Tracer and Non-Tracer Potassium Fluxes in Frog Sartorius Muscle and the Kinetics of Net Potassium Movement

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ABSTRACT Experiments were performed to test the applicability of permeability kinetics to whole frog sartorius muscle using K^{42} ions as tracers of potassium flux. The whole muscle was found to obey closely the kinetic laws expected to hold for single cellular units in which the potassium fluxes are membrane-limited and intracellular mixing is rapid enough not to introduce serious error. In a 5 mM K Ringer's solution, potassium efflux was very nearly equal to influx when the rate constant for K^{42} loss was applied to the whole of the muscle potassium. Over a fairly wide range of external potassium concentration, the assumed unidirectional fluxes measured with tracer K^{42} showed good agreement with net potassium changes determined analytically. The specific activity of potassium lost from labeled muscles to an initially K-free Ringer's solution was measured as a test of the adequacy of intracellular mixing. The results were those expected for a population of cells with uniformly distributed intracellular K^{42}. A small deviation was encountered which can be attributed either to a dispersion of fiber sizes in the sartorius or to a possible small additional cellular compartment in each individual fiber. The additional cellular compartment, should it exist, contains from 0.5 to 1 per cent of the muscle potassium. This is evidently not large enough to interfere seriously with the applicability of permeability kinetics to the whole muscle.

INTRODUCTION

Ion fluxes have been previously measured in whole muscle by many investigators. The work has led to two main kinetic models for whole muscles. One model regards whole muscle as a population of single cellular units each obeying simple first-order permeability kinetics (Keynes, 1954; Creese, 1954). According to this model, the whole muscle will, with possible minor perturbations, obey the same kinetics. The other model proposed regards whole muscle as an assemblage of cellular units in which simple permeability kinetics are not obeyed due to the presence of large perturbations caused by slow diffusion intracellularly (Harris, 1957; Harris and Steinbach, 1956). For con-

venience, the former will be designated the "permeability model," and the latter the "diffusion model." It is understood that the diffusion model may also entail membrane components which introduce permeability concepts into the theory as well.

When applied to measurements of potassium fluxes, the permeability model in its simplest form regards the intracellular potassium as ionized and freely mobile. The model may be modified to include the case where a portion of the intracellular potassium content is bound and, hence, inexchangeable with radioactive isotopic potassium ions. If, however, a significant portion of the cellular potassium is not completely bound but instead displays a much lower mobility than does myoplasmic potassium, the first-order permeability model must be considerably modified if the slow moving potassium affects the rate of the membrane process. In this case, the assumption of essentially instantaneously complete mixing of entering ions once past the membrane permeability barrier is not justifiable. The slow diffusion process must be taken into account and this leads to the diffusion model mentioned.

Experimental evidence has been offered in support of both models. Creese (1954) found it possible to apply the permeability model to rat diaphragm muscle, any deviations being accounted for by assuming an assemblage of cells with different rate constants. Keynes (1954) used various whole muscles from the frog to demonstrate consistency of flux measurements with the permeability model. Using muscles close to the steady state, he showed that potassium influx and efflux were essentially in agreement when measured by the tracer technique assuming permeability kinetics. Agreement was obtained by applying the rate constant for efflux to 100 per cent of the intracellular potassium content. The conclusion to be drawn is that either the permeability model is correct and all of the intracellular potassium is freely mobile or, if a significant fraction of the cell's potassium is bound, the agreement of the data with the permeability model must be fortuitous.

Harris and Steinbach (1956) in a tracer experiment with frog sartorius muscle presented evidence suggesting that all of the intracellular potassium cannot be regarded as "well mixed." These workers found large variations in the specific activity of potassium ions collected from tracer-loaded sartorius muscles into potassium-free solutions. The specific activity differences apparently could not be accounted for by considering a population of cells with different rate constants. The only alternative is to suppose that each individual cell displays a significant gradient in specific activity and, hence, that the assumption of complete mixing within each cell is not justified. In a later investigation, Harris (1957) worked out a model for sartorius muscle cells based on the assumption of membrane permeation combined with a slow intracellular diffusion process. If this model of the muscle cell corresponds closely to the real events in a flux study, Harris and Steinbach (1956)

correctly assert that "the movement of tracer K will not be a measure of the transmembrane flux." The models employed by Keynes (1954) and Harris (1957) thus seem to be mutually exclusive, at least with regard to sartorius muscle. If the diffusion model is correct, the agreement of the flux data obtained by Keynes must be, to a great extent, fortuitous. If the permeability model is correct, the data of Harris and Steinbach (1956) must have been given an erroneous interpretation by these authors and by Harris (1957).

The purpose of the present investigation is to obtain more extensive data on potassium tracer and non-tracer fluxes under a variety of conditions in order to ascertain which of the models discussed is the more applicable to frog sartorius muscle. The plan of the investigation is to measure influx and efflux on the same muscle using K^{42} ions as tracers. The net flux can be obtained by comparison of the final analytical K content of the muscle with the initial K content of a paired muscle from the same animal. These measurements have been made in three ranges of external potassium concentration, one range in which the potassium fluxes are very nearly in balance, one in which considerable net potassium loss occurs, and one in which considerable net potassium gain occurs. Potassium influxes and effluxes reckoned from tracer movement on the assumption of the permeability model should be consistent with the observed net potassium movements in all the above cases if this model is applicable. Marked inconsistencies must be taken as an indication of the non-validity of the permeability model. A variant of the above type of experiment in the case of net potassium losses is to perform the efflux part of the experiment into a potassium-free solution. Under this condition, potassium efflux can be directly measured by counting the tracer in the efflux samples and also by determining the total potassium content in the efflux samples by flame photometry. The specific activity of potassium in the efflux samples can thus be calculated and compared with the average specific activity of the potassium remaining in the muscle. The permeability model applied to this type of experiment would demand that efflux reckoned by the two methods agree, *i.e.* that the rate constants for K^{42} loss and for K loss agree, and that the specific activity of potassium in the efflux samples be close to that of the potassium remaining in the muscle at the termination of the efflux experiment. To be consistent with the permeability model, any deviations in the above type of experiment must be accounted for by a population of muscle cells having different radii and, hence, rate constants.

The influx-efflux type of experiment proposed is essentially the experimental approach of Keynes (1954) using a wider range in external potassium concentration, though Keynes apparently did not attempt to measure net changes in K content precisely. The experiments in which the specific activity of potassium ions lost to an originally K-free solution is measured are similar to the experiments performed by Harris and Steinbach (1956). The difference

is that a K-free Ringer's solution is used whereas the above authors performed efflux into sugar solutions and distilled water, apparently because of analytical limitations imposed by flame photometry.

METHODS

Experimental Methods The experimental methods used were very similar to those previously reported (Sjodin, 1959, 1961; Harris and Sjodin, 1961). Only significant differences will be emphasized. Experiments were performed exclusively on sartorii from *Rana pipiens*. A standard Ringer's solution having the following composition was used: NaCl 110 mM or 105 mM,[1] 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 stable during the experiments. Potassium flux measurements made with this buffer present could not be distinguished from those made using other buffers such as 2 mM $NaHCO_3$. Higher potassium concentrations were produced by adding potassium to the solution of standard composition. The potassium-free Ringer's solution used was the standard solution with the potassium left out. In prolonged experiments in a potassium-free solution, muscles showed a weight loss which could be correlated with a net loss of analytical potassium.

The radioactive isotope, K^{42}, was procured as the chloride salt in acid solution and, after neutralization, was added to the Ringer's solution, the total potassium concentration being adjusted to the values stated. Radioactive counting was performed with a β-scintillation well-type counter.

Muscles were carefully dissected from fresh frogs in good nutritional state. Special care was taken not to damage fibers at the pelvic end of the muscle, a technique being developed which allowed a fine thread to be tied to connective tissue only, at this end of the muscle. The thread ties to the muscle were made in such a way that essentially no distortion of the normal fiber geometry occurred. The freshly dissected muscles were allowed to equilibrate in Ringer's solution for 20 min. Muscles were then carefully wet weighed in a small dish containing Ringer's solution. The muscles upon which flux measurements were to be made were carefully tied to a 21 gauge platinum wire bent to form a frame to support the muscle at its resting length. The platinum frame made a close fit with the test tubes used as efflux tubes so that it was impossible for the supported muscles to come into contact with the sides of the tubes. This procedure was found to be absolutely essential as direct contact of the muscles with tube surfaces causes a substantial net potassium leakage from the muscle.

In a typical influx-efflux type experiment, the paired control muscle was prepared for analysis at a time, t_o, which was also the beginning of the tracer uptake on the experimental muscle. Preparation for analysis consisted of placing the muscle in a dish containing 5 cc of sodium-free Ringer's solution for 10 min., the sodium being replaced with tris(hydroxymethyl)aminomethane neutralized to pH 7.4. This procedure removed essentially all of the extracellular sodium from the muscle, the

[1] There was no detectable difference in K^{42} flux when 105 mM NaCl Ringer results were compared with those in a 110 mM NaCl Ringer's solution. The tendency of muscles to gain sodium ions was somewhat reduced in the 105 mM NaCl Ringer's solutions. All solutions used in which the K concentration was greater than 2.5 mM contained 105 mM NaCl.

cellular sodium efflux from the muscle during this period being negligible for a normal muscle. The procedure allowed the extracellular space to be estimated for each muscle and the intracellular sodium content to be determined. The average value obtained for the extracellular space by analyzing the tris wash solutions for Na was 22.4 ± 4.2 (SD) per cent. After the wash in a sodium-free solution, the muscles were blotted on clean filter paper, weighed, and placed in small platinum crucibles for ashing.

The experimental paired muscle was placed in the radioactive Ringer's solution for known time intervals. Between each interval, the muscle was placed in an empty efflux tube *via* the platinum holder and the tube then placed in the well of the counter. The muscle was counted for 1 min. and returned to the labeled soak solution. Uptake intervals varied with the rate of uptake; initially intervals were 5 and 10 min. and these were increased to 20 and 30 min. as uptake progressed. Uptake was carried on to an extent determined by the desired per cent of tracer equilibration. This varied but accurate measurements of influxes could be made when the degree of tracer equilibration was as little as 10 per cent. At the termination of uptake, muscles were placed into a series of efflux tubes each containing 5 cc of the desired solution. The time intervals for which the muscle was in each tube were generally 20 min. unless a detailed analysis of an initial rapid tracer loss was being made, in which case they were shorter. 3 to 4 hrs. of efflux proved a more than adequate time for the accurate determination of a rate constant for K^{42} efflux. At the termination of efflux, muscles were given a final count in the manner employed for uptake and then prepared for analysis as described above.

