Ultrastructure Study on the T System and the Subcellular Localization of Calcium in Frog Skeletal Muscle

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ULTRASTRUCTURE STUDY ON THE T SYSTEM AND THE SUBCELLULAR LOCALIZATION OF CALCIUM IN FROG SKELETAL MUSCLE

by

Lawrence P. McCallister

A Dissertation Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

June, 1971
BIOGRAPHY

Lawrence P. McCallister was born on March 27, 1943, in Chicago, Illinois.

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During the writing of his dissertation he was awarded a U. S. Public Health Fellowship in the Departments of Medicine and Cardiology at the University of Chicago, Chicago, Illinois.
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**GLOSSARY OF TERMS**

<table>
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<tr>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol bis tetraacetic acid</td>
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<tr>
<td>OsO₄</td>
<td>osmium tetroxide</td>
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I. INTRODUCTION

This study had two goals; (A) to establish whether a direct continuity exists between the T system and the surface membrane in frog skeletal muscle; and (B) to observe and study the localization of calcium in skeletal muscle cells using potassium pyroantimonate as a reactant.

A. PART I: THE T SYSTEM

Transmission electron microscopy has demonstrated the continuity between the membranes of the T system and the sarcolemma in a number of striated muscle cells (Franzini-Armstrong and Porter, 1961; Jasper, 1967; Rayns et al., 1968; Smith, 1966). A noted exception, however, is adult frog skeletal muscle in which the T system forms complex terminations at the fiber surface, obscuring any direct continuity between the wall of the T tubules and the sarcolemma (Peachey, 1965).

On the other hand, several studies have demonstrated the free diffusion of substances between the extracellular space and the central elements of the triads in amphibian muscle. For example, Endo (1954) traced the diffusion of a fluorescent dye, Lisamine Rhodamine B 200, in isolated fibers of the semitendinosus muscle of the frog. In his experiments the dye was consistently observed in the center of the I band, without appearing in the major portion of the fiber. Using autoradiography, Hill (1964) localized the uptake of tritiated serum albumin to the central component of the triads in amphibian muscle; and both Huxley (1964) and Page (1964) have demonstrated the uptake of ferritin in T tubules of frog sartorius muscles. All indicating a possibility of direct continuity between the T tubules and the muscle cell surface.
Despite this indirect evidence, only two investigators, L.D. Peachey (1965) and R.I. Birks (1965), have published transmission electron micrographs of frog skeletal muscle showing terminations of T tubules which could be interpreted as having a possible continuity with the extracellular space. However, due to the complicated termination of the T tubules, and their infrequent occurrence along the cell membrane, such evidence has been subsequently regarded as inconclusive (Bianchi, 1963; Eisenberg and Eisenberg, 1968; Smith, 1966; Franzini-Armstrong, 1970). In fact, while evaluating his own work Peachey (1965) stated, "The evidence presented here for a direct connection of the transverse tubules to the plasma membrane of the fiber is not very convincing."

A similar situation is found in skeletal muscle fibers of the rat. Here, too, the T tubules form extensive convolutions in the subsarcolemmal region, as demonstrated with the transmission electron microscope (Walker and Schrodt, 1965; 1966). However, scanning electron micrographs of this muscle clearly demonstrate rows of apertures along the sides of the muscle fibers corresponding to the transverse tubular system (Carrow, et al., 1969). Hence, the need for a comparative study on the transmission and stereo ultrastructure of the T system in frog skeletal muscle was indicated. It was further assumed, that the greater potential of the scanning electron microscope for observing the surface topography and interrelationships of cells, might reveal new information concerning the surface features of frog skeletal muscle.

Therefore, the purpose of this investigation was to study the ultrastructure of frog skeletal muscle using a number of different techniques, in the hopes of demonstrating morphologically "open" T tubules. The techniques employed include transmission electron microscopy, scanning electron microscopy,
and the use of extracellular markers. Moreover, no reports on the scanning electron microscopy of frog skeletal muscle have as yet appeared in the literature; and while the extensor longus digiti IV muscle of the frog is almost as extensively used for physiological experiments as the sartorius, no detailed electron microscopic study of this muscle is on hand.

3. PART II: THE SUBCELLULAR LOCALIZATION OF CALCIUM

The T system of skeletal muscle is intimately associated with the sarcoplasmic reticulum. The morphology of the sarcoplasmic reticulum was first described by Dobie in 1849 (see Bennett, 1955). More recently, Porter and Palade (1958) investigated the sarcoplasmic reticulum with the electron microscope. These authors proposed that a trigger substance was released from the reticulum for the contraction of the myofibrils. It is now generally accepted that the trigger substance is calcium (Hodgkin and Horowiczs, 1960; Hoyle, 1970; Jobsis and O'Connor, 1966; Winegrad, 1970).

The calcium is believed to be localized in the terminal cisternae of the sarcoplasmic reticulum (Costantin, et al., 1956). According to the physiological model, after excitation of the muscle fiber calcium ions are released from the terminal cisternae and diffuse to the myofilaments where they initiate contraction (Winegrad, 1970). Subsequently, the diffused calcium is reaccumulated by the sarcoplasmic reticulum during relaxation (Weber et al., 1968; Winegrad, 1965; 1968; 1970).

In contrast to calcium, caffeine induces a prolonged state of contraction in skeletal muscle which is referred to as a contracture. According to Ranson (1911), the "rigor" caused by the direct action of caffeine on skeletal muscle was first observed by Voit (1860). More recently, Axelsson and Thesleff(1958)
produced graded caffeine contractures in frog skeletal muscle which were in proportion to the amount of drug administered.

Although the observation of the caffeine contracture is well documented, the mechanism that causes it is still subject to controversy. Bianchi found that caffeine increased both the influx and efflux of calcium in muscle fibers of frog sartorius. He concluded that caffeine "reduces" the binding of calcium in the membrane and the sarcoplasmic reticulum. Horst Weber (1965) observed that caffeine in high concentrations (8 to 10 mM) inhibited the calcium uptake of fragmented sarcoplasmic reticulum isolated from frog skeletal muscle. They also noted that 20 to 40% of the bound calcium released from the reticulum following the application of caffeine, causing one to assume that a rivalry existed between calcium and caffeine. On the other hand, Carvalho (1966) found no evidence of competition between caffeine and calcium for the "anionic binding sites" on fragmented sarcoplasmic reticulum in rabbit skeletal muscle; and Frank (1968) found that caffeine could induce contracture in frog toe muscle that had been inhibited by exposure to calcium-free Ringer's solution.

These previously discussed observations on the effect of caffeine on calcium stores in skeletal muscle are either the results of physiological experiments on whole muscles, or studies of fragmented sarcoplasmic reticulum isolated from whole muscles. As yet no investigator has studied the action of caffeine on calcium in intact muscles with the electron microscope. This is understandable, since up to the present the only method available for determining the distribution of calcium in muscles with the electron microscope involved the laborious technique of removing the sarcolemma in paraffin and precipitating the calcium with sodium oxalate (Costantin, et al., 1969).
However, Legato and Langer (1969) have recently demonstrated a simple method whereby calcium can be precipitated in intact dog papillary muscle as the electron dense salt of calcium pyroantimonate. Therefore, the purpose of this portion of the problem was to extend this technique to frog skeletal muscle, and to use it in studying the effects of caffeine on the intracellular stores of calcium within the tissue.
II. REVIEW OF THE RELATED LITERATURE

The subject of this dissertation is related to the process of excitation-contraction coupling in frog skeletal muscle, or the role of the transverse tubular system in contraction, and its involvement in calcium release and activation. However, excitation-contraction coupling is only one in a series of events which culminate in contraction. Therefore, it will be necessary to begin with a consideration of the theories of contraction, and then to proceed to the role of calcium ion in activation and excitation-contraction coupling.

A. THEORIES OF CONTRACTION

1. THE ROLE OF LACTIC ACID AND ORGANIC PHOSPHATES

Modern theories of contraction have evolved around two foci: (1) the role of lactic acid and organic phosphates in contraction, and (2) the nature of the structural proteins found in muscle. The lactic acid theory of contraction first gained acceptance when Fletcher and Hopkins (1907) demonstrated that lactic acid was produced by contracting muscles under anaerobic conditions. It was later shown that lactic acid was formed from glycogen, and that the process of glycolysis represents the major source of energy for contracting muscles in the absence of oxygen (Farnas and Wagner, 1914). When further studies were conducted to discover the mechanism by which lactic acid was produced, the role of organic phosphates became evident.

In a series of papers, Lundsgaard (1930; 1930; 1934) demonstrated that frog muscles inhibited by sodium iodoacetate could contract without the production of lactic acid provided phosphorylcreatine was present in the perfusate. Subsequently, Lohmann (1934) showed that dialysed muscles were unable to break down phosphorylcreatine in the absence of adenosine diphosphate (ADP).
Thus, the real source of energy during contraction was thought to be the dephosphorylation of adenosine triphosphate (ATP) to ADP, which was then rephosphorylated by phosphorylcreatinine. In this connection it is important to note that Englehardt and Liubimowa (1939) were the first to succeed in demonstrating the enzymatic activity of myosin as an adenosinetriphosphatase. This observation has served to stress the importance of understanding the nature of the structural proteins found in muscle.

2. STRUCTURAL PROTEINS FOUND IN MUSCLE

The structural organization of muscle was first described in detail by Bowman (1860), who characterized the cross-banding of voluntary muscle as consisting of A, I, H, Z, and M bands. This was followed by Englemann's (1873) recognition of the N band, which completed the entire striation pattern observed in both insect and vertebrate muscle. Further, the banding pattern was thought to be localized to bundles of fibrillae or myofibrils (Bowman, 1840); and Kölliker (1883) suggested that the myofibrils were composed of smaller filaments of submicroscopic dimensions.

"Kölliker's filaments" were later confirmed with electron microscopy by Hall, Jakus, and Schmitt (1946). Huxley (1953) refined these concepts, and demonstrated two discrete species of filaments consisting of actin and myosin. The myosin filaments were localized to the A band; whereas, the I band was composed of actin filaments which originated bilaterally at the Z line, traversed the length of the I band, and interdigitated with the myosin filaments in the A band. In cross-sections through the A band, the myosin filaments were arranged in a hexagonal lattice having a spacing of 400 to 450 Å, with the actin filaments located at the trigonal positions of the lattice.
3. SLIDING FILAMENT THEORY OF CONTRACTION

Biochemical, electronmicroscopic, and X-ray diffraction evidence supported this model (Huxley, 1953; Huxley and Hanson, 1954; Huxley and Neidergerke, 1954), and in 1954 Huxley and Hanson proposed the sliding filament theory of contraction. According to the theory the contractile apparatus consists of partially overlapping arrays of filaments of constant length. During contraction the actin filaments are drawn into the A band (sliding past the myosin filaments), and are withdrawn upon relaxation.

Further evidence has shown that the myosin filaments are composed of a backbone of light-meromyosin, to which is attached a molecule of heavy-meromyosin (Huxley, 1960). Isolated heavy-meromyosin molecules examined by shadow casting demonstrate a 400 Å long tail in conjunction with a globular region approximately 50 to 200 Å (Huxley, 1968). Since the globular region bears a striking resemblance to cross-bridges seen linking the actin and myosin filaments in electron micrographs (Huxley, 1953; 1957), and since the adenosine triphosphatase activity associated with contraction was localized to the globular head of the heavy-meromyosin molecule, the events of contraction are now generally interpreted as a result of cyclic interactions between actin, myosin, and ATP at the level of the cross-bridges located on the myosin filaments (Huxley, 1970; Pringle, 1968; Szent-Györgyi, 1968).

B. CALCIUM ION IN CONTRACTION

Parallel with investigations on the molecular basis of contraction, a great effort was put forth to discover the role of calcium in fibrillar activation; namely, this was found to be the only naturally occurring cation which when injected into muscle would bring about a contraction (Neilbrunn and
Further, it was demonstrated that synthetic preparations of actin and myosin had little or no sensitivity for calcium unless a third protein, native tropomyosin, was added (Ebashi and Ebashi, 1964). Native tropomyosin was later separated into tropomyosin and a globular protein, troponin (Ebashi and Kodama, 1965); and it was shown that the calcium sensitivity of actomyosin preparations was directly related to the physico-chemical properties of the troponin moiety (Ebashi and Endo, 1968). Results of antibody staining using fluorescence and electron microscopy, demonstrated that both troponin and tropomyosin were distributed along the length of the actin filaments (Endo et al., 1966; Ohtuski et al., 1967). However, selective removal of these proteins suggested that troponin was bound to tropomyosin but not to actin, since digestion of tropomyosin resulted in a decrease of fluorescence due to a loss of both antitropomyosin and antitroponin (Endo et al., 1966). Finally, it was proposed that the troponin molecule has an inhibitory effect on the interaction of actin and myosin, which is removed by saturation of the troponin molecule with calcium (Ebashi and Endo, 1968).

