

ELECTRON MICROSCOPE OBSERVATIONS ON THE FUSION OF CHICK MYOBLASTS IN VITRO

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ABSTRACT

The process of myoblast fusion was observed in embryonic chick skeletal muscle cells grown in monolayer cultures at the fine structural level. At the first step, the sarcolemmas of cells destined to fuse are closely applied to each other. They are linked in some places by fasciae adherentes; in other places, engulfment of small processes of one cell by another is seen. At a somewhat more advanced stage of myogenesis, vesicles and tubules are formed between the adjacent cytoplasms; presumably, the apposed membranes have opened at several points and their edges have fused to each other. Finally, remnants of cell membranes (vesicles and tubules) disappear completely, and the confluent cytoplasm is formed. The cytoplasmic contents of the multinucleated cells are often poorly admixed, giving the cytoplasm a mosaic appearance in which different zones can be designated as arising from separate cells. This observation suggests, however, that there is slow diffusion of myoblast contents (ribosomes and, possibly, other materials) into the myotube. In agreement with the previous works at the light microscopic level, the present study suggests the occurrence of fusion between mononucleated cells, between mononucleated cells and multinucleated myotubes, and between nascent multinucleated myotubes.

INTRODUCTION

One of the most controversial aspects of muscle differentiation has been the question of how vertebrate skeletal muscle cells become multinucleate. Two hypotheses have been put forward to account for this problem: one, that multinucleated muscle fibers arise by the coalescence of individual cells ("multicellular theory"); and two, that each syncytial fiber arises from one cell by amitotic nuclear division without cytokinesis ("unicellular theory"). The literature on this subject has been reviewed by Godman (15) and Murray (34). The evidence to support the "amitosis" hypothesis was based largely on examination of fixed and stained preparations (1, 15, 32, 38, 44). The evidence in support of the fusion hypothesis has been provided through the use of new techniques: microspectrophotometry (26), hybridi-

zation of myoblasts from different genetic origins (29, 45, 46), the use of inhibitors of DNA synthesis (25) and of mitosis (4), time-lapse cinephotomicroscopic techniques (7, 9), and the use of isotopically labeled nucleic acid precursors (4, 5, 27, 29, 42).

Electron microscope studies on the developing skeletal muscle *in vivo* (3, 8, 10, 13, 17, 23, 39, 40) and *in vitro* (12, 14, 41) strongly favor the multicellular theory. However, a detailed account of the process of myoblast fusion has not been presented. In the present study, this process was investigated by electron microscopy, with the use of embryonic chick skeletal muscle cells grown in monolayer cultures. It has been demonstrated that the developmental sequence of myogenesis *in vitro* closely resembles that *in vivo* (41) except for

