The Potassium Flux Ratio in Skeletal Muscle As a Test for Independent Ion Movement

R. A. SJODIN

From the Department of Biophysics, University of Maryland School of Medicine, Baltimore

ABSTRACT The flux ratio of potassium ions was measured on frog sartorius muscle under conditions in which a substantial net potassium loss occurs. Muscle fiber membrane potentials were measured under identical conditions. The observed flux ratios were compared with values calculated from a theoretical relation derived on the assumptions that the unidirectional fluxes are both passive and occur independently. The results favor the conclusion that the potassium fluxes across skeletal muscle membrane occur along passive electrochemical gradients and obey the independence principle.

INTRODUCTION

Previous studies of potassium movement across the muscle cell membrane indicated that such movement is very likely membrane-limited and that the motion can be described by electrochemical laws (Sjodin, 1959; Hodgkin and Horowicz, 1959 *a*; Sjodin and Henderson, 1964). The latter work strongly supports the notion that, under normal conditions, the potassium fluxes in muscle are passive and it is not necessary to assume the existence of an active transport mechanism to describe the movement. The evidence for this is that the measured unidirectional potassium fluxes are nearly equal when the concentrations and membrane potential predict a state very close to equilibrium.

The most convenient test for passive ion movement is the method of "flux ratio analysis" first developed by Ussing (1949) and Teorell (1949). The unidirectional fluxes of a univalent cation are related to the aqueous concentrations (activities) of the cations on either side of the membrane and to the membrane potential by the following equation:

$$\frac{\phi_o}{\phi_i} = \frac{A_i}{A_o} e^{EF/RT} \qquad (1)$$

where ϕ refers to unidirectional flux, A to ion activity, E to the membrane po-

tential referred to the inside of the cell, the subscript o to outside and i to inside, and where F, R, and T have their usual significance.

Equation (1) is derived on the following assumptions: The membrane is homogeneous and permeability coefficients are equal in the two directions, the net transport is everywhere proportional to the electrochemical gradient, and the unidirectional fluxes are independent of one another. The equation can be used to test the validity of these assumptions for a particular situation. When the unidirectional potassium fluxes are nearly in balance, equation (1) is found to predict the correct or observed flux ratio to within experimental errors in the relevant quantities (Sjodin and Henderson, 1964). This evidence thus favors the view that potassium transport in muscle is normally passive.

The validity of the assumption of independence of influx and efflux, however, cannot be tested critically under conditions where the unidirectional fluxes are nearly equal. The reason for this is apparent from the work of Hodgkin and Keynes (1955) and from considerations developed in the Appendix of this paper. Unidirectional fluxes across membranes are most conveniently measured by means of radioactive tracers. When the fluxes are not independent, an interaction of tracer movement in one direction with non-tracer movement in the opposing direction is possible and leads to a departure from equation (1). Hodgkin and Keynes (1955) treated the case in which ion motion is along a straight path through a pore in a single file so that passing is not permitted. They obtained a modification of the flux ratio equation for this case in which equation (1) occurs raised to the $(n + 1)$ power in which n is the number of single file positions along the pore. In the Appendix, a similar case is treated in which ion movement is across a single barrier of holes (short pores) or sites and where influxing and effluxing ions must use the same pathways. Again, the statistics involved lead to the $(n + 1)$ power law. The flux ratio for non-independent movement thus becomes:

$$\frac{\phi_o}{\phi_i} = \left[\frac{A_i}{A_o}\, e^{EF/RT}\right]^{(n+1)} \qquad (2)$$

where n refers to the number of points in a straight line through the membrane at which an influx-efflux interaction can occur.

When the flux ratio is close to 1, equation (2) does not lead to a great departure from predictions of equation (1). The obvious way to test for the validity of the independent *versus* the non-independent model is to experimentally create conditions such that the observed flux ratio differs greatly from a value of unity. This is the approach of the present work. An experimental operation is performed in which the observed flux ratio for potassium ions is elevated to a value near 9, on the average. The weakest type of interaction in a non-independent situation is the case in which only a single layer of positions occurs at

which flux interactions can occur. In this case $n = 1$ and the theoretical equation describing the flux ratio becomes:

$$\frac{\phi_o}{\phi_i} = \left[\frac{A_i}{A_o} e^{EF/RT}\right]^2 \tag{3}$$

For equation (3) to predict the observed flux ratio of 9, equation (1) would have to predict a flux ratio 1/3 of the observed value. This extent of deviation can easily be detected by the experimental techniques applied in this work.

METHODS

The general methods used for the determination of flux and for cation analyses are the same as those reported by Sjodin and Henderson (1964) and that work can be consulted for details not mentioned here.

Experiments were performed exclusively on sartorius muscles from *Rana pipiens*. A standard Ringer's solution of the following composition was used: NaCl 105 mM, KCl 2.5 mM, $CaCl_2$ 2 mM, tris (hydroxymethyl) aminomethane 1 mM (tris). The tris buffer was acidified with HCl to give a pH of 7.4 which remained at a stable value throughout the experiments. Changes in potassium concentration were made by altering KCl concentrations and maintaining the other salts at their normal concentrations. Radioactive K^{42} Ringer's solutions were prepared in the manner previously described and radioactive counting was performed with a β-scintillation well-type counter as before.

