J. Physiol. (1968), **197**, *pp.* 279–294
With 7 text-figures
Printed in Great Britain

SODIUM MOVEMENTS IN DENERVATED MUSCLE AND THE EFFECTS OF ANTIMYCIN A

BY R. CREESE, A. L. EL-SHAFIE AND GERTA VRBOVÁ*
From the Departments of Physiology, St Mary's Hospital Medical School, London, W. 2, and King's College, London, W.C. 2

(*Received* 3 *November* 1967)

SUMMARY

1. Fibre sodium in rat diaphragm exchanged at a faster rate in muscles which had been denervated 8 days previously. The half-time was 3·7 min for outward movement in denervated muscles and 5·1 min in controls.

2. The inward movement of sodium was also faster in denervated muscle as compared with controls, and the calculated permeability to sodium was approximately doubled.

3. In the presence of antimycin A ($1{\cdot}8 \times 10^{-5}$ M), the outward movement of sodium in denervated diaphragm was slowed and the fibres gained sodium and lost potassium.

4. Antimycin markedly reduced the twitch produced by stimulation of denervated diaphragm, and also the response to acetylcholine. Similar effects were found in denervated tibialis anterior of the cat.

INTRODUCTION

It is well known that the characteristics of the membrane of skeletal muscle fibres change after denervation. Potassium exchange is slowed (Nicholls, 1956; Klaus, Lüllmann & Muscholl, 1960), and the resistance of the membrane is correspondingly increased (Nicholls, 1956; Thesleff, 1963). Although there is no change in potassium content, the resting potential in mammalian muscle is markedly diminished (Lüllmann, 1958; Thesleff, 1963). It is possible that this fall in resting potential of denervated muscle is related to an increase in permeability to sodium ions, and methods have been devised in the present study to compare sodium exchange in normal and denervated muscle.

Sodium movements in frog muscle are affected by a number of metabolic inhibitors (Kernan, 1963). Profound alterations of biochemical processes

* Present address: Department of Physiology, The Medical School, Birmingham 15.

are also known to occur after denervation (Gutmann, 1962). The metabolic inhibitor antimycin A decreases the outward movements of sodium in *Maia* muscle (Bittar, 1966), and some of the effects of this substance on the function of normal muscle have been described by Vrbová (1963). In the present experiments the effects of antimycin A have been examined in denervated muscle of the rat and cat, and a preliminary report has been given (Creese, El-Shafie & Vrbová, 1965).

METHODS

Physiological saline. The composition was that used by Creese & Northover (1961). The temperature was 38° C.

Denervation. Rats weighing 45–55 g were anaesthetized with ether and the left phrenic nerve was avulsed in the neck. The diaphragms were used 6–14 days later, and no twitch was obtained in these muscles when the nerve trunk was cut.

Cats were anaesthetized by pentobarbitone sodium (30 mg/kg intraperitoneally) and the sciatic nerve was sectioned on one side with aseptic precautions. The animal was kept for 14–20 days before the experiment.

Sodium movements in rat diaphragm. The method of Creese (1968) was used. The left diaphragm with its ribs was rapidly removed, attached to a holder and immersed in saline containing ^{24}Na. After 1 or 2 hr the muscle was passed through tubes of inactive saline at 38° C. The washout was stopped after various times and the radioactivity which remained in the muscle was measured. The radioactivity of the original saline was also found, so that the radioactive sodium which remained could be expressed as μmole/g muscle.

For studies of influx the muscles were transferred for 7 min to saline containing ^{24}Na and then passed through tubes of inactive saline for various times. Several muscles were used for each series of experiments and by retropolation an estimate was found of the sodium which had entered in 7 min.

The design of the experiments, the methods of analysis and the corrections which were applied to the results are described in the text.

Analysis of muscles. Sodium, potassium and water content of muscles were measured as described previously (Creese, 1954).

Muscle contraction. In the case of denervated diaphragm the muscle was attached to a holder, set up in saline and stimulated directly by means of steel wires at each end of the strip of diaphragm. Single supramaximal shocks were applied every 10 sec and the contractions were recorded by a strain gauge (Statham) and photographed from the screen of an oscilloscope. Contractures were also produced in denervated muscle by the addition of acetylcholine (5 μg/ml.) to the bath, and recorded by strain gauge.

Cats were anaesthetized by chloralose (70 mg/kg intravenously). The tibia was secured by drills at both ends, and the tibialis anterior muscle was stimulated through the drill nearest the knee and a steel wire which was attached to the tendon. Single twitches were recorded from the denervated muscle by means of a strain gauge and oscilloscope.

Arterial injection in cats. A branch of the artery of the tibialis anterior muscle was cannulated. Acetylcholine was injected every 10 min, and a dose was selected which produced a peak force as large as that given by a single maximal twitch. The response to such a dose was tested before and after injection of antimycin through the artery.

Fibre diameter. Diaphragm muscles were rapidly removed and frozen with solid carbon dioxide. Frozen sections (5–7 μ thick) were cut with a refrigerated microtome at $-20°$ C. The sections were immediately photographed in saline, and the negatives were projected on to a screen with a total magnification of 2000 times. Two diameters at right angles were

measured for each fibre and the mean was recorded. Only fibres were selected which were circular in cross-section. In most cases fifty cells were measured from each muscle.

Drugs. The following drugs were used: chloralose (Roche Products), pentobarbitone sodium (Abbott), acetylcholine chloride (Roche Products), antimycin A (Wisconsin Alumni Research Foundation). The powder was dissolved in ethanol (1 mg/ml.), stored at 0° C and used within 4 weeks. The molecular weight was taken as 548.

