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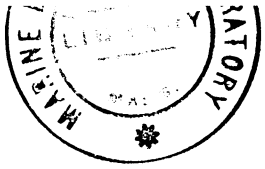

POTASSIUM MOVEMENTS IN CONTRACTING DIAPHRAGM MUSCLE

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Attempts to measure the ionic changes which accompany muscular contraction encounter a number of peculiar difficulties in the case of isolated mammalian tissues. Most preparations are comparatively thick, and the over-all exchange of potassium is believed to be slowed by interfibre diffusion, so that allowance must be made for this when exchange across the cell membrane is calculated. Sodium exchange of rat diaphragm muscle at body temperature is rapid (Creese, 1954), and it was not possible in the present study to record changes in sodium movement accompanying contraction. The oxygen consumption of this muscle is high so that aeration must be well maintained even in the resting state if anoxia of the cells in the interior of the tissue is to be avoided (Creese, Scholes & Whalen, 1958). Perhaps the chief limitation, however, is that rapid stimulation is badly tolerated in these preparations, and even moderate rates of stimulation may produce irreversible impairment of twitch tension. Hence it is desirable to employ only short periods of stimulation at low rates, and the small and transient changes in potassium movements which are produced require sensitive methods for their detection and measurement.

Before tracer substances can profitably be used on this preparation it is necessary that the general effects of stimulation on the muscle be known, and the first part of the present study contains an account of findings in isolated diaphragm muscle after repeated contractions at a moderate rate of 2/sec. A preliminary account of this work has been reported (Creese, Hashish & Scholes, 1958).

METHODS

Diaphragm muscles from albino rats of 120–160 g were secured by the ribs to plastic holders. Dissection, analysis of muscle, estimation of inulin space and measurement of radioactivity were made as described previously (Creese, 1954). The usual corrections were made for background count, resolving time of counter and scaler, and decay of radioactivity. The modified Krebs's

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saline had the same composition as that used before (Creese, 1954) and contained 5·9 mM potassium. It was aerated with a mixture of 5% CO_2, 95% O_2 at 38° C. The muscle holders were suitably wired, and the phrenic nerve was stimulated by a succession of single maximal shocks (duration 0·25 msec) by means of a stimulator designed by Bernstein (1950). In some cases the muscle was stimulated directly through the tendon with shocks lasting 0·5 msec.

Muscle tension was measured with a strain gauge (Statham Laboratories) which was attached to the tendon through a light glass rod. With a d.c. supply of 8 V, a tension of 10 g produced a voltage change of 0·7 mV across the terminals, and this was passed through an amplifier having a

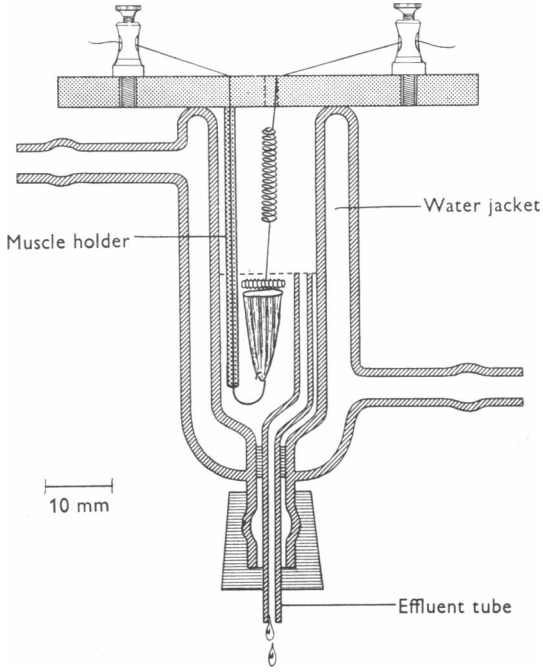

Fig. 1. Arrangement for measuring changes in outward movement of potassium. The muscle, loaded with ^{42}K, is secured to the tissue holder and placed in the small jacketed bath which is fitted with a short overflow tube. The inflow tube for saline is not shown, nor the oxygenation tube. The outgoing saline is collected for counting. The volume of the vessel is 4 ml.

relatively long time constant (over 200 msec) and the deflexions were displayed on an oscilloscope. The terminals of the strain gauge were also connected to a pen writer (Offner, Inc.) which provided a continuous record of twitch tension and resting tension. The deflexions of the oscilloscope beam and of the pen were proportional to the changes in tension. Both methods gave similar results when the twitch tension was diminished either by the use of tubocurarine or by rapid stimulation of the preparation at 2/sec. The natural frequency of the strain gauge was 250 c/s, and the frequency response of the pen writer varied by no more than 10% up to 70 c/s. The resting tension of the muscle was checked at the end of the experiment by cutting the tendon and observing the change in the tension recorded by the pen writer.

Micro-electrodes were used to record resting potentials in various layers of the muscle, the arrangement being similar to that described by Creese, Hashish & Scholes (1958).

Fig. 1 gives the dimensions of the apparatus used for the measurement of changes in the outward movement of ^{42}K. A diaphragm was soaked for 2–4 hr in saline containing ^{42}K, and the

muscle was then transferred (in the dissecting bath) to a holder which was suitably wired for direct stimulation of the muscle. In some cases an isometric arrangement was employed. The muscle holder fitted into a small jacketed organ bath containing 4 ml. of saline. An inflow tube, reaching to the bottom of the bath, brought warm (38° C) saline at a rate of 10 ml./min. The saline was allowed to escape down an overflow tube and was collected in vessels placed beneath the bath. The fluid obtained during intervals of 0·5 or 1 min was adjusted to constant volume and counted for radioactivity. The counts were 3000–8000/min in most cases. Doubling the rate of flow did not affect the outward movement of ^{42}K from the muscle. A fine plastic tube plugged at the tip with fluted glass was used to oxygenate the muscles; the gas bubbles also served to mix the contents of the bath. After a run the muscle was removed, and the radioactivity of the rib, which remained attached to the holder, was measured. The contribution made by the rib was usually less than 10% of the total counts. In some cases a control run with the rib alone was undertaken.

