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INWARD MOVEMENT OF SODIUM IONS IN RESTING AND STIMULATED FROG'S SARTORIUS MUSCLE

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SUMMARY

1. Paired frog sartorius muscles were exposed to Ringer solutions labelled with $^{22}Na^+$ for about 20 min. At the end of this exposure one of them was stimulated supramaximally one hundred to two hundred times. Immediately after the stimulation both members of the pair were washed in a series of tubes filled with a Na^+-free medium containing 3×10^{-5} M strophanthidin.

2. Under the above conditions the intracellular component of the efflux was exponential with an average time constant (τ) of 388 min, that is, approximately four times longer than in the presence of normal Ringer. On the other hand the mean τ for the washout of the interfibre space was 3·2 min.

3. From the extrapolation to time zero of the intracellular component of the washout curve the initial intracellular radioactivity of both muscles was obtained and the resting and extra Na^+ influx were calculated.

4. The mean surface membrane area/muscle weight ratio was found to be 552 $cm^2.g^{-1}$ and the mean fibre diameter 53·4 μm for muscles weighing on the average 60 mg.

5. The average resting Na^+ influx in the presence of normal Ringer was 4·7 p-mole.$cm^{-2}.sec^{-1}$. As the external Na^+ concentration ($[Na^+]_0$) was reduced the Na^+ influx diminished in a non-linear fashion. This non-linearity could be accounted for by the presence in the influx of a Na^+ for Na^+ exchange fraction which saturates at low $[Na^+]_0$.

6. The mean extra Na^+ influx in the presence of normal Ringer was 27·4 p-mole.cm^{-2}.$impulse^{-1}$ and was not significantly affected either by halving $[Na^+]_0$ or by varying the frequency of stimulation. When $[Na^+]_0$ was reduced to 45 mM by partial replacement of Na^+ by $Tris^+$ the extra influx was significantly higher than when $choline^+$ instead of $Tris^+$ was used to substitute for Na^+.

INTRODUCTION

Since Hodgkin & Horowicz (1959*a*) published their measurements of the Na^+ and K^+ fluxes in single muscle fibres from the English frog (*Rana temporaria*) no other determinations of extra Na^+ influx during activity have been reported in the literature. In addition there are no data available on the extra Na^+ influx associated with stimulation in skeletal muscle from the American frog (*Rana pipiens*).

The experiments reported here represent an attempt to measure the extra Na^+ influx per impulse and the resting influx in whole sartorius muscle from *R. pipiens*. It is shown that with a relatively simple technique both types of fluxes can be determined in whole sartorius with a reasonable degree of reliability. The measurements were carried out in the presence of different external Na^+ concentrations and, in the case of the extra influx, at different rates of stimulation. A preliminary report of this work has been published elsewhere (Venosa, 1973).

METHODS

Sartorius muscles from the American frog (*Rana pipiens*) were used throughout this investigation. Paired muscles from a single frog were carefully dissected and used only if they were free from parasites, haemorrhages or any sign of damage. After dissection, each muscle was fastened to a light stainless-steel holder by means of thin surgical threads attached to both tendons. The holders had an elongated C-shape which allowed free access of the solutions to both sides of the muscles while there was no direct contact with the holder. The average weight of the muscles used in the experiments reported here was $59{\cdot}7 \pm 1{\cdot}5$ mg (± 1 S.E. of mean; $n = 82$ pairs) and the mean length $29{\cdot}6 \pm 0{\cdot}2$ mm (± 1 S.E. of mean; $n = 25$ pairs).

The composition of the Ringer fluid was the following (in m-mole/l.): NaCl 115, KCl 2·5, $CaCl_2$ 1·8, Na_2HPO_4 2·15, NaH_2PO_4 0·85 (pH 7·2). The Na^+ free $Tris^+$-Ringer contained (in m-mole/l.): Tris base (Sigma) 120 (neutralized with HCl to pH 7·2), KCl 2·5, and $CaCl_2$ 1·8. Isotonic media containing low $[Na^+]_o$ were prepared by mixing adequate volumes of these two solutions. In some experiments Na^+ was partially replaced by $choline^+$ or sucrose instead of $Tris^+$. In experiments in which one of the solutions contained $choline^+$, tubocurarine chloride (10^{-5} g/ml.) was added to all solutions. In general the experimental procedure was as follows. After dissection, paired muscles were left in normal Ringer solution for about half an hour and then weighed. Each muscle attached to its holder was weighed twice; in one case the muscle was drained (but not blotted) and the holder carefully dried with a filter paper, while in the second case neither was the muscle drained nor the holder dried. Both weighing procedures proved to be quite reproducible. When the resting and stimulated influxes were to be measured in a given medium they were first exposed to that solution for 30–45 min and then transferred to a loading chamber shown in Fig. 1 which contained the same medium labelled with $^{22}Na^+$ (New England Nuclear). The holders were attached to the chamber by clamps which allowed them to be rotated. The muscles remained in the labelled solution for a variable period of time, 23 min on the average ($\pm 0{\cdot}5$ (1 S.E. of mean), $n = 156$). At the end of this loading period both members of the pair were lifted in the air by rotating the holders and one of them brought in

contact with two pairs of electrodes attached to the chamber and stimulated supramaximally between one hundred and two hundred times at frequencies ranging from 3 to 50 shocks/sec. The pulse duration was 0·2 msec in all the experiments and was biphasic in most instances. No obvious differences between rectangular and biphasic pulses with regard to the extra Na^+ influx per impulse, was observed. The frequency of stimulation was usually monitored with a pen recorder (Brush, Model 220). The time spent by the muscles in the air during this procedure was always shorter than 50 sec. Control experiments showed that the rate of water evaporation under those conditions is of the order of 1 mg/min.

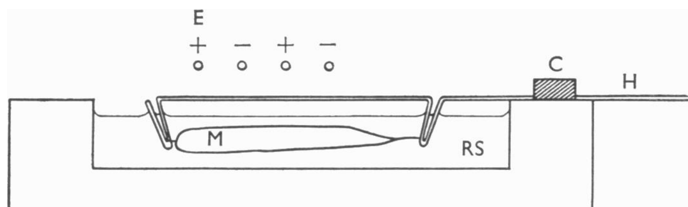

Fig. 1. Diagram of the loading chamber (lateral view). M, sartorius muscle; RS, radioactive solution; E, cross-section of the stimulating electrodes; H, holder clamp. The muscles were brought in gentle contact with the two pairs of stimulating electrodes by rotating the holder 180°.

The washout of radioactivity from both members of the pair was started immediately after stimulation. This was carried out by bathing the muscles in a series of tubes each containing 3·5 ml. inactive $Tris^+$-Ringer to which strophanthidin 3×10^{-5} mole/l. had been added, as a concentrated ethanol solution. The concentration of alcohol in this medium was always less than 0·05 % (v/v). The rotation of the tubes by a motor (60 rev/min) provided adequate stirring. The collecting periods lasted from 0·5 to 5 min at the beginning and were increased to 10 min towards the end of the experiment. The radioactivity released by the muscles was collected during 90–110 min. At the end of the experiment the muscles were removed from the holder and transferred to a tube similar to those used for the washout containing 3·5 ml. distilled water. All the tubes were counted in a crystal well gamma spectrometer. Taking the number of counts/min remaining in the muscle at the end of the experiment and adding back the counts/min in each sample, the total radioactivity in the muscle at the beginning of each collecting period was obtained. All the experiments were carried out at room temperature (21–23° C).

