DIFFERENTIATION OF THE SARCOPLASMIC RETICULUM AND T SYSTEM IN DEVELOPING CHICK SKELETAL MUSCLE IN VITRO

ELIZABETH B. EZERMAN and HARUNORI ISHIKAWA

From the Departments of Anatomy and Biochemistry, the School of Medicine, the University of Pennsylvania, Philadelphia, Pennsylvania 19104

ABSTRACT

The electron microscope was used to investigate the first 10 days of differentiation of the SR and the T system in skeletal muscle cultured from the breast muscle of 11-day chick embryos. The T-system tubules could be clearly distinguished from the SR in developing muscle cells fixed with glutaraldehyde and osmium tetroxide. Ferritin diffusion confirmed this finding: the ferritin particles were found only in the tubules identified as T system. The proliferation of both membranous systems seemed to start almost simultaneously at the earliest myotube stage. Observations suggested that the new SR membranes developed from the rough-surfaced ER as tubular projections. The SR tubules connected with one another to form a network around the myofibril. The T-system tubules were formed by invagination of the sarcolemma. The early extension of the T system by branching and budding was seen only in subsarcolemmal regions. Subsequently the T-system tubules could be seen deep within the muscle cells. Immediately after invaginating, the T-system tubule formed, along its course, specialized connections with the SR or ER: triadic structures showing various degrees of differentiation. The simultaneous occurrence of myofibril formation and membrane proliferation is considered to be important in understanding the coordinated events resulting in the differentiated myotube.

INTRODUCTION

Striated muscle fibers contain two distinct interfibrillar membranous systems: the sarcoplasmic reticulum (SR) and the transverse tubular system (T system). The orderly arrangement, in mature muscle fibers, of the sarcoplasmic reticulum and the T system in close association with the myofibrils has been extensively described (see recent reviews by Peachey, references 25, 26, and Smith, reference 36). In vertebrates, the SR and T system closely associate with each other in structures called triads, at definite levels of the sarcomere. These membranous systems seem to have a role in the intracellular spread of activation for contraction of skeletal muscle fibers.

There have been several electron microscopic observations of developing skeletal muscle in the last few years (Bergman, reference 4; Hay, reference 11; Allen and Pepe, reference 1; Dessouky and Hibbs, reference 8; Heuson-Stiennon, reference 12; Przybylski and Blumberg, reference 29). However, a detailed account of the proliferation and organization of the SR and the T system has not yet been presented. In the present study, the development of the SR and the T system has been studied in cultured, chick embryo, striated muscle fibers, with the electron microscope. Particular attention has been paid to the distinction between the SR and the T system in the developing muscle,

as these have been considered to be different entities both morphologically and physiologically (Franzini-Armstrong and Porter, reference 10; Peachey, reference 24).

Certain advantages of tissue culture material prompted us to use that technique in our study of the development of the SR and T systems in striated muscle. First, muscle cells in vitro differentiate very rapidly under the conditions used (Okazaki and Holtzer, reference 21) and may readily be scanned by phase-contrast microscopy for the selection of different stages of development. Second, there is a degree of synchronizing of cell fusion that cannot be observed with in vivo material. Last, the system is well suited for use in ferritin-diffusion experiments since no dissection of tissue is required.

MATERIALS AND METHODS

Breast muscle tissue was dissected aseptically from 11-day chick embryos according to the method of Holtzer et al. (15). Trypsinized cell populations were grown on glass at a concentration of 1 or 2 × 10^6 cells/milliliter in Eagle medium, horse serum, and embryo extract (8:1:1) with 1% penicillin-streptomycin and 1% L-glutamine. The cultures were grown at 37°C in a 5% CO_2 and water-saturated atmosphere. Each culture was observed, immediately before fixation, with the phase-contrast microscope, to judge degree of development since the development of muscle cells in vitro varies in different cultures and in different areas of the same culture (Okazaki and Holtzer, reference 21).

Cultures of various ages (1–10 days) were fixed for approximately 15 hr in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer containing 2 mM $CaCl_2$ at pH 7.2 at room temperature (22 ± 2°C), were rinsed in the same cacodylate buffer with 10% sucrose for 15 min, then were postfixed for 1 hr in cold 1% osmium tetroxide in the same buffer. Other cultures were fixed in 2.5% glutaraldehyde in Millonig's phosphate buffer at pH 7.2, were rinsed in 10% sucrose, and were postfixed in 1% osmium tetroxide in Palade's Veronal-acetate buffer (Palade, reference 23). All specimens were dehydrated in graded concentrations of ethanol (75, 95, 100%), and then were embedded in epoxy resin (Epon 812 or Araldite). Some specimens were carefully removed with forceps from the glass while in 75% alcohol whereas others were embedded *in situ* on the glass.

Thin sections were cut on a Sorvall Porter-Blum MT-2 microtome, were stained with a saturated solution of uranyl acetate in 50% alcohol and/or lead citrate (Reynolds, reference 31), and were observed with either an AEI EM6B or an RCA EMU3 electron microscope.

FERRITIN EXPERIMENTS: to remove cadmium, crystallized ferritin from horse spleen (Nutritional Biochemicals Corporation, Cleveland, O.) was suspended in 1 mM EDTA and was centrifuged at 40,000 rpm for 120 min at 6°C in a Beckman model L-2 ultracentrifuge. The process was repeated two additional times, after which the ferritin was resuspended in culture medium at a final concentration of 20%. Alternatively, the ferritin was dialyzed against 0.28 M saline for 48 hr, was centrifuged, and was used as a 10% solution in culture medium. The ferritin solutions were warmed to 37°C and were substituted for the culture medium of living muscle cultures of varying ages (4, 6, and 8 days). After 30 or 60 min, the cultures were rinsed quickly with culture medium and fixed, by using the techniques described above. Thin sections of ferritin preparations were stained very weakly with lead citrate or were not stained.

