Three Classes of Filaments in Cardiac Differentiation

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Embryonic chick hearts at representative stages of early development were fixed in phosphate-, cacodylate-, and s-collidine-buffered glutaraldehyde, glutaraldehyde and acrolein, and osmium tetroxide, postfixed in osmium tetroxide and uranyl acetate, and examined with an electron microscope. Comparison of en bloc and en coupe staining with aqueous and alcoholic uranyl acetate revealed the superior preservation of membranous and fibrous components with aqueous en bloc staining, but the disruption and removal of glycogen from free cytoplasmic pools, enlarged mitochondria (cf. 4, 5), membrane-bound glycogen vesicles, and myelin-like whorls associated with degenerating yolk platelets. More important, the presence of a heterogeneous class of 85–130 Å filaments in precardiac cells and cardiac myoblasts, myocytes, fibroblasts, and fibrocytes was revealed by all procedures. Visual comparisons from micrographs and measurements of filament diameters revealed them to be intermediate in diameter between the thin and thick filament of the sarcomere, but consistently heterogeneous with respect to diameters. Large numbers of the intermediate filaments were observed in the myoblasts prior to myofibril deposition, often in the areas of cortical cytoplasm associated with Z band deposition, initial thin filament appearance, and myofibril formation. Unincorporated thin filaments were observed coursing randomly in the peripheral matrix and subsequently associated with presumptive Z band material. Unincorporated but easily recognized thick filaments were observed free in the cytoplasm and in association with free thin filaments and thin filaments with attached Z material in regions of myofibril deposition. As we demonstrate in the accompanying report (106), several extractions were devised to remove specific myofibrillar subunits. The intermediate filaments remained after disruption of the thick filaments, thin filaments, and Z bands, demonstrating that the intermediate filaments cannot be equated with adult myofibrillar constituents, unless major modification of the intermediate filaments occurs preceding sarcomere deposition. Alternative explanations are considered, including interconversion with microtubules associated with protoplasmic streaming and cell extension, interconversion with free thin filaments, and permanent deposition as passive supporting structures.

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However, insufficient data are presently available concerning the newly discovered intermediate filaments to permit conclusive interpretations of structure, composition, or function.

The sliding filament model of muscle contraction, independently proposed by H. E. Huxley and Hanson (70) and A. F. Huxley and Niedergerke (63) in 1954, united the interpretations arising from light and electron microscopies. In the succeeding fifteen years, data from X-ray diffraction of living and of fixed muscle (25, 26, 67, 69, 107), biochemical reconstitution of the myofibrillar subunits (45, 65, 66), and electron microscopy of fixed and unfixed muscle (21, 45, 64–66) have revealed the original sliding filament model to be a reasonably accurate representation of the ultrastructure and mechanism of action of living muscle. There is little consensus, however, on the sequence of events in myofibril deposition, the identity of the various subunits, or the roles of the subunits in sarcomere formation (cf. 2, 31, 47, 48, 61, 62, 84, 100, 134). Though it generally has been assumed that the thick filaments of the developing myocyte are deposited by a coalescence of myosin molecules (2, 31, 100) similar to the in vitro mechanism of thick filament reconstitution (65), no evidence for such deposition in vivo has been presented, nor have individual unincorporated myosin molecules been observed in developing myocytes. The deposition of thin filaments has received even less attention from electron microscopists, though it has been briefly studied by biochemical techniques (94). Unfortunately, the presence of easily recognized filaments thinner than the myosin thick filaments in presarcomere myocytes has been assumed by most investigators (2, 31, 47, 48, 79, 84, 100) to represent the as yet unincorporated actin thin filaments. Recently, however, a third class of myofilaments, intermediate between the thick and the thin filaments, has been demonstrated in developing voluntary muscle (71–75) and cardiac muscle (102, 103). Additional filaments of 40–70 Å have been observed in the peripheral matrix of striated myocytes by Kelly (75) and have been suggested to be the definitive thin filament to be incorporated into the developing myofibril. The origin of the Z band material, too, is unsettled, although attempts have been made to determine the time of synthesis, site of original deposition, relationship to other cellular organelles, and even approximate composition (2, 8, 9, 47, 48, 52, 75, 84, 102). Apparently, the only major areas of agreement concerning myogenesis are that (a) at least two classes of filaments exist in presarcomere stages [but see Manasek (84)]; (b) the earliest primitive sarcomeres possess definitive thick and thin filaments; and (c) myofibrils appear exclusively at the periphery of the early myocyte (but see 84).

Preliminary evidence for three classes of filaments

In 1965, Heuson-Stiennon (52) first noted that unincorporated myofilaments did not conform to either the 50 Å or 110 Å diameter reported in adult sarcomeres fixed
in buffered osmium tetroxide. Because the observed filaments evidenced a range of diameters, it was suggested that the free cytoplasmic filaments increased in diameter by lateral addition of subunits and ultimately formed the thick filaments of the sarcomere. In 1967, Rash et al. (102) presented preliminary evidence based on extractions for more than a single type of free cytoplasmic thin filaments and that they differed significantly from the adult myosin filaments. Subsequently, Ishikawa et al. (72) and Kelly (75) confirmed the presence of a single class of intermediate filaments (100 Å in diameter and over 1.5 μ in length) in the myocytes and myoblasts of voluntary muscle and suggested that the observed intermediate filaments were identical to the “free cytoplasmic thin filaments” observed by others (2, 31, 100). Although these most recent investigations of early myogenesis have clearly demonstrated that the intermediate filaments are not incorporated directly into the myofibril as the actin filament and that the erroneous assumption arose due to incorrect identification of the free filaments (94), only Kelly (75) has been able to demonstrate thinner (30–60 Å) filaments in the cortical matrix of voluntary myoblasts and early myocytes of voluntary muscle.

