Fine Structure and Differentiation of Ascidian Muscle

I. DIFFERENTIATED CAUDAL MUSCULATURE OF *DISTAPLIA OCCIDENTALIS* TADPOLES [1]

MICHAEL J. CAVEY AND RICHARD A. CLONEY
Department of Zoology, University of Washington, Seattle, Washington 98195

ABSTRACT The structure of the caudal muscle in the tadpole larva of the compound ascidian *Distaplia occidentalis* has been investigated with light and electron microscopy. The two muscle bands are composed of about 1500 flattened cells arranged in longitudinal rows between the epidermis and the notochord. The muscle cells are mononucleate and contain numerous mitochondria, a small Golgi apparatus, lysosomes, proteid-yolk inclusions, and large amounts of glycogen. The myofibrils and sarcoplasmic reticulum are confined to the peripheral sarcoplasm.

Myofibrils are discrete along most of their length but branch near the tapered ends of the muscle cell, producing a *Felderstruktur*. The myofibrils originate and terminate at specialized intercellular junctional complexes. These myomuscular junctions are normal to the primary axes of the myofibrils and resemble the intercalated disks of vertebrate cardiac muscle. The myofibrils insert at the myomuscular junction near the level of a Z-line. Thin filaments (presumably actin) extend from the terminal Z-line and make contact with the sarcolemma. These thin filaments frequently appear to be continuous with filaments in the extracellular junctional space, but other evidence suggests that the extracellular filaments are not myofilaments.

A T-system is absent, but numerous peripheral couplings between the sarcolemma and cisternae of the sarcoplasmic reticulum (SR) are present on all cell surfaces. Cisternae coupled to the sarcolemma are continuous with transverse components of SR which encircle the myofibrils at each I-band and H-band. The transverse component over the I-band consists of anastomosing tubules applied as a single layer to the surface of the myofibril. The transverse component over the H-band is also composed of anastomosing tubules, but the myofibrils are invested by a double or triple layer. Two or three tubules of sarcoplasmic reticulum interconnect consecutive transverse components.

Each muscle band is surrounded by a thin external lamina. The external lamina does not parallel the irregular cell contours nor does it penetrate the extracellular space between cells. In contracted muscle, the sarcolemmata at the epidermal and notochordal boundaries indent to the level of each Z-line, and peripheral couplings are located at the base of the indentations. The external lamina and basal lamina of the epidermis are displaced toward the indentations.

The location, function, and neuromuscular junctions of larval ascidian caudal muscle are similar to vertebrate somatic striated muscle. Other attributes, including the mononucleate condition, transverse myomuscular junctions, prolific gap junctions, active Golgi apparatus, and incomplete nervous innervation are characteristic of vertebrate cardiac muscle cells.

The close phylogenetic relationship between the vertebrates and the ascidians was initially established in a detailed embryological and histological study of the tadpole larvae of *Ciona intestinalis* and *Phallusia mammillata* (Kowalevsky, 1866).

Somatic striated muscle in the ascidians is found only in the evanescent lar-

[1] This investigation was supported by NIH Developmental Biology Training grant 5-T01-HD00266 to the University of Washington and NSF Research grant GB 5394.

val tail prior to metamorphosis. Many of the structural characteristics of this muscle more closely resemble vertebrate cardiac muscle than vertebrate somatic striated muscle.

Trypsin treatment of the tadpole larvae of *Boltenia villosa* causes the muscle bands to dissociate into mononucleate cells (Cloney, '61), and careful examination of other ascidian species with the electron microscope verifies the mononucleate condition of the caudal muscle cells. Transverse intercellular junctions that resemble intercalated disks are found between the ends of cells instead of myocommata composed of connective tissue (Jackson, '58; Cloney, '63; Berrill and Sheldon, '64; Pucci-Minafra, '65). Close junctions are prevalent between the lateral surfaces of adjacent cells and only the dorsal row of muscle cells in each band is innervated by axons of the nerve cord (R. A. Cloney, unpublished observations).

Infoldings of the sarcolemma and a system of "vesicular structures" and tubules localized near the myofibrils in *Ciona* larval muscle have been described (Pucci-Minafra, '65), but the observations were based on osmium-fixed tissue. The best preservation of the sarcoplasmic reticulum and T-system has been achieved with glutaraldehyde fixation.

In this report the fine structure of glutaraldehyde-fixed caudal muscle of *Distaplia occidentalis* larvae is described and compared with vertebrate somatic and cardiac striated muscles. A schematic model of the caudal muscle cell, based on serial thin sections, is included. Special attention is given to the organization of the sarcoplasmic reticulum and to details of the myomuscular junctions. This study is the basis for an investigation of the differentiation of the caudal muscle cell.

MATERIALS AND METHODS

Specimen preparation and microscopy

Colonies of *Distaplia occidentalis* (Bancroft) were collected from floating docks on San Juan Island, Washington, and maintained in running sea water at the Friday Harbor Laboratories of the University of Washington. Swimming tadpole larvae were pipetted from the holding tanks directly into the fixative solutions.

Larvae were routinely fixed by one of three methods: (1) 2% osmium tetroxide buffered by 1.25% sodium bicarbonate (pH 7.2) for one hour in an ice bath (Wood and Luft, '65); (2) 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer (pH 7.4; adjusted to 960 milliosmols with sodium chloride) for one hour at room temperature, followed by a rinse in 0.2 M phosphate buffer or filtered sea water and post-fixation with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4; adjusted to 960 milliosmols with sodium chloride) for one hour in an ice bath (Dunlap-Pianka, '66); and (3) 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4; adjusted to 960 milliosmols with sucrose and containing 0.05% unpurified ruthenium red) for six hours at room temperature followed by post-fixation with bicarbonate-buffered osmium as specified above.

Following osmium fixation, the larvae were rinsed briefly in distilled water, dehydrated in a graded series of ethanol at room temperature, transferred through three changes of propylene oxide, and embedded in Epon (Luft, '61).

All larvae were carefully oriented on aluminum slugs before sectioning. One-micron sections for light microscopy were cut with glass knives on a Porter-Blum MT-1 ultramicrotome. Serial thick sections were collected individually and consecutively arranged on glass slides. Sections for light microscopy were stained with a mixture of azure II and methylene blue in 0.5% sodium borate (Richardson et al., '60). The PAS reaction was carried out on sections from which the Epon had been removed by immersing the slides in a fresh solution of one pellet of potassium hydroxide dissolved in 10 ml of absolute ethanol and 40 ml of toluene for 20–30 minutes (J. H. Luft, personal communication). Sections were then hydrated, placed in 1% periodic acid for 15 minutes, washed with distilled water for five minutes, treated with Schiff's reagent for 60 minutes, and quickly transferred through three changes of 0.5% sulfurous acid. After a distilled water wash, the sections were dehydrated, cleared, and mounted.

Sections for electron microscopy were cut with a Du Pont diamond knife on a Porter-Blum MT-2B ultramicrotome at

thicknesses of 500–700 Å, as judged from the interference colors of the sections. Sections were collected on naked 200- and 300-mesh copper grids. Serial thin sections were cut at thicknesses of 700–900 Å and transferred to carbon-coated 75-mesh grids or slot grids. Thin sections were doubly stained for three minutes each in aqueous solutions of uranyl acetate and lead citrate at room temperature (Reynolds, '63; Venable and Coggeshall, '65). Electron micrographs were made with an RCA EMU-3G electron microscope operated at 50 KV or with a Philips EM 300 electron microscope operated at 60 KV. The electron microscopes were calibrated with a carbon grating replica of 28,800 lines/inch.

Analysis of serial sections

Serial transverse and serial longitudinal (1 μ) sections were used in estimating the total number of muscle cells in the tail of the ascidian larva. Since the caudal muscle cells are mononucleate and never fuse to form a syncytium, the total number of nuclei equals the total number of cells.

Larvae were measured before sectioning in order to minimize the error introduced by individual variation; only larvae with muscle bands approximately 1500 μ long were used. The muscle cell nuclei are between 1 and 2 μ wide. In serial longitudinal sections, each nucleus should appear once in each of two consecutive sections. Accordingly, the nuclei in all serial sections were counted and total number was then halved to arrive at the final estimate.

Muscle cell nuclei average about 5 μ in length. If every fifth transverse serial section is examined, each nucleus should appear only once. Nuclei were thus counted in sections at 5 μ intervals along the length of the muscle bands, and the sum of the individual counts was taken as the estimate of total cell number.

The results from the two methods outlined above differ by less than 2%.

OBSERVATIONS

Structure of the larval tail of Distaplia occidentalis

The larval notochord is surrounded on both sides by a longitudinal band of striated muscle (fig. 2). The tubular nerve cord is situated dorsal to the notochord and it contains motor nerve fibers from the visceral ganglion in the trunk. Neuromuscular junctions are established between the motor nerve fibers and the dorsal-most muscle cells in both bands; underlying rows of cells lack nervous innervation (R. A. Cloney, unpublished observations). The endodermal strand (an extension of the gut rudiment) lies along the ventral surface of the notochord. The nerve cord, endodermal strand, notochord, and muscle bands constitute the axial complex.

