CARDIAC MUSCLE OF THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*

I. Ultrastructure

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ABSTRACT

The fine structure of the cardiac muscle of the horseshoe crab, *Limulus polyphemus*, has been studied with respect to the organization of its contractile material, and the structure of its organelles and the cell junctions. Longitudinal sections show long sarcomeres (5.37 μ at L_{max}), wide A bands (2.7 μ), irregular Z lines, no M line, and no apparent H zone. Transverse sections through the S zone of the A band show that each thick filament is ca. 180 A in diameter, is circular in profile with a center of low density, and is surrounded by an orbit of 9–12 thin filaments, each 60 A in diameter. Thick filaments are confined to the A band: thin filaments originate at the Z band, extend through the I band, and pass into the A band between the thick filaments. The sarcolemmal surface area is increased significantly by intercellular clefts. Extending into the fiber from these clefts and from the sarcolemma, T tubules pass into the fiber at the A-I level. Each fibril is enveloped by a profuse membranous covering of sarcoplasmic reticulum (SR). Sacculations of the SR occur at the A-I boundary where they make diadic contact with longitudinal branches of the T system. These branches also extend toward the Z, enlarge at the Z line, and pass into the next sarcomere. Infrequently noted were intercalated discs possessing terminal insertion and desmosome modifications, but lacking close junctions (fasciae occludentes). These structural details are compared with those of mammalian cardiac and invertebrate muscles.

INTRODUCTION

The horseshoe crab, *Limulus polyphemus*, which is a member of the phylum Arthropoda, has a closed circulation and a dorsal tubular heart that contains both venous and arterial valves (11, 40). Since this heart exhibits neurogenic rather than myogenic control (11–14), it has long been of interest to physiologists. Recent studies have shown that, unlike the mammalian heart, the *Limulus* heart has a short refractory period and can be tetanized such that contractile properties may be studied during a steady state (1). The mechanical behavior of the *Limulus* heart has been characterized in terms of its length-tension and force velocity relations, with the demonstration of both length and inotropic (contractility) dependent activity (1). These studies suggest that the comparative physiology of the *Limulus* heart may also provide insights into the functional relationships of the mammalian myocardium.

Despite this interest in the electrical and mechanical properties of the *Limulus* heart, there is little information as to its ultrastructure and no

information as to the relation of its fine structure to mechanical activity. Furthermore, it has previously been suggested that the sliding model of muscular contraction may require some revision when applied to the heart of the horseshoe crab (32). Accordingly, the present studies were undertaken, first to define the ultrastructure of the horseshoe crab heart more precisely, and second to relate this ultrastructure to mechanical activity and the "sliding filament" mechanism of contraction.

MATERIALS AND METHODS

Horseshoe crabs, *Limulus polyphemus*, obtained from the Marine Biological Laboratories at Woods Hole, Massachusetts, were used. Dissection of the dorsal skeleton exposed the living heart which was rapidly excised. The heart was transected such that a ring of muscle about 1 mm wide was obtained. This ring was cut at one point across its width, resulting in a strip of parallel fibers of 3–4 cm in length. A segment of muscle about 15 mm long was removed and suspended vertically in a 15-ml bath containing *Limulus* Ringer's solution aerated with 95% O_2 and 5% CO_2. The lower end of the muscle was held firmly by a spring-loaded lucite clip fixed to a rigid rod extension, through a water seal of a force transducer (Statham model G1-4-250).

Muscles were fixed by rapidly withdrawing the *Limulus* Ringer's from the bath and replacing it with a solution of 3% glutaraldehyde in Sorenson's phosphate buffer (21), pH 7.3 and 550 milliosmols. A selected group of muscles was also fixed by a formaldehyde-glutaraldehyde-picric acid fixation technique[1]; this fixative had a pH of 7.2 and a milliosmol value of ca. 2100. Total time of fixation in the bath was 30 min. The muscle strip was then removed from the bathing chamber, cut into small plates, and placed in a second solution of the fixative for 3 hr. Following this fixation, the tissue plates were placed in a buffer wash solution for 3 hr, and then in a solution of 1% osmium tetroxide in Sorenson's buffer, at 4°C, for 2 hr. After fixation and subsequent rinsing in distilled water, the tissues were carried through dehydration in graded ethyl alcohols. The tissue plates were transferred to a propylene oxide/absolute alcohol mixture and then placed in pure propylene oxide. Subsequent to this, the tissues were placed in a 2:1 propylene oxide-Araldite/Epon mixture for 1 hr (at 25°C), a 1:2 propylene oxide-Araldite/Epon mixture for 1 hr (at 25°C), a pure Araldite/Epon mixture for 3 hr (at 25°C), and an Araldite/Epon mixture for 12 hr (at 60°C). The resin mixture used for embedding was Epon 812-25 ml, dodecenyl succinic anhydride (DDSA)-55 ml, Araldite 502-15 ml, DBP-3 ml, 3.0% benzyldimethylamine (BDM) (41).

Sections were cut with a diamond knife on Porter-Blum MT-2 and Reichert OM-1 ultramicrotomes and double stained in uranyl acetate (5 min) and lead citrate (5 min) solutions. The sections were examined with a Philips 300 electron microscope. All electron microscopic investigation was performed at the facilities of the Cell Biology Laboratories, Harvard College, Cambridge, Mass.

[1] D. Feldman. Personal communication.

OBSERVATIONS

Ultrastructural Organization of the Myocardial Cell

GENERAL COMMENTS: The *Limulus* heart is composed of individual muscle cells (fibers) of interminate length beyond 200 μ that are roughly cylindrical in shape, ranging in diameter from 25 to 65 μ. As is the case with the mammalian myocardium, these fibers are striated, arranged in columns, and surrounded by a sarcolemma (Fig. 1). Occasionally, specializations of this membrane, called intercalated discs, are noted forming fiber-to-fiber "connections." The fiber contains tightly packed myofibrils which run the length of the fiber and are composed of serially repeating sarcomeres (Fig. 2) arranged more or less in register. This variance in sarcomere register, although slight, is reflected in transverse sections cut through the fiber such that micrographs of these sections often show regional differences in substructure characteristic of different band levels of the sarcomere (Fig. 1). Additionally, on cross-section, myofibrils are surrounded by elements of the sarcotubular system; however, as this network is not always seen to be complete, the distinct separation of fibrils may not always be discernible. Mitochondria occur both peripherally and between groups of myofibrils whereas the nucleus is located peripherally in the cell.

THE SARCOLEMMA: A rather stout sarcolemma (Fig. 1), composed of a two-component complex made up of a plasma membrane and a thick extracellular boundary lamina (i.e. basement membrane), surrounds each fiber. The plasma membrane is a typical trilaminar unit membrane, ca. 90–100 A thick. The boundary lamina, which is closely applied to the external surface of the plasma membrane, is quite wide in relation to the plasma membrane and often appears layered. The region closest to the plasma membrane is of lesser density than other areas of the boundary lamina.