OBSERVATIONS

General Remarks

A rapidly multiplying population of cells which will differentiate into striated muscle fibers in vitro can be obtained by trypsinizing 11-day chick embryo, breast muscle. The first few days of culture are characterized by rapid cell division and fusion of myoblasts to form elongated multinucleated units. By the 3rd or 4th day these elongated muscle fibers or myotubes (see Boyd, reference 5; Holtzer, reference 13) can be seen to contain myofilaments and/or myofibrils, either with the electron microscope or with the fluorescence microscope and the fluorescein-labeled antibody technique (Holtzer, et al., reference 16; Okazaki and Holtzer, reference 21). By this time the muscle may be observed, in the phase-contrast microscope, to contract spontaneously. Muscle cells in the same cultures display varying stages of differentiation, but there is generally further development of the myotubes between the 4th and 8th days.

In the present study, muscle cells from the early myotube stage (usually corresponding to 3–5-day cultures) to the well-developed myotube stage (6–10-day cultures) have been observed. In the early days of culture (1–3 days) many cells are mononucleated and show little differentiation; since it is hard to identify them in thin sections as either presumptive myoblasts or fibroblasts, we have not studied these cells extensively.

The SR and the T system will be described separately.

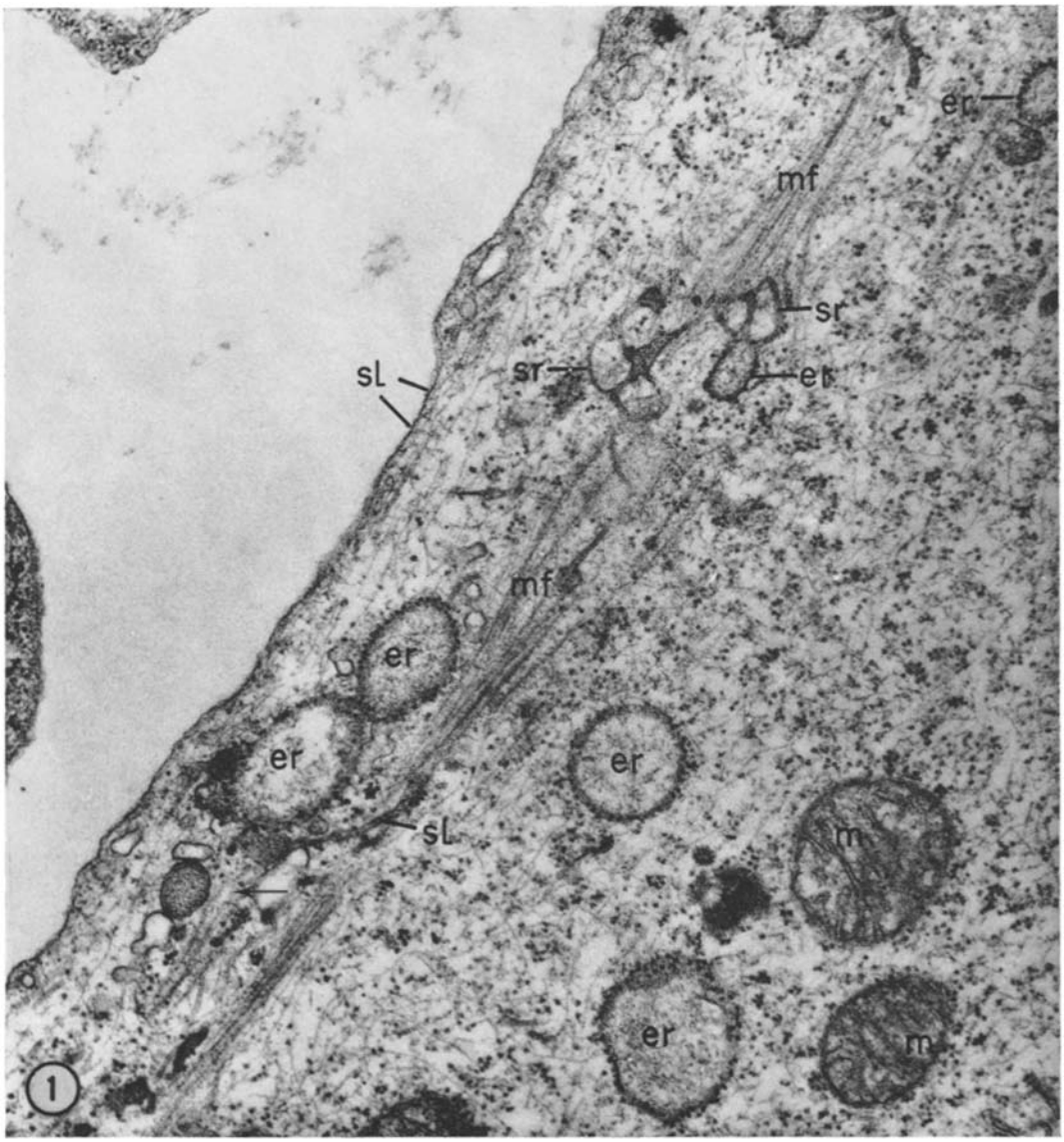

FIGURE 1 Longitudinal section of an early myotube. Distended rough-surfaced endoplasmic reticulum vesicles (*er*) about 0.5 μ in diameter are seen scattered throughout the sarcoplasm. Some of the rough ER vesicles extend multiple tubular projections in various directions in association with a myofibril (*mf*). The projections are usually narrow and smooth-surfaced. Both smooth (*sr*) and rough (*er*) ER have a diffuse dense content, while T-system tubules show sharp limiting membranes and a clear lumen. A close connection (arrow) between membranes of the SR and the T-system tubule is seen beneath the sarcolemma (*sl*). It is the reverse of the definitive triad: the central element is the SR component while the two lateral elements are T-system tubules. *m*, mitochondria. 8 day culture × 32,000.