The simple arrangement shown in Fig. 1 was designed to maintain adequate oxygenation while reducing to a minimum the lag between the appearance of a substance in the bath and its collection in the overflow. Experiments in which dye was suddenly injected at a depth half way between the surface of the saline and the bottom of the bath showed that the dye was removed exponentially with a half-time which was less than 20 sec. The sensitivity was chiefly limited by irregularities in rate of flow, which were liable to produce excessive fluctuations in the counts recorded. A steady flow was only obtained if the apparatus was completely free from grease, and it was necessary that the bubbling produced by the oxygen should not be excessive. The overflow tube was situated eccentrically so that the opening did not lie in the trough of the saline meniscus. Experiments in which the flow became irregular were abandoned, and only muscles which were seen to contract vigorously were accepted. This apparatus was much more sensitive than that used previously (Creese, 1954).

In all experiments in which ion movements were recorded the muscles were allowed to shorten during contraction. Strain-gauge studies were performed on muscles under isometric conditions, and for intracellular recording the muscles were steadied by a tension of 25–50 g wt.

RESULTS

Stimulation rate and twitch tension

The twitch tension produced by isolated diaphragm preparations was usually steady for periods of 12 hr when the rate of stimulation was 4/min. There was, however, a marked decline in performance at moderate stimulation rates of 2/sec or more, as found by Brown, Bülbring & Burns (1948).

Fig. 2 shows the effect on muscle tension of stimulation through the nerve at 2/sec, the tension being plotted at intervals. The peak tension is given by the difference between the total and the resting tension. During the first 2 min the peak tension fell by about 10%, and after 30 min the peak tension was less than half the initial value. There was also an increase in the resting tension. Similar results were obtained by direct stimulation through the tendon. Recovery when the rate of stimulation was reduced again to 4/min was incomplete.

In experiments similar to those of Fig. 2 the mean peak tension after 30 min stimulation at 2/sec was 40% of the initial value $\pm$ 4% (S.E. of seven determinations). The mean initial resting tension in this series was 5·4 g wt. Fig. 3 shows myograms from another muscle before and after stimulation at the

same rate. The rise-time was about 20 msec, which is similar to that recorded by Ritchie (1954), who also used a strain-gauge. The small latency relaxation is not displayed in Fig. 3.

With prolonged stimulation the muscle fails to relax completely, with the result that the resting tension is increased. If the contractions are recorded

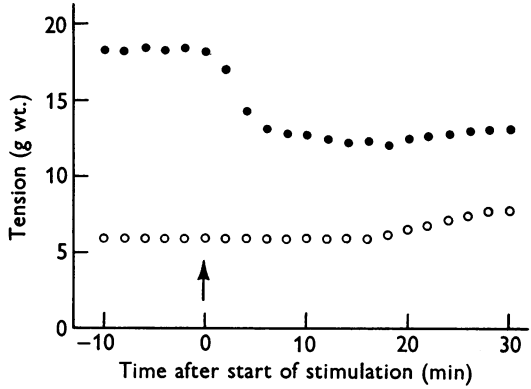

Fig. 2. Isometric twitch tension of diaphragm muscle recorded by strain-gauge. ○ Resting tension; ● total tension: the difference gives the peak twitch tension. Before arrow, stimulation at 4/min; at arrow, stimulation changed to 2/sec. There is a fall in twitch tension, with a later rise in resting tension.

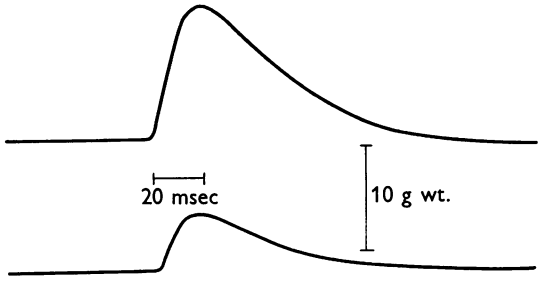

Fig. 3. Twitch tension, recorded by strain-gauge and oscilloscope. Upper trace obtained during stimulation at 4/min, lower trace following stimulation for 30 min at 2/sec. The records have been retouched.

isotonically, a contracture is produced in all cases. Stimulation at 5/sec produced a more severe contracture and a drastic decline in twitch tension. A similar contracture may be produced by depriving the muscle of oxygen, and this suggested that the adverse effects of stimulation at moderate rates might be due to anoxia. This is considered below.

Stimulation and potassium content

Fig. 4 shows the effect of stimulation at 2/sec on the potassium content of diaphragm muscles from rats of 120 ± 3 g (limits). The muscles were soaked in

large saline baths (200 ml.). The precautions described previously (Creese, 1954) were followed in order to minimize potassium losses during dissection. The potassium content of soaked muscles has been expressed for convenience as a percentage of the potassium content of the unsoaked hemidiaphragm, using dry-weight estimations. The upper broken line in Fig. 4 shows values obtained from muscles soaked for 3 hr without stimulation, the figure being 93·7 % ± 1·4 (S.E. of ten determinations). The potassium content of soaked muscles appears to be steady for several hours (Creese, 1954). Expressed in a different way, the potassium content of control soaked muscles was 74 m-mole/kg wet tissue. The other points in Fig. 4 refer to muscles which were soaked for