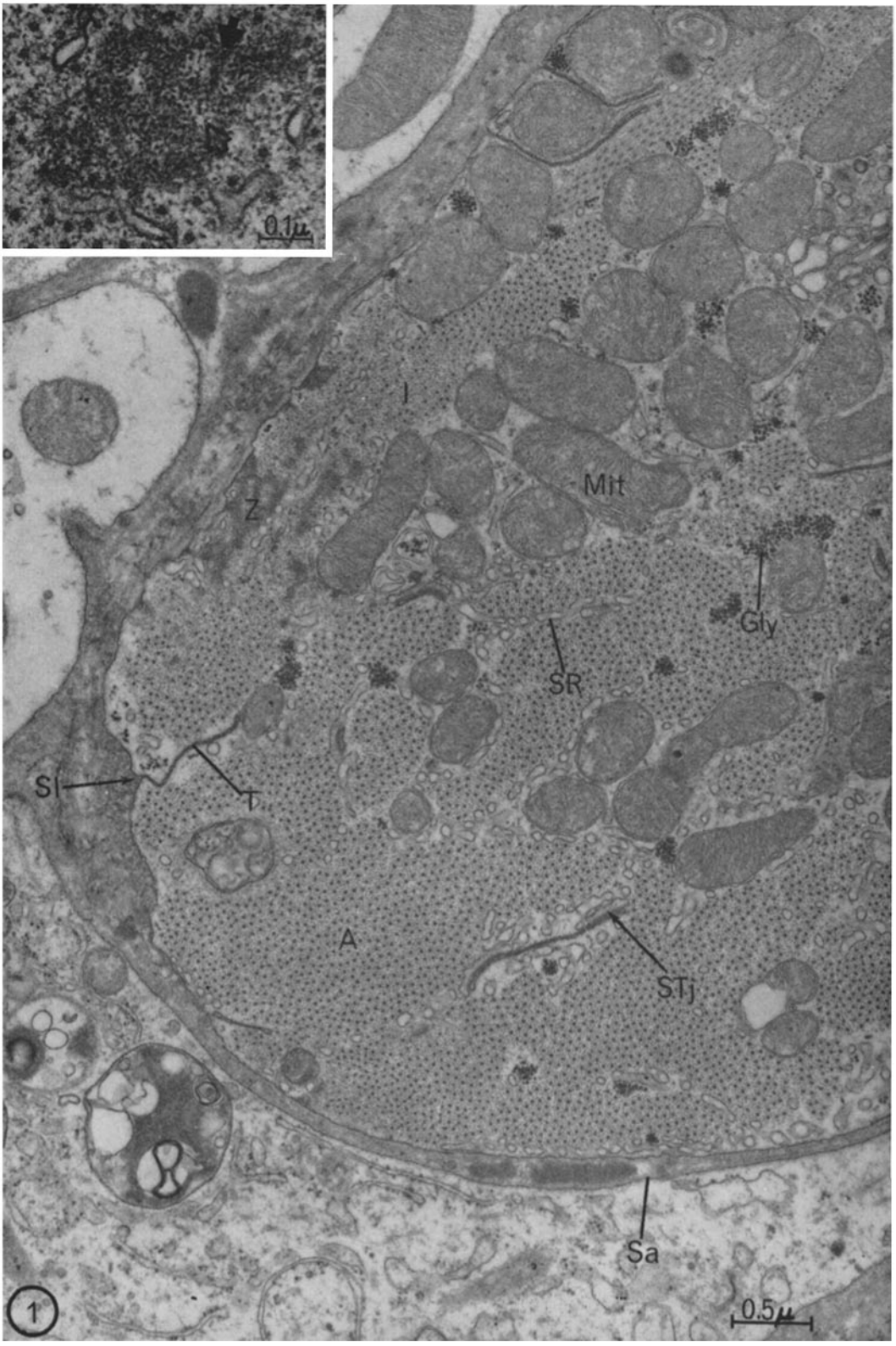

FIGURE 1 Cross-section of a *Limulus* cardiac muscle fiber. The sarcolemma (*Sa*) is relatively thick. Sarcolemmal invaginations (*SI*) give rise to T tubules (*T*) which pass into the fiber forming sarcotubular junctions (*STj*) with the sarcoplasmic reticulum (*SR*). A, I, and Z levels of the fibrils are shown, as are mitochondria (*Mit*) and glycogen (*Gly*). × 23,750. *Insert:* Cross-section cut through the Z line shows its perforate nature. Solid arrow indicate Z rims; open arrow indicate Z perforations. × 76,950.

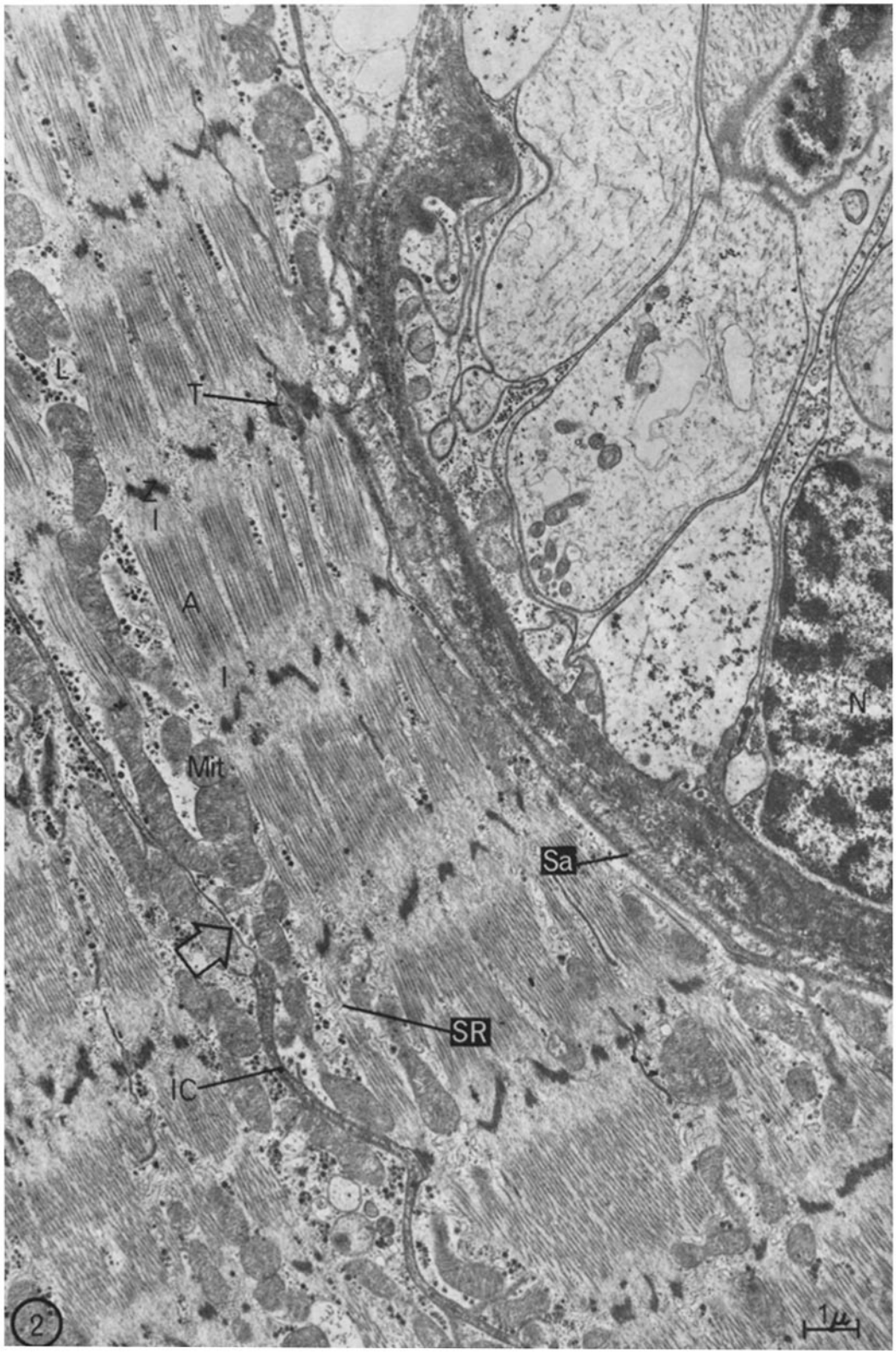

FIGURE 2 Survey electron micrograph of a longitudinal section of *Limulus* cardiac muscle. A, I, and Z bands of the sarcomere are shown. A bands are wide (2.7 μ); Z bands appear irregular in both shape and course across the fibril. M lines are absent. Fragments of SR are found in interfibrillar spaces. T tubules (*T*) are found dilating at the Z and passing from one sarcomere to the next. The sarcolemma (*Sa*) and an intercellular cleft (*IC*) are shown. A point of close apposition of IC membranes is indicated (open arrow). Neural elements are shown in upper right portion of the micrograph. × 8750.

Slight invaginations are observed on both external and internal surfaces of the plasma membrane, suggesting the occurrence of micropinocytosis.

Longitudinal sections (Fig. 2) show the sarcolemma to have a scalloped appearance due to invaginations of this membrane and its apparent attachments at the Z band level of underlying sarcomeres.