This report seeks to demonstrate that (a) three readily identified classes of filaments are present in developing cardiac muscle prior to and concurrent with myofibril deposition; (b) in contrast to data from voluntary muscle development (2, 31, 72, 75) and in support of the data of Heuson-Stiennon (52), the intermediate filaments comprise a heterogeneous population (i.e., a continuum with respect to diameters); (c) the intermediate filaments vary in diameter according to cellular location and degree of cellular differentiation, and (d) contrary to Heuson-Stiennon (52) the intermediate filaments differ biochemically from the thin actin filaments and the thick myosin filaments [see accompanying report (106)]. In addition, we are able to demonstrate unincorporated thick filaments at random in the cytoplasm and thin filaments in the subsarcolemmal matrix in the areas associated with myofibril deposition, both differing markedly from the free intermediate filaments. Last, the origin of the dense material of the Z bands, intercalated disks, and desmosomes is considered in relation to the observed close association and possible alteration of the intermediate filaments immediately preceding or at the time of initial deposition of the Z-like matrix in the cell cortex.

**MATERIALS AND METHODS**

Some 125 fertile eggs of White Leghorn chickens were incubated at 36–38°C and served as the primary source of material for studies of cardiac myogenesis. Embryos were selected at hourly intervals from 0 to 48 hours and 5, 9, and 11 days of incubation and staged according to the chick developmental series of Hamburger and Hamilton (43). Thirty embryos were selected at substage intervals (9 + to 11 +, or 8 to 14 somites) just before and during initial cardiac myogenesis [cf. DeHaan (17)].
Fixation and Embedding. Objections to possible osmotic and pH effects of the fixatives and buffers originally employed (101) prompted a complete reevaluation of the data. This report has incorporated data from tissues fixed in 2%, 2½%, and 3% glutaraldehyde, 2½% glutaraldehyde plus 2½% acrolein, and in 1% osmium tetroxide, each buffered with 0.05 M, 0.10 M, 0.15 M, 0.18 M, and 0.20 M Sørensen’s [Sabatini et al., (111)] phosphate, cacodylate, and collidine buffers (collidine was not used as a buffer with osmium tetroxide) at pH 6.8, pH 7.0, pH 7.2, pH 7.4, and pH 7.6 (15 different procedures). After primary fixation for 1 hour, tissues were rinsed two to five times for 1–24 hours in the corresponding buffer, postfixed for an additional hour in phosphate- or cacodylate-buffered osmium tetroxide. After rinsing for 1 hour in distilled water, most tissues were then placed in 0.5% aqueous uranyl acetate for 16–20 hours. In order to assess the effects of prolonged exposure to uranyl acetate, particularly the effects of glycogen (85), additional tissues were stained with uranyl acetate as a section stain (1% uranyl acetate in 70% ethanol for 5–20 minutes). After dehydration in graded ethanol series and two changes of acetone or propylene oxide, the tissues were embedded in one of three plastic mixtures: A plastic mixture of 10% Epon, 20% Araldite, 70% DDSA with 1.5% DMP-30 accelerator, DER 332 plastic mixture (Polysciences, Inc.), or Spurr low viscosity embedding medium (Polysciences, Inc.).

Electron microscopy. After polymerization for 12–24 hours at 60–80°C, the tissue blocks were examined with a dissecting microscope, mounted parallel or perpendicular to axes of cell migration or organ development (11, 17, 84, 96, 126), and trimmed so that dark gray to gold sections could be cut on Sorval MT-1 and MT-2 ultramicrotomes. The sections were spread with toluene or xylene vapors, picked up on uncoated copper grids, post stained for 1–10 minutes with undiluted Reynolds’ lead citrate (110) or 1–20 minutes in 75% ethanol containing 1% uranyl acetate and counterstained with lead citrate. (All micrographs presented in this report are from tissues block stained with uranyl acetate.) Initial micrographs were made at the University of Texas on a student training Elmiskop I electron microscope operated with variable intermediate lens and double condenser. Because of the heavy student load and the inexperience of the senior author, the resolution of the microscope varied appreciably and micrograph quality was subsequently considered unacceptable for high resolution measurements of filament diameters. Recently all previous data (101) were reevaluated, and additional measurements were made on tissues prepared with additional buffers, stains, and fixatives. Measurements were made from micrographs exposed on Kodak Electron Image Plates and Kodak Electron Microscope Film in a vintage Siemens Elmiskop I electron microscope used solely by the senior author and operated with double condenser and fixed intermediate lens, giving calibrated final magnifications of 20 000 x, 38 000 x, and 80 000 x. It should be noted that contrast of the negatives varied considerably because the scope was not equipped with a photometer or automatic shutter. Exposures, estimated at 3 to 30 seconds, were accomplished by quickly raising and lowering the final view screen. Calibration of the scope was measured using carbon replicas of diffraction gratings and did not vary measurably (less than ±2%) in the 3 months of reevaluation. Micrographs were considered to be in or near focus only when the two layers of “unit membranes” were observed in mutually perpendicular orientations and when the resulting phase contrast grain [cf. Sjöstrand (116)] measured less that 40 Å peak to peak (here defined as the point to point resolution). Linear resolution, therefore, was judged to be one half that of the numerical value of the point to point resolution or approximately 20 Å (or even 15 Å) in those micrographs used for measurements of filament diameters. (When appropriate, linear resolution is indicated in the figure legends.) All measurements were made from micrographs at initial
magnifications of 38,000 and 80,000 × from silver and gray sections. Filament diameters, at first measured directly on the original negatives with a 35 × dissecting microscope, were more consistently and accurately measured on tracings of images projected at 30 × with an Omega photographic enlarger, with final projected magnifications of 1,140,000 × and 2,400,000 × (100 Å = 24.0 mm).

RESULTS

Early morphogenesis of the chick heart

At low magnifications on the electron microscope, morphogenesis of the chick heart corresponds closely to models derived from light microscopy (11, 17, 84, 96, 105). Light and electron microscopies have revealed that the precardiac mesenchyme develops between the epiblast and hypoblast as a loose association of cells with large intercellular spaces and few cell contacts. The cytoplasm initially is filled uniformly with free ribosomes, numerous mitochondria, and an extensive Golgi system, but is almost devoid of endoplasmic reticulum. Anterior portions of the presumptive cardiac rudiments approaching the midline as paired layers approximately two cells in thickness have reduced intercellular spaces and more extensive but poorly differentiated cell contacts. From stage 9 to stage 11+ (7 to 11 somites), a tremendous proliferation of intertwined filaments has been observed in central regions of many cells (79, 101), resulting in large areas devoid of other cytoplasmic organelles (Fig. 1). This early stage, with its tremendous number of filaments, is to be contrasted with all later stages.

