AN ELECTRON MICROSCOPE STUDY OF MYOFIBRIL FORMATION IN EMBRYONIC CHICK SKELETAL MUSCLE

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ABSTRACT

The formation of myofibrils in the developing leg muscle of the 12-day chick embryo was studied by electron microscopy. Myofilaments of two varieties, thick (160–170 A in diameter) and thin (60–70 A in diameter), which have been designated myosin and actin filaments, respectively, on the basis of their similarity to natural and synthetic myosin and actin filaments, appear in the cytoplasm of developing muscle cells. There is a greater than 7:1 ratio of thin to thick filaments in these young myofibers. The free myofilaments become aligned in the long axis of the cells, predominantly in subsarcolemmal locations, and aggregate into hexagonally packed arrays of filaments. The presence of Z band material or M band cross-bridges do not appear to be essential for the formation or spacing of these aggregates of filaments. Formation of the Z band lattices occurs coincidentally with the back-to-back apposition of thin filaments. An hypothesis concerning myofibril growth, based on the self-assembly characteristics of the filaments, is presented.

INTRODUCTION

Myofibril formation in developing skeletal muscle offers a unique opportunity to morphologically detect and trace the organization of a chemically and structurally defined intracellular organelle. The now classical description of the myofibril as an interdigitating, hexagonal array of thick and thin filaments (Huxley and Hanson, 1954) and the subsequent localization of the proteins, actin in the thin filaments, myosin in the thick filaments, and with less certainty, tropomyosin in the Z band (Huxley and Hanson, 1957; Hanson and Lowy, 1963; Huxley, 1963), has provided a basis on which the electron microscopist can identify and follow, on morphological grounds alone, the aggregation and organization of these contractile proteins into the adult sarcomere structure. The appearance of characteristic thin and thick filaments within the cytoplasm of the differentiating myoblast would be direct evidence for synthesis of actin and myosin and their subsequent aggregation into their macromolecular species. Both thick and thin filaments have now been formed in vitro (Huxley, 1963; Hanson and Lowy, 1963) from purified myosin and G-actin, respectively. Furthermore, such filamentous material closely resembles the myofilaments seen in electron micrographs of fixed, thin-sectioned adult muscle, as well as the filaments isolated from disrupted myofibers. By analogy with the aggregation of tropocollagen (Highberger et al., 1951; Gross, 1956) and assembly of viral proteins (Caspar and Klug, 1962), such information strongly supports the view that the tertiary structures of actin and myosin molecules contain much, if not all, the necessary "information" required for aggregation of the protein units into the characteristic myofilaments. Thus, it is

reasonable to assume that, at the ionic strength and ATP[1] concentration within the muscle cell, myosin and actin monomers, once synthesized, aggregate spontaneously into thick and thin filaments, respectively.

In previous electron microscope studies of myogenesis (van Breeman, 1952; Wainrach and Sotelo, 1961; Allbrook, 1962; Bergman, 1962; Hay, 1963; Cedergren and Harary, 1964; Price et al., 1964; Allen and Pepe, 1965; Dessouky and Hibbs, 1965; Heuson-Stiennon, 1965; Przybylski and Blumberg, 1966) the cytology of the developing muscle cell has been extensively covered. Prior to the appearance of cross-striated myofibrils, two types of filaments can be detected in the cytoplasm of these cells, and soon afterward, poorly organized aggregates of the myofilaments can be seen which are presumably immature or early myofibrils. Although many of the authors cited above have presented micrographs of immature myofibrils, there has been relatively little discussion or detailed analysis of various hypothetical models which may be involved in myofibrillar assembly. This is not suprising, since only recently has attention been drawn to the self-assembly characteristics of myofilaments (Hanson and Lowy, 1963; Huxley, 1963).

In the mature myofibril, three transverse structures are known to exist, any one of which might be involved in the positioning of myofilaments in the hexagonal array. These structures are: the Z disc or Z band, the M band cross-bridges between thick filaments in the middle of the sarcomere, and the cross-bridges running between thick and thin filaments in the region of A-I overlap. The suggestion that Z bands may act as an assembly site for myofilaments has been made by Wainrach and Sotelo (1961), Hay (1963), and Heuson-Stiennon (1965), to name just three studies. Allen and Pepe (1965) hypothesized that thick-to-thin filament cross-bridges underly both filament aggregation and their hexagonal spacing. To the author's knowledge, no one has presented evidence that M band cross-bridges play a role in myofibrillar assembly. It is to this problem, the morphological features of myofibril assembly, that the present investigation has been directed.

MATERIALS AND METHODS

Skeletal muscle was obtained from the anterior thigh region of chick embryos ranging in age from 8 to 18 days of incubation. In all experiments, an attempt was made to isolate the sartorius muscle, but at early stages (less than 12 days) the dissection is difficult and isolation of the sartorius muscle cannot be claimed with certainty. The most extensively examined muscle was that from 12-day embryos since, at this stage, the tissue contains cells ranging in development from relatively undifferentiated myoblasts to multinucleated myofibers containing cross-striated myofibrils. Thus, a random section through 12-day embryonic leg muscle presents an almost complete spectrum of myogenesis. Furthermore, at the 12th day of development, the activity of myosin ATPase (Robinson, 1952) and total content of actomyosin (Herrmann, 1952) rapidly increase.

Embryos were removed from the shell, and the legs cut from the body and skinned in Tyrode's solution at room temperature. With cotton thread the legs were tied so that the knees were fully flexed and hips fully extended, the aim being to stretch the anterior thigh musculature. Whole legs were immersed in 2.5% glutaraldehyde buffered to pH 7.4 with 0.1 M phosphate, and fixed for 1 hr at room temperature. Small pieces of muscle were dissected from the leg in glutaraldehyde, fixed for another hour and washed for 30 min. in 0.1 M PO_4 buffer; they were postfixed in 1% OsO_4, 0.1 M PO_4, pH 7.4, followed by rapid alcohol dehydration and immersion in propylene oxide for 15 min, and then embedded in Araldite. Some embryos were fixed by perfusion of glutaraldehyde into the descending aorta, but the results were similar to those obtained by immersion of the whole leg in fixative. Tissues were aligned carefully for both longitudinal and cross-sections; such orientation was checked by light microscope examination of sections cut at 0.5 μ and stained with toluidine blue prior to cutting thin sections from the same block for electron microscopy. Sections showing silver to light-gold interference colors were cut with glass or diamond knives, collected on celloidin-covered 200-mesh, athene grids, and stained with uranyl acetate and lead citrate. But myofilaments unattached to myofibrils were poorly stained by this procedure. The best results were obtained by staining sections for 3 min with 2% phosphotungstic acid dissolved in 95% alcohol followed by the usual uranyl-lead method. Sections were examined with Siemens Elmiskop I and Philips EM 200 electron microscopes.

RESULTS

A. *General Morphology of Embryonic Chick Skeletal Muscle*

In sections of 12-day embryonic leg muscle, embedded in Araldite and stained with toluidine blue (Figs. 1 and 2), the tissue is seen to be composed of

[1] Adenosine-5′-triphosphate.

darkly staining, elongated, mononucleated cells closely apposed to less intensely staining multinucleated myofibers. In this paper, the term "myoblast" will be used to connote the monunucleated cell destined to form a muscle cell, and multinucleate elements will be termed myofibers. The presence or absence of myofilaments is not included in this designation of cell type since myofibrillar proteins have been detected within mononucleated cells in chick somites (Holtzer et al., 1957). These two cell types are ensheathed in a delicate connective tissue network containing fibroblasts oriented circumferentially with respect to the myofibers.

Developing muscle cells aggregate into clusters usually of 3–10 cells, some of which are clearly identified as developing myofibers by the presence of cross striated myofibrils. Many stages of differentiation are apparent in each aggregate. Cytoplasmic basophilia, indicative of ribonucleoprotein (RNP), varies widely. In general, within a given cluster, those cells with greatest cytoplasmic basophilia are situated peripherally, while those cells with least basophilia, most myofibrils, and largest cytoplasmic volume are centrally placed. Myofibrils ranging in diameter from 1 μ to dimensions beyond the resolution of the light microscope appear as granules in cross section (Fig. 1). With the light microscope and toluidine blue staining, it is difficult to differentiate with assurance cross sectioned myofibrils from mitochondria. A peripheral distribution of myofibrils within the myofibers is suggested but required electron microscope confirmation (see also Holtzer et al., 1957).

Mitotic figures are common among the mononucleated cells (*Mit*, in Fig. 2), but none has been observed in cells containing more than one nucleus.