Potassium influx and efflux were performed in a manner identical to that described in the work previously referred to. Influx of potassium from a 0.2 mM K Ringer's solution (the bulk of the experiments to be reported) yields a linear K^{42} uptake relation for periods of hours. In the case of efflux, K^{42} output yields linear semilogarithmic plots for periods of hours. There does not appear to be much experimental ambiguity in the determination of potassium fluxes though some small systematic errors can occur as previously discussed. For a discussion of the possible effects of intracellular and extracellular diffusion, the reader is referred to the work of Sjodin and Henderson (1964).[1]

Experiments were performed as follows: A pair of muscles was carefully dissected from frogs in a good nutritional state. One muscle was then used to determine potassium influx followed by efflux when the external potassium concentration in the Ringer's solution was around 0.2 mM. The potassium flux ratio was thus measured on a single muscle. The other muscle pair member was mounted in a chamber per-

[1] Some uncertainty in the absolute magnitudes of the effluxes reported occurs because of the possible influence of diffusion in the extracellular space (Keynes, 1954). Sjodin and Henderson (1964) present reasons for supposing that the diffusion effect correction factor for medium size sartorius muscles is small by comparing whole muscle fluxes with the single fiber fluxes measured by Hodgkin and Horowicz (1959 *a*). The correction factor is not applied in the present work for two reasons. The factor is not known accurately and can only be estimated and the factor is the same for influx and efflux as pointed out by Keynes (1954). The latter fact makes it clear that the flux ratios measured in this work are insensitive to the magnitude of the effect.

mitting membrane potentials of surface fibers to be measured by means of KCl-filled microelectrodes. After flux and membrane potential measurements were complete, muscles were prepared for cation analysis in the manner previously described. Before flame photometer analysis of the ashed muscles in solution was made, wet weight and dry weight of the muscle were determined. The extracellular space was estimated for each muscle from a knowledge of the extracellular muscle sodium and the sodium concentration in Ringer's solution. Extracellular sodium was estimated by collecting the sodium output from the muscle for 10 minutes into a sodium-free (tris-substituted) Ringer's solution. Extracellular space estimates by this method showed good agreement with values obtained by the output of a non-penetrant, say sucrose labeled with C^{14}.

Influx and efflux of potassium ions were determined in units of micromoles per gram wet weight of muscle per hour. These units suffice for the purposes of these experiments since the flux ratio is the relevant quantity sought and any conversion factors would cancel in the determination. Potassium concentrations were measured on each muscle by dividing the total moles of potassium from analysis by the weight of cellular water. Muscles undergo a net potassium loss while in a 0.2 mM K Ringer's solution for long periods of time. For this reason, initial flux values were determined on the basis of the initial concentrations and weights measured on the control muscle used to obtain membrane potential values. The muscles used to obtain the membrane potential at the 0.2 mM K concentration were in the 0.2 mM K Ringer's solution for a period of 10 minutes.[2]

Membrane potentials were measured by means of 3 M KCl-filled microelectrodes of low-tip potential. Values were recorded to within an accuracy of 1 mv by means of a preamplifier with grid current of less than 10^{-12} amperes and a Tektronix oscilloscope. In a series of measurements, six fibers were penetrated in normal 2.5 mM K Ringer's solution. If the average membrane potential was of the magnitude -93 mv or greater, 20 fibers were penetrated when the potassium concentration was changed to 0.2 mM. If the average membrane potential measured in the normal 2.5 mM K Ringer's solution was less than -93 mv, the muscle pair was rejected. The average membrane potential of 20 fibers from a particular muscle was always found to be within 1 mv of the average value for 20 fibers measured on the other muscle pair member from the same animal.

Experiments were carried out at a temperature of 21°C.

RESULTS

Potassium influx and efflux are very nearly in balance in a normal 2.5 mM K Ringer's solution, the average flux ratio (ϕ_o/ϕ_i) being 1.1 (Sjodin and Henderson, 1964). When the potassium concentration in Ringer's solution is decreased from the normal value of 2.5 mM, influx of potassium ions is observed to de-

[2] If muscles were allowed to remain in the 0.2 mM K Ringer's solution for periods of time comparable to the time required to perform the flux determinations, the membrane potential was found to remain stable. The average membrane potential after 2 hours' contact with the 0.2 mM K solution was within 1 mv of the average value determined after 10 minutes in the 0.2 mM K solution.

crease in proportion to the concentration (Sjodin, 1961). The rate constant for potassium efflux falls under the same conditions but not in proportion to concentration. In fact, a limiting value is reached in a K-free Ringer's solution that is approximately one-half the value in a normal 2.5 mM K Ringer's solution. In going from a 2.5 mM K to a 0.2 mM K Ringer's solution, these factors cause the flux ratio for potassium ions to, on the average, take on the rather high value of 9 found in the experiments to be reported.

TABLE I

THE POTASSIUM FLUX RATIO UNDER CONDITIONS OF NET POTASSIUM LOSS

Muscle	$[K]_o$	$[K]_i$	K^*	ϕ_i	ϕ_o	$-E_m$	ϕ_o/ϕ_i	
							Measured	Calculated
	mM		*hr.*$^{-1}$	*μmoles/gm hr.*		*mv*		
17AP	0.21	151	0.067	0.58	5.57	113	9.6	8.2
17BP	0.20	142	0.098	0.61	8.20	113	13.4	8.1
18AP	0.20	148	0.076	0.72	6.45	113	8.9	8.4
18BP	0.20	163	0.086	0.65	8.24	114	12.7	8.9
114AP	0.21	132	0.068	0.68	5.04	112	7.4	7.4
114BP	0.21	137	0.068	0.69	5.28	113	7.7	7.4
115BP	0.21	147	0.075	0.75	6.13	113	8.2	8.0
218AP	0.20	130	0.076	0.57	5.90	112	10.4	7.6
218BP	0.20	136	0.085	0.74	6.60	113	8.9	7.7
219AP	0.20	127	0.080	0.84	6.01	111	7.2	7.8
219BP	0.20	139	0.089	0.64	6.50	113	10.2	7.9
123AP	0.21	135	0.071	0.68	5.32	112	7.8	7.6
123BP	0.21	145	0.057	0.54	4.80	114	8.9	7.5
317AP	0.21	141	0.084	0.75	6.68	114	8.9	7.3
317BP	0.21	136	0.086	0.73	6.97	114	9.5	7.0
318AP	0.19	130	0.082	0.58	5.87	113	10.2	7.8
324AP	0.21	135	0.075	0.61	5.67	112	9.3	7.6
324BP	0.21	137	0.086	0.89	6.78	115	7.6	6.8
325AP	0.21	120	0.095	0.71	6.77	112	9.5	6.7
325BP	0.21	123	0.094	0.83	7.03	113	8.5	6.7
48AP	0.21	130	0.071	0.66	5.43	118	8.2	5.7
48BP	0.21	142	0.077	0.78	6.68	114	8.6	7.3
Average ± SD							9.2±1.6	7.5±0.7