In the peripheral matrix of the earliest myocytes and in myocytes with only partially formed sarcomeres, partially aligned sarcomere subunits are most commonly associated with the plasmalemma and early myofibrils appear to be formed in the peripheral matrix (Fig. 2). Intermediate magnifications of the electron microscope reveal that discrete thick filaments, and presumptive Z band material are all present concurrent with initial sarcomere deposition. However, the limited resolution in such low magnification micrographs, especially from thicker (pale gold) sections, does not allow clear representation of the nonparallel thin filaments except in limited areas (arrows). Nevertheless, intertwined masses of filaments are clearly revealed, although not exclusively limited to any area of the cytoplasm. These masses of easily resolved intertwined filaments are more common deeper in the cell, but often are

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Fig. 1. Longitudinal section of 11-somite chick ventricle in region devoid of myofibrils. Abundant intertwined filaments present (IF) in areas devoid of additional organelles or associated with the recently formed desmosomes (D). Slightly hypotonic fixation. × 11,000.

Fig. 2. Filament alignment phase in early sarcomere deposition near cell membrane. Compare easily resolved intermediate filaments (IF) with poorly resolved thin filaments (arrows). Amorphous Z material characteristic of fixation in phosphate-buffered glutaraldehyde. × 50,000.
associated with areas of myofilament alignment, perhaps resulting in their general but incorrect designation as thin filaments (2, 31, 79, 84).

Although many investigators (15, 31, 52, 62, 75, 100, 114) have repeatedly indicated that most if not all of the myofibrils are formed near the sarcolemma and subsequently are internalized, only Kelly (75) has investigated the subsarcolemmal matrix of developing striated myocytes. In the heart, too, the peripheral association of the first formed sarcomeres is quickly camouflaged, for within 4 hours after initial myogenesis, virtually all cells of the atria, ventricles, and conus reveal complete myofibrils throughout the cytoplasm and partially formed sarcomeres near the cell surface (Figs. 3a and 3b). [The clear or mottled areas in this 14-somite conus reflect the extraction of glycogen following block staining with uranyl acetate (85).] Higher magnifications of the peripheral areas (Fig. 3b) associated with myofibril deposition, however, clearly revealed the presence of at least three distinct classes of filaments. In addition to the easily resolved thick filaments (M), and superimposed sarcomeric thin filaments (A), a few additional filaments of 55–65 Å are observed in the subsarcolemmal matrix. More important, the free cytoplasmic filaments are clearly revealed as a third class of filaments, intermediate in diameter between the thick and thin filaments. Although the origin of the thin filaments is unknown, they are clearly not identical with the free cytoplasmic filaments and apparently are formed (first appear) near the cell membrane.

In an attempt to clarify the nature of the newly described intermediate filaments, measurements of filament diameters were accomplished directly from the negatives and from tracings of projected images. Graphic representation of the data (Histograms I and II) revealed at least three classes of filaments and the heterogeneous nature of the intermediate filaments. Numerous intermediate filaments (Fig. 3b) are included near the surface, most with diameters of 80 and 95 Å (Histogram II), but with others of 115 Å and 130 Å (arrows). Heterogeneity (or alteration) of the intermediate filaments is thus clearly indicated. Measurements at different stages revealed changes in the relative numbers of intermediate filaments in the various subclasses, with a relative decrease in the number of larger (115 Å) filaments and a concommitant increase in the number of thinner (80 and 95 Å) filaments. Perhaps coincidentally, the appearance of free thin filaments (See Fig. 3b and Fig. 10) is observed, and occasionally

Fig. 3a. Bicellular layer of regularly contracting heart (14 somites) with numerous randomly oriented myofibrils. Clear areas reflect pools of glycogen (GL) extracted by en bloc uranyl acetate post staining. Note the tremendous number of free intermediate filaments (IF) present at this stage. Inscribed area is enlarged as Fig. 3b. × 17 000.

Fig. 3b. Periphery of developing myocyte of 14-somite chick heart. Note the 65 Å thin filaments near the sarcolemma and superimposed in the sarcomeres, thick filaments in the sarcomeres, and various diameters of closely associated and intertwined intermediate filaments (80–85 Å, 95–100 Å, 110–115 Å, and 125–130 Å). (LR = 25 Å). × 105 000.
HISTOGRAM I. Measurements of free filaments encountered in stage 4 to 11 embryos. (Numbers of measurements approximately proportional to numbers of filaments observed in each class.) Free thin filaments (unshaded, see Fig. 10) and free thick filaments (unshaded, see Fig. 6) easily distinguished from the several diameters of intermediate filaments (shaded, see Figs. 3b, 5, 8, and 9).

HISTOGRAM II. Comparison of intermediate filament diameters from precardiac (stage 3-6) stages (striped) and from areas associated with myofibril deposition (shaded). (Not proportional to numbers of filaments observed.) Note the presence of four distinct classes of intermediate filaments, predominantly 110-115 Å at early stages and of 95-100 Å filaments at later stages (see Fig. 3b). Smaller (80-85 Å) filaments usually closely associated with plasmalemma or with presumptive Z material and may represent a class of larger thin filaments.

no clear distinction can be made between thin filaments and smaller intermediate filaments.

Continued early morphogenesis (Fig. 4 and 5) is accompanied at the subcellular level by a significant decrease in the number of free intermediate filaments and an in-

Fig. 4. Myofibril proliferation and alignment evident in 25-somite chick conus. Reduced number of intermediate filaments (arrows) evident, those remaining usually associated with desmosomes (D). Hypertonic fixation. × 27 000.

Fig. 5. Thin section of 5-day ventricular myocyte with well developed sarcomeres, the thick and thin filaments clearly connected by cross-bridges. Note that very few intermediate filaments remain, and none of smaller intermediate filament diameters. No evidence of intermediate filaments as inter-Z band bracing material (75). (LR = 15-20 Å). × 85 000.
crease in the number of myofibrils. By 25 somites few intermediate filaments are observed, those remaining usually associated with desmosomes (Fig. 4). However, a few intermediate filaments can be observed at 5 days (Fig. 5), and throughout cardiogenesis. Nevertheless the abundance of intermediate filaments before and during early myogenesis (Figs. 2, 3a, and 3b) should be contrasted with the much reduced number found at later stages (Figs. 4 and 5). With the tremendous proliferation of myofibrils after stage 11, it was deemed necessary to confine the more detailed examinations of myofibrillogenesis to those cells of the 8–12 somite ventricles which exhibit free filaments or free filaments and rudimentary myofibrils.

