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MEMBRANE POTENTIAL AND CONDUCTANCE DURING TRANSPORT OF SODIUM, POTASSIUM AND RUBIDIUM IN FROG MUSCLE

BY R. H. ADRIAN AND C. L. SLAYMAN*
From the Physiological Laboratory, University of Cambridge

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SUMMARY

1. Muscles with high intracellular sodium concentrations can extrude sodium into solutions which contain 10 m-equiv/l. of either potassium or rubidium. Potassium or rubidium replaces the extruded intracellular sodium. These cation movements take place equally well when the external anion is chloride or sulphate, though muscles deteriorate if left for long periods in sulphate solutions.

2. Measurements of intracellular potentials during extrusion of sodium into solutions containing potassium show:

(*a*) an internal potential more negative than the potassium equilibrium potential (E_K); at 20° C the difference is nearly 20 mV.

(*b*) that a difference between the membrane potential and E_K is dependent on temperature and is abolished by 10^{-5} M ouabain.

(*c*) an internal potential which becomes more negative in the presence of 0·1 % cocaine, a concentration of cocaine which substantially increases the membrane resistance to potassium movement.

In the absence of potassium or rubidium no such hyperpolarization occurs.

3. When muscles extrude into solutions which contain rubidium they have internal potentials which are 10–20 mV more negative than when extruding sodium into corresponding solutions containing potassium.

4. Measurements of electrical conductance in the potassium solution suggest that the electrochemical potential difference for potassium ions may be large enough to account for the measured inward potassium movements during sodium extrusion. The reliability of the measurements does not, however, exclude the possibility that some part of the inward potassium movement is chemically linked to outward movement.

5. Measurements of membrane conductance in solutions containing rubidium, and of net movements of rubidium in the presence and absence of ouabain, lead to the conclusion that at least 90 % of the inward rubi-

* Present address: Department of Physiology, Western Reserve University, Cleveland, Ohio, U.S.A.

dium movement during sodium extrusion must be chemically linked to the sodium movement.

6. The hyperpolarization during extrusion of sodium could be explained on the basis of a fall of the potassium or rubidium concentration in a region of the extracellular space immediately external to the membrane. It is argued that certain characteristics of the hyperpolarization make it difficult to explain the hyperpolarization on this basis alone, though some part of it may be due to extracellular depletion of either potassium or rubidium.

The main conclusion is that the sodium pump is capable of transferring electric charge across the membrane in which it is operating, but that, in a given time, the net charge transferred is less than the charge on the sodium ions that the pump has transported, by an amount that corresponds to the charge on the potassium or rubidium ions chemically transported by the pump.

INTRODUCTION

Dean (1941) first pointed out that, if muscles are not impermeable to sodium ions, they must be able to transport sodium ions against their electrochemical potential gradient. Only by means of a sodium pump could muscles maintain a low internal sodium concentration. Many experiments (Desmedt, 1953; Steinbach, 1940, 1951; Conway & Hingerty, 1948; Frazier & Keynes, 1959) have shown that muscles, loaded with sodium, can produce a net outward movement of sodium, even though the electrochemical potential of sodium is unequivocally greater in the external solution than inside the fibre. It is generally accepted that this sodium transport requires energy from metabolism, but it is not at all clear how the sodium is transported. Potassium from the external solution replaces the sodium pumped out of the fibre, but it is unclear whether the internal or the external electrochemical potential of potassium is the greater. More recent experiments (Kernan, 1962; Keynes & Rybová, 1963; Hashimoto, 1964; Cross, Keynes & Rybová, 1965; Mullins & Awad, 1965; Frumento, 1965) have shown that in frog muscle during replacement of accumulated sodium by potassium the potential inside the fibre may be sufficiently negative to make the electrochemical potential of potassium in the external solution greater than that inside the fibre. It is therefore possible that the net inward movement of potassium is the result of this gradient of electrochemical potential. The observations have been interpreted to mean that the outward movement of sodium is 'electrogenic', that is, that the transport mechanism itself is capable of separating electric charge across the membrane (Kernan, 1962; Mullins & Awad, 1965; Frumento, 1965). Indeed Conway (1964) has gone so far as to suggest that the sodium pump is wholly electrogenic in that sodium, and not potassium,

is involved in the transport mechanism. However, Cross *et al.* (1965) describe experiments in which potassium replaces accumulated sodium under circumstances where the passive movement of potassium would always be outward, and they conclude that the pump mechanism can move potassium as well as sodium. The existence of a hyperpolarization in fibres of muscles which are producing net sodium movements does not necessarily mean that all the potassium is moving passively into the cell across the diffusion barrier in which the pump is operating. The resistance of this barrier might be large enough to make the potassium movement as well as the sodium movement, require energy from metabolism, even though the potassium movement takes place down the gradient of electrochemical potential.

There are also several experimental complications which may invalidate any simple interpretation of the hyperpolarization during pumping. It is not clear, for instance, that the changes in average intracellular concentration have the same time course as the changes of concentration in the surface fibres in which the potentials are measured. It is also possible that the operation of the pump might itself change the concentrations of ions in the immediate vicinity of the cell membrane.

The experiments reported in this paper give additional evidence about the hyperpolarization which occurs during net outward sodium movement. They were done because it seemed that the use of rubidium in place of potassium would help to distinguish between some of the postulated pumping mechanisms. It has been shown that movements of rubidium can accompany the operation of the sodium pump (Solomon, 1952; Lubin & Schneider, 1957; Relman, Lambie, Burrows & Roy, 1957). Adrian (1964) has found that the permeability of muscle fibres to rubidium is much less than their permeability to potassium. If, therefore, muscle fibres exchange internal sodium for an external cation because the sodium pump generates a current of sodium ions, the pump would generate a greater potential when moving rubidium in than when moving in potassium at an equal rate. Alternatively, if the pump could generate only a certain potential, the rate of exchange of rubidium for sodium would be much slower than the rate of exchange of potassium for sodium. A quantitative comparison between pumping hyperpolarization, membrane resistance, and apparent potassium and rubidium movements has been made. The results do not exclude the possibility that all the inward potassium movement during extrusion of sodium could be driven by the potential generated by the sodium pump. However, at least 90 % of the inward rubidium movement must occur by an electrically neutral exchange for sodium.

A preliminary account of this work has already been presented (Adrian & Slayman, 1964).

METHODS

All experiments were done on sartorius muscles of English frogs (*Rana temporaria*). When a muscle was to be used for studies of membrane potential or resistance, the split pelvis was dissected with the muscle attached. Otherwise the tibial and pelvic tendons both were freed and tied with cotton threads. For further handling the muscles were usually tied on to glass frames and were stretched to 120 % of their rest length.

Solutions. The muscles were dissected in a phosphate-buffered Ringer solution (solution *A*, Table 1) described previously (Adrian, 1956). Sodium-loaded muscles were prepared by soaking for 1 or 2 days at 0–2° C in a potassium-free, carbonate-buffered solution (solution *B*) described by Carey & Conway (1954). Up to twelve pairs were kept in 1 l. of solution, which was changed every 12 hr and was stirred with a stream of 95 % O_2 and 5 % CO_2 gas mixture. Care was taken that the gas bubbles did not strike the muscles directly. Muscles for analysis only were usually transferred directly into potassium or rubidium-containing recovery solutions at controlled temperatures. The recovery solutions (solutions *C*, *D*, *F*, and *G*) contained either 10 m-equiv/l. of potassium or 10 m-equiv/l. of rubidium, but the other constituents varied, and depended upon whether chloride or sulphate was the major anion present. The chloride solutions (*C* and *D*) were similar to the soaking solution (*B*), except that the NaCl concentration was reduced by 16 mM to allow for 10 mM-KCl or RbCl and 12 mM-glucose. The sulphate recovery solutions (*F* and *G*) were based on solutions of Hodgkin & Horowicz (1959) and differed from them only in that 13 mM sucrose was replaced by 13 mM glucose. Muscles allowed to recover in sulphate solutions were transferred from the potassium-free soaking solution (*B*) to a low-potassium sulphate solution (*E*) at 1–5° C for 2–3 hr before final transfer to the recovery solutions (*F* and *G*). This step was designed to remove extracellular chloride and to reduce the intracellular chloride. When membrane potentials were to be measured in chloride recovery solutions, muscles were transferred into cold recovery solution for up to 1 hr, in order to allow KCl and the membrane potential to reach steady values before the temperature was raised. In several experiments the transport system was inhibited by 10^{-5} M ouabain, added as an alcoholic solution (5 mg ouabain/ml. ethanol) to the recovery solution. Chloride-free solutions (*H* to *S*, Table 1) containing different potassium or rubidium concentrations were used to determine the membrane potential as a function of external concentration. Most of the solutions were prepared in 1–10 l. quantities and stored at 4° C for up to 3 weeks before use. Solutions containing glucose were prepared at the time of use.

Estimation of intracellular cations. After each experiment the muscles were blotted, weighed, and dried to constant weight at 90° C. They were ashed in platinum crucibles overnight at 540° C. The ash was dissolved in 5·0 ml. of 10 mM-CsCl solution. Sodium (589 mμ), potassium (768 mμ), and rubidium (780 mμ) were estimated in this solution with a Zeiss PMQ II flame spectrophotometer, operating over the range 0·1–1·2 m-equiv/l. Emission was a non-linear function of concentration, so that seven spaced standards were used to permit accurate interpolation of concentrations. The 10 mM-CsCl, which was present in the standard solutions as well as in the sample, enhanced rubidium and potassium by about 100 %, but made potassium emission independent of rubidium concentration, and vice versa.

To calculate the intracellular concentrations, the extracellular space was assumed to be 12·5 % of the muscle wet weight for both freshly dissected and soaked muscles (Desmedt, 1953). The intracellular concentrations were expressed in m-equiv/kg fibre water. A comparison of the sodium content of recently dissected muscles and sodium-loaded muscles, both recovered for 4 hr in solution *C* (see Tables 3 and 4), suggests that this estimate is a reasonable approximation. The sodium content of both sets of muscles was 15·3 m-equiv/kg muscle, so that the maximum extracellular space possible in both sets of muscles would have been only 14·5 % (Na concentration of solution *C* is 105 m-equiv/l.).

Measurements of membrane potential and resistance. Conventional methods employing KCl-filled glass micro-electrodes were used to measure membrane potentials (Adrian, 1956). A potentiometric pen-recorder was used to obtain a record of each fibre impalement. Electrodes were selected with tip potentials less negative than -5 mV. The recording chamber contained channels for two muscles: the channels were arranged so that the temperature and composition of the solution flowing continuously along each groove could be altered independently. The arrangement allowed the membrane potentials in both muscles of a pair to be followed at the same time.

Membrane resistance was measured by the method of Adrian & Freygang (1962), using three intracellular electrodes. The three electrodes were inserted in line at 390, 780 and 1170 μ from the pelvic end of a sartorius muscle fibre. Current pulses were passed through the electrode farthest from the end of the fibre, and the resultant changes in membrane potential were measured at the other two electrodes (V_a at 390 μ, V_b at 780 μ; $V_b - V_a = \Delta V$, $V_a = V$). ΔV is, to a good approximation, proportional to the membrane current causing the membrane potential change V. $\Delta V/V$ is therefore a good approximation to the membrane conductance. Assuming a fibre diameter of 80 μ and an internal specific resistance of 250 Ω cm, a ΔV of 1 mV is equivalent to a membrane current density of 3·5 μA/cm^2. A membrane current density of 1 μA/cm^2, all carried by one species of univalent ion, is equivalent to a net flux of that ion of 10 p-mole/cm^2.sec or, for an 80 μ fibre, a rate of change of internal concentration of that ion of 25·8 m-equiv/kg fibre water.hr.

RESULTS

Recovery of Na-rich muscles in chloride solutions containing K or Rb. Sodium-rich sartorius muscles were prepared by soaking in a K-free solution at 0–2° C (solution *B*, Table 1). These muscles were transferred to warmer chloride solutions which contained 10 m-mole/l. of either KCl or RbCl (solutions *C* or *D*). It is well established that muscles treated in this way will extrude sodium and gain potassium from a K-containing solution (Steinbach, 1940, 1951; Desmedt, 1953; Carey & Conway, 1954; Frazier & Keynes, 1959). In these circumstances both sodium and potassium move against their respective concentration gradients. It is, however, necessary to establish that increases in internal rubidium concentration which occur when muscles are allowed to recover in the Rb-containing solution (*D*) are the result of the activity of the sodium pump and not merely the result of the diffusion of rubidium into the cell which would take place in the absence of any pump activity. The uptake of rubidium by sodium-rich muscles (prepared by soaking for 24 hr in solution *B*) was compared in circumstances in which the sodium pump is active (18° C) and inactive (4° C with 10^{-5} M ouabain). The results are shown in Fig. 1 where mean intracellular rubidium concentrations (m-equiv/kg fibre water) are plotted against time in the Rb-containing solution (*D*). Table 2 gives the experimental results in m-equiv/kg muscle. The uptake of rubidium from solution *D* is greatly reduced by the combined effect of cold and ouabain. The initial uptake rates are 2·5 m-equiv/kg fibre water.hr and 48 m-equiv/kg fibre water.hr.

TABLE 1. Solutions

	K^+	Rb^+	Na^+	Ca^{2+}	Mg^{2+}	Cl^-	SO^{2+}	HCO_3^-	HPO^{2-}	$H_2PO_4^-$	Gluconate	Glucose (mM)	Sucrose (mM)	Gas	Relative* tonicity	Reference
A	2·5	—	120·2	1·8	—	121·1	—	—	2·15	0·85	—	—	—	—	1·00	Adrian (1956)
B	—	—	120·9	0·9	1·2	88·0	2·1	25·0	3·0	—	1·9	—	—	95% O_2.5% CO_2	0·97	Frazier & Keynes (1959)
C	10·0	—	104·9	0·9	1·2	82·0	2·1	25·0	3·0	—	1·9	12·0	—	95% O_2.5% CO_2	0·97	
D	—	10·0	104·9	0·9	1·2	82·0	2·1	25·0	3·0	—	1·9	12·0	—	95% O_2.5% CO_2	0·97	
E	1·0	—	82·0	8·0	—	—	48·2	—	1·08	0·43	—	—	113	O_2	1·00	Hodgkin & Horowicz (1959)
F	10·0	—	73·0	8·0	—	—	48·2	—	1·08	0·43	—	13·0	100	O_2	1·00	
G	—	10·0	73·0	8·0	—	—	48·2	—	1·08	0·43	—	13·0	100	O_2	1·00	
H	—	—	83·0	8·0	—	—	48·2	—	1·08	0·43	—	—	113	—		
I	—	1·0	82·0	8·0	—	—	48·2	—	1·08	0·43	—	—	113	—		
J	2·5	—	80·5	8·0	—	—	48·2	—	1·08	0·43	—	—	113	—		
K	—	2·5	80·5	8·0	—	—	48·2	—	1·08	0·43	—	—	113	—		
L	5·0	—	78·0	8·0	—	—	48·2	—	1·08	0·43	—	—	113	—		
M	—	5·0	78·0	8·0	—	—	48·2	—	1·08	0·43	—	—	113	—		
N	25·0	—	58·0	8·0	—	—	48·2	—	1·08	0·43	—	—	113	—		
O	—	25·0	58·0	8·0	—	—	48·2	—	1·08	0·43	—	—	113	—		
P	50·0	—	111·0	8·2	—	—	86·2	—	2·15	0·85	—	—	—	—		Adrian (1964)
Q	—	50·0	111·0	8·2	—	—	86·2	—	2·15	0·85	—	—	—	—		
R	100·0	—	61·0	8·2	—	—	86·2	—	2·15	0·85	—	—	—	—		
S	—	100·0	61·0	8·2	—	—	86·2	—	2·15	0·85	—	—	—	—		

* Measured by freezing-point depression.
Concentrations given in mg ion/l. soln.

