MITOSIS AND INTERMEDIATE-SIZED FILAMENTS IN DEVELOPING SKELETAL MUSCLE

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ABSTRACT

A new class of filaments intermediate in diameter between actin and myosin filaments has been demonstrated in skeletal muscle cells cultured from chick embryos. These filaments, which account for the majority of free filaments, average 100 A in diameter. They may run for more than 2 μ in a single section and can be distinguished in size and appearance from the thick and thin filaments assembled into myofibrils. The 100-A filaments are seen scattered throughout the sarcoplasm at all stages of development and show no obvious association with the myofibrils. The 100-A filaments are particularly conspicuous in myotubes fragmented by the mitotic inhibitors, colchicine and Colcemid. In addition, filaments similar in size and appearance to those found in myotubes are present in fibroblasts, chondrocytes, and proliferating mononucleated myoblasts. The 100-A filaments are present in cells arrested in metaphase by mitotic inhibitors. Definitive thick (about 150 A) or thin (about 60 A) myofilaments are not found in skeletal myogenic cells arrested in metaphase. Myogenic cells arrested in metaphase do not bind fluorescein-labeled antibody directed against myosin or actin. For these reasons, it is concluded that not all "thin" filaments in myogenic cells are uniquely associated with myogenesis.

INTRODUCTION

A number of recent studies have focused on the ultrastructure of myogenic cells. The appearance of the contractile protein filaments, myosin and actin, and their assembly into myofibrils have received considerable attention despite the uncertainties of identifying filaments in thin sections. Thus far, only two classes of filaments have been described and discussed in developing muscle cells; these are thick filaments (about 150 A) and thin filaments (about 60A), generally considered to be myosin and actin, respectively. The present study reports the occurrence of a class of intermediate-sized filaments in the sarcoplasm of chick skeletal muscle cells maturing in vitro. These intermediate-sized filaments constitute a majority of the free, unoriented filaments. Similar filaments are observed in a variety of cells, including fibroblasts, chondrocytes, and mitotic cells. We wish to describe these free cytoplasmic filaments and to discuss possible interpretations of the nature of these filaments.

MATERIALS AND METHODS

Breast muscle tissue from 10–11-day chick embryos was trypsinized to obtain a monodisperse suspension as has been described by Bischoff and Holtzer (1968). The cells were cultured on fibrin clots or Mylar sheets placed on the bottom of 35-mm plastic Petri dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles). The culture medium consisted of Eagle's minimal essential medium, horse serum, and embryo extract (8:1:1) with 1% antibiotic-antimycotic solution (Grand Island Biological Co., Grand

Island, N. Y.) and 2 mM L-glutamine. Cultures were incubated at 37.5°C in a water-saturated atmosphere of 5% CO_2:95% air.

For the inspection of myogenic cells in metaphase, some early cultures were grown in 10^{-6} M colchicine (Mann Research Labs, Inc., New York) or in Colcemid (Ciba, Products Company, Fair Lawn, N. J.) for varying times. They were then either fixed *in situ* or collected by pipetting and fixed as a pellet of metaphase-arrested cells after centrifugation.

Cultures 1–28 days of age were fixed for approximately 15 hr at room temperature in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) containing 2 mM $CaCl_2$, rinsed in the same buffer with 10% sucrose for 15 min, and then postfixed for 1 hr in cold 1% OsO_4 in the cacodylate buffer. Some cultures were fixed only in OsO_4. All specimens were dehydrated in graded concentrations of ethanol (75, 95, 100%) and embedded in Epon 812. The cells grown on fibrin clots could easily be removed from the plastic dishes in propylene oxide after dehydration. Cells grown on Mylar could be separated from the Mylar after polymerization, leaving the specimens embedded. Sections were cut on a Sorvall Porter-Blum MT-2 microtome and stained with a saturated solution of uranyl acetate in 50% ethanol followed by the application of lead citrate. Specimens were observed with an AEI EM6B electron microscope.

Measurements of filament size were made on the printed micrographs enlarged photographically to 280,000 diameters.

Agar double-diffusion tests (Preer, 1956) for the presence of myosin, heavy-meromyosin, light-meromyosin, and actin followed the procedures described by Marshall et al. (1959) and Holtzer (1961). Antibody in the bottom of a glass tube 2 mm in diameter was overlaid with an agar column 5 mm in length. The extract to be tested was added over the agar. The test extracts were made from pooled cultures consisting of (*a*) well-developed, multinucleated myotubes or (*b*) colchicine-treated metaphase-arrested cells. For extraction, Hasselbach-Schneider solution as modified by Hanson and Huxley (1957) and 0.6 M KI (Szent-Györgyi, 1951) were used. The former solution removes myosin from myofibrils, while KI is known to extract actin in addition to myosin. Undiluted concentrations of protein in the extracts were between 2 and 3 mg/ml. Extracts to be tested for antigen and the antisera were used over a wide range of concentrations.

RESULTS

100-A Filaments in Interphase and Postmitotic Cells

Organized myofibrils can occasionally be seen in early myotubes from 1- to 2-day cultures, but most of the filamentous elements are freely dispersed in the sarcoplasm and show no particular association with the emerging myofibrils (Fig. 1). The free filaments exhibit a wide range of diameters from 130 A down to a size (less than 40 A) that is difficult to measure reliably. To obtain an estimate of the size distribution, a random sample of free filaments was measured on printed micrographs of longitudinally sectioned myotubes. The majority of free filaments fall into a distribution ranging from 80 to 110 A with a prominent peak at 100 A.[1] These filaments are clearly different in diameter from the 50–70-A thin filaments found in the early or definitive sarcomeres (Figs. 1, 3, and 4). It should be emphasized that in both early and well-developed myotubes only a small proportion of the free filaments overlaps the size distribution of definitive actin filaments (Fig. 2). The thin, 50–70-A filaments tend to be arranged as aggregate forms. Actin filaments cannot be identified, however, before the appearance of myosin filaments. Actin filaments can only be identified unequivocally when they are in association with definitive myosin filaments. At no stage of development is there a conspicuous surplus of free 50–70-A filaments as indicated by Allen and Pepe (1965) and Fischman (1967).

The 100-A filaments are usually observed lying parallel to the long axis of the myotube but occasionally are scattered in a more random fashion throughout the sarcoplasm. In cross-section these filaments are usually round or polygonal (Fig. 4). They often appear as tubules in both longitudinal and cross-section (Fig. 4). Many filaments are greater than 1 μ in length, and some are more than 2.5 μ in a single section. They often appear wavy.

The intermediate-sized, 100-A filaments are also seen in material fixed only in OsO_4 (Fig. 6). Thus, their preservation is not restricted to glutaraldehyde-OsO_4 fixation. On the other hand, OsO_4 fixation does not preserve microtubules in these cells, although material fixed in glutaraldehyde-OsO_4 is rich in microtubules.

The 100-A filaments do not show any definite association with the thick and thin filaments of the enlarging myofibril, nor do they form any close associations with each other which suggest the initiation or "nucleation" of new myofibrils.

[1] For convenience, filaments having this size distribution will be referred to as "100-A filaments." It is recognized that this may be a heterogeneous class of filaments.