**Designation of filament classes**

At the high magnifications possible with the electron microscope, visualization of minute structures has been possible for over 15 years, but the fixations, stains, and techniques of section preparation have not been equivalent in quality. Only recently have aldehyde fixations, uranyl acetate block staining, and plastics capable of yielding very thin sections been available to most microscopists. Combining these techniques, it is possible to produce micrographs of sufficient magnification and resolution to demonstrate the presence of the presarcomere subunits in cardiac myoblasts and myocytes.

Using phosphate and cacodylate-buffered glutaraldehyde fixation, a distinct class of 150–165 Å filaments is observed in most myoblasts and myocytes after 8 somites. These easily recognized unincorporated thick filaments (Fig. 6) are observed to possess diameters equivalent to the thick filaments of the sarcomeres, have diffuse margins, tapered ends, and poorly resolved periodic projections assumed to be equivalent to the poorly resolved (by our procedures) cross-bridges of sarcomeric thick filaments (see Fig. 5). Although serial sections demonstrated that the three thick filaments depicted were not arranged in a primitive sarcomere, several thin filaments are closely

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**Figs. 6–10. Three classes of filaments observed during early cardiac myogenesis. Point to point resolution limited to 30–60 Å by phase contrast effects, while linear resolution varies from 15–30 Å (approximately 1/2 point to point resolution). All ×100 000.**

**Fig. 6.** Portions of three thick filaments and three or four closely associated thin filaments attached by poorly resolved cross-bridges. By “sighting” between the opposed large arrows, additional periodic densities can be observed more clearly, possibly representing the truncation in the plane of section of unattached cross-bridges (see text) (LR = 20–25 Å).

**Fig. 7.** Portion of a thick filament with diffuse ends and irregular margins. Very thin (30–40 Å) filaments (arrows) or flocculent material may represent myosin molecules being incorporated into a forming thick filament (LR = 15–20 Å).

**Fig. 8.** Larger (130 Å) intermediate filament with numerous lateral projections of 250–350 Å aperiodicity (see text). From 10-somite chick ventricle (LR = 15 Å).

**Fig. 9.** Larger smooth intermediate filament from precardiac mesenchymal cell (LR = 15–20 Å).

**Fig. 10.** Thin (55–65 Å, small arrows) filaments in cortical “matrix” of 8-somite chick ventricle. Note a single 80 Å filament at lower left (larger arrow) (LR = 15–20 Å).
associated and several cross-bridges (arrows) can be observed linking the thick and thin filaments. By examining the figure at an extreme oblique angle and sighting between the opposed arrows, additional aligned periodic densities can be detected spaced equivalently to the cross bridges normally connecting thick and thin filaments (see inset drawing for a semidiagrammatic representation of Fig. 6). Because cross bridges are less than 150 Å in length and because the section is over 400 Å in thickness, the section cannot contain only the 150 Å region between adjacent thick and thin filaments. Therefore, the regularly spaced cross bridges present between the arrows must either connect to a thick or to a thin filament not included in the plane of section, but not to both. If connected to a thick filament, it must be assumed that cross bridges project from the thick filaments before attachment to thin filaments. Alternatively, if the cross bridges project from a thin filament, it must be assumed that at least some intact myosin molecules may initially attach to the thin filaments, possibly depositing later as the thick filaments by intertwining the freely projecting, heavy meromysin tails. However, numerous very short thick filaments with diffuse borders can be observed (Fig. 7), often with closely associated 20–40 Å filamentous material (arrows). (It should be noted that the limits of resolution do not permit accurate measurements of very small filament diameters.) Such figures may represent the more commonly assumed formative stages of thick filament deposition from individual “native myosin” molecules as proposed by Huxley (65) and searched for by others (2, 31, 100).

In areas which appear to be devoid of cytoplasmic organelles at low magnifications, numerous filaments are observed, often oriented parallel to microtubules, intertwined in apparently homogeneous masses, or randomly coursing through the cytoplasm. Although numerous investigators (2, 31, 94, 100) have equated these filaments with the definitive thin filaments, visual examination of relatively high resolution micrographs reveal them to be intermediate in diameter between the thick and thin filaments. At high magnification, these smooth margined, evenly and densely stained intermediate filaments are observed singly in many cells of the precardiac mesenchyme (Fig. 9a and b) and throughout cardiogenesis (Figs. 2–5 and 8). They possess quantized diameters (Histogram I), most commonly 95 and 110 Å, though very often filaments with diameters of 80 Å or 130 Å are observed (Fig. 3b and 10). In addition, a few of the very long intermediate filaments possess lateral projections with a 230 to 350 Å aperiodicity (Fig. 8b, arrows). (Because the section is significantly thicker than the filament plus lateral projections and because the image is a two dimensional representation of the complex three-dimensional structure, the apparent aperiodicity may be artifactual. Indeed, the marked regularity observed in restricted regions may be assumed to indicate possible periodicity.)

Although the intermediate filaments vary from 80 to 130 Å in diameter (Histogram...
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I), occasionally have lateral projections, and can be observed at all stages from pre-
primitive streak to stages of late myogenesis, the intermediate filaments consistently
demonstrate smooth margins and high electron opacity in tissues block-stained with
aqueous uranyl acetate. None were observed, suggesting the active conversion of
intermediate filaments into thick filaments, either by possessing diffuse margins, by
close association with thin filaments and interconnected by lateral projections, or
by possessing regions corresponding to both intermediate and thick filaments. How-
ever, intermediate filaments were often observed in association with microtubules,
with intermediate filaments of various diameters, and with free thin filaments. In-
stead of a sequential series suggesting thick filament growth by stages as supported
by Heuson-Stiennnon (52), it seems equally plausible (or implausible) to suggest thin
filament formation by sequential breakdown of microtubules into intermediate fila-
ments (113), the intermediate filaments in turn into thin filaments.