The axial complex is enclosed by a simple squamous epithelium. Dorsal and ventral sensory nerves extend from the visceral ganglion and lie within inpocketings

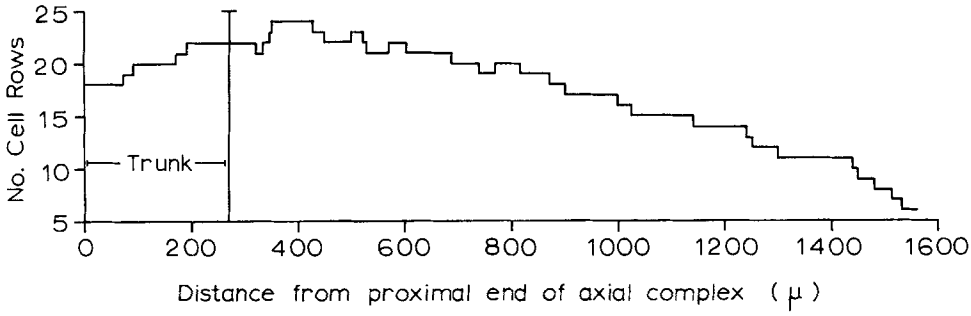

Fig. 1 Number of rows of muscle cells at 5 μ intervals along the length of a single muscle band. The cells are joined end-to-end in longitudinal rows. The number of rows was counted in serial transverse (1 μ) sections and plotted as a function of the distance from the proximal end of the axial complex. The number of rows is seen to increase to a maximum shortly after the tail emerges from the trunk and then to decrease to a minimum at the extreme distal end of the tail.

of the epidermal cells dorsal to the nerve cord and ventral to the endodermal strand. The larva is invested by two cuticular layers of tunic; the outer layer forms the dorsal, ventral, and lateral caudal fins.

During embryonic development the tail of the tadpole larva undergoes a torsion of 90° at its base. The dorsal caudal tissues are thus shifted to the left side of the larva and the ventral caudal tissues are shifted to the right side. Spatial relationships in this report will refer to the *pretorsional* condition.

Morphology of the larval muscle bands

The two muscle bands of *Distaplia occidentalis* larvae are composed of longitudinal rows of flattened cells (figs. 3, 4). The long axis of each muscle cell is parallel to the long axis of the tail, and every cell borders the epidermis and the notochordal sheath at some point along its length.

The rows of muscle cells were counted at 5 μ intervals along the entire length of a single muscle band, and the results are presented in figure 1. The number of rows at the base of the axial complex (18/muscle band) increases to a maximum (24/muscle band) shortly after the axial complex emerges from the trunk and then decreases to a minimum (6/muscle band) at the distal end of the tail.

The muscle cells in the tadpole tail are mononucleate, and the total number of cells was estimated by counting the nuclei in serial transverse and serial longitudinal (1 μ) sections. In larvae whose muscle bands are approximately 1500 μ long, there were about 1500 muscle cells equally distributed between the two bands. (A total of 1552 nuclei were counted in the serial transverse sections and a total of 1528 nuclei in the serial longitudinal sections.) Individual tails vary in length, so it is quite probable that the total number of muscle cells fluctuates from the values given above.

The dimensions of the muscle cells are tabulated in table 1. Measurements of cell height and width were assembled exclusively from light micrographs of transverse (1 μ) sections. The ranges of these measurements reflect a wide variability. The dorsal- and ventral-most cells in each muscle band are considerably flattened and account for the highest values in the height range and the lowest values in the width range. Muscle cells in the medial region of each band exhibit a near parity of height and width; these cells account for the lowest values in the height range and the highest values in the width range. Measurements of cell length were collected from light and electron micrographs of thick (1 μ) and thin (600 Å) longitudinal sections, respectively. Sections were selected in which individual myofibrils

TABLE 1

Muscle cell dimensions

	Mean	Standard deviation	Range	Sample size
	μ	μ	μ	
		All cells		
Length [1]	28.3	±4.9	18.5–46.1	80
Length [2]	30.0	±1.5	27.0–32.8	20
		Dorsal and ventral cells		
Height [3]	16.0	±2.4	12.3–22.3	80
Width [3]	4.8	±1.0	2.7– 7.7	80
		Medial cells		
Height [3]	8.3	±0.9	6.9–10.4	40
Width [3]	9.0	±2.8	4.6–15.0	40

[1] Measurements from light micrographs of longitudinal sections.
[2] Measurements from electron micrographs of longitudinal sections.
[3] Measurements from light micrographs of transverse sections.

spanned the entire length of a cell without leaving the plane of section. Every myofibril originates and terminates at a specialized transverse junctional complex visible with the light microscope. The distance between terminal junctional complexes was measured, and the accuracy of the measurements was checked against the same distances measured on low-power electron micrographs. The average values obtained from these two sources are similar.

Although a considerable variation is evident in muscle cell dimensions, the size of the muscle cell nuclei appears quite uniform. Assuming that the nuclear volume is constant between cells, the sarcoplasmic volume should be constant as well. The fusiform shape of the muscle cells precludes a simple mathematical calculation of volume. If, however, one assumes the cells to have rectilinear proportions, the mean cell dimensions from table 1 can be substituted into a simple equation (Volume = Length × Height × Width). The results for the dorsal and ventral cells (Volume = 2,304 μ^3) and for the medial cells (Volume = 2,241 μ^3) are remarkably close. Such values are obviously larger than the real volumes because of the assumption of rectilinearity.

Morphology of the larval muscle cell

The nucleus, mitochondria, Golgi apparatus, glycogen granules, lysosomes, and yolk inclusions are situated in the muscle cell endoplasm. The myofibrils and sarcoplasmic reticulum reside in the peripheral sarcoplasm a short distance beneath the cell membrane, or sarcolemma (figs. 6, 7). A schematic diagram of a muscle cell is shown in figure 19.

The endoplasmic organelles

The nucleus occupies a central position within the endoplasm (fig. 8). It is irregular in shape and bounded by a perforated nuclear envelope. The chromatin is evenly distributed throughout the nucleus, however a thin layer of condensed chromatin is located immediately beneath the inner unit membrane of the nuclear envelope. Larger patches of condensed chromatin occur infrequently in the nucleoplasm. The small nucleolus is a bipartite structure with a dense outer region and a less dense inner core.

Mitochondria are abundant in the caudal muscle cell and are situated in a single or double layer beneath the myofibrils (figs. 3, 6, 7). Numerous anastomosing cristae create a "serpentine" pattern within these irregularly-shaped organelles.

The Golgi apparatus is an inconspicuous organelle that was only observed in cells fixed with solutions containing glutaraldehyde (fig. 9). It is typically composed of five to ten flattened cisternae and a small number of associated vacuoles. The vesicles and vacuoles contain a dense material. The forming face of the Golgi apparatus is directed toward the myofilaments in the cell periphery.

The largest accumulation of glycogen is in the endoplasm (figs. 6, 7), but its distribution is not limited to this region. Glycogen is also found beneath the sarcolemma and between the myofibrils. Glycogen particles are routinely aggregated into small clusters, or "rosettes" (figs. 11, 12). Single particles or short rows of glycogen also pervade the myofilaments. They can be found between the actin filaments of the I-band, between the myosin filaments of the H-band, and between both types of filaments in the A-band. Particles have never been observed within the M-line of the H-band or within the Z-line of the I-band. The glycogen is intensely PAS-positive (fig. 5).

The caudal muscle cell contains few free or membrane-bound ribosomes. Ribosomes overlap in size with glycogen granules, making an assessment of ribosome distribution difficult. Fortunately, glycogen has a great affinity for the lead stains used in electron microscopy (Biava, '63). By staining very briefly in lead citrate (less than 15 seconds) or by omitting the stain entirely, the staining intensity of the glycogen is diminished and the identification of ribosomes is made easier.

Most free ribosomes occur near the membranes of the sarcoplasmic reticulum in the peripheral sarcoplasm. Short segments of rough-surfaced sarcoplasmic reticulum are localized in both the cell periphery and cell endoplasm. All cisternae of the sarcoplasmic reticulum can

apparently bind ribosomes, but ribosomes are absent from membranes that form peripheral couplings or make intimate contact with the surface of the myofibrils. The outer unit membrane of the nuclear envelope can bind ribosomes on its sarcoplasmic side.

Small, membrane-bounded bodies containing myelinic figures are probably autolysosomes. Some of these bodies have a dense matrix and frequently contain recognizable fragments of organelles. Others are packed with granules (fig. 9) and may represent primary lysosomes (Novikoff, '61). These very small organelles are strongly birefringent in unfixed preparations of the larval tail when examined with a polarizing microscope but are not detected with bright field microscopy. The identification of these organelles is tentative until histochemical tests for hydrolytic activity can be undertaken.

The muscle cells contain sparse quantities of proteid-yolk which were not metabolized during embryonic development. The yolk inclusions are membrane-bounded and are situated near the mitochondria (fig. 6). The yolk is PAS-positive (fig. 5), and its proximity to the mitochondria is consistent with the role of mitochondria in yolk metabolism.

The contractile apparatus and sarcoplasmic reticulum

Swimming movements of the ascidian larva are effected by alternating contractions of the two muscle bands. All contractions are restricted to one plane perpendicular to the long axis of the tail. Myofibrils are at rest length when the tail is straight.

