. Fig. 4a (lower curve) also shows an experimental efflux curve obtained from a muscle not loaded with Na^+, but only with Na^{24}. The efflux was into normal Ringer and under this condition the $[Na]_i$ is quite constant over the efflux period, with a measured value of 7.5 mmole/l.f.w. at the end of the experiment. Fig. 4a shows, therefore, that muscles with an initial $[Na]_i$ of *ca.* 35 mM show an initial plateau of efflux *vs.* time followed by a decline in efflux with time. A muscle with a low $[Na]_i$ and in a steady-state with respect to $[Na]_i$ shows only a simple exponential loss of Na^{24} with a time constant of about 60 min.

In Fig. 4b the solid line of Fig. 2C has been drawn as well as the experimental points for the first part of the efflux curve shown in Fig. 3. The solid line represents the computed efflux from a three compartment system (two spaces plus a constant fiber efflux) and it is clear that the line follows the experimental points closely except that the final value of constant efflux is 500 CPM instead of 1000 CPM. This suggests that while the choice of 1000 CPM may have been correct for an initial value (early plateau), the efflux changed during the washout to the plateau value of 500 CPM.

We cannot attach any accuracy to measurements of this sort nor can we claim that this efflux is even constant between $t = 10$ to 20 min. In all the muscles in which the effect was present, however, an analysis such as that shown in Fig. 4b gives an efflux between two and three times that of the 20 to 40 min. plateau.

From a final analysis for $[Na^+]_i$ and Na^{24} of the muscle whose efflux-time curve is shown in Fig. 3 and the back addition of counts collected during efflux periods in choline Ringer, it is possible to reconstruct a curve for muscle fiber Na^+ concentration vs. time. If the counts known to be extracellular (from a measurement of space) are subtracted and the back addition from $t = 19$ min. to $t = 0$ is made on the basis that the efflux is constant during this period and equal to 1000 CPM[2] then the computed value for the initial $[Na]_i = 63$ mmole/l.f.w. This value is to be compared with a paired muscle that was washed for 15 min. in choline Ringer and then analyzed for $[Na]_i$ as 48 mM. The difference between these figures can be almost accounted for by the loss of Na from the paired muscle to be expected on the basis of a 15 min. wash in choline Ringer. A plot of fiber concentration *vs.* time is shown in Fig. 5. While the initial part

of the curve is not an exponential because it involves a decline in concentration that is linear with time, the time required for $[Na]_i$ to reach $1/e$ of its initial value is 35 min.; this is about half the value for muscles with a normal $[Na]_i$ where the time constant is of the order of 60 min. If time is eliminated from the data, a plot of efflux *vs.* $[Na]_i$ is obtained as shown in the insert of

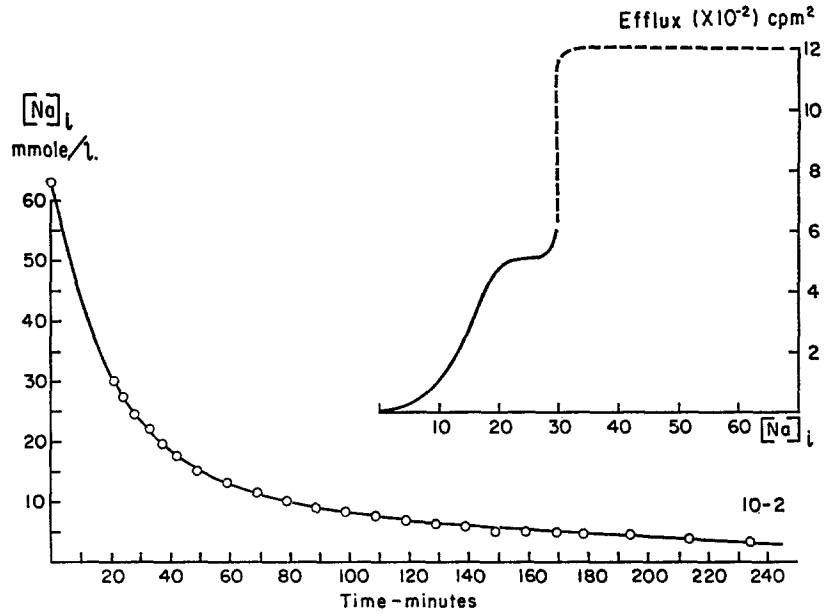

FIGURE 5. The summation of counts lost as a function of time (shown in Fig. 3) results in values for $[Na]_i$ as a function of time. The values of $[Na]_i$ shown between $t = 20$ and $t = 240$ min. have not been corrected for extracellular counts as these are considered negligible. The $[Na]_i$ for $t = 0$ has been corrected for extracellular counts. In the insert, efflux in cpm^2 is plotted against $[Na]_i$. The solid line is for counts not corrected for extracellular space, the dashed line is obtained when extracellular counts are subtracted from efflux measurements.

Fig. 5. This is an S-shaped curve with a half-saturation at about 17 mM $[Na]_i$ and has the further feature of an abrupt transition to a much higher efflux as shown by the dashed line. A reason for this behavior will be considered later.

The efflux-time relationships for some eighteen muscles are shown in Fig. 6. Values of efflux in cpm^2 have been divided by arbitrary factors in order to separate the curves along the ordinate; thus efflux cpm^2 for the top curves have been divided by 3, for the middle curves by 10, and for the bottom curves by 30. Only the first 45 min. of efflux have been plotted in order to spread out the time scale, although the measurements are complete for all curves through 4 hours of efflux. The points of interest regarding these data are the following: all curves show a plateau in efflux from roughly $t = 20$ to

t = 40 min. All curves except those for muscles 7-2, 6-3, 6-2, and 5-1, 5-2, 5-3 show a clear indication of a plateau at a level of efflux higher than that beginning at t = 20 min. (early plateau). This early plateau could have been more clearly resolved if muscles had been held in a Na^{24}-free, K-free but Na-containing Ringer at 4°C after loading so that the extracellular space was free of radio-