In view of the above, the sliding filament theory of contraction can be stated more completely as follows: After stimulation of a muscle cell there is an almost instantaneous increase in the concentration of free calcium ion in the sarcoplasm (Hoyle, 1970; Jobsis and O'Connor, 1966; Winegrad, 1970). The calcium then rapidly attaches to the troponin moiety, reducing its inhibition on interaction of the globular part of the heavy-meromyosin molecule with its attachment to the actin filament (Ebashi and Endo, 1968; Huxley, 1970). The decreased inhibition brings about the hydrolysis of ATP, and the sliding of the actin filaments with respect to the myosin filaments causing the muscle to contract (Huxley, 1970; Pringle, 1968; Szent-Gyorgyi, 1968). During relaxation,
the concentration of free calcium is reduced again to the resting level (Hoyle, 1970; Jobsis and O'Connor, 1966; Winegrad, 1970), and dissociation of the action and myosin filaments is accompanied by a return to their original positions.

C. EXCITATION-CONTRACTION COUPLING

It is well known that the first step in the sequence of events which terminate in contraction is depolarization of the muscle cell membrane (Nastuck and Hodgkin, 1950). However, the mechanism by which surface depolarization is conducted to the interior of the muscle fiber, bringing about a transitory increase in the concentration of free calcium ions, has been the subject of study for recent years. In a series of papers Hill (1948; 1949) demonstrated that the diffusion time for an activation substance from the surface membrane was not sufficiently rapid to account for the abrupt transition from rest to contraction in a striated muscle fiber 50 µ in diameter. Subsequently, it was shown that muscle cells possess an elaborate arrangement of longitudinally running tubules and cisternae called the sarcoplasmic reticulum (Bennett and Porter, 1953; Bennett, 1955; Porter and Palade, 1957). It was suggested that the sarcoplasmic reticulum released a trigger substance for activating the myofibrils (Porter and Palade, 1957). This was identified as calcium (Heilbrunn and Wiercinski, 1947), and localized to the terminal cisternae of the sarcoplasmic reticulum (Costantin et al., 1965).

It was also shown that the sarcoplasmic reticulum was not continuous throughout the length of the muscle cells, but interrupted at regular intervals by transversely oriented tubules (Revel, 1962) called the T system or T tubules (Anderson-Cedergren, 1959). Because the T tubules are opposed on
either side by enlarged elements of the sarcoplasmic reticulum, called terminal cisternae, the three membered structures were collectively referred to as triads (Porter and Palade, 1957). In the most simple models the T system was envisioned as continuous with the surface membrane. Thus, it was thought to play an important role in the inward spread of activation from the cell membrane, bringing about the release of calcium from the sarcoplasmic reticulum.

This hypothesis was tested in the local stimulation studies of Huxley and Taylor (1958). These experiments showed that small depolarizing currents applied to the surface membrane of frog skeletal muscle, although insufficient to generate an action potential, still brought about a local contraction of the underlying myofibrils up to a depth of 10 μ. Moreover, the contractions only occurred when currents were applied to certain sensitive regions located around the periphery of the muscle cells at the level of the Z line. This was later found to correspond to the location of the T tubules in this muscle (Peachey, 1965). Since similar results have been obtained in striated muscle fibers of the lizard and crab (Huxley and Taylor, 1958; Huxley and Staub, 1958), it is now generally accepted that spread of electrical current along the transverse tubular system represents the mechanism by which surface excitation is linked to the triggering of the contractile response, a process which is referred to as excitation-contraction coupling (Smith, 1966).

D. APPROACHES TO THE STUDY OF MUSCLE CALCIUM

1. EARLY WORK

Early approaches to the study of muscle calcium established this element as the major divalent cation involved in contraction. The work of Ringer
(1886), Locke (1894), and Mines (1913) showed that the exclusion of calcium from the bathing solutions of beating hearts uncoupled excitation from contraction. Further, Heilbrunn & Wircinski (1947) demonstrated that calcium injected into muscle cells brought about contraction. This experiment has recently been refined by Gillis (1969) who showed that the amount of calcium necessary to cause contraction in glycerinated crab muscle when applied by means of a microelectrode, was lowest when the calcium was placed near the A-I junction.

2. RADIOCALCIUM EFFLUX

In attempts to characterize the intracellular calcium pools in skeletal muscle several studies have been conducted on the distribution and kinetics of radiocalcium efflux in the frog's sartorius (Curtis, 1966; Gilbert and Fenn, 1957; Shanes and Bianchi, 1959). The results of these studies were difficult to interpret because of complicating efflux from tendon and the extracellular space (Curtis, 1970). However, Curtis (1970) has described a method whereby the effluent of $^{45}\text{Ca}$ can be collected from a single muscle fiber. On the strength of these experiments it appears reasonable to assume that calcium is contained in a three compartment system, each of them having a characteristic efflux time constant of $18 \pm 2$ minutes, $300 \pm 40$ minutes, and $882 \pm 72$ minutes. The compartment with the fastest time constant ($18 \pm 2$ minutes) is thought to represent calcium on the surface of the fiber and on the walls of the transverse reticulum. Efflux from the middle compartment ($300 \pm 40$ minutes) is interpreted as calcium released from the sarcoplasmic reticulum. Calcium in the third compartment ($882 \pm 72$ minutes) was very small, and was difficult to localize to any discrete intracellular stores.
Further information on the intracellular stores of calcium have been obtained from experiments using the dye murexide and a jellfish protein - aequorin, both of which emit a characteristic light in the presence of calcium. Murexide was first employed by Ohnishi and Ebashi (1963) to monitor the rate of in vitro uptake of calcium by particulate factions of sarcoplasmic reticulum. Taking this lead, Jobsis and O'Connor (1966) used murexide to study the movement of calcium during the contraction-relaxation cycle in sartorius muscles of the toad. Their results showed that the free calcium concentration of the sarcoplasm increases within a few milliseconds after stimulation of the muscle fiber. When the muscle reaches peak tension the "calcium signal" ceases, indicating the binding of free calcium to the myofibrils. As the signal does not reappear during relaxation, it is suggested that calcium return to the sarcoplasmic reticulum is slow, and the level of free ionized calcium does not reach a high concentration (i.e., greater than $3 \times 10^{-7}$M) until the next contraction. Similar results have been obtained with the use of aequorin in skeletal muscle fibers of the giant barnacle by Ashley and Ridgeway (see Hoyle, 1970).

4. FRAGMENTED SARCOPLASMIC RETICULUM

Fragmented sarcoplasmic reticulum has been extensively used is the study of muscle calcium. The sarcoplasmic reticulum of muscle cells was first isolated by Marsh (1951) as a "relaxing factor" capable of converting shrunken jels of actomyosin to hydrous form in vitro. It was later shown that this factor could actively accumulate calcium in the presence of ATP (Ebashi, 1961;
Hasselbach and Mackinose, 1961). Ultrastructural studies demonstrated that the relaxing factor had a microsomal nature consisting of vesicular and tubular components of the sarcoplasmic reticulum and transverse tubular system (Muscatella, 1961). Comparative studies on the ultrastructure and calcium transport in heart and skeletal muscle of the rabbit indicate the rate and amount of calcium uptake in heart microsomes is substantially lower than in skeletal muscle (Deamer and Baskin, 1969; Baskin and Deamer, 1969). Thus, both fractions show nodular deposits following precipitation of calcium with oxalate; however, these are only observed in 5-10% of the vesicles isolated from heart muscle, whereas, 20% of the vesicles from skeletal muscle contain such deposits (Baskin and Deamer, 1969). Finally, the mechanism by which calcium is stored in these vesicles is uncertain. It has been suggested that the calcium is deposited in the form of free ions (Hasselbach, 1964), bound to the reticular membrane (Ibashi, 1961), or bound to structures inside the reticulum (Weber et al., 1966).

5. AUTORADIOGRAPHIC STUDIES

Autoradiographic studies with the light microscope have been used to localize $^{45}$Ca in isotopically labelled frog skeletal muscle quickly frozen at rest, and during a maintained tetanus (Vinegrad, 1965; 1968; 1970). These experiments indicate the majority of exchangeable calcium in resting muscles is contained in the terminal cisternae of the sarcoplasmic reticulum, with a small amount present in the A band portion of the myofibrils. During tetanus approximately 0.2 μ moles of calcium/g is translocated from the terminal cisternae to the region of the thin filaments, forming a peak in the I band, just prior to the A-I junction (Vinegrad, 1970). With relaxation the $^{45}$Ca is
reaccumulated by the longitudinal tubules of the sarcoplasmic reticulum, and transported back to the terminal cisternae (Winegrad, 1970).

6. OXALATE PRECIPITATION

Oxalate precipitation of calcium has been employed by Poldolsky and colleagues (Costantin et al., 1965; Podolsky et al., 1970) to study the distribution of calcium in single muscle fibers from which the sarcolemma has been removed in paraffin oil according to the technique of Natori (1954). Electron micrographs of such preparations demonstrate the presence of electron-opaque precipitates localized to the terminal sacs of sarcoplasmic reticulum. In addition, Hasselbach (1964) and Pease et al., (1965) have also observed oxalate precipitates in the longitudinal tubules of the sarcoplasmic reticulum, while no precipitation was associated with the myofilaments.

7. LOCALIZATION OF CALCIUM BY POTASSIUM PYROANTIMONATE

Early studies on the precipitation of cations by potassium pyroantimonate were primarily concerned with the localization of sodium, and were not designed to demonstrate all the possible compounds which could be formed by pyroantimonate precipitation (Bulgar, 1969; Komnick and Komnick, 1963; Zadunaisky, 1966). However, Komnick and Komnick (1963) in their original treatment of the subject noted the solubility characteristics of sodium pyroantimonate, calcium pyroantimonate, and magnesium pyroantimonate are such that any of these salts may form electron dense precipitates under the proper conditions.

Evidence that pyroantimonate forms considerable precipitates in combination with tissue calcium has been obtained from the studies of Bulgar (1969), and by electron microscopy and electron microprobe analysis of Tandler et al.
Subsequently, Legato and Langer (1969) used a modification of Komnick and Komnick's (1963) method for the subcellular localization of calcium in the papillary muscle of the dog's heart. The technique has the advantage that the sarcolemmal membrane is left intact, and the muscle is functional at the time of fixation. Fixation is carried out in 1% OsO₄ containing a 2% concentration of potassium pyroantimonate.

Two discrete types of precipitates were observed in Legato and Langer's (1969) experiments. The extracellular space was marked by large granules of precipitate primarily clustered at the sarcolemmal membrane and in the space bound by the membrane of the transverse tubular system and the intercalated disc. This precipitate was interpreted as sodium pyroantimonate, and thought to reflect the known high extracellular concentration of sodium in muscle.

A second precipitate consisting of smaller 150 Å granules was observed inside the muscle fibers. This precipitate was localized to the region of the A band and along the actin filaments in the I band. The H band, on the other hand, showed a conspicuous lack of precipitation. Precipitate granules were also found filling the lateral sacs of the sarcoplasmic reticulum. Both types of precipitates, i.e., those that associated with the myofilaments and those within the sarcoplasmic reticulum, were interpreted as calcium pyroantimonate.

The assumption that the intracellular precipitate was, in fact, calcium pyroantimonate was tested by perfusing hearts with sodium free solutions, calcium free solutions, and solutions containing the calcium chelating agents EDTA and EGTA. Prior to perfusion with sodium free solution the muscle was perfused with Ringer's solution containing radioactive ²⁴Na (Langer, 1967). When the radioactivity of the muscle reached a asymptotic values, as measured by a Geiger-Muller probe, it was estimated that all the exchangeable muscle
sodium had been replaced by $^{24}\text{Na}$.

The perfusate was then changed to Ringer's solution containing 130 mM choline chloride in place of NaCl. When the tissue activity stabilized at 10% of the asymptotic value, indicating that 90% of the exchangeable tissue sodium had been washed out, the muscle was fixed in the same manner as controls. Electron micrographs of the muscle showed the same distribution of precipitate along the sarcomeres and in the lateral sacs of the sarcoplasmic reticulum as seen in control tissue. Further, since it was known that all but 10% of the exchangeable muscle sodium had been washed out, it was assumed that the intracellular precipitate was not sodium pyroantimonate, but rather magnesium or calcium pyroantimonate.

