Zeitschrift für Zellforschung 59, 6—34 (1963)

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THE FINE STRUCTURE OF DIFFERENTIATING MUSCLE IN THE SALAMANDER TAIL *

By

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With 14 Figures in the Text

(Received September 17, 1962)

Introduction

In reading the vast and controversial literature on the structure of differentiating skeletal muscle, one is tempted to conclude that this was the area of developmental cytology for which the light microscope was the least adequate. Because of the limits of resolution of the instrument, investigators had to rely on special staining techniques and their own imagination to identify cell membranes, cytoplasmic filaments and other minute structures which could barely be detected in their histological preparations. The first major area of controversy was formulated by SCHWANN in 1839 at the time he introduced his theory of the cellular nature of animal tissues. His opinion (see SCHWANN 1847) that multinucleated muscle fibers arise by the coalescence of individual cells ("multicellular theory") was soon contested by REMAK (1845) and others, who believed that each syncytial fiber arose from one cell ("unicellular theory"). In spite of the fact that mitoses are rarely seen in muscle fibers, the unicellular theory was well received. This acceptance was due in part to the great interest in amitosis at the turn of the century, for it seemed reasonable at that time to conclude that nuclei divided directly in developing muscle, without the necessity of chromosome formation (MORPURGO 1898; BARDEEN 1900; GODLEWSKI 1902; MEVES 1909). Subsequently, the problem was studied in tissue culture with the result that some investigators obtained evidence to support the unicellular origin of muscle fibers, others produced evidence in favor of the multicellular theory of SCHWANN, and still others concluded that amitosis and coalescence of cells both occur in developing muscle (see GODMAN 1958; BOYD 1960; MURRAY 1960 for reviews of the literature).

Controversies involving the origin of myofibrils have been equally intensive and equally unresolvable. MACCALLUM's simple idea in 1898 that the fibrils developed "directly by a process of differentiation from the primitive protoplasmic network of the embryonic cell" did not become as popular as GODLEWSKI's (1902) more complicated precursor granule theory. As various cell organelles were discovered, various theories arose attributing to each organelle the primary role in myofibril formation. Thus, mitochondria (MEVES 1909; DUESBERG 1910; NAVILLE 1922) and every other cell component, including nuclear material (EYCLESHYMER 1904; MCGILL 1910) and centrioles (WOLBACH 1927) were believed by one investigator or another to transform directly into myofibrils. None of these opinions

* Supported by grant C-5196 from the United States Public Health Service.

could be really proved or conclusively refuted with the techniques of study employed, and the number of published cytological studies of muscle differentiation began to decline by the middle of the present century.

Recent advances in electron and light microscopic techniques have now reawakened the interest of cytologists in these unsolved problems of muscle differentiation. Autoradiographic studies of DNA synthesis (KONIGSBERG et al. 1960; BINTLIFF and WALKER 1960; STOCKDALE and HOLTZER 1961) in developing muscle have given new support to SCHWANN's multicellular theory of the origin of the syncytial muscle fiber. Cell membranes, cell organelles and myofibrils can be viewed in the electron microscope with the fine resolution that HEIDENHAIN (1899) wished for in his studies of developing muscle. HEIDENHAIN's myofibrils have been shown to consist of the smaller "Molecularfibrillen" he predicted and electron microscopists are now investigating the origin of these minute myofilaments. The increased resolution of the elctron microscope has also added greatly to our understanding of the organelles involved in protein synthesis and a specific role in protein formation has been attributed to the ribonucleoprotein particles in the cytoplasm of cells (SIEKEVITZ and PALADE 1960). Thus, it seems probable that by means of new cytological approaches, answers can now be obtained not only to the question of the multicellular origin of muscle and the mode of formation of myofibrils, but also the problem of the role of cell organelles in myofilament synthesis.

The present report is an attempt to describe the process of skeletal muscle differentiation in a more systematic manner than has been done in past electron microscopic studies. FERRIS (1959b) has described somite development in an unpublished thesis (see also FERRIS 1959a), LINDNER (1957), MUIR (1957), WAINRACH and SOTELO (1961), WEISSENFELS (1962) and others have reported on the fine structure of developing cardiac muscle in embryos and cultures, and ALLBROOK (1962) and HAY (1959, 1962) have described regenerating muscle. Numerous observations of differentiating muscle have also been published which are either incomplete or were made before improved methods of tissue preparation for electron microscopy became available (see VAN BREEMEN 1952; HIBBS 1956; RUSKA and EDWARDS 1957; GILEV 1960). Although many of the questions raised by the light microscopist are answered by these electron microscopic studies, numerous new problems are also created. There is reason to hope, however, that the biochemical and cytological techniques of the present era will soon bring better solutions to these new problems than the techniques available in GODLEWSKI's and HEIDENHAIN's era brought to the fundamental questions raised by the light microscopist.

Material and Methods

Amblystoma opacum larvae (obtained from J.C. NICHOLLS jr., Murphy, N.C.) were fixed in buffered osmium tetroxide (BENNETT and LUFT 1959) at the time of hatching. They were dehydrated in 70% alcohol (5 min.), 95% alcohol (10 min), and 100% alcohol (1 hour). The tails were then removed from the animals and cut into small pieces, which were embedded in n-butyl methacrylate (see HAY 1959 for details). Longitudinal and cross-sections ($\sim$0,1 μ in thickness) were cut on a Porter-Blum microtome, mounted on copper grids, and stained with WATSON's (1958) lead hydroxide stain. Unstained sections were also examined. RCA EMU-3E and Siemens Elmiskope I electron microscopes were used for the observations. The micrographs were taken at original magnifications of 1000—20000 $\times$ and enlarged photographically as desired.

Thicker methacrylate sections ($\sim 1\,\mu$ in thickness) were examined unstained with a Zeiss phase contrast light microscope, or stained with 1% methylene blue (pH 5) and examined in an ordinary light microscope. For general orientation, a few tails were fixed in BOUIN's fluid, stained with HARRIS' hematoxylin and studied as whole mounts, using a light microscope. Various stages of differentiating cells can be found in the distal myotomes and on the dorsal and ventral surfaces of proximal myotomes, but it is important to orient the tails very carefully in order to obtain the younger stages in sections. There is considerable variation from tail to tail in the stages of differentiation encountered. PORTER (1954, 1956) has illustrated in electron micrographs some of the features of muscle fibers in the tails of *Amblystoma* larvae at a similar age (12—15 mm), but apparently his observations were confined to the differentiated muscle that forms the bulk of the tail.

Results

General features of developing muscle in the Amblystoma tail

The myotomes that compose the trunk musculature of the salamander larva are arranged in a regular series from the head of the animal to the tip of the tail. Those at the distal end of the tail are the smallest and the least developed, those in the proximal portion of the tail are larger and more mature, and those over the body of the animal are of even greater size and maturity. In the tail, the myotome extends from the dorsal to the ventral surface of the tail proper, surrounding the spinal cord and the notochord. The distal myotomes are composed of a single layer of multinucleated skeletal muscle fibers, but proximal myotomes have an additional lateral and medial layer. Each muscle fiber extends the length of the myotome (50—200 μ) and is in close contact with adjacent fibers. Nuclei are large and oblong and are located in the center or the periphery of the cell. The junction of the ends of the muscle fibers with the connective tissue separating the myotomes is a musculotendinous junction similar to that observed at muscle origins and insertions elswhere (PORTER 1954).

This material has several advantages for the electron microscopist studying myogenesis. Muscle cells in various stages of differentiation can be found in the myotomes as one proceeds from the distal to the proximal region of the young tail. Thus, it is possible to study myoblasts, early muscle cells and formed muscle fibers in the same preparation and the sampling variations that would be encountered in studying muscle fixed at different times from different embryos are minimized. The great regularity of the arrangement of the myotomes makes it easy to obtain sections that are exactly parallel to the long axis of the developing fibers. Moreover, the tail is so transparent that muscle differentiation can be observed in the living animal with the light microscope. Cells identified as myoblasts, early muscle fibres, and formed muscle fibers in electron micrographs here correspond to the same cell types described by SPEIDEL (1938) in developing amphibian tails *in vivo*. Reconstructions of the sequence of myogenesis in the fixed sections are greatly facilitated by these light microscopy studies of living cells. In the description to follow, the general morphology of myoblasts and muscle fibers will first be considered, and then the characteristic features of myofilament and myofibril formation will be taken up in more detail.

The fine structure of myoblasts

The term "myoblast" was originally used by BARDEEN (1900) to describe a round cell which he believed was a muscle cell precursor. Since then, many

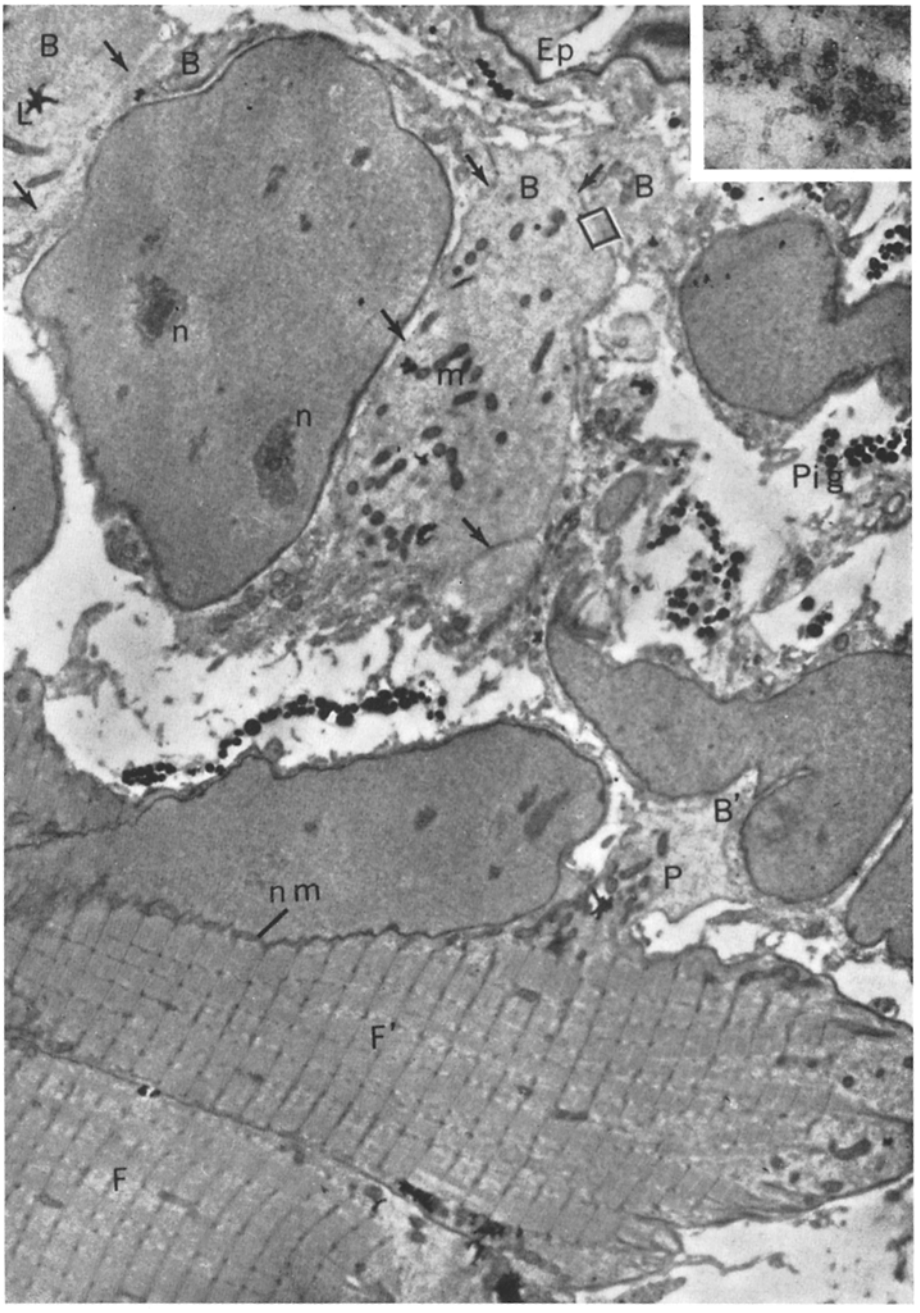

Fig. 1. Electron micrograph of portions of two differentiated muscle fibers (F, F') surrounded by several myoblasts (B, B'). The myoblasts labeled B are separated by ill-defined boundaries (small arrows) which at higher magnifications (inset) appear to be rows of small vesicles (approximate area of inset indicated by small square). The muscle fiber F' has a lateral process P which is intimately related to myoblast B'. *Ep* epidermis, *L* lipid droplet, *m* mitochondria, *n* nucleoli, *nm* nuclear membrane, *Pig* pigment granules in cytoplasm of a melanocyte. × 4000 (inset × 54000)

problems have arisen in the use of the term (see BOYD 1960, for review), due in part to the difficulty of distinguishing this cell type from ordinary mesenchymal cells with the light microscope, and in part to attempts to use the term to define the potency or capacity of undifferentiated cells to become muscle cells. In the present report, the term will be used to define a cell type that can be identified by morphological criteria as a muscle cell precursor.

