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CONTRIBUTION OF AN ELECTROGENIC SODIUM PUMP TO MEMBRANE POTENTIAL IN MAMMALIAN SKELETAL MUSCLE FIBRES

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SUMMARY

1. Relationship between the resting membrane potential and the changes in the intracellular Na and K concentrations ($[Na]_i$ and $[K]_i$) was studied in 'Na-loaded' and 'K-depleted' soleus (SOL) muscles of rats which had fed a K-free diet for 40 and more days.

2. The extracellular space of the muscles was not significantly different between normal and K-deficient rats. The inulin space in both the 'fresh' and 'Na-rich' muscles can be determined by the same function relating the space to the muscle weight.

3. Presence of 2·5–15 mM-K in the recovery solution hyperpolarized the 'Na-rich' muscle fibres at the beginning of recovery. The hyperpolarized membrane potential exceeded, beyond the measured potential of 'fresh' muscle fibres, the theoretical potential derived from the ionic theory, or even beyond E_K. Then, the measured membrane potential declined progressively during the immersion in a recovery solution and returned to the steady-state value. When a considerable Na extrusion and K uptake took place, the measured membrane potential became equal to E_K.

4. The maximal hyperpolarization occurring immediately after immersion in the recovery solution became smaller and had a shorter duration when increasing the external K concentration ($[K]_o$) from 2·5 to 15 mM.

5. The K-sensitive hyperpolarization was completely abolished on exposure to 0 mM $[K]_o$, on cooling to *ca.* 4° C, and in the presence of ouabain (10^{-4} M). The inhibitory effects were reversed on returning to the control conditions.

6. The membrane potential obtained after inhibition of the electrogenic Na-pump with cooling or ouabain agrees well with that predicted by the 'constant-field' equation.

7. The external Cl ions had a short-circuiting effect on the electrogenic Na-pumping activated on adding K ions.

8. The replacement of Na ions in a recovery solution with Li ions resulted in a faster rate of depolarization from the maximal hyperpolarization.

9. It is concluded that the resting membrane potential of 'Na-loaded' and 'K-depleted' SOL muscle fibres is the sum of an ionic diffusion potential predicted by either the Nernst equation or the constant-field equation and of the potential produced by an electrogenic Na-pump.

INTRODUCTION

Desmedt (1953) found that the resting membrane potential of frog sartorius muscles, which were preincubated in a cold Ringer's solution with 0·2 mM-K up to 68 hr, was hardly affected by a decrease in $[K]_i$. According to Sato, Akaike & Nishi (1967), who employed similar experimental methods and materials to those of Desmedt (1953), the alteration of $[K]_i$ from about 120 to 70 m-mole/l. fibre water did not result in the membrane potential expected from the ionic theory, but rather hyperpolarized the fibres beyond the steady-state value. They reported that the hyperpolarization might be attributed to an electrogenic nature of active Na extrusion, as suggested first in 'Na-rich' frog muscles by Kernan (1962*a*). Such an active Na transport contributing directly to the membrane potential of 'Na-rich' frog muscle fibres has been extensively studied by several workers (Kernan, 1962*b*; Cross, Keynes & Rybová, 1965; Frumento, 1965; Hashimoto, 1965; Adrian & Slayman, 1966; Harris & Ochs, 1966).

On the other hand, the method of preparing 'Na-rich' muscles in the frog proved unsuitable in the case of rat muscles because of their homoethermal nature and high O_2 dependence. The mammalian muscles, which had been immersed overnight in cold K-free Krebs solution, failed to excrete Na ions during soaking period in well oxygenated recovery solution containing 10 mM-K (Dockry, Kernan & Tangney, 1966). Conway & Hingerty (1948) observed that feeding of rats with a K-free diet for a long time produced a Na-gain and a K-loss in the skeletal muscles. On restoring the K-deficient rats to either the ingestion of a K-salt or the injection of KCl solution, the 'Na-loaded' and 'K-depleted' rat muscles rapidly took up K ions and excreted Na ions again (Heppel, 1939; Muntwyler & Griffin, 1951; Akaike, 1969). However, little is known about the electrophysiological evidence for an electrogenic Na-pump in the 'Na-rich' muscle fibres of rats after a prolonged K-free diet.

Present study is concerned with three questions. (1) How is the resting membrane potential of the 'Na-rich' soleus muscle fibres affected after K-depletion by an electrogenic Na-pump and how is it activated by

increasing $[Na]_i$? (2) How are the membrane potential and intracellular Na and K concentrations affected in 'Na-rich' muscle fibres during the immersion in a recovery solution with 10 mM-K for 2 hr at 37° C? (3) Can the measured membrane potential be predicted by a 'constant-field' equation under conditions known to abolish the electrogenic Na-pump? A part of this work has already been reported briefly (Akaike, 1974).

METHODS

Materials. The experiments were made on the isolated soleus (SOL) muscle (70–90 mg) of male rats of Wistar strain weighing 100–150 g. The rats had been kept for 40–50 days in 55–60 % humidity at about 22° C and maintained on a rat cake diet or a K-free diet available *ad libitum*.

Solutions. The composition of the test solutions is shown in Table 1. All solutions were made using distilled and deionized water from tenfold concentrated stock solutions of the major ions. Ca-gluconate, glucose and $NaHCO_3$ were added to the solution before each experiment. Solutions were always equilibrated with 95 % O_2 and 5 % CO_2 throughout an experiment, since anoxia produces a rapid loss of K ions and a gain of Na ions in mammalian skeletal muscles (Creese, 1954). Temperature of solutions was maintained at 37° C. The pH of all solutions was 7·2–7·5. Modified Krebs solutions containing varying concentration of K ion were prepared by replacing NaCl by an equivalent amount of KCl. The Na concentration was also altered by replacing it with Li on a mole for mole basis. Cl-free Krebs solution was prepared by replacing NaCl and KCl by equivalent amounts of $NaCH_3SO_4$ and KCH_3SO_4.

Experimental procedure and artifacts. SOL muscles were carefully removed from normal or K-depleted rats, and mounted on a Perspex disk under a slight stretch (1·1–1·2 times the resting length *in vivo*) into a chamber filled with 3 ml. Krebs solution without K ion at 37° C. Therefore, the preparation was superfused with the solution through a heating coil, at a rate of 1·5 ml./min. A rapid change in bathing solution from a K-free to K-rich Krebs solution was done by opening a clamp to the rubber tube leading from the appropriate bottles to the chamber. The entire solution in the bath was changed by flushing through 10 ml. of the new test solution in 30 sec or less. Successively, new test solution surrounding the muscle fibres was renewed at high perfusion rates (25 ml./min) for 1 min, or the preparation was suddenly cooled to 3–4° C within 30 sec by application of cold test solution through a glass tubing coiled in an ice bath containing salt. The temperature was monitored by means of a thermistor transducer, and kept constant within the fluctuation range of ± 1° C during the measurements. Thus, the muscles were allowed to equilibrate with new solution for at least 1·5 min before impalement of the muscle fibres. In preliminary experiments, the time (1·5 min) required for changing the bathing solution at 4 or 37° C was found to be sufficient to record a stable resting membrane potential, while it was found that the maximal hyperpolarization occurred about 1 min after soaking in Krebs solution containing 15 mM-K. In this case, the exchange of bathing medium was made within 1 min by careful treatment of the volume and speed of the perfusion of new test solution.