Figures 2 and 3 show, for muscles soaked for 24 and 48 hr in K-free Ringer at 0° C, the intracellular cation concentrations (m-equiv/kg fibre water) plotted against time of recovery in solutions *C* or *D*. For both groups of muscles the recovery was followed for 4 hr. For the muscles recovering in the Rb-containing solution (filled symbols Figs. 2 and 3) the sums of the potassium and rubidium concentrations are given as well as the concentration of potassium remaining in the muscle fibres. The internal sodium and potassium concentrations of recently dissected

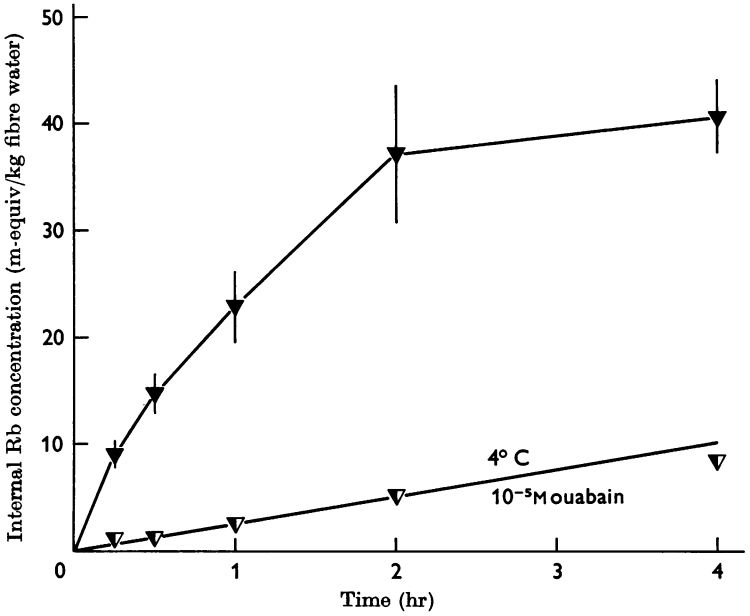

Fig. 1. The uptake of rubidium by Na-rich muscles. Muscles, which were soaked for 24 hr in K-free Ringer solution at 0–2° C, were transferred to a chloride recovery solution with 10 m-equiv/l. of Rb at 18° C (filled triangles). Muscles prepared in the same way were transferred to the same recovery solution at 4° C with 10^{-5} M ouabain (half filled triangles). The internal concentrations are calculated from the results in Table 2 on the assumption that the extracellular space is 12·5 % of the wet wt.

muscles are given at the left of each graph; the intracellular concentrations of sodium and potassium estimated at the end of the soaking in K-free Ringer solution are plotted at zero time. Tables 2 and 3 set out the results from which the intracellular concentrations of Figs. 2 and 3 were derived.

Figures 2 and 3 show clearly that sodium is extruded from the muscle fibres almost equally well in the presence of potassium or rubidium. The rate of replacement of sodium by potassium is not very much greater than

TABLE 2. Recovery of muscles soaked for 24 ± 3 hr in Ringer solution without K. Recovery in Cl^- solutions

Time in zero K at 0° C (hr)	Time of recovery at 18° C (hr)	No. of muscles	Cations in recovery soln. (*C*, *D*) 10 mM	$\frac{\text{Dry wt.}}{\text{wet wt.}} \times 100$	Na m-equiv/kg muscle	K m-equiv/kg muscle	Rb m-equiv/kg muscle	ΣNa + K + Rb
0	0	12	—	21·7 ± 0·4	27·3 ± 0·8	99·3 ± 1·6	—	126·6 ± 2·5
24	0	29	—	22·5 ± 0·2	46·7 ± 1·7	67·8 ± 1·6	—	114·8 ± 1·2
24	0·25	10	K	22·6 ± 0·4	36·5 ± 2·0	76·3 ± 2·5	—	110·7 ± 2·7
24	0·25	10	Rb	24·1 ± 0·3	39·7 ± 2·9	71·2 ± 2·6	6·9 ± 0·9	117·8 ± 3·1
24	0·5	10	K	23·2 ± 0·3	30·1 ± 1·4	83·1 ± 2·0	—	113·1 ± 1·5
24	0·5	9	Rb	23·2 ± 0·4	32·6 ± 1·3	73·5 ± 1·1	10·6 ± 1·3	116·8 ± 9·0
24	1·0	10	K	21·9 ± 0·5	26·7 ± 1·6	93·7 ± 2·5	—	120·4 ± 3·4
24	1·0	6	Rb	22·0 ± 0·6	30·6 ± 3·3	69·6 ± 5·4	16·3 ± 2·1	116·5 ± 4·8
24	2·0	6	K	21·6 ± 1·0	21·0 ± 1·0	96·6 ± 4·0	—	117·6 ± 4·1
24	2·0	6	Rb	22·3 ± 0·6	26·0 ± 2·1	66·4 ± 3·2	25·7 ± 4·6	118·1 ± 4·0
24	4·0	6	K	21·2 ± 0·4	15·8 ± 2·8	96·3 ± 2·0	—	112·1 ± 2·7
24	4·0	6	Rb	22·0 ± 0·5	19·5 ± 1·8	64·4 ± 2·1	27·7 ± 2·4	115·2 ± 2·3
			Muscles 'recovering' in solution *D* at 4° C with 10^{-5} M ouabain					
24	0·25	3	Rb	22·2 ± 0·6	33·2 ± 2·5	67·6 ± 3·1	2·0 ± 0·3	101·9 ± 1·7
24	0·5	3	Rb	22·9 ± 0·2	34·4 ± 4·6	67·4 ± 3·3	2·0 ± 0·1	103·8 ± 1·5
24	1·0	3	Rb	23·4 ± 0·4	37·7 ± 2·7	65·5 ± 0·7	2·9 ± 0·2	106·2 ± 1·7
24	2·0	3	Rb	22·2 ± 0·7	33·3 ± 4·8	69·1 ± 1·5	4·7 ± 0·5	107·1 ± 4·7
24	4·0	3	Rb	22·9 ± 0·5	37·3 ± 5·8	61·0 ± 5·5	6·8 ± 0·6	105·1 ± 0·7

Mean ± S.E. of mean.

the rate of replacement of sodium by rubidium. The internal potassium concentration of the muscles recovering in the rubidium solution does not differ greatly at the beginning and end of the recovery period. Although the intracellular sodium concentration of muscles recovering in rubidium solutions is always somewhat higher than the sodium concentrations of muscles recovering in potassium solutions, the sodium concentration at the end of 4 hr in both recovery solutions falls to values less than those for recently dissected muscles. This effect appears to be a real one, because recently dissected muscles lose sodium when put into the recovery solutions

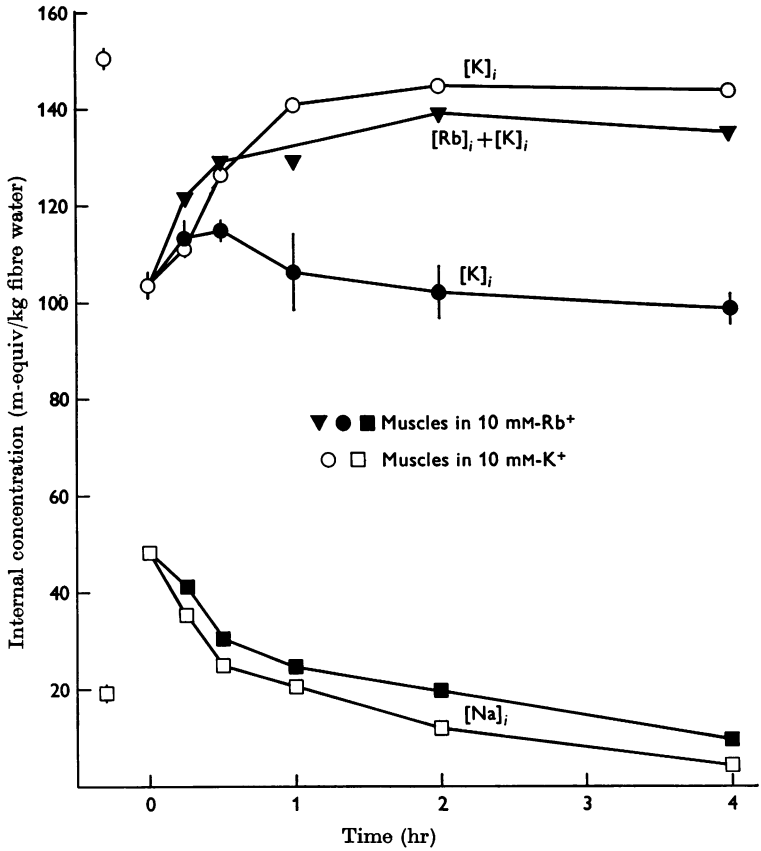

Fig. 2. The change of internal cation concentrations of muscles, prepared by soaking for 24 hr in K-free Ringer solution at 0–2° C, during recovery in chloride solutions (*C* and *D*) with 10 m-equiv/l. of either K (open symbols) or Rb (filled symbols). To the left of 0 hr are shown the internal sodium and potassium concentrations of recently dissected muscles. The internal concentrations are calculated from the results in Table 2. For muscles recovering in Rb-solution the intracellular potassium concentration and the sum of the intracellular concentrations of potassium and rubidium (filled triangles) are plotted.

at 18° C. Three freshly dissected muscles put directly into the potassium recovery solution (*C*) at 18° C, were found to have a mean intracellular sodium concentration of $3{\cdot}2 \pm 0{\cdot}2$ m-equiv/kg fibre water at the end of 4·5 hr, whereas their pairs, estimated directly after dissection, had a mean intracellular sodium concentration of $18{\cdot}5 \pm 3{\cdot}3$ m-equiv/kg fibre water. In a similar experiment the mean intracellular sodium concentrations before and after 4·5 hr in the rubidium recovery solution (*D*) were $16{\cdot}4 \pm 2{\cdot}7$ m-equiv/kg fibre water and $7{\cdot}1 \pm 1{\cdot}2$ m-equiv/kg fibre water (see Table 4). The recently dissected muscles were in a Ringer solution with a potassium

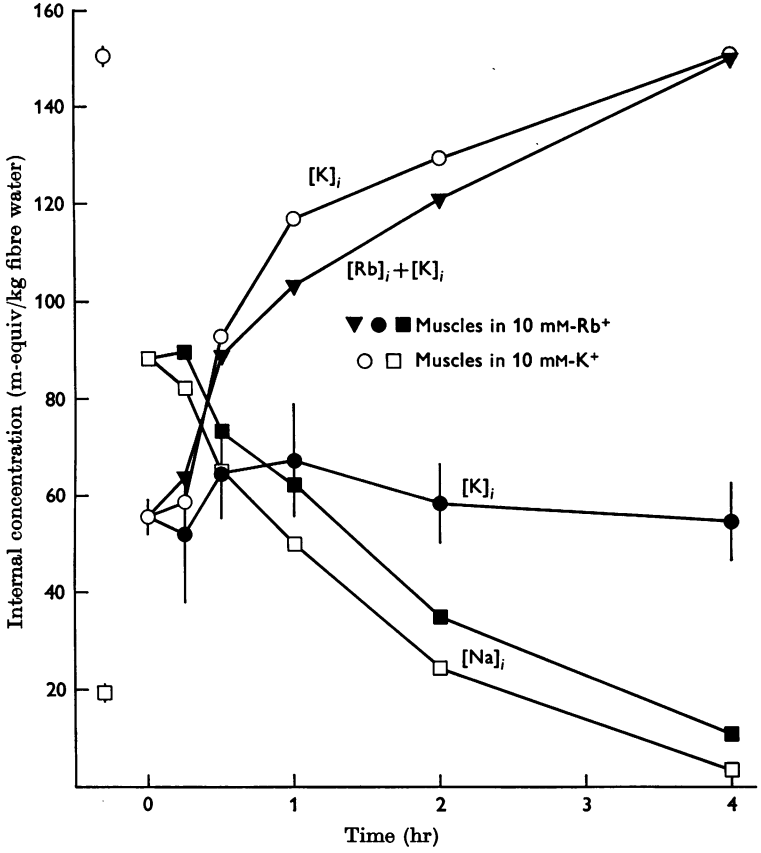

Fig. 3. The change in internal cation concentrations of muscles, prepared by soaking for 48 hr in K-free Ringer solution at 0–2° C, during recovery in chloride solutions (*C* and *D*) with 10 m-equiv/l. of either K (open symbols) or Rb (filled symbols). To the left of 0 hr are shown the intracellular sodium and potassium concentrations of recently dissected muscles. The intracellular concentrations are calculated from the results in Table 3. For muscles recovering in the Rb-solution, the intracellular potassium concentration and the sum of the intracellular potassium and rubidium concentrations (filled triangles) are plotted.