The identification of the free cytoplasmic filaments as a distinct class of 85–130 Å
intermediate filaments necessitated a search for the precursors of the sarcomeric thin
filaments. The peripheral matrix associated with initial myogenesis revealed abundant
filaments of the approximate diameter of thin filaments (Fig. 3b and 10). A layer of
loosely organized free filaments equivalent to diameters of sarcomeric thin filaments
was observed in the peripheral matrix of many cells, even as early as the 8-somite
stage (Fig. 10). In this micrograph of low contrast, many lightly stained filaments of
approximately 60–65 Å diameter (Histogram I) are observed coursing roughly parallel
to the obliquely sectioned plasmalemma. A few larger filaments of 75–80 Å are ob-
served deeper in the cytoplasm and may represent an additional class of filaments.

It is considered unlikely that the isolated 80 Å filaments represent two almost ex-
actly superimposed thinner 60 Å filaments. (The perpendicular orientation and distinct
separation of the bilayers of the vesicle membranes demonstrates that the phase
contrast effects limit point to point resolution to approximately 30–40 Å, and linear
resolution to 15–20 Å.)

The above data indicate clearly the occurrence and general distribution of at least
three major classes of filaments during early cardiac myogenesis. Their possible inter-
actions with the remaining myofibrillar structures (Z bands, intercalated disks, and
desmosomes) must also be considered briefly.

At all stages, the deposition of the Z band material apparently is associated with
the sarcolemma and with the close proximity of intermediate and/or thin filaments.
In tissues fixed with phosphate-buffered glutaraldehyde, the early Z bodies appear
as small clumps of very dense, amorphous material, with numerous attached or closely
associated intermediate and thin filaments (Figs. 2 and 11). The closely associated
intermediate filaments have diameters of 80 and 95 Å, with more distant filaments
of 115 and 130 Å. In tissues fixed with cacodylate or s-collidine-buffered glutaral-
dehydrate (Fig. 12), however, the Z material contains filamentous material in the amorphous matrix, again with attached intermediate and thin filaments. The significance of the difference in Z band appearance after fixation with phosphate, cacodylate, and collidine buffers is unknown, but may represent differential effects of the buffers on different components of the Z material [cf. Rash et al. (104), Douglas et al. (19), and Stromer et al. (119)]. It should be emphasized, however, that despite differences in Z band appearance after different fixation procedures, the attachment of several diameters of filaments to developing and mature Z bands (cf. 29) indicates either a lack of specificity at the attachment site or a similarity of composition of the various associated filaments.

**Cell junctions and associated filaments**

The formation and elaboration of the various types of cell contact characteristic of cardiac myocytes is initiated early in cardiogenesis and has been discussed by several investigators (15, 29). Nevertheless, we have attempted a brief reinterpretation based on the apparent contribution of the newly described intermediate filaments. Recognizable differentiation of intercellular attachment mechanisms [cf. Hay (49)] is detected in cells of the ventricular rudiments of the 6- to 8-somite chick heart (Fig. 13). Initially, closely apposed membranes are observed which may represent the first stages in the development of desmosomes and/or intercalated disks. Microtubules are closely associated and a few linear densities (arrows), possibly representing 60 Å filaments, are observed nearby, not necessarily attached to the minute amounts of dense (predesmosomal?) matrix material. Dense dots of approximately 60 Å are observed in or near the dense matrix and may represent cross sections of additional filaments. Cells with more extensive contacts (Fig. 14) demonstrate numerous filaments of various diameters (45–60, 80, 95, 115, and 130 Å) in continuity or in close association with increasing amounts of desmosome-like material. As is demonstrated

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Fig. 11. Peripheral areas associated with Z band deposition and thin filament deposition. Amorphous appearance of Z material attributed to fixation in phosphate-buffered glutaraldehyde. Note several diameters of filaments from 65–130 Å, associated with early Z band. × 45 000.

Fig. 12. Early Z material with associated thin and intermediate filaments. Filamentous appearance of Z material attributed to fixation in cacodylate-buffered glutaraldehyde. × 38 000.

Fig. 13. Early cell contacts (predesmosome?) of 8-somite chick ventricle. Possible early association of thin or intermediate filament (arrows). × 75 000.

Fig. 14. Early forming desmosomes (9 somites) associated with various diameters (45–130 Å) of thin and intermediate filaments (arrows). Deposition of the desmosomal matrix is at least temporally associated with the attachment of filaments. × 50 000.

Fig. 15. Definitive desmosomes of 8-somite chick ventricle with attached thin and intermediate filaments. × 80 000.

Fig. 16. Early sarcomere with thin filaments inserting into desmosome-like structure. Intermediate filaments attached to densities on opposed membranes, with additional 60 Å thin filaments interconnecting the adjacent desmosome-like structures. × 47 000.
in the micrographs, filaments of less than 100 Å are seen in contact with the desmosomes, usually during what is interpreted as formative stages. Later stages are characterized by the attachment of larger intermediate filaments (Fig. 15).

In support of assumptions by many investigators that the early formed desmosomes of cardiac myocytes are converted into intercalated disks (32, 40, 92, 115), we have demonstrated filaments of several diameters attached to desmosome-like structures and have encountered images which appear to be desmosomes (with attached intermediate filaments) on one side of the apposed membranes (Fig. 16), but with sarcomeric thin filaments and intermediate filaments attached to the densities on the opposite membrane surface. Further, smaller filaments of 60 Å are observed interconnecting the two desmosomes (or intercalated disks). Similar figures demonstrating intermediate size filaments attached to intercalated disks and desmosomes are rather common in the literature [cf. Figs. 46 and 47 of Fawcett and McNutt (29)], and should be reinterpreted in light of the present evidence of the general occurrence of intermediate filaments during cardiac and skeletal myogenesis. Circumstantial evidence suggesting homology of the dense material of the Z bands, intercalated disks, and desmosomes is supported by the rather common observation of continuity of the Z bands and desmosomes, Z bands and intercalated disks, and desmosomes and intercalated disks (40, 115). In the accompanying paper and in recent reports (32, 104, 115), additional biochemical and physical data are discussed which indicate similarity in composition and possible homology of the Z bands, intercalated disks, and desmosomes.

