

Ultrastructure and differentiation of ascidian muscle

II. Differentiation of the caudal muscle cells in the larva of *Diplosoma macdonaldi*

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Summary. The larval muscle cells of *Diplosoma macdonaldi* contain sub-cortical and medullary myofibrils which are invested by fenestrated sheets of the sarcoplasmic reticulum. Cisternae of the sarcoplasmic reticulum are coupled with tubular invaginations of the sarcolemma. To appreciate better such uncommon features of cellular organization, six embryonic stages were selected for an ultrastructural study of myogenesis. The proliferative, synthetic, and elaborative phases of myogenesis were represented by embryos ranging from neurulae to prehatching larvae.

The contractile apparatus originates during the synthetic phase of myogenesis, when thick and thin myofilaments appear in the cortical sarcoplasm at the epidermal and notochordal poles of the cell. The myofilaments promptly aggregate into unstriated fascicles, and the fascicles unite in series to establish the rudimentary myofibrils. All major sarcomeric bands, except the Z-lines, are evident along the myofibrils. Cisternae of the sarcoplasmic reticulum form peripheral couplings with the overlying sarcolemma, and they also form interior couplings with sarcolemmal invaginations from the ends of the cell. The interior couplings localize over the I-bands of the myofibrils.

In the elaborative phase of myogenesis, mitochondria invade the cortical sarcoplasm, and the contractile apparatus passively shifts to the subcortex and medulla of the cell. Relocation of the myofibrils coincides with the disappearance of all peripheral couplings. Cisternae of the sarcoplasmic reticulum anastomose around the myofibrils, creating the fenestrated sheets that extend between sarcomeres. As Z-lines begin to bisect the I-bands, the perifibrillar cisternae become confluent with the cisternae in the precocious interior couplings.

Key words: Ascidian larva – Embryology – Myogenesis – Somatic striated (skeletal) muscle – Ultrastructure

The caudal muscle cells of ascidian larvae differ in the placement of the contractile apparatus and in the method by which the sarcoplasmic reticulum couples to the sarcolemma. In the muscle cells of many larvae, the myofibrils are restricted to the cortical sarcoplasm. Proximity of the myofibrils to the cellular surface facilitates a direct connection of the sarcoplasmic reticulum to the sarcolemma. Cisternae emerge from the perifibrillar networks, span to the overlying membrane, and establish dyadic peripheral couplings. Such features characterize the muscle cells in the larvae of *Clavelina* (Burighel et al. 1977), *Distaplia* (Cavey and Cloney 1972, 1974), *Ciona* (Burighel et al. 1977; Ceresa Castellani et al. 1972), and *Molgula* (Burighel et al. 1977).

In the caudal muscle cells of some ascidian larvae, the myofibrils occupy the subcortical and medullary sarcoplasm. With displacement of the myofibrils from the cellular surface, the sarcoplasmic reticulum tends to make indirect connections with the sarcolemma. Cisternae in the perifibrillar networks adhere to sarcolemmal invaginations, establishing dyadic interior couplings. The sarcolemmal component of the interior coupling can be analogized to the transverse (T) tubule in a vertebrate striated muscle cell. These features distinguish the muscle cells in the larvae of *Diplosoma* (Burighel et al. 1977; Cavey and Cloney 1976), *Botrylloides* (Burighel et al. 1977), *Botryllus* (Schiaffino et al. 1976), *Dendrodoa* (Bone and Ryan 1975), and *Styela* (Burighel et al. 1977).

The initial paper in this series presents an ultrastructural description of the caudal muscle cells in the larva of *Diplosoma macdonaldi* (Cavey and Cloney 1976). This report begins a consideration of larval myogenesis, affording special attention to the construction of the subcortical and medullary myofibrils, the organization of the sarcoplasmic reticulum, and the formation of structural couplings between the sarcoplasmic reticulum and the sarcolemma. Portions of this study have appeared in abstract (Cavey 1978).

Materials and methods

Specimens

Colonies of *Diplosoma macdonaldi* Herdman 1886 were collected from floats at Snug Harbor, San Juan Island, Washington, U.S.A., and removed to marine aquaria supplied with running seawater. The brooded embryos were dissected from the communal tests and transferred to filtered seawater to await further processing. Elapsed time between excision of the embryos and onset of the primary fixation was 1 h or less.

Preparation of specimens for microscopy

Embryos were immersed in a primary fixative containing 2.5% glutaraldehyde, 0.2 M Milonig's phosphate buffer (pH 7.4 or 7.6), and 0.14 M sodium chloride (Cloney and Florey 1968). After 60–90 min in the primary fixative at ambient temperature, the embryos were rinsed with filtered seawater for 30–60 sec and immersed in a secondary fixative containing 2% osmium tetroxide and 1.25% sodium bicarbonate buffer at pH 7.2 (Wood and Luft 1965). After 45–60 min in the secondary fixative at 0–4° C, the embryos were rinsed with distilled water for 15–30 sec, dehydrated with graded solutions of ethanol, cleared with propylene

oxide, and infiltrated with Epon (Luft 1961). The epoxy resin was polymerized at 60° C for 18–24 h.

Light-microscopic sections (1 μm in thickness) were cut with glass knives on a Sorvall MT-2B ultramicrotome, stained with an alkaline solution of azure II and methylene blue (Richardson et al. 1960), and mounted in high-viscosity immersion oil. Electron-microscopic sections (50–70 nm in thickness) were cut with diamond knives, collected on naked copper grids, and serially stained with aqueous solutions of uranyl acetate (saturated) and lead citrate (Reynolds 1963).

Light- and electron micrography

Light-microscopic sections were viewed with Leitz Ortholux and Dialux compound microscopes. Micrographs were made with a Leitz Orthomat-W camera or a Wild MPS20 Semiphoto-mat. Electron-microscopic sections were examined and photographed with a JEOL JEM-100S transmission electron microscope operated at 60 kV. A replica of a carbon grating (2,160 lines/mm) was used to calibrate the microscope.

Results

Staging of embryos

Zooids of *Diplosoma macdonaldi* shed gametes into a cloacal cavity between the apical and basal layers of a communal test. Embryos implant, by an unknown mechanism, in the basal test. When the larvae hatch, they enter the cloacal cavity and leave the colony through a cloacal aperture in the apical test. Ovoviviparity hinders the study of living embryos. They can be dissected from the test, but fibrous material remains firmly attached to the eggs. To visualize an embryo most effectively, it is necessary to strip the fibrous material or divest the egg of its envelopes. The gain in resolution is marginal, because the embryonic cells are heavily laden with pigmented yolk granules.

Since living embryos are ill-suited for study by light microscopy, staging must depend on rather gross anatomical features. Length of the tail is a convenient parameter. It can be expressed as an absolute measurement or as a percentage of final length (Fig. 1). The percentage of final length is a potentially more useful expression for comparative purposes.