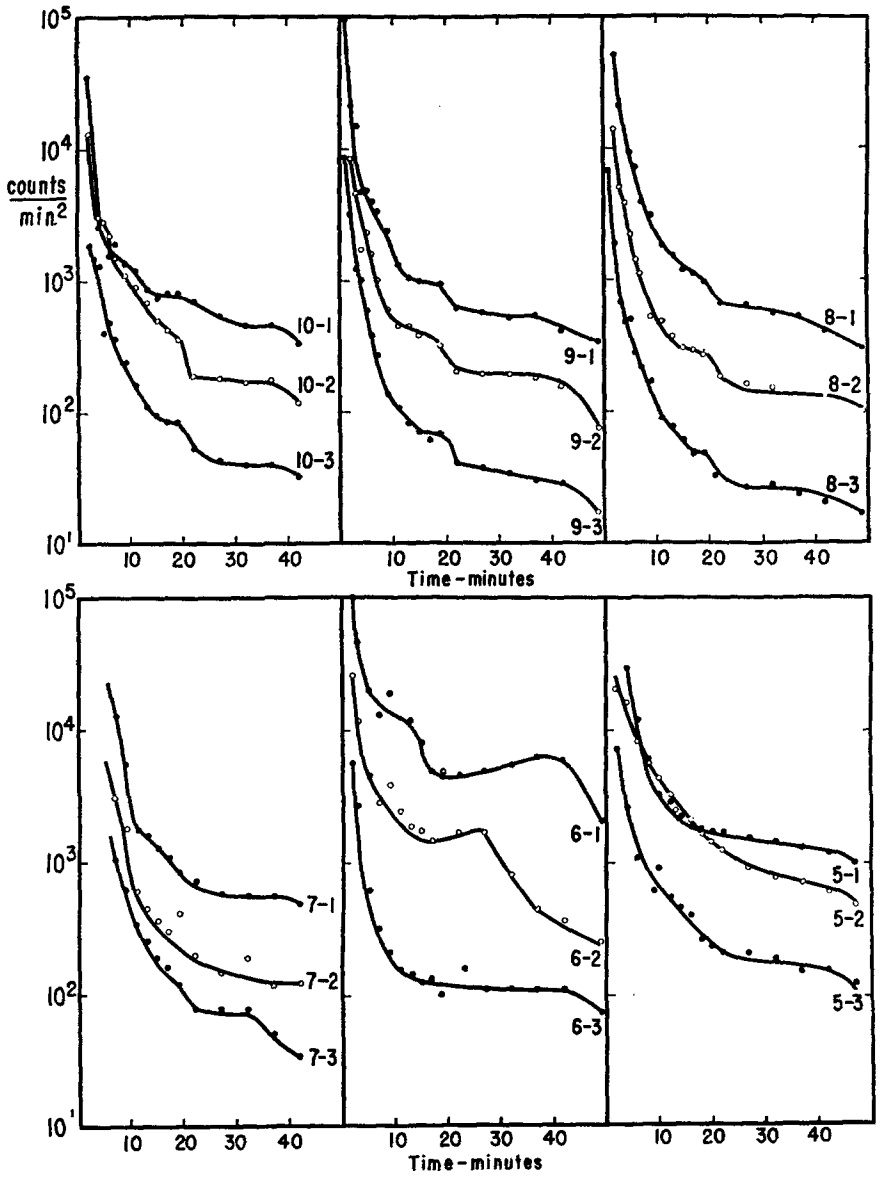

FIGURE 6. The efflux-time relationships for eighteen muscles are shown for the first 40 min. of efflux. The curves have been displaced along the ordinate as described in the text.

activity but $[Na]_i$ was unchanged at the moment of transfer to choline Ringer. This technique was not followed except in a few instances because it decreased very greatly the internal radioactivity of the fibers and thus made it impossible to follow Na efflux over a wide range of $[Na]_i$.

The data in Fig. 6 make it clear that the interesting regions of the efflux-time curves for muscle lie within the first 40 min. of efflux. After this time, the efflux-time curve is not linear (on a semilog plot) but depends on concentration in a way that will be dealt with subsequently. At this point it seems useful to consider the analytical changes in $[Na]_i$ and $[K]_i$ that take place during long soaks in a Na-free Ringer.

TABLE I

CHANGES IN WATER, $[Na]_i$, AND $[K]_i$ FOR MUSCLES IN TRIS OR CHOLINE RINGER

	mmole/kg muscle				mmole/l.f.w.*		
	$[Na]_i$	$[K]_i$	$[Na + K]_i$	Water	$[Na]_i$	$[K]_i$	$[Na + K]_i$
				per cent			
Mean for *A* muscles 4 hrs. in tris or choline Ringer at 20°C	4.6±2‡	52±7	57±7	76	8.5	97	106
Mean for *B* muscles ¼ hr. in tris or choline Ringer at 20°C	31.4±4	47±7	78±6	80	54	81	135
Mean difference between *A* and *B* paired muscles	−26.6±3	+5±3	−21±6	−4	—	—	—

* Based on a measured mean space of 22 per cent and an assumption of no Na^+ in the space.
‡ ±1 SD.

When muscles are loaded overnight in cold K-free Ringer, treated with Na^{24} under the same conditions for 2.5 hours, and then washed 15 min. in choline Ringer and analyzed for $[Na]_i$ and Na^{24} they show a mean $[Na]_i$ of 54 mM and a specific activity of 0.85 that of the soak solution. If, on the other hand, the muscles are soaked 4 hours in choline Ringer and then analyzed for $[Na]_i$ and Na^{24} the mean values are $[Na]_i$ = 8.5 mM and specific activity 0.30 that of the soak solution. This apparent change of specific activity during a long soak in Na-free solution can be understood if it be supposed that there is some Na that does not exchange with Na^{24} under the conditions of equilibration used, as noted by Carey and Conway (1954). The figures cited above would suggest that this amounts to 5.7 mmole/l. so that the final exchangeable $[Na]_i$ in muscle after 4 hours in Na-free solution is (8.5 − 5.7) or 2.8 mM.[1] Our

[1] If SA_1 is initial specific activity and SA_2 is that after 4 hours in Na-free medium, and $[Na]_1$, $[Na]_2$, $[Na]_n$ are the initial, final, and non-exchangeable [Na], then $SA_1 = (CPM)_1/[Na]_1 - [Na]_n$ and $SA_2 = (CPM)_2/([Na]_2 - [Na]_n)$.

attention was drawn to this point because the back addition of counts from muscle to obtain an initial $[Na]_i$ yielded impossibly high concentrations when we took the apparent final specific activity as the basis for our calculation. When the higher specific activity is used, the results obtained are reasonable.