Before analysis for sodium and potassium content, muscles in the platinum crucibles were ashed for 10 hrs. at 550°C. When ashing was complete, 0.2 cc of 1 N nitric acid was added to the crucibles to dissolve the ash. The solution volume was then brought up to 2 cc by addition of deionized water and the solution carefully poured into 10 cc volumetric flasks. The 10 cc solution was then used for analysis and counting. If the muscle contained radioactive ions, 5 cc of the 10 cc was placed in an efflux tube for a final muscle count. The geometry and total volume for this counting were thus identical with those for the counting of the efflux tubes. Corrected counts could be directly back-added to the final muscle count to obtain the kinetics for efflux. The final count plus all the efflux counts gave the tracer content at the end of influx. A sample of the soak solution was then counted in an efflux tube in a total volume of 5 cc. The solution-muscle exchange could be calculated from a knowledge of soak solution specific activity and the potassium content of the muscle.

Cation analyses were performed using a specially constructed flame photometer. The flame photometer consisted of a Beckman atomizer and flame unit and an American Instrument Co. photometer equipped with an RCA 1P22 photomultiplier tube. Potassium and sodium interference filters of 2 mμ band width allowed analyses to be made with no detectable interference. The K filter also had a narrow enough spectral range to permit accurate analyses to be carried out in the presence of Ringer's solution concentrations of sodium ions.

Experiments were performed at a temperature of 21.5°C unless otherwise indicated.

Methods of Treating Data Potassium influx was determined as previously reported (Sjodin, 1961). The tracer uptake points were converted to micromoles of solution potassium per gram muscle using the solution specific activity and the counts per minute in the muscle at the end of uptake as determined by back-adding efflux counts to the final muscle count. The uptake points were plotted *versus* time and could be fitted by a single exponential equation of the form $C_i(t) = C_i(\infty)(1 - e^{-kt})$ when a small correction was applied for extracellular space potassium. In an uptake experiment, the muscle was first dipped in the radioactive solution for a short (10 sec.) period and counted. This count was taken as representing solution adhering to the muscle. This count and the extracellular space counts were always subtracted from each uptake value to obtain the kinetics of cellular penetration. Extracellular space counts were obtained from a knowledge of the volume of the space, determined by the method mentioned, and the activity of the uptake solution. Applying this procedure yielded initial linear portions of the uptake curves which passed through the origin.

The slope of the linear portion or the rate constant fitting the curve times the equilibrium concentration gives the uptake in units of micromoles per gram hour. To convert this uptake rate to units of flux first requires the conversion to uptake per volume of fibers per second. Multiplying the latter by the average volume to surface ratio of the cells and by 10^6 gives units of flux in pmoles/cm^2 sec. Since the type of correlation sought in this investigation involves influx and efflux measurements on the *same* muscle, it is not necessary to make this conversion to absolute flux units and micromoles per gram hour serves as a convenient measure.

The counts per minute remaining in the muscle were then plotted on semilog paper. A straight line was obtained, after about 20 min., which remained straight throughout efflux periods lasting as long as 4 hrs. The initial fast process could be identified with extracellular space washout. In addition, evidence was obtained for a minor perturbation of first-order kinetics lasting for a short period beyond the extracellular space washout period. This may indicate a small additional compartment or may merely reflect the fact that one is dealing with a population of fibers of different diameters and, hence, rate constants. A further discussion of this effect will be given in the text. Any deviations from a straight line on a semilogarithmic plot were minor and occurred early in a 3 or 4 hr. efflux period. It was a simple matter to extract an accurate rate constant for K^{42} loss in each efflux experiment. If the permeability model holds, this rate constant times the muscle potassium concentration at any time should give the potassium efflux at that time.

The potassium concentration of the paired control muscle sacrificed initially is known. This is also taken as the initial concentration of potassium in the experimental muscle. The paired muscle procedure is, of course, subject to the random variations which occur between paired muscles. Several initial measurements indicated that the standard deviation for initial potassium contents of paired muscles was close to 1.5 per cent. In view of other experimental errors, to be discussed later, it appears that good justification exists for taking the initial paired muscle concentration as that for the experimental muscle as well. The rate constant for K^{42} loss times this initial potassium concentration should give the initial potassium efflux, according to

the permeability model. Since influx is also known for the same muscle, a test of the permeability model would be to see whether the presumed unidirectional fluxes measured or calculated agree with the observed net changes in analytical potassium content. To accomplish this requires a knowledge of the kinetics for the net changes so that one can use the initial and final potassium concentrations and the total time interval to obtain an estimate of the initial net flux.

RESULTS

The Kinetics of Net Potassium Movement As indicated in the Methods section, influx and efflux of potassium ions could be represented as essentially first-order single exponential permeability processes. To test the validity of the unidirectional measured fluxes requires the calculation of a net flux from the initial and final analytical potassium contents. This, in turn, requires some knowledge of the kinetics for the net movements.

Two experimental findings suggest the kinetic nature of the net movements: (*a*) influx is constant with time and (*b*) the rate constant for K^{42} loss is constant with time. The majority of the experiments to be reported were performed over a total time interval of some 3 to 5 hrs. During this period, in 2.5 mM and 5.0 mM K solutions, there was no significant departure from the first-order permeability kinetics for either influx or efflux of potassium ions. In addition, influx and efflux performed on one pair member initially and subsequently on the other pair member at a later time did not indicate significant differences in either influx or the rate constant for K^{42} loss. The experimental evidence points to a constant influx and a constant rate constant for K^{42} loss over the 3 to 5 hr. experimental period. It would thus appear that the sole "net flux adjuster" is the internal potassium concentration. This is evident from the following equations, where $\bar{\phi}$ refers to net flux (positive in the outward direction), ϕ_o to efflux, and ϕ_i to influx.

$$\bar{\phi} = \phi_o - \phi_i \tag{1}$$

Formulated as a first-order permeability process, efflux becomes

$$\phi_o = kC_i \tag{2}$$

where k is the rate constant for potassium loss and C_i is the internal potassium concentration. For constant influx and rate constant, the net flux is a function of time if the internal concentration is a function of time. It follows that

$$\bar{\phi}(t) = kC_i(t) - \phi_i \tag{3}$$

Accordingly, if there is an initial net efflux, the internal concentration will

fall, the fall obeying the simple differential equation:

$$\frac{dC_i(t)}{dt} = -\bar{\phi}(t) \tag{4}$$

The drop in internal concentration will take place until influx equals efflux; *i.e.*, until a steady state is reached.[2] The steady-state internal concentration is given in magnitude by the zero net flux solution to equation (3).

Conversely, if there is an initial net inward movement, a rise in internal concentration will occur until a steady state is reached. It is evident that the time required to reach the steady state is a function of the rate constant. The kinetics, in the case of a net loss, can easily be obtained by elimination of the net flux between equations (3) and (4). The following differential equation is obtained:

$$\frac{dC_i(t)}{dt} + kC_i(t) - \phi_i = 0 \tag{5}$$

Since ϕ_i is constant, the solution to equation (5) is easily obtained by separation of variables. The solution is as follows, where $C_i(0)$ is the initial internal concentration:

$$C_i(t) = C_i(0)e^{-kt} + \frac{1}{k}\phi_i(1 - e^{-kt}) \tag{6}$$

It is not possible to obtain a precise check of equation (6) by experiment since one pair of muscles gives only two points, the initial $t = 0$ point and one other. By pooling experiments performed over different time intervals when the initial concentrations, rate constants, and influxes were similar, it was possible to show that the results were entirely consistent with the predictions of equation (6).

Even though a precise check of the validity of equation (6) is not possible for the same muscle pair, using analytical data only, the predicted variation of C_i with time could be calculated for a given muscle using the rate constant and influx from a tracer experiment. For an experiment in a 2.5 mM K Ringer's solution, typical values of the parameters are: $C_i(0) = 80$ μmoles/gm, $k = 0.15$ hr.$^{-1}$, and $\phi_i = 10$ μmoles/gm hr. Plotting equation (6) for these

[2] The term "steady state" is used in this work to designate the condition in which the intracellular concentration of a particular species, potassium, does not change with time. Since concentration changes occurring in muscles can be experimentally detected only within certain limits, as discussed in the text, it is to be understood that the use of the term steady state is subject to the same uncertainty limits. A steady state need not be an equilibrium state in the thermodynamic sense and, because of its greater generality, the use of the term is preferred to that of equilibrium. Arguments are advanced in the Discussion section for regarding the muscle potassium as being essentially in equilibrium with solution potassium at external concentrations of 5 mM and greater. If this is true, as appears likely, steady state and "equilibrium" could be used interchangeably.

values indicates that the loss is linear for the first 2 hrs. Therefore, for this time interval and the above conditions, the net flux could be obtained by dividing the initial-final concentration difference by the time interval with no error resulting. Beyond the 2 hr. interval, the net flux begins to fall quickly below the initial linear value and the concentration difference divided by the time interval becomes a poorer estimate of the initial net flux.

It was desired to estimate the initial net flux using only the analytical K data and the elapsed time during the experiment. In this way, two independent estimates of the net flux could be obtained, one using tracer data and one using analytical data only. For experiments lasting not more than 3 hrs., the net flux was obtained by dividing the concentration difference by the time interval. Many experiments lasted beyond this period, the bulk of the experiments lasting about 4 hrs. In these cases it was found that a closer estimate of the initial net flux could be made by assuming a simple logarithmic relation for the net change. For experiments lasting over 3 hrs., initial and final potassium contents were used to calculate a rate constant for net loss. Applying this rate constant to the initial potassium concentration gave an estimate of the initial net flux. These numerical methods for estimating the initial net efflux can be shown to give results lying within the experimental errors involved in the method of determining concentration differences. For the 2.5 mM and 5.0 mM K experiments, a consideration of the standard deviation in initial K contents of muscle pair members indicates that the net fluxes reported are accurate to within about 30 per cent.

