FINE STRUCTURE AND PHYSIOLOGY OF CARDIAC MUSCLE IN THE SPIDER, DUGESIELLA HENTZI

by

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INTRODUCTION

The contemporary biologist makes extensive use of "model systems" in order to assist his understanding of fundamental processes in biology. Attempts are made to relate the information, which is garnered in studies of these systems, to the specific process in question. A hypothetical, generalized model is constructed and additional investigations are employed to evaluate and perfect the postulated scheme. The model system is selected because it provides advantages and conveniences not found in less suitable material.

Squid axons have served as a model system in studies concerning the propagation of nerve impulses. Hodgkin and Huxley (1952) demonstrated the vital contribution of sodium and potassium to membrane depolarization, using the giant squid fibers. The unusually large size of these axons makes them especially suitable subjects for microelectrodes.

An impressive amount of knowledge concerning fertilization and development has been acquired in studies of sea urchin embryology. Sea urchin eggs satisfy almost all the requirements of an ideal system for delving into problems of fertilization. Monroy (1965) pointed out that they can be collected in large quantities, will develop quite synchronously when fertilized, take up substances from the medium (tracers and inhibitors, e.g.) and are transparent

for in vivo microscopic studies.

Bacterial systems have been utilized as models for the mechanism of messenger-RNA (m-RNA) synthesis, as well as for the control of protein synthesis. The work of Gros. et al. (1961) has demonstrated that m-RNA is a short-lived nucleic acid in bacteria. In striving to explain the control of certain enzyme levels in the bacterial cell, molecular biologists have theorized that once the concentration of an enzyme is sufficient, the transcription of its corresponding m-RNA is repressed. Any active molecules, of this specific messenger existing at the time of repression, soon loose their activity by virtue of their short-lived nature. Thus, the amount of enzyme synthesized following repression is limited and the enzyme concentration is regulated rather precisely. However, the bacterial model for the control of protein synthesis may not be applicable to higher organisms. Marks et al. (1963) have shown that m-RNA is not short-lived in mammalian reticulocytes. Perhaps, this consideration eliminates the efficient and explicit nature of any straightforward transcriptional control. Indeed, certain evidence suggests that a translational control is present in higher organisms. For example, Watson (1965) has described a situation where proteins coded by a single m-RNA molecule (polycistronic messenger) may be produced in unequal numbers.

In terms of the biology of muscle, fast-acting vertebrate striated muscles (like rabbit psoas) have been extensively

employed as models of muscle structure and function. As a result, a general picture of contraction in striated muscle has emerged. In striving to organize preliminary literature dealing with muscle, Hanson and Huxley (1955) theorized that the structural basis of contraction involves a sliding mechanism. They suggested that two types of discontinuous myofilaments exist in a muscle cell or fiber and that these parallel filaments slide past each other during contraction. Since 1955, intense efforts have focused on three aspects of muscle: ultrastructure and molecular architecture, chemistry, and excitation-contraction coupling. These efforts have succeeded in embellishing the fundamental sliding theory of contraction and have led to the development of a well-organized model.

The probable lack of versatility in the bacterial model for controlling protein synthesis, was discussed above. It may eventually develop that this essential process is definitely dealt with in varying ways by markedly different biological systems--systems that possess different requirements, specializations and limitations. Certainly, the same thing may be true of contractility. A tremendous amount of diversity is displayed by muscles throughout the animal kingdom. At best, many of these muscle types are poorly understood. Whether the currently accepted muscle model is appropriate in many of these situations, or whether it needs to be modified, is not known.

As mentioned above, fast-acting (phasic) striated muscle found in vertebrates has been used as a model system for muscle. However, this muscle is highly specialized, and from a physiological standpoint it does not represent a typical muscle class. It is now a well-established fact that due to inherent differences in striated muscle fibers, two fibers may not respond to stimulation in the same way. Those striated muscle fibers that react in the manner of the two extreme responses are termed fast or slow fibers. With fast types, a single motor impulse may initiate the maximal development of contraction. Subsequent impulses primarily influence the duration of the active state. Slow (tonic) fibers respond to each membrane activation with only a partial development of the active state. Repetitive stimulation produces a maximal contraction by means of facilitation and summation.

Very few investigators have chosen to initiate in-depth research programs of striated muscle types other than the fast forms. As a result, we do not possess a meaningful understanding of slow muscle--the counterpart to fast. The studies reported in this thesis have largely been attempts to make us more knowledgeable in terms of the biology of slow muscle. This has involved evaluating the current muscle model in light of findings related to slow striated muscle.

This research deals with an invertebrate cardiac muscle system, in which the muscle may be classified as slow. No

significant examination had previously been made of the structure and function, at all levels of organization, of an invertebrate cardiac muscle. It was hoped that a worthwhile contribution could be made to the understanding of a specific invertebrate heart muscle and to slow muscle systems in general.

Three aspects of spider cardiac muscle have been investigated here. The first involved structural considerations. Light and electron microscope studies were designed to determine the nature of the heart, including the organization of probable myofilaments and membranous systems. Physiological efforts related to excitation-contraction coupling and the requirements of the contraction process. Finally, limited cytochemical procedures were directed to the composition of muscle fiber components. It was believed that these studies of composition would clarify the structure-function interrelationships.

REVIEW OF THE LITERATURE

In the previous section of this thesis, the concept of a model muscle system was introduced. The various aspects of this muscle model will be reviewed here. Because of the extensive literature in this area, an attempt will be made to select investigations which relate directly to the structure and function of fundamental muscle components. Once this foundation is laid, theories of muscle contraction will be presented. In addition, recent developments with regulator proteins will be discussed. A review of muscle physiology will follow pertaining to essential variations between fast and slow striated muscle. Lastly, we will be introduced to what is known of spider heart anatomy and physiology.

Striated Muscle Model

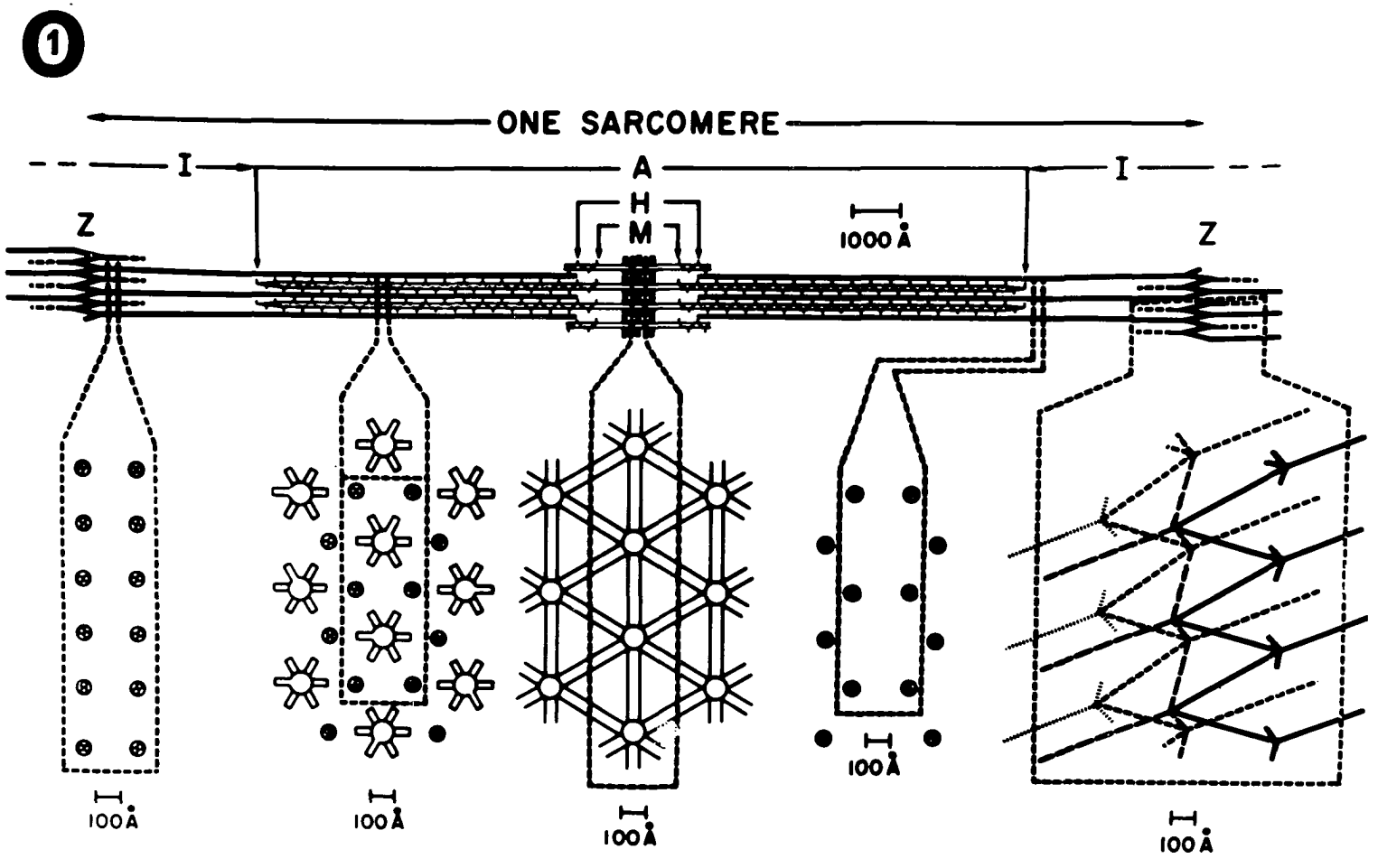
It has long been known that a great many muscles exhibit distinct banding patterns, when viewed with the light microscope. This banding results from the transversely striated appearance of the myofibrils. (A whole muscle may be separated into its component cells or fibers; the fibers are composed of many myofibrils.) In the conventional light microscope, zones of higher refractive index (dark bands) are seen to alternate with zones of lower refractive index (light bands). The dark bands are divided by bands with a lower index of refraction. The light bands are bisected by narrow lines possessing a high refractive index. With the polarizing

microscope, the dark bands of conventional microscopy exhibit a birefringence which is uniaxial with the optical axis parallel to the length of the fiber. Therefore, these bands are termed anisotropic and labeled as "A-bands". The other bands are isotropic and known as "I-bands". A. F. Huxley (1957a) has reviewed the essential facts concerning muscle structure at the light level.

Electron microscopy of ultra-thin sections enabled investigators to demonstrate the structural basis of the striated appearance (Huxley, 1953a; Hanson and Huxley, 1953; Hanson and Huxley, 1955). The A-band was shown to be the result of an orderly array of thick filaments and the I-band a collection of parallel thin filaments.

Figure 1 illustrates the arrangement of the two filament types in the myofibril. Thick filaments are largely composed of the protein myosin, and the protein actin resides in the thin filaments. The muscle unit, the sarcomere, extends from one Z-disc (from the German "Zwischenscheibe") or Z-line to the next. The discontinuous thick filaments are situated in the center of the sarcomere; they do not extend to the Z-lines. The thin filaments extend from the Z-lines and interdigitate with the thick filaments to varying degrees. The greater the interdigitation, the greater is the extent of contraction. The area of the sarcomere occupied exclusively by thick filaments is termed the H-band, (from the German "Hellerscheibe"). Its area is inversely proportional to the

Figure 1. This diagram reviews the molecular anatomy of a sarcomere. The thick filaments of the A-band (A) are shown to interact with I-band (I) thin filaments by means of cross-bridges. These bridges are illustrated in the cross section of the A-band. Thick filaments are linked together at the M-line (M). Thin filaments, which do not extend into the H-band (H), possess either a square or hexagonal arrangement in cross section. The square formation occurs near the Z-line (Z). At the Z-line, thin filaments are believed to give rise to four strands. These strands pass into several different planes. Presumably, two strands are actin and two are tropomyosin. This diagram was devised by R. E. Davies at the University of Pennsylvania.


1
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I
A
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1000 Å
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degree of interdigitation. See Huxley and Hanson (1960) for an appropriate review.

Current theory suggests that the increased interdigitation of thick and thin filaments is due to a sliding mechanism, (Hanson and Huxley, 1955). No shortening of the A-band is believed to occur. The works of Huxley and Niedergerke (1955) and Huxley and Hanson (1954), with interference and phase microscopy respectively, have provided verification of this premise. Both reports indicate that the A-band length remains constant during stretching and various degrees of contraction. Changes in I-band length alone account for the changes in sarcomere length. Electron microscope studies have confirmed this (Hanson and Huxley, 1955; Page and Huxley, 1963).

The sliding filament theory stipulates that the sliding results from the interaction of myosin and actin by means of cross-bridges. A "pulling" has been suggested to result from this interaction. Huxley (1957) has clearly demonstrated these linkages in an electron microscope (EM) study employing sections only 150 Å thick. The bridges occur every 60-70 Å on the thick filament and bind to the actin molecules of the thin filaments. See Figures 1, 4 and 5. It is thought that the heavy-meromyosin fragment of myosin (resulting from digestion with trypsin) acts as the bridge. Szent-Györgyi (1953) showed heavy-meromyosin to be capable of binding with actin and splitting adenosine triphosphate (ATP).

In terms of excitation-contraction coupling, a well-received idea has involved transverse tubules (t-tubules) and the sarcoplasmic reticulum (SR). Presumably, impulses pass to the contractile machinery by way of the t-tubules, which are invaginations of the cell membrane. The local activation experiments of Huxley and Taylor (1958) support this concept. The work of Hasselbach and Makinose (1961) suggests that impulses which reach the fiber interior, affect the release of calcium from the SR. Once freed from its storage compartment in the SR, the calcium is able to activate the contractile proteins.

Muscle filaments and Z-lines

In the previous discussion, it was mentioned that the thick filaments are largely myosin, the thin predominantly actin. This statement is based on a wide variety of investigations.

Hanson and Huxley (1953) and Hasselbach (1953) demonstrated the loss of A-band material from muscle after it had been treated with solvents (high ionic strength salt solutions plus ATP or pyrophosphate) known to selectively dissolve myosin. Electron microscopy confirmed the loss of thick filaments from the A-bands. Huxley and Hanson (1957) and Hanson and Huxley (1957) also used interference microscopy to support the finding that A-band density was decreased following myosin extraction. It should be noted

here that biochemical literature pertaining to muscle was very extensive, before muscle filaments were even known to exist. Therefore, procedures were available for extracting protein which fulfilled the definition of "myosin".

Corsi and Perry (1958) detected an absence of myosin in extractions known to remove I-bands. All the adenosine triphosphatase (ATPase) activity of the isolated myofibrils remained behind in the A-band remnants.

Turik and Holtzer (1961) employed the fluorescent antibody technique to correlate myosin with thick filaments. Further work by Szent-Györgyi et al. (1964) showed thick filaments to bind anti-myosin, anti-heavy-meromyosin (anti-HMM) and anti-light-meromyosin (anti-LMM). Anti-myosin did not bind, however, along the M-line region of the thick filament. Thick filaments are known to be linked together structurally at the level of the M-line, (from the German "Mittelscheibe"). See Figure 1 and the work of Knappeis and Carlsen (1968). The patterns of anti-HMM and anti-LMM binding were similar to that of anti-myosin. This would be expected on the basis of the relatively small size of a myosin molecule. That is, myosin molecules are distributed all along the thick filaments. Pepe and Huxley (1964) have reported the limited binding of anti-actin to I-segments.

Evidence for the hexagonal positioning of six thin filaments around a single thick filament (as in Figure 1), goes beyond the EM studies of Hanson and Huxley (1955).

Huxley's (1953b) low angle X-ray diffraction studies on muscle (in the wet state) had previously indicated that two sets of filaments were present. The well-defined X-ray pattern revealed a hexagonal array of filaments about 450 Å apart. The ordered array was not disrupted during contraction of this fast-acting vertebrate muscle. In addition, the X-ray diffraction efforts of Elliott et al. (1965) have suggested that no appreciable changes in filament lengths are associated with activation and the development of tension.

Thick filaments It has been estimated that a single thick filament, which is approximately 160 Å in diameter and 1.5 μ long, contains 400 myosin molecules. Biochemists have centered attention on this molecule, in hopes of illuminating the entire area of muscle biology.

Numerous investigations (Gergely, 1950; Perry, 1951; Mihalyi, 1953; Szent-Györgyi, 1953) have shown trypsin, among several agents, to be capable of splitting myosin into two unequal particles. The slower sedimenting particle was termed light meromyosin (LMM) and the faster, heavy meromyosin (HMM). HMM retains full ATPase activity and combines with actin. LMM molecules combine with each other. In a kinetic study, Mihalyi and Harrington (1959) interpreted their findings to indicate that trypsin attacks myosin at a point where the peptide chain is unfolded and not in an α-helical conformation. Young et al. (1964, 1965) reported that HMM appears as 65-70 percent of the original myosin weight; the

molecular weight of myosin was given as approximately 600,000.

EM investigations (Huxley, 1963; Rice, 1964; Pepe and Huxley, 1964) have shown LMM to be a rod-shaped particle 600-700 Å long. HMM consists of a small tail attached to a globular head; the total HMM length is 400-600 Å. The unfolded, trypsin-sensitive portion is believed to measure 200-300 Å in length. It lies in the center of the myosin molecule (Figure 2). Biochemical efforts have confirmed the existence of a short tail on the head of HMM (Young et al., 1964, 1965; Lowey, 1964). This tail is a highly helical peptide. These studies of Young et al. also involved the spontaneous dissociation of three subunit polypeptide chains from the globular head of HMM. Therefore, it has been theorized that myosin consists of a coiled-coil over its entire length, with two exceptions. The first is the trypsin-sensitive region dividing LMM and HMM. The second exception is the globular head region of HMM where the three peptide chains of the molecule have an α-helical content of 45 percent. Presumably, each peptide chain has ATPase activity.

The structure of the myosin molecule fits very well into Huxley's (1963) concept of a thick filament. The thick filament shafts appear to have their origin in LMM (these particles adhere together) and the tails of HMM fragments. The globular heads of HMM form the cross-bridges reported first by Huxley (1957). See Figure 3. The ATPase

Figure 2. The light- and heavy-meromyosin (LMM and HMM) portions of the myosin molecule are illustrated. The trypsin sensitive (TS) region is located in the center of the molecule. The dimensions listed are the result of several investigations (Huxley, 1963; Rice, 1964; Pepe and Huxley, 1964).

Figure 3. A theorized arrangement of myosin molecules is presented. It is designed to account for the structure of the central portions of thick filaments. Note the manner in which LMM forms the filament shaft, and HMM heads serve as cross-bridges. Davies (1963) has theorized that the myosin ATPase site is found at the base of HMM. The pseudo-H-band is free of cross-bridges. The hollow core (S) of the filament is exaggerated in the diagram. However, certain thick filaments do possess cores of a low density.

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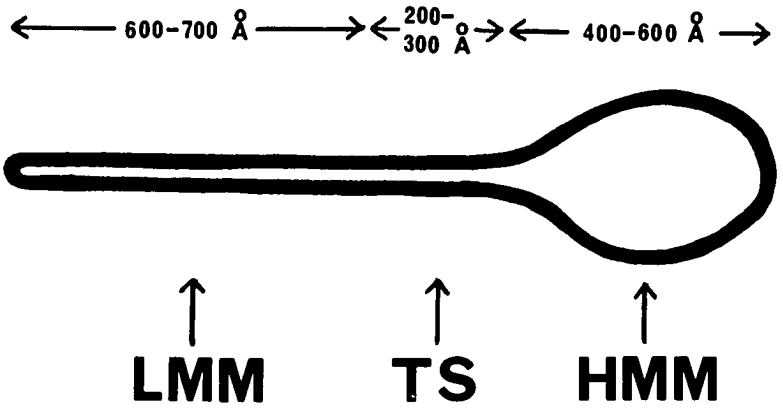

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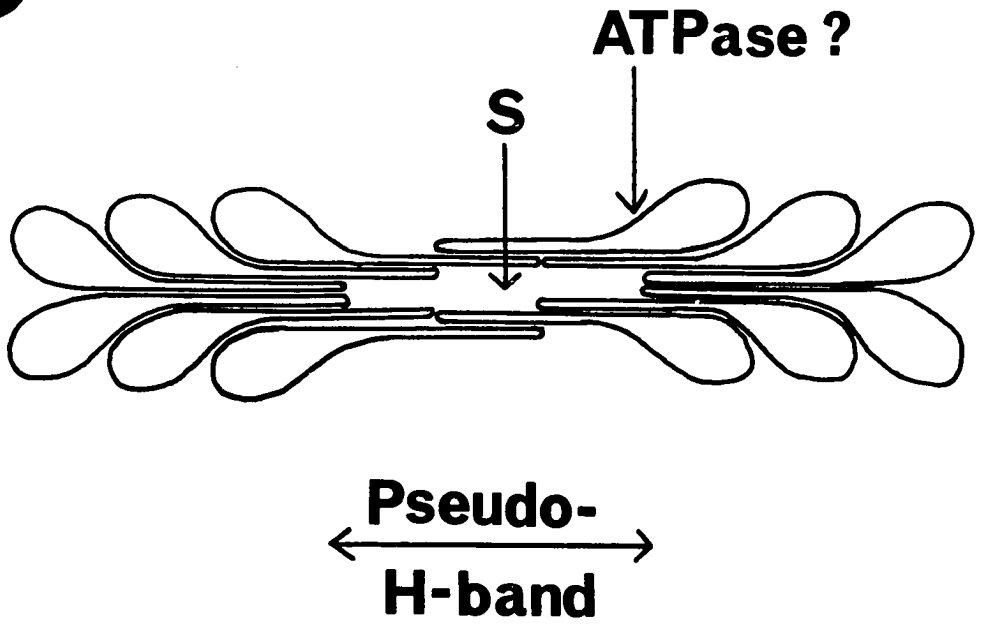


activity demonstrated in the HMM bridges is appropriately located for myosin-actin interaction. Furthermore, the HMM head is the site where myosin displays its ability to bind to actin.

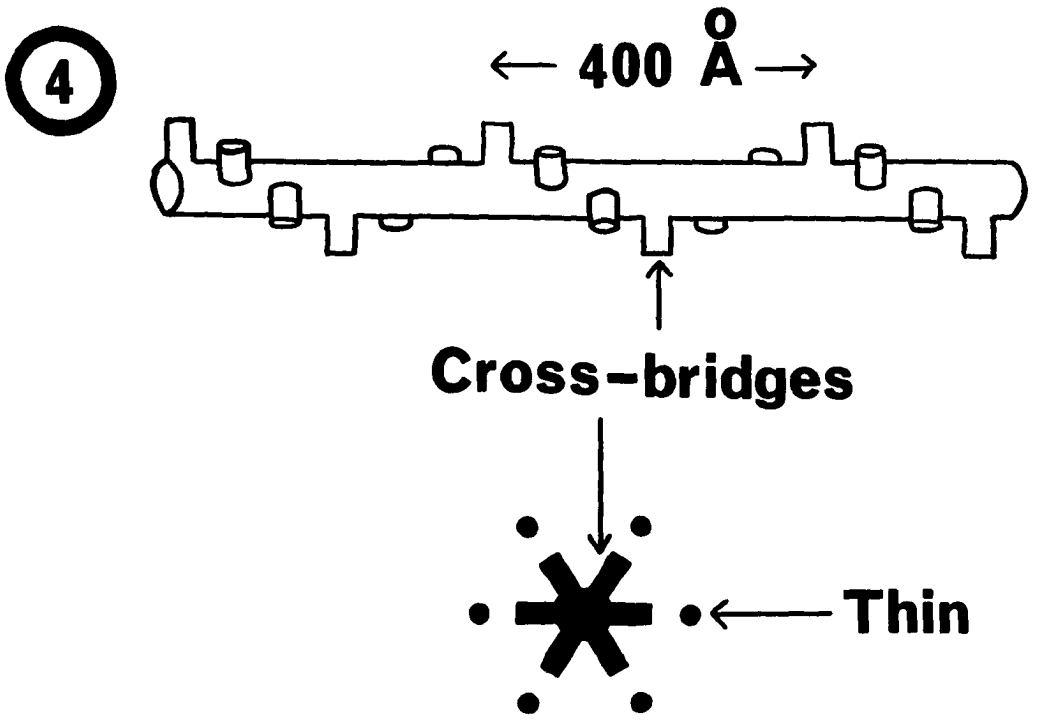
Thick filaments appear to possess cross-bridges in their lateral regions and short, "bare" spots directly in the middle (Huxley, 1963; Rice, 1964). It has been suggested by Huxley (1963, 1965) that myosin molecules in one half, point in an opposite direction to those of the other half. In the middle region, LMM segments of each half overlap and grant the filament its necessary rigidity. This middle region, devoid of projections, is seen in electron micrographs as a light band within the H-band. It has been called the pseudo-H-band (Figure 3).

The work of Huxley (1957) described cross-bridges at intervals of 60-70 Å, six bridges occurring every 400 Å along the filament's length (Figure 4). In cross section, the six bridges were believed to radiate from the filament shaft with approximately 60 degrees separating consecutive bridges. Of course, they would also be staggered along the thick filament length. Thus, it was presumed that these points of intersection would be effectively directed to the hexagonal array of six thin filaments.

Reedy et al. (1965) have also published the results of an examination of cross-bridge orientation in various muscle states. They found bridges to be perpendicular to thick

Figure 4. Huxley (1957) described the presence of cross-bridges every 60-70 Å on thick filaments, six bridges occurring every 400 Å. A thick filament segment is illustrated. In cross section, cross-bridges radiate from the shaft every 60 degrees. They extend towards hexagonally arranged thin filaments. The thick filament is approximately 160 Å in diameter, the thin 50-70 Å. Davies (1963) has illustrated this arrangement of myofilaments.

Figure 5. Reedy (1967) has shown cross-bridges to exist as opposed pairs. Both members of the pair occur at the same point along the longitudinal axis of the thick filament. Each member gives rise to two cross-bridges. Thus, four thin filaments may be bound at a given origin. Origins of this type occur every 146 Å. Each repeat rotates 67.5 degrees. Three possible binding configurations at an origin are illustrated.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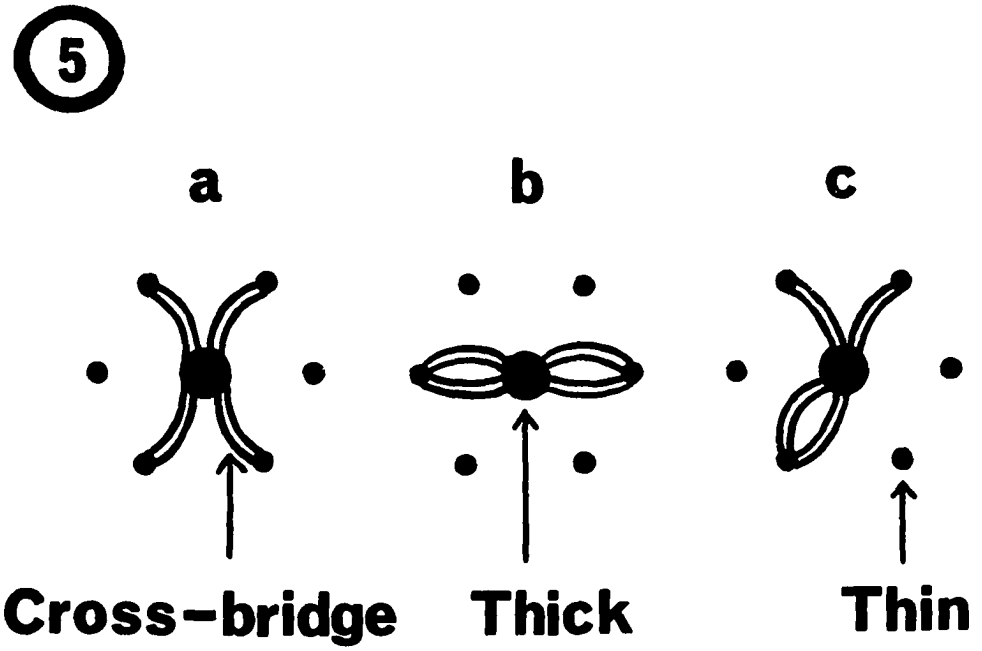
Cross-bridges
Thin


5
a
b
c
Cross-bridge
Thick
Thin

filaments in relaxed muscle. In rigor, the bridges become slanted towards the M-line. They suggested that the cross-bridges may "push" actins inward, thereby producing a tension increase that is associated with rigor induction. This work was carried out in Hugh Huxley's laboratory and it also dealt with other structural features of myosin filaments. These other studies have been reported by Reedy (1967) and appear to contradict the previous findings of Huxley (1957). This is undoubtedly a result of the impressive development, over this ten year period, of the techniques utilized in both studies.