All our analytical data on eighteen muscle pairs are collected in Table I. From this it can be noted that between the 15th min. (when the *B* muscles were analyzed) and the 240th minute when the *A* muscles were analyzed, there was a loss of 26 mmole Na/kg muscle and a gain of 5 mmole/kg muscle for K. The difference between *A* and *B* muscles in the sum of $([Na]_i + [K]_i)$ presumably represents the gain of choline (or tris) by the fibers and this amounts to 21 mmole/kg. A point of some interest is that there is no apparent one to one coupling between the extrusion of Na and the uptake of K. This lack of apparent reciprocity between Na extrusion and K gain may be somewhat artificial as the following calculations indicate. Renkin (1961) has reported that choline penetrates the frog sartorius; his data suggest an influx of about 5 pmole/cm² sec. if the assumption is made that influx increases 20-fold in going from 4 mM $[choline^+]_o$ (where measurements were made) to 110 mM. This assumption is based on experience with Na influx—this increases less than proportionally with concentration (Keynes and Swan). This value for influx is about 7.5 mmole/kg hour so that in 4 hours our muscles should have gained 30 mmole/kg. The value given in Table I (21 mmole/kg) is less than this, but the membrane potential of Na-loaded muscles is considerably lower than for normal muscle (Stephenson, 1957) so that allowing for the diminished driving force acting on choline, the value found seems reasonable. Renkin also noted that there was a negligible efflux of choline from his muscle fibers, suggesting strongly that the pump mechanism does not accept choline. Passive efflux must be very small because even with 21 mmole/kg of choline the system is very far from electrochemical equilibrium.

At the start of an efflux in choline the muscles have a $[K]_i$ of about 80 mM and from this it can be calculated that E_K is about -80 mv. If allowance is made for the very similar permeabilities of $choline^+$ and Na^+, the membrane potential E_m should be -70 mv, $[Cl]_i$ in equilibrium with this potential should be about 7 mM, and the muscle should have increased its water content by about 5 per cent. Our figures for water content (Table I) show that indeed the *B* muscles have an 80 per cent water content and that this decreases after 4 hours in choline. The foregoing figures are necessarily rough because we have not made any potential measurements in Na-loaded muscles. Kernan (1962) has recently reported that during Na extrusion the membrane potential is elevated considerably over that calculated for E_K. In choline, with 10 mM $[K]_o$ he reports, for example, a 34 mv increase in E_m. It is difficult to extrapolate these results to our experimental conditions where $[K]_o = 2.5$ mM, but it is sufficient to note that if the ion conductances g_{Cl} and g_K have the

values, given by Hodgkin and Horowicz, of 200 and 100 μmho/cm² respectively, a very small increase in membrane potential suffices to balance the large Na efflux observed at short times after transfer to choline, thus a membrane potential of −84 mv suffices to balance the large Na extrusion as shown below. Because of the very small amount of Cl^- in the fibers, however, the

Na efflux	−17 pmole/cm² sec.
Choline influx	+5
Net flux	−12
For E_m 4 mv more negative than E_K	
K net flux (in)	+4 pmole/cm² sec.
Cl net flux (out)	+8
Total net flux	+12

continued efflux of Na^+ cannot be balanced for long; when $[Cl]_i$ comes to equilibrium with the potential imposed by the Na pump, either a higher potential must be generated or the efflux of Na must fall. From our data it would appear that Na efflux falls abruptly to about 9 pmole/cm² sec.; this flux can just be balanced by the inward net flux of K and the influx of choline. The initial efflux of Na of 17 pmole/cm² sec. corresponds to 25 μmole/gm hour and if we assume that this rate is maintained for 20 min. then 8.3 μmole Na/gm muscle have been lost. This would correspond to a loss of 5.5 μmole Cl and a gain of 2.8 μmole K. This Cl loss corresponds to a concentration change of 3.7 mM, and with the initial $[Cl]_i$ = 7 mM, the final $[Cl]_i$ would be 4.3. This corresponds with the value (3.8 mM) calculated for the equilibrium distribution of Cl^- at a potential of −84 mv.

If $[Na]_i$ calculated from back addition of efflux counts is plotted against efflux on a log-log scale, curves similar to those shown in Fig. 7 are obtained. The initial slope at low $[Na]_i$ is about 3.5 and is thus similar to the value of 3.0 found by Keynes and Swan. In our experience this slope on a log-log plot is quite variable and may range from 2 to 3.5. In any event, such a slope is not constant but declines with increasing $[Na]_i$ and eventually reaches a value of zero. Such an approach to zero may not be apparent from Fig. 7 unless allowance is made for the compression of the abscissa brought about by the log scale. On each of the three curves shown there are at least three points with a constant efflux value. The curves selected from our data for Fig. 7 show some of the variations in efflux pattern and can be quite complex in structure.

The efflux *vs.* $[Na]_i$ data for fourteen muscles have been plotted on linear coordinates and the curves averaged at the following reference points: efflux = 95, 75, 50, 25, 12.5, and 6.25 per cent of that during the 20 to 40 minute plateau. The standard deviation of the $[Na]_i$ value for each point was computed and is shown as a horizontal bar in Fig. 8. The features of interest in this plot are the following: The efflux from $[Na]_i$ = 3 to 8 mM is fit closely by:

Efflux = k' $([Na]_i)^3$ and is, therefore, the "cubic" region of the curve—it also corresponds to the physiological concentration range for muscle fibers. From $[Na]_i$ = 15 to 25 mM, efflux (m_o) is given approximately by $m_o = k''$ $[Na]_i$; this is the "linear" region of the curve. At about 25 mM the efflux begins to saturate, while above $[Na]_i$ = 35 mM there is the discontinuity in efflux that we have ascribed to $[Cl]_i$; this has been termed the early plateau and is

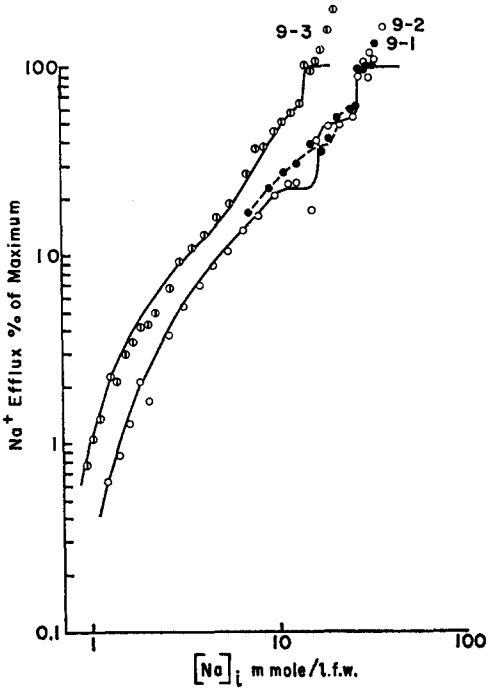

FIGURE 7. A log-log plot of the efflux of Na^{24} from three muscles *vs.* $[Na]_i$. Concentrations have been adjusted to correct for 5 mmole Na/kg muscle that does not exchange with Na^{24} and differences in muscle weight have been compensated for by calculating efflux per cm^2 fiber surface. The maximum efflux has been taken as the efflux shown at 10 min. after the transfer of the muscle to either tris or choline Ringer, corrected for extracellular counts. Muscles 9-2 and 9-3 were held in choline Ringer for 4 hours. The dashed line represents muscle 9-1; this muscle spent only 1 hour in choline Ringer. Although all muscles were loaded in inactive Na for the same length of time, 9-1 and 9-2 had appreciably higher $[Na]_i$ than 9-3.