Perfusion with solutions containing no calcium greatly diminished the intracellular precipitate, but did not eliminate it in all areas. However, perfusion with EDTA and EGTA virtually eliminated the precipitate densely concentrated on myofibrils and the lateral sacs of the sarcoplasmic reticulum. Further, while magnesium can be chelated by EDTA, EGTA is specific for calcium even in the presence of excess magnesium (Ebashi et al., 1960; Ebashi and Endo, 1968; Gillis, 1969; Podolsky, 1968). Thus, it was concluded that the intracellular precipitate was calcium pyroantimonate.
III. MATERIALS AND METHODS

A. THE MUSCLE

Throughout these experiments the extensor longus digitii IV muscle of the frog Rana pipiens was used. This muscle is very small (see Results), having a large surface to volume ratio (Gray, 1957), which facilitates rapid ionic equilibration with Ringer's solution. The Rana pipiens were purchased from the Lemberger Co., Oshkosh, Wisconsin, flown to Chicago, and used within 24 hours of their arrival.

B. EXPERIMENTS ON THE T SYSTEM

The first procedure was a transmission and scanning electron microscopic study of the muscle, with particular emphasis on the T system.

1. FIXATION AND DEHYDRATION

Rana pipiens were pithed, and the legs were removed and placed in Ringer's solution (see 1°- Ringer's solution, section C 2). The extensor longus digitii IV muscle was then dissected and fixed at either (a) rest length and allowed to contract, or (b) at 100 to 150% of its resting length under a tension of 0.5 g for 1 hour. Fixation was carried out at 4° C in phosphate buffered 3.125% gluteraldehyde at pH 7.1 to 7.2 (Peachey, 1965). The muscles were then rinsed for 1 to 3 hours in cold phosphate buffer (0.15%) (Millonig, 1961), and post fixed in phosphate buffered 1° OsO₄ at 4° C for an additional hour (Ledbetter and Porter, 1963). In some cases the OsO₄ was 2-collidine buffered (Bennett and Luft, 1959), and contained a 1% concentration of lanthanum nitrate to serve as a marker of the extracellular space (Revel and
Yarnovsky, 1967). Following fixation all the muscles were rapidly dehydrated in a graded series of chilled ethanol baths. The muscles were then passed through two 10 minute changes of propylene oxide at room temperature; and, subsequently prepared for viewing with either the transmission or scanning electron microscope.

2. **TRANSMISSION ELECTRON MICROSCOPY**

For conventional electron microscopy, the muscles were embedded in Epon 812 (Luft, 1961) and sectioned on a Reichert OM U2 ultramicrotome. The sections were mounted on Formvar coated 200-mesh copper grids; contrast enhanced with uranyl acetate (Swift and Rasch, 1948), or a combination of uranyl acetate and lead hydroxide (Karnovsky, 1961); and examined with an RCA EMU 3F-2 electron microscope at an accelerating voltage of 50 kV.

3. **SCANNING ELECTRON MICROSCOPY**

For scanning electron microscopy, the muscles were allowed to dry for 24 hours in a vacuum dessicator containing a drying agent. The specimens were then coated with a 200 to 400 A layer of evaporated gold, and subsequently viewed with a Cambridge Stereoscan Mark II A electron microscope, courtesy of Ennis Equipment Co., Morton Grove, Illinois.

C. **THE PHYSIOLOGY OF THE CAFFEINE-INDUCED CONTRACTURE**

The purpose of the second procedure was to confirm the caffeine-induced contracture in the presence of calcium-free Ringer's solution as reported by Frank (1960). Subsequently, the caffeine-induced contracture was arrested and the muscles were prepared for electron microscope study (see section D 2).
<table>
<thead>
<tr>
<th>METHOD</th>
<th>NUMBER OF MUSCLES OBSERVED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed in 3.125% phosphate buffered glutaraldehyde, with post-fixation in 1% phosphate buffered CsO₄, and observed with transmission electron microscopy.</td>
<td>17</td>
</tr>
<tr>
<td>Fixed in 3.125% phosphate buffered glutaraldehyde, with post-fixation in 1% 2-collidine buffered CsO₄ containing 1% concentration of lanthanum nitrate, and observed with the transmission electron microscope.</td>
<td>8</td>
</tr>
<tr>
<td>Fixed in 3.125% phosphate buffered glutaraldehyde, with post-fixation in 1% phosphate buffered CsO₄, and observed with the scanning electron microscope.</td>
<td>6</td>
</tr>
</tbody>
</table>
The physiological method was essentially the same as Frank's; however, the contractures were recorded isotonically.

1. MUSCLE PREPARATION AND RECORDING APPARATUS

The extensor longus digitii IV was dissected and vertically mounted in a 10 ml cylindrical bath. The bath was open at the top for the addition of solutions, and contained a valve at the bottom for evacuation of the cylinder. The lower end of the muscle was fixed to the bottom of the bath, and the upper end was tied to the lever arm of a linear motion transducer, Model ST-2, Phipps and Bird Inc. The transducer was calibrated using a Microameter Calibrator, No. 463-K, L.S. Starrett Co. Contractures were recorded with a Texas Instruments Oscilloriter, Model PICAL CAL. In all cases the chart speed was adjusted to .25 mm/sec. Thus each small square in the Figure representations corresponds to a time lapse of 4 seconds. The distance of muscle shortening was measured, and is indicated in the Figure descriptions. The rectangular artifacts before and after the contractures were caused by the changing of solutions in the tissue bath. As such they served as event markers.

2. SOLUTIONS

The primary (10-) Ringer's solution had the following composition mM:
choline chloride 111.8; KCl 2.47; CaCl₂ 1.03; Na₂HPO₄ 0.087; NaHCO₃ 2.38; glucose 11.1 (Frank, 1960). With the exception of CaCl₂, the composition of the calcium-free Ringer's solution was the same. The caffeine Ringer (5mM) was prepared by the addition of anhydrous caffeine (Sigma Chemical Co.) to the
calcium-free solution. The elevated potassium Ringer's (50 mM) was prepared by the addition of solid KCL to both the 1°- and calcium-free Ringer's solutions. Solutions containing calcium chelating agents were prepared by the addition of either 5 mM sodium EDTA or 5 mM sodium EGTA to either the calcium-free, caffeine, or elevated potassium Ringer's solutions. All solutions contained d-tubocurarine $10^{-4}$ g/ml. The Ringer's solutions were adjusted to pH 7.1 - 7.2 by gassing with 95% air and 5% CO₂. Water for the above solution had a minimum purity of one part per million with respect to foreign ions, and was obtained by passing distilled water through a Continental demineralizer.

3. PROCEEDURE

Preceding exposure to caffeine Ringer, a standard potassium contracture was induced using 1°-Ringer + 50 mM K. Following the potassium induced contracture, a calcium washout was accomplished by soaking the muscle in three changes of calcium-free Ringer's. After 10 minutes the bath solution was changed to calcium-free Ringer + 50 mM K. If no contracture occurred, the washout was considered complete and experiments with caffeine Ringer's solution were conducted. The caffeine-induced contracture was subsequently arrested by exposing the muscle to 5 mM caffeine for 2 minute periods, at regular 10 minute intervals. In some cases 5 mM EDTA or 5 mM EGTA was present in all the calcium-free solutions.

D. SUBCELLULAR LOCALIZATION OF CALCIUM

The third procedure was an histochemical study on the subcellular localization of calcium pyroantimonate in muscles exposed to 1°-Ringer's solution,
calcium-free Ringer's solution, Ringer's solution containing chelating agent, or muscles in which the caffeine-induced contracture was arrested.

1. MUSCLES EXPOSED TO 1° RINGER'S SOLUTION, CALCIUM-FREE RINGER'S SOLUTION, OR RINGER'S SOLUTION CONTAINING CALCIUM CHELATING AGENTS

Extensor muscles were dissected in 1°-Ringer's solution and tied to glass bars (Eisenberg and Eisenberg, 1963). The muscles were then placed in glass vials, and covered with three 10 ml changes of calcium-free Ringer's solution, or calcium-free Ringer's solution containing 5 mM EDTA or 5 mM EGTA. After exposure to the solutions for periods of 10 minutes and 1 hour, the muscles were fixed at 4° C in 1.5 OsO₄ containing a 2.5% concentration of potassium pyroantimonate (K₂H₃Sb₂O₇·4H₂O) (Fisher Chemical Co.), pH adjusted to 7.4 with the addition of acetic acid (Legato and Langer, 1969). Control muscles were fixed immediately following their dissection in OsO₄ containing potassium pyroantimonate, or in 1.5 phosphate buffered OsO₄ without potassium pyroantimonate. After fixation, the muscles were dehydrated in graded series of chilled acetones, and prepared for viewing with the electron microscope (see section B 2).

2. MUSCLES IN WHICH THE CAFFEINE-INDUCED CONTRACTURE WAS ARRESTED

The caffeine-induced contracture was depleted in the presence of calcium-free Ringer's solution, or calcium-free Ringer's solution containing 5 mM EDTA or 5 mM EGTA. The contractures were induced and recorded as previously described (section C). At the conclusion of the experiment, the muscles were processed for the electron microscope as above (section D 1).
TABLE II.  
EXPERIMENTS ON THE SUBCELLULAR LOCALIZATION OF CALCIUM

<table>
<thead>
<tr>
<th>METHOD</th>
<th>NUMBER OF MUSCLES OBSERVED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscles fixed in 1% phosphate buffered OsO₄•</td>
<td>6</td>
</tr>
<tr>
<td>Muscles fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>8</td>
</tr>
<tr>
<td>Muscles exposed for 10 min to calcium-free Ringer's solution, and fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>7</td>
</tr>
<tr>
<td>Muscles exposed for 1 hour to calcium-free Ringer's solution, and fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>8</td>
</tr>
<tr>
<td>Muscles exposed for 10 min to calcium-free Ringer's solution + 5 mM EDTA, and fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>6</td>
</tr>
<tr>
<td>Muscles exposed for 1 hour to calcium-free Ringer's solution + 5 mM EDTA, and fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>6</td>
</tr>
<tr>
<td>Muscles exposed for 10 min to calcium-free Ringer's solution + 5 mM EDTA, and fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>6</td>
</tr>
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<td>6</td>
</tr>
<tr>
<td>Muscles in which the caffeine-induced contracture was arrested in calcium-free Ringer's solution, and fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>6</td>
</tr>
<tr>
<td>Muscles in which the caffeine-induced contracture was arrested in calcium-free Ringer's + 5 mM EDTA, and fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>6</td>
</tr>
<tr>
<td>Muscles in which the caffeine-induced contracture was arrested in calcium-free Ringer's + 5 mM EDTA, and fixed in 1% OsO₄ containing 2% potassium pyroantimonate.</td>
<td>6</td>
</tr>
</tbody>
</table>
IV. RESULTS: THE T SYSTEM

A. TRANSMISSION ELECTRON MICROSCOPY

1. GENERAL MORPHOLOGY

The extensor longus digiti IV muscle originates with the tendon of the extensor digitorum communis, and inserts on the fourth digital extensor tendon of the same muscle (Figure 1). The muscle measures 20 to 25 mm in length, and has a cross-sectional diameter of 0.5 to 1.0 mm. The muscle is composed of numerous myofibers 40 µ in diameter, each fiber containing several myofibrils oriented parallel to the long axis of the cell (Figure 2). Within the myofibrils regular cross-striations forming lighter I bands (0 to 1.4 µ) and darker A bands (1.5 µ) are evident. A prominent Z line and H zone containing a well defined H disk is also seen. The A band contains thick myosin filaments approximately 130 Å in diameter, whereas, the I band is composed of smaller actin filaments 60 Å in diameter. The actin filaments originate at the Z line and interdigitate with the myosin filaments in the A band. All these structures can be seen in longitudinal section (Figure 7); however, an analysis of both longitudinal and transverse sections is necessary to determine the distribution of the filaments in relation to the banding pattern, and the changes in the appearance of the banding pattern in contracted and relaxed muscles.