The washout of $^{22}Na^+$ in the presence of Na^+-free medium containing strophanthidin

The influx measurements in the present experiments are based upon the extrapolation of the intracellular component of the washout curve of $^{22}Na^+$ to zero time. Such a procedure can be misleading unless the time constant of this component is much longer than the one of the extracellular component (Huxley, 1960). To meet this requirement little can be done to accelerate the diffusion in the extracellular space, but it is rather easy to slow down the release of $^{22}Na^+$ from the fibres by exposing the muscles to $Tris^+$-Ringer plus strophanthidin (3×10^{-5} M) immediately after the loading period. Most of the active extrusion of Na^+ and the Na^+ for Na^+ exchange are eliminated for those conditions and, therefore, the efflux time constant is increased by a factor of about four with respect to the time constant in the presence of normal Ringer. By means of this procedure a reasonably clear separation between the

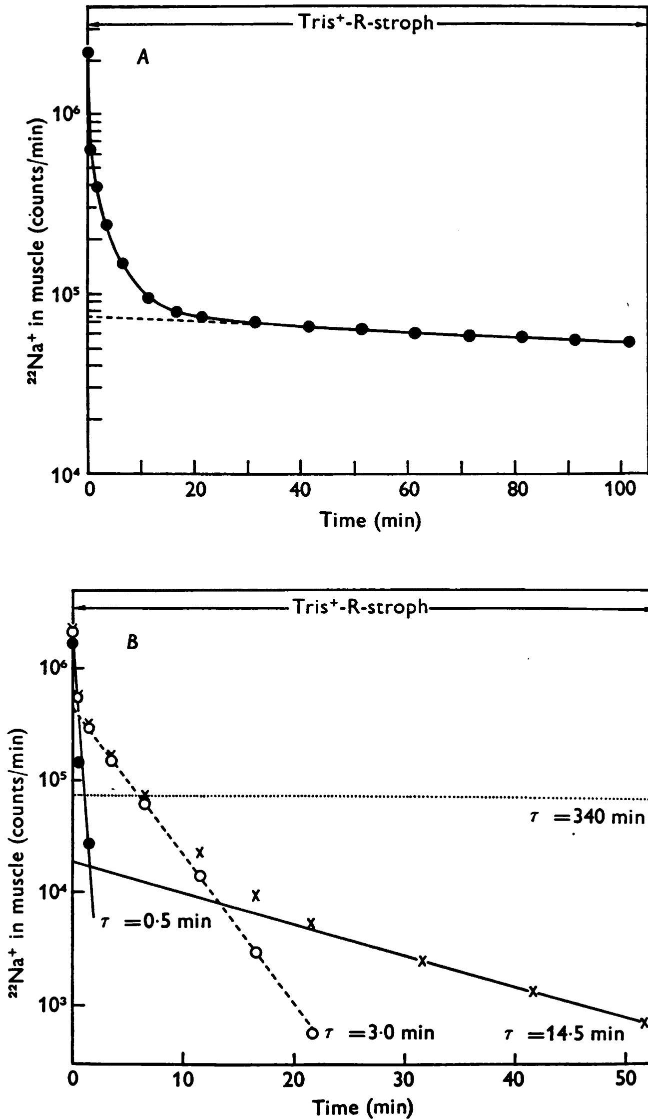

Fig. 2. *A*, washout of $^{22}Na^+$ from a sartorius muscle in the presence of Tris+-Ringer plus 3×10^{-5} M strophanthidin. The muscle had been exposed before to normal Ringer labelled with $^{22}Na^+$ for 17 min. The dashed line represents the extrapolation to time zero of the intracellular component of the efflux. *B*, graphical analysis of the washout curve of curve *A*. The interrupted line represents the intracellular component and the other three exponentials (×, ○, ●) correspond to the extracellular ones (see text). The τ's next to each line indicate their respective time constants. Expt. 22 July 1972.

intracellular and extracellular components can be achieved. A similar approach has recently been used by Sjodin & Beaugé (1973) to measure intracellular Na^+ concentration in frog sartorius.

Fig. 2*A* shows a semilogarithmic plot of the radioactivity remaining in a muscle as a function of the washout time. It can be seen that after about 35 min from the beginning of the washout the slow component is quite apparent and also that between this time and the end of the run all the experimental points fall on a straight line indicating the single exponential nature of the intracellular component under the experimental conditions used in this investigation. A graphical analysis of this curve is shown in Fig. 2*B*. In general, as in this case, most of the extracellular radioactivity can be accounted for by the sum of three exponential terms. The mean time constants ($\pm$ 1 S.E. of mean) of these three terms from twelve similar experiments were $0{\cdot}5 \pm 0{\cdot}0$; $3{\cdot}2 \pm 0{\cdot}2$ and $15{\cdot}6 \pm 0{\cdot}8$ min respectively. The average time constant of the intracellular component from the same muscles was 374 ± 19 min and the total mean from all the muscles used in this investigation was 388 ± 12. The time course of the extracellular washout of Na^+ can also be estimated by recording the decline in twitch tension which follows the sudden replacement of Na^+ by $Tris^+$ in the external medium. For this purpose the pelvic tendon of a sartorius was fastened to the lower extreme of a holder similar to those used for the tracer experiments, while the distal tendon was connected to a displacement transducer whose output voltage was recorded with a pen recorder (Brush, Model 220). The muscles were stimulated by a pair of platinum electrodes attached to the holder and connected to a stimulator. As in the tracer experiments supramaximal pulses of 0·2 msec in duration were used. The solutions were quickly changed by replacing the small beaker in which the muscle was submerged by another one containing the same volume of a different medium. The inset in Fig. 3 illustrates one of those experiments. Usually there was a residual mechanical activity in the presence of $Tris^+$-Ringer due to local contractures under the stimulating electrodes; it remained unchanged even after long exposures to Na^+-free medium. As shown in Fig. 3 practically no twitch tension could be detected at external Na^+ concentrations below 40 mM, at least for the experimental conditions used here. This result is at variance with those of Overton (1902), Edwards, Ritchie & Wilkie (1956), Mashima & Matsumura (1962), Grabowski, Lobsiger & Lüttgau (1972), and Vaughan, Howell & Eisenberg (1972), whose experiments indicate that at $[Na^+]_o$ = 40 mM the twitch tension is still practically normal. This difference is probably due to the fact that these authors used either $choline^+$ or sucrose instead of $Tris^+$ to replace Na^+ in the external medium. In some control experiments where $[Na^+]_o$ was partially substituted by $choline^+$ or sucrose the results were similar to those reported by the workers just mentioned. At the moment it is difficult to assess the roles played by low ionic strength of the sucrose containing solutions, the prolongation of the active state by $choline^+$ containing solutions (Edwards *et al.* 1956), and $Tris^+$ *per se* in explaining the observed differences in twitch tension when various substitutions are made for external Na^+. However, experiments in progress indicate that below 45 mM-$[Na^+]_o$, using $Tris^+$ to replace Na^+, the stimulation threshold increases very steeply as $[Na^+]_o$ is reduced and practically no action potential can be recorded at $[Na^+]_o$ = 35 mM at least by external stimulation.