Cross-sections of this tissue viewed at low magnification with the electron microscope best demonstrate its over-all histological characteristics (Fig. 3). Myoblasts (*Mb*) and myofibers (*M*) are aggregated into multicellular clusters which are separated from each other by the long cytoplasmic extensions of fibroblasts (*F*) and small bundles of collagen fibrils (*C*); no collagen fibrils are seen between cells within the muscle cell clusters. Since the comparative morphology of the fibroblast, myoblast, and myofiber has been amply described by previous workers (Hay, 1963; Price et al., 1964), only the major distinctions between the three cell types will be presented here. The fibroblast generally is separated from the other two cell types by a wide intercellular space; it is a flattened cell possessing long cytoplasmic processes containing lamellae of "rough-surfaced" endoplasmic reticulum. Myoblasts, on the other hand, are spindle-shaped cells with a cytoplasm literally packed with free RNP granules, and are almost devoid of endoplasmic reticulum and cytoplasmic filaments. Myofibers are multinucleated cells containing varying amounts of free RNP granules, vesicles of "smooth-surfaced" endoplasmic reticulum, and myofibrils of varying length, diameter, and number. Free filaments within the cytoplasm of the myofiber are a prominent cytological feature of this cell type.

The close apposition of developing muscle cells within each aggregate, often with cytoplasmic interdigitation, membrane blebbing, and discontinuities of the plasma membranes (Fig. 4), suggests that all cells within a cluster are destined to fuse, forming one large, multinucleated myofiber. Collagen separating adjacent cell aggregates presumably becomes the future endomysial sheath. The present observation regarding cellular fusion of myoblasts is in agreement with the large body of evidence supporting the concept that multinucleation of skeletal muscle fibers arises by the cytoplasmic fusion of mononucleated myoblasts (Lash et al., 1957; Stockdale and Holtzer, 1961; Capers, 1960; Konigsberg, 1965). The amorphous layer of mucopolysaccharide, termed basement membrane or "glycocalyx" (Bennett, 1963), that surrounds mature muscle fibers, can, in embryonic tissue, be seen only on the peripheral surfaces of these cells facing the connective tissue sheath. Thus, the glycoprotein coat outside the mature sarcolemma may be, in embryonic tissues, absent or present in low concentration on cell surfaces between myofibers or myoblasts destined to fuse.

B. *Myofibril Formation*

Upon examination of myofibers in cross-section (Figs. 3 and 4), one of the most prominent features to be noted is the peripheral distribution of newly formed myofibrils (*Mf*). Although the vast majority of these myofibrils are located beneath the sarcolemma, this is definitely not an absolute rule; a few small myofibrils, presumably in early stages of formation, can be seen deep within many cells. It would appear that, although most of the myofilaments aggregate into myofibrils beneath the sarcolemma, this same process can occur, albeit at a lower frequency, within the core of the myofiber.

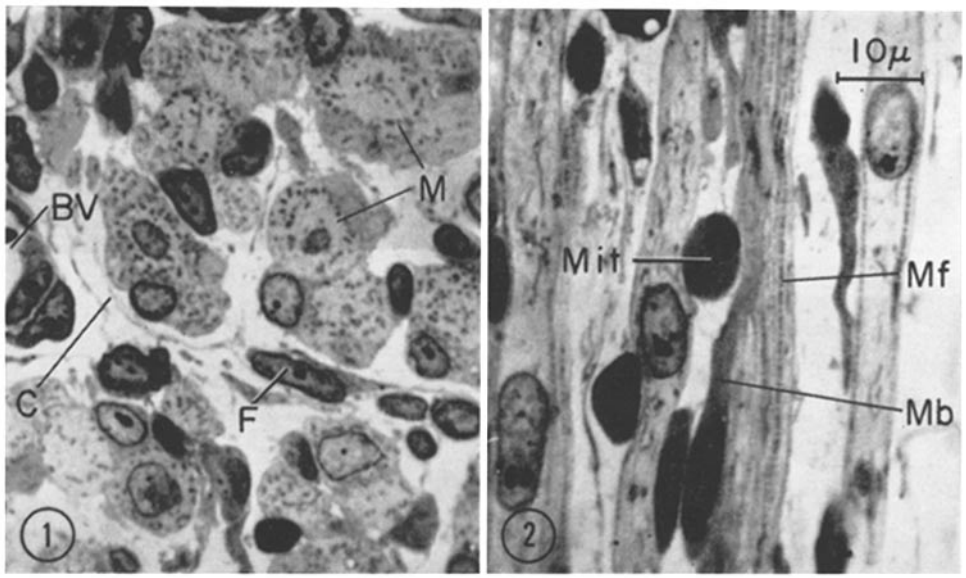
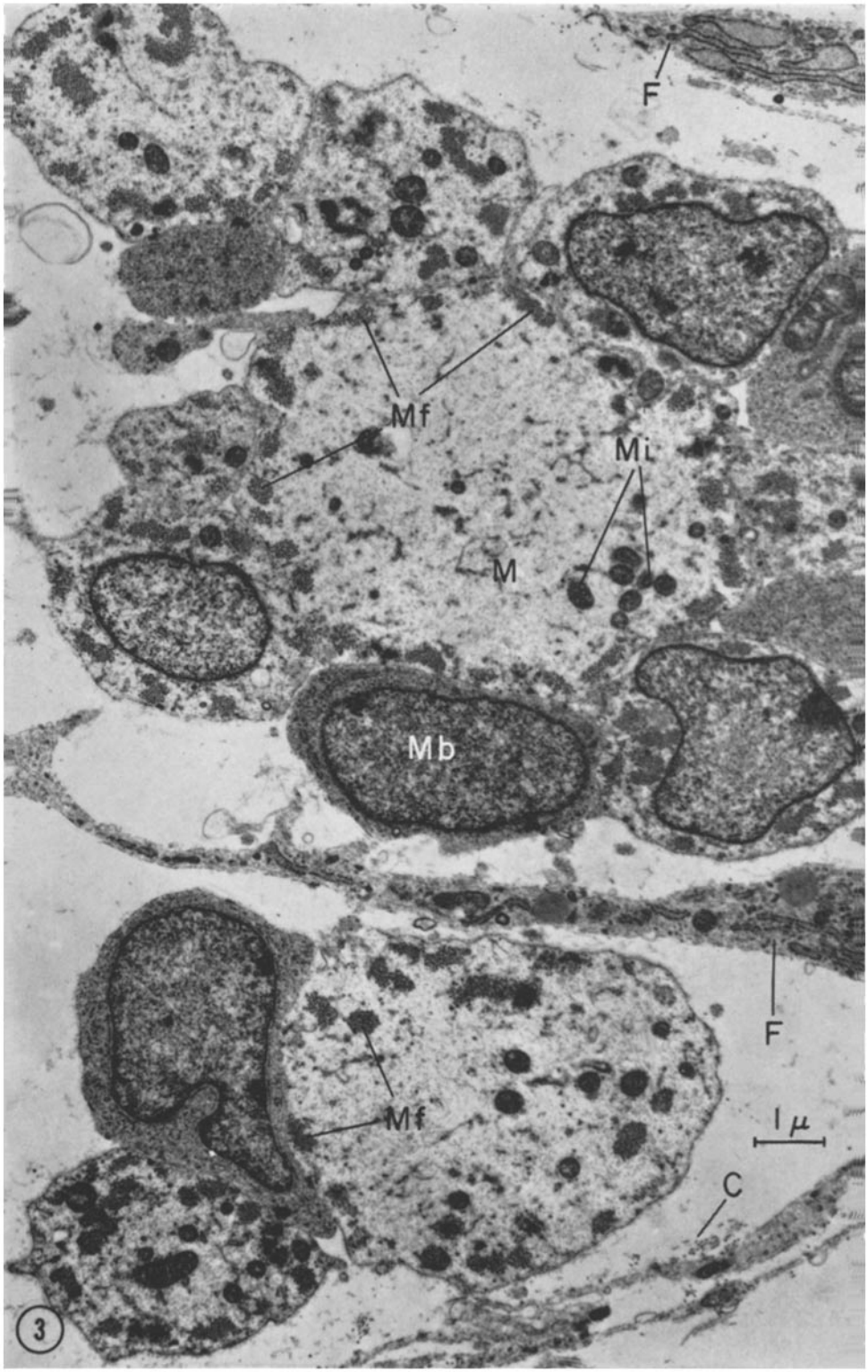


FIGURE 1 Cross-section of the leg muscle of a 12-day embryonic chick. ½-μ-thick section, stained with toluidine blue. Developing myofibers (*M*) and myoblasts aggregate into cell clusters enveloped in a thin areolar, connective tissue (*C*) sheath. Fibroblast (*F*) nuclei are oriented circumferentially with respect to the long axis of the myofiber clusters. A blood vessel (*BV*) is seen at the left of the field. Few capillaries are present in muscle before the 12th day of incubation, but they greatly increase in number as differentiation advances. Same magnification as Fig. 2. × 1,100.