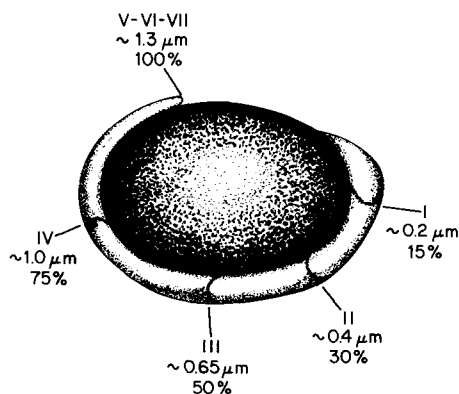


Fig. 1. Schematic diagram illustrating the stages of caudal myogenesis in embryos and larvae of *Diplosoma macdonaldi*. At each stage, designated by a Roman numeral, the length of the tail is expressed as an absolute measurement and as a percentage of final length

Length of the tail was the sole parameter for designating stages I–IV. Additional criteria become necessary in late myogenesis, since the tail achieves its final length in stage V. Stage V is identified by the appearance of rudimentary organs in the trunk of the embryo: the adhesive papillae and the branchial basket of the oozoid. Stage VI is identified by the appearance of a rudimentary organ in the trunk of the prehatching larva: the branchial basket of the first blastozoid. The larva represents stage VII in this study of myogenesis (Cavey and Cloney 1976).

Proliferative phase of myogenesis

The proliferative phase is a period of cellular segregation, division, and rearrangement (Cavey 1982). This phase, encompassing stages I and II, begins at neurulation when the presumptive muscle cells appear in the caudal rudiment. The presumptive muscle cells segregate into paraxial bands between the epidermis and the notochord. An external lamina surrounds each band. The cells in stages I and II will be designated as *early myoblasts* and *late myoblasts*, respectively.

The early myoblasts are polygonal cells with central, spherical nuclei. Each nucleus contains a preponderance of euchromatin and a massive nucleolus. The outer membrane of the nuclear envelope binds ribosomes, and evaginations of this membrane are confluent with the perinuclear cisternae of the granular endoplasmic reticulum. Small mitochondria with simple cristae, an inconspicuous Golgi apparatus, free polysomes, and a few glycogen granules also reside in the perinuclear cytoplasm. In light-microscopic sections, the perinuclear cytoplasm is more basophilic than outlying regions. The stronger basophilia may reflect a higher concentration of ribosomes in the cytoplasm around the nucleus. Mitochondria, polysomes, and glycogen granules can be found in the cortical cytoplasm, but large, membrane-bounded granules of proteid-yolk fill most of the available space.

At the onset of stage I, the muscle anlagen are multilayered bands of cells. The anlagen expand as the early myoblasts multiply. The contours of the myoblasts are relatively smooth, and the intercellular spaces are narrow but irregular in width. Occasionally, the apposed plasmalemmata assume parallel orientations. These sites, where the intercellular spaces are uniform in width, may be the loci of developing junctions. Before the conclusion of stage I, the early myoblasts send blunt processes toward the epidermal and notochordal boundaries of the anlagen, gradually transforming them into unilayered bands.

The late myoblasts are columnar cells with central, somewhat ovoid nuclei. Each nucleus contains a preponderance of euchromatin and several nucleoli. Evaginations of the outer nuclear membrane are numerous, and long cisternae of the granular endoplasmic reticulum mingle with the mitochondria that flank the nucleus. Increasing numbers of free polysomes and glycogen granules appear in the perinuclear cytoplasm. Judging from the electron-dense material in its cisternae and vesicles, the Golgi apparatus is now active. The membrane-bounded, multigranular bodies that accumu-

late near the Golgi apparatus are tentatively identified as primary lysosomes. These putative lysosomes emigrate to the cortical cytoplasm and disperse among the yolk granules.

Near the end of stage II, there is a rapid depletion of proteid-yolk which coincides with the arrival of putative primary lysosomes in the cortical cytoplasm. A causal relationship is suspected but not proved. Degradation of the yolk granules correlates with the accumulation of glycogen granules and multigranular glycogen rosettes. Mitotic divisions of the late myoblasts subside as the muscle anlagen acquire their full cellular complements. Septilaminar junctions, with intercellular clefts measuring 3–4 nm in width, are evident on the lateral and terminal surfaces of apposed myoblasts. These junctions are morphologically indistinguishable from the close junctions between the cells in the larval musculature (Cavey and Cloney 1976).

Synthetic phase of myogenesis

The synthetic phase is a period of cellular growth and elongation (Cavey 1982). This phase, encompassing stages III and IV, highlights the establishment of the contractile apparatus. The cells in stages III and IV will be designated as *early fascicular myocytes* and *late fascicular myocytes*, respectively. Since there are morphological indications of cellular fate, some terminological substitutions are appropriate: sarcolemma for plasmalemma, sarcoplasm for cytoplasm, and sarcoplasmic reticulum for endoplasmic reticulum.

The early fascicular myocytes are tall columnar cells with central, indented nuclei. Each nucleus contains multiple nucleoli. Small mitochondria, free polysomes, and glycogen granules surround the nucleus. The Golgi apparatus, putative primary lysosomes, and diminutive yolk granules also occur in the perinuclear sarcoplasm. Cisternae of the sarcoplasmic reticulum tend to be agranular, although some cisternae exhibit both agranular and granular segments.

Sarcoplasm at the epidermal and notochordal poles of the myocyte is granular in appearance, owing to its content of free polysomes and glycogen granules (Fig. 2). Nascent myofilaments arise in the cortical sarcoplasm at the cellular poles. Thick (myosinoid) filaments, averaging 13 nm in diameter, and thin (actinoid) filaments, averaging 7 nm in diameter, appear simultaneously. The random orientations of the myofilaments obviate accurate determinations of length.

The myofilaments promptly aggregate into fascicles (Figs. 2, 4, 5). The fascicles are randomly disposed in the cortex of the myocyte, and the registration of the myofilaments is poor, accounting for the absence of distinct sarcomeric bands. The aggregated filaments are comparable in length to those in the larval sarcomeres: the thick myofilaments average 1.3 μm in length, and the thin myofilaments average 1.1 μm in length. Inside the fascicles, the arrangement of the myofilaments is seldom hexagonal, so the ratio of thick:thin is rarely 1:6. Fascicular growth occurs by the apposition of myofilaments, either as individuals or in small bundles.

Short cisternae of the agranular sarcoplasmic reticulum (SR) adjoin the fascicles of myofilaments (Figs. 2, 3). Some cisternae lie in subsarcolemmal positions. A few of the structural associations between the SR and the sarcolemma qualify as peripheral couplings: the outer surface of the subsarcolemmal cisterna lies parallel to the inner surface of the sarcolemma; a sarcoplasmic gap of uniform width separates the apposed membranes; and a dense plaque situates in the sarcoplasmic gap, lying equidistant from the membranes (Cavey and Cloney 1972).