shown in the insert to Fig. 5. At the discontinuity around $[Na]_i$ = 35 mM, efflux has a mean value of 17 ± 5 pmole/cm^2 sec. The estimation of this early efflux is subject to many uncertainties; only three fourths of the loaded muscles had an initial $[Na]_i$ high enough to show such an efflux. The mean Na efflux at saturation as shown in Fig. 8 is 9 ± 1 pmole/cm^2 sec.; this value will be that used in the discussion that follows, and corresponds to the efflux shown by muscles 30 min. after their transfer to Na-free solutions.

If the reaction with the membrane "carrier" were $3\ Na^+ + X \rightleftharpoons Na_3X^{+++}$ and if $[Na_3X]$ were rate-limiting, we might expect that a plot of [Na] *vs.* $[Na_3X]$ would properly represent our concentration-efflux data. If we write $[Na_3X]/([Na]^3[X]) = K$, or $[Na_3X]/[1 - Na_3X] = K[Na]^3$ we can then calculate $[Na_3X]$ as a function of $[Na]_i$. The solid line in Fig. 8 has been drawn from the equation given above and it is clear that it fits the experi-

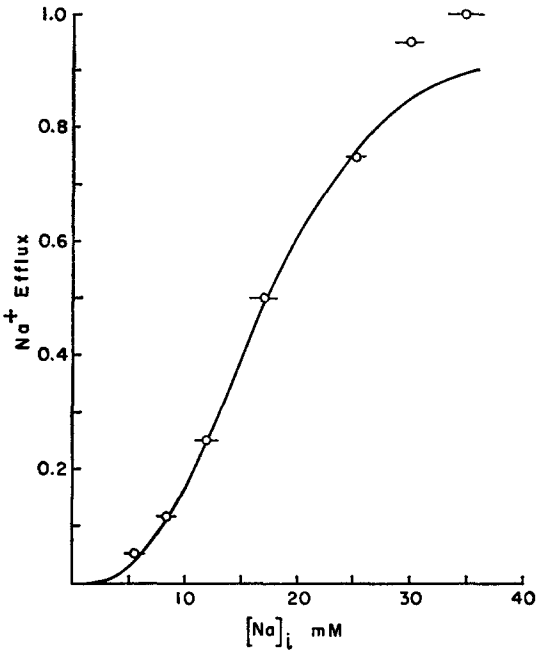

FIGURE 8. This plot is of Na efflux (m_o) as a function of fiber Na^+ concentrations and is a summary of all measurements made. The mean value of efflux at 30 min. after transfer of a muscle to Na-free solution has been set equal to 1.0. The horizontal bars represent ±1 SD in concentration for the particular value of efflux shown. The solid line is a solution of the equations $m_o = [Na_3X]$ and $[Na_3X]/[1\text{-}Na_3X] = K([Na])^3$. This yields $m_o = \dfrac{1}{1 + 1/K[Na]^3}$. If [Na] is in mM, $K = 1/4913\ (mM)^{-3}$.

mental points quite closely up to about 80 per cent of the maximum efflux. At high values of efflux there is more experimental uncertainty because of possible slow components of the extracellular space and the data are regarded as giving a good fit to the curve throughout.

In an effort to resolve the components of the Na efflux curve, muscles previously loaded with Na in the cold were transferred to choline in the cold and the efflux measured for 28 min., followed by a long soak at 20°C. The results of such an experiment are shown in Fig. 9 (left); in muscle 12-1 the transfer

to 20°C produced no obvious effect and the curve shows only the usual plateau. Both muscles 12-2 and 12-3 showed slight increase in efflux after the temperature change but again in one case this was immediate while in the other it was delayed 12 min. In any event, the data do suggest that the 20 to 40 min. plateau has been shifted to 30 to 50 min. On the right in Fig. 9 is shown a similar experiment in which muscles were held 10 min. at low temperature.