In transverse sections through the overlap of thick and thin filaments in the A band, each myosin filament is surrounded by an orbit of six actin filaments (Figure 3). In the I band the actin filaments are randomly oriented and take on an orderly appearance as the Z disk is approached (Figures 3, 4). Just prior to reaching the Z disk the actin filaments form parallel rows with
a spacing of approximately 230 Å between the rows (Figure 4). At the level of the Z disk the parallel rows of actin filaments interdigitate with rows of actin filaments from the adjacent half-sarcomere on the opposite side of the Z disk. The interdigitating rows of actin filaments are further interconnected by Z filaments (Figures 4, 5).

The depth to which the actin filaments penetrate the A band is best seen in longitudinal section. The actin filaments enter the A band from opposite sides, and the distance of separation between the ends of the actin filaments determines the width of the H band. Thus, in relaxed sarcomeres the H band is broad (Figures 6, 11); however, in contracted muscles the H band is narrow (Figures 7, 8) or totally lacking (Figure 9). Similarly, as the myofilaments slide along each other, the length of the I band is of necessity decreased. I bands as wide as 1.4 μ are observed in relaxed muscles (Figure 11), whereas, in contracture the I band is entirely absent (Figure 9).

2. THE SARCOPLASMIC RETICULUM AND T SYSTEM

The sarcoplasmic reticulum and T system of the extensor muscle is composed of longitudinal and transverse elements which abut on one another at the level of the Z band to form triads. The central component of the triad is referred to as the transverse or T tubule, and measures 250 Å in diameter. The T tubule is flanked on either side by larger terminal cisternae of the sarcoplasmic reticulum. Continuous with the terminal cisternae are intermediate cisternae and longitudinal tubules. The longitudinal tubules extend from the margin of the I band to the region of the H zone, where they coalesce to form a continuous fenestrated collar surrounding each sarcomere.
All of these general relationships can be observed in longitudinal (Figure 11) and transverse section (Figure 5). However, the various elements of the reticulum are most clearly seen in face view, when the plane of section passes between two myofibrils. Such a section is shown in Figure 12, in which the convergence of the longitudinal tubules to form the fenestrated collar is clearly evident. The continuity of the intermediate cisternae and longitudinal tubules with the terminal cisternae is shown in longitudinal section in Figure 10. Thus, the terminal cisternae, intermediate cisternae, longitudinal tubules, and fenestrated collar form one continuous compartment with membrane continuity between each of the elements.

The T system, however, is separated from the terminal cisternae of the sarcoplasmic reticulum by a space of approximately 200 Å (Figure 12). This space often contains dense material which distends the luminal aspect of the terminal cisternae giving the cisternal membrane a beaded appearance (Figure 12). At other times, however, particularly in cross-sections, the cisternal membrane appears relatively smooth (Figure 11). Finally, as seen in face view (Figures 10, 12), the T tubules are continuous structures for as long as the triads remain in the plane of section.

3. ASSOCIATION OF THE T SYSTEM WITH THE FIBER SURFACE

As the T tubules approach the muscle surface they become dilated and enlarge from 3 to 5 times their normal diameter. In Figure 13 the innermost tubule measures 250 Å along a line parallel to the long axis of the muscle, whereas, just beneath the fiber surface the T tubule increases to 750 Å in diameter. Further, this portion of the T tubule appears to be running parallel to the muscle cell membrane, and its termination is complicated by pinocytotic
vesicles forming a subsarcolemmal careolae.

The opposite condition, or a termination of a T tubule that approaches the muscle surface at an angle of 90°, is shown in Figure 14. The T tubule in this micrograph also measures 750 Å, and its termination is again complicated by pinocytotic vesicles.

Figures 15 and 16 show terminations of T tubules which combine aspects of both approaches to the muscle surface. The T tubule in Figure 15 measures 1300 Å, and that in Figure 16 is 750 to 800 Å in diameter. Moreover, the T tubule in Figure 16 pursues a highly convoluted course in approaching the sarcolemma. It twice passes out of the plane of section and runs parallel with the sarcolemma before its termination at the fiber surface. Such convoluted terminations of T tubules and their association with pinocytotic vesicles at the fiber surface made it difficult to detect any direct connection of the T tubule wall with the surface membrane, or a direct continuity of the T tubule lumen with the extracellular space in conventional electron micrographs of the muscle (McCallister, 1970).

4. THE ENTRANCE OF LANTHANUM INTO THE T SYSTEM

That the lumen of the T system is, in fact, continuous with the extracellular space may be demonstrated by the entrance of lanthanum into the T tubules. The muscles were fixed in the same way as controls, only 1% lanthanum nitrate was added to the OsO₄ solution. Figure 17 is a low power transmission electron micrograph of such a preparation. The micrograph was taken of the most peripheral fiber in the muscle, and both its superficial and internal aspects can be seen. The lanthanum is seen on the superficial surface of the muscle fiber and adhering to bundles of fibers in the surrounding connective
tissue. On the other hand, the internal surface of the muscle fiber is relatively free of lanthanum. Only occasionally was lanthanum observed in the interstitial spaces beneath the most superficial cells in the muscle.

The intracellular distribution of lanthanum in the muscle is shown in Figure 18. In addition to surface deposition the lanthanum is found filling the pinocytotic vesicles, and in the space bounded by the membranes of the T system. However, the elements of the sarcoplasmic reticulum including the terminal cisternae, intermediate cisternae, longitudinal tubules, and fenestrated collar, are totally free of lanthanum indicative of their independence from the T system. See also Figures 19 and 20.

B. SCANNING ELECTRON MICROSCOPY

1. GENERAL MORPHOLOGY

With the scanning electron microscope the entire surface of whole muscles could be observed, and areas showing good preservation of membrane structure selected. One such area is shown in Figure 21. The surface of this muscle was composed of 18 fibers when viewed from its widest aspect. The fibers had a diameter of 40 to 50 µ, and extended the entire length of the muscle.

Two "spindle-like" structures were also observed in the study. One of these is indicated by the arrow in the center of Figure 21. This structure has a diameter of 120 µ, and could be followed for a distance of 0.45 mm on the muscle surface. As seen in polar view (Figure 22), the "Spindle-like" structure narrows to a diameter of 40 µ. This portion of the structure is then continuous with a connective tissue capsule which eventually disappears in between the connective tissue of the surrounding muscle fibers. This is also
shown in side view in Figure 23. To distinguish these structures from the surrounding muscle cells, the latter are here referred to as extrafusal muscle fibers.

2. THE MUSCLE SURFACE OF EXTRAFLUAL FIBERS

Figure 24 demonstrates the appearance of the extrafusal fibers as seen in scanning electron micrographs of relaxed muscles. Tortuous threadlike structures possibly representing nerves, capillaries, or large collagenous bundles of the perimysium, are seen on the surface of the muscle fibers. Beneath these larger structures, the individual muscle cells are ensheathed in a delicate wrapping of collagenous and reticular fibers of the endomysium. Where the connective tissue elements are sufficiently thin, as shown in the center of Figure 24, the regular striated pattern of the myofibrils is evident at the fiber surface. In these areas the A bands appear as lighter ridges, whereas, the I bands form darker furrows. In many cases a fine line corresponding to the Z disk (Figure 25) could be seen in the center of the "I furrows". So regular was the A-I banding pattern, that often vernier shifts of adjacent myofibrils could be detected on the outside of the muscle cells.

In high power scanning electron micrographs (Figure 25) highly organized parallel rows of openings or apertures corresponding to the level of the T system were seen on the muscle surface. The apertures were always found at the level of the I band in the vicinity of the Z disk. Occasionally more than one aperture was observed to correspond to a sarcomere. These secondary apertures were interpreted as openings of vesicles from subsarcolemmal caveolae.
Of the apertures corresponding to the T system, two types were noted. In cases where the T tubules terminated at an acute angle with reference to the plane of the sarcolemma (lower portion of Figure 25), the apertures appeared as slit-like openings on the fiber surface, making it difficult to estimate the internal diameter of the T tubules. However, in areas where the T tubules more closely approached the sarcolemma at an angle of 90°, the apertures appeared almost uniformly circular (upper portion of Figure 25). Further, since the diameter of a circular opening which is tilted away from the viewer is equal to its greatest diameter, irrespective of the direction of angle, the internal diameter of the circular apertures could be measured without the use of stereo pairs. In these areas the internal diameters of the T tubules varied from an estimated 1000 to 2000 Å.

It should be stressed that on no occasion were the tubular apertures uniformly distributed over the entire length of the muscle. In many instances the parallel rows of openings corresponding to the T system were discontinuous for distances of several microns, and in one muscle evidence of tubular apertures was totally lacking (McCallister and Hadek, 1970).
IV. RESULTS: THE SUBCELLULAR LOCALIZATION OF CALCIUM PYROANTIMONATE

A. PHYSIOLOGY OF THE CAFFEINE-INDUCED CONTRACTURE IN CALCIUM-FREE RINGER'S SOLUTION

Figure 44a, b, c shows the appearance of a standard potassium-induced contracture and calcium washout, followed by exposure of the muscle to 5 mM caffeine. When the 1°-Ringer's solution is changed to 1°-Ringer+50 mM K, (Figure 44a), the tension rapidly rises and reaches a peak at 12 to 16 seconds after initiation of the response. This is then followed by a spontaneous relaxation in which the tension rapidly returns to baseline. If the high potassium Ringer's solution is then followed by three changes of calcium-free Ringer, the potassium-induced contracture is subsequently inhibited.

Figure 44b shows the effect of calcium-free Ringer+50 mM K on the muscle. The muscle was previously exposed to calcium-free Ringer for 10 min before testing with the elevated potassium solution. The rectangular artifacts at the beginning and end of the response are caused by the changing of solutions in the tissue bath. As indicated, the muscle is totally unresponsive to 50 mM K in the presence of calcium-free Ringer's solution.

At this time, however, the muscle will respond to Ringer's solution containing caffeine. Figure 44c shows the response to calcium-free Ringer+5 mM caffeine. After exposure to caffeine the tension rises and is usually maintained until the caffeine Ringer is removed from the tissue bath. However, the development of tension during any portion of the caffeine response is a function of the number of contractures induced, as the response is decreased and eventually arrested upon repeated exposure of the muscle to Ringer's solution containing caffeine.
In Figure 45 the depletion of the caffeine-induced contracture in the presence of calcium-free Ringer’s solution is shown. Prior to depletion experiments, a standard potassium-induced contracture and calcium washout were conducted (Figure 45a, b). The last response in the series was obtained by increasing the gain of the oscillograph by approximately 50% (Figure 44k). This resulted in an increase of magnification from 7 to 15X. If a deflection of 1 mm or less (i.e., a muscle shortening of 1/15th of a mm) was recorded after exposure to caffeine at this magnification, the caffeine-induced contracture was considered arrested. The muscle was then removed from the tissue bath and fixed in a 1% solution of CsO₂, containing a 2% concentration of potassium pyroantimonate.

The caffeine-induced contracture was eliminated by exposing the muscle to calcium-free Ringer + 5 mM caffeine for 2 min periods, at regular 10 min intervals. After each caffeine-induced contracture, the Ringer’s solution containing caffeine was replaced with three changes of calcium-free Ringer. As shown (Figure 45c-k), when the muscle is repeatedly exposed to Ringer’s solution containing caffeine, both the amplitude and duration of the caffeine-induced contracture are decreased. Under these circumstances the muscle reaches peak tension after approximately 35 seconds. The tension then usually declines to the baseline before the end of the 2 min exposure to caffeine Ringer. Of the six muscles fixed for electron microscopy, an average of 7.5 responses or a total 15 min exposure to 5 mM caffeine was necessary to deplete the caffeine-induced contracture in calcium-free Ringer’s solution.

Figures 46 and 47 show the depletion of the caffeine-induced contracture in calcium-free Ringer’s solution containing calcium chelating agents. The procedure was identical to that previously described, except 5 mM EDTA (Figure
or 5 mM EGTA (Figure 47) were added to both the calcium-free Ringer's solution and Ringer's solution containing caffeine. As indicated, the caffeine-induced contracture was always much more rapidly arrested in the presence of calcium chelating agents. An average 3.25 contractures (6.5 min exposure to 5 mM caffeine) was necessary to deplete the caffeine response in the presence of EDTA, and 3.75 contractures (7.5 min exposure to 5 mM caffeine) was necessary to eliminate the caffeine-induced contracture in the presence of EGTA.