At first sight, the uncertainty in net flux would seem to preclude precise correlations. Two experimental ranges minimize the difficulties considerably. For the case in which the unidirectional fluxes are both very large and nearly in balance, the uncertainty in the difference between the unidirectional fluxes will be of the order of the uncertainty in the net flux. The data obtained in a 5 mM K Ringer's solution illustrate this case. Under these conditions the unidirectional fluxes are essentially in balance and are some twenty times greater than the average rate of net change. Another experimental case which minimizes the net flux difficulty is the case in which a potassium-free Ringer's solution is present. In this type of experiment, potassium influx may be taken as zero and equation (6) predicts a simple exponential net K loss. In addition, the net efflux is much larger in this instance and much greater initial-final concentration differences occur. There is correspondingly less uncertainty in the measured net fluxes. The intermediate case of a 2.5 mM K solution is also treated, as well as the case in which considerable net gain of potassium occurs.

The results obtained on twenty muscle pairs using a 5 mM K Ringer's solution are summarized in Table I. The values reported for influx were obtained from K^{42} tracer flux as described. The net fluxes were obtained by the

method stated. Efflux was obtained by multiplying the initial potassium content, as given by the paired control muscle, by the rate constant measured for K^{42} efflux from the experimental muscle. Since the net flux is taken as positive in the outward direction, the influx plus the algebraic value for net flux should equal the efflux. The sum of influx and net flux is to be compared with the efflux value given by tracer found in the adjacent column. Also

TABLE I

POTASSIUM FLUXES IN 5 mM K RINGER'S SOLUTION

Muscle No.	Weight	Rate constant K^{42} loss	Influx ϕ_i^*	Net flux $\bar{\phi}$	$\phi_i^*+\bar{\phi}$	Efflux ϕ_o^*	ϕ_o^*/ϕ_i^*	Equilibration
	gm	$hr.^{-1}$		*μmoles/gm hr.*				*per cent*
411A'S	0.0545	0.201	15.4	1.48	16.9	16.1	1.045	49.6
424A'S	0.0567	0.178	13.8	1.15	14.9	14.4	1.043	8.3
425A'S	0.0490	0.200	16.8	1.66	18.5	17.6	1.048	26.6
52A'S	0.0405	0.226	19.2	1.07	20.3	19.9	1.037	35.7
522A'G	0.0340	0.165	14.6	−0.30	14.3	13.5	0.925	32.0
523A'G	0.0310	0.247	20.0	0.83	20.8	20.3	1.014	54.5
612A'G	0.0308	0.344	26.1	0.86	27.0	27.7	1.062	48.7
613A'G	0.0344	0.346	28.0	−0.66	27.3	26.0	0.927	58.8
627A'G	0.0656	0.137	12.0	−0.35	11.6	12.0	1.000	24.6
73A'G	0.0855	0.161	13.7	0.50	14.2	14.4	1.052	28.6
75A'G	0.0653	0.193	14.9	−1.87	13.0	13.8	0.925	36.2
710A'G	0.0540	0.249	21.2	−1.53	19.7	18.7	0.882	33.3
716A'G	0.0774	0.181	14.4	0.80	15.2	16.3	1.130	26.6
717A'G	0.0635	0.189	16.8	−0.37	16.4	16.2	0.965	43.9
725A'G	0.0610	0.189	16.6	0.26	16.9	16.9	1.018	32.7
731A'G	0.0770	0.182	15.2	2.02	17.2	17.1	1.125	18.3
81A'G	0.0492	0.207	15.4	0.16	15.6	16.9	1.097	42.3
911A'G	0.0130	0.550	46.8	2.13	48.9	50.0	1.069	40.2
43A'H	0.0593	0.180	15.6	0.66	16.3	16.1	1.032	79.0
712A'S	0.0566	0.245	19.0	0.75	19.8	19.9	1.047	12.7
			Average ± SD: 0.462±1.07				1.018	
			Average rate of sodium gain: 0.104					

* Refers to measurement using tracer ions.

included in the table are the efflux rate constants, the muscle weights, the per cent of tracer equilibration at the end of the influx period, and the flux ratio for the unidirectional fluxes. It is evident from Table I that the results show good agreement with the permeability model and any discrepancies noted are well within experimental errors which are discussed in a later section.

The average flux ratio for the twenty muscle pairs is 1.018 indicating that, on the average, muscles in a 5 mM K solution are in a steady state from the time of excision from the animal. Looking at the data as a whole, some muscles are found to undergo modest net losses of potassium while others show modest

gains. Such differences are most likely attributable to the initial conditions of the muscles. As stated in the Methods section, every care was taken to insure that muscles came in contact with solution, only, during the experiment. However, it was necessary to make many transfers from solution to solution during an experiment. In general, twelve to fifteen such transfers were required, about five of which involved counting the muscle for 1 min. out of solution. The unavoidable handling of the muscles may well mean that the observed net fluxes are not necessarily the ones one would have measured had the muscles remained quietly in a dish throughout similar intervals. Differential muscle fragility could easily account for some muscles showing somewhat greater K losses than others.

On the average, however, the fluxes are nearly in balance and a small initial average net efflux of about 0.4 μmole/gm hr. occurs. The initial and final sodium content values on the muscles in Table I indicated that the sodium fluxes are also very nearly in balance. The average value for sodium net flux indicated a gain of 0.1 μmole/gm hr. In no case was the gain greater than 0.3 μmole/gm hr. Thus, of the average 0.4 unit of K net efflux, 0.1 can on the average be regarded as in exchange for sodium ions. The remaining 0.3 μmole/gm hr. of potassium efflux shows good agreement with the net equivalents of phosphate loss from muscles not exposed to excessive experimental handling (L. J. Mullins, personal communication). The question arises as to the statistical significance of the net fluxes reported in Table I. Is the average value of 0.46 μmole/gm hr., for example, significantly different from zero? A statistical analysis of the results was made and the usual "*t*-test" applied. The results of this test indicated that the average net flux is significantly greater than zero at the probability level $p = 0.10$ but not at the level $p = 0.05$. The same test was then applied to the net fluxes obtained from the difference between measured unidirectional tracer fluxes. The average value obtained by this technique was 0.43 ± 1.37 μmoles/gm hr. The statistical test showed that this value is not significantly different from zero and that it is not significantly different from the value arrived at without use of K^{42} ions as tracers. The average values of around 0.4 flux unit do, however, seem representative of the results obtained on muscles in very good condition when experimental technique was especially good.

Considerable variation is noted in the magnitudes of the rate constants and unidirectional fluxes reported in Table I. The rate constants reported contain the surface to volume ratios of the fibers and this factor might be expected to normalize the rate constants and fluxes. That this is the case is suggested by the remarkable correlation observed between muscle weight and flux or rate constant. Efflux is plotted against muscle weight in Fig. 1 for the data in Table I and the correlation is apparent. If the extracellular spaces and water contents of the muscles are comparable, the reported values

should be normalized by multiplying them by the average volume to surface ratio which, for cylindrical cells, is $r/2$ where r is the average fiber radius. From these considerations, Fig. 1 is in qualitative agreement with theory as small muscles are likely to have small average fiber radii. In fact, if the naive assumption be made that average fiber volume is approximately proportional to muscle weight, the fluxes in Fig. 1 are considerably normalized on the basis that the variation in muscle length is much less than the variation in

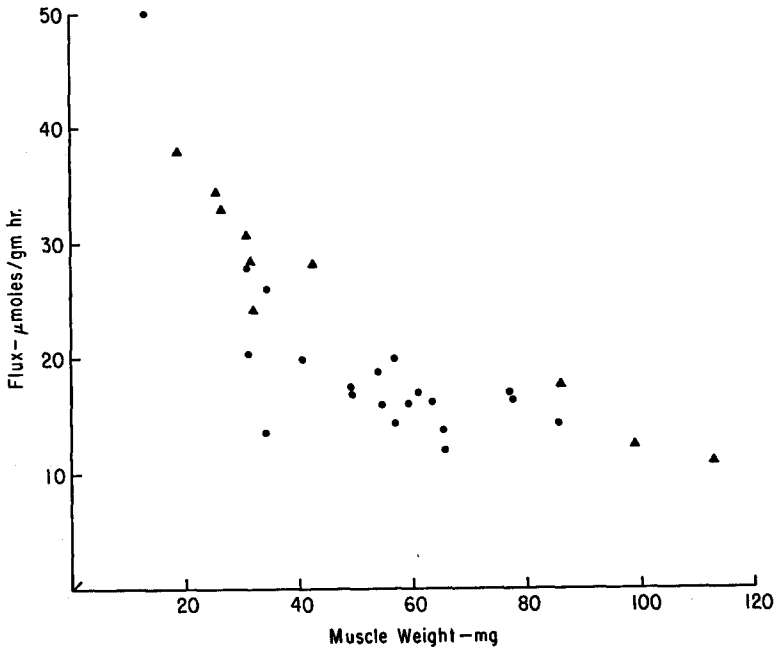

FIGURE 1. Potassium efflux into 5 mM K Ringer's solution is plotted against muscle weight. The data appearing in Table I are plotted and a few additional muscles are included on which either influx or net flux was not measured. Data from Table I are plotted as filled circles. Additional data are plotted as triangles.

fiber diameter.[3] A histological study of the relation of fiber diameter to muscle size is under way to verify these points.