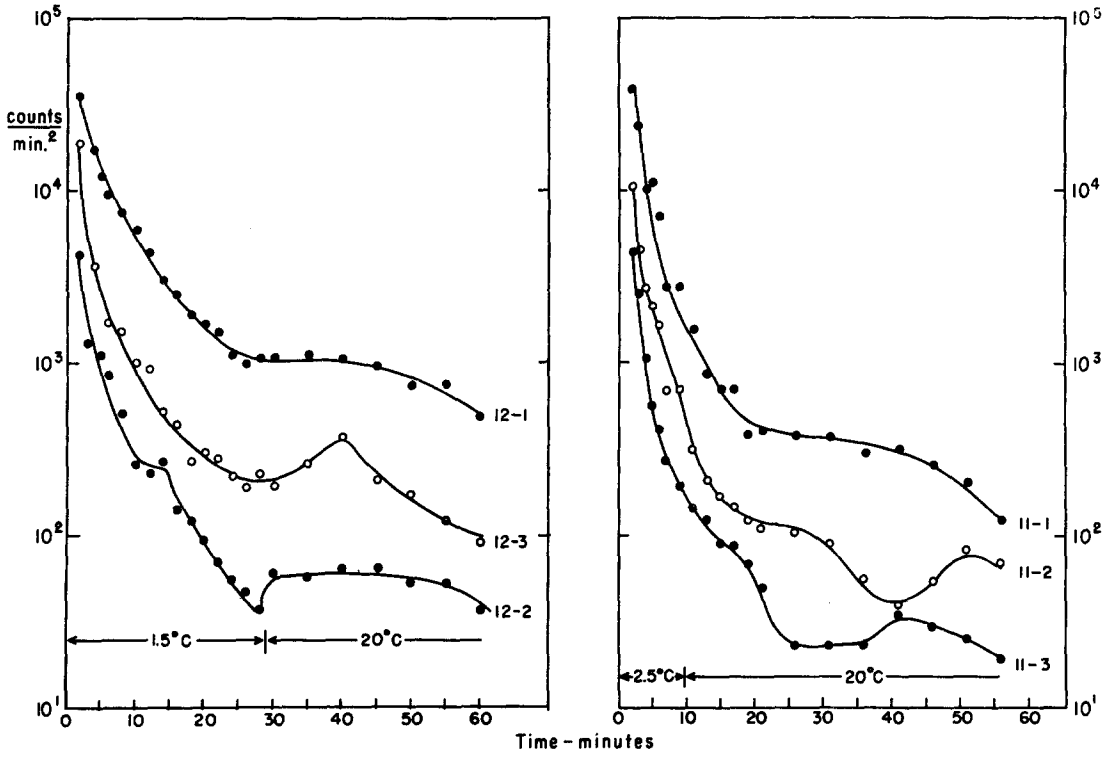

FIGURE 9. Efflux in cpm² is plotted against time for muscles undergoing efflux into Na-free solutions at different temperatures. On the left, temperature was held at 1.5°C for 28 min.; on the right, the temperature was 2.5°C for 10 min.

These curves closely resemble efflux curves measured at room temperatures, and this is to be expected because the low temperature was applied during a time when only space washout was being measured. In these and other curves there are maxima in efflux. Their occurrence is not sufficiently common for us to demonstrate the phenomenon statistically but one may well ask how it is possible for efflux to increase when $[Na]_i$ is continually decreasing. One possibility, of course, is that the membrane potential may have decreased and that this may have had an influence on the pump.

DISCUSSION

In the treatment of the experimental data, it has been assumed that the passive efflux of Na^+ from the fiber represents a negligible fraction of the total Na^+ efflux. While it seems clear that this is so for a muscle with a normal $[Na]_i$, it may not be so for a muscle with $[Na]_i$ = 40 mM. One way of judging whether the permeability of the fiber membrane for Na^+ has been altered by loading the fiber with Na^+ is to determine the extent to which fibers take up Na^{24} subsequent to their loading with Na^+. In our experiments, the fraction of Na^+ not exchanging in 180 min. was 0.15 and our $[Na]_i$ was about 30 mmole/kg. The fraction of Na exchanging may be expected to be given by $1 - e^{-t/\tau}$ and the time constant τ works out to be 95 min. For normal muscle with perhaps 8 mmole/kg $[Na]_i$ the time constant is 60 min. (*cf.* Keynes and Swan, 1959). The $[Na]_i$ of loaded muscles compared with normal $[Na]_i$ is 30/8 or about four times greater, while the time constants are 95/60 or 1.5 times greater. It would appear, therefore, that P_{Na} might be increased by 4/1.5 or 2.6 times. With $[Na]_i$ = 30 mmole/kg the fiber water concentration of Na^+ has been increased about four times over normal and the membrane potential may be expected to be about −70 mv. Flux ratio considerations suggest therefore that passive efflux should have increased ninefold from the combined effects of potential and concentration. The permeability change would increase this factor to 9 × 2.5 or 22-fold. The Na pump, however, has increased its efflux at least eightfold under these conditions so that there is, on a proportional basis, a tripling of the passive efflux. For normal muscle this efflux is a small fraction of 1 per cent of the total efflux; even in the loaded muscle it is negligible.

It seems useful to inquire whether the efflux of Na^+ into choline Ringer may be expected to be quantitatively different from Na^+ efflux into normal Ringer. A point of some importance is the finding of Keynes and Swan that the rate constant for Na^{24} loss is about the same in either choline or normal Ringer (if $[Na]_i$ is constant). This finding suggests that sodium ions on the outside of the membrane do not impede the release of Na^{24} coming from the pump. The supposed coupling of the Na fluxes, termed exchange diffusion, is dealt with separately in the Appendix. Reasons are advanced for doubting that the evidence presently available requires the existence of such a coupling. It seems, therefore, quite likely that the Na pump operates to produce an efflux that is dependent mainly upon $[Na]_i$. Our experimental data suggest that it is possible for the pump to produce an efflux that is approximately twice that found during the long plateau region of the efflux curve, and it has been suggested that this movement may be balanced by an outward Cl^- movement produced by the electrogenic action of the Na pump itself. If this is so, it is

clear that another supposed mode of coupling of the Na pump, that is the coupled exchange of Na, moving outward, for K moving inward (see Keynes, 1954) may not be a coupling in the usual sense. The maintenance of electroneutrality requires that Na^+ efflux be balanced by the movement of other ions and the pump is apparently able to generate a potential difference that leads to a movement of cations inward and anions outward. Without Cl^- inside, the efflux of the pump is dependent upon the movement of cations inward and under our experimental conditions these are K^+ and choline$^+$. If Na efflux is high, these ions may be contributing equally to balancing the outward movement of positive charge; if K^+ is removed from the external medium, the Na efflux must fall or a very much larger potential must be generated to force choline$^+$ to move inward at twice its former rate. As the capacity of any system for generating a potential is limited by the source of free energy available, it is understandable that there appears to be a coupling of the Na-K fluxes. It is unfortunate that detailed potential measurements of the muscle membrane with high $[Na]_i$ are not available. A further difficulty with the notion of a coupled Na-K process in muscle is the common observation that in fresh sartorius muscle, with $[Na]_i = 6$ mM, there is no effect whatever on Na efflux when the muscle is transferred from Ringer to K-free Ringer. The preceding discussion is not designed to demonstrate that there is any conclusive evidence against a coupling of the Na-K movements in muscle but only to suggest that the present evidence for such a coupling is weak.

The fact that the curve for efflux *vs.* $[Na]_i$ follows quite closely a relation to be expected if the Na^+ formed a chemical compound of the sort Na_3X with some sort of complexing substance, and that the efflux is proportional to the concentration of this intermediate makes it tempting to suppose that affairs are just this simple in so far as the selectivity of the pump substance is concerned.

The reasons for supposing that a chemical compound is involved are as follows. In both the squid axon and in sartorius muscle the evidence is clear that Li^+ can substitute for Na^+ in so far as bioelectric phenomena are concerned. In the sartorius, Li^+ have about the same rate of penetration as do Na^+. A calculation of the singly hydrated radius for Li^+ and Na^+ (Mullins, 1961) shows that these are virtually identical so that on the basis that both the passive permeability properties and the bioelectric properties of an ion depend upon steric considerations, Li^+ and Na^+ are handled similarly by the membrane. It is clear, however, (Keynes and Swan, 1959 *b*) that Li^+ efflux from previously loaded fibers is very small indeed, and that the affinity of the Na transport system for Li can be only a few per cent of that for Na. It seems, thus, difficult to suppose that any steric property of Na^+ might be involved in its interaction with the pump, but that the difference in electronic structure between Li and Na is the relevant parameter.