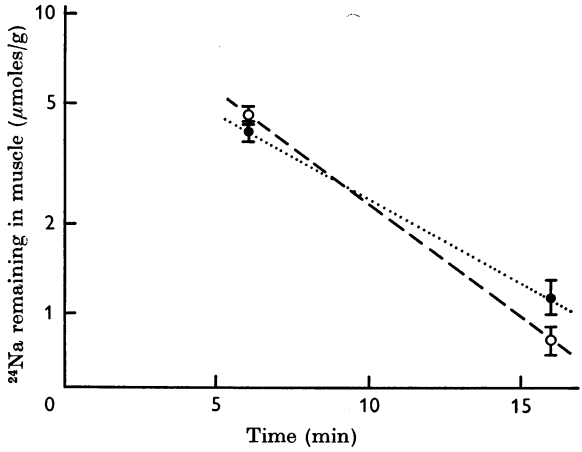

Fig. 1. Outward movement of ^{24}Na. Filled circles show the labelled sodium which remains, plotted on a semi-logarithmic scale. The early part of the curve was not recorded. Each point is the mean of 8 muscles, and the limits are the S.E. Open circles are from diaphragms which had been denervated 8 days previously, and these muscles show a more rapid rate of exchange.

RESULTS

Sodium efflux in denervated diaphragm. Sodium exchange was measured in diaphragm muscles of rats which had been denervated 8 days previously, and in control muscles. The left hemi-diaphragm was immersed in saline containing ^{24}Na at 38 °C, and after 1 hr the tissue with its holder was passed through tubes containing inactive saline for 6 min or for 16 min and the radioactivity which remained in the muscle was measured and expressed as counts min^{-1}. g^{-1}. The specific activity at the start of the washout is equal to that of the saline (Creese, 1968), and hence the radioactive sodium which remained after the washout could be converted to μmole/g wet muscle. Results similar to these of Creese (1968, fig. 2) showed that after 5 min the fast fraction of sodium which corresponded to the extracellular sodium had been removed and the remainder of the ^{24}Na was identified as fibre sodium.

The experiment was designed as a symmetrical 4-point assay. A group of four immature rats was selected from the same batch, each of approximately 50 g, and in two of these the left phrenic nerve was sectioned.

Eight days later the rats weighed 70–90 g, and the four left diaphragms were treated with ^{24}Na as described above. The results obtained from four muscles gave an estimate of the sodium which remained after washouts of 6 and 16 min in the case of normal and denervated muscle.

TABLE 1. Outward movement of labelled sodium in diaphragm of rat (70–90 g)

	Rate constant (min^{-1})	Apparent fibre Na (μmoles/g)	Corrected fibre Na (μmoles/ml.)	Corrected rate constant (min^{-1})
Normal	0·128 ± 0·0156 (16)	8·75	6·22	0·137
Denervated	*0·171 ± 0·0129 (16)	12·9	7·71	0·186
Normal (7 min uptake)	0·117 ± 0·0066 (12)	4·32	3·07	
Denervated (7 min uptake)	*0·184 ± 0·0047 (12)	7·58	4·54	
Denervated	0·163 ± 0·0097 (18)	9·8	6·1	0·175
Denervated + antimycin	*0·109 ± 0·0189 (18)	11·7	8·7	0·117

The first column gives the measured rate of exchange, obtained from the regression, with standard deviation and the number of muscles. Values marked with an asterisk differ significantly from controls ($P < 0·01$, see text). The apparent fibre sodium was obtained by retropolation to zero time. The last two columns give corrected values for fibre sodium (in μmole/ml. muscle) and for the rate constant.

Figure 1 shows the results of thirty-two diaphragms, and each point represents the mean of eight muscles. The points have been plotted on semi-logarithmic paper, for it is known that the radioactivity declines exponentially after the first 5 min (Creese, 1954, 1968). The lines connecting the points give the mean slope. It can be seen that the slope of the denervated muscles (open circles) is steeper than that obtained from control muscles (filled circles), and that the two lines cross.

The thirty-two results were converted into logarithms and analysed by contrast methods (Finney, 1952). In Fig. 1 the slopes have a divergence of 1·517 $\log_{10}$ units, S.E. ± 0·529 unit. This degree of divergence is unlikely to be fortuitous ($P < 0·01$ by t test). Table 1 gives the mean slope for controls and for denervated muscle, expressed as rate constants (Creese, 1954). For controls the rate of exchange was 0·128 min^{-1} (half-time 5·4 min), while the sodium of denervated muscles exchanged more rapidly with a rate constant of 0·171 min^{-1} (half-time 4·1 min). Table 1 also shows the results obtained by retropolation back to zero time, and the apparent fibre sodium at the start of the washout was 8·7 μmole-g^{-1} for controls and 12·9 μmole-g^{-1} for denervated muscle. It is shown below that correction factors need to be applied to obtain values of the true fibre sodium.

Sodium, potassium and water content. Table 2 shows the total sodium, potassium and water content of control and denervated diaphragms *in vivo* and *in vitro*. The water content of muscles which had been denervated 8 days previously was significantly increased in both series. Denervation produced no significant change in the potassium content *in vivo*, and there was some fall in potassium content *in vitro* in all muscles. The sodium

of denervated musle *in vivo* when expressed as μmole. g^{-1} showed little change as compared with controls. All muscles gained sodium *in vitro* and the mean increase in the case of denervated muscles was somewhat greater than the controls. These results are similar to those obtained by Lüllmann (1958) except that he found that the total sodium of denervated muscle was greater than the controls.