Sarcoplasmic Reticulum

In the earliest myotube stage many distended rough-surfaced endoplasmic reticulum (ER) vesicles are scattered throughout the sarcoplasm of the myotube (Fig. 1). The vesicles are usually separated from one another, but occasionally two or more are seen to be continuous with each other through narrow channels.

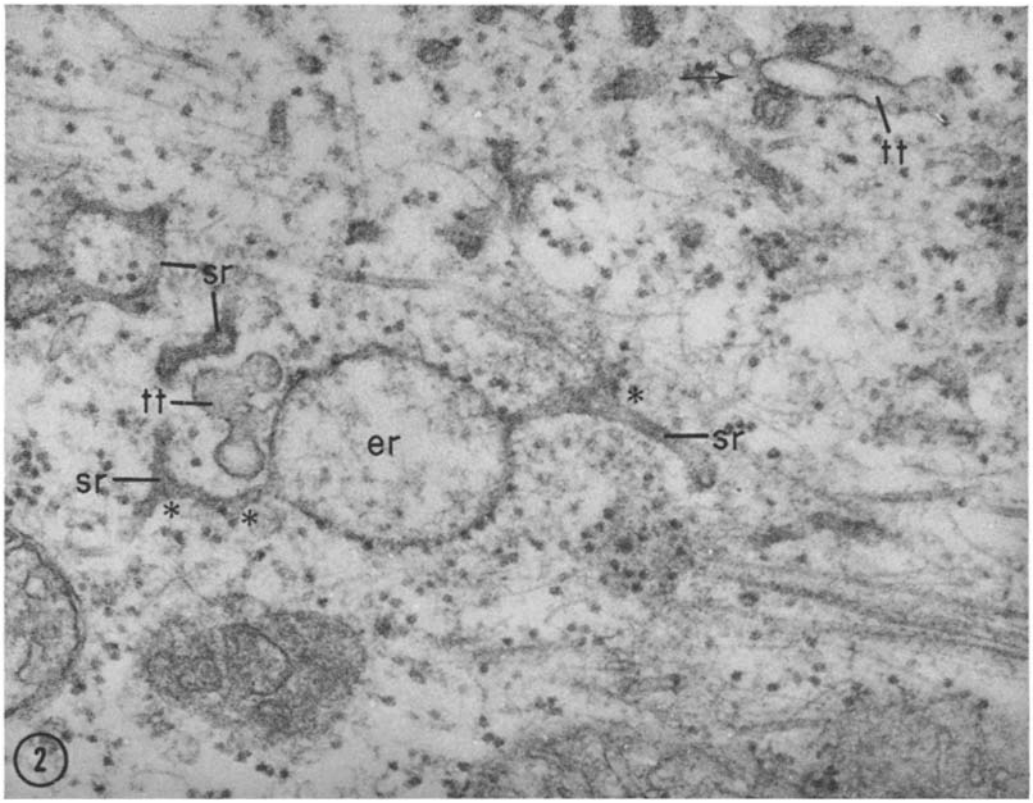

FIGURE 2 Tubular projections extending from rough ER (*er*). These SR projections (*sr*) show varying degrees of elongation and branching (asterisks *). Many profiles of the SR projections and many T-system tubules (*tt*) forming close associations (arrow) with each other are also seen. 8 day culture. × 60,000.

As the myotube develops, forming more myofibrils, the rough-surfaced endoplasmic reticulum (ER) vesicles form multiple tubular projections in various directions (Figs. 1–3). This kind of image was seen far too often to have been due to chance overlap of rough ER and smooth-surfaced tubules. The projections are usually narrow and smooth-surfaced but occasionally have a few attached ribosomes. From the subsequent development described below, it is plausible that these tubular projections represent an early form of the SR. In early myotubes these tubules show varying degrees of elongation and branching and are 300–600 A in diameter. Subsequently, these tubules form characteristic and highly branched honeycomb structures in association with the developing myofibrils (Fig. 3). These honeycomb structures are continuous with rough ER still present in the interfibrillar spaces (Fig. 3, arrows), and may be continuous with more than one rough ER vesicle through thin tubules. In addition, the connection of a honeycomb structure through rough or smooth tubules with another such structure in an adjacent myofibril results in an elaborate network.

The development of these membrane networks in association with the developing myofibrils suggests that they may be regarded as early stages of the development of the SR. The earliest elaborations of the SR may occur without any structural association with the developing myofibrils. The first indication of such an association is the presence of tubular elements around or adjacent to the I or Z region of the myofibril (Fig. 1). In sections, parallel arrays of tubular profiles are often found across successive Z-line regions (see also Allen and Pepe, reference 1). These tubules at the Z line are seen to be the partial profiles of what later become the extensive honeycomb structures.