The time elapsed between the application of Na^+-free $Tris^+$-Ringer and the disappearance of the twitch tension was, on the average, 5·7 min. If one makes the reasonable assumption that the twitch tension falls to zero when $[Na^+]_o$ at the centre of the muscle is around 40 m-mole/l., the apparent diffusion constant of Na^+ in the extracellular space can be estimated by following the treatment used by A. V. Hill (1928). The mean diffusion constant obtained in this way in seven experiments was $1{\cdot}95 \times 10^{-6}$ $cm^2.sec^{-1}$ for muscles of an estimated thickness of 0·7 mm. This value,

although somewhat lower, is not far from those reported previously. Harris & Burn, (1949) obtained a value of $2\cdot6\times10^{-6}$ $cm^2.sec^{-1}$ and Conway (1957) $2\cdot3\times10^{-6}$ $cm^2.sec^{-1}$. Using Hill's equations for the present experiments it can be estimated that the mean $[Na^+]_o$ in the extracellular space at the moment when the twitch disappears is about 26 m-mole/l. which corresponds to a washout time constant of 3·7 min.

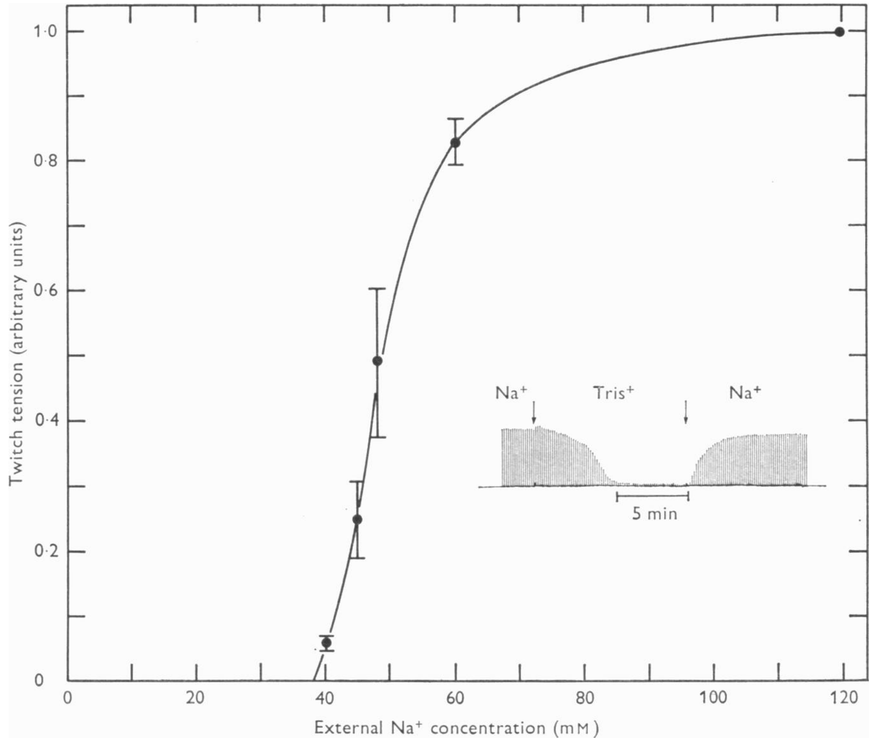

Fig. 3. Relative twitch tension as a function $[Na^+]_o$. The experimental points are the means ± 1 S.E. of mean from the same four sartorii. The tension in normal Ringer was taken as 1. The inset on the right shows the time course of the change in twitch tension of a sartorius upon replacing $Tris^+$ for Na^+ (left arrow) and vice versa (right arrow) in the external medium, see text. Expt. 9 February 1973.

This suggests that the exponential term of the extracellular washout of $^{22}Na^+$ with a time constant of 3·2 min represents the release of this isotope from the intercellular space. In addition, the mean size of this compartment amounts to 25 % of the total extracellular radioactivity in these muscles which were neither blotted nor drained. This is equivalent to 11·7 % of the blotted weights ($\simeq$ 0·75 × unblotted weights) of the same muscles. This figure agrees well with the value obtained by Desmedt (1953) for the extracellular space of blotted muscles (12·5 %).

The slowest of the extracellular components (τ: 15·6 min) represents $1\cdot6\pm0\cdot1$ % (n = 12) of the total initial extracellular radioactivity or about 0·7 cm^3/100 g blotted muscles. The time constant of this component and its small size makes it

reasonable to assume that such a fraction corresponds to the release of $^{22}Na^+$ from the connective tissue present in the extracellular space and tendons. The washout of the layer of active solution covering muscle and holder when they were transferred from the loading chamber to the first tube containing inactive solution is probably the origin of the fastest and largest (70 % of the extracellular radioactivity) of the exponential terms detected in these experiments. From this type of analysis it is clear then that 97 % of the extracellular radioactivity can be accounted for by the three terms described above. The remaining fraction (3 %) is obviously too fast to be picked up by this method.

Mullins & Frumento (1963), identified two extracellular components in the washout of $^{24}Na^+$ from sartorius muscles with time constants of 0·6 and 2·4 min respectively, corresponding the first to the release of the isotope from the threads used to tie the muscles and the second to the washout of the interfibre space. Whatever the significance of the extracellular components may be, the important point to be stressed here is that there is a clear difference between the time constant of the intracellular component and the time constant of any of the extracellular ones. This circumstance makes the extrapolation of the intracellular component a reliable estimate of the amount of Na^+ within the fibres at zero time of the washout. It should also be pointed out that practically no difference was found between the extrapolated value obtained by eye and by regression analysis of the last 50 min of washout. If in the present experiments the mean time constant of the intracellular component and the mean time constant of the slowest extracellular component (15·6 min) are considered, the extrapolated intracellular radioactivity at $t = 0$ would overestimate the true value by about 8 %, while if instead, the extracellular component with a time constant of 3·2 min is taken into account, the over-estimation would be of the order of only 2 %. Since it is apparent that this last component is the one that most likely represents the washout of the interfibre space, no correction factor was applied to the extrapolated figure obtained in these experiments.

The reliability of this method was further investigated by comparing the initial intracellular radioactivity in four identically treated pairs of muscles. Both members of each pair were exposed to the same active solution for 20 min and washed simultaneously. The difference between the initial extracellular radioactivities expressed as percentage of the mean extrapolated value in each pair was on the average $4·5 \pm 2·0$ % (mean $\pm$ 1 S.E. of mean). This result indicates that paired muscles are reasonably well matched and any member of a pair makes a good control for the other.