The results obtained on 22 muscle pairs are presented in Table I. Measured muscle potassium concentrations, measured fluxes, and measured membrane potentials are presented in the table. Flux ratios computed from measured flux values are compared with values calculated from equation (1) assuming that internal and external potassium ion activity coefficients are equal. The results may be summarized by stating that the average values ± 1 SD are: measured 9.2 ± 1.6 and calculated 7.5 ± 0.7. A statistical *t*-test applied to the

results indicated that the difference is significant at the level $P = 0.001$. Though the predictions of equation (1) differ significantly from the measured values, the disagreement is not severe and agreement of the observed values with the theory for independent fluxes is fair. In fact, the results are in much greater agreement with equation (1) than with equation (3) which predicts an average flux ratio of around 60, in marked disagreement with observations. These results appear to favor the conclusion that the potassium fluxes are independent under the stated experimental conditions, at least to a first approximation. More will be said in the Discussion about possible reasons for the discrepancy between average experimental values and average theoretical values.

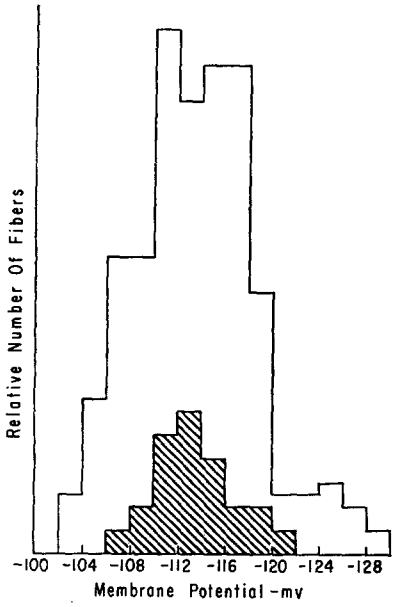

FIGURE 1. The distribution of observed membrane potentials when muscles are in a 0.2 mM potassium Ringer's solution is presented. The relative number of fibers having a given membrane potential is plotted against the membrane potential. The large distribution represents all the membrane potentials measured in these experiments. The bottom inset is for a single muscle and is typical of the distributions seen for individual muscles.

One possible source of the modest discrepancy is the fact that a given muscle displays a dispersion of membrane potential values when in a 0.2 mM K Ringer's solution. A histogram of 0.2 mM K membrane potentials is presented in Fig. 1. The spread in values is greater than in a normal 2.5 mM K Ringer's solution and the possible effect of this factor on the results is discussed later.

It is interesting to consider the potassium unidirectional flux changes in changing from a 2.5 mM K to a 0.2 mM K Ringer's solution. The observed changes in a single muscle pair experiment are presented in Fig. 2. The figure shows the uptake curves and efflux curves obtained when one muscle is in a 2.5 mM K solution and the other pair member is in a 0.2 mM K Ringer's solution throughout. These results bear out the earlier statement that, below a potassium concentration of 2.5 mM, potassium influx is a linear function of the external concentration. Since equation (1) appears to be obeyed at both con-

centrations, it is clear that potassium efflux must change by the factor $e^{\Delta EF/RT}$ where ΔE represents the membrane potential change observed in passing from a 2.5 mM K solution to a 0.2 mM K solution. The value of observed ΔE's is nominally 20 mv of hyperpolarization. The membrane potential change does not appear to affect influx and efflux in a symmetrical manner. One interpretation of this result is that the membrane potential affects efflux only under these conditions. Indeed this is the only possible in-

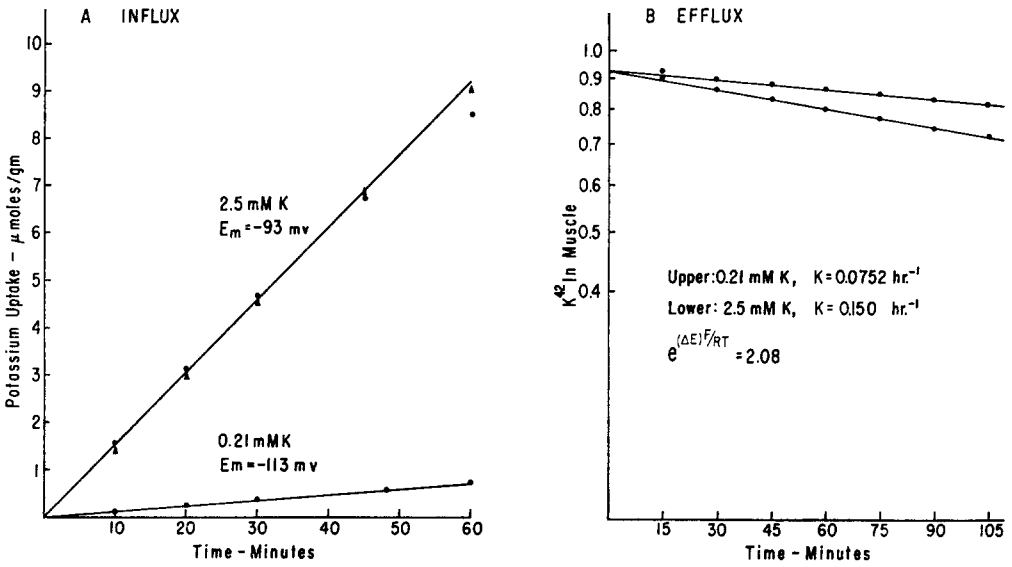

FIGURE 2. Potassium influx and efflux is illustrated at two potassium concentrations, 2.5 mM and 0.21 mM. Influx and efflux were performed on the same muscle at each concentration. Both muscles were pair members from the same frog. The membrane potential in the 2.5 mM K solution was −93 mv while the average value in the 0.21 mM K solution was −113 mv. The triangular points on graph A represent points on the lower curve multiplied by the concentration ratio of 2.5/0.21. The fact that the triangles lie on the line through the upper series of points demonstrates that influx of potassium ions is directly proportional to the external potassium ion concentration under these conditions.

