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CHANGES IN SOME OF THE PROPERTIES OF MUSCLE MEMBRANE PRODUCED BY CHRONIC DENERVATION IN RAT DIAPHRAGM

by

Hilal Ahmad Shaikh

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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FALL, 1971

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

CHANGES IN SOME OF THE PROPERTIES OF MUSCLE MEMBRANE PRODUCED BY CHRONIC DENERVATION IN RAT DIAPHRAGM

Submitted by Hilal Ahmad Shaikh in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Some of the changes produced by chronic denervation in rat diaphragm were investigated. Calcium ion fluxes were studied by using radioactive Ca^{45} and the results showed that the calcium influx, efflux and the exchangeable calcium is more in denervated muscles. Associated with the increase in calcium, the resting membrane potential was reduced by 11 mV in denervated muscles and the duration of the action potential was prolonged after denervation. The possible role of increase in calcium permeability is discussed. Calcium fluxes were increased by electrical stimulation and caffeine. It was also shown that the increase in fluxes paralleled the increase in tension. The effects of blocking the transverse tubules by glycerol showed that glycerol, besides blocking the T-tubules, has other effects by which calcium influxes were increased, in normal and denervated muscles. The possible links between these observations and other known effects of denervation are discussed.

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INTRODUCTION

I. HISTORICAL BACKGROUND

For approximately a century, physiologists have been interested in the interrelationship between nerve and muscle since it was discovered that sectioning a motor nerve caused the innervated muscle to undergo atrophy and subsequent degeneration. Initially this was considered the result of a disturbance in the trophic functions of the nervous system. By trophic functions were meant "a certain regulation of tissue or cell metabolism which is dependent to a certain extent on the connection of these tissues with the nervous system" (Gutmann & Hnik, 1963). Gutmann and Hnik (1963) have suggested that aside from the exclusion of the phenomena of junctional transmission, i.e. nerve impulses producing end plate potentials, which result in muscle impulses, that all other functions are trophic. However, in contrast to this idea, work by Hamburger (1939) and Zelena (1962) showed that embryologically, nerve and muscle develop independent of the other, there being no trophic interaction. Even after the formation of a nerve-muscle junction, the muscle still retains the capacity to grow and differentiate when denervated at this stage. However, this ability is lost with maturation.

The usual technique that has been employed to study the trophic nervous influences on muscle has been to section or damage the nerve and observe the resulting changes in the muscle during nerve degeneration or regeneration. Other procedures have been

developed by subjecting the muscle to disuse by blocking nerve impulses in the absence of degeneration (Tower, 1937a, 1937b; Tower et al., 1941; Eccles, 1941, 1944; Johns & Thesleff, 1961). Similarly, tenotomy and cross-innervation techniques are also available (Tower, 1939; Eccles, 1944; Gutmann & Vrbova, 1952; Hnik & Skorpil, 1962).

In the late 1930's, three changes were considered to be specific in denervated muscle, these being:

(1) an increased sensitivity to acetylcholine;

(2) fibrillation;

(3) changes in electrical excitability.

With regard to the first of these changes, i.e. increased sensitivity to acetylcholine, it was suggested by Brown (1937) and Rosenblueth and Luco (1937) that this increased sensitivity was restricted to the denervated end plate, since it was known that the acetylcholine receptors were localized to the neuromuscular junction. However, Eccles (1941a), Ginetzinsky and Shamarina (1942) as quoted by Eccles (1963) and Kuffler (1943) demonstrated an increase in receptor area along the muscle fiber length. Similarly, Axelsson and Thesleff (1959) showed in cat tenuisimus muscle that, following denervation, the acetylcholine sensitive receptor areas were uniformly distributed over the whole length of the muscle fiber. However, Miledi (1960a), using denervated frog sartorius muscle, showed that, although the entire muscle fiber became more sensitive to acetylcholine after denervation, a preferential sensitivity, about 1,000 times more, still existed at the motor end plate. Katz and Miledi (1964) also showed that although the whole denervated frog muscle developed a high sensitivity to acetylcholine

after 2 weeks of denervation, a remote part of the muscle fiber, distant from the end plate, could independently develop a high drug sensitivity. They also demonstrated that the chemosensitivity of a muscle was labile and subject to long term changes. Using denervated rat hemidiaphragms, Mitchell and Silver (1963) reported that passive acetylcholine release from denervated end plates fell within 2 hours of denervation to 50% of control level with a return to near normal values after 11 weeks. They also observed the passive spontaneous release of acetylcholine at different temperatures and concluded that only a very small portion of the acetylcholine spontaneously liberated can be associated with miniature end plate potentials. Krnjevic and Straughan (1964) observed that acetylcholine liberation from 22 to 24 days denervated rat diaphragms was about 2/3 the value of normal muscle at rest, with there being a small increase after direct stimulation. Aklon et al. (1970) also observed a smaller release of acetylcholine from denervated rat diaphragm upon direct stimulation as compared to normal muscle. Potter (1970) localized acetylcholine synthesis in the region of end plates in normal rat diaphragm, in which it was reduced to a few per cent after 6 weeks of denervation. The mechanical responses of the denervated frog muscle to acetylcholine in sodium-free solutions were shown not to be mediated by membrane depolarization (Lorkovic, 1970), it being proposed that the primary action of acetylcholine was either on the outer membrane, involving no changes in the membrane potential, or was exerted by a change in the properties of the T-tubule membranes at the level of triads. Very recently, Fleming (1971) also studied supersensitivity of the

rat diaphragm to potassium and concluded that the supersensitivity of the diaphragm had two components:

(1) a marked, highly specific supersensitivity to cholinergic agents like acetylcholine; and,

(2) a small, non-specific supersensitivity to potassium which was proposed to be associated with the changes in the membrane and/or excitation-contraction properties.

Since denervation produces a marked increase in the sensitivity to depolarizing drugs in the extra-junctional region of the skeletal muscles, Taylor et al. (1965) found that labelled decamethonium became concentrated in the junctional region of normal rat diaphragm. This had already been shown by autoradiographic studies of Waser (1960). Creese and MacLagan (1970) also showed that decamethonium entered the muscle fibers, while Taylor et al. (1967) have observed injected radioactive decamethonium to remain in muscle in conjunction with a rapid fall of plasma concentration to low levels. Creese et al. (1971) also showed that the uptake of decamethonium could be blocked by tubocurarine, that denervated muscles took up less during *in vivo* than the normal muscles, and that *in vitro* the uptake in denervated muscle was markedly increased, being located in the extrajunctional region. Similar results have also been obtained by Waser and Nickel (1969), and by Cookson and Paton (1969) who proposed that the alteration in permeability produced by the pharmacological action of decamethonium, leads to the entry of organic cations as well as the net movements of inorganic ions.

The second specific change following denervation was observed to be fibrillation, the onset of which has been found to vary with the type of preparation. It has been observed in cockroach muscle (Beránek et al., 1959) and follows 40 to 60 days after denervation in frog muscle (Reid, 1941). Hnik et al. (1958) as quoted by Hnik and Skorpil (1963) have noted fibrillation to occur as early as 40 hours in mice, and 29 to 49 hours in rats. The intensity of fibrillation was found by Brown (1937) to be increased by acetylcholine if applied in small doses, leading him to believe that fibrillation resulted from the action of acetylcholine on the highly sensitized muscle fibers. Rosenblueth and Luco (1937) rejected this idea, since curarine was unable to depress these fibrillations. Curarine, nevertheless, was very effective in depressing the excitation of denervated muscle by applied acetylcholine. As well, after re-innervation, Feinstein et al. (1945) as quoted by Hnik and Skorpil (1963) reported fibrillation to disappear. By means of intracellular recording, Li, Shy and Wells (1957) observed continuous wave-like variations in the membrane potential. These were believed by Ware, Bennett and McIntyre (1954) to be superimposed upon a depolarized membrane, with the generation of a muscle impulse occurring when a depolarizing wave attained a critical level. However, no changes in the resting membrane potential were noted by Nicholls (1956), although Harris and Nicholls (1956) observed an increase in the membrane resistance and a decrease in potassium permeability later. Lüllmann and Pracht (1958) found, in denervated rat diaphragm, that the extracellular space had increased by 5% of its wet weight, that the fiber water had

decreased by 28 gm/kg of its wet weight, and that the membrane potential had decreased by 13 mV. Recently, Belmar et al. (1966) showed in rat muscle that fibrillation action potentials originated at the denervated end plate zone, while acetylcholine and noradrenaline increased the frequency of fibrillation when applied in small doses to the denervated end plate zone surface. Large doses of acetylcholine depressed the fibrillation frequency. They also showed that large doses of decamethonium depolarized the muscle membrane and reduced the amplitude of action potentials. Increased amounts of Mg^{++} and Ca^{++}, and d-tubocurarine had no effect on the fibrillation frequency. By studying the calcium uptake by the sarcoplasmic reticulum obtained from glycerinated rat diaphragm, Howell et al. (1966) concluded that fibrillation could be related to surface membrane instability caused by a persistant sequestration of calcium in the sarcoplasmic reticulum. In a similar study, Brody (1966) observed a greater calcium uptake by the sarcoplasmic reticulum of guinea pig gastrocnemius muscle, and suggested that fibrillation could be explained on the basis of an enhanced ability of the sarcoplasmic reticulum in denervated muscle to release and bind calcium. However, Miledi and Stefani (1970), by their studies on the miniature end plate potentials in denervated slow muscle fibers, in which they observed these to be of a variable time course and amplitude, with a slow frequency that could be increased by hypertonic solutions, suggested following nerve degeneration, that the Schwann cells of small motor nerve fibers release packages of transmitter, resulting in these miniature end plate potentials.

The third specific feature of denervation was noted to be a

change in the electrical excitability of the muscle (Lewis, 1962; Eccles, Eccles & Kozak, 1962). Moreover, Desmedt (1950a, 1950b) demonstrated, shortly after denervation, a progressive increase in chronaxie, reaching a stationary level of approximately 15 mS, as well as a slowing of accommodation, which was associated with the onset and development of fibrillation. Recovery from these excitability changes was shown to begin at an early stage of nerve regeneration, being completed upon restoration of neuromuscular transmission. Nicholls (1956) observed no change in the resting membrane potential of the denervated frog sartorius muscle; however, Harris and Nicholls (1956) observed an increase in the membrane resistance and a decrease in potassium permeability, which was further confirmed by Jenkinson and Nicholls (1961). This increase in membrane resistance was also observed in denervated frog muscle by Hubbard (1962, 1963a, 1963b) who contended it to be mainly the result of a decrease in potassium permeability, there being no effect on chloride permeability. A decrease in resting membrane potential was also observed in rat diaphragm by Lüllmann and Pracht (1957, 1958), and later by Thesleff (1963). A similar study of membrane properties of denervated fast and slow muscles by Albuquerque and Thesleff (1968) showed that the membrane electrical time constants, the transverse resistance, and the action potential threshold were increased in both EDL and soleus muscle, while the rate of rise and amplitude of the spike were significantly decreased. Therefore, it was suggested that there was possibly a reduction in the number of membrane sites connected with the passive ionic transport. More recently, Redfern

et al. (1970, 1971) showed, following denervation, that the action potential generation mechanism became resistant to the blocking action of tetrodotoxin, while between 30 to 40 hours post-denervation, the action potential rate of rise was reduced to one third, the membrane potential decreased by 14 mV, and the electrical time constant and membrane resistance increased by 70% and 50%, respectively.

Because it had been shown by Ringer as early as 1883 that calcium was an important ion for muscle contraction, Hines and Knowlton (1933), Knowlton and Hines (1937) and later Shimizu and Kuriaki (1960) investigated the calcium changes in muscles following denervation, finding the calcium content to be increased. As well, it has been reported by Humoller et al. (1950) that the calcium ion content in rat muscles was unchanged at 10 days, but increased after 20 days denervation. None of these authors showed whether this increase of calcium occurred in the extracellular or intracellular muscle compartment, although Fenn et al. (1937) and Morgulis and Osterhoff (1938), using dystrophic muscles, correlated an increased calcium uptake with the histological observation of calcification and fibrosis.

In the recent years, the ionic fluxes in denervated muscle have also been studied. Jenkinson and Nicholls (1961) found in depolarized denervated rat diaphragm, that the calcium uptake, plus Na^+ and K^+ fluxes, were increased by acetylcholine, which also caused contractures in the depolarized muscle. These contractures could be blocked by tubocurarine or by the absence of calcium, it being concluded that the acetylcholine contractures resulted from an increased cell membrane permeability to calcium ions. There have also appeared

reports concerning the accumulation of Na^+ in denervated muscles (Lüllmann, 1958; Drahota, 1963; Gutmann& Hnik, 1963; Dockry et al., 1966), as well as an observed increase in the Na^+ fluxes in myotonic dystrophic muscle (Hofmann et al., 1968). It has also been shown by Gutmann and Sandow (1965) in rat EDL muscle, that the susceptibility to caffeine contractures was markedly increased after denervation. This suggested that whereas caffeine was unable to release calcium from intracellular sources in normal muscle, after denervation the reticulum became altered such that it readily relinquished calcium in the presence of caffeine, causing a contracture. Howell et al. (1966) followed this with the observation that the isolated sarcoplasmic reticulum in denervated glycerinated rat diaphragm had an increased calcium uptake of 2½ times. Similar results were also obtained by Brody (1966) who found a three-fold increase in calcium uptake by microsomes in the denervated gastrocnemius muscles of the guinea pig. Sandow et al. (1965) and Isaacson and Sandow (1967) further observed in denervated rat EDL muscle, the calcium uptake to be greater than normal muscle with and without the presence of caffeine. Lahrtz and Lüllmann (1967) measured the calcium fluxes in denervated rat diaphragm, observing, however, no change in the Ca^{45} efflux time course between normal and denervated muscle (half time 70 minutes) nor any difference between the total calcium content of the two muscle preparations, thus concluding that the cell membrane contained a freely moveable calcium fraction. Recently, calcium and lanthanumhave been shown by Parsons et al. (1971) in denervated muscle, to decrease membrane excitability, and to depress the following features

of the action potential: the maximum rate of rise, the overshoot and the maximum rate of the falling phase.

The morphological changes associated with denervation were initially studied by light microscopy. Tower (1932, 1935) described atrophic changes occurring in muscle following one year of denervation with an accompanied reduction of nuclei in the nuclear bag and a disintegration of intrafusal fibers. This was confirmed by Chor et al. (1937). Tower (1935, 1939) also observed the I bands to be longer than the A bands, agreeing with the recent finding of irregular variations in the sarcomere length (Gutmann & Zelena, 1963). Later Bowden and Gutmann (1944) observed a decrease in fiber diameter and weight secondary to a decrease in fiber water and proteins, a loss of fiber cross-striation, and an increased number of nuclei and nucleoli. Moreover, after three years of denervation, they observed a loss in the anatomical continuity of the fibers, either by longitudinal splitting into separate fibrils, or by fragmentation into oval segments. The change in nuclear content following denervation has been further clarified by Gutmann and Zelena (1963) to involve an initial 40% increase in DNA for the first month, with a return afterwards to the same content as in normal muscle. Another early observation was the replacement of muscle by fat cells (Chor, 1937; Bowden & Gutmann, 1944; Gutmann & Young, 1944) which, in rabbit EDL and soleus muscle, has been noted to occur in the intracellular spaces.

With the advent of electronmicroscopy, histologic attention has now focussed upon the ultrastructural changes following dener-

vation. As a result of intensive study, the sequence of degenerative changes in muscle after denervation can be described as follows. After sectioning, the distal end of the nerve quickly degenerates, with a decrease in the density of the axonal matrix after 2 to 4 days. Within a few more days, the axon terminal completely degenerates, retracting from the muscle fiber, except for the post-synaptic foldings, still visible and allowing identification of the motor end plate (Miledi & Slater, 1963). Further work by Miledi and Slater (1970) in rat diaphragm has demonstrated complete failure of end plate transmission after 20 hours of sectioning, accompanied by a cessation of spontaneous miniature end plate transmission after 20 hours of sectioning. As well, upon complete failure of transmission, the end plate structure appears abnormal, while the nerve terminal disintegrates into small fragments. However, in frog muscle, the miniature end plate potentials have been shown to persist long after complete axonal degeneration (Birks et al., 1960). In rat leg muscles, during the first 2 weeks of denervation, there has been noted an initial rapid decrease in weight, becoming less later (Pellegrino & Franzini-Armstrong, 1963). Within the muscle, fibers at first loose their contractile material, then mitochondria, and later sarcoplasmic reticulum. Alterations commence at the Z line which becomes bent, resulting in a disturbance of the thick and thin filaments, while the sarcolemma and basement membrane invaginate deep into the fiber substance. Later, the fibril diameter becomes decreased. During the period of contractile material degeneration, the sarcoplasmic reticulum appears to become

over-developed. This has been attributed to:

(1) a proliferation of the tubules derived from the T-system;

(2) the appearance of pentads derived from the triads, being composed of two T-tubules lined and separated by the terminal cisternae of the sarcoplasmic reticulum (Pellegrino & Franzini-Armstrong, 1969).

Furthermore, the hypertrophy of the sarcoplasmic reticulum becomes more obvious as a result of the over-development of tubules and vesicles secondary to an increase of the sarcoplasmic protein content (Margreth, 1966). Along with the contractile material degeneration, the mitochondria progressively decrease, which in rat diaphragm occurs early and extensively.(Miledi & Slater, 1968). However, the rate of atrophy in frog muscle has been observed to be slower, with degenerative changes occurring much later (Muscatello et al., 1965).

Unlike other muscle, rat diaphragm undergoes a temporary hypertrophy, which has been noted to be maximal 7 days after denervation. This hypertrophic state later subsides, the muscle undergoing atrophy as in other types of denervated muscle. Miledi and Slater's (1969) recent electronmicroscopic studies in rat diaphragm during hypertrophy have demonstrated the fiber cross-sectional area and number of myofibrils to be increased. Because no changes in the filament spacings were observed, it was concluded that the number of actin and myosin filaments were also increased. Feng, Jung and Wu (1963) and recently Jurmaniva and Zelena (1970) have also

shown the anterior latissimus dorsi, a slow muscle in the chick, to become hypertrophic after denervation. The latter investigators also observed, in conjunction with this hypertrophy, degenerative changes to occur, similar to those described in other denervated atrophic muscles. Furthermore, in chicken dystrophic muscle, Baskin (1970) observed areas of enlarged sarcoplasmic reticulum, plus a decreased membrane bound creatine phosphokinase in the muscle microsomes. There was no observed increased calcium uptake by the sarcoplasmic reticulum. Hence in summary, the electronmicroscopic observation of the muscle preparation on various types of atrophic muscles, have revealed the following common features: dissociation of the surface membrane complex, a decrease in fiber diameter, the presence of satellite cells and myoblasts, lamellar arrays of membrane-bound cisternae, and fragmentation of the muscle fibers with subsequent degeneration of the fragments and disintegration of the myofilaments (Birks et al., 1959; Pellegrino et al., 1963; Hess & Rosner, 1970; Schrodt & Walker, 1965; Miledi & Slater, 1969).

II. IONIC HYPOTHESIS

1. Resting Membrane Potential:

In 1895, Biedermann discovered that application of an isosmotic potassium chloride solution to a portion of muscle generated a large potential difference between the site of application and the remaining surface of the muscle. Later Hober in 1905 extended this observation, finding that the ability of various cations in altering

the resting potential of muscle increased in the following order: Li, Na, Mg, Cs, NH_4, Rb, K. Similarly, he found that the anions in the order CNS, NO_3, I, Br, Cl, acetate, HPO_4, SO_4, tartrate had the same effect. In 1902, Bernstein published his membrane theory in which he postulated:

(1) that the resting potential was pre-existant at the plasma membrane of the cell;

(2) that the resting potential was maintained by virtue of the semi-permeability of the plasma membrane.

At that time, the pre-existance of ions in electrolyte solutions, and the osmotic phenomena of membranes was already known, Nernst's book on theoretical chemistry having also been published. Later, Bernstein added two more postulates to his theory:

(1) that the resting potential was a diffusion potential resulting from the difference in the molarity of potassium and phosphate ions through the membrane;

(2) that the action potential was caused by a reduction of the resting potential resulting from a non-specific increase of permeability of the membrane during activity.

It was further demonstrated that the relationship between the resting potential and the external potassium concentration could be expressed by the Nernst equation. However, this equation did not consider the effect of potassium permeability in maintaining the resting potential (Field et al., 1959).

The Nernst equation represents the value of the resting potential that would be obtained if the concentration gradient of potassium was the cause of the resting membrane potential. Therefore, when the recorded resting membrane potential was observed to exceed the value calculated by the Nernst equation, it was believed that the resting potential was generated by some electrochemical mechanism other than the simple diffusion of potassium ions. To explain this divergence between the observed and Nernst theoretical resting potential, Hodgkin (1951) used the modified Goldman (1943) equation and showed that at least three ions, K, Na and Cl^- were responsible for the maintenance of the resting membrane potential.

According to recent studies, the cell membrane of mammalian skeletal muscle has been demonstrated to possess three interrelated properties:

(1) that the membrane separates an intracellular fluid, with a high potassium and a low sodium concentration, and an extracellular fluid with a high sodium and low potassium concentration;

(2) that the membrane is permeable to sodium and potassium ions, as shown by their continuous flux across the membrane in radioactive tracer experiments;

(3) that across the membrane there exists an electrical potential difference of -85 to -90 mV, as measured by intracellular microelectrodes.

Despite the cell membrane's permeability to sodium and potassium ions, the internal cellular composition of these ions has been noted to be

constant. This has been shown to be maintained by an active transport "pump" process, involving active sodium efflux across a concentration and electrical gradient and a balancing of the internal potassium concentration, the metabolic energy being derived from ATP breakdown. For the transport of cations, it has been necessary to propose the presence within the membrane of sites capable of transient binding, which appears to be dependent upon ATP, as this active transport process has been shown to be linked to ATP breakdown.

2. Action Potentials:

At present there is one generally accepted theory of action potential production. This theory is based upon the movement of ions across the cell membrane, namely an influx of sodium resulting in depolarization (Hodgkin, Huxley & Katz, 1952; Hodgkin & Huxley, 1952b, c, d, e). As well, most action potentials of skeletal muscles have also been noted to be characterized by after potentials, either negative or positive, appearing late during the repolarization phase. The term 'negative after potential' has been used to describe the slow declining membrane potential change following the initial short duration spike potential (in a monophasically recorded action potential). During this time the membrane is still slightly depolarized. Similarly, 'positive after potential', referred to as an 'undershoot', describes the temporary persistance of the membrane potential, after the spike potential, at a level below that of the resting potential. Various speculations and hypotheses have been proposed to explain these after potentials

in terms of associated ionic movements. Nastuk and Hodgkin (1950) first showed that the action potential of a frog muscle fiber was followed by a negative after potential. Desmedt (1953) noted the membrane potential at the beginning of an after potential was largely independent of the potassium concentration ratio, $[K]_o/[K]_i$, across the membrane. Nevertheless, because the resting membrane potential has been known to depend upon the ratio $[K]_o/[K]_i$, it was suggested that the amplitude of the after potential relative to the resting membrane potential also depended on this ratio. Assuming the time course of the after potential to be exponential (Frank, 1957), it has been further suggested that the membrane was passively recharged by a constant resting leakage conductance during the after potential (Frank, 1957; Hutter & Noble, 1960). However, MacFarlane and Meares (1958a, b) showed that metabolic poisons such as dinitrophenol and cooling reduced or abolished the negative after potential. Moreover, Lubin (1957) demonstrated that replacement of chloride in the extracellular fluid by other halides or thiocyanide increased both the negative after potential and twitch tension. It has also been suggested by Frankenhaeuser and Hodgkin (1956), Frankenhaeuser and Moore (1963) and Persson (1963) that a small delayed increase in sodium permeability could explain most of the properties of the negative after potential. A similar conclusion, i.e. a rise in the sodium component during the action potential plateau, was made by Noble (1962) in frog heart muscle. Recently the observations of Adrian et al. (1970) have suggested that a delayed rectifying channel could be responsible for rapid repolar-

ization during the falling phase of an action potential and the early after potential. The positive after potential has been believed to be associated with a pronounced decrease in the membrane resistance (Grundfest et al., 1953). According to the sodium theory of Hodgkin and Huxley (1952a-e), the undershoot of the squid giant axon could be attributed to an increase in the potassium membrane permeability.

Electrically, the action potential consists of a transient depolarization of the cell membrane, bringing about a sequence of processes enabling the electrical charges to activate a mechanical response. Hence, the action potential not only elicits contraction, but also correlates the nature of the contractile response. This correlation has been demonstrated by the use of substances which produce simultaneous changes in certain features of both the action potential and the twitch mechanical response (Bianchi & Shanes, 1959; Sandow et al., 1965). Thus, specific changes in the features of the action potential during the mechanically effective period, i.e. the duration of the action potential at the mechanical threshold level, could be correlated with subsequent changes in the mechanical response. For example, Sandow et al. (1965) showed that NO_3 and 1 mM caffeine, which only prolong the duration of the action potential by 10% of normal, decrease the mechanical threshold from -50 mV to -65 mV, producing a potentiation and prolongation of both the twitch and rate of rise of tension. Similarly, these workers also demonstrated that, although heavy metal ions (Zn or UO_2) would not change the mechanical threshold, they would tremendously increase the duration of the action potential, thus increasing both the peak

tension and the duration of twitch. However, the increase in rate of rise of tension was delayed, the cause of which was suggested to be a prolongation of the spike, in turn prolonging the mechanically effective period, thus delaying the onset of excitation-contraction coupling till late in the mechanically effective period. This would be projected in the mechanical response as a corresponding delay in the onset of the associated increase in the rate of rise of tension. Similar relationships between the shape of the action potential and the resultant twitch had also been previously proposed by Edwards et al. (1956) and Lubin (1957). Thus, it has been generally agreed that changes in the shape of action potential produce changes in excitation-contraction coupling, in turn resulting in alterations of the contractile properties of a muscle.

III. EXCITATION-CONTRACTION COUPLING

1. Historical Background:

As has been mentioned, Ringer (1883) was the first to note calcium as being necessary for the contraction of heart muscles. Later, Locke and Rosenheim (1907), using a capillary electrometer, demonstrated that removing the external calcium resulted in a disappearance of mechanical beat, concluding that calcium was necessary for the "production of the wave of contraction out of the wave of excitation". Hines (1913) also demonstrated that replacing calcium in the external solution by the addition of Sr^{++} greatly prolonged both the electrical and mechanical responses, compared to those in a

normal calcium medium, this being further supported by others (Daly & Clark, 1921; Bogue & Mendez, 1930). Later, Heilbrunn (1940, 1943) suggested that the release of calcium from the cell cortex lead to the coagulation of intracellular muscle proteins, in turn resulting in muscle contraction. Heilbrunn and Wiercinski (1947) further showed that calcium in a concentration as small as 0.2 mM was able to produce contractions, concluding it to be the only physiological ion capable of this effect, although Ba^{++} and Sr^{++} could also induce contractures. This work of Heilbrunn and Wiercinski was against the theory of Szent-Györgyi (1945), who, working with purified proteins, contended that, because Ca^{++} and Mg^{++} were strongly bound by myosin and thus immobilized, it was the movement of K^{+} which conditioned contraction or relaxation. However, Sandow (1952) pointed out that contraction of the muscle depended upon the excitation of the membrane, thus postulating that, at the surface during excitation, a substance was released which moved inward to the contractile machinery to initiate contraction. He termed this sequence of events "excitation-contraction coupling", further demonstrating by massive stimulation of the muscle to eliminate local currents that longitudinal currents played no role in excitation-contraction coupling. This conclusion of Sandow's was also based on previous work by Kuffler (1946) which had shown that a muscle could not develop local currents during K^{+} induced contractures because the membrane potential was constant over the entire length of each fiber.