Since the results summarized in Table I are entirely consistent with the permeability model, one might ask how inconsistent they are with the diffusion model. As pointed out by Harris and Steinbach (1956), it would not

[3] Some of the variation in flux that occurs over a range in muscle size can be attributed to diffusion of ions in the extracellular space. Applying factors correcting for the "diffusion effect" would also be expected to normalize the fluxes presented in Fig. 1. From arguments previously presented (Sjodin, 1959, 1961), it is not likely that this factor is the sole explanation for the flux variation in Table I and Fig. 1 since the highest value for the correction factor appears to be about 1.5 for very large muscles.

be expected that tracer movements would accurately trace unidirectional transmembrane fluxes if slow intracellular diffusion is a major factor. Since tracer potassium apparently does give the transmembrane fluxes in the 5 mM K case, one would be forced to conclude that the agreement in Table I is fortuitous should the diffusion model actually hold. Such fortuitous agreement seems extremely unlikely as the degree of tracer equilibration extends over a wide range in the experiments reported, the least equilibrated muscle having a value of 8.3 per cent, the most completely equilibrated value being 79 per cent. No apparent difference in the agreement shown by the results was observed to occur over this wide range of tracer equilibration. The diffusion model predicts greater flux discrepancy the less the degree of tracer equilibration. At the lowest tracer equilibration of 8.3 per cent, a flux discrepancy of up to about tenfold would be possible. The majority of the experiments involved tracer equilibrations of around 25 per cent. Fourfold discrepancies in the fluxes could result according to the diffusion model. It appears that the results are not consistent with the diffusion model.

A feature of the permeability model is that uptake follows the relation:

$$C_i(t) = C_i(\infty)(1 - e^{-kt}) \tag{7}$$

in the steady state, where $C_i(\infty)$ is the internal concentration and k is the rate constant for efflux. It is apparent that the uptake curve is mathematically determined if k and $C_i(\infty)$ are known. Since these quantities are determined by methods not involving influx techniques, a further test of the permeability model is available. A muscle pair was selected in which a negligible change in internal potassium content occurred during the experiment; *i.e.*, the muscles were in a steady state and equation (7) should hold for uptake. A constant internal potassium concentration and the rate constant for K^{42} loss were used to calculate the uptake predicted by equation (7). The agreement with the experimental data is illustrated by Figs. 2 and 3. In Fig. 2, K^{42} uptake (curve *a*) followed by output (curve *b*) from the same muscle is plotted against time semilogarithmically. According to the permeability model, the fraction of total muscle K yet unequilibrated with tracer K^{42} during uptake should yield a straight line when plotted semilogarithmically against time. Also, the fraction of the total initial K^{42} remaining in the muscle during efflux should yield a straight line when similarly plotted. If the internal K concentration is not to change, the time courses and fluxes obtained for the two processes must agree. Fig. 2 shows this to be the case. Fig. 3 is a linear plot against time of the uptake data used for Fig. 2 (curve *a*). The curve fitting the points was calculated from equation (7) using the analytically determined potassium content and the rate constant obtained from efflux (Fig. 2, curve *b*). If muscles in the steady state in a 5 mM K Ringer's solution were allowed

to remain in contact with a labeled solution for long periods of time, tracer exchange proceeded to completion (100 per cent equilibration) along a time course given by equation (7). All experiments performed in their entirety in a 5 mM K Ringer's solution thus support the permeability model but not the diffusion model. A non-detectable perturbation due to slow diffusion cannot, of course, be ruled out.

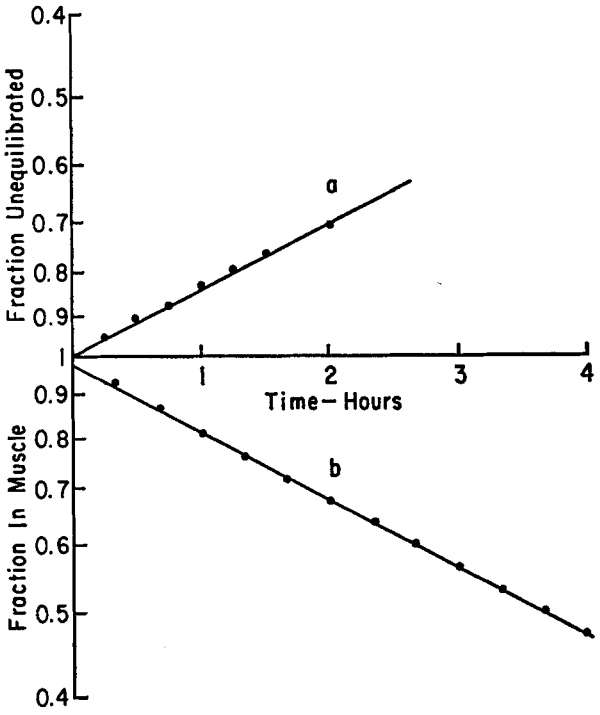

FIGURE 2. Curve *a* is a semilogarithmic plot against time of the fraction of muscle potassium yet unequilibrated with tracer K^{42} during uptake in 5 mM K Ringer's solution. Curve *b* is a similar plot of the fraction of radioactive potassium remaining in the muscle during efflux into 5 mM K Ringer's solution, the muscle being the same as that used to obtain the data for curve *a*. The line fitting the points of curve *a* yields a rate constant of 0.176 $hr.^{-1}$. The line fitting the points of curve *b* yields a rate constant of 0.180 $hr.^{-1}$. The line in *a* passes through the origin because radioactivity residing in the extracellular space has been subtracted from the uptake points.

The next series of experiments was performed in a 2.5 mM K Ringer's solution. Keynes (1954) showed that potassium influx roughly doubled in frog muscle in going from a 2.5 mM to a 5 mM K Ringer's solution. It was subsequently found (Sjodin, 1961), that the factor is closer to 1.8. Muscles resting in dishes of 2.5 mM K Ringer's solution showed slight potassium losses until a lower K concentration was reached and a steady state developed. When the influx-efflux type of experiment was performed on a muscle, the

rate of net potassium loss was somewhat greater due to the greater amount of experimental manipulation required. The results of the influx-efflux experiments in 2.5 mM K Ringer's solution are presented in Table II. The procedure used was identical to that used for the 5 mM K case. It is apparent from the data that influx from tracer plus the net efflux from analysis shows good agreement with the efflux determined by means of tracer. The data in Table II, therefore, also support the permeability model for frog sartorius muscle.

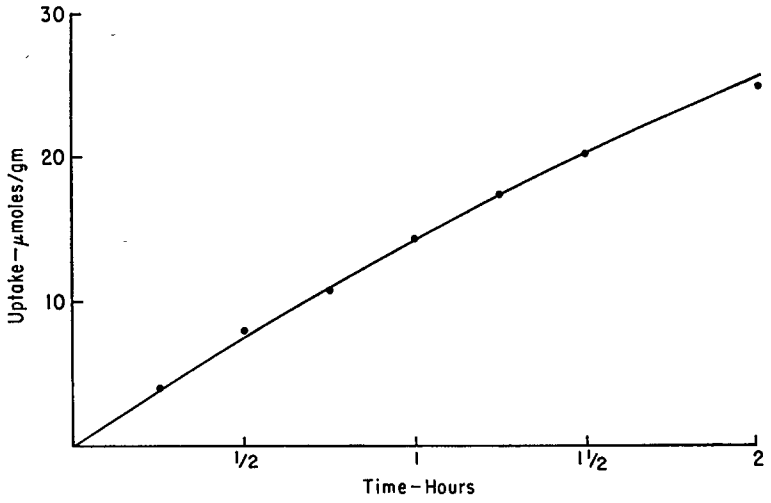

FIGURE 3. Potassium uptake determined by means of tracer K^{42} is plotted against time using a 5 mM K Ringer's solution. The muscle is the same as that giving the data for Fig. 2. The curve fitting the experimental points was calculated from equation (7) using the analytically determined value of $C_i(\infty)$ and the rate constant 0.18 $hr.^{-1}$ obtained from efflux measured on the same muscle. The value of $C_i(\infty)$ by analysis was 84.5 μmoles/gm.

As in the case of Table I, a statistical analysis of the results was made. Results obtained on the first twelve muscles of Table II were used to compute average values for the net fluxes and flux ratios reported. The last two muscles in the table were not included in the averaging because their net flux and flux ratio values lie about three standard deviations away from the mean of the first twelve values. If the average flux ratio and standard deviation are computed for the last two muscles and a *t*-test applied to the hypothesis that this mean differs significantly from the mean for the twelve muscles, a significant difference is indicated at a p level of about 0.037. This information, together with the fact that the experimental procedure itself biases results in the direction of high net fluxes and flux ratios, provides some justification for not including the last two muscles in the averaging. A *t*-test

applied to the average net flux of 0.99 ± 0.64 obtained from Table II indicates that this value is very significantly different from zero ($p = 0.001$). A test was also applied to the net fluxes obtained from the difference between unidirectional fluxes measured with tracer. The test showed that the mean net flux by this method is significantly different from zero at a p level of 0.005. The statistical test also showed that the mean from tracer measurement does not differ significantly from the mean obtained without use of K^{42} ions as tracers.

In going from a 5 mM to a 2.5 mM K Ringer's solution, influx and efflux are observed to drop by approximately the same factor. On the average,

TABLE II

POTASSIUM FLUXES IN 2.5 mM K RINGER'S SOLUTION

Muscle No.	Rate constant K^{42} loss	Influx ϕ_i^*	Net flux $\bar{\phi}$	$\phi_i^*+\bar{\phi}$	Efflux ϕ_o^*	ϕ_o^*/ϕ_i^*	Equilibration
	hr.$^{-1}$		*μmoles/gm hr.*				*per cent*
161AH	0.110	8.90	1.23	10.1	9.1	1.023	13.3
311AH	0.133	9.35	0.93	10.3	10.6	1.133	30.6
26AS	0.158	10.1	1.30	11.4	12.8	1.268	35.8
220AH	0.201	11.8	0.40	12.2	13.0	1.110	4.45
227AS	0.178	12.2	1.22	13.4	13.6	1.115	1.33
312AG	0.163	10.1	1.74	11.8	12.6	1.247	17.0
325AG	0.161	10.0	1.57	11.6	12.6	1.260	17.1
829CH	0.131	9.04	0.46	9.50	10.1	1.117	73.1
73DH	0.0768	6.07	0.043	6.11	6.06	0.999	14.0
710DH	0.1095	8.68	0.00	8.68	9.08	1.047	14.2
716DH	0.129	9.56	1.03	10.6	10.2	1.067	15.6
301AH	0.139	8.50	1.92	10.4	11.0	1.293	15.4
251AH	0.151	8.20	2.82	11.0	11.9	1.450	40.8
327AG	0.199	11.4	3.92	15.3	15.8	1.387	24.1
Average ± SD for first twelve muscles:			0.99±0.64			1.140±0.102	

* Refers to measurement using tracer ions.

however, influx drops by a slightly greater factor than does efflux in making the 5 to 2.5 change. This is borne out by the flux ratios given in Table II, the average flux ratio being 1.140 compared with 1.018 in the 5 mM K solutions.