Diffraction and EM studies enabled Reedy (1967) to illustrate the unexpected features of thick filaments (Figure 5). He showed cross-bridge origins to form opposed pairs every 146 Å. Each repeat rotates 67.5 degrees. The twin helical tracks require 1.5 turns (i.e., 8 x 146 Å) to establish a repeat of 1166 Å. The diffraction patterns exhibit this characteristic value. Each member of the opposed pair at a single origin, on the thick filament, gives rise to two cross-bridges. Thus, each site on the filament involves four bridges. The beauty of this arrangement is that one half a turn of the myosin helix (388 Å) matches exactly the pitch of the actin helix (388 Å) in Reedy's investigation. At first, it appears to be awkward to match 16 myosin sites with 6 actin filaments. However,

the correspondence in pitch tends to minimize axial mismatching between myosin and actin subunits.

Thin filaments The thin filaments measure a micron in length and are 50-70 Å in diameter. A single thin filament contains approximately 600 actin molecules. However, tropomyosin (TM) is also present in appreciable quantities. Perry and Corsi (1958) illustrated this and indicated that the ratio of actin to TM was 2:1. Laki et al. (1962) later suggested the ratio was 3:1. Troponin and β-actinin are also thin filament components and will be discussed with regulator proteins in a later section of this review. A possible function of TM will be dealt with at the same time.

In 1952, Mommaerts published a series of papers which noted that actin occurs in two forms. He believed that actin was in a globular form (G-actin) when extracted (1952a), and in a fibrous form (F-actin) in muscle (1952b). Mommaerts (1952c) demonstrated that during the G to F transformation, following extraction, ATP was dephosphorylated to ADP. He showed that a stoichiometric relationship existed with one mole of ATP combining with one mole of G-actin. Barany et al. (1954) have suggested that actin catalyzes the polymerization itself. However, this ATPase activity is apparently not involved in contraction. Evidence points to a lack of G to F transformation during contraction

(Martonosi et al., 1960). Earlier thought had favored such a transformation as a primary contractile event.

Katz (1963) has proposed a model for the structure of G-actin in solution. This involves a globular sphere (56 Å in diameter) which is partially held together by calcium near an opening in the sphere. The theorized structure is composed of a peptide chain, whose sulfhydryl groups are found on the inner surface of the hollow sphere.

Using the electron microscope, Hanson and Lowy (1963) have examined thin filaments and F-actin from a variety of species. F-actin was always indistinguishable from their "native" thin filaments. (Preparation involved relaxing glycerinated fibrils in an EDTA-ATP-MG^{++} solution.) The filaments appeared to consist of two helically wound strands (Figure 6). Both strands were composed of a single type of subunit. The spherical subunit measured approximately 56 Å in diameter, the filaments possessed diameters of 80 Å. A full turn was shown to require 13 subunits in a distance of 698 Å. Cross-over points created repeats every 349 Å. The 56 Å subunits were considered to be G-actin. Huxley (1963) and DePue and Rice (1965) observed similar structures in their EM studies. The theorized structure is in agreement with moderate angle X-ray diffraction data (Selby and Bear, 1956; Cohen and Hanson, 1956). For example, Selby and Bear had reported that the number of globular subunits per helix turn was either 13 or 15. The cross-overs

Figure 6. Hanson and Lowy (1963) have pictured F-actin as a double helix of 56 Å subunits. A cross-over occurs approximately every 350 Å. The diameter of the double helix is 80 Å. A full turn was shown to require 13 subunits over a distance of approximately 700 Å.

Figure 7. Hanson and Lowy (1963) have theorized that tropomyosin (TM) strands lie in the grooves of the actin double helix. Huxley (1960) has suggested that actin and TM sizes would be comparable. In addition, the work of Ohtsuki et al. (1967) has demonstrated the binding of troponin to TM at intervals along the thin filament. This diagram combines the results of these varied studies. Because troponin binds only at certain intervals, cross sections of the thin filament at other points would only involve the four protein strands.

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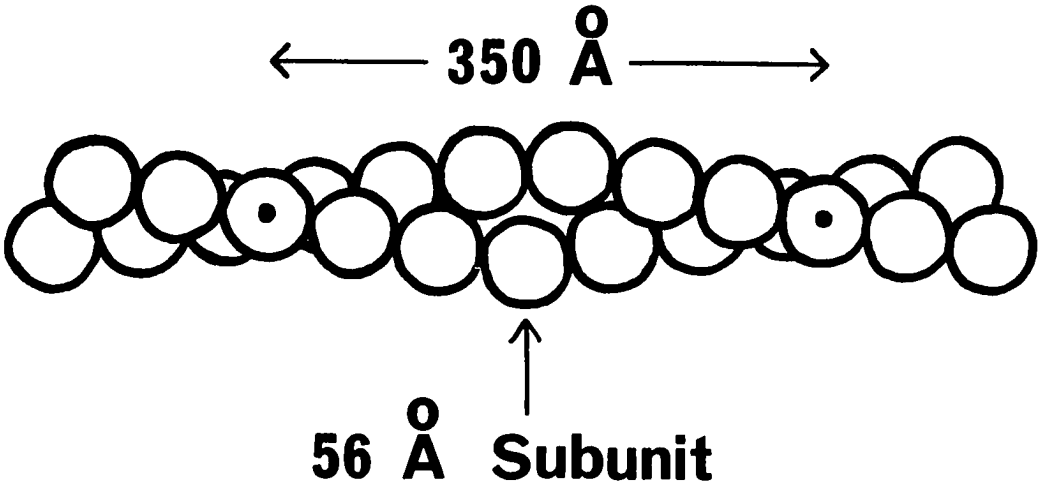

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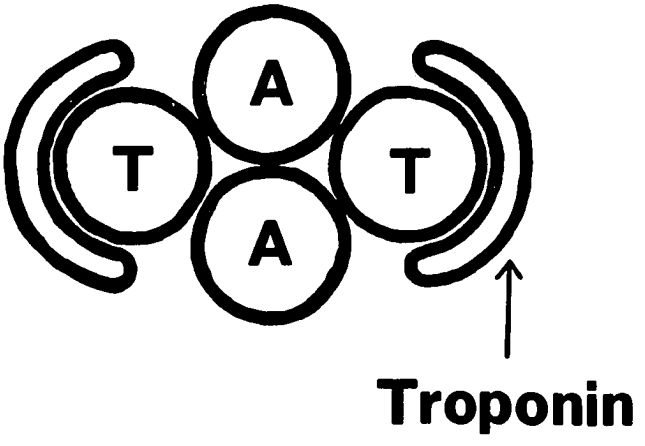


would then occur at either 351 Å or 406 Å, according to their data. Burge's (1963) diffraction evidence further substantiates the F-actin model offered by Hanson and Lowy.

However, Hanson and Lowy (1963) questioned the strict equality of F-actin and the thin filaments found *in vivo*. They based their doubt on the X-ray diffraction studies of Huxley (1953b) and Worthington (1959) and the EM observations of Carlsen et al. (1961). The diffraction efforts described an X-ray reflection at 400 Å in an axial pattern and the EM examination reported fine cross striations every 400 Å on the thin filaments. The structure of actin alone cannot account for this organization. Hanson and Lowy (1963) showed actin subunits to be identical. Therefore, it is not believed that certain subunits every 400 Å would be capable of binding additional thin filament components, while intervening units would be incapable. These workers theorized that tropomyosin (TM) may exist as two threads, each one being situated in one of the two grooves of the F-actin double helix (Figure 7). This could possibly account for the 400 Å periodicity of thin filaments. Hanson and Lowy suggested that TM may be lost during the isolation and negative staining of thin filaments for electron microscopy.

TM could possibly create the periodicity while serving as an internal backbone for actin. However, Huxley (1960) had previously argued that TM would compose a 50 Å core, if all of this protein served as internal support for thin

filaments. Clearly, this should be detected at the EM level and a TM core would be less readily removed than an external filament covering. Still further circumstantial evidence for TM strands in actin grooves comes from Carlsen et al. (1961). They noted in vertebrate striated muscle, that a given thin filament branched into four strands of equal diameter at the level of the Z-line (Figure 1). Presumably, this would involve two of actin and two of TM. On the basis of the TM concentration in muscle, Huxley (1960) has estimated that the sizes of actin and TM strands would be comparable. This assumes a TM strand lies in each of the major actin grooves.

Pepe (1966) and Ebashi and Kodama (1966) supported the fact that thin filaments contain TM. Pepe employed antibody-staining techniques. The results of his experiments indicate not only the presence of TM in thin filaments, but they also suggest that TM molecules, or at least parts of these molecules, are available for antibody binding in situ.

Although the specifics are not yet certain, it is clear that TM is now viewed as an important structural, and perhaps functional, component of thin muscle filaments.

In an attempt to stress the structural specificity of myosin-actin interaction, let us return to the work of Reedy (1967). Recall that he found the pitch of myosin to match appropriately with actin. Thus a juxtapositioning of reactive sites could result. He also showed that cross-bridges bent

toward the M-lines during rigor. Huxley (1963) has obtained a similar effect with a different technique. He has bound HMM to actin filaments and has examined the results with negative staining at the EM level. An "arrowhead" configuration is acquired where HMM binds to actin at an angle. All bound HMM points in the same direction. This is also true at low concentrations where long, "empty" regions of actin are observed. In this situation, the binding of one HMM molecule would not be interfered with by other HMM molecules. When thin filaments still linked to Z-line material are tested, all bound HMM points away from the Z-line. This is true of HMM bound on both sides of the Z-line. The precise reason for this specificity is not known. However, the polarity illustrated here suggests that thin filaments, on opposite sides of a Z-line, are specialized for cooperation in contraction. It was also noted above that the polarity in thick filaments is opposite in different halves.

Remarkable advances have been made in the area of muscle chemistry. The discussion of myosin and actin in this review has reflected this progress. We now await the elucidation of: 1) the manner in which muscle proteins catalyze contractile reactions and 2) the effect of catalysis on these proteins.

Other myofibrillar proteins The fourth major myofibrillar protein is paramyosin. Paramyosin has been implicated in the "catch mechanism" of several invertebrate

muscles. These muscles remain contracted for long periods of time (Bailey, 1957). Elliott (1964) has found "extra-thick" filaments to be paramyosin filaments in a muscle displaying the catch mechanism. Lowy and Millman (1963) do not implicate paramyosin in their alternate hypothesis. They suggest that the active state of holding muscle is related to the formation of myosin-actin linkages and that decreases in tension are represented by the rate of linkage breakage. This breakage rate is proportional to the concentration of a relaxing substance, probably 5-hydroxytryptamine. Some experimental support may exist for this hypothesis. Yet, these unusual muscles and paramyosin remain very poorly understood.

Many other less significant myofibrillar proteins are beyond the scope of this review. See Goll (1965).

Z-lines A prominent feature of muscle structure at the light microscope level is the Z-line--a fine transverse line dividing the I-band. Although it has received considerable attention, it remains a rather mysterious muscle component.

H. E. Huxley's work (1953a, 1955 and 1957) has shown that Z-lines clearly serve as a structural means of linking thin filaments from one sarcomere to those of the adjacent functional unit. In addition, the proper structural relationship between thin filaments is maintained within a sarcomere.

Some evidence suggests that tropomyosin (TM) is the principal component of Z-lines. Corsi and Perry (1958) extracted both actin and TM and removed thin filaments and Z-lines. Structural studies of Knappeis and Carlsen (1962) and Huxley (1963) have noted an approximately square crystal lattice when Z-lines are cut in cross section for electron microscopy. These units possess dimensions which are on the order of 200-250 Å. It is most interesting that Huxley's examination of TM crystals revealed a similar lattice with dimensions of 200-220 Å. In the discussion of thin filaments, it was noted that TM may form strands that lie in the grooves of the actin double helix (Figure 7). If TM is also found as crystals in the Z-line, it must be capable of existing in two structural forms. Cohen and Longley (1966) have obtained two TM forms. A further structural consideration has been reported by Huxley (1964). In fast rabbit muscle, six thin filaments have traditionally been shown to surround a single thick filament in a hexagonal arrangement. Huxley has noted that thin filaments undergo a rearrangement near the Z-lines. Here they are seen in cross section to exist in a more or less square arrangement (Figure 1). This organization is maintained as you follow filaments into the Z-line.

However, Pepe (1966) has not observed any binding of anti-TM to Z-lines in his antibody investigations. Other similar studies, on the other hand, have demonstrated the

presence of ∝-actinin at the Z-line (Masaki et al., 1967). Therefore, it is possible that ∝-actinin is masking Z-line TM.

The work of Stromer et al. (1967a) involved extracting Z-lines from rabbit muscle and fractionating the extracts. They were able to rather effectively reconstitute Z-lines by adding back a specific fraction. Although TM was found in their original Z-line extract it was not contained in the extract fraction that possessed a meaningful ability to reconstitute Z-lines. These investigators are currently trying to characterize the Z-line extracts.

The efforts of Masaki et al. (1967) and Stromer et al. (1967a), which have just been cited, indicate that Z-line composition goes beyond TM. Indeed, it would not be surprising to find several molecular species in this complex structure. This would be expected in view of the very dense Z-lines in cardiac muscle, for example. Kelly (1967) suggests that a matrix may be deposited on Z-line filaments in these cells.

In discussing thin filaments, the work of Carlsen et al. (1961) was cited. They reported that thin filaments branch into four strands at the Z-line (Figure 1). Thus, they considered the Z-line to exist as a filamentous structure. Reedy (1964) also supported a filamentous Z-model in his later work. However, Franzini-Armstrong and Porter have suggested a membranous model for thin filament interconnec-

tions (1964a). Their model proposes that the Z-line lattice, seen in cross sectional electron micrographs, is the product of ridges in the membranes of the Z-disc. The ridges would result from a stretching of the membrane to thin filaments on both sides.

Kelly (1967) believes, on the basis of stereo-analysis at the EM level, that a filamentous fine structural model is most appropriate. Because such a model is rather representative of current thought in this area, his actin-TM model will be presented here. However, this is merely a model. No definitive answer to Z-line structure has yet been achieved.

Kelly theorized that thin filaments are composed of two actin and two TM strands as suggested by Hanson and Lowy (1963). He postulated that actin components terminate at the edge of the Z-band. The TM strands then continue past the Z-I boundary to form the Z-band lattice. Huxley's (1963) observation of a more or less square lattice in the Z-line (with dimensions similar to TM cyrstals) supports Kelly at this point. This hypothesis dictates that actin filaments do not exceed approximately one micron in length. That is, they do not extend into a second sarcomere. Thin filaments isolated by homogenizing muscle do not exceed one micron in length (Allen and Pepe, 1965). This appears to be a feasible in vivo length for thin filaments. If thin filaments extend through Z-lines, they must somehow reverse their polarity as they pass into the second sarcomere. Recall that Huxley

(1963) showed a thin filament polarity on one side of the Z-line to be opposite to that of the other side. Kelly's hypothesis involves a looping TM strand configuration. It is theorized that a TM strand loops back on itself and continues back to the Z-I junction. It ends there. As the TM thread loops back, it intertwines with another TM loop. This second loop is the component of a thin filament entering the Z-line from the adjacent sarcomere. Kelly's micrographs display looping effects and he has also demonstrated filament looping patterns in desmosomes (Kelly, 1966). He believes this similar arrangement fulfills two related structural demands.

Sarcolemma and the sarcoplasmic reticulum

The experiments of Huxley and Taylor (1955, 1958) and A. F. Huxley (1957b) demonstrated that localized contractions were elicited in muscle by placing microelectrodes at specific points on the fiber surface. It was shown that the specific points corresponded to the level of A-I junctions in the crab, and Z-lines in the frog. This seemed to indicate that the muscle cell membrane, the sarcolemma, was specialized at these points. More recently, Podolsky and Constatin (1964) have illustrated sarcolemmal specializations by applying microdroplets of a dilute calcium solution to various regions on the cell surface. Once again, application at distinct points resulted in contractions.

Early EM studies were unable to clearly visualize theorized surface adaptions. A transverse-system (T-system) had been well-described by Andersson-Cedergren (1959), Fawcett and Revel (1961) and Revel (1962). It was shown to course deep into the fiber from near the surface. However, it had not been shown to be directly continuous with the sarcolemma. With the advent of improved EM fixation procedures, Franzini-Armstrong and Porter (1964b) demonstrated that sarcolemmal invaginations constituted the T-system. They observed the sarcolemma, a typical unit membrane, to invaginate as transverse-tubules 150-300 Å in diameter. They suggested that extracellular fluids could readily gain access to the fiber interior, as could electrical impulses, via this pathway.

Furthermore, Peachey and Huxley (1964) later observed transverse-tubules (t-tubules) to occur at the A-I junction in the crab, and Peachey (1965) visualized them at the Z-line in frog muscle. Thus, the localization of t-tubules in these systems corresponds to the sites of local activation (Huxley, 1957b). These results were taken as rather meaningful evidence for a structure-function dualism. It is now believed that a membrane depolarization of the sarcolemma is able to deeply penetrate the muscle fiber by means of the t-tubules. This provides for a greater degree of synchronized contraction than would be otherwise possible. The belief that t-tubules serve to conduct impulses inward, is reinforced

by the work of Gage and Eisenberg (1967) and Eisenberg and Eisenberg (1968). These investigators disorganized t-tubules and, although surface action potentials were detected, contractions did not occur. It was their belief that this substantiated the fact that t-tubules are an essential link between membrane depolarization and the activation of the contractile apparatus.

The T-system was clearly demonstrated by Franzini-Armstrong and Porter (1964b) in gluteraldehyde-fixed tissue. It was shown to consist of tubules, which ran perpendicular to and between myofibrils. In fish muscle, the tubules ran across myofibrils at each Z-line. In the longitudinal area between two fibrils, a second class of membrane elements was present, the sarcoplasmic reticulum (SR). Although these investigators illustrated the SR very beautifully, it had been well described previously.

Porter and Palade (1957) described the SR as a specialized smooth form of endoplasmic reticulum found in muscle cells. They observed it to surround myofibrils and appear as cisternae between fibrils in longitudinal sections. These longitudinal components, now called the L-systems, came in close contact with the L-systems of adjacent sarcomeres at the Z-line. There was no continuity between the two, but a terminal swelling of each was demonstrated. Although less impressively done, an intermediate component was illustrated between the pair of swellings. Work in

several laboratories (Andersson-Cedergren, 1959; Revel, 1962; Fahrenbach, 1963) confirmed the early findings and demonstrated the intermediate component (the T-system) more clearly. No continuity was observed between the T-system and the longitudinal components of the SR. However, the contacting surfaces were often modified to increase their surface area. This formation of two terminal cisternae with the intervening transverse element became known as a "triad". The occurrence of triads is widespread in vertebrate muscle. However, it should be noted that triads are replaced by dyads in certain invertebrate muscles (Smith, 1965a). Here t-tubules lie adjacent to only a single SR element.

The real significance of the close association between t-tubules and the SR resides in the possibility of a scheme for excitation-contraction coupling. It has been shown by means of biochemical methods that the SR serves as a storehouse for calcium (Hasselbach and Makinose, 1961). Furthermore, these workers demonstrated that ATPase activity is associated with calcium uptake by the SR. A "relaxing factor" in the SR was specifically implicated in this active storage process. Cytochemical techniques have attested to the in situ localization of calcium (Hasselbach, 1964) and ATPase activity (Gauthier and Padykula, 1965) in the SR. Most importantly, rather solid evidence suggests that the release of calcium elicits contraction in muscle (Weber et al., 1964). Therefore, it is likely that contraction results

when the t-tubule triggers the rapid release of calcium from the SR. The depolarization of the t-tubule membrane could effectively alter the membrane properties of the SR. As a final stage in the contraction process, it is apparent that the relaxing factor may induce relaxation by lowering the concentration of free calcium (Hasselbach and Makinsoe, 1962; Ebashi and Lipmann, 1962; Weber and Herz, 1962).

Other components of the muscle fiber

Mitochondria or sarcosomes As in other cells, mitochondria provide ATP for the metabolism of muscle fibers. The number of mitochondria generally corresponds to the needs of these active cells. For example, in the flight muscle of insects, the tremendous demand for ATP is met by large numbers of well-developed mitochondria (Smith, 1965b). The metabolism of carbohydrates and lipids is linked to oxidative phosphorylation in the mitochondria. Glycogen is one carbohydrate involved; however, it may also be metabolized anaerobically. Both glycogen particles and lipid droplets are routinely observed in electron micrographs of muscle.

Sarcoplasm The cytoplasm of muscle, the sarcoplasm, contains a variety of globular proteins (myoglobin, some hemoglobin and the enzymes of the glycolytic and citric acid cycles). The ions commonly present include Na^{+}, K^{+}, Cl^{-}, Mg^{++}, Ca^{++} and different forms of phosphorous (Dicker-

son and Widdowson, 1960). Goll (1965) notes that the concentration of free Mg^{++} around myofibrils is always approximately 2×10^{-6} M. However, the level of Ca^{++} increases during contraction from 1×10^{-8} M to approximately 1×10^{-7} M.

Theories of Muscle Contraction

A considerable amount of information has been presented in this review which confirms a sliding theory for muscles that contain interdigitating filaments (beginning with Hanson and Huxley, 1953). In addition, evidence has been presented that suggests t-tubules and the sarcoplasmic reticulum may very well serve as agents of excitation-contraction coupling. Therefore, it is not unusual that the majority of contraction theories should consider the shortening of fibers to involve these two approaches. Variations in these theories exist in the suggested means by which the sliding motion is produced. For example, Weber and Portzehl (1954) suggested that ATP is cleaved at essentially the same time as the fiber contracts. Thus, the liberation of heat should also correspond to the time of shortening. It was believed that the classic work of A. V. Hill (reviewed by Davson, 1964) substantiated such a correlation. On the other hand, a theory based on the work of Morales and Botts (1952) indicates that the binding of ATP to actomyosin leads to contraction. The breakdown of ATP to ADP then

corresponds to relaxation.

However, not all contraction schemes implicate the sliding as a primary event. Podolsky (1961) has discussed contraction in terms of a coiling of I-filaments. This coiling would pull Z-lines toward the A-band, affecting a decrease in sarcomere length. A further possibility is noted by the work of deVillafranca (1961). He has shown that A-band constancy is not maintained during contraction, and that ATP is capable of inducing A-band shortening. Huxley and Gordon (1962) have also detected a lack of A-band constancy.

An early presentation of the sliding theory was offered by A. F. Huxley (1957a). He formulated a theory based on the major experimental findings of muscle structure and energetics. He suggested that a myosin site exists which has a chemical affinity for an actin site. The myosin site would possess a spring-like flexibility. This myosin site could bind to actin at a time when the myosin flexibility was being strained. There would then be a tendency for the myosin to recoil, at the same time pulling the actin deeper into the sarcomere. Due to its spring-like nature, the myosin site would oscillate about its mean position. However, the probability of forming a link with actin is zero on one side of the mean. Thus, a one-way character is imparted to the pull. This theory could explain the fact that maximum tension depends on muscle length. The specific

sarcomere length would determine the extent of proper alignment between myosin and actin sites. Shortening would necessitate a formation-breakage cycle involving the myosin-actin interaction, a sort of ratchet mechanism. Huxley's treatment consists of a two-step activation process. Phosphates are removed from actin, then myosin and actin bind together. Thus, relaxation would also require two stages. That is, relaxation would first involve a cessation of shortening (no more new links) and then a breakage of existing linkages. Jewell and Wilkie (1960) have shown that muscle does briefly maintain its tension, after it has stopped contracting and before it has started lengthening.

A prominent theory of muscle contraction has been offered by Davies (1963). It is based on a sliding scheme. He suggested that in the resting state ATP is bound to an extended polypeptide of heavy meromyosin (HMM). The ATPase site on HMM lies at the base of this extension and a fixed negative charge lies adjacent to the ATPase site. This negative charge repels a similar charge on the ionized ATP, and the extension of the polypeptide chain is thus maintained. When the muscle is activated, calcium is released from the SR and forms chelate links between the bound ATP of the extended HMM peptide and the bound ADP of F-actin. Recall that Mommaerts (1952c) showed ADP to be incorporated into actin upon polymerization. Calcium is bound ionically and this serves to neutralize the ATP of HMM. The former

electrostatic repulsion no longer exists, and the extended polypeptide of the cross-bridge is believed to contract. An α-helix is formed by a process of twisting and hydrogen bond formation. This contraction pulls the thin filament over the thick. The ATP of the HMM arm is brought close to the ATPase site by this process and the terminal phosphate is cleaved. With this hydrolysis, the myosin-actin linkage is broken. Phosphorylation of the HMM-ADP occurs, and the resultant electrostatic repulsion forces the HMM polypeptide into an extended configuration once again. The process is repeated as long as calcium remains available in the myofilament area. Each time, the HMM reacts with an actin subunit closer to the Z-line. The active state is terminated by the passage of calcium back into the SR.

Szent-Györgyi and Johnson (1964) have offered an alternative theory for striated muscle contraction. It is a modification of the simple sliding theory. Unlike the pure sliding theory, the new alternative is believed to account for the generation of forces necessary to rearrange huge molecular aggregates. This scheme has resulted from the studies of Szent-Györgyi et al. (1964) with antibody staining. These investigators noted a possible "reorganization" of myosin-containing structures. This reorganization involved a shortening during isotonic contraction, which was proportional to the shortening of the sarcomere. It was suggested that this process may be the primary event of contraction.

That is, it may precede sliding. This contraction theory follows such a line of reasoning. It describes a contraction of the myosin components that proceeds towards the lateral portions of the A-band. This is the first contractile event. A structural continuity is suggested to exist in the sarcomere by virtue of a connecting piece. This piece joins thick filament halves on the left with thin filaments on the right of the sarcomere (contralateral thin filaments). In other words, the myosin filaments are linked to actin filaments with which they would not associate by means of sliding. Therefore, this model increases myosin-actin interaction, and according to its promoters, the magnitude of the forces generated. In returning to the order of events, the myosin contraction (first event) would bring about the formation of a linkage between the contralateral thin filament and the myosin on that thin filament's side of the sarcomere (second event). This second event corresponds to the traditional myosin-actin interaction involved in sliding. A cycle of first and second events was then proposed. It is stressed that this theory deals effectively with the kinetics of cross-bridge formation and breakage. Also, the theory is not negated by considerations of energy production, and related physiological phenomena.