**INTERPRETATIONS**

The early events in muscle development have received considerable attention for over 50 years. Investigators have suggested virtually every cellular organelle or constituent as the precursor of myofibrils, the elongate mitochondria being the organelle of choice for most light microscopists (23, 81, 91), although additional peripheral "granules" were implication in early sarcomere differentiation (36, 42, 89). In 1957, improved polarization and fluorescence light microscopy combined with electron microscopy allowed Holtzer and collaborators (62) to confirm earlier observations of the formation of myofibrils in close association with the cell surface. Later studies validated these observations, particularly with respect to the peripheral distribution of the first-formed myofibrils (15, 75, 114, 125). It has been suggested, however, that sarcomeres formed later in development may not be dependent on a sarcolemma-associated deposition but may assemble at the ends of the developing myofibrils (13, 52). Nevertheless, it appears that in striated (skeletal) muscle, at least, differentiating muscle fibers are characterized by cortical distribution of myofibrils and central location of nuclei, resulting in the myotube stage. Continued development is charac-
characterized by the lateral displacement of nuclei, resulting in definitive adult muscle fibers with nodular or peripheral nuclei (88).

Early investigators of cardiac muscle, noting that organization and activity at the light microscope level differed greatly from that of voluntary muscle (36, 51, 88), assumed that the differences would be even more apparent on an ultrastructural level. Electron microscopy, however, has revealed a great similarity of the myofibrils in the two types of muscle (28, 30, 58, 82, 92) and demonstrated major differences only in cellular organization and the supportive organelles. Both possess similar cross-striated myofibrils, but because the Z bands of adjacent sarcomeres are not always aligned in cardiac muscle, the striated appearance occurs in limited areas or under high magnification. In addition, early assumptions of syncytial development of the heart (51) have proved to be invalid (17), yet the syncytial nature of voluntary muscle is unchallenged (76). As a counterexample of cellular association and tissue development, therefore, the heart should provide invaluable data for distinguishing basic details of myogenesis from general cytoplasmic development. Cardiac muscle, unlike striated muscle, contracts rhythmically without nervous excitation (myogenic instead of neurogenic contraction) and apparently starts to contract as soon as the first myofibrils appear (17, 84), facilitating the identification of the first stages of muscle fiber formation. However, few studies of myogenesis have capitalized on this easily staged and readily identified tissue for the analysis of sarcomere formation, perhaps because this earliest formed tissue is extremely susceptible to osmotic effects (17), although Manasek (84) has presented evidence for good fixation in hypertonic (0.2 M) cacodylate-buffered glutaraldehyde. Nevertheless, as is demonstrated in this and the accompanying report, variations in osmotic concentration of the primary fixatives is relatively ineffective in altering filament diameters.

An extensive literature on myofibrillogenesis has accrued since 1951, with most of the investigations relying solely on buffered OsO₄ as the fixative and primary stain for electron microscopy. Although cytoplasmic preservation is generally acceptable, the sometimes erratic fixation and the leaching effects of osmic acid have been reported (38, 78, 123, 124). The introduction of glutaraldehyde fixation (111) has prompted a reevaluation of virtually all prior studies. Comparison of the two methods reveals that significant differences occur. Microtubules, for example, measuring 150 Å in diameter after osmium fixation, are 240 Å after glutaraldehyde. As a result of the consistent use of osmium fixation, nearly all the investigators of muscle development reported the general occurrence of 50–80 Å thin filaments, 100–120 Å thick filaments, and 150 Å microtubules in the areas of myogenesis. The possibility of recognizing a third class of filaments, intermediate between the thick and the thin filaments, was therefore minimized. The discrepancies between the two fixation methods, however, cannot be attributed solely to the deleterious effects of osmium. Using essentially
the same techniques of osmium fixation, Heuson-Stiennon (52) observed additional classes of filaments in developing myocytes and suggested a mechanism of thick filament formation based on continued filament growth (possibly by the addition of lateral projections equivalent to the cross-bridge of the adult myofilament). She also suggested that Z band material was derived from the sarcolemma by a process resembling "intracellular blebbing." Although she was unable to account for the origin of the thin filaments, her detailed observations were sufficiently at variance with the literature (2, 31, 47, 100) to necessitate their earnest consideration in any new model of myogenesis.

Biochemistry of sarcomere subunits

The molecular weight of "native" adult myosin is classically reported to be 480 000–500 000 or even 600 000 according to the preparative procedure (22, 83), and the thick filament is said to be composed of approximately 380 of these native myosin subunits (68). However, it has been suggested (22, 50) that the thick filament subunit ("native myosin") may exist as a basic dimer or trimer (mol. wt. 200 000), each portion being associated with three additional minor components. Recently embryonic myosin has been synthesized in vitro in several laboratories by means of a monocistronic polysome (37) of 55–65 ribosomes isolated from embryonic chick and fish (3, 53–57). Because the length of mRNA between successive ribosomes in the polysome is on the order of 300–340 Å (equivalent to 90–100 mRNA bases) and is sufficient to code for a length of polypeptide chain with a weight of 3 000 to 3 500 daltons (18, 117, 129), it can be assumed that the myosin molecule produced by a polysome of 55–65 ribosomes would have a molecular weight of 180 000–210 000, roughly corresponding to the values reported by Woods et al. (133) and Dreizen et al. (22). As predicted, the myosin molecules isolated from the synthesis experiments apparently possessed a molecular weight of 215 000, spontaneously dimerized into the "native myosin" molecule (56), and precipitated as thick filaments with lateral projections, suggesting that the rapid deposition of the embryonic myosin thick filament in vivo resembles the in vitro reconstitution of thick filaments from concentrated salt solution extracts of whole muscle (65, 108). Partly formed filaments have not been rigorously identified in serially sectioned embryonic material (31), although the observed "frayed" thick filaments (Fig. 7) may represent the final stages of thick filament coalescence. The very small filaments nearby may represent additional unincorporated myosin subunits. In the accompanying report (106), we have demonstrated a similar redeposition of thick filaments in situ upon dilution of the 0.6 M KI extraction medium, and thus we support the Huxley hypothesis of thick filament growth. In addition and in contrast to expectations from the Heuson-Stiennon (52) model, heavy meromyosin (HMM) labeling of thin, but not of intermediate filaments (73) in a wide variety of
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cells presumably demonstrates the ability of actin thin filaments to bind HMM but an inability of intermediate filaments to bind or incorporate a portion of the myosin molecule equivalent to the cross bridges. In summary then, none of the present biochemical and physical evidence supports the Heuson-Stiennon (52) model of thick filament growth, either by addition of preformed myosin polypeptide subunits or of complete cross-bridges to preformed "structural myosin" intermediate-sized filaments. However, at least one other mechanism of thick filament growth is logistically plausible. Following assembly at the polysomes, myosin molecules may not coalesce immediately into the thick filament, but instead, may attach to free cytoplasmic thin filaments (Fig. 6) similar to HMM labeling of thin filaments (73). Attachment of the "actomyosin" filaments to early Z material would provide the critical orientation necessary to assure intertwining of the freely projecting LMM tails, resulting in the in situ deposition of thick filaments correctly oriented and interdigitating with the pre-sarcomere thin filaments.