TABLE 2. Sodium, potassium and water content of normal and denervated diaphragm (8 days) from rats of 70–90 g

		Na (μmoles/g)	K (μmoles/g)	H_2O (mg/g)
In vivo	Normal	35·7 ± 2·6 (12)	96·4 ± 3·6 (12)	777 ± 6·0 (12)
	Denervated	35·4 ± 1·2 (12)	97·0 ± 2·0 (12)	*795 ± 2·8 (12)
In vitro	Normal	47·8 ± 4·4 (15)	85·7 ± 6·5 (20)	777 ± 5·6 (10)
	Denervated	55·3 ± 5·9 (12)	85·4 ± 6·5 (20)	*795 ± 5·7 (10)

Mean values are given, with S.D. and the number of muscles. Values marked with an asterisk differ significantly from controls ($P < 0{\cdot}01$).

TABLE 3. Correction factors for fibre sodium and exchange rate

	β/α	Total sodium (μmoles/ml. muscle)	$B/(T-B)$	Correction factor for fibre Na	Correction factor for rate constant	Extracellular Na (μmoles/ml.)
Normal	0·185	51·1	0·224	1·507	0·966	44·9
Denervated	0·247	58·3	0·304	1·764	0·950	50·6
Denervated	0·235	—	0·227	1·707	0·963	—
Denervated + antimycin	0·155	—	0·262	1·414	0·967	—

The table gives values which were used in calculations of correction factors.

Corrected values of fibre sodium and rate constant. The estimate of the fibre sodium obtained by retropolation in Fig. 1 requires correction (Huxley, 1960). The true fibre sodium is apparent value/factor (Creese, 1968) and in this case

$$\text{factor} = 1+2\frac{\beta}{\alpha}+\left(\frac{\beta}{\alpha}\right)^2\left(3+\frac{B}{T-B}\right)+\left(\frac{\beta}{\alpha}\right)^3\left(4+2\frac{B}{T-B}\right)+\ldots, \qquad (1)$$

where α, β are the apparent rate constants of the fast and slow fractions, B is the apparent fibre sodium obtained by retropolation and T is the total sodium, expressed as μmole/g muscle.

For rats of 70–90 g the initial exchange rate in four cases was very similar to the results obtained by Creese (1954), and the half-time of the fast fraction has been taken as 1 min so that α is 0·693 min^{-1}. Table 3 shows the correction factors, and it can be seen that the corrections are large and are over 1·5 and 1·7 in the case of normal and denervated diaphragms from rats of this size. The corrected fibre sodium was found, in μmole/g. In practice the fibre sodium is required in μmole/ml. muscle, and this has been obtained by multiplying by the specific gravity which was taken as 1·07 for normal diaphragm and 1·055 for denervated muscle (Klaus *et al.* 1960). The corrected fibre sodium has been listed in Table 1.

There are also two small corrections to be applied to the rate constants shown in Table 1. During the washout the muscles were in air for approximately 2 sec during each min, and the measured exchange rate has been multiplied by 1·034. There is also a correction for the effect of the fast fraction on the exchange of the slow fraction. If β is the measured rate constant then the true rate constant k is β/factor where

$$\text{factor} \simeq 1 - \frac{\beta}{\alpha} \frac{B}{T-B}. \tag{2}$$

In practice the exact formula for k was employed (equation 32, Creese, 1968). In Table 3 this factor has been listed, and Table 1 gives the corrected values. The control diaphragms had a mean rate constant of 0·137 min^{-1} (half-time 5·1 min), while denervated muscles had a rate constant of 0·186 min^{-1} (half-time 3·7 min).

Fibre diameter. Frozen sections were prepared from the left diaphragm of rats of 70–90 g. In four of the animals the left phrenic nerve had been avulsed 8 days previously and four rats acted as controls. The diameter of fifty fibres was measured from each muscle except in one of the controls, in which twenty-five fibres were measured. For the controls the mean fibre diameter was $40{\cdot}0\mu \pm 4{\cdot}8$ (S.D. of 175), and in denervated muscle the diameter was $41{\cdot}6\mu \pm 7{\cdot}1$ (S.D. of 200). The difference of 4 % is not statistically significant. Klaus *et al.* (1960) measured the ratio of volume to area (which is related to fibre diameter) in normal and denervated diaphragms and found the value after denervation to be 5 % greater than the controls.

Calculation of sodium fluxes and permeability coefficients. These measurements enable the outward sodium fluxes to be calculated. The flux in mole.$cm^{-2}sec^{-1}$ (Keynes & Lewis, 1951) is

$$\text{efflux} = k(V/A)\text{Na}_i, \tag{3}$$

where k is the rate constant for outward movement (sec^{-1}) and is termed k_2 by Harris & Burn (1949), V/A is the ratio of volume to area (cm), and Na_i is the internal concentration of sodium in mole/ml. myoplasm.

The total sodium in normal and denervated muscle *in vitro* is given in Table 2 as μmole/g muscle. The value in μmole/ml. muscle is shown in Table 3, and subtraction of the fibre sodium gives the extracellular sodium. If the sodium content of extracellular fluid is taken as 145 μmole/ml. then it is possible to obtain the extracellular space in ml./ml., and this is given in Table 4. The fraction by volume is 0·31 for normal muscle, and this is similar to values in younger rats (Creese, 1968). The space for denervated muscle is somewhat larger than in normal muscle, as found by Lüllman (1958). If the extracellular space is known then the remaining volume may be taken as myoplasm. From the fibre sodium shown in Table 1, the internal concentration was calculated as μmole/ml. myoplasm (Table 4).