Throughout stage III, the early fascicular myocytes increase in volume and elongate. Length eventually supplants height as the primary cellular axis. Dimensional changes of the myocytes may account, at least in part, for the continued outgrowth of the tail. Close junctions are the only intercellular contacts evident during this stage.

The late fascicular myocytes are relatively long, laterally flattened cells. Each indented nucleus contains several nucleoli. The composition of the medullary sarcoplasm is unchanged, although the mitochondria and the glycogen granules are more prevalent. Rudimentary myofibrils, derived from the fascicles of myofilaments, are under construction in the cortical sarcoplasm at the cellular poles (Figs. 6, 7). The myofibrils, which align longitudinally with respect to the primary cellular axis, exhibit sarcomeric I-bands, A-bands, and H-bands. In some sarcomeres, H-bridges join the central shafts of the thick myofilaments to create M-lines in the middle of the H-bands. A flocculent material associates with the thin myofilaments in the I-bands. There are no discernible Z-lines in the I-bands of the sarcomeres.

As the fascicles align in series, there is a dramatic improvement in both the registration and the arrangement of myofilaments. The number of distinct sarcomeric bands reflects the improved registration, and the regularity of hexagonal packing attests to the improved arrangement. Inconsistencies in hexagonal packing, frequently observed at the edges of the myofibrils,

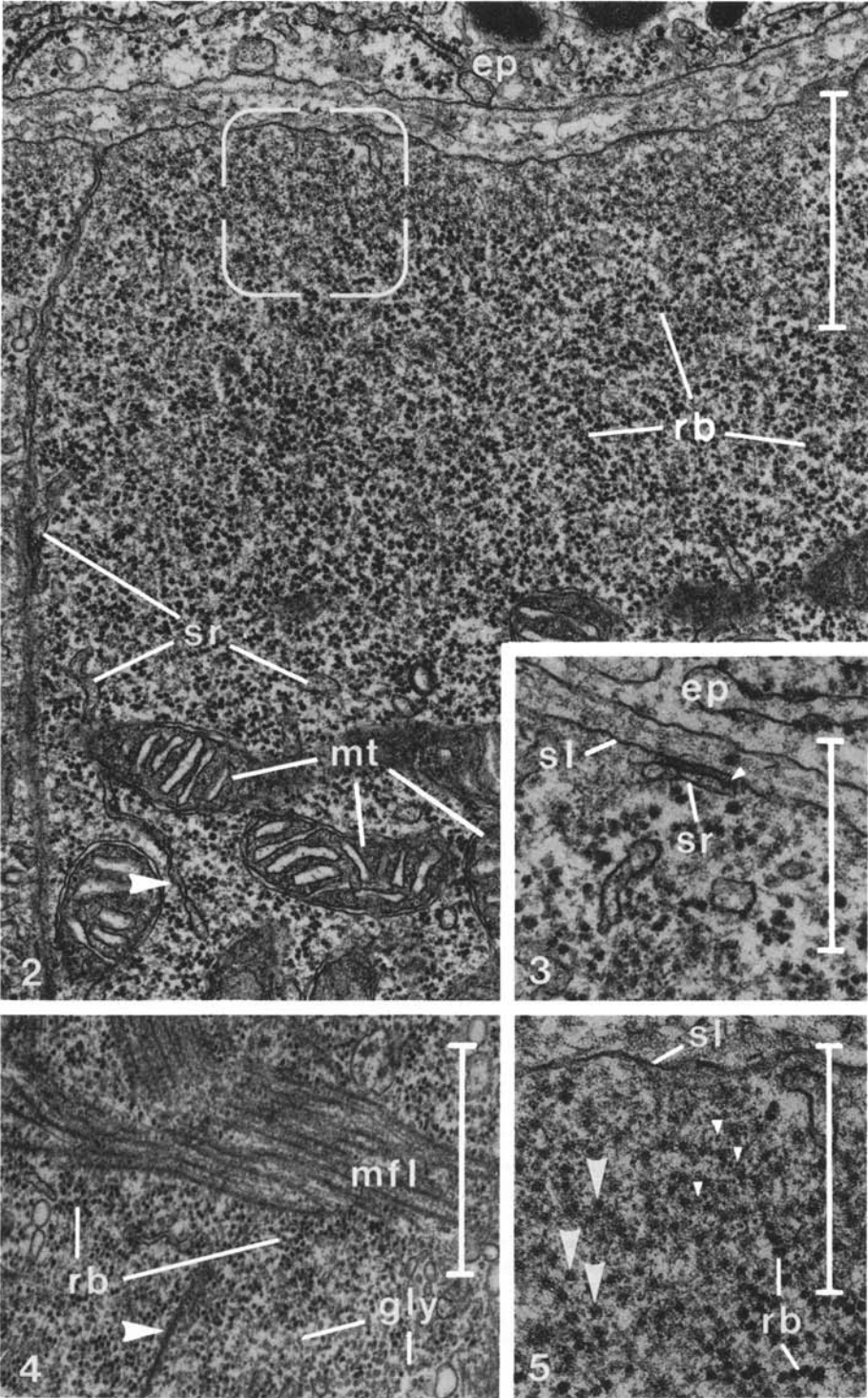
Figs. 2–5. Early fascicular myocytes of *Diplosoma macdonaldi*

Fig. 2. Transverse section through the polar sarcoplasm. Glycogen granules, polysomes (*rb*), and myofilaments account for the granular appearance of the polar sarcoplasm. The circumscribed area is shown at higher magnification in Fig. 5. Most cisternae of the sarcoplasmic reticulum (*sr*) are agranular, but some (*arrowhead*) exhibit both agranular and granular segments; *ep* epidermis, *mt* mitochondrion. Calibration bar = 1 μ m

Fig. 3. Transverse section through a peripheral coupling in the polar sarcoplasm. A gap (*arrowhead*), approximately 15 nm in width, separates the cisterna of the sarcoplasmic reticulum (*sr*) from the sarcolemma (*sl*); *ep* epidermis. Calibration bar = 0.5 μ m

Fig. 4. Longitudinal section through the myofilaments in the polar sarcoplasm. Myofilaments appear individually (*arrowhead*) and in unstriated fascicles (*mfl*); *gly* glycogen granules, *rb* polysomes. Calibration bar = 1 μ m

Fig. 5. Transverse section through a fascicle of myofilaments in the polar sarcoplasm. Thick (*large arrowheads*) and thin (*small arrowheads*) myofilaments can be discerned from ribosomes (*rb*) on the basis of size; *sl* sarcolemma. Calibration bar = 0.5 μ m



can be attributed to the recent addition of myofilaments. The myofibrils, like the fascicles, grow by apposition.