Viewed in the light microscope, myoblasts have been distinguished from mesenchymal cells by their spindle shape, their abundant, basophilic cytoplasm, and their close proximity to muscle. In living regenerating amphibian tails studied with the light microscope, rows of undifferentiated appearing cells with the characteristics of myoblasts can be seen adjacent to the myotome fibers and these cells do differentiate into muscle subsequently (SPEIDEL 1938). With the electron microscope, similar cells can be identified (*B*, *B'*, Figs. 1 and 3) in close proximity to formed muscle fibers (*F*, *F'*, Figs. 1 and 2). Nuclei are large and contain one or two prominent nucleoli. Like the nucleoli of formed muscle fibers in the tail (PORTER 1960), these nucleoli possess an elaborate nucleolonema. Chromatin is highly dispersed, giving the nuclei a "vesicular" rather than a clumped appearance when viewed in the light microscope. Mitoses are occasionally encountered. The cytoplasm contains numerous small granules (*G*, Fig. 4) similar in size (100—150 Å) and arrangement to those originally described in the cytoplasm of basophilic cell types by PALADE (1955) and identified by cytochemical studies as ribonucleoprotein in nature (PALADE and SIEKEVITZ 1956). Since the cytoplasm of developing muscle cells is highly basophilic in fixed sections appropriately stained for light microscopy and the small granules are similar in size, density and arrangement to the Palade granules known to be responsible for cytoplasmic basophilia, the granules can be identified without reasonable doubt as ribonucleoprotein (RNP) granules. The only other known particulate component of the cytoplasm, glycogen, is much larger and occurs in cells which give a positive Schiff stain for mucopolysaccharides (FAWCETT and SELBY 1958). Such larger particulates are only seen occasionally in developing muscle cells. The ribonucleoprotein granules in myoblasts are predominantly "free" in nature, that is, they are not associated with the membranes of the endoplasmic reticulum. Endoplasmic reticulum is variable in amount, but is usually respresented by smooth-surfaced vesicles (*v*, Fig. 4) and poorly defined agranular profiles (*w*, Fig. 4), rather than by what would be termed (PALADE 1956) "granular" or "rough-surfaced" reticulum. Mitochondria (*m*, Figs. 1, 3, and 4), lipid droplets (*L*, Figs. 1—3), and occasional profiles of Golgi material also occur.

The close association between myoblasts (*B*, Fig. 1) and between myoblasts and muscle fibers (*P*, Fig. 1) is an interesting one. SPEIDEL (1938) has described fusion of myoblasts in the living amphibian tail and he has illustrated coalescence of myoblasts with formed muscle fibers by cell processes similar to that illustrated in Fig. 1 (at *P*). In the light microscope, the cells appear completely fused and, indeed, examples of true syncytia among myoblasts can be found with the electron microscope (Fig. 2). It is not uncommon, however, to find rows of myoblasts with ill-defined boundaries between them. These boundaries appear as fuzzy lines at low magnification (arrows, Fig. 1), but at high magnification are seen to consist of rows of small vesicles (inset, Fig. 1). It is tempting to

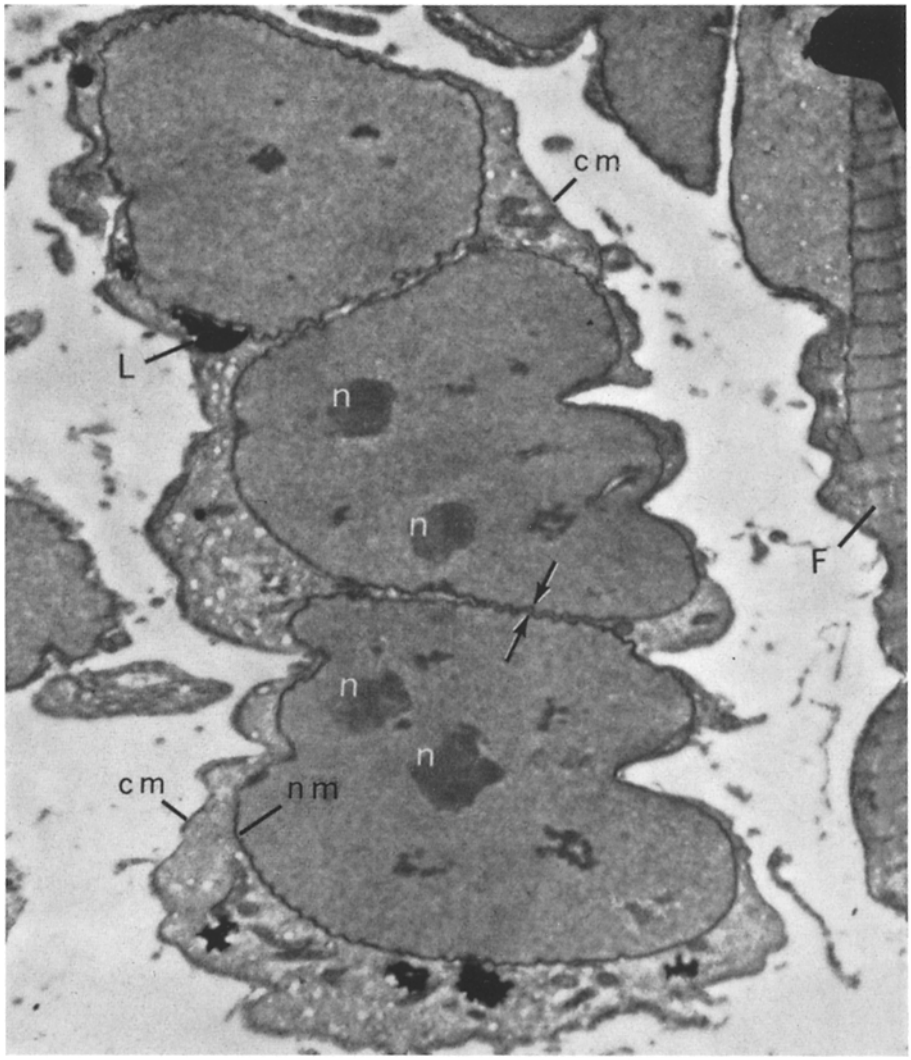

Fig. 2. Electron micrograph of three myoblasts which form a true syncytium. The nuclear membranes (arrows) of adjacent nuclei are in close apposition and the cell membrane (*cm*) is continuous around the nuclei. *F* differentiated muscle fiber, *L* lipid droplet, *n* nucleoli, *nm* nuclear membrane. × 4000

think that the small vesicles are remnants of membranes that previously were continuous between the cells, and that the cell membranes disappear by breaking up into vesicles when the cells fuse (see HAY 1959). The possibility that such vesicles are associated with obliquely cut cell membranes cannot be ruled out, however.

Not all of the cells classified as myoblasts by their shape and location contain recognizable myofilaments (inset, Fig. 4). What all of these cells do have in common is the presence of numerous free ribonucleoprotein granules in their relatively abundant cytoplasm; large nuclei and nucleoli; and a tendency to be closely associated in groups adjacent to formed muscle. If we turn now to the

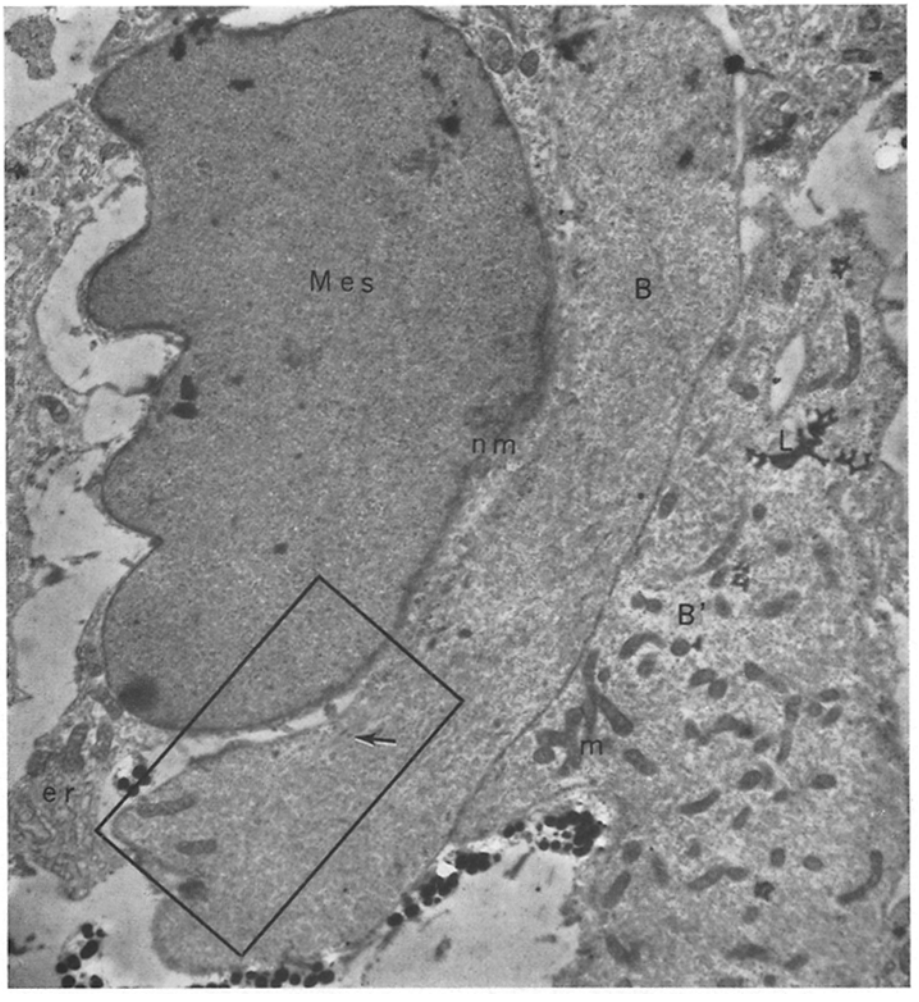

Fig. 3. Electron micrograph of portions of the cytoplasm of two adjacent myoblasts (*B*, *B'*). The cytoplasm of an adjacent mesenchymal cell (*Mes*) contains abundant endoplasmic reticulum (*er*), whereas the cytoplasm of the myoblasts is diffusely granular and contains less reticulum. An aggregation of developing myofilaments can be detected in the myoblast cytoplasm (arrow). *L* Lipid droplet, *m* mitochondria, *nm* obliquely cut nuclear membrane. × 6600

mesenchymal cell, we find that the situation with respect to relative amounts of free ribonucleoprotein granules and granular reticulum is just the reverse of that found in the myoblast; that nuclei and nucleoli are not usually as large as in muscle cells; and that the cells are frequently widely separated by extracellular connective tissue elements. The mesenchymal cells in the developing salamander