Table 4 gives the values required for the calculation of sodium fluxes. The term V/A for a cylinder has been taken as one quarter of the mean diameter of the fibres. The rate constants are from Table 1. It can be seen that the flux for denervated muscle is approximately twice that for normal fibres.

There is evidence that the diaphragms are in a steady state when studied by the methods used above (Creese, 1968). In this case the calculated efflux may also be used as a measure of the influx. If the constant field equation can be applied to the inward movement of sodium then the

TABLE 4. Sodium fluxes, permeability coefficients and fibre sodium

	Extracellular space (ml./ml.)	Fibre sodium (μmoles/g fibre water)	Fibre sodium (μmoles/ml. myoplasm)	Volume/area (μ)	Rate constant ($sec^{-1} \times 10^{-3}$)	Sodium flux (p-mole $cm^{-2}.sec^{-1}$)	Permeability coefficient ($cm.sec^{-1} \times 10^{-6}$)
Normal	0·310	12	9·0	10	2·28	20·5	0·042
Denervated	0·349	15	11·8	10·4	3·2	39·3	0·087

influx, the permeability coefficient P and the resting potential E are related by the expression (Keynes, 1951):

$$\text{influx} = P\frac{EF}{RT}\frac{\text{Na}_o}{1-\exp(-EF/RT)}, \tag{4}$$

where RT/F is 26·8 mV at 38 °C. Thesleff (1963) found that the resting potential of normal diaphragm muscle shortly after immersion in saline containing 5 mM potassium was 87 mV. In the present experiments the external sodium was 145×10^{-6} mole/ml., and with this value the permeability coefficient of normal diaphragm is $0{\cdot}042 \times 10^{-6}$ cm/sec. If the resting potential of diaphragm muscle is taken as 77·7 mV (Kernan, 1963) then P is $0{\cdot}046 \times 10^{-6}$ cm/sec. In denervated muscle the permeability coefficient can only be calculated if the fall in resting potential is known, but since the flux of sodium is almost doubled (Table 4) it is likely that the permeability coefficient is at least twice that of normal muscle. In Table 4 the permeability for sodium in denervated muscle has been calculated on the assumption that the resting potential is reduced by 9 mV in 7–9 days (see Lüllmann, 1958).

The ratio of sodium and potassium permeability of normal muscle *in vitro* is needed for some purposes and can be obtained as follows:

The expression for the influx, analogous with equation (3), is

$$\text{influx} = k_1 (V/A) S_o, \tag{5}$$

where k_1 is the rate constant for inward movement and S_0 is the external concentration of an ion in mole/ml. It follows from (4) and (5) that P the permeability coefficient is proportional to k_1. Now if the fluxes can be considered as proportional to concentration then

$$\frac{k_1}{k} = \frac{S_i}{S_o} \tag{6}$$

(Harris & Burn, 1949), where k is the rate constant for outward movement and S_i, S_o are

the internal and external concentrations of the ion. Therefore P is proportional to $k(S_i/S_o)$, and hence for any one muscle

$$\frac{P_{Na}}{P_K} = \frac{k_{Na}(Na_i/Na_o)}{k_K(K_i/K_o)} \quad (7)$$

(see also Keynes, 1951).

In the case of sodium in normal muscle, k_{Na} is 0·137 min^{-1} (Table 1) and Na_o is 145 μmoles/ml. The water content (Table 2) is 0·770 g/g muscle, and if the specific gravity is 1·07 the water content is 0·824 ml./ml. muscle. The extracellular water is 0·310 ml./ml. muscle (Table 4), and hence the fibre water is 0·514 ml./ml. muscle. The fibre sodium is 6·22 μmoles/ml. muscle (Table 1), and hence the fibre sodium is 12 μmoles/g fibre water, and this has

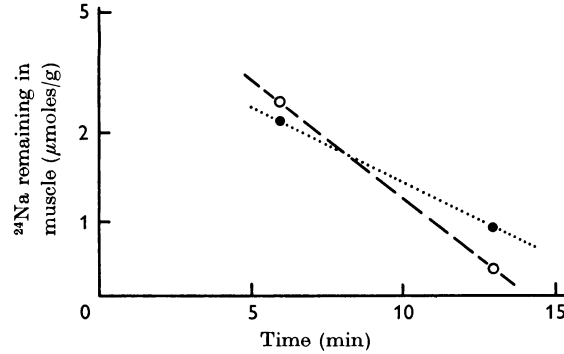

Fig. 2. Experiment on inward movement of sodium. Filled circles show ^{24}Na which remains after 7 min in saline containing ^{24}Na, and washout in inactive saline. Each point is the mean of six muscles. Open circles show results from denervated muscles. Retropolation to zero time gives an estimate of the labelled sodium which entered in 7 min.

been listed in Table 4 together with the values obtained in denervated muscle. The results shown in Table 4 show a small increase in fibre sodium in denervated muscle, though these differences are not statistically significant. Drahota (1962) detected no change in fibre sodium in denervated rat muscle.

In diaphragms from rats of 120 g the potassium exchanges with an apparent rate constant of 1·17 hr^{-1} (Creese, Neil & Stephenson, 1956). A large correction for diffusion is necessary and the corrected rate is 1·17/0·35 which comes to 0·0557 min^{-1}. K_o is 5 μmoles/ml. The extracellular space is 0·310 ml./ml. muscle and contains 1·5 μmoles/ml. muscle. The total potassium *in vitro* (Table 2) is 85·7 μmoles/g or 91·7 μmoles/ml. muscle. The fibre potassium is then 90·2 μmoles in 0·514 ml. fibre water, or 175 μmoles/g. fibre water.

When these terms are inserted into equation (7) the factor P_{Na}/P_K comes to 0·006 for diaphragm muscle *in vitro*.