Later, Niedergerke (1955) showed, by the electrophoretic application of Ca^{+} , that during a current pulse the muscle would

develop a local contraction, followed by partial relaxation when the current was terminated. From this he concluded that calcium was playing a role as a link in the contractile cycle. Similarly, Caldwell and Walster (1963), by the injection of 1 to 50 mM $CaCl_2$, $SrCl_2$, $BaCl_2$ and caffeine into single muscle fibers, obtained contractions, which approximately were of the same duration for low concentrations of these substances with the same molar quantity. The explanation of these results was based on the assumption that such substances caused a release of Ca^{++} from a binding site, one which had also been proposed previously by Frank (1962).

Constantin et al. (1967) also demonstrated in skinned frog muscle fibers that Ca^{++} could induce local contractures.

2. Calcium Fluxes During Contraction:

In 1957, Hodgkin and Keynes showed Ca^{45} to be accumulated in the resting squid giant axon with an increase in influx during electrical stimulation and K^+ depolarization. Similarly, Bianchi and Shanes (1959) showed the influx of calcium per twitch (potentiated by NO_3) to be thirty times greater in muscle fibers than in stimulated nerve. A further observation was that an increased external calcium concentration increased the resting influx, but not the influx during activity, suggesting separate membrane sites of calcium entry for the two processes. Later, Shanes and Bianchi (1960), using frog sartorius muscle, studied Ca^{45} release during electrical stimulation and K^+ contractures, finding the efflux per twitch was the same as the influx per twitch, and that the release of Ca^{45} during a K^+ contracture

was dependent upon the extent of contracture, suggesting the shortening process influenced Ca^{45} efflux. At the same time, Winegrad (1960) demonstrated in the guinea pig heart, that an increased rate of stimulation was parallelled by an increased Ca^{++} uptake per beat. In view of the results of Bianchi and Shanes, and those of Winegrad, it would appear that there was a quantitative correlation between the strength of contraction and the amount of Ca^{++} entering the cell. However, Weiss and Bianchi (1965) showed that although frog sartorius muscle had a greater Ca^{++} uptake in nitrate than chloride Ringers for potassium concentrations between 16 and 32 mM, but not for 48 to 80 mM, the maximum developed tension plus the tension curve area increased over the range 16 to 80 mM K , suggested Ca^{++} influx not to be quantitatively correlated with changes in the tension curve area during K^{+} contractures.

Some evidence has been presented for Ca^{++} being firmly bound on intracellular sites within the muscle fiber (Hodgkin & Keynes, 1957; Harris, 1957; Gilbert & Fenn, 1957; Shanes & Bianchi, 1959). For example, Harris (1957) showed prolonged soaking of frog muscles in Ca^{45} exchanged only 10 to 25% of the total Ca^{++}. Moreover, upon exposing these muscles to an EDTA, Ca^{++} free solution, not all the contained Ca^{45} was observed to elute, thus suggesting a "bound" fraction. However, because these experiments were performed at 4°C, the values of Shanes and Bianchi of 38%, and Gilbert and Fenn of 39%, exchangeable Ca^{++} at room temperature, would probably be more representative. From this demonstration of Ca^{++} compartmentalization,

the theoretical distribution of muscle Ca^{++} has been conceived as follows: a "membrane" or loosely bound Ca^{++}, relatively freely exchangeable, its influx probably being associated with contractures produced by agents such as K^{+}; a store of bound Ca^{++}, not as freely exchangeable, probably being localized on the membrane and/or sarcoplasmic reticulum; and, as suggested by Curtis (1970), an intracellular Ca^{++}, probably being firmly bound in various intracellular stores.

In an attempt to determine the mode by which calcium induced contractures, Hill (1948) calculated the time required for calcium to diffuse from the surface membrane to contractile proteins, noting it to be significantly longer than the time observed between the generation of an action potential and the mechanical response. Winegrad (1961) and Frank (1961) furthermore showed that the amount of Ca^{++} influx during stimulation alone was not sufficient for contraction. Similarly, Weber et al. (1963a, b) and Portzehl (1964) demonstrated the Ca^{++} threshold concentration required for activation to be larger than the Ca^{++} influx concentration during a twitch, as observed by Bianchi and Shanes. Sandow (1965) showed the difference between these two concentrations to be 10 to 100 fold. Therefore, it was suggested that special mechanisms existed to increase the release of Ca^{++} during activity. Even though no net gain or loss of Ca^{++} occurred, it appeared that marked shifts occurred in localized intracellular regions of individual fibers during activity. Hence, Bianchi (1961) suggested that Ca^{++} influx during stimulation was due to an uncharged calcium ion pair. Hodgkin and Horowicz (1960) also postulated

that depolarization initiated contraction by allowing the entry of an activation particle, possibly a negatively charged calcium ion complex. Therefore, it was possible that in fast skeletal muscles, the entry of Ca^{++} involved the release of further larger amounts of Ca^{++} from cellular stores, either on the membrane or on the sarcoplasmic reticulum.

Bianchi and Shanes (1959) also demonstrated the necessity of an external Ca^{++} concentration in relation to its influx during activity. Similarly, Frank (1958, 1960, 1961) showed that K^{+} induced contractures were rapidly inhibited in a Ca^{++} free solution, this inhibition also being shown to occur without a change in the degree of K^{+} induced depolarization or in the resting membrane potential (Frank, 1958, 1964). The above worker further demonstrated that the rate-limiting step in this inhibition of K^{+} induced contracture was the diffusion of Ca^{++} from the extracellular space, as the muscle size, rather than fiber size, determined the time period required for inhibition to occur. This was further proven by the use of EDTA in a Ca^{++} free solution, causing the inhibition of K^{+} induced contractures to occur more rapidly. Frank (1961, 1962) also showed that some multivalent metallic cations (CO^{++} and Ni^{++}) were capable of substituting for Ca^{++}, allowing K^{+} induced contractures to take place in a Ca^{++} free solution. The means by which these ions assisted in producing a contracture was considered to be different from that of Ca^{++}, Frank himself believing that these ions released Ca^{++} from binding sites, in turn resulting in a contracture.

3. Intracellular Organelles and Contraction:

Bozler (1954) noted that the contractile activity of the glycerol extracted muscles depended upon the presence of Ca^{++}, which in relaxed fibers and the presence of ATP, caused contraction, while the addition of EDTA caused relaxation. Similarly, in the presence of ATP and Mg^{++}, Ca^{++} in small concentrations was observed to cause contraction, and in higher concentrations, relaxation. Bozler therefore suggested the action of a relaxing factor and of EDTA were explainable by the inactivation of Ca^{++} by these substances. Later, Eabshi (1961) showed Ca^{++} to be necessary for ATPase activity and the superprecipitating actomyosin, while the Ca^{++} binding activities of various chelating agents directly correlated with their contracture relaxing ability. Weber and Winicur (1961a, 1961b) further showed this contractile activity to be demonstrable in an actomyosin system upon the addition of Ca^{++} at a concentration greater than 10^{-5} M, when ATP, Mg^{++} and one of the several relaxing factors (EDTA) was present. Likewise, they (1961b) found that when the Mg^{++} concentration was greater than 0.01 mM, the absence of Ca^{++} inhibited superprecipitation of actomyosin. Furthermore, they described two possibilities of Ca^{++} action:

(1) Ca^{++} could combine with actomyosin, initiating contraction; or,

(2) Ca^{++} could interfere with a reaction between a relaxing factor and actomyosin.

To clarify this, Weber and Herz (1963a,b) showed Ca^{++} to be exchange-

ably bound to actomyosin and myofibrils, the consequence of this binding being activation of ATPase activity and superprecipitation. It was further noted that upon lowering the Mg^{++} concentration, the binding of Ca^{++} to myofibrils in the presence of EGTA was greater than that bound at higher Mg^{++} concentration in the absence of EGTA, with superprecipitation accompanying increased Ca^{++} binding. These experiments suggested the existence of two or more Ca^{++} binding sites, i.e. in the presence of Mg^{++} or Mg-ATP, only one of these sites was saturated with Ca^{++}, with only half as much Ca^{++} being bound as when Mg^{++} was absent. If the concentration of Mg^{++} was lowered, however, additional Ca^{++} could be bound to a second site.

Ebashi (1963) demonstrated that although extracted actomyosin possessed increased ATPase activity, superprecipitation with ATP, and increased Ca^{++} concentration, the reconstituted actomyosin did not. A protein substance similar to tropomyosin was found to be necessary for the restoration of actomyosin sensitivity to Ca^{++}, therefore indicating that a tropomyosin-like protein called troponin might be the site of Ca^{++} combination on the contractile proteins. Weber et al. (1963a, b) further suggested that the ATPase activity of actomyosin in the presence of Mg^{++} was a resultant of the anzymatic action of actin increasing the relatively low rate of ATP hydrolysis by myosin. Hence, the relaxing agents could prevent actin from activating myosin ATPase activity. Furthermore, the presence of ATP in concentrations greater than 10^{-5} M was found to inhibit ATP hydrolysis, plus contraction. However, in the presence of Ca^{++}, actomyosin and myofibrils were observed to exhibit contractile activity by means of the formation

of a Ca^{++} complex. Ebashi and Endo (1968) have suggested that the formation of a complex containing Ca^{++}, tropomyosin and troponin is involved, and this complex inhibits the interaction of actin and myosin in the presence of Mg, ATP and in the absence of Ca^{++}. The addition of Ca^{++} then produces ATPase activity and superprecipitation.

4. Intracellular Bound Ca^{++}:

From the preceding discussion, the contractile activity of muscle appeared to be controlled by the intracellular Ca^{++} concentration, in turn influenced by Ca^{++} fluxes. From the work of Harris (1957), Gilbert and Fenn (1957) and Bianchi and Shanes (1959), the basis for compartmentalizing intracellular bound stores of Ca^{++} represented a site of flux regulation. Early support of this came from Marsh (1952) who demonstrated that the supernatant fraction of muscle homogenates caused a ten-fold increase in the ATPase activity and contraction of myofibrils, from which he assumed this fraction to contain a relaxing factor. Later, Bendall (1958) found this relaxing factor to be related to the presence of 'granules', which subsequently were demonstrated by Ebashi and Lipmann (1962), and Muscatello et al. (1962) to be vesicles of the endoplasmic reticulum or the sarcotubular system and to exhibit ATP and ATPase dependent Ca^{++} accumulation. This was further supported by the findings of Hasselbach and MacKinose (1961, 1962) that these relaxing granules

of the "endoplasmic reticulum" had a preferential uptake, from a solution containing Ca^{++}, Mg^{++}, oxalate and ATP, of large quantities of Ca^{++}, present in the granules as oxalate. The relationship between Ca^{++} concentration, the activity of the Ca^{++} pump, ATPase splitting, and a phosphate exchange was found to be directly correlated. Furthermore, Weber et al. (1963a, b) noted that during a "contractile" response, fragments of the sarcoplasmic reticulum were capable of removing most of the exchangeable Ca^{++} bound to actomyosin or myofibrils, and consequentally, directly related to the inhibition of contractile activity. Later, Weber et al. (1966) further suggested that Ca^{++} binding within the sarcoplasmic reticulum was in equilibrium with the intracellular Ca^{++} concentration. This was followed by Hasselbach and Seraydarian's (1966) demonstration that, of three types of sulfhydryl groups, one, Type A, located on the external surface of the sarcoplasmic reticulum membrane, was involved in Ca^{++} transport and ATPase splitting. This was confirmed by Costantin et al. (1965), who demonstrated the accumulation of externally applied Ca^{++} in the terminal sacs of the sarcoplasmic reticulum. Furthermore, it was proposed that the proximity of these lateral sacs to the T-tubules implicated them in a role of Ca^{++} release as required for contraction initiation.

By means of autoradiographic techniques, Winegrad (1965) elaborated on the intracellular movements of calcium. He found that, although in resting muscle Ca^{45} was localized to the lateral sacs, during K^{+} induced contractures, it migrated into the region of actin-myosin overlap in amounts increasing as a function of the con-

centration of K^+ and tension produced. Using the same techniques, Winegrad (1968, 1970) recently studied the movement of Ca^{45} during a maintained tetanus, during the declining phase of tetanus, and during the period immediately following. From this he showed that there were three sites at which the isotope was concentrated:

(1) the terminal cisternae;

(2) the intermediate cisternae and longitudinal tubules;

(3) the "A" band portion of the myofibrils.

Moreover, during a tetanus, he observed that the majority of the myofibrillar Ca^{45} was localized within the thin filament region of the sarcomere, the amount varying with the tension developed. As a consequence of this work, Winegrad suggested that Ca^{++} was released from the terminal cisternae by electrical stimulation and rebound by the longitudinal tubules and intermediate cisternae, resulting in muscle relaxation.

5. Caffeine and Ca^{++} Movements:

While trying to define the action of caffeine and its effects on calcium fluxes in normal frog muscle, Bianchi (1961) noted that 5 mM caffeine increased both the resting influx and efflux by three-fold, independent of the external Ca^{++} concentration. He also observed that in the presence of caffeine and independent of the external calcium concentration, the calcium uptake to be increased in K^+ depolarized muscle, but not the rapid transient high Ca^{++} influx at the beginning of a K^+ induced contracture. Furthermore, since caffeine contractures could be elicited in the absence of calcium, Bianchi concluded that

the increased calcium influx was not the basis of a caffeine contracture. Further work by Caldwell and Walster (1963), using the external application of 5 mM caffeine to crab muscle fibers to produce a sustained contracture, demonstrated no initially significant reduction in the membrane potential, and that contractures could be obtained both when the fibers were depolarized with K^+ and during the repolarization phase. Also, the injection of Ca^{++} and caffeine each, was shown to cause a contracture in normal polarized and K^+ depolarized fibers. Moreover, Axelsson and Thesleff (1958) noted that the effect of caffeine on contractures was independent of the external calcium concentration, leaving the resting membrane potential and transverse membrane resistance unaltered. Thus, it was suggested that caffeine acted at a site subsequent to membrane depolarization, i.e. membrane depolarization was not a prerequisite for caffein's contractile activity.

The above observations could be integrated into a plausible explanation if it was assumed that caffeine reduced the binding of Ca^{++} on the surface membrane and/or sarcoplasmic reticulum, thus increasing the intracellular free ionic Ca^{++}, in turn increasing the Ca^{++} efflux. Supporting this, Bianchi (1962), and Isaacson and Sandow (1967) have shown caffeine to enter and freely move through frog and rat muscles, thus being capable of action at internal sites such as the sarcoplasmic reticulum. Moreover, Naylor (1963) showed that caffeine induced a positive inotrophic response in the toad ventricle, causing increased Ca^{++} fluxes and abolution of the 'staircase' phenomenon. The latter could be explained in terms of increased Ca^{++} permeability

of the membrane and sarcoplasmic reticulum, and therefore a functional refractoriness to the signal normally leading to graded increments of Ca^{++} release. Similar actions of caffeine were proposed by Mambrinian and Benoit (1963) and Delga and Foulhoux (1963) to explain the decurarizing action of caffeine at the neuromuscular junction, i.e. that caffeine increased the mobilization of Ca^{++}, in turn releasing more acetylcholine. As well, Nagai and Uchida (1960) had previously demonstrated caffeine-contracted fibers to relax with crude muscle extracts containing relaxing factor. Herz and Weber (1965) later clarified the above observations by finding caffeine in concentrations of 8 to 10 mM to inhibit Ca^{++} uptake by frog sarcoplasmic reticulum, and to release 20 to 40% of the bound Ca^{++} from the sarcoplasmic reticulum after the maximum Ca^{++} uptake had been reached. This meant that caffeine either caused an inhibition of the ability of the sarcoplasmic reticulum to accumulate Ca^{++}, or decreased the efficiency of the Ca^{++} pump.

Caffeine has also been shown to potentiate the twitch in concentrations of 0.05 mM to 4 mM and to produce contractures in concentrations of 2 to 5 mM in frog muscle fibers (Axelsson & Thesleff, 1958; Frank, 1962; Sandow & Brust, 1966). Furthermore, Sandow (1965) has shown that caffeine produces changes in the action potential similar to the lyotrophic anions, i.e. a reduction in the mechanical threshold, and the divalent metallic cations, i.e. a prolongation of the action potential. Although the action of both caffeine and divalent cations result in increased Ca^{++} release during activation, the ions have been believed to act on the surface membrane

in mediating changes in the action potential, while caffeine has been thought to act at an intracellular site. From the recent literature, Sr^{++}, although not physiologically present, has been shown to be similar to Ca^{++} in supporting excitation-contraction coupling. For example, Edwards et al. (1966) demonstrated K^{+} induced contractures in frog muscle to be supported by Sr^{++}, and caffeine contractures to be partially restored by the presence of Sr^{++} in a Ca^{++} free medium. These findings were also correlated with an increased Sr^{++} uptake during activity. In addition, Naylor (1965) reported that Sr^{++} was the only ion capable of substitution for Ca^{++} in cardiac muscle.

Despite these observations in frog muscle, Gutmann and Sandow (1965) reported that normal mammalian skeletal muscle failed to produce contractures even in 20 mM caffeine concentrations. For this to occur, denervation appeared to be a prerequisite. Recently, however, Buss and Frank (1967) and Frank and Buss (1967) reported that normal mammalian skeletal muscle was indeed sensitive to caffeine.

6. Model for Excitation-Contraction Coupling:

From the description given so far, a summary of the role of Ca^{++} in excitation-contraction coupling can be presented with a minimum of histological detail. When a muscle is electrically stimulated, the fiber surface membrane becomes highly permeable to Na^{+} ions with a resultant depolarization, which is transmitted through the T-tubules (invaginations of the surface membrane). This depolarization could permit either the displacement of Ca^{++} across the cell membrane, or the release of bound Ca^{++} from the terminal cisternae of the sarco-

plasmic reticulum. The second is more important in fast muscles. This released ionic calcium could sufficiently raise the free ionic calcium concentration in the sarcoplasm to activate the actomyosin-ATPase and initiate contraction. An alternate possibility is the transmission of the signal propagated along the surface membrane and T-tubules across the junction of the T-tubules and adjacent terminal cisternae with a resultant increased permeability of the terminal cisternae and release of Ca^{++}; one mode of transmission plausibly being an electrotonic pulse. As a consequence, the released Ca^{++} could then diffuse from the terminal cisternae under a large concentration gradient to activate the myofibrils. Regardless of the mechanism, however, the role of Ca^{++} is primary for contraction. Hence, relaxation most probably is governed by the removal of Ca^{++} from the contractile proteins by the Ca^{++} pump of the sarcoplasmic reticulum.

From the above model, it is evident that many details of the processes involved in the excitation-contraction coupling cannot be explained at the present time. For example, the exact mechanism by which intracellularly bound Ca^{++} is released, or by which it is sequestered by the sarcoplasmic reticulum from the contractile proteins remain unknown.

IV. DISRUPTION OF T-TUBULES

In the last few years, a technique has been developed to selectively disrupt T-tubules. The initial observations were made by

Fujino et al. (1961) and Yamaguchi et al. (1962), who noted that a hypertonic solution of glycerol or urea had a different effect on muscle compared to a hypertonic solution of sucrose or NaCl. This effect was the disappearance of mechanical activity with the preservation of normal electrical activity, i.e. the action potential, in preparations exposed for 1 hour to 400 mM glycerol and returned to normal isotonic solutions. By electronmicroscopy, Howell and Jenden (1967) and Eisenberg and Eisenberg (1968) demonstrated the only damage to these preparations was disruption of the T-tubules, the mechanism of which remains clouded. Recently, Howell (1969) suggested that the removal of glycerol, after it penetrated the muscle fibers during their exposure to the hypertonic solution, caused disruption of T-tubules by an osmotic phenomenon. For this he offered two possible mechanisms:

(1) that during exposure, glycerol penetrates the fiber, only to elute in exchange for an influx of water when the isotonic solution is restored, the sites of exchange being between the sarcoplasm and the extracellular space, and the T-system and the extracellular space. If either the glycerol efflux or water influx occurs faster at the former than the latter site, an osmotic gradient could develop across the T-system membranes causing swelling or disruption of the T-tubules.

(2) that in a hypertonic glycerol solution, the fibers shrink, while upon return to an isotonic solution

they swell, the volume changes being due to the efflux and influx of water at rates faster than those of glycerol. Thus, a faster influx of water into the sarcoplasm than efflux of glycerol from the T-tubules could establish an osmotic concentration gradient across the T-system membranes, causing them to disrupt.

V. MECHANICAL PROPERTIES OF DENERVATED MUSCLE

Recently, Padsha (1968) and Padsha and Winchester (1968) studied some mechanical properties of denervated muscle, observing that the mechanical responses of normal and denervated muscle differed from each other, the degree depending upon the environmental temperature. For example, they found denervated muscle developed a maximum twitch tension at 35°C, the tension dropping progressively to very low values at 10°C, whereas normal muscle developed maximum twitch tension at 17°C, the tension decreasing above and below this temperature. They also observed that denervated muscle developed less tetanic tension than normal muscle at all temperatures, although the twitch tension ratio at 35°C was greater in denervated muscles. Moreover, the maximum rate of rise of tension in twitch was always greater in normal muscle for all the temperatures, while the duration of the active state was longer in denervated muscles above 20°C.

Little is known about the relation between changes in the ionic fluxes, changes in the electrical and mechanical properties of

denervated muscle. Since changes in the excitation-contraction coupling may produce changes in the mechanical properties of the muscles, and since Ca^{++} is believed to be one of the most important ions responsible in linking excitation of the membrane to the contraction, it was suspected that changes in Ca^{++} fluxes could be involved in many of the observed mechanical property changes in denervated muscle. Hence, the purpose in undertaking this project was to study in normal and denervated muscle, passive Ca^{++} fluxes, plus some electrical properties in an attempt to correlate changes in these with changes in the mechanical properties of denervated muscle.

MATERIALS AND METHODS

Denervation:

Adult white albino female rats of Wister strain, weighing 200 to 350 g, were used throughout the experiments reported here. For denervation purposes, the rats, under ether anesthesia, were injected intraperitoneally with sodium pentobarbital, 25 mg/kg of body weight. The left hemidiaphragms were then denervated by first lifting the digastric muscle, making a small incision through the sternohyoid muscle to expose the region where the phrenic nerve crosses the brachial plexus, and about 1 to 2 cm of the nerve was excised. The incision was closed with surgical clamps. The reason for denervating the left hemidiaphragm was because of the easier access in exposing the left phrenic nerve as it crosses the brachial plexus. For the purpose of sterilization, the surgical instruments were boiled for 15 minutes prior to denervation. In a few animals, sham-denervation was done by exposing, but not touching, the left phrenic nerve. These animals were kept on a normal diet.

Solutions:

The normal mammalian Krebs solution used was a slightly modified Krebs-Henseleit solution and unless otherwise stated, had the following composition: NaCl, 118 mM; KCl, 4.8 mM; $CaCl_2$, 1 mM, $MgSO_4$, 1.2 mM; KH_2PO_4, 0.8 mM; $NaHCO_3$, 25 mM; glucose, 5.55 mM; pH, 7.3. All the chemicals were obtained from Fisher Scientific Company and were of analytical grade. In order to avoid spontaneous twitching, Procaine

(0.25 mM) was added to the Krebs solution. This use of Procaine corresponded to that of Feinstein (1963) for frog sartorius muscles, who showed that relatively low concentrations of Procaine (i.e. up to 0.367 mM) did not block the release of Ca^{45} produced by 2.5 mM caffeine, and that of Isaacson and Sandow (1967), who found that 0.25 mM Procaine did not interfere with the effect of 20 mM caffeine on rat EDL muscles. Although Procaine has been shown to cause a profound inhibition of active calcium transport by isolated sarcotubular muscles when used in 15 to 20 mM concentrations (Wilcox & Fuchs, 1969), no effect, when used in 2 to 5 mM concentrations, was observed by Carvalho (1968) on either the release or inhibition of calcium from isolated fragmented sarcoplasmic reticulum at rabbit skeletal muscle. Similarly, Kuperman et al. (1968) showed that 7 to 10 ml Procaine was required to produce a measureable and consistant release of Ca^{45} from frog sartorius muscle and desheathed sciatic nerve. As a further check, Tubocurarine chloride (10 mg/l) was used in some experiments, there being found no difference in the calcium uptake or release for either Procaine (0.25 mM) or Tubocurarine (10 ml/l). Thus, although Procaine could cause a block or release of calcium, the concentrations required would be much greater than 0.25 mM, which was used in the Krebs solution. Hence, the concentration 0.25 mM Procaine was chosen as Isaacson and Sandow (1967) have successfully used it for mammalian muscles.

Dissection:

When desired, the animals, under ether anesthesia, were

sacrificed by opening the thorax. The whole diaphragms were then excised and transferred immediately to the normal mammalian Krebs solution.

In those experiments where cut strips were used, 6 to 10 mm wide, slightly triangular strips, four from each hemidiaphragm, were cut lengthwise from tendon to ribs, with a piece of attached rib at the base, and transferred to a bulk (600 ml) of Krebs solution through which a mixture of 95% O_2 and 5% CO_2 was bubbled. In the experiments employing hemidiaphragms, after removal, each was first separated, washed in Krebs solution, and then allowed to equilibrate in a bulk of Krebs solution at room temperature for a minimum of 1 hour. Fifteen minutes prior to any experimentation, these were then transferred to 100 ml Krebs solution at the desired temperature.

Efflux Experiments:

For both the efflux and influx studies of calcium the standard techniques of Shanes and Bianchi (1959) were used, which are described here.

The efflux experiments were done in two series. In one, muscle strips were used with a small piece of rib attached, as it was extremely difficult to excise this without damaging the muscle fibers, which terminate via a short tendon in close conjunction to the ribs. The strips were fixed vertically in either:

(1) a specially constructed chamber with a temperature-controlled water jacket; or,

(2) a test tube placed directly in a water bath.
In both, the lower ends of the muscles were fixed to a siliconized glass rod while the upper end was attached by a stainless steel wire to a force transducer. The resting tension of the muscle was kept 1g. The muscles were loaded for 2 hours in 5 ml Krebs solution containing 1 μc/ml Ca^{45}, obtained from New England Nuclear Corporation, Boston, U.S.A. After loading, those muscles mounted in the chambers were washed quickly 3 times to remove surface adsorbed Ca^{45} and then washed in non-radioactive Krebs solution for 3 hours. The effluents were collected every 10 minutes and replaced by fresh solution. When test tubes were used, after loading, the muscles were rinsed for 5 seconds in non-radioactive Krebs solution and then transferred to test tubes containing 5 ml of non-radioactive Krebs solution every 10 minutes. At the end of 3 hours washing, the muscles were removed from the chambers or test tubes, lightly blotted 3 times, and their wet weights determined.