TABLE 3. Recovery of muscles soaked for 24 ± 3 hr in Ringer solution without K. Recovery in Cl^- solutions

Time in zero K at 0° C (hr)	Time of recovery at 18° C (hr)	No. of muscles	Cations in recovery soln. (*C*, *D*) 10 mM	$\frac{\text{Dry wt.}}{\text{wet wt.}} \times 100$	Na m-equiv/kg muscle	K m-equiv/kg muscle	Rb m-equiv/kg muscle	ΣNa + K + Rb
0	0	12	—	21·7 ± 0·4	27·3 ± 0·8	99·3 ± 1·6	—	126·6 ± 2·5
48	0	5	—	23·3 ± 0·3	71·0 ± 2·1	35·7 ± 2·2	—	106·8 ± 3·3
48	0·25	3	K	23·4 ± 0·6	65·7 ± 5·9	38·8 ± 8·6	—	104·6 ± 3·0
48	0·25	3	Rb	24·1 ± 0·5	69·9 ± 5·7	32·9 ± 8·7	8·1 ± 1·8	110·7 ± 5·5
48	0·5	3	K	23·7 ± 0·5	55·5 ± 7·6	60·2 ± 8·6	—	115·7 ± 1·1
48	0·5	3	Rb	24·2 ± 1·0	59·7 ± 7·5	40·9 ± 5·3	16·6 ± 3·3	117·7 ± 1·3
48	1·0	3	K	22·9 ± 0·7	45·5 ± 7·2	76·5 ± 8·6	—	122·1 ± 2·7
48	1·0	3	Rb	23·2 ± 0·6	53·4 ± 7·3	43·2 ± 7·3	24·4 ± 10·7	120·9 ± 2·1
48	2·0	3	K	23·1 ± 0·1	28·2 ± 5·0	84·4 ± 6·3	—	112·5 ± 2·1
48	2·0	3	Rb	23·7 ± 0·3	35·5 ± 9·1	37·2 ± 5·2	41·0 ± 4·2	113·8 ± 0·5
48	4·0	3	K	22·2 ± 0·5	15·3 ± 1·3	99·6 ± 7·5	—	114·8 ± 6·0
48	4·0	3	Rb	22·9 ± 0·2	20·0 ± 2·5	35·2 ± 4·3	62·9 ± 2·0	118·2 ± 3·7

Mean ± S.E. of mean.

TABLE 4. Recovery of fresh muscles

Time in zero K at 0° C (hr)	Time of recovery at 18° C (hr)	Cations in recovery solutions (*C* + *D*) 10 mM	No. of muscles	$\frac{\text{Dry wt.}}{\text{wet wt.}} \times 100$	K m-equiv/kg muscle	Na m-equiv/kg muscle	Rb m-equiv/kg muscle
0	0	—	3 pairs	21·7 ± 0·2	89·6 ± 1·7	27·3 ± 1·9	—
0	4·5	K	3 pairs	20·1 ± 0·4	93·7 ± 2·6	15·3 ± 0·2	—
0	0	—	3 pairs	21·5 ± 0·4	93·5 ± 1·5	25·8 ± 1·8	—
0	4·5	Rb	3 pairs	21·6 ± 0·4	80·6 ± 2·2	17·7 ± 0·8	16·1 ± 0·7

Mean ± S.E. of mean.

concentration of 2·5 m-equiv/l. The increased potassium or rubidium concentration (10 m-equiv/l.) of the recovery solutions appears to allow the muscles to maintain a lower steady-state internal sodium concentration.

The internal potassium concentration of muscles recovering in the rubidium solution (*D*) seems to rise in the first hour of recovery, though it falls thereafter. This effect is seen in Fig. 2 and in Fig. 3, but in Fig. 3 none of the values of the internal potassium concentration differs significantly from the internal potassium concentration at the beginning of recovery. In Fig. 2, however, the internal potassium concentrations at 15 and 30 min are significantly different from the internal potassium concentration at the beginning of recovery. Since there is no potassium in the rubidium recovery solution, these changes, if real, must reflect changes in the fibre volume.

Recovery of Na-rich muscles in sulphate solutions containing K or Rb. Sodium-rich muscles, with low intracellular chloride concentrations, were prepared by soaking muscles for 48 hr in the K-free Ringer solution and then transferring the muscles to a sulphate solution at 0° C with a potassium concentration of 1 m-equiv/l. (solution *E*, Table 1). The half-time for the loss of chloride from muscles under these conditions was measured with ^{36}Cl and found to be about 2 hr. After the long soak in the K-free Ringer solution, muscles were left in the sulphate solution for 2–3 hr, by which time the intracellular chloride concentration should have dropped to about 1 m-equiv/kg fibre water. (Surface fibres of muscles, soaked for 48 hr in K-free Ringer solution at 0° C, had internal potentials, measured at 0° C, between -80 and -100 mV (see below). The mean intracellular chloride concentration of the fibres at the end of 48 hr in cold K-free Ringer solution was probably about 3 m-equiv/kg fibre water.) After 2–3 hr in solution *E* the muscles were transferred to sulphate solutions at 18° C containing 10 m-equiv/l. of either potassium or rubidium (solutions *F* and *G*, Table 1). Figure 4 shows the intracellular cation concentrations during the first hour in the recovery solutions. The concentrations are plotted in the same way as in Figs. 2 and 3. Table 5 gives the experimental results from which the intracellular concentrations were derived. During the first hour of recovery substantial quantities of sodium were lost from the muscle and replaced by either potassium or rubidium. However, for longer times the recovery was not as complete as for muscles in solutions containing chloride (*C* and *D*) and signs of deterioration appeared after the first hour. Clotted fibres became frequent and the intracellular sodium concentration failed to fall further or even started to rise. Kernan & Tangney (1964) and Mullins & Awad (1965) have reported difficulty in making muscles pump sodium in sulphate solutions, and muscles soaked for 24 hr or longer in cold K-free sulphate solutions do not survive in good

condition. Observations on muscles pumping sodium in sulphate solutions have therefore been confined to the first hour of recovery. During this period they seem to be able to reduce their internal sodium concentration at a rate comparable to muscles in chloride solutions, and potassium or rubidium replaces the lost sodium about equally well. The intracellular potassium concentration of the muscle recovering in the rubidium solution rises initially but subsequently falls. After 15 min in the recovery solution the intracellular potassium concentration is significantly different from that at the beginning of recovery. A similar rise and fall were noted in

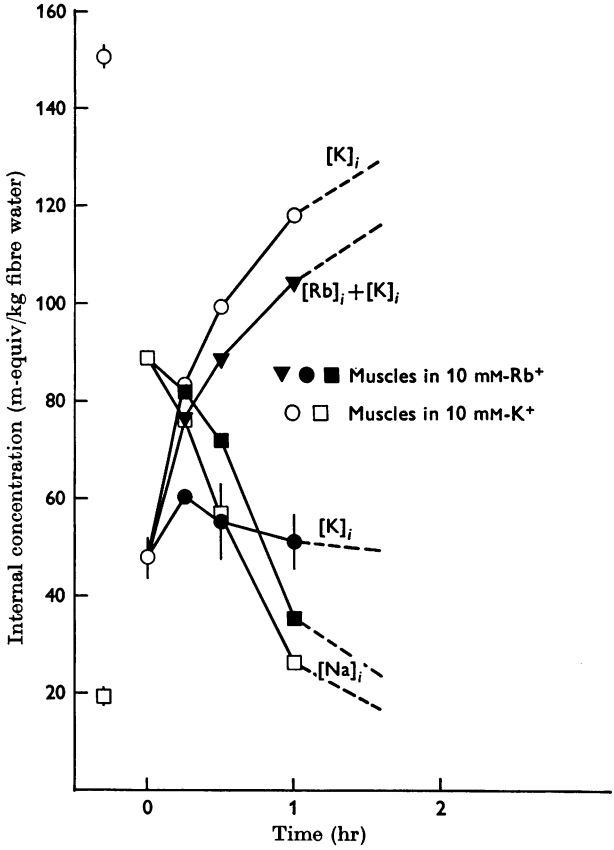

Fig. 4. The change in internal cation concentrations of muscles, prepared by soaking for 48 hr in K-free Ringer solution at 0–2° C, and then for 2–3 hr in solution *E* (1 m-equiv/l. K-sulphate solution), during recovery in sulphate solutions (*F* and *G*) containing 10 m-equiv/l. of either K (open symbols) or Rb (filled symbols). To the left of 0 time are shown the intracellular concentrations of recently dissected muscles. The internal concentrations are calculated from the results in Table 5. For muscles recovering in the Rb-containing solution the intracellular potassium concentration and the sum of the intracellular concentrations of potassium and rubidium (filled triangles) are plotted.

TABLE 5. Recovery of muscles soaked for 48 ± 3 hr in Ringer solution without K. Recovery in SO_4^{2-} solutions

Time in zero K at 0° C (hr)	Time of recovery at 18° C (hr)	No. of muscles	Cation in recovery soln. (*F*, *G*) 10 mM	$\frac{\text{Dry wt.}}{\text{wet wt.}} \times 100$	Na m-equiv/kg muscle	K m-equiv/kg muscle	Rb m-equiv/kg muscle	ΣNa+K+Rb
0	0	12	—	$21{\cdot}7 \pm 0{\cdot}4$	$27{\cdot}3 \pm 0{\cdot}8$	$99{\cdot}3 \pm 1{\cdot}6$	—	$126{\cdot}6 \pm 2{\cdot}5$
48	0	8	—	$25{\cdot}0 \pm 1{\cdot}9$	$64{\cdot}8 \pm 2{\cdot}7$	$27{\cdot}0 \pm 3{\cdot}7$	—	$91{\cdot}9 \pm 4{\cdot}1$
48	0·25	3	K	$25{\cdot}1 \pm 0{\cdot}2$	$56{\cdot}5 \pm 5{\cdot}7$	$52{\cdot}7 \pm 1{\cdot}9$	—	$109{\cdot}4 \pm 4{\cdot}7$
48	0·25	3	Rb	$24{\cdot}6 \pm 0{\cdot}2$	$60{\cdot}8 \pm 6{\cdot}1$	$38{\cdot}2 \pm 0{\cdot}3$	$11{\cdot}4 \pm 0{\cdot}5$	$110{\cdot}5 \pm 6{\cdot}3$
48	0·5	3	K	$25{\cdot}2 \pm 0{\cdot}6$	$44{\cdot}6 \pm 2{\cdot}7$	$63{\cdot}0 \pm 1{\cdot}8$	—	$105{\cdot}7 \pm 2{\cdot}6$
48	0·5	3	Rb	$24{\cdot}5 \pm 0{\cdot}2$	$54{\cdot}1 \pm 3{\cdot}8$	$34{\cdot}6 \pm 4{\cdot}7$	$22{\cdot}2 \pm 2{\cdot}6$	$110{\cdot}9 \pm 3{\cdot}5$
48	1·0	4	K	$25{\cdot}9 \pm 0{\cdot}9$	$25{\cdot}2 \pm 3{\cdot}8$	$73{\cdot}8 \pm 12{\cdot}5$	—	$99{\cdot}2 \pm 6{\cdot}2$
48	1·0	4	Rb	$26{\cdot}1 \pm 1{\cdot}2$	$30{\cdot}8 \pm 5{\cdot}2$	$31{\cdot}3 \pm 5{\cdot}0$	$33{\cdot}7 \pm 5{\cdot}5$	$95{\cdot}9 \pm 4{\cdot}8$

Mean ± S.E. of mean.

Figs. 2 and 3. Since the rise occurs in chloride-depleted muscles recovering in sulphate solutions, it cannot only be due to a loss of sodium chloride during the initial stages of recovery.

Some other points from the results in Tables 2, 3 and 5 deserve brief mention. The conclusions should not be considered as well established, since many of the changes are either not, or are only marginally, significant statistically. The dry to wet weight ratio is increased during soaking in K-free Ringer solution. At the same time the sum of the sodium and potassium concentration falls. This fall is too large to be accounted for by the change in the dry to wet weight ratio, or by any probable change in the extracellular space (Desmedt, 1953). These changes appear to be at least partially reversed during recovery. It seems that soaking muscles in K-free Ringer solution in the cold produces a loss of solutes and water, as well as a reduction in the electric charge associated with each osmole of internal anion. It seems probable from the results in Tables 2, 3 and 5 that there are several simultaneous changes in the state of the muscle in the course of these experiments, but the sum of all these changes appears to be insufficiently large to vitiate the conclusion that large and rapid reciprocal movements of sodium and potassium or of sodium and rubidium can take place in sodium-rich muscles.

Rubidium uptake and external rubidium concentration. A few measurements were made to see how the external rubidium concentration affects the ouabain-sensitive fraction of the rubidium uptake at 20° C. Sodium-rich, chloride-depleted muscles were allowed to recover at about 20° C in sulphate solutions containing 2·5, 5, and 10 m-equiv/l. rubidium (solutions *K*, *M*, and *G*). The results are shown in Table 6. Over the range of concentrations tested the ouabain-sensitive rubidium uptake appears to be nearly proportional to the external rubidium concentration.

The effect of temperature on the uptake of rubidium. Table 7 shows the effect of temperature on the inward movement of rubidium in the pre-

TABLE 6. Uptake of Rb at varying $[Rb]_o$

Muscles soaked for 48 hr in zero K at 0° C (*B*); 1 hr in 1 mM K-sulphate solution (*E*); recovered in solutions *G*, *M* or *J*.

Temp. (° C)	$[Rb]_o$ (mM)	Ouabain M	Recovery time (hr)	$[Rb]_i$ m-equiv/kg fibre water	Rate of Rb uptake m-equiv/kg fibre water.hr	No. of muscles
20	10	10^{-5}	1	4·3 ± 0·4	4·3	3
20	10	—	0·5	31·7 ± 1·5	63·4	3
19	5	10^{-5}	1	1·1 ± 0·1	1·1	3
19	5	—	0·5	14·3 ± 1·5	28·6	3
19	2·5	10^{-5}	1	0·6 ± 0·2	0·6	3
19	2·5	—	0·5	6·9 ± 1·2	13·8	3

Mean ± S.E. of mean.

sence and absence of ouabain (10^{-5} M). Sodium-rich, chloride-depleted muscles were allowed to recover in sulphate solution with 10 m-equiv/l. rubidium (G) at temperatures close to 8, 20, and 30° C. In the absence of ouabain the rubidium uptake was measured after 30 min, in its presence after 1 hr. At all three temperatures the presence of ouabain reduces the uptake of rubidium by 85–95 %. At 30° C the ouabain-sensitive uptake of rubidium during the 30 min period is at a rate of about 100 m-equiv/kg fibre water.hr. Assuming that the sartorious is made up of fibres 80 μ in diameter, and that their internal concentrations change uniformly with time throughout the thickness of the muscle, a rate of change of internal concentration of 100 m-equiv/kg fibre water.hr corresponds to a net inward flux of 39 p-mole/cm^2.sec. However, the assumption that the deep and superficial fibres of the sartorius change their internal concentrations at the same rate is probably wrong, and the actual maximum fluxes may be considerably greater than 39 p-mole/cm^2/sec.

The effect of extracellular diffusion on the measured uptakes of potassium and rubidium. If the uptake of potassium or rubidium by the muscle fibres is sufficiently rapid, diffusion through the extracellular space may not be adequate to maintain the extracellular concentration throughout the depth of the muscle at the same value as the bulk of the solution. The effect is the same as the slowing of the uptake of isotope by extracellular diffusion which has been considered by Harris & Burn (1949) and Keynes (1954). Keynes's treatment will be followed here.