B. **ELECTRON MICROSCOPY OF MUSCLES FIXED IN 1% OsO₄ WITHOUT POTASSIUM PYROANTIMONATE**

Figures 26 and 27 show the appearance of muscles fixed in 1% OsO₄ without the addition of potassium pyroantimonate. The mitochondria and myofibrils are well preserved in such preparations, however, the latter are often found in the contracted state. Thus, the I bands are frequently absent and the A bands extend the entire distance between two Z lines (compare with Figures 7 and 12). When the plane of section passes between two myofibrils, the sarcoplasmic reticulum and T system are observed in face view (Figure 27). The various elements of the sarcoplasmic reticulum are easily identified; however, the entire structure possesses a more vesiculated appearance than seen with aldehyde fixation, which is characteristic of muscles fixed in OsO₄ (Porter and Palade, 1957; see also Peachey, 1965; Smith, 1966).

C. **MUSCLES FIXED IN 1% OsO₄, CONTAINING A 2% CONCENTRATION OF POTASSIUM PYROANTIMONATE**

When examined in the electron microscope these muscles revealed a localized pattern of electron opaque calcium pyroantimonate salts in the
sarcoplasmic reticulum and along the myofibrils. Figure 28 shows the intracellular distribution of the precipitate in contracted cells. Small granules of electron dense material were seen in the terminal cisternae of the sarcoplasmic reticulum, and closely applied to the luminal aspect of the cisternal membrane at its junction with the T system. Along the myofibrils the precipitate was most dense at the overlap of thick and thin filaments near the Z disk, and decreased as the M band was approached. A distinct absence of precipitate was seen in the longitudinal tubules of the sarcoplasmic reticulum, and in the intracellular spaces between the myofibrils.

In more relaxed sarcomeres a slightly different pattern of precipitate was observed. Pyroantimonate granules were still seen in the region of the terminal cisternae; however, the distribution of precipitate on the myofibrils was much more distinct. In these fibers a dense line of precipitate was observed in the I band on the boundary of the A-I junction (Figure 29). The A band contained precipitate, except for the M disk which was relatively free of precipitate compared to the rest of the sarcomere.

Figure 30 demonstrates that when the I bands reach a length of 1.2 μ or greater, the dense line of precipitate located near the A-I junction disappears. It thus became evident that the intensity of the I band precipitate is directly related to the state of contraction in which the muscle is fixed. This was subsequently confirmed in electron micrographs such as that shown in Figure 31. This micrograph shows two muscle cells: one relaxed, and the other contracted. In the relaxed cell at the top of the picture the I bands measure 1.2 to 1.3 μ; in the lower cell the I bands are less than 0.5 μ in length. It is evident that while the I band precipitate is almost totally lacking in sarcomeres of the relaxed fiber, a reasonably heavy deposition of precipitate is seen near the
A-I junction in the contracted cell (bottom).

Figures 31 and 32 show that the extracellular space of the muscles contained only very little precipitate; although, occasionally some precipitate was observed near the sarcolemma.

D. MUSCLES EXPOSED TO CALCIUM-FREE RINGER'S SOLUTION, AND RINGER'S SOLUTION CONTAINING CALCIUM CHELATING AGENTS

The intracellular distribution and pattern of calcium pyroantimonate was not appreciably different in muscles exposed for 10 min and 1 hr to calcium-free solutions and solutions containing calcium chelating agents. Figures 33, 34, and 35 are electron micrographs of muscles exposed 1 hr to calcium-free Ringer's solution, and calcium-free Ringer's solution containing 5 mM EDTA and 5 mM EGTA respectively.

E. MUSCLES IN WHICH THE CAFFEINE-INDUCED CONTRACTURE WAS ARRESTED IN CALCIUM-FREE RINGER'S SOLUTION

In these muscles considerable deposits of pyroantimonate salts were observed in the sarcoplasmic reticulum and along the actin and myosin filaments. Further, the precipitation occurred at the same characteristic loci as that seen in control muscles. A relatively high concentration of precipitate was usually observed in the I band near the boundary of the A-I junction (Figures 36, 37, and 38). The A band also contained some precipitate which was lowest in the region of the M disk. Figures 37 and 38 show that the precipitate in the sarcoplasmic reticulum was concentrated in the terminal cisternae at its junction with the T system, and totally absent from the longitudinal tubules.
Occasional granules of precipitate were sometimes observed in the extracellular space of these muscles, very near the sarcolemma of the fibers (Figures 36, and 39).

F. MUSCLES IN WHICH THE CAFFEINE-INDUCED CONTRACTURE WAS ARRESTED IN CALCIUM-FREE RINGER'S SOLUTION CONTAINING 5 mM EDTA AND 5 mM EGTA

When the caffeine-induced contracture was depleted in the presence of 5 mM EDTA (Figures 40, and 41) or 5 mM EGTA (Figures 42, and 43) a striking decrease in the concentration of precipitate was observed. The precipitate along the myofilaments was almost entirely eliminated (Figures 40 and 42). In addition, the amount of precipitate in the sarcoplasmic reticulum had greatly diminished. Only a few granules of pyroantimonate salts were observed in the terminal cisternae of these fibers, closely applied to the membranes of the T system (Figures 41 and 43).
A. GENERAL MORPHOLOGY

Much of the pertinent physiological information concerning muscle contraction has been derived from studies on frog skeletal muscle. In order to provide morphological data for physiological theories, the sartorius muscle of the frog has been extensively studied with the electron microscope. However, the extensor longus digiti IV muscle which is also widely used for physiological experiments, has not been studied thus far in sufficient depth. Thus, one of the goals of this investigation has been to describe the general morphology of this muscle.

In almost every respect the ultrastructure of the extensor longus digiti IV was comparable to that of the sartorius. The banding pattern, filament lattice, and distribution of the sarcoplasmic reticulum and T system along the myofibrils was identical. On the other hand, occasional branching of the T system previously reported for the sartorius (Peachey, 1965; Peachey and Schild, 1963), was not confirmed in the extensor longus digiti IV. Neither were slow fibers seen, which lack a T system and have a poorly developed sarcoplasmic reticulum. However, since light microscopic studies indicate that as little as 6% of the cells in the extensor muscle may be considered slow fibers (Gray, 1968), their absence in electron micrographs was not surprising.

B. THE T SYSTEM

1. ASSOCIATION WITH THE FIBER SURFACE

Since the local activation experiments of Huxley and Taylor (1953), a great deal of evidence has accumulated suggesting a continuity between the
lumen of the T system and the extracellular space in amphibian muscle. As noted in the introduction, different extracellular markers have been localized to the transverse tubular compartment (Eisenberg and Eisenberg, 1968; Endo, 1964; Hill, 1964; Huxley, 1964; Page, 1964). In addition, physiological experiments indicate that the membrane capacitance of frog muscle is directly related to the connection of the T system with the fiber surface. The capacitance of fast muscle fibers of the frog has been estimated at 5 \( \mu F/cm^2 \) by Katz (1948) and 8 \( \mu F/cm^2 \) by Fatt and Katz (1951). However, slow fibers which lack triads (Peachey and Huxley, 1962) have a membrane capacitance of only 2.5 \( \mu F/cm^2 \). Also, Gage and Eisenberg (1969a) have shown that if fast fibers are "treated" with glycerol which causes selective disruption of the T system (Howell and Jenden, 1967), the capacitance of the muscle cells is reduced to approximately 2.4 \( \mu F/cm^2 \), a value very similar to that for slow fibers.

Since other electrical properties of muscle, including the early after-potential, late afterpotential, and creep potential, have been attributed to the transverse tubular system (Gage and Eisenberg, 1969a; 1969b), it has become a working hypothesis that the T system is open and accessible to ions in the extracellular fluid. Nevertheless, the difficulty of establishing a connection between the T system and the surface membrane in frog skeletal muscle is well documented in the literature (Bianchi, 1968; Ebashi and Endo, 1968; Eisenberg and Eisenberg, 1968; Franzini-Armstrong, 1970; Smith, 1966).

In the present study it was shown that the T system becomes dilated near the fiber surface. In the subsarcolemmal region these enlarged terminations are exceedingly tortuous. Several instances were observed where the T tubules appeared to be running parallel to the plasmalemma of the muscle fibers. Although, some terminations could be followed to within 200 \( \AA \) of the muscle
membrane, still no direct communication between the wall of the T tubule and the sarcolemma was observed.

However, lanthanum was observed to enter the lumen of the T system. The conclusions reached on the strength of lanthanum staining were also verified by scanning electron microscopy. With this technique highly organized parallel rows of apertures at the level of the T system were seen on the most superficial fibers in the muscle. Occasional openings corresponding to large caveolae seen filled with lanthanum were also noted. However, random openings in areas which did not show lanthanum penetration were never observed in the scanning electron microscope. Thus, the results of both techniques complement each other and add new morphological evidence for a direct connection between the T system and the surface membrane.

2. FACTORS INFLUENCING THE CONTINUITY OF THE T SYSTEM WITH THE EXTRACELLULAR SPACE IN CONVENTIONAL ELECTRON MICROGRAPHS

A number of factors may contribute to the paucity of open T tubules seen in conventional electron micrographs of frog skeletal muscle. First, as noted in the results, when the T tubule approaches the sarcolemma its relationship to the surface membrane is complicated by irregular vesicles forming caveolae. A similar situation has been reported for the sartorius of the frog (Peachey, 1965), for rat skeletal muscle (Walker and Schrodt, 1965; 1966), and for skeletal muscle of the guinea-pig (Rayns et al., 1963). In fact, it appears that such complicated terminations of T tubules may be a general feature in skeletal muscles of most higher vertebrates (Franzini-Armstrong, 1970).

Secondly, some evidence suggests that fixation may affect the communication of the T system with the extracellular space in frog skeletal muscle.
It has been demonstrated that fixation in glutaraldehyde prevents the entrance of ferritin into the majority of the T tubules in frog sartorius muscles (Hurley, 1964; Page). However, as noted by Smith (1966), aldehyde fixation still preserves the continuity between the T system and the surface membrane in a number of muscle cells. This investigator successfully employed glutaraldehyde to demonstrate the continuity of the T system with the extracellular space in the flight muscle of the dragon fly (Smith, 1966), as have Franzini-Armstrong and Porter (1964) in the body muscles of the Black Molly fish, and Jasper (1967) in the body muscles of the lamprey. In addition, this study has shown that lanthanum can enter some of the T tubules even after fixation for 1 hour in glutaraldehyde. However, since lanthanum was only observed in the most peripheral fibers, it may be that the T system in these cells is better preserved, as the fixative would reach these areas first before coming in contact with the deeper fibers in the muscle.

Thirdly, physiological experiments indicate that only a portion of the T tubules may actually be accessible to ions in the extracellular space. In the local stimulation experiments of Hurley and Taylor (1958), microdepolarizing currents applied to the cell membrane of frog sartorius fibers were only capable of inducing a localized contraction when the pipette was placed opposite certain sensitive areas around the periphery of the muscle cells. Although, the sensitive areas were always found at the level of the Z disk, they did not correspond to the spaces between the myofibrils. The sensitive areas were separated by an average distance of 5 μm. Since the myofibrils have an average diameter of 0.5 to 1.0 μm, the data suggests that at any one time only a portion of the T tubules remain open.
Finally, during the writing of this dissertation Franzini-Armstrong (1970) has suggested still another reason why the aperture of the T system is so difficult to demonstrate in amphibian muscle. She recently published an electron micrograph of the sartorius of the frog showing that the last portion of the T system narrows and runs parallel to the sarcolemma in such a manner that the wall and mouth of the T tubule are included in the plane of section at its exit from the muscle fiber, making direct visualization of the opening extremely difficult (Franzini-Armstrong, 1970). Although it is clear from her elegant picture that the T tubule in this micrograph does indeed narrow before its termination at the fiber surface, it is difficult to determine whether this accurately depicts the condition in vivo, or represents a T tubule in the process of closing as suggested by physiological experiments (Huxley and Taylor, 1958) or as the result of fixation (Huxley, 1964; Page, 1964).

As mentioned, when observed in the scanning electron microscope, the openings corresponding to the T system did not occur uniformly throughout the muscle. However, with this technique it was possible to observe the entire surface of whole muscles. Hence, small areas showing morphologically open T tubules could still be determined. Obviously, the likelihood of chancing upon such areas with scanning electron microscopy is far greater than in thin sections randomly cut from small tissue pieces and viewed with the transmission electron microscope.

C. THE JUNCTION BETWEEN THE T SYSTEM AND THE SARCOPLASMIC RETICULUM

If frog skeletal muscle is bathed in Ringer's solution made hypertonic with sucrose, the volume of fiber water decreases as if the cells were perfect osmometers (Dydymska and Wilkie, 1963). Cell volume, however, does not
decrease to the extent predicted by the amount of water lost from the muscle (Blinks, 1965). To explain this inconsistency it was suggested that sucrose might enter some compartment within the muscle (Johnson and Simonds, 1962). As the osmolarity of the Ringer's solution was increased, the sucrose compartment was thought to become increasingly dilated.