Since micro-electrodes of high tip resistance are often temperature-sensitive (Sokolove & Cooke, 1971), electrodes employed in the present experiments were carefully checked and ascertained not to be sensitive for changes in temperature from 37 to 4° C. In addition, the penetration of micro-electrodes were made after

TABLE 1. Composition of modified Krebs solution (mM)

	Na	K	Li	Ca	Mg	Cl	H_2PO_4	HCO_3	CH_3SO_4	HPO_4	SO_4	Gluconate	Glucose
Normal Krebs	135	5	0	2·5	1·2	110	1·2	28	0	0·4	1·2	2·5	11
K-free Krebs	140	0	0	2·5	1·2	110	1·2	28	0	0·4	1·2	2·5	11
Recovery Krebs	140	10	0	2·5	1·2	120	1·2	28	0	0·4	1·2	2·5	30
Li Krebs	30	5	105	2·5	1·2	110	1·2	28	0	0·4	1·2	2·5	11
CH_3SO_4 Krebs	135	5	0	2·5	1·2	0	1·2	28	110	0·4	1·2	2·5	11

Bubbled with 95 % O_2 and 5 % CO_2.

balancing out any small changes due to the junction potential between the reference electrode and new test solution.

Electrical measurements. Membrane potentials of muscle fibres were recorded with a glass capillary micro-electrode of Nastuk–Hodgkin type, filled with 3 M-KCl and with a resistance of 10–20 MΩ, the indifferent electrode being Ag-AgCl electrode connected via a Krebs-agar bridge to the bathing medium. The potential difference between the intracellular micro-electrode and grounded reference electrode was displayed on an oscilloscope through a d.c. preamplifier with a cathode follower, and photographed on moving film and recorded simultaneously with a pen-writing recorder. The tip potential was measured before each experiment, and micro-electrodes with tip potentials of less than 5 mV were selected for use. The electrode resistance and tip potential were measured after each experiment again.

Inulin space. The extracellular space was measured with inulin. SOL muscles were excised from both the normal rats and those which had fed a K-free diet for 40 and more days. After a given time of immersion in Krebs solution containing inulin (1 % w/v) at 37° C, each muscle was gently blotted with a filter paper, weighed and then reimmersed for 12 hr at 5° C in 10 ml. Krebs solution without inulin or glucose. The concentration of inulin which had been diffused out from the muscle in the inulin-free solution was determined photometrically according to the method by Kobayashi & Yonemura (1967).

In the case of *in vivo* study, both the normal and K-deficient rats were anaesthetized with ether, nephrectomized, and a warm solution of inulin (15 % w/v) was injected into the femoral vein in a dose of 0·5 ml./100 g body wt. After 3 hr the blood was taken from carotid artery. The muscles were dissected, blotted, cleaned of connective tissues, and weighed. The amount of inulin in muscle and plasma was analysed by the same method described above.

Electrolyte estimation. Chemical analyses of Na and K ions were carried out with a flame spectrophotometer. The intracellular cation concentration ($C_{i.w.}$) were calculated from the cation concentration of the muscle (C_m), the extracellular cation concentration (C_o), the extracellular space (v), and the dry-to-wet-weight ratio (d.w./w.w.) by

$$C_{i.w.} = \frac{C_m - vC_o}{1-(\text{d.w./w.w.}+v)} \quad \text{(m-mole/l. fibre water)},$$

if C_m is expressed as (m-mole/kg wet wt.), C_o as (m-mole/l.) and v as (l./kg wet wt.) (Desmedt, 1953).

The numerical values are given as mean values ± the S.D. of the mean. The significance of difference of means was decided by Student's t test.

RESULTS

Extracellular space

For estimating the intracellular ion concentrations the extracellular space of muscles was measured. Fig. 1*A* shows the time course for the equilibration of inulin when 'fresh' SOL muscles of normal rats were immersed in Krebs solution containing inulin for varying periods at 37° C. After soaking for 2 hr, the uptake of inulin approximately reached a steady state, the inulin space of which was 11·1 ± 1·5 % (twenty-one determinations; muscle weight range, 66·3–127·2 mg). Therefore, the comparison of the inulin space between 'fresh' and 'Na-rich' muscles was

made in a solution with inulin for 2 hr. The results are presented in Fig. 1*B*, in which a great variation was observed among space values in both kinds of muscles. However, the inulin spaces of 'fresh' muscles were nearly the same in amount as those of 'Na-rich' muscles of a similar weight, and the relationship between the inulin space (y) and the wet weight (x) in both the 'fresh' and 'Na-rich' muscles can be expressed by an equation,

$$y = 659{\cdot}80/x + 2{\cdot}67, \tag{1}$$

as shown by Kobayashi & Yonemura (1967).

The inulin space was also obtained *in vivo*. The amount of inulin in muscle and plasma was determined 3 hr after intravenous injection of inulin (Sréter & Woo, 1963). There exists no significant difference in the inulin space between 'fresh' muscles ($11{\cdot}71 \pm 1{\cdot}29$ %, nine observations) and 'Na-rich' muscles ($11{\cdot}21 \pm 1{\cdot}68$ %, seven observations) at $P > 0{\cdot}05$, as seen in Fig. 1*B*, whereas the dry-to-wet-weight ratio was smaller in 'fresh' muscles ($0{\cdot}220 \pm 0{\cdot}0068$, thirty-six determinations) than in 'Na-rich' muscles ($0{\cdot}226 \pm 0{\cdot}0077$, twenty-eight determinations).

From the results the relationship between the extracellular space and the muscle weight was considered to be the same in both 'fresh' and 'Na-rich' muscles, and the eqn. (1) was applied to both kinds of muscles. The dry-to-wet-weight ratio was assumed to be 0·220 for 'fresh' muscles and 0·226 for 'Na-rich' muscles. These values were employed when the intracellular ion concentrations were calculated.

$[Na]_i$, $[K]_i$, *resting membrane potential and* E_k *during K reaccumulation*

Fig. 2 shows changes in K-equilibrium potential (E_k) and in the membrane potential (dots) during K reaccumulation in a recovery solution containing 10 mM-K, together with the intracellular cation concentrations. The E_k values were calculated from the following Nernst equation using analytical data of K ion, determined with eight to fifteen muscles at various time after the immersion of muscles in the recovery solution.

$$E_k = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i}, \tag{2}$$

where F, R and T have their usual significance. The recovery caused a decrease of $[Na]_i$, while $[K]_i$ increased. During the enhanced active Na-pumping at the beginning of recovery the measured membrane potentials hyperpolarized beyond the calculated E_k value. During first 3–6 min of the recovery the membrane potentials ($-83{\cdot}0 \pm 3{\cdot}8$ mV, thirty muscle fibres) significantly exceeded the E_k ($-58{\cdot}1 \pm 1{\cdot}6$ mV, eleven muscles) at $P < 0{\cdot}001$. Thereafter, the hyperpolarization of the membrane potential

diminished and reached the steady-state value 30 to 60 min after the beginning of recovery, at which time no significant difference was found between the measured membrane potential and E_K ($P > 0.05$), although E_K value increased slightly during the recovery.

