SODIUM EXCHANGE IN MOUSE AND RAT MUSCLE

by

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ABSTRACT

The outward movement of sodium was studied in isolated diaphragm muscle of the rat and in a toe muscle (flexor digitorum brevis IV) of the mouse by means of the isotope ^{24}Na. The sodium exchanged in rat diaphragm with a half-time of approximately five minutes, and the fibre sodium was estimated by compartmental analysis.

Strophanthin (10^{-4}g/ml) slowed the outward movement of sodium in rat diaphragm and caused an increase in the sodium content of the muscle and a decrease in its potassium content. It was found that these effects were reversible and it was therefore possible during the recovery to demonstrate net extrusion of sodium from the diaphragm.

Strophanthin also slowed the outward movement of sodium in mouse muscle and increased its sodium content while the potassium content was decreased. Insulin (0.1 unit/ml.) increased the rate of outward movement of sodium in mouse muscle.

It was found that the exchange of sodium in diaphragm muscle which had been denervated (8 days) was more rapid, the half-time being 4.1 min. for denervated muscles as compared with 5.4 min. for controls. The experiments were conducted on

a factorial design based on a 4-point assay, and these differences are significant ($P < 0.01$).

CONTENTS

CHAPTER I

SODIUM IN SKELETAL MUSCLE

In this chapter, an account of the development of ideas and concepts regarding sodium in muscle is given. As sodium and potassium are the main cations in muscle, and as there is an intimate relation between their behaviour, so the role of potassium will be mentioned when required. This chapter is mainly about muscle, though other tissues will be referred to when necessary.

Sodium pump and concept of active transport

Before the introduction of radioactive isotopes it was generally accepted that the muscle membrane was permeable to potassium but not to sodium ions. This concept of the selective permeability of the muscle membrane towards these two cations arose from the low concentration of sodium inside the cells as compared to that in plasma. Fenn and Cobb (1935), Fenn (1936) and Boyle and Conway (1941) all showed that muscles could gain and lose potassium. Fenn (1936) in a review of the subject considered that the muscle membrane is impermeable to sodium, but referred to the difficulty of applying this concept to explain electrolyte changes during recovery after stimulation. Boyle and Conway (1941) also indicated that sodium ions could accumulate in frog muscles *in vitro* without

a raised potassium concentration in the outside medium.

This concept of the impermeability of the muscle membrane to sodium persisted until the exciting experiment of Heppel (1939) who showed that muscles of rats deprived of potassium took up sodium in large quantities and lost potassium *in vivo*. Soon after that, Heppel (1940), using radioactive tracers, showed that both extracellular and intracellular fractions of muscle sodium exchanged readily with sodium in plasma, and thus it was concluded that the muscle membrane was apparently permeable to sodium. After that physiologists faced the question of how to explain the low internal sodium concentration, although the ion had been proved to penetrate into the fibre. To explain that, Dean (1941) postulated the well known "sodium pump". In this mechanism he stated that sodium ions are continuously pumped out actively at the same speed as they enter. As a matter of fact, Heppel's findings were further confirmed by Conway and Hingerty (1948) who showed that sodium which accumulated during potassium deficiency was slowly extruded on placing the rats on potassium-rich diets. Other investigators proved the presence of the active "sodium pump" in tissues other than muscle, e.g. Maizels (1951) for red cells, and Hodgkin and Keynes (1955) for cephalopod axons.

The subject of the active "sodium pump" mechanism was discussed in the review by Hodgkin (1951) who showed that it is necessary to consider that sodium is continuously pumped out of

excitable cells by a process which depends on metabolism.

Sodium and potassium exchange in muscle

By the use of radioactive compounds it had been shown that sodium could penetrate the muscle membrane. It should be mentioned, moreover, that the rate at which ions leave and enter the muscle cells could not be studied before the availability of radioactive isotopes. This point had been fully discussed by Harris and Burn (1949). Using radioactive tracers, these authors showed that ^{24}Na was lost from frog sartorius muscle *in vitro* in two stages, a fairly rapid sodium diffusion from the extracellular space, followed by a relatively slower exchange of fibre sodium, and it was thus possible to construct curves relating this loss to time which could be fitted by a double exponential curve. Harris (1950) obtained similar results for sodium exchange in frog muscle, but he observed that the slower portion sometimes showed more than one fraction. Similar results were obtained by Keynes (1954) in frog toe muscle *in vitro*.

Concerning potassium exchange in frog muscle, Harris and Burn (1949) showed that it also consisted of diffusion and permeation but there was no convenient separation into "fast" and "slow" fractions as in the case of sodium. Keynes (1954) also showed that in frog muscle, potassium exchanged according to a single exponential.

In mammalian muscle, the exchange of sodium and potassium resembles that in frog muscle but the exchange is more rapid in mammalian muscle. This can be shown by comparing the rate constants of exchange. Thus Keynes (1954) found that sodium exchanged in frog sartorius muscle with a rate constant of 0.81 hr^{-1} and that potassium exchanged in frog toe muscle with a rate constant of 0.20 hr^{-1}. In mammalian muscle, Creese (1954) showed that sodium exchanged in rat diaphragm with a half-time of 10.7 min. corresponding to a rate constant of about 3.9 hr^{-1}, and that potassium exchanged with a rate constant of 1.1 hr^{-1}.

Net sodium extrusion in muscle

From the "sodium pump" concept it appears that the demonstration of a net outward movement of sodium by muscle would be a good representation for the ability of muscle to extrude sodium against an electrochemical gradient. Actually, it was shown by Steinbach (1940) prior to the postulation of the "sodium pump" concept, that when frog muscle was soaked in potassium-free Ringer, potassium left the muscle and sodium entered in exchange, and that this exchange was reversible on condition that the muscle does not lose more than half its original potassium content. Steinbach (1951) emphasized his previous findings and showed that it depended on the sodium content of the fibres at the start of the recovery period. The rate of net sodium extrusion in frog muscle was

demonstrated by Desmedt (1953) using methods similar to those of Steinbach (1940) with modified solution to imitate frog's plasma. He showed that frog muscle extruded sodium with a half-time of about half an hour, and could nearly restore normal cation distribution pattern. Using Desmedt's conditions, a net sodium extrusion in frog muscle was confirmed by Carey, Conway & Kernan (1959), who, moreover, emphasized the dependence of the value of net sodium extrsuion on the sodium content of the initial and the recovery fluids, and found a marked extrusion up to 17.1 meq/kg muscle when the sodium content was 120 and 104 mM in the initial and the recovery fluids respectively. An interesting finding was shown by Kernan (1962b) by using the same sodium concentrations in both the initial and the recovery fluids. He enriched the frog muscle by immersion during the night in cold potassium-free Ringer-Conway fluid containing 120 or 104 mM sodium and reimmersed it in the same fluid at room temperature with the addition of 2.5 or 10 mM-K and in this solution the muscle could not extrude sodium. When insulin or lactate, alone or together, were added to the reimmersion fluid, up to 27 meq-Na/kg muscle was extruded over a 3 hr. period.

Net sodium extrusion in mammalian muscle was shown by Dockry, Kernan and Tangney (1966). They showed that net sodium and potassium transport by extensor digitorum and soleus muscles of rat was increased when the nervous innervation was intact as compared with muscles whose nerves had been recently sectioned.

Sodium and potassium movements and the electrogenic nature of the sodium pump

Since the demonstration of du Bois Reymond (1848), it was known that if nerve or muscle is cut at one end, and electrodes placed one on the cut end, and the other on the undamaged surface, a current flows from the intact surface to the damaged end, showing that the "cut" or "damaged" end is electrically negative to the intact surface. Although this "injury" or "demarcation" potential is not exactly identical to the resting membrane potential, as measured by the microelectrode technique, yet its discovery was an important step in the study of membrane potentials and the electric properties of excitable tissues in general.

In 1902, Bernstein explained the development of the resting membrane potential as being due to the difference in concentration of potassium ions across the membrane, and to the relatively high permeability to potassium as compared with other ions. This followed from the experimental fact that the demarcation potential could be lowered by external potassium. Accordingly, if the potassium concentration difference is maintained across the membrane, then the potential can be obtained from the equation:

$$E = -60 \log_{10} \frac{[K]_i}{[K]_o} \quad \ldots\ldots\ldots \quad (1)$$

where $[K]_i$ and $[K]_o$ refer to the activities of potassium ions inside and outside the membrane respectively, and E is the potential in mV inside relative to an electrode in the outer fluid. It is usually assumed that the ratio of activities equals that of concentration. The dependence of the resting potential on the external potassium concentration was shown in cephalohod axon by Hodgkin & Keynes (1955b), and Baker, Hodgkin & Shaw (1961) found that the resting potential was altered by varying the internal potassium concentration.

In practice, it has been shown that this equilibrium potassium potential, calculated from the above equation, is usually higher <u>in vitro</u> than the measured resting membrane potential. Thus, the membrane potential of frog muscle is about -92 mV while the calculated value of E_r is -102 mV (Adrian, 1956). This deviation shows that, at least <u>in vitro</u>, more complex equations are required in which other ions can be involved especially the sodium ions which represent the other dominant cation in excitable tissues. Thus the effect of sodium can be obtained from the following equation (see Hodgkin, 1958):-

$$E = -60 \log_{10} \frac{[K]_i + b[Na]_i}{[K]_o + b[Na]_o} \quad . \quad . \quad . \quad . \quad (2)$$

where $[Na]_i$ and $[Na]_o$ are the internal and external concentrations of sodium and b is a factor which expresses the

relative permeability of sodium as compared to potassium.

As the resting membrane potential depends on the ratio $[K]_i/[K]_o$, it is thus important to discuss how the internal potassium concentration is maintained. Boyle and Conway (1941) described a double Donnan equilibrium where the non-penetrating cation sodium is in high concentration outside, and the non-penetrating anions like proteins and phosphate esters are in high concentration inside, and consequently, the penetrating ions, potassium, chloride, etc., will be distributed in a manner such that electroneutrality is maintained on both sides of the membrane. However, sodium ions were found to penetrate the membrane and some modification of this concept proved necessary.

Another concept regarding the maintenance of $[K]_i$ and $[Na]_i$ was stated by Hodgkin (1958) who suggested that the potassium ratio is maintained by metabolism by means of a neutral sodium-potassium pump in the sense that for each sodium ion actively extruded a potassium ion is actively absorbed. He suggested that this coupled sodium-potassium exchange, which depends on metabolism, could keep a cell in a steady state with its characteristic ionic distribution unchanged, and that for this steady state to be achieved there should be a balance between such metabolically driven movements of sodium and potassium, and between the sodium and potassium leak down their concentration gradients. According to Hodgkin's view, such a pump involving "one for one" exchange will not itself be

responsible for the generation of any potential difference across the membrane.

The question whether the sodium pump is neutral or electrogenic in nature was tested experimentally by different authors. Thus Kernan (1962a) measured the membrane potential in frog sartorii made sodium-rich by overnight soaking in potassium-free fluid, and immediately on reimmersion in recovery fluid containing 10 m.equiv. potassium per litre. At the end of the experiment muscles were analysed for potassium from which the potassium equilibrium potential was calculated by means of equation 1. From this Kernan found that the observed potential is greater than the calculated potassium equilibrium potential during sodium extrusion, and that both potentials were in good agreement when extrusion declines after 3 hrs. Similar results were obtained by replacing sodium by choline chloride in the recovery fluid. Moreover Kernan proved that the increased potential is not due to chloride movement, by reimmersing the sodium-rich muscles in chloride-free recovery fluid and measuring the membrane potentials under these conditions. All these results show that the sodium pump is not neutral, but that during its operation the interior of the fibre became more negative with respect to the exterior. Potassium ions are presumably attracted electrostatically into the fibres. Keynes (1965) referred to the coupling between sodium and potassium movements. It does not appear to be a one-to-one

process and it varies in a complicated fashion with the internal sodium concentration.

It is interesting to note that Kernan (1962b) found that the membrane potential measured during sodium extrusion was significantly greater than the calculated potassium equilibrium potential, and that after the inhibition of sodium extrusion by adding O-phenanthroline (an inhibitor for lactate dehydrogenase) to the recovery fluid, the two potentials were in better agreement. From these results, Kernan gave the same conclusion, namely that the sodium pump is electrogenic in nature and that potassium enters the fibres freely to restore electrical neutrality.

Saturable ion fluxes and the concept of carriers

The relation between the flux and the ionic concentration is of importance in the study of the mechanism of ion transport. Thus if the influx when plotted against the external ionic concentration is linear, then the rate of exchange is proportional to the concentration gradient, and the mechanism may be diffusion-limited; if the flux is not a linear function of the external concentration but levels off at high concentrations, then the flux may be said to be saturated at these high concentrations, and in this case a mechanism other than diffusion may be involved. Curves showing saturable ion

fluxes plotted against concentration are similar to those showing the relation between enzyme velocity and substrate concentration. These curves are of hyperbolic nature and the relation can often be expressed by the following equation:

$$v = V \frac{s}{s + K_m} \quad \ldots\ldots\ldots \quad (3)$$

where v is the velocity when the substrate concentration is s, V is the maximum velocity obtained when the substrate concentration is high enough to saturate the enzyme and K_m is a constant known as Michaelis constant of the enzyme for this substrate (it is the value of s which is experimentally found to give half the maximum velocity).

In case of saturable ion fluxes, similar equations can be used to identify these fluxes whereby the "half saturation value" of the flux can be calculated and this value is very useful in the characterization and identification of different saturable fluxes.

It was mentioned that when a flux is proved to be "saturable" a special mechanism should be involved. The most attractive and reasonable one is the "carrier mechanism". In this mechanism, a substance, possibly a constituent of the cell membrane, with specific affinity to the ion is supposed to complex with it at one side, the complex thus formed travels across the membrane and at the other side it dissociates and

releases the ion. The condition of saturable ion fluxes had been extensively studied by Glynn (1956) in red cells, and he gave examples of sodium efflux and potassium influx as representatives for saturable ion fluxes and he suggested that there is a correlation between these two fluxes. In frog skeletal muscle, Keynes and Swan (1959) showed that a portion of the sodium efflux was dependent on external sodium concentration, and this efflux was maximal at 130 mM with an apparent half saturation value of 38 mM. These authors also suggested that there is a degree of coupling between sodium efflux and potassium influx. The existence of such coupling was explained by different authors in different tissues by the help of the carrier concept. Thus, it is supposed in the case of red cells (see Glynn 1956) that potassium and sodium can cross the membrane combined with the carriers x and y, x being potassium-specific and y being sodium-specific. x and y are interconvertible and are in equilibrium at the outer surface of the membrane, while at the inside surface, x is converted to y with the expenditure of energy. Keynes (1965) has further explored the relation between the internal sodium concentration and the movements of sodium in frog muscle, and he confirmed that there is a saturable component of the efflux. Potassium affected the movements of sodium in a complicated manner and did not show a one-to-one relation. The other component of sodium efflux has been interpreted in terms of exchange diffusion

(Keynes & Swan, 1959), but the complexities of the experimental results do not yet allow a satisfactory kinetic treatment.