A single row of 15–30 myofibrils is located in the peripheral sarcoplasm of each caudal muscle cell (figs. 3, 7). The myofibrils are irregular in diameter and appear to be discrete structures along most of their length. Near the tapered ends of the cell, however, interconnections between the myofilaments do occur (fig. 6) and the myofilaments thus constitute a myofilament field (*Felderstruktur*).

Depending on its position, each myofibril spanning the length of the cell consists of 14–19 sarcomeres. The sarcomere is an ordered array of hexagonally-packed thick (myosin) and thin (actin) filaments (fig. 15). The sarcomere is delimited by two consecutive Z-lines (fig. 11), but the zig-zag pattern of the Z-lines in longitudinal section is often obscured by amorphous material associated with the Z-filaments; the amorphous material is weakly PAS-positive (fig. 5). Thin myofilaments comprise the remainder of each I-band and overlap with the thick myofilaments in adjacent A-bands. Bridges between the thick and thin myofilaments are evident (fig. 11). Thick myofilaments constitute the central H-band, and the center of the sarcomere is marked by a narrow M-line.

At the ends of the muscle cell the myofibrils insert at specialized junctional complexes. These intercellular junctions are transversely oriented to the myofibrils and superficially resemble the intercalated disks of vertebrate cardiac muscle. To denote their presence in somatic striated muscle and to distinguish them from other junctional complexes between sarcolemmata, they will be referred to as *transverse myomuscular* (TMM) *junctions* (fig. 12).

Several myofibrils may insert at a single TMM junction. In the region of juncture, the apposed sarcolemmata of adjoining cells are separated by a gap of approximately 550 Å. On the sarcoplasmic surface the sarcolemma is bounded by an area of low electron density which, in turn, is adjoined by an amorphous layer of material contributed by the myofibril. The amorphous layer occupies a level of sarcomere equivalent to a Z-line and will be referred to as *Z-matrix*. The intracellular deposition of Z-matrix almost doubles the width of the terminal half-I-bands, so that the TMM junction can be resolved with the light microscope (fig. 4). The TMM junction is relatively flattened in comparison with most vertebrate intercalated disks, and within the extracellular junctional space filaments are oriented perpendicular to the sarcolemmata and parallel to the long axes of the myofilaments.

Thin filaments cross the area of low electron density, interconnecting the Z-matrix and sarcolemma. These thin filaments often align with the filaments in the junctional space and give the impression of myofilament continuity between

cells (fig. 12, inset). The diameter of the thin filaments in the area of low electron density matches the diameter of the actin filaments (approximately 50 Å). The diameter of the extracellular filaments (approximately 65 Å) does not correspond to the diameter of either the thick or thin myofilaments. In transverse section through the extracellular junctional space, the filaments are found to be randomly disposed within an amorphous substrate.

The preceding details of the TMM junctional space are based upon sections from larvae fixed with bicarbonate-buffered osmium. In larvae fixed with glutaraldehyde, the substrate and oriented filaments of the junctional space are not well preserved. The constant separation of the sarcolemmata is usually lost, and the extracellular space is enlarged. Myofilaments are well preserved, however, as are the filaments bridging the area of low electron density. These observations appear to preclude the possibility of myofilament continuity across the TMM junctional space in *D. occidentalis* larvae.

Many myofibrils terminate without traversing the full length of the muscle cell. In frontal sections of the muscle band, a stepped pattern of TMM junctions is visible along many cellular interfaces (fig. 13). Frequent branching of the myofibrils occurs in these regions, and the insertion of a single myofibril at two or more TMM junctions is common.

Most caudal muscle cells form irregularly-spaced, lateral outpocketings of sarcoplasm. Glycogen granules fill the majority of these outpocketings, but short segments of myofibrils can be found in them as well. Outpocketings in which a single sarcomere inserts at two TMM junctions (fig. 14) are not uncommon.

The sarcoplasmic reticulum (SR) establishes contact between the myofibrils and the sarcolemma. In the caudal muscle cell there is no evidence of a T-system. Instead, close associations between the sarcolemma and cisternae of the sarcoplasmic reticulum are present; following the terminology of Johnson and Sommer ('67), these associations will be called *peripheral couplings*. Peripheral couplings occur on all cell surfaces (figs. 8, 15, 17) but are conspicuously absent from areas of sarcolemma involved in the formation of either gap junctions or TMM junctions. Since myofibrils lie in close register across cell boundaries, the peripheral coupling in one cell is often paired with a peripheral coupling in an adjacent cell. The arrangement of muscle cells in each band obviates paired peripheral couplings at lateral cell surfaces.

The cisterna of the SR applied to the sarcolemma, or *subsarcolemmal cisterna* (after Fawcett and McNutt, '69), is a flattened bulbous structure. The inner surface of the sarcolemma and the outer surface of the subsarcolemmal cisterna are parallel and separated by a gap of 150–200 Å (fig. 15). A dense material is deposited in the gap parallel to the two membrane surfaces and equidistant from both. Approximately four subsarcolemmal cisternae extend to the level of each H-band and approximately three extend to the level of each I-band.

The sarcoplasmic reticulum surrounding each myofibril consists of longitudinal and transverse components. The longitudinal component is a meandering tubule which runs at a slight angle to the long axis of the myofibril. It occasionally leaves the surface of the myofibril and returns at a point farther along its length. Two or three longitudinal tubules, depending on the diameter of the myofibril, interconnect consecutive transverse components (fig. 7). The transverse components encircle the myofibrils at the level of each I-band and H-band (figs. 8, 16). Cisternae leave the transverse components and make contact with corresponding transverse components on the two closest neighboring myofibrils (figs. 7, 15).

Close examination reveals a morphological difference between the transverse components at the two levels of the sarcomere. The transverse component at the I-band consists of three anastomosing tubules applied to the surface of the myofibril as a single perforated layer. The perforated layer is centered on the Z-line and extends laterally almost to the edge of the I-band. The transverse component centered on the M-line and embracing the H-band is a more intricate structure (fig. 15). Approximately four anastomosing tubules contribute to this component. The tubules are applied as a bilayered sheet over most of the surface of the myofibril.

The bottom layer lies in intimate contact with the myofilaments. At various points around the myofibril, the layers appear to exchange their relative positions.

The perforated sheet of sarcoplasmic reticulum encircling the I-band was relatively easy to reconstruct. At the H-band, however, the arrangement of tubules is more complex and two models of its structure are proposed (fig. 18). In one model ("interlacing" model) tubular cisternae coupled to the sarcolemma extend to the H-band and wrap around it one-and-three-quarters times. This model requires that the tubules in each layer interlace, i.e., pass through fenestrations in the opposite layer. An alternate and less complex model ("interdigitating" model) can also be proposed. The anastomosing tubules in this model could surround and embrace the myofibril in one complete layer, recurve to form a double layer, and then extend peripherally to become the subsarcolemmal cisternae. The two layers of tubules would interdigitate extensively where they lie in contact over the myofibril and at the raphe formed at the zone of recurvation.

The sarcolemma

The sarcolemma is irregularly infolded along all surfaces of the caudal muscle cell (fig. 12). A thin external lamina of homogeneous material surrounds each muscle band, but it does not parallel the irregular cell contours nor does it penetrate the extracellular space between cells. Membrane separation between cells is not uniform except at TMM junctions or close junctions. Close (gap) junctions are prevalent (fig. 10) between laterally apposed sarcolemmata. A granular material often occupies the gap of the close junction; similar junctions have been described in the statocyte of *Distaplia* larvae (Eakin and Kuda, '71).

The infolded sarcolemma changes in appearance when the muscle cells are fixed in the contracted state (fig. 17). Sarcolemmata at the borders with the epidermis and the notochordal sheath are deeply indented to the level of each Z-line, and peripheral couplings are located at the base of each indentation. In this instance, the external lamina does follow the muscle cell membrane into the indentations. The basal lamina of the epidermal cell layer is also displaced toward the indentations. It would appear that the association between the external lamina and the basal lamina is stronger than the association between the basal lamina and the epidermis. A firm attachment between the sarcolemma and subsarcolemmal cisterna is also indicated.

DISCUSSION

After escaping from the parental colony, the tadpole larva of *Distaplia occidentalis* swims for a few minutes or several hours before it settles and undergoes metamorphosis. The most striking event in metamorphosis is the resorption of the tail. Contractile filaments in the caudal epidermis are implicated in effecting this morphogenetic movement (Cloney, '66). The axial complex is passively pushed into the trunk of the larva and later phagocytized.

Each caudal muscle cell of the larval ascidian contains numerous mitochondria with well developed cristae. Mitochondrial ATPase activity is high in the larva, and there is a large increase in actomyosin-like ATPase activity upon hatching, the event which coincides with the onset of muscle activity (D'Anna and Metafora, '65). Large glycogen stores provide a potential energy source for contraction.