The terminal sarcomeres of the myofibrils adjoin the sarcolemma at the ends of the myocyte (Fig. 10). Intercellular alignment of the myofibrils is a common occurrence. The thin myofilaments and flocculent material from the half-I-bands contact the sarcolemmata, and the intercellular cleft widens. Filamentous strands span the intercellular cleft at right angles to the facing membranes. Such intercellular contacts foreshadow the intermediate junctions by which the myofibrils anchor to the sarcolemmata. The nascent intermediate junctions orient obliquely to the myofibrils.

Numerous cisternae of the agranular sarcoplasmic reticulum occur in the polar sarcoplasm. Some cisternae assume subsarcolemmal positions, incorporating into the peripheral couplings (Fig. 8), and others engage with sarcolemmal invaginations to establish a network of interior couplings (Fig. 7). The structural prerequisites of an interior coupling are threefold: the outer surface of the coupled cisterna must lie parallel to the side of the sarcolemmal invagination; a sarcoplasmic gap of uniform width must separate the apposed membranes; and a dense plaque must situate in the sarcoplasmic gap, lying equidistant from the membranes (Cavey and Cloney 1976).

There is no morphological evidence suggesting that the interior couplings originate from internalization of the peripheral couplings, since the sarcolemmal invaginations arise exclusively from the ends of the myocyte. Sarcolemmal segments within and near the intermediate junctions are especially

Figs. 6–10. Late fascicular myocytes of *Diplosoma macdonaldi*

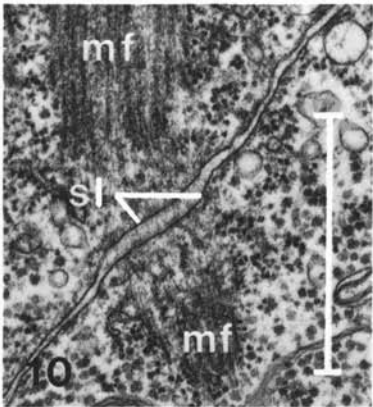
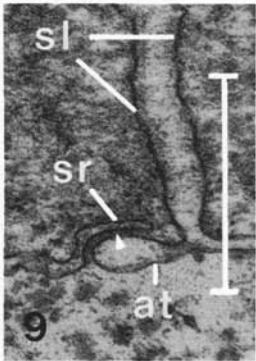
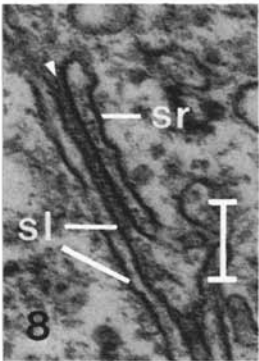
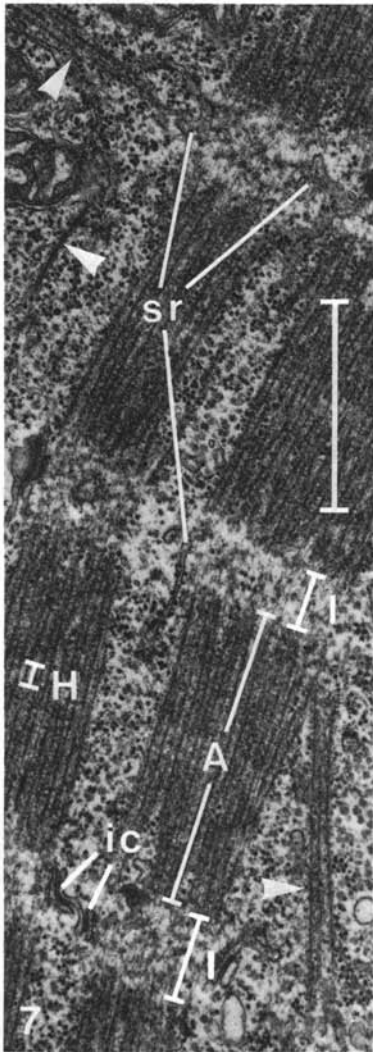
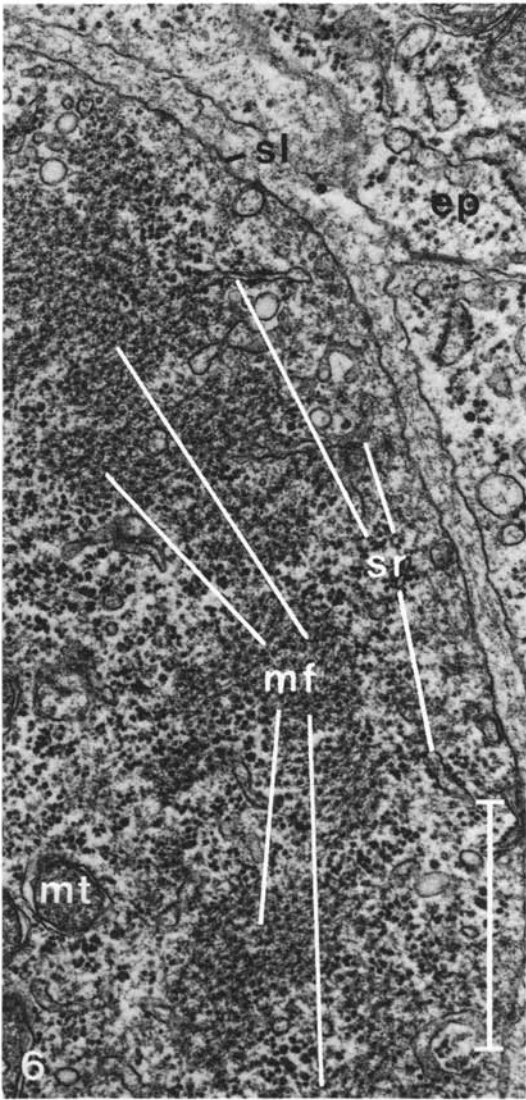
Fig. 6. Transverse section through the polar sarcoplasm. Cisternae of the sarcoplasmic reticulum (*sr*) mingle with the indiscrete myofibrils (*mf*); *ep* epidermis, *mt* mitochondrion, *sl* sarcolemma. Calibration bar = 1 μ m

Fig. 7. Longitudinal section through the myofibrils in the polar sarcoplasm. Sarcomeric I-bands (*I*), A-bands (*A*), H-bands (*H*), and occasional M-lines are evident. Free myofilaments, occurring individually or in small fascicles (*upper arrowheads*), flank the myofibrils. Growth of the myofibrils is appositional (*lower arrowhead*); *ic* interior coupling, *sr* sarcoplasmic reticulum. Calibration bar = 1 μ m