FIGURE 2 Longitudinal section of the same tissue as in Fig. 1. Cross-striated myofibrils (*Mf*) are visible within some myofibers and generally occupy a peripheral location within the developing muscle cells. Myoblasts (*Mb*) are oriented parallel to the myofibers to which they are closely opposed. Mitotic figures (*Mit*) are common within the mononucleated cells but have not been seen in multinucleated cells. The 10-μ calibration mark indicates magnification for both Figs. 1 and 2. × 1,100.

FIGURE 3 Electron micrograph, at low magnification, of a cross-section of leg muscle from a 12-day embryo. Glutaraldehyde-OsO_4 fixation; Araldite embedding; PTA, UrAc, Pb citrate staining. Developing muscle forms by an aggregation of myoblasts (*Mb*) and myofibers (*M*) into roughly spherical, multicellular clusters. These clusters are separated from each other by a thin connective tissue sheath composed of collagen (*C*) and fibroblasts (*F*). The cytoplasmic processes of the fibroblasts are oriented circumferentially with respect to aggregates of muscle cells. There is no clear cut orientation of collagen fibrils at this developmental stage, although most fibrils parallel the myofibers. Within the myofibers, myofibrils (*Mf*) can be discerned, usually at the periphery of the cells beneath the sarcolemma. Even the smallest fibrils show an hexagonal, lattice structure. Mitochondria (*Mi*), quite distinct from myofibrils, appear circular in cross-sections of muscle, but sausage-shaped in longitudinal sections. Within each multi-cellular aggregate, many stages of differentiation are apparent. One adult myofiber is thought to form in each aggregate by the cytoplasmic fusion of all the component cells. It is suggested that the connective tissue visible in this micrograph will become endomysium. × 10,000.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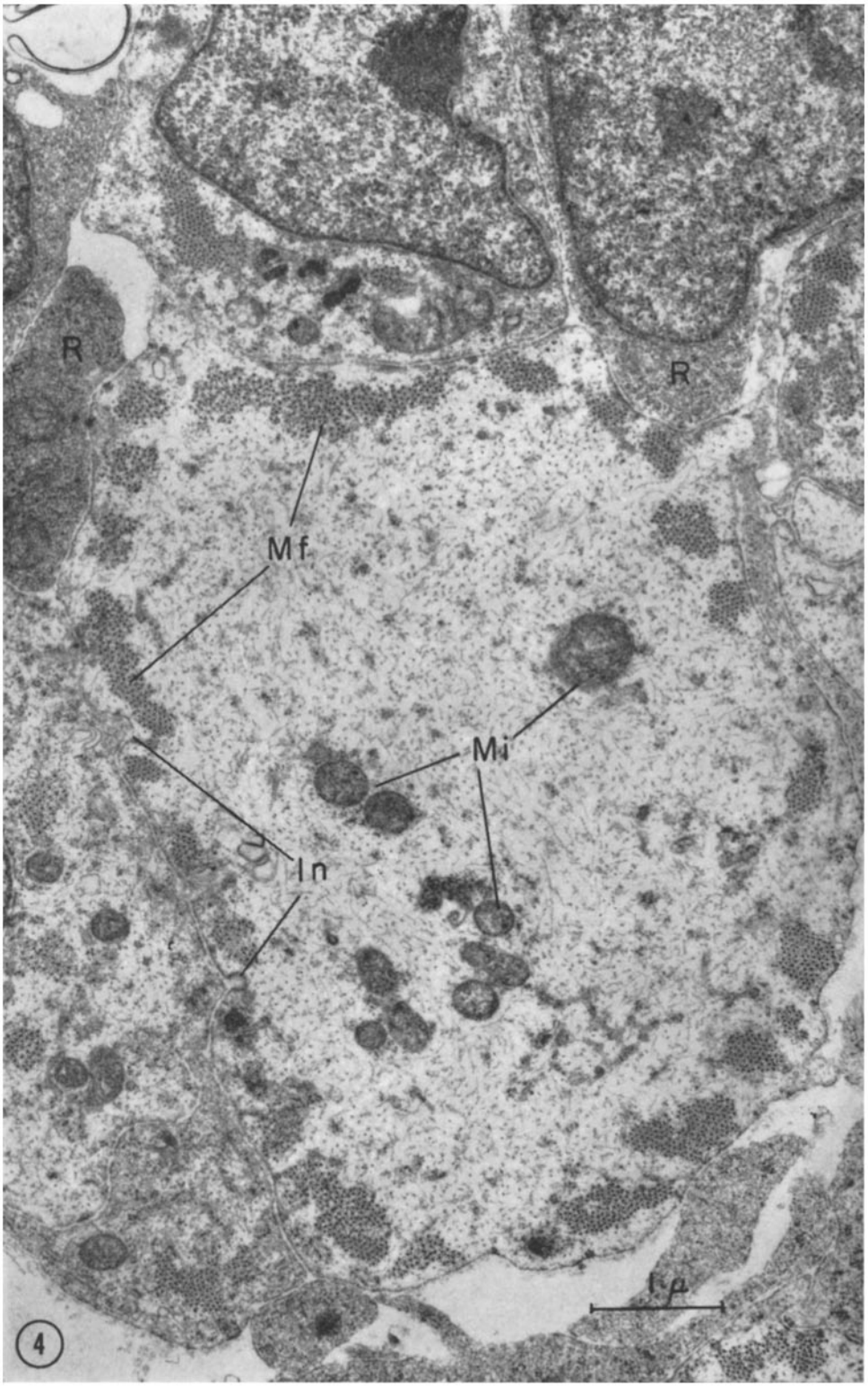

FIGURE 4 Cross-section through developing myofibers; materials and methods the same as for Fig. 3. Invaginations of the plasma membrane (*In*) are frequently encountered at the surface of myofibers. Mitochondria (*Mi*) and myofibrils (*Mf*) are seen. Adjacent myoblasts packed with ribonucleoprotein granules (*R*) also are visible. Numerous free myofilaments can be seen in the cytoplasm of the large myofiber. Most of these myofilaments are actin and are oriented longitudinally. This alignment of filaments is most obvious beneath the sarcolemma and least near the core of the cell. × 19,500.

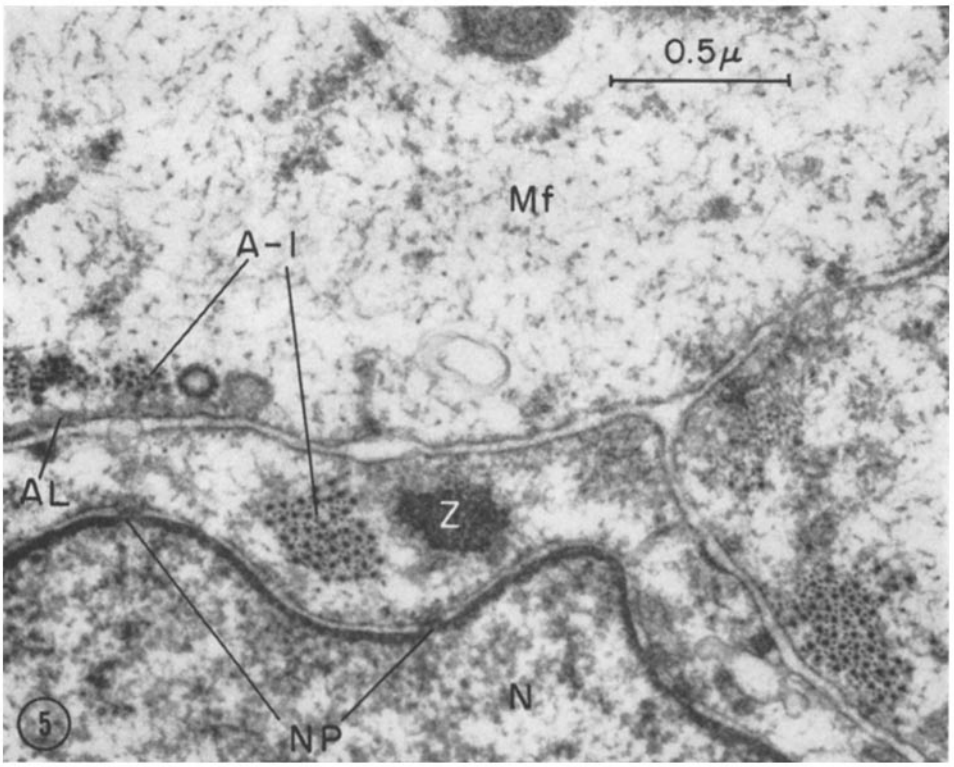

FIGURE 5 Higher magnification of a cross-section through regions of three adjacent myofibers. Fixation and staining as for Figs. 3 and 4. Myofibrils have been cut through various sarcomere regions; sections through thin and thick filament overlap in the A band (*A–I*) and the Z band (*Z*) are seen. All myofibrils contain myofilaments packed in the hexagonal array. An amorphous layer (*AL*) between the assembling myofibrils and the sarcolemma can be seen in the upper cell at the left of the micrograph. Free myofilaments (*Mf*) are seen in the myoplasm. A nucleus (*N*) with prominent nuclear pores (*NP*) is visible in the lower cell. × 47,000.