Fig. 4. Higher magnification electron micrograph of the region of myoblast cytoplasm enclosed by the rectangle in Fig. 3. Numerous ribonucleoprotein granules are found free in the cytoplasm (*G*) and closely associated with developing myofilaments (*G'* inset). The aggregation of myofilaments labelled in Fig. 3 is shown at the arrow and is enlarged further in the inset. Isolated myofilaments (*F*) also occur (see Fig. 9). The primitive *Z* band material (*Z* inset) differentiates at about the same time as the initial myofilaments and is quite distinctive in its appearance. *m* mitochondrion, *v* small vesicles associated with obliquely cut cell membrane, *w* wisps of membranous material. × 22000 (inset × 54000)

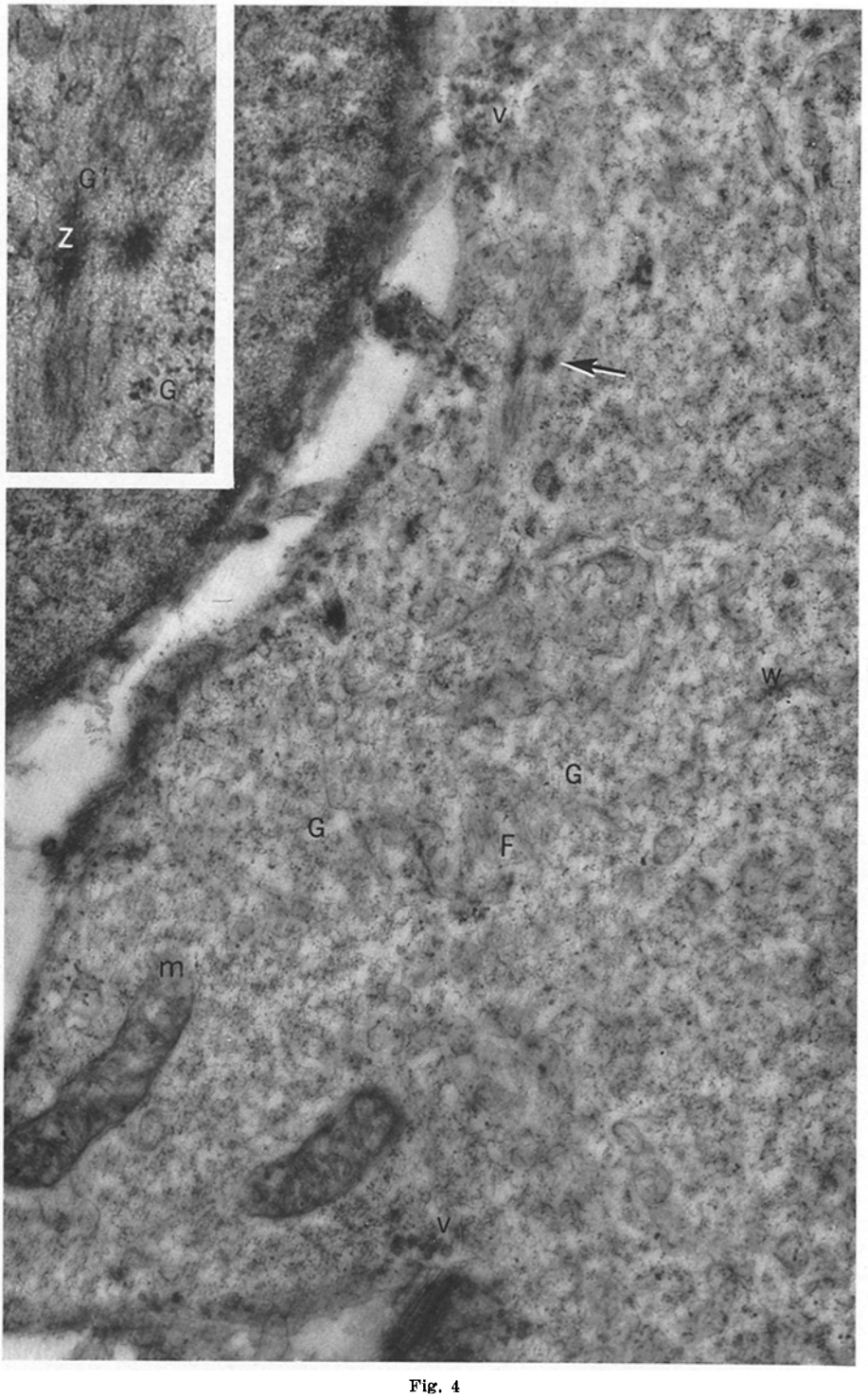

Fig. 4

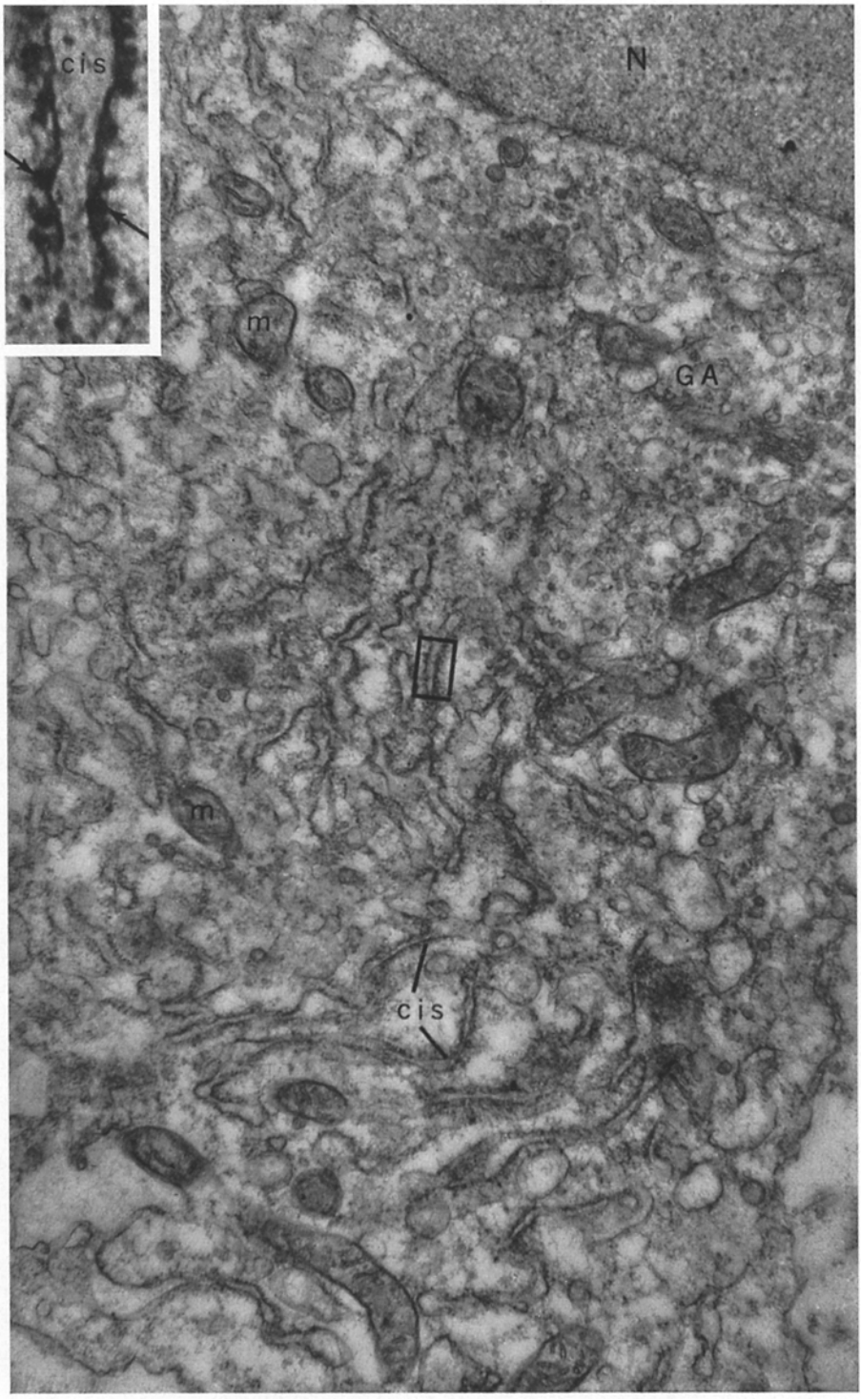

Fig. 5

tail are variable in shape. They may occur as stellate shaped cells with extremely attenuated cell processes or they may have more abundant perinuclear cytoplasm (Fig. 5). The entire cytoplasm, including that extending into the cell processes, is filled with reticulum of the type described by PORTER and PALADE in gland cells (PALADE 1956). The reticulum is primarily of the "granular" or "rough-surfaced" type (PALADE 1956), that is, the limiting membranes are covered with ribonucleoprotein granules (arrows, inset, Fig. 5). Numerous cisternae or flattened sacs occur (Fig. 5) which are sometimes arranged in parallel rows. This elaborate form of aggregated granular reticulum was originally called "ergastoplasm", but the term is unfortunately often used now for small amounts of dispersed granular reticulum (ALLBROOK 1962). The mesenchymal cells also resemble gland cells in the high degree of development of the Golgi apparatus (*GA*, Fig. 5).

Cells with some of the morphological features of mesenchymal cells (well developed Golgi apparatus and ergastoplasm) are occasionally seen in close association with myoblasts (*Mes*, Fig. 3). Some of the cells which are clearly muscle may possess a few granular cisternae. A transformation of mesenchyme to muscle has been suggested by earlier workers (BARDEEN 1900; MCGILL 1910; SCHMIDT 1927; KATZNELSON 1934) and a static population of cells stereotyped in morphology and function would hardly be expected to occur in a growing system. Nevertheless, those cells actively making the intracellular muscle proteins, the typical myoblasts, can easily be identified *in the stage of development studied here* as distinctly different in their fine structure from those cells primarily involved in the production of connective tissue, the mesenchymal cells.

Fine structure of muscle fibers

It is difficult to distinguish stages in a process as dynamic as muscle differentiation. It is useful for descriptive purposes, however, to try to define an intermediate stage between the relatively undifferentiated myoblast, on the one hand, and the formed muscle fiber, on the other. Elongate cells, which may be mononucleated or multinucleated, can be observed which have the general form of a "fiber", but the myofibrils do not fill the cytoplasm. These cells will be called *early muscle fibers*. They can usually be identified as muscle precursors without any difficulty using the light microscope. There may be only one or two myofibrils in some of the early muscle fibers, whereas in others the myofibrils may form groups oriented parallel to the long axis of the fiber (Fig. 6). The Z bands of the adjacent fibrils may not be in close alignment and there are other irregularities in these "primitive myofibrils" which will be described later. The sarcoplasmic reticulum (*er*, Fig. 6) has not yet assumed the form characteristic of the mature fiber. Ribonucleoprotein granules are found scattered throughout the cytoplasm and in close relation to the myofibrils (*G*, Fig. 6).