It is assumed that the net movement of potassium or rubidium into the fibre is directly proportional to the extracellular concentration of potassium or rubidium. If the muscle is considered to be a plane sheet of thickness $2b$ exposed to stirred solution on both sides, then the extracellular concentration of potassium or rubidium (C_x) at any depth from the surface of the muscle is given by the solution of the partial differential equation

$$\frac{\partial C_x}{\partial t} = D' \frac{\partial^2 C_x}{\partial x^2} - \frac{(1-\epsilon)}{\epsilon} \cdot \frac{A}{V} . M . C_x \qquad (1)$$

where ϵ is the extracellular fraction of the muscle volume;

A/V is the ratio of the surface area to the volume of a muscle fibre. For an 80 μ fibre, $A/V = 5 \times 10^2$ cm^{-1};

D' is the diffusion coefficient of potassium or rubidium, making allowance for the geometry of the extracellular space;

M is the net rate of inward transport for unit surface area of membrane and unit external concentration.

The intracellular compartment is treated as effectively infinite, and a steady extracellular concentration $(\partial C_x/\partial t) = 0$ is assumed to be established in a short time. The boundary conditions are that $C_x = C_b$ at $x = \pm b$, and that at $x = 0$, $\partial C_x/\partial x = 0$. For the steady state

$$C_x = \frac{C_b}{\cosh b/\lambda} \cosh \frac{x}{\lambda},$$

where C_b is the concentration of potassium or rubidium at the surface of the muscle, and

$$\lambda^2 = \frac{D' V \epsilon}{AM(1-\epsilon)}.$$

The ratio of the measured uptake (U') to the rate of uptake which would be measured if C_x were everywhere equal to C_b (U) is given by

$$\frac{U'}{U} = \frac{\lambda}{b} \tanh \frac{b}{\lambda}.$$

TABLE 7. Comparison of Rb uptake with and without ouabain; at different temperatures; SO_4^{2-} solutions

Temp. (° C)	Ouabain (M)	Recovery time (hr)	$[Rb]_i$ m-equiv/kg fibre water	Rate of Rb uptake m-equiv/kg fibre water . hr	$\frac{\text{Dry wt.}}{\text{wet wt.}} \times 100$	K m-equiv/kg muscle	Na m-equiv/kg muscle	No. of muscles	Pairs
8	10^{-5}	1	$2{\cdot}1 \pm 0{\cdot}5$	2·1	$25{\cdot}9 \pm 0{\cdot}4$	$25{\cdot}1 \pm 3{\cdot}2$	$71{\cdot}5 \pm 3{\cdot}5$	3	*a*
8	—	0·5	$8{\cdot}1 \pm 1{\cdot}3$	16·2	$25{\cdot}3 \pm 1{\cdot}2$	$28{\cdot}2 \pm 2{\cdot}0$	$63{\cdot}5 \pm 4{\cdot}3$	3	*a*
20 (Cl-solution, *D*)	10^{-5}	4·0	$13{\cdot}6 \pm 0{\cdot}8$	3·4	$20{\cdot}8 \pm 0{\cdot}3$	$21{\cdot}1 \pm 0{\cdot}5$	$90{\cdot}9 \pm 0{\cdot}7$	3	*b*
20	10^{-5}	1	$4{\cdot}3 \pm 0{\cdot}4$	4·3	$25{\cdot}8 \pm 0{\cdot}4$	$26{\cdot}7 \pm 2{\cdot}6$	$58{\cdot}2 \pm 3{\cdot}0$	3	*b*
20	—	0·5	$31{\cdot}7 \pm 1{\cdot}5$	63·4	$23{\cdot}5 \pm 0{\cdot}4$	$15{\cdot}6 \pm 3{\cdot}5$	$70{\cdot}0 \pm 1{\cdot}0$	3	*c*
30·5	10^{-5}	1	$5{\cdot}6 \pm 0{\cdot}8$	5·6	$24{\cdot}6 \pm 0{\cdot}4$	$18{\cdot}9 \pm 0{\cdot}4$	$56{\cdot}4 \pm 2{\cdot}0$	3	—
30	—	0·5	$53{\cdot}3 \pm 2{\cdot}2$	106·6	$23{\cdot}3 \pm 0{\cdot}9$	$15{\cdot}2 \pm 2{\cdot}6$	$53{\cdot}8 \pm 1{\cdot}8$	3	*c*

Mean ± S.E. of mean.

The diffusion coefficient D' is assumed to be 4×10^{-6} cm²/sec. This value is between the mean value given by Keynes (1954) for sartorius exposed on one side only, and the value found by Keynes for toe muscles of the frog. Since the diffusion coefficients for potassium and rubidium chloride are almost identical in free solution (Robinson & Stokes, 1959) it seems likely that they will be so in the extracellular space. If $M = 5 \times 10^{-6}$ cm/sec, $\lambda = 148\,\mu$. This value of M, when the external concentration of potassium or rubidium is 10 m-equiv/l., corresponds to a net influx of 50 p-mole/cm².sec or a rate of change of internal concentration of 129 m-equiv/kg fibre water.hr. If the half thickness of the muscle is 350 μ, then U'/U is equal to 0·41, so that the rate of change of internal concentration measured on the whole muscle would be only 53 m-equiv/kg fibre water.hr.

It appears that the actual rate of potassium or rubidium movement in the surface fibres could be 2–3 times the maximum measured rates for the whole muscle. For measured rates less than the maximum the correction factor will be less. The correction factors derived above cannot be regarded as giving more than an idea of the magnitude of the effect of extracellular diffusion. The assumptions involved in writing equation (1) are uncertain as is the value of D'. Despite these, and other, uncertainties, the conclusion is that at the pumping rates found in these experiments extracellular diffusion cannot be ignored, and may cause the transport rate in surface fibres to be underestimated by as much as three-fold.

If λ is comparable to the diameter of a muscle fibre, the potassium or rubidium concentration at the deep surface of the superficial fibre could be appreciably less than at the surface of the muscle. Unstirred layers at the surface of the muscle could exaggerate this reduction of the effective external potassium or rubidium concentration.

The membrane potential during ionic movement. When a muscle is put into a rubidium-recovery solution (solutions D and G), the only direction in which potassium can move is out of the fibres. Equally the only direction in which rubidium can move initially is into the fibres, and a rubidium entry would be expected to take place in the absence of any metabolically driven ion movements. For a muscle which is recovering in a potassium-recovery solution there is always a clearly defined potassium equilibrium potential (E_K) with which the membrane potential (E_m) can be compared. But for a muscle which is extruding sodium into a rubidium-recovery solution, it is not very helpful to compare the membrane potential with the potassium equilibrium potential which is always infinite and negative (related to the inside of the fibre). As soon as any rubidium has entered the fibre there will be a clearly defined rubidium equilibrium potential (E_{Rb}), but this will in general be less negative than the membrane potential until most of the internal potassium has been replaced by rubidium. The potassium and rubidium equilibrium potentials are given by the two following equations:

$$E_K = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i}, \quad E_{Rb} = \frac{RT}{F} \ln \frac{[Rb]_o}{[Rb]_i}.$$

Strictly the internal and external activities of the ions should be used rather than the concentrations. If the internal and external activity coefficients are the same no error will be introduced by using concentrations in place of activities. It is however possible to define a potential ($E_{K,\,Rb}$) as the potential for any given set of concentrations and per-

meabilities, and in the absence of metabolically driven ion movement, at which the sum of the currents carried by potassium and rubidium across the membrane is zero. This potential is given by

$$E_{K,Rb} = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Rb}[Rb]_o}{P_K[K]_i + P_{Rb}[Rb]_i}$$

and it is a bi-ionic potential of the kind studied by Sollner, Dray, Grim & Neihof (1954) in cation permselective membranes. P_K and P_{Rb} are the permeabilities of the membrane to potassium and rubidium.

Adrian (1964) has studied the permeabilities of potassium and rubidium in each other's presence and concluded that in the absence of external potassium P_K is reduced (Hodgkin & Horowicz, 1959), and that rubidium in the presence of external potassium also reduces P_K. When there is external rubidium but no external potassium, the ratio of the permeabilities of potassium and rubidium appears to be greater than 1 and less than 2, though both permeabilities are less than the value of P_K when potassium but not rubidium is present in the external solution. The mechanism of these complex permeability changes is not understood, but they must be taken into account when considering the expected membrane potential of a muscle recovering in a rubidium solution. Because of the reduced potassium permeability and the low rubidium permeability in these circumstances, it is not safe to ignore contribution of sodium to the cation currents in the membrane. Making the assumptions that the anion currents are zero and that potassium, rubidium, and sodium carry practically all the current through the membrane, the sum of the currents resulting from the electrochemical gradient of each cation will be zero when the membrane potential is equal to $E_{Na,K,Rb}$. This poly-ionic potential will be given by

$$E_{Na,K,Rb} = \frac{RT}{F} \ln \frac{P_K[K]_o + P_{Rb}[Rb]_o + P_{Na}[Na]_o}{P_K[K]_i + P_{Rb}[Rb]_i + P_{Na}[Na]_i}.$$

In the equations for $E_{K,Rb}$ and $E_{Na,K,Rb}$ it must be realized that the permeability factors cannot be considered constants but are functions of the concentrations on either side of the membrane and of the potential difference across the membrane.

It is reasonable to assume that $E_{Na,K,Rb}$ will be close to the measured membrane potential when (1) the activities of metabolic pumps are negligible, and (2) permeant anions such as chloride are absent, or if present are in equilibrium. In practice it is assumed that the first condition is met in the presence of 10^{-5} M ouabain, and also in freshly dissected muscles which have a low internal sodium concentration. When the sodium content is low the activity of the sodium pump will be much less than in sodium-rich muscles which are producing net outward sodium

movement (Keynes & Swan, 1959; Mullins & Frumento, 1963). It is important to have a measure of the initial and final values of $E_{Na, K, Rb}$ and of E_K for muscles recovering in rubidium and potassium solutions respectively, and an estimate of how these two potentials vary during the recovery.

Figure 5 shows the relation between the internal potential of sartorius muscle fibres and the external concentration of potassium or rubidium in chloride-free solutions (solutions *E* to *T*, Table 1). The points represent averages from many fibres in several muscles. The micro-electrode measurements were generally made by changing the external solution and then

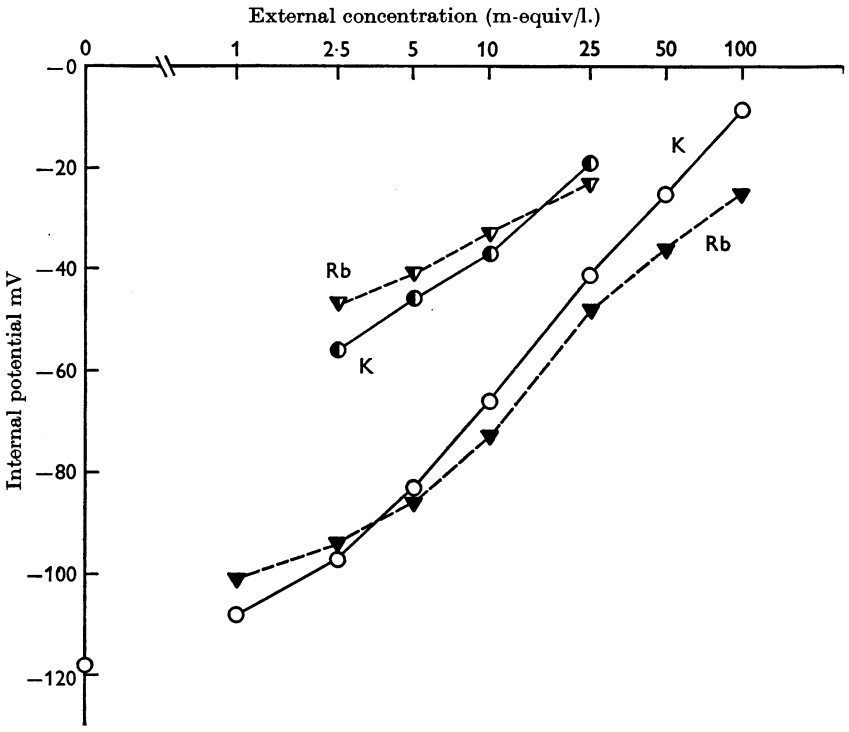

Fig. 5. The internal potential of surface fibres of muscles in sulphate solutions plotted against the external concentrations of either K or Rb. The two lower curves (open circles and filled triangles) are from recently dissected muscles. Measurements were made at 18° C. The upper curves (half filled circles and triangles) are measurements on muscles which had been soaked for 48 hr in K-free Ringer solution and then for 2–3 hr in solution *E*. The mean intracellular potassium concentration of muscles treated in this way is 43 m-equiv/kg fibre water. The measurements were made at 18° C in the presence of 10^{-5} M ouabain. The point at 0 m-equiv/l is the mean of ten measurements on one muscle. The remaining points are each the mean of a total of between twenty-two and eighty measurements on three muscles. At 10 m-equiv/l.-K, E_K for fresh muscles is −68 mV, and E_K for the depleted muscles is −37 mV.

sampling several fibres, but in some experiments the solutions were changed while the micro-electrode was left in a fibre. The lower pair of lines in Fig. 5 (open circles, filled triangles) was obtained from recently dissected muscles; the upper pair (half-filled circles and triangles) was obtained from sodium-rich muscles at 20° C in the presence of 10^{-5} M ouabain. The mean potassium content of muscles prepared in the same way (see Table 5) was 27 m-equiv/kg muscle, equivalent to 43 m-equiv/kg fibre water, and an E_K in 10 m-equiv/l.-K of -37 mV. In both the fresh and the sodium-rich muscles, below a certain external concentration, the internal potential in a rubidium solution is less negative than the potential in the solution with the same potassium concentration. For large concentrations the internal potential in a rubidium solution is more negative than the internal potential in the solution with the same potassium concentration. In fresh muscles the lines relating internal potential to external concentration of potassium and rubidium cross at an external concentration of just less than 5 m-equiv/l., but for individual fibres changing from 5 m-equiv/l. of potassium to the same rubidium concentration (solutions *L* and *M*) may either depolarize or hyperpolarize the membrane by a few mV. For the sodium-rich muscles the cross-over concentration is between 10 and 25 m-equiv/l.

If the sodium pump were to operate in a manner which did not affect the membrane potential, except in so far as it alters the internal cation concentrations, then one would expect the potential during the recovery of sodium-rich muscles to change between the limits set by the values for an external concentration of 10 m-equiv/l. in Fig. 5. Further the change of potential should be in the same direction throughout the recovery period. For muscles recovering in solutions containing 10 m-equiv/l. potassium, one would expect the membrane potential to go from -37 to -67 mV. Between these limits the membrane potential would follow the instantaneous value of the potassium equilibrium potential (E_K). For muscles recovering in solutions containing 10 m-equiv/l. rubidium, one would expect on the basis of Fig. 5 the potential at the beginning of recovery to be -33 mV and at the end of recovery to be -74 mV. The magnitude of the potential change during recovery in rubidium would be somewhat greater than during recovery in potassium, though the shape of the potential change should be similar in the two solutions. The use of fresh muscles probably over-estimates the magnitude of the expected potential change, especially in the case of muscles recovering in rubidium, which will have substantial internal rubidium concentrations, at the end of recovery.