High-energy phosphates and sodium efflux

Evidence for the intimate relation between sodium efflux and high-energy phosphates had been brought about mainly by the work of Caldwell, Keynes, Hodgkin & Shaw. Thus it was found by Caldwell (1960) that a fall in the concentration of arginine phosphate and adenosinetriphosphate (ATP) occurred when squid giant axon was poisoned with cyanide or dinitrophenol (DNP) and that resynthesis took place after washing the poison (the effect of DNP towards both phosphate esters depended on the pH). These changes appeared to run in parallel with the changes which these inhibitors bring about in the sodium efflux, and so, these authors suggested that ATP and arginine phosphate may play a part in the active transport of sodium. It was also shown by Caldwell, Hodgkin, Keynes & Shaw (1960) that injection of solutions of ATP and other phosphates into the giant axons of Loligo poisoned by cyanide partially restored sodium efflux while injection of the same solutions after being hydrolyzed by boiling had no effect. This is strong evidence that the energy of sodium transport is utilized from such high-energy phosphate compounds. Caldwell *et al.* (1960) also found that injection of ATP and arginine phosphate had a restorative effect in the same axons poisoned with 0.2 mM DNP. Moreover, Caldwell *et al.* (1960)

found that these two phosphate esters had no effect when applied externally to fibres poisoned with cyanide.

Another sort of link between sodium transport and high energy phosphates was demonstrated in red cells by Dunham and Glynn (1961). These authors found that the ATPase component which requires sodium and potassium for its action, is completely inhibited by cardiac glycosides in concentrations sufficient to inhibit ion transport in intact cells. Thus they concluded that the close resemblance between glycoside-sensitive ATPase and the ion pump suggests that they are intimately connected.

The effect of cardiac glycosides

Cardiac glycosides have in common a specific and powerful action on the myocardium which has been recognised since the time of Withering (1785).

The effect of cardiac glycosides on ion movements was first demonstrated by Schatzmann (1953). He found that cardiac glycosides prevented the uptake of potassium and elimination of sodium that normally occur when cold-stored red cells are incubated at 37°C. Since then the inhibitory effect of cardiac glycosides was shown by other authors in different tissues. Thus Hatchett and Johnson (1954) depleted frog sartorii from their intracellular potassium and increased their intracellular sodium by soaking in low-potassium high-sodium Ringer. These

muscles were then allowed to recover in Boyle-Conway fluid, and to the recovery fluid of one set of muscles, ouabain was added. There was a significant difference in the final potassium and sodium concentrations; control muscles achieved nearly normal sodium and potassium concentration while ouabain-treated muscles failed to show such recovery. In similar experiments on frog sartorii Johnson (1956) obtained comparable results when ouabain appeared to inhibit the net transport of sodium from, and of potassium into, the cells of these muscles under conditions where transport would otherwise occur. Sodium extrusion was also inhibited in frog muscle by ouabain (Kernan, 1962[b]; Keynes, 1965).

The rate of exchange of sodium was also shown to be affected by cardiac glycosides. Thus Edwards and Harris (1957) using radioactive sodium as a tracer, found that sodium output from frog muscle was depressed 1.6-2.2 times under the effect of strophanthin, and that the same effect could be obtained by omission of potassium from the external solution.

In nerve, Caldwell and Keynes (1959) showed that ouabain added to sea water bathing squid axons markedly reduced the rate constant of ^{22}Na exchange. In mammalian muscle, Page et al. (1964) found that ouabain caused large losses of cell potassium and increase of cell sodium in cat papillary muscles.

Possible mechanism of the action of cardiac glycosides. It is

firstly important to mention that Schatzmann (1953) noted that concentrations of glycosides, sufficient to affect ion movement in red cells, had no effect on oxygen consumption or lactic acid formation and he concluded that the drugs could affect ion movements without alterations in energy production. It was found by Glynn (1957) that digoxin greatly reduced the size of the saturable component of potassium influx in red cells, this effect being much greater than the effect of glucose deprivation. These results have been interpreted as evidence that digoxin acts directly on the carrier mechanism responsible for the saturable part of the influx. Glynn (1957) also found that digoxin caused a large reduction in sodium efflux, both active and passive components, and he therefore considered that the drug can act without disconnecting the energy supply. It can thus be concluded that as long as cardiac glycosides show pronounced effect on the saturable ion fluxes, and on fluxes which are known to be "downhill" as well, this means that they act, not by disconnecting the pump from the energy supply, but on the transport or "carrier" mechanism itself. The work of Dunham and Glynn (1961) on red blood cells showed a close resemblance between the properties of the glycoside-sensitive adensinetriphosphatase and the ion pump - both being inhibited by cardiac glycosides - and the authors have suggested that they were intimately connected.

Reversibility of the action of cardiac glycosides. Glynn (1957) found a slow recovery in potassium influx of red cells after removal of the cardiac glycoside and he attributed this to the slow loss of inhibitor from the cells. Edwards and Harris (1957) also observed a small degree of recovery of sodium efflux after withdrawal of strophanthin in the case of frog muscle.

Possible competition between cardiac glycosides and potassium ions. Support for the concept that cardiac glycosides act on the carrier mechanism and not on the energy supply was obtained when it was shown that the glycosides also inhibit fluxes which are "downhill" and which are not affected by the absence of glucose in the case of red cells (Glynn, 1957). With low concentrations of the glycoside, inhibition can be completely reversed by increasing the external potassium concentration. These results can be taken as evidence for the possibility that the inhibitor molecules and potassium ions compete for the same carrier mechanism. It is interesting to mention here that Caldwell and Keynes (1959) obtained a marked slowing effect on sodium efflux in squid giant axons when ouabain was applied externally but not when injected internally. They explained the apparent failure of ouabain to act internally by assuming that it competes with potassium for the same carrier, the internal potassium concentration being thirty times higher than that outside.

In mammalian muscle, Page et al. (1964) showed that the inhibition of cation transport produced by ouabain in cat papillary muscle is dependent on external potassium concentration and they took that as an evidence for competition between ouabain and potassium ions at the outer surface of the membrane.

The action of insulin on muscle

Insulin has a widespread effect in different tissues and include actions on metabolic processes and on ionic movements. In this section an attempt is made to list briefly some of these actions, and this is followed by a summary of concepts which have been employed to explain the mechanism of the action of this hormone. References which are not specifically given can be found in the book by Krahl (1961).

Effects on carbohydrate utilization:

Insulin causes an increased glucose uptake by mammalian muscle which is correlated to increased rate of carbohydrate transport by the hormone. At the same time, the glycogen synthesis is stimulated in muscle and this is due partly to increased glucose entry and partly to an increased rate of formation of glucose-6-phosphate. In liver, glucose utilization is increased and at the same time the glycogen level is raised. These effects on liver occur after an interval following the administration of the hormone.

Effects on protein metabolism:

Insulin was found to stimulate incorporation of amino acids into peptides in muscle, a process which is independent of the observed stimulatory effect of insulin on glucose transport or on amino acid transport. Accordingly, insulin is thought to stimulate protein synthesis in muscle. An increase in protein synthesis in liver also occurs but again, an interval of time elapses before the effect is manifested.

In connection with the effects of insulin on protein metabolism and synthesis, its parallel effect on growth should be mentioned. Thus, in muscle, insulin and growth hormones supplement each other with respect to anabolism, while growth hormones act oppositely to insulin with respect to glucose utilization.

Effects on lipids:

In the adipose tissue of rat, insulin stimulates the conversion of glucose to glycogen, carbon dioxide, glycerol, and fatty acids. Moreover, it increases the incorporation of fatty acids into lipids in the presence of glucose. Fatty acid synthesis in the liver is also increased after an interval following the administration of the hormone.

Insulin and oxygen consumption:

The effect of insulin on oxygen consumption seems to differ in different species. Thus in frog muscle, insulin directly stimulates oxygen consumption *in vitro*. In mammalian muscle, Gemmill (1940) found that insulin caused no increase in oxygen consumption in normal rat diaphragm in presence of glucose. Hall (1960) however found that in rat diaphragm and some other skeletal muscles of alloxan diabetic rats, the oxygen consumption was originally low, and was increased by insulin *in vitro*.

The effect of insulin on electrolytes and the resting potential:

It was observed long ago that serum potassium decreased when insulin was administered to intact animals (Briggs *et al.*, 1924). This drew the attention towards the role which might be played by that hormone on the potassium ions, and on electrolytes in general. Thus Kamminga *et al.* (1950) showed a net movement of potassium out of the extracellular fluid and into isolated rat diaphragm muscle.

Under the impression that insulin might hyperpolarize the membrane (due to its above mentioned effects on potassium movements), Zierler (1957) studied the effect of insulin on the resting membrane potential of the isolated peroneus longus muscle of the rat. His experiments lasted for one hour and he obtained a significant increase in the resting potential under

the effect of insulin as well as an increase in the ratio $[K]_i/[K]_o$. Zierler considered that insulin can act by its association with muscle membrane and that this membrane complex results in spatial changes in the barrier to diffusion, thus increasing the membrane permeability and simultaneously increasing the potential difference across the membrane. In response to the increased potential difference across the membrane, potassium moves into the muscle towards a new equilibrium concentration.

The effect of insulin on the resting potential and on potassium content in muscle was further studied by Zierler (1959) on the extensor digitorum longus muscle of the rat. The potassium content was measured after 1, 2 and 3 hours. Zierler (1959) found no increase in the ratio $[K]_i/[K]_o$ after 1 hr in insulin solutions, whereas after 2-3 hours there was an increase of about 10%. Again the resting potential was found to be increased significantly after 1 hr in insulin solutions, and thus Zierler suggested that the hyperpolarization produced by insulin is the cause of the potassium shift. In an attempt to explain how the net change in potassium concentration occurred, Zierler (1960) studied the effect of insulin on potassium efflux and influx in the extensor digitorum longus of the rat by the use of the radioactive isotope ^{42}K. He found that insulin decreased both fluxes but the effect on the efflux was greater. Zierler (1960) suggested that insulin increased the

positive fixed charge within the muscle membrane.

Potassium movement in excitable tissues is closely related to sodium movement, and sodium exchange should not be ignored in such studies. Creese et al. (1961) showed that rat diaphragm soaked in saline containing insulin had a lower sodium content than that of control muscles similarly treated but without insulin. Moreover, the potassium content of insulin-treated muscles was found to be higher than that of controls without insulin. These findings regarding the maintenance of muscle sodium and potassium at values which were similar to those found in vivo lead to attempts to detect the effect of insulin on sodium movements in muscle. Thus Creese (1964) using the radioactive tracer ^{24}Na, measured sodium efflux in rat diaphragm muscle treated with insulin and in control muscles without insulin. It was found that the exchange rate was increased under the effect of insulin. At the same time, the potassium content was found to be increased by insulin. Referring to the previous findings that insulin increased the potassium content of rat muscle as well as the resting potential (Zierler, 1957 and 1959), Creese (1964) thus concluded that these effects appear also to be associated with an increase in the rate of turnover of sodium.

Possible biochemical mechanisms of insulin action:

It is not easy to explain insulin action by one process

which can be the basic step leading to all these widespread variety of effects, until decisive experimental evidence is in hand, and which should be, moreover, undoubtedly confirmed. In what follows a summary of the most important theories put down to explain insulin action are given.

(1) Randle and Smith (1957, 1958a and b) suggested that insulin acts by restricting access of energy-rich phosphate to the process concerned with the regulation of glucose entry. It was suggested by Randle and Smith (1960) that the carrier - with which glucose is supposed to combine before its entry into the muscle cell - this carrier is capable of such combination when it is in its non-phosphorylated form. It was therefore suggested that insulin either stimulates dephosphorylation of the carrier or inhibits its phosphorylation, thus accelerating glucose transport. In a survey of the subject, Randle and Young (1960) referred to this mechanism.

This theory does not account for those effects of insulin which are glucose independent.

(2) Chain et al. (1956) suggested that insulin raises the energy level of the cell whereby insulin acts by making energy available for the reactions observed. Chain (1959) referred to this mechanism and that these authors consider that, at least in part, this effect may be achieved by making the TPN-TPNH (triphosphopyridine nucleotide, oxidised or reduced form respectively) system more effective for synthesis. That insulin

acts by making energy available for energy-requiring reactions was also suggested (Chain, 1960).

The actual mechanism by which insulin raises the energy level and makes energy available for the observed reactions, is not understood.

(3) Krahl (1956, 1957) suggested that insulin acts by alteration of the fine structure of responsive cells resulting in decompartmentation that favours anabolic processes. It is difficult to produce experimental evidence in support of this concept.

(4) Kernan (1962b) suggested that insulin stimulates the sodium pump through the stimulation of oxiditative metabolism and the associated electron transfer; thus the stimulated sodium pump increases the resting potential, and the potassium ions enter passively to restore electrical neutrality. This arose from the finding (Kernan, 1962b) that insulin and lactate stimulated sodium excretion in frog muscle, and that the addition of O-phenanthroline (an inhibitor for lactate dehydrogenase) markedly inhibited this excretion.

(5) Levine (1965) considered that the demonstrated effects of insulin are not due to a primary effect on glucose transport, but he gave a fundamental importance to the cell membrane as a primary site of insulin action. Insulin is thought to act on some specific and still unknown receptor located at the membrane.

This interaction of insulin with the cell membrane initiates a set of signals which, in turn, lead to the other demonstrated effects of insulin including stimulation of glucose transport.

CHAPTER II

METHODS

Rat diaphragm preparation:

Rats were stunned, decapitated and the left hemidiaphragm was exposed by two parallel cuts including rib and tendon. A cotton thread was passed through the rib, and a hole was made in the tendon through which the platinum hook of the glass holder was passed. The diaphragm was then removed and immersed in the saline and a clip weighing 4 g was attached. Gassing was effected by means of a polythene tube attached to the glass holder so as to give a fine spray of gas from underneath the muscle (Fig. 1). This method was described by Creese and Northover (1961) and it has the advantage of preventing deterioration and permitting good oxygenation. The time from decapitation to immersion was about 100 sec. so that the preparation could be set up within a short time of the cessation of circulation.

Denervation:

Denervation of the left hemidiaphragm was carried out under ether anaesthesia. The brachial plexus and phrenic nerve were exposed by a low anterior cervical incision, and then the trunk of the phrenic nerve was avulsed. The denervated muscles were used 7-9 days later.

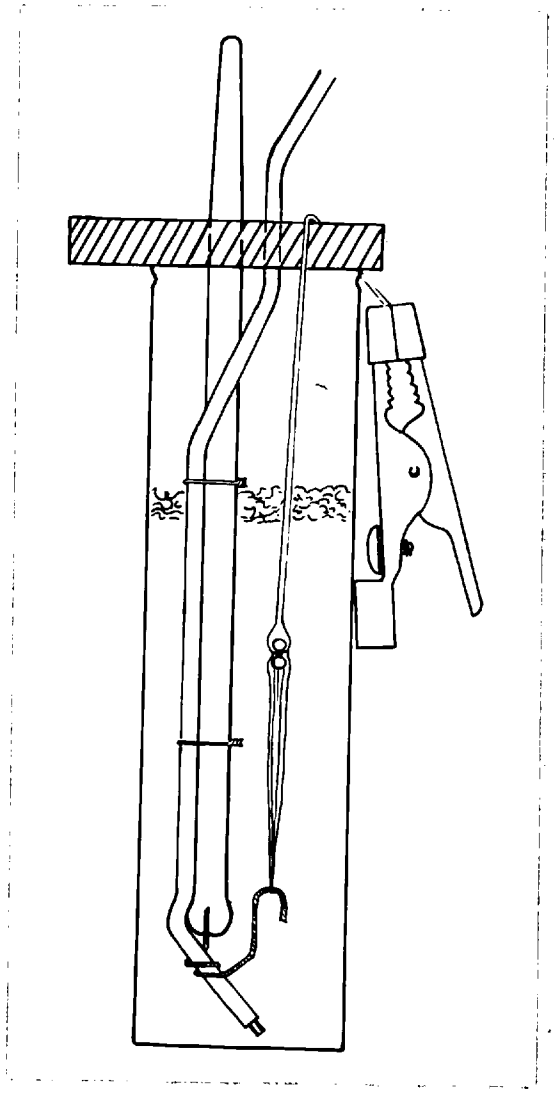

Fig. 1. Holder used for diaphragm muscle. The tendon is attached by a hook of platinum wire which has been fused to a holder made of glass rod with a plastic cross-piece. The holder also includes an oxygen tube. The thread passes round the rib and is steadied by a clip. The holder is in a tube which holds 10 ml saline. The top of the holder is shaped so that it can be transferred when necessary to the shaft of the motor for vertical movement during washout experiments, as shown in Fig. 2.