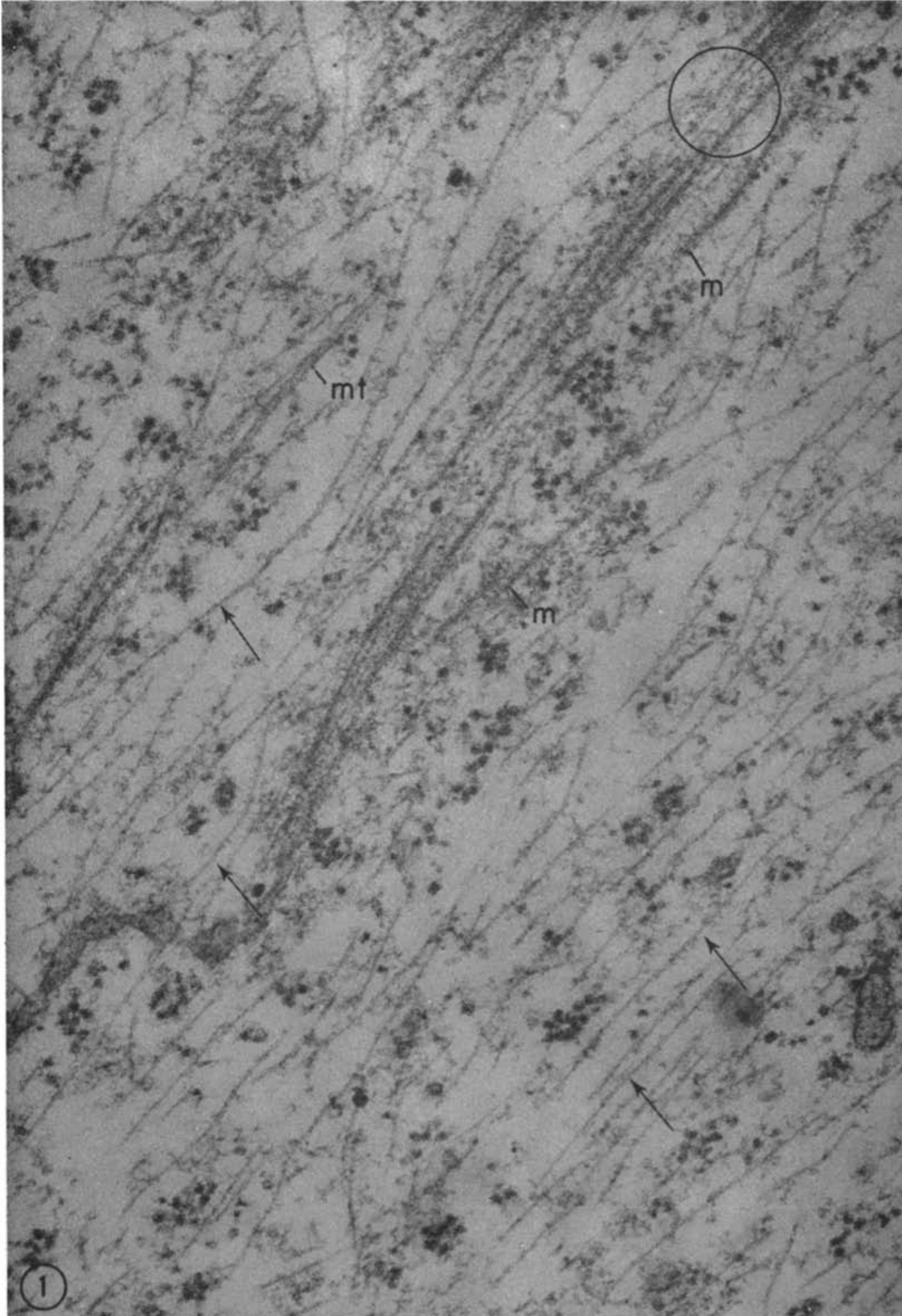

FIGURE 1 Longitudinal section of myotube from a 2 day culture. The majority of the free filaments (arrows) are 80–100 A in diameter. Compare these with the thin filaments (circle) associated with thick filaments in the early myofibrils. *m*, thick filament. *mt*, microtuble. × 72,000.

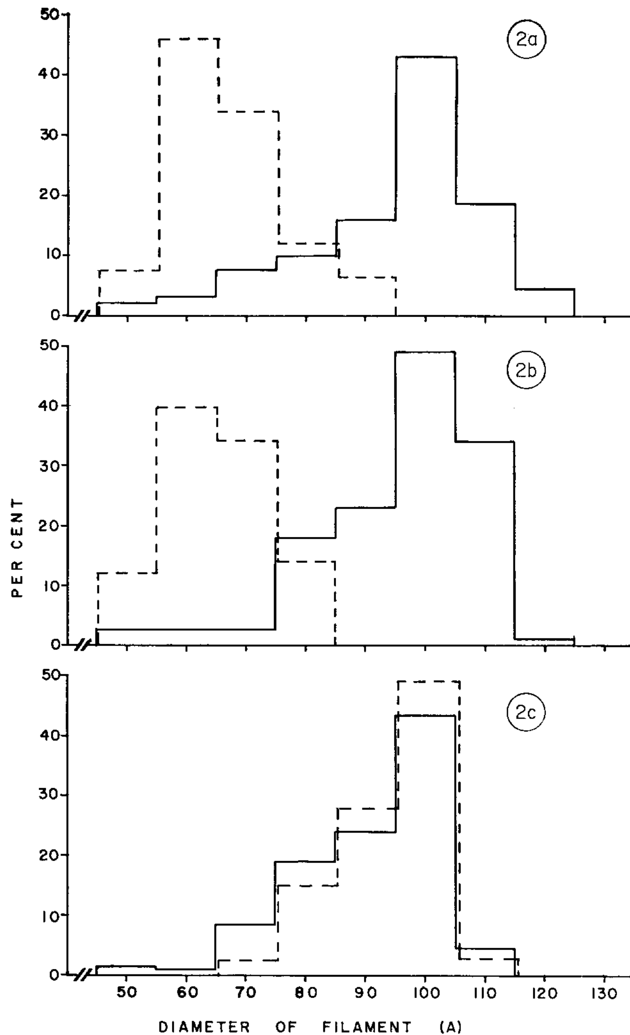

FIGURE 2 Size distribution of the various filaments. Measurements were made on longitudinally sectioned filaments from printed micrographs enlarged to 280,000 diameters. Filaments occurring in aggregates are not included in the data for the free filaments. The figures in parentheses indicate the number of filaments measured. *a*, early myotubes from 2-day culture. Solid line, free sarcoplasmic filaments (250). Broken line, thin filaments within sarcomeres (68). *b*, well-developed myotubes from 13–16-day culture. Solid line, free sarcoplasmic filaments (119). Broken line, thin filaments within sarcomeres (50). *c*, Solid line, free filaments in fibroblastic cells (202). Broken line, free filaments in metaphase-arrested cells (106).

Their length, smooth surface, and uniform width make it unlikely that the 100-A filaments represent growing or enlarging myosin filaments (see Firket, 1967; and also Heuson-Stiennon, 1965). Myosin filaments in developing muscle cells do not exceed 1.5 μ in length and show a fuzzy surface and tapered ends.

In well-developed myotubes from older cultures (5 days and older), most of the sarcoplasm is filled with highly organized myofibrils. In the interfibrillar spaces one can still see variable numbers of intermediate-sized free filaments (Fig. 5). These wavy filaments tend to course parallel to the long axis of the myotube. No definite connections or relationship can be seen between the intermediate-sized filaments and any elements of the sarcomeres. As in early myotubes, in a single section some filaments are greater than 2 μ in length. The filaments do not appear to be continuous with microtubules, although the two elements may show a close association spatially (also see Inoué and Sato, 1967). The observations that the 100-A filaments are different in size and appearance from both the thick and thin myofilaments and that they do not appear to be associated with sarcomere formation suggested that such filaments may be a general cell constituent. Consequently, we examined other types of cells for the presence of these filaments.

In addition to multinucleated myotubes, our cultures contain many mononucleated cells. Free filaments identical in size with those seen in myo-

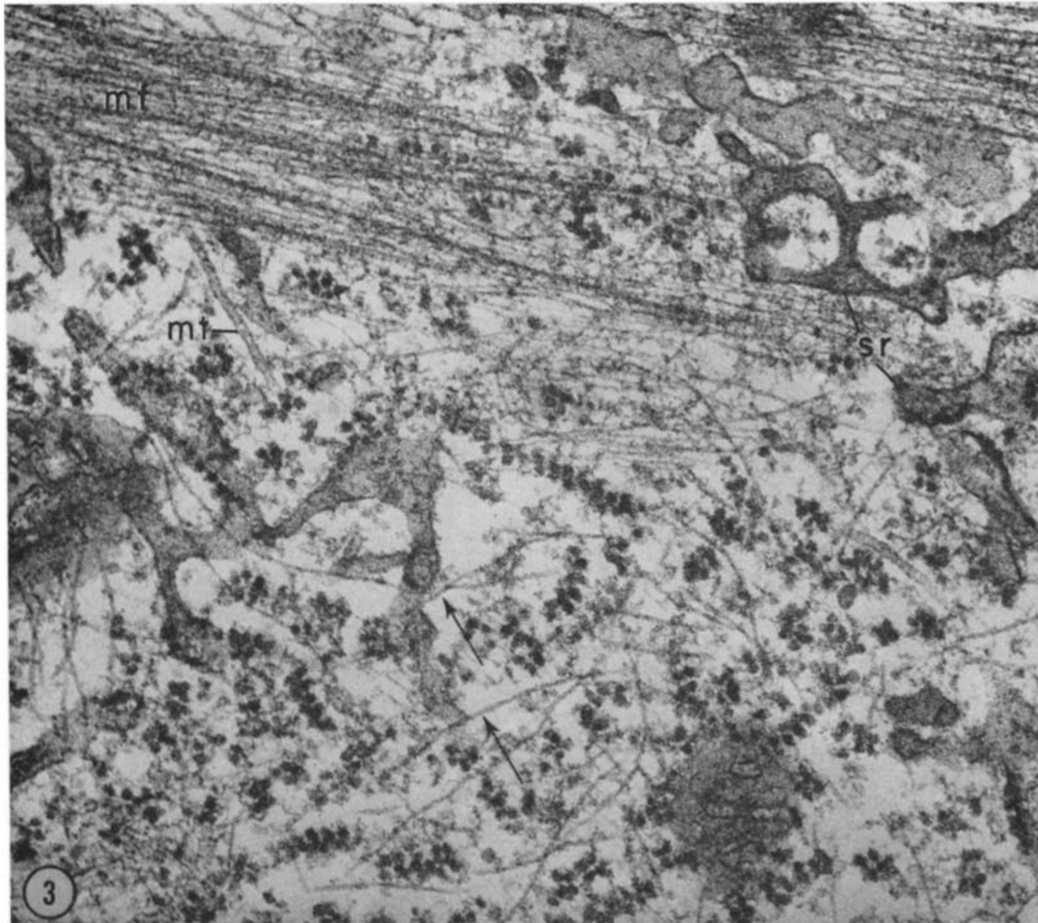

FIGURE 3 Longitudinal section of myotube from a 7 day culture. The free 100-A filaments (arrows) are clearly different in diameter from the thin filaments in the myofibril (*mf*) and show no obvious association with the emerging myofibril. *sr*, sarcoplasmic reticulum. *mt*, microtubule. × 60,000.