INTERCELLULAR CLEFTS: Deep clefts that appear to be continuous with the sarcolemma pass deeply into (Fig. 3) the interior of the fiber. These intercellular clefts contain both a plasma membrane and external lamina, appear to be identical to the sarcolemma in composition, and are ca. 650 A wide. Intermittently, these clefts appear to narrow with a loss of external lamina (Fig. 2). At this point, the membrane interspace is ca. 180–250 A. Despite this close approximation of cleft membranes, at no point was a fusion of opposing membranes observed.

INTERCALATED DISCS: Although not frequently observable in *Limulus* cardiac muscle, these specialized membranes transect the cardiac cells from one intercellular cleft to the next. Generally, they are seen crossing the myofibrils in a plane where the Z is usually found (Fig. 4). The discs are comprised of two dense, opposed membranes separated by a relatively clear space of uniform width, and are surrounded on their inner surface by an irregular band of electron-opaque material. As is the case of mammalian cardiac muscle, there is an apparent alteration of: (*a*) longitudinal segments extending between the myofibrils, and (*b*) transverse segments which intersect the myofibrils. Only two structural modifications of the intercalated disc are evident in *Limulus* cardiac muscle, the fascia adherens and the macula adherens. The former modification, also termed the terminal insertion plaque, is found at the point of end-to-end cell attachment. Here, apposing membranes are separated by an intercellular space of uniform width (ca. 200 A). Along the inner surface of these membranes is an area of high density into which thin filaments of the I band insert and terminate. Further, in areas of the disc between the sites of filament insertion, a macula adherens (desmosome)-type modification is often noted. Areas compatible with the fascia occludens of mammalian cardiac muscle were not observed.

THE SARCOTUBULAR SYSTEM: Interfibrillar spaces of *Limulus* cardiac muscle fibers contain a complex and profuse (Fig. 5) network of membrane-limited tubules, the sarcotubular system. As in other striated muscles, this network is comprised of a transverse component, the T system, and a more or less longitudinal component, the SR (sarcoplasmic reticulum). An examination of the relationship of these components is seen in cross-section (Figs. 1, 3, 6–8). At various points over the muscle fiber surface the sarcolemma (and the intercellular clefts) forms invaginations. These sarcolemmal invaginations, lined by both plasma membrane and a discrete external lamina, are generally seen at the A-I level of the sarcomere and occasionally at the Z line. The T system originates from these sarcolemmal invaginations; its tubule wall appears to be continuous with the wall of the sarcolemma (or intercellular cleft) and its invagination. As the T tubule passes into the fiber, its membrane wall shows small vesicular depressions or invaginations similar in nature to those observed in the sarcolemma. As in the case of the sarcolemma, this observation may suggest cellular transport across the T tubule membrane wall by micropinocytosis. Progressing into the fiber interior, the T tubule membrane wall appears to gradually lose its external lamina.

The SR is a nonfenestrated system of more or less longitudinally oriented tubules which form a profuse, membrane-limited network around each myofibril and which exhibit an apparent: (*a*) longitudinal continuity at all levels of the sarcomere and myofibril (Fig. 9), and (*b*) lateral continuity between adjacent myofibrils. Unlike the T system, this tubule system does not display a continuity with the sarcolemma. Additionally, numerous SR and SR-like vesicles (Fig. 6) have been observed at the inner margin of the sarcolemma (i.e. at its cytoplasmic surface) and in close approximation to the sarcolemmal invaginations, displaying a vesicular distribution similar to that described in mammalian cardiac muscle. However, specific diadic-type junctions between the SR subsarcolemmal cisternae and the sarcolemma were not observed.

As noted above, the T tubule system extends in from sarcolemmal invaginations, most often at the sarcomeric level of the A-I boundary. At this level, it passes between two myofibrils each of which is surrounded by an SR tubule system. At this point, the T system gives off a longitudinally oriented component which lies parallel to the longitudinal sarcomeric axis. The two systems come into close approximation at this point, forming a diadic

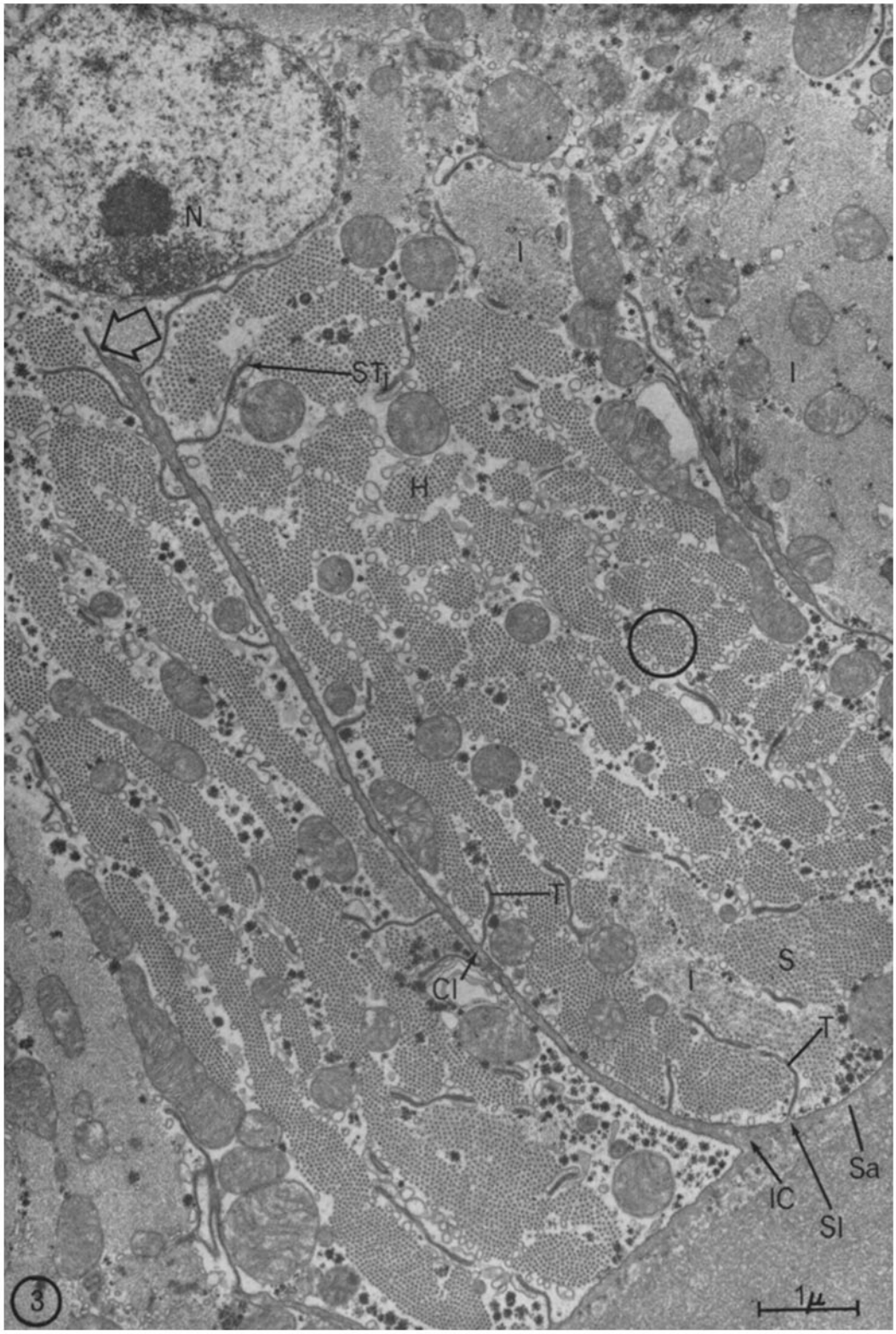

FIGURE 3 Survey cross-section of stretched fiber. The relationship between sarcolemma (*Sa*), sarcolemmal invaginations (*SI*), and intercellular clefts (*IC*) is shown. Depth of IC penetration within the fiber is indicated (open arrow). T tubules arise from sarcolemmal invaginations (*SI*) and cleft invaginations (*CI*), forming sarcotubular junctions (*STj*) with elements of the SR tubules. Myofilaments are shown (within circle) grouped into a discrete myofibril by membranes of the SR. I band, S and H zones of the A band, and the nucleus (*N*) are designated. × 15,200.