Mouse toe muscle:

The muscle used is the flexor digitorum brevis IV which is a fusiform muscle containing a variable amount of fibrous tissue. It was dissected under anaesthesia with pentobarbitone (0.1 mg Sodium Pentobarbitone per g) with the aid of a low power microscope. The anaesthetised mouse was laid on a wooden board with tail and one of the toes secured with thread. The skin of the foot was cut laterally and removed so that the foot muscles were exposed. The heel tendon at the origin of the muscle was freed and a ring of platinum wire was pushed through it; also the insertion tendon of the muscle was freed and a nylon thread tied around it. Then the looped end of a platinum wire was pushed through the platinum ring of the heel tendon, and finally and as quickly as possible, the muscle was freed, removed and immersed in the required solution. A weight of 0.3 g was clamped to the thread and gassing was effected by a polythene tube running to the bottom of the soaking tube so as to give a fine spray of gas from underneath the muscle. In most of the experiments, left and right muscles from the same animal were used, one serving as a control.

Solutions:

Modified Krebs saline was used (Krebs & Henseleit, 1932; Creese & Northover, 1961) as shown in Table 1.

Table 1

(a)

Modified Krebs Solution

Substance	Concentration	Quantity taken
NaCl	4.5%	20 ml.
H_2O		80.75 ml.
$NaHCO_3$	1.3%	21 ml.
K Cl	1.15%	4.25 ml.
$CaCl_2$"dried 70%"	1%	3 ml.
$Mg\ SO_4, 7H_2O$	3.82%	1 ml.
$NaH_2PO_4, 2H_2O$		0.024 g.
Glucose		0.260 g.
	Total	130 ml.

(b)

Ion Concentration in Modified Krebs Solution

Ion	Concentration (mM)	Ion	Concentration (mM)
Na^+	145	Cl^-	125
K^+	5	HCO_3^-	25
Ca^{++}	1.3	$H_2PO_4^-$	1.2
Mg^{++}	1.2	SO_4^{--}	1.2

Radioactive solutions:

A proportion of sodium was replaced by the radioactive isotope ^{24}Na which was delivered as an isotonic solution (0.9% w/v) $^{24}NaCl$.

For exchange measurements in mouse muscle, it was necessary to prepare a small volume of solution with a high specific activity. Double ion saline without calcium chloride was first prepared, and then the radioactive saline was made up as shown in Table 2.

Bath and equilibration:

After removal from the animal, muscles were transferred to tubes containing the soaking saline for equilibrium. These tubes were suspended in a "PERSPEX" water-bath containing a heater and a thermostat. Gassing of saline in such tubes was effected by polythene tubes connected to the gas cylinder by means of rubber tubing. For the control of temperature in the tubes during exchange measurements a small accessory jacketed water-bath was connected so that water circulated from the main reservoir (Fig. 2). All the tubes of saline were kept gassed for about 15 minutes in the water-bath before use so that the saline acquired the desired temperature. The temperature was maintained at 38°C.

Uptake of ^{24}Na

The holder containing the tissue was soaked for 1 hour in

Table 2

(a)

Double ion saline without calcium chloride

Substance	Concentration	Quantity taken
NaCl	4.5 %	20 ml.
H_2O		57.5 ml.
$NaHCO_3$	1.3 %	42 ml.
KCl	1.15 %	8.5 ml.
$MgSO_4,7H_2O$	3.82 %	2 ml.
$NaH_2PO_4,2H_2O$		0.048 g.
Glucose		0.52 g.
	Total	130 ml.

(b)

Radioactive saline made up from the above double ion saline for mouse muscle

Substance	Quantity taken
Double ion saline without calcium chloride (above)	1 ml.
$^{24}NaCl$ 0.9%	1 ml.
$CaCl_2$ 1% (w/v) of the dried 70% salt	0.05 ml.
Total	2.05 ml.

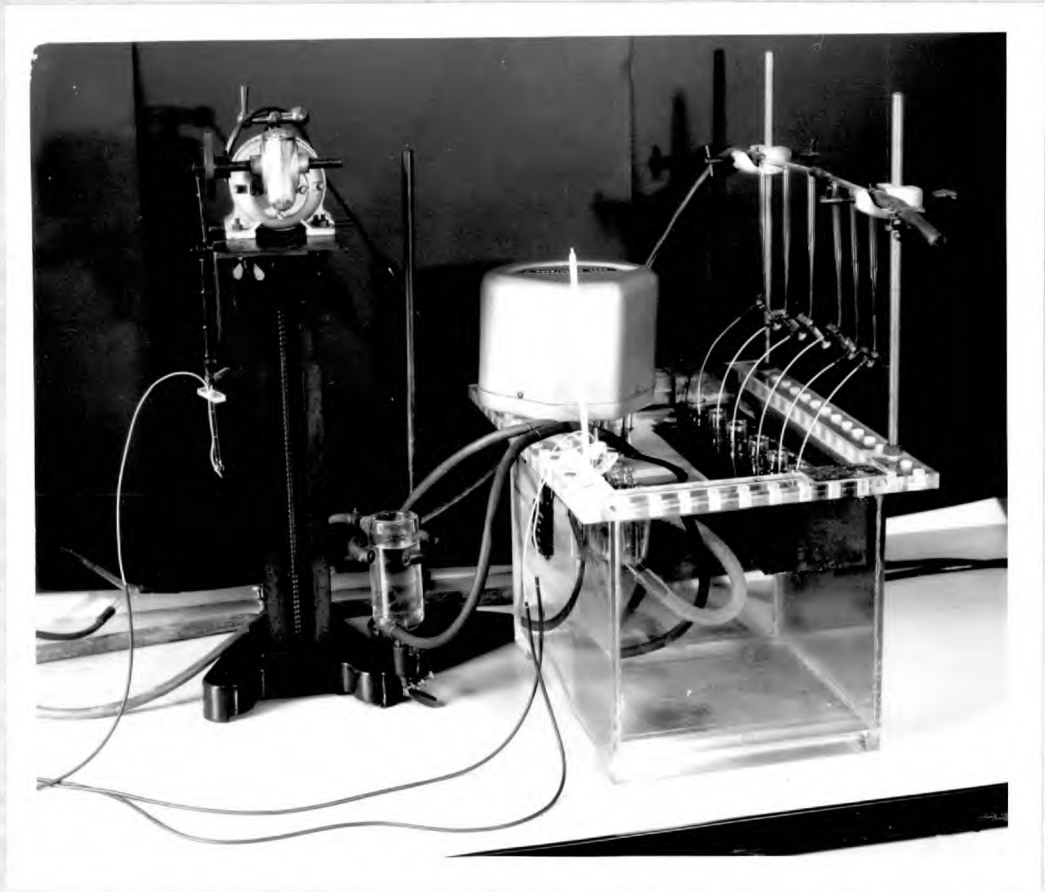

Fig. 2. Photograph showing the bath used for equilibration (to the right) containing soaking and washout tubes, heater, thermostat and thermometer. The small jacketed bath is used for keeping the temperature constant during the washout. The motor used for vertical movements during washout is on the left, with the holder and its attached oxygen tube in place.

the solution containing ^{24}Na at 38°C. In the case of diaphragm muscle this is sufficient to make the specific activity of the muscle equal to that of the external saline (Creese, 1964), and the uptake for mouse muscle is similar (see below).

In some cases the muscles were loaded by intraperitoneal injection of 1 ml 0.9 % saline containing ^{24}Na into the rat 1 hour before the muscle was used for measurements of outward movement.

Measurement of outward sodium exchange

The muscle was attached to the glass holder. After equilibration in the radioactive saline the holder with its muscle was quickly attached to a metal shaft which was moved in an up-and-down direction by means of a rotating cam driven by a small motor (Fig. 2). The muscle was steadied by the thread which was passed over a projecting screw and attached to a weight. The muscle was passed through the series of tubes each containing 10 ml of oxygenated inactive saline at 38°C, so that the fluid around the muscle was changed each minute. This method is similar to that described by Keynes & Swan (1959).

Washout was stopped after definite periods, the rib and tendon were cut away and the muscle quickly wiped on a clean, dry tile to remove the adhering saline (Creese, 1954). The muscle was then weighed in a small dry stoppered glass tube.

The radioactivity in the muscle was then measured (below). The rib and tendon were discarded.

After the end of the washout, one ml. of the radioactive saline was taken and diluted 1250 times. Then the radioactivity in 2 mls from this diluted saline was measured.

In some cases the saline through which the muscles had been passed was preserved and counted.

The outward movement of sodium was measured in mouse muscle by similar methods, except that the muscle was transferred to a simple platinum holder fused to a glass rod which was then fixed into the moving shaft of the motor shown in Fig. 2. Also in the case of this muscle, small tubes containing 2 ml. saline were used both for the initial soak in radioactive saline and for the washout in unlabelled solution.

Measurement of radioactivity:

The muscle was placed in a small glass tube graduated to 2 ml. 0.1 ml concentrated nitric acid + 0.1 ml distilled water were added and the tubes were boiled in a water-bath for 10 minutes, after which about 1 ml of distilled water was added and the tube boiled for a further 10 minutes. Then distilled water was added to give a total volume of 2 mls.

The tube containing 2 ml of muscle solution, the washout tubes (in the case of mouse muscle), and the tubes containing the 2 mls of the diluted radioactive saline, were then wiped

and counted using a well scintillation counter and a scaler.

In rat diaphragm experiments where the radioactivity in the washout saline had to be measured, the muscle solution was made up to 10 ml. Then the 10 ml of solution as well as the 10 ml saline of each of the washout tubes, were transferred to plastic cups and counted by means of another scintillation counter and scaler.

In all radioactivity measurements, corrections were made for the background contribution and decay. The half-time for ^{24}Na was taken as 15.0 hours.

Analysis of muscle tissue:

(1) H_2O %. Muscles were dried overnight in the oven at 105°C, and reweighed, the difference between this weight and the wet weight thus giving the water content of the muscle. From this, and the wet weight of the muscle, the H_2O was calculated as a percentage.

In the case of the mouse muscle, it was found that complete dryness of the muscle occurred after three hours at 105°C.

(2) Sodium and potassium. Sodium and potassium ions were estimated quantitatively by means of an EEL flame photometer against sodium and potassium standards respectively. The method used for dissolving the muscles has been previously described. Measurements were made in duplicate, and the results were expressed in the first instance as parts per million in the solutions.

The following precautions were taken to minimize, as far as possible, the exchange between the ions of the glassware and the solution in which these ions were to be estimated.

All the glassware used in such analyses was thoroughly rinsed in distilled water and then the glass tubes and the cups of the flame photometer were boiled in distilled water. The volumetric flasks were washed and filled with distilled water overnight.

The appropriate sodium and potassium standards were also made up fresh for every experiment.

Moreover, handling of such glassware was carried out by means of forceps. By this means contamination with sodium from the hands was avoided.

Blank values were estimated in exactly similar tubes but without tissue. These blank values were subtracted from the values obtained for the tubes containing the muscle solutions.

CHAPTER III

SODIUM EXTRUSION IN RAT DIAPHRAGM AND THE REVERSIBLE ACTION OF STROPHANTHIN

Schatzmann (1953) found that cardiac glycosides prevent the uptake of potassium and the elimination of sodium by cold-stored red cells. Since then similar studies were carried out on red cells and other tissues.

Strophanthin, which is a member of the cardiac glycoside group, was found to depress the sodium output in frog muscle (Matchett & Johnson, 1954; Edwards & Harris, 1957). In the frog muscle Edwards & Harris have found a small degree of recovery after removal of the drug.

These results have now been extended to mammalian muscle and considerable effects were found on sodium movements in rat diaphragm, and it became of importance to see whether these effects were reversible. It was possible to prove this reversibility in rat muscle, and thus to show that mammalian muscle can extrude sodium.

The first part of this chapter deals with the effects of strophanthin on total sodium and potassium, and on sodium exchange and fibre sodium. The next part demonstrates the reversible action of strophanthin on these processes. Finally, the effects of diffusion on fibre sodium are dealt with and values of fibre sodium, corrected for diffusion, are calculated.

RESULTS

Effect of strophanthin on total sodium and potassium

Muscles were soaked in Krebs saline containing strophanthin (10^{-4}g/ml) for 1 hr. after which they were freed from the rib and tendon, and analysed for total sodium and potassium as described in Chapter II. Control muscles were treated similarly but in strophanthin-free saline. (Strophanthin saline was prepared by dissolving the requisite amount of the drug in a few drops of 80% ethyl alcohol and then into the appropriate volume of normal Krebs saline to give the required drug concentration.)

The second and third rows of Table 3 show the results. It is clear that strophanthin after 1 hr. caused an increase in total sodium by 38 μ mole/g wet tissue, and a decrease in total potassium by 25.8 μmole/g wet tissue. The first row in this table also shows some values for total sodium and potassium *in vivo*.

Effect of strophanthin on sodium exchange

Fig. 3 shows the general pattern of outward sodium movement as measured by ^{24}Na. In this experiment, 1 ml. isotonic ^{24}NaCl containing 1 m C was injected intraperitoneally, and after 1 hr. the muscle was dissected out and passed through

Table 3. Effect of strophanthin 10^{-4} g/ml and recovery on total Na and K in rat diaphragm

	Sodium Content	S.D.	No.	Potassium content	S.D.	No.
In vivo	42.2	±3.44	10	93.6	±4.11	10
In vitro	+44.4	±1.53	10	+85.7	±6.48	20
After strophanthin	+82.4	±4.46	11	+59.9	±5.74	13
After recovery	*65.9	±2.73	11	*79.9	±4.45	18

Mean values for total Na and K are given in μmole/gm wet weight ± S.D.

+ soaked 1 hr.

* soaked 1 hr. in strophanthin and then allowed to recover for 35-80 min. in saline free from strophanthin. Values obtained after recovery differ significantly from muscles treated with strophanthin without recovery ($P < 0.01$ for Na and for K).