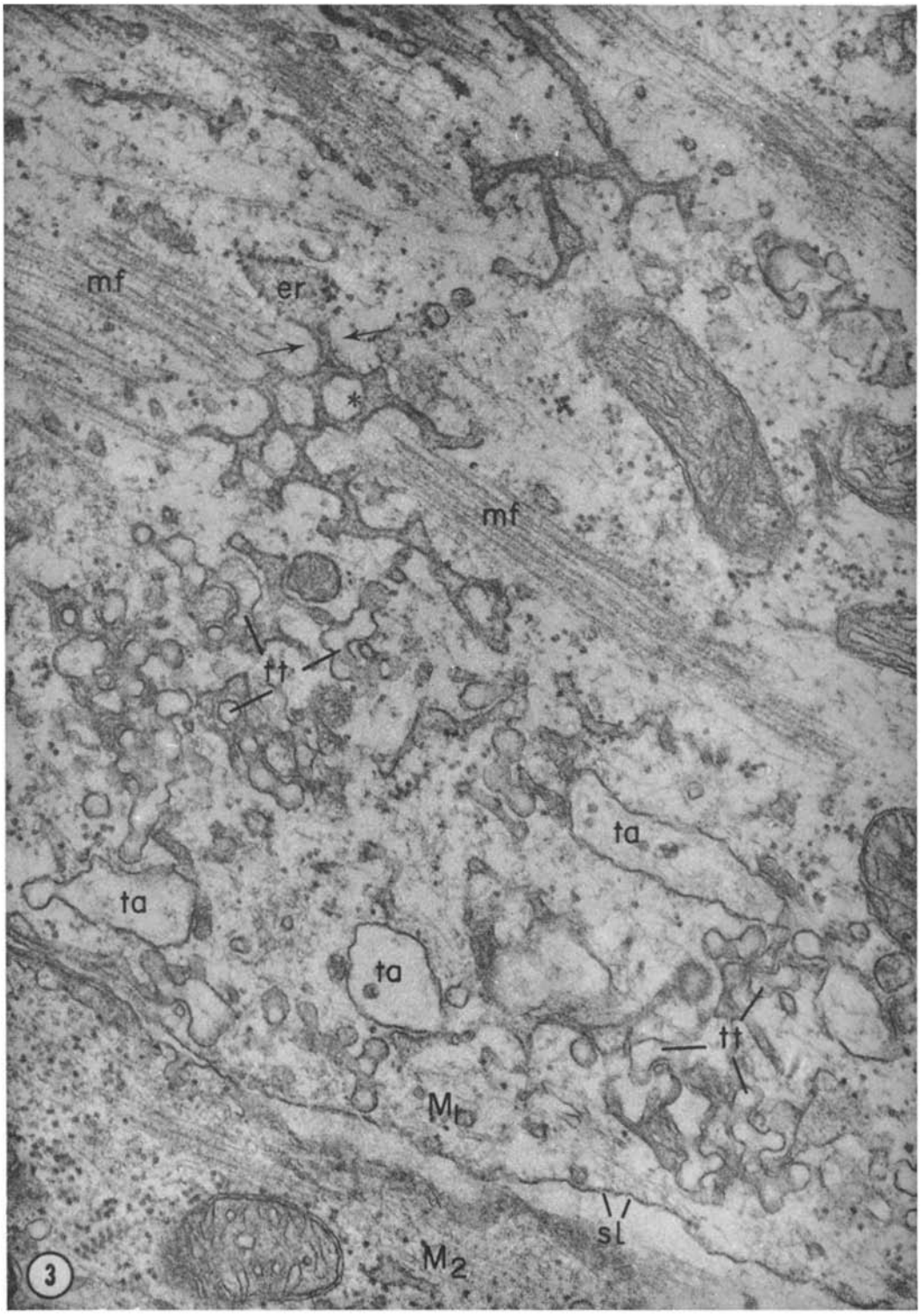

FIGURE 3 Longitudinal section of two myotubes (M_1, M_2). SR honeycomb structures (*) continuous with (arrows) rough ER (*er*) is seen around myofibril (*mf*). Beneath the sarcolemma (*sl*) T-system tubules (*tt*) can clearly be distinguished from the SR. Some T-system tubules show ampulla-like dilatations (*ta*). That both systems are in intimate association with each other results in a very complicated relationship. 6 day culture. × 50,000.