Kernan (1962), Keynes & Rybová (1963), Mullins & Awad (1965), and Frumento (1965), have all shown that when a muscle is producing a net

outward movement of sodium into a solution containing 10 m-equiv/l. potassium, the internal potential (E_m) may appear to be considerably more negative than the potassium equilibrium potential given by

$$E_K = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i}.$$

Under these conditions the electrochemical potential of the potassium ions is greater outside than inside the fibre and a net inward movement of potassium ions will result. The following experiments confirm these observations on muscles recovering in potassium solutions and extend them to muscles recovering in rubidium solutions.

The potential of muscles recovering in solutions containing chloride. Muscle fibres have a high permeability to chloride so that the chloride equilibrium potential can under certain circumstances cause the membrane potential to differ from E_K and unless this can be allowed for the interpretation of the potential changes during pumping may be difficult. Keynes & Rybová (1963) have pointed out the importance of controlling the internal chloride concentration when studying the potential during net pumping of sodium. In the present experiments sodium-rich muscles were placed in a measuring cell which was arranged to allow independent changes of the composition and temperature of the solutions surrounding the muscle (see Methods). The internal potential of the fibres in the K-free soaking solution (*B*) at 1–3° C was measured first. The solution was then changed to the potassium recovery solution (*C*, 10 m-equiv/l.-K) but the temperature was kept low. The muscle remained at 1–3° C for up to 2 hr until the internal potential of the fibres had reached a new steady level. The slow approach to the final level in this solution is attributable to an inward movement of KCl to bring E_{Cl} to the final value of the membrane potential in the cold recovery solution. In the experiment shown in Fig. 6 a pair of muscles were treated in this way. Towards the end of the equilibrating period in cold recovery solution, one muscle of the pair was transferred to a rubidium recovery solution (*D*, 10 m-equiv/l.-Rb). This change of solution produced little change in membrane potential (cf. Fig. 5). After sufficient time for the extracellular potassium to have been replaced by rubidium the temperature of the muscle in the rubidium solution and of its pair which had remained in the potassium solution was raised as rapidly as possible to about 20° C. The mean internal potentials of the fibres in both muscles fell steadily to reach -85 mV in the muscle recovering in the potassium solution and -100 mV in the muscle recovering in the rubidium solution. Although the temperature change was complete in less than 2 min, the maximum hyperpolarization was not reached for 30 min. After the initial 30 min of recovery the internal potential of both muscles rose

until after 2–3 hr the potentials were about equal and neither differed greatly from the predicted value of the potassium equilibrium potential for the muscle recovering in the potassium solution. The predicted change in E_K for the muscle recovering in the potassium solution is shown in Fig. 6. It was estimated from the time course of the change in internal

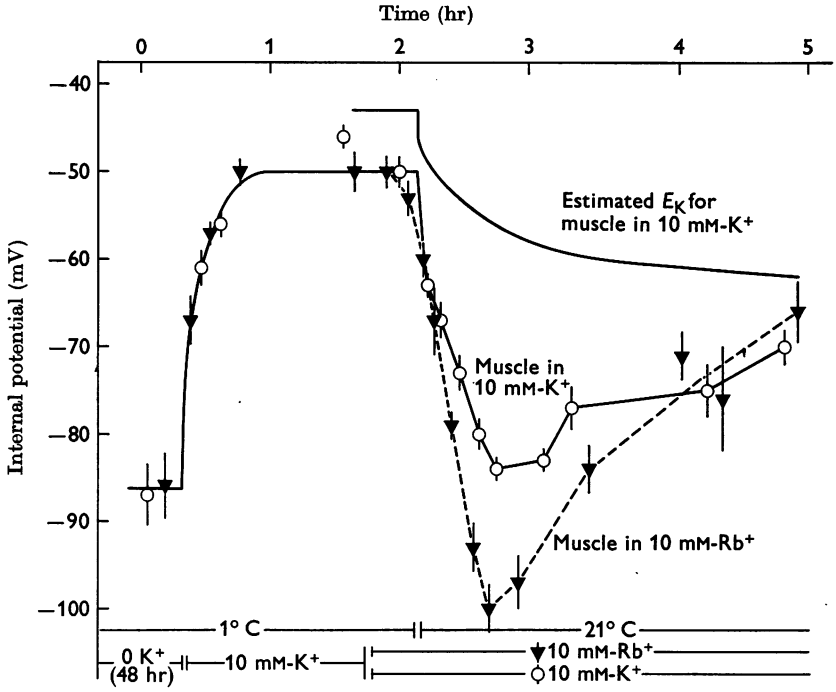

Fig. 6. The internal potential of surface fibres of sodium-rich muscles recovering in chloride solutions containing 10 m-equiv/l. of either K or Rb (solutions *C* or *D*). Potential measurements were made in the solution and at the temperature indicated at the bottom of the figure. The muscles which were a pair were analysed at the end of the experiment. The muscle recovering in K-solution (open circles) had an internal potassium, concentration of 118 m-equiv/kg fibre water, and the muscle recovering in Rb-solution (filled triangles) had an internal potassium concentration of 61 m-equiv/kg fibre water. These concentrations have been used to estimate the initial and final values of E_K. The sodium concentrations of the two muscles were 18·4 and 19·4 m-equiv/kg muscle, and the rubidium taken up by the one muscle was 43 m-equiv/kg muscle. Each point is the mean (±S.E. of the mean) of not less than six measurements.

potassium concentration given in Fig 2. When the internal ionic concentrations were changing most rapidly, the internal potential of the muscle recovering in the potassium solution was nearly 30 mV more negative than E_K, and about 15 mV less negative than the muscle recovering in the rubidium solution.

A similar experiment is shown in Fig. 7. In this experiment the muscle recovering in the potassium solution (open circles) was transferred to the rubidium solution when the hyperpolarization in the potassium solution was nearly maximal. The internal potential fell rapidly to the same value as the internal potential of its pair which had been in the rubidium solution throughout its recovery. The two muscles had the same internal potential in the rubidium solution even though after recovering for about 1 hr the internal cation concentrations of the two muscles must have been different

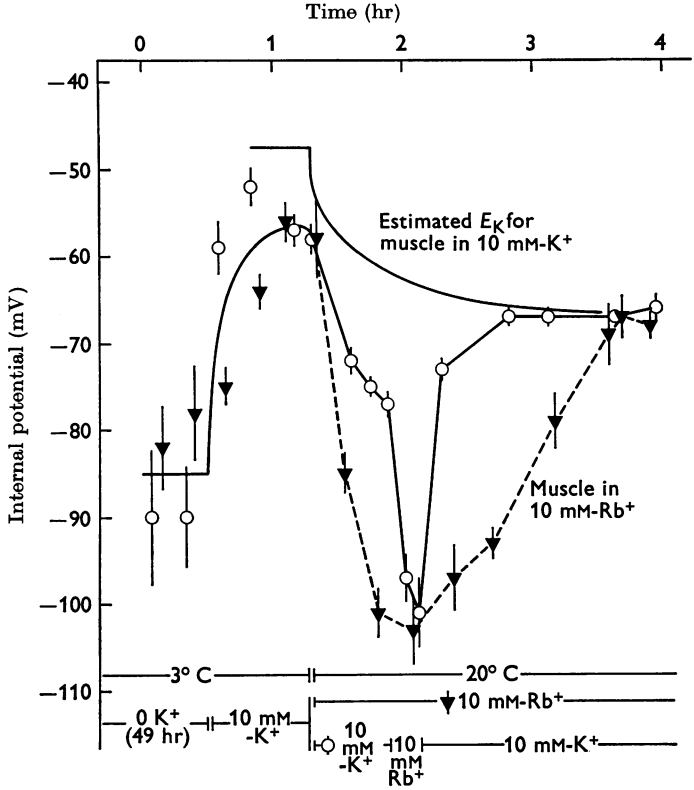

Fig. 7. The internal potential of surface fibres of sodium-rich muscles recovering in chloride solutions containing 10 m-equiv/l. of either potassium or rubidium (solutions *C* or *D*). The muscle recovering in the K-solution was transferred for a short time to the Rb-recovery solution. At the end of the experiment the muscles which were a pair were analysed. The muscle recovering in K-solution (open circles) had an internal potassium concentration of 142 m-equiv/kg fibre water. The other muscle had an internal potassium concentration of 75 m-equiv/kg fibre water. These concentrations have been used to estimate the initial and final values of E_K. The sodium concentrations of the two muscles were respectively 18 and 28 m-equiv/kg muscle. The rubidium concentrations were 6 and 46 m-equiv/kg muscle. Each point is the mean (± S.E. of the mean) of not less than six measurements.

TABLE 8. Potential during recovery in chloride solutions with 10 m-equiv/l. of K or Rb

		Before recovery			Recovery: Initial			Recovery: Final		
Muscle	Solution	Temp. (0° C)	V_m (mV)	Est. 'E_K' (mV)	Temp. (° C)	V_m (mV)	Est. 'E_K' (mV)	Temp. (° C)	V_m	Est. 'E_K'
a	*C*	5	$-60 \pm 1{\cdot}7$	-59	19	$-74 \pm 1{\cdot}6$	-64	19	$-70 \pm 0{\cdot}9$	-69
b	(KCl)	4	$-61 \pm 1{\cdot}6$	-56	20·5	$-75 \pm 2{\cdot}1$	-62	1	$-66 \pm 1{\cdot}2$	-65
c		3	$-57 \pm 1{\cdot}6$	-56	21	$-77 \pm 1{\cdot}4$	-64	21	$-67 \pm 0{\cdot}5$	-67
d		3	$-60 \pm 1{\cdot}9$	-53	21	$-89 \pm 1{\cdot}2$	-63	22	$-72 \pm 2{\cdot}0$	-67
e		1	$-50 \pm 1{\cdot}8$	-43	21	$-84 \pm 1{\cdot}1$	-57	21	$-70 \pm 2{\cdot}0$	-63
—		4	$-59 \pm 1{\cdot}6$	-50	21	$-74 \pm 2{\cdot}8$	-63	—	—	—
	Mean difference between V_m and E_K -5 mV					-17 mV				-3 mV
a	*D*	5	$-64 \pm 2{\cdot}4$	-59	19	$-85 \pm 3{\cdot}0$	-65	19	$-76 \pm 1{\cdot}9$	-69
b	(RbCl)	4	$-61 \pm 1{\cdot}1$	-56	20·5	$-87 \pm 2{\cdot}1$	-63	1	$-67 \pm 1{\cdot}5$	-65
c		—	—	—	21	$-103 \pm 4{\cdot}0$	-64	21	$-68 \pm 1{\cdot}4$	-67
d		3	$-64 \pm 1{\cdot}9$	-53	21	$-106 \pm 4{\cdot}4$	-63	22	$-70 \pm 1{\cdot}5$	-67
e		1	$-53 \pm 1{\cdot}9$	-43	21	$-100 \pm 2{\cdot}7$	-57	21	$-66 \pm 3{\cdot}4$	-63
—		1	$-43 \pm 3{\cdot}0$	-32	20	$-82 \pm 5{\cdot}0$	-57	—	—	—
	Mean difference between V_m and E_K -8 mV					-32 mV				-3 mV

Letters indicate paired muscles. All muscles were soaked at 0° C in K-free Ringer solution for 24–48 hr. In this Table 'E_K' $= 58 \log 10/([K]_i + [Rb]_i)$ $[K]_i$ before recovery was estimated from the unrecovered soaked pair, or from the pair recovered in RbCl (solution *D*). $[K]_i$ and $([K]_i + [Rb]_i)$ at time of measuring potential was estimated by interpolation between the final value of $[K]_i$ or $([K]_i + [Rb]_i)$ and $[K]_i$ before recovery.

(see Fig. 2). Table 8 summarizes the membrane potential measurements made on muscles recovering in chloride solutions containing either potassium or rubidium.

Cooling a muscle recovering in the potassium solution, during the hyperpolarization associated with the large net sodium movements returned the potential to near E_K. For a muscle recovering in the rubidium solution, cooling at the same stage returned the internal potential to a similar value, that is to a value between the initial and final potentials in the rubidium recovery solution and as much as 40 mV more positive than the internal potential at the peak of pumping hyperpolarization. Cooling after 3–4 hr, when the internal potential of both muscles had returned to about the value of E_K in the muscle recovering in the potassium solution, produced only small effects on the membrane potential. Though not investigated in detail, it was obvious that the membrane potential during pumping was very sensitive to temperature.

The potential of muscles recovering in solutions containing sulphate. Chloride-depleted sodium-rich muscles, prepared in the way previously described, show the same increase in membrane potential while they are pumping sodium into warm sulphate solutions containing 10 m-equiv/l. of either potassium or rubidium. Figure 8 shows an experiment, similar to the experiment in Fig. 7, in which the internal potential was measured initially in a sulphate solution at 2° C with a potassium concentration of 1 m-equiv/l. Increasing the external potassium concentration to 10 m-equiv/l. without raising the temperature caused a relatively rapid depolarization to an internal potential of -37 mV. Warming to 20° C altered the internal potential rapidly to -63 mV, so that immediately after warming the internal potential was 27 mV more negative than the potassium equilibrium potential. This was estimated from the internal potassium concentration of the pair of the experimental muscle which was similarly treated but prepared for analysis from the cold recovery solution. Substitution of rubidium for the potassium in the sulphate recovery solution produced a further hyperpolarization to -72 mV, which was reversible. In the sulphate recovery solutions, as in the chloride solutions, the internal potential at the peak of the pumping hyperpolarization, was 10–20 mV more negative in the rubidium-containing solution than in the potassium-containing solution.

Figure 9 shows for four fibres the time course of change in the internal potential and the change in temperature of the external solution. The temperature of the solution was followed with a thermocouple in the solution close to the muscle. The records of the internal potential were made with a potentiometric pen-recorder, and the initial downward step in each record is produced by the penetration of the micro-electrode into

the fibre. In the chloride recovery solutions only a slow hyperpolarization followed the rapid rise in temperature of the external solution. Figure 9*A* shows a record from a fibre of a muscle warmed in the chloride recovery solution containing rubidium. In sulphate recovery solutions containing either potassium or rubidium, warming the solution produced a hyperpolarization which did not lag behind the temperature change appreciably (Fig. 9*B*, *C*, *D*). Cooling the solution depolarized the membrane and caused contracture (Fig. 9*D*). The irregularities in the record in Fig. 9*D* were observed to be accompanied by contractures in the surrounding fibres.