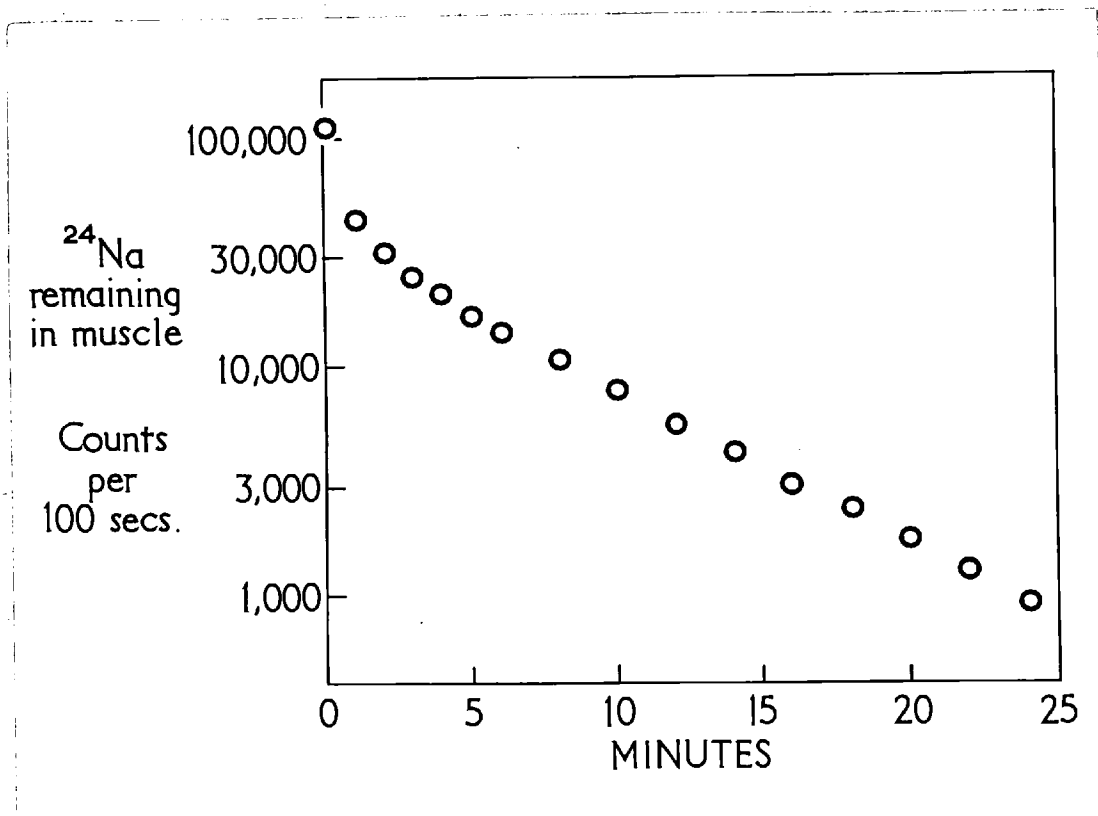

Fig. 3. Plot of the outward movement of ^{24}Na in diaphragm muscle. The muscle was loaded by intraperitoneal injection of 1 ml 0.9% saline containing ^{24}Na into the rat 1 hour beforehand. The muscle (and ribs) were passed through tubes of saline which were later counted. The cumulative counts which remain are plotted on semi-logarithmic paper against time. The early portion of the curve, which is completed in the first 5 minutes, represents the extracellular sodium, while the remaining portion is identified as fibre sodium. The latter exchanges with a half-time of 4.6 minutes.

a succession of tubes containing inactive saline, so that the saline was changed every minute. After the end of the washout, the radioactivity was measured in the tubes as described in Chapter II, and Fig. 3 shows the radioactivity which remains in the tissue as obtained from the cumulative counts. When plotted on semilogarithmic paper the curve shows two portions. The early part is rapid and corresponds to the loss of extracellular sodium which is largely completed in the first five minutes; the second portion is slower and corresponds to the fibre sodium extrusion. It can be seen that the curve can be fitted by a double exponential curve, and the present study is mainly concentrated on the second portion which is believed to represent the outward movement of fibre sodium. In Fig. 3 the slow portion has a rate constant of 0.151 min^{-1} ($T_{\frac{1}{2}}$ = 4.6 mins.).

Figure 3 gives the shape of the wash-out curve, but this method in its unmodified form is not suitable for experiments on sodium movements. This is due to the presence of the rib, which produces variable effects for which corrections cannot be applied. For this reason it was decided to adopt a modified procedure. The muscles were loaded with ^{24}Na by soaking in radioactive saline and they were then passed through a succession of tubes as above. After known intervals the washout was stopped, the rib and tendon were removed and the radioactivity remaining in the muscle was measured. By the

use of many muscles it was possible to construct a curve similar to that of Fig. 3, and the circles in Fig. 4 show the use of this method.

In the results shown in Fig. 4 the diaphragms were soaked for 1 hr. in radioactive saline containing ^{24}Na. Creese (1964) has shown that in this time the specific activity of the tissue becomes equal to that of the surrounding saline. After washout for known times in inactive saline the activity in the muscle was measured (see Chapter II) and expressed as counts per min. per gram wet muscle. Since the specific activity was known by analysis of the radioactive saline it was possible to express the result as μ mole/g and this was a measure of that portion of the fibre sodium which remained in the muscle. The open circles of Fig. 4 show the results from several muscles from which the slope can be obtained and also, by retropolation, an estimate of the original fibre sodium. This estimate requires to be corrected for the effects of diffusion as described below.

Muscles were also equilibrated for 1 hr. in radioactive saline in the presence of strophanthin (10^{-4} g/ml), and then washed out in inactive saline which also contained strophanthin. The closed circles in Fig. 4 show the result of this experiment. Two distinct effects due to strophanthin can be seen from this figure. The outward movement of sodium is markedly slowed: from the slope of the two regression lines,

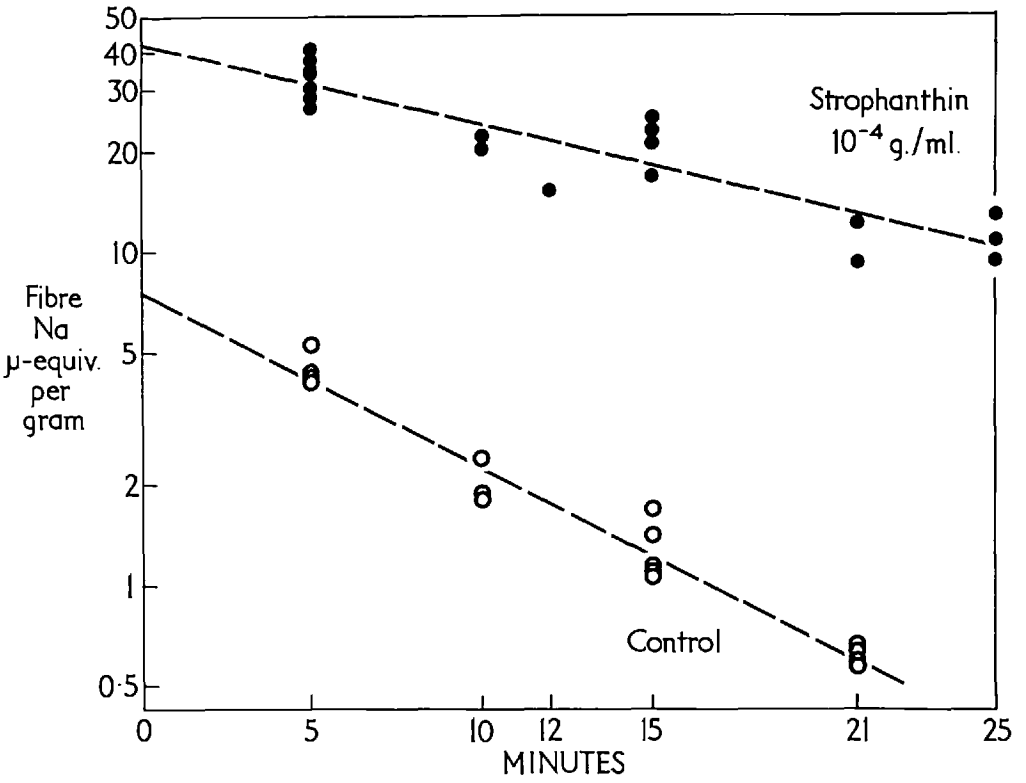

Fig. 4. Outward movement of sodium in diaphragm which had been loaded with ^{24}Na *in vitro* (1 hour). Each point represents a separate muscle which was counted after being exposed to inactive saline for a known time. The counts which remained have been expressed as μ-equiv g^{-1} (see text) and plotted on semilogarithmic paper against time. The rapid initial component (extracellular) is not shown because the washout lasted at least 5 minutes. The slope obtained from the control muscles (open circles) corresponds to a half time of 5.8 min. The closed circles show muscles treated throughout with strophanthin, which slowed the rate of sodium exchange, the mean half-time being 12.6 min. When the regression lines are retropolated to zero time it can be seen that the fibre sodium at the start of the washout is considerably increased by strophanthin.

the rate constants for controls (open circles) is 0.120 min^{-1} ± 0.0065 (S.D. of 16) and for strophanthin (closed circles) is 0.055 min^{-1} ± 0.0072 (S.D. of 19), these corresponding to half-times of 5.8 and 12.6 mins. respectively. The difference in these rate constants is significant ($P < 0.01$).

By retropolating the two curves back to zero time, a value of fibre sodium could be obtained which corresponds to the initial fibre sodium. By so doing, it is clear from Fig. 4 that the fibre sodium is highly increased by strophanthin, acquiring a value of 41.6 μ mole Na/g. wet tissue in contrast to a value of 7.5 for controls. These values require corrections as shown below.

The reversible action of strophanthin - total sodium and potassium

From these effects of strophanthin on sodium movement in rat diaphragm, it seemed that if such effects were proved to be reversible, this would throw light on the behaviour of this muscle towards sodium ions, and show whether or not mammalian muscle is really able to extrude sodium ions.

Muscles were soaked in strophanthin saline for 1 hr., and then allowed to recover for 1 hr. in strophanthin-free saline, the latter being renewed after 10 minutes to remove the strophanthin that might have accumulated into the medium used for recovery. After that the muscles were removed and

analysed for total sodium and potassium. These values were compared with values for muscles soaked in strophanthin saline for 1 hr., but which were not allowed to recover.

The third and fourth rows of Table 3 show the result of this experiment. It is clear that total sodium decreased by 16.5 μ mole/g wet tissue, and that total potassium increased by 20 μ mole/g wet tissue.

As will be seen later, fibre sodium recovery was followed for different periods of recovery, and it was desirable to follow total potassium recovery after different periods as well. The circles in Fig. 6 show the time course of total potassium recovery.

The potassium acquired its optimal recovery value after about 35 minutes and then remained unchanged for a further 40 min. The half-time was approximately 10-15 min., and it was difficult to make a more accurate estimate because of uncertainty about the origin (see Fig. 6). Potassium value after recovery for 35-80 mins. (79.9 μ mole/g wet tissue) is shown in the fourth row in Table 3.

In Table 3 the changes in sodium and potassium produced by recovery for 1 hour are significantly different from values obtained in the presence of strophanthin ($P < 0.01$ in each case).

Effect of recovery on sodium exchange and fibre sodium

The closed circles in Fig. 5 show results similar to the

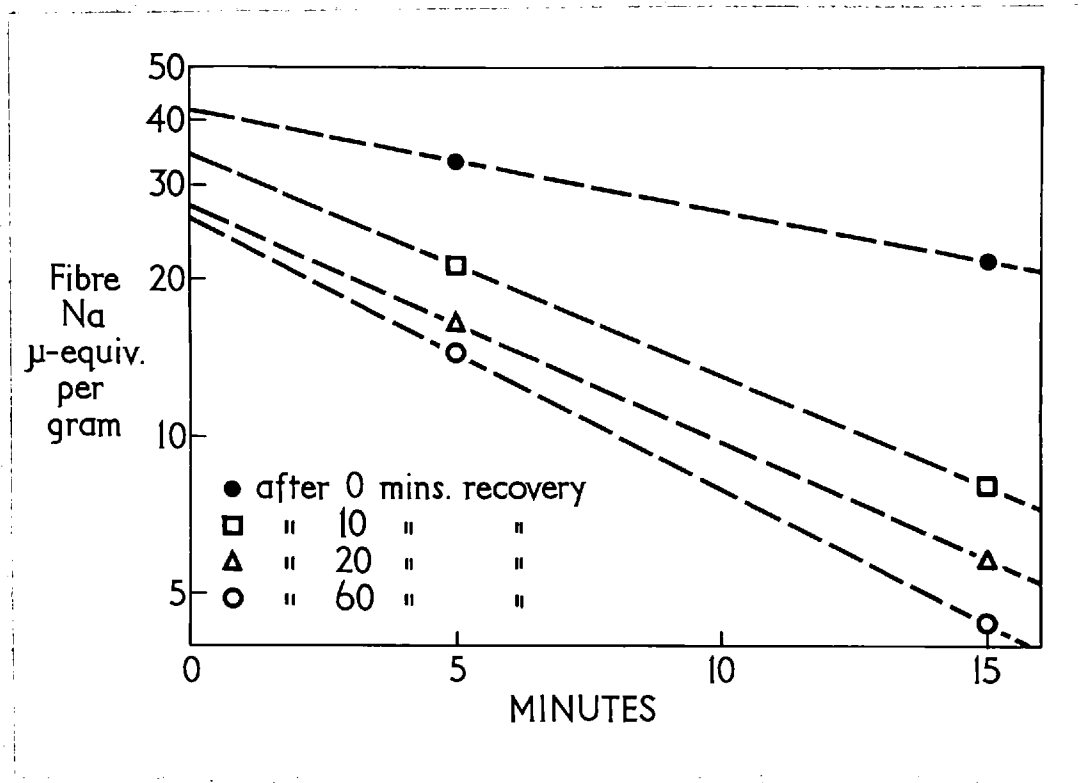

Fig. 5. Experiment to show net outward movement of fibre sodium in diaphragm muscles which were treated with strophanthin (1 hr) and allowed to recover. Each point represents the mean of 4-7 diaphragms. The muscles showed by the closed circles were treated with strophanthin-saline throughout. They were loaded with ^{24}Na and were passed through inactive saline for 5 or 15 min. The sodium which remained in the muscle has been plotted on semilogarithmic paper as in Fig. 4. The slope is similar to that of the closed circles in Fig. 4 and the fibre sodium, estimated by retropolation, is high. The other points represent muscles treated in strophanthin-saline (with ^{24}Na) and allowed to recover (in saline-^{24}Na but no strophanthin) for various times. These muscles show after recovery a faster rate of sodium exchange and a progressive diminution in fibre sodium (estimated by retropolation).

Table 4. Recovery of fibre Na and Na efflux in strophanthin-treated diaphragms

Recovery time min.	No. of muscles	Fibre Sodium (uncorrected) μmole g^{-1}	Time after changeover, (for rate measurement) min.	Half-time min.	Rate constant $min.^{-1}$
0	11	41.4	0	15.8	0.044
10	8	34	15-25	7.2	0.096
20	8	27.3	25-35	6.7	0.103
60	10	26.1	65-75	5.8	0.120

The values are those shown in Fig. 5.