Equation (6) for net potassium efflux can be tested using the 2.5 mM K data, even though a precise test was not possible using analytical data alone, for reasons stated. If a combination of tracer and analytical data is used, it is apparent that a test of the theory is possible. Influx from tracer, the rate constant for tracer loss, the elapsed time interval, and the initial potassium content given by the control muscle determine the final potassium content according to equation (6). The values so calculated can be compared with

the final measured analytical potassium content. The comparisons are made in Table III where all quantities required for the calculation are presented. For the most part, the experiments presented were either performed over long time intervals or involved muscles with somewhat large net fluxes. This was done to make the potassium content changes as large and, hence, significant as possible. The agreement of the data with equation (6) is satisfactory and within random variations between pair members and experimental error. The available experimental evidence, therefore, seems to support the theoretical basis for equation (6).

The data in Table II encompass a range in tracer equilibration of from 1.33 per cent to 73.1 per cent. No significant differences are apparent in the results of experiments performed on lightly K^{42}-loaded muscles when compared with experiments where tracer equilibration was more complete. In an effort to see how low a tracer equilibration would still lead to consistent flux results, a pair member was loaded with K^{42} in a 2.5 mM K Ringer's solution for 5 min. Efflux was then performed on this muscle by the usual technique. The semilogarithmic efflux plot is shown in Fig. 4. No departure from a first-order output curve is apparent throughout the experiment, after an initial extracellular space washout. The rate constant extracted has a value of 0.178 $hr.^{-1}$ which is well within the range for the other 2.5 mM K experiments. The degree of tracer equilibration for the 5 min. loaded muscle was 1.3 per cent. Since the single 5 min. uptake point does not provide a very certain estimate for influx, the other pair member was used to obtain an accurate estimate of influx. The 5 min. points for both muscles were nearly the same and it seems reasonable to suppose that both muscles had the same value for influx. The

TABLE III

NET POTASSIUM LOSSES COMPARED WITH THEORETICAL PREDICTION

Muscle No.	$C_i(0)$	Measured $C_i(t)$	Calculated $C_i(t)$	Rate constant k^*	Influx ϕ_i^*	t
		μmoles/gm		*hr.*$^{-1}$	*μmoles/gm hr.*	*hrs.*
311AH	79.8	73.0	74.0	0.133	9.35	7.43
26AS	81.5	71.3	69.3	0.158	10.1	7.57
301AH	86.5	75.0	73.0	0.139	8.50	5.50
211AS‡	74.4	50.4	50.7	0.199	8.19	6.33
161AH	82.5	79.0	81.6	0.110	8.90	5.50
221AS	80.5	66.7	68.8	0.182	10.7	4.28
3110AH	79.2	57.9	55.7	0.230	8.86	3.75
1912AS‡	77.3	66.3	66.9	0.184	10.5	4.00

* Refers to measurement using tracer ions.
‡ Experiment performed at 25.5°C.

net flux was obtainable since the pair members were exposed to Ringer's solution for significantly different total time intervals. The flux values obtained were: influx from tracer = 12.2 μmoles/gm hr., efflux from tracer (5 min. load time) = 13.6 μmoles/gm hr., and net efflux = 1.22 μmoles/gm hr. It is evident that good flux agreement is obtained for the case in which tracer equilibration was of the order of 1 per cent.

Experiments in a Potassium-Free Ringer's Solution When the concentration of potassium ions in the external solution bathing the muscle is zero, potassium influx may be taken to be zero and equation (6) reduces to a single

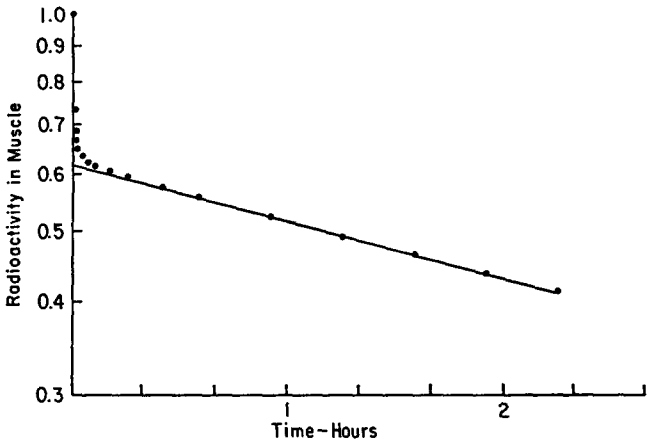

FIGURE 4. Radioactive potassium remaining in the muscle during efflux is plotted against time semilogarithmically. The muscle had been previously loaded with K^{42} ions for a period of 5 min. and the degree of tracer equilibration at the end of this period was 1.33 per cent. The K^{42} loss was to a 2.5 mM K Ringer's solution. The straight line drawn through the points yields a rate constant of 0.178 $hr.^{-1}$. An early rapid washout from the extracellular space is apparent. An additional rapid process not due to the extracellular space is also detectable (see text).

exponential relation for efflux which is now all a net efflux. According to the permeability theory being tested, the rate constant for tracer K^{42} ion loss should now be equal to that for total analytical potassium loss.

The experiments were performed as follows. A member of a pair was loaded in a 2.5 mM K solution containing K^{42} ions. The time of loading was 30 min. or greater. At the end of the uptake period, the other muscle of the pair was prepared for analysis and its potassium content served as an initial value. An efflux experiment using a potassium-free Ringer's solution was then performed with the loaded muscle. As in the previous cases, K^{42} efflux yielded a straight line for several hours when the logarithm of the counts per minute remaining in the muscle was plotted against time. The rate constant for K^{42}

ions so obtained can be compared with the rate constant for total potassium loss obtained from initial and final potassium content values assuming a single exponential process for net loss. Some later experiments provide direct justification for this procedure.

The K^{42} *versus* total K rate constant comparison is made in Table IV. Since the technique for deducing net potassium changes is subject to the errors previously discussed, the agreement between the rate constants appears to be within experimental error, as demanded by the permeability model. Further, a statistical analysis indicated that there is no significant difference between the mean of the tracer K^{42} rate constants and the mean of the rate constants obtained without the use of tracer. The diffusion model, on the other hand, predicts a multiexponential process for K^{42} loss and a marked

TABLE IV

TRACER AND NON-TRACER RATE CONSTANTS FOR K LOSS TO A K-FREE RINGER'S SOLUTION

Muscle No.	Rate constants		Equilibration
	K^{42} loss	K loss	
	hr.$^{-1}$		*per cent*
815AG	0.078	0.067	10.9
313AS	0.089	0.080	4.0
327AS	0.070	0.059	5.6
319BG	0.103	0.097	8.9
1113AS	0.062	0.067	5.6
1113BS	0.048	0.057	60.0
49AG	0.069	0.068	9.3

disparity between the kinetics for K^{42} loss and analytical K loss when tracer equilibration is far from complete.

An obvious variant of the above type of experiment suggests itself. If the Ringer's solution into which efflux occurs contains no potassium initially, any potassium acquired during an efflux interval must be derived from the muscle. Subject to possible interference from other ions, this potassium is analyzable by flame photometry. The filter used permitted accurate potassium analyses to be made in the 0 to 50 μM range in the presence of Ringer's solution concentrations of sodium and other ions. With deionized water used as a zero and 50 μM K Ringer's solution set at full scale, K-free Ringer's solution gave a deflection of 5 per cent of scale. Potassium, under these circumstances, can be determined to within the usual flame photometer error of ± 1 per cent. Standard solutions were made up in a range about 50 μM and the unknown readings were always within 10 per cent of a standard value.

The technique outlined permits some additional measurements to be made.

The kinetics of net potassium loss can be ascertained without the use of K^{42} ions as tracers. It was found that potassium loss to a K-free Ringer's solution can be described by a single exponential term as demanded by equation (6) under these conditions. If the muscle has also been loaded with K^{42}, the K^{42} and the total K rate constants can be obtained for the same muscle and compared. Since the K^{42} and total K content of the efflux samples is known, the specific activity can also be calculated and compared with the average specific activity of potassium remaining in the muscle at the end of the experiment. This is essentially the experiment of Harris and Steinbach (1956) with the exception that a physiological solution is employed in the present experiments.

It is relevant to examine the outcome for the above type of experiment predicted by the permeability model and by the diffusion model. Permeability theory applied to a single cell with a single well mixed intracellular compartment and an extracellular space predicts that the specific activity of the potassium collected from the muscle will initially have the value of that for the loading solution and will fall rapidly, with a time constant for extracellular space washout, to the constant value holding in the cell's interior. For a population of such cells showing a dispersion of rate constants, the fall to a constant specific activity could be delayed if a small portion of the fibers had radii much lower than the average and hence equilibrated with tracer at a rate much greater than the average. From the known radius dispersion occurring in forg's sartorius (Carey and Conway, 1954), it can be shown that this effect would be rather small and could not account for the specific activity remaining severalfold higher than the average value in the muscle for long periods, say hours. The diffusion model, difficult to distinguish from the case of two or more intracellular compartments, makes a rather different prediction. For a muscle loaded with K^{42} (2.5 mM K) for 30 min., the average tracer equilibration is about 10 per cent. A small completely equilibrated outer region of fiber would thus have a specific activity ten times greater than the average value holding within the fiber. The diffusion model predicts that a gradient in specific activity occurs within each fiber beginning with a high value at the surface and falling to low values at the center or in a central well mixed region. It is just this gradient that Harris and Steinbach (1956) claim to have demonstrated.

The first of these experiments performed employed an early 6 min. collection and five, 30 min. collection intervals. The kinetics of extracellular space washout indicate that the space should be largely cleared of tracer in the first collection interval. The results are shown in Fig. 5A for the case in which a 30 min. K^{42} loading was used and the average specific activity indicated an equilibration of 6.1 per cent. The specific activity in the efflux samples is observed to remain significantly higher than the average of the potassium remaining in the muscle (horizontal line on graph) for a period of 1 hour.