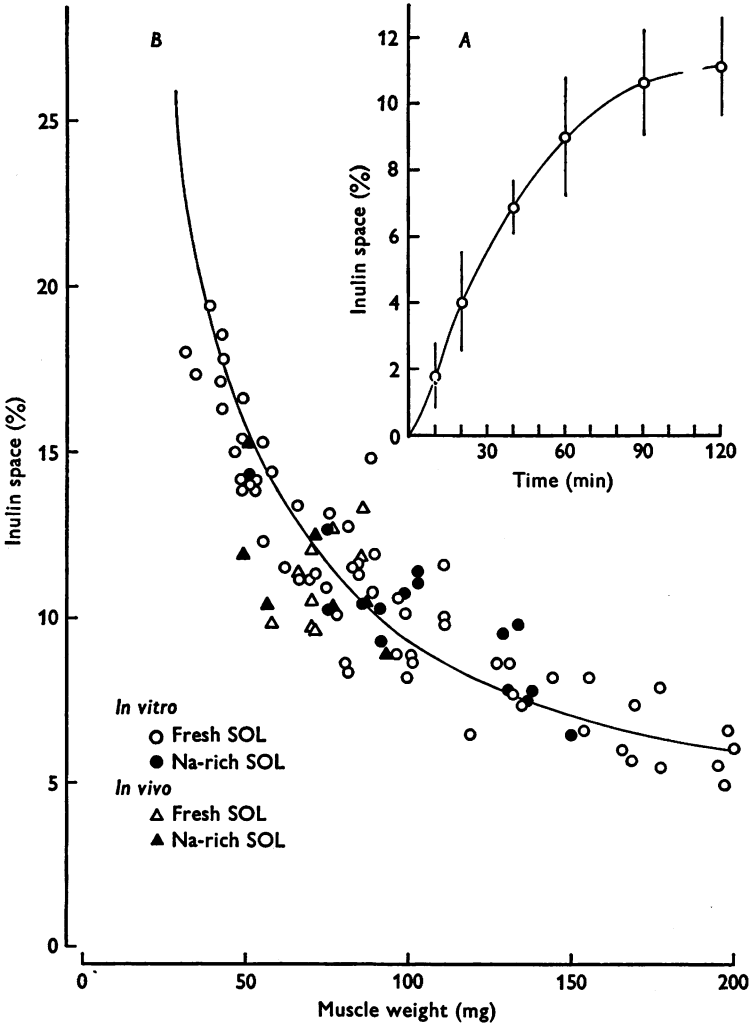

Fig. 1. Panel *A*: uptake of inulin in isolated 'fresh' SOL muscles in inulin Krebs solution. The symbols give the mean of eleven to twenty-one experiments, and the limits indicate the S.D. Abscissa: time of exposure in min. Panel *B*: relationship between the inulin space of either 'fresh' and 'Na-rich' SOL muscles which were equilibrated for 2 hr in inulin Krebs solution or 'fresh' and 'Na-rich' muscles *in vivo* 3 hr after injection of inulin and the muscle weight. Each point represents individual estimation. The curved line was drawn according to the equation, $y = 659{\cdot}80/x + 2{\cdot}67$, in which y represents the space and x the weight.

After exposure to a recovery solution for 2 hr at 37° C the 'Na-rich' muscles were capable of restoring their $[Na]_i$ from $69{\cdot}4 \pm 9{\cdot}1$ to $42{\cdot}8 \pm 3{\cdot}1$ m-mole/l. f.w. (thirteen to fifteen muscles) and $[K]_i$ from $84{\cdot}1 \pm 9{\cdot}8$ to $115{\cdot}0 \pm 3{\cdot}5$ m-mole/l. f.w. towards values in 'fresh' muscles in normal rats ($[Na]_i$, $24{\cdot}2 \pm 3{\cdot}2$; $[K]_i$, $134{\cdot}1 \pm 7{\cdot}4$ m-mole/l. f.w.; thirty muscles). The amounts of Na and K in SOL muscles transported actively during the recovery were 27·6 and 30·9 m-mole/l. f.w., respectively. The difference in $[Na]_i$ or $[K]_i$ between control and recovered muscles was highly significant at $P < 0{\cdot}001$, though the ionic recovery was incomplete since in these recovered muscles Na content was still great and K content was small as compared with those in 'fresh' muscles.

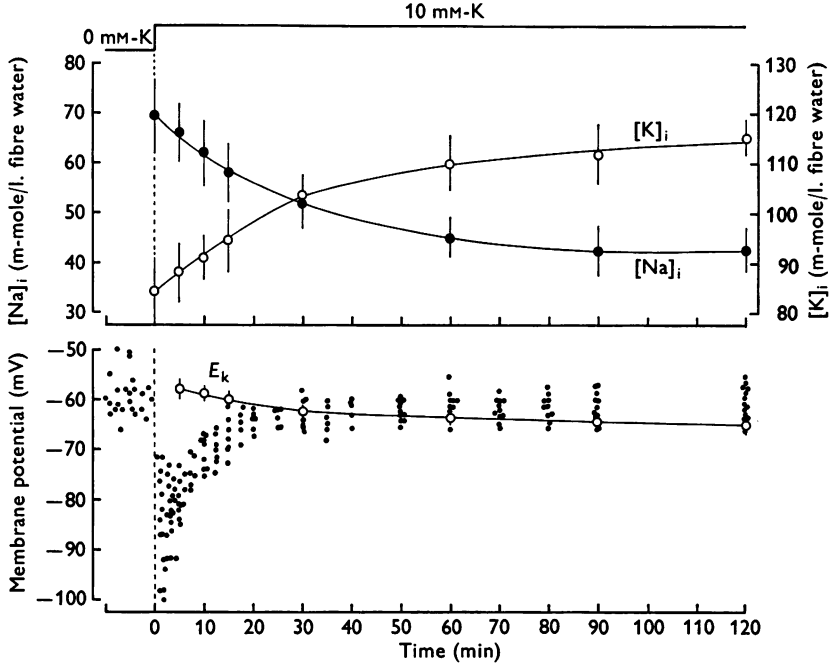

Fig. 2. Upper panel: the intracellular Na (●) and K (○) concentrations ($[Na]_i$ and $[K]_i$) of 'Na-rich' SOL muscles of rats, which had been maintained on a K-free diet for 40–50 days, during recovery at 37° C. At zero time, Krebs solution containing 10 mM-K was added to the muscles. Each point represents an average of eight to fifteen muscles and the vertical bar indicates ± S.D. of the mean. Note the scales of the left and right ordinate: $[Na]_i$ and $[K]_i$, respectively (m-mole/l. fibre water). Lower panel: membrane potential (●) and K equilibrium potential (○) of 'Na-rich' muscles after transference to a recovery Krebs solution containing 10 mM-K at 37° C. The E_k values were calculated from $[K]_i$ data in the upper panel, the vertical bar being ± S.D. of the mean. Each filled circle represents a single penetration from six experiments. Left ordinate: membrane potential and E_k (mV). Abscissa: time (min).