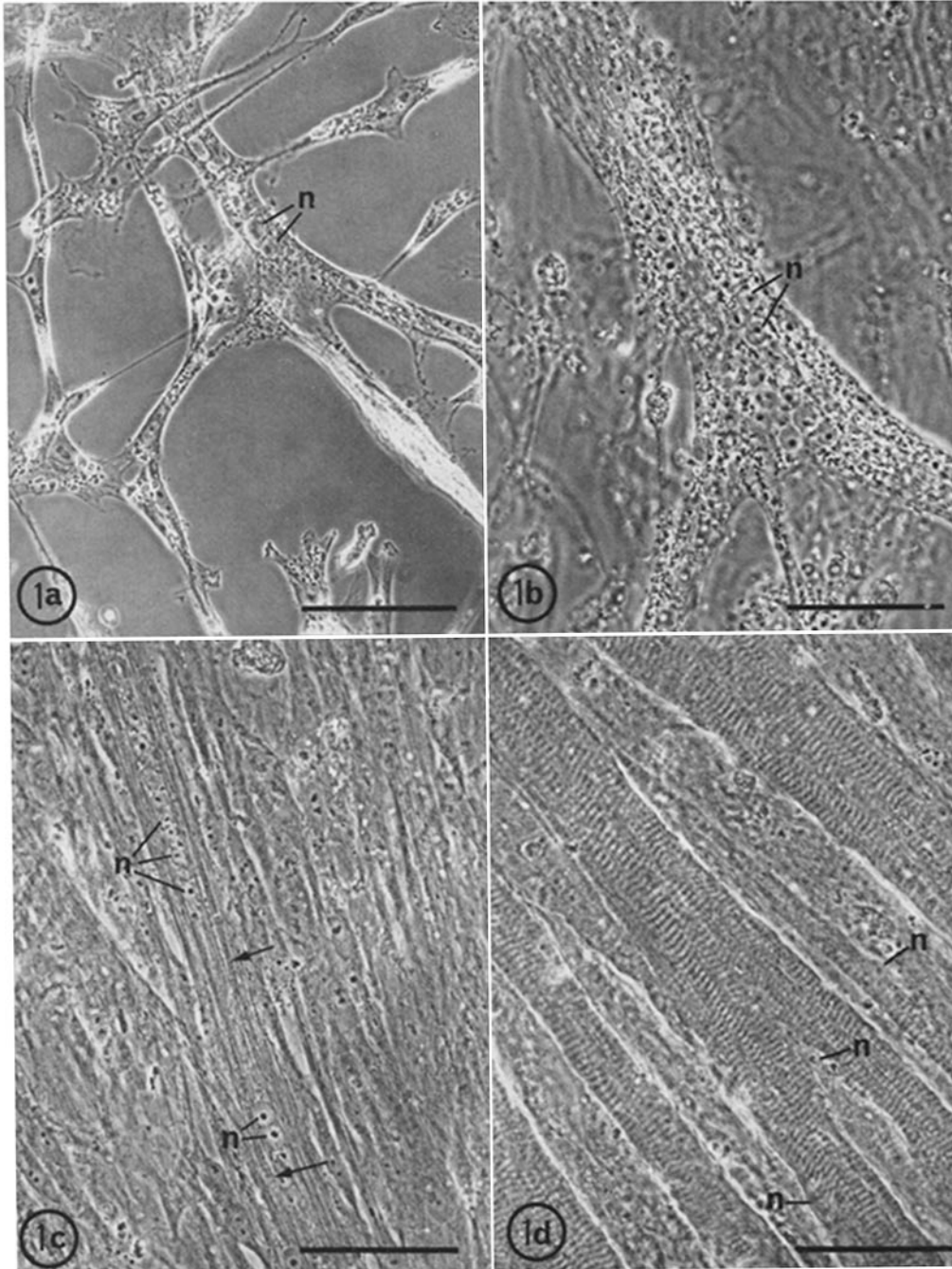


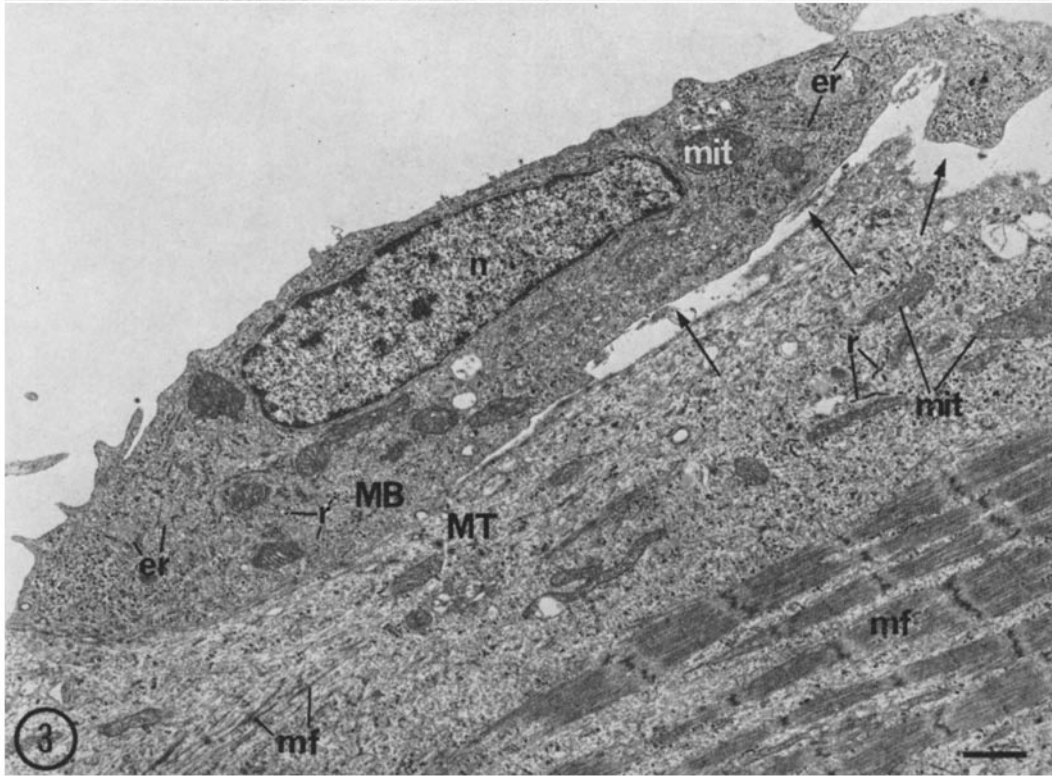
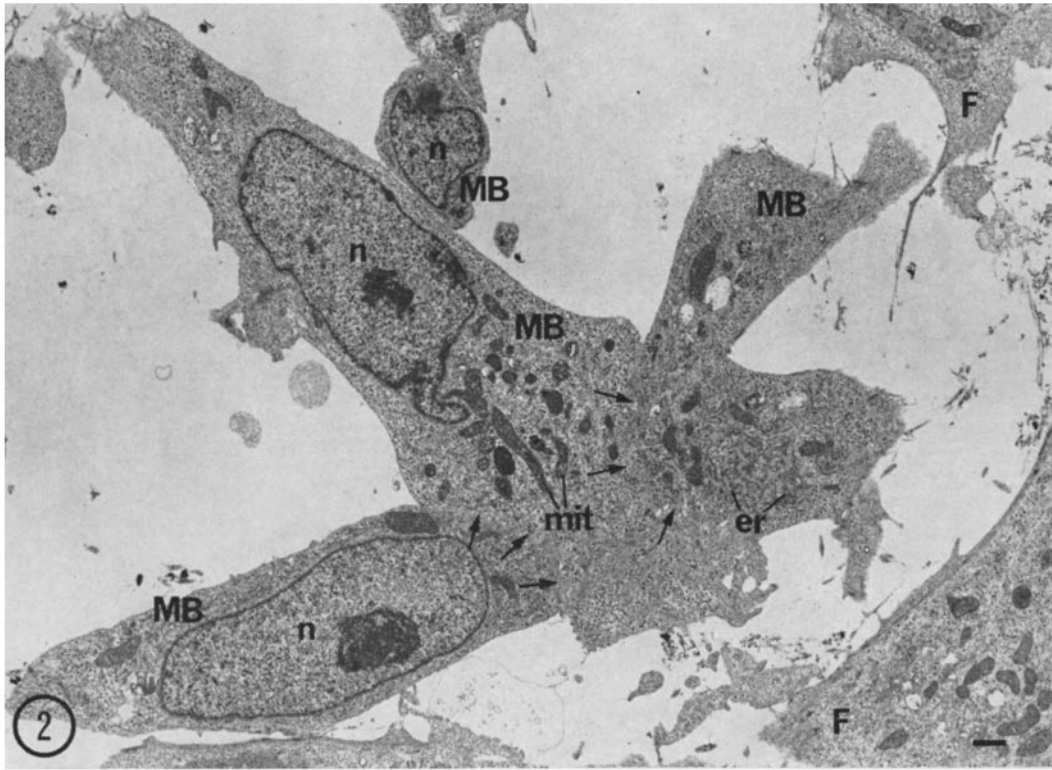
FIGURE 1 Four phase-contrast photomicrographs of myogenic cells after varying times in monolayer cultures. Calibration bar = 50μ . $\times 450$.

Fig. 1 *a* 4 day culture. Clustered myoblasts are seen. Cells in such aggregates undergo cytoplasmic fusion to form multicellular, elongated myotubes. Nuclei, *n*.

Fig. 1 *b* 6 day culture. The typical morphology of the multinucleated myotubes is seen. Anastomoses of myotubes are observed. Nuclei, *n*.

Fig. 1 *c* 8 day culture. Cross-striations begin to appear (arrows). Nuclei (*n*) are arranged lengthwise in the central axis of fibers.

Fig. 1 *d* 10 day culture. Mature muscle straps. Myofibrils with characteristic cross-striations fill almost the entire cytoplasm of the muscle fibers. Nuclei (*n*) are seen at the cell periphery.



hypernucleation and branching of myotubes (36) and an overproduction of T-system tubules (19). But, certain advantages of tissue culture material prompted us to use this technique in this study. First, muscle cells in vitro differentiate very rapidly under the conditions used (36) and may be readily scanned by phase-contrast microscopy for the selection of different stages of muscle development. Second, there is a degree of synchronizing of cell fusion that cannot be observed in in vivo material (37). Last, the plane of sections is automatically parallel to the longitudinal axis of developing muscle cells when they are embedded in flat face.

MATERIALS AND METHODS

Suspensions of embryonic skeletal muscle cells were obtained from thigh muscles of 12-day chick embryos by using the standard trypsinization procedure of Moscona (33). The cells were dispersed in culture medium (Eagle's basal medium with glutamine, 10% horse serum, 10% embryo extract, and 1% penicillin-streptomycin, Microbiological Associates, Inc., Bethesda, Md.) and distributed into 35-mm plastic culture dishes (Falcon Plastics, Los Angeles, Calif.) with round glass coverslips (15 mm in diameter) on the bottom, at a concentration of 4×10^6 cells per dish. The coverslips were precoated with silicone, then covered with evaporated carbon (41), and overlaid with collagen (16). All cultures were maintained at 38°C in an atmosphere of 5% CO₂ in air, at saturation humidity. The culture medium was changed every 2 days. Cultures of various stages were fixed in phosphate-buffered 2.5% glutaraldehyde and postfixed in similarly buffered 1% osmium tetroxide (pH 7.4). After rapid dehydration, the cultures were embedded in Araldite, split off the coverslips, and cut with an LKB 4800 Ultratome ultramicrotome. Thin sections were stained sequentially with phosphotungstic acid, uranyl acetate, and lead citrate (13), and were examined with a Hitachi HU-11E electron microscope operated at 75 kv. Coverslip cultures fixed in 2.5% glutaraldehyde were

also examined as whole mounts by phase-contrast microscopy.