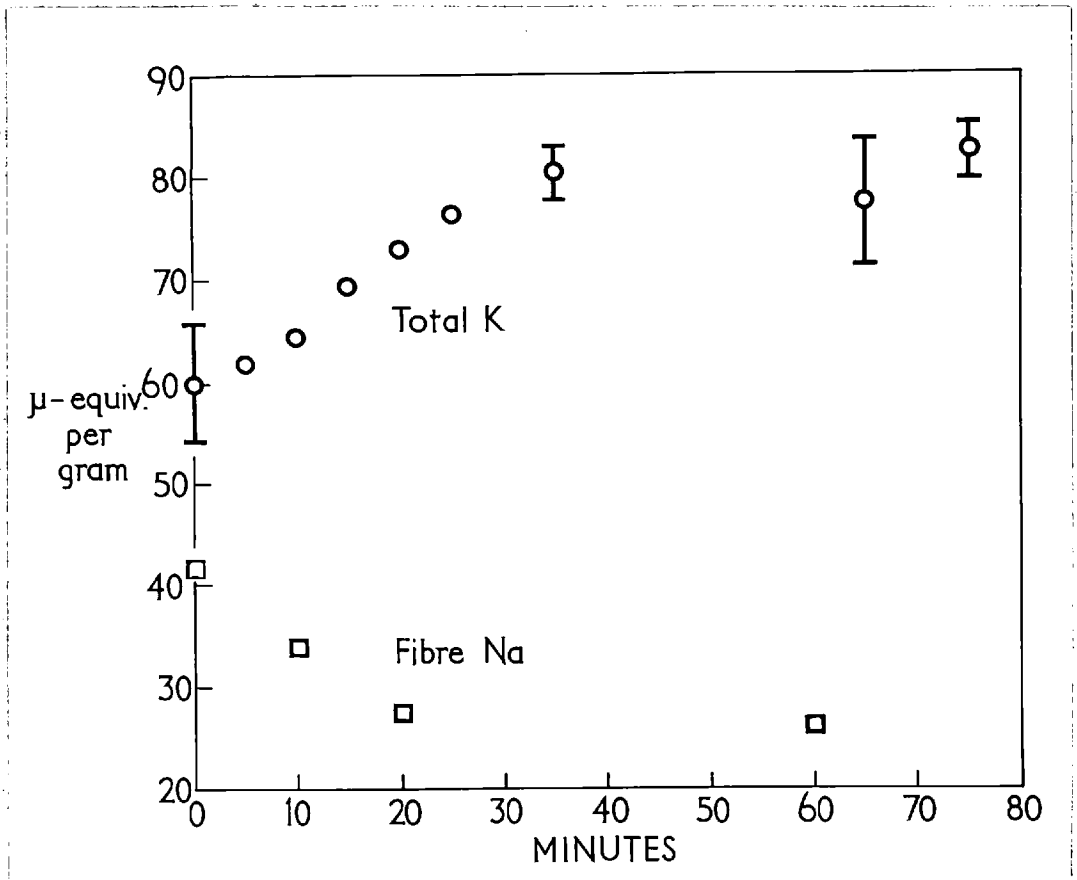

Fig. 6. Potassium and sodium in diaphragm muscles which had been treated with strophanthin (1 hr) and allowed to recover. The circles show total potassium (μ equiv g^{-1}) and the squares show fibre sodium obtained from the results of Fig. 5 (μ equiv g^{-1}). At zero time the potassium is low and the fibre sodium is high. The abscissa gives the time after the muscles were placed in strophanthin-free saline. Each point is the mean of 4-13 muscles and the limits give the standard deviation. There is a rise in total potassium and a fall in fibre sodium.

closed circles in Fig. 4 and represent the outward movement of labelled sodium from muscles in the presence of strophanthin (10^{-4} g/ml.). Each point gives the mean of 4-7 diaphragms and the washout periods (in inactive saline with strophanthin) were 5 or 15 min. On retropolation the fibre sodium was 41.4 μ mole/g and this represents the value after strophanthin.

The other curves in Fig. 5 were obtained after varying periods of recovery, and they show a progressive fall in value of fibre sodium and an increase in the rate of the outward movement of sodium. The squares in Fig. 5 represent muscles soaked for 1 hr. in radioactive saline with strophanthin, then allowed to recover for 10 min. in radioactive saline with no strophanthin, and finally passed through a succession of tubes containing inactive saline with no additions. The other curves give the results for recovery for 20 min. and 60 min.

Fig. 5 and Table 4 show the effect of recovery on sodium movements and fibre sodium studied by this experiment. It is clear that after 60 mins. recovery, the rate of sodium extrusion returned back nearly to its normal value. This can be seen from the increase of the rate constant from 0.044 $min.^{-1}$ at no recovery to 0.120 $min.^{-1}$ after 60 mins. recovery. This is close to the value for untreated muscles shown in Fig. 4. From the values of Fig. 5 and Table 4 it can be said that normal rate constant for sodium extrusion was achieved soon after 20 mins. recovery.

The squares in Fig. 6 show the time course of recovery of fibre sodium, obtained by retropolation from Fig. 5. It can be seen from this latter curve that fibre sodium recovery occurred with a half-time of approximately 10 mins.

The fibre sodium shows a progressive fall and these values require certain corrections as shown below.

Diffusion correction for fibre sodium

It was shown early in this chapter that ^{24}Na washout consisted of 2 portions, an initial fast portion followed by a slower one. These are believed to represent the exchange of extracellular and intracellular sodium respectively (Fig. 3). The second part of the curve was retropolated so that its intersection with the y axis was taken as an estimate for the fibre sodium at the beginning of the washout.

It has been shown by Harris & Burn (1949) and by Huxley (1960) that in this kind of experiment, fibre sodium obtained by such retropolation has to be corrected for the effects of diffusion. The true value of the fibre sodium is less than the apparent value.

The true values of fibre sodium are estimated as follows (Huxley, 1960).

The curve in Fig. 3 can be fitted by a double exponential curve, and the activity remaining in the muscle can be

described as the sum of two exponential values.

$$\text{Labelled sodium remaining} = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t} \quad \ldots\ldots (4)$$

where A and B are the fast and slow fractions, and λ_1 and λ_2 are the rate constants for the fast and slow fractions respectively.

The true slow fraction, which in the nomenclature of Huxley (1960) is termed P_{30}, is

$$P_{30} = \frac{AB(\lambda_1 - \lambda_2)^2}{A\lambda_1^2 + B\lambda_2^2} \quad \ldots\ldots\ldots (5)$$

and if P_{30} = apparent slow fraction/factor

$$\therefore \text{factor} = \frac{A\lambda_1^2 + B\lambda_2^2}{A(\lambda_1 - \lambda_2)^2}$$

$$= 1 + 2\frac{\lambda_2}{\lambda_1} + \left(\frac{\lambda_2}{\lambda_1}\right)^2\left(3 + \frac{B}{A}\right) + \left(\frac{\lambda_2}{\lambda_1}\right)^3\left(4 + 2\frac{B}{A}\right) \ldots \text{etc} \quad \ldots\ldots (6)$$

It can be seen that the value of $\frac{B}{A}$ has in practice little effect on the correction factor.

The mean of λ_1 was found to be 1.01 min.$^{-1}$ and this value has been accepted for calculation of the diffusion correction.

In Figs. 4 and 5, λ_1 will be taken as 1.01, also λ_2 and B are obtained from the curve. A, the apparent fast fraction is not known because the early part of the curve was not obtained. The total sodium (T) was, however, measured, and the fast fraction A can be put equal to T-B in equation 6. The corrected values of the fibre sodium are shown in Table 5.

DISCUSSION

Sodium extrusion in mammalian muscle without the use of abnormal solutions

The ability of a muscle to extrude sodium has been demonstrated by different methods. Probably the most widespread procedure involved the loading of muscle with sodium by immersion in K-free saline containing high sodium in the cold. When such muscle was reimmersed in a recovery saline containing the normal amounts of potassium and sodium, the muscle could extrude its extra sodium which had been accumulated during the 1st immersion. This method had been mostly used with amphibian muscle. Thus Dermedt (1953) had shown a net sodium extrusion in frog sartorius using this procedure. Carey,

Table 5. Fibre sodium corrected for diffusion

Experiment	Apparent Fibre Na B μ mole ml^{-1} wet muscle	Total Na T μ mole ml^{-1} wet muscle	λ_2 min^{-1}	$\frac{\lambda_2}{\lambda_1}$	A	$\frac{B}{A}$	Factor	Corrected Fibre Na P_{30} μ mole ml^{-1} wet muscle
Muscles in saline (Fig. 4)	7.91	46.8	0.120	0.1190	38.89	0.203	1.28	6.2
Strophanthin (Fig. 4)	43.9	86.9	0.055	0.0545	43.0	1.02	1.12	39.2
No recovery from strophanthin (Fig. 5 and Table 4)	43.7	86.9	0.044	0.0436	43.2	1.01	1.10	39.7
10 min. recovery (Fig. 5 and Table 4)	35.9	(79.1)	0.096	0.095	43.2	0.831	1.22	29.4
20 min. recovery (Fig. 5 and Table 4)	28.8	(71.7)	0.103	0.102	42.9	0.671	1.25	23.0
60 min. recovery (Fig. 5 and Table 4)	27.5	69.5	0.119	0.1178	42.0	0.655	1.30	21.2

The apparent fibre sodium B was obtained by retropolation to zero time in Figs. 4 and 5 (some terms are listed in Table 4). The values in μ mole g^{-1} have been converted to μ mole ml^{-1} by multiplying by 1.055, the specific gravity (Creese, 1954). The values of total sodium are taken from Table 3, where they are shown as μ mole g^{-1}. Interpolated values are shown in brackets. λ_1 was taken as 1.01 min^{-1} throughout. The following formulae are used, being adapted from Huxley (1960):

Fibre sodium = B/factor

$$\text{Factor} = 1 + 2\frac{\lambda_2}{\lambda_1} + \left(\frac{\lambda_2}{\lambda_1}\right)^2\left(3 + \frac{B}{A}\right) + \left(\frac{\lambda_2}{\lambda_1}\right)^3\left(4 + 2\frac{B}{A}\right)$$

A was taken as (Total sodium - B)

Conway & Kernan (1959) also demonstrated a net sodium extrusion in frog muscle under similar conditions, and they have emphasised the importance of reducing sodium and increasing potassium concentrations in the recovery solution so that sodium could be extruded.

In mammalian muscle, Dockry, Kernan and Tangney (1966) demonstrated net sodium extrusion by sodium-rich extensor digitorum and soleus muscles of rat by the use of similar methods.

In the present study net sodium extrusion has been demonstrated in mammalian muscle by the use of strophanthin 10^{-4}g/ml. From Table 3 it can be seen that the sodium content in the diaphragm muscle increased from 44.4 to 82.4 μeq/g in strophanthin after 1 hr, i.e. there was a gain of 38 μeqNa/g muscle. When the muscle was allowed to recover in exactly similar saline but without strophanthin, the sodium content fell from 82.4 to 65.9 μeq/g, i.e. there is a loss of 16.5 μeqNa/g wet muscle. This means that there is a net extrusion of sodium produced by the muscle when the drug was removed. This net sodium movement provides further evidence for the active extrusion of sodium in mammalian muscle against the electrochemical gradient.

These experiments show that the net sodium extrusion in mammalian muscle can be demonstrated without the use of abnormal solutions and without altering the ionic concentrations of the external saline. It is clear that with strophanthin, there was

an accumulation of sodium, and that removal of the drug from the outside solution caused the restoration of active extrusion.

Reversibility of the action of strophanthin on sodium exchange

In this study the effect of strophanthin on the outward movement of sodium in mammalian muscle has been shown to be reversible. Formerly there had been doubt concerning this reversibility of cardiac glycosides in muscle. Thus Edwards & Harris (1957) showed that the outward movement of sodium in frog muscle was decreased in strophanthin-saline, and increased after the removal of the drug. On the other hand, Johnson (1956), although he had demonstrated quite clearly an accumulation of sodium in frog muscle in ouabain saline, yet, in his procedure, no reversibility of ouabain action was shown.

The effect of strophanthin, and the reversibility of this effect in rat diaphragm muscle are shown in Figs. 4 and 5, and Tables 4 and 5. Normally sodium leaves the muscle with a rate constant of 0.120 mins.$^{-1}$ corresponding to a half-time of 5.8 mins, and when strophanthin 10^{-4}g/ml was applied, the rate constant was decreased to 0.055 min^{-1} corresponding to a half-time of 12.6 mins (see Table 5 and Fig. 4). Comparing these two values for sodium exchange after and before strophanthin we find that this compound reduced the outward movement nearly to

half. Edwards & Harris (1957) obtained a somewhat comparable value for frog muscle where strophanthin reduced the efflux of sodium by a factor of 1.6-2.2.

By the removal of the drug, the rate of sodium exchange increased gradually according to the time of recovery. This gradual increase in the rate constant can be seen in Fig. 5 and in Tables 4 and 5. The measurement of sodium exchange was done by stopping the washout in inactive saline (with or without strophanthin) after 5 or 15 mins. By this procedure it was shown that the effect of strophanthin on sodium exchange in mammalian muscle is reversible, and that after the removal of the drug the rate constant was largely restored after 20 mins of recovery.

There is a time lag in the recovery of the rate constant of sodium extrusion, and this is partly due to the interval required to wash the drug from the tissue. A lag can also be observed in the recovery of potassium (Fig. 6).

It can be seen from Fig. 6 that the fibre sodium decreased with a half-time of 5-10 mins, and that it became constant after 20 mins. recovery. Here it is interesting to mention that Desmedt (1953), by studying the time course of intracellular sodium recovery in frog muscle at room temperature, found nearly comparable results. He found that fibre sodium in frog muscle recovered with a half-time of 30 mins. and that it became steady after one hour.

Measurement of fibre sodium by retropolation

Fibre sodium can be obtained by subtracting the value for extracellular sodium from that of total sodium. This has considerable difficulties chiefly due to uncertainty about the extracellular space, and in the present study a different method has been adopted based on retropolation of washout of labelled sodium.

From Fig. 3 it can be seen that the curve of outward movement of sodium is composed of two parts, an initial rapid portion which represents the exchange of extracellular sodium, and a slower portion which presumably represents the exchange of fibre sodium. The method used to estimate fibre sodium involves the retropolation of the slow fraction back to zero time. This can be seen in Fig. 4 in which the radioactivity remaining in the muscle was converted into μmole/g wet muscle. This is possible because the specific activity of the muscle at the start of the washout was equal to the specific activity of the soaking saline (Creese, 1964), whence the counts could be converted into μmole sodium. The points have been plotted on a semilogarithmic scale against time, and by retropolation of the slow portion in Fig. 4 to zero we obtain the value of fibre sodium in μmole/g wet muscle at the time of removal of the muscle from the soaking saline. As mentioned in other places (see results and next section of discussion) such values

need correction for the effect of diffusion, and this has been done and shown in Table 5.

The usefulness of this method can be observed in Fig. 5 where it has been used to follow the changes occurring in the fibre sodium during the study of recovery, and of course it can be used in other comparable situations.

Double exponential curves and the measurement of fibre sodium

During the present study it has been shown that the radioactive sodium remaining in the muscle during a washout in inactive saline can be represented as a double exponential when plotted on semilogarithmic paper. Retropolation of the slower portion of the curve to zero time gives the apparent fibre sodium in μmole/g.

Huxley (1960) has shown that such estimate of fibre sodium is subject to an error due to interaction between the compartments. For this reason, a correction (Huxley, 1960) has been used in values of fibre sodium obtained by retropolation.

From Huxley (1960) it can be shown (see results) that the value of the correction can be obtained by dividing the value of the apparent fibre sodium by a factor which is equal to:

$$1 + 2\frac{\lambda_2}{\lambda_1} + \left(\frac{\lambda_2}{\lambda_1}\right)^2\left(3 + \frac{B}{A}\right) + \left(\frac{\lambda_2}{\lambda_1}\right)^3\left(4 + 2\frac{B}{A}\right) \ldots \text{etc} \ldots \quad \text{(equation 6)}$$

where A is the fast fraction, B is the slow fraction λ_1 and λ_2 are the rate constants for the fast and slow fractions respectively. This factor comes to 1.1 - 1.3 and its treatment and application in the present study are given in detail at the end of the section of results, and the figures before and after such a correction are given in Tables 4 and 5. Table 5 contains figures of fibre sodium corrected for the effect of diffusion. From this table it can be seen that the faster the exchange, the bigger is the term $\frac{\lambda_2}{\lambda_1}$, and consequently the correction is larger. For this reason it may be said that such a correction is less serious when studying ion movements in amphibian muscle where this movement is slower.

It seems that the need for such a correction depends on the thickness of the preparation and of course, it can be avoided altogether by working on a single fibre where there will be no extracellular space for the ion to diffuse through and in this case the movement of the ion will be directly between the cell interior and the outside solution through the cell membrane.