Extracellular space

A measure of the extracellular space (Na^+ space) was obtained from the difference between the total and the intracellular radioactivities at $t = 0$ and the radioactivity per unit volume of the labelled solution (i.e. c.p.m./μl.). The washout was always started with the muscles undrained in order to minimize the transfer time from the loading solution to the first tube containing unlabelled solution. Knowing the drained and undrained weights of the muscles (see above), the extracellular space in the drained situation could be easily calculated. The average value from seventy-eight muscles was $0·23 \pm 0·01$ ($\pm$ 1 S.E. of mean) cm^3/g muscle. In the remainder of this paper 'muscle weight' refers to the drained condition.

Estimation of the surface membrane area per unit weight of muscle

In order to compare the flux data of this work with those obtained in single fibres (Hodgkin & Horowicz, 1959*a*), or in sartorii whose surface area per unit weight is known (Keynes & Swan, 1959), it was necessary to have an estimate of that relation

in the muscles used here. Six muscles representative of the ones used in these experiments were weighed, and their lengths were measured. Later on they were fixed with glutaraldehyde (3%) followed by osmium (2%) and embedded in Epon. Their cross-sections were photomicrographed and the fibres in each muscle were counted in the prints so obtained. With these data and taking an extracellular space of 0·23 cm^3/g muscle (see above), the surface area (T-system excluded) and the mean fibre diameter of each muscle was computed. The results, in Table 1, show a mean surface area of 552 cm^2/g and an average fibre diameter of 53·6 μm. This is close to the mean value of 51 ± 1 μm (± 1 S.E. of mean, $n = 88$) obtained by direct measurement of fibres from the inner surface of several sartorii by means of a stereomicroscope (Wild) with a calibrated eye piece scale and a total magnification of $\times 100$. The number of fibres per muscle varies within a narrow range (602–710) despite the wide spectrum of muscle weights (43–81·5 mg) and, as expected, these two parameters show no correlation. The mean surface/weight ratio (552 cm^2/g) was used to express the fluxes in terms of surface membrane area. It can be seen that the largest deviation from this value in Table 1 is (635–552) 100/552 = 15%.

TABLE 1. Some anatomical parameters of muscles representative of the ones used in the flux measurements

Muscle reference	Length (mm)	Weight (drained) (mg)	Number of fibres	Mean fibre diameter (μm)	Surface membrane area per unit weight ($cm^2.g^{-1}$)
A	30	67·5	602	59·1	497
B	31	81·5	682	60·0	489
C	28	43·0	670	46·3	635
D	29	53·0	636	51·8	566
E	28	62·0	710	54·0	544
F	27	49·5	672	50·5	582
Mean	28·8	59·4	662	53·6	552
± 1 S.E. of mean	± 0·6	± 5·7	± 15	± 2·1	± 22

Extracellular space: 0·23 $cm^3.g^{-1}$

Influx measurements

The resting Na^+ influx can be readily obtained, if in addition to the initial intracellular radioactivity, the loading time, the specific activity of the labelled solution and the weight of the muscle are measured. It would appear that the period of time spent by the muscles in the loading solution does not represent the loading time because the equilibration of the extracellular space with the labelled solution is not instantaneous. But it is also true that at the beginning of the washout the extracellular space does not equilibrate instantaneously with the external inactive solution either. In this way, the deficit in the extracellular specific activity at the beginning of the loading period tends to be compensated by the excess of specific activity at the beginning of the washout.

The resting Na^+ influx was calculated by means of eqn. (3). The rate of change of the intracellular concentration of $^{22}Na^+$, during the loading time is given by eqn. (1) (Keynes & Lewis, 1951):

$$\frac{d[^{22}Na^+]_i}{dt} = \frac{A}{v}(J_i s_0 - J_0 s_i), \tag{1}$$

where

A fibre surface area in cm^2,
v fibre water volume in cm^3,
J_i Na^+ influx in p-mole.$cm^{-2}.sec^{-1}$,
J_0 Na^+ efflux in p-mole.$cm^{-2}.sec^{-1}$,
s_0 specific activity of the external labelled solution in c.p.m./p-mole Na^+,
s_i specific activity of the internal medium in c.p.m./p-mole Na^+,
$[^{22}Na^+]_i$ = intracellular concentration of $^{22}Na^+$ in c.p.m./cm^3 fibre water.

It is assumed here that the intracellular concentration of Na^+ $[Na^+]_i$, is in steady state ($d[Na^+]_i/dt = 0$), therefore, $J_i = J_0$ and that s_0 remains constant. The intracellular specific activity will change according to eqn. (2):

$$s_i = s_{i\infty}(1-e^{-k_0 t}) = s_0(1-e^{-k_0 t}), \tag{2}$$

where $s_{i\infty}$ represents s_i at time = ∞, k_0 is the Na^+ efflux rate coefficient (in min^{-1}) and t the loading time (in min). Combining eqns. (1) and (2), eqn. (3) is obtained:

$$J_i = \frac{[^{22}Na^+]_i v}{A s_0 60}\frac{k_0}{1-e^{-k_0 t}} = \frac{^{22}Na^+_i}{A_T s_0 60}\frac{k_0}{1-e^{-k_0 t}}, \tag{3}$$

where $^{22}Na^+_i$ is the amount of $^{22}Na^+$, in c.p.m., taken up by all the fibres of a given muscle during the loading time t and A_T represents the total surface membrane area of that muscle (i.e. 552 $cm^2.g^{-1}$ × muscle weight in g). This equation which is essentially similar to eqn. (9) of Hodgkin & Horowicz (1959*a*) was used to calculate the resting Na^+ influx. The value of k_0 in normal Ringer solution is about 0·01 min^{-1} and in control experiments did not change significantly when 50 or 75 % of $[Na^+]_0$ was replaced by $Tris^+$. In addition, the term containing k_0 in eqn. (3) does not change much in spite of relatively large changes in k_0. In consequence k_0 = 0·01 min^{-1} was used in all the resting influx computations.

RESULTS

The resting Na^+ influx

The resting Na^+ influx was measured in the presence of 120, 60, 45 and 30 mM-$[Na^+]_0$. Table 2 shows the values obtained for these conditions. In normal Ringer solution the average Na^+ influx obtained in this series of experiments was $4·7 \pm 0·3$ p-mole.$cm^{-2}.sec^{-1}$ (mean ± 1 S.E. of mean).