Fig. 5. Electron micrograph of a portion of the cytoplasm and nucleus (*N*) of a mesenchymal cell in the salamander tail. Endoplasmic reticulum is more highly developed than in the myoblast. Cisternae, or flattened sacs, are particularly prominent (*cis*) and at higher magnification (small rectangle) ribonucleoprotein granules can be seen adhering to the membranes of this reticulum (arrows, inset). The Golgi apparatus is prominent (*GA*) and consists of smooth-surfaced vesicles and flattened lamellae. *m* mitochondria. × 26000. Inset × 100000

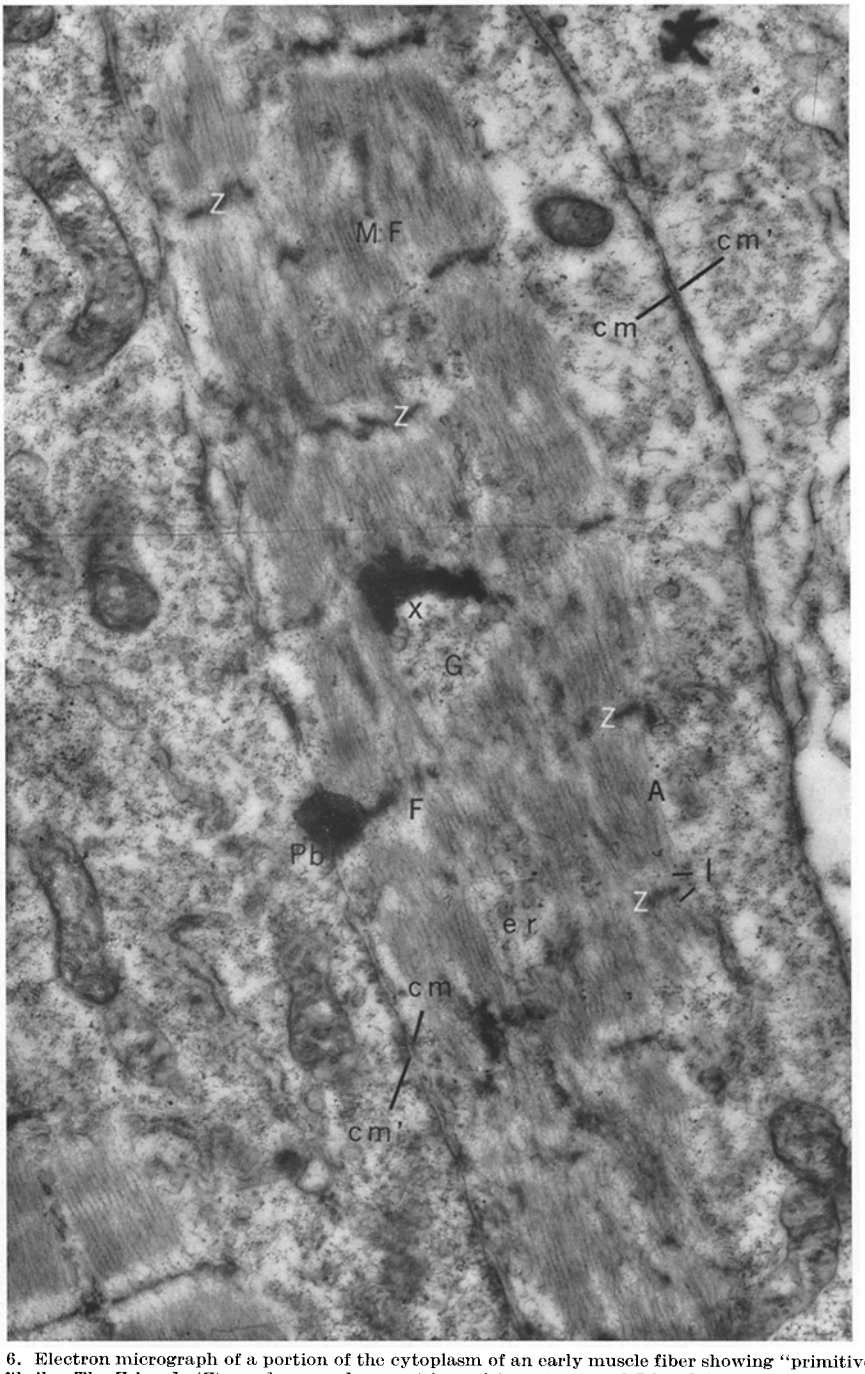

Fig. 6. Electron micrograph of a portion of the cytoplasm of an early muscle fiber showing "primitive" myofibrils. The Z bands (*Z*) are dense and are not in register. An A and I band (*A*, *I*) can be distinguished, however, and both thick (*MF*) and thin (*F*) myofilaments are present. Ribonucleoprotein granules are found in the interfibrillar cytoplasm (*G*) and smooth-surfaced endoplasmic reticulum (*er*) is developing near the myofibrils. *cm* cell membrane of the early muscle fibre, *cm'* cell membrane of adjacent muscle cells, *Pb* lead precipitate from the stain, *x* unknown dark body. × 17 000

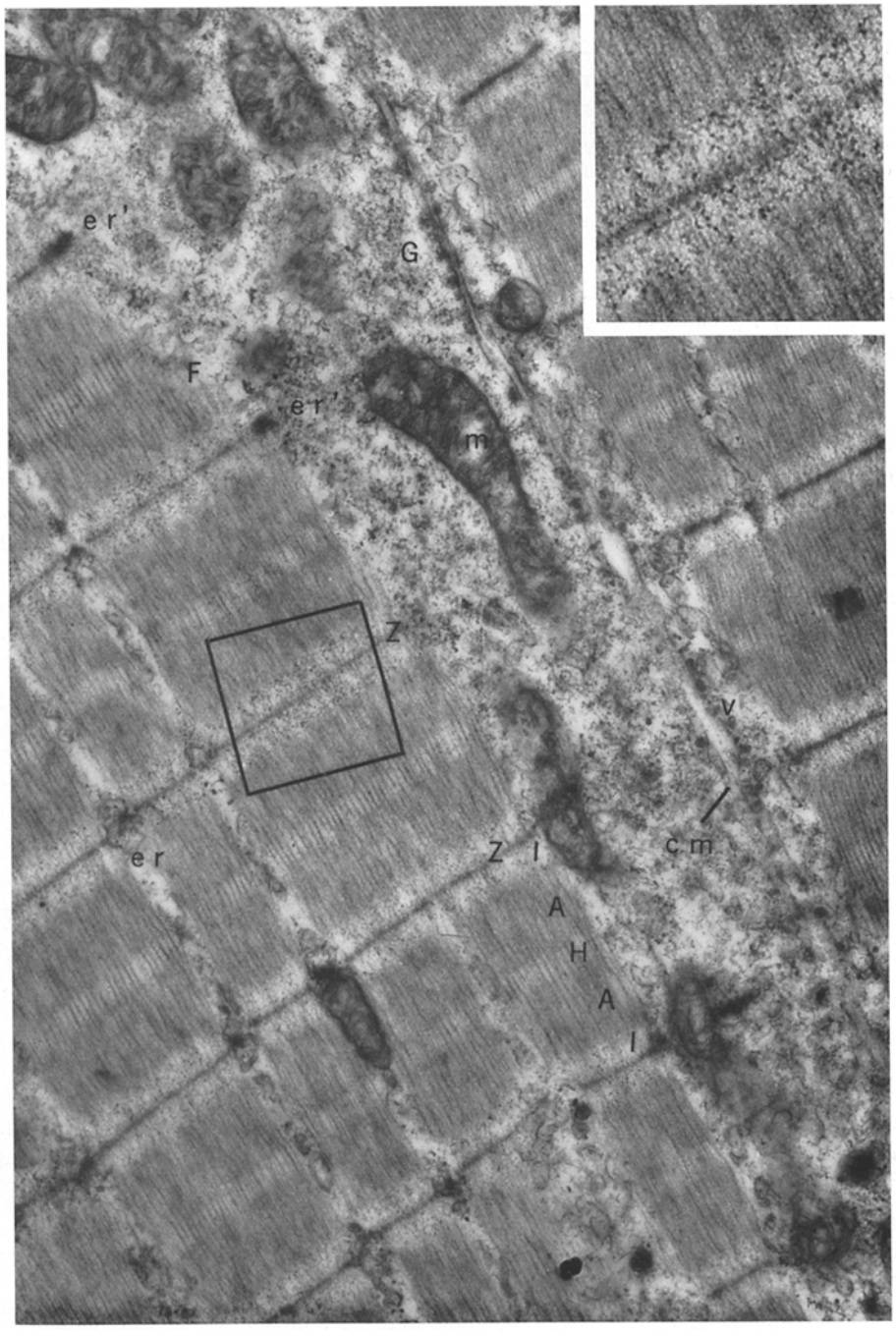

Fig. 7. Electron micrograph of portions of the cytoplasm of two formed muscle fibers. The "cross-striations" (*A*, *H*, *I*, and *Z*) of adjacent myofibrils are closely aligned and the endoplasmic reticulum (*er*) characteristic of the mature muscle fiber is well developed. Ribonucleoprotein granules (*G*) are still prominent. Small granules are found in the I bands (inset). Developing triads of reticulum (*er'*) appear on the lateral surfaces of the myofibrils. Incomplete myofilaments (*F*) can be seen in these areas. *cm* cell membrane, *m* mitochondrion, *v* vesicles. × 22000. Inset, × 50000

The formed muscle fiber in the tail is defined here as a cell in which myofibrils fill most of the cytoplasm and are regularly aligned (Fig. 7). The peripheral cytoplasm may still contain ribonucleoprotein granules (*G*, Fig. 7), but the relative number of ribonucleoprotein granules decreases as the fiber becomes more mature. This is in keeping with the well-known fact that the basophilia of the cytoplasm decreases as the cell differentiates and is consistent with the premise that ribonucleoprotein granules account for most of the cytoplasmic basophilia observed in fixed tissues appropriately stained for light microscopy. Vesicles and tubules of endoplasmic reticulum, occasional lamellae resembling Golgi material, and scattered mitochondria are also found in the peripheral cytoplasm. Mitochondria between the myofibrils are located at random, but the sarcoplasmic reticulum forms triads (*er*, Fig. 7) at the level of the Z bands. Longitudinal elements connect the triads, as described by PORTER (1956) and PORTER and PALADE (1957) in salamander muscle. In some of the formed muscle fibers, small granules similar to ribonucleoprotein granules may be found localized preferentially in the I band (inset, Fig. 7). However, the I bands do not give a strong basophilic reaction in sections stained with methylene blue at pH 5 for the light microscope, and firm identification of the granules must await more refined histochemical techniques[1]. In the tails of very young salamanders, the ends of the formed fibers contain areas of undifferentiated cytoplasm (Fig. 8). Similar undifferentiated terminal cytoplasm was described by SPEIDEL (1938) in living muscle and this observation can also be confirmed by cross sections. Ribonucleoprotein granules (*G*, Fig. 8), mitochondria (*m*, Fig. 8), and disorganized myofilaments (inset, Fig. 8) may be found in these regions. In older salamander larvae, the myofibrils seem to extend to the cell membrane for the most part (MUIR 1961).