Fig. 8. Transverse section through a peripheral coupling. Note the apposed sarcolemmata (*sl*) of adjoining cells and the sarcoplasmic gap (*arrowhead*) of the peripheral coupling; *sr* coupled cisterna of the sarcoplasmic reticulum. Calibration bar = 100 nm

Fig. 9. Longitudinal section through the origin of an axial tubule. Widely separated sarcolemmata (*sl*) lie within an intermediate junction (cf. Fig. 10). Outside the junction, the intercellular space narrows, and the sarcolemma of the left myocyte invaginates to form an axial tubule (*at*). Observe the adherent cisterna of the sarcoplasmic reticulum (*sr*) and the sarcoplasmic gap (*arrowhead*) of an interior coupling. The axial tubule bends sharply, leaving the plane of section. Calibration bar = 0.5 μ m

Fig. 10. Longitudinal section through an oblique intermediate junction. Terminal sarcomeres of the myofibrils (*mf*) send thin myofilaments to the sarcolemmata (*sl*). Widening of the intercellular space is a consistent feature of junctional development. Calibration bar = 1 μ m



prone to invagination (Fig. 9), creating the axial tubules that wend longitudinally through the sarcoplasm, crisscrossing between the myofibrils and coupling to the sarcoplasmic reticulum. The interior couplings, although formed precociously, are spatially restricted in distribution: the couplings overlie the sarcomeric I-bands near the future levels of the Z-lines.

By the end of stage IV, the late fascicular myocytes have certain characteristics associated with the larval muscle cells: a system of indiscrete myofibrils (myofilament field or *Felderstruktur*); an array of intermediate and close junctions; and a network of interior couplings. Feeble, infrequent contractions of the myocytes are evident when living embryos are examined under the compound microscope.

Elaborative phase of myogenesis

The elaborative phase is a period of subtle alterations in cellular shape and sarcoplasmic organization (Cavey 1982). This phase, encompassing stages V and VI, highlights the relocation of the contractile apparatus and the investment of the myofibrils with the sarcoplasmic reticulum. The cells in stages V and VI will be designated as *early fibrillar myocytes* and *late fibrillar myocytes*, respectively.

The early fibrillar myocytes are laterally flattened, terminally truncated, fusiform cells. They interdigitate terminally and subterminally, having attained the size and the approximate shape of the larval muscle cells. The shrunken, indented nucleus of the myocyte contains one nucleolus, and condensed chromatin mottles the nucleoplasm. A thin layer of heterochromatin adheres to the inner membrane of the nuclear envelope. The mitochondria in the vicinity of the nucleus often exhibit angular cristae. The number of free polysomes declines sharply, with the remnants restricted

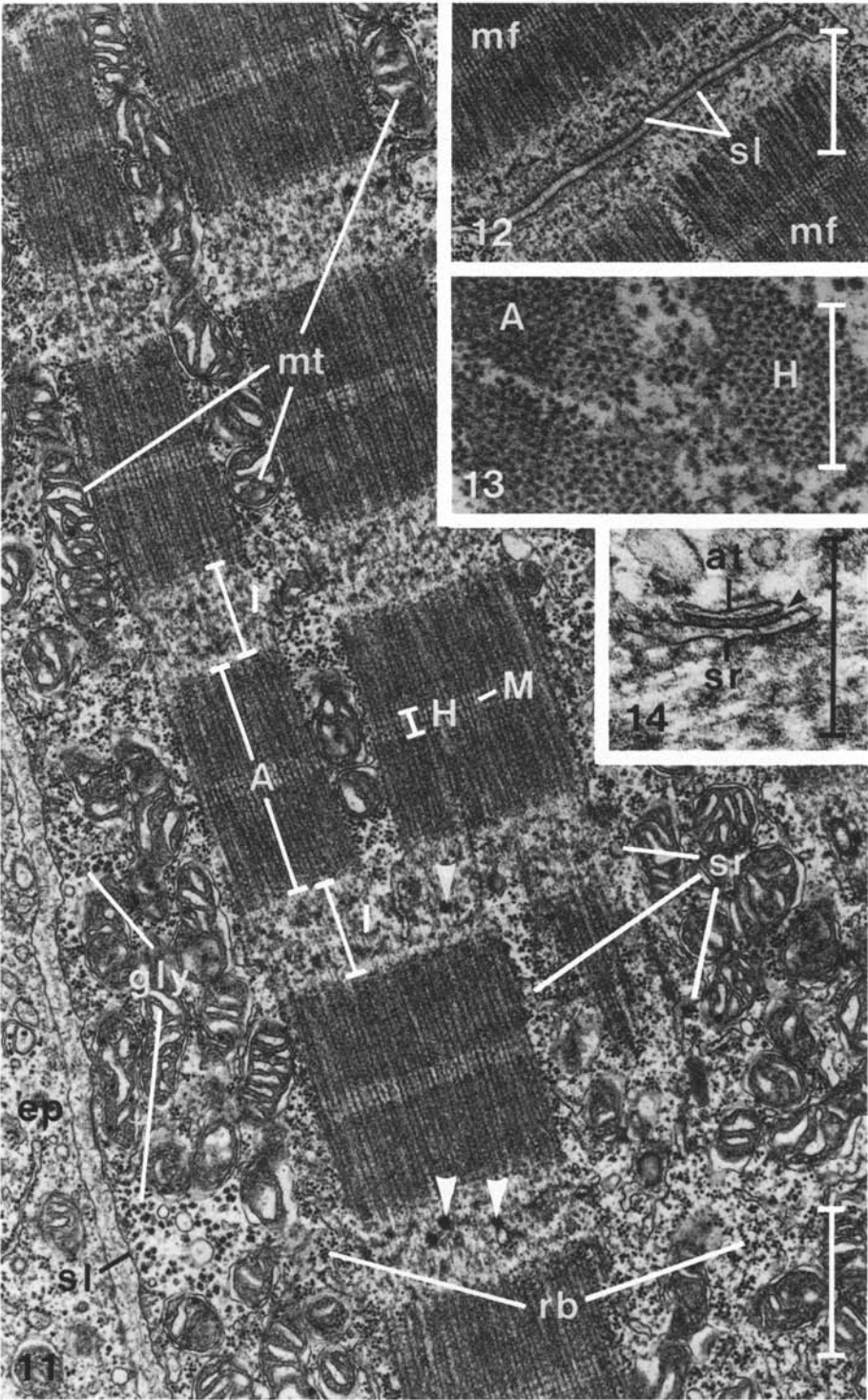
Figs. 11–14. Early fibrillar myocytes of *Diplosoma macdonaldi*