Inward movement of sodium. An increased rate of inward movement was also found in denervated muscle. Figure 2 shows results obtained in normal muscles and in muscles which had been denervated 9 days previously. The muscles were equilibrated for 1 hr in physiological saline, transferred for 7 min to saline with ^{24}Na, and then passed through tubes containing inactive saline for 6 min or for 13 min. Jenkinson & Nicholls (1961) have used a similar method for diaphragm muscles.

The experiment was designed as before as a 4-point balanced assay, and

each point in Fig. 2 represents the mean of six muscles. The control diaphragms are shown as filled circles, and on retropolation to zero time an estimate can be obtained of the sodium which entered the fibres during 7 min.

Table 1 gives the rate constants for the outward movement shown in Fig. 2, and also the apparent fibre sodium obtained by retropolation. The open circles in Fig. 2 give the values obtained for denervated diaphragms, and in these muscles the slope is steeper and the value on retropolation is greater. Analysis by contrast methods (Finney, 1952) gave a divergence of 1·340 $\log_{10}$ units, with a standard error of 0·309 unit, and the degree of divergence is unlikely to be fortuitous ($P < 0{\cdot}01$ by t-test). The same correction factors have been used as for the results in Fig. 1, and the corrected values of fibre sodium in μmoles/ml. are given in Table 1. In 7 min the uptake of normal muscle was 3·07 μmoles/ml. and the total fibre sodium was 6·22 μmoles/ml., so the fractional uptake was 0·494. For denervated muscle the fraction exchanged in 7 min was 4·54/7·71 or 0·589. The fraction f which has exchanged may be expressed as $1-\exp(-k't)$, where k' is the apparent rate constant for inward movement into the fibre. The term k' for normal muscle is 0·0972 min^{-1}, while for denervated muscle the value comes to 0·127 min^{-1}.

Sodium appears to enter denervated fibres at a rate which is 0·127/0·0972 or 1·31 times faster than controls. From Table 1 the uncorrected rate constants for outward movement are 0·171 and 0·128 min^{-1}, so the ratio is 1·34. It is not practicable to measure absolute rates of inward movement in diaphragm muscle, but the results in Fig. 2 are consistent with a more rapid entry of sodium in denervated muscle, and the ratio of rate constants for inward movement is similar to that obtained for outward movement.

Effect of antimycin on sodium movements and potassium content of denervated muscle. Creese *et al.* (1965) were unable to show an effect of antimcyin on the exchange of sodium in normal diaphragm, though denervated muscle appeared to be affected. Figure 3 shows the effect of antimycin (10^{-5} g/ml.) on denervated muscle. The experiment was performed on groups of six rats whose left phrenic nerves had been avulsed 9 days previously, and the design was similar to that described by Bliss (1952) for a 6-point factorial assay. The rats weighed 70–90 g and the muscles were soaked for 2 hr in saline containing ^{24}Na and then passed through inactive saline for 5, 10 or for 15 min. The fibre sodium which remained was measured and plotted on semi-logarithmic paper. Each point in Fig. 3 represents the mean of six diaphragms. The open circles show results from denervated muscles (without antimycin), and the other points are from denervated muscles which had been soaked for 2 hr in saline containing ^{24}Na plus antimycin (10^{-5} g/ml.). The solutions were changed twice during this soak in radioactive saline, and the muscles were subsequently passed through tubes of inactive saline. It can be seen that the points in Fig. 3 appear to fall on linear regressions and that the out-

ward movement is slowed in muscles which had been treated with antimycin.

The results are listed in Table 1. The thirty-six values of fibre sodium were converted to logarithmic units and an analysis of variance was carried out (Bliss, 1952), and the error variance was found to be 0·00702 $\log_{10}$ units. By factorial analysis the variance due to deviations from parallelism was 0·0806 $\log_{10}$ units, and by the ratio test this is significant ($P < 0{\cdot}01$). This indicates that the difference in the slopes of Fig. 3 is unlikely to be fortuitous. The variance due to combined curvature is small (0·0113 $\log_{10}$ units) so that the deviations from linearity are within the experimental error. This gives some support for the use of linear regressions as applied to the semi-logarithmic plot of fibre sodium against time in experiments such as that of Fig. 3.

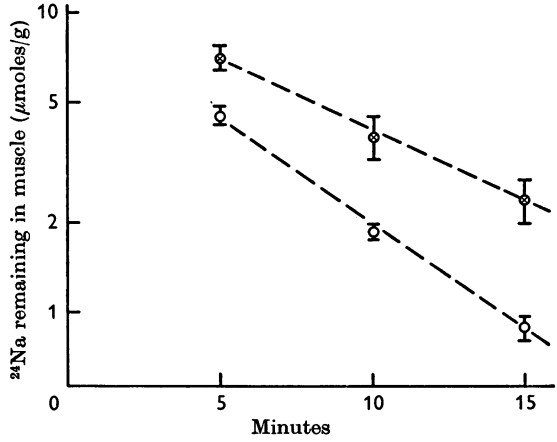

Fig. 3. Effect of antimycin ($1{\cdot}8 \times 10^{-5}$ M) on outward movement of sodium in denervated muscle. Each point is the mean of six, and the limits give the S.E. Open circles show denervated muscle, and circles with crosses show diaphragms which have been treated with antimycin, and in these muscles the exchange is slowed.