terpretation unless a potassium permeability change occurs. If both unidirectional fluxes occur independently along electrochemical gradients, the potassium permeability coefficient must undergo a decrease in passing from a 2.5 mM K to a 0.2 mM K Ringer's solution. This conclusion is arrived at by applying the constant-field solution of the differential flux equation for potassium ions to the unidirectional fluxes independently; that is, on the assumption that each unidirectional flux can be looked upon as having its own independent driving force. On the basis of this assumption, P_K must undergo a drop to 80 per cent of the value observed in a 2.5 mM K Ringer's solution if the data for the

0.2 mM K case are to be fit. The constant-field relation is applied to the observed net fluxes in the Discussion section.

The electrochemical flux ratio relation (equation 1) has been seen to hold when the potassium fluxes are in balance and also when a large net outward movement of potassium occurs. It is also possible to test the relationship when a net inward movement of potassium takes place. Reference to Sjodin and Henderson (1964, Fig. 6) indicates that the intial ratio of influx to efflux ob-

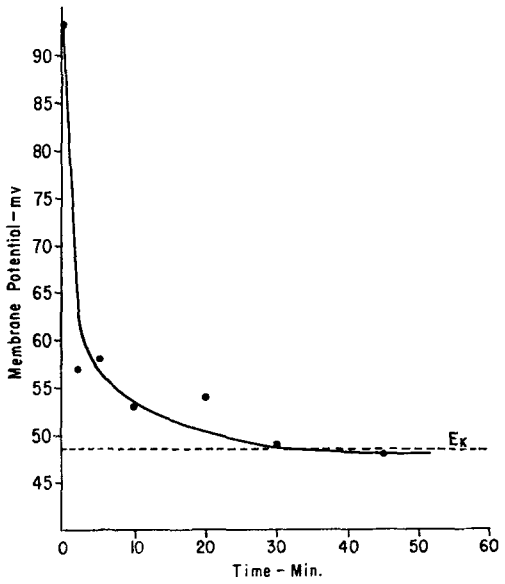

FIGURE 3. The membrane potentials observed when muscles are placed in a 20 mM K Ringer's solution containing chloride ions at a concentration of 130 mM are plotted as a function of time. At time = 0 on the graph, the solution bathing the muscle was changed from a normal 2.5 mM K Ringer's solution to the 20 mM K solution. The dotted line labeled E_K represents the potassium equilibrium potential in the 20 mM K solution as calculated from the Nernst relation using the measured internal potassium concentration. Each point on the graph represents the average value obtained from four microelectrode penetrations on different surface fibers within a 1 minute interval.

served when muscles are placed in a 20 mM K Ringer's solution is 1.5. The flux ratio remains at this value for approximately 15 minutes and then approaches the value of 1.0, reaching that value after about 30 minutes. During this period the muscle is gaining KCl until the internal [K] [Cl] product equals the external value. The membrane potentials of muscle fibers were measured on a pair member in a 20 mM K Ringer's solution during the same time interval. The results are shown in Fig. 3 where the average membrane potential is plotted as a function of time in contact with the 20 mM K solution. The membrane

potential is observed to reach the potassium equilibrium potential in about 30 minutes, agreeing with the flux kinetics. For the first 10 minutes, the membrane potential has an average value of around -56 mv, E_K having the value of -48 mv. The 8 mv departure from E_K leads to a flux ratio (ϕ_i/ϕ_o) of 1.4 calculated from equation (1) which agrees favorably with the measured value of 1.5.[3] Equation (1) has thus been shown to yield correct results, probably to within experimental errors, over a 100-fold variation of the external potassium ion concentration.

Experimental Errors

These are the same as the magnitudes presented and discussed by Sjodin and Henderson (1964). Influxes are accurate to within ± 2.5 per cent and effluxes to within ± 3.5 per cent. The flux ratio thus has an experimental error of ± 6 per cent.

DISCUSSION

For calculations involving flux equations where the ratio of two fluxes does not occur, it is convenient to express fluxes in absolute units. To accomplish this, the present flux unit (micromoles per gram hour) must be first expressed as uptake per volume of fibers per second. Subsequent multiplication by 10^6 times the average volume to surface ratio of the fibers yields flux in units of pmoles/cm^2 sec. Katz (1948) reports an average fiber diameter of 75 μ for frog skeletal muscle. For cylindrical cells the volume to surface ratio is r/2 where r is the fiber radius. Taking the volume of fibers to be 80 per cent of the muscle volume, it can be shown that a flux of 1 μmole/gm hr. is equivalent to a flux of 0.65 pmole/cm^2 sec. using the above average diameter.

It can be inferred from the work presented that potassium movement across the membrane of frog skeletal muscle meets the criterion demanded by the theory for independent ion movement under the stated experimental conditions. One possibility is that potassium ions in muscle move in aqueous channels that are wide enough to permit free passage of ions in a single channel. For such a membrane the differential flux equation integrated across a classical Nernst-Planck region might be expected to hold. A flux equation commonly applied to biological membranes where free electrochemical diffusion of ions is thought to occur is the flux equation integrated with a constant elec-

[3] The actual kinetics of the non-steady state potential and flux changes occurring during the 30 minutes following a step change in external potassium ion concentration from 2.5 mM to 20 mM in a chloride Ringer's solution are admittedly complicated. Numerical precision cannot be claimed for the flux ratio calculation presented in the case of a net KCl gain because of the rapidity with which the relevant quantities are changing with time. The calculation suffices, however, to show that the observed flux ratio is in approximate agreement with equation (1).

trical field (Goldman, 1943; Hodgkin and Katz, 1949). This equation is presented below for potassium ions:

$$\bar{\phi}_K = P_K \frac{EF}{RT} \left(\frac{[K]_i \, e^{EF/RT} - [K]_o}{e^{EF/RT} - 1} \right) \quad (4)$$

where $\bar{\phi}$ refers to net flux, P is the permeability coefficient, bracketed quantities refer to concentration, and other symbols have the same significance as in the previous equations.