The biochemical identification of actin as the primary component of the thin filaments (45, 65, 66, 72) is generally accepted, but the origin and assembly of the thin filaments in vivo are not clearly established. A recent report by Kelly (75) has demonstrated the presence of very thin (30-60 Å) filaments in the cortical cytoplasm of developing striated muscle and has proposed their direct incorporation into the sarcomere as the Z material or possibly as the actin thin filaments. The nearby intermediate filaments were relegated to a cytoskeletal function because they were observed at later stages and in positions resembling inter-Z band bracing material. We have demonstrated that (similar?) 60 Å filaments occur in cardiac myoblasts and myocytes, predominantly in the cortical cytoplasm, either free or associated with Z-like material and partially aligned thick filaments. In skeletal myoblasts and myocytes (and a variety of other cells), Ishikawa et al. (73) have been able to label similar filaments with heavy meromyosin, presumably demonstrating identity with sarcomeric actin filaments. Although limited evidence is presented in the accompanying report (106) which suggests that the peripheral thin filaments share similar biochemical properties with sarcomeric actin filaments, no conclusive evidence is available which demonstrates either an actin composition for these filaments or their direct incorporation into the sarcomere as the thin filaments. Therefore, alternative concepts of sarcomeric thin filament formation must still be considered.

Evidence of the origin and biochemical nature of the intermediate filaments and Z bands and of the sites of tropomyosin deposition is considered less definitive than for the formation and composition of thick and thin filaments. Although other as yet unidentified proteins may occur in cross-striated muscle, we are tempted (perhaps unwisely) to correlate those structures of undetermined composition with those molecules of undetermined location, namely tropomyosin with Z bands and/or inter-
mediate filaments. Perhaps coincidentally, purified tropomyosin spontaneously forms long filaments of variable diameter \textit{in vitro} (1, 7, 10, 127). Moreover, tropomyosin has been implicated in the thin filaments by procedures that remove complexed actotropomyosin, by X-ray diffraction data, and by fluorescent antitropomyosin labeling of the thin filaments (44, 45, 65, 111, 112). Indeed, recent biochemical data suggest that tropomyosin reacts with and binds strongly to actin (16, 20, 45, 77, 86, 87, 98) and may be present in the grooves of the adult thin filament (45). Despite evidence that much more tropomyosin is present in the myofibril than can be attributed to the nonactin component of the thin filaments (45), the localization of tropomyosin in regions other than the thin filaments has not been adequately demonstrated. However, as indicated previously (104), much circumstantial evidence (58, 60, 65) suggests that tropomyosin is in the Z bands, possibly combined with lipids. The presence of unidentified protein(s) and lipid(s) in the Z bands has been adequately demonstrated by digestion with protease, trypsin, and detrypsinized lipase (21, 41, 119, 128). Later biochemical investigations supporting tropomyosin as a Z band protein included observations of a marked susceptibility of tropomyosin and of Z bands to trypsin digestion (6, 41, 119) and a significant release of tropomyosin after disruption of Z bands with acetone (10, 20, 45, 77, 86, 128), lipase (31, 41), borate buffer (16), and Tris buffer (120). Original attempts to localize tropomyosin with fluorescent antitropomyosin indicated that tropomyosin was not in the Z bands (97, 112), but later fluorescent labeling experiments (27, 46) following prolonged storage of the myofibril in glycerol (possibly yielding lipid removal) reportedly demonstrate the presence of tropomyosin in the Z bands. Although it may be suggested that the tropomyosin observed may have migrated to the Z bands during glycerol storage, it seems unlikely that the tropomyosin should bind preferentially to the glycerol altered Z bands but not to Z bands during normal development. We favor the suggestion that Z band lipids may normally mask the tropomyosin during fluorescent labeling and that lipids and mucopolysaccharides [cf. Douglas \textit{et al.} (19)] may contribute to the amorphous appearance observed after phosphate buffered glutaraldehyde fixation as contrasted with the filamentous appearance following cacodylate and collidine-buffered fixation [cf. Kelly (75)]. The identities of the proposed Z band lipids and mucopolysaccharides are not yet established. Regardless of the true composition we suggest that Z-like material may constitute an intracellular cement, passively or actively interconnecting the lipoprotein plasmalemma with various sizes of cytoplasmic filaments. Tropomyosin is known to react with and bind both lipids (10, 33, 41) and actin (20, 45, 77, 86, 99) and may provide a portion of the hypothesized intracellular cementing substance of the Z bands, intercalated disks, and desmosomes.

The role of the Z bands in myogenesis and muscle contraction must be of great significance, for muscles of rapid, long distance contraction are characterized by the
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possession of numerous short subunits (sarcomeres) linked by relatively strong binding material (Z bands and intercalated disks). Most recent reports of cross-striated muscle formation [with the notable exception of Mañasek (84)] favor a cortical deposition of myofibrils and a sarcolemmal association of the newly formed Z material. It should be noted, however, that Allen and Pepe (2) consider the Z bands to be the last skeletal sarcomere subunit to appear, forming only after recognizable myofibrils are observed. In contrast, we suggest that cardiac myofibrils are usually (if not exclusively) formed near the sarcolemma, that recognizable Z-like material can be observed before and during all phases of myofibril deposition, and that the Z material is derived from (or deposited initially in close association with) the sarcolemma (15, 47, 52). Further, the subsarcolemmal association of Z-like material with thick and thin filaments in various stages of alignment resembles an ordered series in the development of sarcomeres. [Because several investigators have attempted to analyze the stages of myogenesis in older tissues (2) or have stated that early myofibrils can be observed deep in the cytoplasm of some cells (84) it seems necessary to state that partially formed myofibrils may provide no clue to the understanding of Z band deposition or of initial myofilament alignment, particularly if Z formation and initial myofilament interaction occur at an earlier period or in a different cellular location.] Before the deposition of Z material can be clarified, it appears necessary to examine the interactions of the presarcomere subunits (and intermediate filaments) present just before and during myofibril deposition.