Because of the possibility of Ca^{45} contamination from the attached rib, in the second series of experiments, the muscle strips were cut as above, four per hemidiaphragm, and loaded for 2 hours in 60 ml Krebs solution containing 1 μc/ml Ca^{45}. These were washed for different lengths of time in a 100 ml Krebs solution which was changed every 10 minutes, the effluents being discarded. Instead, at the end of each period, a small piece of muscle was excised from the center of the strips and weighed. Usually sixteen strips from four different hemidiaphragms, either normal or denervated, were used to construct one single efflux curve. Thus, by this technique, what was being

measured was the Ca^{45} remaining in the muscle itself at the end of each washing time, not that coming out during this period.

In using cut strips, there was also a possibility that some of the calcium may not elute through the sarcolemma but through the cut edges of the strips. In order to avoid this, whole hemidiaphragms were loaded for 2 hours in 100 ml Krebs containing 1 μc/ml Ca^{45}, then washed for different time periods in 100 ml Krebs solution which was changed every 5 minutes during the first 30 minutes and then every 10 minutes thereafter. At the end of each washing period, a small piece from the center of each hemidiaphragm was excised, blotted lightly 3 times and weighed. Ca^{45} remaining in this central piece was then measured by liquid scintillation counting. The results were presented as Ca space (ml/g) which was obtained by dividing the radioactivity remaining in 1 g of muscle by the specific activity of 1 ml of loading solution. The reason for presenting them in terms of Ca space was to enable them to be expressed independently of the external calcium concentration. The time constants were derived from a regression line, drawn by least square method using I.B.M. computer.

Influx Experiments:

For calcium influx studies, both the cut strips and whole diaphragms were used. For influx rate measurements, these muscles were loaded in 60 ml Ca^{45} Krebs solution for 5, 10 and 15 minutes, and then washed for 1 hour to remove the extracellular Ca^{45}. Radioactivity remaining in the central piece of the muscles was then

measured and converted into Ca space as described previously. Since the molarity of calcium in our solutions was 1 mM/liter, the actual amount of calcium/ml is 1 μmole. It also means that whenever the Ca space is 1 ml/g, the actual amount of calcium is 1 μmole/g. Thus, for the calculations of rates of influx, the Ca space in ml/g was divided by loading time in seconds.

For Ca space measurements, the muscles were loaded at varying time periods up to 480 minutes. If the total (intracellular plus extracellular) Ca space was to be measured, the muscles immediately after loading were stirred vigourously for a few seconds in two changes of 50 ml of 400 mM sucrose solution to remove the surface adsorbed Ca^{45}, blotted lightly 3 times on filter paper, and weighed. When the intracellular Ca space was to be measured, the muscles after loading were washed either for 60 or 90 minutes in 100 ml Krebs solution to remove the extracellular Ca^{45} and then proceeded as usual.

In Vivo Experiments:

In another experimental series for studying the efflux and influx of calcium, the loading of the muscles was done *in vivo*. The animals were kept under nembutal anesthesia (6 mg/kg) and the left external jugular vein was exposed, and 1 ml of normal saline containing 10 μc/ml Ca^{45}, was injected. After 15 minutes of injection, the diaphragms were excised, rinsed in two changes of 100 ml of 400 mM sucrose solution, blotted and weighed. For intracellular calcium uptake or for efflux studies, the diaphragms were washed in 100 ml

of non-radioactive Krebs solution for a desired time, the bathing medium being changed every 10 minutes, and the Ca^{45} remaining in the muscles after this time was measured.

Extracellular Space:

For the measurement of the extracellular space, Inulin-C^{14} was used. Whole diaphragms were loaded for various time periods from 2 to 120 minutes in 60 ml Krebs solution, containing 0.25 µc/ml Inulin-C^{14}, at the end of which, the muscles were quickly rinsed in two changes of 50 ml of 400 mM sucrose solution, blotted and weighed. The radioactivity remaining in the central piece of the diaphragms was then measured and Inulin-C^{14} space was calculated as described previously for the Ca^{14} space determination. Since the Inulin-C^{14} intracellular space saturation curves reached a plateau between 60 and 90 minutes, the muscles were loaded for 2 hours as a precaution. Hence, the results were presented as Inulin-C^{14} extracellular space (ml/g) of muscles at the end of 2 hours loading.

Digestion and Counting Fluor:

At the end of each experiment, the muscles were transferred to the standard 20 ml liquid scintillation counting vials and digested directly in NCS. NCS, a product of Nuclear Chicago Corporation, Illinois, U.S.A., is a 0.6 N toluene-soluble quaternary ammonium base. Hansen (1967) has shown its superiority as a solubilizer for biological materials. One ml of NCS was used for digesting 20 mg or less of muscle, complete digestion occurring within 2 hours at

60°C with continuous shaking. At the end of digestion, to each vial 14 ml of counting fluor was added as well as 0.25 ml 30% hydrogen peroxide as a bleaching agent to remove any extraneous color which might alter the counting efficiency. Similarly, to the effluents from each efflux experiment, 14 ml of the counting fluor was added.

Although numerous methods were available for the preparation of liquid scintillation counting fluors (Hansen & Bush, 1966; 1967; Radkin, 1967; Radim, 1964), the choice was made of that given by Bruno and Christian (1961) because it can take more than 30% water. This counting fluor (also known as XDC fluor) was prepared according to the following formula.

PPO (2, 5-Diphenyloxazole)	=	10.0 gms
POPOP (1, 4-Bis-[2-(5-phenyloxazolyl)]-benzene)	=	0.5 gms
Naphthalene	=	80.0 gms
Xylene	=	143.0 ml
1, 4-Dioxane	=	428.0 ml
Ethylene Glycol monobutyl Ether (Butyl cellosolve)	=	428.0 ml

PPO and POPOP were that of scintillation grade and were obtained from Fraser Medical Supplies, Ltd., Vancouver, B.C. The rest of the chemicals were Fishers' certified reagents. This fluor took 2 to 3 days to prepare.

Counting of Radioactivity:

The estimation of both the radioactive Ca^{45} and C^{14} was

done by counting the samples on Picker Nuclear Scintillator Model Liquimate 220. The samples from each experiment were counted for 10 minutes, corrected for background and quenching (according to the procedure given in manufacturer's manual) and the data was then used for further calculations.

Recording of Twitch Tension:

For the recordings of isometric twitch tensions, the muscles were placed horizontally in the upper part of a muscle chamber, the lower part through which water circulated from a water bath (LO-Temptrol, Model 154 - Precision Scientific Company). The bath's temperature was controlled by a Tele-thermometer, Model 73 (Yellow Springs Instrument Company, Inc., U.S.A.). By stainless steel hooks, one end of the muscle was attached to a fixed stainless steel rod which also was used as a stimulating electrode. The other end of the muscle was attached to a Statham G1-4-250 force transducer whose output was amplified by a Sanborn transducer amplifier - Indicator, Model 311A. A platinum electrode was placed on the muscle, 1 to 2 cm away from the fixed stimulus electrode. Both the electrodes were connected to a Grass Stimulator, Model S4, through a stimulus isolation unit, also from the same company. All the recordings were made on Tektronix Type 549 storage oscillo-scope and photographed by a Kymograph Camera, Model C4J. The muscles were always kept immersed in the running oxygenated Krebs solution. For this purpose, oxygenated Krebs solution was drawn from a reservoir through a coil of stainless steel tubing passing

through the water-filled lower part of the muscle chamber. Thus, when it emptied into the upper part of the chamber, it had equilibrated to the desired temperature. The excess of the fluid was removed by suction into a collecting jar at the other end of the chamber.

The muscles were always stimulated supramaximally. Usually a 10 msec pulse was used for this purpose. Before each experiment, the resting length of the muscle was determined by stimulating the muscle at zero resting tension and then gradually increasing the length while recording the corresponding twitch. This was continued until a maximum twitch tension was obtained, the corresponding length being taken as the resting length of the muscle, and was used for further calculations.

Recording of Action Potentials:

For recording the action potentials intracellularly, glass microelectrodes, pulled from 0.9 to 1.1 mm Kimex tubing with a microelectrode puller, Model M1 (Industrial Science Associates, Inc., New York) and filled with 3M KCl, were used. Microelectrodes only with 5 to 30 megaohm resistance and low tip potentials (< 25 mV) were used. The microelectrodes were connected, by means of a platinum wire loosely hung floating from a Leitz micromanipulator to a Medister microelectrode probe and amplifier, Model A-35. When the microelectrode was dipped in the Krebs solution, usually a tip potential was observed. If this tip potential was 25 mV or less, it was adjusted to zero; the potential change that occurred when the microelectrodes entered the

fiber was then recorded, providing it remained stationary for at least 30 seconds. The problem of mechanical and electrical disturbances during the action potential recording were overcome by stimulating the muscle with a very small current of short duration (0.1 msec) and floating microelectrodes.

RESULTS

I. Ca^{45} EFFLUX FROM NORMAL AND DENERVATED RAT DIAPHRAGM

The purpose of this set of experiments was to determine the efflux half times of Ca^{45} from the slow and fast compartments of muscles and to determine if there was a difference between the normal and denervated muscles.

1. From Cut Strips:

In the experiments studying the Ca^{45} efflux from rat diaphragm strips, the technique of Shanes and Bianchi was used. The cut strips from both the normal and 16 to 46 days denervated muscle to which was attached a small piece of bone and some connective tissue, were placed in separate chambers containing 5 ml of Krebs solution to which 1 μc/ml of radioactive Ca^{45} was added. The muscles were loaded for 2 hours and washed in non-radioactive Krebs solution for 3 hours, the solution being changed every 10 minutes. At the end of 3 hours washing, the muscles were dissected free of bone and tissue, blotted lightly three times on tissue paper and their wet weight determined. The radioactivity remaining in the muscles was then measured as described in the methods. For the construction of efflux curves, the counts remaining in the muscles were added to the effluent counts to get the total Ca^{45} at zero washing time, which was the total Ca^{45} uptake at the end of 2 hours loading. Taking this value as 100%, the percentage of activity remaining in the

muscle was calculated and plotted against washing time on a semilog paper. Figure 1 shows efflux curves for Ca^{45} at 35°C. The data to construct this figure was obtained by averaging the points from individual experiments. The curve constructed from individual experiments looked like one presented in Figure 1 with exceptions in the half times of Ca^{45} efflux from the slow compartments (see text for Figure 2). These efflux curves showed at least 2 components, an initial fast component, which most probably represented the extracellular compartment, and a slow component, which most probably was the intracellular compartment (see Discussion).

Figure 1 showed that the average rate of efflux of Ca^{45} from the slow compartment of denervated muscles was faster with a half time of 67 minutes, as compared to the normal muscles, which had a half time of 113 minutes. After denervation, the rate of Ca^{45} efflux became almost double. The half times were calculated in a region of the curve between 90 and 180 minutes of washing when most of the extracellular space was washed out. Although wide fluctuations were observed in the half times obtained from individual efflux curves of normal muscles, they usually ranged from 64 to 177 minutes, except in two experiments where extreme values of 267 and 445 minutes were obtained. These two experiments were not included in the construction of efflux curves or in taking the means of half time. Similarly, the half times obtained from individual efflux curves of denervated muscles ranged from 40 to 95 minutes. Figure 2 shows frequency histograms of half times obtained from individual Ca^{45} efflux curves of normal and denervated muscles. In normal muscles, the maximum number of values fell either at 97 or 107 minutes; however, since the distribution was larger on the right side, the mean shifted slightly to the right, giving a slightly higher value of 113 minutes. In denervated muscle, the maximum number of values fell either at 62 or 72 minutes,

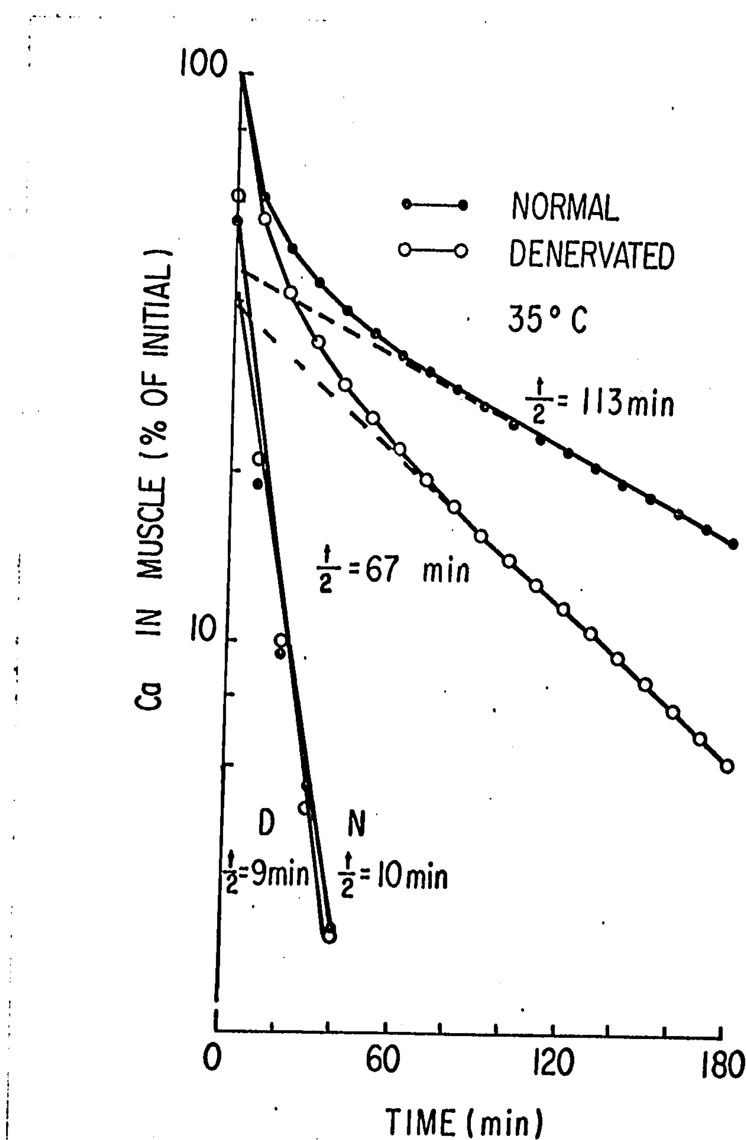

Figure 1 Efflux curves for Ca^{45} obtained from normal and 16 to 45 days denervated rat diaphragm strips at 35°C. The muscles were loaded for 2 hours in 5 ml radioactive Krebs solution containing 1 μc/ml Ca^{45}. The curves were the means of 44 experiments for normal muscle and 28 experiments for denervated muscle.

(N) Normal R^2 for (N) = 0.992 (D) Denervated R^2 for (D) = 0.988

t/2 = half time in minutes.

Ordinate = Ca space on log scale Abscissa = washing time (min)

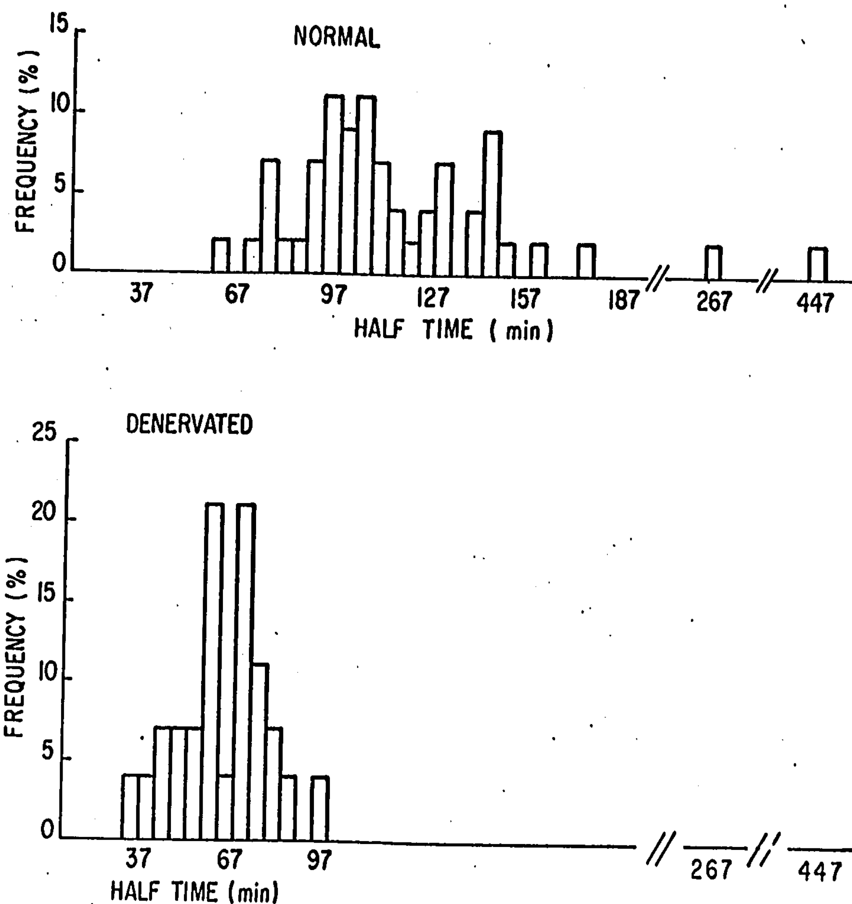

Figure 2. Frequency histograms constructed from half times obtained from Ca^{45} efflux curves of both the normal and 16 to 46 days denervated rat diaphragm strips at 35°C.

Number of normal muscles = 44

Number of denervated muscles = 28

the rest being distributed almost equally on either side, giving a mean at 67 minutes. Although the histograms showed an overlap of half times obtained from normal and denervated muscles, in no one experiment did normal muscle show a half time smaller than its contralateral denervated side. There was a significant difference in the mean half times between the normal and denervated muscles.

The half times for the efflux of Ca^{45} from the fast compartment were measured by plotting on a semilog paper the difference between the total and the intracellular calcium (extrapolated value) at different washing times (Fig. 1). The values obtained gave a straight line relationship with correlation coefficient R^2 0.979 for normal and 0.982 for denervated muscles. The half times for Ca^{45} efflux from the fast compartment calculated from the slopes of these lines were 10 and 9 minutes for normal and denervated muscles, respectively. This difference in time courses of Ca^{45} efflux from the fast compartments of normal and denervated muscles was negligible.

In the experiments described above, all the muscles used had attached a small piece of bone and some connective tissue. Thus there was a possibility of calcium contamination from them. Therefore, an attempt was made to remove at least all the connective tissue completely so that only a very small, thin piece of bone was left.

Six such muscle strips were used, the results shown in Figure 3 and summarized in Table I. Again, there was a difference in the time course of Ca^{45} efflux from the slow compartments of the normal and denervated muscles. The half times of Ca^{45} efflux from

normal muscles ranged from 120 to 191 minutes with an average of 147 ± 10 minutes, while those from the denervated muscles ranged from 104 to 134 minutes with an average of 115 ± 4 minutes. There was a significant difference between the half times for the normal and denervated muscles.

When the slope of slow compartments in the efflux curve was extrapolated back to zero washing time, the zero time intercept gave the amount of calcium taken up by this compartment in 2 hours loading time. The amounts of intracellular calcium in μmole/g in normal and denervated muscles is given in Table 1. The average amount of intracellular calcium in normal and denervated muscles was 2.7 ± 0.2 μmole/g and 3.2 ± 0.3 μmole/g, respectively. There was no significant difference in the amounts of intracellular calcium between the normal and denervated muscles ($P > 0.05$), but the amount of calcium remaining in the denervated muscles after 4 hours washing was less than that in the normal muscle (Table I). This difference was significant ($P < 0.005$). This was because most of the calcium was washed out at a faster rate in the denervated muscles.

2. From Whole Diaphragms:

Since in the previous sets of experiments cut strips were used, another possibility or error was leakage of Ca^{45} from the cut edges. Thus, to avoid this possibility, whole diaphragms were used in the following experiments. The diaphragms were loaded with radioactive Ca^{45} for 15 minutes and then washed for different time periods. At the end of each experiment, the radioactivity remaining in the

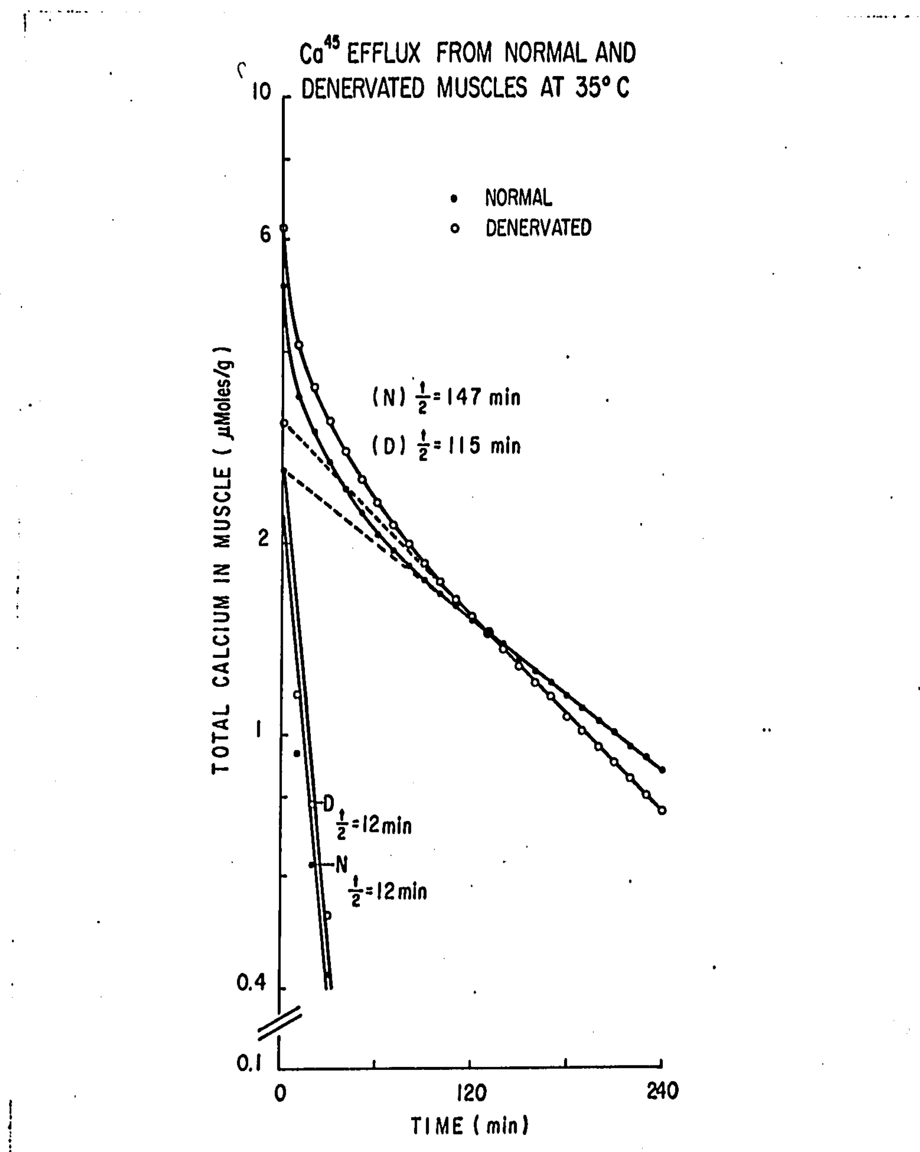

Figure 3. Efflux curves for Ca^{45} obtained from normal and 27 to 38 days denervated rat diaphragm strips at 35°C. The muscles were loaded for 2 hours in 5 ml radioactive Krebs solution containing 1 μc/ml Ca^{45}. The curves were the means of six experiments, each, of normal and denervated muscles. Here the amount of Ca in μmole/g remaining in the muscles was plotted against washing time.
(N) Normal (D) Denervated t/2 = half time in minutes

TABLE 1

Half times of efflux of Ca^{45} and the uptake of calcium in the slow compartments during 2 hours loading in normal and 27 to 38 days denervated rat diaphragm strips at 35°C.

Muscles	Half times (min) Mean ± S.E.		Amount of calcium at zero time intercept (µmole/g) in Slow compartment	Ca remaining at the end of 4 hours washing (µmole/g)
	Slow compartment	Fast compartment	Mean ± S.E	Mean ± S.E.
Normal (6)	147 ± 10	12.0	2.7 ± 0.2*	0.29 ± 0.05
Denervated (6)	115 ± 4	12.0	3.2 ± 0.3*	0.08 ± 0.01

This data was obtained from Figure 3. The figures in parentheses represent the number of muscles used. P denotes the probability that there is no significant difference between the two populations.

* $P > 0.05$

central piece of the muscle was measured while the effluent samples were discarded.

In this series of experiments, 19 to 38 days denervated rat diaphragms were used. Both normal and denervated muscles were taken from the same animal. Figure 4 shows the rate of efflux of Ca^{45} from the slow compartments of both the normal and the denervated muscles at 35°C. The half times for the efflux of Ca^{45} were calculated from the regression lines. Thus, the half time for the efflux of Ca^{45} from the slow compartment of the normal muscles was 158 minutes, the regression line having a correlation coefficient R^2 0.4196. Similarly, the half time for the slow compartments of the denervated muscles was 77 minutes, the regression line having a correlation coefficient R^2 0.833.