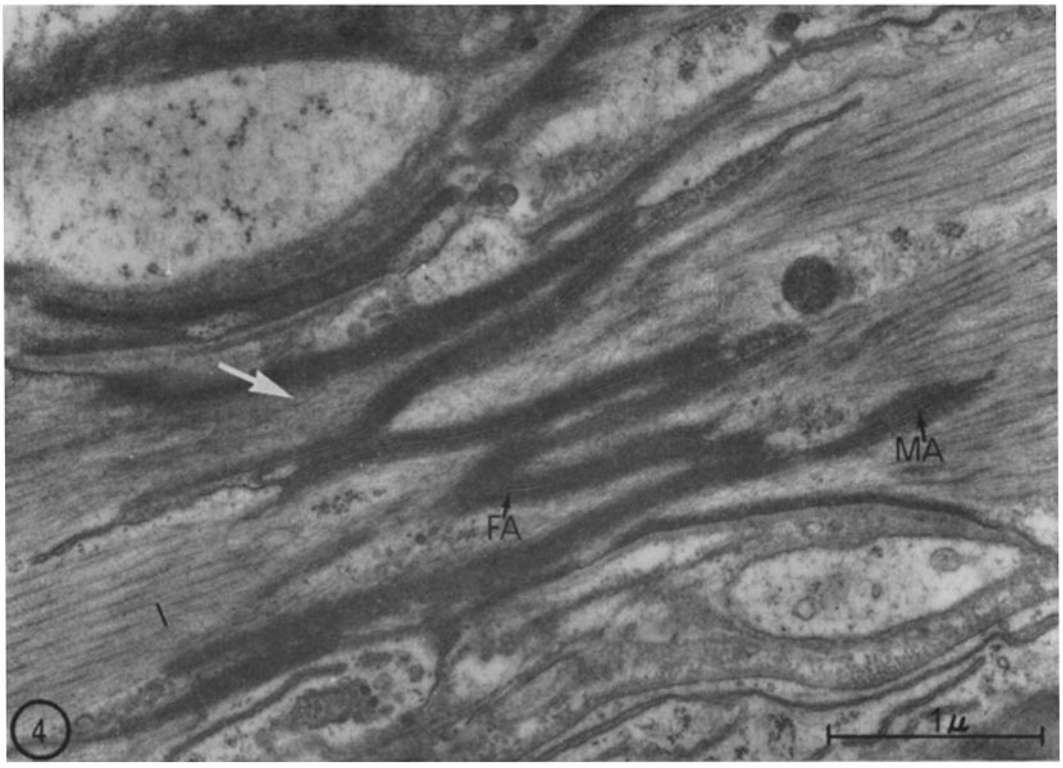

FIGURE 4 Longitudinal section of a portion of an intercalated disc. The cell surfaces are separated by an interspace of uniform width. Thin filaments appear to enter (white arrow) accumulations of dense material subadjacent to the cell membrane. Fascia adherens (*FA*) and macula adherens (*MA*)-like modifications are indicated. × 20,000.

junction (Figs. 1, 3, 5, 7, 8). A single T tubule seen in cross-section at the diad is elliptical in shape, measuring ca. 260 × 1500 A, and is apparently shared by the SR tubule system of two adjacent myofibrils (Fig. 8). Hence, the T tubule forms a diadic junction first with the SR of one myofibril and then with the SR of an adjacent myofibril. Transverse sections reveal the SR tubules to be clear and roughly circular (ca. 465 A in diameter) except at diadic junctions. At this point, the tubules appear elliptical (ca. 360 A × 1200–4800), and are filled with a granular substance. Although the limiting membranes of the T component generally appear to be of greater density than those of the SR, the latter undergo an increase in electron opacity at the diadic junction. Furthermore, a high degree of granularity also exists between the two tubules comprising the diadic junction. This granularity appears at points to form dense, nontubular connections between the membranes of the T system and the SR.

In addition to forming diadic junctions with the SR, the longitudinal components of the T system extend, parallel to the long axis of the fibril, in the opposite direction, toward the Z line. At the Z line, the T-tubule membranes thicken while the tubules dilate markedly (Fig. 9, inset) and are seen to contain a granular substance. These tubules then decrease in diameter and extend to the next in-series sarcomeres. Hence, successive T tubules may be connected, or, alternatively, in-series sarcomeres may thus be "in contact" with a specific T tubule.

MITOCHONDRIA: The mitochondria of *Limulus* cardiac muscle occur throughout the cell, appearing variable in size and shape. Generally, they appear either circular or elliptical in shape (circular profiles are ca. 0.5 μ in diameter) (Figs. 1, 3). As in the mammalian myocardium, mitochondria of *Limulus* cardiac muscle are bounded by a double unit membrane complex (Fig. 1, 8), separated by an intermembrane space. The inner unit membrane of this complex gives rise to numerous

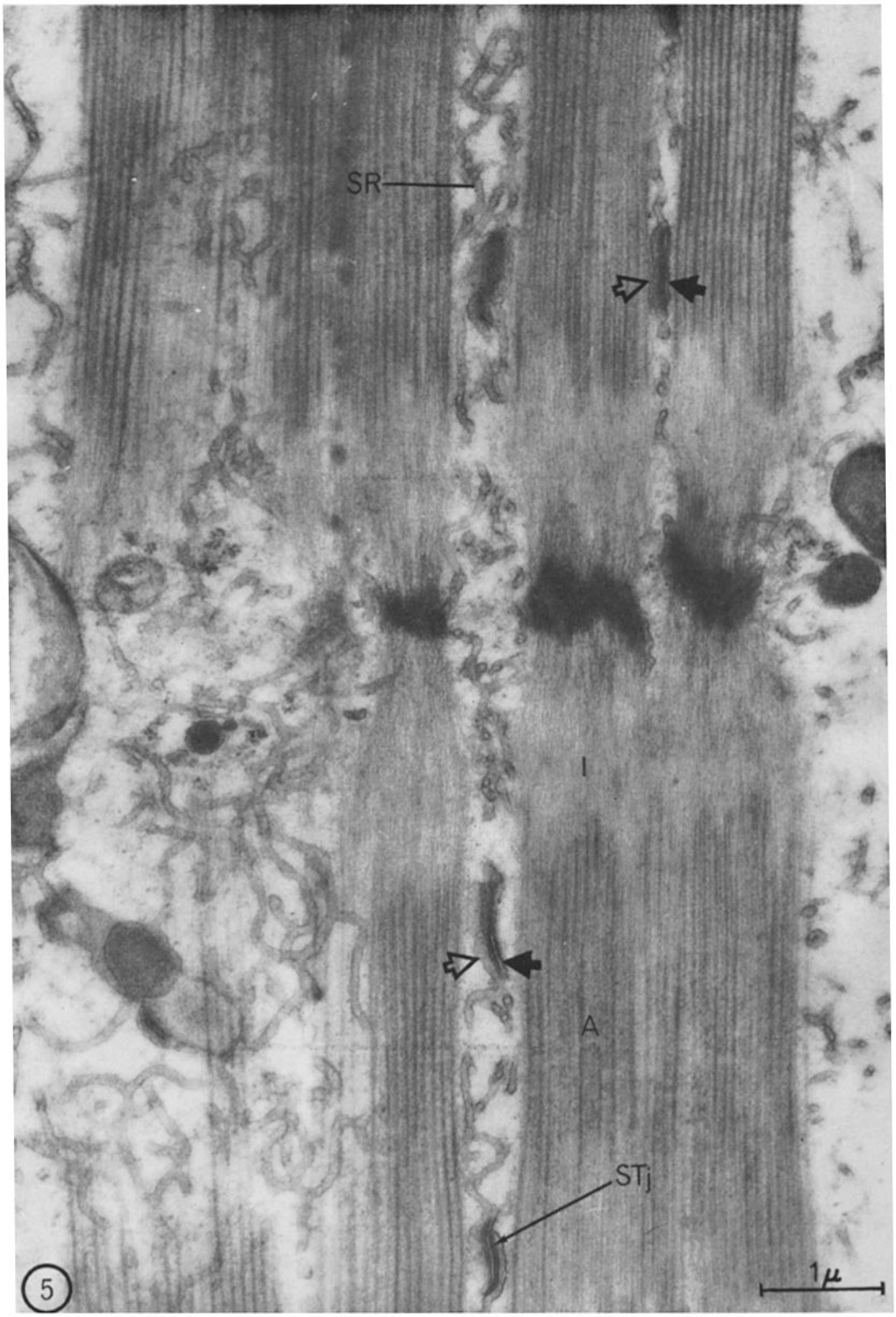

FIGURE 5 A portion of a longitudinal section of the cardiac muscle fiber, depicting some longitudinal branches of the transverse tubule system, SR tubules, and the sarcotubular junction (*STj*). T tubules (closed arrow) are readily differentiated from SR elements (open arrow) at the STj by the greater density of their membranes. The relation of the tubular system and STj to the sarcomere band pattern is indicated. × 18,500.