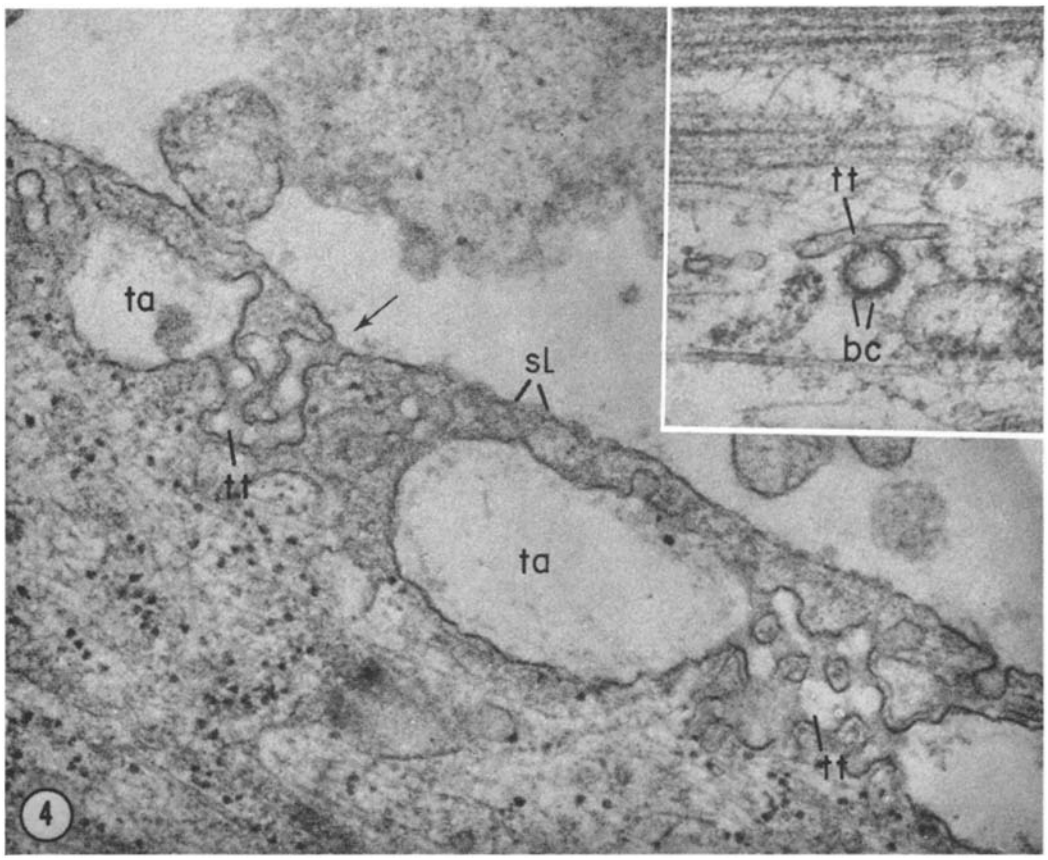

FIGURE 4 Longitudinal section. A T-system tubule (*tt*) is seen to be continuous (arrow) with the sarcolemma (*sl*). Occasionally the T system forms ampulla-like dilatations (*ta*) beneath the sarcolemma. Gray areas in the T-system tubules are regions where the membrane forming the tortuous wall of the tubule lies obliquely in the section. 3 day culture. × 54,000. Insert, longitudinal section showing bristle-coated projections (*bc*) of T-system tubule (*tt*) × 57,000.

T System

In mature skeletal muscle, the T system (Andersson-Cedergren, reference 2) is oriented transversely at regular intervals along the sarcomere. In the tissue culture materials studied, a membranous system similar in some respects to the T system of mature muscle is observed (Fig. 4). In contrast to that of the mature muscle, however, the arrangement and distribution of this system is very irregular. From the point of view of the subsequent differentiation of the system and its continuity with the sarcolemma, this membranous system is clearly the precursor of the definitive T-system.

The T-system tubules appear first in the sarcoplasm beneath the sarcolemma (subsarcolemmal region) as inpocketings and invaginations of the sarcolemma. Subsequently they extend deeper within the myotubes (Fig. 5). Their membranes are *rarely* flat (like the sarcolemma) but are always highly curved, showing a beaded appearance. In sections, finger-like tubular invaginations of varying lengths are seen to be continuous with the sarcolemma along the sides of the myotubes. The T-system tubule has a smooth, sharp, limiting membrane and a clear lumen, while the lumen of the SR has a diffuse dense content similar to that of the lumen of the rough ER. The membrane of the SR does not appear so sharply defined as that of the T-system tubule.

After entering the sarcoplasm radially, the T-system tubules often turn their course longitudi-

nally or obliquely with respect to the myotube axis; only a few tubules are seen to run directly transversely with respect to the long axis. The T system near the sarcolemma often takes a very tortuous form with ampulla-like dilatations from which several budding tubules extend in various directions (Fig. 6). Occasionally the T-system tubules are seen to branch directly. Because of their irregular course, the tubules appear to pass in and out of the plane of section. Tangential sections of sarcolemma show round or slightly oval openings 300–500 A in diameter (Fig. 7). Since T-system tubules can be seen in subsarcolemmal regions, most if not all of these openings may be regarded as those of the T-system tubules. In addition, the diameters of the openings are similar to those of the profiles of the connections of T-system tubules with the sarcolemma in saggital sections. Occasionally, inpocketings of the sarcolemma and of the T-system tubules have bristle coats about 200 A wide on the convex sarcoplasmic side (Fig. 4, insert) and are similar in fine structure to pits or vesicles in other mononucleated cells in our materials (see also Heuson-Stiennon, reference 12) and to those in oocytes described by Roth and Porter (34).

In the early stages, the T-system tubules seem to invaginate and proliferate without any particular relation to the SR or the myofibrils. Subsequently, the T-system tubules encounter the SR or rough ER membranes and make intimate contact with them. Especially in subsarcolemmal regions, the T-system tubules and SR intermingle; the result is a very complicated relationship.