Changes in the membrane potential induced by different $[K]_o$

Fig. 3 summarizes the time courses of the resting membrane potentials in 'Na-rich' muscles in Krebs solution containing 0·5, 2·5, 5, 10 and 15 mM-K. The 'Na-rich' muscle fibres in K-free Krebs solution showed the membrane potentials of $-61{\cdot}5 \pm 6{\cdot}5$ mV (312 fibres). Upon adding 0·5, 1, 2·5 and 5 mM-K to the K-free solution bathing 'Na-rich' muscles, the membrane potential hyperpolarized slowly and attained the maximum of $-74{\cdot}0 \pm 7{\cdot}5$ mV (142 fibres), $-88{\cdot}1 \pm 9{\cdot}6$ mV (104 fibres), $-97{\cdot}5 \pm 3{\cdot}5$ mV (170 fibres) and $90{\cdot}1 \pm 4{\cdot}0$ mV (190 fibres), respectively. The membrane potentials were then reduced in magnitude and reached the steady state. The addition of 10 and 15 mM-K caused a rapid hyperpolarization, the membrane potential reaching $-83{\cdot}0 \pm 3{\cdot}8$ mV (thirty fibres) and $-69{\cdot}0 \pm 2{\cdot}3$ mV (forty-two fibres) within 1 to 3 min. The maximal hyperpolarization occurred earlier and was more short-lived with higher $[K]_o$ than with low $[K]_o$, and therefore the membrane potential reached the steady state earlier in high $[K]_o$. The exposure of the muscles to the solution containing 30 mM-K or higher concentration of K produced a rapid depolarization of the fibres without a transient hyperpolarization, and the membrane potentials were almost identical to E_K. On reducing $[K]_o$ to 0 mM at the beginning of recovery, the 'Na-rich' muscle fibres were again depolarized rapidly.

The average membrane potentials in both 'fresh' and 'Na-rich' muscle fibres were plotted in Fig. 4 as a function of $[K]_o$ on a logarithmic scale. The membrane potential in 'fresh' muscles was $-76{\cdot}7 \pm 3{\cdot}2$ mV (126 fibres, ten muscles) in the solution containing 5 mM-K at 37° C. Exposure of the 'fresh' muscles to solutions with various $[K]_o$ was accompanied by changes of the membrane potentials, which settled at a steady-state level within a few minutes. Over the $[K]_o$ range from 10 to 120 mM the membrane potential in the muscle fibres changed by 57·0 mV for a tenfold change in $[K]_o$. This is in approximate agreement with the theoretical slope of 61·5 mV predicted by the Nernst equation. However, between 0 to 10 mM $[K]_o$ the fibres depolarized and the slope relating the membrane potential to $[K]_o$ became smaller than that predicted from the Nernst equation. On the other hand, the maximally hyperpolarized membrane potentials of 'Na-rich' muscle fibres, measured in Krebs solution containing 2·5–15 mM-K, exceeded beyond the membrane potential of 'fresh' muscle fibres, or even beyond the calculated E_K values. At concentrations below 1 mM-K, the membrane potentials were less negative.

Under these conditions, it is important to determine whether the behaviour of the membrane potentials in both 'fresh' and 'Na-rich' muscle fibres is described by a constant-field equation (Goldman, 1943).

If the muscle fibre membrane is poorly permeable to ions other than Na, K and Cl ions, in which Cl ions are distributed passively, and if P_K $[K]_i$ is much greater than P_{Na} $[Na]_i$, P_{Na}/P_K $[Na]_i$ may be neglected (Hodgkin & Horowicz, 1959), the following simplified equation can be derived from the constant-field theory:

$$V_m = \frac{RT}{F} \ln \frac{[K]_o + (P_{Na}/P_K)\,[Na]_o}{[K]_i}, \qquad (3)$$

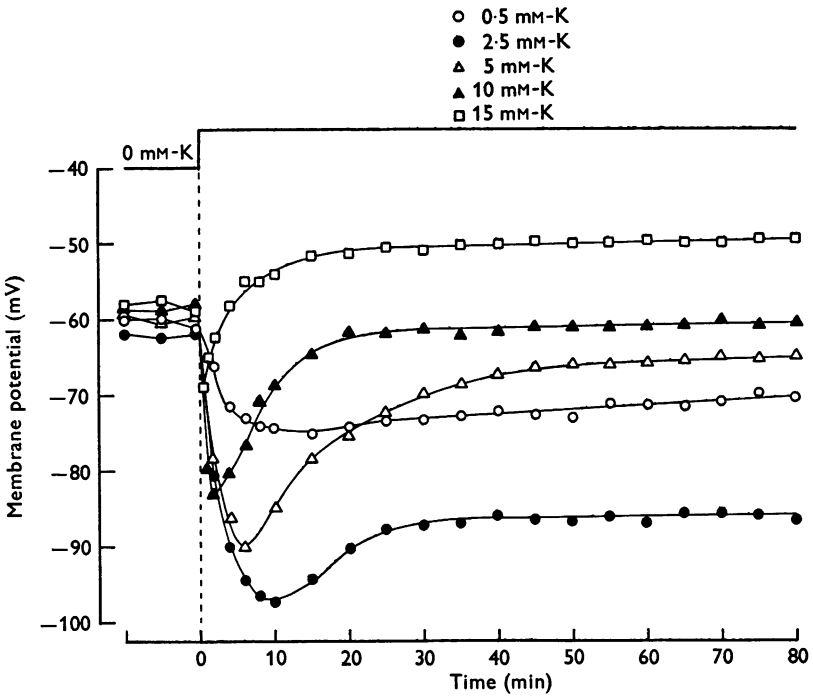

Fig. 3. The membrane potentials of 'Na-rich' muscle fibres during reaccumulation of K ions from the Krebs solutions containing different K concentrations at 37° C. Each point represents an average value of eight to thirty muscles, in which the membrane potentials were measured in several muscle fibres.

where V_m is the membrane potential, P_K and P_{Na} are permeability constants for K and Na ions, and P_{Na}/P_K represents the relative permeability ratio for Na ion to that for K ion.

The curve of 'fresh' muscles in Fig. 4 shows the theoretical relation, calculated from eqn. (3) by choosing the values 129·9 mM and 0·018 for $[K]_i$ and P_{Na}/P_K respectively, which indicates that all of the experimental points at 37° C lie on a theoretical curve. Accordingly, the membrane

potentials of 'fresh' muscle fibres, deviated from eqn. (2) below 10 mM-$[K]_o$, can be accounted for by eqn. (3). However, the membrane potentials of 'Na-rich' muscle fibres obtained between 0 to 15 mM-$[K]_o$ did not fit to a theoretical curve calculated by eqn. (3) using $[K]_i$ (93·8 mM) and P_{Na}/P_K (0·02), which fit the experimental values at 3 to 4° C.