Sequence of development of the myofilaments

The most striking feature of the development of myofilaments in myoblasts and early muscle fibers is the irregularity of the process. Small clumps of filaments associated with primitive Z band material can be detected in the cytoplasm of some myoblasts (Fig. 4). A single observation of this kind might not attest to the discontinuous nature of the myofibril components illustrated; incomplete myofibril elements or groups of filaments, however, are seen so often in developing salamander muscle and in differentiating muscle of other animals (see FERRIS 1959a and b; HAY 1962; WEISSENFELS 1962), that it must be concluded that the various components of the fibril are not always laid down as a single, long, uninterrupted unit. Clumps of filaments and discontinuous myofibrils probably correspond to the myofibril precursors interpreted as granules, short threads, and

[1] Since this paper went to press, our attention has been called to a recent report by BERGMANN describing similar granules in the I bands of developing mammalian skeletal muscle. [BERGMAN, R. A.: Observations on the morphogenesis of rat skeletal muscle. Bull. Johns Hopk. Hosp. **110**, 187—201 (1962)].

Fig. 8. Longitudinal section through the ends of several adjacent muscle fibers. The myofibrils taper off into filaments (*F*) which insert at the cell membrane. The terminal Z bands (*Z'*) may be incomplete. As in most musculotendinous junctions, collagen fibers (*coll*) are found on the extracellular surface of the cell membrane (*cm*) in such areas. In developing muscle, regions of undifferentiated cytoplasm containing ribonucleoprotein granules (*G*) and mitochondria (*m*) occur at the ends of some of the fibers. The inset shows disorganized myofilaments (*F'*) and ribonucleoprotein granules (*G*) in an area of terminal cytoplasm equivalent to that shown in the square. *ne* nerve ending. × 8000. Inset, × 50000

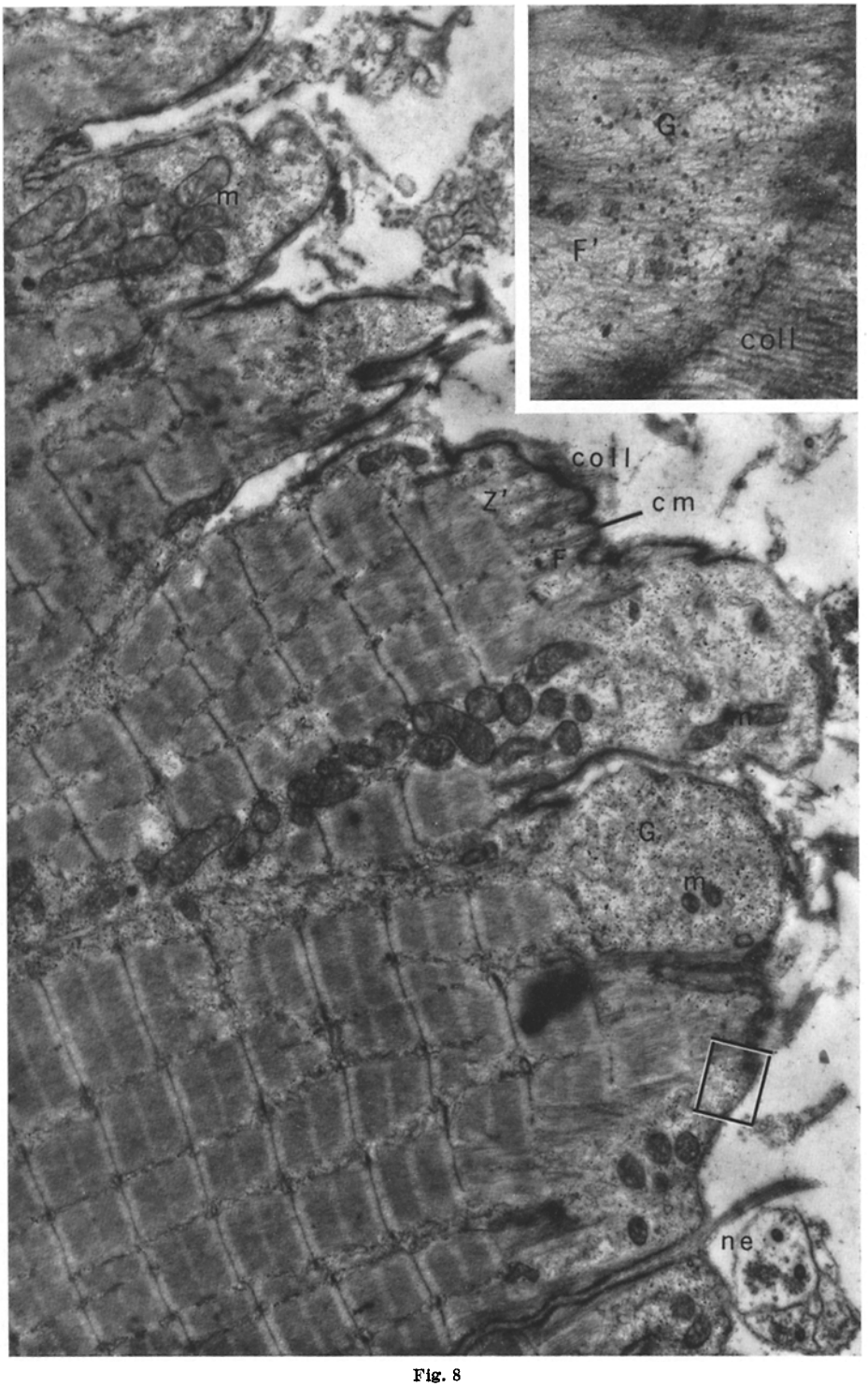

Fig. 8

"centrioles" by light microscopists (GODLEWSKI 1902; SPEIDEL 1938; WOLBACH 1927). The story of myofibril differentiation is made even more complicated by the presence of scattered filaments disposed more or less at random in the cyto-

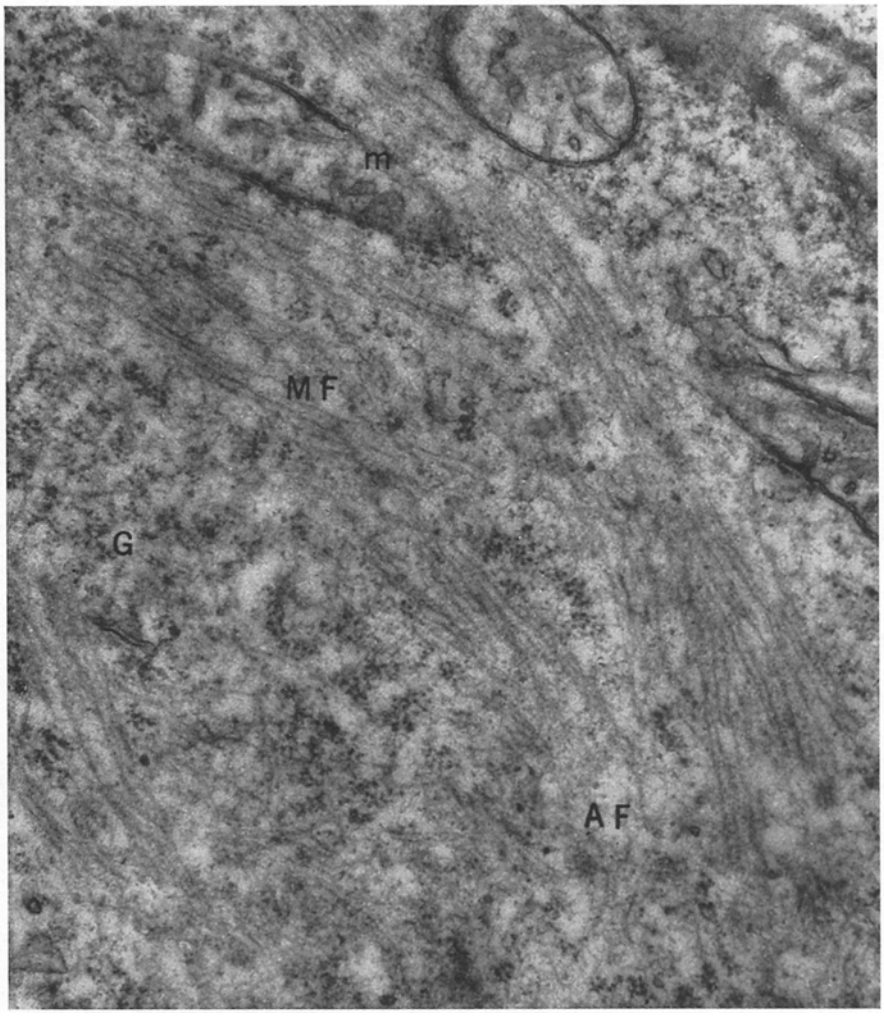

Fig. 9. Electron micrograph of a small portion of the cytoplasm of a myoblast showing thick filaments (*MF*) arranged in a random fashion in the cytoplasm. Numerous free ribonucleoprotein granules (*G*) are present in the vicinity of the developing myofilaments. The smaller filaments at *AF* are similar in diameter to actin filaments (see Fig. 10). *m* mitochondrion. × 45000

plasm of some developing muscle cells. These filaments may or may not be associated with Z bands, but it seems fair to classify them as myofilaments because such prominent filaments occur in the cytoplasm of muscle precursor cells, never in mesenchymal cells, and, therefore, by a literal interpretation of the definition are myofilaments ("muscle" filaments). Whether or not they are the actual precursors of the definitive myofilaments of the organized myofibril remains to be established. These filaments are variable in length and fall into two categories