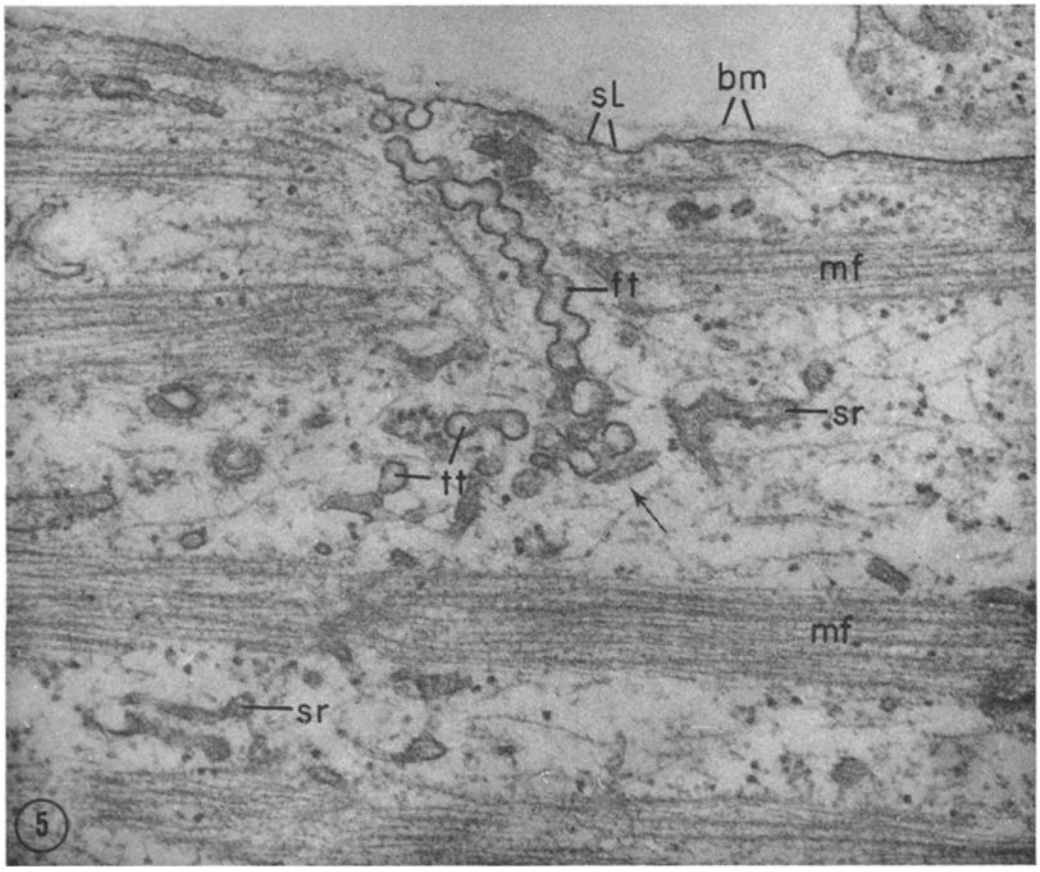

FIGURE 5 Longitudinal section of a developing myotube. That a transversely running T-system tubule takes a very tortuous form results in a complicated interrelationship with the SR (arrow). In this area T-system tubules (*tt*) could not be found deep within the cell. *mf*, myofibrils; *sl*, sarcolemma; *bm*, basement membrane; *sr*, sarcoplasmic reticulum. 6 day culture. × 50,000.