RESULTS

By trypsinizing chick embryonic skeletal muscle, a cell population is obtained which differentiates in vitro into striated muscle fibers (24, 41, 42). Freshly dispersed muscle cells in suspension are spherical. In the culture dishes, the cells settle rapidly out of suspension, attach to the substrate, and within hours assume elongated shapes. The first few days of culture are characterized by prominent mitotic and migratory activity. Fusion of myoblasts¹ and beginning of myotube² formation are observed already by the end of the 2nd day of culture. These myotubes continue to grow by repeated cytoplasmic fusion with myoblasts and/or other myotubes and assume the characteristic shapes of long, branched myotubes (Figs. 1 *a* and *b*). Within the cytoplasm of these myotubes, cross-striated myofibrils begin to appear after 7 days of culture (Fig. 1 *c*). By the 10th day, myofibrils fill the cytoplasmic space within the developing muscle fibers (Fig. 1 *d*), and spontaneous contractions of individual myofibers are evident. In the present study, the attention was focused on early stages of myogenesis when adhesion and fusion of myoblasts and myotubes are most prominent (between the 4th and 8th days in culture).

An electron micrograph of a cluster of mononucleated myoblasts (*MB*) similar to that in Fig. 1 *a* is shown in Fig. 2. These cells are spindle-shaped and show little structural evidence of muscle differentiation; the cytoplasm contains

¹ A myoblast denotes a mononucleated muscle precursor cell not yet fused with another myoblast. ² A myotube is the term given to the cell with two or more nuclei.

FIGURE 2 An electron micrograph of a cluster of myoblasts (*MB*) similar to that in Fig. 1 *a*. Myoblasts have bipolar shape. Cell boundaries appear as fuzzy lines (arrows), because they are cut obliquely. Mitochondria, *mit*; rough-surfaced endoplasmic reticulum, *er*; fibroblastic cell, *F*; nucleus, *n*. 4 days in vitro. Scale marks on electron micrographs equal 1 μ . $\times 4,500$.

FIGURE 3 A mononucleated cell (*MB*), presumably a myoblast, is apposed to a myotube (*MT*). The left part of the mononucleated cell is contiguous with the myotube, while a wide space is seen between the right part of the mononucleated cell and the myotube (arrows). Myofibrils (*mf*) at various developmental stages are seen in the myotube. Mitochondria, *mit*; ribosomes and polysomes, *r*; rough-surfaced endoplasmic reticulum, *er*; nucleus, *n*. 6 days in vitro. $\times 8,100$.

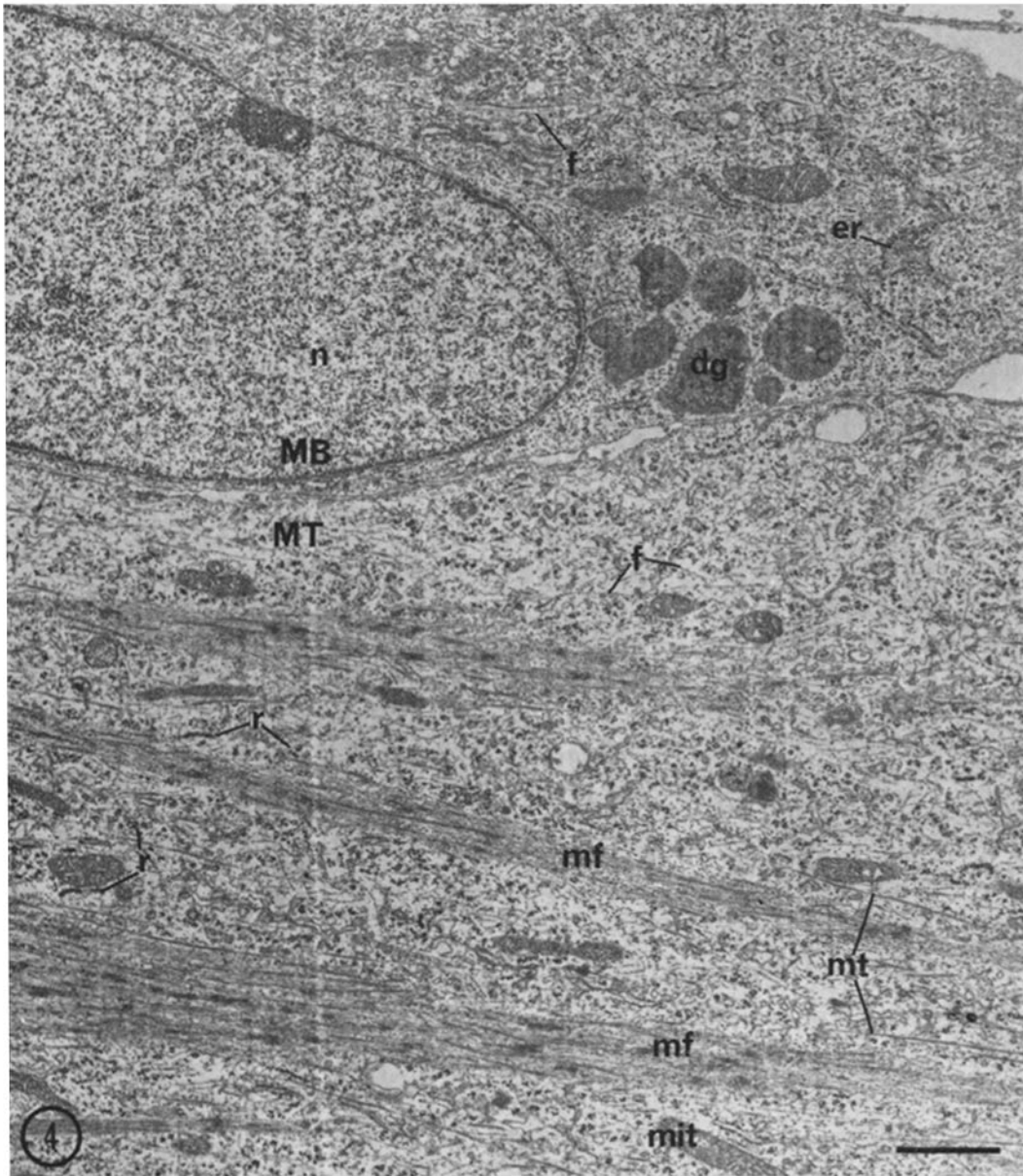


FIGURE 4 An electron micrograph of a mononucleated cell (*MB*) adjacent to a myotube (*MT*). The plasmalemmas of the contiguous cells run parallel. Dense granules (*dg*) are seen, which are tentatively identified as an autophagic vacuole variety of lysosomes (see Shimada et al., 41). Myofibrils, *mf*; microtubules, *mt*; ribosomes, *r*; 100-A filaments, *f*; nucleus, *n*. 6 days in monolayers. $\times 13,000$.

numerous free ribosomes, rod-shaped mitochondria (*mit*), and a small amount of granular endoplasmic reticulum (*er*), but are devoid of myofibrils. The close apposition of myoblasts within a cluster suggests that they either undergo or soon undergo

cytoplasmic fusion, forming a large multinucleated muscle fiber. Parts of irregularly shaped, isolated cells (*F*) are seen. Observing monolayer cultures of embryonic leg muscle cells under phase-optics, Konigsberg (24) has reported that the isolated

cells which give rise to fibroblast-like colonies are extremely flattened and irregular in outline. Thus, although little fine structural evidence to classify between the cytoplasm of a myoblast and a fibroblastic cell is found, these cells are interpreted as fibroblastic judging from their irregular contours.