Fig. 11. Longitudinal section through the polar and juxtamedullary sarcoplasm. Mitochondria (*mt*) now congregate in the cellular cortex and wedge between the cross-striated myofibrils (*A* A-band, *H* H-band, *I* I-band, *M* M-line). Flocculent material and electron-dense patches (*arrowheads*) intersperse with the thin myofilaments of the sarcomeric I-bands; *ep* epidermis, *gly* glycogen rosettes, *rb* polysomes, *sl* sarcolemma, *sr* sarcoplasmic reticulum. Calibration bar = 1 μ m

Fig. 12. Longitudinal section through an orthogonal intermediate junction. Half-I-bands of the terminal sarcomeres adjoin the junctional sarcolemmata (*sl*); *mf* myofibril. Calibration bar = 0.5 μ m

Fig. 13. Transverse section through myofibrils after relocation. Hexagonal packing of the thick and thin myofilaments becomes increasingly evident once appositional growth has concluded; *A* A-band, *H* H-band. Calibration bar = 1 μ m

Fig. 14. Longitudinal section through an interior coupling. Flocculent material from the sarcomeric I-band contacts the undersurface of the cisterna of the sarcoplasmic reticulum (*sr*). A sarcoplasmic gap (*arrowhead*), measuring 15–20 nm in width, separates the coupled cisterna from the axial tubule (*at*). Calibration bar = 0.5 μ m



to small clusters throughout the sarcoplasm. On total elimination of the proteid-yolk, putative primary lysosomes begin to accumulate near the Golgi apparatus.

The fully grown myofibrils are seemingly passive in relocation to the cellular subcortex and medulla. Relocation of the contractile apparatus is correlated with the movement of mitochondria into the cortical sarcoplasm (Fig. 11). The invasive mitochondria lodge with the glycogen rosettes in the cortex and wedge between the indiscrete myofibrils. The registration and the arrangement of myofilaments are excellent, and the ratio of thick:thin normalizes at 1:6 (Fig. 13). Sarcomeric I-bands, A-bands, H-bands, and M-lines are well delineated, but Z-lines are still missing from the myofibrils (Fig. 11). The I-bands exhibit flocculent material and small, electron-dense patches among the thin myofilaments.

Between the ends of the myocytes, the intermediate junctions are orthogonal in orientation (Fig. 12). Since the contractile apparatus is a myofilament field, several myofibrils insert at each junction. Myofibrils can also split near the cellular boundary, with the branches inserting independently at contiguous junctions. Flocculent material and electron-dense patches infiltrate the half-I-band of each terminal sarcomere. Some of the flocculent material collects into a layer beneath the junctional sarcolemma.

Figs. 15–20. Late fibrillar myocytes of *Diplosoma macdonaldi*

Fig. 15. Transverse section through the subcortical and medullary sarcoplasm. Note the fenestrated sheets of the sarcoplasmic reticulum (*sr*) around the myofibrils and the latticework of the sarcomeric Z-lines (*Z*); *A* A-band, *gly* glycogen rosettes, *I* I-band, *mt* mitochondrion, *nu* nucleus. Calibration bar = 1 μ m

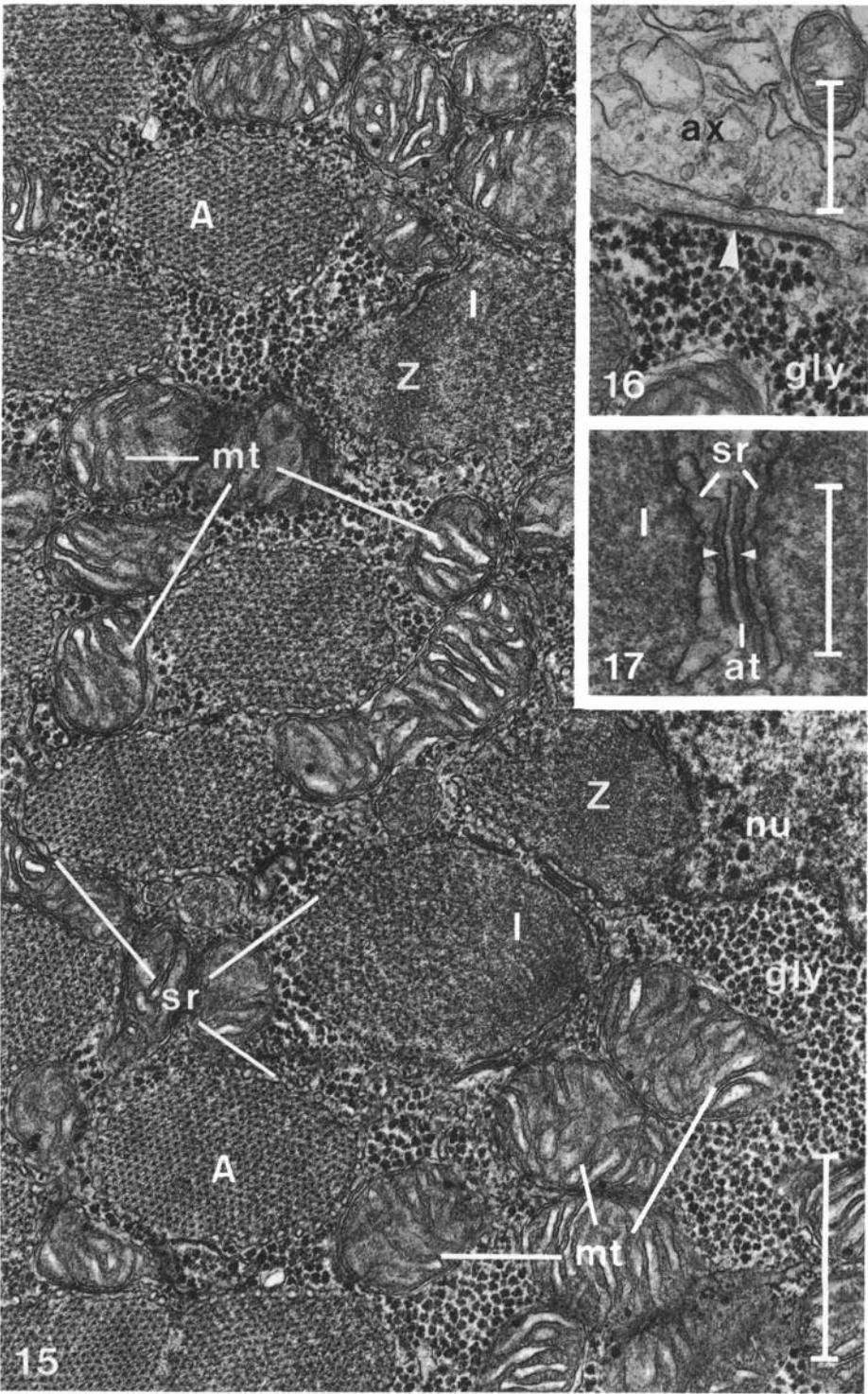
Fig. 16. Transverse section through a developing neuromuscular junction. A subsarcolemmal deposit (*arrowhead*) reveals the junctional locus; *ax* motor axon, *gly* glycogen rosettes. Calibration bar = 0.5 μ m