No clearly acceptable theory of contraction has yet been presented. Davies' (1963) rather sophisticated model clarifies the roles of calcium and ATP. However, many biophysicists

consider cross-bridges incapable of generating sufficient forces. Furthermore, Reedy et al. (1965) have indicated that cross-bridges "push"; they presumably do not "pull" thin filaments over thick. The theory of Szent-Györgyi and Johnson (1964) also has met with problems. They postulated certain myosin lattices and myosin-actin contralateral connections. These have not been observed, as would be expected.

Regulator Proteins in Muscle

A common criticism of the majority of muscle investigations is that they relate *in vitro* experiments to the question of contraction in living fibers. These *in vitro* studies typically involve glycerinated muscle or even actomyosin superprecipitates. Clearly, many *in vivo* factors are eliminated in these efforts. However, recent studies with regulator proteins are granting more *in vivo* properties to *in vitro* systems. These investigations will be discussed here.

Troponin

Ebashi and Ebashi (1964) demonstrated the presence of a new protein in the *in vitro* "contraction" or superprecipitation of actomyosin. This new protein appeared to grant their system a sensitivity to calcium, a behavior that had not been previously observed. In the presence of this protein, calcium stimulated actomyosin activity and calcium-removing agents affected a relaxation. Previously, magnesium had been

employed as a stimulant, although calcium was believed to serve in the normal physiological situation (Ebashi, 1961). Thus, an important characteristic was granted to these in vitro preparations. The new protein resembled tropomyosin (TM), yet usual TM preparations did not possess the same abilities. They postulated that a "native tropomyosin" had been isolated.

Ebashi and Kodama (1965) treated this native TM with trypsin and isolated a globular protein in addition to classic TM. The globular species promoted the aggregation of TM. These same workers later (1966) demonstrated that the new protein, troponin, interacted with F-actin in the presence of TM. Laki et al. (1962) had previously shown TM to be capable of binding to actin. Interest then arose in localizing native TM within the muscle fiber. Endo et al. (1966) used fluorescent microscopy (fluorescent proteins and fluorescent antibodies) to demonstrate the presence of TM and troponin in the I-bands of chicken muscle (Figure 7). Both studies showed a lack of fluorescence in the A-band. Thin filaments were seen to bind ferritin-labeled anti-troponin in the EM study of Ohtsuki et al. (1967). A periodicity of 400 Å was noted here and it was suggested that TM determines this effect and the binding of troponin at intervals along the thin filament.

∝-Actinin

Work in Ebashi's laboratory also led to the discovery of ∝-actinin. This protein was isolated from usual actin preparations by Ebashi and Ebashi (1965). They noted that it remarkably promoted the superprecipitation of actomyosin. It not only allowed superprecipitation to occur at higher ionic strengths, but it also increased the degree of actomyosin shrinkage in the process (reflecting a greater "contraction"). Once ∝-actinin was removed, normal actin preparations were found to interact very weakly with myosin at usual concentrations of ATP. These workers theorized that ∝-actinin served as a regulator protein in muscle.

Maruyama and Ebashi (1965) reported that the polymerization rate of G-actin was accelerated by ∝-actinin. The gelation of F-actin was also evoked by this protein. It was shown that ∝-actinin failed to interact with myosin. The studies of Seraydarian et al. (1967) partially support the work of Ebashi.

Masaki et al. (1967) have shown ∝-actinin to reside at the Z-lines. They believe that it constitutes from one-half to one-third of the Z-line material. Briskey et al. (1967) also proposed that ∝-actinin represents Z-line material, as did Goll et al. (1967). This work of Briskey has shown 0.9 parts of ∝-actinin to bind to one part of actin.

β-Actinin

A third regulatory protein has been described by Maruyama (1965a, 1965b). He reported that this protein hinders the formation of F-actin networks in solution. In the presence of this "actin factor", F-actin particles are obtained which measure about one micron in length. Without β-actinin, the actin filaments routinely reach lengths of eight microns. Thin filaments in situ generally measure approximately one micron. One part of β-actinin was shown to bind to ten parts of actin. It is theorized that this newly described protein binds near the ends of thin filaments, thereby controlling filament length. Its globular nature possibly restricts its binding along the entire length of the filament.

Differences Between Fast and Slow Muscle

Physiological variations

Neurophysiologists have demonstrated two types of muscle fiber response to stimulation (see Wiersma, 1961 for a review; Atwood, 1963; Atwood, 1965). It has been shown in certain situations, that the type of response is due to an inherent property of the muscle fiber. That is, two types of striated muscle appear to exist. (Actually, intermediate forms are also present.) The first fiber type is termed fast or phasic. These muscle fibers, like the frog gastrocnemius, can respond maximally to a single stimulus. If the muscle

is stimulated again without being given an opportunity to relax, the contracted state or active state is maintained. The muscle is said to enter tetany. Fast fibers are generally believed to contract in an "all-or-none" manner. However, these fibers are rather rare in the animal kingdom. The second class of fibers is the slow or tonic type (e.g., opener muscles in crayfish claws or vertebrate postural muscles). Here repetitive stimulation is required to achieve a maximal contraction via a graded response. The graded response typically involves summation and facilitation (Florey, 1966). Summation is defined as the addition of local depolarizing potentials (or the subtraction of local hyperpolarizing potentials) to an ongoing local postsynaptic potential. Facilitation involves synaptic transmissions which cause an increase in subsequent postsynaptic responses. Tetany results only after very extensive stimulation. It may last for considerable periods of time.

Metabolic pathways

Smit (1958) observed that slow insect fibers possessed more lipid and a stronger reducing capacity for methylene blue. Gauthier and Padykula (1966) later detected a similar variation in vertebrate material. The early work encouraged George and Bhakthan (1961) to investigate lipase activity in fast and slow fibers. They showed fast fiber lipase activity to be close to half that of certain slow fibers.

These findings have led to the suggestion that fast fibers utilize carbohydrates, slow fibers lipids (Hoyle, 1967). However, the evidence is fragmentary.

Myosin ATPase activity

A most striking study has been reported by Barany et al. (1965). They have isolated myosin from fast (gastrocnemius) and slow (soleus) rabbit muscle and have examined the ATPase activities of each. The slow muscle had a two to three times lower actin-activated, EDTA-activated and calcium-activated ATPase. Since contraction time in the soleus is 2.5 times that of the gastrocnemius, it was suggested that shortening is proportional to the rate of ATP hydrolysis. Also, the slow myosin ATPase activity, unlike the fast, failed to increase at alkaline pH. Although myosin and actin concentrations were equal in the two fiber types, the fast muscle contained more sarcoplasmic and less stromal proteins. Seidel et al. (1964) have also reported a higher ATPase activity in fast myosin.

Barany et al. (1964) have noted a close similarity between slow rabbit myosin and rabbit cardiac myosin. They suggested that this may represent a common physiological demand. That is, over reasonably long periods both may be subjected to considerable activity. The heart contracts only briefly, but in rather rapid succession. Slow muscle may maintain a single contraction for long periods.

Ultrastructure of slow muscle

The early work of Kruger (1929) suggested that an unusual histological appearance was displayed by certain frog muscle fibers. The proportion of these fibers was similar to the fraction of fibers able to yield typical slow contractions. The unusual appearance was termed "Feldenstruktur" in contrast to the "Fibrillenstruktur" of twitch fibers. Peachey and Huxley later (1962) reported a comparative EM study of fast and slow frog fibers in which they dealt with fibers that had been examined physiologically prior to fixation. They found fast myofibrils to be more clearly delineated by sarcoplasmic elements, and triads and M-lines were absent in tested slow forms. Z-lines were thicker and less regular in all of the slow muscles studied. Thus, a definite ultrastructural variation did appear to exist between the two fiber types.

Page (1965) extended these observations of frog material, and she found the slow Z-line to possess no ordered structure. Possibly related to this, was the finding that thin filaments lacked a regular order even close to the Z-line. Since M-lines were absent in slow muscle, no cross-links occurred between thick filaments as in fast fibers. Although she observed triads in slow forms, the number was considerably less than in twitch fibers. Sarcomere lengths were comparable in the two fiber types.

Fahrenbach (1967) has found arthropod slow fibers to

possess greater sarcomere lengths and greater thick filament diameters. Because the slow properties of tonic muscles vary, certain structural characteristics may likewise differ. However, it appears that slow arthropod muscles always exhibit rather long sarcomeres (8-12 μ in contrast to 1.5-3 μ). Fahrenbach has also shown that a given thick filament is surrounded by as many as 12 thin filaments--not just 6 as in fast muscle.

On the basis of the fact that slow fibers had longer sarcomeres and more thin filaments, Fahrenbach (1967) proceeded to search the literature for reports of slow fibers. He found this muscle type to be present in a wide variety of cases as either a postural musculature (body wall, "locking" and proprioceptor muscles) or a visceral musculature (digestive, reproductive and circulatory systems).

Anatomy and Physiology of the Spider Heart

Heart anatomy

The spider heart is a rather muscular tube, lying dorsally in the abdomen (Snodgrass, 1952). Its anterior opening is continuous with the anterior aorta, as is the posterior opening with the posterior aorta. The heart is attached to the integument covering the dorsal surface of the abdomen and it is surrounded by a pericardium. Suspensory ligaments assist in supporting the heart. Paired openings in the heart wall, the ostia, allow the heart lumen to communi-

cate with the pericardial cavity.

Wilson (1967) has reported a dorsal cardiac nerve in association with a spider heart.

Circulation

Petrunkevitch (1910) has shown the spider to possess an open circulatory system. Hemolymph passes from the heart by means of the anterior and posterior arteries. It permeates the tissue areas of the organism and is eventually aerated at the book lungs. Vessels direct the fluid to the pericardial cavity and it enters the heart by way of the ostia. Certain spiders have been shown to possess hemocyanin (Florey, 1966). This respiratory pigment is believed to function in oxygen transport in a manner similar to hemoglobin in vertebrates.

Cardiac innervation

Heart action may be initiated by either a neurogenic or myogenic mechanism. In a neurogenic system (Florey, 1966), rhythmic activity originates in cardiac ganglion cells. These cells in turn activate the myocardium. In myogenic hearts, however, rhythmic activity resides in the muscle itself. In each type, regulatory nerves (excitatory and inhibitory) may arrive at the heart from the central nervous system.

Rijlant (1933) first theorized that the spider heart was neurogenic in nature, although he possessed no meaningful experimental support. Kanungo (1957) agreed with Rijlant, in

spite of the absence of further information.

The first significant indication of a neurogenic means of innervation in the spider came from Wilson (1967). He described for the first time a dorsal cardiac nerve, which contained nerve cells and sent lateral branches into the heart muscle at various intervals. In another arachnoid, the horseshoe crab, a system had previously been described which the spider heart resembles very much (Carlson, 1905). A cardiac nerve, containing ganglion cells, also sends branches into the crab heart. Prosser (1942) has shown the heart of the mature horseshoe crab to be neurogenic.

However, the presence of a cardiac nerve may not imply that the heart possesses a neurogenic nature. For example, Kanungo (1957) has contributed a myogenic nature to the scorpion heart, on the basis of acetylcholine inhibition and ether insensitivity. And this arachnoid has been shown to possess a cardiac nerve (Police, 1902). Thus, the scorpion would be said to display an innervated myogenic heart. However, Zwicky and Hodgson (1965) claim that the scorpion heart is neurogenic. This claim is based on surgical and electrophysiological techniques. Presumably, the beating of portions of the heart is halted when the cardiac nerve is separated from the associated myocardium.

The work of Kadziela and Kokocinski (1966) suggests that spider hearts may exist in the myogenic class. They

showed acetylcholine to inhibit and adrenalin to accelerate the spider heart. Prosser (1942) has shown acetylcholine treatment to be a means of analyzing modes of innervation. Neurogenic hearts are accelerated by this neurohumor, and innervated myogenic hearts are inhibited. However, it is difficult to compare the work on the spider heart with previous investigations on other invertebrate hearts. Not only were the methods for acetylcholine treatment different, the concentrations were apparently higher than those of Prosser. Furthermore, Needham (1950) has found ether to be a more precise tool for classifying pacemaker types. The ether treatment of a spider heart has not been reported.

Thus, the horseshoe crab and the scorpion, each related to the spider, may possess different mechanisms for triggering heart beat. Kanungo (1957) has observed that modes of innervation also vary within the crustaceans and within the insects. He has noted that the type of innervation bears no relationship to the taxonomic position of the individual crustacean, for example. Therefore, in view of this consideration and the lack of definitive information, it appears that additional studies are required to convincingly place the spider heart in either a myogenic or neurogenic class.

MATERIALS AND METHODS

Earlier in this thesis, the lack of an extensive examination of invertebrate cardiac muscle was noted. With this in mind, an invertebrate system was sought which was: 1) representative of organisms with open circulatory systems, 2) sufficiently large for physiological studies, 3) adaptable to laboratory existence and 4) not too difficult to obtain. Tarantula spiders were believed to satisfy these requirements. Male and female tarantulas, Dugesiella hentzi (Chamberlin, 1940), were obtained from northern Texas, Arkansas and southern Missouri.

Procedures for Structural Examinations

Light microscopy

Spider hearts and leg muscles were dissected out in a physiological spider saline (Chichibu, 1961). They were fixed in Bouin's for 24 hours and then transferred to 80 percent alcohol for 8 hours. This was followed by 95 percent (1 hour) and three changes of 100 percent (10 minutes each). Three changes of methylbenzoate (4 hours, 4 hours and 16 hours) were followed by three methylbenzoate-benzene treatments (3:1 for 15 minutes, 1:1 for 10 and 1:3 for 5). After three 5-minute benzene changes, tissues were placed in benzene-paraffin (1:1) for 15 minutes. Embedment was preceded by three 2-hour paraffin exposures. This procedure

(Martignoni, 1960) prevented excessive hardening of the tissues.

The procedures of Humason (1967) were employed for staining with Delafield's Hematoxylin and Mallory's Triple Stain. Her instructions were also followed for the periodic acid-Schiff (PAS) reaction and Golgi's Rapid Method of staining nerve fibers.

Electron microscopy

Thin sectioning techniques A hybrid procedure was employed for fixation and embedment of EM material (Peachey, 1965; Smith, 1966). A three-hour fixation in cold 2.5 percent gluteraldehyde (pH 7.4 with 0.05 M cacodylate buffer and 0.17 M sucrose) was followed by an overnight rinse in several changes of cacodylate buffered (0.05 M at pH 7.4) sucrose (0.34 M) at approximately 4°C. Post-fixation in an initially cold 1 percent solution of osmium tetroxide (buffered at pH 7.4 with veronal-acetate) was carried out for one hour at room temperature. Dehydration was in a graded ethanol series (50, 80, 95, 100). Treatment was for 5 minutes each, with the absolute alcohol being changed three times (5, 5 and 10 minutes). Dehydration was insured with two 10-minute exposures to 100 percent propylene oxide. Material was then transferred to a mixture of propylene oxide and embedding medium (2:1) for one hour. Epon 812 and Epon-Araldite (Anderson and Ellis, 1965) were the media used in this study. Tissues were finally exposed to pure

embedding mixtures overnight, and transferred to fresh media and polymerized at 60°C for 36 hours.

Thick and thin sections were cut with glass knives on a Reichert Om U2 Ultramicrotome. Thick sections were studied by means of phase microscopy and thin sections were placed on EM grids. Generally, grids were coated with parlodion or formvar and stabilized with carbon. Pease (1964) has reviewed these techniques, as well as most other EM procedures mentioned in this discussion. Grids were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined at either 50 or 100 KV in an RCA EMU-3F electron microscope.

Freeze-etching procedures A Varian vacuum evaporator (VE-30) equipped with a Berkeley freeze-etch device (McAlear and Kreutziger, 1967) was employed in these investigations. Spider heart muscle was fixed overnight in 3 percent gluteraldehyde (buffered with phosphate at pH 7.2) at 4°C. It was rinsed twice with buffer (10 minutes each) and exposed to two 15-minute changes of 30 percent ethylene glycol. The material was inserted in a rolled 400-mesh EM grid and frozen in liquid propane, which had been cooled with liquid nitrogen. The frozen muscle was transferred to a cooled fracturing block and then to the vacuum evaporator. When the vacuum had entered the 10^{-6} Torr range, the specimen was fractured and allowed to etch for from 2.5 to 5.0 minutes. The fracture was shadowed with platinum-paladium wire and replicated with carbon. The replica was retrieved

by placing the specimen in 50 percent ethylene glycol and gradually increasing the Clorox concentration to 100 percent. The replica was rinsed in five changes of distilled-deionized water, picked up on an EM grid and examined by means of transmission electron microscopy.

Rotational printing of thick filaments The Markham rotation technique (Markham et al., 1963) was used to enhance the detail exhibited by selected thick filament cross sections.

Ultrastructural Cytochemistry

Tryptic digestion of Z-lines

Spider leg muscle and rabbit psoas were dissected out and placed in buffered salines. Slow spider leg muscle was substituted for cardiac muscle because: 1) it is more readily oriented for EM sectioning, 2) it can easily be separated into single fibers and 3) its cell surfaces and general organization are more closely related to the rabbit skeletal fibers. Chichibu's (1961) spider saline was used (8.00 grams NaCl, 0.10 KCl, 0.20 $NaHCO_3$, 0.10 $CaCl_2$, and 0.01 NaH_2PO_4 in a liter of water at pH 7.6). Rabbit muscle was placed in a solution that was 0.03 M KH_2PO_4, 0.03 M K_2HPO_4, 0.05 M KCl and 5 mM $MgCl_2$ at pH 6.8. Fibers of these two muscle types were treated simultaneously at 28°C in buffered trypsin (Type III, Sigma; 50 µg trypsin per ml, 100 mM Tris and 100 mM KCl at pH 7.6). Following treatments ranging from 2 to 15 minutes, digested fibers were quickly transferred to soy-

bean trypsin inhibitor (Sigma; 100 µg inhibitor per ml and 100 mM Tris at pH 7.7). After several minutes, fibers were fixed in gluteraldehyde and processed for electron microscopy. Goll et al. (1967) have utilized this trypsin method in their studies of rabbit muscle.

Polysaccharide-specific EM staining

The procedures of Hay (1966) were followed for a theorized polysaccharide-specific staining. Thin Epon-Araldite sections on grids were stained with 1 percent periodate (several minutes), 2.5 percent sodium chlorite (one-half the periodate time) and 2 percent uranyl acetate (4 minutes).

Procedures for Physiological Studies

Investigations with isolated fibers

Spider heart and leg fibers were isolated and placed in a magnesium-containing salt solution (MgSS) devised by Aronson (1962). The MgSS was an aqueous solution of 0.1 M KCl, 5 mM KH_2PO_4, 2 mM EDTA and 4 mM Mg at a pH of 7.0. Fibers were then placed on microscope slides in an excess of one of the solutions being studied. The preparations were examined by means of phase microscopy and photographs were taken on a Wild phase-contrast photomicroscope. When the first reaction solution was followed by a second, the second was added at the edge of the coverslip. Fibers were studied in the following solutions:

1. Relaxing medium -- MgSS made 5 mM with respect to ATP
2. Calcium-ATP solution -- 0.1 M KCl, 4 mM $CaCl_2$, 20 mM ATP and 5 mM phosphate buffer at pH 7.5
3. $CaCl_2$ -- 20 mM at pH 6.8
4. EDTA -- 30 mM at pH 7.0
5. KCl -- 1.0 M
6. ATP -- 50 mM

ATPase activity of spider heart homogenates

The orthophosphate cleaved from ATP and ADP by muscle homogenates was measured according to Mutchmor and Richards (1961). A standard curve was constructed when various volumes of KH_2PO_4 (0.20 mg/ml) were treated with 2.0 ml of 10 percent trichloroacetic acid (TCA), 1.0 ml of 2.5 percent ammonium molybdate and 0.4 ml of an aminonaphtholsulfonic acid solution. (This was prepared by dissolving 6.0 grams of $Na_2S_2O_5$ and 1.2 of Na_2SO_3 in 50 ml of water, adding 0.1 gram aminonaphtholsulfonic acid, filtering to remove insoluble particles and storing in a red bottle.)

Spider and frog muscle were homogenized in a tissue grinder, stored on ice and added to reaction tubes containing 0.2 ml 0.02 M $MgCl_2 \cdot 6\ H_2O$, 1.56 ml 0.038 M barbitol buffer (pH 7.8) and 0.2 ml of 0.02 M ATP or ADP. Incubations at 27°C ranged from 5 to 60 minutes. Enzymatic activity was halted with 2.0 ml 10 percent TCA. Colored products were achieved as with the standards.

Ionic analysis of muscle

Spider and frog muscle were dissected out and washed in isotonic sucrose (0.4 M) for 30 seconds. They were blotted to remove extracellular fluid and weighed before and after oven-drying (65°C) to a constant weight in order to determine water content. The dried tissues were dissolved in hot concentrated nitric acid and diluted appropriately with distilled-deionized water (Atwood and Dorai Raj, 1964). A Jarrell-Ash 82-700 Flame Spectrometer was employed to determine the concentrations of calcium in the two muscle types. For these purposes, standards were prepared from 0.001 M solutions and methods outlined in the "Flame Absorption Methods Manual" (compiled by the Jarrell-Ash Co.) were followed.

For these calcium determinations, a 20 percent glycerol solution of 0.2 N perchloric acid was added in a ratio of 1:1. This volatilizer protects against the interference of phosphates and sulfates in calcium absorption. The H_2-flame is not sufficiently hot to break down bonds between calcium and either of these anions. Therefore, calcium is not volatile and low values are acquired without the volatilizer.

Ether and acetylcholine treatments of spider hearts

Spiders were placed in the ether-saturated atmosphere of a fiberglass box equipped with a glass top. Temperatures within the box were recorded. An incision in the integument on the dorsal surface of the abdomen exposed the heart, and

observations were made with a dissecting microscope. Heart rates and comparisons of cardiac and somatic activity were recorded. In one instance, an EKG was run on the heart of an etherized animal.

Exposed hearts were also treated with acetylcholine (Nutritional Biochemicals, Inc.) at concentrations ranging from 10^{-8} to 10^{-5} M. The acetylcholine was removed with washes of spider saline (Chichibu, 1961). EKG records (Physiograph Four, E and M Instruments) of these experiments were obtained by placing one silver wire electrode on the surface of the heart and one elsewhere on the organism.

RESULTS AND DISCUSSION

Structural Studies of Spider Cardiac Muscle

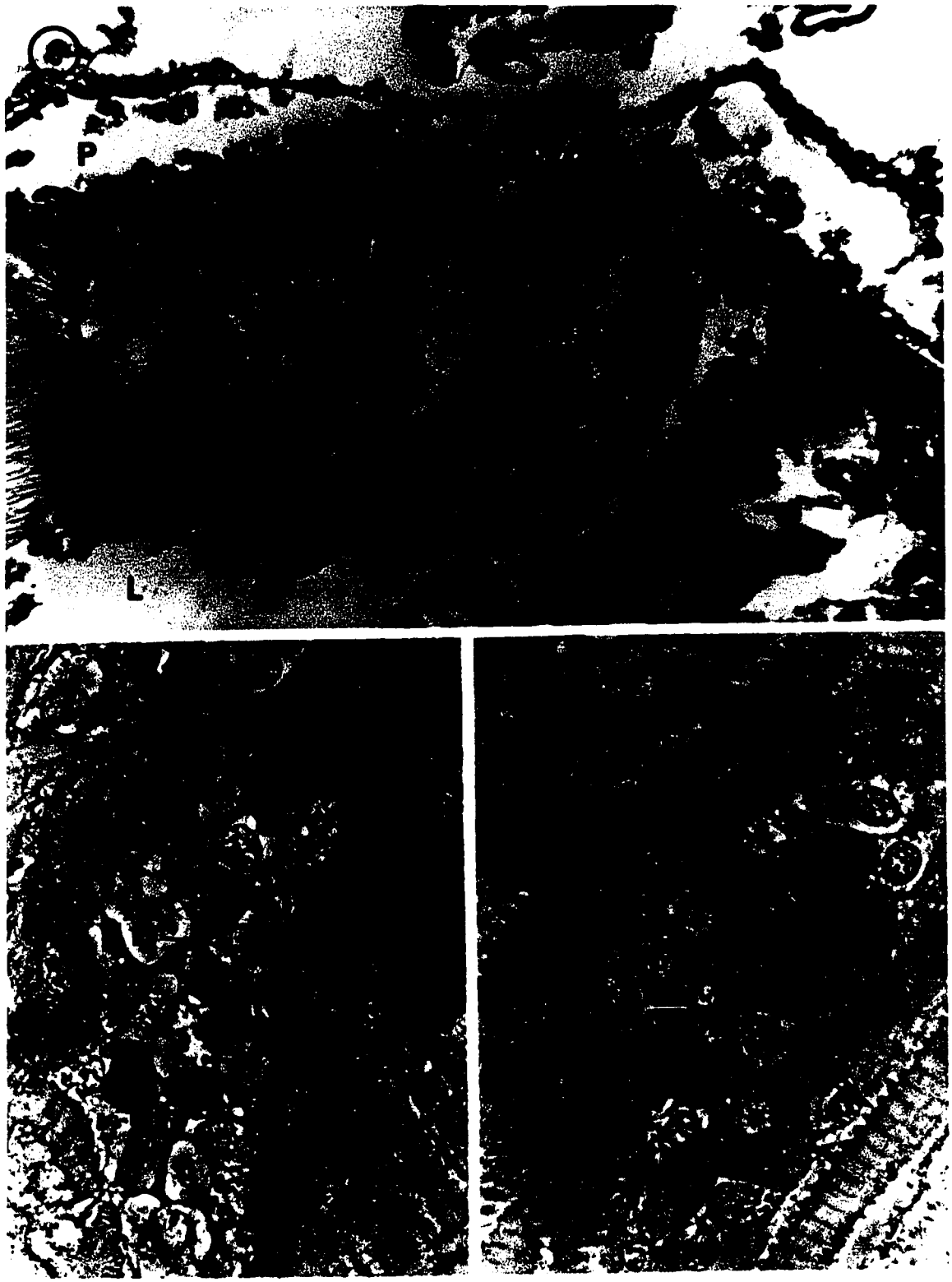
Anatomy and histology

In light microscope studies, the tubular nature of the spider heart is very apparent. In the tarantula, the heart generally measures 10 mm in length and approximately 3 mm in diameter. In cross sections, longitudinal muscle ridges appear as finger-like projections into the heart lumen (Figure 8). These ridges are prominent and routinely reach lengths of 0.4 mm. As a result of this structural pattern, the internal surface area of the heart is extensively increased. Thus, the metabolic needs of these active cells may be served rather effectively. The longitudinal ridges, when closely packed together, create the illusion of a thick heart wall. However, the wall is actually very thin, often measuring on the order of a hundred microns. The heart possesses no internal covering, instead the muscle is directly exposed to the hemolymph. In thick sections cut from EM blocks, the outer surface of the heart appears to be covered by smooth muscle cells. Zwicky and Hodgson (1965) have also observed a thin, superficial layer of smooth muscle covering the scorpion heart. The pericardium surrounding the spider heart is composed of epithelial and connective elements. Limited strands of muscle are also present. Blood cells and blood protein are not only seen in the pericardial cavity,

Figure 8. Photomicrograph of a heart cross section. A pericardial cavity (P) is formed by the pericardium surrounding the heart. Longitudinal muscle ridges (R) extend into the lumen (L) of the heart and vessels (V) are often observed, which possibly are directed to the pericardial space. X 460.