Examination in the electron microscope demonstrated that the sarcoplasmic reticulum was consistently dilated in muscles exposed to hypertonic sucrose solutions (Huxley, et al., 1963). It was postulated that the reticulum was extracellular in nature, and in continuity with the lumen of the T system (Birks, 1965). The most probable site of continuity was thought to be the cross-bridges located at the junction of the T tubules with the terminal cisternae. These were described by Birks (1965) as possible semi-permeable pores providing for the exchange of fluid between the extracellular space and the sarcoplasmic reticulum.

More detailed studies with the electron microscope, however, have yet to reveal the presence of pores at the triad junction (Franzini-Armstrong, 1963; 1970; Kelly, 1969; Kelly and Cahill, 1963; Peachey, 1965). In general the membrane of the sarcoplasmic reticulum is separated from the T tubules by the distance of 120 to 200 Å. At regular intervals, however, the membrane of the reticulum is scalloped forming projections (the cross-bridges of Birks, 1965) which come into close apposition with the T tubules. The projections have been referred to as "dimples" by Kelly (1969) and as "SR feet" by Franzini-Armstrong (1970). Whether the projections actually contain pores linking the lumen of the reticulum to that of the T tubules is dependent on the degree of continuity between the projections and the wall of the T tubules. Unfortunately, the depth of the projections varies considerably with different preparations.
Both direct contacts, and remaining gaps of 50 to 80 Å have been reported in the literature (Franzini-Armstrong, 1970; Kelly, 1969).

In this study the extracellular space was made opaque using colloidal lanthanum. In these preparations electron dense material was observed in pinocytic vesicles and in the lumen of the T system. However, the sarcoplasmic reticulum was entirely free of lanthanum. Nor was lanthanum observed in any other compartment within the muscle. Since Revel and Farnovsky (1967) have previously shown that lanthanum can penetrate spaces as small as 10 to 20 Å, these results cast further doubt on the presence of pores in the cross-bridges.

The results are significant in terms of the current mechanisms used to explain electrical coupling between the T system and the sarcoplasmic reticulum. These include (1) low-resistance pathways for direct flow of ions between the sarcoplasmic reticulum and the T system, and (2) capacitative coupling of the membranes (Eisenberg and Gage, 1969). In the absence of pores (present data), or close apposition of the junctional membranes (Franzini-Armstrong, 1970) both hypotheses appear unlikely. Alternatively, the data does not exclude the possibility of chemical coupling brought about by the release of a substance from the T system at the time of activation which could increase the permeability of the sarcoplasmic reticulum to calcium ions.

D. SPINDLE-LIKE STRUCTURES

According to the literature the extensor longus digiti IV muscle of the frog is richly supplied with muscle spindles of several different types. Single spindles, tandem spindles, and spindle systems have all been reported (Barker and Cope, 1962; Gray, 1957; Jahn, 1959). The individual spindle units may consist of 1 to 8 intrafusal fibers; however, 3 to 5 fibers are the most
common (Barker and Cope, 1962; Gray, 1957). In the equatorial region the intrafusal fibers are encapsulated in several lamellae of connective tissue (Barker and Cope, 1962; Gray, 1957). These encapsulated regions extend for 1 mm or less within the muscle (Barker and Cope, 1962).

In the present study two spindle-like structures were observed with the scanning electron microscope. The one described had a diameter of 120 μ in the encapsulated region, which could be followed for 450 μ on the muscle surface. The size of these structures, the fact that they were encapsulated, and their relationship to the extrafusal muscle fibers suggest they represent single muscle spindles. However, they could be encapsulated units of either tandem spindles or larger spindle systems as reported by Barker and Cope (1962).

3. THE LOCALIZATION OF CALCIUM BY POTASSIUM PYROANTIMONATE

1. INTRACELLULAR PRECIPITATE

When the caffeine-induced contracture was arrested in Ringer's solution containing EDTA or EGTA, a striking decrease was observed in the intracellular concentration of precipitate in muscles fixed in OsO₄ containing potassium pyroantimonate. Since EGTA is specific for calcium even in the presence of other divalent cations (Schwarzenbach, et al., 1957; Ebashi, et al., 1960; Sillen and Martoll, 1964; Ebashi and Endo, 1963; Gillis, 1970), these observations support those of Legato and Langer (1969) who were the first to use pyroantimonate for the subcellular localization of calcium in muscle.

In addition, the intracellular loci of pyroantimonate salts observed in the present study is in good agreement with the known distribution of calcium stores in skeletal muscle. The localization of pyroantimonate was essentially in the terminal cisternae of the sarcoplasmic reticulum. This result
corresponds with the deposits of oxalate observed in the terminal cisternae of the sarcoplasmic reticulum of skinned muscle fibers (Constantin, et al., 1965; Poldolsky, 1968; Poldolsky et al., 1970), and in the vesicles of fragmented sarcoplasmic reticulum isolated from skeletal muscle (Baskin and Deamer, 1969).

However, Hasselbach (1964) and Pease et al. (1965) have also observed oxalate precipitates in the longitudinal tubules. Nevertheless, the majority of studies only report the presence of calcium oxalate in the terminal cisternae (Constantin, et al., 1965; Poldolsky, 1968; Poldolsky, et al., 1970), and in the present investigation the only pyroantimonate precipitate in the sarcoplasmic reticulum was localized to the terminal cisternae. As suggested by Legato and Langer (1969) this would fit with the idea that the longitudinal portion of the reticulum acts as a pump, removing calcium from the actin and myosin filaments and shunting it to the terminal cisternae where it is stored in an inactive form. Thus, the calcium in the longitudinal tubules is not present for prolonged periods of time or in sufficient amounts to form electron opaque salts.

The presence of pyroantimonate granules in the A band portion of the myofibrils was not surprising. It has been demonstrated that the concentration of $^{45}$Ca in the A band increases after stimulation of the muscle fiber (Winegrad 1968; 1970). However, much of the A band calcium is apparently unrelated to contraction, as $^{45}$Ca was also observed in the region of the H zone where the thick and thin filaments do not overlap (Winegrad, 1968; 1970).

Moreover, the autoradiographic studies of Winegrad (1965; 1968; 1970) also support the results obtained here indicating that the region of the M band is relatively free of calcium compared to the rest of the sarcomere. In muscles quickly frozen during a maintained tetanus, a declining tetanus, or during the period immediately following a tetanus or contracture, the myofibrilar concen-
tration of $^{45}$Ca was always lowest at the M band (Winegrad, 1965; 1968; 1970). It has been suggested that the cross-bridges located on the myosin filaments in the M band may prevent the diffusion of calcium ions from one Z line to the next (Legato and Langer, 1969).

The dense line of calcium pyroantimonate in the I band, and its absence in sarcomeres with I bands longer than 1.2 μ, suggests that this locus of intracellular calcium may play an important role in the contractile process. Recent evidence indicates that the ability of isolated acto-myosin preparations to contract is dependent on the calcium sensitivity of troponin molecules located along the actin filaments (Ebashi and Endo, 1968; Endo, et al., 1966; Ohtsuki, et al., 1967). Although on morphological grounds alone it is difficult to demonstrate any special regions of calcium binding in the actin filaments (Legato and Langer, 1969), nevertheless, certain physiological data are in agreement with the present results.

Winegrad (1970) has observed that during tetanus the majority of myofibrillar calcium is localized to the region of the thin filaments forming a peak in the I band adjacent to the A-I junction. In addition, the amount of calcium in the I band varies with the amount of tension being developed. During a declining tetanus or in muscles at rest the calcium in the I band is markedly reduced, even below that present in the A band.

The experiments of Gillis (1970) in which calcium was applied by micro-iontophoresis at different areas along the sarcomeres of glycerinated crab myofibrils, also support the existence of calcium sensitive regions on the myofilaments. Using this technique the threshold of iontophoretic current needed to produce contraction was always lowest when the calcium was applied at the level of the A-I junction. Thus, the dense localization of calcium
pyroantimonate observed near the A-I junction in our muscles, may actually reflect the increased ability of the actin filaments in this portion of the sarcomere to actively bind calcium ion. As the precipitate was observed in contracted cells and lacking in cells with I bands broader than 1.2 µ, it is further suggested that this region of the thin filaments corresponds to the intracellular site of calcium activation during contraction.

2. **EXTRACELLULAR PRECIPITATE**

Large plaques of precipitate have been reported in the extracellular space and filling the T tubules of muscles fixed in solutions containing potassium pyroantimonate. These large granules of precipitate have been interpreted as sodium pyroantimonate, and have been thought to reflect the high known extracellular concentration of sodium ions in muscle (Legato and Langer, 1969; zadunaisky, 1966). However, in a statistical analysis Eisenberg and Eisenberg (1968) reported that only 1 out of 10 sartorius muscles contained precipitate in the sarcotubules, and then only 20% of the T tubules were filled. In addition, Legato and Langer (1969) observed that in general the T system of dog papillary muscle lacked precipitate, suggesting that the electron dense material was mechanically washed out during preparation of the tissue for electron microscopy.

In the present experiments the extracellular space and the lumen of the T system were almost totally free of precipitate. Only a small amount of precipitate was observed in association with the fiber surface. Thus, the removal of the extracellular precipitate during dehydration and embedding is entirely possible. However, it should be noted that 111.8 mM choline chloride was used in place of sodium chloride in the Ringer's solution. This was
necessary to eliminate the spontaneous contractures which occur when a muscle is transferred to calcium-free Ringer's solution (Frank, 1960; 1962). Therefore, it may be that the extracellular compartment was free of sodium at the time of fixation.

3. PRECIPITATE IN MUSCLES EXPOSED TO CALCIUM-FREE SOLUTIONS AND SOLUTIONS CONTAINING CALCIUM CHELATING AGENTS

Studies on the efflux of radiocalcium from frog skeletal muscle indicate that calcium is contained in a three compartment system. The first compartment is believed to represent calcium in the extracellular space; whereas, calcium in the second compartment is thought to correspond to the sarcoplasmic reticulum and exchangeable calcium bound to the myofilaments during contraction. Calcium in the third compartment is almost non-exchangeable, and may represent many intracellular stores (Curtis, 1970; Bianchi, 1968; Gilbert and Fenn, 1957; Shanes and Bianchi, 1969).

The time constant for the desaturation of \(^{45}\text{Ca}\) from the first compartment, or the time necessary for the radioactivity of the muscle to reach \(1/e\) of the amount originally present at zero time (Bianchi, 1968), has been estimated at 3 minutes by Shanes and Bianchi (1959) and 13 minutes by Curtis (1970). Thus, calcium in the extracellular space is rapidly exchanged, and it has been suggested that calcium lost from this compartment accounts for the abrupt change in membrane function which takes place when a muscle is transferred to calcium-free Ringer's solution (Curtis, 1970; Frank, 1960; 1962; 1965).

Time constants for the efflux of \(^{45}\text{Ca}\) from the second compartment have been calculated at 300 minutes (Curtis 1970; Gilbert and Fenn, 1957), 438 minutes (Bianchi, 1968), and 500 minutes (Shanes and Bianchi, 1959). Further,
in frog skeletal muscle efflux from the second compartment is not significantly different in muscles exposed to normal Ringer's solution, or Ringer's solution containing calcium chelating agents (Curtis, 1970). Thus, using the intermediate value of $t_c = 438$ minutes, one can predict that only 13% of the calcium in the second compartment would be lost after a 1 hour soak in zero-calcium Ringer's solution.

In the present study the subcellular distribution of calcium pyroantimonate was not greatly decreased in muscles exposed to calcium-free Ringer's solution, or Ringer's solution, or Ringer's solution containing 5 mM EDTA or 5 mM EGTA for 10 min to 1 hour. However, it is unlikely that such a small decrease in the intracellular calcium as predicted by efflux studies would have been detected in the present investigation. It appears that the intracellular stores of calcium in skeletal muscle are much less affected by conditions which deplete calcium in cardiac muscle (Legato and Langer, 1969).