SUMMARY OF CHAPTER III

1. Rat diaphragm muscles soaked in saline containing strophanthin (10^{-4} g/ml) for 1 hr, showed an increase in total sodium from 44 to 82 μ mole/g and a decrease in total potassium from 86 to 60 μ mole/g wet.
2. The outward movement of sodium, as measured by ^{24}Na was slowed in strophanthin saline, the rate constant being 0.055 min^{-1} as compared with 0.120 min^{-1} for controls.
3. The measurements with ^{24}Na enabled the fibre sodium to be estimated as the slow fraction obtained from the washout of labelled sodium. After strophanthin the muscles had a higher fibre sodium, which was 39.2 μ mole/ml as compared with 6.2 μ mole/ml for controls (both values corrected for the effects of diffusion).
4. These effects of strophanthin were partly reversible:
   (a) After recovery for 1 hr, total sodium decreased from 82 to 66 μ mole g^{-1}. This represents net sodium extrusion.
   (b) Following recovery for different periods, fibre sodium decreased with a half-time of approximately 10 mins. and reached a final value of 21.2 μ mole/ml after 60 mins. recovery. After 35-80 mins. recovery total potassium increased to a final value which was within 10% of the initial value without strophanthin.
   (c) The rate of exchange of fibre sodium gradually returned and approached its initial value.

CHAPTER IV

SODIUM MOVEMENTS IN MOUSE TOE MUSCLE AND THE EFFECTS OF INSULIN AND OF STROPHANTHIN

Zierler (1959) found that insulin 0.1 u/ml increased the resting potential of rat extensor digitorum longus muscles *in vitro*, and caused a small rise in intracellular potassium. Kernan (1962b) found that insulin increased the net extrusion of sodium during recovery of frog sartorius muscles which had been loaded with sodium. Creese (1964) found that insulin increased the normal rate of sodium exchange in rat diaphragm.

The effect of insulin has been examined in the foot muscle of the mouse. This is a small fusiform muscle which can be studied without the complications produced by bone or rib. It contains however a variable amount of fibrous tissue.

It was also decided to see whether the effect of strophanthin on sodium movement could be demonstrated on this mouse toe muscle.

RESULTS

Muscle thickness and weight:

The muscle was dissected from anaesthetised mice as described in the methods. After dissection the muscle was laid quickly with its platinum wire, on a tile and its thickness

measured under a microscope after the divisions of its objective had been calibrated against a mm. ruler. The mean thickness was found to be 0.62 mm. (mean of 6, range 0.40 to 0.75). To obtain the weight, muscles were dissected, and quickly transferred to small stoppered glass tubes which have been previously weighed. The difference gave the weight of the muscle. The mean weight was found to be 2.5 mg (mean of 12, range 1.27 to 4.88).

Equilibration of ^{24}Na in mouse toe muscle in vitro:

Before studying sodium movement, it was essential to estimate the time required for its equilibration. For this reason, sodium uptake was estimated using ^{24}Na as follows: After dissection, the muscle was transferred to radioactive saline containing ^{24}Na. After a definite time it was dissolved and assayed for its radioactivity and total sodium. The soaking saline was also assayed for radioactivity and total sodium. From this, the uptake of ^{24}Na was estimated by dividing the specific activity of the muscle by that of the soaking saline, i.e.

$$\text{Uptake} = \frac{\text{Specific activity of muscle}}{\text{Specific activity of soaking saline}}$$

$$= \frac{\text{Counts per min. per mg. sodium in muscle}}{\text{Counts per min. per mg. sodium in soaking saline}}$$

After 1 hr. the uptake was 98.2% ± 2.39 (S.D. of 5) and after 2 hrs. it was 102% ± 1 (S.D. of 3).

During the present study the muscle was soaked for 2 hrs. to ensure complete equilibration.

Effect of insulin on the outward movement of sodium

Muscles were equilibrated in radioactive saline containing insulin (0.1 u/ml). (Insulin saline was prepared by dissolving the requisite amount of the drug in a few drops of 0.1 N HCl then into the appropriate volume of normal saline to give the required drug concentration. After equilibration, muscles were washed out in inactive saline containing insulin. The outward movement was measured on the same muscle whereby the radioactivity of the effluent in successive tubes, and finally the radioactivity remaining in the muscle, was estimated. Then the radioactivity in the muscle was added to the radioactivity of the effluent in each tube in a reversed direction to give the radioactivity remaining in the muscle after the elapse of a definite time of washout (cumulative). By this method, a curve similar to that in Fig. 3 was constructed by plotting the radioactivity remaining in the muscle against time on a semilog scale. Control muscles were used from the other foot of the same animal, right and left alternatively, and these were treated in the same manner but without insulin additions.

Fig. 7 shows the result of this experiment. The crosses show the insulin treated muscles, and the open circles show the controls. It is seen from this figure that the rate of outward movement of sodium is more rapid in insulin-treated muscles than in control muscles. These experiments were done on muscle pairs, right and left, from the same animal, one serving as a control alternatively. The individual figures for the half-times of these pairs of muscles are shown in Table 6. From this table it is clear that the half-time for outward sodium movement was decreased from 10.7 mins. for controls to 8.2 mins under the effect of insulin, which means that insulin had increased the rate of outward movement of sodium. The difference between the two half-times is significant ($P < 0.01$).

Fig. 8 also shows the difference in sodium movements between muscles treated with insulin (crosses) and controls (open circles), as well as the effect of strophanthin (closed circles) which will be described in the next section.

Effect of strophanthin on sodium, potassium and water content

Muscles were equilibrated either in normal saline, or in saline containing strophanthin (10^{-4} g/ml). They were then weighed, dried up and reweighed, the difference giving the water content which was then estimated as a percentage. The muscles were then analysed for total sodium and total

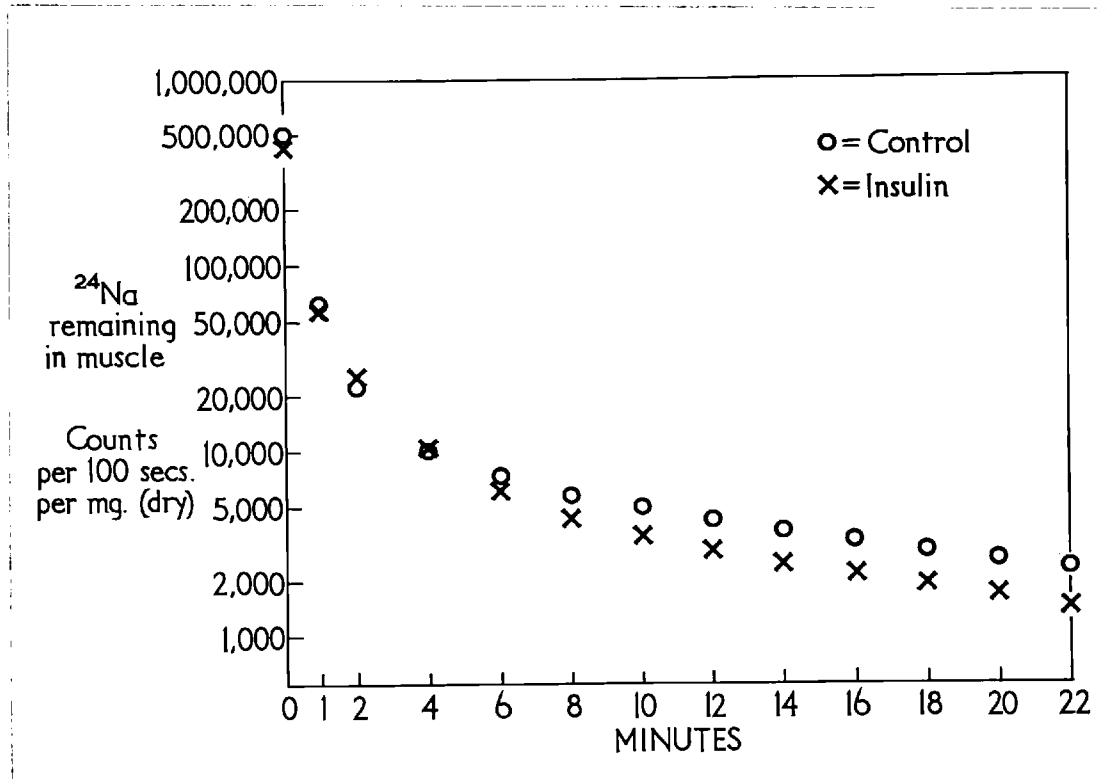

Fig. 7. Outward movement of sodium in right and left flexor digitorum brevis IV muscles of mouse. The muscles were loaded with ^{24}Na, and passed through tubes containing inactive saline. The tubes were later counted and the points show the cumulative counts/100 secs./mg. plotted on semilogarithmic paper against time. The open circles show the control muscle while the crosses represent the muscle from the other foot which was treated with insulin. Compared to Fig. 3 it is seen that the early part of the curve, which represents rapidly exchanging sodium, is large. The final slope which represents the exchange of fibre sodium is somewhat steeper in the case of the insulin-treated muscle (half-time 10.6 min) as compared to the control (half-time 12.8 min).

Table 6. Effect of insulin on sodium exchange in flexor digitorum brevis IV of mouse

	Half-times in minutes		
	Control	Insulin 0.1 u/ml	Difference
	14.7	12.7	- 2.0
	12.8	10.6	- 2.2
	6.7	6.9	+ 0.2
	14.2	10.0	- 4.2
	11.1	7.0	- 4.1
	9.2	4.8	- 4.4
	7.3	6.5	- 0.8
	9.6	6.8	- 2.8
Mean	10.7	8.2	= - 2.5

Toe muscles from the right and left limbs were dissected from each animal under anaesthesia, one muscle being treated with insulin and the other was used as a control. Insulin was used alternatively on the right and left muscle on 8 consecutive occasions. The half-time for sodium exchange was less in muscles treated with insulin (standard error of difference = 0.59, $t = 4.3$, $P < 0.01$).

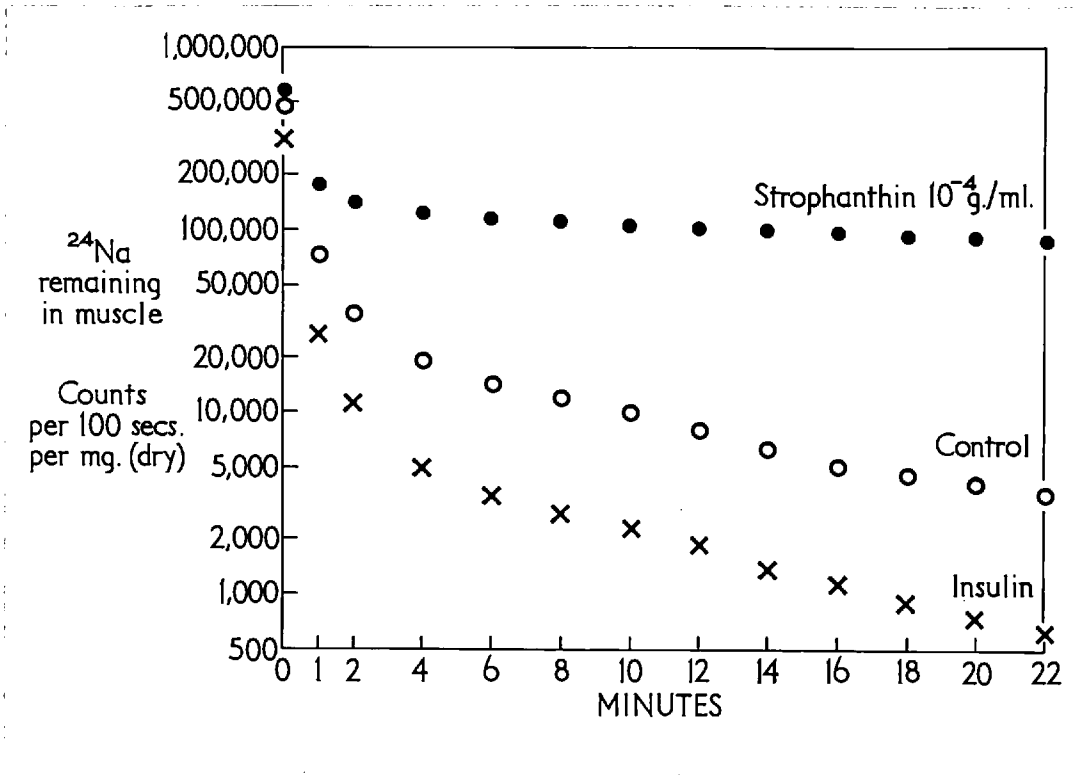

Fig. 8. Semilog plot as in Fig. 7 with an extra curve showing the movement of sodium in a mouse muscle treated throughout with strophanthin (closed circles). In presence of strophanthin, fibre Na exchanged at a slow rate, the half-time being 43.8 min. The exchange of the insulin-treated muscle (crosses, half-time 6.8 min) was more rapid than that of the control muscle from the other foot (open circles, half-time 9.6 min.).

potassium. Table 7 shows the results, and the values *in vivo* as well. It can be seen that strophanthin, after 2 hrs., caused an increase in water content by 2.3%, an increase in total sodium by 33.3 µmole/g wet and a decrease in total potassium by 37 µmole/g wet.

Effect of strophanthin on sodium movements in muscle and tendon

Muscles were equilibrated in radioactive strophanthin saline (10^{-4} g/ml), and then washed out in the same manner described before (see effect of insulin, and also Chapter II), and this washout was carried out in inactive saline containing strophanthin.

The closed circles in Fig. 8 show the effect of strophanthin on the outward movement of sodium, which is greatly slowed. The mean half-time for sodium exchange in the presence of strophanthin was found to be 34.8 mins. ± 9.3 (S.D. of 8). The mean half-time for control muscles was taken from the controls of insulin experiments, namely 10.7 mins. Fig. 8 also shows the effect of insulin, as described above.

Sodium exchange was studied in the tendon of the flexor digitorum longus IV of the mouse. This tendon is larger than that of the flexor digitorum brevis IV. Experiments were carried out as in the case of the muscle except that the tendon was dissected *in vitro*.

Fig. 9 shows outward movement of sodium in a control

Table 7. Effect of strophanthin 10^{-4} g/ml on water, sodium and potassium content in mouse flexor digitorum brevis IV

	Water %	S.D.	No.	Sodium Content	S.D.	No.	Potassium content	S.D.	No.
In vivo	75.8	±1.8	6	71.5	± 7.8	6	65.9	±10.0	6
In vitro	77.9	±2.7	18	108	±26.8	18	52	± 8.4	18
After strophanthin	80.2	±1.2	9	141.3	±24	9	15	± 4.04	9

Mean values for sodium and potassium are given in µmole/g wet weight ± S.D.

Muscles in vitro and after strophanthin were soaked for 2 hrs.

Sodium and potassium values after strophanthin were obtained from values for dry weight by multiplying by a factor to give the values for wet weight.