Newly formed myofibrils are usually spherical in cross-section, but wide variation is seen.

When viewed at higher magnification (Figs. 5 and 6), myofibrils are consistently seen to be composed of hexagonally packed thick (160–170 A in diameter) and thin filaments (60–70 A in diameter). This hexagonal packing of myofilaments is seen even in the smallest myofibrils examined. For example, in Fig. 5 a myofibril containing only nine thick filaments is present, yet myofilaments fit an hexagonal pattern. The regularity of the packing is least perfect at the outermost layer of filaments in each myofibril. It is concluded that when myofilaments aggregate, they do so in an hexagonal pattern; the adult lattice structure forms coincidentally with the myofibrils, and rearrangement of the myofilaments subsequent to filament aggregation would appear to be unnecessary and unlikely. This finding suggests that the properties of myofilaments which lead to their aggregation may also be responsible for their spatial arrangement.

Membranous or filamentous connections between myofibrils and the sarcolemma have not been detected, although an amorphous layer (*AL*, Fig. 5) interposed between the plasma membrane and myofibrils is sometimes visible. Whether or not such a layer provides an attachment site for myofibril assembly remains a matter for speculation. No evidence has been obtained to support the hypothesis that Z band material is derived from a pinching-off of invaginations of plasma membrane as suggested by Heuson-Stiennon (1965).

Dispersed throughout the cytoplasm of the developing myofibers are numerous filaments, some

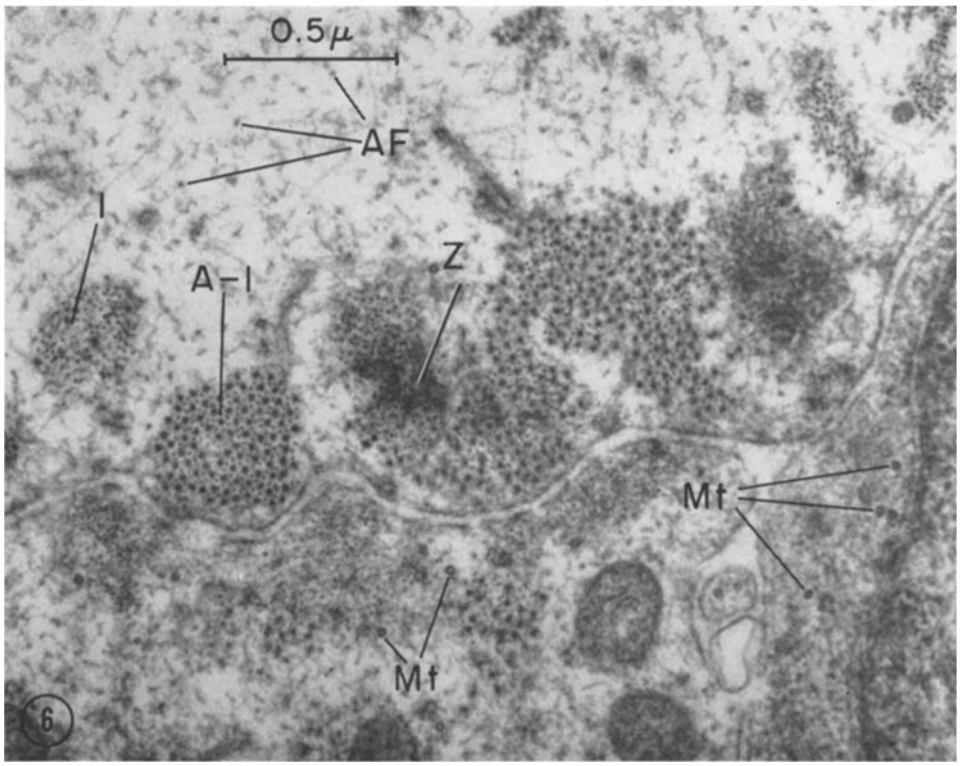

FIGURE 6 Transverse sections through the I band (*I*), A band (*A–I*) and Z band (*Z*) are shown. Same material, orientation and methods used as in Fig. 5. Free actin filaments (*AF*) in the myoplasm of the upper cell can be recognized by their diameter. Most free myofilaments in Figs. 5 and 6 have been cut transversely, but short stretches of longitudinally sectioned myofilaments also can be seen. Many microtubules (*Mt*) are visible in myoblasts and growing myofibers. No correlation has been noted between the positions of the microtubules and of the thick filaments, except that both structures are oriented longitudinally, particularly beneath the plasma membrane. × 47,000.

microtubules and many RNP granules. The filaments fall into two distinct classes: thin, measuring 60–70 A, and thick, measuring 160–170 A in diameter (Figs. 7 and 8). These free[2] thick filaments (*MF*) have a marked similarity to synthetic myosin filaments and thick filaments within adult myofibrils (Huxley, 1963), in that all of these filaments possess lateral projections along the shaft, have tapered ends, and show similar dimensions. Neither the tapered ends or lateral projections are seen with the free thin filaments (*AF*) or microtubules (*Mt*, in Fig. 8). This similarity in appearance of the free thick filaments to the A band filaments within the sarcomere strongly supports their identification as myosin filaments. The ends of unaligned thick filaments often appear frayed and somewhat granular, but no clear pattern of thick filament attachment to ribosomes or polyribosomes is apparent (Cedergren and Harary, 1964). Individual thick filaments are almost straight or gently curved in longitudinal section (Figs. 7–10), but thin filaments are often sinuous, forming twisted, interwoven patterns within the cell. These appearances support the conclusion that thick filaments are more rigid or, conversely, less flexible than the thin filaments. Both of these conclusions are compatible with the suspected substructure of myosin and F-actin filaments (Hanson and Lowy, 1963; Huxley, 1963).

Unaligned thin filaments have the same diameter as I band myofilaments, 60–70 A in this chick

[2] A free myofilament is the term given to that myofilament not yet attached to another myofilament or any visible intracellular structure.