The effect is not nearly so pronounced as in the Harris and Steinbach (1956) experiments in which the initial specific activities were severalfold higher than the average value. In Fig. 5A, the specific activity is observed to fall rapidly to a value 1.5 times the average final value. A slower decline to values close to the average is then observable. In the second 30 min. interval the specific activity is about 1.2 times the average final values. Plotting the back-added counts per minute semilogarithmically yielded a straight line beginning with

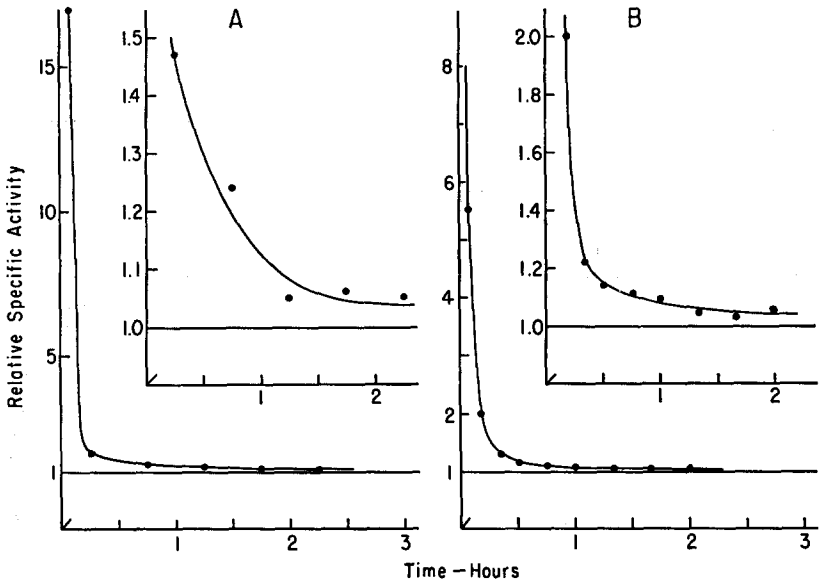

FIGURE 5. The relative specific activity of potassium ions lost from muscles loaded for 30 min. with K^{42} ions (2.5 mM K) to an originally K-free Ringer's solution is plotted against time of collection. Graphs A and B were obtained from different muscles as described in the text. The experimental conditions were the same for A and B except that more and shorter collection intervals were used to obtain graph B. The inset graphs in both A and B show the data plotted with expanded specific activity scales. The horizontal lines drawn at Relative Specific Activity = 1 represent, in each case, the final average specific activity of potassium remaining in the muscle.

the second interval. At 30 min., the specific activity discrepancy is evidently not of sufficient magnitude to cause a significant departure from single exponential potassium 42 output.

It is possible that some of the specific activity elevation observed in the first 30 min. interval in Fig. 5A is due to incomplete extracellular space clearance in the initial 6 min. interval. Since the specific activity of potassium in the extracellular space is about sixteen times higher than the muscle K specific activity, very little carry-over of space potassium into the second collection period could significantly raise the specific activity in the second

efflux sample. The next series of experiments allows a closer examination of the early kinetics by making the early collection intervals much shorter. Because of the great importance of this type of experiment in establishing the validity or non-validity of the permeability model, a typical protocol of experimental data is presented in Table V. The table indicates the time intervals of potassium collection, the counts per minute of K^{42} in each efflux sample, the total analytical potassium content in each collection sample, and the final counts per minute and the total potassium remaining in the muscle at the termination of the experiment. The specific activities obtained from Table V are plotted against time in Fig. 5B. Again, some early specific activity elevation is noted but the effect is less pronounced than in Fig. 5A due to the shorter early intervals. The loading time for the muscle giving the data in

TABLE V

SPECIFIC ACTIVITY OF POTASSIUM LOST FROM MUSCLES TO K-FREE RINGER'S SOLUTION

Collection sample	Collection time interval	Counts/min. in sample	K in sample	Specific activity	Apparent rate constant for total K loss
	min.		*μmoles*	*(counts/min.)/μmole*	*hr.*$^{-1}$
1	5	48,500	0.0510	951,000	0.194
2	5	8,450	0.0247	342,000	0.0948
3	10	7,860	0.0374	210,200	0.0727
4	10	6,930	0.0353	196,500	0.0694
5	15	9,750	0.0510	191,100	0.0678
6	15	9,670	0.0515	188,000	0.0697
7	20	11,010	0.0611	180,300	0.0633
8	20	11,150	0.0630	177,000	0.0665
9	20	11,450	0.0630	181,700	0.0682
Final remaining in muscle		471,500	2.740	172,000	

Table V was 30 min. and tracer equilibration was 9.2 per cent at the end of uptake. Several experiments affirmed that Fig. 5B may be taken as the true time course of the potassium specific activity decline when efflux is performed into an initially K-free medium and muscles have been loaded with K^{42} for 30 min. The early elevation of the specific activity is well within the range that can be accounted for by the extracellular space and a population of fibers having a dispersion of radii and, hence, rate constants.

By back-adding the analytical potassium lost by the muscle to the final potassium content, the logarithm of the potassium remaining in the muscle can be plotted against the time to obtain the kinetics of net potassium loss. The straight line so obtained affirms the single exponential nature of net total potassium loss after an initial period of about 15 min. The rate constant for K loss obtained by plotting is 0.0678 $hr.^{-1}$ which compares with the rate

constant for K^{42} loss of 0.0690 hr.$^{-1}$. These values can be compared with the figures in the last column of Table V which gives the apparent rate constant for total K loss in each interval. Though the agreement is almost within experimental error, several experiments indicated that the tracer rate constant is significantly higher under these experimental conditions by up to 5 per cent. It seems reasonable to conclude that a systematic error in potassium efflux determined by the tracer method, in the 30 min. loading time and zero potassium concentration case, can occur. The error is estimated to be not greater than 5 per cent and is in the direction of an estimate of efflux that is somewhat too high. With potassium present in the efflux medium, the rate constants are higher and the slight early specific activity elevation would be expected to introduce systematic errors that are below 5 per cent and, hence, well within experimental error. Tracer loading times larger than a half-hour would, of course, reduce these modest discrepancies even more. The 30 min. tracer loading technique followed by efflux into a potassium-free medium would seem to provide a rather severe test of the permeability model.

Experiments with a Net Gain of Potassium Ions When frog muscle fibers are placed in a Ringer's solution containing potassium chloride at an elevated concentration, they are found to rapidly gain KCl until the external and internal KCl products are equalized (Boyle and Conway, 1941, and Adrian, 1960). The rate of the additional KCl entry is dependent on the external KCl concentration. Adrian (1960) studied inward net chloride movement when the KCl concentrations were 100 mM and 50 mM. In the 100 mM KCl solution, the additional entry was complete after 3 hours. In the 50 mM KCl solution, the initial entry rate was lower but the entry was complete after 2 hours. As these were the only concentrations studied, the still lower concentration of 20 mM was used in this investigation. The technique employed was identical with that of Adrian (1960), except that potassium movement was followed instead of chloride movement. Muscles whose initial potassium contents were the same, as indicated by the paired control, were analyzed for potassium after being exposed to the high KCl solution for varying time intervals. The time course of the KCl entry was similar to that reported by Adrian (1960) for *R. temporaria*, the initial entry rate being about 60 per cent that of the 50 mM rate reported by Adrian. The final value, however, was reached in one-half the time, the rapid entry being complete after 1 hour. The average gain at the new steady state was to a K value 1.096 times the initial value (average of eleven muscle pairs). This is in good agreement with the approximately 10 per cent KCl gain required to equalize internal and external KCl products.

The question now arises as to the ability of K^{42} ions to trace unidirectional

potassium fluxes which are consistent with the observed net potassium gains, as demanded by the permeability model. As might be expected, the constant influx assumption which led to equation (6) for net potassium loss is not applicable to the case of rapid KCl gains. The increment of K inward movement associated with the rapid KCl gain would be expected to be in addition to the K-K exchange component of influx (Sjodin, 1961; Harris and Sjodin,

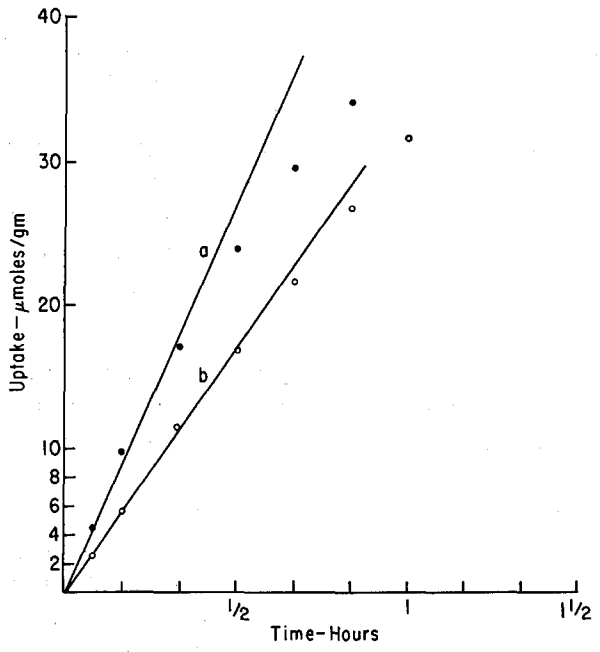

FIGURE 6. Potassium uptake from a 20 mM K Ringer's solution determined from tracer K^{42} movement is plotted against time. Curve *a* was obtained by placing a muscle directly into a labeled 20 mM K solution from a 2.5 mM K Ringer's solution. Curve *b* was obtained using the other member of the pair by pretreating it for a period of 1 hr. in an unlabeled 20 mM K Ringer's solution prior to measurement of K^{42} uptake. The lines drawn through the points are determined by the initial uptake points in each case. Extracellular potassium has been subtracted in each case. The initial slope of curve *a* is 54 μmoles/gm hr. The initial slope of curve *b* is 35 μmoles/gm hr.