In many ways the simplest hypothesis concerning the mechanism of a Na pump is that the carrier responsible for Na transport and the enzyme catalyzing the energy release necessary for the transport are a single compound. Such an enzyme is the ATPase from crab nerve described by Skou (1957, 1960). While this enzyme shows a complex series of interactions between Ca^{++}, Mg^{++}, Na^{+}, and K^{+} it does show, at physiological concentrations of the ions concerned, an activation by Na and an inhibiton by K (and also by Li). Because of the complexity of such interactions it is not possible to show a compound formation of the sort Na_3X, as suggested by our data.

APPENDIX

The Exchange Diffusion Effect

L. J. MULLINS

The curve shown in Fig. 8 relating $[Na]_i$ to Na efflux has an application to the phenomenon of exchange diffusion described by Ussing (1947) and demonstrated in frog sartorius muscle by Keynes and Swan (1959). For purposes of the discussion that follows, exchange diffusion will be described as positive if the transfer of a muscle from Na-containing to Na-free Ringer solution results in a decrease in the efflux of isotopically measured Na. Correspondingly if efflux increases upon transfer to Na-free medium, exchange diffusion is called negative. While the mechanism of exchange diffusion, as originally proposed, was an enforced one-for-one exchange between Na and radioactive Na, a somewhat different model for accounting for the effect is proposed below. Some revision in the original proposal is clearly needed in view of the experimental demonstration by Keynes and Swan that exchange diffusion in muscle can be zero or, in the squid axon, even negative (Hodgkin and Keynes, 1955; Mullins, Adelman, and Sjodin, 1962).

The purpose of this discussion is to consider a model which can display the properties of exchange diffusion in a positive sense at low $[Na]_i$, a zero exchange diffusion at increased $[Na]_i$, and a negative exchange diffusion at high $[Na]_i$. Such behavior depends upon the existence of a spatial separation between the intake for the Na pump and the point at which Na^{+} from outside are discharged into the fiber water. The result of this separation is that a concentration gradient for Na^{+} must exist between the intake of the pump and the fiber interior, except where the Na fluxes are in balance. Because the Na pump responds to increases in $[Na]_i$ with an increase in efflux approximately as the cube of $[Na]_i$ at low $[Na]_i$, a small change in concentration at the intake of the sodium pump results in a large change in efflux, and it is clear from the mathematical analysis that follows that even though non-radioactive Na^{+} are used to bring about the increase in [Na] at the pump intake, there follows an actual increased output of radioactive ions. Further, in that region of the efflux

vs. $[Na]_i$ curve where efflux is only linearly proportional to $[Na]_i$, an increased concentration of Na^+ derived from outside the fiber, will contribute to the efflux in a linear way and the efflux of radioactive ions will remain unchanged (*i.e.* the exchange diffusion will be zero).

The model to be discussed is shown in Fig. 10; although a number of possibilities exist for bringing about the required separation between pump intake and fiber interior, one of the simplest has been chosen for this discussion. It is supposed that the intake for the Na pump is at the inner face of the membrane and that immediately adjacent to this region inside the fiber there is a region where diffusion is not as free as in the fiber water. One could just as well suppose that the intake for the pump was located in the membrane interior and that a part of the membrane thickness

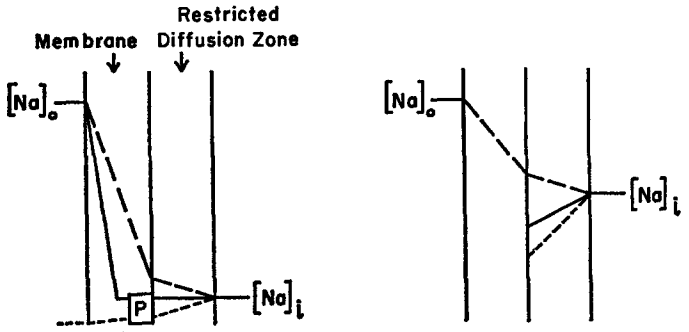

FIGURE 10. This shows the membrane and an immediately adjacent zone of restricted diffusion. The left hand drawing is for the Na fluxes in balance (*e.g.* $[Na]_i$ low). The dashed line is a concentration profile for Na influx and the dotted line is for efflux from the pump into a Na-free medium. The solid line is the net flux concentration profile. As drawn, the solid line is $[Na]'_p = [Na]_i$ and the dotted line as it enters the Na pump P is $[Na]_p$. On the right is a similar diagram for the case in which $[Na]_i$ is high. The solid and dotted lines are, again, $[Na]'_p$ and $[Na]_p$.

between this intake and the inner face of the membrane acted as the region of restricted diffusion. No allowance is made in the treatment that follows for the effect of membrane potential on the Na fluxes because any such treatment would be rather arbitrary. It may be noted, however, that in the region of $[Na]_i$ of particular interest (from 0 to 8 mM), the membrane potential of muscle fibers is not changed in going from Ringer to choline Ringer solution and therefore the membrane potential constitutes a constant force acting on the fluxes under discussion. An analysis of the changes in efflux to be expected from this model upon transfer from Na-free to Na-containing solutions is set forth below.

Let $[Na]_i$ be the measured internal [Na] and $[Na]_p$ be the calculated [Na] at the intake of the sodium pump in the absence of external Na while $[Na]'_p$ is the calculated [Na] at the pump intake in the presence of 110 mM $[Na]_o$. If we assume that Fick's law applies across the zone of restricted diffusion and that the diffusion constant and the thickness of the zone are fixed, the diffusion constant D and the thickness x can be combined into a single constant k and the relationship between the net

flux $\bar{m}$ and concentration difference is given by equation (1). This equation can be used to evaluate $[Na]_p$ and $[Na]'_p$ as shown in equations (2) and (3).

$$\bar{m} = D\, dc/dx = k\,([Na]_p - [Na]_i) \tag{1}$$

$$[Na]_p = [Na]_i + k^{-1}\,\bar{m} \tag{2}$$

$$[Na]'_p = [Na]_i + k^{-1}\,\bar{m}' \tag{3}$$

Now the experimental findings given in Fig. 8 can be represented analytically by the following equations (4, 5, 6) for the cubic, linear, and "saturated" regions of the concentration-efflux curve. The treatment below divides the efflux-concentration curve for muscle into regions in which relatively simple relations apply and it then considers the response of the efflux to a small change in $[Na]_p$. As we are concerned with radioactive ion effluxes, let m_o be the efflux of inactive Na in Na-free solution and m_o^* the efflux of Na^{24} under similar conditions. In normal Ringer, the efflux of Na is denoted by m'_o and the efflux of Na^{24} by $m_o^{*\prime}$. As later calculation will show, $[Na]_p$ and $[Na]'_p$ do not differ from $[Na]_i$ by more than 1 to 2 mM which is below

$$\text{For } [Na]_i = 3 \text{ to } 8 \text{ mM}, \qquad m_o = k'\,([Na]_p)^3 \tag{4}$$

$$\text{For } [Na]_i = 15 \text{ to } 25 \text{ mM}, \qquad m_o = k''\,[Na]_p \tag{5}$$

$$\text{For } [Na]_i = > 35 \text{ mM} \qquad m_o = k''' \tag{6}$$

the experimental precision with which $[Na]_i$ can be estimated. It is consistent with experimental findings to write equations (4) and (5) in terms of $[Na]_p$.