It is of interest to apply this equation to the net fluxes of potassium occurring in a 2.5 mM K Ringer's solution and also in a 0.2 mM K Ringer's solution. In both cases the potassium net flux is in an outward direction and the average magnitude is known. Potassium permeability coefficients can be calculated from equation (4) in both cases and the values compared. From the work of Hodgkin and Horowicz (1959 *a*) and Sjodin and Henderson (1964), the net potassium leakage in a 2.5 mM K Ringer's solution under normal conditions is close to 1 pmole/cm² sec. The net potassium leakage (as observed in this work) increases to about 4 pmoles/cm² sec. when the concentration of potassium ions in Ringer's solution is lowered to 0.2 mM. These flux values lead to the following permeability coefficient values from equation (4): $[K]_o$ = 2.5 mM, $P_K = 0.3 \times 10^{-6}$ cm/sec., and $[K]_o$ = 0.2 mM, $P_K = 0.6 \times 10^{-6}$ cm/sec. Constant-field theory applied to the net potassium fluxes thus predicts a permeability increase for potassium ions moving outward during the membrane hyperpolarization occurring in a Ringer's solution having a low (0.2 mM) potassium concentration. Hodgkin and Horowicz (1959 *b*) found the constant-field potassium permeability coefficient to assume low values for outwardly directed driving forces compared to permeability coefficients calculated for cases when potassium net movement is inward. The present values compare favorably with values calculated by Hodgkin and Horowicz for the case in which the direction and magnitude of the net potassium flux are similar. For example, fiber reference *m* in their Table 8 indicates a net outward potassium flux of 5.8 pmoles/cm² sec. and a P_K equal to 0.4×10^{-6} cm/sec. The conditions in the present experiments are certainly different than those holding in the experiments reported by Hodgkin and Horowicz. For net fluxes of similar magnitude and direction, however, the potassium permeabilities seem to be consistent with those calculated and presented by Hodgkin and Horowicz (1959 *b*).

The finding that P_K apparently rises, under the present experimental conditions when the external potassium concentration is changed from 2.5 mM to 0.2 mM, may indicate that the potassium permeability coefficient is somewhat concentration-dependent as well as dependent on the magnitude of the driving force as deduced by Hodgkin and Horowicz. The fact that the P_K change deduced from net potassium movements does not seem to agree with the P_K

change suggested by each unidirectional flux independently, may indicate that it is incorrect to apply the constant-field flux equation to individual unidirectional fluxes in an independent manner. The reason for this may be that the potassium fluxes across the skeletal muscle cell membrane occur in spatially separated regions of membrane as suggested by Adrian and Freygang (1962). The basis for the separation may be found in the model proposed by the latter authors or it may be found in a more general property of mosaic type membranes.

Some factors in the experiments reported could modify the results of this investigation somewhat. One factor has to do with a possible concentration build-up just outside the membrane. Since a considerable potassium leakage takes place under the experimental conditions employed, any external region in which diffusion is restricted could allow an accumulation of potassium just outside the membrane. The tubular lumen of the endoplasmic reticulum could be the site of such a region. Adrian and Freygang (1962) postulate that this region of the muscle cell may be approximated by an additional membrane, in series with the cell membrane, which separates a small well mixed compartment[4] from the external solution. Since the capacity of the additional compartment is small, about $\frac{1}{500}$ of the fiber volume, it is of negligible size compared to the intracellular volume. Single cellular compartment kinetics are expected in such a system agreeing with the observations of Hodgkin and Horowicz (1959 *a*) and Sjodin and Henderson (1964). The concentration build-up that can occur in the small external compartment when a given net potassium efflux takes place depends on the permeability coefficient assigned to the second membrane. Adrian and Freygang are able to account for their membrane conductance data by assuming the outermost membrane to be some 18 times more permeable to potassium ions than the cell membrane across which the membrane potential is developed. For the same relative permeabilities, the net potassium losses in the present experiments would lead to a potassium accumulation in the intermembrane space amounting to about 0.2 mM (Mullins and Noda, 1963). Such a calculation is arbitrary, however, since it depends on the value assigned to a constant. If the outermost membrane is assumed to be twice as permeable as in the above example, the concentration build-up would be 0.1 mM rather than 0.2 mM. On the other hand, the build-up would be greater should the outer membrane be less permeable. These values suffice to at least give the possible order of magnitude of the effect.

Another means is available to estimate the actual average potassium concentration with which the outer membrane surface is in contact in these ex-

[4] It is very likely that diffusion gradients would exist in the small space postulated. The assumption of complete mixing is made only to facilitate the estimation of possible concentrations within the space.

periments. Hodgkin and Horowicz (1959 *b*) find that the membrane potential of frog skeletal muscle fibers is given to a good approximation by the relation:

$$E = \frac{RT}{F} \ln \frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[Na]_i} \quad (5)$$

where all symbols have their usual or previous significance. The quantity α is a constant to be assigned or evaluated. Hodgkin and Horowicz found a satisfactory fit to data could be obtained by assigning α the value 0.01. The equation is now applied in the following way. The average membrane potential when $[K]_o$ is known to be 2.5 mM is used to calculate a value for α. With $[K]_i$ = 140 mM and $[Na]_i$ = 10 mM, which are reasonable representative values, α turns out to be between 0.010 and 0.012 in good agreement with Hodgkin and Horowicz. An average membrane potential of −113 mv when fibers are in a 0.2 mM K Ringer's solution is now used to calculate a value for $[K]_o$ just outside the membrane, other quantities retaining their previous values. The values calculated for $[K]_o$ by this means are between 0.3 mM and 0.5 mM depending on the particular value of α. The implication of the analysis is that, on the average, a concentration build-up of potassium amounting to 0.1 mM to 0.3 mM has taken place just outside the membrane. The two methods for estimating the actual potassium concentration in contact with the membrane thus give results in fair agreement.