A mononucleated cell (*MB*), presumably a myoblast, apposed to a myotube (*MT*) is shown in Fig. 3. At the left part of this micrograph, these two cells are closely applied, while at the right part they are separated by a wide intercellular space (arrows). The myotube contains myofibrils (*mf*) at various stages of development, elongated mitochondria (*mit*), and helical aggregates of ribosomes (*r*; see Waddington and Perry, 43). The mononucleated cell is spindle-shaped, and its prominent cytological features are as follows: abundant ribosomes and polysomes (*r*), round and rod-shaped mitochondria (*mit*), and the ab-

sence of myofibrils. The endoplasmic reticulum (*er*) in the mononucleated cells has ribosome-studded profiles, and there is a moderate amount of fine granular material in its distended lumen (Figs. 2-4).

A somewhat more advanced stage of myogenesis is presented in Figs. 4-7. A mononucleated cell (*MB*), presumably a myoblast, is closely applied to the surface of a multinucleated myotube (*MT*). The plasmalemmas of the adjoining cells run parallel and are separated by an intercellular space of uniform width (300-450 Å), leaving occasionally wide spaces between them. Adjacent cells are linked in some places by attachment plaques (fasciae adherentes) with symmetrical aggregations of electron-opaque material towards the membranes (arrows, Figs. 5 and 6). In other places, the membrane of a mononucleated cell shows small finger-like projections towards a

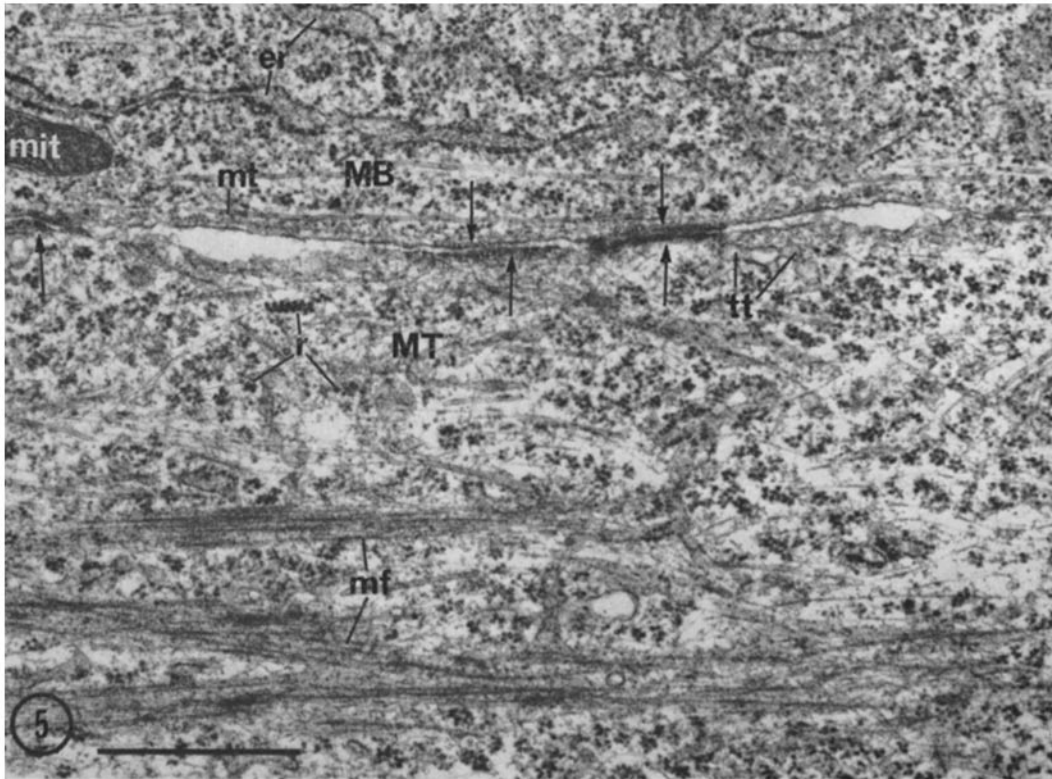


FIGURE 5 A higher magnification view of the site of attachments of a myoblast (*MB*) with a myotube (*MT*). The sarcolemmas generally run parallel. Sarcolemmal thickenings (fasciae adherentes) are seen (arrows). Myofibrils, *mf*; granular endoplasmic reticulum, *er*; ribosomes, *r*; T-system tubules, *tt*; microtubules, *mt*; mitochondria, *mit*. 4 day culture. $\times 28,000$.