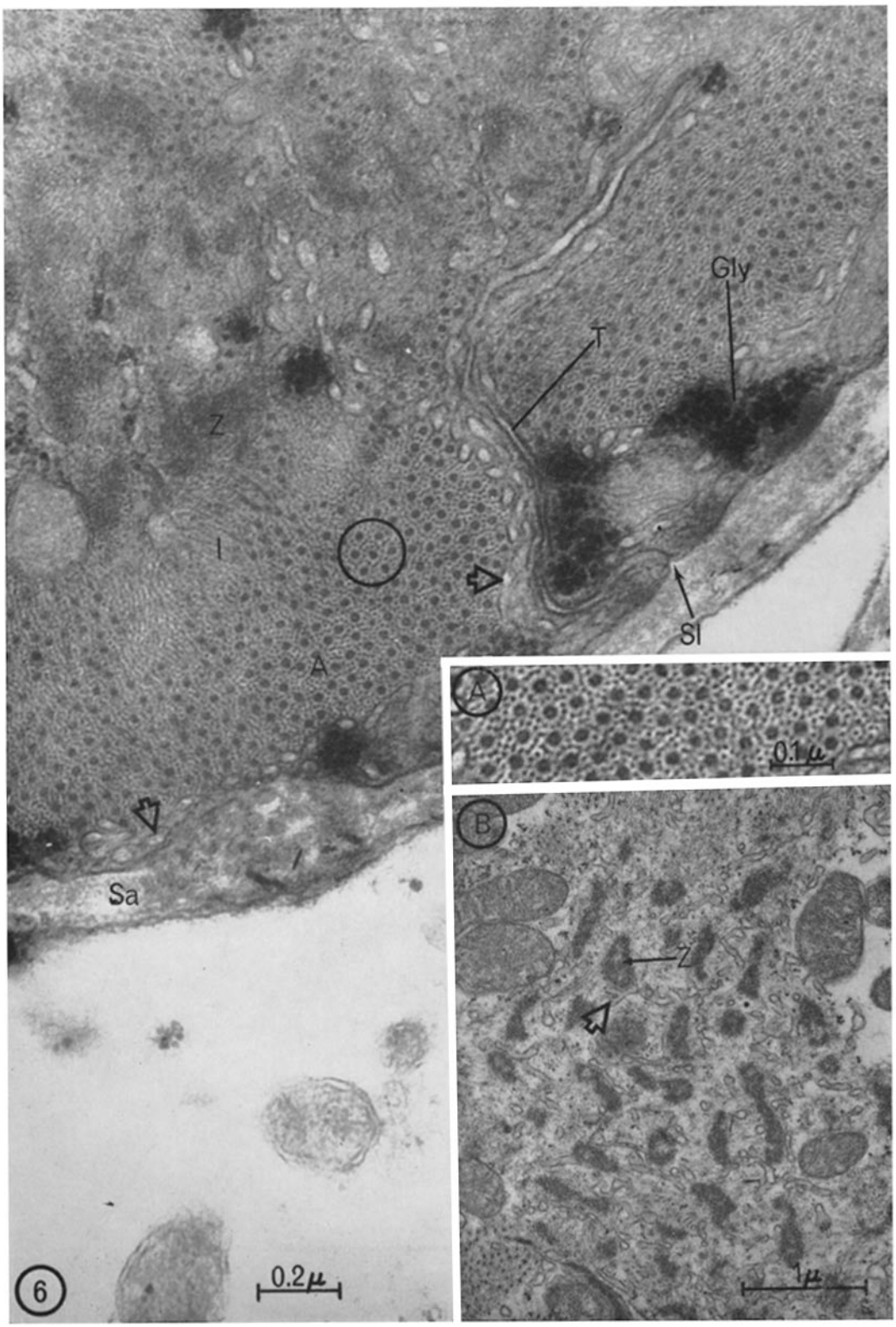

FIGURE 6 Cross-section showing A, I, and Z bands of the fiber. Thick filaments are circular in shape with a central core of low density, and are arranged in a hexagonal array (within circle). 9–12 thin filaments surround each thick filament. The relation of T tubule and sarcolemmal invagination (*SI*) is shown. Open arrows indicate close relation of SR tubules to T tubule and to inner surface of sarcolemma. × 60,000. *Insert A* is higher magnification of cross-section cut through the A band showing the thick-thin filament relation in greater detail. × 95,000. *Insert B* shows the Z band as individual dense bodies. Open arrow indicates SR tubule lying in close apposition to and between neighboring Z bodies. × 18,750.