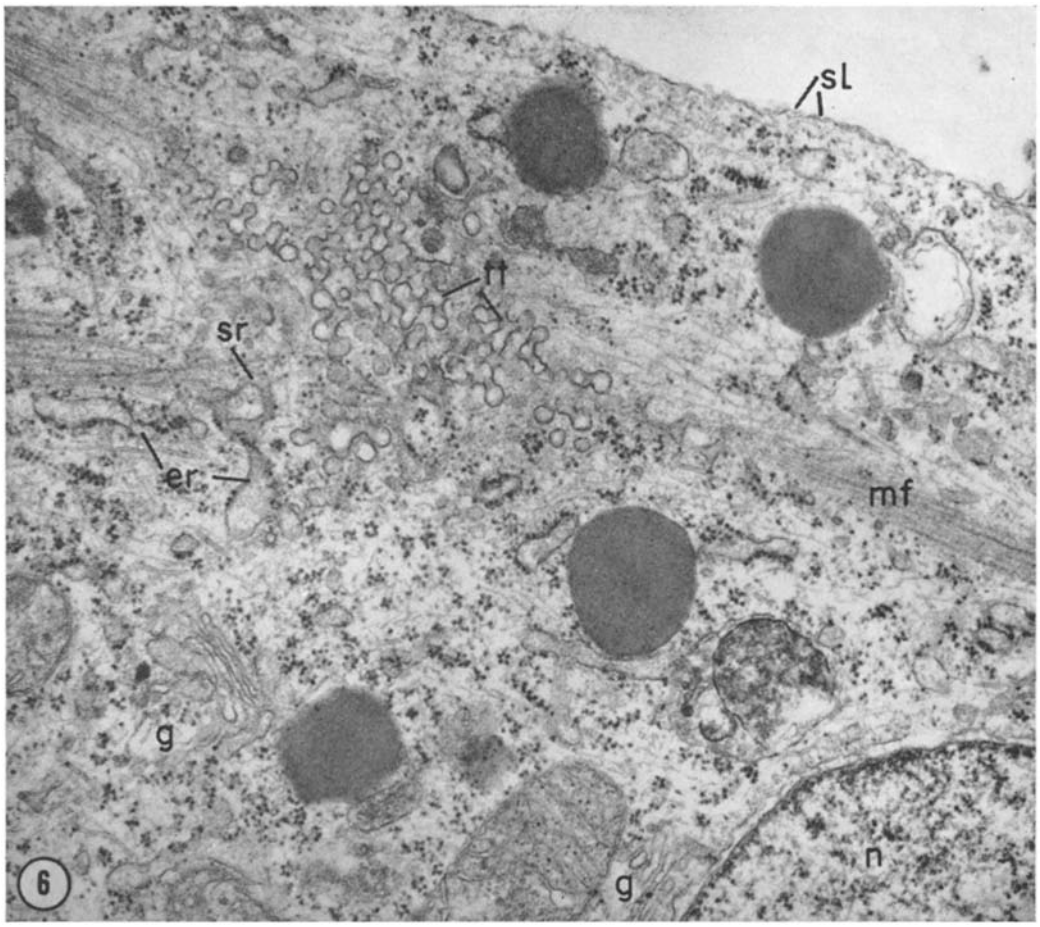

FIGURE 6 Longitudinal section of an early myotube. T-system tubules (*tt*) are seen to branch and fuse and result in a network formation. The T-system tubule can be easily distinguished from the SR (*sr*), rough ER (*er*), or Golgi membranes (*g*). *n*, nucleus; *mf*, myofibrils; *sl*, sarcolemma. 3 day culture. × 30,000.

As development of the myotube proceeds and more myofibrils form, the T-system tubules can be observed deeper in the myotube. Some T-system tubules are seen to run longitudinally beside the myofibrils for considerable distances (see Fig. 10). Occasionally, virus-like particles are found in the T-system tubules (see Fig. 9). These particles are never found in the SR, and we have no information on their origin. Since the differentiation of the myotubes appears in many respects to be similar to that which takes place in vivo, these particles do not seem to interfere with or alter the development of the myotubes in vitro.

The Triad

In mature skeletal muscle fibers, localized regions of the membranes of the SR and the T system are in close connection in a complex called a triad (Porter and Palade, reference 28). In early myotubes in vitro, one occasionally observes connections or close contacts between T-system tubules and vesicles apparently representing rough-surfaced ER (Fig. 8 *a*). More commonly, such connections are seen between T-system tubules and smooth-surfaced SR vesicles (Fig. 8*b*); the later are dilated and contain more diffuse, dense material than the rough-surfaced ER, and are very similar to the terminal cisternae in mature muscles (Porter and Palade, reference 28). These connections appear to be randomly distributed without any association with the developing myofibrils. The simplest and presumably earliest connections show only close apposition (100–120 A) of the membranes of the SR and

T-system tubule (Fig. 8 *a*, *b*). More specialized and presumably more developed connections appear with increasing frequency in more advanced myotubes (Fig. 8 *c*, *d*). These connections are similar to those described in mature muscle, i.e. Revel (30), Franzini-Armstrong (9), Peachey (24), Walker and Schrodt (40), Smith (37); they consist of dense zones in the spaces between the apposed membranes of the two systems. The spacing between the membranes is not altered by the presence of these dense structures. Even in the same section some of the spaces between apposed membranes have these dense continuous or interrupted septal zones whereas others do not. Thus there may be various degrees of differentiation into the triad in a single myotube. The dense zones are most clearly demonstrated in sections stained with both uranyl acetate and lead citrate.

In both transverse and longitudinal sections, several terminal cisternae of the SR may be apposed to a single dilated T-system tubule, and appear as multiple dyads (Fig. 9). When the myotube is further developed, connections are seen between the T-system tubules and the well-developed honeycomb structures deep within the cell. Subsequently, the part of the SR honeycomb associated with the T-system tubule seems to be reorganized into dilated regions corresponding to terminal cisternae of mature muscle. In well-developed myotubes, triads are frequently observed adjacent to I bands or A–I junctions; however, the distribution and direction of the triads are still irregular.

Ferritin Diffusion

Confirmation of the observation that the T system is morphologically distinguishable from the SR and has a distinct origin was provided by experiments with ferritin. Although various methods were tried, the best results were obtained by soaking the living muscle cultures for 30 min in 10 or 20% ferritin in culture medium in an incubator. Ferritin particles about 110 A in diameter were found within the T-system tubules only, not in the