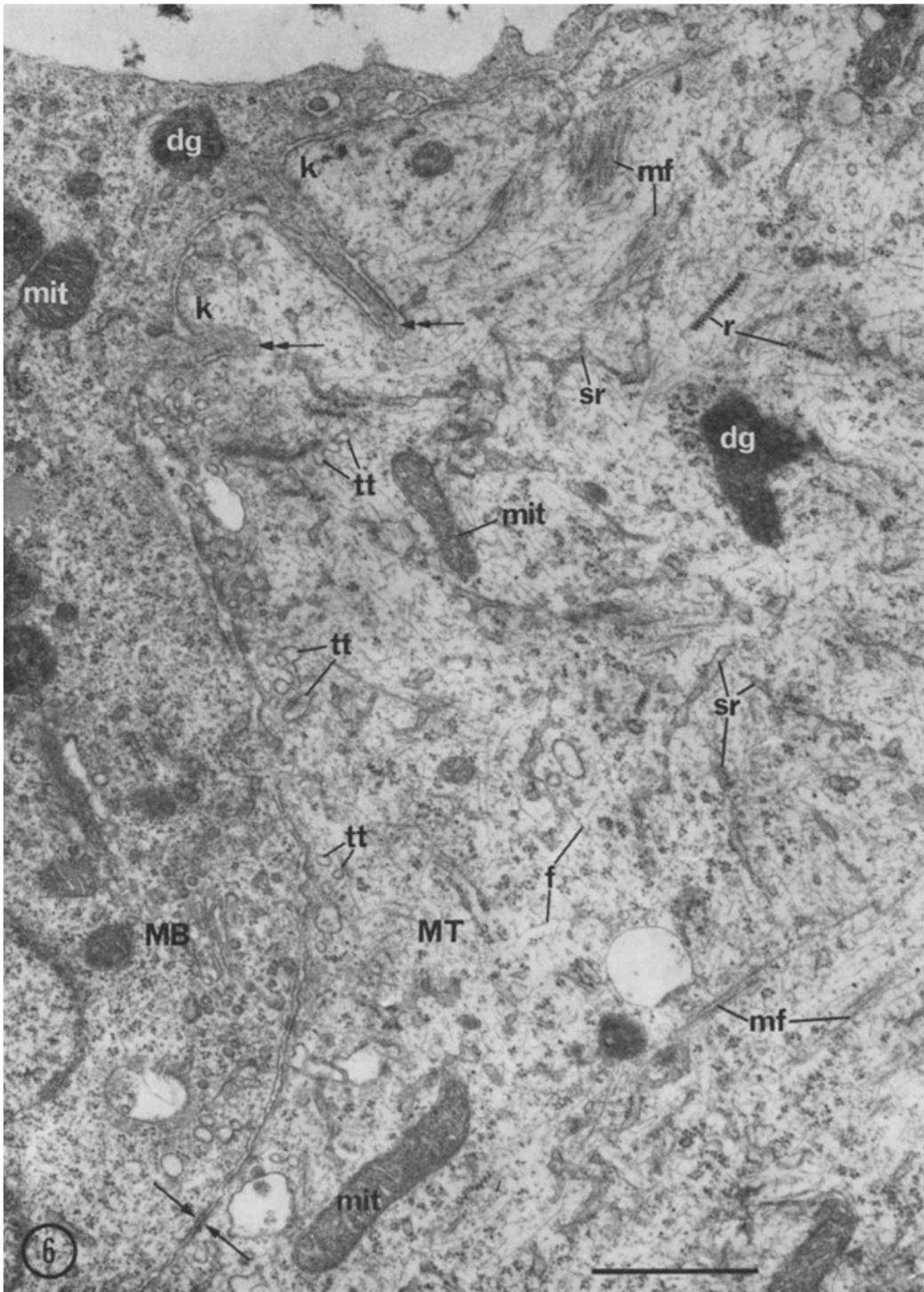


FIGURE 6 An electron micrograph similar to that in Fig. 4. The sarcolemmas of the adjacent cells run parallel. Sarcolemmal thickenings (arrows) are observed at the area of the apposed membranes. Finger-like projections (double arrows) of a myoblast (*MB*) membrane are seen, which are embedded in caveolae (*k*) of a myotube (*MT*). T-system tubules, *tt*; myofibrils, *mf*; ribosomes and polysomes, *r*; sarcoplasmic reticulum, *sr*; mitochondria, *mit*; dense granules, *dg*; nucleus, *n*; 100-A filaments, *f*. 4 day culture. $\times 25,000$.

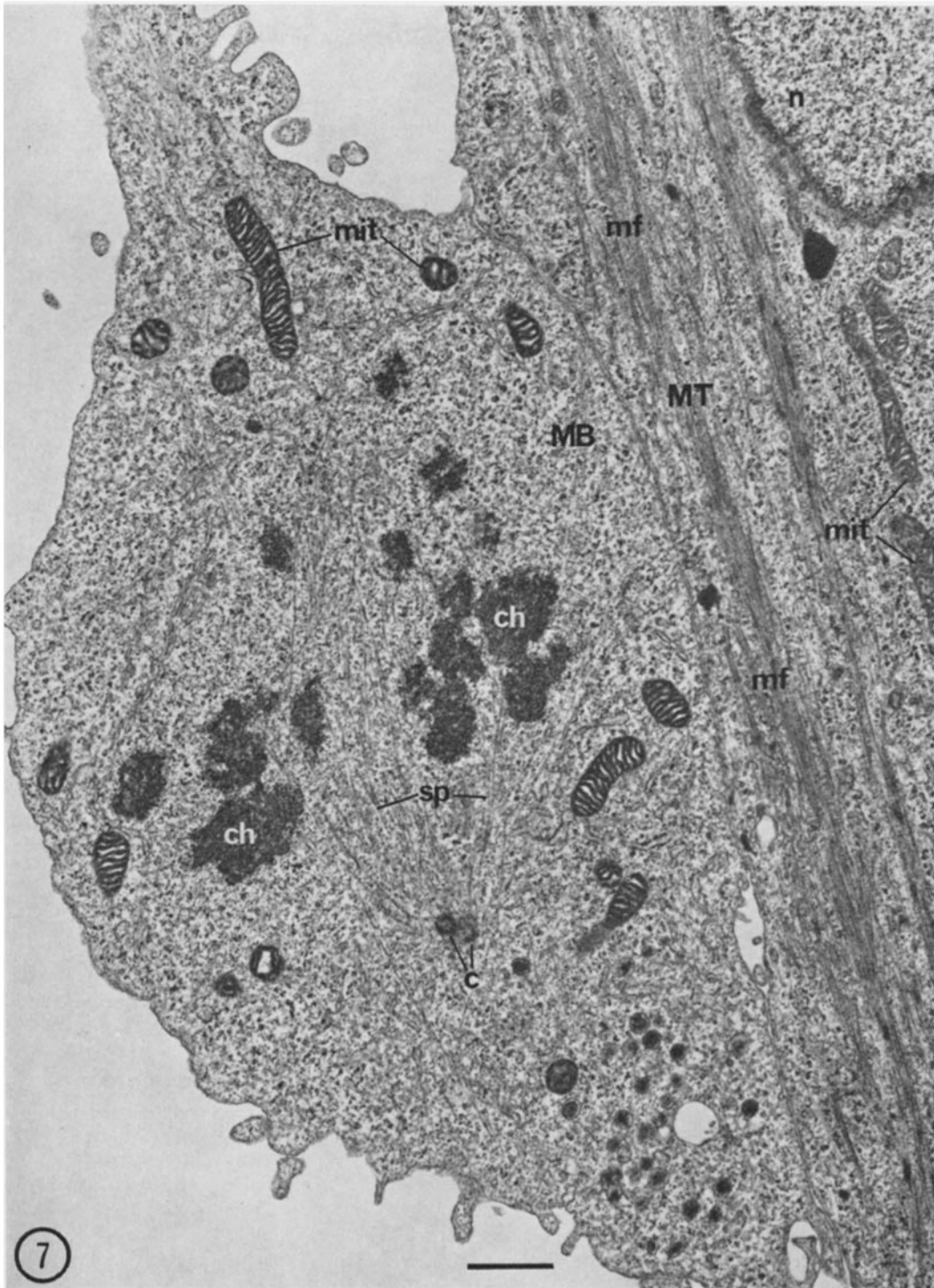


FIGURE 7 Dividing myoblast (metaphase) from a 6 day muscle culture. This cell (*MB*) is in close apposition to a myotube (*MT*). Sarcolemmas of the adjoining cells run parallel. Young myofibrils (*mf*) are seen in the myotube, while actomyosin has not been synthesized in the cell in mitosis. In the dividing cell, chromosomes (*ch*), spindle fibers (*sp*), and centrioles (*c*) are seen. Mitochondria, *mit*; nucleus, *n*. $\times 12,500$.