Figure 9. Phase contrast micrograph of a longitudinal section of fibrils in the muscle ridge. A typical banding pattern is observed with A-bands (A), I-bands (I) and Z-lines (Z). H-bands (H) lack the distinct qualities of phasic muscle fibers. A possible pool of mitochondria (M) is illustrated. A scalloped border (S) is apparent. Blood cells are seen between muscle ridges. X 630.

Figure 10. Phase contrast micrograph of a muscle ridge. A shorter sarcomere length is shown here in contrast to Figure 9. Note that the scalloped borders (S) of the muscle cells have been compressed as a result of contraction. X 630.


P
L

but also between longitudinal muscle ridges. Deevey (1941) has characterized tarantula blood cells. Ostia are not prominent features in the histology of the spider heart.

The spider heart is cellular and multinucleated, a finding supported by EM observations. The striated nature of the cardiac muscle is evident throughout the heart (Figure 9). Z-lines are prominent in their division of the I-bands and the H-bands are less discrete than in fast muscle systems. The scalloped border of a muscle ridge is frequently observed (Figure 9). The base of each scallop is seen to correspond to the level of the Z-line. Mitochondria are concentrated within the scallops (supported by EM studies). Since not all longitudinal sections display scallops, this configuration was presumed to be the result of an orderly, linear distribution of pit-like sarcolemmal invaginations. It was believed that sections could be cut between the pits and the scalloped effect would be lacking. However, freeze-etch studies suggest that the invaginations are grooves, not pits. This surface modification is often apparent in muscle ridges, which are seen to correspond to single muscle fibers (Figure 10). Pools of mitochondria lie between the myofibrils of each surface. Although this appearance occurs as the result of a heart cross section, the sections of the muscle fibers are clearly longitudinal.

Along the mid-dorsal line of the tubular heart, lies a

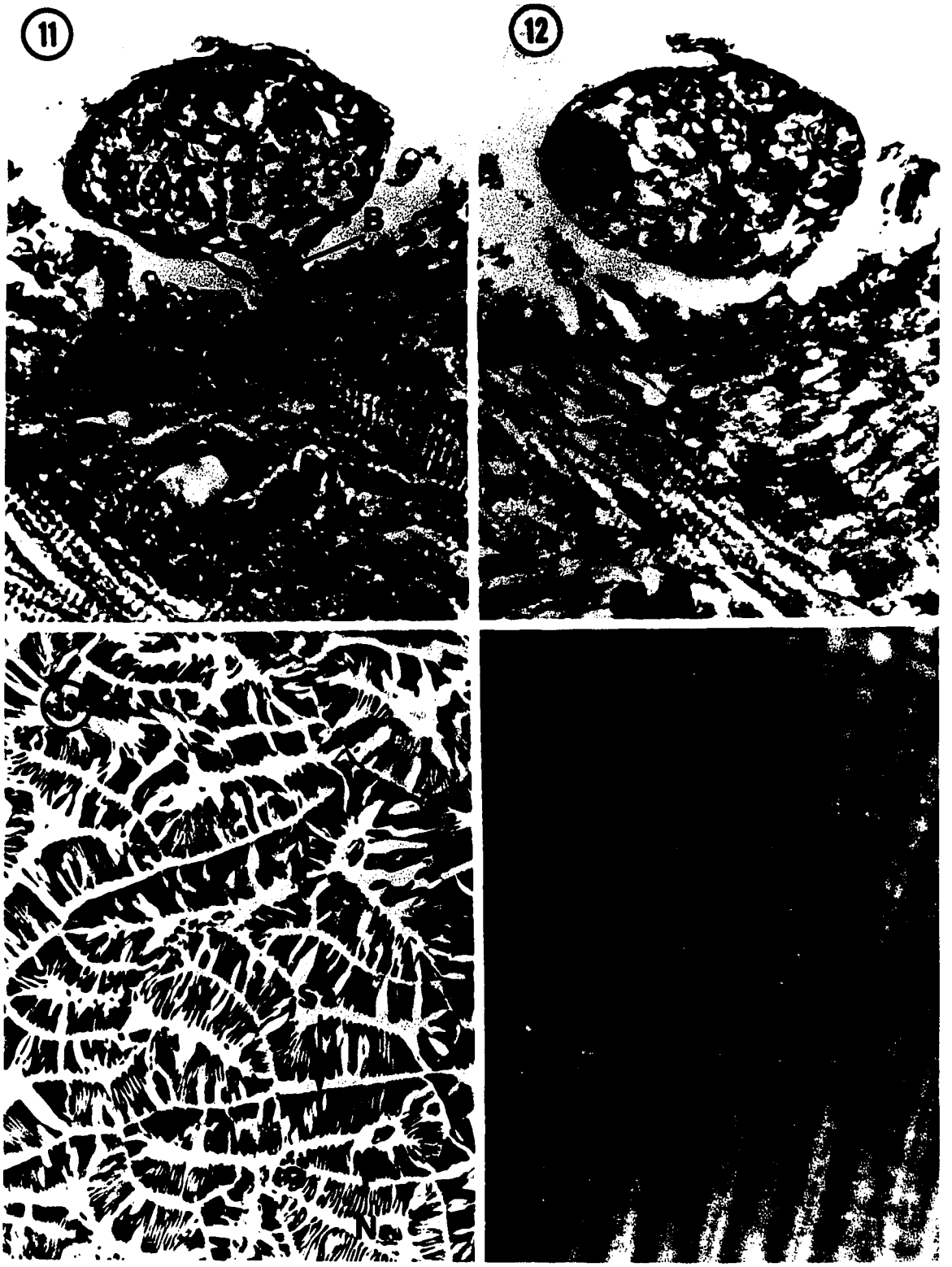
dorsal cardiac nerve. Wilson (1967) has reported the existence of this nerve in the spider, although he has not supported his findings with structural evidence. In the tarantula, the cardiac nerve runs along 80 percent of the heart and sends numerous branches into the myocardium (Figure 11). Because the nerve often lies in very close contact with the heart, it is difficult to determine the precise number of these branches. Certainly, the number exceeds 15. The nerve contains at least two different cell types. The majority of cells are characterized by small, elliptical nuclei. The second cell class consists of much larger cells with large, round nuclei (Figure 12). It is believed that the small cells are Schwann cells (supported by EM studies) and the large ones ganglion cells. Generally, the ganglion cells are associated with the nerve branches which enter the heart, the ganglion cells lying in the nerve slightly anterior to the branches. There are approximately 40-55 ganglion cells in the nerve. Zwicky and Hodgson (1965) have reported 60-100 similar cells in the scorpion cardiac nerve. It appears that ganglion cells may be concentrated in five regions along the spider cardiac nerve; however, these regions are not crisp. Snodgrass (1952) suggests that the tarantula heart possesses four pairs of ostia. This would divide the heart into five segments and five ganglion-rich regions would be most appropriate. The possible involvement of the ganglion

Figure 11. A branch from the cardiac nerve passing towards the heart. This photomicrograph of the dorsal heart surface illustrates a nerve branch (B) directed towards the myocardium. X 840.

Figure 12. Photomicrograph of a ganglion cell in the dorsal cardiac nerve. A large ganglion cell (Ga) with a correspondingly large nucleus is illustrated slightly anteriorly to a nerve branch. Smaller elliptical nuclei (N) are believed to be those of Schwann cells. X 840.

Figure 13. Photomicrograph of a cross section of spider leg muscle. In this modified tubular type of muscle, the myofibrils (M) extend from the sarcolemma (Sa) into the center of the fiber, which contains the nuclei (N). The sarcolemma does not form a strict cylinder, and folds (F) in the surface are common. X 1080.

Figure 14. Phase contrast micrograph of leg muscle longitudinal section. The typical striations of muscle fibers are present: A-bands (A), I-bands (I), H-bands (H) and Z-lines (Z). The sarcomere lengths in this relaxed muscle average 5.4 μ. X 3500.


11
12
B
S
N

cells in initiating the heart beat will be discussed later with related physiological investigations.

Spider leg muscle appears to be a modified tubular type of striated muscle (Figure 13). Myofibrils are plate-like and extend from the sarcolemma into the cell interior, where nuclei are found. These long plates run parallel to the axis of the fiber. Striated fibers with short and long sarcomeres are found in the spider leg. The great majority are long (Figure 14) and probably "slow" physiologically (Fahrenbach, 1967). Fibers with short sarcomeres contain many more mitochondria. This morphological recognition of both fast and slow muscle could possibly be expected, since Chichibu (1961) has detected fast and slow responses in the spider leg.

Ultrastructure of cardiac muscle

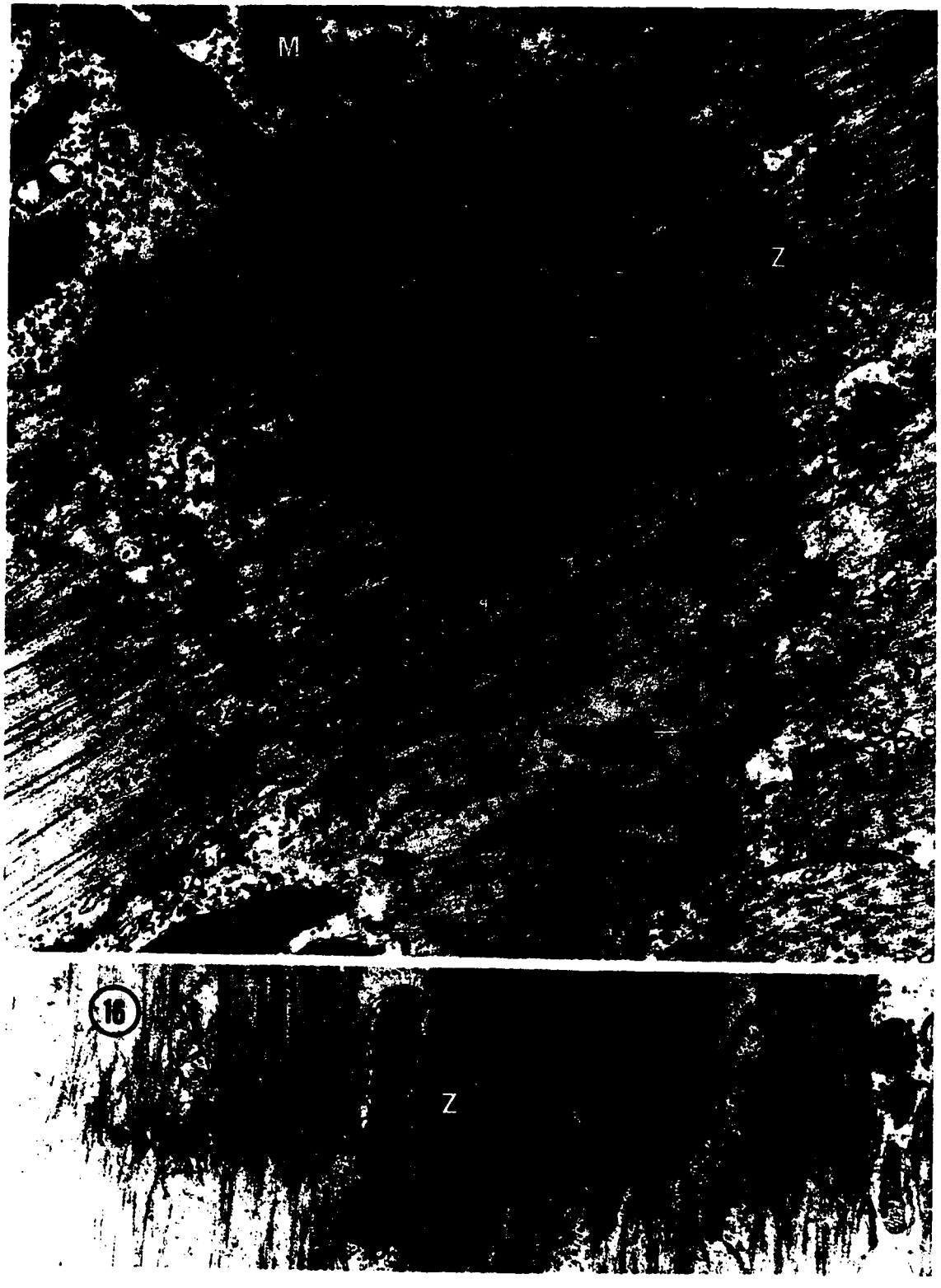
Banding patterns and general features. The ultrastructure of longitudinal sections of spider cardiac muscle (Figure 15) is characterized by two classes of myofilaments and broad Z-lines. The diameters of thick filaments average 230-240 Å; thin filament diameters measure 60-70 Å. In addition, H-bands are generally not apparent and M-lines are absent.

Recall, however, that H-bands were visualized with the light microscope (Figure 9). They were not clearly delineated and, therefore, it is believed that thin filaments in

Figures 15 thru 44. Electron micrographs of spider cardiac muscle. All of the micrographs involve sections which were stained with uranyl acetate and lead citrate.

Figure 15. Longitudinal section of cardiac muscle. This 3 μ sarcomere displays broad Z-lines (Z) and two classes of myofilaments. The central A-band (A) is flanked by limited I-bands in this moderately contracted sarcomere. Mitochondrial (M) aggregates and dyads (D) are present. The fenestrated collar of the SR (SR) appears to surround the myofibril. The longitudinal section is adjacent to a more oblique section (O). X 33,300.

Figure 16. Z-line region of supercontracted muscle. Thick filaments (AF) can be observed to pass through the Z-line (Z). Hoyle et al. (1965) have shown thick filaments to extend through the Z-discs of barnacle muscle during neurally evoked supercontractions. However, the majority of filaments were bent back in non-neurally evoked contractions. Whether this interpretation is valid in spider heart muscle is not known. X 21,200.


M
Z
16
Z

this cardiac muscle do not all terminate at the same level of the sarcomere. Because the H-band "begins" at a large number of different levels, reinforcement must occur before the H-band may be visualized. It is suggested that reinforcement exists in thick light microscope sections, but not in thin EM sections. This reinforcement would result from the greater depth of field involved with the light microscope. In a section 2 μ thick, there would be over 100 layers of thin filaments. Thin sections (approximately 500 Å) would only contain several thin filament layers (Figure 1). This number would not be sufficient to significantly reinforce the precise borders of the H-band, but it would be able to obscure the H-band on a given filament level. Only a possible low density of thin filaments in the center of the H-band would be displayed. It would be necessary to cut sections approximately 150 Å in thickness to insure clear demonstration of thin filament terminations.

A second factor may have also contributed to the inability to demonstrate H-bands at the EM level in this study. Almost all muscle examined was in a contracted state. On several occasions, supercontracted muscle was even observed (Figure 16). With contraction, H-bands areas would be very limited. It should be noted that H-bands were correspondingly scarce at the light level. Serious restrictions were placed on attempts to physically maintain the relaxed state. In future efforts with this system, attention should be directed

to a physiological means of guaranteeing relaxation. Investigations with isolated muscle, to be reported later, indicate that placing EDTA in the fixative may eliminate contracted states. North (1963) was plagued with a similar problem in his investigation of the fine structure of the snail heart.

deVillafranca (1961) and deVillafranca and Philpott (1961) have observed H-bands in horseshoe crab striated muscle with the light microscope. However, as in this study of spider cardiac muscle, H-bands were not apparent at the EM level. (Essentially the same thing is true of spider leg muscle.) One of their micrographs demonstrated the presence of a thin filament in the approximate center of the A-band. Immediately adjacent to this point were several regions containing only thick filaments. They interpreted these findings to indicate that crab thin filaments ran through the center of the A-band and, perhaps, through the entire sarcomere. It is theorized here that this represents the lack of a uniform termination of thin filaments. In both the crab and spider muscle, the A-I junctions are less discrete than in vertebrate fast muscle. There is a certain staggering of thick myofilaments. It would, therefore, not be at all unexpected to find a similar effect with actin filaments as they end near the A-band center. Furthermore, Z-lines are "wavy" and not straight as in vertebrate muscle; if thin filaments were of a uniform length, some would necessarily extend further into the A-band.

All banding features described above have been contributed to slow vertebrate muscle by Peachey and Huxley (1962) and Page (1965). In addition, Fahrenbach (1967) has observed these features in slow arthropod muscles. Clearly, the larger class of filaments is not bound together at an M-line as in fast muscle. In addition, the smaller class of filaments is attached to a broad Z-line. When the slow Z-line is cut transversely or obliquely (Figure 28), the Z-line material appears to lack any fine organization which is found in fast forms. The thin filaments neither appear to display any ordered arrangement close to the Z-line, nor are "sleeves" apparent on the filaments at this level. In fast muscle, thickenings are noted on thin filaments immediately adjacent to the Z-line (Knappeis and Carlson, 1962; Franzini-Armstrong and Porter, 1964a; Page, 1965). Spider cardiac muscle Z-lines reflect this generally amorphous nature of the slow Z-lines, in contrast to filamentous fast forms.

In contrast to the sarcomeres of the other slow arthropod muscles studied by Fahrenbach (1967), which measured 8 to 12 μ in length, spider heart sarcomeres are rather short (Figure 15). Sarcomeres average 3 μ in length. Other slow cardiac muscles in invertebrates also possess short (1-3 μ) sarcomeres (Edwards and Challice, 1960; North, 1963). Cross sections reveal the presence of 8 to 12 thin filaments in an unorderly array around a given thick myofilament (Figures 17 and 28). This is characteristic of slow muscle.

Since the thick spider filaments measure approximately 240 Å in diameter, they are significantly larger than typical fast arthropod filaments. Fahrenbach (1967) found fast arthropod thick filaments to measure 160 Å, and slow 240 Å in diameter. Spider heart thin filaments (60-70 Å) are comparable in diameter to fast I-filaments. Hagopian and Spiro (1968) suggest that larger thick filament diameters generally imply rather extensive filament lengths. This does not appear to apply to spider thick filaments, in that they are only approximately two microns long.

Since Krüger (1929) first theorized that slow or tonic muscle is characterized by a "Feldenstruktur", numerous workers have substantiated this correlation in vertebrate (Peachey and Huxley, 1962; Hess, 1965; Page, 1965) and arthropod muscle (Fahrenbach, 1967). Therefore, there is considerable support for the belief that slow striated muscle is morphologically distinct from fast. Furthermore, the extensive structural similarities between typical slow muscle and spider cardiac muscle have been presented. As a result, spider cardiac muscle has been assumed to be a slow striated muscle system in this study.

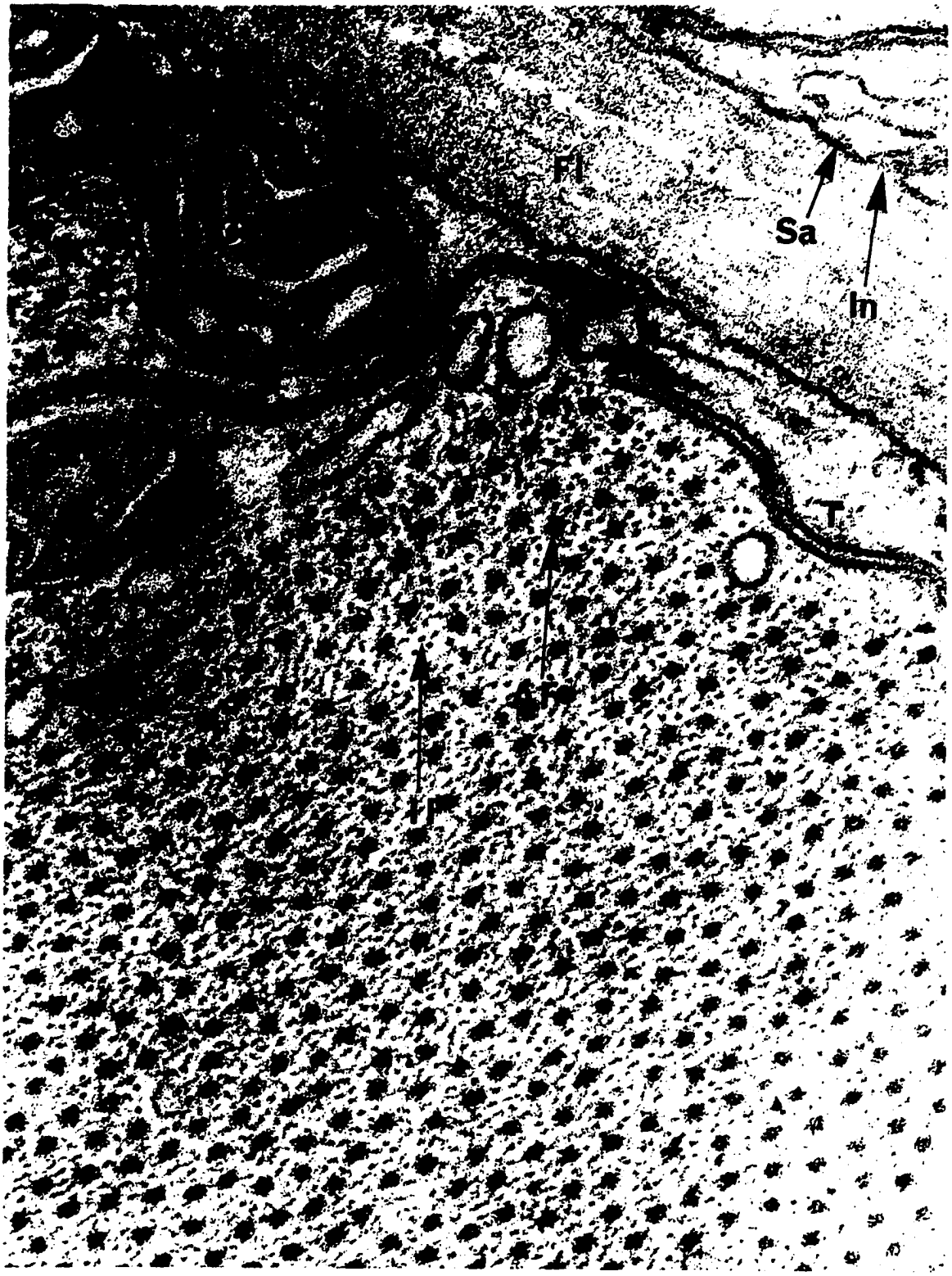
There is a need for clarifying the exact nature of the physiological slow quality in cardiac muscle. North (1963), Kawaguti (1963b) and Nisbet and Plummer (1966) have reported ultrastructural studies on snail cardiac muscle and their evidence suggests that this too is a slow muscle system. The

last investigation clearly shows the snail heart to be innervated, and it is known to be myogenic (Krijgsman and Divaris, 1955). Furthermore, the myogenic nature of the innervated mammalian heart is well documented (Florey, 1966), and it is apparently a fast striated muscle system (Stenger and Spiro, 1961). Therefore, fast or slow features are not necessarily related to a given mode of rhythmic activity. Instead, as with other slow striated muscles, the slow feature is reflected in the cardiac muscle's inherent inability to respond to nervous stimulation in a phasic manner. Presumably, a slow myogenic cardiac muscle would also be restricted to a graded contraction in association with its rhythmic functions, where nerves need not participate.

The sarcoplasmic reticulum (SR) is prominent in spider cardiac muscle, and dyads are commonly observed (Figure 15). The SR is characterized by its formation of fenestrated collars around myofibrils. These collars are very similar to those described by Peachey (1967) in his study of crab fibers. This distribution of the SR creates a most effective means of providing sites for the theorized storage and release of calcium. Mitochondria are well-developed and rather abundant. They tend to be present in pools of what is believed to be glycogen.

Filaments At high levels of magnification, thick filament cross sections display centers of a slightly lower electron density than the peripheries (Figure 17). As mentioned

Figure 17. Filament cross sections. Thick filaments (AF) are shown to be surrounded by 8 to 12 thin filaments (IF) in this cross section of an A-band. Thick filaments are somewhat less electron-dense in their centers. The sarcolemma (Sa) possesses a unit membrane structure and is observed to invaginate (In). These invaginations form t-tubules (T) that pass into the cell. Cristae (C) are prominent in mitochondria underlying the cell surface. This surface possesses a fibrous covering (Fi). X 94,100.


Fl
Sa
In
T
P

above, these 230-240 Å filaments are surrounded by 8 to 12 thin filaments (60-70 Å). Not only do slow I-filaments lack the typically ordered fast muscle distribution, but thick filaments also fail to lie in long straight rows. The degree of order is typically lower in this slow muscle system, than in fast fibers.

Because the thick filament diameters are increased approximately 50 percent over fast diameters, a corresponding alteration in thick filament substructure was questioned. Since Barany et al. (1965) have shown differences in fast and slow myosin ATPase activities, some possible support for an alteration of molecular architecture did exist. Therefore, selected thick filament cross sections were rotationally printed so that any filament substructure would be reinforced. There was a clear enhancement of detail with n=6 (Fahrenbach, 1967), while this enhancement did not exist with n=5, 7, or 9 (Figures 18 thru 27). The enhanced substructure consists of six pairs of spheres, each located at one corner of the hexagonal core. It is theorized that the peripheral spheres correspond to thick filament cross-bridges (largely HMM), and the core would then represent the filament shaft (largely LMM). If this interpretation is correct, slow thick filaments show close similarities with their fast counterparts. Huxley (1957) and Reedy (1967) showed neighboring cross-bridges to be separated by approximately 60 degrees, when viewed in cross section. Thus, the increased size of slow thick fila-

Figures 18 thru 27. Rotationally printed thick filament cross sections. The first five prints represent early attempts at enhancing the substructure of thick spider filaments. It seemed that six bridges or units were visualized in this cross sectional treatment. These branches presumably correspond to the cross-bridges of Huxley (1957). However, more sophisticated efforts resulted in the demonstration of a more complex arrangement. Although the six bridges continued to be present, the peripheral spheres now numbered 12. It appeared that each bridge gave rise to a pair of arms. This surprising demonstration may relate to the cross-bridge organization theorized by Reedy (1967).

Figure 18. Printed with n=6, and a reinforcement occurs. X 353,000.

Figure 19. Printed with n=6. Substructure is enhanced. X 353,000.

Figure 20. No significant enhancement occurs with n=5. X 253,400.

Figure 21. A reinforcement occurs once again with n=6. X 253,400.

Figure 22. Printed with n=7. X 253,400.