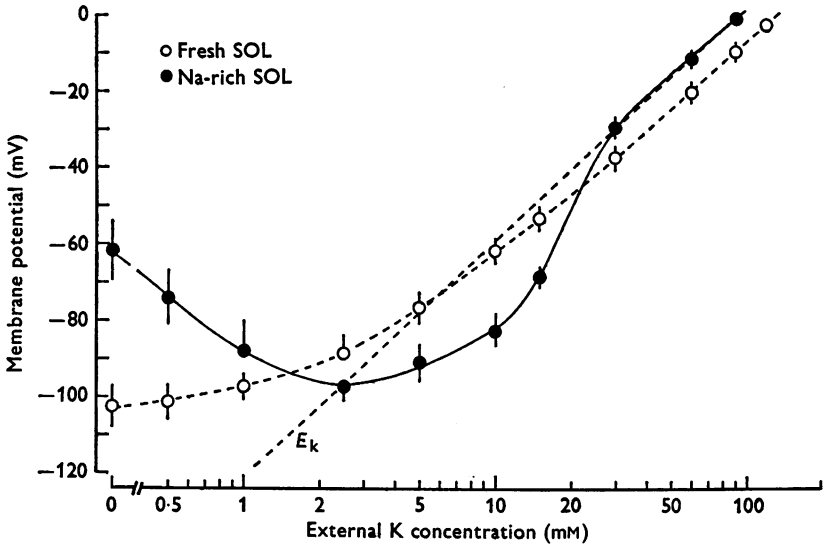

Fig. 4. Relationship between the membrane potential and log $[K]_o$ at 37° C in 'fresh' and 'Na-rich' muscles, in which internal cation concentrations were modified by K depletion for 42–46 days. Each point represents an average value of thirty to three hundred and twelve muscle fibres in eight to thirty muscles. The straight line represents a slope of 61·5 mV predicted by the Nernst equation for a K electrode at 37 °C. A smooth curve was drawn from the modified constant-field eqn. (3) at 37° C for 'fresh' muscle. The vertical bar for each point shows ± S.D.

Effect of temperature on the membrane response to $[K]_o$

At 3–4° C the membrane potentials of the 'Na-rich' muscle fibres in K-free Krebs solution were hardly affected by adding 5 mM-K. As the temperature was increased from about 4° C to 37° C in the presence of 5 mM-K, there was a rapid hyperpolarization, the membrane potentials reaching −85 to −100 mV within 5 min (Fig. 5). The membrane potentials then declined gradually with time and decreased to about −65 mV in 55 min after beginning of rewarming.

Fig. 6 represents the effect of adding 5 mM-K to the K-free solution bathing 'Na-rich' muscles at five different temperatures (3 to 4, 20, 25, 30 and 37° C). When the addition of K ions was tested at 20° C the hyperpolarization developed very slowly over a period of 15 min. The transient

increase in the membrane potentials during K reaccumulation became smaller with decreasing temperature, and, in addition, the steady state of the membrane potential occurred later. The response of membrane potentials to low temperature (3–4° C), shown in Fig. 6, indicates that the hyperpolarizing effect of 5 mM-K is completely eliminated at 3–4° C.

When the membrane potentials of 'Na-rich' muscle fibres were measured over a wide range of $[K]_o$ at 3–4° C, that is, when the activity of the metabolically driven Na-pump is much reduced (Keynes & Swan, 1959; Skou, 1965), the membrane potentials of cooled muscle fibres fit well to a curve calculated from eqn. (3) by choosing values of 93·8 mM and 0·02 for $[K]_i$ and P_{Na}/P_K, respectively (Fig. 7). Thus, it appears that an electrogenic Na-pump does not contribute to the membrane potential at low temperatures. In addition, the slope of a line relating to $[K]_o$ and the membrane potential is 49·5 mV for a tenfold change in $[K]_o$ over 5–60 mM-$[K]_o$ at 3–4° C. This is close to the slope of 54·9 mV predicted by the Nernst equation.

Effect of ouabain on the membrane response to $[K]_o$

Ouabain is well known as the most specific inhibitor of the Na^+-K^+ dependent ATPase and not as a general metabolic poison (Skou, 1965). In the present experiments, the membrane potential was measured in ouabain before there is any substantial drop due to decreased $[K]_i$. Fig. 8 shows the effects of ouabain on the recovery of the membrane potentials of 'Na-rich' muscle fibres at 37° C. 6×10^{-6}, 10^{-5} and 3×10^{-5} M ouabain (Fig. 8*A*, *B* and *C*) had no or little effects on the development of the hyperpolarization in 'Na-rich' muscle fibres after adding 5 mM-K to K-free Krebs solution. 10^{-4} M ouabain (Fig. 8*D*) prevented completely the quick hyperpolarization of the muscle fibres that followed exposure to test solutions containing 0·5–15 mM-K at 37° C. The effect of ouabain on the electrogenic Na-pump activity was reversible, since a rapid appearance of hyperpolarization occurred immediately after washing it out (Fig. 8*C* and *D*).

Fig. 7 also shows the membrane potentials in 'Na-rich' muscle fibres in response to changes in $[K]_o$ at 37° C in the presence of 10^{-4} M ouabain. The non-linear behaviour of the membrane potential between 0 and 30 mM $[K]_o$ at 37° C, as shown in Fig. 4, was eliminated after an application of ouabain to the bathing medium, and over a full range of $[K]_o$ the experimental points of ouabain-treated muscle fibres fell on a smooth curve as predicted by eqn. (3), in which the values of $[K]_i$ and P_{Na}/P_K were 86·2 mM and 0·038 respectively, based on the determination from Fig. 10.

Effect of Li on the membrane potential

In order to study the membrane potential in Li Krebs solution, the 'Na-rich' muscles were first exposed in K-free Krebs solution. Subsequently, the immersion of the muscles in Li Krebs solution containing 5 mM-K, in which Li was used as a substitute for Na, was started. Within 1–2 min the maximal hyperpolarization appeared, though the hyperpolarization of the membrane potentials is reduced in magnitude earlier

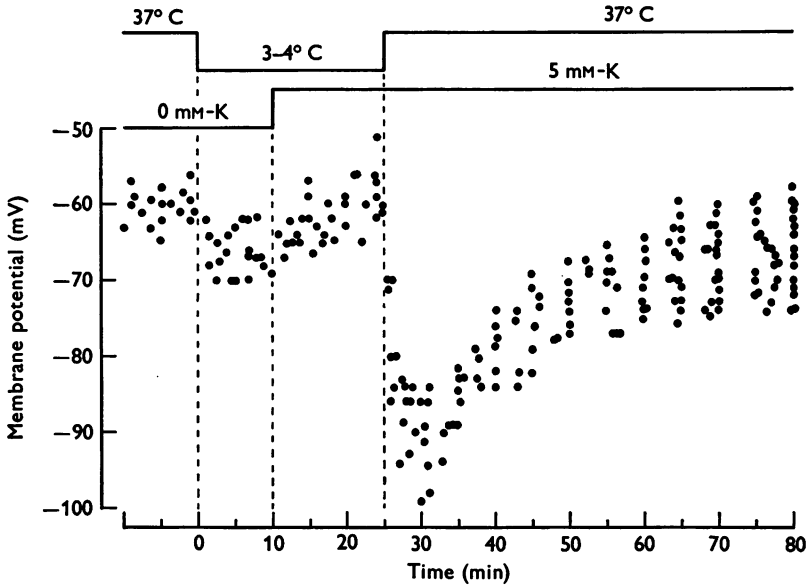

Fig. 5. Effects of temperature increase and K addition on the membrane potentials of 'Na-rich' muscles. The membrane potentials of 'Na-rich' muscle fibres in K-free Krebs solution were determined as the temperature was decreased from 37 to about 4° C. Then Krebs solution containing 5 mM-K was added to the tissues under cooling. After a further 15 min, the temperature was increased to 37° C. Each point represents a single penetration, the data being plotted from six preparations. Notice a huge rise in the membrane potential after the sudden rewarming.

than is in normal Krebs solution. The rate of depolarization from the maximal hyperpolarization was also greater in Li Krebs solution than in normal Krebs solution, as is illustrated in Fig. 9. The membrane potentials gradually declined over 1 hr. One hr after the beginning of the recovery period, the mean membrane potential fell to $-45{\cdot}8 \pm 2{\cdot}5$ mV (twelve fibres) in Li Krebs solution compared to $-65{\cdot}4 \pm 4{\cdot}8$ mV (fourteen fibres) in normal Krebs solution; the significance of the difference could be established at $P < 0{\cdot}001$.