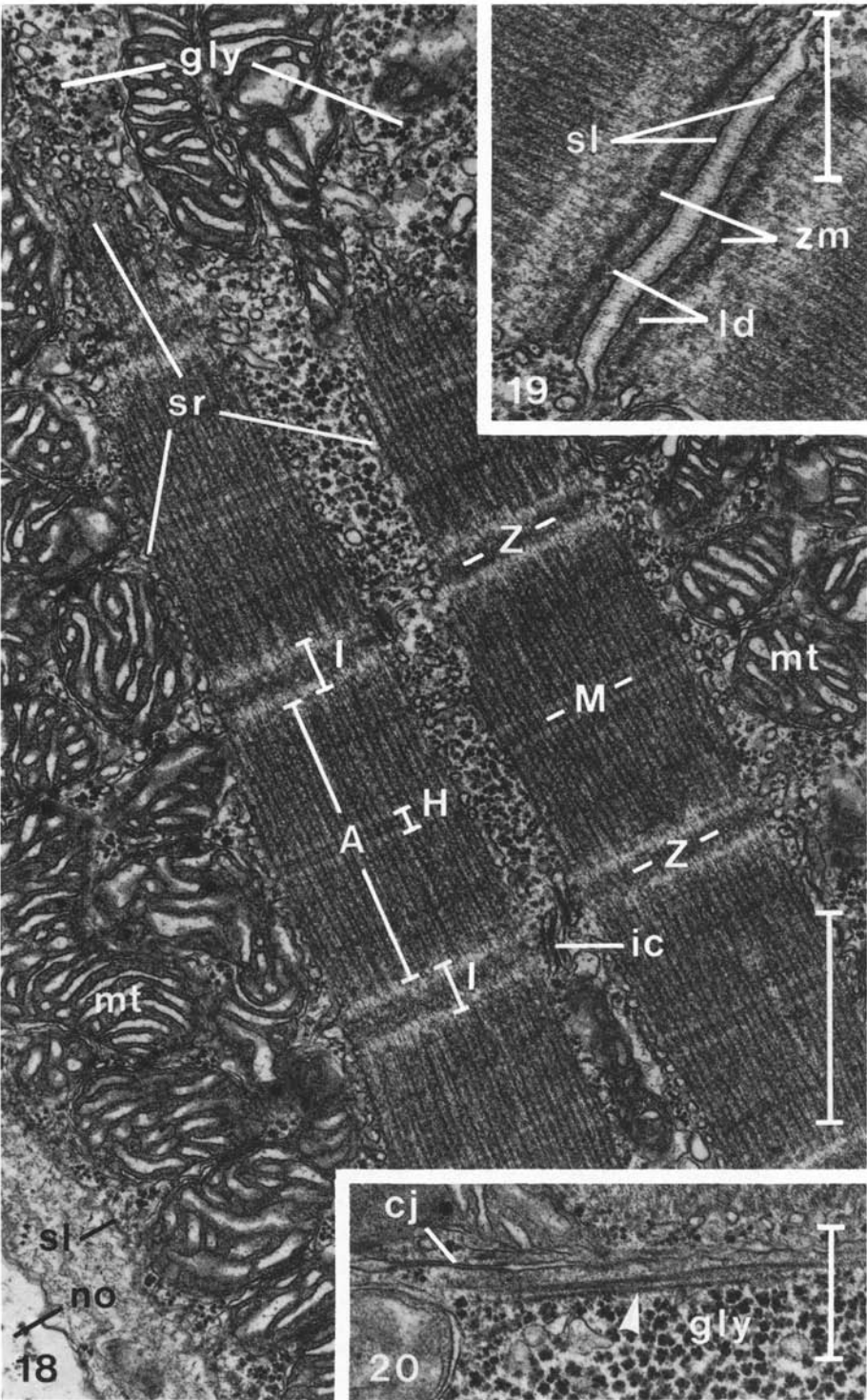
Fig. 17. Transverse section through apposed interior couplings. Note the sarcoplasmic gaps (*arrowheads*) of the interior couplings, one on each side of the axial tubule (*at*). Such “triadic” associations arise when the myofibrils lie close together; *I* I-band, *sr* coupled cisterna of the sarcoplasmic reticulum. Calibration bar = 0.5 μ m

Fig. 18. Longitudinal section through the cortical, subcortical, and medullary sarcoplasm. Note the abundant mitochondria (*mt*) with angular cristae between the sarcolemma (*sl*) and the cross-striated myofibrils (*A* A-band, *H* H-band, *I* I-band, *M* M-line, *Z* Z-line); *gly* glycogen rosettes, *ic* interior coupling, *no* notochord, *sr* fenestrated sheet of the sarcoplasmic reticulum. Calibration bar = 1 μ m

Fig. 19. Longitudinal section through an intermediate junction in the transverse myomuscular complex. Z-matrices (*zm*) interact with the thin myofilaments from the half-I-bands of terminal sarcomeres. Filamentous strands, averaging 5 nm in diameter, interconnect the sarcolemmata (*sl*) across the junctional cleft; *ld* intracellular zone low electron density. Calibration bar = 0.5 μ m

Fig. 20. Transverse section through a close junction. Sarcolemmal undulations are lost at the site of the close junction (*cj*), and the intercellular space narrows, leaving a uniform cleft that is 3–4 nm in width. Observe the fascicle of myofilaments (*arrowhead*) beside the membrane of the lower cell; *gly* glycogen rosettes. Calibration bar = 0.5 μ m





In the early fibrillar myocyte, there are no peripheral couplings between the sarcoplasmic reticulum and the sarcolemma (Fig. 11). A few electron micrographs do reveal the presence of cortical vesicles with adherent cisternae. While vesiculation of the sarcolemma might eliminate the peripheral couplings, there are too few observations to dismiss other possibilities. Interior couplings between the SR and the axial tubules are commonplace (Fig. 14). Pillars or sheets of flocculent material from the I-bands contact the undersurfaces of the coupled cisternae.

Early in stage V, the elongation of the myocyte subsides, and the outgrowth of the tail ceases. The developing tail twitches as groups of myocytes contract sporadically and in unison.

The late fibrillar myocytes tend to purge their polysomes, both from the sarcoplasmic clusters and from the outer membrane of the nuclear envelope. Further condensation of the chromatin and a diminution of the nucleolus are noted in the nucleus. Large mitochondria with angular cristae and multigranular glycogen rosettes occupy all sectors of the cell (Fig. 15).