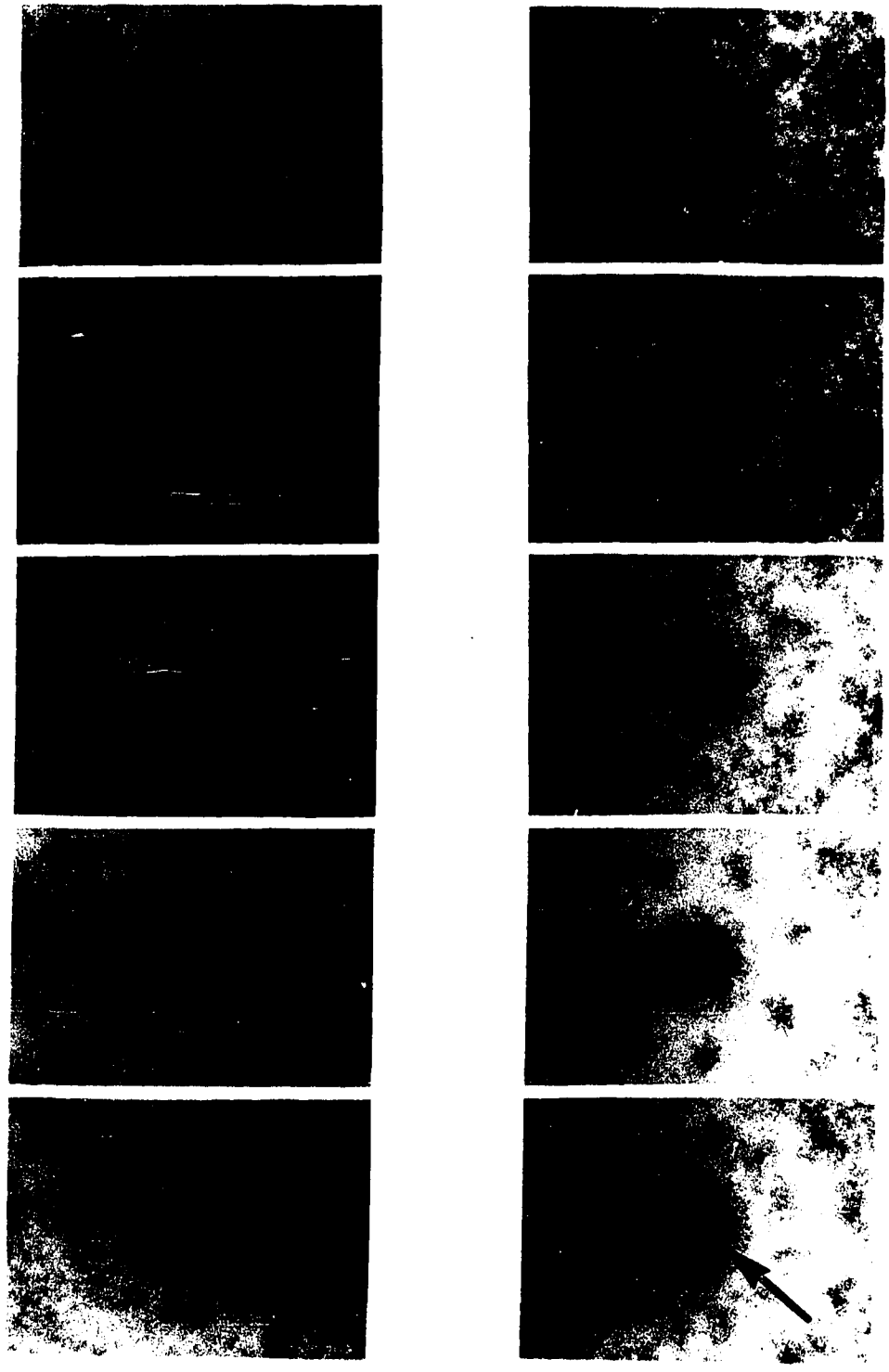
Figure 23. Printed with n=5. X 398,000.

Figure 24. Printed with n=6. The thick filament shaft displays a sixfold substructure as noted previously in Figure 18. However, there appears to be an electron-dense unit adjacent to each side of each cross-bridge. X 398,000.

Figure 25. Printed with n=7, and no enhancement appears significant. X 398,000.

Figure 26. Printed with n=9. X 398,000.

Figure 27. Printed with n=12. A clear twelvefold peripheral organization is exhibited. Twelve bridges are also displayed. However, it is suggested that each of the six cross-bridges is printed twice. This suggestion is based on the lack of a twelvefold bridge pattern when rotated with n=6. Reedy's (1967) concept of paired arms at a single origin has been presented (Figure 5). X 398,000.



ments does not influence an extensive, associated alteration in substructure. Therefore, it is suggested that other factors may contribute more significantly towards granting a slow character to tonic muscle. The increased number of slow thin filaments, and their lack of a fine organization, could be such factors. That is, if slow thick filament substructure is sixfold as is fast, a non-hexagonal thin arrangement would not as effectively provide for an alignment of reactive sites. Since thick filaments link with thin during contraction by means of cross-bridges, the processes of contraction would be promoted by an alignment of HMM-bridges and actin filaments.

Sarcolemma, t-tubules and SR When spider heart muscle is sectioned transversely or obliquely, the intracellular membrane systems are well displayed (Figure 28). The myofibrils are surrounded by membrane spheres or flattened sacs. These are the cross sectional attributes of the fenestrated collar of the SR, which was noted earlier in longitudinal section (Figure 15). These components of the L-system are associated with tubules in structures termed dyads. Spider cardiac muscle dyads generally lie close to the A-I junction. It was noted previously in reviewing the literature, that dyad and triad positioning was a constant feature of a given muscle system.

The muscle cell membrane, the sarcolemma, displays the typical unit membrane structure. The sarcolemma is frequently

Figure 28. An oblique-section of the myofibril. An A-band (A), I-bands (I) and Z-lines (Z) are characterized in this electron micrograph. A possible tapering of thick filaments occurs near their terminations at the A-I junction. Longitudinal components of the SR (SR) are displayed, and dyads (D) are formed by elements of the L-system and t-tubules (T). The t-tubules may form more than one dyad as they course into the cell. Mitochondria (M) are pictured, and probable glycogen (G) particles are seen as single granules and rosettes. X 37,400.


G
Z
Z
G

seen to invaginate as 200-400 Å tubules (Figures 29 and 30). These tubules are observed to be identical with those noted to associate with the SR as dyads (Figure 29). T-tubules course deep into the muscle, forming numerous dyads along the way. Smith (1966), among several workers, has reported a similar observation from his investigations of a slow insect muscle system. In addition, numerous efforts were previously reviewed which indicated that this association provided a pathway for excitation-contraction coupling in fast muscle. Considerable support exists for the fact that membrane depolarization of t-tubules (in reality the sarcolemma) effects the release of calcium from the SR. The work of Weber et al. (1964) has shown calcium to activate the contractile apparatus. Since dyads in spider muscle appear as the counterpart to fast muscle triads, it would seem likely that the means of excitation-contraction coupling in these systems is similar. Selverston (1967) supports this hypothesis. It will be shown later that calcium elicits contraction in spider muscle, but a demonstration of t-tubule function is lacking in this system. Future experiments could effectively employ the t-tubule disorganization procedure of Gage and Eisenberg (1967). Disorganizing the tubules should eliminate contraction, or at least tremendously reduce it.

Surface specializations A fibrous material covers the cardiac muscle fibers (Figures 31 thru 33), and it has been observed on several occasions to contain unusual

Figure 29. Formation of t-tubules. The sarcolemma (Sa) is observed to invaginate at several points to form t-tubules (T). These tubules approach elements of the SR to create dyads (D). It is theorized that dyads serve as an essential link in the process of excitation-contraction coupling. X 37,400.

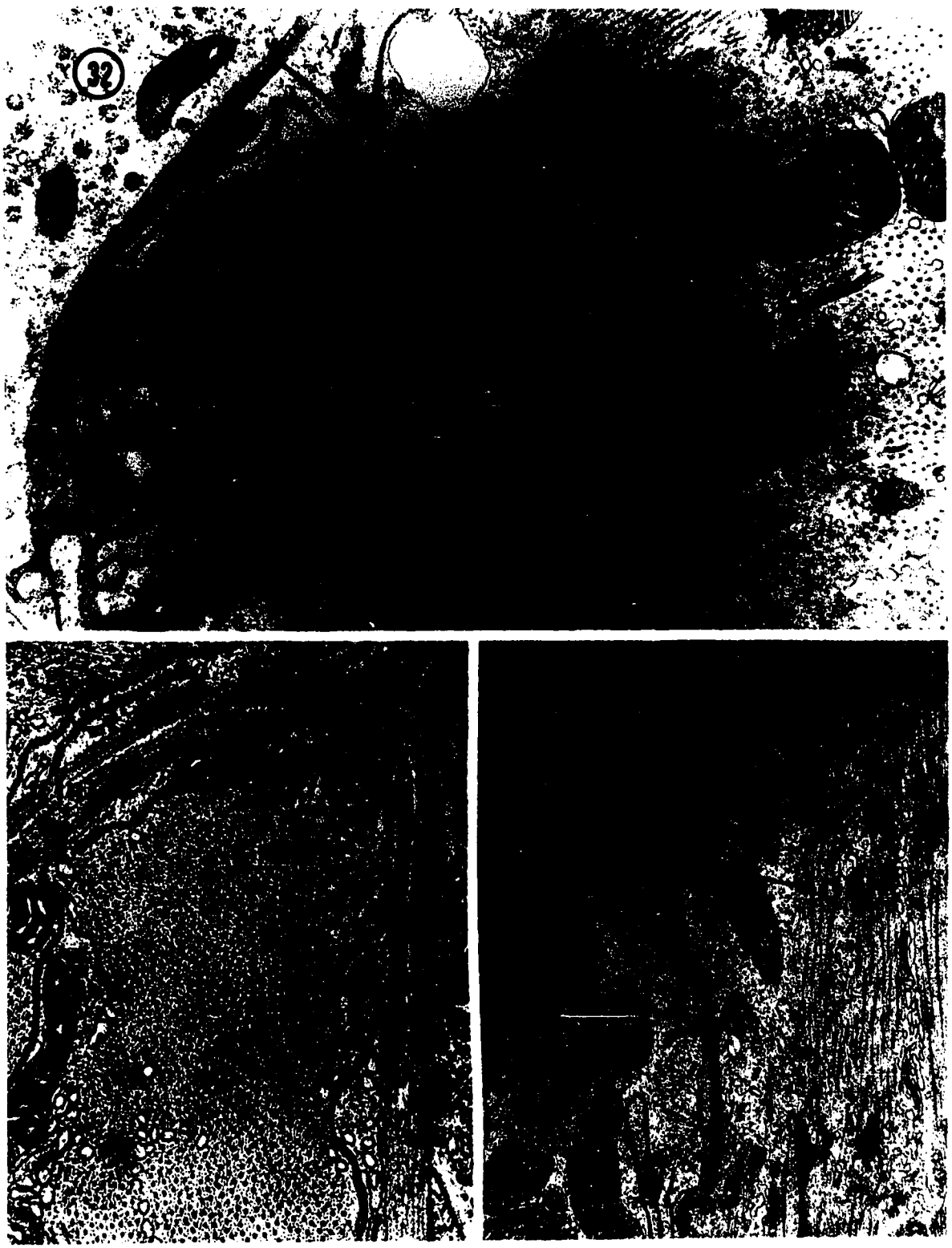
Figure 30. Sarcolemmal invaginations. The initiation of two t-tubules (T) is seen here at a high magnification. The two cells displaying t-tubules appear to be separated by an extension (E) of a third cell. This strip of the third cells' cytoplasm is surrounded by an extracellular space. The extracellular space is continuous with the t-tubule lumen. X 50,580.

Figure 31. Organization at the surface of the cardiac muscle cell. Unusual structures (U) exist in the fibrous cell coat. The large-scale invagination (In) of the cell surface may represent an oblique-section through a scallop. The underlying filaments have been sectioned obliquely. Dyads (D) are present. X 40,400.

Figure 32. Scattered elements of the SR (SR) at the muscle fiber surface. Another view of the cell surface is presented. X 28,300.

Figure 33. Junction of three muscle cells. The cellular nature of the spider heart is illustrated by this junction involving three cells. The fibrous coat of each cell appears to be distinct from that of adjacent cells. Numerous tubules (T) are displayed, and certain probable initiation points (In) suggest they are t-tubules. X 25,600.

Figure 34. Membrane densities on intracellular surfaces. Electron-dense plaques (DP) are frequently observed internally on membrane surfaces. It is possible that they are involved in the adhesive properties of these muscle cells. However, intermembranous distances are often not reduced. These distances are reduced in the intercalated discs of vertebrate cardiac muscle (Stenger and Spiro, 1961). X 18,860.


32

cylindrical structures (Figure 31). They uniformly measure several hundred angstroms in diameter and their length is often seen to be several times their diameter. The nature of these structures is unknown. A fibrous surface material has been noted in crab muscle by Peachey (1967) and in crayfish cardiac muscle by Kawaguti (1963a). This cover may influence permeability to a limited degree; however, it does not appear to pass down into t-tubules.

The heart of the spider is not syncytial, but clearly cellular (Figure 33). Single cells have been observed to contain several sections through nuclei, separated by considerable distances. Therefore, the cells are believed to be multi-nucleated. It was just noted that a fibrous material is found on the cell surfaces. This covering could conceivably assist in holding cells together. No comparable covering exists in vertebrate muscle, however, a basement membrane material and a network of reticular fibers covers the muscle fiber surface (Bloom and Fawcett, 1962). Cellular adhesion in the spider heart may also be enhanced by electron-dense membranous plaques (Figures 33 and 34). Several workers have observed similar structures in a variety of invertebrate and vertebrate muscle systems (snail heart, North, 1963; crayfish muscle, Brandt et al., 1965; lamprey muscle, Jasper, 1967). The densities have either been considered to be desmosomes or intercalated discs. Desmosomes are thought to provide cell-to-cell adhesive forces in epithelial tissues (Overton, 1962),

and intercalated discs appear to perform a similar function in the cardiac muscle of vertebrates (Stenger and Spiro, 1961). The membranous plaques in the spider do not appear to be identical with either of these surface specializations. All three may, nevertheless, serve the same functional role.

Light microscopy revealed the presence of a scalloped border on surfaces of fibers found in the heart's longitudinal ridges. Electron microscope efforts have also demonstrated this rather unusual surface specialization (Figure 35). The extracellular fibrous coat adheres all along the scalloped border. T-tubules frequently are displayed here and the scallops are filled with mitochondria and what is presumably glycogen. This may reflect a rather high degree of cellular activity at the membrane surface. Certainly, active transport must be involved in maintaining the intracellular concentrations of several ions.

Densities often are found internally on the membranes of the scallop base. Studies at the light level showed each base to correspond to the level of a Z-line. The same is true of EM findings. Perhaps, the membrane density represents an adhesion of Z-line material to the sarcolemma. Such an attachment would protect the cell's structural integrity during contraction. Distances between scallop bases are reduced during contraction (Figures 9 and 10).

Edwards and Challice (1960) have investigated the fine structure of the insect heart, and they also have observed a

Figure 35. Scalloped border of the muscle cell. The cell surface is often seen to form a series of large-scale invaginations. In many sections, this surface modification assumes a scalloped appearance. The base of each scallop has been shown to correspond to the level of the Z-line. Densities (De) are observed intracellularly on the membranes of the scallop base. Mitochondria (M) crowd into the scallop. Unusual structures (U) are noted in the fibrous coat which covers the sarcolemma throughout the scalloped effect. The fenestrated collar of the SR (SR) has been sectioned obliquely here. A possible early lysosome (Ly), either a phagosome or autophagic vacuole, is observed near the cell surface. X 46,500.


R
R
U

scalloped border on these fibers. Scallop bases are aligned with Z-lines. Some evidence even suggests that surface scallops may be present in vertebrate cardiac muscle (Bloom and Fawcett, 1962). In the insect heart, the scalloped effect also is prominent on the outer surface of the heart, not just the internal surface areas as in the spider. It is possible that this structural modification allows for an extensive stretching of the heart wall by extra-cardiac factors. Systole would then decrease heart volume most significantly. Alary muscles could qualify as such an extra-cardiac factor. They are known to be important, often times vital, in normal insect heart function (Wigglesworth, 1965). Such a mechanism would provide for the transport of large blood volumes by a heart, the cardiac muscle system of which is not extensively developed. The spider heart possesses muscles similar to insect alary muscles. It should also be noted that a hydraulic mechanism exists for the extension of the spider leg (Parry and Brown, 1959). This extension may be rapid due to tremendous hydrostatic pressures. At times such as this, extra-cardiac spider heart muscles could be called on to stretch the heart beyond the normal volume (drawing in large quantities of blood). Freeze-etch studies indicate that grooves may lie transversely on the cell surface. The scallops created by these grooves would possibly be eliminated during a "super-relaxation". Contraction could then move a correspondingly large volume of blood into the open

circulatory system. Parry and Brown (1959) theorize that the heart is involved in producing the ninefold increase in leg pressures which is observed during locomotion.

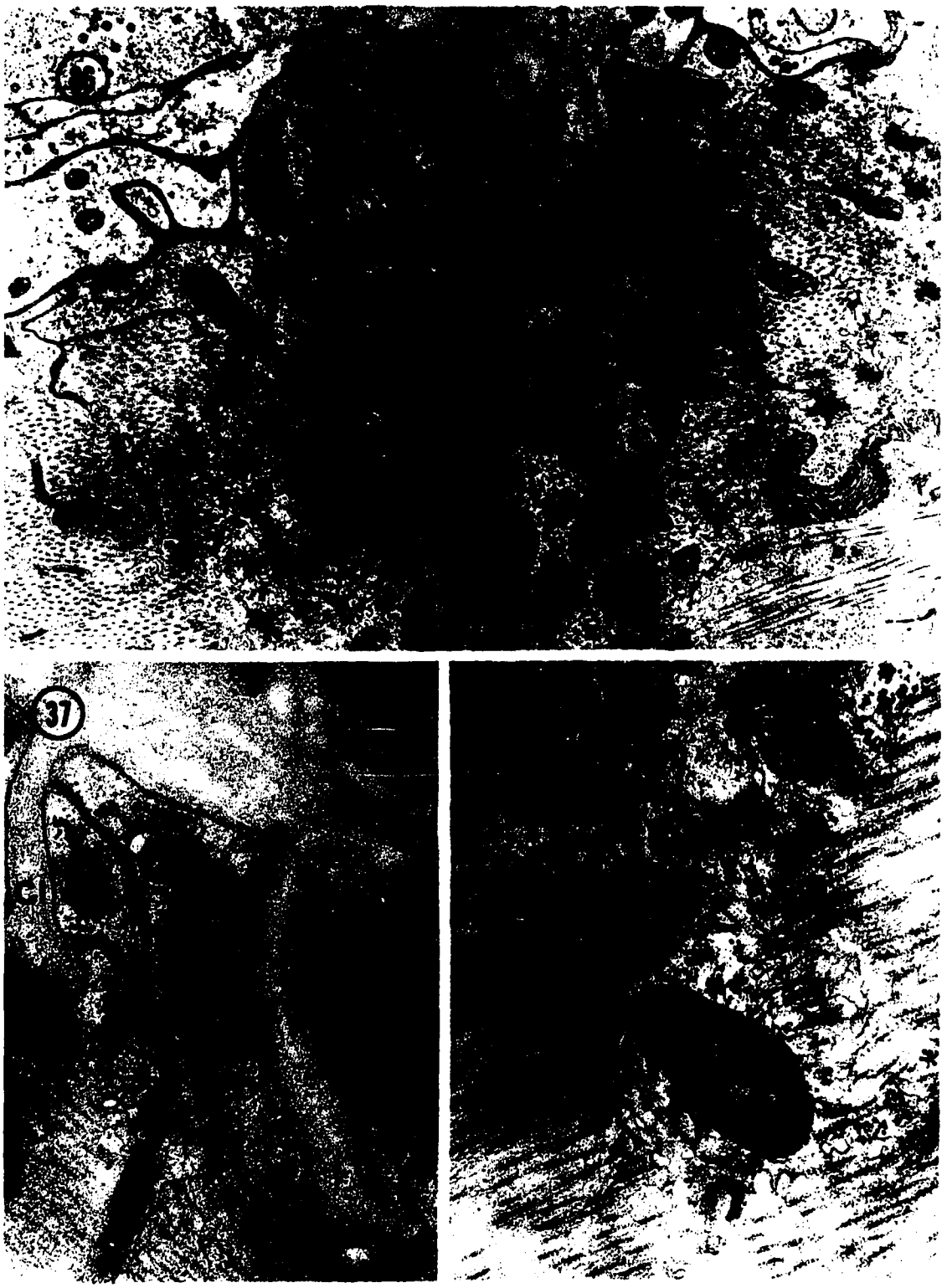
Vacuoles of varying densities are frequently seen in areas adjacent to the scalloped border (Figures 35, 36 and 38). Without the results of any biochemical or cytochemical investigations, it is not possible to identify the nature of these vacuoles or dense bodies. However, evidence does exist for the presence of lysosomes and lysosomal enzymes in vertebrate cardiac muscle (Munnell, 1967; Abraham et al., 1967). Similar organelles could serve invertebrate systems, and the morphology of spider heart dense bodies does resemble that of lysosomes. Shibko et al. (1963) have even detected lysosomal enzymes in crab muscle, where activities are comparable to those of the vertebrate muscles studied. Lysosomes in muscle are believed to either phagocytize various materials or more probably, assist in breaking down components of the muscle cell. Related to the latter is the finding that there is a limited turnover of protein in muscle, myofibrils have been shown to have a life time of 30 days (Dreyfus, et al., 1960).

According to deDuve's scheme (1963), early phagocytic vacuoles are termed phagosomes. Vacuoles serving to degrade cell components are called autophagic vacuoles. Each type of vacuole, by virtue of its lysosomal enzymes, digests its contents and concentrates any residues. The resultant

Figure 36. Ultrastructure of region underlying cell surface. A limited invagination corresponds to the level of the Z-line, which is enveloped by significant concentrations of SR elements. An electron-dense vacuole (Ly) is observed to close to the cell surface. X 16,500.

Figure 37. Clefts in the cell surface. Clefts (Cl) in the surface are often observed in possess reduced "mouths" and terminal swellings. T-tubules (T) appear as invaginations of the cleft wall. This wall possesses no fibrous cover. A density (De) at the base of one cleft is aligned with a Z-line (Z). X 36,100.

Figure 38. Dense body in muscle. Electron-dense vacuoles are frequently seen in this muscle system. These bodies are membrane-limited. X 44,400.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lysosomal vacuole in this theorized scheme is very electron-dense and is termed a residual body by deDuve. Residual bodies may be extruded by cells. If the dense bodies in the spider muscle are lysosomes, their presence near the cell surface could be related to a discharge activity.

Another type of major cell invagination may also exist (Figure 37). The rounded appearance of the scallop is not present in these sarcolemmal invaginations. These invaginations may represent another aspect of scallops, but such an interpretation is doubtful. The features of these two invagination types appear dissimilar, when the underlying muscle is sectioned longitudinally in both instances. Structures resembling the second class of invaginations have been reported by Peachey (1967) and Selverston (1967) in crustacean material. These infoldings they have termed clefts. Selverston observes that the clefts markedly increase fiber surface area and produce a corresponding increase in membrane-capacitance values.

Large-scale cell invaginations (not including t-tubules) may serve a most important function in tonic muscle. Atwood et al. (1965) suggest that a graded contraction in slow muscle generally results from a non-propagating, post-synaptic potential (PSP)--not an all or none action potential as in fast fibers. As a result, the PSP decays exponentially as it travels away from the synapse. In addition, slow fiber diameters average ten times those of fast fibers. Therefore,

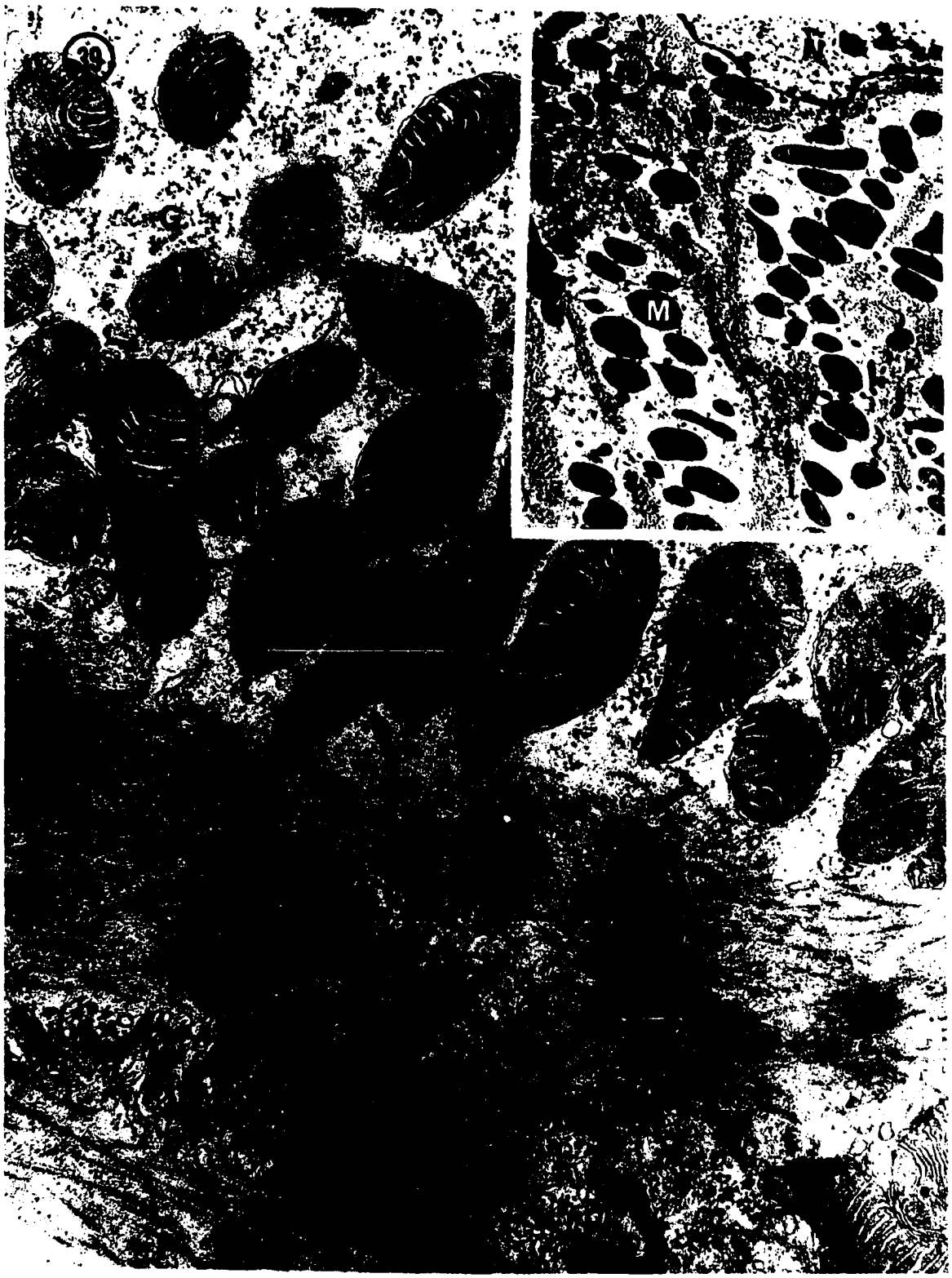
the efficiency of slow fibers would be increased by any factor that assisted the tonic fiber in alleviating or circumventing the difficulties associated with the non-propagating PSP. Selverston (1967) has observed motor axons to follow clefts inward and form myoneural junctions deep within the muscle fiber. This significantly decreases the distance which non-propagating potentials are required to travel. The resultant contractions are more extensive and more highly synchronized than would be otherwise possible.

Mitochondria The mitochondria in this muscle system tend to lie in pools of what is believed to be glycogen (Figures 39 and 40). Possible evidence is presented later for the fact that the cytoplasmic granules are glycogen. The locations of mitochondrial concentrations do not reflect a high degree of organization. Instead, mitochondrial groupings appear to be randomly squeezed in between myofibrils. The mitochondrial density is rather high and not strictly typical of slow muscle. Edwards and Challice (1960) reported an unusually high concentration of mitochondria for tonic muscle in their study of the insect heart. The impression of Stein et al. (1966) was similar in their work with Daphnia. It is possible that this indicates a general specialization of slow cardiac muscle.

The spider mitochondria display double unit membranes and a typical internal structure. The cristae are abundant and of the lamellar form (Figure 30). Dense intramitochondrial

Figure 39. Concentration of mitochondria in a glycogen pool. Mitochondria (M) aggregate in pools of what is believed to be glycogen (G). Glycogen occurs as single granules and as rosettes. The abundant lamellar cristae in the organelles are routinely observed to surround very dense granules (IM). The intramitochondrial granules may display transparent cores (arrow). These granules may contain calcium and phosphate (Greenawalt et al., 1964). Note the well-developed SR (SR). X 38,400.