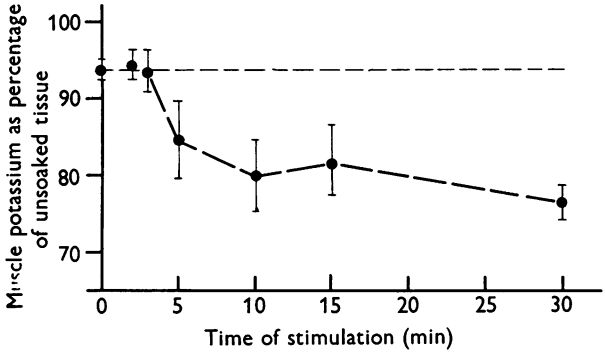

Fig. 4. Effect of stimulation on muscle potassium, expressed as a percentage of the potassium content of the unsoaked hemidiaphragm. The value at zero time, which is also shown by the upper broken line, gives the mean of ten muscles which were soaked and not stimulated. Other points refer to the mean of four muscles after stimulation for various times. The limits give 2 × S.E.

3 hr and then stimulated for various periods at 2/sec. Each point represents the mean of four muscles, and the limits give twice the S.E. There seems to be little change during the first 2–3 min, though a fall in potassium content is apparent if stimulation is maintained for longer periods.

The average value for muscles stimulated for 10, 15 and 30 min is 79·3 % ± 2·1 (S.E. for twelve muscles), expressed as a percentage of the potassium content of the unsoaked muscles. This is a fall of about 15 % compared with the mean of the ten muscles which were soaked but not stimulated, and this difference is unlikely to be fortuitous ($t = 5{\cdot}5$, $P < 0{\cdot}01$). It should be noted that these results refer to diaphragm muscles of about 550–600 μ thickness; a few measurements obtained on diaphragms of 400 μ thickness showed that stimulation produced a much smaller effect in these cases. From Figs. 2 and 4 it can be seen that a comparatively small loss of potassium was associated with a considerable decrease in twitch tension. There was a small and statistically insignificant gain of sodium in the case of muscles stimulated at 2/sec.

Resting potentials

Micro-electrodes were pushed slowly through the muscle by a motor, and the resting potentials of individual fibres were recorded by means of a pen writer having a large excursion. Fig. 5 shows a record obtained from an unstimulated muscle. Several such controls were obtained in each case at various positions along the muscle.

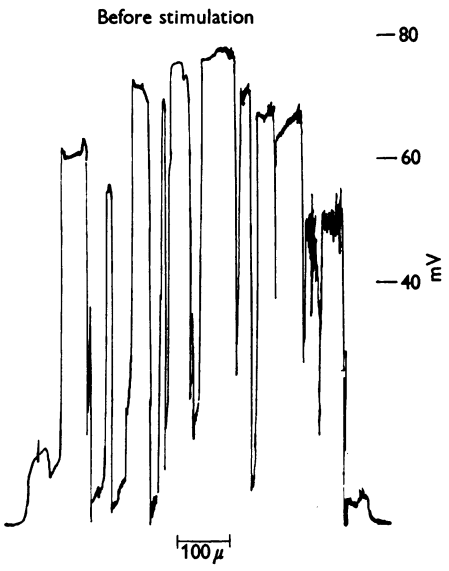

Fig. 5

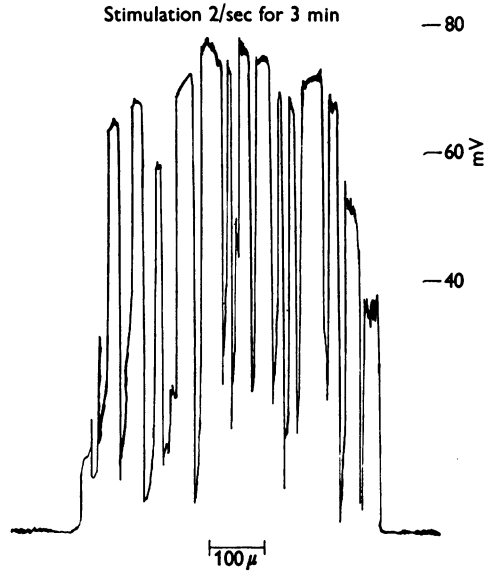

Fig. 6

Fig. 5. Resting potentials of unstimulated diaphragm. The micro-electrode was driven slowly through the muscle, and the potentials of the fibres were recorded by a pen writer. The cells at the right (pleural) side may have been damaged during stripping of the pleura. Upward deflexion represents a negative potential with reference to the earthed saline.

Fig. 6. Same muscle as that of Fig. 5, after stimulation for 3 min at 2/sec. The muscle was impaled at a nearby position. There is little apparent change.

In Fig. 5 the cells at the right side show potentials which are low, presumably owing to damage during removal of the pleura. The distance between vertical movements of the pen record measures the apparent fibre thickness. Owing to the arrangement whereby chords are measured instead of diameters, this apparent thickness is in most cases somewhat smaller than the true diameter and may be corrected as described previously (Creese, Scholes & Whalen, 1958). Fig. 5 shows cells with an apparent thickness up to about 65μ, and this range is similar to that found previously. In the present series the uncorrected mean fibre diameter was 23μ from rats of 120–160 g, and the corrected mean value was 27μ. This may be compared with a corrected value of 32μ from rats of 200–300 g found by Creese, Scholes & Whalen (1958). Creese (1954) found a mean of 16μ in the fibres of the left diaphragm and a mean of 20μ from the diaphragm as a whole in rats of 120 g, using a different strain of animals. Krnjević & Miledi (1958) state that the fibre diameters in diaphragm muscle are mostly between 25 and 50μ, with a range extending up to 80μ, in rats of 100–200 g.