If we assume that all the ions inside the fiber are Na^{24} and denote the specific activity CPM/$[Na]_i$ as one, then equations (4) and (7) describe the efflux of total Na

$$m_o^* = k'\,([Na]_p)^3 \times 1 \tag{7}$$

and Na^{24} in the absence of $[Na]_o$. When $[Na]_o$ is 110 mM, the efflux of total Na is given by equation (8), but the specific activity of the emerging Na^+ has

$$m'_o = k'\,([Na]'_p)^3 \tag{8}$$

been diluted by the contribution made by $[Na]_o$ to $[Na]'_p$. As $[Na]'_p$ is $[Na]_p + \Delta\,[Na]_p$ where $\Delta\,[Na]_p$ is the contribution made by inactive Na to the total concentration, the fractional dilution of the specific activity is $[Na]_p/([Na]_p + \Delta\,[Na]_p)$ or $[Na]_p/[Na]'_p$. The efflux of radioactive Na in the presence of $[Na]_o$ is therefore given by equation (9). Exchange diffusion can be represented as the fractional change

$$m_o^{*\prime} = k'\,([Na]'_p)^3\,[Na]_p/[Na]'_p \tag{9}$$

in Na^{24} efflux in going from Na-free to Na-containing solutions and is, for the cubic region of the efflux curve, given by the ratio of equations (7) and (9), as shown in equation (10).

$$\frac{m_o^*}{m_o^{*\prime}} = \frac{k'([\mathrm{Na}]_p)^3}{k'([\mathrm{Na}]'_p)^3[\mathrm{Na}]_p/[\mathrm{Na}]'_p} = \frac{([\mathrm{Na}]_p)^2}{([\mathrm{Na}]'_p)^2} \tag{10}$$

If $[\mathrm{Na}]_i$ has a value such that equation (5) represents total efflux, the radioactive ion efflux in the absence of $[\mathrm{Na}]_o$ will be identical with (5), while the equation for radioactive ion efflux in the presence of $[\mathrm{Na}]_o$ will require a term to correct for specific activity that is identical with that used in equation (9). Exchange diffusion for the linear region of the concentration-efflux curve is given by equation (11). The result is, by definition, zero exchange diffusion.

$$\frac{m_o^*}{m_o^{*\prime}} = \frac{k''([\mathrm{Na}]_p)}{k''([\mathrm{Na}]'_p)[\mathrm{Na}]_p/[\mathrm{Na}]'_p} = 1 \tag{11}$$

It is of interest to locate the point on the concentration-efflux curve where exchange diffusion is zero; *i.e.*, the point where exchange diffusion changes from positive to negative. To do this it is necessary to equate (5) which is an equation for a line passing through the origin with the equation given in the legend to Fig. 8 for Na efflux over the complete range of concentrations studied. This equation is written below as (12) with $\bar{k}$ a constant to give efflux in pmole/cm² sec.

$$m_o = \frac{\bar{k}}{1 + 1/K[\mathrm{Na}]_p^3} \tag{12}$$

When the line represented by (5) is tangent to (12) we locate the point on (12) where exchange diffusion vanishes because equation (11) demonstrates that the line represented by (5) is a case for zero exchange diffusion. Equating (5) and (12) and their derivatives we have equations (13) and (14). Equation (13) can be solved for k'' and this equated to (14), as shown in (15). From the value of K, the equilibrium con-

$$k''[\mathrm{Na}]_p = \frac{\bar{k}}{1 + 1/K[\mathrm{Na}]_p^3} \tag{13}$$

$$\frac{dm_o}{d[\mathrm{Na}]_p} = \mathrm{k}'' = \bar{k}\, d\left\{\frac{1}{1 + 1/K[\mathrm{Na}]_p^3}\right\}/d[\mathrm{Na}]_p \tag{14}$$

stant, previously found, $[\mathrm{Na}]_p$ is 21.5 mM at the zero exchange diffusion point.

$$\frac{K[\mathrm{Na}]_p^2}{K[\mathrm{Na}]_p^3 + 1} = \frac{3K[\mathrm{Na}]_p^2}{(K[\mathrm{Na}]_p^3 + 1)^2}; \qquad [\mathrm{Na}]_p = (2/K)^{1/3} \tag{15}$$

In the region of the concentration-efflux curve where equation (6) holds, it is clear that m_o and m'_o will always be equal but the radioactive Na efflux ratio is given by equation (16) and must be greater than 1 as $[\mathrm{Na}]_o$ will always contribute some

$$\frac{m_o^*}{m_o^{*\prime}} = \frac{k'''}{K'''[\mathrm{Na}]_p/[\mathrm{Na}]'_p} = \frac{[\mathrm{Na}]'_p}{[\mathrm{Na}]_p} > 1 \tag{16}$$

Na^+ to the pump intake; exchange diffusion is, therefore, negative.

To demonstrate that the model can give reasonable values for exchange diffusion in the region of the concentration-efflux curve where such a phenomenon is observed, a few examples have been worked out below. The data chosen are not necessarily those giving a closest fit to experimentally measured values but have been selected

for numerical convenience in calculation. Thus it is supposed that Na fluxes are in balance at $[Na]_i = 6.2$ mM and with a value of 3.5 pmole/cm^2 sec. (both these values are experimental). The rest of the data follow from an assumption of a value of $k = 2.5 \times 10^{-6}$ cm/sec. This has units of a permeability constant; it cannot be compared with membrane permeability constants both because we have no idea of the fraction of membrane thickness it might represent and because we do not allow for membrane potential. It may be noted that with $[Na]_o = 110$ mM and $[Na]_i = 6.2$ mM, there is a Δ [Na] of 104 mM across the whole membrane and zero Δ [Na] across the restricted diffusion zone. Upon changing $[Na]_o$ to zero, there is only a 1.0 mM Δ [Na] across the restricted diffusion zone. Thus, to make the model work only a relatively small diffusion resistance is required.