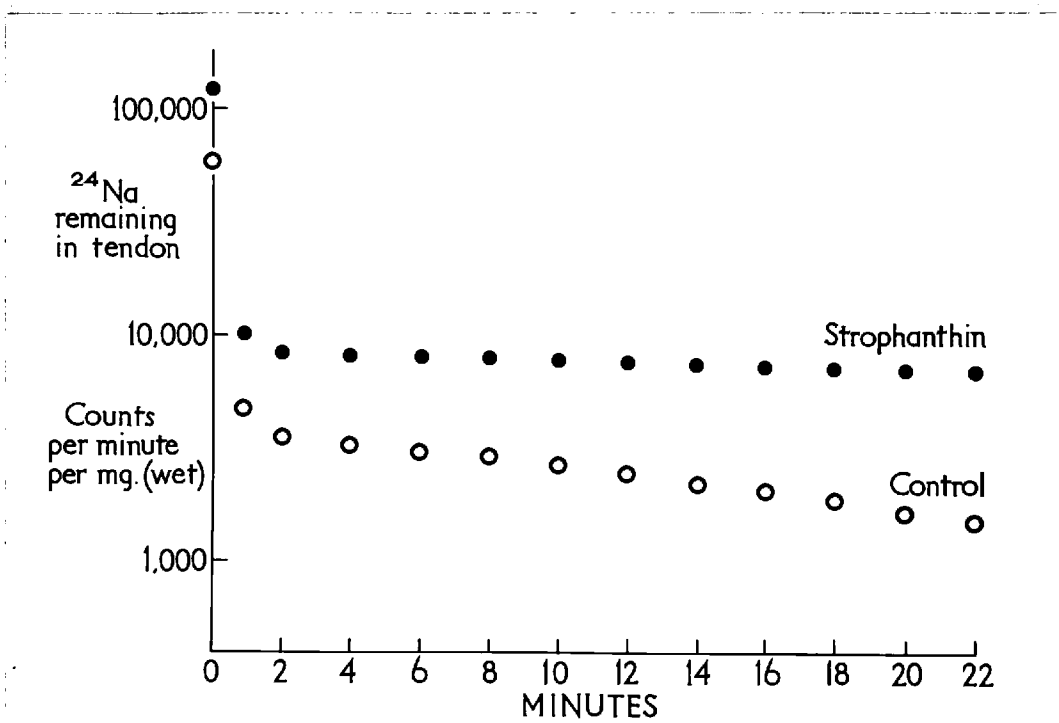

Fig. 9. Effect of strophanthin 10^{-4} g/ml on the outward movement of ^{24}Na from the tendon of the flexor digitorium longus IV of the mouse. Open circles represent the curve of normal washout in a control tendon. Fibre Na exchanged with a half-time of 12.1 min. Closed circles represent the curve of a tendon equilibrated in ^{24}Na saline with strophanthin, then washed out in inactive saline also with strophanthin. Strophanthin markedly slowed the outward movement of sodium and the half-time was 67.6 min.

(open circles) and a strophanthin-treated tendon (closed circles). The normal washout curve (open circles) resembles that of the mouse toe muscle except that the initial fraction which exchanges rapidly is smaller.

This figure shows that the exchange of sodium had been markedly reduced under the effect of strophanthin (closed circles), the half-time being 67.6 mins, as compared to the control tendon (open circles), the half-time being 12.1 mins.

DISCUSSION

Use of mouse toe muscle

There are two main advantages in the use of this muscle. Firstly the absence of rib which made it possible to measure the sodium exchange on the same muscle and secondly, it was also possible to use control muscles from the same animal, from the right and left feet alternatively.

This muscle contains a variable amount of fibrous tissue and this probably accounts for the high sodium and low potassium content. Thus from Table 7 it can be seen that sodium content is 71.5 μmole/g and potassium content 65.9 μmole/g. These values can be compared with the sodium and potassium content of rat diaphragm which are 42.2 and 93.6 μmole/g respectively (see Table 3).

Open circles in Figs. 7 and 8 show sodium movements in this muscle from which it can be seen that the initial fast

fraction is large and prolonged, and that the exchange of fibre sodium is slower than in the case of rat diaphragm, the half-time being 10.7 min here as compared to 5.8 min in the diaphragm. These curves of normal washout of sodium show that it is difficult to decide how to retropolate the slow fraction, and hence there is uncertainty about the sizes of both intracellular and extracellular compartments. Keynes (1954) has found similar results in the case of the frog toe muscle and he gave several possible reasons for this but referred to the difficulty of deciding between these possibilities. It seems that the fibrous tissue makes the separation between extracellular and intracellular portions of the curve less clear. In order to get the most reliable estimate for sodium exchange of the slow fraction the half-time was calculated between 14 and 22 min. and in this interval there was no change in the slope.

Effect of Strophanthin on sodium exchange in mouse muscle

Strophanthin in high doses has been shown to give effects on this muscle similar to those described in the rat diaphragm. Thus from Table 7 it can be seen that sodium content increased from 108 to 141.3 μmole/g, and potassium content decreased from 52 to 15 μmole/g. The sodium exchange was also affected. From Fig. 8 it can be seen that sodium movement is markedly slowed under the effect of strophanthin 10^{-4} g/ml (closed

circles). The half-time was increased from 10.7 mins to 34.8 min. Comparing these values with those of rat diaphragm (see Chapter III), namely 5.8 min (controls) and 12.6 min (strophanthin), it can be said that strophanthin approximately doubled the half-time in the rat diaphragm, and increased it three times in the toe muscle of the mouse.

As mentioned above, the rapidly exchanging fraction in the mouse toe muscle is large and prolonged as compared to the rat diaphragm. This is attributed to the fibrous tissue, and this concept is in agreement with Keynes (1954) who included the effect of fibrous tissue when discussing the interpretation of outflow curves in toe muscles of the frog.

From Fig. 8, it can be observed that strophanthin diminished the rapidly exchanging fraction of sodium in the whole muscle. This may be due to the fact that strophanthin also acts to slow the exchange of sodium in the fibrous tissue (see Fig. 9).

The effect of insulin on sodium movements

Fig. 7 shows that insulin (0.1 u/ml) increased the rate of sodium exchange in the toe muscle of the mouse. Table 6 gives the half-times in muscle pairs for both insulin-treated and control muscles. From this table it can be seen that insulin decreased the half-time from 10.7 min to 8.2 min. These results are in agreement with those of Creese (1964)

who found that insulin decreased the half-time for sodium in rat diaphragm from 5.7 min to 4.2 min. He also observed an increase in potassium content in insulin-treated diaphragm. It is interesting that Creese et al. (1961) found that insulin maintained sodium content at a low level in rat diaphragms. From these results on rat diaphragm and the mouse toe muscle, and from the results of Zierler (1957, 1959) on the effect of insulin on the resting potential and potassium content in rat muscle, it can be said that there is an inter-relation between the outward movement of sodium, the resting potential, and potassium content in mammalian muscles. Kernan (1962b) found that insulin increased the extrusion of sodium during the recovery of sodium-rich frog muscle, and this shows a direct effect of insulin on the sodium pump mechanism.

SUMMARY OF CHAPTER IV

1. Analysis of the flexor digitorum brevis IV of the mouse *in vivo* showed a sodium content (71.5 μ mole/g) which was high, and a potassium content (65.9 μ mole/g) which was low as compared to rat diaphragm.
2. Sodium uptake measured by ^{24}Na was found to be complete after 1 hour, and the specific activity of the tissue became equal to that of the external solution.
3. In the presence of insulin radioactive sodium exchanged more rapidly, the half-time being reduced from 10.7 min to 8.2 min (mean values from 8 pairs of muscles, tested by differences).
4. In the presence of strophanthin (10^{-4} g/ml) the sodium content was increased from 108 to 141 μ mole/g, and the potassium decreased from 52 to 15 μ mole/g. The rate of exchange of sodium was slowed, the half-time being increased from 10.7 min to 35 min (mean of 8). Strophanthin (10^{-4} g/ml) also markedly slowed the exchange of sodium in the tendon of flexor digitorum longus IV.

CHAPTER V

OUTWARD MOVEMENT OF SODIUM IN DENERVATED DIAPHRAGM MUSCLE OF THE RAT

In denervated mammalian muscle a fall in membrane potential has been observed (Lüllmann, 1958; Thesleff, 1963), and according to Klaus, Lüllman and Muscholl (1960) there was no change in potassium content after denervation. The latter authors showed that the rate of potassium exchange in denervated muscle is decreased. It seemed that the fall in the resting potential in mammalian muscle might be due partly to an increase in sodium permeability (see equation 2 in Chapter I), and experiments were designed to compare the outward movement of sodium in normal and in denervated rat diaphragm muscle.

METHODS

For the study of sodium movement in normal and denervated rat diaphragm, the design of the experiment had to be modified to obtain statistically reliable results. In what follows, a brief account of the methods used for this study is given, the other general procedures being fully described in Chapter II.

To study the effects of denervation, the experiment was

designed as a symmetrical 4-point assay. Four rats, each weighing about 50 g were selected from the same batch, and in two of them, the left phrenic nerve was avulsed as described in Chapter II. After 8 days, the rats weighed 70-90 g. The left hemi-diaphragm was dissected out, equilibrated in ^{24}Na saline for 1 hour and then passed through tubes containing inactive saline for 6 or 16 min. The radioactivity remaining in the muscle was then measured and expressed as counts/min/g. As the specific activity at the start of the washout was equal to that of the soaking saline (Creese, 1964), this radioactivity could then be converted into μmole/g wet muscle as in the previous results, and this gave a measure of the fibre sodium which remained after 6 or 16 min in the case of normal and denervated muscle. This process was repeated, and the mean values then plotted on semilogarithmic paper. The statistical procedures used to analyse the results are described later.

RESULTS

Effect of denervation on water, sodium and potassium content

Table 8 shows the effect of denervation of rat diaphragm (8 days) on water, sodium, and potassium content both *in vivo* and *in vitro*. The water content was significantly increased *in vivo* and *in vitro*. The sodium content was little affected

Table 8. Water, sodium and potassium content of normal and denervated diaphragm muscle (8 days) from rats of 70-90 g.

Experiment		Water content mg/g.	S.D.	No.	Sodium content µmole/g	S.D.	No.	Potassium content µmole/g	S.D.	No.
In vivo	Normal	777	±6.0	12	35.7	±2.6	12	96.4	±3.6	12
	Denervated	*795	±2.8	12	35.4	±1.2	12	97.0	±2.0	12
In vitro	Normal	777	±5.6	10	47.8	±4.4	15	85.7	±6.5	20
	Denervated	*795	±5.7	10	55.3	±5.9	12	85.4	±6.5	20

* Values marked with an asterisk are significantly different from controls ($P < 0.01$)

in vivo but marked gain of sodium was observed *in vitro*. The potassium content was not affected by denervation, but all muscles showed some fall *in vitro*.

Effect of denervation on outward movement of sodium

Fig. 10 shows the result of denervation on the outward movement of ^{24}Na where the radioactivity remaining in the muscle is plotted against time. Each point gives the mean of 8 muscles, so that this figure shows the result of 32 diaphragms. The open circles represent denervated muscles while the closed circles represent controls. In Fig. 10 the slope shown by the open circles (denervated muscles) is steeper than that formed by the controls, and the two lines cross each other. In Table 9 are shown the rate constants for sodium exchange in normal and denervated muscles which are 0.128 min^{-1} (half-time 5.4 min) and 0.171 min^{-1} (half-time 4.1 min) respectively. In the same table the fibre sodium obtained by retropolating both regression lines back to zero is also given. In denervated muscles, fibre sodium appears to be greater than in controls, the values being 12.9 and 8.7 $\mu mole\ g^{-1}$ respectively. These values represent the apparent fibre sodium at the start of the washout and need to be corrected for the effect of diffusion. This correction has been done by methods similar to those described in detail in Chapter III, and will be referred to later.

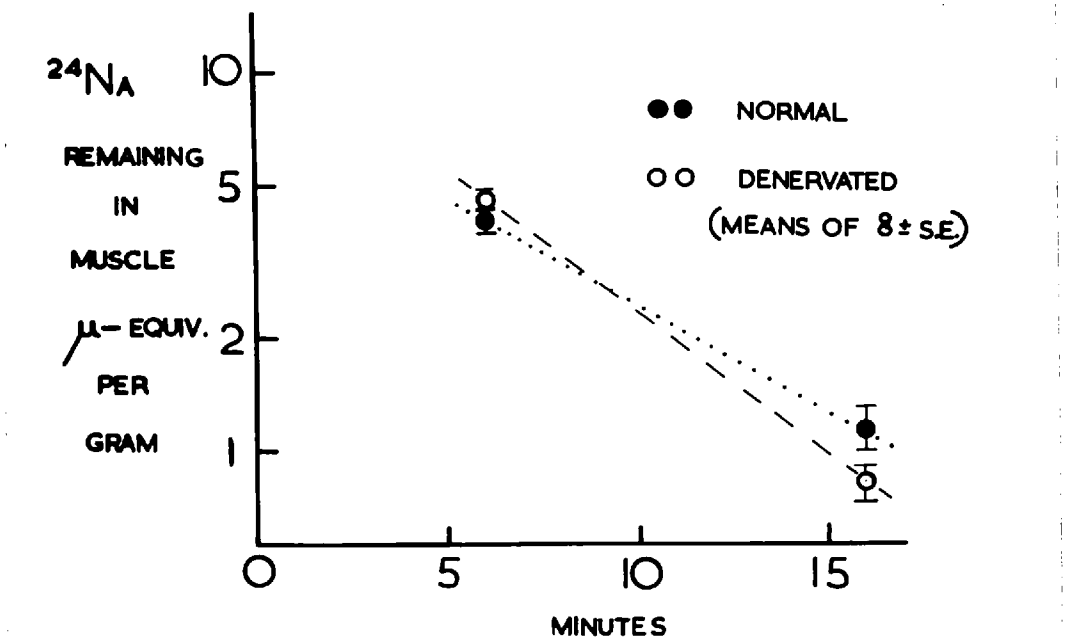

Fig. 10. Effect of denervation on outward movement of ^{24}Na from rat diaphragm. The circles show the ^{24}Na remaining in the muscles (as described in Chapter III) after washout for 6 or 16 min plotted on a semilogarithmic scale. The early part of the curve is not recorded. Each point is the mean of 8 muscles and the limits are the S.E. Closed circles show control diaphragms, and open circles show diaphragm which were denervated 8 days previously. The outward movement of ^{24}Na is more rapid in denervated muscles (open circles), the half-time being 4.1 min, as compared to 5.4 min for control diaphragms (closed circles).

Table 9. Effect of denervation on the outward movement of sodium in diaphragm of rat (70-90g)

Experiment	Rate constant min^{-1}	S.D.	No.	Apparent fibre Na µmole/g	Corrected fibre Na µmole/ml
Normal	0.128	±0.0156	16	8.75	6.22
Denervated	*0.171	±0.0129	16	12.9	7.71

The rate constants were obtained from the regression lines. For normal muscle, the summed values for 8 muscles were 4.877 and 0.446 log_{10} units for 6 min and 16 min respectively (see Table 10). This gives a slope of -0.05539 log_{10} units/min. The rate constant k is 2.3 times the slope or 0.128 min^{-1}. The half-time is 0.693/k or 5.4 min. Values for denervated muscle were obtained similarly. The calculationsof the S.D's are described in the text and shown in Tables 11 and 12.

The apparent fibre sodium was obtained by retropolating the regression lines to zero time, and the corrected fibre sodium is given in µmole/ml muscle. The method of calculating the corrected fibre sodium is described in detail in Chapter III.

Values marked with an asterisk differ significantly from controls ($P < 0.01$).

Statistical analysis of the effect of denervation on the outward movement of sodium

It has been mentioned previously that the experiment was designed as a symmetrical 4-point assay. The 32 results were converted into logarithms and analysed by contrast orthogonal method (Finney, 1952).