tubes were found in virtually all mononucleated cells included in the muscle cultures. These mononucleated cells are fibroblasts, presumptive myoblasts, and myoblasts. The myoblasts, few in number in the younger cultures (Okazaki and Holtzer, 1965), are readily identified by the presence of definitive myofilaments. It is not, on the other hand, always possible to distinguish replicating fibroblasts from replicating presumptive myoblasts. Many large mononucleated cells from 1- to 5-day-old cultures, however, exhibit abundant rough-surfaced endoplasmic reticulum and are probably functional fibroblasts. These cultures are rich in collagen. Cells with rough-surfaced endoplasmic reticulum contain numerous free filaments dispersed throughout the cytoplasm (Fig. 7). The free filaments consist of a single type, 70–110 A in diameter (Fig. 2). In addition, massive aggregates of much thinner (55 A or less) filaments are often observed immediately beneath the plasma membrane in these same cells. Aggregates of these thin filaments are associated with cytoplasmic projections or spike formation in the mononucleated cells and myotubes (see also Taylor, 1966). It is worth stressing that when these myogenic cultures are treated with fluorescein-labeled antibodies against myosin, the meromyosins, or actin, there is no binding of the antisera in regions corresponding to the location of the 100-A filaments (Holtzer, 1961; 1969).

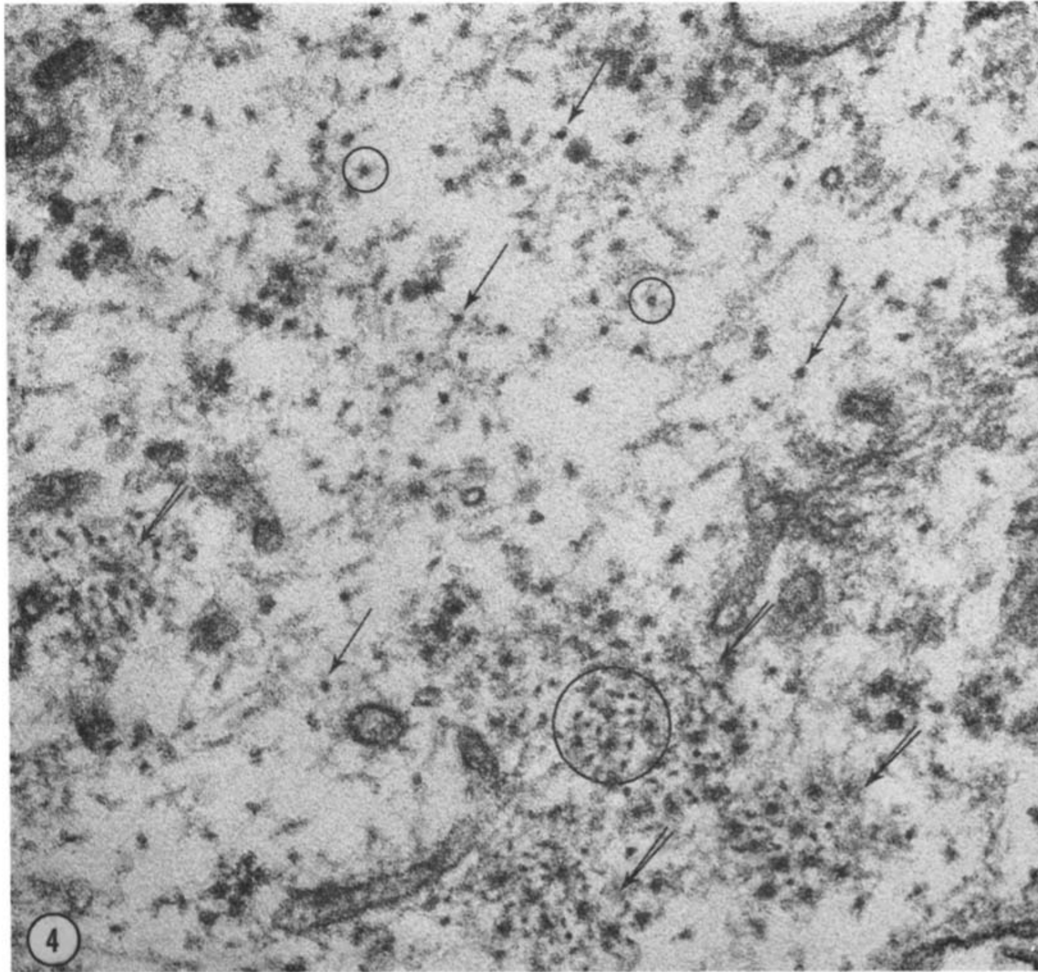

FIGURE 4 Cross-section of myotube from a 2 day culture. The 100-A filaments (arrows) are freely distributed throughout the sarcoplasm and occasionally appear tubular in cross-section (small circles). Compare these with the thin filaments (large circle) within the developing myofibrils (double-stemmed arrows). × 107,500.

Cultures of myogenic cells grown in the presence of 5-bromodeoxyuridine (BUdR) do not fuse or synthesize myosin, but they do replicate (Stockdale et al., 1964; Okazaki and Holtzer, 1965; Coleman and Coleman, 1966). The resulting mononucleated cells are exceedingly flattened and enlarged. When inspected under the electron microscope, the BUdR-treated myogenic cells display goodly numbers of elongated microtubules and large numbers oı 100-A filaments.

Because of the possibility of confusing replicating fibroblasts with replicating presumptive myoblasts, cultures of chondrogenic cells, all derived from a single chondrocyte (Abbott and Holtzer, 1968), were inspected for the 100-A filaments. As this material will be reported in detail elsewhere, it is sufficient to state that many functional and dedifferentiated chondrocytes display large numbers of 100-A filaments as well as bundles of thin filaments less than 60 A. Clearly, the intermediate-sized filament is not uniquely associated with myogenic cells, nor are there greater numbers of such filaments in myogenic cells.

100-A Filaments in Metaphase Cells

Dividing, mononucleated cells are often observed in thin sections of early muscle cultures.