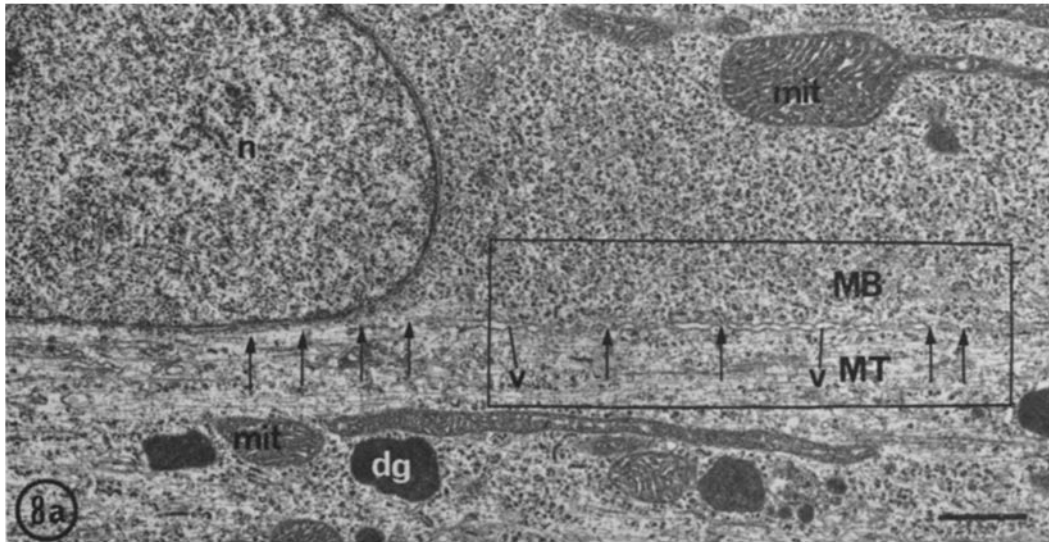


FIGURE 8 *a* An electron micrograph of cells in the process of cytoplasmic fusion. The cell border of a myoblast (*MB*) is partially disrupted, showing cytoplasmic continuity (arrows) with the adjacent myotube (*MT*). Mitochondria, *mit*; nucleus, *n*; dense granules, *dg*. 6 days in vitro. $\times 11,000$.

myotube (double arrows, Fig. 6). Complex tubular networks (*tt*) are often seen (Figs. 5 and 6). Such structures are considered to represent the first stages of T-system tubules (11, 19).

Dividing, mononucleated cells are often observed in early muscle cultures. In Fig. 7, a myoblast (*MB*) in metaphase is shown which is in close apposition with a myotube (*MT*). Chromosomes (*ch*) on the metaphase plate, spindle fibers (*sp*), and centrioles (*c*) are seen in the myoblast. Myofibrils are not visible in the cells in mitosis; they are considered to be synthesized after the completion of cell division. Thus, our observations agree with the concept that cell division and myofibril differentiation are mutually exclusive events in skeletal muscle differentiation (36, 42).

Somewhat later phases of muscle differentiation are shown in Fig. 8 *a*. Observing the boundaries between the closely associated cells (a myoblast [*MB*] and a myotube [*MT*]), one can see that the apposed sarcolemmas have disappeared in several regions (arrows). In Fig. 8 *b*, the apposed cell membranes of the squared-off area in Fig. 8 *a* are shown at a higher magnification. At several points indicated by arrows, the plasma membrane of the myoblast (*MB*) is continuous with that of the myotube (*MT*). Thus, vesicles and tubules (*v*; 800–1,000 Å or more in diameter) are found be-

tween these cells. Such cells which show interrupted cell membranes and the establishment of cytoplasmic continuity are considered to represent an intermediate stage of cytoplasmic fusion of a myoblast with a myotube. This observation clearly indicates that the muscle-forming cells at different developmental stages are capable of fusing to form multinucleated fibers. Although the two cytoplasms become confluent by fragmentation of sarcolemmas, the cytoplasmic contents are very different; the ribosome content, per unit area, in the myoblast is much greater than that in the myotube.

In Fig. 9 *a*, two multinucleated myotubes (*MT*₁ and *MT*₂) are seen, the sarcolemmas of which are closely applied (arrows). Such close apposition suggests that fusion might occur between nascent multinucleated myotubes. A mononucleated myoblast (*MB*) which has recently fused with a myotube (*MT*₁) is observed at the upper part of this micrograph. The cytoplasm which belonged to a myoblast is clearly distinguishable from that of a multinucleated myotube. The ribosomal concentration is much higher in the former cytoplasm than in the latter; myofibrils have not been formed in the former, while they are already visible in the latter. Thus, the cytoplasmic contents of this newly formed myotube appear as a mosaic. In Fig. 9 *b*,

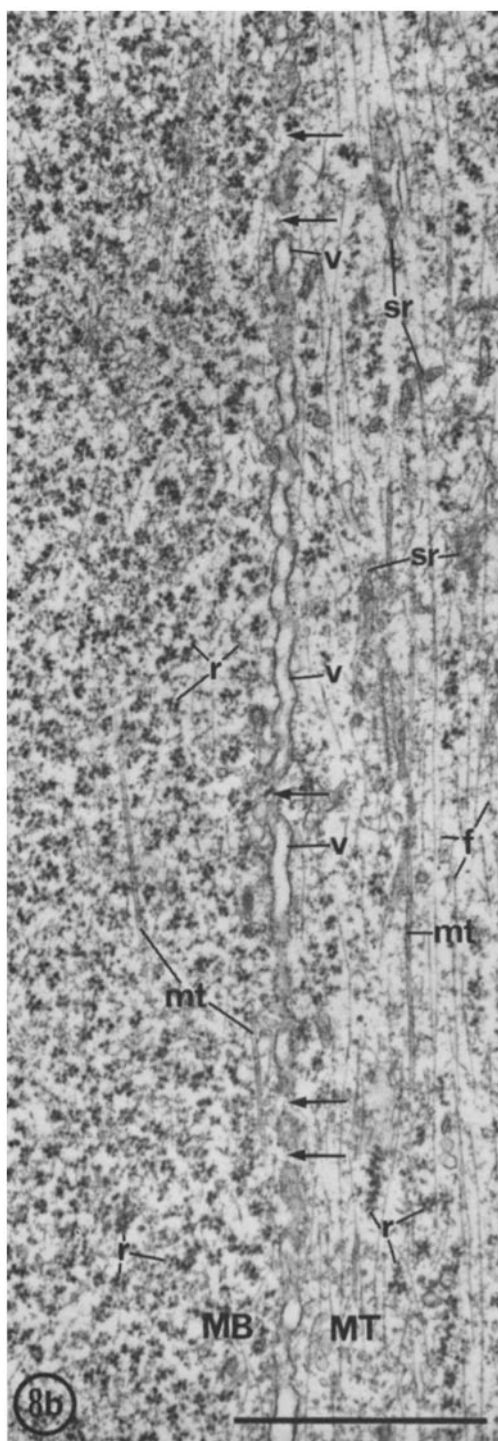


FIGURE 8 *b* A higher magnification view of the rectangle in Fig. 8 *a*. Note the cytoplasmic continuity

the squared-off area in Fig. 9 *a* is shown at a higher magnification. Because of the difference in the ribosomal concentration, the left part of this sarcoplasm (*MB*) appears darker than the right part (*MT*₁). In the former part, numerous free and aggregated ribosomes (*r*) and a few thin filaments (*f*) are seen. In the latter portion, a developing myofibril (*mf*), microtubules (*mt*), and thin filaments (*f*) are visible. These thin filaments measure approximately 100 Å in diameter, are gently curved or wavy, and generally run parallel to the longitudinal axis of the cells (*f*, Figs. 8 *b* and 9 *b*; see Ishikawa et al., 20, 21, Kelly, 22). The gradual decrease in the amount of ribosomes near the previous boundary between the two adjacent cells (arrows) suggests that there is slow diffusion of myoblast contents into the myotube.