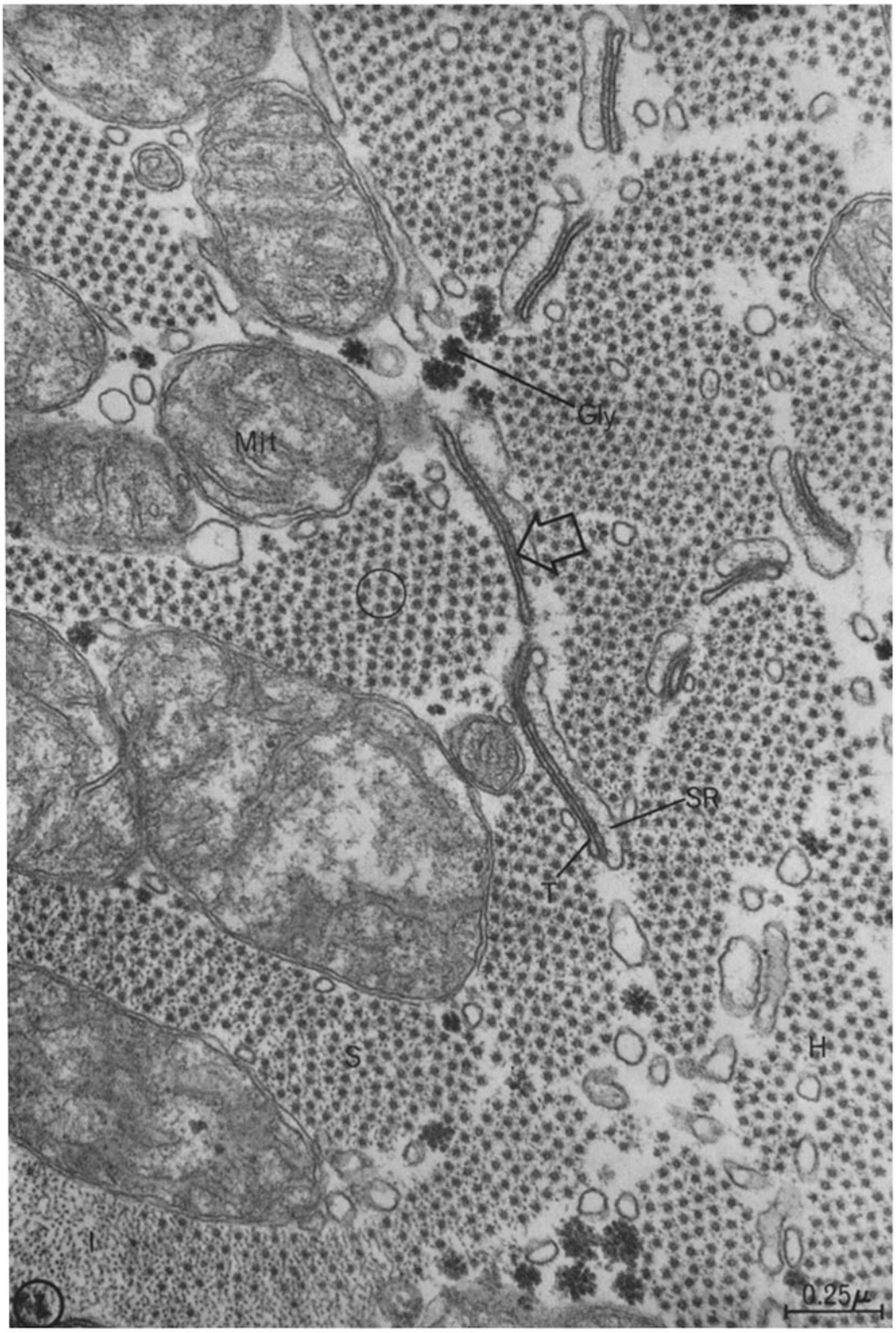

FIGURE 7 Cross-section of stretched fiber. I band and S and H zones of the A band are shown. An irregular thin filament array is seen in the I band, while the hexagonal array of thick filaments is seen in the H zone (within circle). T-SR tubule relationships are seen at the sarcotubular junction. At this point, the SR tubule appears elongated and contains a granular material. Granular densities between the T and SR tubule membranes are indicated (open arrow). × 57,000.

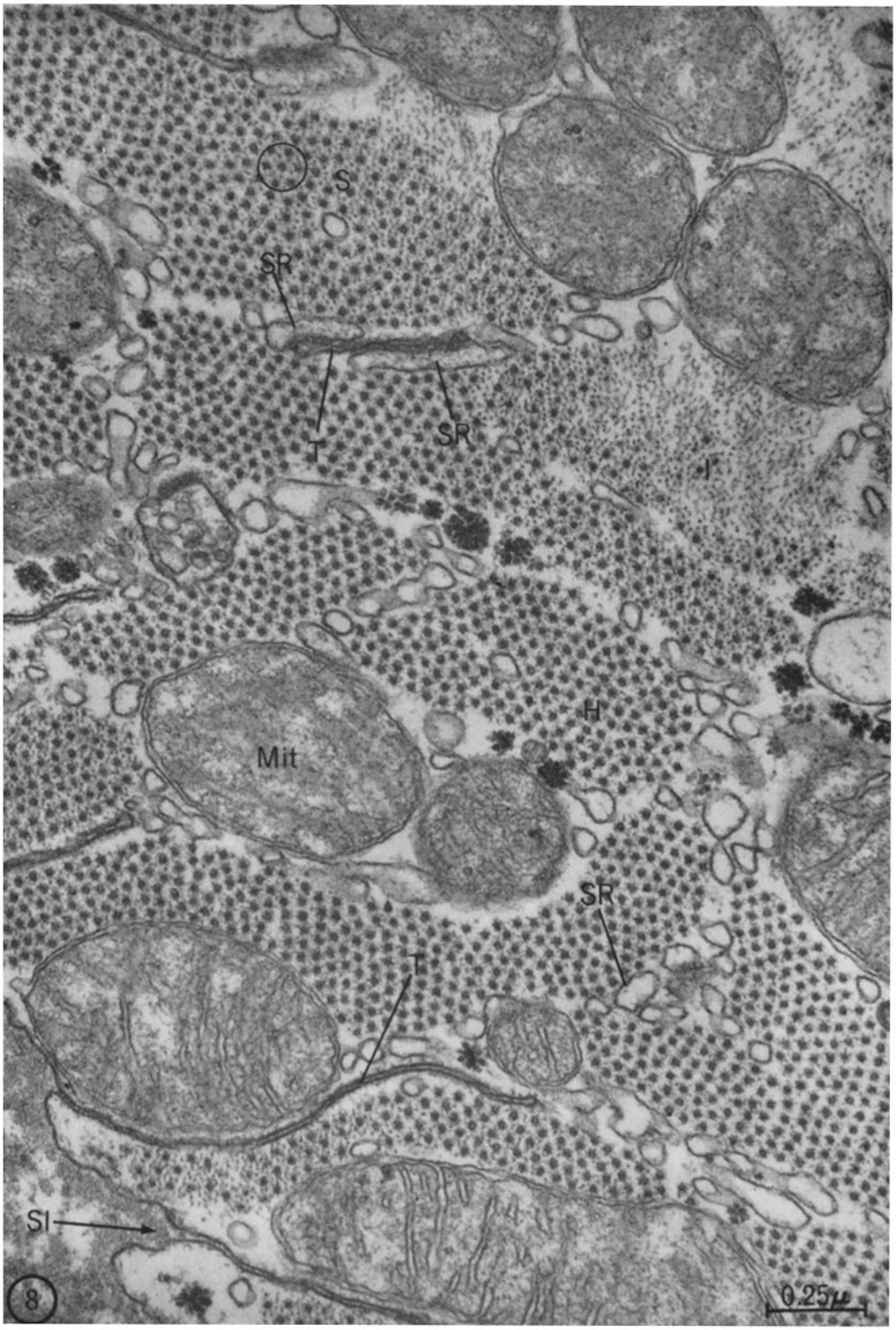

FIGURE 8 Cross-section of stretched fiber. A single transverse tubule (*T*) is shown first associated with the SR of one myofibril and subsequently with the SR of an adjacent myofibril. At the STj, the SR tubules are elliptical and contain a granular substance, while at other sarcomere levels SR tubules have a circular profile. SI and T tubule relations are shown. I band and H and S zones of the A band are designated. The thick filament hexagonal array and S zone thick/thin filament relationship is shown (within circle). × 57,000.

FIGURE 9 Longitudinal section showing A, I, and Z bands of the sarcomere. The sarcoplasmic reticulum is a profuse network of anastamosing tubules, continuous across the Z line, with no apparent local specialization at any band level. × 20,500. *Insert* shown a portion of two tubular branches of the T system crossing the Z line. At this point, an increase in membrane thickness and a dilation of the tubule (white arrow) is evident. The extension of this tubule from the sarcotubular junction with its SR (open arrow) and T (closed arrow) components can be noted. × 12,250.

cristae, which project into the mitochondrial cavity. These cristae are of variable length, often exhibit angulations, and are closely packed in a relatively ordered arrangement. The intercristal matrix is of moderate density containing intramitochondrial granules.

SARCOMERE STRUCTURE AND FILAMENTOUS ORGANIZATION: Longitudinal sections (Fig. 2, 9) show the sarcomere to have a banding pattern generally similar to that observed in typical vertebrate cardiac muscle (36, 62, 63, 65, 66),[2] yet significantly different in several details. As in the case of extensor leg muscles of *Limulus polyphemus* (15) and in cardiac muscle of the Japanese horseshoe crab, *Tachypleus tridentatus* Leach,[3] only three cross-striations are apparent: A, I, and Z bands.