In the peripheral matrix associated with myofibril deposition, numerous filaments of approximately 60 Å diameter are observed, often independent of Z-like densities (Fig. 10), a state to be contrasted to recent evidence presented by Kelly (75). Additional Z-like densities are observed with attached filaments of various diameters (Figs. 11 and 12). The attached thin filaments are presumably to be incorporated into the sarcomeres as the actin thin filaments, but what is the fate of the attached and closely associated smaller intermediate filaments?

The intermediate filaments are consistently observed in areas of initial Z appearance (defined here as areas in which Z material is observed without associated thick or thin filaments), are measured to possess smaller diameters when closely associated with apparently forming Z bands (Histogram II), and apparently decrease in number during myogenesis (compare Figs. 2 and 3 with Figs. 4 and 5). A role in myogenesis is assumed, possibly of Z deposition or Z stabilization. Further, deposition of the dense material of the desmosomes and intercalated disks is seen as resembling the proposed deposition of Z material, for the original cell attachment sites [zonula occludens or macula adhaerens diminuta, (49)] initially lack dense material and associated filaments. Subsequent development of the desmosomes is associated with attachment of thinner intermediate filaments and the deposition of the characteristic
desmosomal matrix, possibly reflecting a transformation or removal of material from the intermediate filaments. Maturity is reflected by the appearance of cardiac desmosomes which possess intermediate filaments of predominantly larger (115 and 130 Å) diameters. An evolutionary and developmental model of muscle formation is derived from this basic premise of Z-material deposition plus the observations that relatively large amounts of muscle-like actin (G and F forms) and myosin have been isolated from myxomycetes (46) and a variety of plant and animal tissues (35, 46, 50, 78, 90, 93, 109, 130).

Evolutionary and developmental considerations

Muscle and muscle-like systems occur in virtually every phylum of the animal kingdom and therefore presumably evolved from the basic contractility and/or motility systems found in primitive unicellular organisms [cf. for example, the filamentous contractile systems in Spirostomum (80) and Stentor (12)]. The development of several different types of cross-striated and smooth muscle systems in various phyla indicates divergent evolution at a very early period, but with each phylum utilizing basic cell constituents in the evolution of each new muscle type. The de novo development of similar complete muscle systems in each case (i.e., multiple parallel evolution) seems unlikely. In the vertebrates at least, evolution of the cross-striated myofibril apparently utilized materials associated with cellular attachment mechanisms as sites for myofibril insertion. Concomitant incorporation and elaboration of the desmosomes as myofibril attachment sites is seen to have provided discrete points for the application and transmission of force. The first primitive Z bands may have developed as the loosely draped peripheral protomyofibrils were pulled away from the cell surface [see ref. 118 and see Fig. 3 in the accompanying report (106)], while the desmosomes remaining at the ends of the myofibril are seen to have been elaborated as the intercalated disks (Fig. 16). The attendant formation of myofibrils with discrete sarcomeres would, thus, have created the highly contractile cross-striated muscle systems associated with rapid, long distance contraction.

Other mechanisms which may explain the appearance and possible disappearance of the intermediate filaments include the possibility that the numerous intermediate filaments and microtubules noted shortly after fusion of the cardiac rudiments represent remnants of the cellular machinery of protoplasmic streaming, mesenchymal movement, and intracellular mixing (34, 39, 80, 90, 95, 122, 131, 132). This hypothesis is supported by a great increase in intermediate filaments during or just after the period of maximum cell movement and extension. The increase in intermediate filaments may reflect either a transition phase in the breakdown or transformation of the previously abundant microtubules (24, 113) or of a structural (passive) and/or contractile (active) element associated with cytoplasmic streaming. Although it
appears that much more material is present in the masses of intermediate fila-
ments than could be derived from the number of microtubules observed earlier, simi-
lar statements have been made concerning the intermediate size filaments ob-
served after microtubule disruption with bicarbonate and acrolein (113). If microtubules consist of actin (40, 109, 130), the intermediate filaments may then rep-
resent a transitory phase between microtubular and thin filament states. It
should be noted, however, that Echandia and Piezza (24) have demonstrated
thermal disruption and reorganization of neural microtubules apparently with-
out affecting the number or distribution of intermediate size (but not necessarily equivalent) filaments. A passive (cytostructural) or active (contractile) role in intra-
cytoplasmic transport was indicated. In possible support of the proposed passive (structural) function for the intermediate filaments is Kelly’s (75) suggestion that intermediate filaments have no active role in myofibril formation, but remain as inter-Z band bracing material.

With all the contradictory evidence, we should bear in mind the suggestion of Hay (48) concerning possible biochemical heterogeneity of the intermediate filaments. Assumptions of homogeneity based solely on apparent physical similarity (2, 31, 100) may be unwise, particularly in view of the wide range of intermediate filament diam-
eters and the uncertainty introduced by various fixation and embedding techniques (78, 123). Our evidence of several intermediate filament diameters, possible differences in reactivity, and differences in extractability in various media suggest such hetero-
geneity.

The above considerations lead us to conclude that bimodal distributions of myo-
filament diameters (2, 31, 100) should be superseded by polymodal distributions (71, 72, 74, 75). The evidence on striated muscle development in recent reports and the present supplementary evidence from cardiac muscle development indicate that earlier interpretations of myogenesis can no longer be accepted as accurate. Future analyses of myogenesis must consider the origin and role of at least four contributors to the myofibril: thick filaments, thin filaments, Z bands, and the newly described inter-
mediate filaments.

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