The muscles in Figs. 6 and 7, recovering in chloride solutions, show a slow hyperpolarization on warming which reaches its maximum after about 30 min. These muscles had been equilibrated, before warming, in a chloride solution in which their internal potentials were

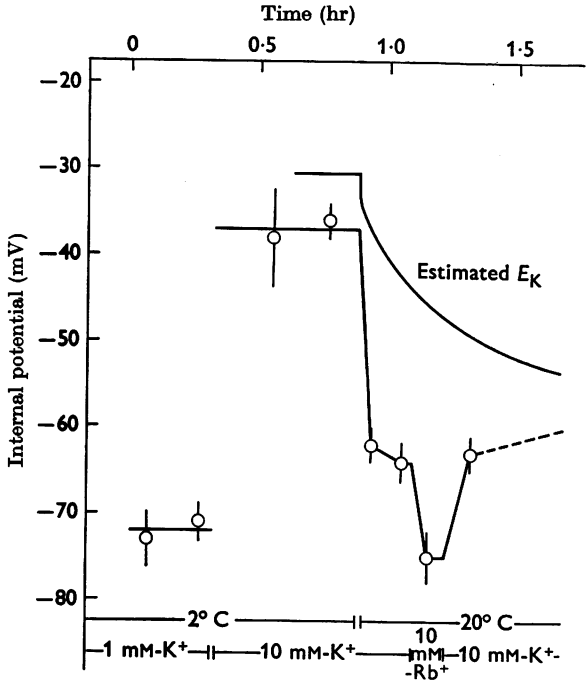

Fig. 8. The internal potential of surface fibres of sodium-rich, chloride-depleted muscles in sulphate solutions containing 10 m-equiv/l. of either K or Rb. The muscle had been soaked for 42·5 hr in K-free Ringer solution and then for 2 hr in solution *E* before any potential measurements were made. K or Rb concentrations, and the temperature of the solution are indicated at the bottom of the figure. At 1·6 hr the experimental muscle had an internal potassium concentration of 66 m-equiv/kg fibre water. Its pair, analysed at 0 hr, had an internal potassium concentration of 36 m-equiv/kg fibre water. These values have been used to estimate the initial and final values of E_K. The sodium concentrations of the control and experimental muscle were 70 and 45 m-equiv/kg muscle. The rubidium concentration of the experimental muscle was 5·4 m-equiv/kg muscle. Each point is the mean (±S.E. of mean) of not less than six measurements.

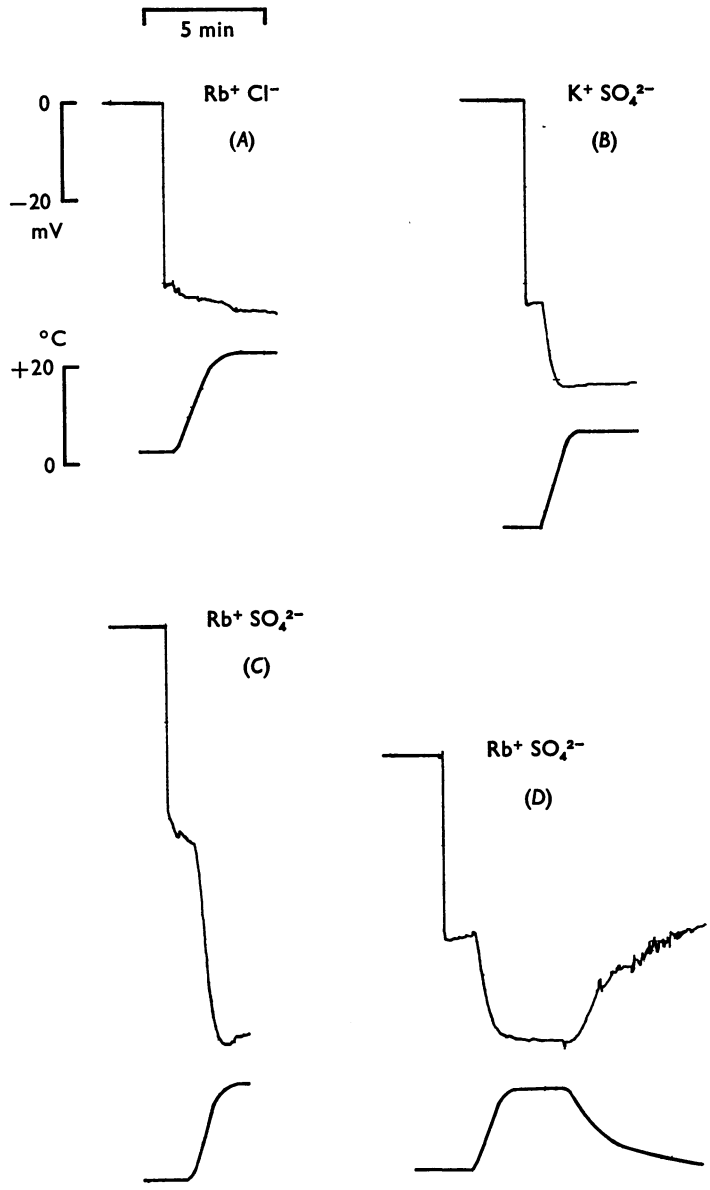

Fig. 9. Records from individual fibres from sodium-rich muscles in solutions *D*, *F* and *G* showing the internal potential in mV and the temperature of the external solution. The internal potential was recorded on a potentiometric penwriter recorder. The temperature was read from a galvanometer and plotted on the same time scale as the records of potential. The initial downward step represents the penetration of the recording micro-electrode into the fibre.

between −50 and −60 mV. If the chloride ion had been in equilibrium just before warming the internal chloride concentration would have been about 8 m-equiv/kg fibre water: a hyperpolarization produced by any mechanism would cause a passive outward movement of chloride in order to equalize the electrochemical potential of chloride inside and outside the fibre. During the period after warming, while there is still a net outward movement of chloride, the hyperpolarization will be less than it would be if there were no chloride ions present. An approximate calculation shows that the time taken for hyperpolarization to reach its maximum in chloride solutions (30 min) is consistent with the chloride permeability of the membrane estimated by other methods ($P_{Cl} = 3 \times 10^{-6}$ cm/sec: Hodgkin & Horowicz, 1959; Adrian & Freygang, 1962).

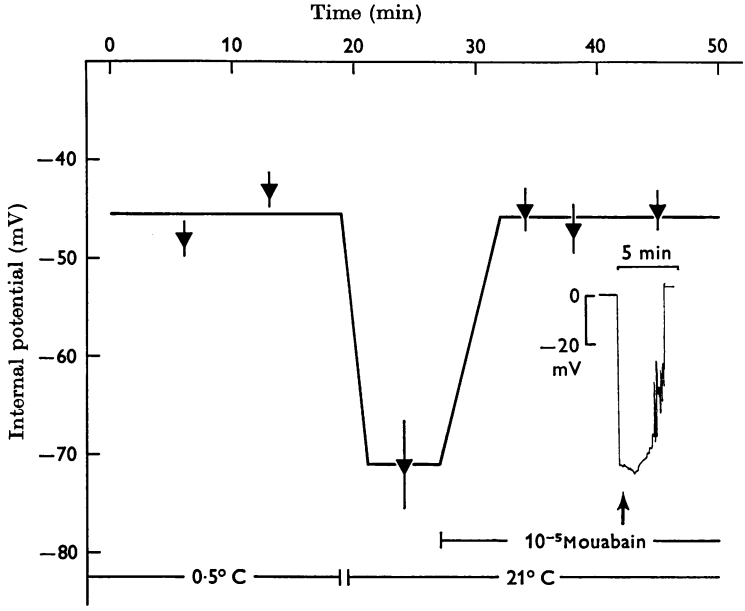

Fig. 10. The internal potential of surface fibres of a sodium-rich, chloride-depleted muscle in a sulphate solution containing 10 m-equiv/l.-Rb (*G*). The muscle had been soaked for 47 hr in K-free Ringer solution and 2·5 hr in solution *E* before it was transferred to solution *G* at 0 hr. At 20 min the temperature was raised from 0·5 to 21° C; at 28 min 10^{-5} M ouabain was added. After the potential measurements the intracellular potassium concentration was 66 m-equiv/kg fibre water. Had the muscle been in a solution with 10 m-equiv/l. of *K*, E_K would have been −44 mV at 0·5° C, and −48 mV at 21° C. In the inset record from a fibre of a second muscle at 22° C, the arrow indicates the addition of 10^{-5} M ouabain.

Figure 10 shows that the hyperpolarization is also abolished by 10^{-5} M ouabain. The inset record in Fig. 10 is the record of the potential change in a fibre, and shows that the action of the ouabain is rapid. The instability of the recorded potential just before withdrawal of the electrode was associated with contracture in the surrounding fibres.

The greater hyperpolarization in fibres exchanging rubidium for sodium than in fibres exchanging potassium for sodium is qualitatively

consistent with an electrogenic pump and the greater membrane resistance to the movement of rubidium. The larger hyperpolarization in rubidium is difficult to reconcile with the operation of a neutral pump which causes hyperpolarization by altering the concentration of either potassium or rubidium just outside the fibre membrane. Whether one supposes that the region of diminished potassium or rubidium concentration is an unstirred layer in the superficial sarcolemma, the extracellular space just deep to the superficial fibres, or the transverse tubular system of the sarcoplasmic reticulum, it is unlikely that the diffusion constants for potassium or rubidium in that region are very different. Since it appears that rubidium and potassium are transported into the cell at approximately the same rate, the concentrations of potassium and rubidium in the region should be about the same during net outward sodium movements. If this were the case it should be possible to find a potassium concentration, and an equal rubidium concentration, at which the membrane potentials are approximately equal to those recorded during net movements of sodium. In particular the internal potential at the rubidium concentration should be 10–25 mV more negative than at the same potassium concentration. Figure 5 shows that in recently dissected muscles at concentrations between 1 and 10 mM, the potential at a particular rubidium concentration is never more than 7 mV more negative, and at most concentrations is less negative than the internal potential of fibres in the same potassium concentration. In Fig. 5 the sodium-rich muscles, at all rubidium concentrations between 10 and 2·5 mM, have internal potentials less negative than at the same potassium concentrations. It is possible that extracellular depletion of potassium or rubidium accounts for some part of the hyperpolarization seen in fibres pumping sodium. But it is difficult, without special assumptions, to see how it can account for the very different magnitudes of the hyperpolarizations observed in muscles recovering in potassium and rubidium solutions.

In the surface fibres, during extrusion of sodium, the membrane potential, which starts at or around the initial value of E_K falls to a minimum, and then rises towards the final value of E_K as recovery becomes complete. The fact that the potential passes through a minimum, also observed by Frumento (1965), is important because it disposes of the possibility that the difference between the measured internal potential and E_K during pumping is due to the much more rapid extrusion of sodium in the surface fibres than in the deep fibres of the muscle. If the membrane potential had not fallen to a value more negative than the final E_K, it could have been supposed that the surface fibres achieved a normal potassium concentration substantially before the internal potassium concentration averaged over all the fibres of the muscle had recovered. The very rapid hyper-

polarization in sulphate solutions is also difficult to explain on the basis of more rapid recovery of surface fibres.

The effect of temperature on the potential changes associated with sodium loss and rubidium uptake in sulphate solutions. Figure 11 shows an experiment in which the temperature was changed from 4 to 32° C. The sodium-rich, chloride-depleted muscle was in a sulphate solution containing 10 m-equiv/l.-Rb (*G*). The points in Fig. 11 represent individual measurements of internal potential, and the inset record is the potential change that was recorded in one fibre during the change of temperature. The

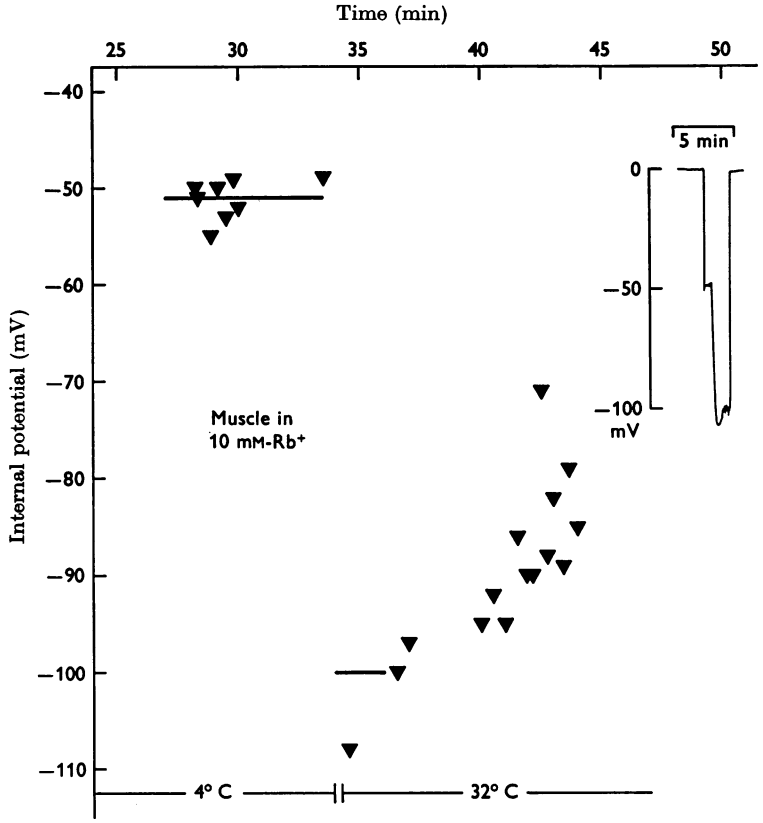

Fig. 11. The internal potentials of surface fibres of a sodium-rich, chloride-depleted muscle at 4° C and then at 32° C in a sulphate solution containing 10 m-equiv/l. Rb (solution *G*). The muscle had been in K-free Ringer solution for 42·5 hr and then in a sulphate solution without potassium for 2 hr. Each point is the internal potential of one fibre, with the exception of the last point before and the first point after the change in temperature, which are both from the same fibre. The inset record shows the potential change (−49 to −109 mV) which occurred when the temperature was raised. The muscle was analysed at 45 min. The internal potassium concentration was 63 m-equiv/kg fibre water.

internal potential of this fibre changed from -49 to -108 mV. After the potential measurements the intracellular potassium concentration was found to be 63 m-equiv/kg fibre water. In a similar experiment, the maximum hyperpolarization, also seen immediately after the change in temperature, was to -111 mV, and the mean internal potential before warming was -47 mV. The intracellular potassium concentration in this second experiment was 55 m-equiv/kg fibre water. In both these muscles the internal potential immediately after warming was substantially more negative than would be expected if the concentration of rubidium had been zero outside the fibre. In Fig. 5 a recently dissected muscle had a mean internal potential of $-118 \pm 1{\cdot}4$ mV in the absence of external potassium or rubidium. If the only difference between the muscles of Figs. 5 and 11 was in their internal potassium concentrations, one would expect the internal potential of the muscle in Fig. 11 at 32° C, and in the absence of external potassium or rubidium, to be given by

$$\frac{60{\cdot}5}{58}\left(58\log_{10}\frac{150}{63}-118\right) = -100 \text{ mV}.$$

A similar calculation for the second muscle gives a potential of -97 mV. This method of calculating the potential in the absence of both potassium and rubidium is obviously doubtful, because it depends on the unjustified assumption that the ratio of the sodium and potassium permeabilities is the same in fresh and soaked muscles. However, the calculation probably estimates a potential more negative than would be measured. Indeed, when a muscle, soaked for 48 hr in K-free Ringer solution, which had a mean internal potential of $-81 \pm 1{\cdot}9$ mV at the end of the soaking, was warmed to 20° C in the K-free Ringer solution, the mean internal potential was $-36 \pm 2{\cdot}3$ mV, and this muscle showed a typical hyperpolarization when potassium (10 mM) was added. Hyperpolarization beyond the estimated internal potential for zero external potassium or rubidium does not always occur. It was seen in two out of six experiments. In the four other experiments the hyperpolarizations were to or just short of the estimated potential. Nevertheless it is difficult to account for the large hyperpolarizations seen in the experiments at 32° C only on the basis of depletion of the extracellular space of rubidium ion.