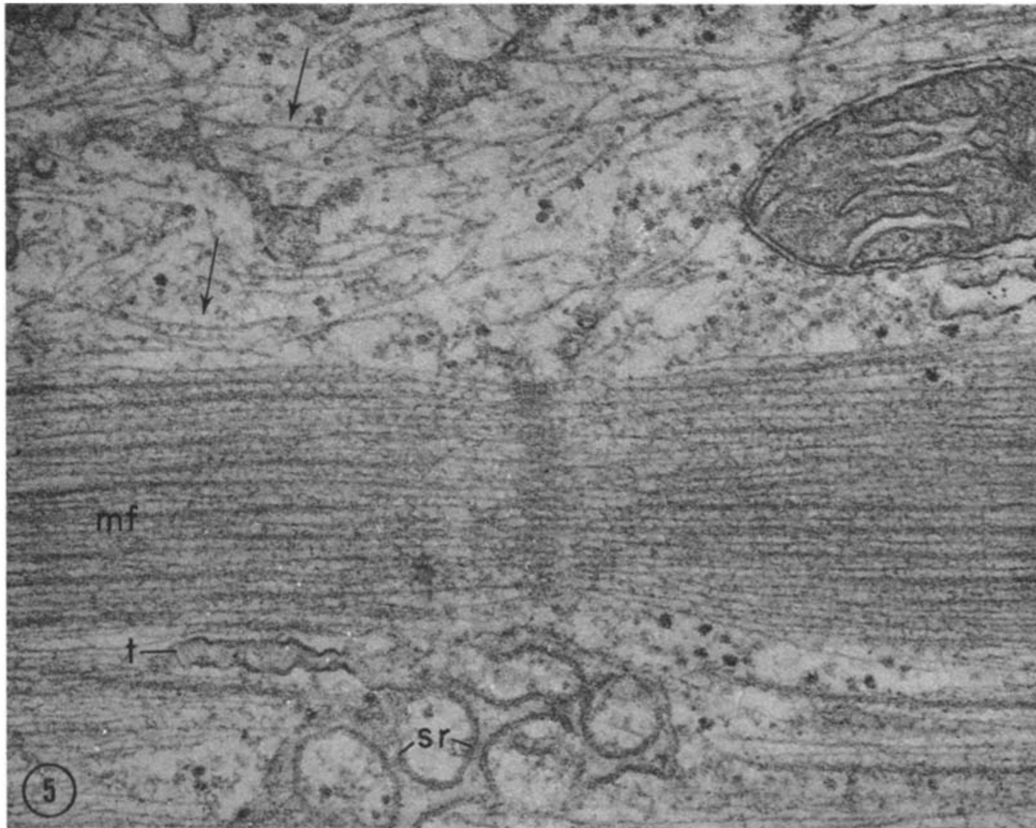

FIGURE 5 Longitudinal section of a well-developed myotube from a 14 day culture. Most of the sarcoplasm is filled with organized myofibrils (*mf*). Free 100-A filaments (arrows) can still be seen pursuing a wavy course in the interfibrillar spaces. No connections can be detected between the free filaments and any elements of the sarcomeres. *sr*, sarcoplasmic reticulum; *t*, T-system tubule. × 70,000.

Cells in various stages of mitosis contain filaments approximately 100 A in diameter and similar in morphology to both the cytoplasmic filaments in mononucleated interphase cells and the intermediate-sized filaments in myotubes (Fig. 8). In order to provide a larger sample of mitotic cells, 1–2-day cultures were incubated in 10^{-6} M colchicine or Colcemid, and the resulting metaphase-arrested (MA) cells were collected. Over 90% of all the cells in these cultures may be arrested in metaphase after 24 hr in the mitotic inhibitors. As controls, we used normal metaphase cells and interphase cells from the inhibitor-treated cultures.

Our observations agree with those of Brinkley et al. (1967) and Robbins and Gonatas (1964) on the effect of mitotic inhibitors on dividing and interphase cells. We found no significant difference between the effects of the two inhibitors despite the observation that cells arrested in metaphase by Colcemid can recover, while those arrested by colchicine do not (Bischoff and Holtzer, 1968). The MA cells have a more rounded contour than have normal metaphase cells, owing to the apparent retraction of all cytoplasmic extensions (Fig. 9). The chromatin is condensed into a central mass, and this tends to become more compacted with increasing exposure to the inhibitors. In occasional sections, paired centrioles can be seen lying adjacent to the chromatin. Spindle tubules are totally lacking in the MA cells.

The most prominent feature differentiating MA cells from normal metaphase cells is the abundance of concentric arrays of membranes surrounding the central chromatin mass (Fig. 9). These mem-

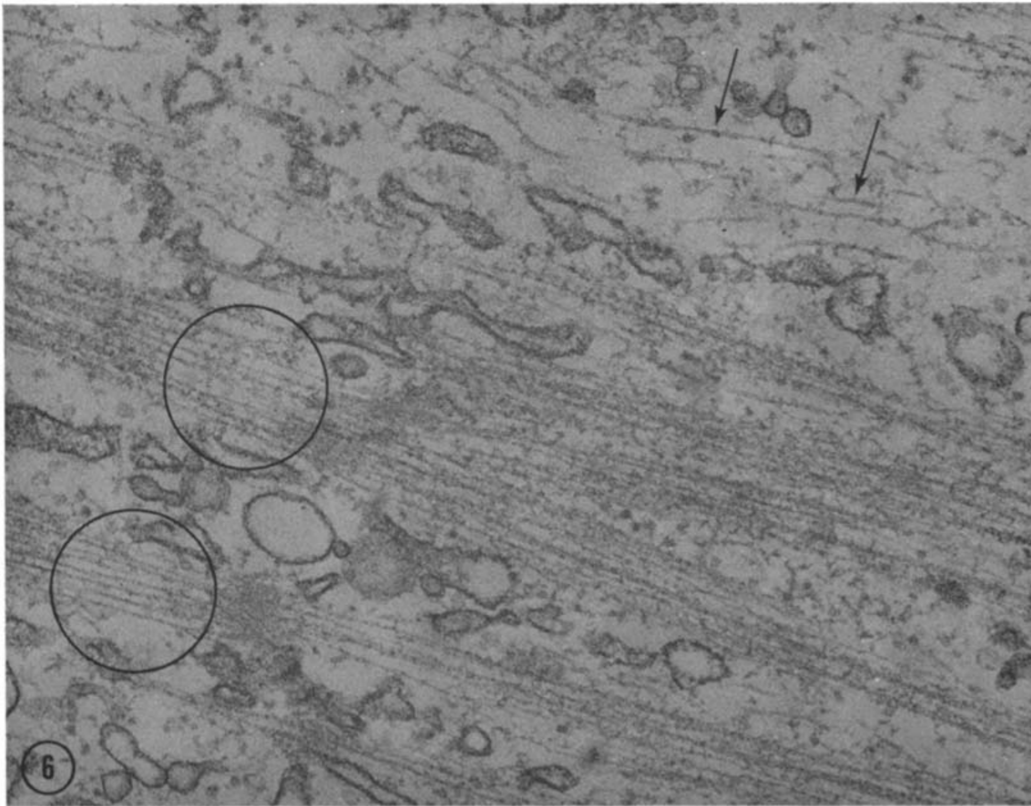

FIGURE 6 Longitudinal section of an early myotube fixed only in OsO_4. The 100-A free filaments can also be seen (arrows) in this preparation, demonstrating that their preservation is not restricted to glutaraldehyde-OsO_4 fixation. Compare with the thin filaments (circles) in the I-bands of the sarcomeres. 4 day culture. × 68,000.

branes are usually smooth-surfaced but occasionally bear attached ribosomes and from a multilayered reticulum of fenestrated lamellae. Interphase cells from inhibitor-treated cultures show no increase in membranous elements. Since metaphase cells are metabolically depressed (Mazia, 1961; Robbins and Scharff, 1966) and protein and RNA synthesis are minimal, the concentric lamellae may be formed by reorganization of preexisting membranes or by reassembly of membrane precursors. It would be of interest if these represent abortive attempts to reform a nuclear envelope.

There is no indication of a developing T system (Ezerman and Ishikawa, 1967; Ishikawa, 1968) in myogenic cells in normal metaphase or in metaphase-arrested cells. The formation of the T system, as of the definitive myofilaments, appears to be a property not of presumptive myoblasts, but of their postmitotic daughter myoblasts (Holtzer and Abbott, 1968; Holtzer, 1969).

The 100-A filaments are found in normal metaphase cells and in MA cells. The 100-A filaments appear to be more prominent in the MA cells than in normal metaphase cells, suggesting an increase in number or grouping of the filaments (Fig. 10). Again, if these filaments are not synthesized during metaphase, then presumably they are assembled from a pool of preexisting molecules. The size distribution of the filaments in the MA cells is similar in pattern to that seen in fibroblasts, chondroblasts, and developing muscle cells (Fig. 2). These filaments are quite different in size and appearance from the definitive thick and thin filaments found in myofibrils.

More than 50 cells in normal metaphase and more than 100 cells in metaphase arrest have now