Cl contribution on the membrane potential

According to Kernan & Tangney (1964), the muscle fibre in a Cl-containing solution Cl ion leaves passively during the active Na excretion and has a short-circuiting effect on an electrogenic Na-pump. In order

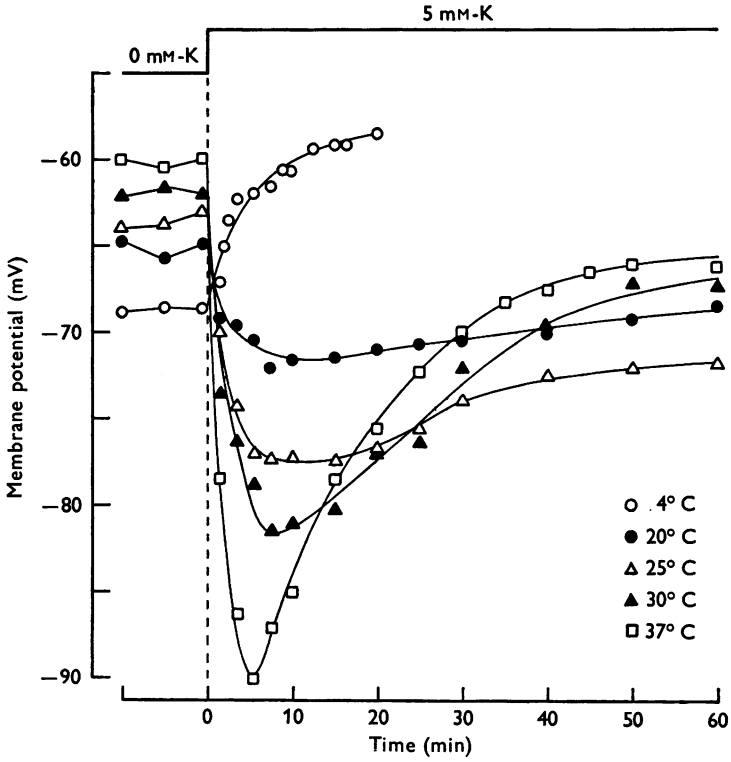

Fig. 6. The membrane potentials of 'Na-rich' muscle fibres during reaccumulation of K ions in Krebs solution containing 5 mM-K at different temperatures. Each symbol shows the mean value of six to eight muscles, in which membrane potential measurements were carried out on several muscle fibres. The hyperpolarization was inhibited by low temperature (3–4° C) and was promoted by high ones.

to eliminate any contribution of Cl ions to the membrane potential and to make sure that no Cl was left in the extracellular space, the immersion of 'Na-rich' muscles in CH_3SO_4 Krebs solution containing no Cl ions and K ions was made over a period of 2 hr at 37° C. The maximal membrane potentials produced by the addition of Cl-free solution to which 5 mM-K had been added reached as much as about −110 mV after 3 min of the recovery, as shown in Fig. 9. Such a high value was measured only in

CH_3SO_4 Krebs solution. Then, the membrane potentials were followed by a slow progressive depolarization, though the membrane potentials remained more negative throughout the period of recovery than those in normal Krebs solution. The membrane potential of 'Na-rich' muscle fibres recovered in CH_3SO_4 Krebs solution with 5 mM-K for 1 hr was $-74{\cdot}9 \pm 3{\cdot}6$ mV (seventeen fibres), which was significantly greater than $-65{\cdot}4 \pm 4{\cdot}8$ mV (fourteen fibres) in normal Krebs solution at $P < 0{\cdot}001$.

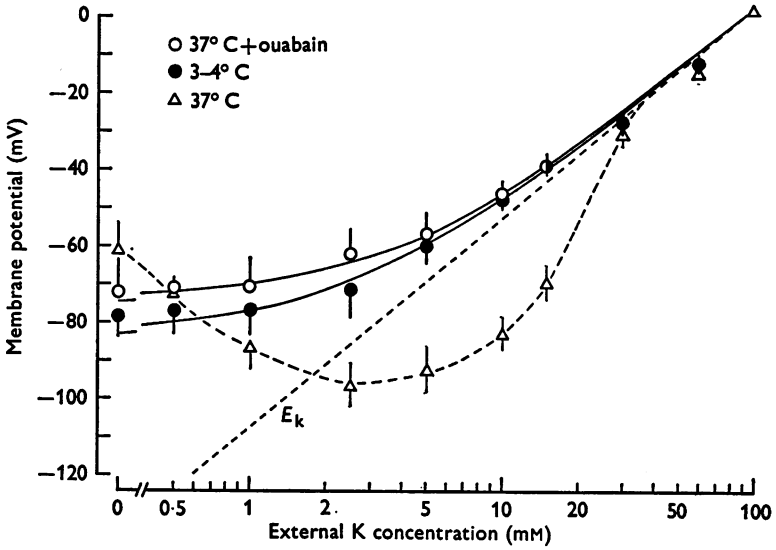

Fig. 7. Effects of cooling and ouabain on the relationship between the membrane potential and log $[K]_o$ of 'Na-rich' muscles in rats which had fed a K-free diet for 40–49 days. Each point is the average value of fifty-five to sixty muscle fibres in six to eight muscles. Each vertical bar is the ± S.D. of the mean. Two smooth curves through the experimental points were drawn by the modified constant-field eqn. (3). Both the cooling and ouabain abolished the 'hyperpolarizing curve' (△) at 37° C, which was quoted from Fig. 4 as a reference curve.

DISCUSSION

A marked transient hyperpolarization occurred soon after the addition of 2·5–15 mM-K to 'Na-rich' SOL muscles in K-free Krebs solution. The observed membrane potential exceeded the membrane potential of 'fresh' muscle fibres, the calculated K equilibrium potential (E_k) and the theoretical potential expected from the constant-field type eqn. (3) during the hyperpolarization. However, such a hyperpolarization was abruptly abolished by lowering the temperature to 3–4° C, or in the presence of 10^{-4} M ouabain. Under such conditions as known to inhibit the electrogenic Na-pump the membrane potentials of 'Na-rich' muscle fibres were

in good agreement with those predicted by eqn. (3), indicating that in such tissues the membrane potential is maintained mainly by a process of electrodiffusion potential.