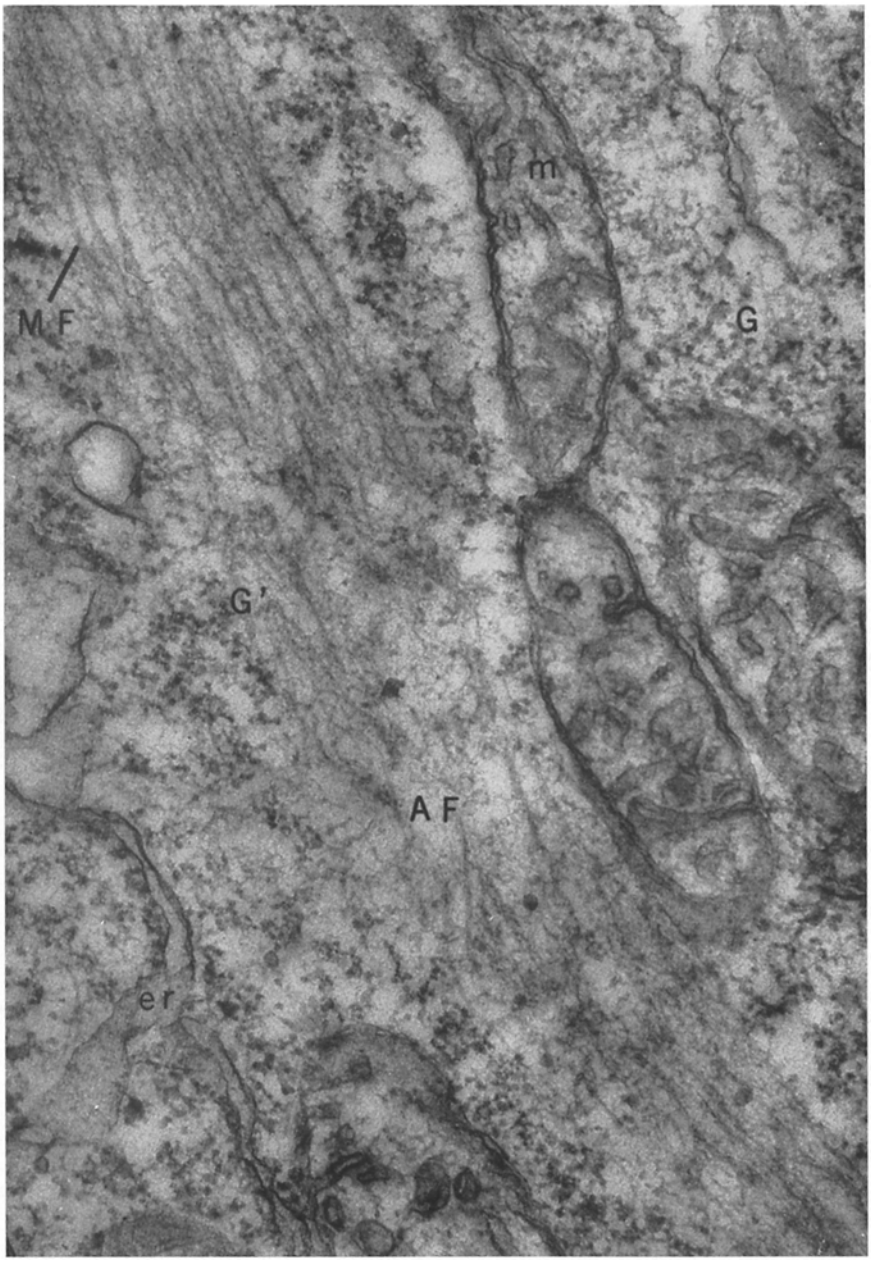

Fig. 10. Electron micrograph of a small portion of the cytoplasm of another developing muscle cell, showing the thin filaments (*AF*) and associated ribonucleoprotein granules (*G'*). Thick filaments (*MF*) resembling the myosin filaments of the mature myofibril (*MF*, Fig. 12) are also present. Ribonucleoprotein granules are predominantly free in the cytoplasm (*G*). A few granular cisternae of endoplasmic reticulum (*er*) may occur. *m* mitochondrion. × 70000

with respect to diameter. Some are ~100 Å in diameter (*MF*, Figs. 9—11) and resemble the thick filaments (*MF*, Fig. 12) thought to be composed of myosin

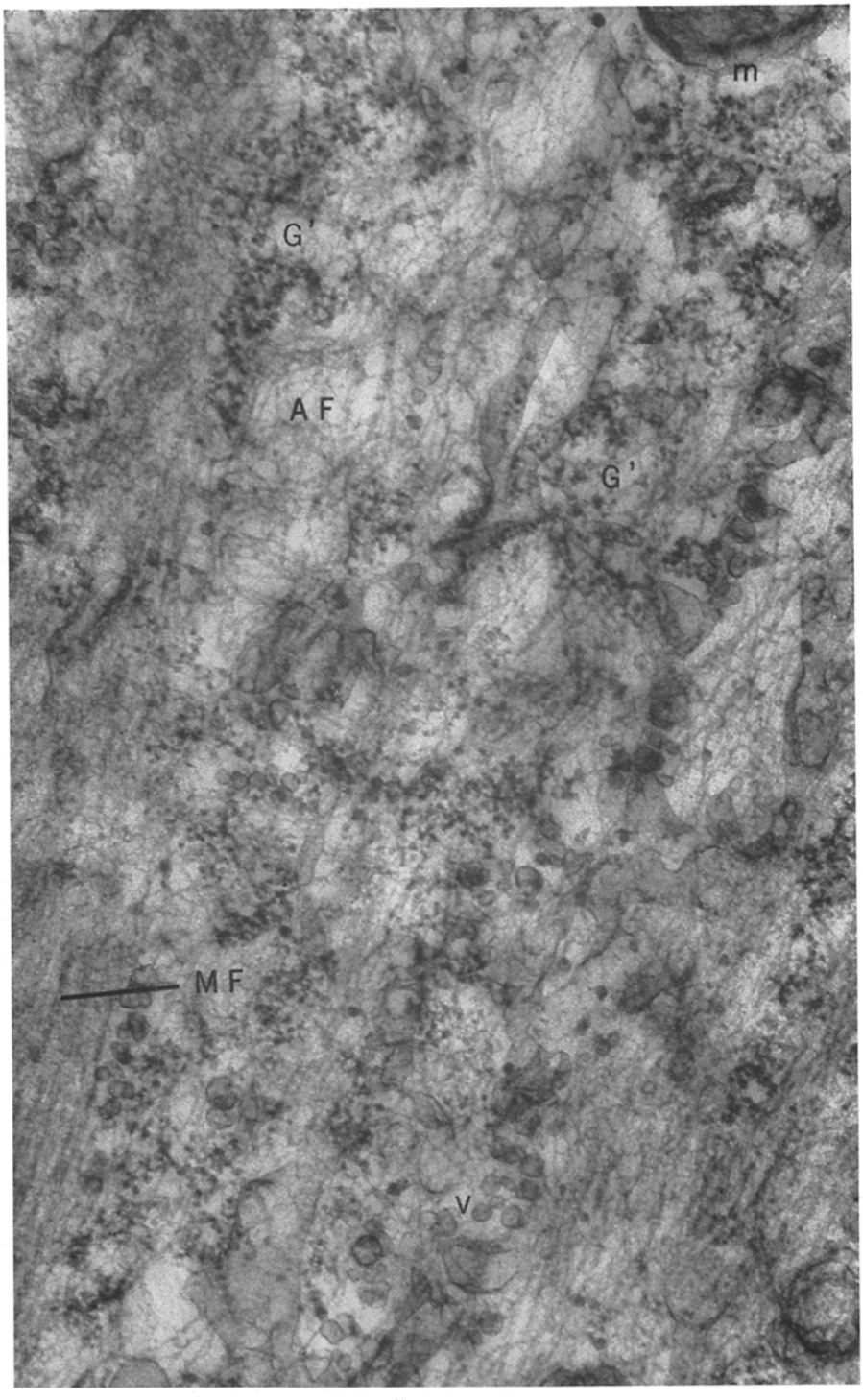

Fig. 11

in formed myofibrils (HUXLEY and HANSON 1959). The thick filaments in developing muscle cells may appear to have a less dense central core, particularly in very thin sections (arrows, Fig. 13). Cross sections of the A band of formed myofibrils (Fig. 14) confirm the impression that the central portion of the "myosin" or thick filament in these young muscle fibers may be less dense than the cortex. The ends of these thick filaments sometimes appear frayed (Fig. 10) and it is not impossible that they are forming by polymerization of individual thinner units. A second type of filament is seen in developing muscle cells, a thin filament (*AF*, Figs. 9—11) which is not necessarily associated with the thick filaments. These thin filaments are similar in diameter (~50 Å) to the "actin" filaments which in formed myofibrils extend from the Z band through the I band and into the A band (HUXLEY and HANSON 1959). Thin filaments of this type may be extremely irregular in their disposition (*AF*, Fig. 11; see also FERRIS 1959b; HAY 1962).

It is not easy to imagine how scattered myofilaments could become assembled into myofibrils. It is possible that some of them are replaced subsequently, and it should be noted that they are not present in all of the early muscle cells. It must be emphasized, however, that although it is difficult to believe that some of the filaments are synthesized "helter-skelter" in the cytoplasm of myoblasts, there can be no real doubt that both thick and thin filaments *can be laid down in a very disorganized fashion.* The disposition of the filaments depicted in Figs. 9—11 is too irregular to be explained on the basis of oblique sectioning of formed myofibrils. Moreover, the same kinds of randomly oriented filaments have been described in developing muscle cells of other forms of striated muscle (FERRIS 1959b; HAY 1962).

Not only is the development of the filaments highly irregular, but also the formation of Z band material is variable. The Z band may be partly continuous in the regions where filaments insert (inset, Fig. 4), or it may be seemingly absent in regions which appear to be future I band areas (*AF*, Fig. 10). It may form dense, irregular plaques in "primitive myofibrils" (*Z*, Fig. 6). There may be two Z bands in the future I bands of the fibrils, and the Z bands are not always denser than those of mature myofibrils. The sarcomeres delimited by the Z bands may be shorter or longer (Fig. 6) than the definitive sarcomere (lower left, Fig. 6; Fig. 7). The sarcomeres contain thick filaments (*MF*, Fig. 6), forming "primitive" A bands (*A*, Fig. 6). The I band is a region, sometimes poorly delimited, into which thick filaments do not extend (*I*, Fig. 6). Thin filaments may be present in these I bands and between the thick filaments of the A bands in early muscle fibers.

In formed muscle fibers with undifferentiated peripheral cytoplasm (Fig. 7), Z bands may appear on the lateral surfaces of the myofibrils adjacent to the Z bands of the myofibrils themselves. Triads of endoplasmic reticulum may also appear in this location (*er'*, Fig. 7). In the distal ends of the myofibrils (Fig. 8),

Fig. 11. The thin filaments (*AF*) may be very disorganized in their appearance in developing muscle cells. Here, they seem to interconnect, forming a network with which ribonucleoprotein granules are associated (*G'*). The thick filaments (*MF*) in this cell are somewhat more organized (they are arranged parallel to one another) than in other cells (*MF*, Fig. 9). Ramifying profiles of smooth-surfaced reticulum occur and a number of agranular vesicles (*v*) are seen at the lower half of the micrograph. Granular reticulum (Fig. 5) is not prominent in myoblasts and muscle fibers. *m* mitochondrion. × 70000