Figure 40. Distribution of mitochondria between filament groupings. Mitochondria (M) are here concentrated in an area close to a nucleus (N). X 6700.


M

granules are frequently observed in the matrix of the organelle. These granules are spherical or oblong and often reach a size of 600-700 Å. Ishikawa and Pei (1965) have reported observing beta glycogen particles (single granules) in the mitochondrial matrix of retinal cells. These particles resembled glycogen in structure (crudely spherical and rather evenly electron-dense) and size (approximately 200 Å). However, the inclusions of spider mitochondria do not conform to this description. Certainly, the larger and varying sizes of the spider inclusions are of note here. Furthermore, numerous reports of lipids and crystalline arrays in mitochondria also fail to relate to the question of spider muscle mitochondria.

Possible sources of illuminating information come from the work of Peachey (1964) and Greenawalt et al. (1964). By allowing mitochondria to accumulate ions in vitro, granules are formed during respiration which are greater than 500 Å. "Loaded" mitochondria display numerous granules at or near the cristae and electron-transparent cores are often visible. The first study suggests that loaded mitochondria possess larger than normal granules, but not any more than usual. Thus, it is theorized that the normal granules act as nuclei for the growth of larger deposits. Mitochondria are believed to accumulate Ca^{++}, Mg^{++}, Mn^{++}, Ba^{++}, Sr^{++} and $HPO_4^{=}$. Rossi and Lehninger (1963a, 1963b) have shown mitochondria to

accumulate calcium and phosphorus in a molar ratio of approximately 1.67. This value is close to that of hydroxyapatite (the basic structure of bone mineral). The accumulation of calcium and phosphate is related to electron transport and oxidative phosphorylation. As a pair of electrons proceeds through each phosphorylation site of the respiratory chain, calcium (1.67 molecules) and phosphorus (1 molecule) are accumulated.

These physiological accumulations of ions may relate to the intramitochondrial spider inclusions. These granules vary in size, as would be expected. Maximum sizes of the spider inclusions approach the lower levels of loaded mitochondria. Lighter cores are often seen, yet the granules are very electron-dense.

It is frequently noted that myofilaments on opposite sides of a mitochondrial pool are approximately at right angles to each other (Figure 41). Clearly, no cellular borders separate the two groups of filaments. This unusual filament arrangement has, perhaps, a logical basis. Contraction in the two arrays would reduce both the length and width of the cardiac muscle cell, thereby reducing both the length and diameter of the heart. As a result, the heart is able to transport larger quantities of blood than would be otherwise possible. It may be noted that entire cells also are oriented in a variety of planes.

Figure 41. Right angle filament arrangement. Only a collection of mitochondria (M) separate the two filament groups, which are aligned approximately at right angles to each other. X 40,400.


41

Cardiac nerve Because histochemical procedures for nerve fibers were not positive when applied to the theorized cardiac nerve, EM sections were cut. Electron micrographs display large numbers of myelinated axons (Figures 42 and 43). These axons are apparently derived from the central nervous system of the spider. Electron-dense granules (Figure 42) and possibly synaptic vesicles (Figure 43) are seen in the cytoplasm of the axons. Nisbet and Plummer (1966) have observed these inclusion types in separate nerve endings in the auricle of the molluscan heart. In cross sections of the spider cardiac nerve, the electron-dense granules may be more abundant. The endoneurium contains a fibrous material displaying a periodicity; this material is possibly collagen. The Schwann cell nuclei represent the elliptical nuclei of light microscopy.

The outer surface of the heart is shown to possess deep pits into which nerve processes extend (Figure 44). An electron-dense material is deposited on the intracellular surface of the membrane lining the invagination. Similar densities are typical of vertebrate myoneural junctions.

Electron microscopy of freeze-etch replicas

The technique of freeze-etch enables the electron microscopist to achieve a view of biological material, not possible in section studies. This new view has a third dimension to it, and possible artifacts due to dehydration

Figure 42. Axonal fibers in the cardiac nerve. Bundles of fibers are surrounded by the fibrous endoneurium (En). The fibrous material possesses a periodicity. The endoneurium also contains cellular elements (arrow). A large number of axons (Ax) are myelinated (My). The nuclei of Schwann cells (N) are displayed. Axons often display electron-dense granules (Gr). X 7500.

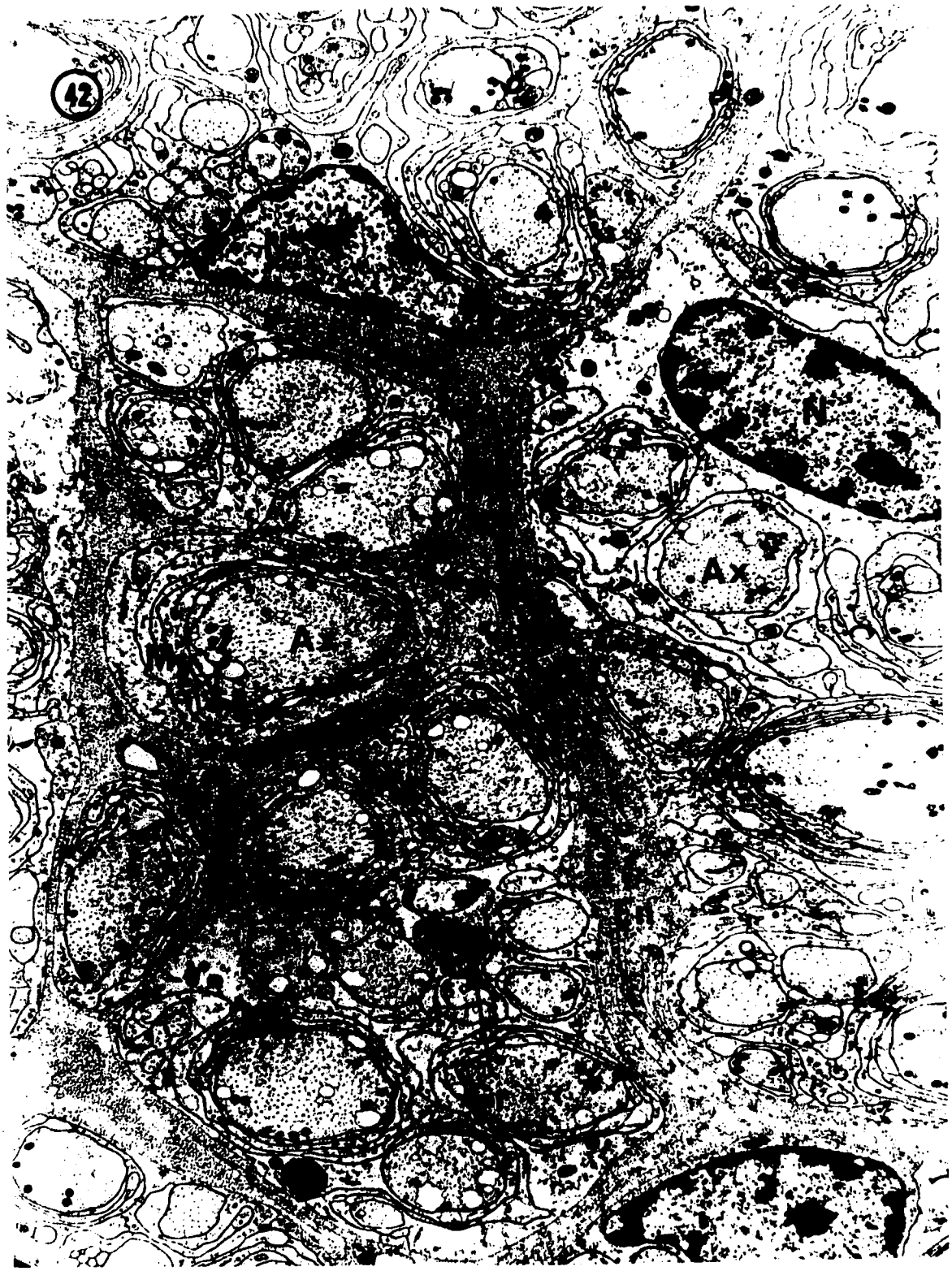
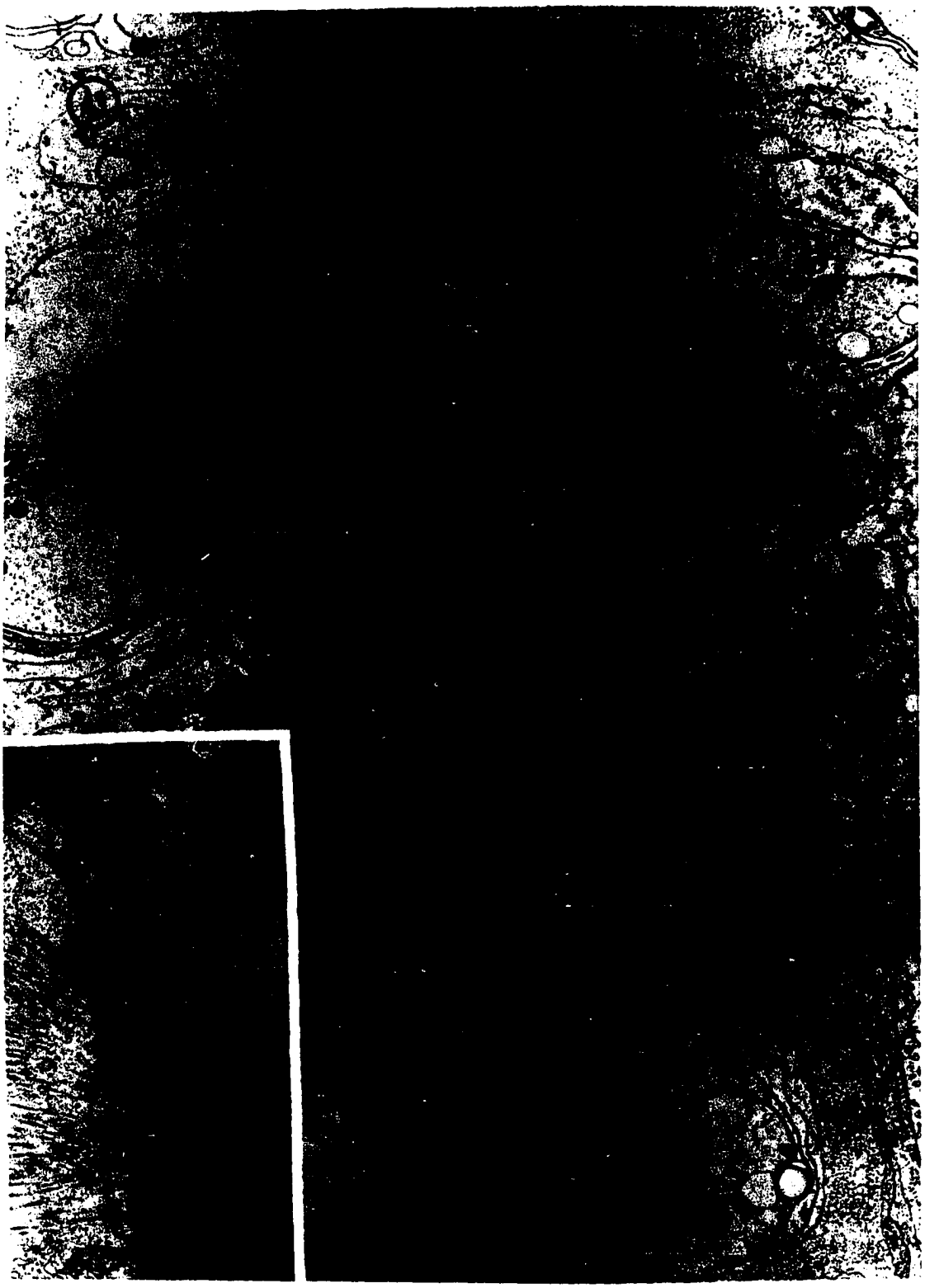

42
Ax
Ax
M

Figure 43. Specializations of axonal fibers. The myelin sheath of an axon (Ax) is seen to be due to a wrapping of Schwann cell cytoplasm around the fiber. The nucleus (N) of another Schwann cell is also observed. Axons may contain synaptic vesicles (Sr) or electron-dense granules (Gr). X 13,900.

Figure 44. Invaginations of the outer heart surface. Nerve processes (NP) extend into surface invaginations (In). This probably represents an area close to a myoneural junction. A dense material is deposited intracellularly on the membrane lining the invagination. X 16,500.



and embedment (even fixation in certain investigations) are eliminated. Freeze-etch replicas were sought in this study to improve the understanding of surface specializations and to clarify the general ultrastructure of these cells.

Typical banding was observed (Figure 45) with the I-band being more extensive than in sectioned material. Perhaps, part of the contraction problem in this tissue results from dehydration and embedment. The rather amorphous character of the Z-lines is apparent. The A-band is seen to etch more extensively than the I-band and Z-line. Mitochondria are fractured in a variety of ways, and the cristae are prominent. In oblique fractures through the filaments (Figure 46), elements of the SR can possibly be observed.

Fractures near cell surfaces yield replicas with a high information density, although interpretation is often difficult. Fractures may occur over, under and through membranes covering the surfaces of cells. Fractures through the surface may follow any of an infinite number of planes into the cell interior.

Replicas, that possibly represent the scalloped border of these muscle cells, indicate that the scalloped effect is not due to an orderly, linear array of pit-like invaginations (Figure 47). Instead, grooves appear to run across cell surfaces at right angles to the filaments which lie beneath the surface. Sectioned material showed the grooves to correspond to the level of Z-lines. The interpretation

Figures 45 thru 47. Freeze-etch micrographs of spider cardiac muscle. The material has been shadowed with platinum-paladium wire. These micrographs are positive prints.

Figure 45. Banding patterns in a longitudinal fracture. Mitochondria (M), which have been fractured in a variety of ways, are grouped adjacent to a relaxed sarcomere. A-bands (A), I-bands (I) and Z-lines (Z) are displayed. Small vacuoles represent either SR components or mitochondria. X 20,200.

Figure 46. An oblique fracture of myofilaments. See Figure 45 for a legend. Membrane bodies are probably elements of the SR (SR). X 21,200.

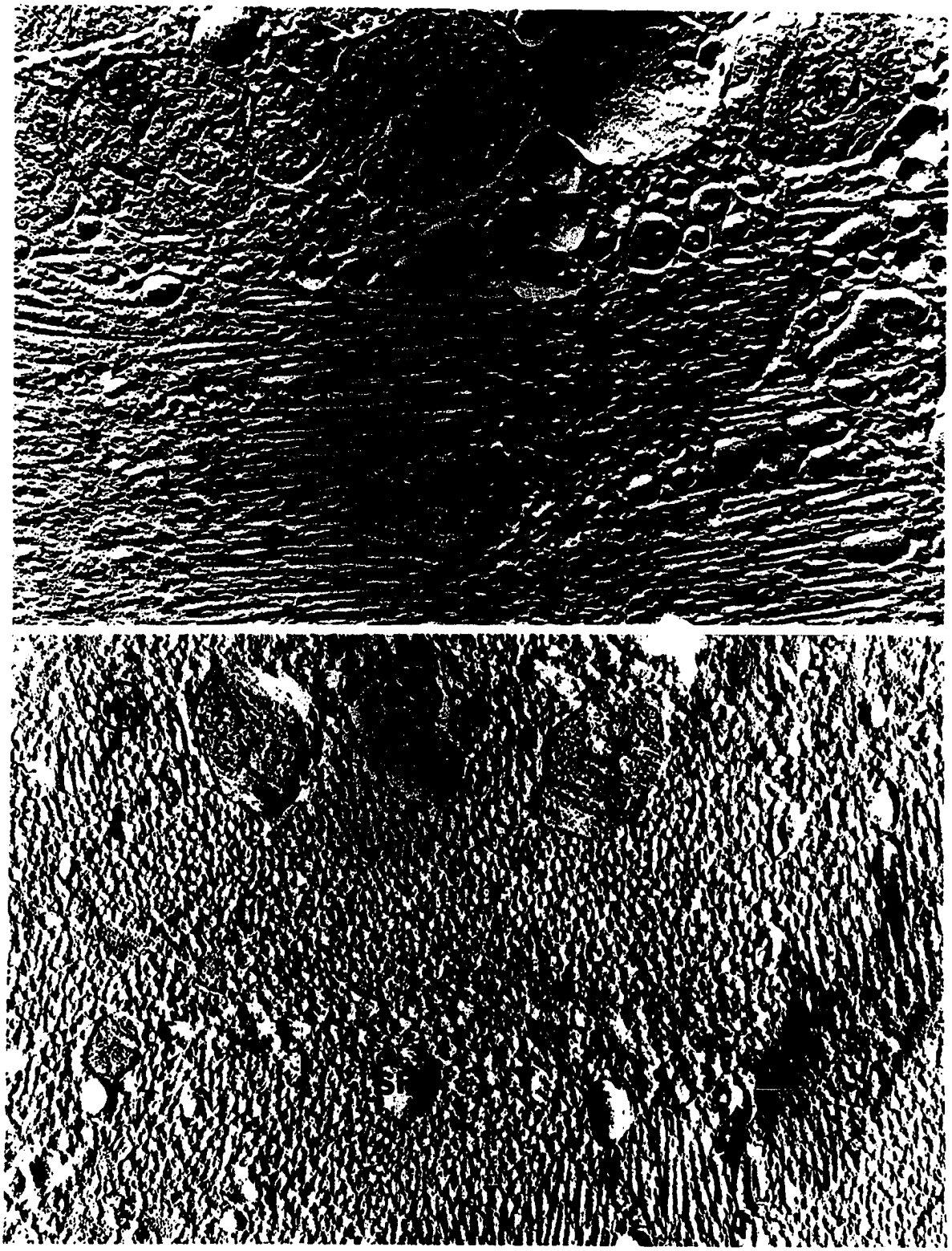
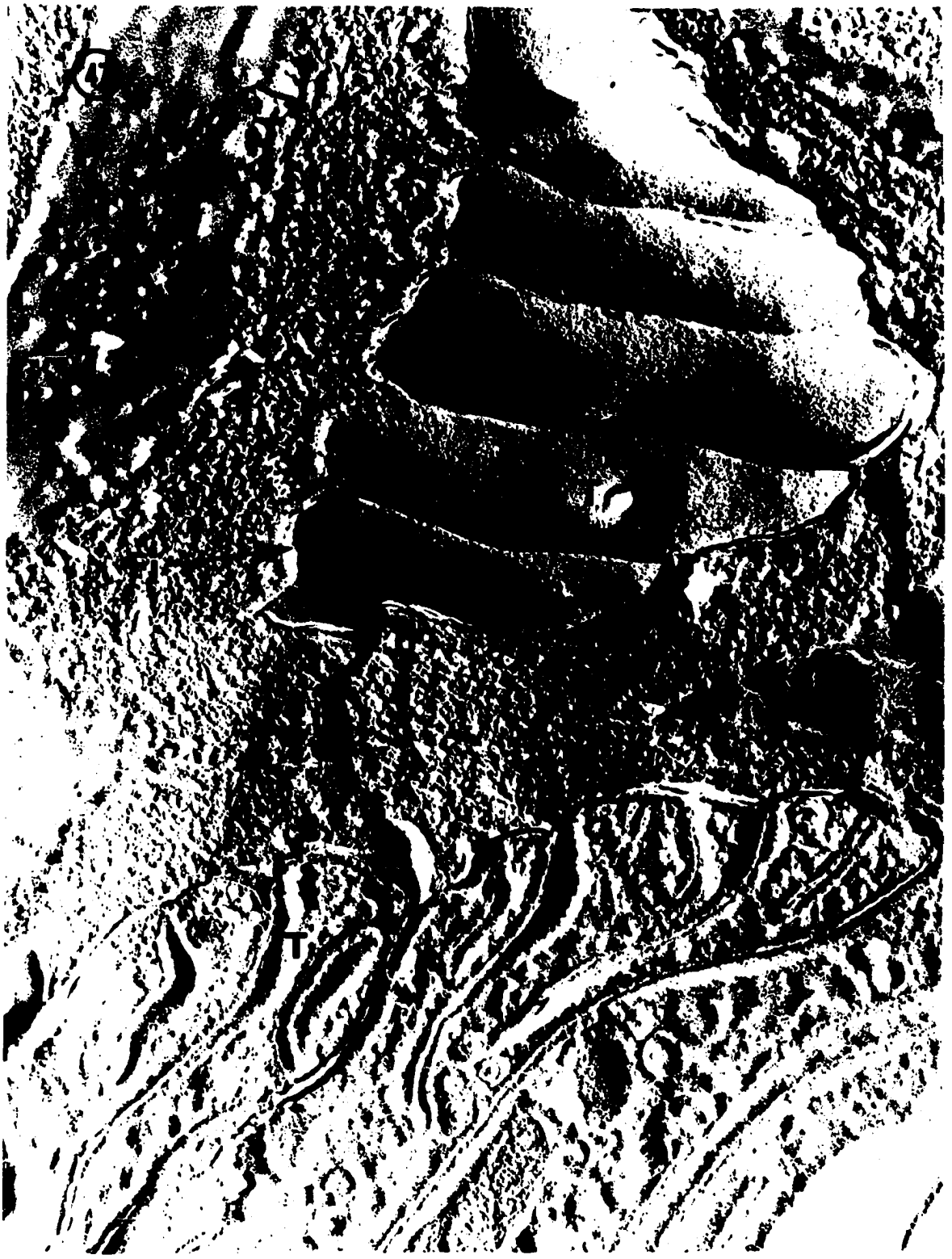


Figure 47. Fracturing of the muscle cell surface. The theorized grooved-nature of the scalloped border is noted (Gv). The view of this membrane is believed to be from the intracellular aspect. A fibrous (Fi) material covers this membrane surface. A possible t-tubule aperture (T) is displayed. Rayns and Simpson (1967) have reported similar structures in their study of the vertebrate myocardium. This aperture is larger than the tubule diameter, when measured at a deeper point in the cell. This is logical since section studies indicate the tubule origin is often rather broad. An extensive array of t-tubules (T) is displayed. X 27,300.


A
T

that grooves produce the scalloped effect is based on the observation of numerous, similar structures in the replicas studied. These structures appear to be fractures along membranes, and it is believed that the fractures are specifically under sarcolemmal membranes. If grooves do exist and if the fracture were seen from the intracellular surface, one would expect to observe a structure very similar to what has been frequently seen (Figure 47). This would represent a stripping of part of the membrane from the scalloped surface. A fibrous material can be seen to cover membranes, as in section studies.

Fractures often display an impressive number of t-tubules (Figure 47). It is likely that the tubules represent a fracture plane of lower resistance than the surrounding sarcoplasm.

Ultrastructural Cytochemistry

Trypsinizing Z-lines

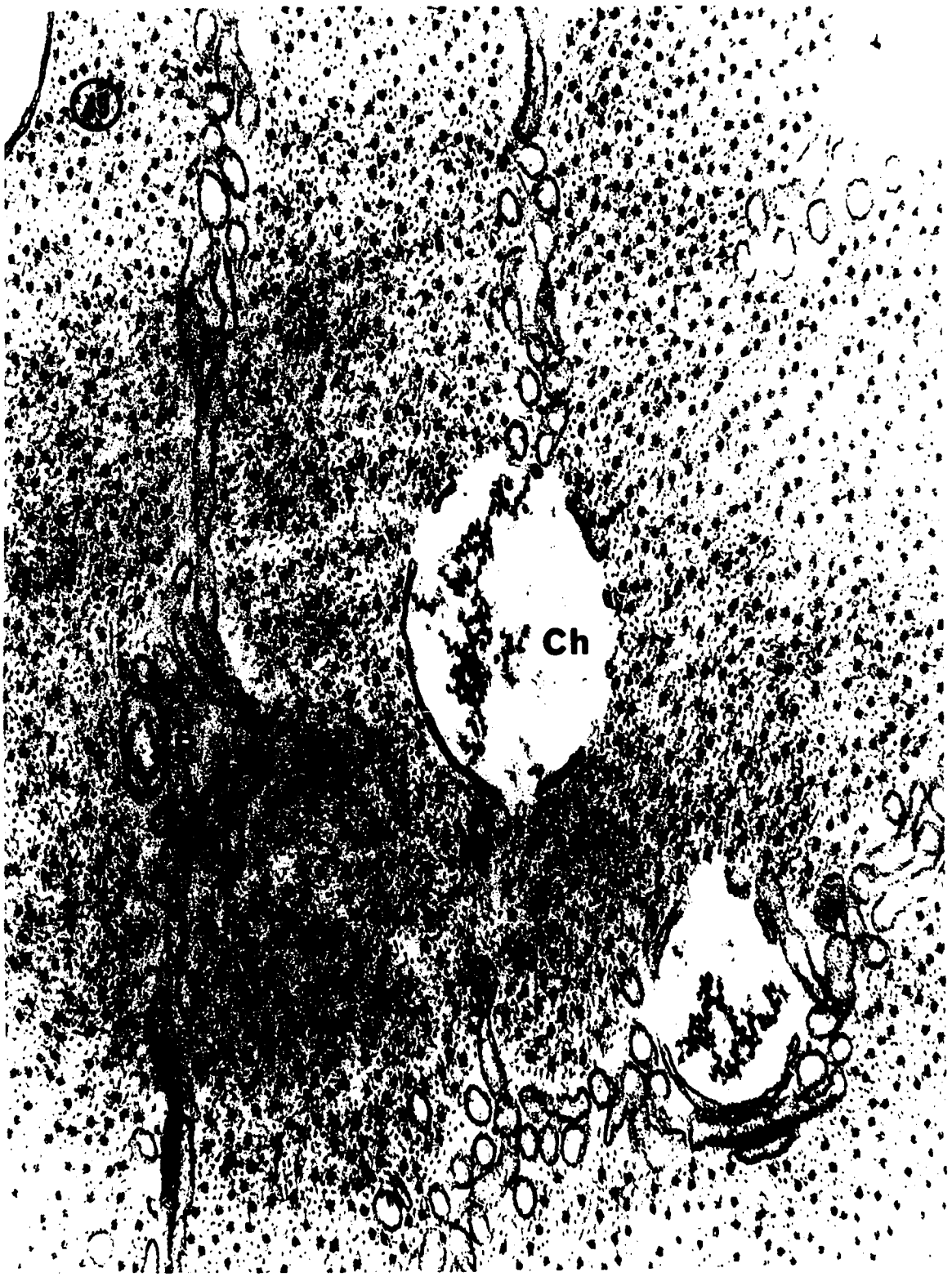
As indicated in the section on methods, slow spider leg muscle was substituted for heart muscle in the trypsin experiments. Therefore, a micrograph of a longitudinal section of leg muscle and one of a cross section are included here (Figures 48 and 49). Longitudinal sections reveal the typical banding of slow muscle (Fahrenbach, 1967), with two classes of filaments being present. The myofibrils are more concentrated than in cardiac muscle, and the SR is more

Figures 48 thru 56. Electron micrographs of muscle stained with uranyl acetate and lead citrate.

Figure 48. Electron micrograph of a spider leg muscle in longitudinal section. The A-bands (A), I-bands (I) and Z-lines (Z) are illustrated. Mitochondria (M) lie between myofibrils. Lengthy arrays of the SR are characterized by dyads (D) near the A-I junctions. X 38,400.