Fig. 6 was obtained from the same muscle as that used for Fig. 5 following stimulation for 3 min at a rate of 2/sec through the nerve, the record being obtained with the minimum delay from a site in the central strip of the muscle. The record shows no obvious abnormality as compared with Fig. 5. In Fig. 7 the same muscle was stimulated for 20 min before exploration with the micro-electrode at an adjacent site. The cells in the depths of the tissue appear to show lower potentials than those situated more superficially. This effect was often more pronounced. Fig. 8 shows a more striking example from a muscle

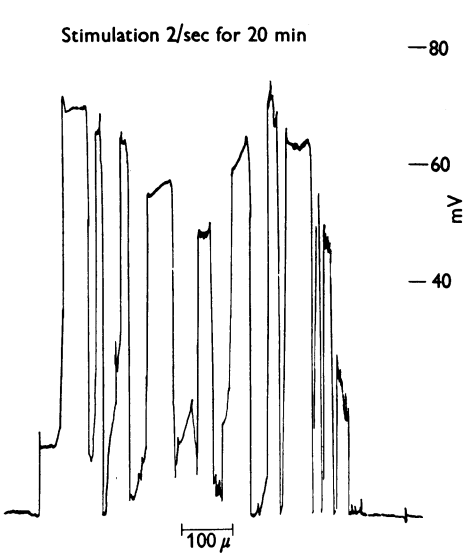

Fig. 7

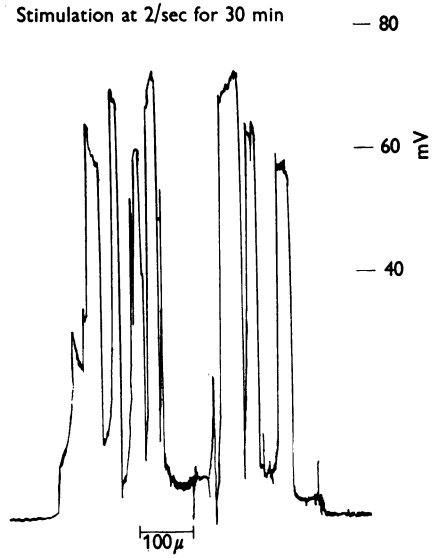

Fig. 8

Fig. 7. Same muscle as that of Figs. 5 and 6, after stimulation through the nerve for 20 min at 2/sec. The cells in the depths of the tissue show depolarization.

Fig. 8. Resting potentials from another diaphragm stimulated for 30 min at 2/sec. The region in the centre shows small potentials or none.

stimulated for 30 min in the same manner. Similar records were obtained from adjacent regions of the muscle. Five diaphragms were used for these experiments, and normal controls were obtained initially. Stimulation for 20–30 min was accompanied by a zone of low or absent potentials when the central portion of the diaphragm was explored with micro-electrodes in each of this small number of experiments. Recovery seemed to be incomplete, and in some cases exploration 30 min after cessation of stimulation still disclosed a zone of depolarized fibres. The regions within 2 mm of the cut edges of the muscle appeared to show only marginal effects, presumably owing to better oxygenation. A few experiments in which stimulation at 5/sec was employed

produced more drastic effects than those of Fig. 8, especially when thick muscles were used.

The results revealed with micro-electrodes after stimulation resemble in general those obtained in resting muscles by diminishing the oxygen content of the gas mixture used to aerate the tissue (Creese, Scholes & Whalen, 1958). The records are consistent with the view that isolated muscles which are stimulated at moderate rates may be damaged by anoxia of the cells in the depths of the tissue, and this possibility is discussed further below.

In some cases the electrode was driven right through the muscle. This manœuvre may result in breaking the tip of the electrode, and usually the motor was stopped when deflexions corresponding to fibres seemed to have ceased. The horizontal movement of the paper used for recording was also stopped. The electrode was then withdrawn back into saline, and usually a downward deflexion occurred as the base line was reached again. The record was then continued with the needle in saline. This explains the abrupt fall in p.d. on the left side of Fig. 7. If the final base line differed from the initial level it was concluded that the tip of the needle was blocked or broken and the records were discarded.

Diffusion of inulin during stimulation

Diaphragm muscles from rats weighing 120 ± 5 g (limits) were mounted on a holder and attached to a spring-loaded lever. The muscles were soaked for 2 hr and the solution was then changed to saline containing inulin (0·5%, w/v). During their period of immersion in the inulin-saline the muscles were stimulated through the nerve at 2/sec. The uptake of inulin in contracting muscle was determined by methods used previously (Creese, 1954), and was expressed as the ratio inulin/100 g muscle: inulin/ml. saline. The inulin space after 30 min stimulation was 24·6 ml./100 g $\pm$ 1·7 (S.E. of eleven determinations). This differed little from the value of 26·4 ml./100 g obtained in resting diaphragm (Creese, 1954). The uptake of inulin after stimulation for 5 min was 11·5 ml./1009 $\pm$ 1·1 (S.E. of ten determinations), so that the fractional uptake during this time was 0·468. Assuming the thickness of the diaphragm to be 0·6 mm, the apparent diffusion coefficient of inulin in stimulated muscle was $5{\cdot}1 \times 10^{-7}$ cm^2/sec, using the same method of calculation as before. The value previously found for resting muscle was $5{\cdot}6 \times 10^{-7}$ cm^2/sec at 38° C, so that contractions at this slow frequency produced no great change in diffusion rate.