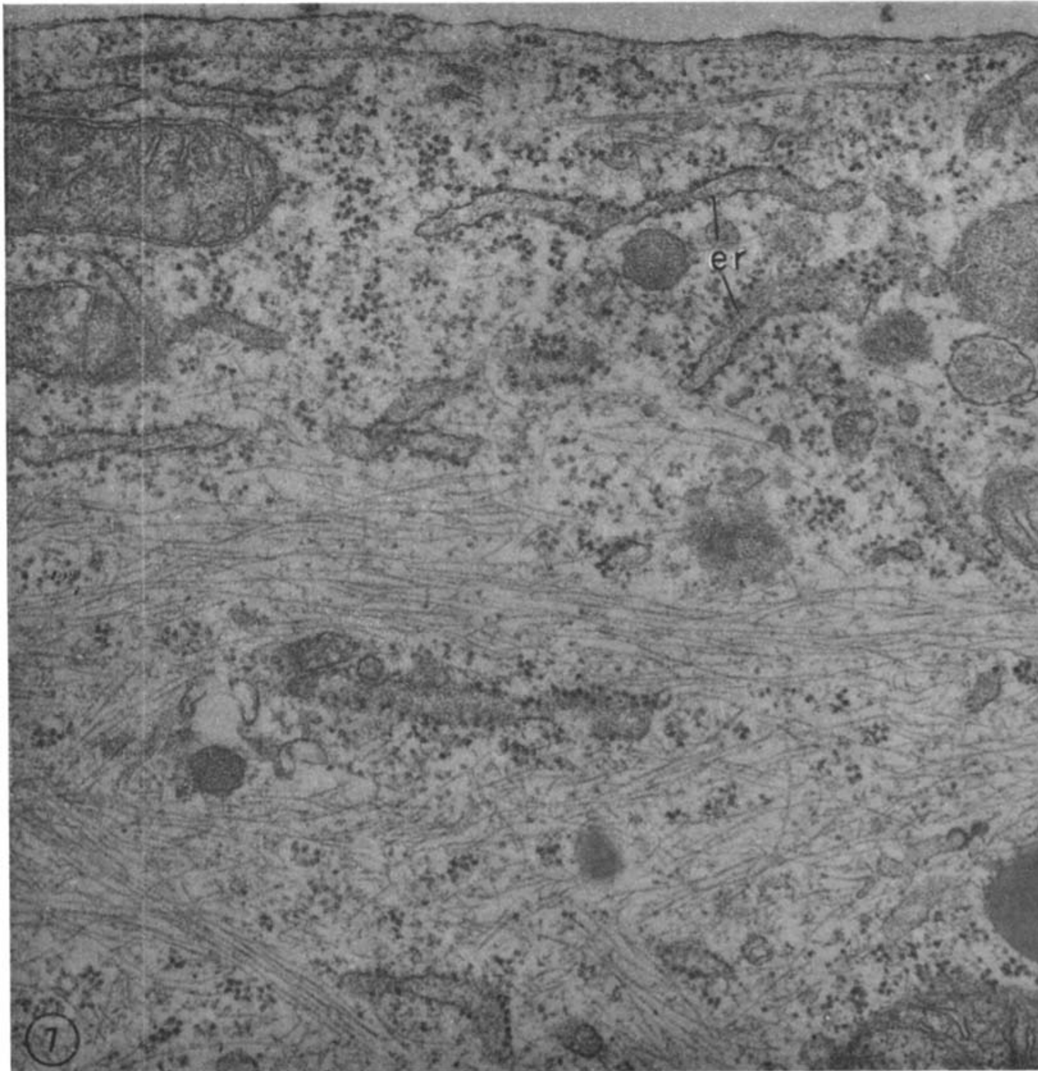

FIGURE 7 Mononucleated cell from a 2 day muscle culture. The cell is rich in rough-surfaced endoplasmic reticulum (*er*) and also contains numerous filaments. These cytoplasmic filaments are similar in diameter and appearance to the intermediate-sized free filaments found within myotubes. × 42,000.

been examined under the electron microscope. In no instance among these 150 cells have definitive thick or thin myofilaments been observed. Metaphase cells from cultures of skeletal muscle do not bind fluorescein-labeled antibody directed against myosin, heavy- and light-meromyosin, or actin (Okazaki and Holtzer, 1965). The agreement between observations based on the labeled-antibody technique and those reported in this study is striking.

Failure to detect positive reactions for myosin or actin by the use of fluorescein-labeled antisera on cells might be attributable to low concentration of antigen per unit area of cytoplasm (Holtzer et al., 1957). Accordingly, the possibility that MA cells contain small quantities of myosin or actin was tested immunologically by double agar diffusion (Marshall et al., 1959, Holtzer, 1961). Pooled cultures of 10^8 cells, consisting of approximately 70% MA cells and 30% mononucleated inter-

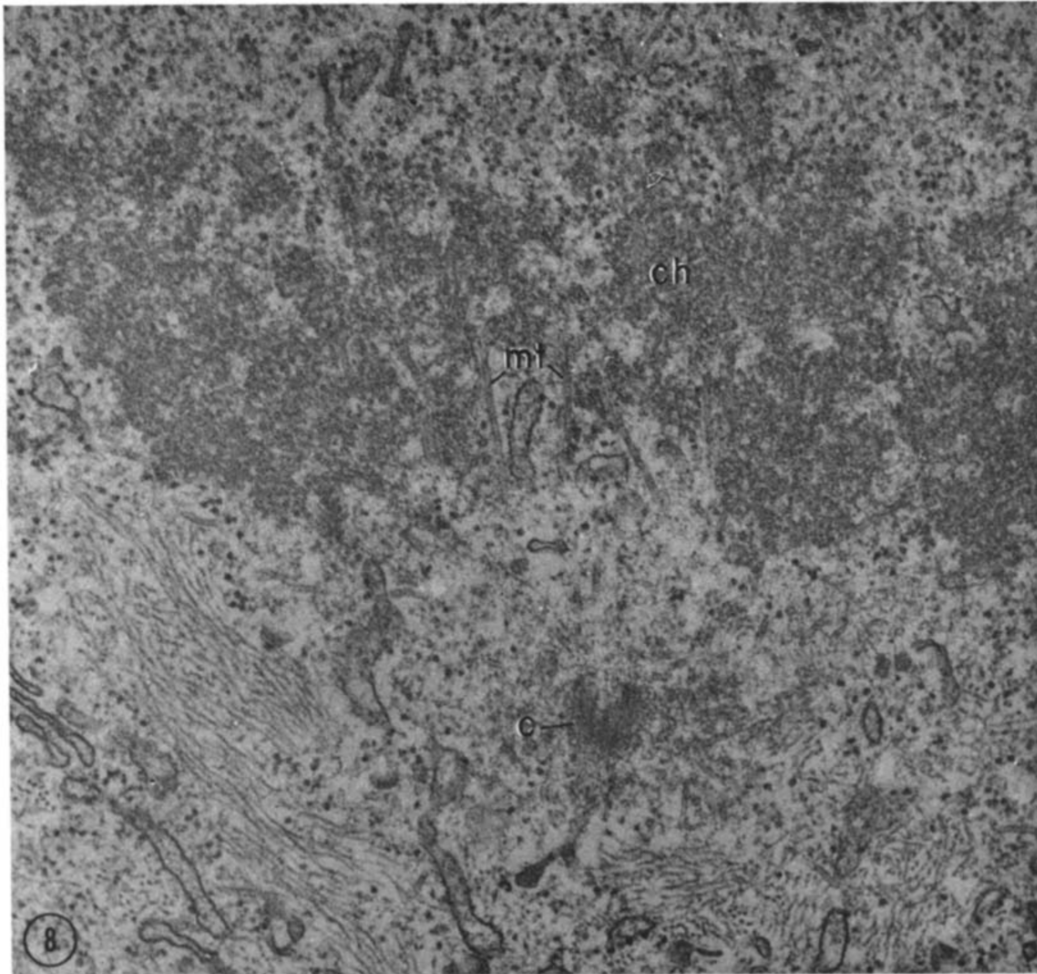

FIGURE 8 Dividing cell (anaphase) from a 6 day muscle culture. Clusters of filaments, 70–110 A in diameter, are disposed around the centriole (*c*). *mt*, spindle tubules. *ch*, chromosome. × 46,500.

phase cells, were extracted with a modified Hasselbach-Schneider solution or 0.6 M KI. Similar extracts of the same numbers of cells from 10-day-old cultures consisting of well-developed myotubes were prepared as positive controls. The extracts were layered over various concentrations of antisera against myosin, heavy-meromyosin, light-meromyosin, or actin. Precipitin bands did not appear in tubes with extracts from MA and mononucleated cells. Precipitin bands did form in tubes with extracts from 10-day-old cultures when these were tested against the four different antisera.

Taken together, these experiments with MA cells suggest that proliferating skeletal myogenic cells have not begun to translate or polymerize the contractile proteins in quantities sufficient to be detected by using the electron microscope or by immunological techniques.