The means listed in Table 2 include all the measurements of the resting influx in the presence of normal Ringer and Na^+ deficient solutions containing either $Tris^+$ or $choline^+$ whether they were performed as a part of the extra Na^+ influx determinations or in paired muscles. The Na^+ influx as a function of $[Na^+]_0$ when $Tris^+$ is used to replace Na^+ is shown by curve A in Fig. 4 which is a normalized plot obtained from paired muscles. In these experiments one member of the pair was loaded in radioactive normal Ringer while the other was exposed to a low $[Na^+]_0$ containing solution also labelled with $^{22}Na^+$ and for the same period of time. It is clear from this figure that for a given change in $[Na^+]_0$ the corresponding change in Na^+ influx is more pronounced at low levels of $[Na^+]_0$ than a concentration close to normal. This non-linearity of the Na^+ influx has already been

pointed out by Keynes & Swan (1959). In normal Ringer about 38 % of the total Na^+ efflux is Na^+-activated and strophanthidin-insensitive (Horowicz, Taylor & Waggoner, 1970; Venosa & Horowicz, 1973) showing the characteristics of the exchange diffusion mechanism proposed by Ussing (1949) and first demonstrated in muscle by Keynes & Swan (1959). Since in normal Ringer the muscle fibres are close to steady state, the net Na^+ flux being practically zero, one should expect that about 38 % of the Na^+ ions moving inward do so via exchange diffusion. This is in good agreement with the results of Erlij & Leblanc (1971). They estimate that 33 %

TABLE 2

$[Na^+]_0$ (m-mole.l.$^{-1}$)	Resting influx (p-mole.cm^{-2}.sec^{-1})	Extra influx (p-mole.cm^{-2}.impulse^{-1})
120 (normal Ringer)	4·7 ± 0·2 (36)	27·4 ± 2·8 (21)
60	2·8 ± 0·2 (30) T	25·3 ± 3·3 (16) T
	3·0 ± 0·3 (5) Ch	24·2 ± 1·8 (3) Ch
45	2·9 ± 0·2 (19) T	32·3 ± 2·6 (13) T
	3·2 ± 0·8 (4) Ch	17·0 ± 4·3 (3) Ch
30	1·3 ± 0·2 (8) T	—
	2·6 ± 0·4 (5) Ch	15·2 ± 2·5 (5) Ch

± 1 S.E. of mean.
T, partial replacement of Na^+ by $Tris^+$.
Ch, partial replacement of Na^+ by $choline^+$.
Number of experiments in parentheses.

of the total Na^+ influx would take place through that mechanism. The rest of the influx is very likely all passive and according to the constant field equations should be directly proportional to $[Na^+]_0$. (If one allows for the small hyperpolarization in Na^+ deficient solutions, the relationship between this fraction of the Na^+ influx and $[Na^+]_0$ is still practically linear.) When the linear passive component (Fig. 4*B*) is subtracted from the total Na^+ influx, the remaining influx, presumably the exchange diffusion fraction, appears to be close to saturation even at relatively low $[Na^+]_0$. It is apparent that halving the normal $[Na^+]_0$ produces a reduction of less than 10 % in this fraction as depicted by curve *C* in Fig. 4. Also, the reduction of this component to one half of its value in normal Ringer is reached only when $[Na^+]_0$ falls to about 26 mM. The simple scheme just described, which can account for the non-linearity of the Na^+ influx with respect to $[Na^+]_0$, seems the most reasonable in view of the current evidence available. It would appear from Table 2 that the non-linearity of the resting Na^+ influx with respect to $[Na^+]_0$ is also present when $choline^+$ instead of $Tris^+$ is used to replace Na^+.

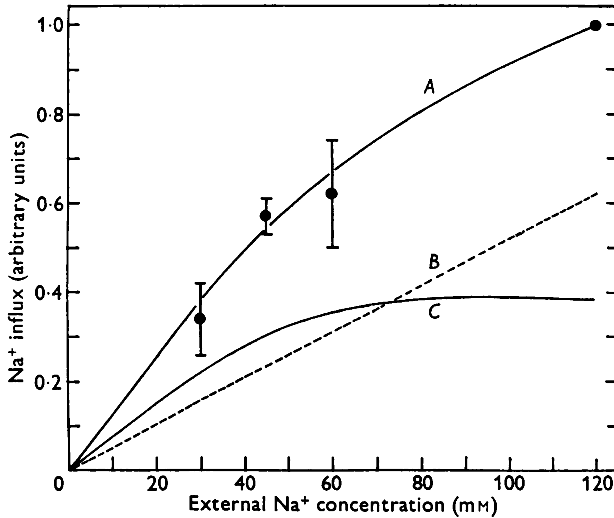

Fig. 4. *A*, normalized plot of Na^+ influx as a function of $[Na^+]_o$ (Na^+ replaced by $Tris^+$). The experimental points are means of influx in x mM-$[Na^+]_o$/influx in 120 mM-$[Na^+]_o$ ratios obtained in paired muscles (see text). The number of pairs used were: 6 in 60 mM-$[Na^+]_o$, 6 in 45 mM-$[Na^+]_o$ and 8 in 30 mM-$[Na^+]_o$. The vertical bars denote ± 2 S.E. of mean. *B*, passive component of the Na^+ influx which according to the constant field flux equation should be directly proportional to $[Na^+]_o$. *C*, this curve was obtained by subtracting *B* from *A* and would represent the exchange diffusion component of the Na^+ influx.

The stimulated Na^+ influx

Most determinations of the resting Na^+ influx were obtained in experiments in which pairs of muscles were exposed to the radioactive solution and one of them stimulated at the end of the loading period as described in the Methods section. From the difference in intracellular radioactivity between the stimulated and the resting muscle at time zero of the washout and the number of impulses, the extra Na^+ influx per impulse was readily obtained. Fig. 5 illustrates the washout curves from a pair of muscles one of which was stimulated at a frequency of 5·4/sec for a total of 216 impulses in the presence of labelled normal Ringer just before starting the washout. It is apparent that the intercept of the intracellular component from the stimulated muscle with the ordinate is appreciably higher than the one from the resting muscle. In this particular experiment the extra Na^+ influx was 30·7 p-mole.cm^{-2}.$impulse^{-1}$ and the resting influx 3·9 p-mole.cm^{-2}.sec^{-1}. Similar experiments were performed in the

presence of 60 and 45 mM-$[Na^+]_o$-$Tris^+$. In some experiments the choline ion was used instead of $Tris^+$ as a partial replacement for Na^+ in the external medium. As shown in Table 2 the extra influx in the presence of 60 mM-$[Na^+]_o$-choline$^+$ is similar to that in the presence of 60 mM-$[Na^+]_o$-$Tris^+$ and both are not different from the value obtained in normal Ringer. In 45 mM-$[Na^+]_o$, however, the extra influx was considerably greater in $Tris^+$ containing solution than in choline-containing medium. In principle, any change in extra influx upon reduction of $[Na^+]_o$ to half (60 mM) of its

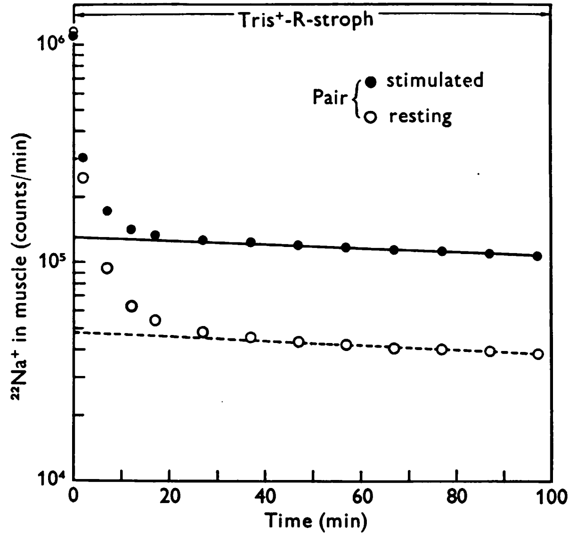

Fig. 5. $^{22}Na^+$ content of a pair of muscles as a function of time in the presence of $Tris^+$-Ringer plus 3×10^{-5} M strophanthidin. Before the washout both muscles were exposed to normal Ringer labelled with $^{22}Na^+$ for 18 min and at the end of that period one of them (●) was stimulated 216 times at a frequency of 5·4 impulses/sec. From the intracellular radioactivity of the unstimulated muscle (○) at time zero of the washout, a resting influx of 3·9 p-mole.cm^{-2}.sec^{-1} was calculated. An extra Na^+ influx of 30·7 p-mole. cm^{-2}.$impulse^{-1}$ was computed from the difference in $^{22}Na^+$ content of the two muscles at t = 0. Expt. 29 September 1972.