Table 1 also gives the apparent fibre sodium obtained by retropolation of the lines in Fig. 3 back to zero time. These values require correction by equation (1), and Table 3 gives the correction factors. The total sodium was not measured and the correction factor was obtained by iteration as follows. The term $(T-B)$ in equation (1) is given by the expression [extracellular sodium $-(B-P_{30})$], where B is the apparent fibre sodium and P_{30} is the corrected fibre sodium. The nomenclature of Huxley (1960) has been retained whenever possible. In practice the extracellular sodium of denervated muscle was taken as 50·6 μmoles/ml. (see Table 3) or 48·0 μmoles/g. A preliminary value for the correction factor was obtained by putting 48·0 in place of $(T-B)$ in equation (1). This give an initial estimate of P_{30}, and with this a better value of $(T-B)$ was found so that a second estimate of the correction factor was obtained. Further iteration was not necessary. Table 3 gives the values for the correction factors for both the fibre sodium and for the rate constants.

From Table 1 the exchange rates for denervated muscle in all three series of experiments are similar, the means being 0·171, 0·184 and 0·163 min^{-1}. Muscles treated with antimycin contained a somewhat higher

fibre sodium than the corresponding controls, and the outward exchange of sodium was slowed.

The potassium content of the denervated muscles which were used as controls was also measured and was found to be 89·5 μmoles/g $\pm$ 10·5 (S.D. of 18). In the case of denervated muscles treated with antimycin the value was 68·5 μmoles/g $\pm$ 16·2 (S.D. of 18, $P < 0{\cdot}01$), so a large loss of potassium was also produced.

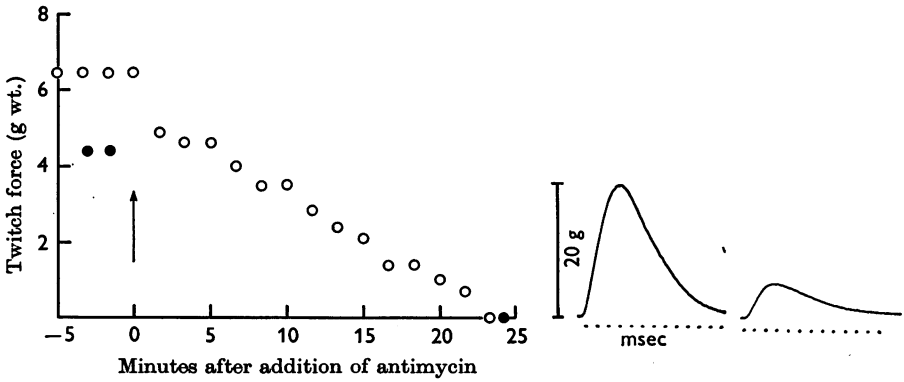

Fig. 4. Effect of antimycin on contraction of denervated diaphragm elicited by direct stimulation and recorded by strain gauge (open circles). At arrow, antimycin was added to the bath ($0{\cdot}7 \times 10^{-5}$ M). Filled circles show responses produced by acetylcholine (5 μg/ml.).

Fig. 5. Similar experiment to that of Fig. 4. Left side shows single twitch of denervated diaphragm. Right side shows myogram 20 min after addition of antimycin ($1{\cdot}8 \times 10^{-5}$ M). Time marks show msec.

Effect of antimycin on contractions and contractures of denervated diaphragm. Anticymin A has been found to produce neuromuscular block in rat diaphragm and in tibialis anterior muscle. It had, however, no effect on contractions elicited by direct stimulation of normal muscles (Vrbová, 1963). In the present experiments the effect of antimycin A was tested on denervated muscle.

Six hemidiaphragms which had been denervated 7 or 8 days previously were used. Contractions produced by electrical stimulation, and also the responses obtained when acetylcholine was added to the bath were reduced or abolished by antimycin A (Fig. 4). This effect could be obtained by concentrations of antimycin A as small as $0{\cdot}7 \times 10^{-5}$ M. Figure 5 shows another experiment in which the myogram was recorded before and after antimycin (10^{-5} g/ml., or $1{\cdot}8 \times 10^{-5}$ M).

Effect of antimycin on denervated muscle of the cat. Similar results were obtained with tibialis anterior muscle of the cat which had been dener-

vated 14 or 21 days previously. Contractions were elicited by electrical stimulation, and responses were also obtained by intra-arterial injection of acetylcholine which was administered at intervals of 10 min so that desensitization was avoided.

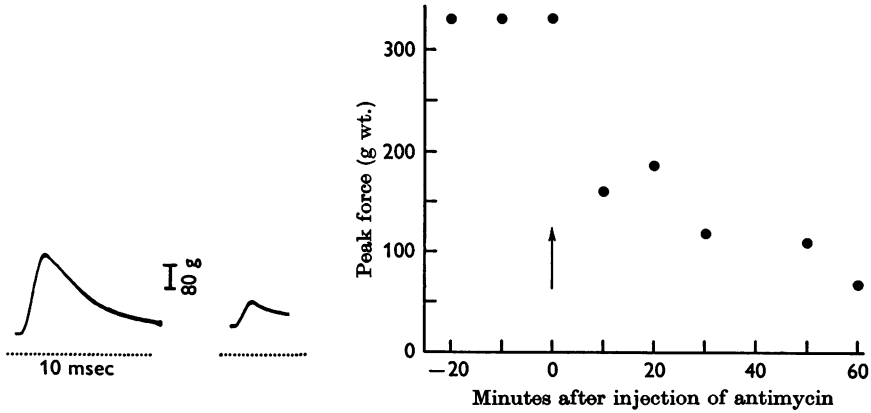

Fig. 6. Left side shows twitch of denervated tibialis anterior muscle of cat, recorded by strain gauge. The muscle had been denervated 14 days previously. Right-hand side shows myogram 20 min after intra-arterial injection of antimycin (0·2 mg). Time marker shows 10 msec.