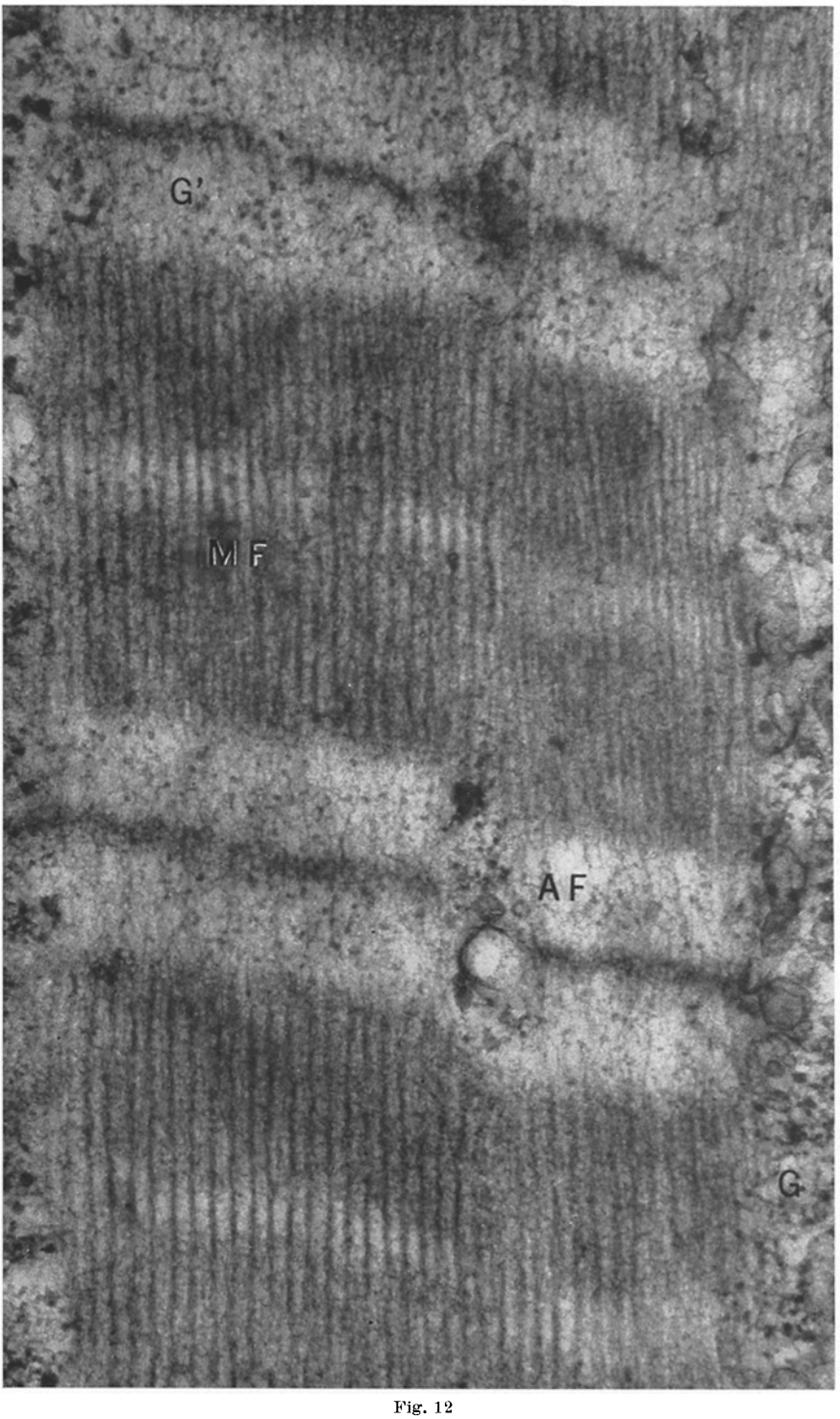

Fig. 12

Z bands may be incomplete, but they are usually located a sarcomere distance from the last complete Z band. The sarcomeres of the formed muscle fiber are in close register for the most part (Figs. 7 and 8) and their length is now relatively constant.

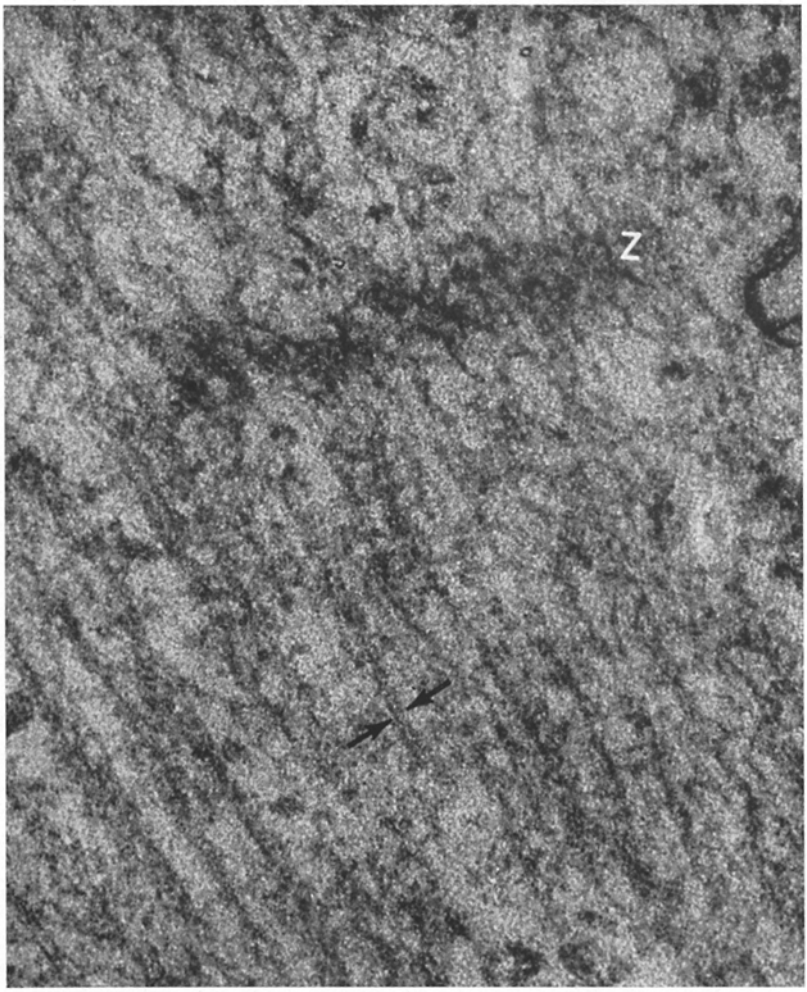

Fig. 13. Higher magnification electron micrograph of a differentiating myofibril, showing a thick filament with a central core that is less dense than the periphery (arrows). The "hollow center" of the thick filament is shown to better advantage in cross-section (Fig. 14). *Z* Z band. ×200000

The increase in sarcomere length that occurs in developing invertebrate muscle (ARONSON 1961) does not occur here. The formed muscle fiber is remarkably regular in its morphology and subsequent growth at the ends of the fiber or in other regions of the cytoplasm must take place without disrupting the overall structure and function of the cell.

Fig. 12. Formed myofibrils in the cytoplasm of partially differentiated muscle fibers have well developed thick filaments (*MF*) in A bands, but the thin filaments (*AF*) may not have the precise arrangement of mature actin filaments (HUXLEY 1957). Small granules (*G'*), which are similar in size to the free ribonucleoprotein particles (*G*) in the cytoplasm, occur in the I bands (see Fig. 7). × 75000

An interesting feature of developing muscle cytoplasm which has not received much attention in the past is the presence of numerous small particles in the immediate vicinity of the differentiating myofilaments. The cytoplasm is basophilic and these particles have the morphological characteristics of the free ribonucleoprotein granules described in the first section of the results. The granules surround the filaments (*G*, Fig. 9 and inset, Fig. 4) and are often in contact with them (*G'*, Figs. 10 and 11). The granules are sometimes arranged in short linear rows (HAY 1962), but often they simply form clumps near the myofilaments. We have searched for more precise arrangements, as sometimes occur on the membranes of the reticulum, but like everything else in early muscle cells, the granules are extremely disorderly. Free ribonucleoprotein granules are consistently seen in the vicinity of forming muscle proteins, and are inconspicuous in mesenchymal cells. The significance of this observation will be considered in the discussion.

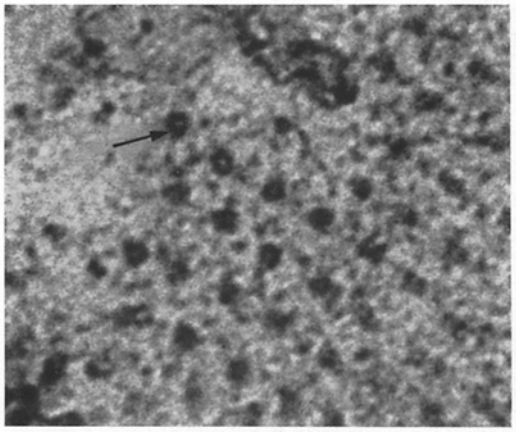

Fig. 14. Cross-section through the A band of a myofibril in a formed muscle fiber. The thick filament (arrow) appears to have a dense cortex and a central core lacking in electron density × 250000

Discussion

The observations presented here provide an outline of the fine structural events that occur during muscle differentiation, a groundwork, so to speak, for future investigation into the mechanisms underlying this remarkable example of cell specialization. There are many aspects of the process that clearly will require much additional study before it will be fruitful to attempt to understand them, but several problems arising from earlier light and electron microscopic work can be discussed to some advantage at the present time. The electron microscopic observations of FERRIS (1959a and b) and others, demonstrating that the earliest filaments in muscle cell precursors are of both the thick and the thin types described by HUXLEY (1957), have been confirmed. Contrary to the belief of many light microscopists, these early fibrous proteins of muscle cells are not always arranged in the form of definitive myofibrils. Some implications of this observation will be considered in the first part of the discussion.

The basophilia of myoblast cytoplasm as viewed with the light microscope is probably due, in large measure, to free ribonucleoprotein granules. Recent cytochemical studies (SIEKEVITZ and PALADE 1960) make it tempting to conclude that these granules are directly involved in the synthesis of proteins. The fine structure of the myoblast will be compared with that of the secretory gland cell and the mesenchymal cell in the second part of the discussion, and the theory of myofibril formation proposed by WEED in 1936 will be revised and amplified in the light of more recent cytological and cytochemical studies.

Sequence of events in myofibril differentiation

The first detectable filaments that are synthesized in the cytoplasm of myoblasts are not necessarily laid down in formed myofibrils, but may be scattered throughout the cytoplasm, sometimes associated with "primitive" Z bands. Filaments which do not seem to be associated with Z bands also occur, and sometimes these are arranged in a very disorderly fashion. Such filaments are found in muscle cells and not in mesenchymal cells and will be called myofilaments, although their exact relation to the filaments of formed myofibrils has not been established. Thick (~100 Å) filaments similar in morphology to the thick filaments thought to be composed of myosin in the mature fibril (HUXLEY 1957, 1962) are found, and thin filaments (~50 Å in diameter) similar to the actin filaments decribed by HUXLEY (1957) and HUXLEY and HANSON (1959) also occur. FERRIS (1959b) described the simultaneous appearance of thick and thin filaments in developing chick somites and LINDNER (1960) is of the opinion that actin and myosin filaments appear simultaneously in developing chick heart. Thick and thin filaments appear at the same time in differentiating skeletal muscle of regenerating salamander limbs (HAY 1962). We initially suggested that myosin filaments appeared before the smaller actin ones (HAY 1961a) and PORTER (1958) has suggested that actin appears before myosin. It now seems more likely that both types of myofilaments polymerize simultaneously or in such close temporal relation that small differences in various muscles are of minor consequence. There has been considerable disaggreement between biochemical and immunochemical studies (EBERT 1954; ENGEL and HORVATH 1960; HERRMAN 1952; HOLTZER et al. 1957; OGAWA 1962; WINNICK and GOLDWASSER 1961) regarding the sequence of development of muscle proteins. It seems reasonable to conclude from electron microscopic studies that both thick and thin filaments appear in the cytoplasm at a much earlier stage than would have been appreciated in some of these light microscopic and biochemical investigations; if these filaments are indeed composed of myosin and actin, as seems very possible, then much of the disagreement in previous studies may stem from the fact that the methods employed were too gross to detect chemically the first myofilaments that form in the cytoplasm of myoblasts.

The initial stage of myofibril formation may in some cases consist of a coalescence of the disorganized clumps of myofilaments and Z bands. These elements appear to come together in longitudinal array to form primitive sarcomeres of variable length. The short threads in the cytoplasm of myoblasts described by SPEIDEL (1938), the granules observed by GODLEWSKI (1902), HEIDENHAIN (1899), MOSCONA (1955) and others, and the centrioles of WOLBACH (1927), probably correspond to the early aggregations of myofilaments and Z bands observed in the electron microscope. GODLEWSKI believed that the granules he visualized fused to form homogeneous myofibrils. His concept of a homogeneous fibril has, unfortunately, persisted to the present day. Although occasional investigators (HÄGGQVIST 1920; LEWIS 1919) believed striation of myofibrils was a primary phenomenon, most workers in the field (see JORDAN 1920; WEED 1936) concluded that striations developed subsequent to the formation of the fibril, with the Z band appearing first. Uniform birefringence (see ARONSON 1961; GODMAN 1958) and uniform fluorescence with myosin antibodies (HOLTZER et al.