When the regression line was extrapolated back to zero washing time, the zero time intercept gave the amount of calcium taken up by the slow compartment during 15 minutes of loading. The amounts of calcium in the slow compartments in the normal and the denervated muscles (as obtained from Fig. 4) were 0.034 μmole/g and 0.074 μmole/g of muscle, respectively. When the efflux curve for Ca^{45} from the slow compartment was plotted on an ordinary graph paper, it yielded an exponential curve. This exponential curve can be represented by the equation:

$$Y(t) = Y(o)e^{-t/t.c.} \quad [1]$$

where Y(t) = amount of Ca remaining in the slow compartment of the

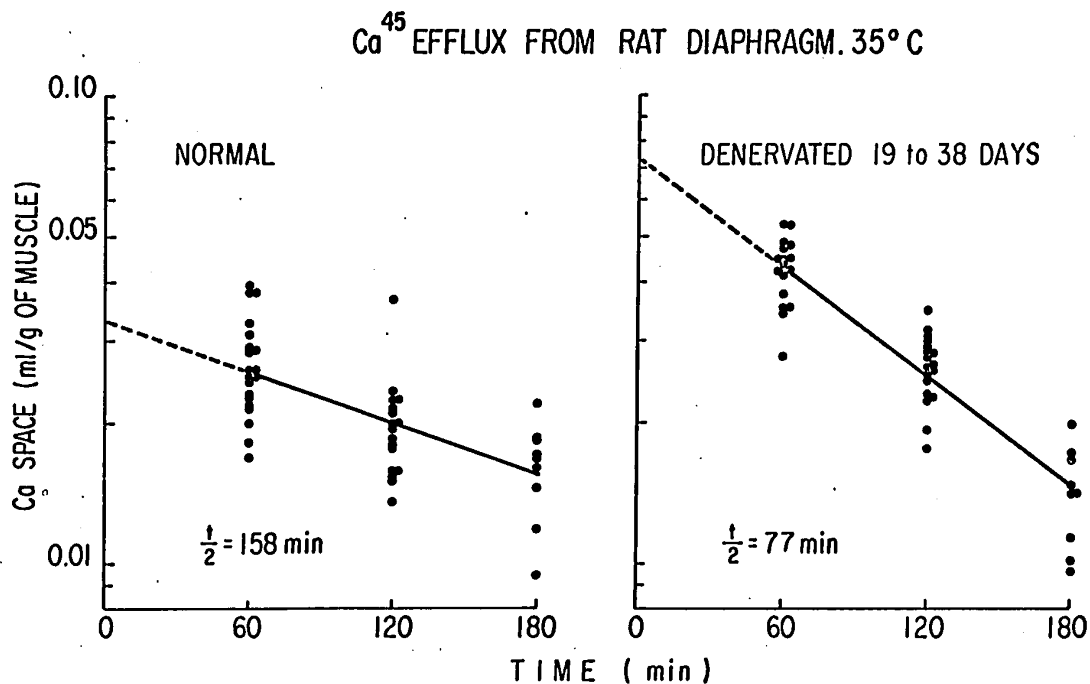

Figure 4. Efflux curves of Ca^{45} from the slow compartments of both the normal and 19 to 38 days denervated rat diaphragms at 35°C. The muscles were loaded in 60 ml radioactive Ca^{45} containing Krebs for 15 minutes and washed for 60, 120 and 180 minutes. The half times were measured from the regression lines. In each case 49 muscles were used.

Ordinate = Ca space on log scale

Abscissa = washing time (min)

muscle after t minutes of washing.

$Y(o)$ = amount of Ca in the slow compartment of the muscle at zero washing time.

e = exponential.

t = washing time in minutes.

$t.c.$ = efflux time constant in minutes.

In order to find the value of $Y(o)$, i.e. the amount of calcium in the slow compartment at zero washing time, the above equation can be rewritten as follows:

$$Y(o) = Y(t)/e^{-t/t.c.} \qquad [2]$$

However, upon plotting the efflux curve of Ca^{45} from the slow compartment on semilog paper, a straight line was yielded, with equation [2] being rewritten as follows:

$$\log Y(o) = \log Y(t) + t/t.c.$$

The time constants used for these calculations for normal and denervated muscles were obtained from the regression lines in Figure 4. The calculated average Ca uptake by slow compartments (for 49 normal and denervated muscles) was 0.034 ± 0.001 µmole/g and 0.075 ± 0.002 µmole/g of muscles, respectively. There was a significant difference between the two.

3. Effect of Denervation Days on Ca^{45} Efflux:

In the course of experiments on Ca^{45} efflux from the whole diaphragms, it was observed that the half times for the rate of efflux also depended on the number of denervation days. The results were arranged in four groups depending upon the days of denervation as follows:

Group I	7 to 8 days
Group II	14 to 20 days
Group III	19 to 38 days
Group IV	40 to 61 days

Experiments of Groups I, II and IV will be explained here. In each set of experiments, the denervated muscles were always compared with their own normal sides. The results of all these experiments are summarized in Table II.

a) Group I:

In this set of experiments, 7 to 8 days denervated hemidiaphragms were used. The muscles were loaded with Ca^{45} for 2 hours and then washed for different time periods. The effluents were discarded and Ca^{45} remaining in the central piece of the muscle after each washing was measured. Figure 5 shows the rate of efflux of Ca^{45} from slow compartments of both the normal and denervated muscles at 35°C. The half times for the efflux of Ca^{45} were calculated from the regression line. The half time for the efflux of Ca^{45} from the slow compartment of the normal muscle was 176 minutes, the regression

TABLE II

Half times of Ca^{45} efflux and calcium uptake by slow compartment during 2 hours loading in normal and different days denervated rat diaphragms at 35°C.

Muscles	Days of Denervation	Half Time (min)	Ca uptake by slow compartment (µmole/g)
Normal (24)	7 to 8	176	0.3
Denervated (24)		134	0.64
Normal (50)	14 to 20	141	0.33
Denervated (50)		86	0.51
Normal (35)	40 to 61	162	0.31
Denervated (35)		67	0.72

This data was obtained from Figures 5, 6 and 7. The figures in parentheses represent the number of muscles used.

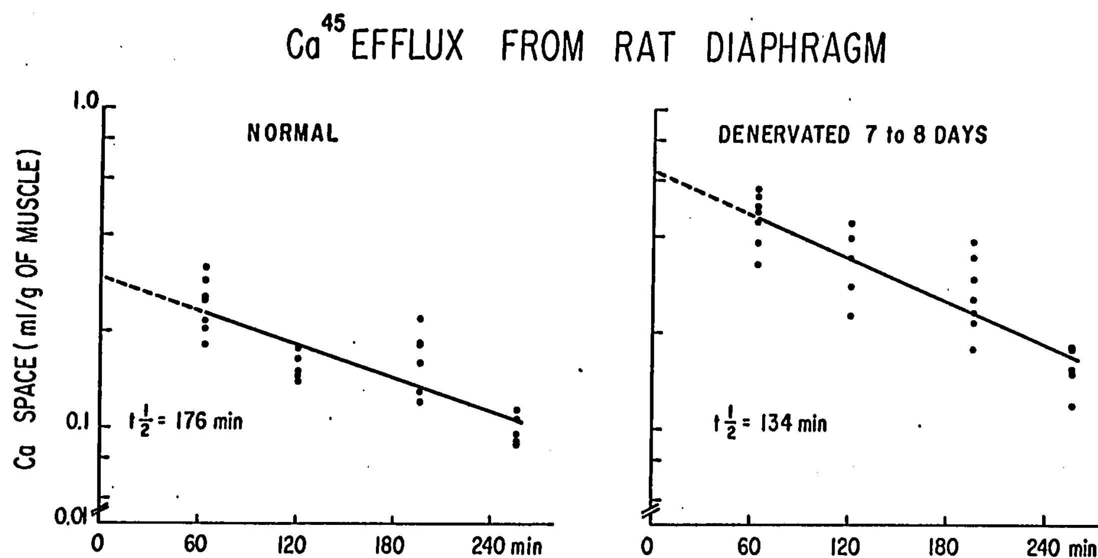

Figure 5. Efflux curves of Ca^{45} from the slow compartments of both the normal and 7 to 8 days denervated rat diaphragms at 35°C. The muscles were loaded in 60 ml radioactive Ca^{45} containing Krebs solution for 2 hours and washed for 64, 121, 196 and 256 minutes. The half times were measured from the regression lines.

Ordinate = Ca space on log scale

Abscissa = washing time (min)

line having a correlation coefficient R^2 0.668. In comparison, the half time for the efflux of Ca^{45} from the slow compartments of the denervated muscle was 134 minutes, the regression line having a correlation coefficient R^2 0.7317.

When the regression line was extrapolated to zero washing time, the zero time intercept gave the amount of calcium taken up by the slow compartment at the end of 2 hours of loading, these being 0.3 ml/g Ca space for normal and 0.64 ml/g Ca space for the denervated muscles. Since the molarity of Ca in the solution was 1 mM/liter, the actual amount of Ca in 1 ml of solution was 1 µmole. This meant that 1 ml of Ca space represented 1 µmole of Ca. Thus, the above slow compartment calcium spaces in normal and denervated muscles could also be represented as 0.3 µmole/g and 0.64 µmole/g of muscle, respectively. There is a significant difference between the two.

b) <u>Group II</u>:

In this set of experiments, 14 to 20 days denervated hemidiaphragms were used, the procedure having been previously described. Figure 6 shows the rate of efflux of Ca^{45} from the slow compartments of both the normal and denervated muscles. The half times for the efflux of Ca^{45} were calculated from the regression lines which are 141 minutes for the normal and 86 minutes for the denervated muscles. The zero time intercept gave the calcium uptake by the slow compartment at the end of 2 hours loading in normal and denervated muscles: 0.33 µmole/g and 0.51 µmole/g of muscle, respectively. The difference between the normal and denervated muscles was significant.

A comparison of the Ca uptake by the slow compartment in

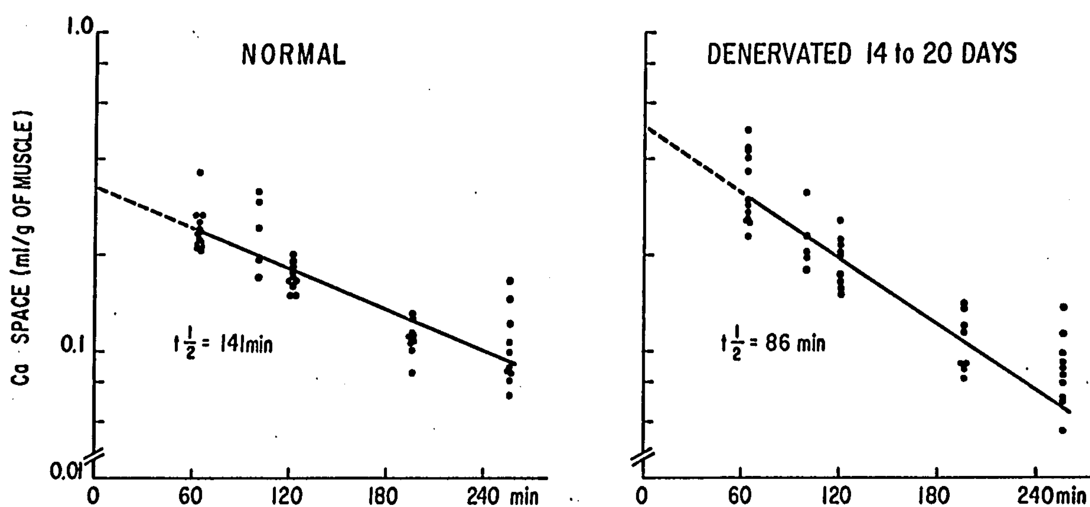

Figure 6. Efflux curves of Ca^{45} from the slow compartments of both the normal and 14 to 20 days denervated rat diaphragms at 35°C. The muscles were loaded in 60 ml Ca^{45} containing Krebs for 2 hours and washed for 64, 100, 121, 196 and 256 minutes. The half times were measured from regression lines having correlation coefficient R^2 for normal muscles 0.7732 and for denervated muscles, 0.7316.

Ordinate = Ca space on log scale

Abscissa = washing time (min)

normal muscles of Groups I and II showed that there was no significant difference between the two groups ($P > 0.05$). A similar comparison in denervated muscles showed that the slow compartment calcium uptake in 7 to 8 days denervated muscles was about 0.11 μmole/g of muscle more on the average than 14 to 20 days denervated muscles. There was a significant difference between the two groups ($P < 0.005$).

c) Group III:

Group III experiments, in which the muscles were loaded only for 15 minutes, have already been described in detail.

d) Group IV:

In this series of experiments, 40 to 61 days denervated hemidiaphragms were used. Figure 7 shows the rate of Ca^{45} efflux from the slow compartments of both the normal and denervated muscles. The half time for the efflux of Ca^{45} from the slow compartments of the normal muscles was 162 minutes and that for the efflux of Ca^{45} in the denervated muscles was 67 minutes. The calcium uptake by the slow compartment in normal and denervated muscles, as obtained from Figure 7, were 0.31 μmole/g and 0.72 μmole/g of muscle, respectively. There was a significant difference between the normal and denervated muscles.

A comparison of calcium uptake by the slow compartment in normal muscles in these experiments showed that there was no significant difference ($P > 0.05$). However, the calcium uptake by the slow compartment in 40 to 61 days denervated muscles was 0.08 μmole/g more than the average uptake in 7 to 8 days denervated muscles, the difference between these two groups being significant ($P < 0.05$).

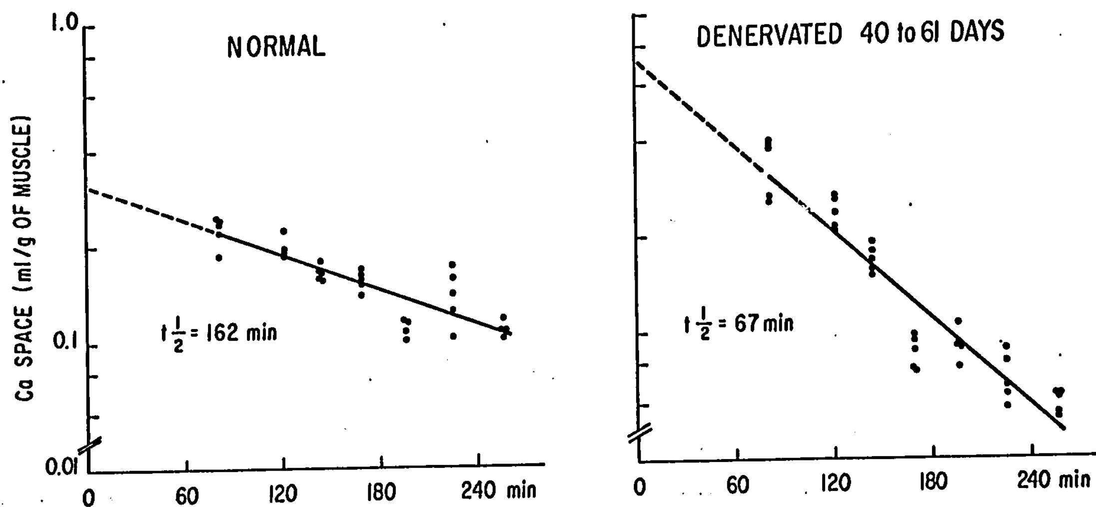

Figure 7. Efflux curves of Ca^{45} from the slow compartments of both normal and 40 to 61 days denervated rat diaphragms at 35°C. The muscles were loaded in 60 ml Ca^{45} containing Krebs for 2 hours and washed for 81, 121, 144, 169, 225 and 256 minutes. The half times were measured from regression lines having a correlation coefficient R^2 for normal muscles 0.7458 and for denervated muscle, 0.8873.
Ordinate = Ca space on log scale
Abscissa = washing time (min)

Similarly, when a comparison was made between 14 to 20 days denervated and 40 to 61 days denervated muscles, it was found that the calcium uptake by the slow compartment in 40 to 61 days denervated muscles was 0.19 μmole/g muscle more than the uptake in 14 to 40 days denervated muscle. There is a significant difference between these two groups.

e) Conclusion:

In these experiments, the calcium uptake in normal muscles showed no significant difference ($P > 0.05$). The calcium uptake in the denervated muscles, however, depended upon the number of days of denervation: 40 to 61 days denervated muscles took up 0.08 μmole/g calcium more than do 7 to 8 days denervated muscles ($P < 0.05$), and correspondingly, 0.19 μmole/g more than the 14 to 20 days denervated muscles.

4. Ca^{45} Efflux at 25°C:

In this series, Ca^{45} effluxes were measured at 25°C, the reason for using this temperature being that the muscles survived better and longer than at 35°C. Although normal muscles produce more twitch tension at 25°C than at 35°C, it had been shown that in denervated muscles the tension decreases (Padsha, 1965; Padsha, 1968; Padsha & Winchester, 1968). Thus, the purpose was to determine if there was any correlation between tension changes and calcium fluxes in normal and denervated muscles. The denervated preparations were 10 to 30 days denervated rat diaphragms. From the experiments using 34 normal muscles and 27 denervated muscles, the following were obtained:

(1) that half times for Ca^{45} efflux, calculated from the regression lines, were 217 minutes for normal and 125 minutes for denervated muscles;

(2) that the calcium uptake by the slow compartments, obtained by extrapolation of the regression lines to zero washing time, were 0.15 μmole/g for normal and 0.4 μmole/g for denervated muscles;

(3) that it was concluded from this that the half times showed the same difference as that at 35°C except for being prolonged.

5. Effect of Caffeine on Ca^{45} Efflux:

In this experimental series, it was attempted to study the relationship between the produced tension and the calcium fluxes in muscle preparations. Caffeine was used to produce both these changes. The technique for the efflux study has been described. In these experiments, muscle strips were loaded for 2 hours with 20 mM caffeine being added at the desired times during washing. The caffeine solutions were prepared by the method of Frank et al. (1970). The results are presented in Figure 8. To summarize for contracture tension, it was noted that:

(1) at 15°C the addition of caffeine induced a contracture in both normal and denervated muscles;

(2) in both preparations, the contractures were sustained;

(3) the produced contracture in normal muscle was

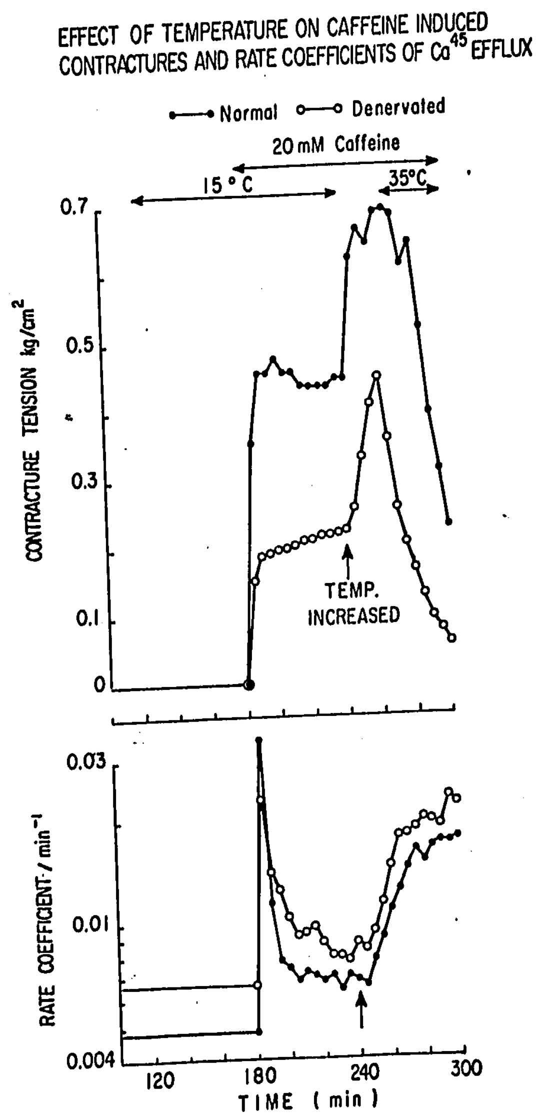

Figure 8. Effect of temperature change on caffeine induced contractures and rate coefficients of Ca^{45} efflux from normal and a 25 days denervated rat diaphragm strip. The muscles were previously loaded for 2 hours at 15°C. Arrows indicate where the solutions were changed or the temperature was increased.

greater than that in denervated muscles;

(4) an increase in temperature to 35°C resulted in a transitory increase in contracture tension for both preparations.

With regard to the rate coefficients, caffeine caused in both preparations an initial transitory increase, which decreased to a level greater than the previous resting value. The difference between these two values was greater for the normal than denervated muscle, corresponding to the greater contracture tension produced in normal muscle. Upon increasing the temperature to 35°C, the rate coefficients showed an initial rapid increase, corresponding with contracture tension, and continued to slowly increase while the tension decreased. From this, it appeared that caffeine induced Ca^{45} efflux was also dependent upon temperature.

II. Ca^{45} UPTAKE BY NORMAL AND DENERVATED RAT DIAPHRAGM

The purpose of this series of experiments was to measure the calcium uptake by denervated muscles. Since in these experiments left and right hemidiaphragms were used, it was necessary to determine the possibility of differing Ca uptake in the two sides of a normal diaphragm. In a few experiments sham-denervation was done by exposing the phrenic nerve as explained in the methods. The following comparisons were made for calcium uptake:

(1) right and left sides of normal hemidiaphragms

(2) normal and sham-denervated hemidiaphragms

(3) normal and denervated hemidiaphragms

1. Ca^{45} Uptake *In Vitro*:

Three diaphragms were used for each set of experiments. The whole diaphragms were loaded in Ca^{45} Krebs solution for 10 minutes at 25°C, then washed for 64 minutes to remove the extracellular Ca^{45}. The Ca^{45} remaining in the central portion of the muscle was then measured by means of the procedure given in the methods. The results are presented in Figure 9 and Table III.

When the normal hemidiaphragms were compared, although the left had a slightly higher Ca uptake, there was no significant difference between the two sides ($P > 0.05$). Similarly, when the right normal side was compared with the left sham-denervated side, there was no significant difference between the two ($P > 0.05$). Although there was no significant difference in the calcium uptake in these sets of normal diaphragms (left, right or sham-denervated), comparison of the right normal side to the left 29 to 37 days denervated side showed that the denervated side took up about 2½ times more Ca. There was a significant difference ($P < 0.01$).

2. Rates of Ca^{45} Influx:

The efflux experiments showed that denervated muscles take up more Ca than normal muscles at a faster rate. The purpose of the following experiments was to measure the rates of Ca influx in normal and denervated muscles. For this it was necessary to keep the loading time very short to avoid any back flux of the isotope,

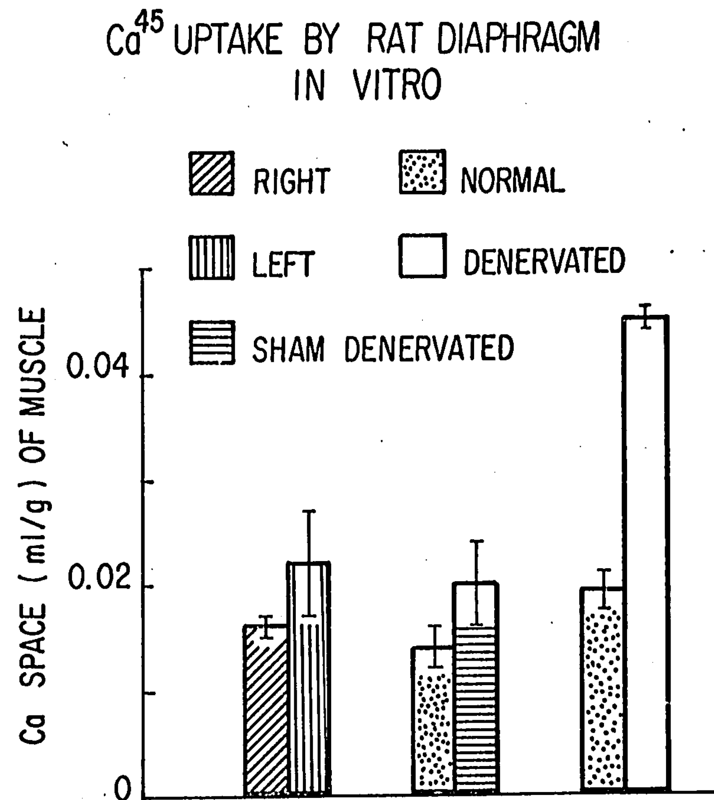

Figure 9. Ca^{45} uptake by rat hemidiaphragms *in vitro* at 25°C. The hemidiaphragms were loaded in 60 ml Ca^{45} containing Krebs solution and washed for 64 minutes. Data used to construct this figure was taken from Table III and calcium remaining in the muscles was shown as Ca space (ml/g). Bars indicate standard error of means.

TABLE III

Ca^{45} uptake by normal, sham-denervated and 29 to 37 days denervated rat hemidiaphragms *in vitro* at 25°C.

Hemidiaphragms	Ca space (ml/g) of Muscles. Mean ± S.E.	P*
Normal (rt. side) (3)	0.016 ± 0.001	P > 0.05
Normal (lt. side) (3)	0.022 ± 0.005	
Normal (3)	0.014 ± 0.002	P > 0.05
Sham-denervated (3)	0.020 ± 0.004	
Normal (3)	0.019 ± 0.002	P < 0.01
Denervated (3)	0.045 ± 0.001	

Data from this table was presented in Figure 9. The figures in parentheses represent the number of muscles used.

* P denotes the probability that there is no significant difference between the two populations.

rt = right

lt = left

which would otherwise reduce the rates of influx. Thus, strips of normal and 19 to 38 days denervated diaphragms were loaded in 60 ml Ca^{45} containing Krebs solution for 5, 10 and 15 minutes and then washed for 1 hour to remove the extracellular Ca^{45}. At the end of washing, the radioactivity remaining in the center of the muscle strips was measured. This amount, divided by the loading time in seconds, gave the rate. This is summarized in Table IV and presented in Figure 10. The data shown in Figure 10 was not corrected for any loss of Ca^{45} from the intracellular compartment that might have occurred during 1 hour of washing. All the experiments were done at 35°C.

After 5 minutes loading and 1 hour washing, the calculated rates of influx in normal and denervated muscles were 47.4 ± 2 μμmole/g/sec and 78.7 ± 5 μμmole/g/sec, respectively. After 10 minutes of loading and 1 hour washing, the rates of influx in normal and denervated muscles were 42.3 ± 2 μμmole/g/sec and 68.4 ± 2.7 μμmole/g/sec, respectively. When the muscles were loaded for 15 minutes and washed, the calculated rates of influx in normal and denervated muscles were 37 ± 2.8 μμmole/g/sec and 54 ± 2 μμmole/g/sec, respectively.

The calculated average rates of influx did not decrease significantly up to 15 minutes of loading in normal muscles, but they did decrease in denervated muscles after 10 minutes. Thus, for the calculation of rates of influx, 10 minutes loading time was chosen.

The influx rates described so far were not corrected for Ca^{45} loss during 1 hour washing. This data has been corrected for

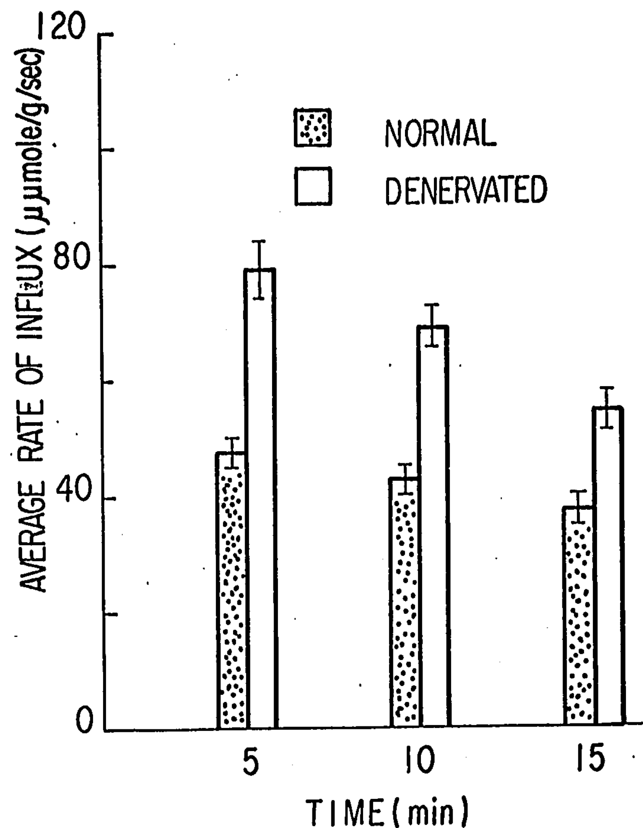

Figure 10. Average rates of Ca^{45} influx in normal and 19 to 38 days denervated rat diaphragm strips at 35°C, after 5, 10 and 15 minutes of loading and 1 hour washing. The data shown was not corrected for any loss of Ca^{45} from the intracellular compartments during washing. Bars indicate standard error of means.