It is relevant to ask how this effect would affect the results of this investigation. Since K influx is directly proportional to the outside concentration for the sartorius when $[K]_o$ is below 2.5 mM, the measured flux ratios and the calculated flux ratios are both affected in exactly the same way by any change in $[K]_o$. The effect discussed above, should it be present, would thus not affect the general conclusion of this investigation that the potassium fluxes obey the principle of independence. The presence of the effect would mean that the measured and calculated flux ratios are lower than the values presented in Table I by the same factor. Allowing for a concentration build-up of 0.2 mM, which seems to be near the upper limit, the effective potassium concentration would be close to 0.4 mM rather than 0.2 mM. The result would be a halving of the presented flux ratio values. The mean values would then be: measured, 4.6 and calculated, 3.8. The mean value computed from equation (3) is about 14 under these conditions and this is some 3 times the measured values. The method is thus adequate to test for independent *vs.* non-independent ion movement even with a generous allowance for concentration build-up of potassium in a region just outside the membrane. If a "single-file" mechanism is present, such as that postulated by Hodgkin and Keynes (1955) for giant nerve cell membrane, the exponent in equation (2) becomes higher than 2. If n = 2, for example, the independent flux ratio occurs to the 3rd power in the case of non-independence. The computed value in the case of concentra-

tion build-up to a total value of 0.4 mM then becomes 12 times the measured ratio. The method is thus a very powerful test for a single-file mechanism.

Another factor that could influence the results is the dispersion of membrane potentials among the individual fibers of a muscle when in a 0.2 mM K Ringer's solution. The distribution of observed membrane potentials is seen in Fig. 1. In the calculation of the theoretical flux ratio for each muscle, the arithmetic mean of the observed potentials was used. One must now examine the effect of the membrane potential distribution on such calculations. Since the membrane potential enters the flux ratio calculation in an exponential term, use of an arithmetic mean membrane potential may introduce a bias and it is of interest to determine the direction of the bias. To accomplish this, the following average values are used in a representative calculation: $[K]_i = 140$ mM, $[K]_o = 0.2$ mM, and $E_{av} = -113$ mv. These values in equation (1) yield a calculated flux ratio of 7.95. Next, the distribution in Fig. 1 is separated into two equal groups with means 5 mv on either side of the -113 mv mean for the whole group. The calculated flux ratio for the group with a mean membrane potential of -108 mv is 9.72 while that for the group with a mean of -118 mv is 6.48. The arithmetic mean of the calculated flux ratios for both groups is 8.10 which is to be compared with the value of 7.95 obtained by using the arithmetic mean for the whole group. Use of the arithmetic mean for the whole group is seen to bias the calculation very slightly in the downward direction. If this analysis is continued for wider separations of subgroups, the effect becomes more pronounced. For two equal groups 10 mv on either side of the whole group mean, the average of the two calculated flux ratios is 8.6. Dividing the distribution into a greater number of subgroups and averaging in a similar manner indicate that the dispersion effect leads to flux ratios that are on the average 3 per cent higher than the calculated values in Table I. The effect is a small one and is in the direction of improving the agreement between measured and calculated values. The calculations also make use of an average potassium concentration for the whole muscle while there is undoubtedly a distribution of individual fiber values within a muscle.

Another small correction that can be applied stems from a systematic error in tracer efflux determinations when tracer equilibration is very low. The effect is noted and discussed in the work of Sjodin and Henderson (1964). Potassium efflux reckoned from tracer movement when equilibration is very slight is about 5 per cent too high for the whole muscle. The tracer equilibrations in the experiments reported are of the order of 1 per cent and it should be appropriate to lower the effluxes measured in this work by 5 per cent.

The activity coefficients for potassium ions have been assumed to be equal on both sides of the membrane throughout this work. Mullins and Noda (1963) present some evidence that the intracellular activity coefficient for potassium ions may be somewhat lower than that for Ringer's solution. The ac-

tivity coefficient in a small region just outside the membrane may be of more relevance than the activity coefficient in Ringer's solution. In view of other uncertainties, the assumption of equal activity coefficients appears to be justified.

It has been assumed that any component of active inward transport of potassium ions that does not lead to a departure from equation (1) can be neglected in this work. At normal Ringer's solution potassium ion concentrations, any deviations from equation (1) are small and the deviations remain small when the external potassium ion concentration is lowered as in this work. The agreement of the data with equation (1) could, of course, be a coincidence due to the fortuitous interplay of several complicating factors, including active transport. That this is not the case is supported by the fact that in several experiments performed in the presence of 5 μg/ml of strophanthidin or 0.2 mM DNP at pH 6.8, agreement of observed flux ratios with those calculated from equation (1) remained within 15 per cent.

Another process that has been assumed not to significantly affect the results presented is exchange diffusion of potassium ions. This process, like active inward transport, could if present, lead to a fortuitous agreement of the data with the passive independent flux ratio formulation. The justification for assuming the absence of exchange diffusion of K is that there is no compelling evidence for its presence. The drop in potassium tracer efflux that occurs when the external potassium is removed from solution or lowered in concentration can easily be accounted for on a passive diffusion basis by the increased negativity developed within the cells under these conditions. It does not seem wise at present to assume the operation of a special mechanism unnecessarily.