The K concentration in serum of the low K rats was $2{\cdot}0 \pm 0{\cdot}2$ m-mole/l. serum (seventeen observations after feeding a K-free diet for 40–45 days), which was less than $4{\cdot}6 \pm 0{\cdot}4$ m-mole/l. serum of normal rats (sixteen

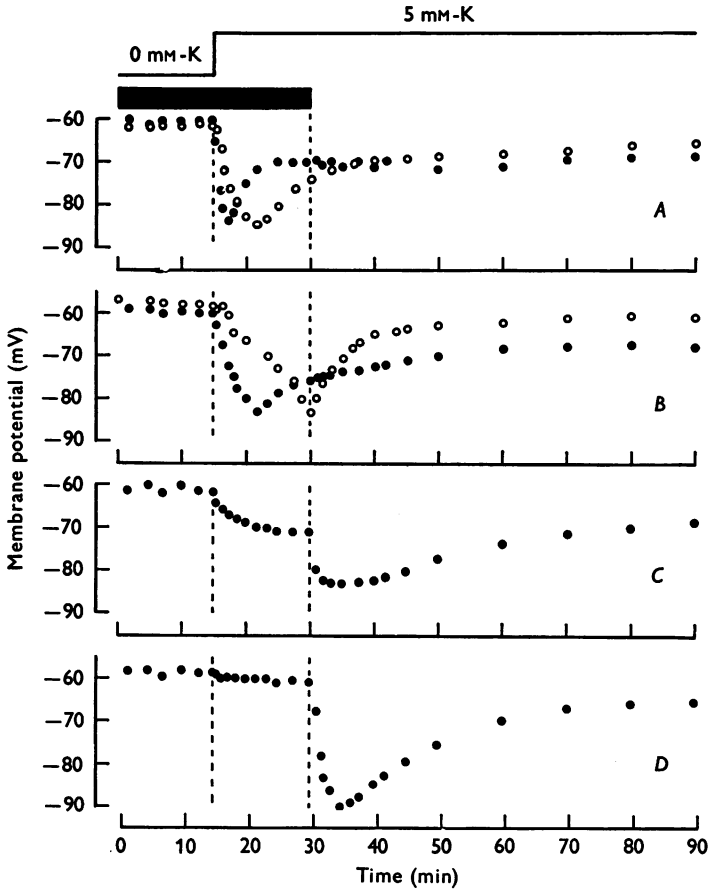

Fig. 8. Changes in the membrane potentials during immersion of 'Na-rich' muscle fibres in 5 mM-Krebs solutions containing 6×10^{-6} M (*A*), 10^{-5} M (*B*), 3×10^{-5} M (*C*) and 10^{-4} M (*D*) ouabain concentrations at 37° C. The 'Na-rich' muscles were soaked in K-free Krebs solution containing ouabain for 15 min (●) or 60 min (○), and then 5 mM-K Krebs solution containing ouabain was added to the tissues. After a further 15 min, ouabain was washed out. A large horizontal bar above graph A shows the experimental manoeuvre of ouabain. Each point is the mean value of four to six muscles, in which membrane potential measurements were carried out on several muscle fibres.

observations). Therefore, if the K concentration in the extracellular space immediately surrounding the fibre membrane of 'Na-rich' muscles was significantly less than that of 'fresh' muscles, the measured membrane potential of the former would be greater than that of the latter. Moreover, the theoretical potential calculated using the $[K]_o$ in the bathing medium would underestimate the actual value of the theoretical potential. In

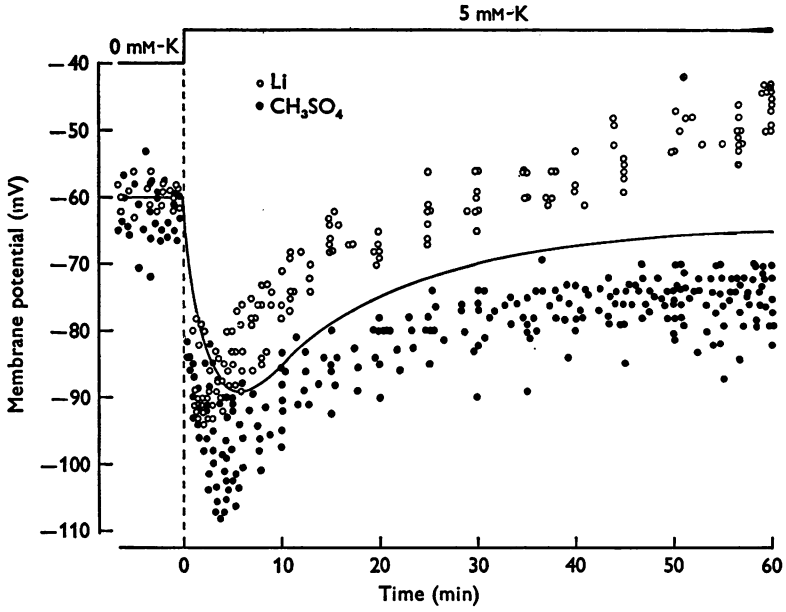

Fig. 9. Changes in the membrane potentials of the 'Na-rich' muscle fibres during soaking-in period in either Li Krebs or CH_3SO_4 Krebs solution containing 5 mM-K. The time course of membrane potential in normal Krebs solution with 5 mM-K is shown by a continuous curve which is quoted from Fig. 3. Each point represents a single penetration from six to seven experiments.

fact, the 'fresh' muscle fibres were hyperpolarized by a removal of K ions and depolarized by high K, and the behaviour of membrane potentials can be predicted by the Nernst equation or the constant-field equation. It is evident that the membrane potential of 'fresh' muscle fibres is maintained by the passive ion fluxes dependent on the electrochemical potentials of various ions. However, the removal of K ions from the external solution depolarized the 'Na-rich' muscle fibres, and conversely a transient hyperpolarization, which cannot be accounted for in terms of an electroneutral Na-pump, occurred on adding 2·5 to 15 mM-K to the solution. Therefore, the findings suggest that the hyperpolarizing response of

'Na-rich' muscle fibres is not due to local K depletion in the extracellular space but rather to an activation of an electrogenic Na-pump.

The membrane potential of 'Na-rich' frog sartorius muscle fibres, which were allowed to extrude Na ions and absorb K ions in Ringer solution containing 10 mM-K at room temperature, was greater than E_k during an active Na extrusion (Kernan, 1962*a*; Frumento, 1965). The potential

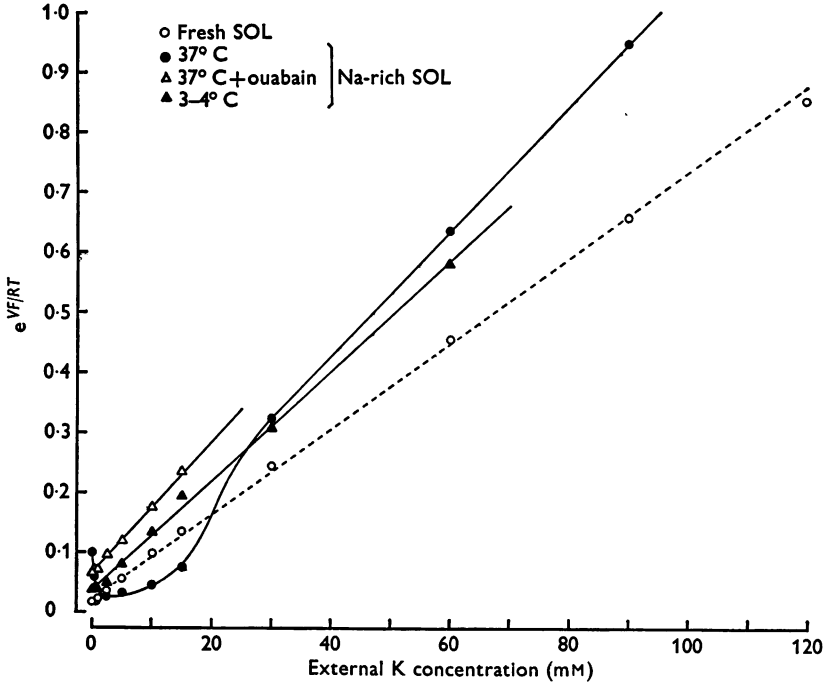

Fig. 10. The relationship between $e^{VF/RT}$ and $[K]_o$ in the 'fresh' and 'Na-rich' muscles from normal and K-deficient rats. The data were calculated from Fig. 4 and 7. The experimental points of 'fresh' muscles to $[K]_o$ fell on a straight line at 37° C, while the non-linear behaviour of $e^{VF/RT}$ plots *vs.* $[K]_o$ was seen in 'Na-rich' muscles at 37° C. On cooling or upon adding 10^{-4} M ouabain, the straight line was drawn through the experimental points of 'Na-rich' muscles.