M

Figure 49. Cross section of spider leg muscle. More than six thin filaments surround a given thick myofilament. Large channels (Ch) are observed in the muscle, and they are located among elements of the SR (SR). Brandt et al. (1968) suggest that similar structures in crustacean muscle are terminal swellings of t-tubules at the diadic junction. However, in spider muscle, the channels are not completely membrane-limited and possible glycogen particles (G) often fill the channels. This would probably indicate the cytoplasmic nature of the channel lumen. X 73,500.


Ch

ordered in the leg. Mitochondria lie between myofibrils, not in large pools scattered throughout the muscle. The Z-lines resemble those of the heart muscle in that an ordered filamentous structure is lacking. No sleeves are noted on thin filaments as they approach the Z-lines. The SR is striking in cross section, and large channels are observed. Selverston (1967) has observed similar channels in crustacean muscle. Glycogen apparently is found in these channels.

When the ultrastructure of spider leg (slow) and rabbit psoas (fast) muscle is examined following a simultaneous six-minute treatment with trypsin, a most unusual observation is made. While rabbit Z-lines are almost totally removed, spider Z-lines display a near normal fine structure (Figures 52 and 56). Typical Z-lines are found in each group of controls (Figures 50, 51 and 53). Several reports have illustrated the removal of Z-lines by trypsin (Ashley et al., 1951; Endo et al., 1966; Stromer et al., 1967b).

The membranes of both trypsinized rabbit and spider muscle have been altered rather extensively. While these changes may be results of the enzyme activity, tonicity stresses may also be involved. Typical rabbit Z-lines are definitely absent in the treated material, and M-lines are also apparently digested. This latter finding is similar to the report of Stromer et al. (1967b). Since Z- and M-lines support <u>in situ</u> thin and thick filaments respectively, their

Figure 50. Rabbit psoas muscle in longitudinal section. This constitutes the exposed surface of a control fiber in the trypsin experiments. Note the zigzag-nature of the Z-line (Z). X 38,400.

Figure 51. Rabbit control for trypsin studies. This micrograph was taken at a point slightly under the exposed surface. X 43,400.

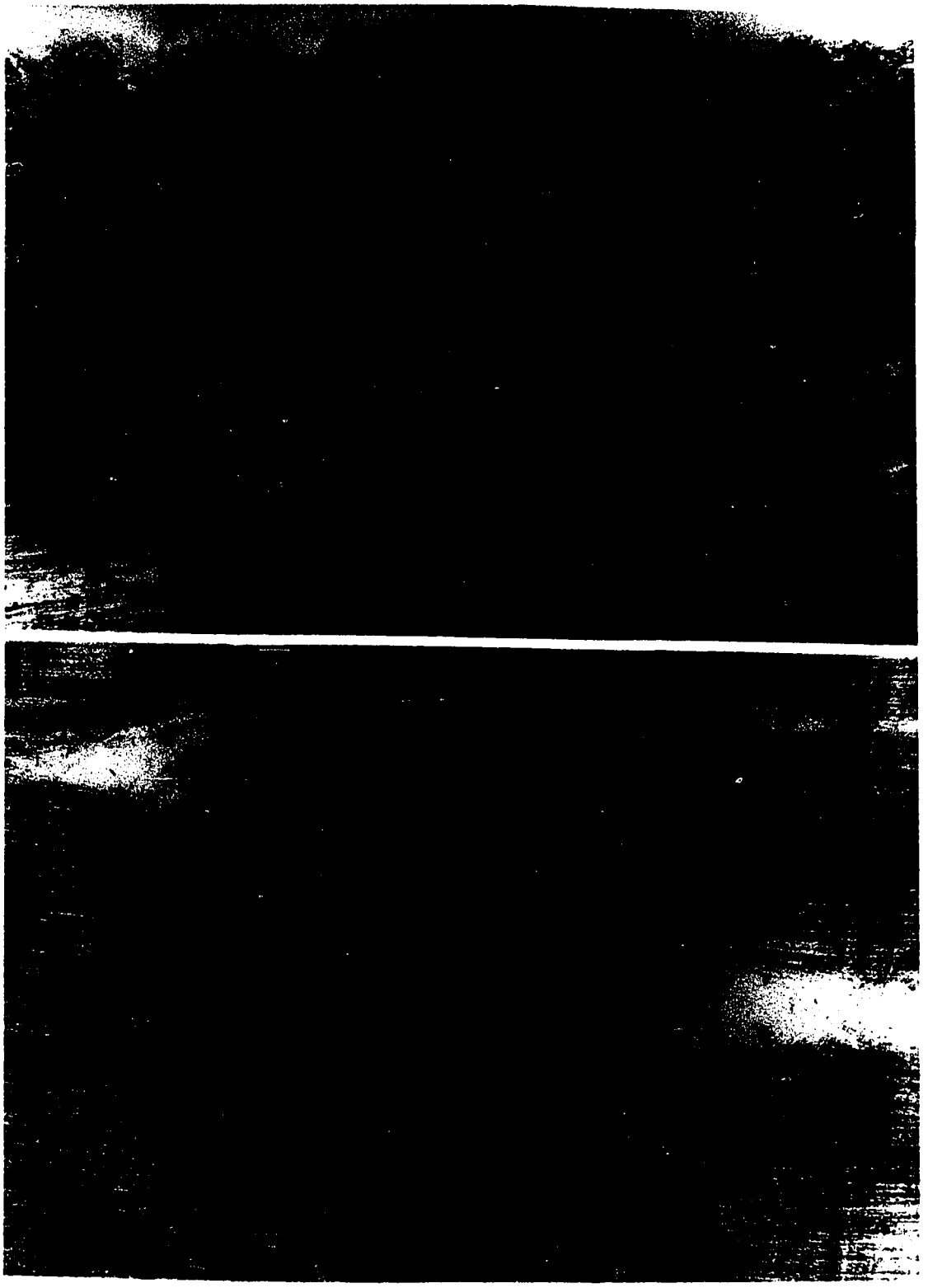


Figure 52. Rabbit psoas treated with trypsin for six minutes. Z- and M-lines (Z and ML) have been significantly removed at this exposed surface of the fiber. Probable glycogen (G) particles remain. X 47,500.

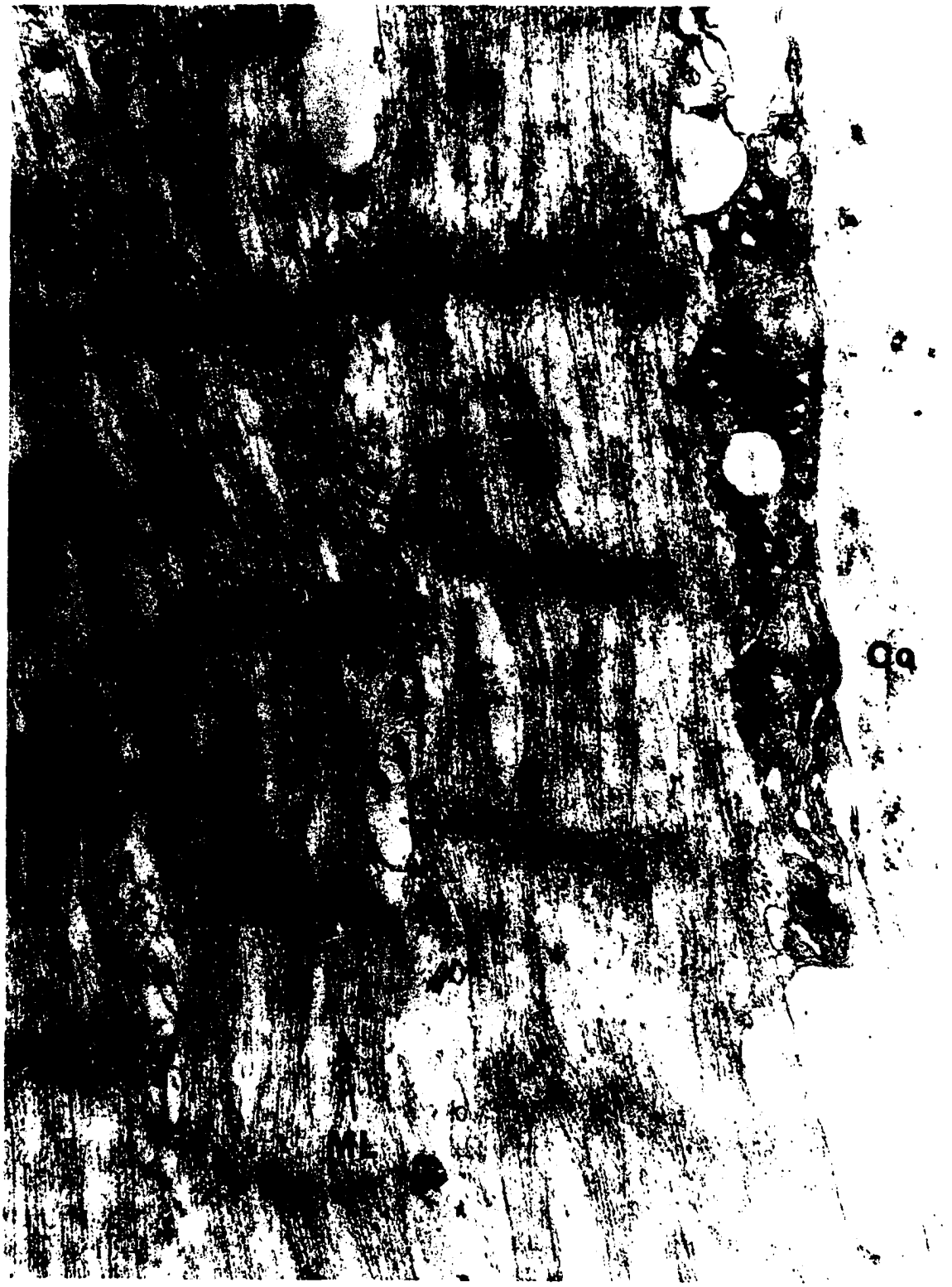

Qa

Figure 53. Spider leg muscle trypsin control. The typical Z-lines and SR are illustrated. X 24,200.

Figure 54. Spider leg muscle treated with trypsin for 15 minutes. This area is not close to the exposed fiber surface. Partial removal of Z-line material has occurred. Membranes of the SR have lost their orderly, linear distribution. The section is a glancing one over the myofibril surface. X 38,400.

Figure 55. Fifteen-minute trypsinizing of spider muscle. Z-line material (Z) has been totally removed at this point close to the fiber surface. X 28,300.

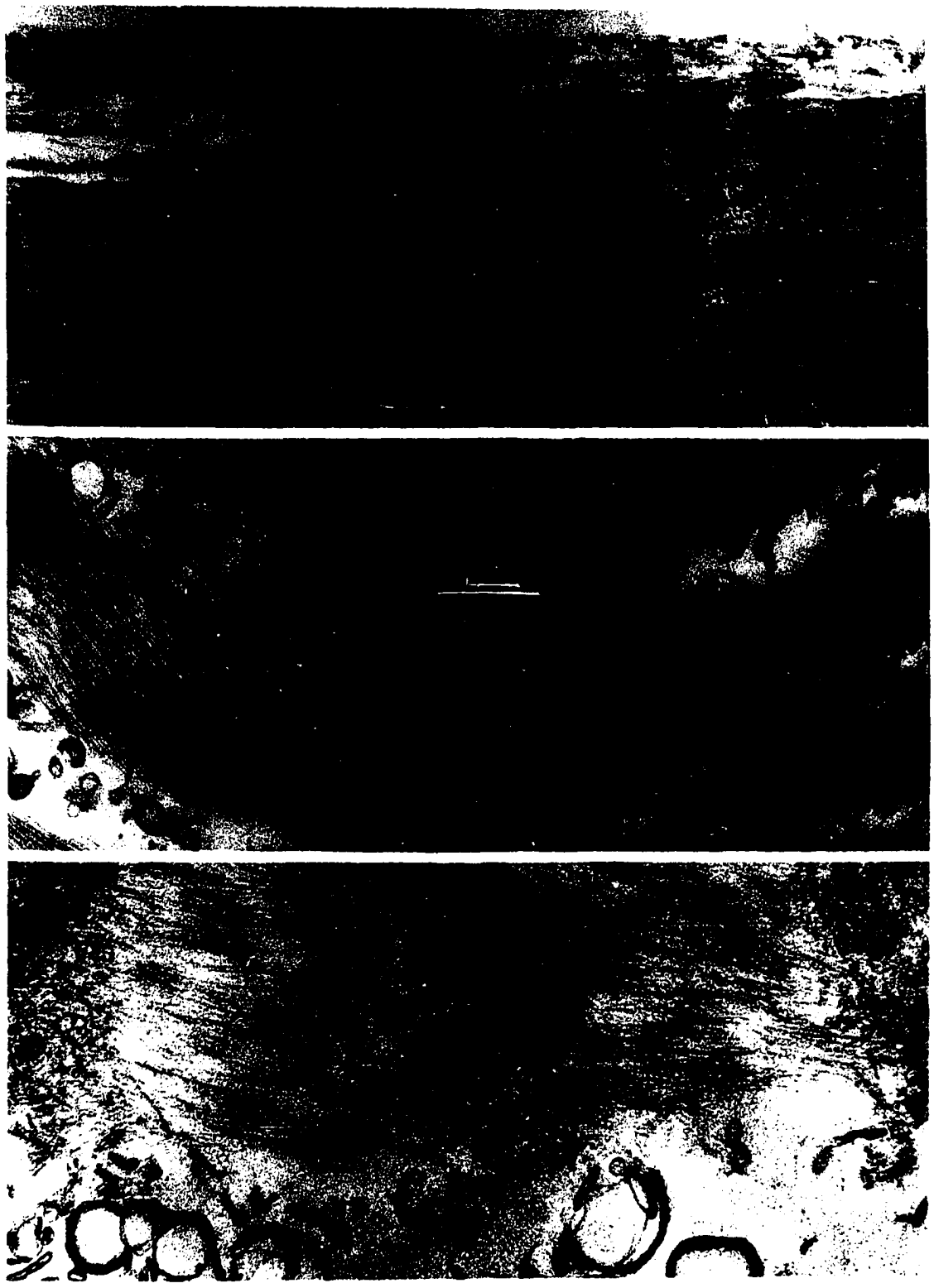


Figure 56. Spider leg muscle treated with trypsin for six minutes. Z-lines (Z) display a near normal fine structure at this exposed cell surface. A moderate amount of material (Co) covers the fiber surface, as was the case with rabbit muscle (Figure 52). Mitochondria (M) have been altered. X 24,200.


56
Co

removal could account for the tendency towards disorganization in the treated rabbit fibrils. This is in contrast to the spider filaments which bear the usual arrangement, even after six minutes of trypsin. A certain amount of material is seen to cover both the rabbit and spider fiber surfaces. Possible glycogen particles are observed in each group of treated fibers.

Spider Z-lines were eventually removed in experiments involving 15-minute exposures to trypsin. At this time, myofibrils near the exposed fiber surface were observed to be free of Z-line material (Figure 55). However, fibrils deeper into the cells still displayed some Z-material (Figure 54). Membranes in each case appeared to have been disrupted. There was an apparent tendency for the membranes to "ball-up", in contrast to the cisternae of normal spider muscle (Figure 48).

The significance of these experiments is uncertain. Certainly, rabbit Z-lines were more susceptible to trypsin. Yet, the basis for this susceptibility is not known. At least two possibilities exist, and there is plausible support for each. The first possibility indicates that surface permeabilities to trypsin vary, and thus more trypsin may have reached rabbit Z-lines. Differences in permeability could be due to cell membrane coats or to the membrane itself. Outstanding differences were not noted in cell membrane coats,

and comparable amounts of material were deposited on the two fiber surfaces after hydrolysis. Yet, a critical interpretation is probably not possible. Selverston (1967) has reviewed the membrane permeability differences that do exist between fast and slow muscle. However, it is rather difficult to see how differences in ionic permeabilities could create a differential permeability to a molecule as large as trypsin. In future experiments, the permeability factor could be eliminated by substituting isolated myofibrils for fibers.

The second possibility relates to an actual variation in the Z-lines, when contrasted in fast and slow muscle fibers. This variation could conceivably have a structural or compositional basis. As noted previously, Page (1965) has characterized fast Z-lines as filamentous and slow as amorphous. However, this level of structure may not genuinely pertain to the molecular level at which the trypsin would operate. A compositional variation may exist between the two Z-line types, and the fundamental differences in fine structure may be suggestive. In addition, the generally broader nature of slow Z-lines is worth noting. In this respect, Kelly (1967) has theorized that substances may be deposited on an elementary Z-line framework.

If a compositional basis exists for the contrasting responses to trypsin, α-actinin may be one Z-line species involved. Goll et al. (1967) have shown trypsin to remove

rabbit Z-lines, and ∝-actinin is present in the digest. Fluorescent microscopy also localizes ∝-actinin within or close to the Z-lines (Masaki et al., 1967). The role of this trypsin-labile protein in promoting actomyosin superprecipitation was noted earlier (Ebashi and Ebashi, 1965). Since ∝-actinin influences the rate of actomyosin activity, a rather naive model for slow muscle may be constructed. This model proposes that ∝-actinin is absent or in lower concentrations in tonic muscle. Among possibly numerous factors that contribute to the slow quality of tonic muscle, the ∝-actinin factor could partially explain the differences in rates that characterize fast and slow muscle.

Glycogen

Glycogen routinely is observed in muscle as single granules (beta particles) or rosettes (alpha particles). Fawcett (1966) has described the presence of characteristic glycogen in several systems.

To confirm the presence of this polysaccharide in spider muscle, a newly developed EM cytochemical technique (Hay, 1966) was employed. However, results obtained with the procedure were inconclusive. Yet, Revel (1964) suggests that glycogen exhibits a rather characteristic fine structure. This structure is reflected in sectioned material, as well as in isolated preparations. The beta particles are roughly sherical and measure 150-300 Å. The particle edges may be

smooth or somewhat irregular. Tiny grains may be observed in the individual particles.

Spider heart muscle contains particles fulfilling this description (Figures 15, 28 and 39). Granules are rather uniform in diameter and measure slightly less than 200 Å. Alpha particles may also be present (Figure 28); they consist of aggregations of particles resembling the beta type. The rosette sizes vary. The presence of these two particle types lends credence to the view that they are indeed glycogen. Considering the extensive presence of glycogen in vertebrate (Peachey, 1965; Fawcett, 1966) and invertebrate muscle (Hagopian and Spiro, 1968), the glycogen-nature of the spider particles appears rather likely. Furthermore, regions in the spider heart, known to contain the particles, were found to be PAS-positive. This would support the carbohydrate character of the particles.

On the basis of the work cited in reviewing the literature, Hoyle (1967) has suggested that fast muscle metabolism may derive its energy from carbohydrates, and slow from lipids. However, in the slow spider cardiac system, glycogen appears to be plentiful. Lipids have not been observed in the form of free droplets or membrane-limited vacuoles. These findings would strongly suggest that spider cardiac muscle utilizes carbohydrates as a source of metabolic energy. The glycogen is presumably broken down to glucose, and ATP is generated in the metabolism of this hexose.

Physiological Investigations of Spider Muscle

Contraction of isolated fibers

The results of *in vitro* contraction experiments with leg fibers and heart muscle are presented in Figures 57 through 64. All but one of these photomicrographs present the results of investigations with isolated leg fibers. Since the heart exhibits a fibrous nature much less than leg muscle, it was found to be difficult to achieve preparations that were sufficiently thin for significant phase microscopy. However, the general conclusions which are to be presented appear to apply to both spider leg and heart muscle. Ideally, myofibril preparations should be employed in future studies. The photomicrographs included here involve areas of the fibers close to the teased end. Thus, it is believed that reacting solutions were given ample opportunity to reach the myofibrils, and membrane barriers were not a factor.

The lengths of sarcomeres (Figure 57) in Aronson's (1962) magnesium-containing salt solution (MgSS) are decreased by addition of $CaCl_2$ (Figure 58). In contrast, EDTA (a chelating agent) causes an elongation in both the heart (Figure 59) and the leg muscle (Figure 60). The replacement of MgSS with the ATP solution possibly causes a slight shortening of the sarcomere (not illustrated). The Mg-ATP relaxing medium increases the sarcomere length somewhat (Figure 61), while Ca-ATP shortens the MgSS length (Figure

Figures 57 thru 64. Phase contrast micrographs of isolated spider muscle.

Figure 57. Leg muscle in MgSS. The sarcomere length is 4.6 μ, with the A-band measuring 2.6 μ, the I-band 1.6 μ and the Z-line 0.4 μ. X 2160.

Figure 58. Leg muscle in $CaCl_2$. The sarcomere length is 3.7 μ. X 1080.

Figure 59. Cardiac muscle in EDTA. The sarcomere length is 3.5 μ. X 2160.

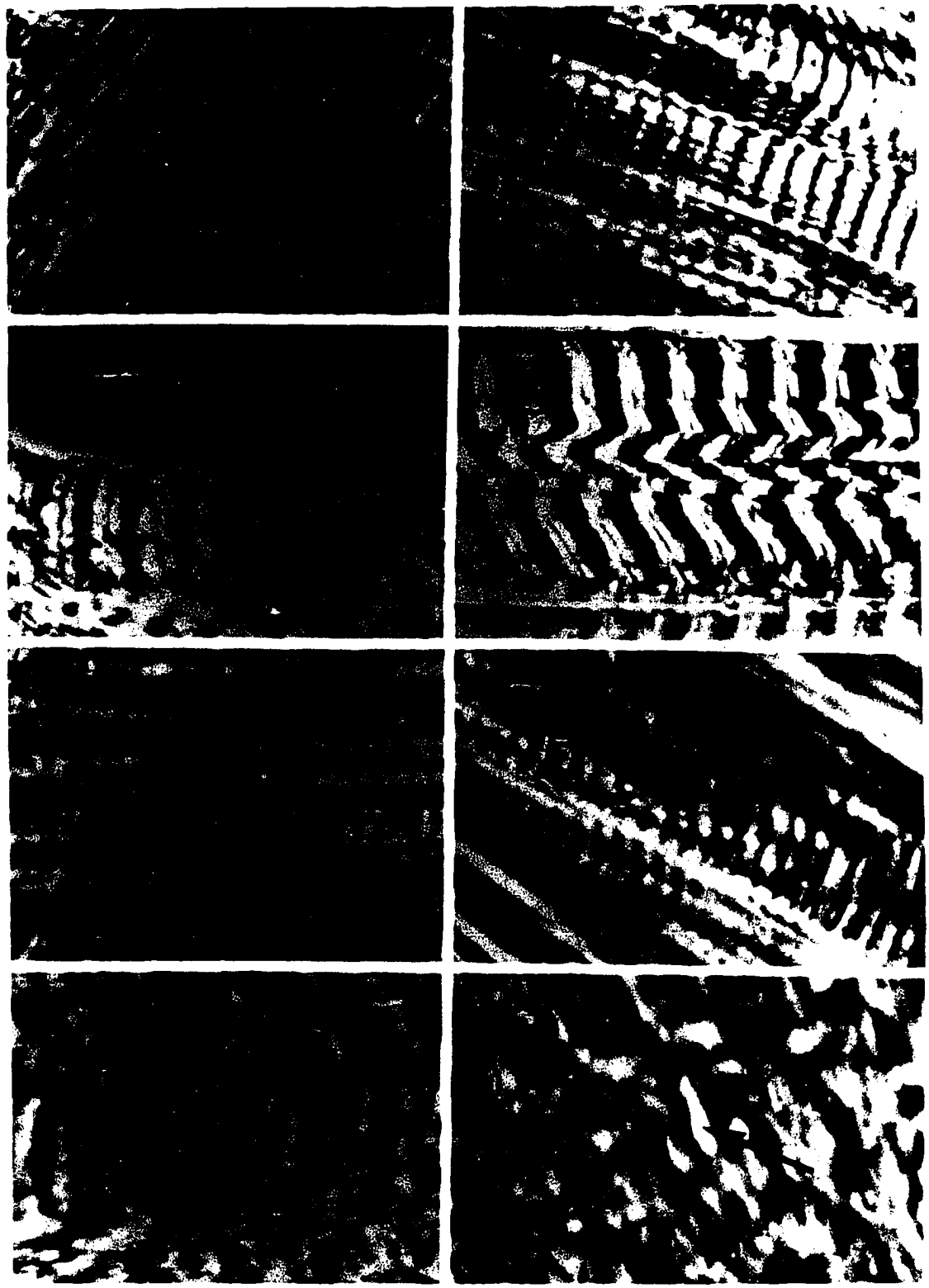
Figure 60. Leg muscle in EDTA. The sarcomere length is 5.4 μ. X 1470.

Figure 61. Leg muscle in a relaxing solution (Mg-ATP). The sarcomere length is 5.3 μ. X 2160.

Figure 62. Leg muscle in Ca-ATP. The sarcomere length is 4.1 μ. The A-band does not appear to have been decreased to one-half its resting length as deVillafranca (1961) and deVillafranca and Marschhaus (1963) have observed with glycerinated horseshoe crab muscle. Instead, the length appears to remain rather constant (Aronson, 1967). X 1080.

Figure 63. Leg muscle in 1 M KCl. The Z-lines (Z) have widened considerably. Sarcomere length is 5.5 μ. X 2160.

Figure 64. Leg in 1 M KCl. This photomicrograph was taken after a longer KCl treatment than in Figure 63. The A-band has been completely removed at one point, and I-band density has increased. X 2160.



62). This latter shortening is almost as extensive as contraction in $CaCl_2$. The response to the relaxing solution also resembles that associated with EDTA.

In these studies, calcium appears to induce a shortening of sarcomeres that is reflected in the banding patterns exhibited by the myofibrils. A theorized role of calcium in contraction has been presented by Davies (1963), whereby Ca^{++} is said to ionically link the ADP of actin to the ionized ATP of a HMM peptide. The experimental evidence of Ebashi (1961) and Weber et al. (1964) points to the involvement of calcium, although the specific manner of participation is not known. Goode (1967) has shown calcium to induce contraction in the isolated fibrils of insect flight muscle. Chelating agents such as EDTA presumably influence an elongation by binding calcium. Bendall (1958) has illustrated this with vertebrate material. Recall also that Podolsky and Constantin (1964) produced a localized contraction of muscle by applying microdroplets of a dilute calcium solution to certain points on the sarcolemma.

Aronson (1962) has reported an elongation of myofibrils from indirect flight muscle in Drosophila. This presumed relaxation was induced with the MgSS-ATP relaxing medium. Since EDTA does not affect an elongation (nor does calcium cause a shortening) in this system, the EDTA present in the MgSS is probably not involved in elongation. Instead, Aronson suggested that ATP and magnesium ions are required

for the lengthening. The participation of ATP was shown to be specific, since pyrophosphate and several nucleoside triphosphates failed to produce an elongation. He was not able to present a concise mechanism for this relaxation.

In the spider studies, the EDTA component of the MgSS-ATP solution could possibly contribute to the observed elongation. Along these lines, the calcium ions in the Ca-ATP may significantly participate in the shortening, which did occur. The concentrations of EDTA and calcium in the respective solutions are lower than in the pure EDTA and $CaCl_2$ preparations. Therefore, it is reasonable to consider length changes in the mixtures to be somewhat less than in the pure solutions. It is difficult to determine the contribution of ATP in these studies. It is possible that calcium and EDTA reached the myofibrils more readily.