Later in development, myofibrils increase in number and diameter until their aggregate volume fills the sarcoplasmic space within the developing myotube. Well-developed muscle fibers possess a structure typical of vertebrate skeletal muscle (41).

DISCUSSION

This electron microscope study of developing chick skeletal muscle cells *in vitro* demonstrates intermediate stages of cytoplasmic fusion between developing muscle cells. Mitosis is often seen in mononucleated myoblasts, but neither amitosis nor mitosis within multinucleated myotubes has been detected. These results support the concept that multinucleation in muscle fibers arises by fusion and coalescence of myoblasts and myotubes (4-7, 9, 25-27, 29, 36, 37, 42, 45, 46). In agreement with the previous works at the light microscopic level (36, 37, 42), the present study suggests the occurrence of fusion between mononucleated cells, between mononucleated cells and multinucleated myotubes, and probably between nascent multinucleated myotubes.

Vesicles or tubules seen in the process of cytoplasmic fusion are presumably formed by opening of the apposed sarcolemmas at several points and fusion of their edges to each other. The width of such vesicles or tubules are much wider than the intercellular space of the closely applied sarcolemmas prior to fusion. Wide spaces are occasionally

through the pores (arrows) between vesicles or tubules (*v*) formed by fragmentation of the adjoining sarcolemmas. Ribosomes and polysomes, *r*; sarcoplasmic reticulum, *sr*; microtubules, *mt*; 100-Å filaments, *f*. × 34,000.

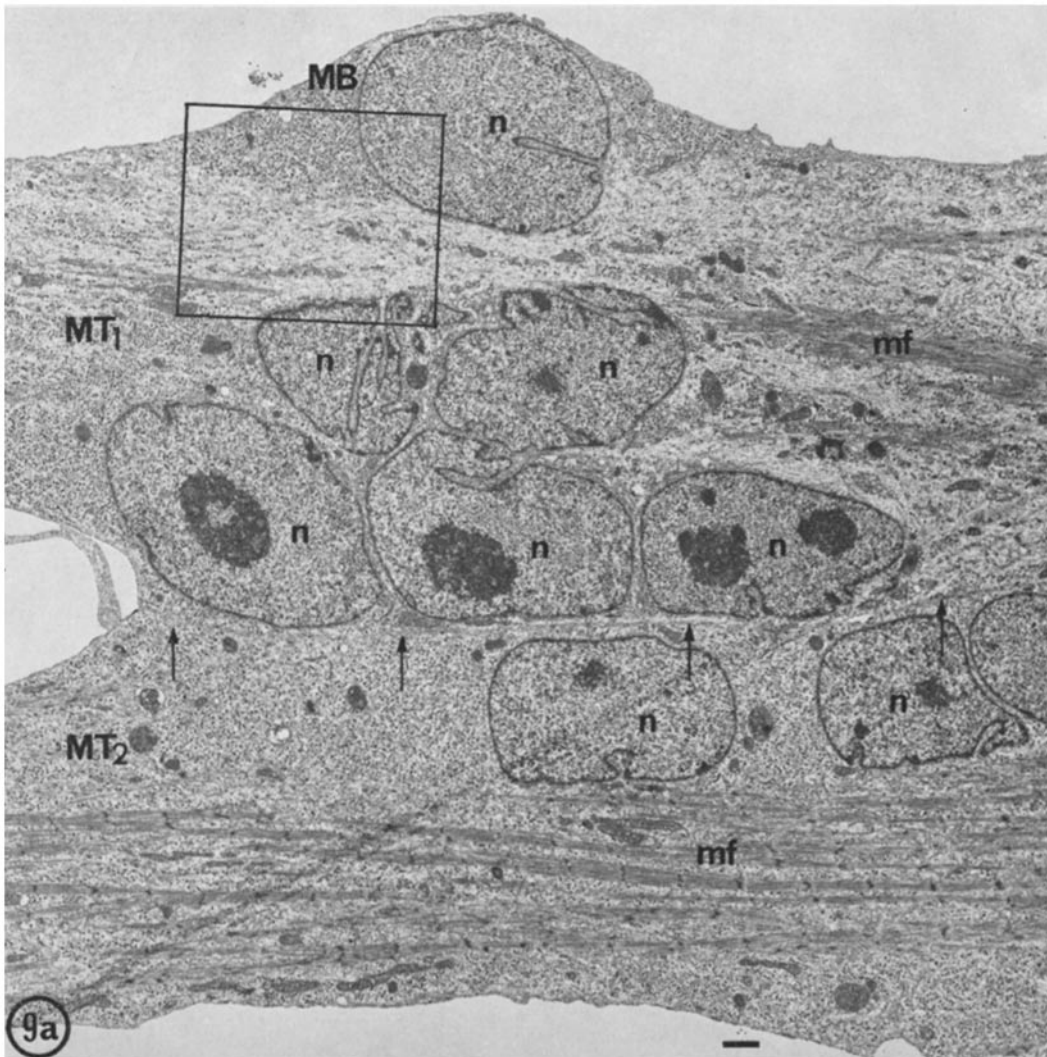


FIGURE 9 *a* An electron micrograph of myotubes (MT_1 and MT_2) which are closely applied (arrows). Such a close apposition suggests that they are destined to fuse. A myoblast (MB) which has recently fused with a myotube (MT_1) is seen at the upper part of this micrograph. The cytoplasmic contents of this newly formed myotube ($MB + MT_1$) are poorly admixed, giving the cytoplasm a mosaic appearance in which different zones can be designated as arising from separate cells. Myofibrils, *mf*; nucleus, *n*. 8 day culture. $\times 4,900$.

seen between such closely associated membranes. Thus, it is conceivable that membrane dissolution occurs at the closely associated areas and that the membranes facing wide spaces contribute to the formation of the walls of such vesicles and tubules. Similar structures have been reported in developing muscle cells *in vivo* (10, 17, 39) and *in vitro* (12,

14). Observing differentiating muscle in the salamander tail, Hay (17) stated: "the possibility that such vesicles are associated with obliquely cut cell membranes cannot be ruled out." To exclude such a possibility, serial sections are needed. In the present study, however, such structures are found between cytoplasm at different developmental