Creese (1954) concluded that the apparent diffusion coefficient (D') of inulin in diaphragm muscle at 38° C was about a quarter of that in free solution. McLennan (1956) also finds that D' for thiocyanate at 20° C in isolated leg muscle of the rat is about one quarter of the value in free solution ($2{\cdot}2 \times 10^{-4}$ cm^2/min compared with $8{\cdot}7 \times 10^{-4}$ cm^2/min), whereas D' for inulin at 20° C was $7{\cdot}96 \times 10^{-5}$ cm^2/min compared with the value in free solution of $9{\cdot}6 \times 10^{-5}$ cm^2/min. McLennan (1957, Table 4) has also calculated from Conway's data that D' for inulin in frog sartorius muscle is $6{\cdot}0 \times 10^{-5}$ cm^2/min at 20° C, so that the ratio of D' to the value in free solution is here about 60%.

Inward movement of ^{42}K during stimulation

No consistent change was detected in the rate of inward movement of ^{42}K during the first minutes of stimulation at moderate frequencies. Diaphragms

from rats of 120 g were attached to holders and soaked in non-radioactive saline for 2 hr. They were then placed in saline containing ^{42}K for varying times, the total potassium content of all saline solutions being similar. Alternate muscles were stimulated through the nerves while in radioactive saline. At the end of the appropriate period the muscles were quickly removed and the percentage exchange was determined, as expressed by the ratio radio-activity/mg K in tissue: radioactivity/mg K in saline. Table 1 shows the results of stimulation at 5/sec, for 5 and for 10 min. The differences between stimulated muscles and controls are small and inconsistent.

TABLE 1. ^{42}K uptake in stimulated diaphragm

Stimulation time (min)	5	10	30
K % exchanged			
Control	10·7±0·56 (6)	20·6±2·13 (7)	43·6±1·77 (20)
Stimulated	11·0±0·88 (7)	20·4±1·36 (7)	—

Muscles were stimulated through the nerve at 5/sec. ^{42}K uptake is expressed as the percentage of potassium which has exchanged, ±S.E. (with no. of expts.). Values for 30 min controls are from data used by Creese, Neil & Stephenson (1956).

If the time allowed for entry of ^{42}K is small, an appreciable fraction of the potassium exchanged is attributable merely to extracellular potassium. Some 2% of the total potassium in soaked diaphragm muscle is extracellular (Creese, 1954). No corrections have been applied to the figures in Table 1.

Outward movement of ^{42}K during stimulation

Fig. 9 shows the results obtained on resting muscle, using the apparatus shown in Fig. 1, with the precautions described above. The muscle, previously loaded with tracer, was deprived of its rib before being secured in the organ bath. A stream of normal saline was allowed to pass over it and the effluent was collected and counted. The results appear to give a linear relation between counts/min and time, when plotted on semilogarithmic paper, though longer runs usually revealed a mild degree of curvature of the semilogarithmic plot as found in previous studies. The slope gave rate constants k varying between 0·9/hr and 1·5/hr. In Fig. 9 k is 1·2/hr (half-time 34 min), and this is consistent with the results obtained with other apparatus (Creese, 1954; Creese, Neil & Stephenson, 1956).

Calkins, Taylor & Hastings (1954, Table 1) found the average rate constant k_p for exchange of ^{42}K in diaphragm to be 0·016/min at 37° C, or 0·96/hr. These results seem to be a little lower than those reported here, and this may be attributed to the lower potassium concentration (3·65 mM) used by Calkins *et al.* (1954).

Stimulation at a relatively rapid rate of 5/sec gave the result shown in Fig. 10. There was an immediate and rapid increase in the tracer found in the

effluent fluid, reaching a peak after about 3 min of stimulation. After the experiments the rib was removed and a separate run was made under similar conditions so that the contribution made by the rib could be calculated.

Attempts were made to detect and measure an increase in outward movement of tracer during the first few minutes following stimulation at 2/sec. It has been shown above that during this time there is no evidence of depolarization, no detectable change in the total potassium content, and a small

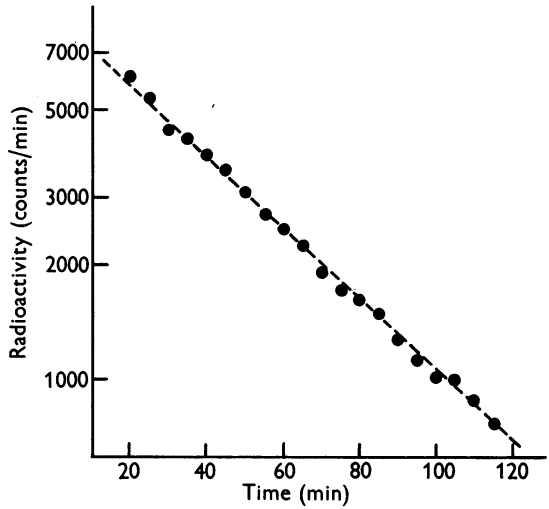

Fig. 9. Outward movement of labelled potassium from resting diaphragm. Saline from the organ bath was collected during 1 min periods, and the radioactivity in counts/min is plotted on semilogarithmic paper. The abscissa gives the time in min during which the muscle, previously loaded with ^{42}K, has been in contact with inactive saline. The rib was removed. The slope has a half-time of 34 min.

diminution in the twitch tension. Particular precautions were taken to produce an even flow of saline. Fig. 11 shows a result obtained from a muscle stimulated at 2/sec. The scale is enlarged compared with the other graphs. There is a detectable increase in the tracer collected after stimulation. A correction for the effect of the rib was made in the usual manner before plotting.

The increase in outward movement of ^{42}K is given by the difference between the points after stimulation and the corresponding points on the extrapolated initial regression line. The curve of Fig. 11 shows that there is an immediate increase in the output of ^{42}K, but there was frequently a lag of some 2–3 min before this increase reached a maximum. This lag is partly due to apparatus delay. It may also be due to the time taken by the extra potassium released by stimulation to diffuse through the interspaces and reach the external saline. In practice the increase was measured after 3 min stimulation. In the experi-

ment of Fig. 11 the value is 23% greater than the figure obtained by extrapolation of the regression line. In ten experiments an increase was obtained in each case, the values ranging from +10 to +33%, with a mean increase of 22·6% ± 3·4 (S.E. of 10).