difference between the measured membrane potential and E_k in 'Na-rich' frog muscles was increased with an increase in the rate of Na extrusion (Hashimoto, 1965). When the ionic recovery was complete after 2 hr in 10 mM-K the measured membrane potential became equal to the E_k. Similarly, in the 'Na-rich' SOL muscles of rats the measured membrane potential exceeded significantly the E_k during the early recovery period in 10 mM-K at 37° C, at which time little K had been reaccumulated intracellularly. The ion gradients in the cells recovered as the hyperpolarization dis-

appeared, although the recovery of the membrane potentials of the cells at the muscle surface preceded slightly the net ion movements found by chemical analysis of the whole muscle. After 2 hr recovery the recovered muscle fibres gave an agreement between the measured membrane potential and E_k. It is assumed that the difference between the measured membrane potential and E_k in 'Na-rich' SOL muscles at the beginning of the recovery is due apparently to the activity of an electrogenic Na-pump. The results also prove that the operation of an electrogenic Na-pump is stimulated by increased $[Na]_i$.

The following equation rewritten from eqn. (3) in an exponential form was applied to the membrane potentials of 'fresh' and 'Na-rich' SOL muscle fibres (Gorman & Marmor, 1970):

$$e^{VF/RT} = \frac{[K]_o}{[K]_i} + \frac{(P_{Na}/P_K)\,[Na]_o}{[K]_i}, \tag{4}$$

where $e^{VF/RT}$ is a linear function of $[K]_o$. The slope estimates $[K]_i$, the y-intercept allows an estimate of P_{Na}/P_K in the fibre membrane, and a straight line relationship confirms the constant-field equation. In Fig. 10 $e^{VF/RT}$, calculated from the measured membrane potentials, was plotted as a function of $[K]_o$. In the 'fresh' muscles the function $e^{VF/RT}$ was linear over the whole range of $[K]_o$ as predicted by eqn. (4). In the 'Na-rich' muscles the deviation from the straight line was observed at concentrations below 30 mM-K at 37° C. After cooling or in the presence of 10^{-4} M ouabain, however, the non-linear behaviour of the membrane potentials found in 'Na-rich' muscle fibres in response to changing $[K]_o$ at 37° C disappeared, and $e^{VF/RT}$ now varied linearly at all $[K]_o$. Hence, the membrane potentials in 'fresh' muscle fibres can be explained as the diffusion potential by passive ionic movements, while those in 'Na-rich' muscle fibres reflects the contribution of an electrogenic Na-pump.

The theoretical $[K]_i$ value in 'fresh' muscles, calculated from a slope of the linear portion in Fig. 10, is 129·9 mM, which is in good agreement with $134{\cdot}1 \pm 7{\cdot}4$ m-mole/l. f.w. found by analysis of thirty 'fresh' muscles by flame photometry. The value of P_{Na}/P_K calculated from y-intercept is 0·018. On the other hand, the estimation of $[K]_i$ and P_{Na}/P_K in the 'Na-rich' muscles at 37° C was impossible, because of the inflexion between 0 and 30 mM-$[K]_o$. The value of $[K]_i$ in 'Na-rich' muscles was therefore calculated from the full plots of $e^{VF/RT}$ versus $[K]_o$ on cooling, the value being 93·8 mM. This is close to $85{\cdot}5 \pm 3{\cdot}9$ m-mole/l. f.w. of sixteen 'Na-rich' muscles estimated by chemical analysis. Knowing the $[K]_i$, the value of P_{Na}/P_K estimated from the y-intercept is 0·02. Similarly, the values of $[K]_i$ and P_{Na}/P_K are 86·2 mM and 0·038 in the presence of 10^{-4} M ouabain, respectively.

As described above, the theoretical value of $[K]_i$ could be estimated from the slope of $e^{VF/RT}$ as a function of $[K]_o$. Therefore, in Fig. 10 the similarity of the slope in two straight lines, which are found in 'Na-rich' muscles after cooling and adding ouabain, suggests that the effect of ouabain would not result from a reduction of $[K]_i$ but rather occurs by abolishing the contribution of an electrogenic Na-pump to the membrane potential. Accordingly, ouabain had no effect on the membrane potentials in 'Na-rich' muscle fibres once K ion was removed from the bathing medium. It appears from the result that both ouabain and removal of K ions act upon the same membrane process, as postulated for a molluscan neurone (Gorman & Marmor, 1970).

By increasing $[K]_o$ in the recovery solution, the maximal hyperpolarization of 'Na-rich' muscle fibres came to appear earlier and the total duration of the hyperpolarization came to be shortened. The hyperpolarization disappeared completely with recovery solution with more than 30 mM-$[K]_o$. The results confirm the observations of Rang & Richie (1968) who found that post-tetanic hyperpolarization of non-myelinated nerve fibres of the rabbits declines significantly at $[K]_o$ value greater than 2 mM, and also those of Casteels, Droogmans & Hendrickx (1971) who demonstrated that a maximal hyperpolarization of 'Na-rich' taenia coli of the guinea-pigs is less and disappears sooner with increasing $[K]_o$. In contrast, Taylor, Paton & Daniel (1970) observed an increasing hyperpolarization for increasing $[K]_o$ (e.g. even at very high value such as 120 mM $[K]_o$) in the 'Na-rich' myometrium cells of the rats. The differences might be produced by the degree of Na accumulation and K depletion or by different contribution of an electrogenic Na-pump to the membrane potentials.

The cell membrane of frog muscle fibre is permeable to Li ion (Keynes & Swan, 1959; Yonemura & Sato, 1967). Carmeliet (1964) reported that the resting membrane permeability of the heart muscle fibre to Li ion was high and that the net inward movement of Li ions was found to be equal to the sum of outward movements of Na and K ions. From these results the gradual depolarization of 'Na-rich' SOL muscle fibres in Li Krebs solution can be accounted for by an accumulation of Li ions and the depletion of K ions in the cell interior.

Rang & Ritchie (1968) found that the replacement of external Cl ions by a large impermeable anion increased the post-tetanic hyperpolarization in mammalian non-myelinated nerve fibres which could be due to the activity of an electrogenic Na-pump. In the present experiments, a transient hyperpolarization of the 'Na-rich' muscle fibres was found to be greater in CH_3SO_4 Krebs solution than in normal Krebs solution containing Cl ions during recovery after adding 5 mM-K. The results suggest

that Cl ion would also contribute to the transient hyperpolarization during active Na excretion and short-circuits an electrogenic Na-pump.

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