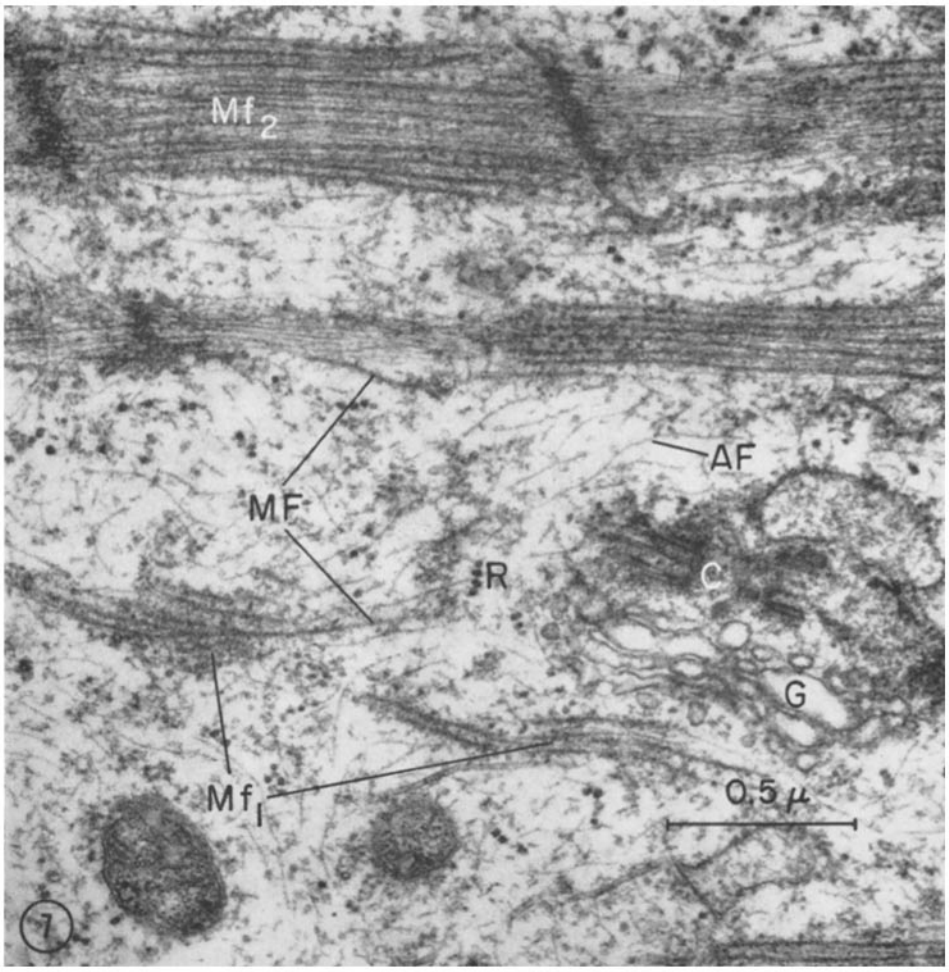

FIGURE 7 Longitudinal section of the same material prepared as for previous micrographs. Stages of myofibril formation, ranging from free myofilaments to complete myofibrils, are seen. Actin filaments (*AF*) and myosin filaments (*MF*) are clearly distinguishable. Ribosomes (*R*), Golgi apparatus (*G*), and centrioles (*C*) also are visible. There does not appear to be any direct involvement of the Golgi apparatus or centrioles in the synthesis or aggregation of myofilaments. Two stages of myofibril formation labeled Mf_1 and Mf_2 are seen. The first stage (Mf_1) contains thick and thin myofilaments in roughly parallel, longitudinal aggregation but without visible Z bands. A later developmental stage containing Z, I, and A bands is seen above (Mf_2). It should be noted that adjacent sarcomeres have contracted or been stretched independently of one another, probably during fixation. × 50,000.

muscle. Since I band filaments are largely composed of actin (Hanson and Lowy, 1963), it is suggested that the free thin filaments be termed actin filaments. In contrast to the thick filaments, the thin variety appear smooth surfaced and no inner structure can be resolved; neither globular subunits nor helical patterns are visible.

Gradations in the diameter of myofilaments have not been observed in this study; as mentioned above, two distinct size classes without statistical overlap have been noted. These results support the findings of Przybylski and Blumberg (1966), but should be compared with the somewhat different conclusions of Heuson-Stiennon (1965).

Without the use of serial sections and myofilament reconstruction, it is impossible to measure

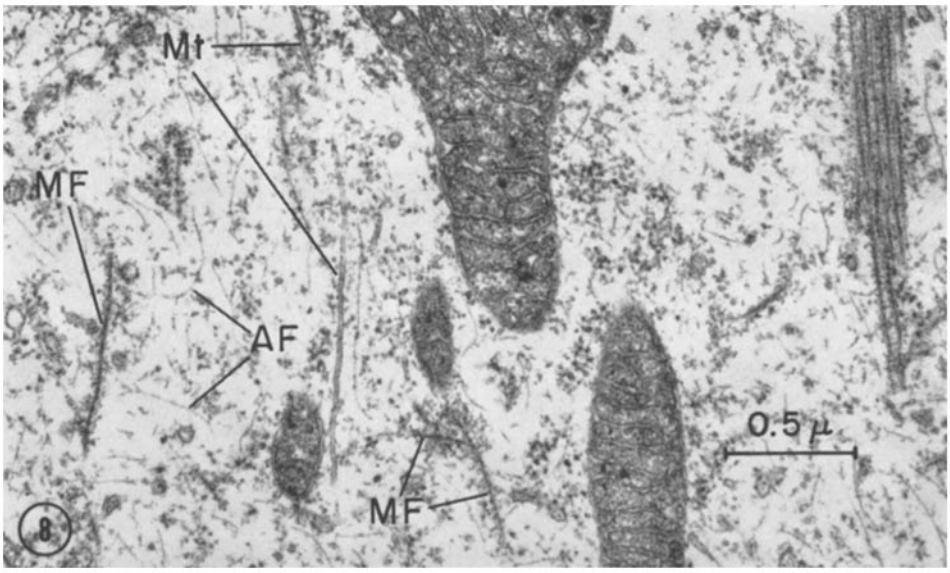

FIGURE 8 Longitudinal section. Materials and methods as in Fig. 3. Myosin filaments (*MF*), actin filaments (*AF*), and microtubules are all clearly distinguishable. The identity of free myofilaments can be verified by comparison with aligned myofilaments in the myofibril at the right of the field. From an examination of longitudinal sections such as those in Figs. 7 and 8, it is estimated that thin filaments outnumber thick filaments by approximately 9:1. × 34,000.

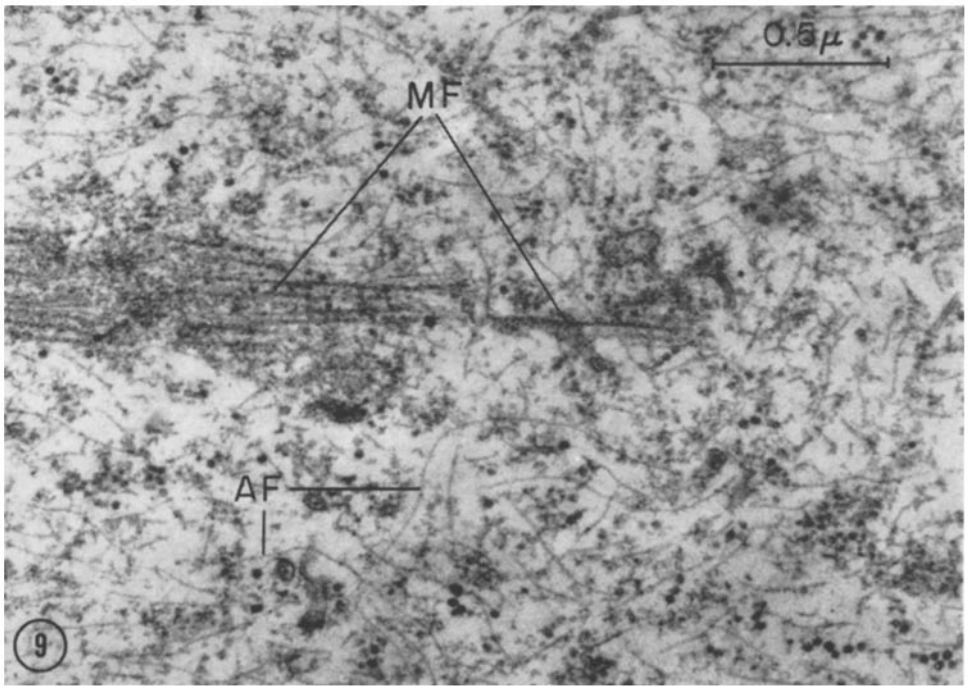

FIGURE 9 Longitudinal section. Materials and methods as in Fig. 3. Thick filaments (*MF*) can be seen assembling into a myofibril. Numerous thin filaments (*AF*) meander through the cytoplasm; some measure up to 1 μ in length. Note the irregular granularity of the Z band. × 46,000.