When treated with 1 M KCl (Figures 63 and 64), spider muscle A-bands are seen to decrease in density. These observations are in agreement with those of Huxley and Hanson (1957), which relate to the removal of myosin from the A-bands. However, the first change is noted in the Z-lines (Figure 63). They widen significantly and appear rather filamentous. This is followed by a noticeable decrease in A-band density, with a simultaneous increase in the density of I-bands. Garamvolgyi and Kerner (1966) have shown dissolved myosin to attach to thin filaments in the fibril ghosts of insect flight muscle. The Z-lines were finally

seen to break from the myofibrils (at the A-bands) and drift into the potassium solution. I-band material continued to flank the Z-lines. Considerable dissolution of Z-lines was then observed. This would suggest that Z-lines in the spider contain a component sensitive to 1 M KCl, although possibly less sensitive than myosin. Bailey (1948) has shown tropomyosin to be removed by molar KCl, and it is believed to be a Z-line component (Corsi and Perry, 1958; Stromer et al., 1967a).

Enzymatic activity of muscle

The results of studies involving the incubation of muscle with ATP and ADP are summarized in Table 1. The activities of spider heart and frog gastrocnemius homogenates were investigated in order to contrast apyrase

Table 1. Enzymatic activity in muscle

Muscle	Mean values of mg phosphate cleaved per hour per gram of muscle during incubation with: ATP[a]	ADP[a]	ATP minus ADP
Spider heart	11.1 (1, 12)[b]	7.4 (1, 12)	3.7
Frog gastrocnemius	139.3 (2, 14)	110.7 (2, 14)	28.6

[a]Residual phosphate values have been subtracted.

[b]The number of samples are listed, followed by the number of determinations.

activity in a slow and a fast system. There is no concentration of fast fibers in the spider. This eliminates the possibility of making the logical comparison of phasic and tonic spider muscle. The frog gastrocnemius represents a well-characterized fast system (Florey, 1966). However, certain difficulties do arise in comparing muscle types in these two cold-blooded organisms.

Approximately 12 times as much orthophosphate was cleaved by frog muscle, in contrast to that of spider heart, during an incubation with ATP. This can possibly be explained on the basis of differences in the ATPase activities of myofibrils, the SR and mitochondria. Barany et al. (1965) have shown a two- to three-fold increase in fast over slow myosin ATPase, and Pellegrino and Franzini (1963) and Harigaya et al. (1968) have suggested that at least a twofold difference exists in fast over slow SR. Studies with the relaxing factor (Hasselbach and Makinose, 1962) and the cytochemical investigations of Gauthier and Padykula (1965) have demonstrated ATPase activity in the SR. Finally, observations on spider cardiac muscle ultrastructure indicate that the density of myofibrils and mitochondria may combine to lower associated ATPase activities by one-half. The twelvefold difference would then generally reflect a higher activity in several aspects of fast muscle metabolism.

However, on a closer inspection, it is noted that Mg^{++} (present in the incubation mixture) may preferentially

activate mitochondrial ATPase activity (Sacktor, 1953). Maximum activation occurs at a Mg^{++} concentration of 6×10^{-4} M, and inhibition is caused by concentrations greater than 6×10^{-3} M. Mg^{++} levels in this study were approximately 2×10^{-3} M. Although mitochondria normally dephosphorylate ten molecules of ATP for every one of ADP (Gilmour, 1961), Sacktor (1953) indicates that 1×10^{-3} M Mg^{++} may increase the dephosphorylation of ADP eightfold. All of these studies relate to insect systems, and strict applications should probably not be made to spider muscle. However, it is likely that Mg^{++} activates spider sarcosomes as well. If this is true, a large part of the activity during ATP and ADP incubation may be due to a mitochondrial ATPase.

A portion of the orthophosphate cleaved on incubation with ADP is likely due to an ATPase activity. Sacktor (1953) has demonstrated the presence of an adenylate kinase in insect muscle, specifically in the sarcosomes. This enzyme converts two molecules of ADP to one of ATP and one of AMP (Kielley and Kielley, 1951). The ATP would then be available for dephosphorylation.

Once values from ADP incubations are subtracted from ATP values, an approximately eightfold difference still exists (Table 1) between frog (fast) and spider (slow) muscle. The adjusted values do not, however, indicate specific ATPase action due to the probable presence of adenylate kinase in spider cardiac muscle. Whether the

eightfold difference reflects a variation in extra-mitochondrial ATPase activities is not known. It is possible that the adjusted values suggest certain differential activities in the myosin and SR of fast and slow muscle.

The rather atypically high concentrations of mitochondria in slow cardiac muscle have been discussed. If these observations are valid, the low level of ATPase activity in slow muscle cannot be solely related to low mitochondrial numbers. Instead, actual differences in mitochondrial rates must exist, as well as probable variations in other muscle components.

Calcium concentrations in fast and slow muscle

The results of calcium determinations on spider heart and frog gastrocnemius muscle are reviewed in Table 2.

Table 2. Calcium concentrations in muscle

Muscle system	Average value of mM calcium per kg of fiber water	Range of values
Spider heart	14.9	14.4-15.3
Frog gastrocnemius	10.4	9.6-11.1
Crab tonic	11.0	10.0-11.6
Crab phasic[a]	6.1	5.0-7.2

[a]These results are from a study of Atwood and Dorai Raj (1964).

The frog results are derived from two muscle preparations, and the spider from only one. Therefore, it is not possible to place a high degree of confidence in these values. Yet, 6 determinations were made on the spider material and 12 on the frog. As a result, the difference in calcium noted (ranges do not overlap) may be valid for the preparations analyzed here, but not generally for all spider hearts and frog gastrocnemius muscles.

Atwood and Dorai Raj (1964) have noted a difference in the calcium concentrations of fast and slow muscle. The level in slow crab muscles is almost twice that in fast. The determinations reported here possibly indicate a higher calcium concentration (close to 1.5 times) in the spider heart (slow), in contrast to the fast gastrocnemius. Fast frog muscle was employed in this study in an attempt to contrast a fast with a slow system. Yet, this comparison is fraught with certain difficulties, as indicated previously.

In discussing studies with isolated fibers, the importance of calcium in muscle contraction was noted. Higher concentrations of calcium may be required in the sustained contractions which are typical of tonic muscle. The increase in thin filaments in slow muscle may demand additional calcium for "linking" actin to myosin.

In considering calcium levels in fast and slow muscle, a recent paper concerning binding activity is appropriate.

Harigaya et al. (1968) have studied this activity in red rabbit muscle. Generally, red muscle is considered to be "slow" and white to be "fast". However, exceptions to this rule do exist. These workers found the calcium binding capacity of red muscle microsomal fractions to be one-third that of white. The yield of red microsomal material was further found to be one-half that of white. The EM efforts of Pellegrino and Franzini (1963) and Page and Slater (1965) had previously indicated that the SR of fast muscle was twice as extensive as that in slow. Thus, the total calcium binding of slow muscle is suggested to be one-sixth of fast. In addition, Harigaya's group acquired evidence that the rate of calcium uptake in fast material was two times that of the slow. The lower rate of binding activity in slow muscle suggests that calcium may remain free in the sarcoplasm for a period of time greater than that of phasic muscle. This would facilitate sustained contractions, as would the generally higher calcium content of slow muscle.

Atwood and Dorai Raj (1964) have also noted differences in sodium, although potassium concentrations are very similar. The sodium content of phasic muscle is approximately two-thirds that of tonic. These workers have theorized that sodium extrusion may be less efficient in tonic fibers.

Source of spider heart rhythmicity

Ether Three spider hearts were shown to be resistant to ether. After a period in excess of 60 minutes in an ether-saturated environment, the heart rate has been observed to measure 66 percent of the basal rate. At this point, somatic activity was very, very restricted even after intense physical stimulation. Within 10 minutes after removal from the ether, a basal heart rate (48 beats per minute) was again detected. Yet, somatic activity exhibited no significant reversal. During a period of ether exposure when the heart rate was near normal, a leg was removed. This typically results in an extensive twitching, but in this instance no such activity was observed. The work of Needham (1950) suggests that the response described here is typical of myogenic hearts.

An EKG run after a 25-minute exposure to ether (Figure 66) is similar to a typical, normal EKG (Figure 65).

Acetylcholine In one series of experiments, acetylcholine (Ach) at a concentration of 10^{-8} M was shown to possibly decrease heart rate by 17 percent (48 to 40). This rate decrease occurred in four minutes and was accompanied by a drop in amplitude. In two minutes, 10^{-7} M Ach lowered the heart rate by 25 percent (40 to 30) and decreased the amplitude. Following treatment with 10^{-5} M Ach, the rate was increased from 17 to 42 beats per minute in two minutes by addition of spider saline.

Figures 65 thru 72. EKG records of spider heart activity with the time interval equal to one second.

Figure 65. Normal spider EKG. A single major spike is observed. Neurogenic hearts often display additional major spiking activity (Prosser and Brown, 1962).

Figure 66. EKG after 25-minute exposure to ether.

Figure 67. EKG before exposure to 10^{-7} M Ach.

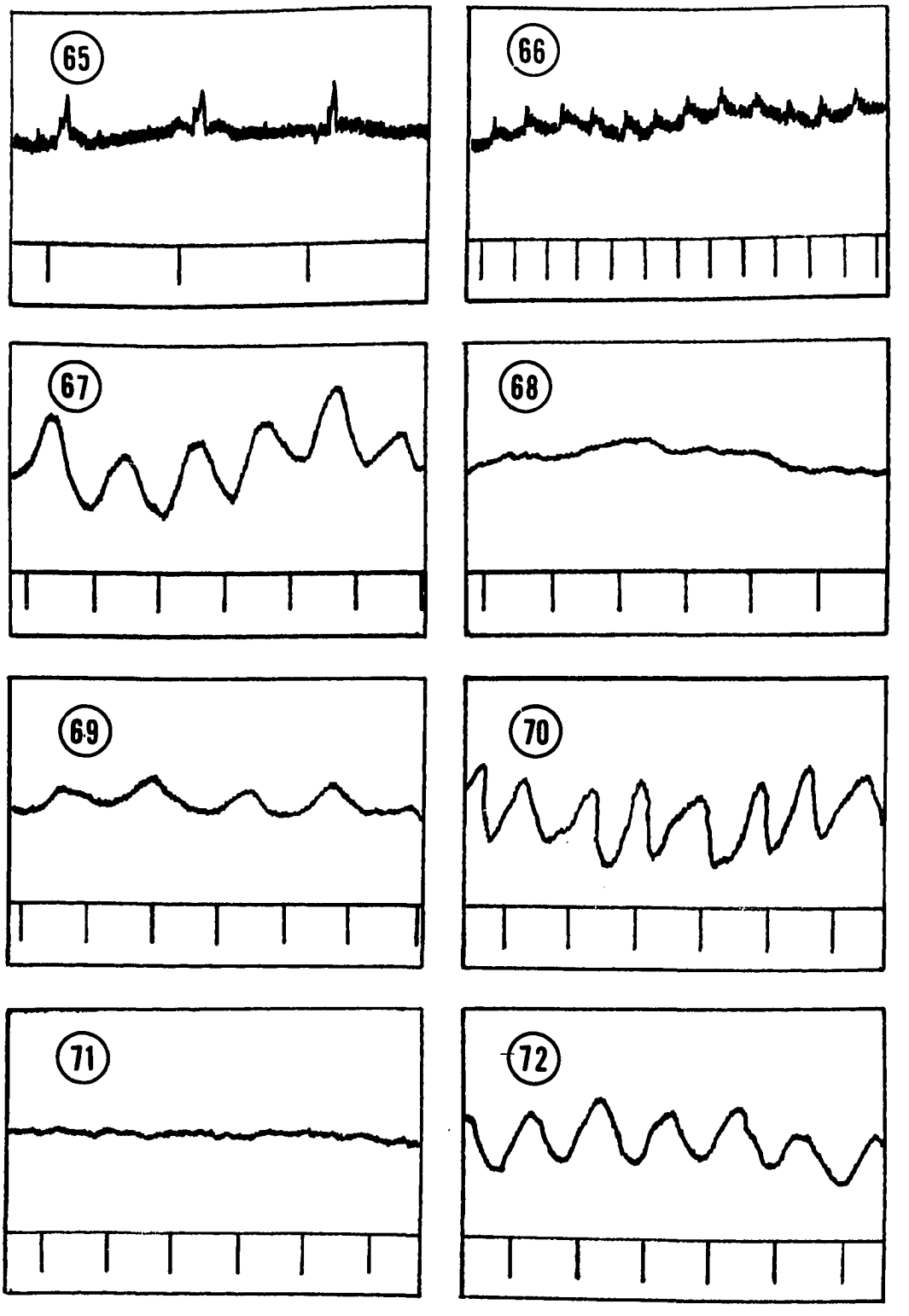
Figure 68. EKG one minute after exposure to 10^{-7} M Ach.

Figure 69. EKG six minutes following removal of the drug with saline.

Figure 70. EKG before exposure to 10^{-5} M Ach.

Figure 71. EKG one minute after exposure to 10^{-5} M Ach.

Figure 72. EKG following removal of the drug with saline, (eight minutes after first wash).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EKG's of spider heart activity reflected the influence of 10^{-7} M Ach, in another series of experiments (Figures 67 thru 69). Rate and amplitude both are visibly decreased. A more pronounced Ach inhibition is produced by 10^{-5} M concentrations (Figures 70 thru 72). Prosser (1942) has characterized this type of Ach inhibition as a feature of myogenic hearts. Kadziela and Kokocinski (1966) have reported the Ach inhibition and adrenalin acceleration of spider hearts. Their concentrations were higher; however, their methods were somewhat different.

Cardiac nerve removal Swicky and Hodgson (1965) have suggested that the scorpion heart is neurogenic. They based their claim on surgical removal of the cardiac nerve and electrophysiology. Removal of the nerve immediately stopped the associated portions of the heart. However, Kanungo (1957) had previously reported pharmacological results that suggest the myogenic nature of this heart. His results resemble those acquired here with the spider. Yet, if Zwicky and Hodgson (1965) did not injure the scorpion heart in the process of removing its nerve, the heart may actually possess a neurogenic character.

Wilson's work with the spider heart (1967) has a definite relevance at this point. He has totally removed the nerve and the heart contractions did not stop. However, because they became irregular, he considered the spider heart to be neurogenic. If the pharmacological studies on

the spider reported here are valid, another interpretation may be necessary. The spider studies represent the results of investigations with only a half dozen organisms, yet certain findings may be cautiously considered. It is theorized that the spider heart is myogenic, in contrast to Wilson's conclusion. The Ach inhibition (with concentrations as low as 10^{-7} M, possibly even 10^{-8}) and ether insensitivity support this, as does the lack of dependence on cardiac ganglia. Also, extensive spiking does not appear in a normal spider EKG (Figure 65). Extensive spiking is seen, however, in the scorpion (Zwicky and Hodgson, 1965) and crayfish hearts (Prosser and Brown, 1962). The latter is known to be neurogenic. Investigations relating to generator potentials across cardiac muscle cell membranes would do much to clarify the nature of the pacemaker.

In the vertebrate heart, the parasympathetic portion of the vagus serves to regulate (slows the heart) cardiac activity. Preganglionic fibers end in the cardiac ganglia of the auricles (Ranson and Clark, 1959). Postganglionic fibers then extend into the heart. If spider hearts are myogenic, their cardiac ganglion cells may also be interpreted as regulators--not pacemakers.

General Conclusions

References to other invertebrate muscle systems

The similarities between spider cardiac muscle and typical slow arthropod muscles (Fahrenbach, 1967) have been noted. In addition, this spider muscle also resembles a number of other invertebrate muscle systems which are believed to be physiologically slow. Certainly, spider cardiac muscle resembles the cardiac muscle of insects (Edwards and Challice, 1960), crustaceans (Kawaguti, 1963a) and gastropods (North, 1963). This resemblance is based on filament organization, general sarcomere appearance and surface specializations. Although the structural similarities may also be extended to striated muscle in the horseshoe crab arterial wall (Dumont et al., 1965), there is nothing especially striking about these observations. All of these striated muscles are involved in similar circulatory functions.

Comparable morphological features are observed in the visceral muscles of the insect (Smith et al., 1966) and slow leg muscles of the insect (Auber, 1967), scorpion (Auber, 1963) and crayfish (Brandt et al., 1965). Furthermore, Fahrenbach (1967) has reported that features, similar to those of spider cardiac muscle, are present in muscles of invertebrate reproductive systems and in postural muscles. Thus, these structural characteristics appear to be present in a wide range of invertebrate muscle types. It is here that a probable significance lies. It would appear that

structure and function are closely related in these systems. In this intimate relationship, structure does or does not grant certain functional capabilities to the muscle and a functional "slow" quality is reflected in fiber structure.

This wide range of muscle types appears to involve situations where slow, sustained contractions are suitable, or at least acceptable. The slow muscle fibers generally participate in functions requiring no rapid physiological response, but prolonged activities. Certainly, slow muscle systems in the invertebrates appear to be concerned with processes dealt with by vertebrate smooth muscles. However, striated slow muscles are also found in the vertebrates (Florey, 1966). Rosenbluth (1967, 1968) has suggested that the obliquely striated muscle of Ascaris exists as an intermediate between cross-striated and smooth muscles. His contraction mechanism for this system implicates a sliding, as with the thick and thin filaments of striated muscle. In addition, a theorized shearing is believed to account for the great extensibility of non-striated muscles.

Factors contributing to the "slow" quality of tonic muscle

Studies of fast and slow striated muscles strongly suggest that a large number of factors are involved in determining whether a given muscle is fast or slow. In reviewing the literature, it was noted that myosin ATPase activities vary in these two muscle types (Barany et al., 1965).

Certain differences were also noted in the sources of metabolic energy. Furthermore, Harigaya et al. (1968) detected a much lower capacity and rate of calcium binding in the microsomal fractions of slow muscle, where calcium concentrations may be higher (Atwood and Dorai Raj, 1964). Finally, the membrane properties of fast and slow muscles vary (Wiersma, 1961).

Investigations reported here with spider cardiac muscle indicate that, although thick filament diameters are 50 percent greater than typical fast diameters (Fahrenbach, 1967), the substructure of cross sections is essentially sixfold. The same thing is true of fast thick filaments. However, when the ratio of thin to thick filaments becomes greater than 2:1, thick filament lengths generally increase (Hagopian and Spiro, 1968). Thus, the number of cross-bridges along a given region of a slow thick filament may equal the number along a similar length of a fast thick filament, but slow sarcomeres may possess more cross-bridges because of longer thick filaments. These additional cross-bridges may assist in the typical sustained contractions of tonic muscle. On the other hand, the lack of a hexagonal arrangement of thin filaments in the slow fiber could prevent a fast response to stimulation. That is, since thick filament cross-bridge organization is hexagonal, an immediate response may be restricted by an unordered distribution of I-filaments.

If a compositional variation exists between fast and

slow Z-lines (as possibly suggested by the trypsin experiments), α-actinin could be one of the protein components involved. The newly-described regulator proteins in muscles have been implicated in several situations. Maruyama's (1965a, 1965b) work indicates that β-actinin regulates thin filament lengths. Since thin filaments from different systems vary in length (Hagopian and Spiro, 1968), β-actinin may vary qualitatively and/or quantitatively in different muscle systems. Similar variations could then conceivably occur in regards to α-actinin in fast and slow muscle. As suggested, variations in this promoter of superprecipitation could possibly endow tonic muscle with its slow quality. Certainly this recently uncovered area of regulator proteins offers exciting opportunities for muscle research. Possibly an understanding of slow muscle, as well as fast, will be enhanced by these future investigations.

To summarize, fast and slow muscle may vary with respect to almost every muscle fiber component--filaments, Z-lines, membrane systems and the sarcoplasm.

Contraction model for spider cardiac muscle

On the basis of investigations of spider cardiac muscle and other studies in the literature, a theorized contraction model for spider cardiac muscle is briefly reviewed here.

Rhythmic contractions may originate in the muscle itself, or as a result of neural activation. If the heart is myogenic,

specialized muscle cells may be involved; if it is neurogenic, nerve impulses would be implicated in the muscle activation. Regardless of the source of rhythmicity, a membrane depolarization of the sarcolemma is able to pass deeply into the cell interior by following the t-tubules. These tubules come in close contact with the SR at the diadic associations. It is suggested that t-tubule depolarization causes the release of Ca^{++} from the fenestrated collars which surround the myofibrils. Ca^{++} has then been shown to activate the contractile apparatus. The slightly higher concentration of calcium in slow muscle and the lower calcium binding activities of slow muscle may contribute to the tonic contractions. However, free calcium is eventually returned to its storage site in the SR. During contraction, calcium bridges could be formed between myosin and actin as Davies (1963) has theorized. The ATP required by spider cardiac muscle is presumably generated by the mitochondria as the vital step in the metabolism of glycogen. A sliding of thin over thick filaments is theorized in the traditional manner whereby only the I- and H-bands are reduced.

SUMMARY

1. The spider heart is a rather well-developed muscular tube with internal, longitudinal muscle ridges. The myocardium, which is surrounded by a pericardium, displays Z-lines and A-, I- and H-bands. The membrane borders of the muscle cells often display a scalloped appearance. The heart is cellular and multi-nucleated, and it possesses a dorsal cardiac nerve. The nerve contains ganglion cells and sends branches into the heart.
2. Electron microscopy of thin sections has demonstrated the presence of thick (230-240 Å) and thin (60-70 Å) filaments. The 3 μ sarcomeres possess no M-lines, and H-bands are not generally apparent. Z-lines are broad, and a highly filamentous character to these components is not observed. When sectioned transversely, 8 to 12 thin filaments can be seen to surround a single thick myofilament. All of these findings suggest that spider cardiac muscle is a slow striated muscle system (Fahrenbach, 1967). Rotationally printed thick filament cross sections display a hexagonal core or shaft with a pair of electron-dense spheres at each of the six corners. The spheres are interpreted as representing the arms of HMM cross-bridges.
3. The sarcolemma of these cardiac muscle cells invaginates as t-tubules at the level of the A-I junction. These

tubules associate with longitudinal components of the SR to form dyads. This is the theorized pathway for excitation-contraction coupling. The SR involves fenestrated collars which surround the myofibrils.

4. A fibrous coat generally covers the sarcolemma. The scalloped border observed by light microscopy is seen to involve orderly, periodical surface invaginations which occur at the level of Z-lines. Z-lines are apparently attached to scallop bases, and scallops are compressed during contraction. Electron-dense, membrane-limited bodies (suggested to possibly be lysosomes) are frequently seen near the cell surface.
5. Mitochondria are concentrated in pools of what is believed to be glycogen. These aggregations are randomly squeezed in between myofibrils. The rather abundant mitochondria possess lamellar cristae and intramitochondrial granules. It is theorized that these muscle mitochondria accumulate calcium and phosphate ions and that this respiratory-linked accumulation produces the electron-dense granules (Greenawalt et al., 1964).
6. The dorsal cardiac nerve involves a large number of myelinated axons containing limited amounts of electron-dense vesicles and synaptic vesicles. Branches are observed to pass into invaginations of the external heart surface.

7. Freeze-etch replicas demonstrate the typical spider cardiac muscle ultrastructure, and indicate that scallops may be due to grooves running transversely across the cell surfaces.
8. The Z-lines of rabbit psoas fibers (a fast system) are removed after a six-minute exposure to trypsin. However, simultaneously-treated spider leg fiber Z-lines display a near normal fine structure after six minutes. The resistance of these slow muscle Z-lines to trypsin may be based on a permeability factor. An alternative suggestion considers an actual variation, either structural or compositional, in the two Z-line types. If a compositional difference does exist between fast and slow Z-line material, α-actinin could be one protein species involved. This trypsin-labile protein (Goll et al., 1967) promotes the superprecipitation of actomyosin (Ebashi and Ebashi, 1965), and it may be present in or near the Z-line (Masaki et al., 1967). A lack of or low concentration of α-actinin in tonic muscle could possibly grant a physiological "slow" quality to this muscle.
9. PAS-positive regions of the spider heart contain abundant granules that measure slightly less than 200 Å. These isodiametric granules occur singly or as rosettes. It is suggested that these are glycogen particles. The particles form large pools at cell surfaces and between

myofibrils, which contain groups of mitochondria.

10. Isolated leg muscle fibers and cardiac muscle shorten in the presence of Ca^{++} and lengthen when exposed to EDTA. 1 M KCl removes A-band material from isolated muscle. This same treatment causes an increase in I-band density and a "swelling" and dissolution of Z-lines.

11. When homogenates of spider heart and frog gastrocnemius muscle are incubated with ATP, 12 times as much orthophosphate is detected following incubation with the fast frog muscle. The incubations contain Mg^{++} and therefore, a majority of the activity may be due to a Mg^{++} -activated mitochondrial ATPase (Sacktor, 1953). The ADPase activity of mitochondria is also increased with Mg^{++}. When homogenates are incubated with ADP and these orthophosphate values are subtracted from the ATP values, frog muscle is still 8 times more active than the spider muscle. Whether this reflects a variation in extra-mitochondrial ATPase activities is not known. If so, differences in the ATPase properties of fast and slow myosins (Barany et al., 1965) and the two SR forms (Harigaya et al., 1968) could be involved.

12. Preliminary efforts in measuring calcium concentrations in fast and slow muscle have suggested that slow values are approximately twice the fast (Atwood and Dorai Raj, 1964). In the study reported here, a spider heart value was approximately one and a half times that of two frog

gastrocnemius values.

13. Spider hearts appear to be insensitive to ether and to be inhibited by acetylcholine. Typical EKG records display a single major spike, and isolated hearts beat regularly. In addition, Wilson (1967) has reported that complete removal of the cardiac nerve does not stop heart contractions. While these latter observations would suggest that the spider heart is myogenic, electrophysiological studies of the heart are necessary before definite conclusions can be drawn.

14. A sliding model for contraction in spider cardiac muscle is presented.

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DEDICATION --

C. Judson Herrick[1] of the University of Chicago has charged all men to meet the responsibilities imposed upon them by the Universe:

> "We are citizens of the universe. Our cosmos is dynamic and intrinsically creative at all levels of organization. This native creativity is amplified in the domain of organic evolution.... The sublimity of this conception...commands...our utmost effort to meet the demands imposed upon us by that nature which is our alma mater."

However, as we as scientists strive to achieve understanding in our own restricted centers of interest, we often lose sight of the magnitude and scope of the entire Universe. To lose this vision, suggests Theodosius Dobzhansky[2], is to underestimate and minimize the breadth and majesty of His Creator:

> "A common foible of scientists is to suppose that the little truths which they discover explain everything rather than only something. The kindest way to regard this foible is to say that scientists are only human. A less kind way is to suspect that a fondness for explaining highly complex situations by one simple cause is an earmark of a mediocre mind. This is, however, besides the point, since believing that the evolution of the Cosmos is the method whereby the Creator actuates His idea of creation has neither the defects nor the virtues of simplicity."

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[1]C. J. Herrick. 1960. Science, faith and human nature. In Shapely, H., ed. Science ponders religion. Pp. 289-308. Appleton, Century and Crofts, New York.

[2]T. Dobzhansky. 1960. Man consorting with things eternal. In Shapely, H., ed. Science ponders religion. Pp. 117-135. Appleton, Century and Crofts, New York.

As this thesis is dedicated, the reader is encouraged to recognize and appreciate the intensely beautiful organization of living cells. Yet, he is cautioned to recall that this represents but one segment of our vast Universe, and one facet of His magnificent Creation. We must all strive to identify our responsibilities in the wide range of arenas in which we should participate. This is not to say that a biologist should also be a physicist, and a physicist also a psychologist. It is meant to suggest that the concerns of a scientist should extend far beyond the laboratory. This thesis is dedicated to the hope that all men hasten to achieve an understanding of the relationships they bear with their fellow man, their Universe and their God.

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