100-A Filaments in Myosacs and Colchicine-Treated Interphase Cells

In addition to their effects on dividing cells, mitotic inhibitors also produce fragmentation of myotubes formed in vitro (Godman, 1955; Bischoff and Holtzer, 1968). These rounded, multinucleated structures which result from the action of mitotic inhibitors on myotubes have been termed

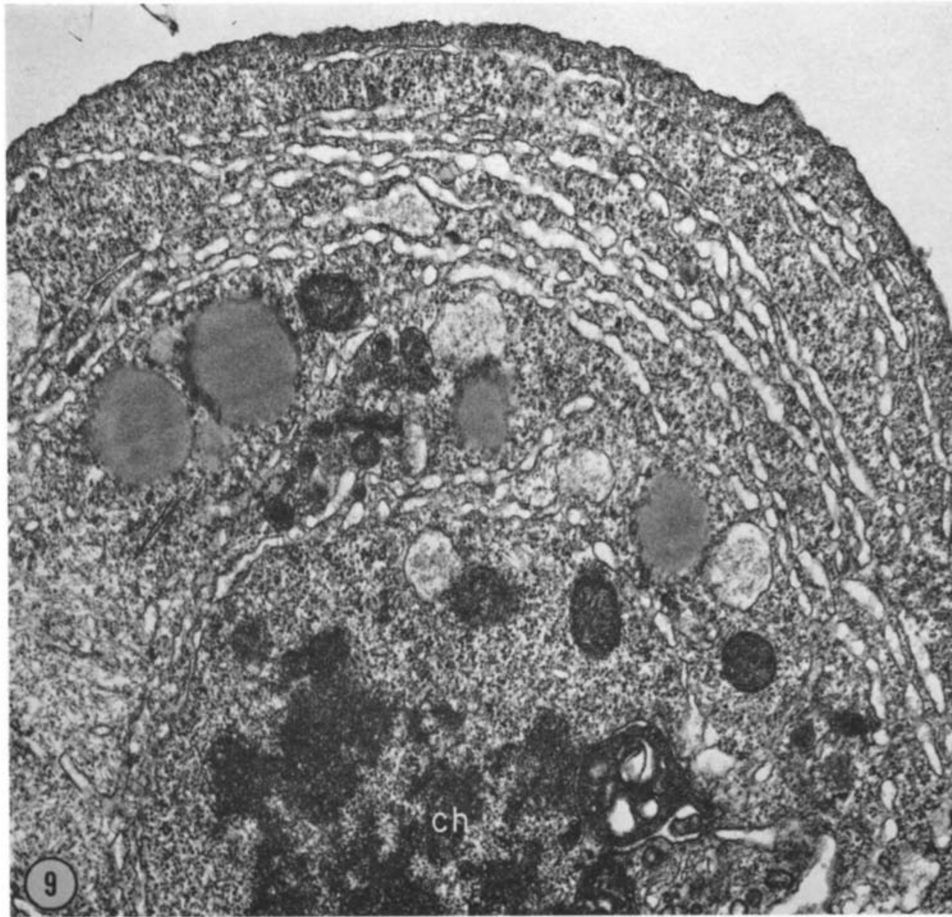

FIGURE 9 Dividing cell in MA collected from an early muscle culture after incubation in 10^{-6} M colchicine for 17 hr. The MA cell has a more rounded contour than normal metaphase cells. Note the abundant concentric arrays of membranes surrounding the central chromatin mass (*ch*). These membranes are smooth-surfaced and form a multilayered reticulum of fenestrated lamellae. Cytoplasmic filaments are also present in this cell (arrow). × 19,500.

"myosacs." Comparison of the effects of the inhibitors on myotube fragmentation and on mitosis in the same cultures indicates that the drug may be acting on similar cytoplasmic elements, presumably microtubules, in both cases. Examination of myosacs in the present study shows that collapse of the myotube is accompanied by a considerable loss of cytoplasmic microtubules. The few microtubules that do survive in the myosacs can be followed only for short distances and appear more convoluted than those in normal myotubes.

In contrast to the microtubules, the 100-A filaments appear more abundant in the myosacs, particularly around the nuclei. Living myosacs examined with the phase-contrast microscope often exhibit peculiar phase-dense structures directly adjacent to the nuclei (Fig. 11). Such structures are not evident in fixed and stained material prepared for light microscopy or for fluorescent microscopy after treatment with labeled antibodies. Upon examination with the electron microscope, these perinuclear regions are found to contain dense populations of intermediate-sized filaments (Figs. 12 and 13). Other cytoplasmic organelles are largely excluded from the areas of packed filaments. These filaments are indistinguishable from the free filaments found in normal myotubes and other cells studied.

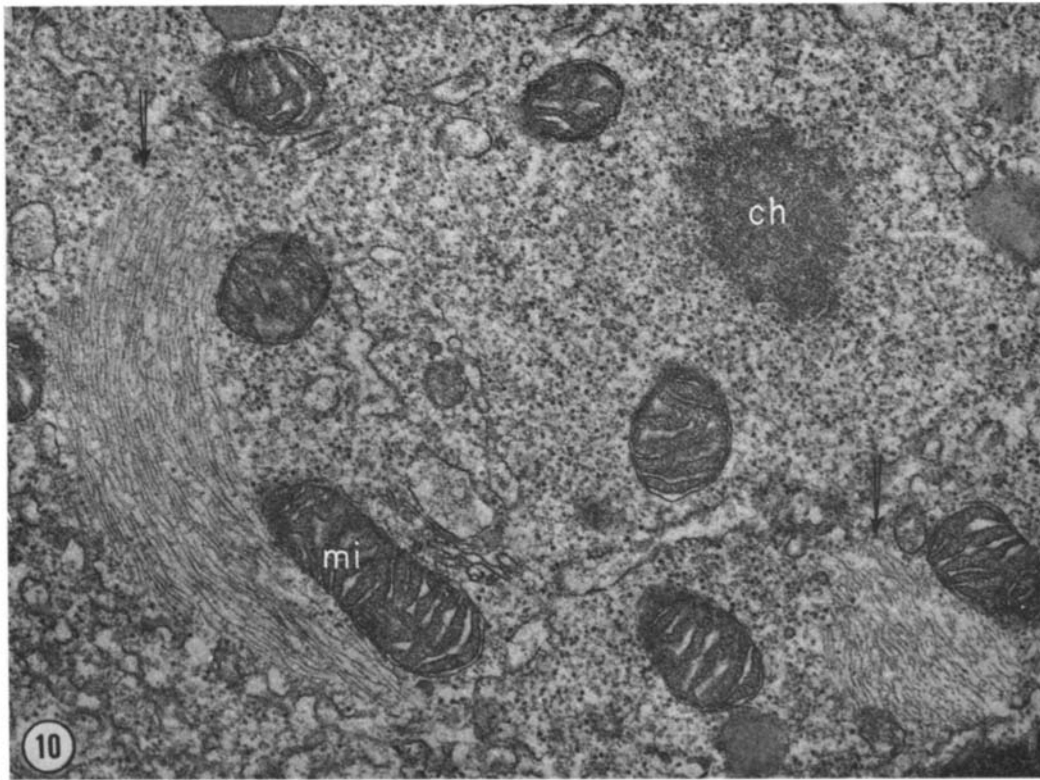

FIGURE 10 MA cell from an early muscle culture exposed to 10^{-6} M Colcemid for 6 hr. 100-A cytoplasmic filaments can be seen scattered or in bundles (arrows). *mi*, mitochondria. *ch*, chromatin mass. × 30,000.

Although myofibrils are reduced in length after colchicine treatment, presumably as a result of mechanical disruption (see Bischoff and Holtzer, 1968), the fine structure of the myofilaments themselves and their association into sarcomeres are not noticeably affected. Indeed, myotubes remain contractile during and after treatment. The incidence of free, thick and thin myofilaments does not increase during myotube fragmentation.

Some mononucleated interphase cells from the colchicine-treated cultures also contain large aggregates of 100-A filaments (Fig. 14). The aggregates are similar to those described in myosacs. In contrast to myosacs, however, the mononucleated cells show no evidence of myofibril formation and contain abundant rough-surfaced endoplasmic reticulum.

DISCUSSION

Many investigators have described free, fine filaments in developing muscle. Opinions differ, however, as to the fine structure, nature, and time of appearance of these free filaments. For example, some investigators claim that (*a*) "actin" filaments appear before myosin filaments (Price et al., 1964; Allen and Pepe, 1965; Obinata et al., 1966; Shafiq et al., 1967), (*b*) "myosin" filaments appear before actin filaments (Firket, 1967), (*c*) there is no detectable difference in time of appearance between the two types of filaments (Hay, 1963; Przybylski and Blumberg, 1966; Fischman, 1967), and (*d*) there is a great excess of actin filaments with respect to myosin filaments (Allen and Pepe, 1965; Przybylski and Blumberg, 1966; Fischman, 1967). Probably some of these differences stem from the problem of rigorously identifying the nature of the "fine" filament and of identifying the type of cell inspected. The diameter reported in the above literature for fine or "thin" filaments varies from 55 to 110 A. Differences in preparing material for microscopy might account for some of the reported variations in diameter of the fine

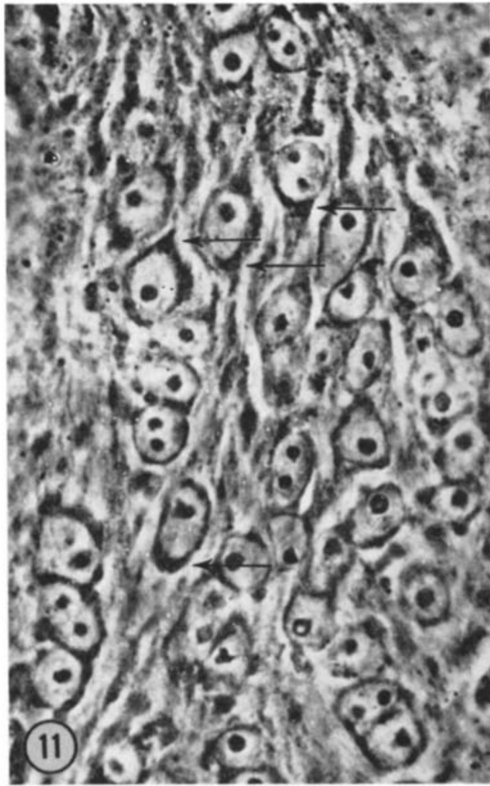

FIGURE 11 Phase-contrast micrograph of a myosac from a living 3 day culture exposed to 10^{-6} M colchicine for 24 hr. The dense "caps" (arrows) on most of the myosac nuclei correspond to areas which, in the electron microscope, are found to contain massive aggregates of the 100-A filaments. × 850.

filaments. However, careful inspection of the published micrographs in these reports[2] strongly suggests to us that the free filaments described by these authors are, in fact, of greater diameter than definitive actin filaments, and that they are the same as the 100-A filaments described in this paper. A recent abstract by Kelly (1968) on developing salamander muscle would also support this view. Franzini-Armstrong (Personal communication.) has also observed, in developing tadpole muscle fibers, filaments which are thinner than thick myofilaments and thicker than thin myofilaments.