The A band, extending approximately 2.7 μ in width, occupies the central region of the sarcomere. On longitudinal sections, variations in A band density are not noted (Figs. 1, 9); thus, no H zone or M-L complex is observed. This uniform density persists independent of sarcomere length.[3] However, an examination of transverse sections cut through various levels of the fibers reveals the presence of H zones at longer sarcomere lengths (Figs. 7, 8).[3] Thick myofilaments, ca. 180 A in diameter, run the length of the A band. Cross-sections reveal these filaments to have a circular profile, and frequently they appear to have a central core of lower density. Further, these thick filaments are generally arranged in a fairly well ordered hexagonal array (Figs. 1, 8, 9). Surrounding each thick filament is an orbit of 9–12 thin filaments, each 60 A in diameter. Examination of cross-sections cut through the S zone region of the A band (regions of thin and thick filament overlap) shows the presence of lateral projections (i.e. cross-bridges) extending between thick and thin filaments. In line with the absence of an M line in longitudinal section, bridges between thick filaments (i.e. M bridges) at the center of the A band were not discerned in cross-sections.[3]

The I band, flanking and of lower density than the A band (Figs. 2, 5, 9), contains only thin filaments ca. 60 A in diameter. These thin filaments originate at the Z band, extend through the I band, and pass into the A band between the thick filaments. Since H zones could not be discerned in longitudinal sections, thin filaments might be thought to be continuous across the length of the sarcomere. However, transverse sections of elongated sarcomeres show that such is not the case (Figs. 7, 8).[3] In such elongated sarcomeres, it is apparent that the A band is composed of: (*a*) a true H zone, found in the central region of the band, in which only thick filaments are noted, and (*b*) S zones, flanking this central H zone, in which both thick and thin filaments are found (Figs. 7, 8).[3] Further, although transverse sections show that thin filaments are found in an ordered array within the S zone of the A band, no regular pattern of filaments is found within the I band (Figs. 1, 7, 8). Unlike that of vertebrate cardiac muscle, the I band of *Limulus* cardiac muscle does not exhibit an N zone.

Electron micrographs of longitudinal sections (Figs. 2, 5, 9) do not reval the Z band to be a disc-like structure. In the *Limulus* heart, the Z appears as an irregularly shaped band lacking the typical zig-zag configuration noted in vertebrate cardiac muscle. Moreover, while in cross-section Z bands of mammalian cardiac muscle show a basket weave–like pattern (35), such is not seen in the *Limulus*. Transverse sections through the Z disc show it to be comprised of filaments that appear to be embedded in or surrounded by a dense matrix (Fig. 1). These filaments, ca. 50–60 A in diameter, appear in cross-section to be virtually identical to thin filaments of the I band. Some sections through the Z show it to have a perforate structure (Fig. 1) similar to that observed in striated depressor fibers of the barnacle (34, 37–39), while other sections, cut at a slightly different orientation, show the Z structure as isolated blocks of electron-opaque material (Fig. 6). These "Z bodies" appear similar to the J rods of oblique striated muscle of the earthworm (29) and to the dense bodies observed in studies of visceral muscle (31, 59). Between these dense bodies in the Z line, tubules of the sarcoplasmic reticulum are seen in profusion (Fig. 6).

DISCUSSION

Previous electron microscope investigations have served to establish a generalized concept of cardiac muscle ultrastructure. Recently, there has

[2] R. A. Leyton, H. M. Spotnitz, and E. H. Sonnenblick. 1970. Relation of ultrastructure to function in the intact heart: Sarcomere structure relative to pressure volume curves of the intact canine right ventricle. Submitted.

[3] R. A. Leyton, W. W. Parmley, and E. H. Sonnenblick. 1969. Cardiac muscle of the horseshoe crab, *Limulus polyphemus*. II. Correlation of ultrastructure and function. In preparation.

been a growing awareness of the nonhomogeneity of cardiac ultrastructure as reflected by reports of structural variations in fiber types within a species and from one species to the next. In describing the fine structure of *Limulus* cardiac muscle, we pointed out numerous and distinct differences observed between this structural pattern and that of typical mammalian cardiac muscle. Specifically, these differences involve: (*a*) sarcomere banding, length, and substructure, (*b*) the sarcotubular system, and (*c*) the intercalated disc.

The Sarcomere

The typical sarcomere found in mammalian cardiac muscle (18, 36, 62, 63, 66)[2] is delimited by a pair of Z lines within which, in longitudinal section, a definite arrangement of alternating light and dark areas is observed. The central region of the sarcomere, the A band, is 1.5 μ long, is comprised of an M-L complex and two S zones, and is flanked by two I bands. Within the I band, at a point close to the Z, is a region of slightly greater density called the N zone. Studies of isolated cat and dog papillary muscle (33) and intact canine left and right ventricles (64),[2] relating sarcomere structure to the length-tension curve, have shown that at the point of maximum active tension (L_{max}) the sarcomere length is ca. 2.20–2.25 μ.

Sarcomeres of *Limulus* cardiac muscle fibers also exhibit three general band areas: A, I, and Z. As in mammalian cardiac muscle, two Z bands delimit each sarcomere. Moreover, there is a central A band flanked by two I bands. However, the A band of *Limulus* cardiac muscle differs from that of the mammal both in its width and in its sub-banding. The width of the *Limulus* A band, ca. 2.7 μ, is almost twice that of the A band of the cat and dog. Additionally, in longitudinal sections of *Limulus* fibers, no M-L sub-banding is discerned. The A band thus appears to be of uniform density throughout its entire length. This uniform density of the A band persists at all sarcomere lengths.[3] Accordingly, discrete H zones are not seen in longitudinal sections, although thin filaments are shown to be absent from the center of the A band in elongated sarcomeres stretched beyond L_{max}.[3] Further, the sarcomere length of *Limulus* cardiac muscle measured at L_{max} was 5.39 μ,[3] ca. 2.5 times longer than that of mammalian cardiac muscle (61, 64).[2]

Sarcomere Substructure

Several studies (17, 24, 34, 43) have indicated a correlation between sarcomere length and: (*a*) the length of thick and thin filaments, (*b*) thick filament diameter, (*c*) thin/thick filament ratio, (*d*) the architecture of the Z line, and (*e*) the M-L-H complex.

While sarcomere lengths in vertebrate cardiac and skeletal muscles are found to fall within a rather narrow range (1.6–2.6 μ), such is not the case in invertebrates. As compared with mammalian cardiac muscle fibers, invertebrate fibers exhibiting long sarcomeres are associated with longer thin and thick filaments, thick filaments of greater diameter, and a higher thin/thick filament ratio, while an M band and a discrete Z-line structure are generally lacking (17, 24, 34, 43).

An examination of sarcomeric substructure of *Limulus* cardiac muscle reveals that the above morphological generalizations apply to these fibers. Accordingly, while the absence of an H zone in longitudinal section prevents the determination of thin filament length, thick filaments (2.7 μ long) are about 1.8 times the length of those found in mammalian cardiac muscle (i.e. 1.5 μ). Moreover, while thick filaments of mammalian cardiac muscle in cross-section appear somewhat triangular in shape, are of uniform density, and measure 100–120 A in diameter, thick filaments of *Limulus* cardiac muscle appear circular in profile, with a central core of lesser density, and measure ca. 180 A in diameter.

Unlike mammalian cardiac muscle, *Limulus* cardiac muscle has no observable M line. In mammalian cardiac muscle the M line is characterized by thick filaments, arranged in hexagonal array, and connected by bridges. Although transverse sections of stretched *Limulus* fibers reveal the presence of H zones,[3] at no point in this zone were bridges or "links" between thick filaments discerned. Studies of the M line in vertebrate cardiac (63) and skeletal muscle (33)[4] have suggested that the M line may serve to maintain the thick filaments in register and counteract alterations in cross-sectional area of the sarcomere with changes in sarcomere length. The apparent absence of this structure in *Limulus* cardiac muscle is consonant with similar observations in other fibers that have

[4] R. A. Leyton, J. Moss, and W. W. Parmley. 1969. Correlation of ultrastructure and function in tail muscle of *Amblystoma*. In preparation.

long sarcomeres (10, 16, 34, 51) and with alterations in sarcomeric substructure in relation to its length-tension curve.[3]

While the arrangement of thick filaments is more or less hexagonal in array in all striated muscles, within the S zone of the A band, the orbit of thin filaments around each thick filament has been observed to vary (24, 27, 34, 48). Thus, in the S zone of mammalian cardiac muscle, six thin filaments surround each thick filament (a 2:1 thin/thick filament ratio), whereas in *Limulus* cardiac muscle 9–12 thin filaments surround each thick filament, providing a 4–5:1 filament ratio. Thin filaments in excess of six around each thick filament have also been observed in many invertebrate striated muscles (2, 5, 7, 8, 10, 22, 50, 52, 54, 55) that are characterized by long sarcomere lengths and elongated thick filaments with diameters greater than 110 A. A question exists as to how the lateral filament stability is maintained and as to the mode of interaction between thick and thin filaments in these longer sarcomeres. The low density of the central core of the thick filaments in *Limulus* cardiac muscle may indicate a molecular arrangement which is different from that found in thick filaments of vertebrate cardiac muscle fibers. Additionally, thick filaments of greater length and diameter may have an increased content of myosin monomers. This may provide an increased number of cross-bridges per unit of length in line with the increased thin/thick filament ratio of *Limulus* cardiac muscle.