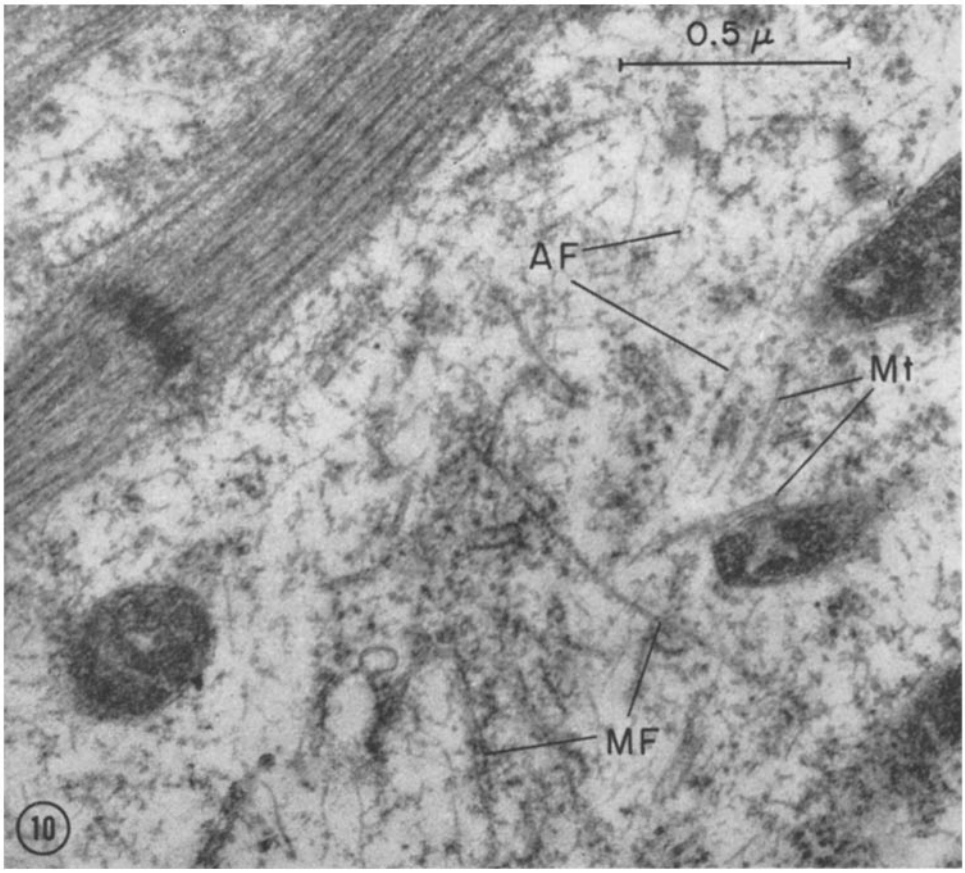

FIGURE 10 Longitudinal section. Materials and methods as in Fig. 3. Free actin (*AF*) and myosin (*MF*) filaments are seen; a cluster of thick filaments is present intermixed with a number of cytoplasmic granules. It is suggested that this may represent a focus of thick filament growth following myosin synthesis. Microtubules (*Mt*) are also visible. × 60,000.

accurately the total length of individual, free myofilaments. However, the extremes of filament length within a given section can be measured, and it has been found, after an examination of 400 filaments of each type, that free thick filaments measure up to, but do not exceed, 1.6 μ, whereas the longest free thin filaments measure 1.1 μ in length. As seen in Fig. 11, the dimensions of a fully developed myofibril from chick muscle are identical with those measured for the skeletal muscles of rabbit (Huxley, 1963) and frog (Page and Huxley, 1963). Thus, thick and thin filaments apparently can grow to full lengths while still free in the cytoplasm. Further lengthwise growth of the filaments while included in a sarcomere is considered unlikely since the earliest myofibrils possess sarcomeres of adult length. Evidence has been presented, from two laboratories, suggesting that in insect flight muscle the sarcomere length increases during the growth of the myofibrils (Shafiq, 1963; Auber, 1965 *b*). In contrast to the elongation of myofibrils in dipteran muscle, sarcomere length remains constant in growing chick fibrils; presumably different growth processes occur in the two species.

Upon examination of longitudinal sections of developing myofibers (Figs. 7–10), it is apparent that the number of thin filaments greatly exceeds that of thick filaments. Although precise figures are difficult to derive in the absence of serial reconstruction, it is estimated that there is at least a 7- to 10-fold excess of thin over thick filaments. This

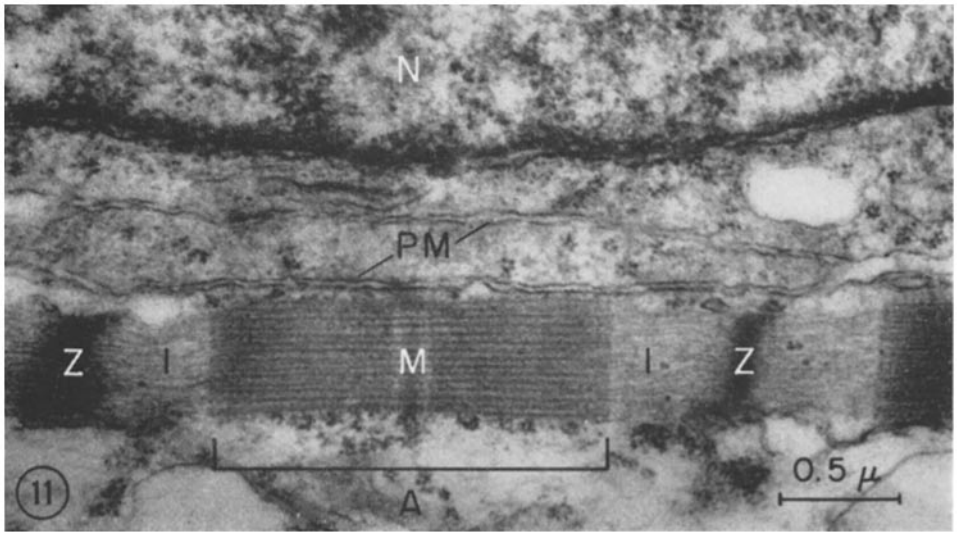

FIGURE 11 A fully formed myofibril with characteristic adult morphology is shown. Materials and methods as in Fig. 3. Sarcomere bands (A, I, Z, and M) are well seen. Total sarcomere length measures 2.90 microns and the A band 1.68 microns. The plasma membranes (*PM*) separating adjacent cells are labeled, as well as the neighboring nucleus (*N*). × 32,000.

imbalance of filament number has been seen in all young myofibers examined for this study.

Since there has been some disagreement in the literature as to whether or not actin is synthesized before myosin during muscle differentiation (Ogawa 1962; Hay, 1963; and Allen and Pepe, 1965), it was of interest to examine developing myofibers and myoblasts in the 12-day limb musculature to see whether any temporal sequence in the appearance of the respective myofilaments could be detected. In more than 200 cells examined during this study, no cell has been found to contain one filament variety but not the other. Clearly, in this material there does not appear to be any temporal sequence for the formation of thin and thick filaments. Assuming that actin and myosin molecules aggregate into their respective myofilaments immediately after the proteins are synthesized, one must conclude that the syntheses of actin and myosin begin simultaneously or within a very short time interval of one another.

Upon comparing longitudinal and cross-sections (Figs. 4–6 with Figs. 7 and 8) of developing myofibers, it is apparent that myofilaments (both thick and thin), before they are packed in hexagonal array, are oriented predominantly in the longitudinal axis of the cell. Such an orientation of the filaments is particularly evident near the periphery of the cell, where myofibrils are usually detected first. Of the two types of filaments, the thin variety is more randomly oriented, the filaments often appearing as wavy or curved lines in longitudinal section. The mean length of thin filaments in Fig. 9 (a longitudinal section) is 0.30 μ, with a range of 0.08 to 1.1 μ (73 measurements), while in a cross-section (Fig. 4) the mean thin filament length was found to be 0.15 μ with a range of 0.04 to 0.80 μ (82 measurements). Thus, distinctly shorter lengths of thin filaments pass in the cross-sectional plane of the myofiber than in the longitudinal plane; in other words, thin filaments are preferentially oriented in the longitudinal axis of the myofiber.

An interesting finding is the fact that in cross-sections longer portions of thin filaments can be measured in the central regions of the myofiber than at the periphery. If the cross-sectional area of the myofiber (Fig. 4) is divided into a series of concentric circles and if filament lengths are measured in each ring, it is usually found that longer fragments of filament are present in the center of the fiber than near the plasma membrane. From examination of both longitudinal and cross-sections, it is clear that actual lengths of the filaments do not differ significantly between the periphery and the center, but rather, the orientation of a thin filament with respect to the longitudinal axis of

the cell is more perfect the nearer the filament is to the plasma membrane. Whatever force aligns the filaments, it appears to be most effective beneath the sarcolemma.

When cells are cut transversely, thick myofilaments rarely are seen in longitudinal section, and it appears that thick filaments are aligned more strictly in the longitudinal axis than the thin ones.

Previous workers (Wainrach and Sotelo, 1961; Hay, 1963) have postulated that Z bands first appear as amorphous, densely staining bodies to which myofilaments become secondarily attached. Although careful search was made, no examples of Z bands without attached thin filaments have been found in the present study. Furthermore, the author has not seen any examples of Z band densities spaced at regular intervals within the cytoplasm prior to myofibril appearance (Heuson-Stiennon, 1965), nor have Z band densities been found a sarcomere distance ahead of a longitudinally growing myofibril. Thus, there is no support for the suggestion that sarcomere spacing is regulated by the prior spatial deposition of Z band material in the myoplasm onto which thin filaments are then inserted.