[2] For examples, see Obinata et al., 1966, p. 197, Fig. 4; Fischman, 1967, p. 566, Fig. 9; Shafiq et al., 1967, p. 570, Fig. 4.

The majority of free sarcoplasmic filaments observed in the present study are 80–110 A in diameter and may have a continuous length of 2.5 μ in a single section. These filaments are present in the sarcoplasm throughout myogenesis, and there is no evidence that they participate in the formation of the myofibrils. In contrast, thin filaments associated with the thick myofilaments invariably measure 50–80 A in diameter and are approximately 1 μ in length. A small number of free filaments also fall into the 50–70 A range; these may represent grazing sections of nascent myofibrils and possibly free actin filaments. In any case, there is no evidence of an excess of free definitive actin filaments at any stage of myotube development (see, however, Allen and Pepe, 1965; Fischman, 1967). Definitive actin filaments can be identified only in association with myosin filaments. Caution must be used in identifying filaments only on the basis of size. Any thin filament need not be identical with actin since thin cytoplasmic filaments are found in many types of cells (see also Hay, 1963; Taylor, 1966; Buckley and Porter, 1967). In practice, developing muscle cells can be identified by the appearance of myosin filaments and proliferation of the membranous systems (Ezerman and Ishikawa, 1967; Ishikawa, 1968) but not simply by the apperance of thin filaments.

The occurrence of 100-A filaments in various kinds of cells also casts doubt on the unique involvement of these filaments in myofibrillogenesis. Perhaps the most compelling evidence that the 100-A filaments are not associated in an obligatory manner with myofibrillar proteins is the finding that metaphase cells are negative for myosin and actin. Metaphase cells collected from muscle cultures include large numbers of presumptive myoblasts, i.e. cells which will begin to synthesize contractile proteins within a few hours after completing mitosis (Okazaki and Holtzer, 1966; Bischoff and Holtzer, 1966). Virtually all MA cells contain large numbers of the 100-A filaments, yet actin or myosin is not detectable by immunologic tests, including fluorescent antibody staining and gel diffusion; also MA cells do not contain definitive thick or thin myofilaments. These observations taken together make it clear that the 100-A filaments constitute a class of intracellular elements separate and distinct from the thick and thin myofilaments.

The prominence of these filaments in cultured

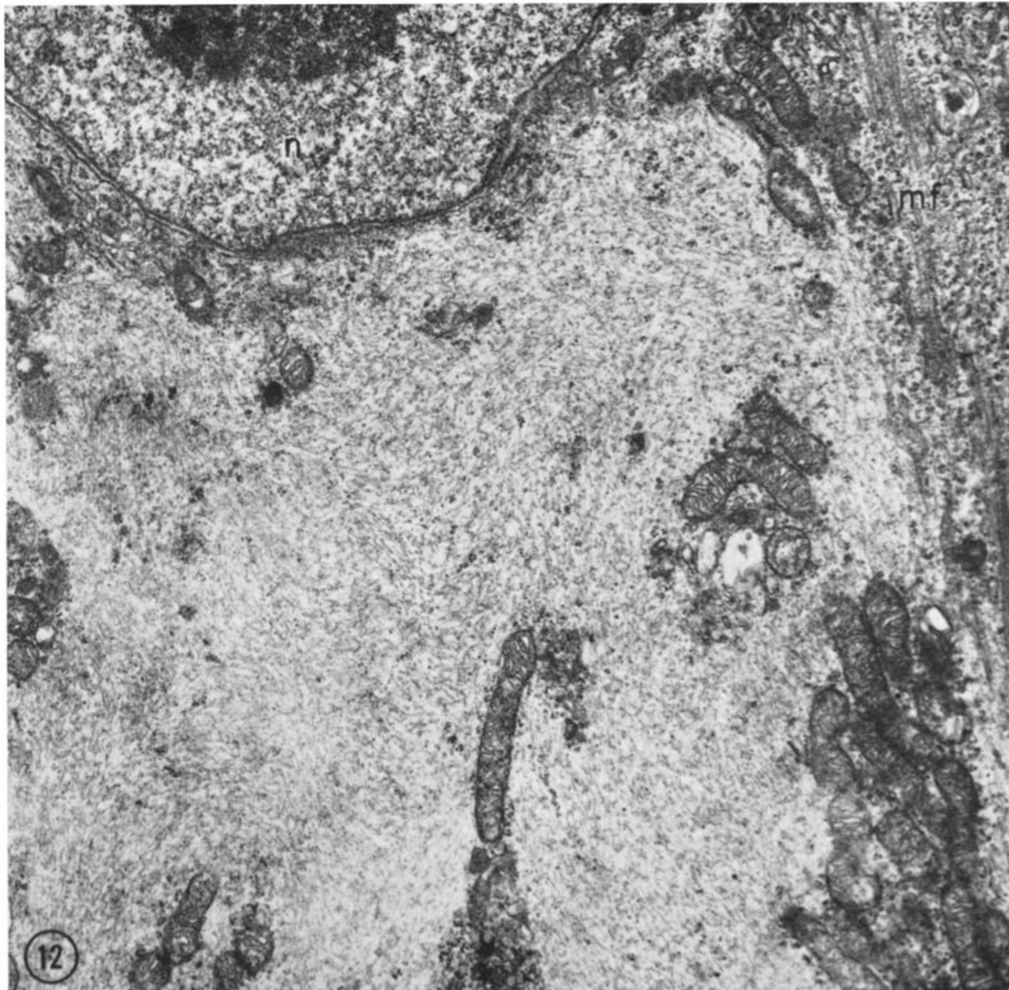

FIGURE 12 Portion of a colchicine-fragmented myotube (myosac). The light area forming a cap around one end of the nucleus (*n*) is packed with 100-A filaments. Compare with Fig. 11. Other cytoplasmic organelles are excluded from the region of packed filaments. *mf*, myofibril. 3 day culture exposed to 10^{-6} M colchicine for 12 hr. × 12,000.

cells is not a peculiar adaptation to the conditions in vitro. Examination of chick embryo skeletal muscle in vivo reveals the presence of filaments similar in size to those described here (Ishikawa, Unpublished observations.).

Presumptive myoblasts in mitosis do not contain definitive thick or thin myofilaments or contractile proteins identifiable by immunological techniques. This mutual exclusivity of proliferation and contractile protein synthesis in normal myogenic cells supports earlier reports (Holtzer et al., 1957; Holtzer, 1958; Stockdale and Holtzer, 1962; Strehler et al., 1963; Bassleer, 1963; Okazaki and Holtzer, 1965, 1966; Przybylski and Blumberg, 1966; Bischoff and Holtzer, 1966). On the other hand, the report by Manasek (1968) unequivocally demonstrates that some myocardial cells in metaphase exhibit thick and thin myofilaments. This may represent a significant difference in the relation between proliferation and the synthesis of contractile proteins in the two types of myoblasts. However, before accepting this interpretation, further studies are needed to determine whether cell division in cardiac myoblasts is a normal