Although differences in thick filament diameter have been detailed in different species and different fiber types within a species, it appears that thin filament diameters are relatively constant (ca. 60 A). The high thin/thick filament ratio of the *Limulus* heart may help to explain both the difficulty in discerning H zones in longitudinal sections and the structural nature of the Z disc. The greater the thin/thick filament ratio, the more difficult it is to resolve individual thin filaments. Additionally, in longitudinal sections the Z line appears as an irregular band, frequently coursing unevenly across the fibril. As a result, if the thin filaments are, as in other muscle fibers, of uniform length, they may, owing to the unevenness of the Z band, terminate out of register in the A band. As the sarcomeres of *Limulus* cardiac muscle lack an observable M line, stretching of the fiber and the sarcomeres results in some loss of register of thick filaments. This, coupled with the possible lack of thin filament register, would further tend to obscure the H zones in longitudinal sections.

As noted above, the thin/thick filament ratio may be related to Z-line structure. That an increase in the number of thin filaments for a given cross-sectional area should be reflected in Z-line architecture has previously been discussed (3, 4, 6, 17, 34, 43). Micrographs of mammalian cardiac muscle show the Z line in longitudinal section to have a zig-zag appearance and in cross-section to have a basket-weave effect (35). Longitudinal sections show that the Z line of *Limulus* cardiac muscle appears as an irregular intersarcomeric structure that seems to consist of a simple interdigitation of thin filaments from adjacent sarcomeres. Cross-sections show the Z line to appear either as a perforate plate structure or as separate unconnected Z bodies, depending on the angle of section. A similar description of the Z line has previously been reported in arthropod striated muscle (37–39).

The Sarcotubular System

Studies of the morphology of the sarcotubular system in various types of muscles have served to establish a general topographical pattern of the T and SR components and their structural interrelationships. As in the case of sarcomere structure, investigations have demonstrated specific and significant inter- and intraspecies variations in the arrangement of the sarcotubular system.

The sarcotubular system of mammalian cardiac muscle is comprised of a system of T and SR tubules. The T system originates from sarcolemmal invaginations and passes into the Z-band level of the sarcomere. The T system is continuous with the cell surface membrane (sarcolemma) and forms an extension of this membrane into the fiber by which the extracellular environment may be distributed within the cell. The SR is essentially a simple plexus of tubules of relatively uniform diameter, continuous from sarcomere to sarcomere (18, 48). As the SR comes into close apposition with the T tubules in the general region of the Z line, the SR tubules undergo a slight saccular expansion and form a diadic coupling with the T tubule. Diadic rather than triadic junctions have previously been reported in arthropod skeletal muscle (10, 22, 23, 42, 48, 56–58). Additionally, in mammalian cardiac muscle, subsarcolemmal cisternae are observed along and in close apposition to the

inner margin of the sarcolemma and the close junctions of the intercalated disc (44).

Unlike mammalian cardiac muscle, *Limulus* heart muscle contains a system of sarcolemmal infoldings (intercellular clefts) which are open to the extracellular surface. As in the case of the crayfish (10, 20) and the crab (47, 49), this results in the sarcolemma not only forming the cylindrical outer "covering" of the fiber but also a network of membranes throughout the fiber interior that contains material seemingly identical to the extracellular coating of the fiber. T tubules emanate from the sarcolemma both at the fiber surface (Fig. 1) and from the walls of the intercellular clefts (Fig. 3).

Unlike mammalian cardiac muscle and seemingly unlike *Limulus* extensor muscle, *Limulus* cardiac muscle appears to have two sets of invaginating T tubules. As has been noted in skeletal muscle fibers of the crab (47, 49), these tubules exist in addition to the intercellular clefts. Those entering at the level of the Z band (i.e. Z tubules) are rarely seen, and no direct connections with the SR have been discerned. Those entering at the level of the A-I boundary pass into the fiber and, unlike the case in mammalian cardiac muscle, they form diadic junctions with the SR in a plane more or less parallel to the long axis of the myofibrils. While the tubules of the T system of mammalian cardiac muscle have been observed to be of large diameter relative to those of the SR (18), such is not the case in the *Limulus*, where the SR and T tubule diameters appear to fall within the same range.

The increase in electron opacity of the SR contents and of the apposing SR membrane at the point of diadic junction with the T tubule observed in the *Limulus* has also been noted in other species (23, 28, 45, 46). Thus, the presence of a granular substance in the saccular expansion of the SR at the diad is consonant with similar findings in other muscles. Accordingly, it, therefore, seems likely that, as in other muscles, this dense material may contain calcium-binding sites and that the SR of *Limulus* cardiac muscle is a major site of variable calcium release and uptake.

The presence of nontubular densities or granules apparently connecting T and SR tubules at the diad of *Limulus* cardiac muscle has also been noted in vertebrate and arthropod striated muscles (10, 19, 23, 26, 28, 45, 53, 57, 67). It has been suggested that these contact areas may be similar to septate junctions between epithelial cells (26, 45). It is thought that ionic coupling may take place between cells through such junctions.

The T tubules that penetrate the fiber at the level of the A-I boundary not only form diadic connections with the SR but also extend longitudinally in the opposite direction toward the Z lines. At the A, the tubule dilates significantly with an accompanying increase in membrane thickness. The tubule then crosses the Z line and passes to the A-I region of the next in-series sarcomere where it participates in another diadic junction. Such is not the case in mammalian cardiac muscle but has been noted in insect femoral muscle (23). The increase in membrane thickness at the Z line is due to the presence of an electron-opaque material and appears somewhat similar to the desmosome.

As in mammalian cardiac muscle, subsarcolemmal cisternae are present in *Limulus* cardiac muscle. These cisternae are in close apposition to the inner margin of the sarcolemma and contain a dense granular substance similar in appearance to that found in the saccular expansions of the SR at the diad (thus suggesting the presence of calcium-binding sites). However, diadic junctions are not seen at this point.

The fact that *Limulus* cardiac muscle is characterized by longer sarcomeres and myofilaments, by a greater thin/thick myofilament ratio, and by thick myofilaments of greater diameter as compared with vertebrate cardiac muscle may explain the nature of SR and T distribution. These structural features indicate that *Limulus* cardiac muscle may possess a greater number of myofilament reactive sites than does mammalian cardiac muscle. Accordingly, to effect calcium binding and movement commensurate with the contractile activity characteristic of *Limulus* muscle, the particular SR and T tubular pattern may be essential.

The Intercalated Disc

Like mammalian cardiac muscle, the *Limulus* heart is composed of separate cellular units. Intercellular junctions in both of these muscles exhibit membrane specializations called intercalated discs. In mammals, the disc structure is frequently seen and exhibits four structural modifications (60) which: (*a*) subserve intercellular mechanical connection via desmosomes, (*b*) fix myofibrils and myofilaments at the ends of the cell by terminal insertion plaques, and (*c*) provide intracellular,

low-impedence pathways via so-called "tight junctions" (areas of close membrane apposition).

The intercalated disc of *Limulus* cardiac muscle is infrequently seen, exhibits two membrane modifications seen in mammalian cardiac muscle discs, but appears to lack the close or "tight" junctions. Of interest is the fact that studies of this disc modification in mammalian cardiac muscle tend to support the possibility of the involvement of these sites in cell-to-cell conduction (9, 25, 30). The fact that such a structural modification is lacking in the *Limulus* disc and that electrotonic interactions between fibers cannot be demonstrated (1) lends further support to the fact that such specialized junctions are necessary for low-impedence pathways in cell-to-cell conduction.

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