As stated above, evidence obtained from cross-sections, such as Figs. 5 and 6, strongly suggests that the earliest aggregates of thick and thin filaments are arranged in hexagonal packing. Cross-sections, however, provide no information as to whether or not thin filaments in early myofibrils are attached to a Z band lattice. Fig. 7, a longitudinal section, shows what appear to be early stages in the aggregation of thick and thin filaments without visible Z band material. Serial sections are required to establish this point firmly, but present evidence supports the hypothesis that free thick and thin filaments can aggregate without the prior synthesis or formation of a Z band lattice; in fact, Z band densities in early myofibrils (Fig. 7) are rather diffuse, irregular, and granular in longitudinal section. The characteristic crystalline pattern of the adult Z band (Knappeis and Carlsen, 1962; Reedy, 1964) is not seen in early stages of fibril formation.

The cross-bridges interconnecting thick filaments in the M band of the sarcomere (Franzini-Armstrong and Porter, 1964; Page, 1965) are also seen in mature myofibrils of this embryonic muscle (Figs. 11 and 12). However, M bands are not seen consistently, particularly in smaller fibrils; thus, the middle myofibril in Fig. 7 is well stretched, yet no M band is visible. Whatever the function of the M band cross-bridges may be, the present results support the conclusion that the hexagonal packing of myofilaments can occur without such cross-bridges being present. That slow fibers in frog muscle lack M band cross-bridges (Page, 1965) yet possess hexagonal packing of myofilaments lends further support to this conclusion.

Microtubules (*Mt*) measuring 235–250 A in outside diameter are frequently seen in developing myofibers (Figs. 6 and 8) beneath the sarcolemma. These microtubules appear to have no structural relationship to free myofilaments or to developing myofibrils; they are approximately 100 A wider than the thick filaments, and they show no signs of lateral projections or tapered ends. Furthermore, no microtubules have been seen attached to, or contiguous with any myofilaments. The microtubules are always oriented with their long axis parallel to that of the cell; there does not appear to be any strict relationship between the plane of the lattice formed by thick filaments in a myofibril and the position of an adjacent microtubule (Fig. 6). It has been stated in the literature that thick filaments in growing insect muscle may have a developmental relationship to microtubules (Auber and Couteaux, 1963; Auber, 1964), that is, thick filaments may be derived from microtubles or form on them. In a recent publication (Auber, 1965 *a*), Auber has altered his opinion on this subject and no longer considers microtubules to be precursors of the thick filaments. Our results support this latter position; no evidence has been found suggesting any microtubule-myofilament interconversion.

It is clear that in developing chick myofibers the myofibrils precede the formation of the sarcoplasmic reticulum and transverse tubules. Intracellular membranes with attached RNP granules are occasionally seen in myoblasts, but it should be stressed that intracellular membranes, either with attached RNP granules or smooth surfaced, are poorly developed in myofibers containing large numbers of free myofilaments, and that the vast majority of ribosomes are nonmembrane bound. Recently, Cohen and Longley (1966) have suggested that the intracellular divalent cation concentration may be important in the aggregation of tropomyosin molecules during myogenesis, and further, that this divalent cation concentration may be regulated locally by the movement of ions into or out of the sarcoplasmic reticulum. That

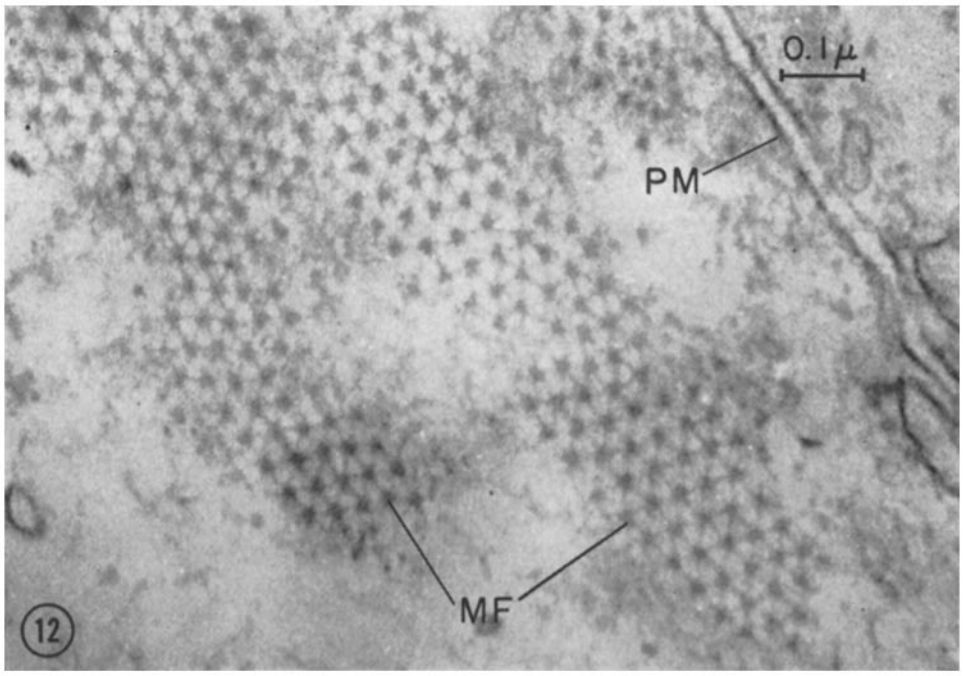

FIGURE 12 Cross-section through a myofibril in the M band region. This section has been stained with uranyl acetate and lead citrate, but prior phosphotungstic acid staining has been omitted. Cross-bridges between thick filaments are visible (see Franzini-Armstrong and Porter, 1964; Page, 1965); the function of these cross-bridges is unknown. × 110,000.

the mode of aggregation of tropomyosin is strongly dependent on the divalent cation concentration is clear from the work of Cohen and Longley (1966), but the absence or sparsity of membranous elements during early stages of myofibril formation suggests that if the intracellular divalent cation concentration changes during myogenesis, it is not likely to be caused by the sarcoplasmic reticulum. As seen in Figs. 5–8, myofibrils have formed in myofibers containing little or no sarcoplasmic reticulum, and it is concluded that neither the aggregation of myofilaments into myofibrils nor the sarcomere spacing of these fibrils is dependent upon the presence of any structured intracellular membrane system.

DISCUSSION

The formation of skeletal muscle can be subdivided conveniently into the following overlapping stages: 1. The transformation of undifferentiated mesenchyme cells into mononucleated myoblasts. 2. The aggregation and cytoplasmic fusion of myoblasts, resulting in the formation of multinucleated, syncytial myofibers. 3. The initiation and continuing regulated synthesis of myofibrillar proteins. 4. The organization of myofibrillar proteins into characteristic, cross-striated myofibrils. 5. The formation of the sarcoplasmic reticulum and transverse tubular system. 6. Innervation and the functional integration of contractile activity.

The present study has focused attention predominantly on stage 4, but the other topics will be discussed briefly before a more detailed analysis of myofibril formation is entered upon.

This investigation has not provided any new information about mesenchyme-myoblast transformations. Hay (1959, 1963) has discussed the pertinent ultrastructural changes in such transformations as seen in regenerating limb and developing tail musculature of amphibians.

That myoblasts can be recognized and distinguished from fibroblasts on the basis of shape and future development has been demonstrated elegantly by Konigsberg (1963) in his in vitro studies of myogenesis. In chick muscle tissue, myoblasts can be clearly differentiated from fibroblasts on the

basis of their position in the tissue, their shape, and intracellular morphology. Clear examples of both cell types have been presented in Fig. 3. Mitotic figures have been noted only in the mononuclear cell populations; no multinucleated myofibers have been seen to divide. No evidence has been found bearing on the problem of whether fibroblasts can be transformed into myoblasts; intracellular morphology and synthetic activity support the conclusion, however, that both cell types are developmentally determined and probably arise from a more undifferentiated cell population. When the dichotomy of cell types arises remains unsettled.

That multinucleation arises by cytoplasmic fusion of myoblasts has been demonstrated conclusively in a number of previous studies (see review by Konigsberg, 1965), and the present electron microscopic results are in complete agreement with this conclusion. Previous electron microscope studies of myogenesis (Hay, 1963; Price et al., 1964; Dessouky and Hibbs, 1965; Heuson-Stiennon, 1965; Przybylski and Blumberg, 1966) also support this view. Presumably, DNA synthesis is already inhibited in those myoblasts about to fuse, since 4N DNA concentrations are not observed in nuclei within multinucleated myofibers, but only within the mononuclear population (Lash et al., 1957; Firket, 1958; Bassleer, 1962, Strehler et al., 1963). Holtzer et al. (1957) have stated that some mononucleated cells in embryonic chick somites stain with fluorescein-labeled antimyosin and presumably contain myosin. In embryonic leg muscle of the chick, used in the present study, myofilaments have been seen in cells which appear mononucleated, but this evidence is inconclusive without serial sections to prove mononucleation. On the basis of Holtzer's evidence, it must be assumed that multinucleation is not an essential prerequisite for myosin or actin synthesis. However, in developing chick leg muscle certainly the vast majority of myofilaments demonstrable with the electron microscope are found in multinucleated cells.