The muscle in the experiment of Fig. 11 was analysed immediately after the potential measurements had been complete. The internal rubidium concentration was then 16 m-equiv/kg fibre water, which represents an inward movement of 96 m-equiv/kg fibre water.hr, measured in the first 10 min, and averaged over all the fibres in the muscle. Suppose however that the extra potential is proportional to the rate of sodium pumping which is itself proportional to the internal sodium concentration. Then one

can estimate the rate of sodium movement in the surface fibres of the muscle in Fig. 11. The initial rate of loss of sodium is

$$\left(\frac{d[Na]_i}{dt}\right)_{t=0} = -\frac{1}{\tau}[Na]_{i_{t=0}}$$

where τ is the time constant for the exponential fall in sodium concentration. After 5 min at 32° C the rate of loss of sodium is

$$\left(\frac{d[Na]_i}{dt}\right)_{t=5} = -\frac{1}{\tau}[Na]_{i_{t=0}} e^{-5/\tau}.$$

By hypothesis the ratio of these two rates is equal to the ratio of the hyperpolarizations; (108–50)/(92–50) = 1·38. The time constant for the loss of sodium is therefore 15·5 min. If the initial intracellular concentration of sodium is 70 m-equiv/kg fibre water, the initial rate of loss would be 270 m-equiv/kg fibre water.hr. If the movement of rubidium is equal and opposite to the movement of sodium, the rubidium movement is 2·8 times the initial rate measured on the whole muscle, which agrees well with the calculated effect of extracellular diffusion (see above p. 987).

Table 9 collects the experimental results for sodium-rich muscles recovering in the sulphate solution containing 10 m-equv/l. rubidium (solution *G*). On any individual muscle measurements of internal potential were made at the low temperature (*ca.* 3° C) and then after warming to one of the higher temperatures (20 or 30° C). The internal potassium concentration was estimated and from it an E_K was calculated as if there had been 10 m-equiv/l. potassium in the external solution. Figure 5 suggests that at 18° C in the presence of 10^{-5} M ouabain and 10 m-equiv/l. rubidium the internal potential is about 4 mV less negative than in the same external potassium concentration. Therefore 4 mV has been added to the calculated E_K in order to arrive at an estimate of the internal potential which would have been measured in the absence of any pump activity ($E_{K, Rb, Na}$). The measured internal potentials at 20 and 30° C in Table 9 are the means of the potentials measured, during the first 10 min after warming. It is clear from Table 9 that at 3, 20, and 30° C the internal potential is more negative than the estimated potential for a fibre in which there is no active transport of sodium. The significance of the difference at 3° C is hard to estimate. If it does represent a residual activity of the sodium pump, the difference at 3° C should be sensitive to ouabain. Unfortunately this point was not tested. At 30° C the potential associated with activity of the sodium pump is very probably underestimated, because this potential declines considerably in the first 10 min (see Fig. 11). In two muscles warmed to 32° C (*f* and *g*, Table 9) where the potential of a particular fibre was measured before and immediately after the change in

temperature the hyperpolarizations associated with warming were 59 and 64 mV. It seems reasonable to conclude that the immediate hyperpolarization associated with sodium pumping in these muscles at 30° C in the presence of 10 m-equiv/l. rubidium does not greatly exceed 60 mV.

TABLE 9

Muscle	Temp ° C	$[K]_i$ m-equiv/kg fibre water (estimated after recovery)	$E_K + 4$ mV (mV)	V_m mV ± 1 S.E. of mean	$V_m - (E_K + 4$ mV) mV	No. of fibres
a	1·0	69	−43	−44 ± 1·3	−1	10
b	3·5	59	−38	−55 ± 1·2	−17	8
c	0·5	67	−41	−46 ± 1·3	−5	15
d	2·0	80	−46	−34 ± 2·3	+12	20
e	4·0	35	−26	−27 ± 1·4	−1	14
f	4·0	63	−40	−51 ± 0·9	−11	10
g	5·0	55	−37	−46 ± 3·7	−9	12
h	2·0	25	−19	−36 ± 3·2	−17	5
Mean	2·8			−42·4 mV	−6 mV	
a	20·0	69	−45	−75 ± 1·4	−30	13
b	20·5	59	−41	−95 ± 2·1	−54	10
c	21·0	67	−44	−71 ± 4·4	−27	12
d	22·0	80	−48	−76 ± 6·8	−28	7
i	20·0	36	−29	−75 ± 2·9	−46	10
Mean	20·7			−78·5 mV	−37 mV	
e	32·0	35	−29	−68 ± 2·8	−39	9
f	32·0	63	−44	−102 ± 3·2	−58	3
g	32·0	55	−39	−104 ± 3·7	−65	3
h	30·0	25	−20	−65 ± 2·2	−45	18
j	30·0	25	−20	−70 ± 4·1	−50	7
Mean	31·2			−81·8 mV	−51 mV	

It is possible, from the results in Tables 7 and 9 to estimate how fast rubidium would move into a muscle if the only forces acting on it were its concentration gradient and the measured membrane potential. In the presence of ouabain at 18° C sodium-rich muscles have a mean internal potential of −33 mV (Fig. 5, $[K]_i$ = 43 m-equiv/kg fibre water). Table 7 gives the measured uptake of rubidium in the presence of ouabain at 8, 20, and 30° C. Ignoring the small change in potential due to the change of temperature, one can assume that these measured rubidium uptakes represent the influxes of rubidium at the three temperatures and at an internal potential of −33 mV. Adrian (1964) has shown that the current carried across the membrane by rubidium is linearly related to the membrane potential. The rubidium current does not behave like the potassium current, which is non-linear and rectifies strongly. Since the rubidium does not deviate markedly from the predictions of the constant field equation assuming a constant rubidium permeability, the constant field assumption may be used to predict the magnitude of the inward rubidium movement at the potential measured during outward sodium movement. Table 9

can be used to estimate these potentials at 8, 20, and 30° C. By linear interpolation between 2·8 and 20·7° C, the additional potential associated with sodium pumping would be −15 mV at 8° C; at 20·7° C the additional potential is −37 mV; at 30° C it is estimated to be −60 mV (see above). The internal potentials during maximal outward sodium movement are therefore assumed to be: −48 mV at 8° C, −70 mV at 20° C, and −93 mV at 30° C. The ratio of the influxes (M_i) of an ion, from a fixed external concentration, at two potentials V_1 and V_2 if the permeability is indpendent of the membrane potential, is given by the equation

$$\frac{(M_i)_{V_2}}{(M_i)_{V_1}} = \frac{V_2}{V_1}\frac{\exp(V_1F/RT)-1}{\exp(V_2F/RT)-1}.$$

The values of V_2/V_1 at the three temperatures are 1·45, 2·12, and 2·82; the values of the ratios $(M_i)_{V_2}/(M_i)_{V_1}$ are 1·25 at 8° C, 1·66 at 20° C, and 2·08 at 30° C. It is clear that at all three temperatures the potential observed during the large outward sodium movement is inadequate to account for the 10 to 20-fold difference between the rate of rubidium entry in the presence and absence of ouabain.

An alternative way of considering the discrepancy is to calculate the change in rubidium permeability (P_{Rb}) required to account for the difference in the inward movements of rubidium in the presence and absence of ouabain

$$P_{Rb} = \frac{RT}{VF}\frac{M_i(\exp(VF/RT)-1)}{[Rb]_i}.$$

In Table 7 the rubidium influx at 30° C in the presence of 10^{-5} M ouabain is 2·2 p-mole/cm².sec and it takes place at an internal potential of −33 mV. In the absence of ouabain the influx, uncorrected for the effects of extracellular diffusion, appears to be 41 p-mole/cm².sec and this influx takes place at an internal potential of −93 mV (estimated). P_{Rb} at −33 mV appears to be $0{\cdot}12 \times 10^{-6}$ cm/sec but at −93 mV it would have to be $1{\cdot}0 \times 10^{-6}$ cm/sec if the large inward rubidium movement is produced only by concentration and potential gradients. Changes in the potassium permeability of muscle membrane have been observed (Hodgkin & Horowicz, 1959; Adrian & Freygang, 1962), but measurements of rubidium conductance as a function of membrane potential suggest that the rubidium permeability is both relatively constant and less than the potassium permeability (Adrian, 1964).

The measured rates of rubidium uptake in Table 7 are uncorrected for the effects of extracellular diffusion, and the uptake rates which apply to the superficial fibres in which potential measurements were made are likely to be substantially greater (see p. 987). Since the observed potential changes cannot account for more than about 10% of the measured

rubidium uptake, it seems safe to conclude that more than 90% of the inward rubidium movement is linked to the outward sodium movement by a means other than the membrane potential. It seems hardly likely that a muscle cell would have a special mechanism for transporting rubidium which does not, to some extent at least, transport potassium. It is therefore reasonable to suppose that this same mechanism, whatever it proves to be, can link part of the inward potassium movement to the outward sodium movement. However, because the potassium permeability is not independent of the membrane potential it is not possible to make a quantitative estimate of the fraction of the observed inward potassium movement that could be driven by the potential measured during pumping. The membrane permeability to potassium is much greater than to rubidium, and therefore the current of potassium which could be driven in by the potentials associated with large outward sodium movements is likely to be considerably greater than the rubidium current under similar circumstances. Since the measured net movements of potassium and rubidium are nearly equal, one must conclude that much more than 10% of the inward potassium movement could be driven in by the observed potential difference.

Membrane resistance measurements. A second, but probably less reliable, method is available for estimating the fractions of the inward cation movements that are driven as electric currents. If the sodium pump produced these inward potassium and rubidium movements only by altering the membrane potential, the electrically measured resistance to the movement of these ions should be such that the observed potentials would produce the observed movements. Resistance measurements were therefore made on both recently dissected muscles and on sodium-rich muscles in the presence of 10^{-5} M ouabain. The muscles were in sulphate solutions containing 10 m-equiv/l. of either potassium or rubidium (solutions *F* and *G*). A three electrode method was used (Adrian & Freygang, 1962). This method provides records of the membrane potential displacement (V) and a potential difference (ΔV) which is very nearly proportional to the membrane current producing the membrane potential change. The membrane current (ΔV) and the membrane potential change (V) were measured at the end of long (5 sec) current pulses, in order to imitate, as far as possible, the steady current which would be produced by the operation of an electrogenic pump.

Figure 12 plots, for individual fibres, an internal potential (V plus the internal potential in the absence of applied current) against ΔV. Each point therefore shows the steady current necessary to maintain a particular internal potential. For fibres in the rubidium solution (*G*) V and ΔV are related linearly, and the short line through each point is drawn so that it

projects to the internal potential in the absence of applied current. For a fibre in the potassium solution (*F*) whose membrane conductance rises as the current is increased, the short line is drawn as a tangent to the curve relating V and ΔV. Several points on the curve for each fibre were measured, but to avoid confusion only one point from each fibre has been plotted.

The membrane current density (I_m) in $\mu A/cm^2$ is related to ΔV in mV by the following approximate equation

$$I_m/\Delta V = \frac{r.10^3}{3a^2R_i},$$

where r is the fibre radius (40 μ), R_i is the internal specific resistance (250 Ω cm), and a is the longitudinal spacing of the electrodes measuring V and ΔV (390μ). Using these values, $I_m/\Delta V = 3{\cdot}5\ \mu A/cm^2.mV$. A current of 3·5 $\mu A/cm^2$ would be carried by a net flux of a univalent ion of 35 p-mole/cm^2.sec, or a rate of change of the internal concentration in a fibre with a 40 μ radius of 90 m-equiv/kg fibre water.hr.

In Fig. 4 the initial rates of change in the internal concentration of potassium and rubidium appear to be 140 and 116 m-equiv/kg fibre water.hr, but these high rates and the small change in the sodium concentration during the first 15 min seem likely to be the result of volume changes during the beginning of the recovery period. At the end of the first 15 min recovery the internal concentration of rubidium was 16 m-equiv/kg fibre water, and the difference between the potassium concentrations of muscles recovering in potassium and rubidium solutions was 22 m-equiv/kg fibre water. The initial rates of potassium and rubidium entry from sulphate solutions (*F* and *G*) will be taken to be 88 and 64 m-equiv/kg fibre water.hr. As has been stressed already these rates are average rates for all the fibres in a muscle, and the rates in the surface fibres are likely to be much greater. Applying the correction outlined on p. 987 ($D' = 4 \times 10^{-6}$ cm^2/sec, $b = 350\ \mu$), the rates of change of internal concentration in the surface fibres would be for potassium 349 m-equiv/kg fibre water.hr and for rubidium 187 m-equiv/kg fibre water.hr. These rates are comparable to the rate estimated from the experiment in Fig. 11. The currents necessary to produce these rates of change correspond to values of ΔV of 3·8 mV for potassium and 2·1 mV for rubidium. If the membrane resistance is low enough to allow the total inward cation movement to be in the form of an electric current, the points in Fig. 12, which have been selected at internal potentials comparable to those measured in muscles extruding sodium, should have values of ΔV corresponding to the values calculated above from the measured cation movements. The horizontal lines in Fig. 12 are drawn at a vertical position

corresponding to the calculated values of ΔV. The lengths of the lines indicate the range of peak membrane potentials measured in muscles exchanging sodium for potassium and sodium for rubidium.