4. **PRECIPITATE IN MUSCLES IN WHICH THE CAFFEINE-INDUCED CONTRACTURE WAS ARRESTED**

According to the literature the contractility of frog muscle induced by the action of caffeine is a function of the concentration used. At a concentration of 1 mM, caffeine acts as a twitch potentiator, reducing the concentration of potassium necessary to induce a contracture (Sandow, et al., 1964). At concentrations of 2.5 mM caffeine can cause either a graded contracture or prolong a potassium contracture which disappears when caffeine is removed from the bathing solution (Axelsson and Thesleff, 1968; Bianchi, 1961; Frank, 1960). At concentrations greater than 5 mM caffeine causes irreversible contraction or rigor (Bianchi, 1968).
That caffeine can induce a contracture in both polarized and depolarized muscles (Axelsson and Thesleff, 1958), has suggested that the mechanism of caffeine-induced release of calcium from the sarcoplasmic reticulum bears an intimate relationship to that of calcium release under physiological conditions (Ebashi and Endo, 1968). Although numerous studies have been conducted to describe this mechanism, there is still no general agreement as to whether caffeine brings about the release of internal calcium stores through its affect on (1) the transverse tubular system (Lüttgau and Oetliker, 1968), (2) coupling between the T system and the sarcoplasmic reticulum (Fairhurst and Hasselbach, 1970), or by (3) inhibition of calcium-reuptake by the sarcoplasmic reticulum (Weber, 1966).

Several lines of evidence indicate that the T system may play an important role in the production of the caffeine-induced contracture. Gordon and Grodt (1970) showed that frog skeletal muscle bathed in hypertonic solutions, known to dialate the T system (Huxley, et al., 1963; Freygang, et al., 1967) and interrupt excitation-contraction coupling (Hodgkin and Horowicz, 1957), generate only 10% of the normal tension when exposed to 5 mM caffeine. Further, Lüttgau and Oetliker (1968) demonstrated that the increase and fall in tension after the addition or removal of caffeine takes place with a half-time to 2 to 4 seconds. This was thought to be too slow if caffeine acted directly on the cell membrane, but probably too fast to affect the sarcoplasmic reticulum. It was suggested, therefore, that caffeine most probably affects the membranes of the transverse tubular system. However, in this connection it is important to note that caffeine can induce a diminished contracture in "glycerol-treated" muscles in which only 2% of the T tubules remain connected to the extracellular space (Eisenberg and Eisenberg, 1968). Thus, although contact of the tubular
membrane with the sarcoplasmic reticulum may be necessary for the caffeine response, the action of caffeine is not totally dependent on the continuity between the T system and the muscle cell membrane.

The direct action of caffeine on the sarcoplasmic reticulum, and coupling between the sarcoplasmic reticulum and the T system is not simple. That the sarcoplasmic reticulum is necessary for the action of caffeine has been demonstrated, since glycerinated fibers in which the reticulum is lacking are not modified by caffeine unless relaxing factor is present (Nagai and Uchida, 1960). However, still some studies show that caffeine inhibits calcium re-uptake of isolated sarcoplasmic reticulum (Herz and Weber, 1965; Weber, 1968; Weber and Herz, 1968), while others report no evidence of decreased calcium binding capacity and relaxing activity in the presence of caffeine (Carvalho, 1966; Takeda, 1965).

It has also been shown that the caffeine-induced release of calcium from the "heavy" fractions of centrifuged muscle homogenates is stronger than from "lighter" ones (Herz and Weber, 1965; Weber, 1968). And since it seems likely that the heavy fraction contains a great deal of intact triads, and the lighter fraction more incomplete triads, it has been suggested that complete triads are necessary for the action of caffeine (Lüttgau and Oetliker, 1968).

This hypothesis has recently gained further support from the work of Fairhurst and Hasselbach (1970). These investigations compared the calcium efflux of heavy and light sarcotubular fractions isolated from rabbit skeletal muscle. Efflux from the heavy sarcotubular fraction was stimulated by caffeine, whereas, the lighter fraction was much less sensitive. They also showed that the action of caffeine on both fractions was completely blocked by 5 mM magnesium. Since muscle contains about 8 mM magnesium, the level of free mag-
nesium in the myoplasm would probably be great enough to antagonize the action of caffeine. Thus, they proposed that in vivo caffeine acts at the junction of the transverse tubules and the terminal cisternae, where the concentration of external magnesium is much lower (Fairhurst and Hasselbach, 1970).

The fact that caffeine can induce unmodified contractures in frog skeletal muscle inhibited by exposure to calcium-free solutions was first described by Frank (1960). In a later paper he has shown that after repeated exposure to caffeine, the response gradually fails and is finally lost (Frank, 1962). At this stage the failure can only be relieved by the addition of calcium or strontium (Frank, 1962). Based on Bianchi's studies of caffeine-induced radiocalcium efflux in skeletal muscle of the frog (1961), and Herz and Weber's hypothesis that caffeine inhibits calcium-reuptake of the sarcoplasmic reticulum (1965), it was assumed that the loss of the caffeine response was due to the depletion of the calcium stores available for contraction (Frank, 1962; Edwards, et al., 1966). However, as pointed out by A. F. Huxley (1964) and others (Ebashi and Endo, 1968), that the calcium stores had been depleted has not been proven.

In the present study considerable deposits of pyroantimonate salts were still present in muscles in which the caffeine-induced contracture was arrested in calcium-free Ringer's solution. Moreover, the intracellular distribution of the precipitate was identical to that of the controls. Of particular interest was the finding of calcium pyroantimonate in the terminal cisternae. If caffeine acted directly on the sarcoplasmic reticulum, it would be expected that the cisternae would have been depleted of calcium when the caffeine response failed. Thus, the action of caffeine at some intermediary site such as the T system or the junction between the the T system and the sarcoplasmic reticulum...
Further, the results obtained in the dissertational studies suggest that caffeine increases the permeability of the muscle membrane. An average 45 minute exposure to Ringer's solution containing EGTA was necessary to eliminate the caffeine response. Although these muscles showed a significant diminution of precipitate, control muscles exposed for 60 minutes to Ringer's solution containing EGTA, but without the addition of caffeine, still retained abundant precipitate. Thus, caffeine apparently altered the membrane in such a manner that the large EGTA molecule could more easily enter the cell. Such an interpretation would also explain the physiological data, as the caffeine response in the presence of EGTA was eliminated in approximately 1/2 the time necessary to arrest the caffeine-induced contracture in zero-calcium Ringer's solution without calcium chelating agents.

The results are in agreement with those which show that conditions having a stabilizing action on the permeability of excitable membranes (Frankenhaeuser and Hodgkin, 1957; Frankenhaeuser and Lannergren, 1967; Luttgau, 1963), also decrease the responsiveness of the muscle to caffeine (Luttgau and Oetliker, 1968; Fairhurst and Hasselbach, 1970). Further, if caffeine acted at the wall of the T system or at its junction with the sarcoplasmic reticulum, the mechanical failure in calcium-free solutions would not be due to the depletion of calcium from the sarcoplasmic reticulum, but as suggested by Ebashi and Endo (1968) it could also be caused by some type of inactivation of depolarization-Ca-release coupling, which develops very slowly and is only relieved by the presence of calcium or strontium in the extracellular space.
A combined ultrastructural and histochemical study were conducted on the extensor longus digiti IV muscle of the frog to (A) establish whether a direct continuity exists between the T system and the surface membrane; and (B) to observe and study the localization of calcium pyroantimonate in skeletal muscle cells.

(A) Transmission and scanning electron microscopy, as well as lanthanum nitrate, were employed to demonstrate the continuity of the T system with the plasmalemma of the muscle fibers.

Transmission electron microscopy revealed the presence of T tubules near the sarcolemma and their considerable dilation, 3 to 5 times their usual diameter, in the subsarcolemmal region. These enlarged terminations often produced complex images in association with pinocytotic vesicles forming subsarcolemmal caveolae. Due to the complicated termination of the T tubules, their continuity with the extracellular space was never clearly evident in conventional electron micrographs of the muscle.

That the lumen of the T system is continuous with the extracellular space was demonstrated by the entrance of lanthanum into the T tubules. In addition to surface deposition, lanthanum was found filling pinocytotic vesicles and in the space bounded by the membranes of the T system. However, the elements of the sarcoplasmic reticulum, including the terminal cisternae, intermediate cisternae, longitudinal tubules, and fenestrated collar, were totally free of lanthanum despite their close association with the T system and the vesicles at the fiber surface.

The results of lanthanum staining were also verified by scanning electron
microscopy. With this technique parallel rows of circular apertures corresponding to the level of the T system were seen on the surface of the muscle fibers. The apertures measured approximately 1000 to 2000 Å in diameter, and were always located at the level of the I band in the vicinity of the Z disk. Occasional openings corresponding to large lanthanum filled caveolae were also noted. However, random openings in areas which did not show lanthanum penetration were never observed. Thus, the results of both techniques complement each other and add new morphological evidence for a direct connection between the T system and the surface membrane.

A number of factors may contribute to the paucity of open T tubules seen in conventional electron micrographs. These include (1) the complicated termination of the T tubules at the fiber surface; (2) evidence that the majority of T tubules close with fixation (Huxley, 1964; Page, 1964); and (3) physiological evidence that at any one time only a portion of the T tubules may actually remain open (Huxley and Taylor, 1958).

(B) The intracellular precipitation of calcium was effected by fixation of muscles in 1% OsO4 containing 2% potassium pyroantimonate (K2H2Sb2O7·4H2O). In these preparations granules of electron dense pyroantimonate salts were observed in the terminal cisternae of the sarcoplasmic reticulum, closely applied to the cisternal membrane at its junction with the T system. Along the myofibrils the precipitate was most dense in the I band just prior to the A-I junction. Further, the intensity of the I band precipitate appeared to be directly related to the state of contraction in which the muscle was fixed. In cells with I bands longer than 1.2 µ, the dense line of precipitate usually located near the A-I junction was no longer observed. Pyroantimonate granules were
also present in the A band, while, the M disk was relatively free of precipitate. The intracellular precipitate was not significantly decreased in muscles exposed to calcium-free solutions or calcium-free solutions containing 5 mM EDTA or 5 mM EGTA for periods of 10 minutes and 1 hour. Neither was the precipitate decreased in muscles in which the caffeine-induced contracture was depleted in the presence of calcium free Ringer’s solution. However, depletion of the caffeine-induced contracture in Ringer’s solution containing calcium chelating agents was accompanied by a striking decrease of precipitate in all areas. The precipitate along the myofilaments was almost entirely eliminated, and only a few granules of pyroantimonate salts remained in the terminal cisternae of the sarcoplasmic reticulum.

The observed intracellular loci of pyroantimonate salts is in good agreement with the known distribution of calcium in skeletal muscle. Further, the reproducible pattern of I band precipitate localized near the A-I junction and its relation to sarcomere length suggest that this pool of calcium may represent the intracellular site of calcium activation during contraction. The distribution of precipitate in muscles exposed to Ringer’s solution containing caffeine indicate that this alkaloid acts at some site other than the sarcoplasmic reticulum, such as the T system of the junction between the T system and the sarcoplasmic reticulum.
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Figure 1. Color micrograph showing a portion of the foot and lower leg of the frog; the skin has been removed and the extensor longus digiti IV muscle (large arrow) is dissected free except for its tendons of origin and insertion (small arrows). As indicated on the mm rule (mm), the muscle measures 2.4 cm in length, and has a cross sectional diameter of less than 1 mm. X 1.65.