There is no biochemical information pertaining to the "switching-on" or regulation of myosin or actin synthesis. In vitro, embryonic skeletal muscle differentiates in monolayer culture (Lewis and Lewis, 1917, Konigsberg, 1963), and recent evidence would suggest that a collagen substrate strongly promotes such differentiation (Hauschka and Konigsberg, 1966). Myofibrils continue to form in vitro for several days in the presence of sufficient Actinomycin D to inhibit RNA synthesis (Yaffee and Feldman, 1964), and these authors have suggested that long-lived "messenger RNA's" may be involved in the synthesis of myofibrillar proteins. Large polyribosomes containing 60-80 ribosomes have been isolated from the postnatal skeletal muscle of the rat (Breuer et al., 1964) and large aggregates of particles of ribosomal dimensions have been seen in sections of embryonic chick and rat muscle (Heuson-Stiennon, 1964; Allen and Pepe, 1965; Fischman, 1965). Although it has been suggested that such large polyribosomes may mediate myosin synthesis (Breuer et al., 1964; Allen and Pepe, 1965), as yet no supporting evidence exists. Until more is known about genetic subunits of myosin (see Woods et al., 1963), caution must be exercised in ascribing dimensions to messenger RNA involved in the coding for this protein.

Myofilaments first become visible in the electron microscope in elongated cells containing abundant quantities of free RNP granules. In the present study, no clear-cut time interval separating the synthesis of thin from that of thick filaments has been apparent; this observation agrees with that of Hay (1963) but disagrees with the conclusions of Ogawa (1962) and Allen and Pepe (1965). It is the author's conclusion that the large difference in relative thin and thick filament concentrations may explain this apparent conflict. It has been demonstrated in the present study that the number of thin (actin) filaments greatly exceeds that of thick (myosin) filaments. Free myofilaments are not completely visualized in our material after uranyl and lead stains used by previous workers; the addition of phosphotungstic acid staining greatly increases the electron opacity of the myofilaments and thus improves their electron microscopic visualization. It is conceivable that some free thick filaments were not visualized in the preparations of Allen and Pepe (1965), thus explaining why those authors reached conclusions somewhat different from our own. It is suggested, also, that the immunological method used by Ogawa (1962) may not have been sensitive enough to detect small concentrations of myosin in a large excess of actin. Assuming that actin and myosin molecules aggregate into their respective myofilaments immediately after their synthesis, one must conclude that the cell initially synthesizes substantially more actin than myosin. The implications of a large excess of thin over thick filaments

in regard to myofibril growth are discussed below, when possible models for sarcomere formation are considered.

It is clear that myofilaments once synthesized are oriented in the myofiber without visible physical contact between any intracellular organelles and the filaments. The vast majority of RNP granules are nonmembrane bound, and smooth-surfaced membranes of the sarcoplasmic reticulum are not present in sufficient quantity to be an important factor in the synthesis, alignment, or aggregation of myofilaments. These results indicate that the morphogenesis of the sarcoplasmic reticulum and transverse tubular system is secondary to myofibril formation, and definitely not the reverse.

Results have been presented above which indicate that free myofilaments become oriented in the long axis of the myofiber before becoming incorporated into a myofibril. Furthermore, this filament orientation is most pronounced in subsarcolemmal regions of the cell in which myofibrils first develop and microtubules are most numerous. Evidence has recently been presented and discussed (Slautterback, 1963; Tilney and Porter, 1965) indicating that microtubules may be involved in cytoplasmic streaming, intracellular fluid movement, and cellular elongation. Most of this evidence is circumstantial, i.e., microtubules are found in great numbers in cells and regions of cells showing pronounced cytoplasmic streaming and cellular elongation. The experimental studies of Tilney et al. (1966) on the axopodia of *Actinosphaerium nucleofilum*, a protozoan, provide the strongest supporting evidence for this hypothesis. If microtubules play a similar role in developing muscle, then alignment of myofilaments might be expected in the vicinity of microtubules, provided the cytoplasmic streams set up by the microtubules had produced a sufficient velocity gradient to orient these highly asymmetric filaments. If this were so, then one would predict more active cytoplasmic streaming beneath the plasma membrane than in the core of the myofiber. The author is not aware of any published data concerning the presence or rate of cytoplasmic flow in developing muscle cells; this problem should be open to experimental analysis by means of phase or interference microscopy with in vitro systems of myogenesis (Konigsberg, 1963).

Once filaments are oriented in the long axis of the myofiber, what are the ensuing stages in myofibril formation? Of the many hypothetical models of myofibril formation that might be suggested, three are worthy of full discussion. (1) The first model necessitates the formation of a Z band lattice (Knappeis and Carlson, 1962; Reedy, 1964), presumably composed, in large part, of tropomyosin, which provides an attachment site upon which thin filaments can be spatially positioned. Thick filaments are spaced secondarily by cross-bridge attachments to the already positioned thin filaments. In this model, Z band lattice formation is a necessary prerequisite for myofibril initiation. (2) The second model would require thick filaments to be packed in an hexagonal array spaced and held in position by the cross-bridges between thick filaments in the M band (Franzini-Armstrong and Porter, 1964; Page, 1965). Thin filaments either attached or unattached to Z band material would then be aligned with already organized thick filament lattices. (3) The third model postulates that thick and thin filaments are packed in an hexagonal pattern solely as a consequence of the cross-bridges linking the two sets of filaments. A Z band lattice would form at the free ends of thin filaments which already contain Z band material at their free ends. The second and third models would not require a prior synthesis of Z band material for hexagonal placement of the myofilaments. All of the models would require Z band material for longitudinal myofibrillar growth, for it is assumed that this material is always interposed between thin filaments in back-to-back sarcomeres. For purposes of clarity, these models have been presented as mutually exclusive possibilities; this is not a necessity since filament packing and Z band formation could occur as simultaneous events.

Of these three models, the present investigation lends greatest support to the third. Z band material has not been detected unattached to thin filaments, nor have isolated I band segments (Huxley, 1963) without attached thick filaments been observed in any myofiber. Examples have been seen of aggregates of thin and thick filaments which show no signs of M band cross-bridges or attached Z band material. Knappeis and Carlsen (1962) and Reedy (1964) have demonstrated that thin filaments are branched at their Z band insertion. It remains to be established whether or not such branching is an integral part of a newly synthesized thin filament, i.e. a free thin filament which has not yet inserted into a Z band.

Embryonic muscle of the sort used in this study

containing many free thin filaments should provide a useful system for analyzing this question in detail. If it is demonstrated that free thin filaments branch at one end into four subfilaments, then an obvious conclusion is that these branches represent the attachment fibrils at the Z band first noted by Knappeis and Carlsen (1962). If these fibrils are composed of tropomyosin (Huxley, 1963), this would indicate that tropomyosin is an integral component of the thin filament prior to Z band formation. The back-to-back apposition of such branched thin filaments would automatically result in the square lattice of the Z band, eliminating the necessity for synthesis of a Z band lattice separate from the myofilament arrays. The suggestion that some tropomyosin may be located in thin filaments is not new (see Huxley, 1963; Hanson and Lowy, 1963), and there is increasing biochemical evidence that tropomyosin interacts strongly with F-actin (Martonosi, 1962; Drabikowski and Gergely, 1964). Allen and Pepe (1965) also have come to the conclusion that the aggregation and spacing of myofilaments results from the cross-bridge attachments between the two sets of filaments. This step when combined with the hypothetical placement of branched thin filaments set back-to-back supplies all the stages needed for the assembly of myofibrils. Allen and Pepe (1965) have suggested that the large excess of thin myofilaments in developing muscle may saturate the available attachment sites on each thick filament. Such a system also would be ideal for the development of many separate fibrils, rather than an increasing growth of a few large myofibrils. The factors regulating the eventual diameter or length of the myofibrils remain to be clarified.

The author would like to express his gratitude to Dame Honor B. Fell, F.R.S., for her encouragement and advice during early stages of this work and particularly for her careful reading of the manuscript. For training in electron microscopy and facilities provided, the author thanks Drs. Audrey Glauert, Leonard Ross, and Roy C. Swan. The technical assistance of Miss Barbara Zimmer and Mr. Robert Michalak is gratefully acknowledged. This investigation was generously supported by postdoctoral fellowship 1 F 2 NB 19, 842-01 and research grant GM 12518-01.

Received for publication 20 June 1966.

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