In the contractile apparatus, Z-lines finally bisect the I-bands (Figs. 15, 18). Formation of the Z-line coincides with the disappearance of the electron-dense patches from the I-band. Flocculent material, formerly interspersed with the thin myofilaments, congregates centrally to obscure the architecture of the Z-line. In the half-I-band of a terminal sarcomere, an electron-dense layer occurs in the normal position of a Z-line (Fig. 19). This layer, or Z-matrix (Cavey and Cloney 1972, 1976), is located 60–65 nm beneath the sarcolemma in the intermediate junction. Thin myofilaments interact with the Z-matrix, and filaments of comparable size emerge from the Z-matrix and extend to the junctional sarcolemma. The filaments encroaching on the sarcolemma frequently align with the filamentous strands in the junctional cleft.

Cisternae of the sarcoplasmic reticulum anastomose on the surfaces of the myofibrils, creating the fenestrated sheets that stretch between the sarcomeres (Figs. 15, 18). There are no local specializations of the fenestrated sheets in relation to the sarcomeric bands. Perifibrillar cisternae of the SR presumably connect to existing cisternae within the interior couplings (Fig. 17). Two, three, or four interior couplings flank each Z-line.

During myogenesis, motor axons from the visceral ganglion in the trunk grow into the tail. These axons lie in lateral concavities formed by the ependymal cells of the nerve cord. Since axons never breach the developing muscle bands, few cells receive direct innervation (Cavey and Cloney 1976). The late fibrillar myocytes adjacent to the axons have filamentous deposits beneath areas of the sarcolemma that participate in the formation of neuromuscular junctions (Fig. 16). The external laminae of the muscle band and the nerve cord cross the synaptic clefts without interruption. Shortly before hatching, synaptic vesicles aggregate beneath the axolemma in the neuromuscular junctions. Numerous close junctions on the sides and at the ends of the myocytes may facilitate the intercellular spread of excitation from the directly innervated cells (Fig. 20).

As stage VI of myogenesis is ending, concerted contractions of the mus-

cle bands become more and more pronounced. Muscular activity may assist the larva in its escape from the egg envelopes (chorion and follicular epithelium) at the time of hatching.

Discussion

In *Diplosoma macdonaldi* the thick and thin myofilaments appear simultaneously in the myocytes and aggregate into unstriated fascicles. Actomyosin bridges are likely responsible for fasciculation, because there are no demonstrable H-bridges between the thick myofilaments or Z-lines for attachment of the thin myofilaments. The staggered configuration of the myofilaments in the fascicles reconciles easily with this mechanism of aggregation. A similar mechanism has been ascribed to the somatic striated muscle fibers of amphibians and birds (Fischman 1972) and to the caudal muscle cells of other ascidian larvae (Terakado 1972, 1975). In the larval muscle cells of one ascidian, *Distaplia occidentalis*, fasciculation may involve two mechanisms: actomyosin-bridging of the thick and thin myofilaments as well as H-bridging of the thick myofilaments (Cavey and Cloney 1974).

In the myocytes of *D. macdonaldi*, the fascicles of myofilaments align in series to form the rudimentary myofibrils. Interconnection of the fascicles involves the participation of the thin myofilaments, but it seems doubtful that they are solely responsible. In the myocytes of other ascidian larvae, precursory Z-lines appear on the free ends of the thin myofilaments and conceivably interconnect the fascicles (Cavey and Cloney 1974; Terakado 1972, 1975). Precursory Z-lines are not evident in the differentiating muscle cells of *D. macdonaldi*, so several unanswered questions remain. Could the thin myofilaments be solely responsible for the interconnection of the fascicles? Does the flocculent material interspersed with the thin myofilaments play a role in the interconnection of the fascicles? Might the flocculent material represent the precursory Z-lines in this species? Are the electron-dense patches, which do resemble precursory Z-lines, derivatives of the flocculent material in the I-band?

Regardless of the answers to the questions posed above, it is quite evident that the rudimentary myofibrils of *D. macdonaldi* hastily anchor to the sarcolemma by their terminal sarcomeres. The first, feeble contractions of the myocytes correlate with these anchorages, the future intermediate junctions of the transverse myomuscular complexes. Contraction and relaxation of the myofibrils, involving breakage and reformation of the actomyosin bridges, may improve the registration of the myofilaments.

The contractile apparatus consistently appears in the cortex of the ascidian myocyte (Cavey and Cloney 1974; Terakado 1972, 1975). In some species, like *D. macdonaldi*, the myofibrils must secondarily shift to the sub-cortex and medulla. As illustrated in this paper, relocation is an elegantly simple, conservative event in which the myofibrils are passive. The myofibrils assume their new positions when mitochondria infiltrate the cortical sarcoplasm. Relocation involves no obvious alterations to the myofibrils or the intermediate junctions.

Elimination of the peripheral couplings during relocation of the contractile apparatus is rather surprising since the subcortical myofibrils are not far removed from the sarcolemma. In a closely related European species, *Diplosoma listerianum*, the larval muscle cells also contain interior couplings centered on the Z-lines of the sarcomeres. Shallow invaginations of the sarcolemma, arising from the lateral surfaces of the cell, incorporate into the interior couplings that serve the subcortical myofibrils (Burighel et al. 1977). Such lateral invaginations of the sarcolemma have never been observed in the differentiating or functional muscle cells of *D. macdonaldi*. The only sarcolemmal segments that invaginate consistently are those within and near the intermediate junctions at the transverse cellular boundaries.

In differentiating cells of *D. macdonaldi*, sarcolemmal invaginations of the axial tubular system appear during early myofibrillogenesis, and interior couplings are present long before the fenestrated sheets of the perifibrillar sarcoplasmic reticulum. The precocious interior couplings are highly localized, residing in their definitive positions next to the I-bands of the myofibrils. Several questions immediately come to mind. Why do the interior couplings arise so far in advance of the perifibrillar sheets? How do the interior couplings achieve such accurate centration on the I-bands? Does the axial tubule or the adherent cisterna dictate the placement of the interior coupling?

It is tempting to speculate that the cisterna of the sarcoplasmic reticulum might specify the position of the interior coupling. Flocculent material from the I-band projects to the undersurface of the coupled cisterna. Might the flocculent material ensnare and immobilize cisternae in the vicinity of the I-band? If true, interior couplings would form adjacent to the I-band because available cisternae are tethered to it. This hypothesis is not unique to ascidians. There are cases in vertebrate myogenesis for structural linkages between the sarcoplasmic reticulum and the differentiating Z-lines (Walker et al. 1971, 1975; Warren 1973). If the corollary holds, the flocculent material in the ascidian I-bands may indeed equate with precursory Z-lines. It should then be possible to find cisternae of the sarcoplasmic reticulum tethered to the I-bands, or even to the thin myofilaments in the fascicles, before the appearance of the interior couplings.

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