TABLE IV

Average rates of Ca^{45} influx in normal and 19 to 38 days denervated rat diaphragm strips at 35°C after 5, 10 and 15 minutes loading.

Muscles	Loading Time (min)	Rates of Ca^{45} influx (μμmole/g/sec) Mean ± S.E.	
		"Uncorrected"	"Corrected"
Normal (20)	5	47.4 ± 2	61.7 ± 3
Denervated (20)	5	78.7 ± 5	135 ± 0.5
Normal (49)	10	42.3 ± 2	55 ± 3
Denervated (49)	10	68.4 ± 2.7	117 ± 6
Normal (20)	15	37 ± 2.8	48 ± 3.7
Denervated (20)	15	54 ± 3	92.9 ± 5.6

"Uncorrected" means the loss of Ca^{45} during 1 hour washing was not added. "Corrected" means the loss of Ca during 1 hour washing was added as explained in the text. The figures in parentheses represent the number of muscles used.

Ca^{45} loss during washing by using the time constants derived from efflux curves shown in Figure 4. The last column in Table IV gives these values. In this calculation, it was assumed that the resting influx and efflux were the same.

3. Effect of Denervation Days on the Rates of Ca^{45} Influx:

The purpose of this series was to study the effect of denervation days on the rates of influx in normal and denervated muscles. The whole diaphragms were loaded for 10 minutes and washed for 1 hour at 35°C, and Ca^{45} remaining in the central portion of the muscle was measured. These experiments were grouped according to the number of denervation days, the results being given in Table V and Figure 11. Those shown in Figure 11 were not corrected for the loss of Ca^{45} during 1 hour washing. The last column in Table V gives the rates of Ca influx which were corrected for the loss of Ca^{45} during washing. The method used for correction has been described in the previous section.

a) Group I:

In this group, 7 to 9 days denervated diaphragms were used. Table V shows that the average rates of influx in denervated muscles were 25% faster than in normal muscles. After correction for the loss of Ca^{45} during washing time, the rates of influx in denervated muscles were 31% faster than in normal muscles.

b) Group II:

In this group, 21 to 25 days denervated diaphragms were

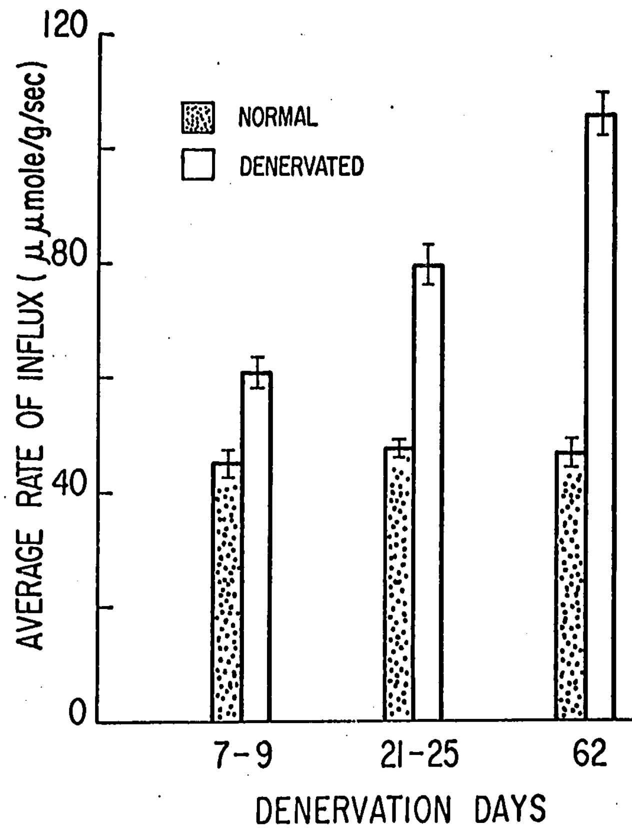

Figure 11. Effect of denervation days on the average rates of Ca^{45} influx in normal and different days denervated rat diaphragms at 35°C. The muscles were loaded for 10 minutes and washed for 1 hour. The data shown here has not been corrected for the loss of Ca^{45} during 1 hour washing. Bars indicate standard error of means.

TABLE V

Average rates of Ca^{45} influx in normal and different days denervated rat diaphragms at 35°C after 10 minutes loading.

Muscles	Denervation	Average rates of Ca^{45} influx (μμmole/g/sec) Mean ± S.E. "Uncorrected"	"Corrected"	Time constants (min) used for "correction"
Normal (20)	7 to 9	45 ± 2	57 ± 3	254 *
Denervated (20)		61 ± 2	83 ± 3	193 *
Normal (15)	21 to 25	47 ± 1	61 ± 2	228 **
Denervated (15)		79 ± 4	136 ± 6	1111 **
		47 ±		
Normal (18)	62	47 ± 3	60 ± 3	234 ***
Denervated (6)		105 ± 4	197 ± 8	96 ***

"Uncorrected" means the loss of Ca^{45} during 1 hour washing was not added. "Corrected" means the loss of Ca^{45} during 1 hour washing was added. The figures in parentheses represent the number of muscles used.

* derived from Figure 5

** derived from Figure 4

*** derived from Figure 7

used. Table V shows that the average rates of influx in denervated muscles were 41% faster than in normal muscles. After correction for the loss of Ca^{45} during washing, the rates of influx in denervated muscles were 55% faster than in normal muscles.

c) Group III:

In this group, 62 days denervated diaphragms were used. Table V shows that the average rates of influx in denervated muscles were 56% faster than in normal muscles. After correction for Ca^{45} loss during washing, the rates of influx in denervated muscles became 69% faster than the normal muscles.

These experiments showed that in denervated muscles, the rates of calcium influx gradually increased with the number of denervation days, whereas there was no significant difference in the normal muscles.

4. Ca^{45} Uptake *In Vivo*:

The studies so far done *in vitro* show that the denervated muscles took up more calcium at a faster rate. The question arose whether the same was true in the living animals. To answer this, a few experiments were done *in vivo*.

Animals were injected with 10 µC Ca^{45} in 1 ml normal saline through the left external jugular vein. After 15 minutes of isotope injection, the animals were killed and the diaphragms were excised immediately, and washed in non-radioactive normal Krebs for 64 minutes at 25°C to remove the extracellular Ca^{45}. The Ca^{45} remaining in the muscles was measured as explained in the methods. The results are

presented in Figure 12 and Table VI, Set 1.

Figure 12 shows that there was no significant difference in the calcium uptake in the right and left sides in normal diaphragms ($P > 0.025$). Similarly, when normal sides were compared with sham-denervated sides, there was no significant difference ($P > 0.05$). However, the 14 to 42 days denervated sides took up twice as much calcium as the normal.

Table VI, Set II, gives the total calcium uptake in normal and denervated muscles, the denervated muscles taking up 61% more Ca^{45} than the normal. Note that these muscles were not washed to remove the extracellular Ca^{45}.

The above experiments showed that even in living animals the denervated side took up more calcium than the normal.

5. <u>Effect of Stimulation on Ca^{45} Influx:</u>

It has been known that stimulation increases the Ca influx in skeletal muscles (Bianchi & Shanes, 1959). The studies of Padsha (1968) and Padsha and Winchester (1968) demonstrated that the mechanical response of denervated rat diaphragm to stimulation differed from the normal, and that this difference depended upon the environmental temperature. The purpose of this series of experiments was to study the effects of stimulation on Ca influx in normal and denervated muscles at different temperatures, and to determine if there was a correlation between the Ca influx and mechanical response of these muscles.

The muscles were loaded in Ca^{45} Krebs solution for 10 minutes

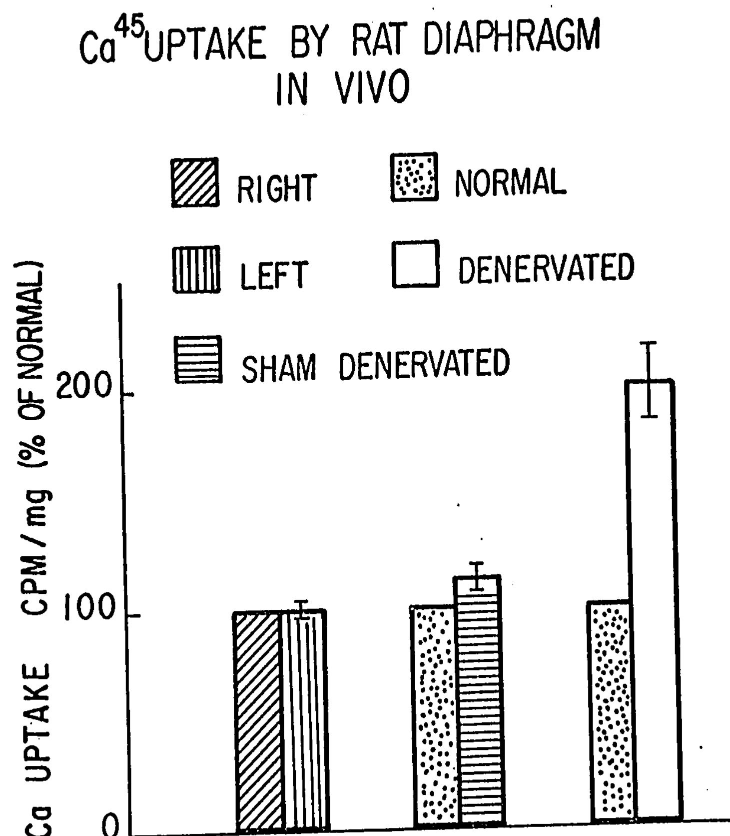

Figure 12. Ca^{45} uptake by rat hemidiaphragms *in vivo* at 25°C. The animals were injected with 10 µc Ca^{45} in 1 ml normal saline through left external jugular vein. After 15 minutes, the hemidiaphragms were excised and washed for 64 minutes. Data used to construct this figure was taken from Table VI, Set I. Bars indicate standard error of means. Ca uptake is given as CPM/mg (% of normal).

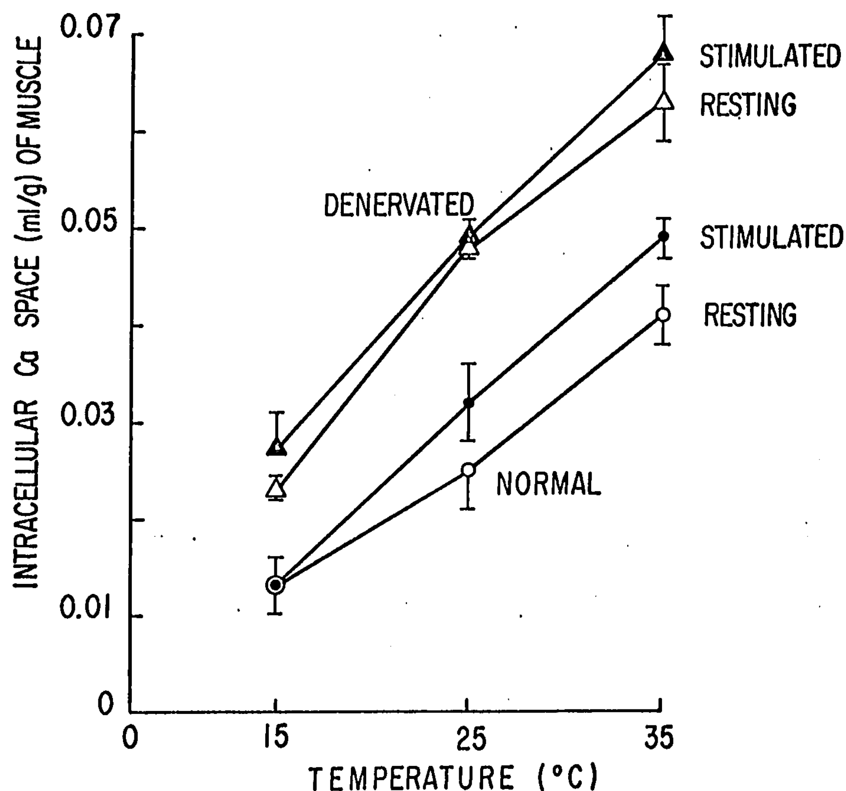

Figure 13. Effect of stimulation on Ca uptake in normal and 19 to 38 days denervated rat diaphragm strips at different temperatures. The muscles were loaded for 10 minutes and tetanized for 1 second per minute for the last 8 minutes, and then washed for 1 hour. No corrections were made for the loss of Ca during washing. Bars indicate standard error of means.

TABLE VI

Intracellular and total Ca^{45} uptake by normal, sham-denervated and 14 to 42 days denervated rat hemidiaphragms *in vivo*.

Hemidiaphragms	Ca^{45} CPM/mg of muscle % of normal or right side, Mean ± S.E.
Set I:	Intracellular
Normal (left side) (3)	100.083 ± 3.581%
Sham-denervated (3)	112.92 ± 6.004%
Denervated (5)	199.652 ± 16.34%
Set II:	
Denervated (7)	161.079 ± 8.958%

The figures in parentheses represent the number of muscles used.

at 35°C, 25°C and 15°C. After the first two minutes of loading at rest, the muscles were tetanized isometrically for 1 second per minute for the last eight minutes. At the end of each loading, the muscles were washed for 1 hour to remove the extracellular Ca^{45}. The rest of the procedure was the same as described previously. The results of these experiments are shown in Figure 13. No corrections were made for the loss of calcium during 1 hour washing. From the experiments using 9 normal and denervated muscles, each at the above temperatures, the following were obtained:

(1) that stimulation increased the Ca uptake in both the normal and denervated muscles at all temperatures except for normal muscles at 15°C, where there was no increase;

(2) that this increased uptake on stimulation was significant only for normal muscles at 35°C;

(3) that when the temperature was lowered from 35°C to 15°C, the Ca uptake was less in both muscle preparations.

III. Ca^{45} SPACE

When the rates of calcium influx were measured in the denervated rat diaphragms, the loading time was kept as short as possible, i.e. 10 minutes, in order to reduce the back flux of Ca^{45}, the results showing that the rates of Ca influx increased in denervated muscles.

The question arose as to what was the total Ca space in denervated muscles when they were equilibrated with the loading solution for a long time.

In order to study this, cut strips of 19 to 38 days denervated muscles were equilibrated with Ca^{45} Krebs solution for varying times up to 480 minutes. The details of the procedure were the same as described earlier. Figure 14 shows the results of these experiments. The total Ca space (intracellular plus extracellular) in denervated muscles was more than that in the normal muscles at all loading times. After 480 minutes, the denervated muscles had 31% more calcium than the normal muscles (0.813 ± 0.045 ml/g and 1.234 ± 0.062 ml/g for normal and denervated muscles, respectively).

The next question arose whether this increased Ca uptake was in the intracellular or the extracellular compartment. In order to study this, the muscles were washed for 1 hour to remove the extracellular Ca^{45}, the results being shown in the lower two traces in Figure 14. After 480 minutes of loading and 1 hour washing, the intracellular Ca space in denervated muscles was, on the average, 6% more than in normal muscles. The average values for normal and denervated muscles were 0.494 ± 0.027 ml/g and 0.524 ± 0.036 ml/g, respectively. These values did not show a significant difference ($P > 0.25$), but they were not corrected for the loss of Ca from the intracellular compartment during washing. When this correction was made using the time constants, 228 minutes for normal and 111 minutes for denervated muscles which were derived from the efflux experiments (Fig. 4), the corrected values for normal and denervated muscles were

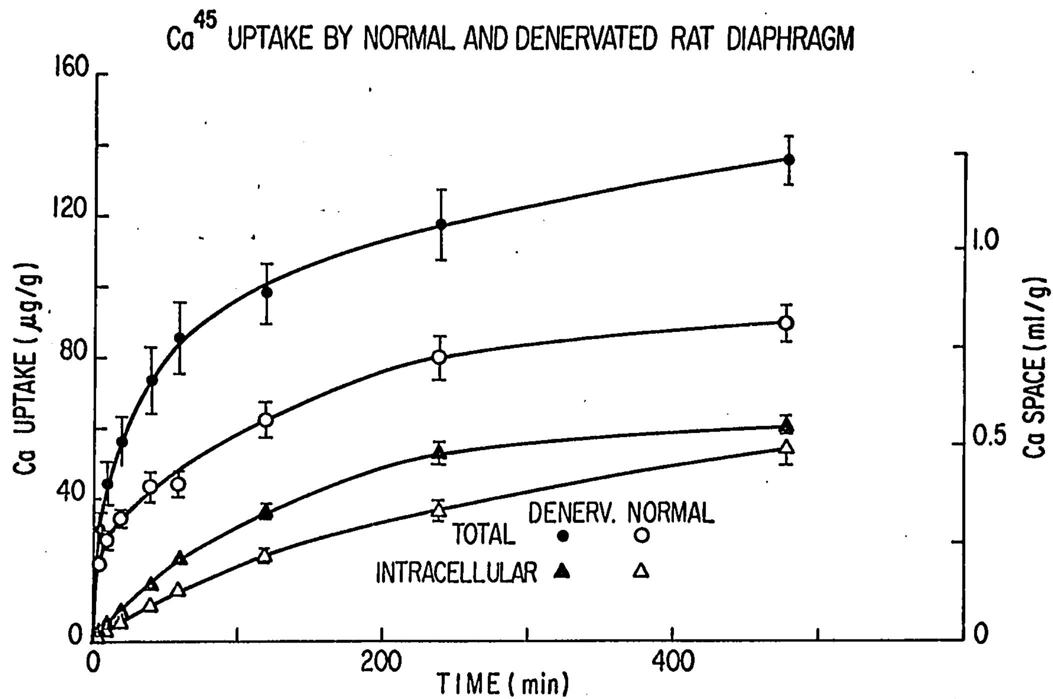

Figure 14. Intracellular and total Ca space in normal and 19 to 38 days denervated rat diaphragm strips at 35°C. The intracellular Ca space was not corrected for loss of Ca during 1 hour washing. Bars indicate standard error of means. Where bars are not present, the standard error falls within the points.

0.643 ± 0.035 ml/g and 0.899 ± 0.061 ml/g, respectively, i.e. the intracellular space in denervated muscles was 29% more than in normal muscles. This difference in the corrected values of the intracellular Ca spaces in normal and denervated muscles was significant.

In these experiments, the extracellular Ca^{45} space was derived by substracting the "corrected" intracellular Ca space from the total Ca space. The results showed that the extracellular Ca space after 480 minutes of loading was 50% more in the denervated muscles (0.17 ml/g for normal and 0.335 ml/g for denervated).

IV. INULIN-C^{14} SPACE

Ca^{45} space measurements showed that denervated muscles have a larger extracellular space than normal muscles. It was therefore useful to measure the extracellular space in denervated muscles by using a substance which was impermeable to the cell membrane. Inulin was chosen because it is an inert substance with a negligible cell permeability. Whole diaphragms were equilibrated with Krebs solution containing radioactive inulin-C^{14} from 2 to 120 minutes, rinsed for a few seconds in 0.4M sucrose solution to remove the surface adsorbed radioactivity, and inulin-C^{14} in the muscles was measured. The details of the procedure were given in the methods. Figure 15 shows the results of the extracellular spaces in normal and denervated rat diaphragms after 2 hours loading only.

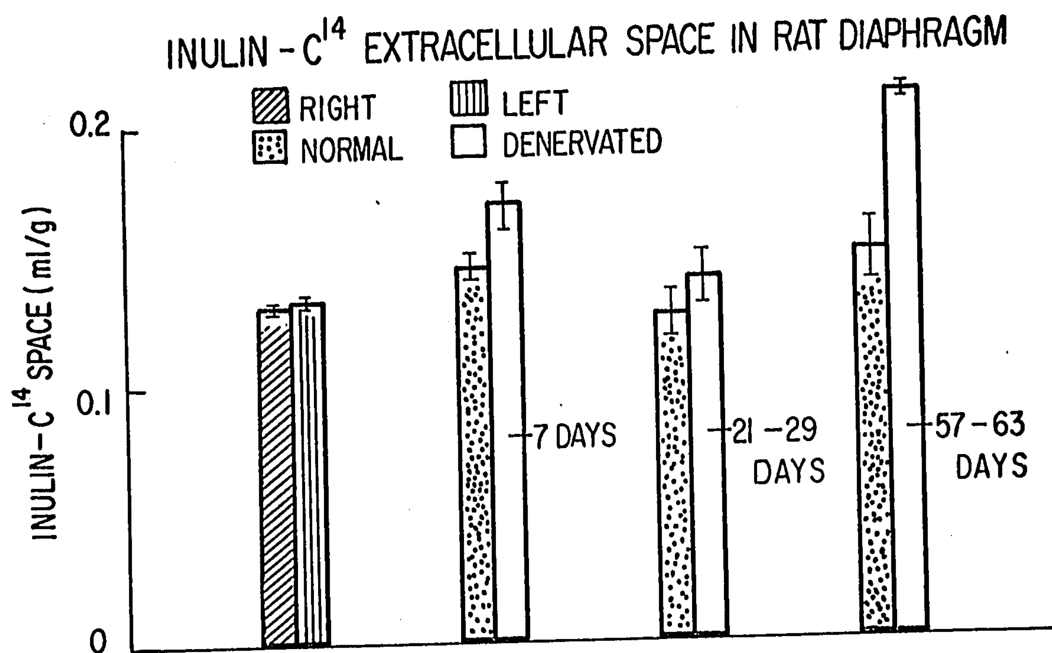

Figure 15. Measurements of Inulin-C^{14} extracellular spaces in normal right and left, and normal and different days denervated rat hemidiaphragms at 35°C. The muscles were loaded with Inulin-C^{14} in normal Krebs for 2 hours. Bars indicate standard error of means.

1. Right and Left Normal Hemidiaphragms:

Before examining the differences between normal and denervated muscles, it was necessary to see if there was any difference in the extracellular spaces in the left and right sides of normal diaphragms. The results showed there was no significant difference ($P > 0.25$) between either sides of the normal diaphragms (0.132 ± 0.008 ml/g and 0.13 ± 0.008 ml/g for left and right sides, respectively).

2. Effect of Denervation Days:

The results of the measurements of extracellular spaces for denervated muscles were arranged in three groups according to the number of denervation days.

a) Group I:

Five experiments were done using normal and 7 days denervated hemidiaphragms. The average Inulin-C^{14} extracellular spaces in normal and 7 days denervated muscles were 0.145 ± 0.0037 ml/g and 0.176 ± 0.0079 ml/g, respectively ($P < 0.005$). The extracellular space was increased in denervated muscles by 19.72%.

b) Group II:

In this case, 10 experiments were done using normal and 21 to 29 days denervated hemidiaphragms. The average Inulin-C^{14} extracellular spaces in normal and denervated muscles were 0.127 ± 0.0072 ml/g and 0.144 ± 0.0106 ml/g, respectively. There was no significant difference between the two preparations ($P > 0.10$).

c) Group III:

In 10 experiments of this series, normal and 57 to 63 days

denervated hemidiaphragms were used. The average Inulin-C^{14} extracellular spaces in normal and denervated muscles were 0.162 ± 0.0129 ml/g and 0.215 ± 0.0034 ml/g, respectively ($P < 0.005$). The extracellular space had increased in denervated muscles by 32.71%.

V. ELECTRICAL PROPERTIES

The results of the experiments on calcium fluxes in denervated muscles suggested the possibility that there may be changes in some of the electrical properties of the muscles after denervation.

The purpose of the following experiments was to study the resting membrane potentials and action potentials in normal and 11 to 55 days denervated rat diaphragms. The resting membrane potentials and action potentials were recorded with intracellularly placed glass capillary microelectrodes filled with 3M KCl. The recording equipment and experimental procedures were described in the methods. The effect of temperature on the electrical properties was studied by decreasing the temperature from either 35°C to 15°C or increasing it from 15°C to 35°C. Whether the temperature was lowered or increased produced no difference in the results obtained.

In order to show some of the differences between the normal and denervated muscles, samples of selected observations are given in Figure 16 and the measurements in Table VII.

(1) The denervated muscle had a smaller membrane potential than the normal (65 mV and 91 mV, respectively) at 35°C (Fig. 16A and B). This differences was less at 25°C

TABLE VII

Membrane potentials and action potentials from normal and 11 to 55 days denervated rat diaphragms at 35°C, 25°C at 15°C.

Temperature	Muscle	Membrane Potential (mV)	Action Potential (mV)	Action Potential Duration (mS)
35°C	Normal	91.2	120.9	1.1
35°C	Denervated	65.1	75.8	2
25°C	Normal	86.6	116.4	1.8
25°C	Denervated	83.7	105.5	2.2
15°C	Normal	87.6	128.7	6.6
15°C	Denervated	82.7	110.9	6.3

This data was obtained from recordings presented in Figure 16 (A to F)

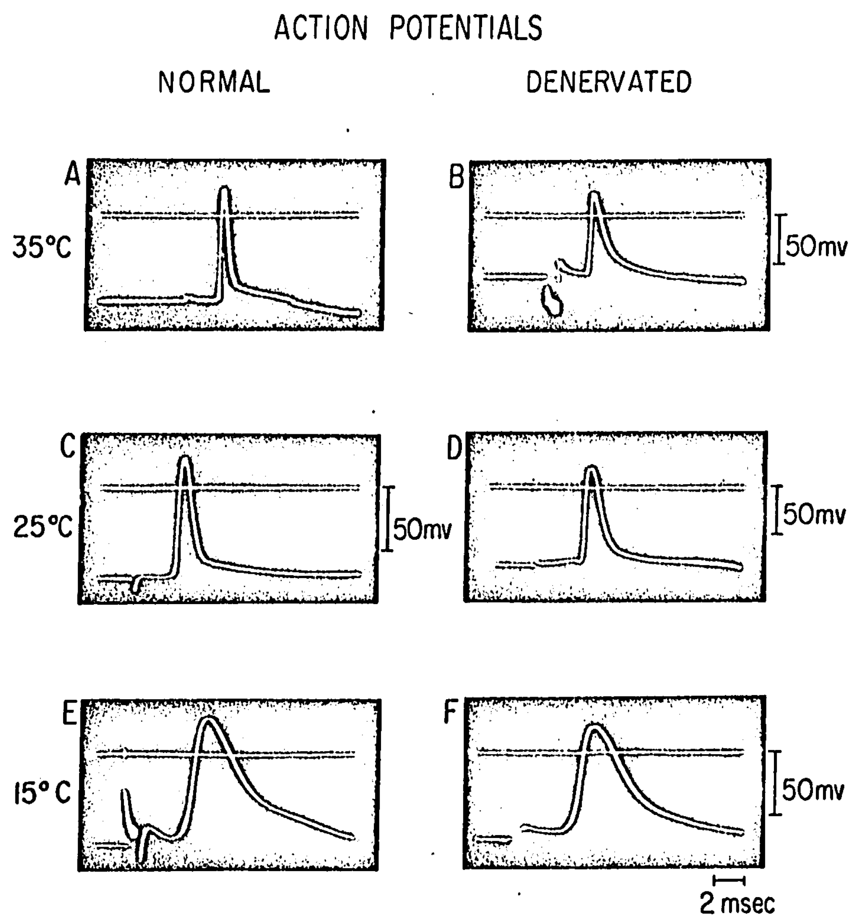

Figure 16. Intracellularly recorded action potentials from normal and denervated rat diaphragms. A - B at 35°C, C - D at 25°C and E - F at 15°C. In B, record was obtained from an 11 days denervated muscle. In D, muscle was 55 days denervated and in F, the muscle was 43 days denervated.

and 15°C.