Figure 2. Light micrograph of a longitudinal section from the extensor longus digiti IV of the frog showing a portion of 2 myofibers. The myofibers measure 42 μ in diameter and are composed of numerous myofibrils oriented parallel to the long axis of the cells. Within the myofibrils regular cross-striations forming lighter I bands and darker A bands are evident. A dense line, the Z disk, is seen in the center of the I band. X 1,300.
PIATE II

EXPLANATION OF FIGURE

Figure 3. High power transmission electron micrograph taken from a transverse section showing the overlap of thick and thin filaments in the A band. The large myosin filaments (130 A) are surrounded by an orbit of six actin filaments approximately 60 A in diameter. M, mitochondrion. X 131,000.
Figure 4. Electron micrograph showing the orderly arrangement of actin filaments as they approach the Z disk (white I--Z). Note the interconnection of actin filaments with Z filaments at the level of the Z disk (black Z). black I, I band; white A, A band. X 131,000.
PLATE IV

EXPLANATION OF FIGURE

Figure 5. Transverse section of the extensor longus digiti IV muscle. A, A band; I, I band; Z, Z disk; Tt, T tubule. X 60,300.
PLATE V

EXPLANATION OF FIGURE

Figure 6. Longitudinal section of a relaxed fiber in the region of the nucleus (N). M, mitochondrion; g, glycogen. X 25,300.
PLATE VI

EXPLANATION OF FIGURE

Figure 7. Low power electron micrograph of a slightly contracted cell. A, A band; black Z, Z disk. X 16,000.
Figure 8. High power electron micrograph of a slightly contracted cell. N nucleus; white m, mitochondrion; black m, M line. X 46,700.
PLATE VIII

EXPLANATION OF FIGURE

Figure 9. Electron micrograph of a fiber fixed in a state of contracture. Note the thick myosin filaments in the A band (A) extend the entire distance between two Z lines (black Z). \( \times 34,000 \).
Figure 10. Electron micrograph showing the convergence of the longitudinal tubules (LT) to form the terminal cisternae (TC) of the sarcoplasmic reticulum. TT, T tubule. X 58,500.
Figure 11. Longitudinal section of the extensor longus digiti IV muscle showing the various elements of the sarcoplasmic reticulum. A, A band; H, H band; Tt, T tubule; Tc, terminal cisternae; Ic, intermediate cisternae; Lt, longitudinal tubule; fc, fenestrated collar. X 50,000.
Figure 12. Longitudinal section of the extensor longus digiti IV muscle. The plane of section has passed between two myofibrils and the sarcoplasmic reticulum is seen in face view. Note the convergence of the longitudinal tubules (Lt) to form the fenestrated collar (Fc). Tt, T tubule; Tc, terminal cisternae. X 58,700.
Figure 13. Electron micrograph showing the increase in size of the T system as it approaches the sarcolemma (S). The T tubule (Tt) at the bottom of the micrograph measures 250 Å; whereas, just beneath the sarcolemma the T tubule (Tt) increases to 750 Å in diameter parallel to the long axis of the cell. Note the termination of the T tubule is running parallel to the sarcolemma and is complicated by pinocytotic vesicles. X 40,700.
PLATE XIII

EXPLANATION OF FIGURE

Figure 14. Termination of a T tubule (TT) at an angle of 90° to the sarcolemma (S). The T tubule measures 750 Å, and its termination is again complicated by pinocytotic vesicles (P). TC, terminal cisternae. × 46,600.
Figure 15. Termination of a T tubule (TT) 1300 Å in diameter, and intermediate in appearance between Figures 13 and 14. The T tubule enters the plane of section (lower arrow) and runs perpendicular to the sarcolemma before turning away at the fiber surface (upper arrow). Note the association of this portion of the T tubule with pinocytotic vesicles (P). X 77,000.

Figure 16. Convoluted termination of a T tubule (TT) at the fiber surface. Arrows indicate the areas in which the T tubule passes out of section and runs parallel with the sarcolemma (S). X 68,000.
Figure 17. Electron micrograph showing the extracellular distribution of lanthanum in the muscle. The micrograph was taken of the most peripheral fiber in the muscle, and both its superficial (S) and internal (I) aspects can be seen. While lanthanum (L) is seen on the superficial surface of the muscle fiber and adhering to bundles of fibers in the surrounding connective tissue, the internal surface of the muscle fiber is relatively free of lanthanum. Only occasionally was lanthanum observed to penetrate beneath the most superficial cells in the muscle. X 7,300.
Figure 18. Electron micrograph showing the intracellular distribution of lanthanum in the muscle. The lanthanum is seen filling pinocytotic vesicles and in the space bounded by the membranes of the T system. Note the elements of the sarcoplasmic reticulum, including the terminal cisternae (tc), intermediate cisternae (ic), longitudinal tubules (lt), and fenestrated collar (fc) are totally free of lanthanum. X 68,000.
Figure 19. Electron micrograph showing the penetration of lanthanum into two T tubules near the fiber surface. Note again the absence of lanthanum from the sarcoplasmic reticulum. tc, terminal cisternae; lt, longitudinal tubule; fc, fenestrated collar. X 41,300.

Figure 20. Electron micrograph similar to Figure 19. TT, T tubule; FC, fenestrated collar. X 53,000.
Figure 21. Low power scanning electron micrograph showing the muscle surface of the extensor longus digiti IV. The arrow indicates a "spindle-like" structure situated between the extrafusal muscle fibers. The "spindle-like" structure measures 120 µ in diameter, and extends 450 µ on the muscle surface. X 330
Figure 22. Polar aspect of the "spindle-like" structure shown in Figure 21. Arrows indicate the lamellae of connective tissue which surround this structure and disappear in between the connective tissue of the extrafusal muscle fibers (E). X 1,320.

Figure 23. Scanning electron micrograph showing the "spindle-like" structure (S) in side view. E, extrafusal muscle fiber. X 770.
Figure 24. Scanning electron micrograph showing a portion of 3 extraneous muscle fibers. The striated pattern of the myofibrils is evident at the fiber surface; the lighter ridges corresponding to the A bands, and the darker furrows corresponding to the I bands. X 2,500.
Figure 25. High power scanning electron micrograph. Arrows indicate the parallel rows of openings or apertures corresponding to the T system. The apertures are found at the level of the I band in the vicinity of the Z disk (Z). X 22,500.
PLATE XXII

EXPLANATION OF FIGURE

Figure 26. Electron micrograph of a muscle fixed in OsO$_4$. Note the contracted appearance of the myofibrils. M, mitochondria; Z, Z disk. × 29,300.
PLATE XXIII

EXPLANATION OF FIGURE

Figure 27. Micrograph showing the vesiculated appearance of the sarcoplasmic reticulum characteristic of muscles fixed in OsO₄. TC, terminal cisternae; LT, longitudinal tubule; FC, fenestrated collar. X 50,000.
Figure 28. Electron micrograph showing the distribution of pyroantimonate in contracted cells. Electron dense granules are seen in the terminal cisternae (TC) of the sarcoplasmic reticulum, and closely applied to the luminal aspect of the cisternal membrane at its junction with the T system. Along the myofibrils the precipitate is most dense at the overlap of thick and thin filaments near the Z disk (Z). X 58,600.
Figure 29. Sarcomere showing the distribution of pyroantimonate in slightly contracted cells. Note the dense line of precipitate in the I band on the boundary of the A-I junction (arrows). The A band also contains precipitate which is lowest in the region of the M band (white m). X 64,000.

Figure 30. Sarcomere of a muscle fiber with I bands longer than 1.2 μ. Note the absence of I band precipitate (arrows). X 64,000.
Figure 31. Electron micrograph showing the difference in I band precipitate in contracted (C) and relaxed (R) cells. In the contracted cell the I bands measure less than 0.5 μ, and a dense line of precipitate is present on the boundary of the A-I junction (lower arrows). Note the striking diminution of I band precipitate in the relaxed fiber (upper arrows). X 16,000.
Figure 32. Longitudinal section of 4 muscle fibers showing the low concentration of pyroantimonate in the extracellular space of the muscle. N, nucleus. x 7330.
PLATE XXIX

EXPLANATION OF FIGURE

Figure 34. Electron micrograph showing a portion of two fibers from a muscle exposed to calcium-free Ringer's solution containing 5 mM EDTA for 1 hour. Arrows indicate precipitate in the region of the terminal cisternae; S, sarcolemma; M, mitochondrion. X 28,600.
Figure 35. Electron micrograph of a muscle exposed 1 hour to calcium-free Ringer's solution containing 5 mM EGTA. Arrows indicate precipitate in the region of the A-I junction. TT, T tubule; M, mitochondrion. X 36,200.
Figure 36. Low power micrograph of a muscle in which the caffeine-induced contracture was arrested in calcium-free Ringer's solution. Arrows indicate precipitate in the I band. S, sarcolemma, X 15,000.
PLATE XXXII

EXPLANATION OF FIGURE

Figure 37. Muscle in which the caffeine-induced contracture was arrested in calcium-free Ringer's solution. Precipitate is present along the actin and myosin filaments and in the region of the terminal cisterna. Note the absence of precipitate in the longitudinal tubules of the sarcoplasmic reticulum (LT). TT, T tubule. X 32,000.
Figure 38. Muscle in which the caffeine-induced contracture was arrested in calcium-free Ringer's solution. Note the dense concentration of precipitate in the I band on the boundary of the A-I junction. Abundant precipitate was also observed in the terminal cisternae of these muscles (Tc). Note the absence of precipitate in the longitudinal tubules of the sarcoplasmic reticulum. (m) mitochondrion; L, lipid. X 33,700.
Figure 39. Muscle in which the caffeine-induced contracture was arrested in calcium-free Ringer's solution. Note the presence of precipitate along the actin and myosin filaments and in the terminal cisternae (TC). L, lipid; S, sarcolemma. X 32,000.
Figure 40. Muscle in which the caffeine-induced contracture was arrested in calcium-free Ringer's solution containing 5 mM EDTA. Note the striking diminution of precipitate in the A band, and along the actin filaments in the I band. L, lipid; S, sarcolemma; M, mitochondrion. X 34,000.
Figure 41. Muscle in which the caffeine-induced contracture was arrested in calcium-free Ringer's solution containing 5 mM EGTA. The precipitate along the myofilaments is almost totally eliminated, and only a few granules of pyroantimonate remain in the terminal cisternae closely applied to the luminal aspect of the cisternal membrane at its junction with the T tubule (arrows). Z, Z disk; TC, terminal cisternae. X 46,500.
PLATE XXXVII

EXPLANATION OF FIGURE

Figure 42. Electron micrograph of a muscle in which the caffeine-induced contracture was arrested in Ringer's solution containing 5 mM EGTA. Note the absence of precipitate at the boundary of the A-I junction (arrows). The precipitate in the terminal cisternae is also markedly reduced (TC). S, sarcolemma. 26,600.
Figure 43. Muscle in which the caffeine-induced contracture was arrested in Ringer's solution containing 5 mM EGTA. Note the precipitate along the myofilaments is lacking. A few granules of pyroantimonate (arrows) can still be seen in the terminal cisternae of the sarcoplasmic reticulum. L, lipid; TT, T tubule. × 48,600.
Figure 44. (a) Standard potassium-induced contracture and (b) calcium wash-out, followed by (c) exposure of the muscle to calcium-free Ringer's + 5 mM caffeine. Arrows indicate the changing of solutions in the tissue bath; pH 7.1 to 7.2; Temp. = 22 ± 2°C; chart speed = 25 mm/sec; 7 mm on the ordinate corresponds to a muscle shortening of 1 mm.
EXPLANATION OF FIGURES

Figure 45. (a) Standard potassium-induced contracture and (b) calcium wash-out, followed by (c-k) depletion of the response to 5 mM caffeine in zero-calcium Ringer's solution. Arrows indicate the changing of solutions in the tissue bath; pH 7.1 to 7.2; Temp. = 22 ± 2° C; chart speed = 25 mm/sec; (a-j) 7 mm on the ordinate corresponds to a muscle shortening of 1 mm; (k) 15 mm on the ordinate corresponds to a muscle shortening of 1 mm.
Figure 46. (a) Standard potassium-induced contracture and (b) calcium washout followed by (c-e) depletion of the response to 5 mM caffeine in zero-calcium Ringer's solution containing 5 mM EDTA. Arrows indicate the changing of solutions in the tissue bath; pH 7.1 to 7.2; Temp. = 22 ± 2°C; chart speed = .25 mm/sec; (a-d) 7 mm on the ordinate corresponds to a muscle shortening of 1 mm; (e) 15 mm on the ordinate corresponds to a muscle shortening of 1 mm.
Figure 47. (a) Standard potassium-induced contracture and (b) calcium wash-out, followed by (c-f) depletion of the response to 5 mM caffeine in zero-calcium Ringer's solution containing 5 mM EGTA. Arrows indicate the changing of solutions in the tissue bath; pH 7.1 to 7.2; Temp. = 22 ± 2° C; chart speed = .25 mm/sec; (a-e) 7 mm on the ordinate corresponds to a muscle shortening of 1 mm; (f) 15 mm on the ordinate corresponds to a muscle shortening of 1 mm.
Figure 48. Drawing of the sarcoplasmic reticulum of the extensor longus digiti IV muscle of the frog. Included are two terminations of T tubules continuous with the surface membrane at the right of the figure. Other openings in the membrane are of pinocytotic vesicles from underlying caveolae. The drawing is consistent with the findings in the results section except that, in general, the terminal portion of the T tubules appear more convoluted in transmission electron micrographs.
The dissertation submitted by Lawrence P. McCallister has been read and approved by members of the faculty of the Graduate School of Loyola University of Chicago.

The final copies have been examined by the chairman of the thesis committee and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Date: 01/17/72

Signature of Advisor: [Signature]