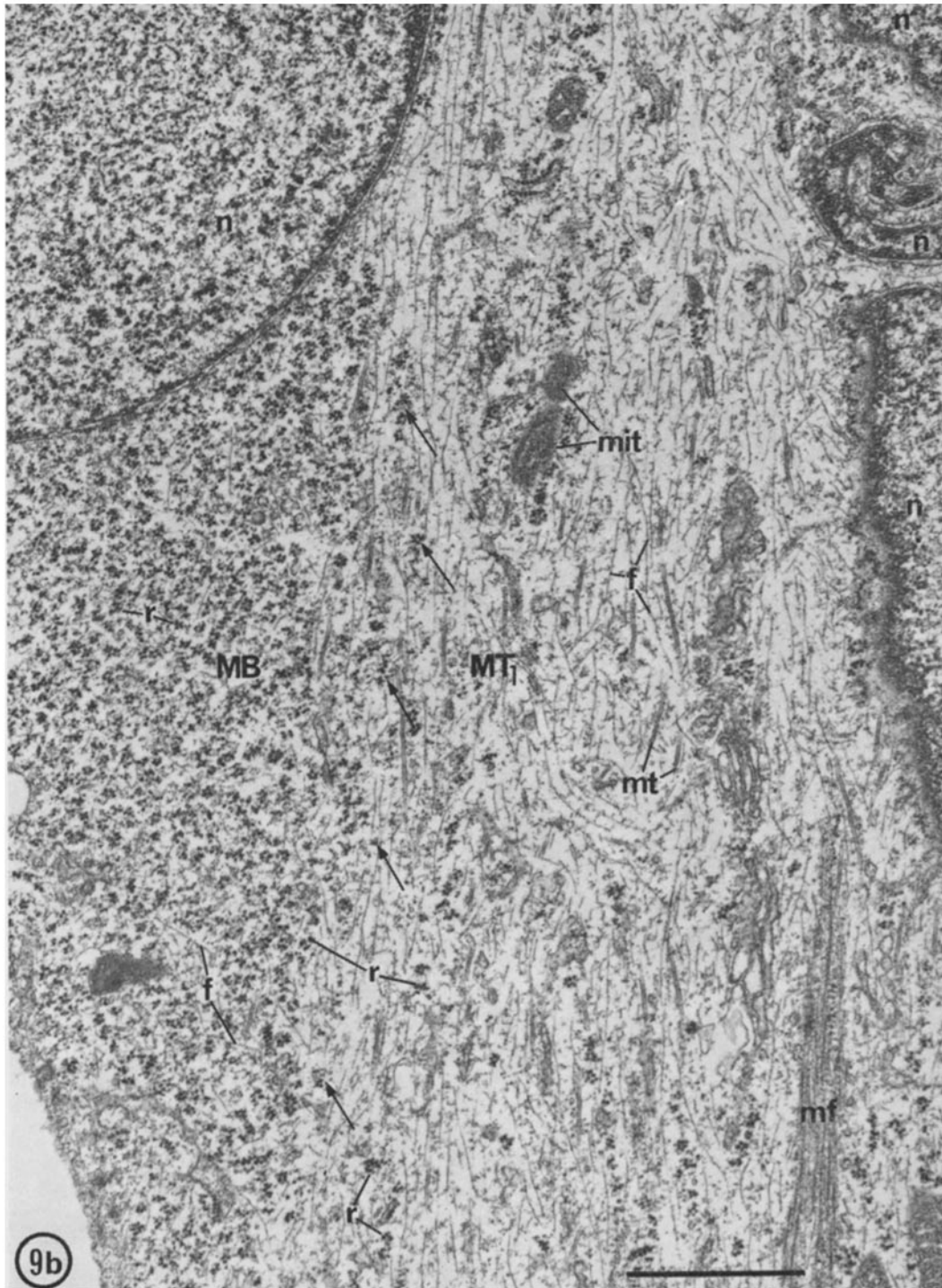


FIGURE 9 *b* An enlargement of the squared-off region in Fig. 9 *a*. Cell membranes of apposed cells (myoblast, *MB*, and myotube, *MT*₁) have completely dissolved. Somewhat slow diffusion of myoblast contents into the myotube is seen (arrows). Myofibrils, *mf*; ribosomes, *r*; mitochondria, *mit*; microtubules *mt*; nucleus, *n*; 100-Å filaments, *f*. $\times 26,000$.

stages (i.e., between myoblasts and myotubes). This observation strongly supports a cellular fusion hypothesis.

When tubules formed by fragmentation of apposed sarcolemmas are found between two cytoplasms at the same developmental stage, they may sometimes be difficult to distinguish from T-system tubules which run longitudinally (11). Diffusion of chemicals such as ferritin will not distinguish both kinds of structures. The inside of the present structures belonged formerly to the extracellular space, and some are perhaps still continuous with it; T-system tubules are also continuous with extracellular fluid (11). Whether the structures seen in the present study contribute to the formation of longitudinal tubules is unknown. Thus, the identity of a difference between such similar structures has yet to be clarified. But the endoplasmic (sarcoplasmic³) reticulum is readily distinguishable from the present structures. The lumen of the present tubules is electron-lucent, while that of the sarcoplasmic reticulum is finely granulated. Furthermore, the endoplasmic reticulum in younger cells is sometimes studded with ribosomes.

In the process of fusion, cytoplasmic continuity is established between the apposed cells through the pores formed between fragmented and vesiculated sarcolemmas. After the complete disappearance of the intervening plasma membranes, the cytoplasmic contents of the multinucleate cells are often poorly admixed, giving the cytoplasm a mosaic appearance in which different areas can be designated as arising from separate cells. This observation reveals that the amount of ribosomes decreases gradually from the myoblast to the myotube at the area of the previous cellular boundary. This finding suggests that there is somewhat slow diffusion of myoblast contents (ribosomes and, possibly, other materials) into the myotube (14).

Holtzer et al. (18) have stated that some mononucleated cells in embryonic chick somites stain with fluorescein-labeled antimyosin, and demonstrated that multinucleation is not an essential prerequisite for myosin or actin synthesis. In this study, no attempt was made to correlate multinucleation with the presence or absence of myofilaments in mononucleated cells, since conclusive evidence cannot be obtained without serial sections

³The term sarcoplasmic reticulum is given to the agranular reticulum seen in muscle-forming cells.

to prove mononucleation and myofilament existence. However, as in the developing chick leg muscle *in vivo* (13), examination of the same muscle *in vitro* has demonstrated that the vast majority of myofilaments demonstrable by electron microscopy are found in multinucleated cells.

Thickenings of apposed membranes of cells destined to fuse are often observed in the present study. Similar structures have been seen in developing rat intercostal muscle (23) and in chick embryo myocardium (28). Those in the embryonic heart are found to undergo modification to form intercalated discs (28). After completion of cell fusion, such membrane specializations are not found in the skeletal muscle. Thus, they appear to represent a temporal attachment device.

Myotubes which, by electron microscopy, do not appear fused may sometimes appear confluent by phase-contrast optics (compare Figs. 4, 6, or 9 *a* with Fig. 1 *a* or *b*). Therefore, it is necessary to determine by the higher resolution technique whether or not fusion has occurred.

It is pointed out that fusion is initiated by the "recognition" of homotypic cells, and is presumably mediated by unique or subtle molecular markers on the cell surface (36). "Recognition" is followed by interaction leading to breakdown of the intervening membranes. This mechanism thus far has not been documented. It is not clear whether the behavior of the membranes is more analogous to activity of other cell membranes engaged in phagocytosis (2), fertilization (30), syncytial trophoblast formation in some mammalian placentas (31), or giant polynuclear cell formation caused by HJV virus (35). In the present study, engulfment of the myoblast processes by the myotube is observed. Whether this structure shows initial phases of phagocytic activity which leads to cell fusion is unknown. Further investigation of the membranes relating to myoblast and myotube association may be expected to broaden our understanding of myogenesis, and possibly, too, it may clarify some problems about contact or adhesion in other cell systems.

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