1961). It proved an easy matter to demonstrate this experimentally. One member of a pair of muscles was allowed to remain in a 20 mM K Ringer's solution for a period of 1 hour prior to the determination of influx. The other member of the pair was transferred immediately from a 2.5 mM K solution to a 20 mM K solution labeled with K^{42} ions and influx was performed in the usual manner. The muscle pretreated with 20 mM K Ringer's solution should be in a steady state after 1 hour and should have a K influx below the initial uptake slope for the non-pretreated muscle. The data plotted in Fig. 6 show

this to be the case. The muscle undergoing rapid KCl gain has a very significantly greater initial K influx than the paired member previously brought to the steady state.

It must next be shown that these fluxes are in quantitative agreement with the permeability model. Influx was measured on muscles pretreated in 20 mM K Ringer's solution. Since potassium contents do not change after 1 hr. in the 20 mM K solution, muscles are in the steady state and efflux should equal influx. After K^{42} uptake, efflux was measured at the same external concentration on the same muscle. The rate constant for K^{42} efflux obtained was multiplied by the potassium content determined analytically and these values are compared with the influx values as before. The comparison is made in Table VI where the agreement with permeability theory is adequate. In view of Fig. 6 it is obvious that influx and efflux would not agree had influx as initially determined on non-pretreated muscles been used.

TABLE VI

POTASSIUM FLUXES IN 20 mM K RINGER'S SOLUTION

Weight	Rate constant K^{42} loss	Influx	Efflux
gm	$hr.^{-1}$	*μmoles/gm hr.*	
0.062	0.440	35.2	36.2
0.039	0.638	53.0	51.7
0.051	0.343	28.4	27.8
0.049	0.380	34.2	32.8

The increased initial rate of K^{42} uptake in the muscle not pretreated in inactive 20 mM K Ringer's solution must reflect the rapid net inward KCl movement. To verify this quantitatively, an influx experiment was performed on non-pretreated muscles followed by efflux determination as in previously described experiments. The net quantity of potassium gained was obtained from the difference between the steady-state potassium content in the 20 mM K solution and the initial value as given by the paired control muscle exposed only to 2.5 mM K Ringer's solution. The kinetics applied were obtained from the time course of the net movement previously determined. If the net movement is subtracted from the total uptake under these conditions, the influx due to exchange alone should be obtained. Since the efflux rate constant does not change with time in the 20 mM K solution, the total initial uptake rate minus the net uptake rate should equal the initial value for efflux and these fluxes should be approximately the steady-state fluxes. The kinetics of the exchange or steady-state uptake should follow equation (7). The efflux rate constant and the final potassium content specify the uptake curve. The net uptake is subtracted from the total uptake in Fig. 7 and

the points obtained are compared with predictions of equation (7). The good agreement obtained is typical of several experiments of this type and supports the permeability model for frog sartorius muscle.

Experimental Errors The possible errors associated with net flux determinations when these fluxes are small have been discussed. In the potas-

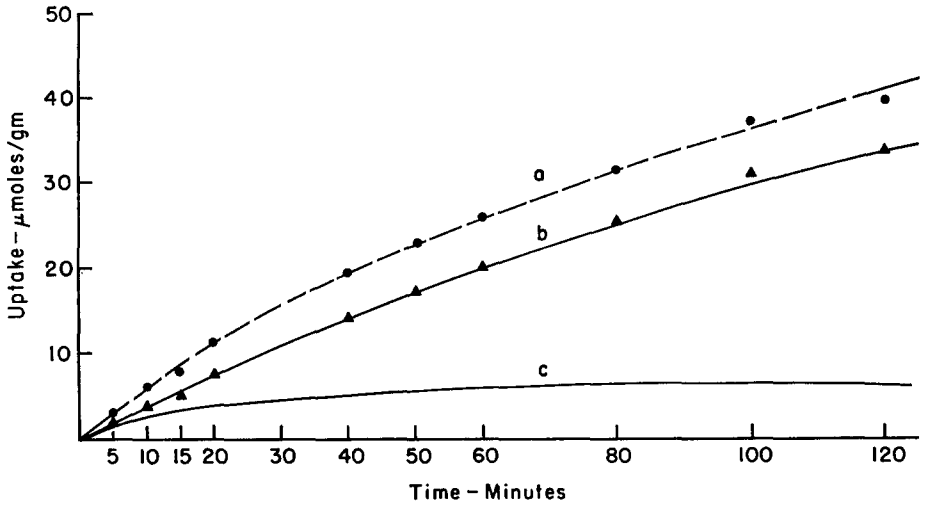

FIGURE 7. Curve *a* is a plot of tracer-measured uptake from a 20 mM K Ringer's solution on a non-pretreated muscle. The curve drawn is fit to the points. The significant amount of potassium residing in the extracellular space at this concentration is subtracted from the total measured uptake before plotting. The total K in the space is known accurately since the extracellular space fraction is known. The time course of space equilibration is known and the kinetics are applied to the initial portion of the curve in subtracting space counts from total counts. After 10 min. the space counts remain constant at the equilibrated value. Curve *c* is the net gain of potassium ions obtained by the method discussed in the text. No points occur on curve *c* because only the final value and the known time course are used in constructing the curve. Curve *b* is obtained by subtracting the net potassium uptake at each point from the total uptake. That is, curve *b* = curve *a* − curve *c*. The line drawn through the points in curve *b* is a plot of equation (7) using the measured value of $C_k(\infty)$ (the initial K content of the paired control muscle) and the rate constant for K^{42} loss to a 20 mM K Ringer's solution determined on the same muscle. The values are: $C_i(\infty) = 75.1$ μmoles/gm and $k = 0.30$ hr.$^{-1}$.

sium-free solution experiments, the net fluxes are considerably larger and experimental error is estimated to be ±6 per cent for the initial-final value method. This value was arrived at by comparing the "difference method" with the more direct method of determining the potassium lost to a K-free solution analytically. The accuracy of the latter method is simply that of

flame photometry or ±1 per cent. Net flux in the KCl gain experiments is also estimated to be accurate to ±6 per cent.

Influx from K^{42} movement is accurate to the extent that a straight line can be drawn through some five or six initial uptake points or, alternatively, to the extent that a longer uptake can be fitted by a single exponential relation. The limit of error was arrived at by having two individuals plot the same data independently and report the best value of influx and the range of possible values. The ranges for the two individuals were comparable and the "best" values chosen by each were always within this range of each other. The most frequent value of the range was 5 per cent and influx is taken to be accurate to ±2.5 per cent.

Determining efflux from K^{42} movement involves two factors, a rate constant and the potassium content. The determination of the rate constant for K^{42} efflux was unambiguous since straight lines were obtained for periods of hours. The "two individuals method" indicated that the rate constants could be determined to within ±1 per cent. The potassium content used, though accurate to within ±1 per cent, is that of the control member of a pair. The standard deviation of potassium contents of pairs initially was ±1.5 per cent. This uncertainty leads to a total possible error in potassium content of about ±2.5 per cent. These errors lead to an error of ±3.5 per cent in the product which is thus the error estimated for efflux.

It is evident that influx and efflux agree experimentally if they agree to within (2.5 + 3.5 = 6) 6 per cent. Most of the agreement reported in this work is to within this range. In the section dealing with experiments in a potassium-free medium, it was stated that, for 30 min. K^{42} loading periods, a systematic error of about +5 per cent can occur as a consequence of treating the population of sartorius fibers as a single fiber having an average radius and an average uniform specific activity. This error is just within the limit of experimental error. For more complete tracer equilibrations, the systematic error becomes less than experimental error.

Radioactive counting rates were such that tens of thousands of counts were made as routine. In an uptake experiment muscles acquired as routine radioactivity up to 500,000 counts per min. The activity lost during an efflux interval was usually sufficient to give a total count of at least 20,000 counts in a 10 min. counting period. At this counting level, the error is less than 1 per cent and this represents the maximum error possible from this source.

DISCUSSION

The results of this investigation are in general agreement with those of Keynes (1954), and of Hodgkin and Horowicz (1959*a*) on frog sartorius fibers. The whole frog sartorius muscle, to a very good approximation, obeys single exponential permeability kinetics. The experimental demonstration of this is

that influx, as determined by the initial linear slope of the K^{42} uptake curve, agrees with efflux when the rate constant for efflux is applied to the whole of the muscle potassium and the cells are in the steady state as determined by direct chemical analysis for potassium. Flux agreement with the permeability model has been demonstrated when muscles are in the steady state, when muscles are undergoing considerable potassium loss, and when they are gaining significant quantities of potassium. It seems extremely unlikely that the agreement obtained is fortuitous in all concentration ranges.

If single fiber units follow a single exponential law, as deduced by Hodgkin and Horowicz (1959*a*), it should not be surprising that a population of such units also follows the exponential law. It has been emphasized that the dispersion of fiber sizes that occurs in whole muscle will not cause significant departures from the kinetics shown by the individual units (Carey and Conway, 1954; Creese, Neil, and Stephenson, 1956; and Harris, 1957).

Since Hodgkin and Horowicz (1959*a*) measured the rate constant for K^{42} loss on several sartorius fibers of varying diameter, a direct comparison with the present whole muscle values is available. For a 74 μ fiber at 16°C in a 2.5 mM K Ringer's solution, they obtained a time constant of 398 min. For a 90 μ fiber at 20°C, a value of 394 min. was obtained. The mean value for several fibers of various diameters and temperatures close to 20°C was around 400 min. This value, converted to rate constant dimensions, is equivalent to 0.15 $hr.^{-1}$. The fourteen muscles in Table II were in a 2.5 mM K Ringer's solution at 21.5°C and the average value for the rate constant for K^{42} loss is 0.146 $hr.^{-1}$ which is in good agreement with the average value reported for single muscle fibers. Hodgkin and Horowicz also performed influx and efflux measurements on the same fiber, as in the present work with whole muscle, and obtained agreement to within their experimental error.