Protochordate striated muscle cells have thus far all proven to be mononucleate, even if they constitute the somatic musculature. The TMM junctions of ascidian caudal muscle are similar to the intercalated disks of vertebrate cardiac muscle (Sjöstrand et al., '58; Sjöstrand and Andersson-Cedergren, '60). Both junctions are oriented normal to the primary axes of the myofibrils, and both are interposed between two sarcomeres at the level of the Z-lines. The stepped arrangement of vertebrate intercalated disks and the ascidian TMM junctions are similar. Although most transverse cellular boundaries in cardiac muscle are highly convoluted, the cell membranes in the caudal muscle of ascidian larvae are relatively straight and produce a flattened TMM junction. The flat TMM junction resembles the intercalated disks of the ascid-

ian myocardium (Kalk, '70; Oliphant and Cloney, '72) and the simplified intercalated disks of the turtle atrium (Fawcett and Selby, '58).

The Golgi apparatus in *Distaplia* larval muscle cells appears to be functional. Membrane-bounded secretory granules are found within and near the Golgi apparatus. The significance of the granules is unknown but they could, for example, represent primary lysosomes. Following metamorphosis primary lysosomes might interact with autophagosomes to form autolysosomes during the period of muscle cell degeneration. Cytochemical tests for hydrolytic activity could be used to test this hypothesis. An active Golgi apparatus is usually found in vertebrate myocardial cells (Fawcett and McNutt, '69; McNutt and Fawcett, '69; Jewett et al., '71).

The prolific infoldings of the sarcolemma on all surfaces of the ascidian caudal muscle can be assigned several functions. The folds provide a flexible cell surface during tail flexion and, in addition, provide a surface onto which cisternae of the sarcoplasmic reticulum are coupled. The indentations in contracted muscle cells demonstrate the membrane flexibility and the firm attachment between the sarcolemma and subsarcolemmal cisternae. The images are similar to the "incisures" reported in muscle cell surfaces before it was clearly demonstrated that the T-tubule was continuous with the sarcolemma (Revel, '62).

Stereo electron microscopy of larval newt intercalated disks has provided evidence of the discontinuity of myofilaments between cardiac muscle cells. The actin filaments intermix with the matrix in the region of the Z-line, and arching profiles suggest that the myofilaments loop into and out of the Z-line matrix (Kelly, '67). The wide extracellular space of the TMM junction and the oriented filaments within it have been a subject of speculation. Berrill and Sheldon ('64) postulate there is "functional continuity" between the myofibrils of adjoining caudal muscle cells in *Dendrodoa grossularia* tadpoles. Such "functional continuity," it is argued, could represent a continuity of unbroken myofilaments or the end-to-end joining of myofilaments by a structure which bridges the extracellular junctional space. No continuity of myofilaments is evident across the TMM junctional space in *Ciona intestinalis* tadpoles (Pucci-Minafra, '65).

In *D. occidentalis* muscle fixed with bicarbonate-buffered osmium, thin filaments emerge from the Z-matrix and extend to the sarcolemma. From measurements of filament diameter, the thin filaments contacting the sarcolemma are apparently actin filaments. The diameter of the thin myofilaments, however, is about 25% smaller than the diameter of the extracellular filaments. In larvae fixed with glutaraldehyde, the material of the extracellular space is not well preserved, but the integrity of the intracellular structures is maintained. The constant membrane separation at the junction is lost concurrently with the failure to preserve the extracellular material in the junctional space. (Cacodylate-buffered glutaraldehyde more faithfully preserves the detail than phosphate-buffered glutaraldehyde.) It would appear more reasonable to assign the extracellular filaments and substrate a function in maintaining membrane adhesion; the substrate in which the filaments are disposed may have adhesive properties. Preliminary results show the junctional space to be weakly PAS-positive, suggesting it may contain a mucopolysaccharide.

An "atypical system of vesicles" was originally thought to constitute the sarcoplasmic reticulum of the ascidian myocardium (Kalk, '70). A subsequent study revealed peripheral couplings on the lateral and basal surfaces of the myocardial cells and a well developed SR in the myofilament field (Oliphant and Cloney, '72). In the caudal muscle of the ascidian larva, an early ultrastructural investigation revealed "vesicular structures" occasionally found between the myofibrils (Pucci-Minafra, '65). The extent of the sarcoplasmic reticulum can usually be correlated with size of a muscle fiber and its speed of contraction; the SR is prominent in large and/or fast-acting muscle cells (Peachey and Porter, '59). The vigorous activity of the tadpole larva would suggest a well-ordered system of intracellular membranes, and such a system is indeed present. In *D. occidentalis* tadpoles, the cross-striated myofibrils are encircled by transverse components of SR at each I-band and H-band, and these

components are interconnected by longitudinal tubules. The arrangement of the transverse component at the H-band in ascidian larval muscle is evidently unique but its significance cannot be assessed with our current knowledge of the function of the sarcoplasmic reticulum.

In the few available studies of protochordate striated muscle, there has been no report of the presence of a T-system. Both the ascidian myocardium (Oliphant and Cloney, '72) and the caudal muscle of the ascidian larva apparently rely on peripheral couplings to link the events of excitation and contraction. The peripheral coupling is common to muscle cells of small diameter and to muscle cells with tonic responses. It has been described in annelid body wall muscle (Rosenbluth, '68; Mill and Knapp, '70), in oyster adductor muscle (Hanson and Lowy, '61), and in muscle of the squid chromatophore organ (Cloney and Florey, '68). Among the vertebrates, peripheral couplings are found in certain cardiac muscle fibers of mammals (Johnson and Sommer, '67; Sommer and Johnson, '68; Fawcett and McNutt, '69; McNutt and Fawcett, '69), in the cardiac muscle of birds (Jewett et al., '71) and amphibians (Sommer and Johnson, '69; Gros and Schrével, '70), and in developing stages of mammalian skeletal muscle (Kelly, '69; Schiaffino and Margreth, '69). The subsarcolemmal cisterna of the peripheral coupling corresponds to the terminal cisterna of the triad in muscle cells with T-systems; in either case, the result is a specific association between the sarcoplasmic reticulum and the sarcolemma. The proximity of myofibrils to the sarcolemma facilitates a direct contact with the SR over relatively short distances (a few microns), so invaginations of the sarcolemma (T-tubules) become unnecessary.

The apparent need for a functional contact between the SR and the sarcolemma provides a rationale for the reexamination of muscles in which there is no report of either T-tubules or peripheral couplings. In the protochordate amphioxus, for example, membrane profiles are seen adjacent to the sarcolemmata of cells in the body wall musculature (Peachey, '61; Flood, '68). Although described as infoldings of the cell membrane by Peachey, these membranes may yet prove to be the subsarcolemmal cisternae of peripheral couplings.

ACKNOWLEDGMENT

A large part of this investigation was carried out at the Friday Harbor Laboratories of the University of Washington. The cooperation and helpful suggestions of the Director, Dr. Robert L. Fernald, are gratefully acknowledged.

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Abbreviations

A, A-band
BL, basal lamina
EL, external lamina
Ep, epidermis
ES, extracellular space
Gly, glycogen granules/rosettes
Go, Golgi apparatus
H, H-band
HSR, sarcoplasmic reticulum encircling the H-band
I, I-band
ISR, sarcoplasmic reticulum encircling the I-band
LDA, area of low electron density of transverse myomuscular junction
LSR, longitudinal tubule of sarcoplasmic reticulum
Ly, lysosome
M, M-line
Mb, muscle band
Mf, myofibril
Mt, mitochondrion
Nl, nucleolus
No, notochordal cell
Nu, nucleus
PC, peripheral coupling
Sl, sarcolemma
SR, sarcoplasmic reticulum
SSC, subsarcolemmal cisterna
T, tunic
TJ, transverse myomuscular junction
Yo, yolk inclusion
Z, Z-line
Zm, Z-matrix

PLATE 1

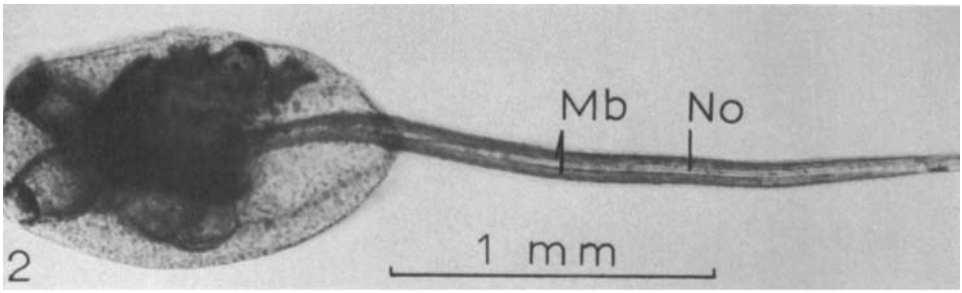
EXPLANATION OF FIGURES

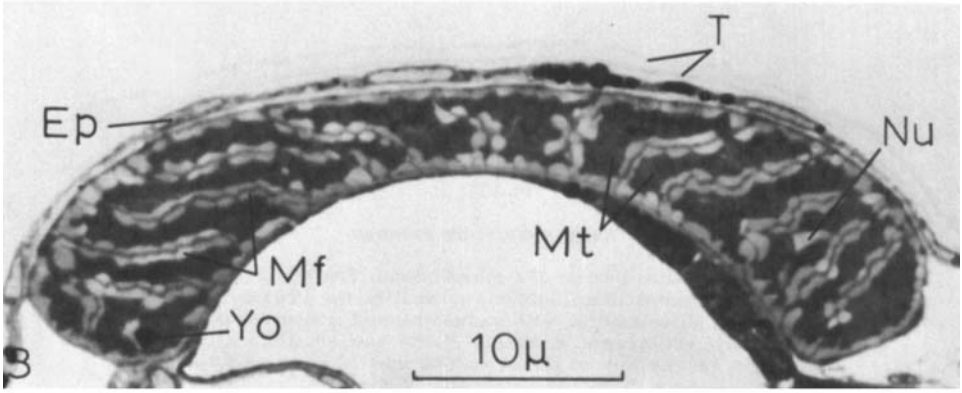
2 Photomicrograph of the right side of a living tadpole larva of *Distaplia occidentalis*. The two muscle bands are visible above and below the notochord, since the tail has rotated 90° to the left during development. The muscle bands are anatomically lateral to the nerve cord and notochord. The caudal fins lie in a plane normal to this optical section. × 97.