The results of this investigation can be summarized by stating that after all possible corrections have been applied, the measured flux ratio is on the average some 15 per cent higher than the theoretical value calculated on the basis of independent ion movement. A model which assumes a minimal degree of non-independent type interactions (equation 3) predicts a flux ratio some 6 times the observed value. In view of this, it seems reasonable to conclude that the potassium unidirectional fluxes in sartorius muscle are independent to a good approximation. The modest elevation of 15 per cent observed on the average could be explained by assuming that 3 per cent of the efflux pathways are of a non-independent type. Alternatively, equation (2) can be used in an empirical manner to give a more precise fit to the data. If n is assigned the value 0.1, the data are adequately fit. A statistical model which assumes a weaker type of interaction than that assumed in the Appendix may account for such an exponent. Due to other experimental uncertainties it does not appear worth while at present to seek such a model. It appears to be of more value to seek explanations for why potassium fluxes do not seem to follow the independence principle in the presence of foreign cations such as rubidium

and cesium (Sjodin, 1961). An experimental approach to this problem is currently underway in this laboratory.

Appendix

Consider a single layer of holes through which ions must pass in order to cross a membrane. The holes in the model can be replaced by a single layer of sites if it is assumed that each site can associate only one ion at a time and that an ion must associate with a site for some finite interval of time to pass through the region. For convenience in the derivation, the ionic positions will be referred to as "holes." When the holes are used for transit by ions on one side of the membrane only, the flux from that side to the other is considered to be independent since no flux in the contrary direction takes place. Such independent fluxes can be represented as follows:

$$\begin{aligned} \phi_{12} &= C_1 P_1 \\ \phi_{21} &= C_2 P_2 \end{aligned} \tag{1A}$$

where C refers to concentration and P is proportional to the permeability coefficient times the driving force in that direction. When ions cross in both directions using the same holes through which one and only one ion can pass at a time, the fluxes to be evaluated are not independent of one another and are designated m_{12} and m_{21} where m refers to actual observed flux.

The treatment to be used makes use of the Poisson distribution for a random variable x. The probability that a random variable will have some definite value x is given by the equation for the Poisson distribution:

$$P(x) = \frac{e^{-\mu}\mu^x}{x!} \tag{2A}$$

where the quantity μ is the average value of the random variable x. In the model, the random variable x will be the number of "successful" collisions which ions on one side of the membrane make with the membrane, a "success" being a collision with a hole and leading to crossing of the membrane. In a given collision event, the only two possibilities are reflection from the membrane or penetration through a hole since, thus far, ions are present on one side of the membrane only. If unit area of membrane is considered, the number of independent ion passages in a time interval t is, on the average, equal to $\phi t = \mu$ since this is also the average number of "successes" occurring in the time interval t. Thus, for the two directions:

$$\begin{aligned} \mu_1 &= \phi_{12} t = C_1 P_1 t \\ \mu_2 &= \phi_{21} t = C_2 P_2 t \end{aligned} \tag{3A}$$

From the Poisson distribution, the probability that no ions passed through a hole in a given direction in the time interval t is $p(0) = e^{-\mu}$. This is also the probability that a given gate will be open to the passage of an ion in the opposite direction for the entire time interval t. Now a time interval, τ, exists such that a given gate must be

open for the entire interval, τ, in order to be effectively open to the passage of an ion in the opposite direction. This probability will be given by $e^{-\mu} = e^{-CP\tau}$ if it be assumed that the Poisson distribution holds as t approaches τ. Subject to these assumptions, the probability that a given ion attempting to cross the membrane from side 1 to side 2 will find a given gate open is $e^{-\mu_2} = e^{-C_2P_2\tau}$. The corresponding probability for the 21 direction is $e^{-\mu_1} = e^{-C_1P_1\tau}$.

The non-independent fluxes m can now be formulated as follows: Let the total number of collisions per gate per unit time by ions on side 1 be $N_1 = C_1K_1$ and on side 2 be $N_2 = C_2K_2$. The fraction of these collisions that will actually result in a transfer in a given direction will be the probability for penetration in the absence of ions on the opposite side times the probability that the gate is not being used by ions on the opposite side; *i.e.*, the probability that the gate is open. Thus, the total actual number of transfers from 1 to 2 and from 2 to 1 in time t will be:

$$N_{12} = \sigma N_1 t(1 - e^{-\mu_1})e^{-\mu_2}$$

and (4A)

$$N_{21} = \sigma N_2 t(1 - e^{-\mu_2})e^{-\mu_1}$$

where σ is the number of membrane holes per unit area. The flux in each direction is the corresponding number of transfers occurring per unit time. Making substitutions from relations given above, the non-independent fluxes become:

$$m_{12} = \sigma C_1K_1(1 - e^{-C_1P_1\tau})e^{-C_2P_2\tau}$$

and (5A)

$$m_{21} = \sigma C_2K_2(1 - e^{-C_2P_2\tau})e^{-C_1P_1\tau}$$

The flux ratio for the non-independent fluxes can easily be formulated using the above relations. Simplifying the resulting ratio yields:

$$\frac{m_{12}}{m_{21}} = \frac{C_1\,K_1(e^{C_1P_1\tau} - 1)}{C_2\,K_2(e^{C_2P_2\tau} - 1)} \tag{6A}$$

This equation can now be compared with the theoretical equation first proposed by Teorell (1949) and by Ussing (1949) for the ionic flux ratio when fluxes are independent; *i.e.*, equation (1) in the text:

$$\frac{\phi_{12}}{\phi_{21}} = \frac{C_1}{C_2}\,e^{zEF/RT} \tag{7A}$$

where E refers to the electrical potential, z to ion valence, and R, T, and F have their usual meanings. For fluxes in balance, the flux ratio is unity and the thermodynamic relation (7A) must hold for both the independent flux ratio and the flux ratio for the non-independent fluxes. In the present notation:

$$\frac{\phi_{12}}{\phi_{21}} = \frac{C_1 P_1}{C_2 P_2} \tag{8A}$$

Comparing equations (7A) and (8A), it follows that:

$$\frac{P_1}{P_2} = e^{zEF/RT} \tag{9A}$$

In the equilibrium situation, $C_1P_1 = C_2P_2$ by equation (8A). The flux ratio for the non-independent fluxes given in equation (6A), at equilibrium, becomes:

$$\frac{m_{12}}{m_{21}} = \frac{C_1 K_1}{C_2 K_2} \tag{10A}$$

It follows from equation (10A) that $K_1/K_2 = e^{zEF/RT}$ and the final form for the non-independent flux ratio becomes:

$$\frac{m_{12}}{m_{21}} = \frac{C_1}{C_2} e^{zEF/RT} \left[\frac{e^{C_1P_1\tau} - 1}{e^{C_2P_2\tau} - 1}\right] \tag{11A}$$