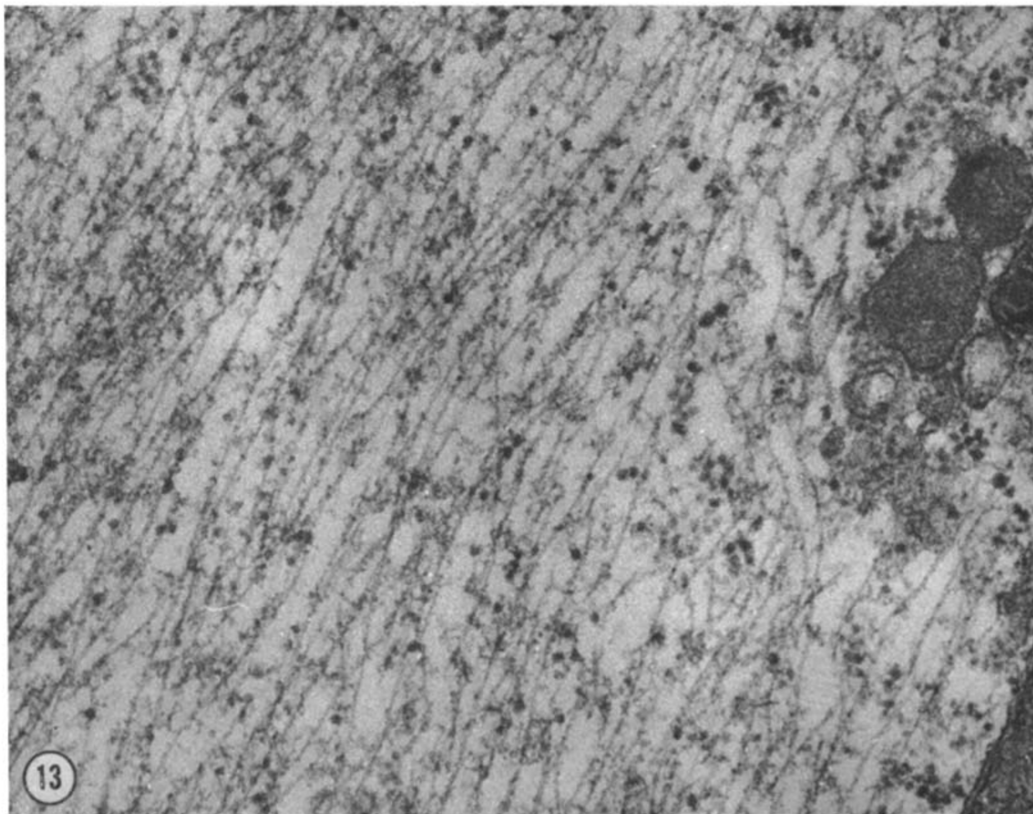

FIGURE 13 Myosac sarcoplasm containing numerous 100-A filaments. Microtubules are rarely found in myosacs but are frequent in myotubes (compare with Figs. 1, 3, and 4). 3 day culture exposed to 10^{-6} M colchicine for 3 hr. × 60,000.

growth process or if dividing cardiac myoblasts degenerate, become polyploid, or form binucleate cells. If any of these conditions are obtained, then differences between cardiac and skeletal muscle cells are minor variations on the same basic theme. Presumptive myoblasts undergo a "quantal" mitosis (Holtzer and Abbott, 1968), leading to the formation of daughter cells which initiate the coordinated translation of myosin and actin. This mitotically-dependent decision to synthesize contractile proteins is coupled with a decision to withdraw from the mitotic cycle. In skeletal muscle cells the decision to synthesize contractile proteins and withdraw from the mitotic cycle is put into effect in the same G_1; in cardiac cells it may take another division or two, after the quantal mitosis, before the decision to withdraw from the mitotic cycle takes effect.

Cytoplasmic filaments, either free or in bundles, have been observed in a great variety of cells. The 90-A filaments described by Claude (1961) in renal tumor cells occur in bundles near the nucleus and in cytoplasmic processes and correspond to areas of birefringence in these cells (Inoué and Sato, 1967). Similar filaments have been described in other vertebrate cells as well (De Petris et al., 1962; Tanaka, 1964; Biberfeld et al., 1965). Wolpert et al. (1964) found masses of 120-A filaments in the isolated cytoplasm of *Amoeba proteus* after contraction was induced by ATP. Fibrous aggregates of fine filaments, 50–70 A in diameter, are present in the cytoplasm of *Physarum* (Wolfarth-Botterman, 1964), *Nitella* (Nagai and Rebhun, 1966), and oat coleoptile cells (O'Brien and Thimann, 1966). Fine filaments are also a prominent constituent of microspikes and cytoplasmic extensions at points of cell-substrate at-

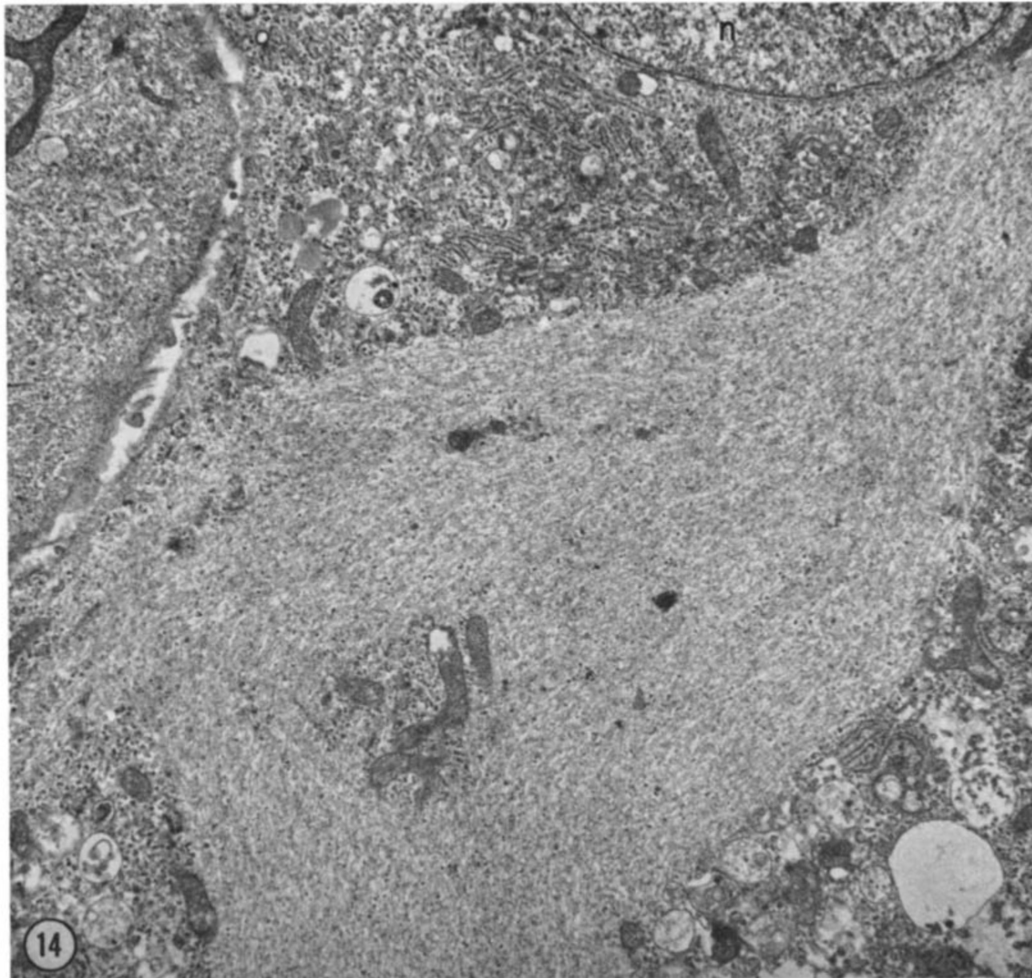

FIGURE 14 Mononucleated interphase cell from 3 day culture exposed to 10^{-6} M colchicine for 12 hr. This cell is rich in rough-surfaced endoplasmic reticulum and contains an area tightly packed with 100-A filaments. *n*, nucleus. × 7,500.

tachment both in vitro (Taylor, 1966; Buckley and Porter, 1967) and in vivo (Ishikawa, 1965).

O'Brien and Thimann (1966) suggested that microtubules and filaments may be alternate states of assembly of the same subunit particle. Our observations may lend support to this theory. The disappearance of microtubules in both MA cells and myotubes after colchicine treatment is accompanied by an apparent enhancement in number of 100-A filaments. Whether the filaments actually increase in number or become locally concentrated through rearrangement has not been determined. The striking increase in phase-dense material in myosacs, however, suggests that new filaments are being formed. These dense areas correspond in shape and in location to regions which contain massive aggregates of 100-A filaments. It is interesting that 100-A filaments are more conspicuous in various kinds of cells after exposure to colchicine. For example, Robbins and Gonatas (1964) report that HeLa interphase cells treated with colchicine show a diminution in microtubules and an increase in cytoplasmic filaments. Normal nerve cells contain cytoplasmic filaments seemingly identical with those described in this paper. Bunge and Bunge (1968) report that after exposure

to colchicine these filaments are more prominent and that the microtubules disappear. Further experiments are required to determine the nature and function of these 100-A filaments which appear in a variety of cell types.

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