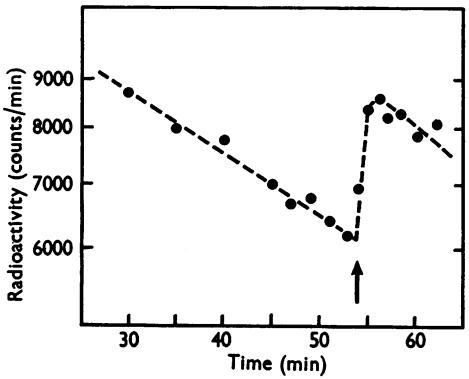

Fig. 10. Outward movement of ^{42}K as before. At the arrow, stimulation was started at a rate of 5/sec. A large increase in the radioactivity of the saline coming from the organ bath is apparent on stimulation of the muscle. A separate run was made later with the rib alone and the appropriate correction was made before plotting.

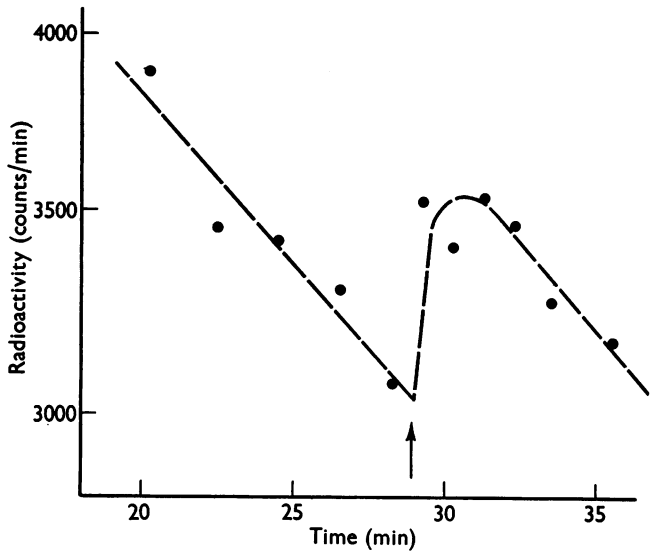

Fig. 11. Outward movement of ^{42}K on larger scale. At the arrow, stimulation was started at a rate of 2/sec. After 3 min the radioactivity has increased by some 23% (see text). A correction for the activity of the rib has been made before plotting.

Calculation of potassium loss accompanying a twitch

Since no change in inward movement of potassium was detected during stimulation, the observed increase in the outward movement was taken to indicate the net outward movement. The figures showed considerable scatter and the means of the various groups of measurements have been used for purposes of calculation. The potassium flux for resting diaphragm from rats of 120 g under similar conditions has been calculated as 45 pmoles/cm²/sec (Creese *et al.* 1956), and this estimation has been used to obtain the potassium loss per contraction.

The calculation is complicated to some extent by diffusion factors. In most cases it is likely that interfibre diffusion slows the over-all exchange of potassium, so that for any given circumstance the true rate of exchange across the cell membrane is greater than the observed rate of exchange between the tissue and its environment. If M is the mean flux across the cell membrane and M' is the apparent flux, then for a flat muscle we have, using a similar nomenclature to that of Keynes (1954):

$$\frac{M'}{M} = \frac{\lambda}{d} \tanh \frac{d}{\lambda}, \tag{1}$$

where d is half the thickness of the muscle and is taken as 0·03 cm (Creese, 1954), and λ is a constant which depends on the apparent diffusion coefficient of potassium. For resting diaphragm we have λ^2 equal to $5{\cdot}0/M \times 10^{-15}$, M' is 15·7 pmoles/cm²/sec and M is 45 pmoles/cm²/sec (Creese *et al.* 1956).

It was found that stimulation at 2/sec produced an increase, after 3 min, of 22·6% in the outward movement of ^{42}K. Hence M'_s, the apparent flux during stimulation, is 122·6% of 15·7 or 19·2 pmoles/cm²/sec. M_s, the outward potassium flux during stimulation, was found by trial and error from the above equation in the usual way: the value is 66·5 pmoles/cm²/sec. It may be noted that for resting muscle M'/M is 0·35, whereas for the increased flux of stimulated muscle the effect of diffusion is even more marked and M'_s/M_s is 0·29. Stimulation at 2/sec increased the outward flux from 45 to 66·5 pmoles/cm²/sec, or a difference of 21·5 units, so that the net loss is 10·7 pmoles/cm²/contraction.

A few experiments were made with stimulation at a rate of 5/sec. The increase after 3 min varied from 25 to 43%, with a mean of 31·3% (four expts.) Calculations based on similar methods to those used above gave the loss per contraction as 5 pmoles/cm², which is less than half the value obtained at 2/sec. Stimulation at 5/sec was found previously to produce a rapid fall in twitch tension and gross depolarization, and for this reason the results obtained at the slower rate of stimulation were preferred as an estimation of the loss for a single twitch.

It is also desirable to estimate this quantity by measuring the potassium content of the muscle before and after stimulation, for such a procedure would give the net change and would serve as a check on the tracer method. Unfortunately, it was found that a rapid rate of stimulation was accompanied by serious depolarization of a portion of the muscle, whereas a slow rate gave small differences with large statistical fluctuations. It is of interest, however, to see whether the methods both give results which are of the same order of magnitude, and the following calculations have been made with these admitted limitations.