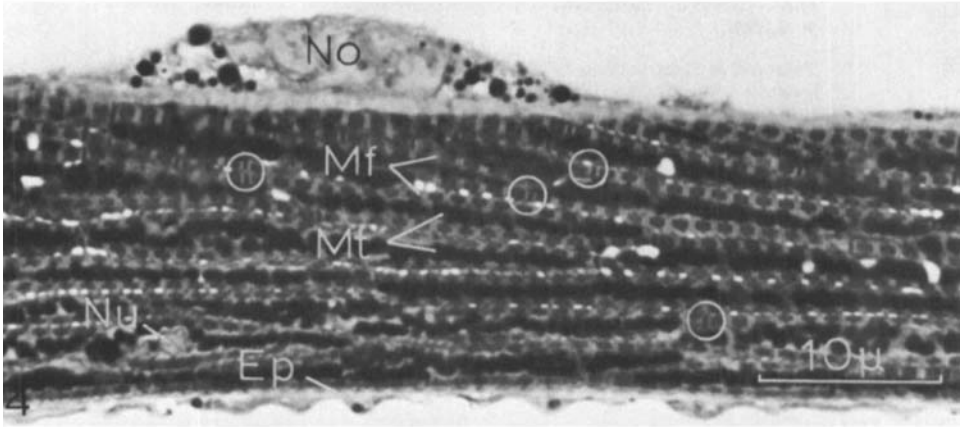
3 Transverse thick section of a muscle band. The flattened muscle cells contain a peripheral row of myofibrils. The nucleus, mitochondria, and yolk inclusions are situated in the endoplasm. Bicarbonate-buffered osmium fixation. × 2,730.

4 Longitudinal thick section of a muscle band. A single layer of squamous cells lines the notochordal cavity. Several transverse myomuscular junctions are encircled. Bicarbonate-buffered osmium fixation. × 2,360.

5 Longitudinal thick section of a muscle band illustrating the effects of the PAS reaction. Glycogen granules and yolk inclusions are intensely PAS-positive. Amorphous material associated with the myofibrils is weakly positive, and the nuclei and mitochondria are PAS-negative. Bicarbonate-buffered osmium fixation. × 2,470.


Mb
No
1 mm
2


T
Ep
Nu
Mt
Mf
Yo
10μ
3


No
Mf
Mt
Nu
Ep
10μ
4

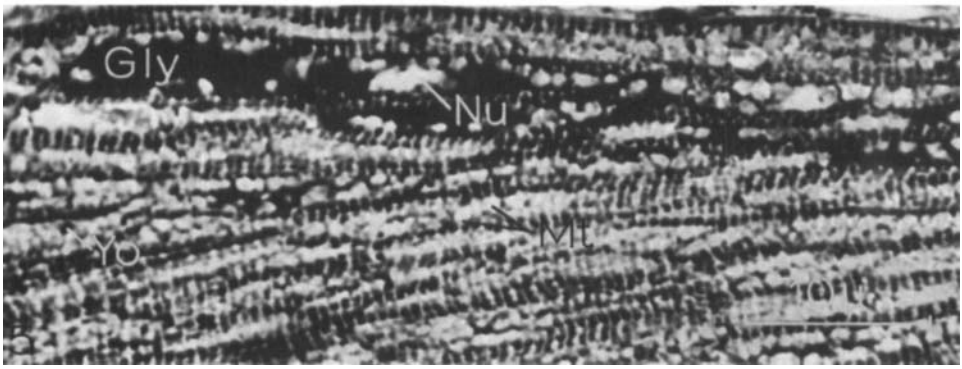

Gly
Nu
Mt
Yo

PLATE 2

EXPLANATION OF FIGURES

6 Longitudinal thin section of a muscle band. The peripheral myofibrils insert at myomuscular junctions normal to the primary axes of the myofibrils. Mitochondria, yolk inclusions, and lysosomes are restricted to the cell endoplasm. Elements of the sarcoplasmic reticulum are found in the vicinity of the myofibrils and in close association with the sarcolemma. Note the branching of the myofibril (arrow). Phosphate-buffered glutaraldehyde/phosphate-buffered osmium fixation. × 8,290.

7 Transverse thin section of the caudal muscle cells. The myofibrils are located immediately beneath the sarcolemmata. Sarcoplasmic reticulum encircles several of the myofibrils and is continuous between them (arrows). Cacodylate-buffered glutaraldehyde/bicarbonate-buffered osmium fixation. × 15,320.