Fig. 7. Responses produced by intra-arterial injections of acetylcholine (2·5 μg) into tibialis anterior muscle of cat which had been denervated 20 days previously. At the arrow antimycin (0·2 mg) was injected into the artery.

Intra-arterial injection of antimycin A (0·2 mg) produced a progressive fall in contractions elicited by electrical stimulation (Fig. 6). The responses to intra-arterial injections of acetylcholine were also reduced (Fig. 7).

DISCUSSION

It is possible to maintain the sodium and potassium content of thin diaphragm muscle at constant values during measurements of ion fluxes (Creese, 1954, 1968). Lüllmann (1958) found a steady fall in potassium and a rise in sodium in normal and denervated diaphragm *in vitro*, although the resting potential of superficial fibres remained constant for 3 hr. However Lüllmann (1958) used diaphragms from rats which weighed up to 280 g, and the calculations of Creese, Scholes & Whalen (1958) raise doubts regarding the oxygenation of such thick muscles. If the depths of the tissue become hypoxic this would account for the progressive deterioration in the ion content of the muscle, although the superficial fibres would be expected to maintain their characteristics for long periods.

The results of the present investigation show that exchange of sodium is

more rapid in denervated diaphragm, and the calculated permeability coefficient is approximately doubled. There remains the question whether the measured changes in ion fluxes are sufficient to account for the fall in the resting potential. The effect of sodium ions on the resting potential at 38 °C can be represented as follows (see Hodgkin, 1958):

$$E = 62 \log_{10} \frac{K_i + bNa_i}{K_o + bNa_o}, \quad (8)$$

where the terms with subscripts indicate concentrations in moles/g water on the assumption that the internal and external activities are similar; E is in mV and b is P_{Na}/P_K, the ratio of sodium and potassium permeability.

The term b can be obtained from equation (7) and comes to 0·006 for normal muscle *in vitro*. K_o and Na_o are 5 and 145 μmoles/ml. respectively, and it has been shown above that K_i and Na_i are 175 and 12 μmoles/g fibre water. The calculated resting potential from equation (8) is then 91 mV. In denervated muscle P_{Na} is doubled while P_K is one-half or one-third, measured at room temperature (Klaus *et al.* 1960). The term b in denervated muscle is therefore increased by a factor which is at least four. If b is put as 0·024 and Na_i at 15 μmole/ml. fibre water (see Table 4), then E is 82 mV and the calculated drop in resting potential is 9mV in diaphragms which have been denervated for 7–9 days. According to Lüllmann (1958) the resting potential is 88 mV in normal diaphragms *in vitro* and 79 mV in muscles which had been denervated 9 days previously, so that the measured fall in resting potential is 9 mV. Lüllmann (1958) used Tyrode solution having a potassium content of 2·7 mM. Thesleff (1963) also found a change in the resting potential in denervated diaphragm from 87 to 72 mV when measured in a solution containing 5 mM potassium. This indicates a fall of 15 mV in 12–14 days, and from other results obtained by Thesleff (1963) it appears that the chloride ion should also be included in equation (8). In spite of several uncertainties it can be concluded that the measured fluxes are probably sufficient to account for the observed fall in resting potential. No account has been taken of the effects of exchange diffusion (Keynes & Swan, 1959) which might modify the calculations in the case both of denervation and of antimycin. The formula for the permeability (equation (4)) is used with the reservations expressed previously (Creese, 1968) to indicate the relative nature of the changes which are produced.

Denervated muscles show fibrillation and such activity might affect sodium exchange. However, it is known that fibrillation soon ceases *in vitro* (Thesleff, 1963; Belmar & Eyzaguirre, 1966) and in the present experiments the muscles were soaked for at least 1 hr before measurements were made. In denervated muscle a higher proportion of the resting ex-

change is contributed by sodium ions. In heart muscle Noble (1962) has shown that certain combinations of ion permeability can produce rhythmic oscillatory discharges in excitable tissues. Comparable calculations are not available for skeletal muscle, but the changes in permeability which occur in denervation presumably contribute to the instability of the membrane and the occurrence of fibrillation *in vivo*.

Many biochemical changes are known to occur in denervated muscles (see Gutmann, 1962). An initial hypertrophy and an increase in protein content has been shown in diaphragm muscles (Stewart, 1955). There is evidence for increased oxygen consumption in denervated muscle *in vivo* and *in vitro* (Bass, 1962). A change of permeability following denervation is suggested by the increased uptake of labelled decamethonium which has been found in denervated guinea-pig diaphragm (Taylor, Creese, Nedergaard & Case, 1965). The present experiments show an increased permeability to sodium ions with only a small increase in fibre sodium, and the more rapid extrusion of sodium ions may represent the response of the tissue to the increase in permeability.

Antimycin A has been found to inhibit the cytochrome chain in a number of tissues (see Slater, 1958). Greengard & Straub (1962) showed that this compound prevented the hyperpolarization which normally followed repetitive stimulation of mammalian unmyelinated nerves and they suggested that antimycin inhibits the electrogenic extrusion of sodium. In the present experiments antimycin reduced the outward movement of sodium in denervated muscle, and reduced the contraction to zero in concentrations which had no detectable effect in normal diaphragm. In normal muscle the twitch produced by direct stimulation was not changed, although the neuromuscular junction appeared to be affected (Vrbová, 1963). After denervation the whole surface of the muscle fibre acquires some characteristics which are similar to those of the end-plate region of normal muscle (Miledi, 1962). It is possible that sodium permeability is higher at sites which are chemo-sensitive, and that at such sites the extrusion is more vulnerable to interference with oxidative metabolism. An alternative explanation is that antimycin penetrates more readily at sites which are chemosensitive.