normal value should not be very large or at least large enough to be detected with this method. In 60 mM-$[Na^+]_o$ E_{Na}, the Na^+ equilibrium potential across the cell membrane would shift from about +52 mV in normal Ringer (assuming $[Na^+]_i$ = 15 mM) to near +35 mV and since the peak of the action potential follows approximately E_{Na} (Nastuk & Hodgkin, 1950) its value will fall about 17 mV. On the other hand, the resting membrane potential, V_m, given by

$$V_m = 58 \log \frac{[K^+]_o + 0{\cdot}01[Na^+]_o}{[K^+]_i} \quad \text{(Hodgkin \& Horowicz, 1959}b\text{)},$$

where $[K^+]_o = 2{\cdot}5$ m-mole/l. and $[K^+]_i = 140$ m-mole/l., will change from $-91{\cdot}5$ mV in normal Ringer to $-95{\cdot}5$ mV in $[Na^+]_o = 60$ mM. Therefore, the amplitude of the action potential will decrease some 13 mV. Assuming a capacitance of about 5 μF/cm² of surface membrane for fibre diameters close to 54 μm (Adrian, Chandler & Hodgkin, 1970; Hodgkin & Nakajima, 1972), the minimum amount of Na ions that should cross the cell membrane to produce such a difference in the amplitude of the action potential would only be about 0·7 p-mole/cm². Moreover, this amount could be even smaller since the peak of the action potential is lower than E_{Na}. This is a net flux difference, the inward flux difference would be slightly higher but still very small. These rather approximate computations indicate that one should not expect any large reduction in extra Na^+ influx even when $[Na^+]_o$ is considerably diminished. Hodgkin & Huxley (1952*a*, *b*) observed in voltage clamped squid axons that the Na^+ inward currents in Na^+ deficient solutions are 20–60 % larger than expected according to the 'independence principle'. They showed that this is due to the small hyperpolarization produced by low $[Na^+]_o$ solutions which, in turn, significantly reduces the Na^+ inactivation. The lack of any significant change in extra Na^+ influx when $[Na^+]_o$ was altered in the experiments reported here might be partly due to the presence of a similar mechanism in twitch muscle fibres from the frog. However, this seems unlikely because the sodium system in muscle appears to be little inactivated at the normal resting potential (Adrian *et al.* 1970). The smaller action potentials usually seen in the presence of Na^+ deficient solutions are also longer than those in the presence of normal Ringer (Nastuk & Hodgkin, 1950; Ferroni & Bianchi, 1965; Akaike & Noda, 1972). Experiments in progress suggest that such a prolongation of the action potential in low $[Na^+]_o$ is more pronounced when $Tris^+$ rather than $choline^+$ is used to replace Na^+ in the external solution. This perhaps could account for the high extra Na^+ influx observed in 45 mM-$[Na^+]_o$-$Tris^+$ and will be further commented on later. The relatively high extra Na^+ influx in the presence of 30 mM-$[Na^+]_o$-$choline^+$ (15·2 p-mole.cm².impulse^{-1}) is consistent with the fact that the action potential is not impaired in this solution and the twitch tension still well maintained (Mashima & Matsumura, 1962).

The frequency of stimulation was also varied between 3 and 50 impulses/sec. No effect of the frequency by itself on the extra Na^+ influx could be detected. The total number of impulses applied was in general close to two hundred except when the frequency of stimulation was high (50 impulses/sec) and the medium contained low $[Na^+]_o$ (60 m-mole/l.). In these cases the number of impulses was reduced to about 100. The reason for this reduction being that, as observed in the course of previous work (Bezanilla, Caputo, González-Serratos & Venosa, 1972), in low $[Na^+]_o$ solutions after

about 130–150 stimuli at 50 impulses/sec the fibres start to respond to alternate stimuli. This was reflected in the present experiments in eight muscles which were stimulated at 50 shocks/sec in the presence of 60 mM-$[Na^+]_o$-$Tris^+$ for a total of 200 impulses. The mean extra Na^+ influx was $16{\cdot}0 \pm 1{\cdot}4$ p-mole.cm^{-2}.$impulse^{-1}$, significantly lower ($0{\cdot}001 < P < 0{\cdot}01$) than $29{\cdot}2 \pm 4{\cdot}4$ p-mole.cm^{-2}.$impulse^{-1}$; the mean obtained by applying

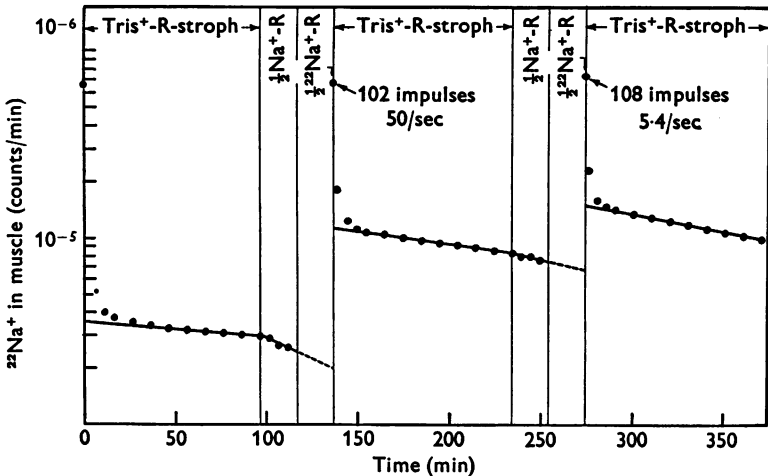

Fig. 6. Resting and extra Na^+ influx in a single sartorius. The muscle was exposed three times to 60 mM-$[Na^+]_o$ – $Tris^+$ labelled with $^{22}Na^+$ (½ $^{22}Na^+$-R) for 20 min and then washed out in the usual way. In the first run the muscle was not stimulated and from the initial intracellular radioactivity a resting influx of 2·7 p-mole.cm^{-2}.sec was calculated. In the second run the muscle was bathed in 60 mM-$[Na^+]_o$ – $Tris^+$ (½ Na^+-R) for 20 min, the remaining radioactivity in the muscle being monitored during this period of time. In the next 20 min it was again exposed to ½ $^{22}Na^+$-R as before except that the muscle was stimulated 102 times at 50 impulses/sec at the end of this exposure. From the initial intracellular radioactivity in this run minus the radioactivity remaining in the muscle from the previous one and assuming that the resting influx was identical to that in the first run, an extra influx of 37·0 p-mole.cm^{-2}.$impulse^{-1}$ was calculated. The third run was similar to the second one except that the stimulation frequency was 5·4 impulses/sec for a total of 108 impulses. In this case the calculated extra influx was 38·8 p-mole.cm^{-2}.$impulse^{-1}$. Expt. 10 November 1972.