From Fig. 4 the main loss of potassium occurs during the first 15 min. If the loss of potassium is plotted against time, the regression slope indicates that the muscle loses about 1·2%/min, with reference to the potassium of the control muscles which are soaked but not stimulated. It is necessary to obtain the potassium associated with each cm^2 of fibre surface. From Creese *et al.* (1956) there are $1{\cdot}15 \times 10^{-4}$ moles/c.c. of myoplasm. Also V/A, the ratio of cell volume to surface area, is 4·2 μ which is equivalent to 2380 cm^2/c.c. Therefore, the cell potassium may be expressed as $(1{\cdot}15 \times 10^{-4})/2380$ moles/cm^2, or 48300 pmoles/cm^2. Then each cm^2 loses 1·2% of this or 580 pmoles in 60 sec, which correspond to 120 contractions. Hence the loss is 4·8 pmoles/cm^2/contraction.

This figure is uncorrected for interfibre diffusion, and it is likely that the true value is greater than the apparent figure as calculated above. If a contraction is accompanied by a net release of potassium from all the fibres simultaneously, then there will be, first, a time lag before this potassium appears from the tissue, and secondly, some of the potassium will be reabsorbed into the cells from the interfibre spaces. If previous calculations are taken as a guide, the true figure may well be two or three times the apparent value. It is concluded that the results obtained by direct analysis of muscle are not inconsistent with the estimates made by means of tracers.

The loss of potassium following a single twitch is small in relation to the potassium content of the whole cell. After mixing has occurred following a contraction, the loss per unit area may be expressed as 10·7/48300 or about 0·02%. If the cell potassium is 158 m-moles/kg fibre water, the change in concentration is about 0·035 mM.

DISCUSSION

If gross deterioration of the preparation is to be avoided, considerable limitations on the stimulation procedure must be accepted in the case of isolated diaphragm preparations maintained at body temperature. The muscles are known to give a steady response for several hours providing the stimulation rate is low, say 6/min. Tetani have usually been given only for short periods of a few seconds (Hajdu, 1950; Ritchie, 1954). If stimulation is continued for

several minutes then even moderate rates produce a marked fall of twitch tension, as was found by Brown *et al.* (1948) who stimulated at 2/sec, and also by Weeks & Chenoweth (1952). A rise in the resting tension may also occur, and this appears to resemble the 'fatigue contracture' which has been considered by Gasser (1930).

This behaviour is easily explicable in terms of the diffusion of oxygen into the muscle. The critical depth b to which oxygen penetrates in a sheet of muscle may be obtained from the simple formula (Warburg, 1923; Hill, 1928):

$$b = \sqrt{(2Dy_0/a)}, \tag{2}$$

where D is the diffusion coefficient of oxygen in muscle and may be taken as $1{\cdot}64 \times 10^{-5}$ cm^2/min at 37° C (Krogh, 1919*a*), y_0 is the external oxygen tension in atmospheres, and a is the oxygen consumption of the muscle, which was found to be 0·027 c.c./c.c. resting muscle/min under somewhat similar conditions (Creese, Scholes & Whalen, 1958), the gas volumes being reduced to 0° C and one atmosphere, as usual. This value for oxygen consumption is somewhat higher than that usually reported for diaphragm muscle, and will be used in the present calculation. Since the gas mixture contained 95% oxygen, a may be taken as 0·95 (760–47)/760 or 0·89 atmosphere. Then b, the critical depth, is 0·0329 cm, so that on this basis a muscle of thickness $2b$ or about 660μ would be adequately oxygenated under resting conditions.

It appears from the results of Whalen (1957) that a diaphragm which is stimulated maximally at a rate of 2/sec shows an increased oxygen consumption of 85% at least (Fig. 2 of Whalen's paper). This is a minimum value as it does not allow for the effects of lag in the recording system. The calculated critical depth is then only 242μ, and may be smaller. Hence a muscle of thickness 600μ would be expected to have a slice over 100μ wide in the depth of the tissue devoid of oxygen following stimulation at 2/sec. It is, therefore, not surprising that after 20–30 min the cells in the centre are found to be depolarized when the muscles are explored by micro-electrodes. The localized depolarization produced by simple anoxia has been studied by Creese, Scholes & Whalen (1958).

Depolarization of a proportion of fibres during moderate rates of stimulation would be accompanied by some net loss of cellular potassium, and the loss of 15% of potassium content shown in Fig. 4 may be due partly to the secondary effects of central anoxia. The experiments of Calkins *et al.* (1954) have demonstrated the rapid loss of potassium which occurs when diaphragm muscle is made anoxic, and a similar effect was noticed by Creese (1954).

Once potassium has passed into the interspaces, then the raised extracellular potassium would itself serve to enhance the depolarization of the fibres unless it were removed quickly by intercellular diffusion to a large external reservoir of saline. The appropriate value for the diffusion coefficient is not

immediately apparent. Creese (1954) found that ^{42}K in the interstitial fluid exchanged with the external saline with a half-time of 0·81 min (after the first min), which gave the apparent diffusion coefficient D' as $5{\cdot}2 \times 10^{-6}$ cm^2/sec at 38° C. Similarly, Harris (1952) found the half-time for loss of extracellular potassium to be 1·2–1·5 min in the case of frog sartorius muscle at 18° C, from which Hill & Macpherson (1954) calculated D' to be about 4×10^{-6} cm^2/sec. McLennan (1955) performed experiments similar to those of Harris (1952) and found that the potassium contained in 0·1–0·15 of the volumes of leg muscles of the rat left the interspaces with a value for D' of $5{\cdot}6 \times 10^{-6}$ cm^2/sec at 20° C. Later experiments of a similar nature have been interpreted by McLennan (1956, 1957) as indicating much lower diffusion coefficients of potassium through the interspaces.