Taken together, the experiments in the present investigation lend support to the notion that whole sartorius muscle follows closely the kinetics obeyed by single constituent fibers and that the kinetics need be no more complicated than those for a first-order permeability process. The experiments reported do not necessarily rule out more complicated kinetics if these lead to results within experimental error of the predictions of the simple kinetics. Harris (1953), for example, concluded that at least two exponential terms are required to fit K^{42} uptake data. This is certainly true in the present investigation if the extracellular space of the muscle is taken into account. The effect of K^{42} washout from this space is clearly seen in Fig. 4, where K^{42} loading was very light. The early process reflects, in the main, a space washout with a time constant of 3 to 4 min. The space should be essentially cleared of K^{42} after 15 min. There is some indication that another departure from single exponential kinetics is involved as the first two points after 15 min. lie significantly (about the width of the dots) above the straight line. This effect was

repeatedly noticed in cases in which K^{42} loading was light, say half an hour or less. A straight line was always obtained from 30 min. to termination, however, and the early deviation is slight. It might well be associated with the early specific activity elevation noted in the K-free experiments (Fig. 5).

With regard to influx, the initial uptake was always approximately linear and the uptake line intersected the concentration axis at a point showing good agreement with the quantity of K in the extracellular space which was estimated on the control muscle in each case. Harris (1953) reported that a deviation from exponential kinetics occurs that cannot be accounted for by the extracellular space. Part of this difficulty may be due to the use of too low an extracellular space value which was apparently not measured in each case. It should also be emphasized that first-order uptake kinetics can only be expected if muscles are in the steady state. Muscles not in the steady state, however, were shown to lose K in accordance with a relation derivable from the permeability model.

It is somewhat surprising to find that muscle fibers obey a simple kinetic law with regard to ion movements in view of the possible structural complexity of the fibers (Huxley and Taylor, 1958; Porter and Palade, 1957; Adrian and Freygang, 1962). It should be noted, however, that simple kinetics for ion movements do not constitute evidence for a simple structural model. Permeability kinetics may be expected when one membrane offers by far the greater resistance to passage. Any number of additional membranes and compartments may be present as long as their diffusivities are much higher than that occurring at the permeability barrier.

The experiments reported, in which the specific activity of potassium ions lost to an originally K-free Ringer's solution was measured, are interesting because they point to a possible second intracellular compartment. The early elevation of specific activity observed is qualitatively similar to the behavior noted by Harris and Steinbach (1956). Quantitatively, however, the effect is considerably less and no explanation is available for the difference. The experiments reported here were consistently reproducible and were performed in a physiological medium. Harris and Steinbach used rather unphysiological solutions, including distilled water. It does not seem likely, however, that this is the sole explanation. To obtain first-order output curves, it was necessary to take the precautions mentioned in the Methods section. Muscles could not be allowed to come into contact with material surfaces without expecting injury of some surface fibers. When the work was in an early stage, it was found that muscles mounted on frames which allowed contact with tube surfaces during uptake showed evidence of multicompartment behavior during efflux. In some early experiments, a high bicarbonate Ringer's bubbled with 5 per cent CO_2 was used. When the bubbling rate was high, muscles showed a multiexponential K^{42} output curve which could

only be attributed to injury and elevated permeability of a fraction of the fibers.

It is difficult to decide whether the early specific activity elevation noted in this investigation is due to a small proportion of rapidly equilibrating fibers or, alternatively, whether it is due to a small more rapid component of initial K^{42} efflux from each individual fiber. The experiments of Hodgkin and Horowicz (1959*a*) on very lightly K^{42}-loaded single fibers hint that the first efflux point (about 5 min.) may be somewhat above the logarithmic line, though counting errors preclude a definite conclusion. The time constant for single fibers for the first 25 min. after a short exposure to K^{42} was of the same order as that for more complete K^{42} loadings and longer efflux periods. The early rate constant, however, would only have to be 10 to 20 per cent higher to make the presently observed specific activity elevation attributable to each individual fiber. The single fiber values are perhaps not precise enough to discover the effect, should it be present. At any rate, efflux would have to be followed on the same fiber for periods beyond the 25 min. point to see whether there was any tendency to "flatten" to a somewhat lower rate.

It is tempting to conjecture that the early specific activity elevation does occur in each single fiber and, thus, that each fiber does contain a small additional cellular compartment. Such a compartment might be associated with the system of tubules known as the endoplasmic reticulum. On the basis that individual cellular compartments are involved, the data in Table V can be used to separate two processes, one slow and having a single rate constant representing the bulk of the muscle potassium at uniform specific activity, and a faster process responsible for the initial elevation of specific activity. As the nature of the compartment, should it exist, is unknown, it is assumed to be in parallel with the main compartment to facilitate calculation. The analytical potassium loss in Table V is first back-added and the results used at each point to calculate the amount of potassium lost with the rate constant of the bulk of the potassium. These amounts of potassium are then used to calculate the number of counts per minute associated with the assumed constant specific activity fraction using the final values of specific activity. The extra initial counts and the extra initial total potassium are then obtained by subtraction from the total early output. The extra total potassium is summed and the quantity residing in the extracellular space is subtracted to obtain the fast cellular potassium. The fast cellular fraction amounts to about 0.5 per cent of the total muscle potassium. The extra tracer counts, when plotted semilogarithmically, yield a continuous curve with monotonically decreasing slope. This type of curve is indicative of a multiexponential process typical of several compartments or of a diffusion-limited process. The curve can be fairly well approximated by two single exponential proc-

esses with time constants of 2.6 min. and 20 min. The time constant of 2.6 min. can be identified with the washout of the extracellular space. The time constant of 20 min. approximates the process which elevates the specific activity in the early efflux samples. The process would be largely complete in 40 min. agreeing with the curves presented in Fig. 5.

The somewhat crude compartmental analysis made is not offered as a demonstration for the existence of complicating cellular compartments. The deviations noted in this investigation could, as easily, be attributed to a dispersion of fiber sizes. Clearly, more tracer investigation on single muscle fibers would be desirable. Should additional cellular compartments exist, they must have very nearly the quantitative properties discussed, with regard to K^{42} movement. The volume of space required to hold 0.5 per cent of the muscle potassium on a single fiber basis depends, of course, on the concentration of potassium. At the bulk concentration of cellular potassium, the space would have to be about 0.5 per cent of the fiber volume. This is somewhat larger than the space postulated by Adrian and Freygang (1962), to account for anomalous rectification in muscle. Also, the space postulated by Adrian and Freygang is supposed to contain potassium at Ringer's solution concentrations. Such a space could not accommodate the rapidly moving potassium presently observed. A region of comparable volume having a high concentration of fixed negative charges, however, could easily accommodate the additional potassium (Harris, 1963). The data do not by any means rule out the possibility that a small fraction of the cellular potassium is involved in a diffusion process within the lumen of the fiber reticular system. The point to be emphasized is that, whatever the nature of the effects observed they are not of sufficient magnitude to seriously disturb the method of treating the whole sartorius muscle as a single fiber with average properties.

Some mention should be made of the flux ratios observed in this work. The average flux ratio in a 5 mM K Ringer's solution was 1.018. This corresponds to a membrane potential within 0.5 mv of the equilibrium potential for potassium ions, on the basis that no potassium ions are actively transported. This is of the order of experimental error in measurements of membrane potential and, hence, on the average, sartorius muscle fibers should, for the case of no active potassium transport, have a membrane potential in 5 mM K Ringer's solution that corresponds to the equilibrium potential for potassium ions. For a fiber kept in the steady state in a 5 mM K solution from the time of excision, the value of E_K should be about 83 mv. Adrian (1956) reports a measured average value that is somewhat lower than this, very nearly 80 mv. Hodgkin and Horowicz (1959*b*) report a range of values of from 74 to 80 mv, but the external chloride concentrations were less than in the present work. A 3 mv departure from E_K would give a passive flux ratio of 1.128. Values this high were rarely encountered in this work as can be seen

from Table I. The obvious conclusion would appear to be that a small component of active inward potassium movement occurs in most muscles.

Mullins and Noda (1963), however, have obtained some evidence that there may be another explanation. The major internal anions of muscle fibers are derived from salts of phosphate esters. Activity coefficients for potassium ions at a concentration of 140 mM in the presence of creatine phosphate ions were measured using a potassium-selective electrode. A value of around 0.6 was obtained. As the value for the activity coefficient in the external solution is about 0.75, the calculated value for E_K is seriously affected. For a fiber in good condition, E_K becomes about 79 mv in a 5 mM K solution, using the lower activity coefficient for the interior of the fiber. This value of E_K is almost within error of Adrian's (1956) average value, and hence, is consistent with the present data on flux ratios without assuming active transport for potassium ions.

When the Ringer solution concentration of potassium ions is 2.5 mM the average flux ratio observed in this work is 1.140. The average membrane potential at this potassium concentration is close to 92 mv. Using equal activity coefficients for internal and external potassium ions, E_K should be about 101 mv for a fiber in good condition. The 9 mv departure from E_K deduced leads to a flux ratio of 1.43 which is well beyond the average ratio measured for fibers in good condition. Again one would have to conclude that an active inward transport process for potassium ions is operative. Using the Mullins and Noda (1963) value for the internal activity coefficient, however, one calculates a value of 94 mv as the E_K for a fresh sartorius fiber. The resulting 2 mv departure of the membrane potential from E_K indicates a passive flux ratio of around 1.1, in good agreement with the average measured value. Again, the use of the probably more accurate value for the internal activity coefficient obviates the need to postulate an inwardly directed potassium pump.

When a substantial net efflux of potassium ions occurred, it was found that equation (6) described the loss of potassium adequately. This equation was derived on the assumption that influx is constant. The assumption would be theoretically valid if the outside concentration, the potassium permeability coefficient, and the membrane potential were constant for muscles kept in Ringer's solution. Outside concentrations did not change and efflux data indicated that P_K does not vary significantly with time. Mullins and Noda (1963) showed that the membrane potential of fibers kept in Ringer's solution did not vary over long periods of time. What does vary with time is the internal potassium concentration which was termed earlier the net flux adjuster. The mechanism for the action is presumably that the internal concentration determines the electrochemical gradient for potassium movement. A very fresh muscle in 2.5 mM K Ringer's solution will have an E_K more

negative than the membrane potential and the electrochemical gradient will favor potassium loss. The loss occurs in accordance with equation (6) until E_K corresponds with the constant membrane potential, at which point a steady state ensues.

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