Comparing this result with equation (7A) shows that in the non-equilibrium case, the non-independent flux ratio is simply the theoretical independent flux ratio perturbed by the factor on the right involving concentrations in the exponentials. For small x, e^x is approximated by $1 + x$. From the formulation of non-independent fluxes, $CP\tau$ will be small and the first term in the expansion of e^x should be sufficient. Using this expansion, equation (11A) becomes:

$$\frac{m_{12}}{m_{21}} = \left[\frac{C_1}{C_2} e^{zEF/RT}\right]^2 \tag{12A}$$

in view of equation (9A).

The model developed predicts that the actual non-independent flux ratio will be the square of the flux ratio expected for independent ion movements. It is of interest to compare this result with the prediction of the model proposed by Hodgkin and Keynes (1955) which consists of a long pore or chain of sites. The present model is obviously the limiting case of the long pore model obtained when the number of "file positions" is one. An equation derived by Hodgkin and Keynes contains the flux ratio assuming independent movement raised to the $n + 1$ power where n is the number of file positions. In the case treated here, n is 1 and their equation reduces to equation (12A). The present results are, therefore, consistent with the Hodgkin-Keynes model.

Hodgkin and Keynes, however, are able to show by more elaborate statistical methods that the exponent in the flux ratio equation for non-independent fluxes should be n rather than $n + 1$ where n is the number of sites in a row. This result is in marked disagreement with the conclusions drawn from the present model. The reason may lie in the assumptions and approximations used in the various derivations.

The Hodgkin-Keynes derivation required that all sites be occupied. The present

derivation does not require this assumption but it is clear that the model developed is plausible only if the number of sites or holes is limited compared to the number of ions available in the solutions near each site. This is equivalent to assuming that the membrane is near saturation and, hence, near the condition demanded by Hodgkin and Keynes. Were this not the case, rather large "dead-times" could exist at a hole in which the gate would be open simply because there were no "trials" for entry from a particular side.

The treatment employed assumes that the Poisson distribution holds in the limit as t approaches a value τ. If τ assumes small enough values the Poisson formula will, of course, fail to hold. Even in such a case, however, it is likely that the probabilities used would be simply proportional to the Poisson values holding for some larger τ where the Poisson formula is valid. Such proportionality factors would obviously cancel in the formation of the flux ratio. Equation (12A) would still be expected to be a good approximation.

If the membrane is not near saturation, it becomes empirically clear that the exponent in the flux ratio equation should be less than 2. In the limiting case of a membrane very far from saturation, the value of the exponent should approach 1 and the theoretical equation (7A) should begin to hold. For the other limiting case of a saturated membrane, which was also assumed by Hodgkin and Keynes, it is concluded that the exponent should be 2 which is equal to $n + 1$ and not n. On this basis, it is concluded that the exponent in the Hodgkin-Keynes formulation should be $n + 1$ and not n.

This investigation was supported in whole by Public Health Service Research Grant No. GM-08426 from the Division of General Medical Sciences.
Received for publication, December 15, 1964.

REFERENCES

1. ADRIAN, R. H., and FREYGANG, W. H., The potassium and chloride conductance of frog muscle membrane, *J. Physiol.*, 1962, **163**, 61.
2. GOLDMAN, D. E., Potential, impedance, and rectification in membranes, *J. Gen. Physiol.*, 1943, **27**, 37.
3. HODGKIN, A. L., and HOROWICZ, P., Movements of Na and K in single muscle fibres, *J. Physiol.*, 1959 *a*, **145**, 405.
4. HODGKIN, A. L., and HOROWICZ, P., The influence of potassium and chloride ions on the membrane potential of single muscle fibres, *J. Physiol.*, 1959 *b*, **148**, 127.
5. HODGKIN, A. L., and KATZ, B., The effect of sodium ions on the electrical activity of the giant axon of the squid, *J. Physiol.*, 1949, **108**, 37.
6. HODGKIN, A. L., and KEYNES, R. D., The potassium permeability of a giant nerve fibre, *J. Physiol.*, 1955, **128**, 61.
7. KATZ, B., The electrical properties of the muscle fibre membrane, *Proc. Roy. Soc. London, Series B*, 1948, **135**, 506.
8. KEYNES, R. D., The ionic fluxes in frog muscle, *Proc. Roy. Soc. London, Series B*, 1954, **142**, 359.

9. MULLINS, L. J., and NODA, K., The influence of sodium-free solutions on the membrane potential of frog muscle fibers, *J. Gen. Physiol.*, 1963, **47**, 117.
10. SJODIN, R. A., Rubidium and cesium fluxes in muscle as related to the membrane potential, *J. Gen. Physiol.*, 1959, **42**, 983.
11. SJODIN, R. A., Some cation interactions in muscle, *J. Gen. Physiol.*, 1961, **44**, 929.
12. SJODIN, R. A., and HENDERSON, E. G., Tracer and non-tracer potassium fluxes in frog sartorius muscle and the kinetics of net potassium movement, *J. Gen. Physiol.*, 1964, **47**, 605.
13. TEORELL, T., Membrane electrophoresis in relation to bio-electric polarization effects, *Arch. sc. physiol.*, 1949, **3**, 205.
14. USSING, H. H., The distinction by means of tracers between active transport and diffusion, *Acta Physiol. Scand.*, 1949, **19**, 43.