(2) By extrapolating the rising and falling phases of the action potential to the base line and measuring the distance between the two where they cross it, the duration of the action potential was able to be measured. Hence, at 35°C, the duration of action potential was longer in the denervated muscle than that in the normal muscle (2 mS and 1.1 mS respectively). This difference was less at 25°C and 15°C. In both the normal and denervated muscles, decreasing the temperature from 35°C to 15°C increased the duration of the action potentials, but this increase was more in normal muscle (1.1 mS to 6.6 mS) as compared with denervated muscle (2 mS to 6.3 mS).

1. Resting Membrane Potentials:

Table VIII summarizes the results of the experiments done at 35°C, 25°C and 15°C. The average resting membrane potentials in normal and denervated muscles at 35°C were 84 ± 0.9 mV and 73.8 ± 0.7 mV, respectively. This showed that the membrane potentials after denervation had decreased on the average by about 11 mV. When the temperature was 25°C, the average membrane potentials in normal and denervated muscles was 85.8 ± 1.1 mV and 71.2 ± 2.4 mV, respectively. Thus, reducing the temperature to 25°C caused a decrease of membrane potentials in normal muscles by about 8 mV. There was no significant change in the resting membrane potentials of the denervated muscles

TABLE VIII

Membrane potentials and action potentials from normal and 11 to 15 days denervated rat diaphragms at different temperatures.

Temperature	Muscle	No. of Fibers Used	Membrane Potentials (mV)	Action Potentials (mV)	Action Potential Durations (mS)
35°C	Normal (4)	88	84 ± 0.9	90.91 ± 1.5	1.3 ± 0.02
35°C	Denervated (6)	112	73.8 ± 0.7	80.3 ± 1.6	2.1 ± 0.04
25°C	Normal (8)	65	75.8 ± 1.1	97.4 ± 1.7	2.3 ± 0.05
25°C	Denervated (4)	18	71.2 ± 2.4	88.7 ± 3.8	2.5 ± 0.1
15°C	Normal (2)	50	73.5 ± 1.5	103 ± 2	7 ± 0.5
15°C	Denervated (2)	16	72.4 ± 2	103 ± 3	7 ± 0.17

The figures in parentheses represent the number of muscles used. The values are given as Means ± S.E.

at 35°C and 25°C ($P > 0.025$). Comparison of the membrane potentials of normal and denervated muscles at 25°C showed a small difference between the two, with potentials about 4.5 mV higher in normal muscle ($P < 0.05$). When the temperature was 15°C, the average membrane potentials in normal and denervated muscles were 73.5 ± 1.5 mV and 72.4 ± 2 mV, respectively. This difference was not significant ($P > 0.25$). There was no significant difference ($P > 0.10$) in resting membrane potentials in normal muscles at 25°C and 15°C. Similarly, there was no significant difference ($P > 0.25$) in resting membrane potentials in denervated muscles at 25°C and 15°C.

2. Duration of Action Potentials:

The average duration of action potentials at 35°C in normal and denervated muscles was 1.3 ± 0.02 mS and 2.1 ± 0.04 mS, respectively, with the difference in the duration between normal and denervated muscles being about 0.7 mS. When the temperature was lowered to 25°C, the average duration of action potentials in normal muscles was increased to 2.3 ± 0.05 mS and in denervated muscles to 2.5 ± 0.1 mS. The increase in normal muscles was about 1.1 mS, whereas the increase in denervated muscles was only 0.4 mS. There is no significant difference in the duration of action potentials in normal and denervated muscles at 25°C. When the temperature was further reduced to 15°C, the average duration of action potentials in both the normal and denervated muscles increased to 7 ± 0.15 mS and 7 ± 0.17 mS, respectively.

VI. GLYCEROL TREATMENT

The observation of an increase in calcium efflux and influx in denervated muscles lead to an investigation of the pathway of calcium entry into rat diaphragm. In skeletal muscle, ions pass through the surface membrane and the invaginations of the membrane known as the transverse tubules (T-tubules). If some of the calcium fluxes occurred through the T-tubules, then this portion of the fluxes would be blocked if the T-tubules were disrupted. In recent years, a technique has been developed which selectively disrupts the T-tubules in frog skeletal muscle (Howell & Jenden, 1967; Gage & Eisenberg, 1967; Eisenberg, 1968; Howell, 1969). This technique was used in these experiments and is to be described.

First it was necessary to determine if this technique worked in rat diaphragm. For this purpose, diaphragm strips were equilibrated in normal Krebs solution, then transferred to Krebs solution containing 400 mM glycerol (glycerol-Krebs) for various time periods, after which they were returned to normal Krebs solution for at least 1 hour. Twitch tension and intracellular action potentials were recorded as described in the methods.

A few experiments demonstrated that the duration of glycerol treatment required was influenced by the temperature at which the experiment was being done, the best results being obtained for 25 to 30 minutes treatment at 35°C. A longer glycerol treatment than 30 minutes at 35°C completely and irreversibly abolished both the twitch tension and action potentials when the muscles were returned to normal

Krebs solution. Similarly, at 25°C, the optimum duration of treatment was 45 to 50 minutes, with complete irreversible abolishment of twitch tension and action potential for treatments longer than this. For treatments less than 45 to 50 minutes, it was observed that the twitch recovered to about 75% of its initial value upon return of the muscles to normal Krebs solution. In most of the preparations, these glycerol treatment times were satisfactory, but occasionally a muscle would lose both its electrical and mechanical properties after treatment, or recovered almost completely. For this, there was no explanation.

There was no direct evidence that the T-tubules within these muscles were disrupted after glycerol treatment at the above mentioned temperatures because electronmicroscopy was not done on these preparations (Fatt, 1964; Falk & Fatt, 1964; Eisenberg, 1967; Gage & Eisenberg, 1969). However, an observable similarity was noted between the rat diaphragm preparations following glycerol treatment and those of frog sartorius muscle used by others, namely, the disappearance of the twitch tension and a recordable action potential with an abolished negative after potential. Thus, it could indirectly be inferred that glycerol treatment of rat diaphragm would disrupt the T-tubules as in frog skeletal muscle.

1. <u>Effect of Glycerol Treatment on Twitch Tensions:</u>

The results of two experiments in which the twitch tensions were measured before, during and after glycerol treatment are plotted in Figure 17. Within 5 minutes after exposure to 400 mM glycerol-Krebs, the maximum twitch tension dropped to about 43% of its initial

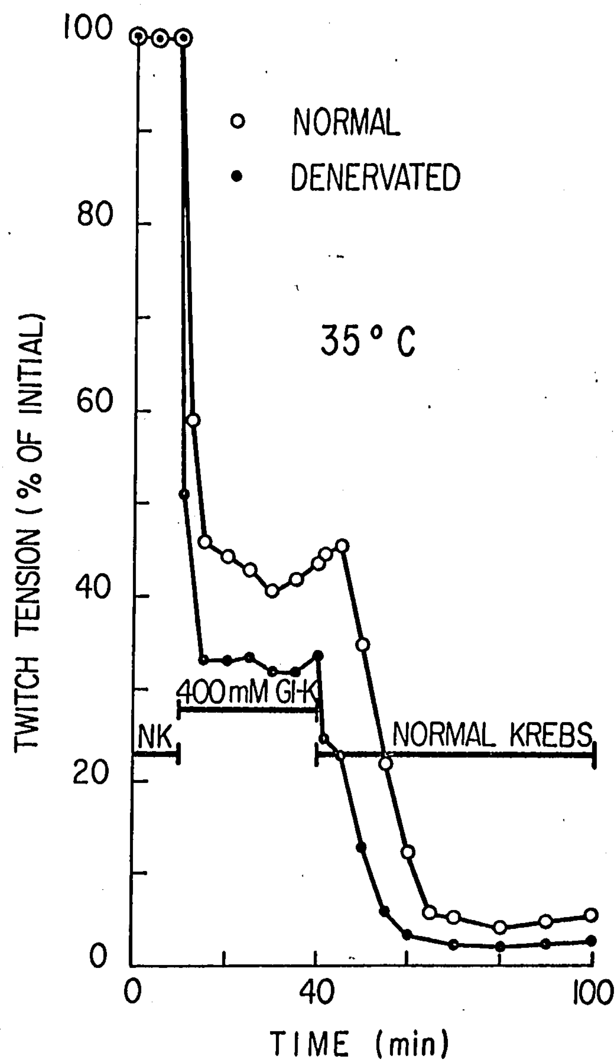

Figure 17. Effect of glycerol treatment on the twitch tension in normal and 7 to 11 days denervated rat diaphragms at 35°C. The muscles were exposed to 400 mM hypertonic glycerol-Krebs for 30 minutes and then returned to normal Krebs for 60 minutes.

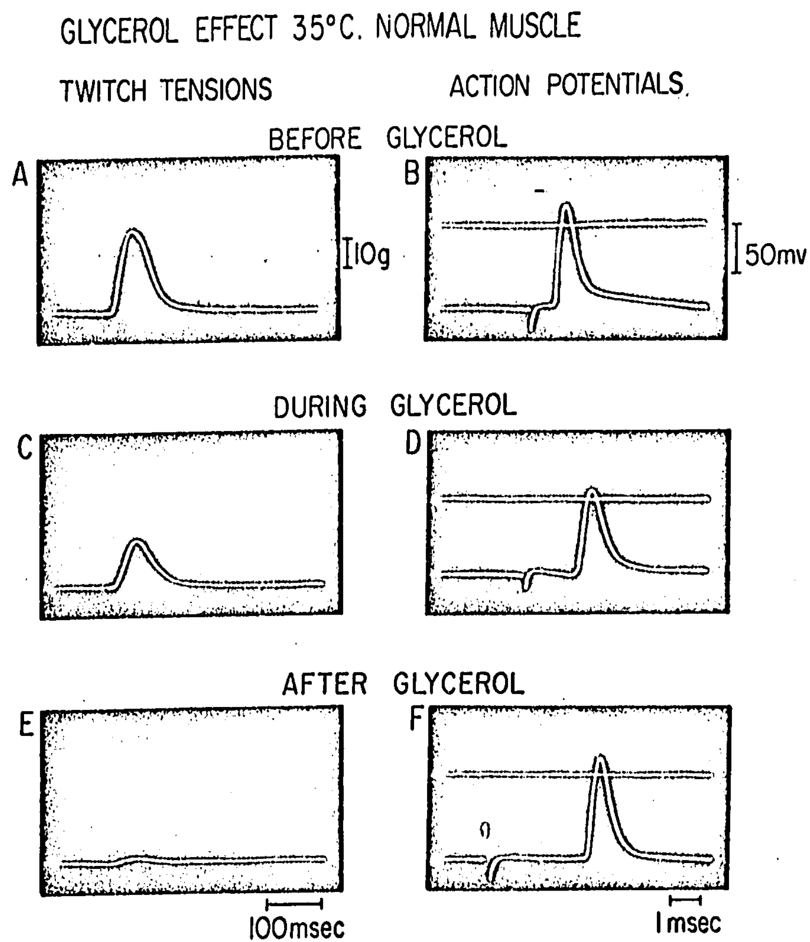

Figure 18. Twitch tensions and action potentials recorded from a normal rat diaphragm before, during and after glycerol treatment at 35°C. For glycerol treatment, the muscle was exposed to 400 mM hypertonic glycerol-Krebs for 30 minutes and then returned to normal Krebs for 1 hour. Action potentials were recorded with intracellularly placed glass capillary microelectrodes filled with 3M KCl.

value in normal and about 33% in denervated muscles. When the muscles were returned to normal Krebs solution, there was a sharp decrease in the twitch tensions in both normal and denervated muscles. Within 25 to 30 minutes, only 2 to 5% of the initial twitch remained. Although both muscles showed a large decrease in twitch tension during and after glycerol treatment, the normal muscle always possessed a greater percentage of remaining twitch tension at any of these periods during the experiments.

2. Effect of Glycerol Treatment on Twitch Tension and Action Potentials in Normal Muscles at 35°C:

Figure 18 presents the twitch tensions and intracellularly recorded action potentials from a normal rat diaphragm before, during and after glycerol treatment at 35°C. Figure 18A shows a record of twitch tension. After recording the twitch tension, the action potentials were recorded using intracellular 3M KCl filled glass capillary microelectrodes. Figure 18A and B presents a typical twitch tension and action potential before glycerol treatment. The membrane potential was 86.5 mV, the action potential 109.3 mV, with a negative after potential of 17.6 mV. Duration of the action potential was 1.2 mS.

After the above recordings, the muscle was exposed to 400 mM glycerol-Krebs for 30 minutes. Figure 18D depicts an action potential recorded after 22 minutes exposure to 400 mM glycerol-Krebs. The membrane potential was 79.1 mV, the action potential 89.3 mV, while the negative after potential had disappeared. The action potential duration was 1.4 mS. Figure 18C shows the recorded twitch tension

that followed.

At the end of 30 minutes exposure, the muscle was returned to normal Krebs solution for 1 hour to remove the glycerol. The twitch tension was then recorded (Fig. 18E) followed by recording the action potential (Fig. 18F). The membrane potential was 87.9 mV, the action potential 107.1 mV, while the negative after potential remained absent. The action potential duration was 1.3 mS.

A comparison of twitch tensions in Table IX showed that when normal muscle was exposed to 400 mM hypertonic glycerol-Krebs solution, the twitch tension was reduced from 39.25 gms to 21.25 gms, i.e. a decrease of about 45.86%. Moreover, after 1 hour of washing, almost all the twitch tension was gone, only 2.0 gms remaining, i.e. about 94.9% of the initial twitch tension was lost (Fig. 18A, C, E).

Similarly, a comparison of the action potentials showed that the membrane potential in normal rat diaphragm was not altered by glycerol treatment, although during treatment there was a small reduction of about 7 mV, which recovered after washing. Similarly, the action potential was reduced by 19 mV during glycerol treatment but returned to its original value after washing in normal Krebs solution. A negative after potential was present only before glycerol treatment, being absent thereafter. The action potential duration was the same before and after glycerol treatment, but was slightly increased (0.22 mS) during treatment.

3. Glycerol Treatment in Denervated Muscles:

The same procedure as for normal muscle was used to record

twitch tension and action potentials before, during and after glycerol treatment, the results being shown in Figure 19 and Table IX. A comparison of the twitch tensions in Table IX showed that when the muscle was exposed to 400 mM hypertonic glycerol-Krebs solution, the twitch tension was reduced from 19.75 gms to 12.25 gms, i.e. a decrease of about 37.97%. However, after 1 hour washing, almost all the twitch tension had disappeared with only 0.875 gms remaining, i.e. about 95.66% of the initial twitch tension was lost.

A comparison of action potentials in Table IX showed that the membrane potential in denervated rat diaphragm was not altered by glycerol treatment at 35°C. The action potential was the same before and during glycerol treatment, but in this particular experiment, it decreased by 9 mV after 1 hour washing. A negative after potential was present before and during glycerol treatment, but after treatment became greatly diminished. Duration of the action potential was not affected by glycerol treatment.

The same experiments were also repeated at 25°C with 45 minutes exposure to 400 mM glycerol-Krebs solution and 1 hour washing in normal Krebs solution. The results showed that the effects of glycerol were the same in both normal and denervated muscles.

Table X summarizes the effects of glycerol treatment in normal and denervated muscles. The results of these experiments showed that:

(1) glycerol treatment abolished the twitch tension but preserved the action potential in both normal and denervated muscles;

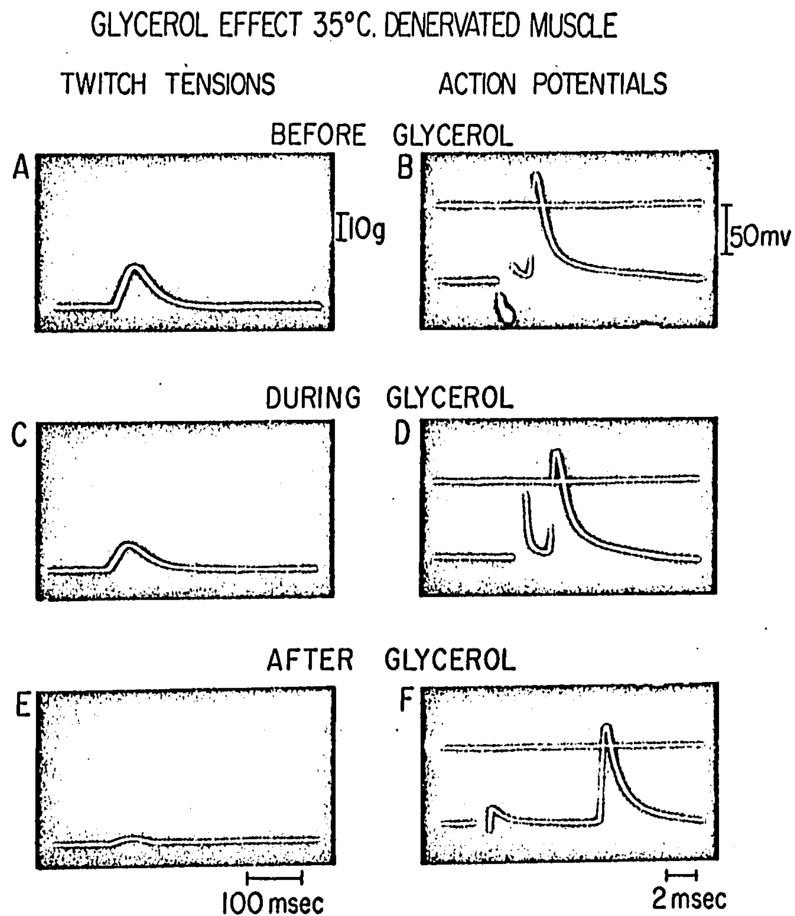

Figure 19. Twitch tension and action potential recorded from an 11 days denervated rat diaphragm before, during and after glycerol treatment at 35°C. For glycerol treatment, the muscle was exposed to 400 mM hypertonic glycerol-Krebs for 30 minutes. The action potentials were recorded with intracellularly placed glass capillary microelectrodes filled with 3M KCl.

TABLE IX

Twitch tensions and action potentials from normal and 11 days denervated rat diaphragms before, during and after glycerol treatment at 35°C.

Muscles	Treatment	Twitch Tension (gm)	Membrane Potentials (mV)	Action Potentials (mV)	Action Potential Durations (mS)
Normal	before glycerol	39.25	86.5	109.3	1.2
Denervated	before glycerol	19.75	76.92	110.44	1.9
Normal	during glycerol	21.25	79.1	89.3	1.4
Denervated	during glycerol	12.25	78.3	109.9	2.1
Normal	after glycerol	2.00	87.9	107.1	1.3
Denervated	after glycerol	0.875	76.8	100	1.9

This data was obtained from the experiments shown in Figures 18 and 19.

TABLE X

Membrane potentials and action potentials from normal and 11 to 55 days denervated rat diaphragms before, during and after glycerol treatment at 35°C.

Muscles	Treatment	No. of Fibers Used	Membrane Potentials (mV)	Action Potentials (mV)	Action Potential Durations (mS)
Normal (4)	before glycerol	88	84 ± 0.9	90.9 ± 1.5	1.3 ± 0.02
Denervated (6)	before glycerol	112	73.8 ± 0.8	80.3 ± 1.6	2.1 ± 0.04
Normal (1)	during glycerol	2	77.9 ± 1.3	94.4 ± 2.8	1.4 ± 0.04
Denervated (3)	during glycerol	12	76.4 ± 3	92.7 ± 4.6	2.4 ± 0.1
Normal (2)	after glycerol	31	86.9 ± 1.2	91 ± 2.1	1.3 ± 0.03
Denervated (3)	after glycerol	34	75.1 ± 1.3	86.2 ± 2.6 [22]	2.1 ± 0.08 [22]

The figures in parenthesis represent the number of muscles used and the figures in square brackets represent the number of fibers studied. The values are given as Means ± S.E.

(2) the resting membrane potentials and the action potentials were not effected by glycerol treatment but the negative after potentials were abolished or reduced in both normal and denervated muscles;

(3) the differences in the membrane potentials and action potentials in normal and denervated muscles were not altered by glycerol treatment.

4. Effect of Glycerol Treatment on Ca^{45} Uptake:

The purpose of this series was to study the effect of disruption of the T-tubules on Ca influx in normal and denervated muscles. The muscles were exposed to 400 mM glycerol-Krebs solution for 45 minutes, washed for 1 hour in normal Krebs solution to remove the glycerol, then loaded for 5, 10 and 15 minutes, followed by a 90 minute washing to remove the extracellular Ca^{45}. The rest of the procedure was the same as described earlier. The data obtained is summarized in Table XI for normal muscles and in Table XII for denervated muscles. The same data, shown in Figure 20 and Figure 21, was not corrected for the loss of Ca^{45} during 90 minutes washing.

These results showed that:

(1) glycerol treatment increased the uptake of calcium in both the normal and denervated muscles;

(2) glycerol treated normal muscles took up more calcium during the first 5 minutes of loading than the glycerol treated denervated muscles (Fig. 20);

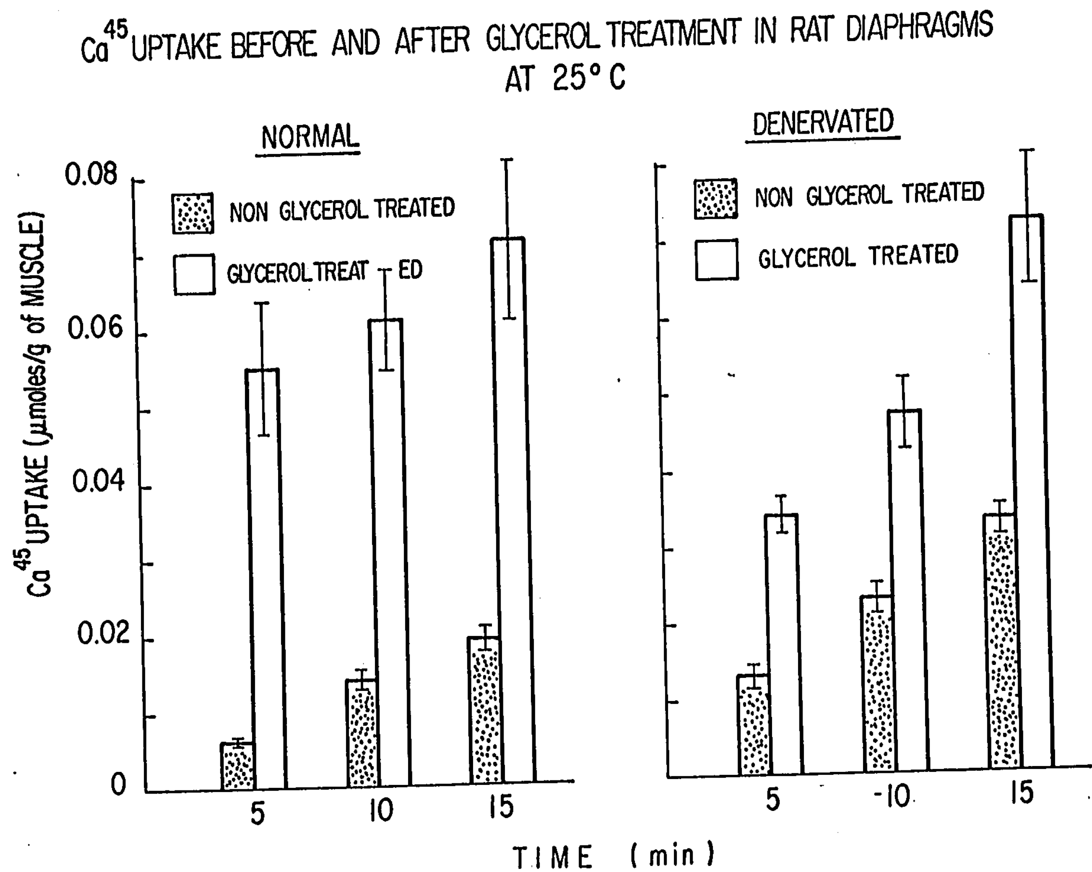

Figure 20. Ca^{45} uptake by normal and 7 to 9 days denervated rat diaphragm strips before and after glycerol treatment at 25°C. The muscles were loaded for 5, 10 and 15 minutes and washed for 90 minutes. No correction was made for the loss of Ca^{45} during 90 minutes washing. Bars indicate standard error of means.

TABLE XI

Ca uptake and the average rates of Ca influx in normal rat diaphragms before and after glycerol treatment at 25°C after 5, 10 and 15 minutes loading.

Muscles	Treatment	Loading Time (min)	Ca Uptake (μmole/g) Mean ± S.E.	Average rates of Ca influx (μμmole/g/sec) Mean ± S.E.
Normal (10)	----------------	5	0.006 ± 0.0006	21 ± 2
Normal (10)	400 mM Glycerol	5	0.055 ± 0.0088	183 ± 29
Normal (10)	----------------	10	0.014 ± 0.0012	24 ± 2
Normal (10)	400 mM Glycerol	10	0.061 ± 0.0066	101 ± 11
Normal (10)	----------------	15	0.019 ± 0.0018	21 ± 2
Normal (10)	400 mM Glycerol	15	0.071 ± 0.0104	79 ± 12

The figures in parentheses represent the number of muscles used.

TABLE XII

Ca uptake and the average rates of Ca influx in 7 to 9 days denervated rat diaphragms before and after glycerol treatment at 25°C after 5, 10 and 15 minutes loading.