The table below has been worked out by taking values for $[Na]_p$ and $[Na]'_p$ and calculating effluxes. Figures for $[Na]_i$ were then obtained from efflux and net flux. Values of $[Na]_p$ and $[Na]'_p$ corresponding to a particular $[Na]_i$ were obtained and are tabulated below. Exchange diffusion expressed as m_o/m'_o is given in the last column and varies from 0.40 to 0.78. The large value (represented by 0.40) is in contrast to experimental values of 0.6 to 0.7 but is to be expected because at $[Na]_i = 2$ mM, efflux is likely to be of the form $m_o = k''''[Na]_i + k'[Na]^3$ since pump efflux and leak of Na^+ (represented by k'''') are of the same order of magnitude. The theoretical treatment presented here would demand that exchange diffusion continue to increase as $[Na]_i$ decreases were it not for the presence of a leakage efflux of Na^+. If leakage decreases linearly with $[Na]_i$ while pump efflux decreases as $[Na]_i^3$, it is clear that leakage efflux will ultimately predominate. At high $[Na]_i$, the efflux ratio will approach 1. The magnitude of the negative exchange diffusion can be calculated from the constants given in the table. For $[Na]_i = 25$ mM, exchange diffusion as defined by equation (16) is 1.07, or there is a 7 per cent increase in Na efflux in going from Na-containing to Na-free Ringer. In squid, where the effect is observed, its magnitude is *ca.* 1.25.

Numerical Values Used for Calculation:

$$m_i = \text{constant} = 3.5 \text{ pmole/cm}^2 \text{ sec.}$$

$m_o = k'([Na]_p)^3$; $k' = 1.46 \times 10^{-8}$ cm^7/μmole^2 sec.; m_o in pmole/cm^2 sec., $[Na]_p$ in μmole/cm^3

$[Na]_p = [Na]_i + k^{-1} m_o$; $k = 2.5 \times 10^{-6}$ cm/sec.; $[Na]_i$ and $[Na]_p$ in μmole/cm^3

$$[Na]'_p = [Na]_i + k^{-1}\bar{m}'$$

$[Na]_p$	m_o	$[Na]_i$	$[Na]'_p$	$\bar{m}$	$\frac{([Na]_p)^3}{([Na]'_p)^3}$
2.0	−0.12	2.05	3.2	+3.02	0.40
2.5	−0.23	2.59	3.7	+2.75	0.46
3.0	−0.39	3.16	4.2	+2.40	0.52
3.5	−0.63	3.75	4.6	+2.08	0.58
4.0	−0.93	4.37	5.0	+1.70	0.64
4.5	−1.33	5.00	5.6	+1.25	0.67
5.0	−1.82	5.73	5.9	+0.46	0.71
5.5	−2.44	6.50	6.4	−0.30	0.73
6.0	−3.14	7.25	6.8	−1.05	0.78

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BIBLIOGRAPHY

1. CAREY, M. J., and CONWAY, E. J., Comparison of various media for immersing frog sartorius at room temperature, and evidence for the regional distribution of fiber Na^+, *J. Physiol.*, 1954, **125**, 232.
2. DEAN, R. B., Theories of electrolyte equilibrium in muscle, *Biol. Symp.*, 1941, **3**, 331.
3. DESMEDT, J. E., Electrical activity and intracellular Na concentration in frog muscle, *J. Physiol.*, 1953, **121**, 191.
4. HODGKIN, A. L., and HOROWICZ, P., The influence of potassium and chloride ions on the membrane potential of single muscle fibers, *J. Physiol.*, 1959 *a*, **148**, 127.
5. HODGKIN, A. L., and HOROWICZ, P., Movements of Na and K in single muscle fibers, *J. Physiol.*, 1959 *b*, **145**, 405.
6. HODGKIN, A. L., and KEYNES, R. D. Active transport of cations in giant axons from *Sepia* and *Loligo*, *J. Physiol.*, 1955, **128**, 28.
7. JOHNSON, J. A., Kinetics of release of radioactive Na, SO_4, and sucrose from frog sartorius muscle, *Am. J. Physiol.*, 1955, **181**, 263.
8. KERNAN, R. P., Membrane potential changes during sodium transport in frog sartorius muscle, *Nature*, 1962, **193**, 986.
9. KEYNES, R. D., The ionic fluxes in frog muscle, *Proc. Roy. Soc. London, Series B*, 1954, **142**, 359.
10. KEYNES, R. D., and SWAN, R. C., The effect of external sodium concentration on the sodium fluxes in frog skeletal muscle, *J. Physiol.*, 1959 *a*, **147**, 591.
11. KEYNES, R. D., and SWAN, R. C., The permeability of frog muscle fibers to lithium ions, *J. Physiol.*, 1959 *b*, **147**, 626.
12. MULLINS, L. J., The macromolecular properties of excitable membranes, *Ann. New York Acad. Sc.*, 1961, **94**, 390.
13. MULLINS, L. J., ADELMAN, W. J., and SJODIN, R. A., Sodium and potassium ion effluxes from squid axons under voltage clamp conditions, *Biophysic. J.*, 1962, **2**, 257.
14. RENKIN, E. M., Permeability of frog skeletal muscle cells to choline, *J. Gen. Physiol.*, 1961, **44**, 1159.
15. SKOU, J. C., The influence of some cations on adenosinetriphosphatase from peripheral nerves, *Biochim. et Biophysica Acta*, 1957, **23**, 394.
16. SKOU, J. C., Further investigations on a Mg + Na activated ATP-ase possibly related to the active linked transport of Na and K across the nerve membrane, *Biochim. et Biophysica Acta*, 1960, **42**, 6.
17. STEINBACH, H. B., Sodium and potassium in frog muscle, *J. Biol. Chem.*, 1940, **133**, 695.

18. STEINBACH, H. B., Na extrusion by the sartorius of *Rana pipiens*, *J. Gen. Physiol.* 1961, **44**, 1131.
19. STEPHENSON, W. K., Membrane potential changes and ion movements in frog sartorius muscle, *J. Cell. and Comp. Physiol.*, 1957, **50**, 105.
20. USSING, H. H., The interpretation of the exchange of radiosodium in isolated muscle, *Nature*, 1947, **160**, 262.