This work was supported by grants from the Medical Research Council to R.C. and G.V. The authors also wish to thank the Board of Governors of St Mary's Hospital for providing technical assistance, and Mrs R. Jones, who prepared the sections of muscle.

REFERENCES

BASS, A. (1962). Energy metabolism in denervated muscle. In *The Denervated Muscle*, ed. GUTMANN, E., pp. 203–272 and table 12. Prague: Czechoslovak Academy of Sciences.

BELMAR, J. & EYZAGUIRRE, C. (1966). Pacemaker site of fibrillation potentials in denervated mammalian muscle. *J. Neurophysiol.* **29**, 425–441.

BITTAR, E. E. (1966). Effect of inhibitors and uncouplers on the Na pump of the *Maia* muscle fibre. *J. Physiol.* **187**, 81–103.

BLISS, C. I. (1952). *The Statistics of Bioassay*, pp. 496–499. New York: Academic Press.

CREESE, R. (1954). Measurement of cation fluxes in rat diaphragm. *Proc. R. Soc.* B **142**, 497–513.

CREESE, R. (1968). Sodium fluxes in diaphragm muscle and the effects of insulin and serum proteins. With an Appendix by Jenden, D. J. *J. Physiol.* **197**, 255–278.

CREESE, R., NEIL, M. W. & STEPHENSON, G. (1956). Effect of cell variation on potassium exchange of muscle. *Trans. Faraday Soc.* **52**, 1022–1032.

CREESE, R., SCHOLES, N. W. & WHALEN, W. J. (1958). Resting potentials of diaphragm muscle after prolonged anoxia. *J. Physiol.* **140**, 301–317.

CREESE, R. & NORTHOVER, J. (1961). Maintenance of isolated diaphragm with normal sodium content. *J. Physiol.* **155**, 343–357.

CREESE, R., EL-SHAFIE, A. L. & VRBOVÁ, G. (1965). Effects of antimycin A on denervated muscle. *J. Physiol.* **178**, 18–19*P*.

DRAHOTA, Z. (1962). Electrolytes in denervated muscle. In *The Denervated Muscle*, ed. GUTMANN, E., pp. 151–171. Prague: Czechoslovak Academy of Sciences.

FINNEY, D. J. (1952). *Statistical Method in Biological Assay*, pp. 148–152. New York: Hafner.

GREENGARD, P. & STRAUB, R. W. (1962). Metabolic studies on the hyperpolarization following activity in mammalian non-myelinated nerve fibres. *J. Physiol.* **161**, 414–423.

GUTMANN, E. (1962). *The Denervated Muscle*. Prague: Czechoslovak Academy of Sciences.

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.

HUXLEY, A. F. (1960). In *Mineral Metabolism*, ed. COMAR, C. L. & BRONNER, F., vol. 1, part A, pp. 163–166. New York: Academic Press.

HODGKIN, A. L. (1958). Ionic movements and electrical activity in giant nerve fibres. *Proc. R. Soc.* B **148**, 1–37.

JENKINSON, D. H. & NICHOLLS, J. C. (1961). Contractures and permeability changes produced by acetylcholine in depolarized denervated muscle. *J. Physiol.* **159**, 111–127.

KERNAN, R. P. (1963). Spectroscopic studies of frog muscle during sodium uptake and excretion. *J. Physiol.* **169**, 862–878.

KEYNES, R. D. (1951). The ionic movements during nervous activity. *J. Physiol.* **114**, 119–150.

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 sodium concentration on the sodium fluxes in frog skeletal muscle. *J. Physiol.* **147**, 591–625.

KLAUS, W., LÜLLMANN, H. & MUSCHOLL, E. (1960). Der Kalium-Flux des normalen und denervierten Rattenzwerchfells. *Pflügers Arch. ges. Physiol.* **271**, 761–775.

LÜLLMANN, H. (1958). Über die Konstanz des Membranpotentials bei spontanen Änderungen der Ionengradienten am normalen und denervierten Rattenzwerchfell. *Pflügers Arch. ges. Physiol.* **267**, 188–199.

MILEDI, R. (1962). Induction of receptors. In *Enzymes and Drug Action*, CIBA Foundation Symposium, ed. MONGAR, J. C. & DE REUCK, A. V. S., pp. 220–235. London: Churchill.

NICHOLLS, J. G. (1956). The electrical properties of denervated skeletal muscle. *J. Physiol.* **131**, 1–12.

NOBLE, D. (1962). A modification of the Hodgkin–Huxley equations applicable to Purkinje fibre action and pace-maker potentials. *J. Physiol.* **160**, 317–352.

SLATER, E. C. (1958). The constitution of the respiratory chain in animal tissues. *Adv. Enzymol.* **20**, 147–199.

STEWART, D. M. (1955). Changes in protein composition of muscles of the rat in hypertrophy and atrophy. *Biochem. J.* **59**, 553–558.

TAYLOR, D. B., CREESE, R., NEDERGAARD, O. A. & CASE, R. (1965). Labelled depolarizing drugs in normal and denervated muscle. *Nature, Lond.* **208**, 901–902.

THESLEFF, S. (1963). Spontaneous electrical activity in denervated rat skeletal muscle. In *The Effect of Use and Disuse on Neuromuscular Functions*, ed. GUTMANN, E. & HNIK, P. Prague: Czechoslovak Academy of Sciences.

VRBOVÁ, G. (1963). Effects of a specific metabolic inhibitor on neuromuscular transmission, *J. Physiol.* **167**, 54–55*P*.