Muscles	Treatment	Loading Time (min)	Ca Uptake (μmole/g) Mean ± S.E.	Average rates of Ca influx (μμmole/g/sec) Mean ± S.E.
Denervated (6)	---------------	5	0.013 ± 0.0016	43 ± 5
Denervated (6)	400 mM Glycerol	5	0.034 ± 0.0024	114 ± 9
Denervated (6)	---------------	10	0.023 ± 0.002	39 ± 3
Denervated (6)	400 mM Glycerol	10	0.047 ± 0.0048	78 ± 8
Denervated (4)	---------------	15	0.033 ± 0.002	36 ± 2
Denervated (4)	400 mM Glycerol	15	0.072 ± 0.0085	80 ± 10

The figures in parentheses represent the number of muscles used.

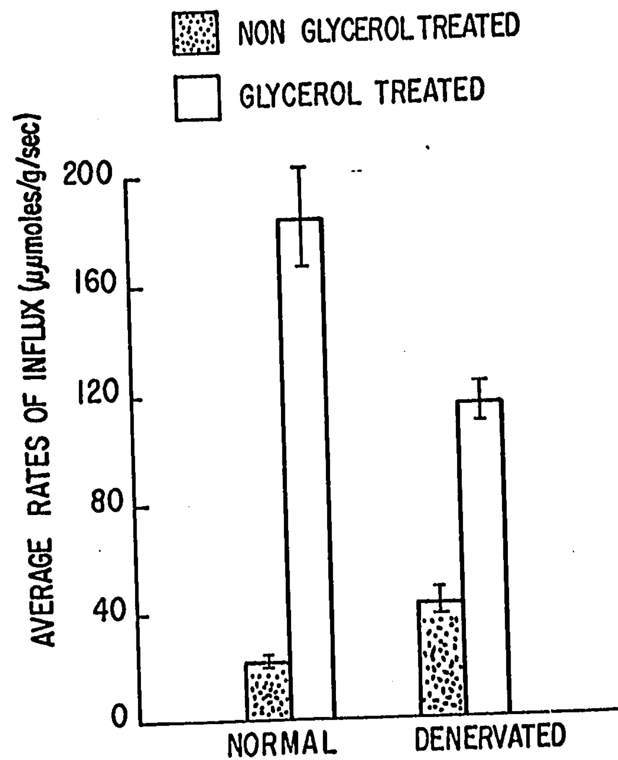

Figure 21. Average rates of Ca^{45} influx in normal and 7 to 9 days denervated rat diaphragm strips at 25°C, before and after glycerol treatment. Loading was for 5 minutes and washing for 90 minutes. No correction was made for the loss of Ca^{45} during 90 minutes washing. Bars indicate standard error of means.

(3) within 15 minutes of loading, the calcium uptake in glycerol treated normal and denervated muscles was almost equal (Fig. 20) (0.071 ± 0.0104 μmole/g and 0.071 ± 0.0085 μmole/g for normal and denervated muscles, respectively);

(4) the average rates of Ca influx at 5 minutes loading (Fig. 21) in both the glycerol treated normal and denervated muscles were 183 ± 29 and 114 ± 9 μμmole/g /sec, respectively.

DISCUSSION

1. Control Experiments:

In all the experiments, the left side of the diaphragm was denervated. Because anatomical differences in the blood supply to the right and left rat hemidiaphragms have been demonstrated by Green (1935), and the work of Sola and Martin (1953) and Thomson (1955) has shown the left hemidiaphragm to be lighter in weight, it was first necessary to compare the two hemidiaphragms for differences in calcium fluxes *in vitro* and *in vivo*. Sham-denervated left hemidiaphragms were also compared. Hence, from the results presented in Figures 9 and 12, no significant differences in the rates of Ca^{45} influx in the two hemidiaphragms, either normal or sham-denervated, were observed. Figure 22 shows there to be no significant difference between the left normal and sham-denervated hemidiaphragms and the right side. A similar comparison between the two diaphragm halves is presented in Figure 15 for Inulin-C^{14} extracellular space measurements with no significant difference being observed. Thus, the conclusion from these results was that the calcium uptake by normal and sham-denervated hemidiaphragms was not significantly different.

2. Extracellular Space:

If the extracellular space is considered as being solely the interstitial space, it would then be absent in an isolated single muscle fiber. However, the single muscle fiber calcium flux studies by Curtis (1970) showed, in the efflux curves, an initial fast com-

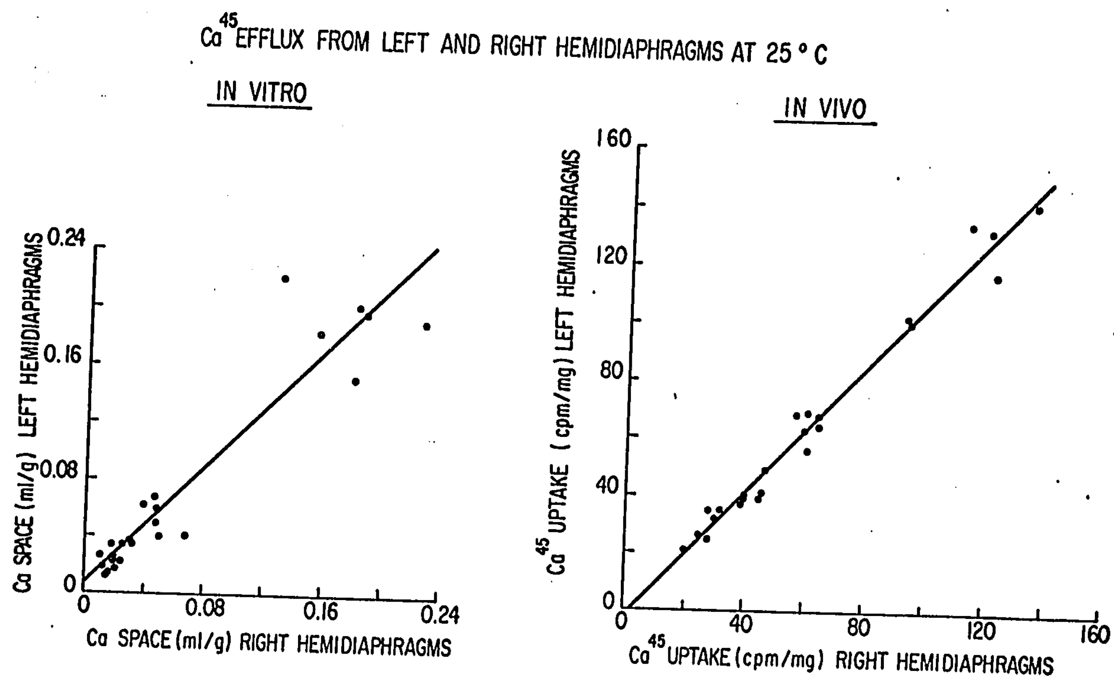

Figure 22 Ca^{45} efflux from left and right hemidiaphragms. The muscles were loaded for 10 minutes *in vitro* and 15 minutes *in vivo*, washed for 0, 16, 36 and 64 minutes, with the remaining Ca^{45} in the two hemidiaphragms at the end of each washing plotted against that in the other. The slope of regression line in *in vitro* was 0.995 and *in vivo* 1.073.

ponent with a time constant of 17 minutes. This inferred an extension of the extracellular space beyond the interstitial space and/or the possible release of adsorbed calcium from bound sites. In view of the electronmicroscope studies of Franzini-Armstrong and Porter (1964) and Huxley (1964) on fish and frog muscle fibers, proposing the possible existence of tubular invaginations (T-tubules) of the sarcolemma into muscle fibers, the former could be considered a plausible explanation. To elaborate, Huxley (1964) demonstrated the passage of ferritin, a protein molecule of 110 Å diameter, into the T-tubule system, and hence a direct continuity between the fluid of the T-tubules and the interstitial space; in other words, that the T-tubules opened directly onto the fiber surface. On this basis, therefore, the T-tubule system could also be considered as part of the extracellular space. Hence, Curtis (1970) explained the fast component of his efflux curves as representing the efflux of calcium from the T-tubules as well as from the surface adsorbed sites. Finally, it has been noted that the measurement of the extracellular space, depending upon the radioactive substance used, could be fictitiously increased by membrane adsorption or permeation. Because of the ill-defined nature of these factors, i.e. the interstitial space, the T-tubular volume, and the degree of membrane adsorption or permeation by radioactive substances, it would be extremely difficult to absolutely define or limit the extracellular space on a morphological basis. Instead, for the Ca^{45} space experiments here, it was elected to define the extracellular space as that compartment of the muscle into and from which ions or molecules could diffuse at rapid rates.

Thus, from the Ca^{45} space measurements, the total calcium was noted to be larger in denervated than in normal muscle. Moreover, after 1 hour washing, it was observed that the majority of the Ca^{45} was rapidly eluted out at rates which were approximately equal in both preparations ($t\frac{1}{2}$ for normal muscle being 10 minutes, $t\frac{1}{2}$ for denervated muscle, 9 minutes). This represented the fast component of the calcium efflux curve, the size of this component being found to be larger for denervated than normal muscle. As a result, it was concluded that the majority of the Ca^{45} was external to the muscle fibers, with the fast component of the efflux curve thus representing the "extracellular space", the definition of which has been discussed.

In the measurement of the extracellular space, a variety of substances relatively impermeable to the cell membrane (Ca^{45}, radioactive Na_2SO_4, Inulin-C^{14}, etc.) have been used (Boyle et al., 1941; Creese, 1965; Johnson, 1955; Niedergerke, 1963; Levine, 1970). Niedergerke (1963) showed that the relative extracellular space volumes in frog heart ventricle, according to the substances used were in the order $Ca^{45} > SO_4 >$ Inulin. Because Inulin is an inert substance and least permeable to the cell membrane, Inulin-C^{14} was used here as a means to measure the extracellular space. From the results, it was found that the average inulin space in normal rat diaphragm was 0.145 ± 0.0079 ml/g of muscle. In denervated muscle it varied with the number of denervation days. At 7 days denervation, the peak of increased muscle weight and hypertrophy, the inulin space increased 19.72% more than normal. However, for 21 to 29 days denervation, when the muscle was within the normal weight range, the

average inulin space decreased to near normal values (0.144 ± 0.016 ml/g). For 57 to 63 days denervation, the period of marked atrophy and weight loss, the inulin space increased 32.71% more than normal. In the stage of hypertrophy following denervation, Miledi and Slater (1969) have demonstrated an increase in the cross-section fiber area. Thus, concerning the extracellular space in hypertrophy, the following could be proposed:

(1) if the increase in muscle weight and cross-sectional area was in the same proportion to that of fiber weight and cross-sectional area, then the extracellular space per gram of muscle would remain unchanged;

(2) if the increase in muscle weight and cross-sectional area was correspondingly less than that of the fibers, then the extracellular space per gram of muscle would decrease;

(3) if the increase in muscle weight and cross-sectional area was correspondingly greater than that of the fibers, then the extracellular space would increase.

For 7 days denervation, it appears that the increase in muscle weight and cross-sectional area was correspondingly greater than that of the fibers, causing the observed increase in extracellular space. Similarly, to explain the increase in the extracellular space in extreme denervation atrophy, it could be proposed that the decrease in muscle weight and cross-sectional area was correspondingly less than that of the fibers. The results for 57 to 63 days denervation

tend to support this.

From the Ca^{45} extracellular space measurements for normal and 19 to 38 days denervated muscle, higher values were obtained for both (0.17 ml/g and 0.335 ml/g, respectively) in comparison to those of the inulin spaces (0.127 ml/g and 0.144 ml/g, respectively). However, because calcium ions possess a double positive charge as well as a greater membrane permeability than inulin molecules, these ions may possibly be adsorbed either on the cell membrane surface, or in some other readily accessible region of the muscle, causing the Ca^{45} measured extracellular space to be increased. Thus, if the inulin adsorption is considered as negligible, the difference between the Ca^{45} and inulin measured extracellular space yields the amount of extracellularly absorbed calcium, calculated to be 0.043 µmole/g and 0.191 µmole/g for normal and denervated muscle, respectively.

Our results of inulin space in normal rat diaphragm did not correspond with those of Creese (1954), who found the average inulin space to be 0.264 ml/g compared to our value of 0.145 ± 0.0079 ml/g. Our curve (not presented in the results) for inulin uptake resembled that of Creese with one exception. It reached a plateau after 1 hour loading, indicating equilibration of inulin-C^{14} between the extracellular space and the bathing medium, while Creese's curve reached equilibration between 30 and 45 minutes. Unlike Creese, cut strips were not used in our extracellular space measurements. It could be speculated that the use of cut diaphragm strips would allow inulin-C^{14} to pass through both the membrane sheath and cut

edges of the muscle to occupy the extracellular space. On the other hand, in our experiments intact hemidiaphragms were used, allowing the inulin-C^{14} to pass through only the muscle's membrane sheath, hence taking a longer time to reach equilibrium. The inulin space values in normal rat diaphragm were compatible with those of Lüllmann (1958) for normal rat diaphragms, although a decrease of inulin space to near normal values after 21 to 29 days denervation was observed whereas Lüllmann observed an increased inulin space in 18 days denervated muscles.

3. Efflux Experiments:

Throughout the experiments, the standard technique of Shanes and Bianchi (1950) was used in order to study the calcium fluxes from whole muscles. Using their analytical methods in the efflux study, the Ca^{45} efflux curves were divided into two components:

(1) a fast component occurring at the onset of efflux with a very small half time of 9 to 10 minutes for normal and denervated muscles, as observed in the experiments; and,

(2) a slow compartment occurring after 60 to 90 minutes with a relatively longer half time, i.e. 113 and 67 minutes for normal and denervated muscles, as reported.

The fast component has been taken to represent the Ca^{45} coming from the extracellular space, while the slow component to be that of the Ca^{45} from within the intracellular space, from which the uptake and

release of Ca^{45} has been shown by Bianchi and Shanes (1960) to have the same kinetics. Similarly, Isaacson and Sandow (1967) found that their results could be described by a two component system. However, Gilbert and Fenn (1957), and recently Langer (1964) have used more than two compartments in describing their efflux curves of Ca^{45} from frog sartorius and dog heart muscle, respectively. As well, recent studies of calcium fluxes in single muscle fibers (Curtis, 1970) have demonstrated in frog semitendinosus muscle there to be three calcium compartments, each with different Ca^{45} efflux time constants. The first, having a very small time constant, has been attributed to the surface adsorbed Ca^{45}, and the Ca^{45} from the extracellular space (T-tubule space within the fibers). The second intermediate compartment has been attributed by Curtis to the membrane-bound Ca^{45} on sarcoplasmic reticulum, while the third, having the longest time constant, to the very poorly exchangeable Ca^{45} which probably is bound within the many intracellular stores. Moreover, Niedergerke (1963), in cardiac muscle, has shown the calcium extracellular space to be larger than that of sulfate of inulin, suggesting that calcium may be bound either on the fiber surface or on some other accessible region within the extracellular space. Hence, it could be conceivable that this bound calcium could elute with a different time constant than the calcium within the extracellular fluid. The slow component of the Ca^{45} efflux curves of Shanes and Bianchi neither showed three compartments, as had been found by Curtis (1970), nor did it exclude the possibility of bound Ca^{45} eluting from the extracellular compartments at a slow rate.

Since the technique of Shanes and Bianchi was used, the slow compartment of our efflux curve does not exclude the possibility that some of the calcium was eluted from the extracellular compartment. However, because the difference in the half time of Ca^{45} efflux from the fast and slow components was fourteen- and seven-fold in normal and denervated muscles, a two-component analysis might not result in too much an error in the intracellular Ca^{++} estimation. Furthermore, assuming that the extracellular compartment would be equilibrated with the external bathing medium during 2 hours loading then after 1 hour washing it would be expected that 0.7 to 1.5% of the initial extracellular calcium would remain, the rest being washed out as six half times are gone during washing. Moreover, as the half times were measured from regression lines obtained from points after 90 minutes washing, the possibility of error caused by Ca^{45} remaining in the extracellular compartment would be reduced.

As mentioned, this technique using whole muscle could not be used for the further analysis of different calcium compartment sizes within the slow component, nor for the rate at which calcium entered and left these compartments. For this, a single fiber study would be more useful. However, for the calcium flux measurements here, where the interest was in the total intracellular uptake and the rates at which it moved in and out from the cell, this technique was suitable. Similarly, for the determination of the 'total' extracellular space for different durations of denervation, a whole muscle was more suitable.

Although in most of the experiments diaphragms denervated

for 19 to 38 days were used, during the course of experimentation it was observed that the Ca fluxes also depended upon the number of denervation days. Thus, to study this effect, denervated muscle preparations were catalogued into four groups based upon the observed condition of the diaphragm after denervation. Initially following denervation, rat diaphragm shows a temporary hypertrophy with an increase in both wet and dry weight (Martin & Sola, 1948; Sola & Martin, 1953; Thomson, 1955), the maximum being reached within 6 to 8 days. These hypertrophied muscles formed the first group. Another grouping consisted of 14 to 20 days denervated muscles in which the weight decreases to almost that of normal. However, because the transitional changes in weight from that of hypertrophy to normal to atrophy were difficult to define, this group was studied only in one series of efflux experiments. The next group consisted of muscles denervated longer than 3 weeks in which atrophy occurred with a decrease in weight. Finally, within the last group were those muscles of long term denervation, which undergo marked atrophy to about half the weight of normal. Because Thomson (1955) has shown that prolongation of denervation beyond 42 days results in loss in wet and dry weight of the right normal hemidiaphragm, for each set of experiments the denervated muscles were compared with the corresponding normal side.

From the data on resting calcium fluxes, two important conclusions were made. First, both the rates of calcium efflux and influx were increased in denervated muscle. Second, these rates were dependent upon the number of denervation days, gradually in-

creasing with prolongation of the denervation period, from 7 to 61 days, and approaching a maximum beyond two months of denervation. How long these fluxes would continue increasing with prolonged denervation is difficult to state since no preparations were studied beyond 62 days denervation. The data on calcium influx showed that the total exchangeable calcium was more in denervated muscle, while the data from the efflux experiments showed that in 2 hours calcium uptake by denervated muscles was greater when the muscle was denervated for 40 to 61 days, as compared to 7 to 8 days. Because Bianchi and Shanes (1960) have shown that the kinetics of calcium uptake and release were about the same in passive conditions, this increased uptake of calcium in 40 to 61 days denervated muscles would be expected on the basis of the faster rates of calcium fluxes in these muscles. To estimate the total exchangeable intrafiber calcium, the amounts of calcium taken up by normal and these two groups of denervated muscles are calculated for infinite loading time (with the time constants derived from the efflux curves of each group, see Table II) using the equation:

$$y(t) = Y(\infty)\ (1 - e^{-t/t.c.})$$

where $y(t)$ = amount of intracellular calcium after t minutes of loading

$y(\infty)$ = amount of intracellular calcium after infinite loading

e = exponential

t = loading time in minutes

t.c. = time constant for the rate of influx in minutes (it was assumed that the rates of influx and efflux were the same in resting muscle)

By this, the calculated amounts of intracellular calcium uptake at infinite loading are:

(1) normal muscles, 0.772 ± 0.036 μmole/g

(2) 7 to 8 days denervated muscles, 1.359 ± 0.106 μmole/g

(3) 40 to 61 days denervated muscles, 1.038 ± 0.062 μmole/g

To explain the first value, if a normal muscle is placed in a solution of 1 μmole/ml Ca, then after equilibrium has been reached, the amount of exchangeable calcium in the muscle/g would be less than that amount/ml which is equilibrated in the fluid volume of the muscle. In other words, assuming that 75 to 80% of muscle is water, then the maximum amount of exchangeable calcium in the muscle/g at equilibrium would be between 0.75 to 0.8 μmole/g. Within this range is the calculated amount of exchangeable calcium in the intracellular compartment of normal muscle. Continuing with the above calculations, in the 7 to 8 days denervated muscles, although the fluxes are slower than those of 40 to 61 days denervation, it is apparent that the exchangeable calcium intracellular compartment size has increased in comparison to that of the 40 to 61 days denervated muscles in which it has decreased. It is to be noted that these experiments do not quantitatively measure the non-exchangeable calcium fraction in any of these muscle preparations.

Recently, Lahrtz and Lüllmann (1967) measured the total calcium content and Ca^{45} efflux from normal and 8 to 10 days chronically

denervated rat diaphragm. They observed no significant difference in the total calcium content nor in the rates of efflux between normal and denervated muscle, i.e. for a 1.8 mM calcium solution, the total calcium content was 2.61 μmole/g and 2.8 μmole/g in normal and denervated muscle, respectively. However, the total calcium content of a muscle has been shown to depend upon the external calcium concentration of the bathing medium (Gilbert & Fenn, 1957; Bianchi, 1968), and since in these experiments a 1 mM solution was used, and no attempt made to estimate the total calcium content of the preparations, it was not possible to compare data with that of Lahrtz and Lullmann. Whereas they found no difference in the half times of Ca^{45} efflux from normal and 8 to 10 days denervated muscle (i.e. 70 minutes for both), our experiments showed that the half time for Ca^{45} efflux was not only shorter in denervated muscle, but also dependent on the number of denervation days. A possible reason for the discrepancy of results could be the difference in techniques, as here the remaining Ca^{45} in a central piece of muscle was measured, not the effluent. The difficulties involved in measuring directly the Ca^{45} efflux was being discussed in the results.

However, Isaacson and Sandow (1967), using 1 mM calcium bathing solution, estimated the total calcium content of normal rat EDL muscles to be 1.78 μmole/g. Assuming this value to be the same for normal rat diaphragm, then our value of the exchangeable calcium in normal muscles (0.77 μmole/g) would indicate that 44% of the total calcium of the whole muscle was exchangeable, approximating Isaacson and Sandow's value of 50% for EDL muscle, and Curtis' (1966) value of

47% for single frog muscle fibers. Furthermore, the value of exchangeable calcium in whole muscle (0.77 μmole/g) obtained here was in close estimation to the values of Gilbert and Fenn (1957), Shanes and Bianchi (1959) and Curtis (1970) as quoted in a table by Curtis (1970).

The exchangeable calcium in whole muscle discussed so far represented the slow component of the efflux curves. This large amount of calcium would be expected to be in some form of bound complex since free ionic calcium at rest in concentrations greater than 10^{-5} mM, would cause a contracture in the muscle (Bianchi, 1968). Supporting this, the recent autoradiographic localization experiments of Winegrad (1968, 1970) show this fraction of bound calcium to be present in the sarcoplasmic reticulum (including the terminal cisternae, the intermediate cisternae and the longitudinal tubules) which has a volume of 13% of the total muscle (Peachy, 1965). Calcium in the sarcoplasmic reticulum may also exist in free, ionized form but it must be very low. Thus the intracellular calcium would be expected to be bound or sequestered in the sarcoplasmic reticulum. Since the time Porter and Palade (1957) described the fine structure of the sarcoplasmic reticulum, and the local activation experiments of Huxley and Taylor (1958), it has been suggested that the triads of sarcoplasmic reticulum are involved in the coupling of surface excitation to contraction within striated muscle. The development of this hypothesis has been reviewed several times in the recent years (Huxley, 1959; Huxley & Peachey, 1964; Huxley, 1964; Porter, 1961; Peachey, 1965; Franzini-Armstrong & Porter, 1964). It is generally believed that the T-tubules are electrically coupled to the terminal cisternae, and that the depolarization of the fiber surface spreads inwards along the T-tubules to the terminal cisternae, causing the release of calcium. Furthermore, recent studies of Winegrad (1968,

1970) have demonstrated that the resting exchange of calcium occurs primarily between the terminal cisternae and the T-tubules, 80% of the surface of which has been shown by Peachey (1965) to be covered by the flattened surfaces of the terminal cisternae. Winegrad also studied the movement of calcium during tetanus, namely its release from the sarcoplasmic reticulum and its association with the contractile proteins. Thus we could assume that although in the normal muscle preparations most of the intracellular calcium would be present in the bound form within the sarcoplasmic reticulum, it could nevertheless be slowly exchanged to the extent of 44%. Similarly, from the results using different durations of denervation, we would again assume that the increased amount of exchangeable calcium was stored within the sarcoplasmic reticulum of these muscles. It is to be noted again that evidence is lacking to show whether the total Ca content of the whole denervated muscle was increased. It could only be stated that the exchangeable fraction was increased and that the latter may also be increased.

The question arises what could be the possible causes for an increased exchangeable Ca fraction in denervated muscles. Two apparently conflicting suggestions have been made:

(1) Pellegrino and Franzini-Armstrong (1963) have shown that in rat soleus and gastrocnemius muscle following denervation, the myofibrillar elements disappear faster than the reticular elements, thus increasing the amount of sarcoplasmic reticulum relative to the amount of contractile material. They also reported

that the first change observed in the sarcoplasmic reticulum is a process of fragmentation into isolated vesicles by which the longitudinal elements lose their continuity over the sarcomere length. This has been confirmed by Schrodt et al. (1965) who observed lamellar arrays of membrane enclosed cisternae, suggesting that they could be derived from the elements of sarcoplasmic reticulum. It is possible that these morphological changes in the sarcoplasmic reticulum structure could account for the differences in calcium accumulating activity of the denervated muscles.

(2) Recently, on the other hand, Howell and Fairhurst (1966), using glycerol extracted isolated sarcoplasmic reticulum from rat diaphragm and gastrocnemius muscles, showed that the calcium accumulating capability of the sarcoplasmic reticulum increased as a result of denervation, suggesting that denervation by itself could result in other changes in the muscle besides altering the ratio of the amount of sarcoplasmic reticulum and the contractile elements. In this regard, one cannot rule out the possibility that alterations in the kinetic characteristics of the calcium pump may occur as a result of denervation.

Since the characteristics of the cell membrane change after denervation (Nicholls, 1956; Harris & Nicholls, 1956; Lüllmann, 1958;

Hubbard, 1962, 1963), it would not be surprising if the properties of the intracellular membranes were also changed. The fact that caffeine is more effective in releasing calcium from the sarcoplasmic reticulum in denervated muscles as compared to the normal (Gutmann & Sandow, 1965) also suggested that biochemical as well as morphological changes could take place. This may again account for the differences in the Ca accumulating activity of the denervated muscles.

With regard to the first possibility mentioned, the results obtained from 7 to 8 days denervated muscles are difficult to interpret in view of the hypertrophy which is reported to occur in these muscles (Sola & Martin, 1953; Thomson, 1955). The increase in weight in denervated diaphragm is accompanied by an increase in the fiber's cross-sectional area and the number of myofibrils in them (Miledi & Slater, 1969). Stewart (1955) also has reported hypertrophy to be accompanied by an increase in the amounts of sarcolemmal and contractile proteins, being greater in the former (36%) than in the latter (27%). Whether or this increase in the sarcoplasmic to contractile proteins ratio reflects a greater hypertrophy of the sarcoplasmic reticulum has not yet been shown by electronmicroscope investigations. Our results show the largest exchangeable calcium fraction to be in 7 to 8 days denervated muscles which could thus be due to either such a hypertrophy of the sarcoplasmic reticulum or its altered pumping properties.