around one hundred impulses under otherwise identical conditions. The similarity between the extra Na^+ influx at high and low stimulation frequencies in the presence of 60 mM-$[Na^+]_o$-$Tris^+$ is illustrated by the experiment shown in Fig. 6 in which resting influx and extra influx at 50 impulses/sec and 5·4 impulses/sec were measured in a single muscle. In

this experiment the resting influx was 2·7 p-mole.cm^{-2}.sec^{-1} and the extra influx 37·0 p-mole.cm^{-2}.$impulse^{-1}$ at 50 impulses/sec and 38·8 p-mole.cm^{-2}.$impulse^{-1}$ at 5·4 impulses/sec. These two values are high compared with the average obtained under similar conditions, nevertheless they clearly show that the extra influx in half Na^+-Ringer as in normal Ringer, is independent of the frequency of stimulation.

DISCUSSION

The aim of this work was to investigate whether it would be possible to obtain a reliable estimate of the resting and stimulated Na^+ influx in whole sartorius muscle by extrapolating to $t = 0$ the intracellular component of the washout curve of $^{22}Na^+$. The main requirements of this procedure are the following: (*a*) an exponential intracellular component of the washout much slower than any of the extracellular ones, and (*b*) paired muscles should behave similarly when they are identically treated. The results show that these requirements are fairly met under the experimental conditions chosen here. The resting Na^+ influx has been measured by Hodgkin & Horowicz (1959*a*) in single fibres from the semitendinosus muscle of the English frog (*Rana temporaria*) by Keynes & Swan (1959) in whole sartorius from the same species and by Horowicz & Gerber (1965) in bundles of fibres from the semitendinosus of the American frog (*Rana pipiens*). Hodgkin & Horowicz obtained a mean value of 3·5 p-mole.cm^{-2}.sec^{-1}, Keynes & Swan reported an average influx of 4·2 p-mole.cm^{-2}.sec^{-1} and Horowicz & Gerber 3·7 p-mole.cm^{-2}. sec^{-1}. The mean influx obtained in this investigation was 4·7 p-mole.cm^{-2}.sec^{-1}. Hodgkin & Horowicz (1959*a*) observed that the extra Na^+ influx per impulse was equivalent to 5–6 sec of resting influx. In close agreement with their finding the results from this investigation indicate an extra influx per impulse equivalent to 5·9 sec of resting influx, but on the other hand, both the resting and the stimulated influx are 34 and 41 % higher respectively than those reported by them. The comparison of these values suggests that the Na^+ influx in sartorius muscle might be slightly higher than in the semitendinosus despite the differences in frog species. For instance, the P value for the difference between the resting Na^+ influx reported by Hodgkin & Horowicz (1959*a*) and the one obtained in this work falls within the 0·02–0·05 interval.

The changes in the Na^+ influx upon reduction of $[Na^+]_0$ is that expected if the total Na^+ influx in normal Ringer were composed essentially of two fractions, one of them about 62 % of the total, passive and obeying closely the constant field flux equation and the other one about 38 % of the total mediated by an exchange diffusion mechanism saturated at a relatively low $[Na^+]_0$. As shown in Fig. 4 (curve C) if that were the case, the exchange diffu-

sion fraction would be half saturated at $[Na^+]_o = 26$ mM. It is worth mentioning here that Keynes & Swan (1959) found, in sartorii from *R. temporaria*, a $[Na^+]_o$ dependent component of the Na^+ efflux which, from their Fig. 5, appears to fall to 50% of its value in normal Ringer at $[Na^+]_o$ of about 25 mM. Taking 62% of the total Na^+ influx (2·9 p-mole.cm^{-2}.sec^{-1}) as passive, the value of the Na^+ permeability, P_{Na}, calculated by using the constant field flux equation would be $6{\cdot}5 \times 10^{-9}$ cm.sec^{-1}. However, this estimate of P_{Na} should be taken cautiously in view of the perhaps oversimplified assumptions on which it is based. Recently, Sjodin & Beaugé (1973) have pointed out the difficulties involved in the measurement of the purely passive Na^+ influx and hence of P_{Na}.

The average extra Na^+ influx in the presence of normal Ringer obtained in these experiments was 27·4 p-mole.cm^{-2}.$impulse^{-1}$. Hodgkin & Horowicz (1959*a*) reported a mean value of 19·4 p-mole.cm^{-2}.$impulse^{-1}$. Although the significance of this difference is not high ($0{\cdot}5 < P < 0{\cdot}10$) it nevertheless suggests that the extra Na^+ influx in sartorius from *R. pipiens* might be larger than in semitendinosus fibres from *R. temporaria*; this parallels the situation with regard to the resting influx. The extra influx is much less sensitive to changes in $[Na^+]_o$ than the resting influx in the range 60–120 mM. In the case of the resting influx the leak of Na^+ down its electrochemical gradient into the fibres which represents a substantial fraction of the total influx decreases with reduction in $[Na^+]_o$. On the other hand, in the case of the extra influx the minimum amount of Na^+ ions that should cross the membrane to displace the membrane potential from its resting value to about E_{Na} at the peak of the action potential does not change much when, for instance, $[Na^+]_o$ is halved, partly because E_{Na} is a logarithmic function of $[Na^+]_o$. The small hyperpolarization in the presence of low $[Na^+]_o$ would not produce any significant reduction in Na^+ inactivation since it is already low at the normal resting potential (Adrian *et al.* 1970). Therefore, in low $[Na^+]_o$ the Na^+ currents should not be larger than those expected on the basis of the 'independence principle'. It is clear from Table 2 that in Ringer containing half of the normal $[Na^+]_o$ the extra Na^+ influx is not significantly different from the value obtained in the presence of normal Ringer regardless of the cation used to substitute Na^+. In 45 mM-$[Na^+]_o$-$Tris^+$, it is apparent that the extra influx remains high and, if anything, tends to be higher than in normal Ringer. On the contrary, in 45 mM-$[Na^+]_o$-$choline^+$ the tendency is toward lower values than in normal Ringer. This difference is statistically significant ($P = 0{\cdot}02$). One may suspect that in low $[Na^+]_o$ particularly when $Tris^+$ replaces Na^+ the inactivation of the Na^+ current could be slower than in normal Ringer. In those conditions the Na^+ current would be correspondingly smaller although the charge transferred during the action potential would not be necessarily

smaller than in normal Ringer. Therefore, the extra Na^+ influx would not change much despite the substantial reductions in $[Na^+]_o$ as observed in the present experiments.