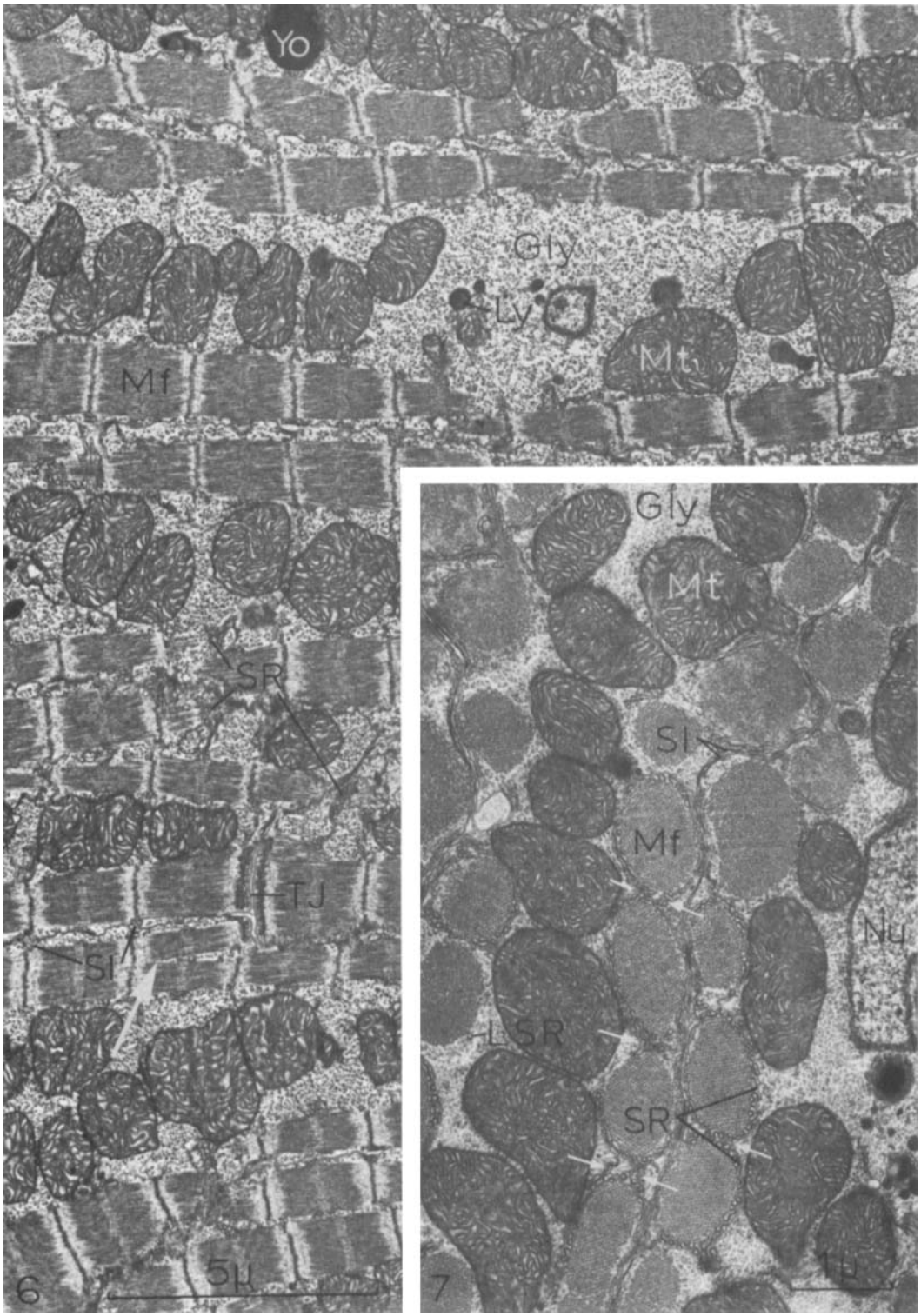

Yo
Gly
Ly
Mt
Mf
SR
TJ
SI
5μ
6
Gly
Mt
SI
Mf
Nu
SR
SR
1μ
7

PLATE 3

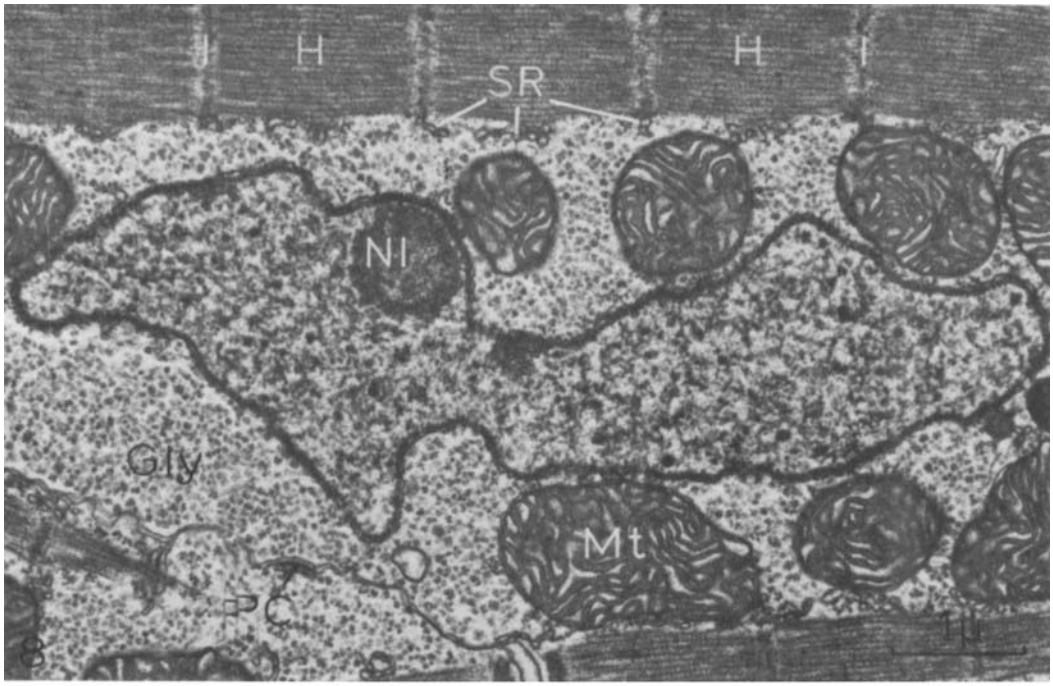
EXPLANATION OF FIGURES

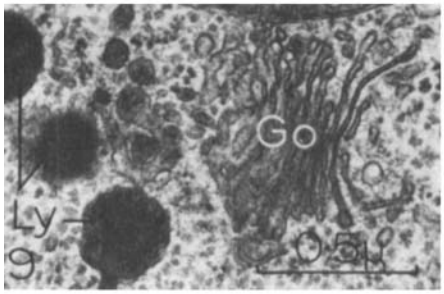
8 Longitudinal thin section of the nucleus. A thin layer of condensed chromatin is seen beneath the inner unit membrane of the nuclear envelope. The nucleolus is a bipartite structure. Membranes of the sarcoplasmic reticulum are found at the level of the myofibrillar I-bands and H-bands. Phosphate-buffered glutaraldehyde/phosphate-buffered osmium fixation. × 17,960.

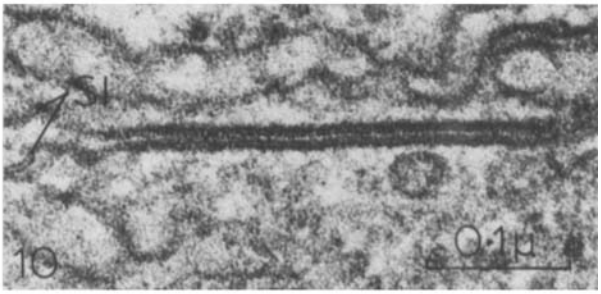
9 Transverse thin section of the Golgi apparatus. The Golgi apparatus is composed of flattened cisternae and a few vacuoles; its forming face is directed toward the muscle cell periphery. Cacodylate-buffered glutaraldehyde/bicarbonate-buffered osmium fixation. × 42,750.

10 Transverse thin section of a close junction between muscle cells. Granular material is frequently found in the gap between the outer leaflets of the apposed sarcolemmata. Cacodylate-buffered glutaraldehyde/bicarbonate-buffered osmium fixation. × 196,990.

11 Longitudinal thin section of a sarcomere. Bridges between the thick and thin myofilaments in the A-band are evident. Only disorganized remnants of sarcoplasmic reticulum are visible following this fixation. Bicarbonate-buffered osmium fixation. × 39,940.


I
H
H
I
SR
I
NI
Gly
Mt
PC
8
1μ


Go
Ly
9
0·5μ


Si
10
0·1μ

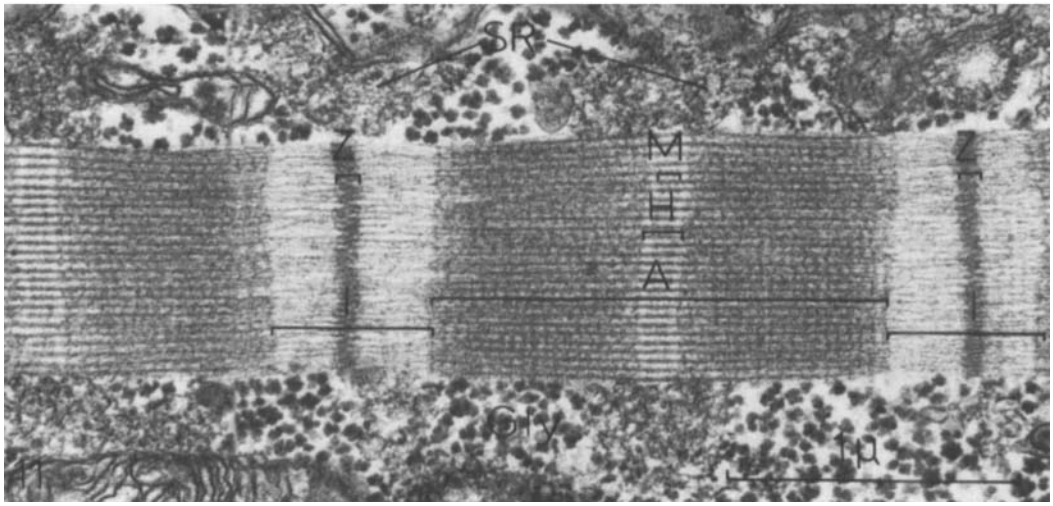

SR
Z
M
H
A
Z
I
I
Gly
11
1μ

PLATE 4

EXPLANATION OF FIGURES

12 Longitudinal thin section of a transverse myomuscular junction. At the region of juncture, the sarcolemmata of the adjoining cells assume a constant separation of about 550 Å. Z-matrix is deposited intracellularly, separated from the sarcolemma by an area of low electron density. The inset is a longitudinal thin section of the extracellular junctional space at a higher magnification. Thin filaments (arrows) extend from the Z-matrix to the sarcolemma and are frequently aligned with other filaments in the junctional space. Bicarbonate-buffered osmium fixations. × 44,120. *Inset:* × 88,090.

13 Frontal thin section of the caudal muscle cells. Myofibrils insert at transverse myomuscular junctions. At the tapered ends of the cells, a stepped pattern of junctions is evident. Bicarbonate-buffered osmium fixation. × 14,750.

14 Longitudinal thin section of a lateral cell outpocketing. A single sarcomere within the outpocketing inserts at two transverse myomuscular junctions. Bicarbonate-buffered osmium fixation. × 14,880.