Csapo & Wilkie (1956) have pointed out that two different diffusion coefficients must be distinguished. First, there is the situation described by Harris (1952) in which the muscle is placed in saline containing a high concentration of potassium phosphate. The extracellular potassium is largely prevented from exchanging with that of the fibre, and this gives the apparent diffusion coefficient D' which is usually about 25–50% of that found in free solution. Net movement of potassium in the interspaces of a muscle which is placed in physiological saline will be described by an effective diffusion coefficient, which may be designated by D_e, such that D_e is smaller than D'. This is due to loss of potassium from the interspaces by exchange across the cell membrane, so that the amount transferred in the interspaces in unit time is diminished. Csapo & Wilkie (1956) show that D_e is given approximately by aD' where a is the fraction by volume of the interspaces. Now a for diaphragm is 0·28 (Creese, 1954) and this argument leads in the present experiments to a value of $1{\cdot}5 \times 10^{-6}$ cm^2/sec for the effective diffusion coefficient for net transport of potassium. Since Dt/b^2 is 0·196 where t is the half-time and b half the thickness (0·03 cm), it follows that the excess potassium in the interspaces would be reduced by half in about 2 min, provided that this excess potassium was evenly distributed initially. If the excess potassium was concentrated in a narrow strip 100 μ wide in the depths of the muscle, then its removal would be correspondingly delayed. From these considerations it is likely that some of the depolarization seen in Figs. 7 and 8 could be attributable to increased potassium in the interspaces. This argument seems unlikely to apply to the results of Creese, Scholes & Whalen (1958) who waited 3 hr (with changes of external saline) before records were taken.

The poor performance of isolated mammalian muscles to prolonged stimulation as compared with those with circulation intact has been recognized previously (e.g. Brown *et al.* 1948). In the case of isolated tissues the oxygen may have to diffuse perhaps hundreds of microns to the deepest fibre, whereas the distance between a capillary and the fibres which it supplies is much shorter

(Krogh, 1919*b*). The difference in the length of the diffusion path in the two cases seems a sufficient explanation. The presence of some fibres which have become depolarized may explain the increase in asynchrony after stimulation as described by Brown *et al.* (1948). Isolated muscles which have been shocked until their performance has been impaired are often said to be 'fatigued'. It is questionable whether it is at all helpful to apply the term 'muscular fatigue' to the tissue from which Fig. 8 was taken, in which a region of cellular damage in the centre of the diaphragm may be demonstrated. Perhaps the term 'fatigue' should be reserved for conditions in which it may be employed in its original context, for example, in the analysis of the behaviour of the adductor pollicis muscle in human subjects as described by Merton (1954).

The limitations discussed above necessitate the use of low rates of stimulation for brief periods. Under these conditions there was no detectable increase of potassium influx, while the rate of outward movement increased by some 23%. Noonan, Fenn & Haege (1941) also found no change in the rate of inward movement of labelled potassium in isolated frog muscles stimulated once per second for 1 hr. Keynes (1951) used 100 impulses/sec in the case of *Sepia* axons and found a small increase of inward movement of 0·39 pmoles/cm^2/impulse, accompanied by a larger increase in outward movement of 4·7 pmoles/cm^2/impulse. Hodgkin & Keynes (1955) have obtained evidence that the potassium movements in these two directions are not independent, and they have considered that '...the interaction between the influx and the efflux of potassium...makes it difficult for external ions to enter against a predominant stream coming in the opposite direction.' Such considerations may well apply also to mammalian muscle.

The interpretation of the quantitative results is complicated by large corrections which are necessary to allow for the effects of interfibre diffusion. Two independent methods of correcting the results have been published, one by Harris & Burn (1949), the other by Keynes (1954). It was at one time thought that the two methods gave different results, but it can be shown that the two formulas are algebraically very similar (Creese *et al.* 1956). In the case of resting diaphragm muscle the mean exchange rate across the cell membrane is likely to be some 2 or 3 times the exchange rate for the muscle as a whole, and this large correction introduces considerable uncertainty into the estimates of the resting potassium flux and also into the net potassium loss per contraction.

The estimated net potassium loss accompanying a twitch is 11 pmoles per unit area, and this value is somewhat larger than that obtained in the case of the action potential of crustacean nerve axons (Keynes, 1951), though of the same order of magnitude. In muscle the impulse appears to travel not only along the surface of the fibre but also radially along the *Z* membrane or other transverse structure to excite the contractile elements (Huxley, 1957) and it is possible that this longer path may contribute to the comparatively large

net change of potassium. Since the net loss of potassium is of the same order of magnitude per unit area as that found for certain nerve axons, it is possible that simple conduction of the electric disturbance is sufficient to account for this ionic change, without invoking the contractile process as a possible cause of potassium loss. The actual fall in concentration of potassium when averaged for the whole fibre is very small, of the order of 0·035 mM for a single twitch.

SUMMARY

1. Isolated rat diaphagm when stimulated at 2/sec showed an increase of 23% in the rate of outward movement of ^{42}K, this being measured in the saline coming from the muscle during the first few minutes of stimulation. No change in inward movement of ^{42}K was detected during stimulation.

2. The calculated net outward movement of potassium at 38° C was 11 pmoles/cm^2/contraction. This estimation is dependent on large corrections for the effect of interfibre diffusion.

3. During stimulation at 2/sec the extra-fibre space and the apparent diffusion coefficient of inulin were similar to values obtained for resting muscle.

4. Continued stimulation at 2/sec produced a fall in twitch tension, loss of potassium, contracture, and depolarization of fibres in the depths of the muscle, as shown by intracellular electrodes. These findings resembled the effects of anoxia.

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