The simplest symmetrical design for parallel line assays is the 4-point assay in which only two treatments of each kind of muscle, namely the standard (normal) and the unknown (denervated), are included. The four treatment combinations may be designated as N_1, N_2, D_1 and D_2 respectively for the effects obtained from 6 min and 16 min washout of the standard (normal muscles) N_1 and N_2, and for the unknown (denervated) D_1 and D_2 respectively. Finney (1952) uses symbols S_1, S_2, U_1 and U_2 respectively. The values are treated as in Table 10, and the contrasts are denoted by the letter L with some distinguishing affix. As shown in Table 10 the contrasts are obtained for each group of 4 rats (2 normals and 2 denervated). These contrasts, L_p, L_1 and L_1' give the contrasts for the amounts, combined slope and divergence respectively. The contrasts are then summed, and it can be seen that the total divergence of the regressions is -1.517 $\log_{10}$ units.

For the estimation of the standard error (S.E.) the contrasts are squared, summed and the error variance calculated, from which the standard error is estimated. The calculations

Table 10. Fibre sodium in normal and denervated rat diaphragm in an assay of sodium exchange

	Values for fibre sodium (μmole/g, in $\log_{10}$ units)				Contrasts			Contrasts squared		
	Normal		Denervated		L_p $-N_1-N_2$ $+D_1+D_2$	L_1 $-N_1+N_2$ $-D_1+D_2$	L_1' N_1-N_2 $-D_1+D_2$			
	6 min N_1	16 min N_2	6 min D_1	16 min D_2	N and D amounts	Combined slope	Divergence	$(L_p)^2$	$(L_1)^2$	$(L_1')^2$
(1)	0.750	0.114	0.812	-0.012	-0.064	-1.460	-0.188	0.00410	2.13160	0.03534
(2)	0.593	0.223	0.677	-0.048	-0.187	-1.095	-0.355	0.03497	1.19902	0.12602
(3)	0.643	0.328	0.629	0.127	-0.215	-0.817	-0.187	0.04622	0.66749	0.03497
(4)	0.622	0.021	0.640	0.072	0.069	-1.169	0.033	0.00476	1.36656	0.00109
(5)	0.456	-0.149	0.603	-0.173	0.123	-1.381	-0.171	0.01513	1.90716	0.02924
(6)	0.648	-0.123	0.634	-0.312	-0.203	-1.717	-0.175	0.04121	2.94809	0.03063
(7)	0.687	0.053	0.684	-0.154	-0.210	-1.472	-0.204	0.04410	2.16678	0.04162
(8)	0.478	-0.021	0.613	-0.156	0.000	-1.268	-0.270	0.0000	1.60782	0.07290
$S(N, D, L \text{ or } L^2)$	4.877	0.446	5.292	-0.656	-0.687	-10.379	-1.517	0.19049	13.99452	0.37181
$S^2(L)$					0.47197	107.72364	2.30129			
$S^2(L)/8$					0.05900	13.46545	0.28766			
$S(L^2)-S^2(L)/8$								0.13149	0.52907	0.08415

Sum of squares due to squared contrasts = 0.13149 + 0.52907 + 0.08415 = 0.74471

Degrees of freedom = 21. The error variance is s where

$4s^2 = 0.74471/21 = 0.035462$ and $s^2 = 0.008866$ $\quad s = 0.09416$

S.E. (standard error) of L_p and L_1' = $sN^{\frac{1}{2}} = 0.09416 \times (32)^{\frac{1}{2}} = 0.09416 \times 5.657 = 0.533$

Divergence = -1.517 $\log_{10}$ units ± 0.533 $\quad t = 1.517/0.533 = 2.85$, degrees of freedom = 21 ∴ $P < 0.01$.

are given in Table 10. It can be seen that the divergence L'_1 totals -1.517 $\log_{10}$ units and the standard error is 0.533. Hence by applying a t-test, a divergence of this magnitude is unlikely to be fortuitous ($P < 0.01$).

Calculation of the standard deviation (S.D.) of the rate constants.

Since there were only two points in each regression the mean slope is easily calculated and the rate constants are shown in Table 9. The variation from regression is (Bernstein & Weatherall, 1952):

$$s^2_{y.x} = \frac{S(y-Y)^2}{n-2}$$

where y is the variable (in $\log_{10}$ units), Y is the value of the regression and n is the number of observations. Since there were only two points in the x axis (6 min and 16 min), Y is equal to $\bar{y}$ the mean value at any time, and the variance from regression is calculated as in Tables 11 and 12.

The variance of the mean slope is:

$$\text{variance from regression} / S(x-\bar{x})^2$$

and this has been calculated in Tables 11 and 12. The square

Table 11. Calculation of standard deviation of the rate constant of outward movement of sodium in normal rat diaphragms

	6 min washout			16 min washout		
	y	$y-\bar{y}$	$(y-\bar{y})^2$	y	$y-\bar{y}$	$(y-\bar{y})^2$
1)	0.750	-0.140	0.019600	0.114	0.058	0.003364
2)	0.593	0.017	0.000289	0.223	0.167	0.027889
3)	0.643	0.033	0.001089	0.328	0.272	0.073984
4)	0.622	0.012	0.000144	0.021	0.035	0.001225
5)	0.456	-0.154	0.023716	-0.149	0.205	0.042025
6)	0.648	0.038	0.001444	-0.123	0.179	0.032041
7)	0.687	0.077	0.005929	0.053	0.003	0.000009
8)	0.478	-0.132	0.017424	-0.021	0.077	0.005929
	$S(y) = 4.877$		$S(y-\bar{y})^2 = 0.069635$	$S(y) = 0.446$		$S(y-\bar{y})^2 = 0.186466$
	$\frac{S(y)}{8} = 0.610$			$\frac{S(y)}{8} = 0.056$		
	$= \bar{y}$			$= \bar{y}$		

Variance from regression $s^2_{y.x} = \frac{S(y-\bar{y})^2}{n-2}$ for 6 min + $\frac{S(y-\bar{y})^2}{n-2}$ for 16 min.

$= 0.069635/14 + 0.186466/14 = 0.004974 + 0.013319 = 0.018293$

Variance of mean slope $= \frac{s^2_{y.x}}{S(x-\bar{x})^2} = \frac{0.018293}{400} = 0.00004573$

S.D. $= \sqrt{0.00004573} = 0.006762$ $\log_{10}$ units $= 0.006762 \times 2.3036 = 0.0156$ $\log_e$ units

∴ Rate constant for normal diaphragms $= 0.128 \text{ min}^{-1} \pm 0.0156$

N.B. y = Fibre Na remaining in muscle after washout for 6 or 16 min (μequiv/g) expressed as $\log_{10}$ units (N_1 and N_2 values in Table 10). $\bar{y}$ = mean of y values. n = no. of observations = 16. $n-2$ = no. of degrees of freedom = 16-2 = 14. x = time of washout (6 or 16 min). $\bar{x}$ = mean of time of washout = $[(6 \times 8) + (16 \times 8)]/16 = 11$. $S(x-\bar{x})^2 = 8(6-11)^2 + 8(16-11)^2 = 16 \times (5)^2 = 400$.

Table 12. Calculation of standard deviation of the rate constant of outward movement of sodium in denervated rat diaphragms

	6 min. washout			16 min washout		
	y	$y-\bar{y}$	$(y-\bar{y})^2$	y	$y-\bar{y}$	$(y-\bar{y})^2$
1)	0.812	0.150	0.022500	-0.012	0.070	0.004900
2)	0.677	0.015	0.000225	-0.048	0.034	0.001156
3)	0.629	-0.033	0.001089	0.127	0.209	0.043681
4)	0.640	-0.022	0.000484	0.072	0.154	0.023716
5)	0.603	-0.059	0.003481	-0.173	-0.091	0.008281
6)	0.634	-0.028	0.000784	-0.312	-0.230	0.052900
7)	0.684	0.022	0.000484	-0.154	-0.072	0.005184
8)	0.613	-0.049	0.002401	-0.156	-0.074	0.005476
	$S(y) = 5.292$		$S(y-\bar{y})^2 = 0.031448$	$S(y) = -0.656$		$S(y-\bar{y})^2 = 0.145294$
	$\frac{S(y)}{8} = 0.662$			$\frac{S(y)}{8} = -0.082$		
	$= \bar{y}$			$= \bar{y}$		

Variance from regression $s^2_{y.x} = \frac{S(y-\bar{y})}{n-2}$ for 6 min + $\frac{S(y-\bar{y})^2}{n-2}$ for 16 min

$= 0.031448/14 + 0.145294/14 = 0.002246 + 0.010378 = 0.012624$

Variance of mean slope $= \frac{s^2_{y.x}}{S(x-\bar{x})^2} = \frac{0.012624}{400} = 0.00003156$

S.D. $= \sqrt{0.00003156} = 0.005618$ $\log_{10}$ units $= 0.005618 \times 2.3036 = 0.0129$ $\log_e$ units

$\therefore$ Rate constant for denervated diaphragms $= 0.171$ min$^{-1} \pm 0.0129$

N.B. The meaning of the symbols are the same as those in Table 11.

root gives the standard deviation, and when this is multiplied by 2.3 the S.D. of the rate constant is obtained and has been listed in Table 9.

Corrected values of fibre sodium:

It was shown in Chapter III (Diffusion correction for fibre sodium) that the apparent fibre sodium obtained by retropolation back to zero needs to be corrected for the effect of diffusion whereby the true fibre sodium was accordingly calculated using Huxley's nomenclature (1960). In this Chapter, also the fibre sodium obtained by such retropolation (Fig. 10) had to be corrected for the effect of diffusion, and the same nomenclature (Huxley, 1960) was used, and the same equations applied. The animals weighed 70-90 g at the time of the study, and the half-time of the fast fraction was similar to the values of Creese (1954), namely 1 min, and so the initial rate constant was considered as 0.693 min^{-1} (0.693/1). The specific gravity of the muscles was taken as 1.07 and 1.055 for normal and for denervated diaphragm respectively (Klaus et al., 1960). The corrected values of fibre sodium in normal and denervated muscles are included in Table 9.

DISCUSSION

In the present study the rate of outward movement of sodium has been shown to be increased in denervated rat

diaphragm and this was associated with some alterations in water and ion content. The water content was increased in denervated muscle *in vivo* and *in vitro*. Humoller *et al.* (1950) and Suftin *et al.* (1954) have shown an increase in water content in denervated muscle *in vivo*. The denervated diaphragm muscle shows a small increase in sodium content *in vitro* and this is in agreement with the results of Lüllmann (1958). Table 9 shows values of fibre sodium estimated by compartmental analysis, and it appears that there is an increase in fibre sodium in the case of denervated muscles. These estimates of fibre sodium are probably more reliable than other methods based on the difference between total sodium and the extracellular sodium as obtained by inulin and other markers.

In denervated rat diaphragm the rate of potassium exchange was slowed and there was no change in potassium content (Klaus *et al.*, 1960). At the same time there was a fall in membrane potential of denervated muscles. From the calculated flux values, the intracellular potassium concentration, and the membrane potential, the potassium permeability in denervated muscles was found to be decreased, the value of P_k (the permeability constant of potassium) being $\frac{1}{2}$ - $\frac{2}{3}$ that of control muscles. Denervated muscles show fibrillation which might affect the sodium exchange, but it was shown that fibrillation stops after twenty minutes *in vitro* (Thesleff, 1963). In the present study on denervated diaphragm, the outward movement of

sodium was measured after soaking the muscle at least for 1 hr.

The oxygen consumption is increased in denervated muscle *in vivo*, and probably *in vitro* (Bass, 1962), and it was shown by Stewart (1955) that the protein content of denervated diaphragm is increased. The uptake of the depolarizing drug decamethonium was shown to be increased in denervated guinea-pig diaphragm (Taylor, Creese, Niedergaard and Case, 1965). The present experiments showed an increased rate of outward movement of sodium in denervated rat diaphragm, together with some increase in the fibre sodium (Table 9). The efflux is (see Keynes and Lewis, 1951):-

$$\text{Efflux} = k \frac{V}{A} [Na]_i \quad \cdots \quad (7)$$

where k is the rate constant for outward movement, $\frac{V}{A}$ is the ratio of volume to area of the fibre, and $[Na]_i$ is the internal concentration of sodium. Since k, $[Na]_i$ and also $\frac{V}{A}$ (Klaus *et al.*, 1960) are increased in denervated muscle, the sodium efflux is also increased. If a steady state occurs *in vitro*, this may be taken to indicate an increase also in the influx of sodium and hence it is likely that the sodium permeability is also increased. The detailed calculation of permeability also includes a term for the resting potential, which was not measured in the present study. It therefore appears that, in denervated muscle, sodium permeability is increased while potassium permeability is decreased.

Now, the resting potential can be obtained from equation (2) in Chapter I as follows (see also Hodgkin, 1958):-

$$E = -60 \log_{10} \frac{[K]_i + b[Na]_i}{[K]_o + b[Na]_o} \quad . \quad . \quad . \quad \text{(equation 2)}$$

where E is the resting potential in mV, $[K]_i$ and $[K]_o$ are the internal and external concentrations of potassium and $[Na]_i$ and $[Na]_o$ are the corresponding concentrations of sodium, and b is a factor giving the relative permeability of sodium to potassium (P_{Na}/P_K). The factor b is likely to be much increased after denervation and consequently there will be a fall in the value of E. Thus it can be concluded from the present experiments that the observed increase in the rate of exchange of sodium in denervated rat diaphragm accounts at least in part for the fall in the resting membrane potential which has been reported by Lüllmann (1958) and Thesleff (1963).

SUMMARY OF CHAPTER V

1) The outward movement of sodium in denervated rat diaphragm muscle (8 days) was shown to be faster than in controls, the half-times being 4.1 min and 5.4 min in denervated and control muscles respectively. A 4-point assay was used, and statistical analysis showed that these results were significant ($P < 0.01$).

2) After denervation of rat diaphragm (8 days) there was an increase in water content *in vivo* and *in vitro*. The fibre sodium was also increased in denervated muscles *in vitro* as compared with control muscles.

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Outward movements of sodium in rat and mouse muscle

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Sodium exchange has been followed in toe muscles of the mouse and in rat diaphragm. Flexor digitorum brevis IV was dissected from mice, under anaesthesia with pentobarbitone, with the aid of a low-power microscope, and fusiform preparations which weighed 3–7 mg were obtained. They contained a variable amount of fibrous tissue, and this is consistent with the high sodium and low potassium content which was found (sodium 72 μmoles/g $\pm$ 7·8, potassium 66 μmoles/g $\pm$ 10·0, mean and S.D. of six estimations in each case). The muscles were transferred to saline containing ^{24}Na, as used by Creese & Northover (1961), and when uptake was complete the tissues were passed through a series of tubes containing inactive saline (Keynes & Swan, 1959). A large fraction of the sodium was found to exchange rapidly and this was followed by a slower fraction with rate constant of 0·072 min^{-1} (mean of 8, range 0·047–0·103), so that the mean half-time of this fraction was 9·6 min. After treatment with strophanthin-g 10^{-4} g/ml. for 2 hr the rate constant was reduced by 80 %.

Diaphragm muscles were also used in conditions which were found to give a low sodium content (Creese & Northover, 1961). The diaphragms were dissected from rats of 50–60 g, mounted on holders and then loaded with ^{24}Na and passed through tubes of saline as above. These muscles had a thickness of approximately 0·35 mm. After 5 min the washout of ^{24}Na became exponential and at various times the muscles were removed for counting, the rib and tendon being discarded. The points were plotted and the mean half-time for exchange of fibre sodium was 5·7 min (mean value from twenty muscles).

These two preparations could be set up within a short time of the cessation of circulation, and they provide results which are sufficiently consistent for further studies. The rate of turnover of sodium is more rapid than that found by McLennan (1957, 1958) in leg muscles of the rat.

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