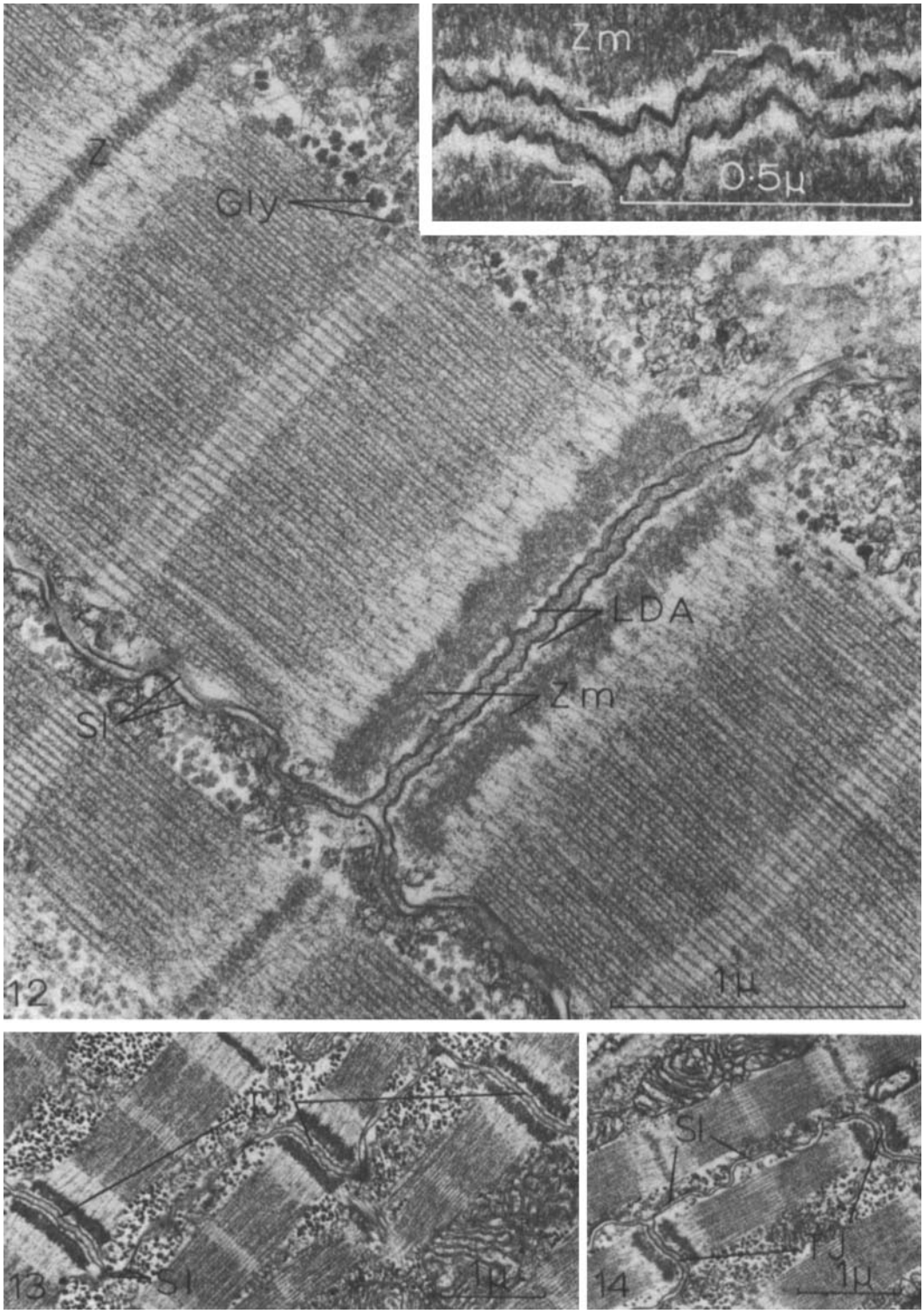

Z
Gly
Zm
0·5μ
LDA
Zm
Sl
1μ
12
Tj
Sl
1μ
13
Sl
Tj
1μ
14

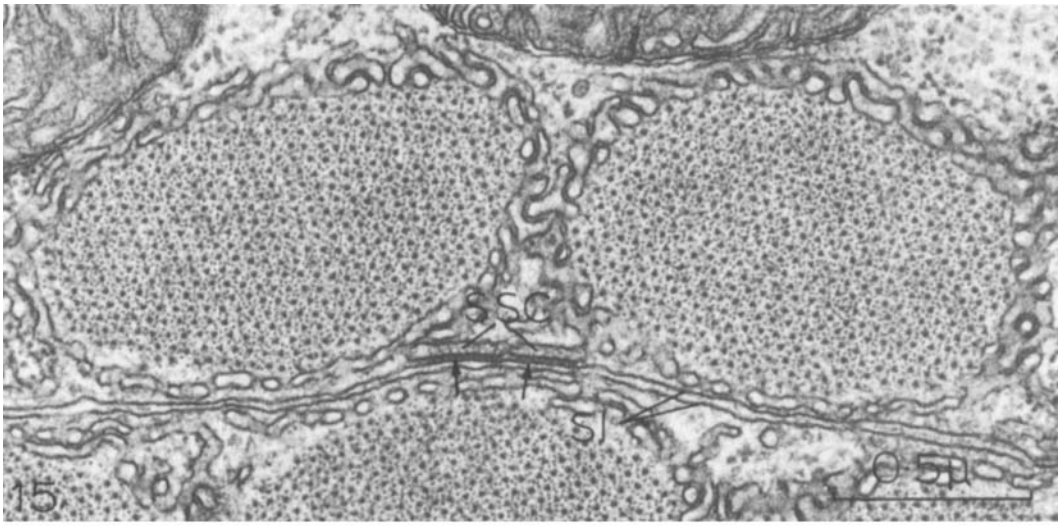
PLATE 5

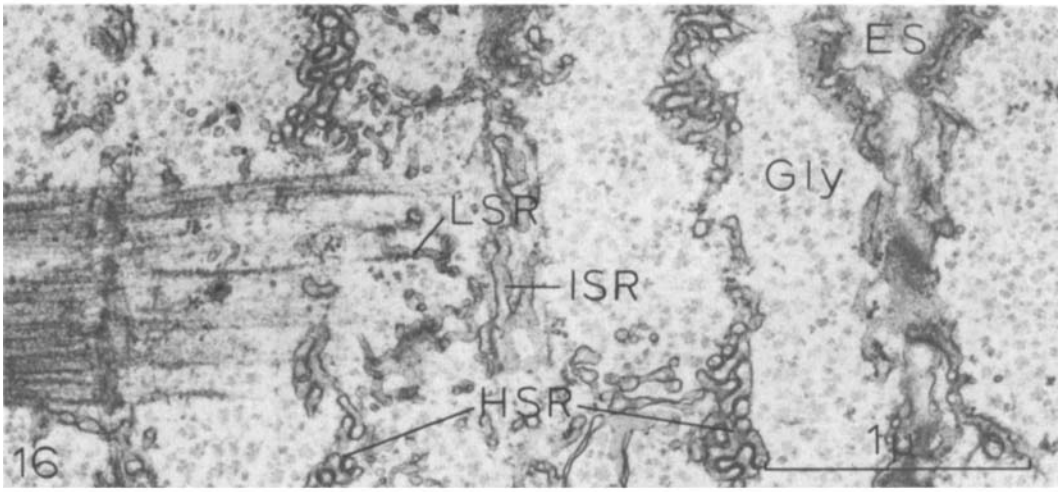
EXPLANATION OF FIGURES

15 Transverse thin section through the transverse component of sarcoplasmic reticulum centered on the M-line. Anastomosing tubules are applied as a double or triple layer around the myofibril, except on the side proximal to the sarcolemma where there is only a single thickness. A dense material (arrows) is deposited in the gap of the peripheral coupling. Cacodylate-buffered glutaraldehyde/bicarbonate-buffered osmium fixation. × 53,010.

16 Longitudinal grazing thin section of the transverse components of sarcoplasmic reticulum. Note the complexity of the transverse component over the H-band in comparison to the component over the I-band and the paucity of longitudinal tubules between the components. Cacodylate-buffered glutaraldehyde/bicarbonate-buffered osmium fixation. × 35,520.

17 Longitudinal thin section through the apical surface of a contracted muscle cell. The sarcolemma is indented to the level of each Z-line, and peripheral couplings are located at the base of the indentations. The external lamina of the muscle band and basal lamina of the epidermis are displaced toward the indentations. Phosphate-buffered glutaraldehyde/phosphate-buffered osmium fixation. × 28,090.