The result for muscles in the rubidium recovery solution (*G*) is clear. All the experimental points lie above the line, and none of the lines through the points projects to cross the horizontal line. The membrane resistance is too high for the membrane potential measured during pumping to be the only means by which rubidium is exchanged for sodium. Nevertheless, these resistance measurements are not as unequivocal as the results of Tables 7 and 9 (see p. 1004). In that comparison the rubidium uptake rates, uncorrected for the effect of extracellular diffusion, were 8- to 10-fold too large for the measured potential to be the only means for the exchange of rubidium for sodium. This difference reflects a discrepancy between the rate of inward movement of rubidium in the presence of ouabain as measured by flame photometry, and the inward current measured under the same circumstances and assuming that all the current is carried by rubidium ions. If permeabilities (P_{Rb}) are derived from both sets of results, using the constant field assumption, the analytic results suggest a value of about $0{\cdot}1 \times 10^{-6}$ cm/sec whereas the electrical measurements suggest about $0{\cdot}5 \times 10^{-6}$ cm/sec. Apart from the uncertainties involved in the calculation of the membrane current density from the electrical results, there are several reasons why the electrical measurements should over-estimate the inward rubidium current. It is improbable that rubidium is the only ion present capable of carrying current across the membrane. Indeed if the sodium current is calculated at -100 mV using the constant field equation ($P_{Na} = 0{\cdot}01 \times 10^{-6}$ cm/sec) it is equivalent to a ΔV of 0·1 mV. Since the membrane resistance is high in rubidium solutions, the effects of imperfect sealing around the tips of the three micro-electrodes may not be negligible.

For muscles in the potassium recovery solution (*F*) it is much harder to draw any conclusion from the comparison of membrane resistance and the calculated inward potassium movement. First, the current–voltage curves of a few of the fibres in Fig. 12 would clearly cross the horizontal line, though the majority probably would not. But if the correction for extracellular diffusion had been over-estimated then there could be reasonable agreement between the net potassium movement and the current measured at potentials prevailing during sodium pumping. Secondly, the reasons for supposing that the rubidium current is over-estimated by the electrical measurements do not apply to the potassium current because the membrane resistance to the inward movement of potassium is much less than to the inward movement of rubidium.

Because of these uncertainties it is not possible to say whether all the

inward potassium movement could be driven in by the difference between E_K and the measured internal potential. Assuming that the value of E_K across the membrane in which the pump operates is that calculated from the intracellular concentration and the concentration in the external solution, something between 50 and 100 % of the inward potassium movement could be driven by the potential measured during sodium pumping. The fact that so large a proportion of the inward rubidium movement cannot be driven by the membrane potential makes it seem probable that part of the potassium movement is linked to the sodium movement in some other way. But the evidence certainly suggests that the fraction of the total movement so linked is substantially less for the exchange of potassium for sodium than for the exchange of rubidium for sodium.

Changes of P_K and hyperpolarization with cocaine. Previous experiments (Adrian & Freygang, 1962) have demonstrated that cocaine reduces the potassium conductance of the muscle membrane and makes it relatively insensitive to changes of membrane potential. Since a large fraction of the inward potassium movement during sodium extrusion appears to be driven by the electrical potential difference, cocaine should, if its only action is on potassium conductance, increase the amount of hyperpolarization produced by the pump in muscles recovering in potassium solutions. Preliminary experiments have confirmed this expectation. The recovery phases of these experiments were carried out in solution *F* of Table 1,

Legend to Fig. 12.

Fig. 12. The abscissa represents the internal potential during applied current of fibres from recently dissected (open symbols) and sodium-rich muscles (half-filled symbols). The ordinate represents the magnititude of the steady membrane current (proportional to ΔV) necessary to alter the internal potential from its resting value. The sloping lines are short segments of the current–voltage relation for each fibre. This relation was measured for each fibre, but only one experimental point per fibre is plotted. The sodium-rich muscles were in the presence of 10^{-5} M ouabain. In the upper graph muscles were in a sulphate solution with 10 m-equiv/l. rubidium (*G*). In the lower graph muscles were in a sulphate solution with 10 m-equiv/l. potassium (*F*). Horizontal lines are drawn at ΔV values of 2·1 mV in *A* and 3·8 mV in *B*. These values represent the calculated inward current equivalent to the measured inward movements (corrected for diffusion delay) of rubidium and potassium which take place when sodium-rich muscles are allowed to recover in solutions *G* and *F* at 18° C (see text). The extent of the horizontal lines indicates the range of internal potentials observed during the net outward movements of sodium. In *A* the sodium-rich muscle had an internal potassium concentration of 34 m-equiv/kg fibre water, and the mean internal potential in the absence of applied current was −32 mV. In *B* the sodium-rich muscle had an internal potassium concentration of 69 m-equiv/kg fibre water, and the mean internal potential in the absence of applied current was −42 mV. The electrical measurements were made at 22° C.

containing 10 m-equiv/l. of potassium with sulphate as the anion. Several muscles were soaked for 1 day in cold K-free solution (*B*) and had average internal potentials near -57 mV ($E_K = -53$ mV) in the recovery solution at 2° C. As the solution was warmed in the absence of cocaine the internal potentials fell to a minimum of -70 to -75 mV. When cocaine was added (0·1 %, 3 mM) the average internal potential fell another 15–20 mV to a level of about -90 mV over a time of 10 min. When changes of E_K are taken into account, the amount of hyperpolarization produced by the pump was multiplied by a factor of 2–3. Resistance measurements of fibres from soaked muscles maintained in ouabain gave also an increase of two- to three-fold, with the ratio $V/\Delta V$ (see above) going from about 30 in the absence of cocaine to about 80, again in a time of 10 min. Unfor-

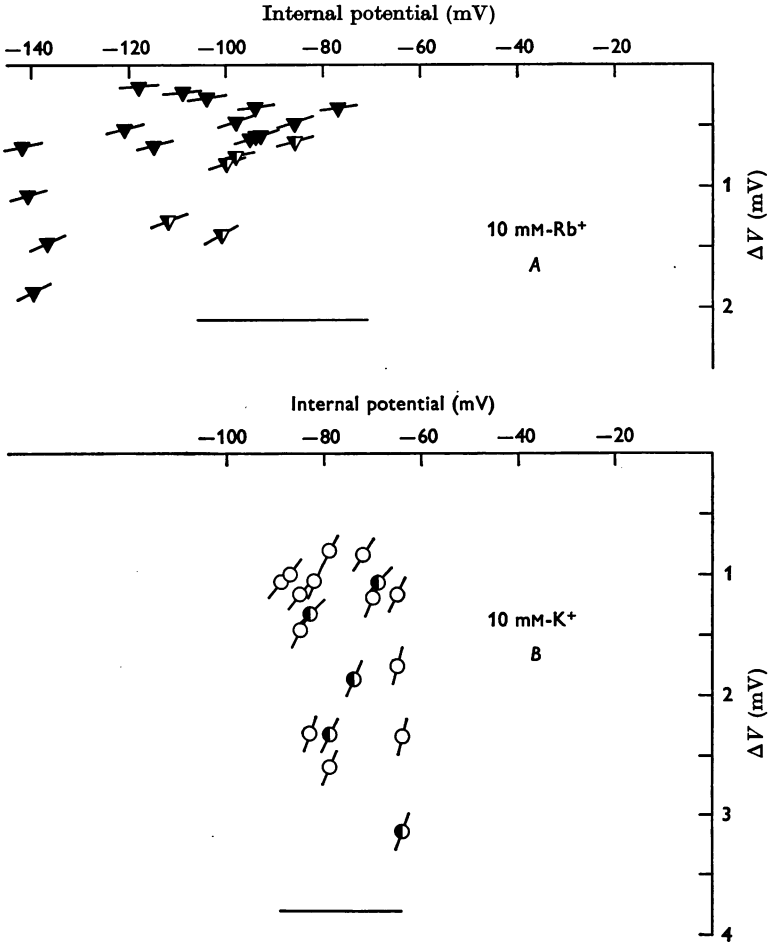

Fig. 12. For legend see opposite page.

tunately, the experiments have not been done to compare the exact time courses of the potential and resistance changes.

It is not certain that cocaine has no effect on the sodium pump itself during potassium uptake, but recovering muscles take up rubidium in the presence of 3 mM cocaine nearly as well as in its absence. Two soaked muscles with internal potassium concentrations of about 85 m-equiv/kg fibre water took up Rb (from solution *G*, Table 1) at rates of 45 and 31 m-equiv/kg fibre water.hr during the first half hour of recovery, while their pairs recovering in the presence of cocaine gained Rb at rates of 43 and 26 m-equiv/kg fibre water.hr, respectively. A precise comparison between net potassium uptake, membrane resistance, and hyperpolarization during pumping would be useful in indicating whether the apparent amount of non-electrical coupling between potassium and sodium movements increases in the presence of cocaine.

DISCUSSION

The experiments described in this paper show clearly that in fibres producing large outward movements of sodium, the internal potential may be substantially more negative than in fibres not moving sodium. The large negative internal potential, associated with sodium movement, differs in several ways from the resting potential. It is much more sensitive to temperature, ouabain, and cocaine than the normal resting potential, and it behaves differently when rubidium is substituted for potassium in the external solution. These observations suggest that the hyperpolarization during net outward movement of sodium could be the result of an outward current generated by the sodium pumping mechanism.

Numerous authors have shown that the extrusion of sodium from frog sartorius muscle is associated with a potential difference between the inside and outside of the fibre which could move potassium into the cell to replace the sodium pumped out. Similar observations have been made on a number of other tissues. Page & Storm (1965) have shown a hyperpolarization beyond the likely E_K in superficial fibres of cat heart papillary muscle while it is extruding excess internal sodium. Kirkut & Thomas (1965) have shown that a lasting hyperpolarization, sensitive to ouabain, temperature, and external potassium, is produced by the injection of sodium salts (but not potassium salts) into the neurones of *Helix*. Connelly (1959) has interpreted the post-tetanic hyperpolarization of frog sciatic nerve and of *Maia* nerve in terms of a sodium extruding mechanism which can separate charge. Somewhat similar findings in mammalian C fibres were initially explained by Ritchie & Straub (1957) by an electrically neutral pump mechanism and an extracellular depletion of potassium during pumping,

but this interpretation cannot account for the fact (Straub, 1961) that the hyperpolarization produced by the pump is always proportional to membrane resistance and sometimes significantly exceeds the value expected if the effective potassium concentration were reduced to zero. Koefoed-Johnsen & Ussing (1958) postulated a neutral sodium and potassium exchange mechanism as the basis of sodium pumping by frog skin. However, Frazier & Leaf (1963) consider that under certain circumstances the potential at the serosal surface of the toad bladder mucosa, which like frog skin transports sodium, cannot be simply a diffusion potential produced by the potassium concentration gradient. They consider that the sodium transporting mechanism in toad bladder is able to separate charge.

The existence of a potential difference between the inside and outside of the cell which is associated with metabolically driven movements of sodium has been taken as evidence that the pumping mechanism is not neutral. However, the presence of this potential difference cannot by itself establish the non-neutrality of the sodium pumping mechanism. Apart from a purely operational use, the term 'electrogenic pump' has come to mean a mechanism which by separating electric charge contributes directly to the ionic currents across the diffusion barrier in which the pump operates. It has been postulated that the pump is capable of moving only sodium ions (Conway, 1964), and that compensatory movements of other permeant ions occur only in so far as the altered membrane potential alters their electrochemical gradients. Such a mechanism constitutes the extreme type of electrogenic pump, but any cation pumping mechanism which drives unequal movements of cations in opposite directions will directly affect the membrane potential. Measurements of the potential difference between the inside of the cell and the external solution cannot distinguish between neutral and electrogenic mechanisms until it has been established that the operation of the pump does not affect the concentration gradients across the barrier in which the pump operates, and does not create either concentration or potential differences across other diffusion barriers in series with the pump.

In general, electrical signs of pump activity are seen only when the rates of sodium movement are large. In frog muscle with a normal internal sodium concentration the ouabain-sensitive outward sodium movement is not large enough to produce an easily measurable potential drop across the electrical resistance of the membrane. Recently isolated frog muscle, for instance, would not be expected to show much immediate alteration of resting potential if sodium pumping was suddenly inhibited. Since the rate of sodium movement and therefore of potassium movement has to be large to detect any electrical sign of pumping, the interpretation of observed hyperpolarizations is always complicated by the effects of extra-

cellular diffusion. Even the interpretation of experiments on isolated fibres might be complex if pumping took place in the walls of the transverse tubules of the sarcoplasmic reticulum. It is certainly not clear how far the hyperpolarizations reported in the present experiments and those reported by other authors are to be accounted for by electrogenic pumping and how far by diffusional depletion of the immediately extracellular potassium. As has been argued on p. 1001 diffusion effects do not seem able to account for all the observations reported here, but they certainly cannot be ignored.

There are other lines of evidence which suggest that inward potassium movement is linked to outward sodium movement by chemical forces, that is by forces other than the potential across the membrane. The observation that the outward movement of sodium is reduced by the absence of external potassium is suggestive, but not of course conclusive since the sodium pumping mechanism could be activated by external potassium but not itself transport potassium. However, in squid nerve, Hodgkin & Keynes (1954, 1955) have shown that the influx of potassium is greatly reduced by the metabolic inhibitors DNP, and cyanide, and these reductions take place without any large alterations in the membrane potential. They have also shown that the influx of potassium has a larger Q_{10} than the efflux.

Relman *et al.* (1957) have shown that for rat muscles *in vivo* in a steady state, the ratio of the potassium concentrations on each side of the membrane is not equal to the ratio of the rubidium concentrations when both ions are present. If a permeant ion is not pumped then in the steady state its electrochemical potential must be the same on both sides of the membrane. In Relman's experiments potassium and rubidium cannot both be in equilibrium and so, if the possibility of differential binding is ignored, one or the other, and therefore probably both must be pumped.

A pump moving potassium as well as sodium, but producing a separation of charge across the membrane, is one which produces unequal movements of potassium and sodium. It is, however, not self-evident that such a pump should produce a fixed ratio of sodium and potassium movements under all circumstances. Mullins & Awad (1965) mention the possibility of a variable linking ratio. Cross *et al.* (1965) suggest how such a variable linking ratio could be produced by a carrier whose affinity for sodium and potassium was altered by metabolic reactions. If the carrier, in its potassium-specific form but uncombined with potassium, could move across the membrane, but the uncombined sodium-specific form could not cross the membrane, more sodium would move out with the carrier than potassium would move in. If the carrier, in its potassium-specific form, had an affinity for rubidium higher even than for potassium, a much smaller

back movement of uncombined carrier might occur under any given circumstances when rubidium rather than potassium was the external exchanging cation. Since some, at least, of the mobile components of such a pump system must carry an electric charge, the over-all pumping rate as well as the linking ratio could be affected by the membrane potential difference. In this connexion Horowicz & Gerber (1965*a*, *b*) have shown that the sodium efflux of isolated muscle fibres is increased by agents which depolarize the membrane.

It seems likely that the pumping rate and the ratio of the pumped movements of sodium and potassium depend on the conditions at any moment. Probable factors influencing these pumping parameters include: the availability of energy-rich phosphate compounds; the membrane potential; the identity (K, Rb, Cs, NH_4?) of the external exchanging ion and its concentration; the internal sodium concentration. It is possible that in the course of any individual experiment of the kind considered in this study the linking ratio may vary over a wide range just as does the pumping rate.

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