4. Influx Experiments:

The influx data showed that the rates of influx were faster in denervated muscle as compared to normal. It was also observed that the rates of influx depended on the number of denervation days. This data was presented in terms of μμmole/g/sec, which does not consider the fiber surface area. Since the movement of ions occurs through the cell surface membrane, a change in the cell surface area would largely alter the movement of ions per unit area of the membrane, although the weight/volume ratio would remain constant. If, in addition, the surface area of the T-tubules, calculated to be seven times the cell surface area (Peachey, 1965; Bianchi, 1968) is considered then the surface area available for diffusion would be tremendous. The muscles investigated here were used after various periods of denervation, where they are reported to be either in extreme hypertrophic or atrophic conditions. Thus, it became necessary to present the results in terms of influx per unit surface area to see if the faster rates of influx in these muscles were due to an increased or decreased cell surface area, or an effect of denervation itself.

Bianchi and Shanes (1959) have calculated the outer surface membrane area to be 300 cm^2/gm, taking the average fiber diameter to be 100 μ in frog sartorius muscles. The fibers constitute a fraction of the total muscle as given by the ratio (total water - extracellular water)/(total water), which is approximately equal to 0.8 (Bianchi, 1968). Thus, if it was assumed that the volume of the fibers present in 1 gram of muscle was equal to 0.8 cm^3, then the length of the fibers the surface area could be calculated using

the equation:

$$\text{Surface Area} = 2\pi rL$$

where length, $L = \text{volume}/\pi r^2$. Hence, knowing the fiber diameter, the surface area could be calculated. Since the fiber diameters were not measured in rat diaphragms during these experiments, for the purpose of the above calculations, these were derived from the work of Zolovick et al. (1970) and Miledi and Slater (1969). The average fiber diameter taken from Zolovick's work was 40 μm for normal muscles, the calculated fiber surface area being 800 cm^2/gm, and the average rate of influx at rest calculated to be 0.075 μμmole/cm^2/sec. The average fiber diameter calculated from the work of Miledi and Slater (1969) for normal, 9 days denervated and 46 days denervated muscles were 15 μm, 21.4 μm and 18.2 μm, respectively; the calculated fibers' surface areas were 2133 cm^2/gm, 1495 cm^2/gm and 1758 cm^2/gm, respectively, and the average rates of influx were calculated to be 0.028 μμmole/cm^2/sec, 0.055 μμmole/cm^2/sec and 0.112 μμmole/cm^2/sec. The value thus calculated for normal rat diaphragm (0.028 μμ mole/cm^2/sec) is about 3½ times smaller than that reported by Bianchi and Shanes (1959) for whole frog muscle (0.094 μμmole/cm^2/sec) and about 10 times smaller than that reported by Curtis (1966) for frog single twitch muscle fibers (0.26 μμmole/cm^2/sec). Despite these calculations being based on many assumptions, they, nevertheless, indicate that the calcium influx per unit area of the fiber increased with the prolongation of the denervation period.

5. Electrical Properties:

From the data on action potential recording at 35°C, two important differences between normal and denervated muscle were noted. First, the resting membrane potential in denervated muscle as compared

to normal, decreased on the average by 11 mV. Second, the duration of the action potential in denervated muscle increased to about twice that in normal muscle.

Concerning the first observation, the question arose as to the possible cause for the decreased potential. It is known that biological membranes are semipermeable, the permeability depending to different extents on the nature of the ions present on both sides. Hence Goldman (1943) showed, with the assumption that the electric field inside a cell was constant, that the membrane potential was represented by the log of a sum of products at permeabilities and concentrations, or in mathematical terms:

$$M.P. = \frac{RT}{F} \log_e \frac{P_K [K]_o + P_{Na} [Na]_o + P_{Cl} [Cl]_i}{P_K [K]_i + P_{Na} [Na]_i + P_{Cl} [Cl]_o}$$

where M.P. = Transmembrane potential

R = Universal gas constant

T = Absolute temperature

F = the Faraday (number of coulombs per mole of charge)

e = Base of natural logarithms = 2.718

P_K, P_{Na}, P_{Cl} = Permeabilities of K, Na and Cl

The same equation was derived by Hodgkin and Katz (1949) under more general assumptions. According to this equation, there are at least three different ions, i.e. K^+, Na^+ and Cl^- which can appreciably cause a change in membrane potential, as was observed in denervated muscle, if the membrane permeabilities were altered. Using the denervated frog sartorius muscle, Nicholls (1956) showed

an increase in the membrane resistance, while Harris and Nicholls (1956) found the membrane permeability to K^+ to be decreased in these preparations. In addition, Klaus, Lüllmann and Muscholl (1960) have shown by radiotracer techniques, that in denervated rat diaphragm, potassium fluxes were reduced to ½ to 2/3 of the normal value. Furthermore, Thesleff (1963) also observed an increased membrane resistance in denervated rat diaphragm, suggesting this to be due to decreased potassium conductance. Lüllmann (1958) have also observed a net loss of potassium from 18 days denervated rat diaphragms, as well as a greater loss of K^+ compared to normals when placed in normal saline. Thus, in the light of the above studies, it may be assumed that a decreased membrane permeability to K^+ could cause a decreased membrane potential in denervated rat diaphragm. According to the Goldman equation, a reduction in the K^+ conductance would enhance the influence of Na^+ and Cl^- on the resting membrane potential and cause depolarization. In accord with this, Drahota and Gutmann (1963) have shown that denervated EDL muscles could not maintain normal internal Na^+ and K^+, and that they accumulated Na^+. Recently, Hofmann and De Nardo (1968) reported that the accumulation of Na^+ in the intercostal muscle fibers of myotonic dystrophy patients appeared due to an increase in Na^+ conductance of the muscle fiber membrane. Furthermore, Dockry et al. (1966) reported the promotion of Na^+ excretion and K^+ uptake in rat muscles with an intact nerve supply. Hence it could be assumed that in denervated muscle an increased permeability of the membrane to Na^+ or a malfunctioning of the Na^+ pump would cause an intracellular accumulation of Na^+ and so decrease the membrane potential. Concerning

the effect of Cl^- on resting membrane potential, Hubbard (1962, 1963) measured the Cl^- conductance in denervated frog sartorius muscle, and found it remained unchanged after denervation. Hodgkin and Horowicz (1959) have shown in frog skeletal muscle that variation in P_{Cl} would not have a long term effect on the membrane potential if the chloride ion is distributed in equilibrium. If the same was assumed true for denervated skeletal muscle, then Cl^- ions would not be involved in decreasing the resting membrane potential.

The Goldman equation does not consider calcium because of the small amount of current carried by this ion. However, in denervated muscle, where K^+ permeability has been shown to be decreased and calcium fluxes increased, perhaps via both the external and internal membranes, the permeability of calcium would be expected to be increased. Therefore, it could be stated that in denervated muscle where the K^+ permeability has been shown to be decreased and the calcium fluxes to be increased, that calcium ions could play a small role in conjunction with other ions, to create a decrease in resting membrane potential.

From this, another question arises, i.e. are the increases in Ca^{++} fluxes the cause of a low membrane potential or the result of a decrease in membrane potential. The first part, discussed above, is very difficult to answer from our experiments, although it is well known that adsorption of Ca^{++} can influence the membrane permeability to other ions (Frankenhaeuser & Hodgkin, 1957). Concerning the second part, in general the reduction of the resting membrane potential causes an increase in calcium influx and efflux even in normal muscle (Bianchi & Shanes, 1959; Bianchi, 1960). The event normally leading to contraction in the muscle is the depolarization of the resting membrane potential (Kuffler, 1946; Sten-Knudsen, 1954; Sandow, 1955;

Sandow et al., 1965). Once the membrane potential is depolarized to threshold level, the tension versus membrane potential curve follows an S-shaped process (Hodgkin & Horowicz, 1960). The same general shape of curve has been observed when calcium influx in frog sartorius muscle is measured at various K^+ concentrations (Weiss & Bianchi, 1965). Similarly, acetylcholine contractures during potassium depolarization have been shown to cause an increased calcium uptake by denervated rat diaphragm as compared to depolarized muscles not treated with acetylcholine (Jenkinson & Nicholls, 1961). Besides, isolated sarcoplasmic reticulum from denervated muscle has been shown to be capable of accumulating 2½ to 3 times more calcium (Howell et al., 1966; Brody, 1966), however, here no membrane potential or barrier was involved. It is therefore suggested that besides decreased membrane potential, some other post-denervation changes in the membrane properties appear to be responsible for the increased calcium fluxes. This point may perhaps be clarified by a study of calcium fluxes from normal and denervated muscle under conditions in which the membrane potential could be decreased or increased by about 10 to 15 mV.

Following denervation, the duration of action potential in rat diaphragm was observed to be increased from 1.3 mS to 2.1 mS. Since the action potential could be roughly divided into a rising and a falling phase, a prolongation of one of these two phases, or both would prolong the duration of action potentials. Katz (1947) and Hodgkin and Katz (1949) showed the rate of rise and peak amplitude of the action potential to be related to the external sodium concen-

tration. Recently, Albuquerque and Thesleff (1968), and Redfern and Thesleff (1971) have shown that after denervation, the rate of rise of the action potential decreased by 40% and 50% in EDL and soleus muscles of rats.

Moreover, Redfern and Thesleff (1970) showed that when the sodium concentration of the bathing fluid was reduced from 145 mM to 45 mM in the presence of tetrodotoxin, the rate of rise of the action potential in denervated muscle was reduced by one third, with a further reduction of external sodium concentration to 17 mM almost abolishing the spike generation. Although the duration of rising phase of the action potential in our experiments was not measured, if the same was true for rat diaphragm as in rat EDL muscle, then a small increase in the duration of action potential could also be caused by a decreased rate of the rising phase. However, the most obvious change in the action potential after denervation noted in our records was the prolongation or the repolarization phase, this most probably being the major factor for the increase in the duration of action potential. From the studies of Katz (1947) and Hodgkin and Katz (1949) on action potentials, it has become obvious that a sudden decrease in sodium conductivity and reciprocal increase in the potassium conductance results in an increased K^+ efflux and decreased Na^+ influx causing repolarization. There has been ample evidence to show that after denervation, the membrane resistance increases and the potassium permeability decreases (Nicholls, 1956; Harris & Nicholls, 1956; Lüllmann, 1958; Klaus et al., 1960; Thesleff, 1963). This would suggest that potassium efflux, although momentarily increased

during the repolarization phase, may be less than that in normal muscle, therefore taking a longer time to repolarize the membrane despite the already low membrane potential. Furthermore, there has been some evidence that Na^+ conductance is increased in myotonic dystrophic muscles (Hofmann et al., 1968). If this was also true for denervated muscle, then at the peak of depolarization, Na^+ conductivity would be higher in denervated than in normal muscle, further accounting for a longer duration of the repolarization phase. Shanes (1958) has further suggested that the influx of Na^+ and the efflux of K^+ could interact when the two became of the same order of magnitude, such interaction occurring if Na^+ and K^+ moved through the same membrane pores but in opposite directions (Hodgkin & Keynes, 1955). Therefore, an increased conductivity of Na^+ in denervated muscle at the peak of depolarization, could interact with the outcoming K^+, further increasing the repolarizing phase of action potential.

Reduction of the temperature to 25°C caused two important changes in normal muscle. One, a reduction in the membrane potential by 8 mV, two, an increase in duration of the action potential by twice its value at 35°C. However, in denervated muscle, the reduction in temperature did not appear to effect either the membrane potential or the duration of the action potential. Thus, the difference between normal and denervated muscle at 25°C became very small.

According to the Nernst equation, the potential across semipermeable membranes is a positive linear function of absolute temperature. In this regard, Ling and Woodbury (1949), Jenerick and

Gerard (1953) were successful in applying this equation to muscle membranes; however, Hodgkin and Katz (1949) were unable to demonstrate a similar relationship for nerve axons. On the other hand, Goldman (1943) has theoretically indicated that temperature could alter the permeability of cell membranes to K^+, Na^+ and Cl^- ions, making the potential a complex function of temperature. However, neither the Nernst nor Goldman equation has been completely able to account for all the experimental data. For example, a great reduction (Tobias, 1950) or an increase (Grundfest et al., 1954; Folk & Gerard, 1954) in the intracellular K^+ concentration has been shown not to alter membrane potential to the extent predicted by these equations. Therefore, it has been suggested (Apter, 1960) that the concentration of calcium in or at the cell surface controls the membrane potential. Further, Apter suggested that the effects of other ions and temperature on membrane potential were produced by changes in the concentration of membrane calcium.

It has not been possible here to comment on the validity of the Nernst or Goldman equations in describing the effect of temperature on ionic fluxes as such a study was not done. Nevertheless, at least two possible explanations can be proposed for the decreased membrane potential in normal muscle for a reduction in temperature from 35°C to 25°C. At low temperatures, the ionic fluxes would be expected to be decreased as those of calcium in our experiments.

A relatively greater decrease in permeability to K^+ than Na^+ would then account for our results.

The second possibility would be the slowing of enzyme

systems. To maintain the resting membrane potential constant, say at -85 mV inside, a sodium pump is required, which uses energy from ATP breakdown. Because most chemical reactions are slowed at low temperatures, a slower breakdown of ATP would therefore reduce the functioning of the sodium pump, with a resultant accumulation of Na^+ within the cell from the continual passive leakage from its high extracellular concentration, plus a loss of K^+. This would cause the membrane potential to be decreased. The same mechanism has been proposed by Harris (1940) for red blood cells, in which storage at low temperatures reduces the membrane potential as a result of loss of K^+ and accumulation of Na^+. The same may also be true for normal rat diaphragm at 25°C. However, further study of different ionic fluxes at 25°C in rat diaphragm would be required to clearly understand the causes of decreased membrane potential.

The prolongation of action potentials at 25°C in normal muscles presumably results from decreases in permeability to potassium and sodium ions. The reasons for these differences between normal and denervated muscle are unclear.

6. Glycerol Treated Muscles:

Since most of the work using 400 mM glycerol to disrupt T-tubules has been done on frog muscle (Eisenberg & Gage, 1967, 1969; Gage & Eisenberg, 1969; Henderson, 1970; Howell, 1969; Fujino et al., 1961, 1962; Sakai et al., 1970), very little has been known in using it for mammalian skeletal muscle (Niemeyer & Forssmann, 1970). We have tried this technique, finding that it works in rat diaphragm

in the sense that twitch almost completely disappeared while the action potential could be recorded from almost all the surface fibers. However, the condition of the deeper fibers was not known as no action potentials were recorded from them. The only difference between frog and rat muscle noted here was that the duration of glycerol treatment for rat muscle also depended upon the temperature.

Upon exposing a muscle to a hypertonic medium, the twitch tension immediately decreased, this being believed to be caused by a rapid outward movement of water from the muscle, thereby causing an increased ionic strength inside the cells (Hodgkin & Horowicz, 1957; Gordon & Godt, 1970). A similar effect was seen in rat diaphragm when it was exposed to 400 mM hypertonic glycerol-Krebs, but, unlike frog skeletal muscle (Fujino et al., 1961, 1962; Howell, 1969), there did not appear to be any recovery of twitch tension in either normal or denervated muscle at 35°C. In both, the twitch tension remained decreased after the initial sharp decline. The membrane potential was not effected by the glycerol treatment, but the negative after potential in most of the action potential recording was absent. The other differences between normal and denervated muscle remained unchanged.

The disappearance of negative after potential observed in rat diaphragm after glycerol treatment, has also been reported previously for frog sartorius muscle by Gage and Eisenberg (1967, 1960). They believed that the early after potential had a reversal potential within a few millivolts of the resting membrane potential. Moreover, because its time course was very small, the early after potential

was almost absent in glycerol treated muscles. Nevertheless, their polarization experiments showed the negative after potential still to be present in glycerol treated muscles. Henderson (1970), also reported similar results, showing the muscles to regain a negative after potential. However, in our experiments, a decrease in the membrane potential in either normal or denervated muscle after glycerol treatment was not observed, while the negative after potential, in almost all the recorded action potentials was noted to be either absent or greatly diminished. In a few cases, a positive after potential, particularly in the denervated muscles, was noted. However, polarization experiments would be required to determine the equilibrium potential, as well as the time course of the after potentials, if still present.

The most striking effect observed in the glycerol treated preparations, was the increased calcium uptake. After treatment, this increase was, on the average, nine-fold in normal and three-fold in denervated muscle during the first 5 minutes, with the calcium uptake becoming equal in both normal and denervated muscles within 15 minutes. These results are quite puzzling in view of the results obtained from non-glycerol treated muscles where denervated muscles always took up more calcium at a faster rate as compared to the normal muscles. If it is assumed that the glycerol treatment does something to these muscles, then perhaps a few speculations can be made to explain these results. If the T-tubules were disrupted after glycerol treatment, then most of the calcium fluxes would be expected to be blocked. From our experiments there was neither direct evidence

to show that the T-tubules were broken, except that the twitch irreversibly disappeared while normal action potentials were recordable from the surface fibers, nor could a block be shown in the calcium influx. Instead, what was observed was a large calcium uptake by both normal and denervated muscle after glycerol treatment possibly due to some kind of damage to the muscle by glycerol, for which there was no evidence. However, the thing to note was the faster uptake of calcium by the normal muscle. If it was assumed that glycerol treatment disrupted the T-tubules as in frog muscles, and that the damage done by glycerol to both normal and denervated muscle was comparable, then it could be concluded that after glycerol treatment more calcium moves through the surface membrane in normal muscle, and less in denervated muscle (Fig. 21). If the same conclusion is applicable for the non-glycerol treated muscles, then it must be assumed that more calcium fluxes occur through the T-tubules in denervated muscle and less in normal muscle.

7. Effect of Caffeine:

The experiments at 15°C on the effects of caffeine on rat diaphragm showed that the normal muscles developed greated contracture tension than the denervated ones, and that this tension was sustained as long as they were exposed to caffeine (60 minutes). The normal muscle, which developed greater tension, showed a parallel increase in the calcium efflux. Similarly, the denervated muscle, which showed a small contracture tension, also showed a smaller difference between the resting calcium efflux and the efflux during contracture.

8. Effect of Electrical Stimulation:

Electrical stimulation was seen to increase the calcium influx in both normal and denervated muscle. Normal muscles produced greater tension than denervated muscle. Likewise, the difference between the calcium influx during rest and during stimulation was more in normal muscle. Although this indicated that the increase in calcium fluxes in rat diaphragm were associated with an increase in the development of tension, a possibility of error cannot be neglected in these experiments. The muscles were loaded here for a very short time, i.e. 10 minutes only, which is the half time of efflux of Ca^{45} from the fast or extracellular compartment of these muscles. This would mean that the extracellular compartment of these muscles is not saturated during 10 minutes loading and possibly all the fibers are not exposed to the radioactive Ca^{45}. In such a situation, a little contraction might have acted as a pumping source to fill the intracellular space quickly and exposing more fibers to the radioactive solution. This would mean that the increased Ca uptake during stimulation might have been due to the exposure of more fibers to the radioactive solution instead of the Ca entry into the cells as a result of membrane depolarization during action potential. Although the muscles used were very thin and the tension was recorded isometrically, this possibility of error still remains.

9. Correlation with Mechanical Properties:

The primary purpose of this project was to study the effect

of denervation on passive calcium fluxes and on some related electrical properties in muscle membrane, although in two sets of experiments, the effect of stimulation and 20 mM caffeine was also studied in conjunction with the related calcium fluxes. During the course of experimentation no attempt was made at correlating calcium fluxes with the observed electrical property changes or with the change in mechanical properties previously described in denervated muscle (Padsha, 1968; Padsha & Winchester, 1968). Hence, within the following discussion, the possible relationships between these passive calcium flux studies and some of the mechanical property changes in denervated muscle will be discussed in terms of excitation-contraction coupling, the role of electrical property changes, and possibly pathways of calcium movement.

From the data presented, it was shown that calcium fluxes were greatly increased as a result of denervation, suggesting some resultant alteration in muscle membrane with an increase in its permeability to calcium. For this, there could be at least three possible mechanisms:

(1) that a decreased K^+ permeability, as shown by others, greatly increases P_{Ca}, driving calcium into the cell.

(2) that increased Na^+ permeability secondary to denervation may result in intracellular Na^+ accumulation, with a correspondingly smaller increase in Na^+ pump activity, leading, if a reciprocal association between Ca^{++} influx and

Na^{+} efflux exists, to increased calcium fluxes;

(3) that a greatly increased calcium pump activity in the sarcoplasmic reticulum would not only increase Ca^{++} fluxes but also accumulate more Ca^{++} within the sarcoplasmic reticulum.

The results also showed that the calculated amounts of intracellular calcium increased in denervated muscle, suggested the existence of some bound complex form of calcium. Supporting this, evidence from electronmicroscope studies has demonstrated in denervated muscle during atrophy, a relatively large sarcoplasmic reticulum (Pellegrino & Franzini-Armstrong, 1963) and membrane enclosed cisternae (Schrodt et al., 1965), possibly accounting for a large sarcoplasmic reticulum storage capacity for calcium as per gram of muscle. Furthermore, Winegrad (1968, 1970) has demonstrated the accumulation or binding of calcium in this region, and its release as free ions during an action potential. Thus, it could correspondingly be assumed that in denervated muscle, large amounts of calcium could be bound in the sarcoplasmic reticulum.

With the generation of an action potential, the depolarizing current extends through T-tubules, releasing free ionic calcium within the vicinity of the contractile machinery. Hence, from the results in which the duration of the action potential in denervated muscle was found to be twice that of normal, it would be expected that the membranes would be depolarized for a longer time, releasing more calcium. Consequently, this could lead to an increase in the duration of the active state, as observed by Padsha

and Winchester (1968), and a longer duration of isometric twitch (Padsha, 1968). Both the above could possibly be accounted for in terms of the relaxing factor, that is, if in denervated muscle there was a delay in the rebinding of calcium to the sarcoplasmic reticulum, there would remain more calcium in the ionic form at the actin-myosin junction, resulting in a prolongation of the duration of both active state and twitch. It has also been shown by Padsha and Winchester (1968) in denervated muscle, that the twitch tension, although smaller than in normal muscle at low temperatures, became approximately equal to normal at 35°C, the reason being that in normal muscle twitch tension decreased for temperatures greater than 20°C while in denervated it increased linearly. This could be explained by assuming at higher temperatures that:

(1) in normal muscle calcium becomes more firmly bound with less released during the duration of an action potential and/or it becomes rebound more rapidly;

(2) in denervated muscle, more calcium becomes released during a longer duration of the action potential and/or its rebinding becomes delayed. Hence, this could result in the decrease in twitch tension in normal muscle, and the increase in twitch tension and duration in denervated muscle at 35°C.

A further observation reported on the effect of temperature at 35°C

was the increase in the twitch tetanus ratio of denervated muscle to almost double that or normal (Padsha & Winchester, 1968; Padsha, 1968), again possibly being explained by assuming that more calcium would be made available by a longer duration of the action potential as well as by a delay in its rebinding.

In the stimulation experiments, a direct attempt was made to relate the calcium influx with the mechanical properties of the muscle. Excluding the possibilities of error in these experiments as previously discussed, it could be stated that denervated muscle produced a smaller tetanic tension for all the temperatures used, and that although the related calcium influx slightly increased, this was insignificant compared to resting values. However, for normal muscle at 35°C, the increase in calcium influx was significant. Thus, the sum of this plus other observations of normal as compared to denervated muscle, i.e. the slower resting calcium fluxes, the smaller intracellular content of calcium, and the shorter duration of the action potential, lead to the possible conclusion that at 35°C, for the maintenance of normal mechanical activity, additional calcium may be needed from the external environment. However, in normal muscle at 25°C, where a prolongation in the duration of the action potential has been demonstrated, an additional extrinsic source of calcium need not be required for maintaining normal mechanical activity, as enough would be released from the sarcoplasmic reticulum during a prolonged action potential. Similarly, this could apply to denervated muscle at 25°C and 35°C, where the duration of the action potential has been shown to be prolonged.

In the caffeine experiments, a similar association between tension and the related calcium movements was observed. At 15°C it was shown that after the addition of 20 mM caffeine, the tension produced was greater in normal than in denervated muscle, as was the difference between the resting and contracture calcium fluxes, i.e. a greater net release of calcium. The same effect was duplicated upon raising the temperature to 35°C. Hence, these results support the premise that in normal muscle, as compared to denervated, for more tension to be produced, more free ionic calcium must be available, in this case being supplied from the sarcoplasmic reticulum by the action of caffeine.

A large amount of calcium was shown to be present in denervated muscle, this being readily released by caffeine, suggesting its presence in the sarcoplasmic reticulum. This posed the question as to the possible link between an increased calcium content and the numbers of myofilaments, which vary with the number of denervation days (Miledi & Slater, 1969), in producing tension. It would be expected with an increased number of myofilaments and a large amount of calcium available that the generated tension would be greater than normal. On the contrary, Miledi and Slater (1969) demonstrated in 7 to 8 days denervated muscle, the extreme of hypertrophy, that the resultant tension was less compared to normal muscle. Moreover, the studies of Feng and Lu (1965), quoted by Miledi and Slater (1969, page 260), indicated that in rat diaphragm hypertrophy involved only the smaller fibers, which histologically appear to be specialized for oxidative metabolism, the larger, appearently anaerobic fibers

being decreased in size. Therefore, the selective hypertrophy of slower fibers, causing them to predominate over faster ones, could possibly contribute to decreased tension development and prolonged contraction. Besides this, there could be two other factors accounting for decreased tension development:

(1) an alteration in the contractile apparatus, i.e. a decrease in the fraction of muscle space occupied by myofibrils, or a fragmentation and degeneration of myofibrils, more distinctive in atrophy (Miledi & Slater, 1969);

(2) an alteration in the calcium activated ATPase activity, i.e. as shown by Hajik, Hanikova and Gutmann (1967) in rat diaphragm, that the ATPase activity becomes decreased 3 days after denervation. Furthermore, in denervated muscle, the above workers have also suggested the newly synthesized contractile proteins to be functionally inferior, thus accounting for a decreased tension and prolonged contraction speed in the presence of large amounts of calcium.

From the glycerol treatment experiments, some light could be reflected upon the pathways of calcium movement, assuming:

(1) that the majority of the T-tubules were disrupted in both muscle preparations, for which there was no direct evidence;

(2) that damage to the muscle fibers by glycerol was equivalent in both preparations.

Thus, the greater calcium uptake by normal muscle within the first 5 minutes following glycerol treatment, suggested, if the T-tubules were disrupted, that most of the calcium had entered through the surface membrane. Conversely, this inferred that in resting conditions in which calcium fluxes were greater for denervated muscle, that more calcium possibly entered through the T-tubules of denervated than normal muscle. In addition, recent studies by Winegrad (1968, 1970) have suggested most of the calcium efflux to occur through the T-tubules. Hence, providing the T-tubules were disrupted, most of the efflux of calcium would be blocked, explaining, if applicable for rat diaphragm, the higher calcium uptake in normal and denervated muscle after glycerol treatment.

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