This idea is consistent with the fact that as $[Na^+]_o$ is decreased the action potential is prolonged (Nastuk & Hodgkin, 1950; Ferroni & Bianchi, 1965; Akaike & Noda, 1972), this effect being more pronounced in $Tris^+$ containing solutions that in $choline^+$ containing media (R. A. Venosa, unpublished). It would be interesting to measure the extra Na^+ influx at different $[Na^+]_i$ in view of the results in squid axon reported by Rojas & Canessa-Fischer (1968). They made the interesting observation that the extra influx and the extra efflux change linearly with $[Na^+]_i$ at constant $[Na^+]_o$ while the net influx per impulse remains constant.

The lack of any obvious effect of the frequency of stimulation on the extra Na^+ influx per impulse deserves some comment. Recently, Bezanilla *et al.* (1972) have shown that the tetanic tension in the presence of solutions in which Na^+ is partially replaced by $Tris^+$ is not well sustained but rather falls to a low level after an initial peak. Their results suggest the presence of a Na^+ dependent regenerative process in the walls of the transverse tubular system. When $[Na^+]_o$ is low and the frequency of stimulation is high the Na^+ concentration in the tubular lumen would fall appreciably in the T-system particularly near the centre of the fibres, possibly below the level needed either for the production of an action potential or for the production of an action potential capable of activating the contractile elements. Both mechanisms would stop the inward spread of activation leaving an inner core of inactivated myofibrils, which become wavy under isotonic conditions (Bezanilla *et al.* 1972), and therefore making the tetanic tension significantly lower than in the presence of normal Ringer. The fact that in the present experiments the extra Na^+ influx per impulse in the presence of 60 mM-$[Na^+]_o$-$Tris^+$ was not affected by the frequency of stimulation would favour the second of the two possibilities just mentioned.

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REFERENCES

ADRIAN, R., CHANDLER, W. K. & HODGKIN, A. L. (1970). Voltage clamp experiments in striated muscle fibres. *J. Physiol.* **208**, 607–644.

AKAIKE, N. & NODA, K. (1972). Effect of local anaesthetic quatacaine on the membrane potential and sodium conductance of frog muscle fibers. *Jap. J. Physiol.* **22**, 281–293.

BEZANILLA, F., CAPUTO, C., GONZÁLEZ-SERRATOS, H. & VENOSA, R. A. (1972). Sodium dependence of the inward spread of activation in isolated twitch muscle fibres of the frog. *J. Physiol.* **223**, 507–523.

CONWAY, E. J. (1957). Nature and significance of concentration relations of potassium and sodium. *Physiol. Rev.* **37**, 84–132.

DESMEDT, J. E. (1953). Electrical activity and intracellular sodium concentration in frog muscle. *J. Physiol.* **121**, 191–205.

EDWARDS, C., RITCHIE, J. M. & WILKIE, D. R. (1956). The effect of some cations on the active state of muscle. *J. Physiol.* **133**, 412–419.

ERLIJ, D. & LEBLANC, G. (1971). The effects of ethacrynic acid and other sulphydryl reagents on sodium fluxes in frog muscle. *J. Physiol.* **214**, 327–347.

FERRONI, A. & BIANCHI, D. (1965). Maximum rate of depolarization of single muscle fiber in normal and low sodium solutions. *J. gen. Physiol.* **49**, 17–23.

GRABOWSKI, W., LOBSIGER, E. A. & LÜTTGAU, H. CH. (1972). The effect of repetitive stimulation at low frequencies upon the electrical and mechanical activity of single muscle fibres. *Pflügers Arch. ges. Physiol.* **334**, 222–239.

HARRIS, E. J. & BURN, G. P. (1949). The transfer of sodium and potassium ions between muscle and the surrounding medium. *Trans. Faraday Soc.* **45**, 508–528.

HILL, A. V. (1928). The diffusion of oxygen and lactic acid through tissues. *Proc. R. Soc.* B **148**, 39–96.

HODGKIN, A. L. & HOROWICZ, P. (1959*a*). Movements of Na and K in single muscle fibres. *J. Physiol.* **145**, 405–432.

HODGKIN, A. L. & HOROWICZ, P. (1959*b*). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HODGKIN, A. L. & HUXLEY, A. F. (1952*a*). Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*. *J. Physiol.* **116**, 449–472.

HODGKIN, A. L. & HUXLEY, A. F. (1952*b*). The dual effect of membrane potential on sodium conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 497–506.

HODGKIN, A. L. & NAKAJIMA, S. (1972). The effect of diameter on the electrical constants of frog skeletal muscles fibres. *J. Physiol.* **221**, 105–120.

HOROWICZ, P. & GERBER, C. (1965). Effects of external potassium and strophanthidin on sodium fluxes in frog striated muscle. *J. gen. Physiol.* **48**, 489–514.

HOROWICZ, P., TAYLOR, J. M. & WAGGONER, D. M. (1970). Fractionation of sodium efflux in frog sartorius muscles by strophanthidin and removal of external sodium. *J. gen. Physiol.* **55**, 401–425.

HUXLEY, A. F. (1960). Appendix 2 of: SOLOMON, A. K. (1960). Compartmental methods of kinetic analysis. In *Mineral Metabolism*, ed. COMAR, C. L. & BRONNER, F., vol. 1, part A, pp. 163–166. New York: Academic Press.

KEYNES, R. D. & LEWIS, P. R. (1951). The resting exchange of radioactive potassium in crab nerve. *J. Physiol.* **113**, 73–98.

KEYNES, R. D. & SWAN, R. C. (1959). The effect of external sodium concentration on the sodium fluxes in frog skeletal muscle. *J. Physiol.* **147**, 591–625.

MASHIMA, H. & MATSUMURA, M. (1962). Roles of external ions in the excitation-contraction coupling of frog skeletal muscle. *Jap. J. Physiol.* **12**, 639–653.

MULLINS, L. J. & FRUMENTO, A. S. (1963). The concentration dependence of sodium efflux from muscle. *J. gen. Physiol.* **46**, 629–654.

NASTUK, W. L. & HODGKIN, A. L. (1950). The electrical activity of single muscle fibers. *J. cell. comp. Physiol.* **35**, 39–74.

OVERTON, E. (1902). Beitrage zur allgemeinen Muskel- und Nervenphysiologie. *Pflügers Arch. ges. Physiol.* **92**, 346–386.

ROJAS, E. & CANESSA-FISCHER, M. (1968). Sodium movements in perfused squid giant axons. *J. gen. Physiol.* **52**, 240–257.

SJODIN, R. A. & BEAUGÉ, L. A. (1973). An analysis of the leakages of sodium ions into and potassium ions out of striated muscle cells. *J. gen. Physiol.* **61**, 222–250.

USSING, H. H. (1949). Transport of ions across cellular membranes. *Physiol. Rev.* **29**, 127–155.

VAUGHAN, P. C., HOWELL, J. N. & EISENBERG, R. S. (1972). The capacitance of skeletal muscle fibers in solutions of low ionic strength. *J. gen. Physiol.* **59**, 347–359.

VENOSA, R. A. (1973). Resting and stimulated sodium influx in frog's sartorius muscle. *Biophys. Soc.* 17th Ann. Meeting, p. 197*a*.

VENOSA, R. A. & HOROWICZ, P. (1973). Effects on sodium efflux of treating frog sartorius muscles with hypertonic glycerol solutions. *J. Membrane Biol.* **14**, 33–56.