SSC
SI
0.5μ
15


ES
Gly
LSR
ISR
HSR
1μ
16

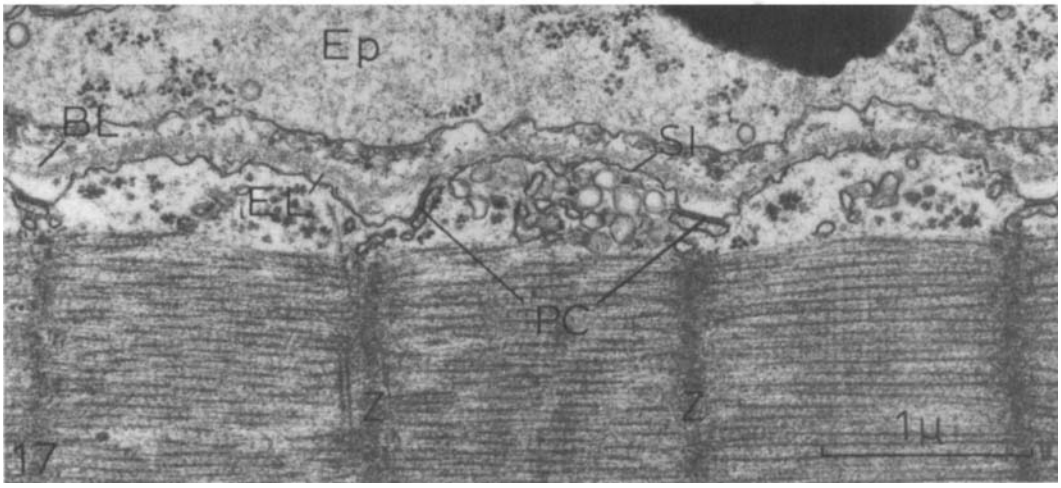
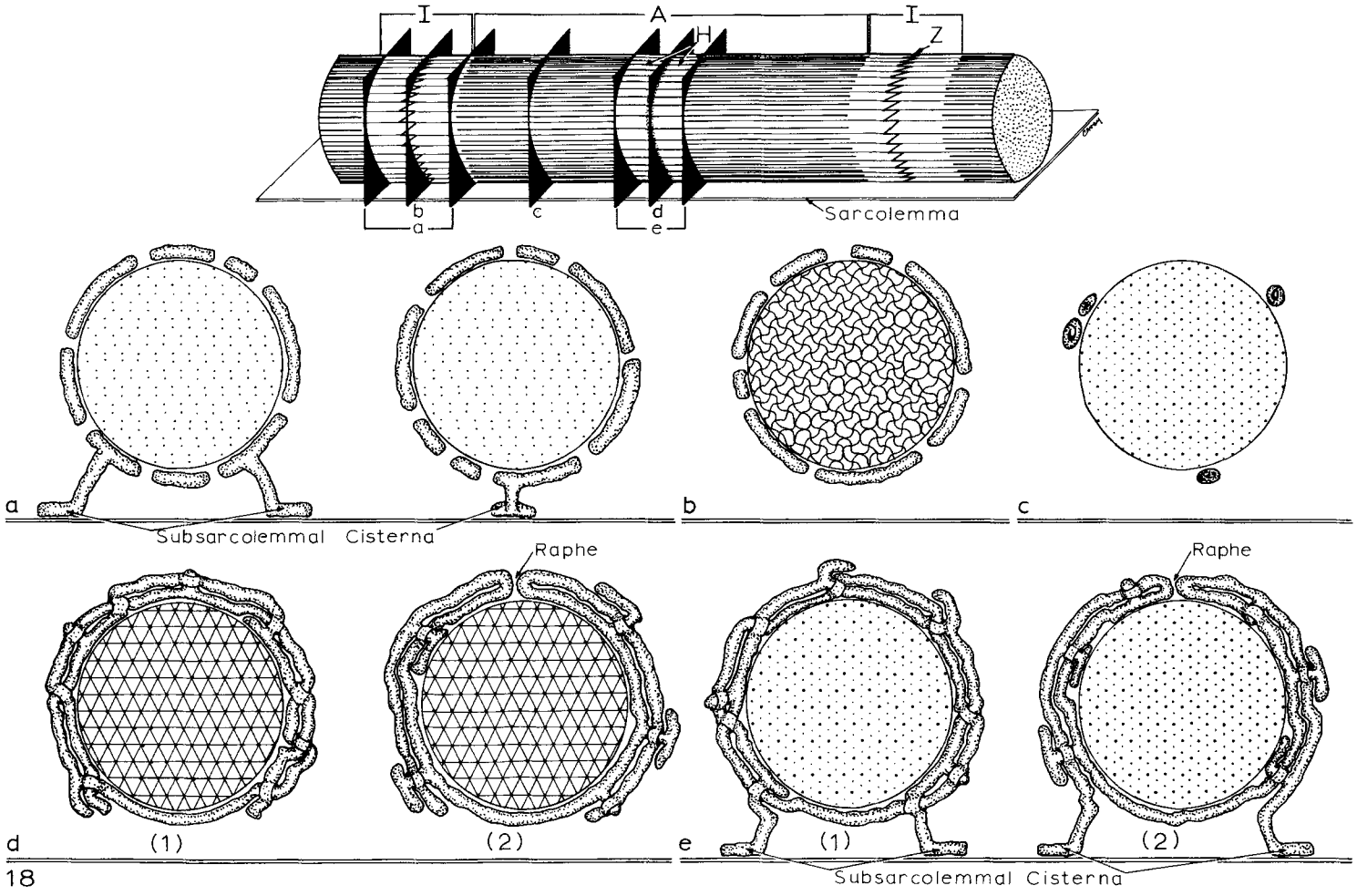

Ep
BL
SI
EL
PC
Z
Z
1μ
17

PLATE 6

EXPLANATION OF FIGURE

18 Schematic diagram of the distribution of sarcoplasmic reticulum at different levels of the sarcomere. Lettered planes through the myofibril correspond to the accompanying lettered figures. The transverse component of SR encircling the I-band is basically a perforated sheet formed by the anastomoses of approximately three tubular cisternae. Near the edge of the I-band (plane a), one or two subsarcolemmal cisternae are usually found, whereas none are found in regions nearer the Z-line (plane b). Two or three longitudinal tubules interconnect consecutive transverse components (plane c); the longitudinal tubules are often observed to bifurcate and later rejoin. The transverse component of SR centered on the M-line extends brief distances over adjacent A-band regions. Subsarcolemmal cisternae are absent from the central regions of this component (plane d), but two subsarcolemmal cisternae are usually present at either juncture with the A-band (plane e). Two models can be proposed for the arrangement of membranes of the sarcoplasmic reticulum encircling the H-band: an "interlacing" model is shown in d(1) and e(1) and an "interdigitating" model is shown in d(2) and e(2). Both models appear to conform to the images seen in the electron microscope, but neither could be conclusively eliminated after several attempts at reconstruction from serial thin sections. Consult the text for additional details.

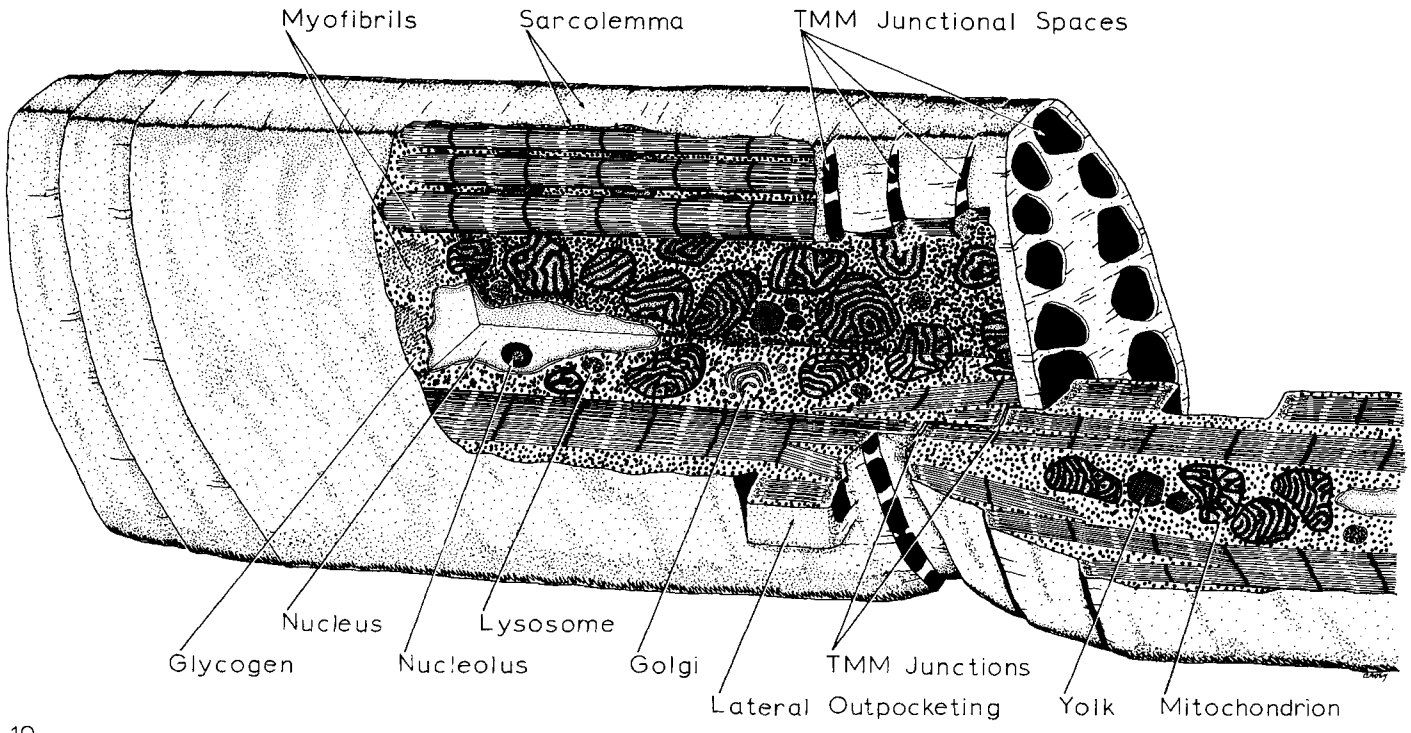

I
A
I
H
Z
b
a
c
d
e
Sarcolemma
a
Subsarcolemmal Cisterna
b
c
Raphe
Raphe
d
(1)
(2)
e
(1)
(2)
Subsarcolemmal Cisterna

18

PLATE 7

EXPLANATION OF FIGURE

19 Schematic diagram of the caudal muscle cells. The sarcoplasmic reticulum has been omitted. The general cell shape and distribution of organelles were verified with serial thin sections, and the organelles were drawn approximately to scale. The shape of each cell is variable, depending on its position within the muscle band (table 1). The diagram represents typical cells in the dorsal- or ventral-most aspects of the muscle band. These cells have the configuration of a flattened spindle with truncated ends. The subterminal lateral surfaces are characteristically stepped at the loci of the transverse myomuscular junctions.


Myofibrils
Sarcolemma
TMM Junctional Spaces
Nucleus
Lysosome
Glycogen
Nucleolus
Golgi
TMM Junctions
Lateral Outpocketing
Yolk
Mitochondrion

19