1957) have been attributed to the so-called homogeneous myofibrils. It now seems quite clear that the initial myofibrils are never homogenous, but are longitudinal aggregations of heterogeneous myofilaments and primitive Z bands. The Z bands may or may not be prominent and they are not always in register with the Z bands of adjacent fibrils and so would be difficult to detect with the light microscope. Both thick and thin myofilaments are present in the "primitive" sarcomeres, however, and there is a suggestion of an A and I band. As the arrangement of myofilaments becomes more orderly, A and I bands become more prominent. It is misleading to continue to speak this early myofibril as a "homogeneous fibril" in view of the fact that the several components of the heterogeneous mature fibril are actually present from the outset. The term "primitive myofibril" used here is perhaps more descriptive from a structural point of view than the light microscopist's term, "homogeneous fibril". The possibility that this fibril is also "primitive" in the sense that it transforms into more orderly myofibrils is suggested by the earlier light microscopy studies. Some of the disagreement in these earlier studies probably stems from the fact that all myofibrils do not pass through a primitive stage; some of them may be organized from the beginning (see HÄGGQVIST 1920; WEED 1936).

The attracting forces which might cause the dispersed myofilaments and myofibrils to aggregate and bring about the amazingly well-ordered final product of myofibril differentiation are completely unknown. It is tempting to think that the Z band material might play a role in the process. In developing cardiac muscle, "Z centers" with numerous attached myofibrils are observed (WAINRACH and SOTELO 1961; WEISSENFELS 1962). In skeletal muscle, however, fibrils consisting of partially aggregated myofilaments can be found in which Z bands are either absent or just beginning to form. Yet primitive I bands can be detected and regions of interdigitating thick and thin filaments occur. The Z band material probably does serve as an important region of attachment for functioning myofilaments and may play a role in directing the subsequent orderly formation of new myofilaments. It is interesting that the primitive Z band material is often denser and more prominent than that found in mature myofibrils. Z bands of increased density are also seen in dedifferentiating muscle (HAY 1959) and it seems probable that the primitive Z band is composed of a material that is distinct from the filamentous components of the myofibril. In its electron density and its disposition with relation to the cytoplasmic filaments, this Z band material resembles that composing the intercalated discs of cardiac muscle and the osmiophilic plaques of desmosomes in epithelial tissues (see FAWCETT 1958).

The similarity of the Z band material to the dense component of the desmosome invites a comparison of the filamentous components in muscle and epithelial cells. Fine filaments $\sim$50 Å in diameter, thought to be composed of keratin ("tonofilaments"), have been described in the cytoplasm of many epithelial cells and larger intracytoplasmic fibers comprise the rootlets of many cilia (see FAWCETT 1958, for review). In some columnar epithelia, the filaments may aggregate to form a dense, terminal web (PALAY and KARLIN 1959) and in certain epidermal cells they aggregate to form a peripheral net (HAY 1961 b). Such filaments are often associated with desmosomes and desmosome-like specializations, the terminal bars of columnar epithelia. In epidermal cells, there is a gradual transformation

of what appear to be scattered keratin filaments in the basal cells into the keratin proper of the outer epithelial layer, and the chemical properties and organization of the two forms of the protein are different in the two locations. The variety of forms assumed by "keratin" in the epithelial cell is no less impressive than the heterogeneous forms of myofilaments, or what we are assuming are myosin and actin precursors in the present report. Moreover, there are certain superficial resemblances between tonofilaments and myofilaments. In cardiac muscle (FAWCETT and SELBY 1958), the myofilaments insert into desmosomes and desmosome-like specializations, the intercalated discs, in a manner reminiscent of the insertion of tonofilaments into desmosomes in epidermal cells. The myofilaments of smooth muscle cells are disposed in a disorganized fashion not unlike that of some tonofilaments. Myofibrils have certain staining reactions in common with the terminal web of epithelial cells (LEBLOND et al. 1960), and x-ray diffraction data suggest that native fibrous proteins fall into only two general classes, the extracellular collagen class and the intracellular keratin-myosin class (ASTBURY 1949). It well may be that the superficial similarities of some of the diverse forms assumed by myofilaments and tonofilaments, and the parallelisms in the modes of organization in the two cases, reflect underlying physicochemical likenesses among the proteins of the keratin-myosin group. It would be reassuring if this were true, for the scattered filaments in developing muscle cells (FERRIS 1959b; HAY 1962) and cells derived from muscle in tissue culture (WEISSENFELS 1962) bear many superficial resemblances to the filaments of epithelial cells, and yet they occur in a cell which is clearly muscle in type. Certainly, more study is required before we can hope to understand the significance of the irregular disposition of myofilaments in early muscle cells, and the mechanisms underlying the orderly formation myofibrils in the differentiated muscle fiber.

Cell components participating in myofilament synthesis

The last really detailed cytological analysis of muscle differentiation was made by WEED in 1936 and it was not until the latter half of that decade that the first important histochemical studies were published which suggested a relation between cytoplasmic basophilia, ribonucleoprotein, and protein synthesis (see BRACHET 1950). WEED was unaware that the basophilic staining reaction of cytoplasm was caused by ribonucleic acid. She failed to emphasize the striking basophilia of myoblast cytoplasm in her report and she overemphasized the development of the Golgi apparatus. In spite of these limitations, however, WEED formulated a theory of myofibril formation which was far ahead of its time in its insight into cell mechanisms. She compared the large nuclei and nucleoli of developing muscle cells to those of secreting glandular cells, and stated (p. 535): "It is suggested, therefore, that the myofibrils are produced in a way similar to that by which secretory materials are elaborated. In muscle, however, the products formed are not eliminated as is the case in glandular tissue, but are retained as permanent structures."

In recent years, electron microscopic and cytochemical studies have provided a clearer idea of the process of protein synthesis and secretion in gland cells. These studies and *in vitro* biochemical investigations of the role of ribonucleic acid in protein synthesis now permit us to extend WEED's speculation about the manner

in which "secretory materials are elaborated". It has been demonstated that small particles 100—150 Å in diameter as visualized in the electron microscope are responsible for the basophilia of cytoplasm in fixed sections studied with the light microscope and that these particles or granules contain ribonucleoprotein (PALADE 1955; PALADE and SIEKEVITZ 1956). In gland cells, the majority of the ribonucleoprotein particles are attached to the membranes of the endoplasmic reticulum. Cell fractionation studies using amino acids labeled with radioactive isotopes to trace protein formation have demonstrated that newly synthesized protein first appears in association with the ribonucleoprotein particles. On the basis of their cytochemical studies, SIEKEVITZ and PALADE (1960) have suggested that the proteinaceous enzymes produced by the pancreatic acinar cell are synthesized on the ribonucleoprotein particles attached to the endoplasmic reticulum and move through the channels of the reticulum into the Golgi complex where the zymogen granules to be extruded by the cell are formed. This hypothesis has now been partly confirmed by autoradiography (CARO 1961).

The theory outlined above suggests that the primary role in protein synthesis belongs to the ribonucleoprotein granules and that the endoplasmic reticulum itself has a secondary function, possibly in the transport of the substances synthesized. If, indeed, the function of the elaborate reticulum of gland cells is to sequester a protein product that will subsequently be transferred to the outside of the cell, and if the association of the ribonucleoprotein granules with the reticulum reflects this specialized role, then in a cell which retains the product synthesized, *the ribonucleoprotein granules might be free in the cytoplasm,* rather than associated with membranes. This, indeed, appears to be the case. Actively growing cells synthesizing intracellular proteins have more abundant free ribonucleoprotein particles than gland cells and less granular reticulum (see SLAUTTERBACK and FAWCETT 1959). The apparent exceptions to this generalization are actively growing epithelial cells which are at the same time synthesizing extracellular products and certain mesenchymal cells, such as those described in the present report, which are probably also involved in the synthesis of extracellular proteins. These "embryonic" cells have a large amount of granular reticulum arranged in the form of cisternae (ergastoplasm) and, in a sense, resemble secretory gland cells. Muscle cells produce large amounts of *intracellular* proteins and the myoblast does have *abundant free ribonucleoprotein granules.* There is not as much granular endoplasmic reticulum in the typical myoblast as in the gland cell and the smooth-surfaced, or agranular, reticulum that occurs is quite different in appearance and probably in function from the granular reticulum (see PALADE 1956). The difference in morphology of the reticulum and RNP particles in muscle and gland cells synthesizing protein may actually reflect the fact that in muscle, as WEED suggested, "the products formed are not eliminated as is the case in glandular tissue, but are retained as permanent structures".

The observations of myoblast fine structure, considered together with recent biochemical studies (see ZAMECNIK 1960) of protein and nucleic acid synthesis, make the following hypothesis of muscle protein synthesis seem reasonable. The amino acids destined to form the characteristic proteins that compose the myofilaments may be assembled on the free ribonucleoprotein granules ("ribosomes") of the myoblast cytoplasm and some of the newly forming protein molecules may

polymerize into visible myofilaments shortly thereafter. The RNP particles do not form rows or geometrical arrays with any great regularity and the idea that the orientation of the filaments depends on the particles does not seem attractive. It has been proposed (see WOODS 1959) that some cytoplasmic RNA may come from the nucleus in multicellular organisms, but the role of the nucleus and DNA-dependent RNA in cytoplasmic protein synthesis in vertebrates is not understood at present. Large nuclei and prominent nucleoli are characteristic of many cells synthesizing proteins actively (gland cells, muscle cells, various growing and differentiating cells). Indeed, it was this striking development of nuclei and nucleoli that called WEED's attention to the possibility that myofibrils might be produced in the same manner as secretory materials. In the interval since her publication in 1936, while much has been discovered about the role of the cytoplasm in secretion, we have learned little about the significance of the nuclear morphology on which WEED based her foresighted comparison of muscle and gland cells. It is to be hoped that this area of developmental cytology will be explored in the future, and the functional significance of the structural similarities between gland and muscle cells elucidated more fully.

Summary

Thin methacrylate sections of developing tails of *Amblystoma opacum* larvae were examined in the electron microscope and a series of stages in the differentiation of the myotome musculature was reconstructed from electron micrographs and earlier light microscopic studies of living muscle. The earliest muscle cell precursor that can be clearly identified is a round or oval cell with abundant cytoplasm containing scattered myofilaments and free ribonucleoprotein granules, but little endoplasmic reticulum. These cells sometimes form a syncytium and they may also be fused with adjacent formed muscle fibers by lateral processes. Nuclei are large and nucleoli are prominent. This cell, called a "myoblast" here, is distinctly different in its appearance from the adjacent mesenchymal cells which have abundant granular endoplasmic reticulum. The earliest myofilaments are of both the thick and thin varieties and are distributed in a disorganized fashion in the cytoplasm. These filaments are similar to the actin and myosin filaments described by HUXLEY and they are present in the cytoplasm at an earlier stage of differentiation than heretofore suspected from light microscopy studies. The first myofibrils are a heterogeneous combination of thick and thin filaments and dense Z bands and are not homogeneous as so many light microscopists have contended. As development progresses, cross striations become more orderly and definitive sarcomeres are formed. Thereafter, new myofilaments and Z bands seem to be added to the lateral surfaces and distal ends of existing myofibrils.

Free ribonucleoprotein granules are a prominent part of the myoblast cytoplasm and are found in close association with the differentiating myofilaments in all stages of development. In early muscle fibers and some of the formed fibers, similar granules are often concentrated in the I bands. A theory of myofilament differentiation based on current concepts of the role of ribonucleoprotein in protein synthesis is presented in the discussion. Stages in myofibril formation and possible relationships of the filaments in developing muscle cells to other types of cytoplasmic filaments are also discussed.

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