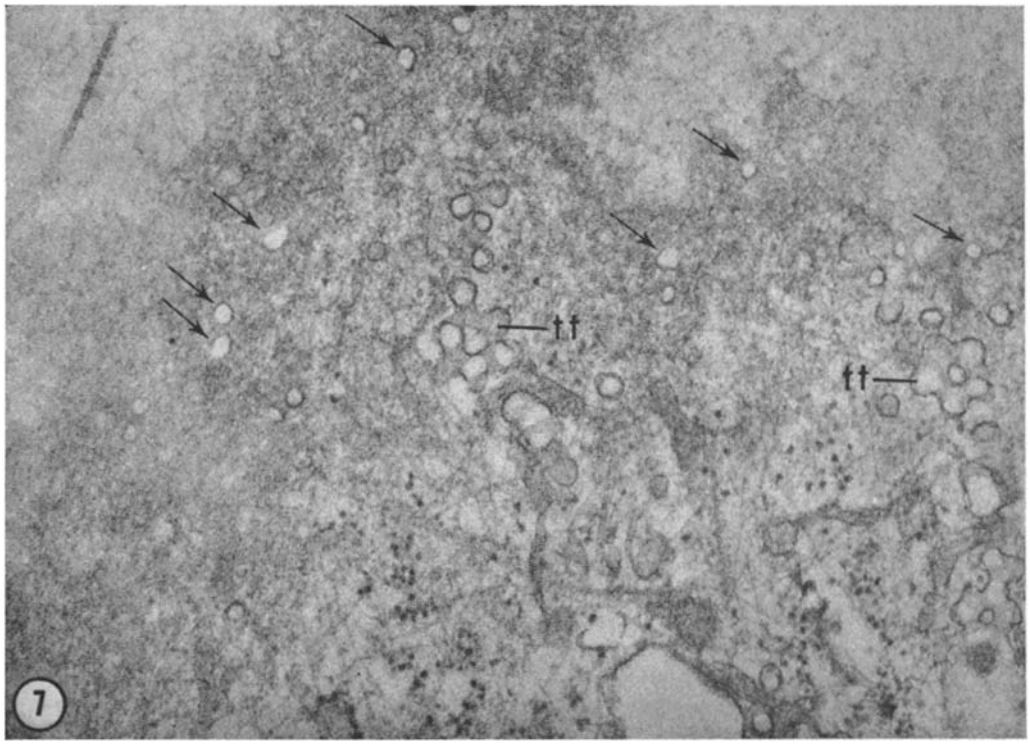

FIGURE 7 Tangential section of the sarcolemma showing round or slightly oval openings (arrows) 300–500 A in diameter. Most of these may be regarded as openings of the T-system tubules. T-system tubules (*tt*) can be seen in the subsarcolemmal regions. 4 day culture. × 48,000.

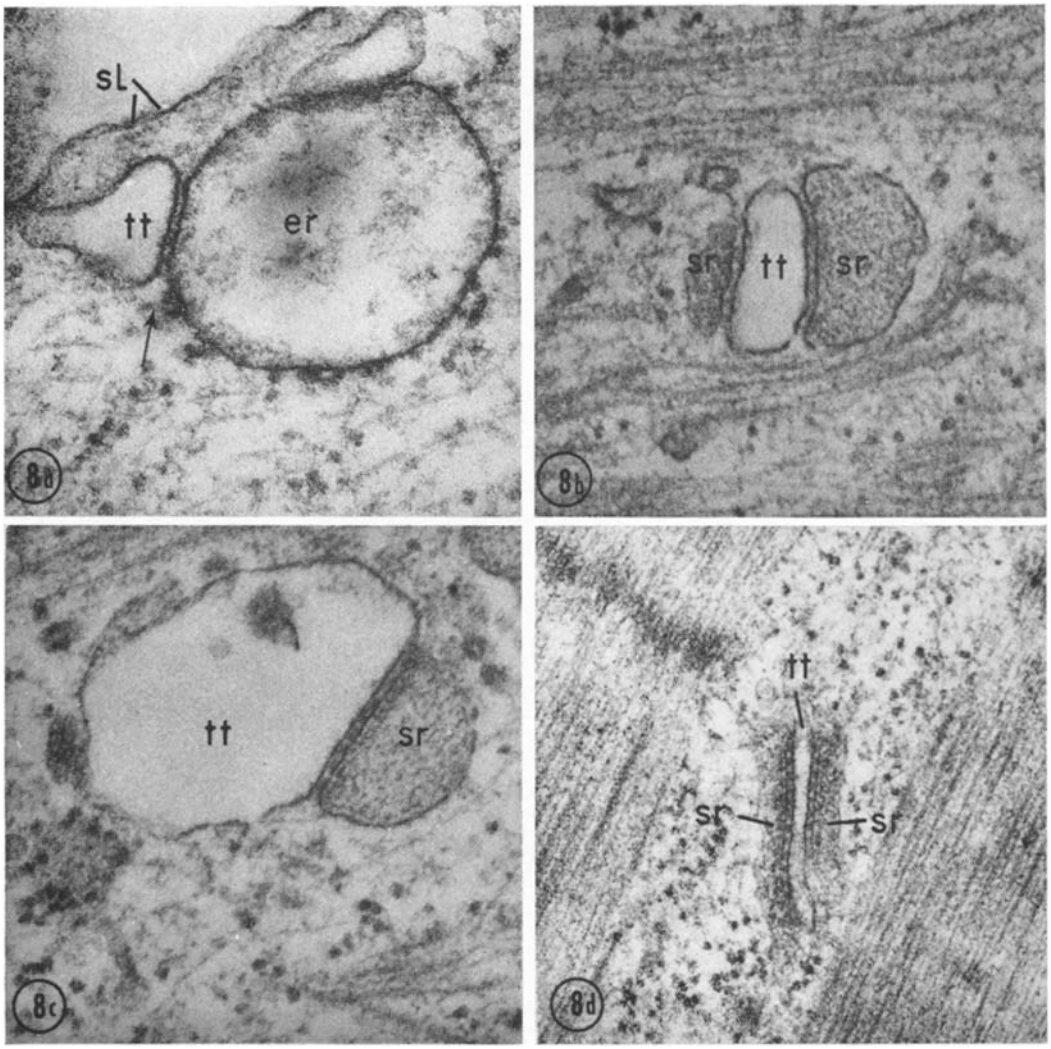

FIGURE 8 Triadic structures showing various degrees of differentiation. Fig. 8 *a*, earliest connection showing a simple, close apposition of membranes of the rough-surfaced ER (*er*) and T-system tubules (*tt*) just beneath the sarcolemma (*sl*). The intermembranous space (arrow) is approximately the same (about 120 A) as that of a mature triad. 8-day culture. Fig. 8 *b*, earliest specialized connection showing a triadic configuration; central T-system element (*tt*) and lateral SR cisternae (*sr*). The spaces do not show any prominent dense lines characteristic of a mature triad. 6 day culture. Fig. 8 *c*, specialized connection similar to that in mature muscle. Dense interrupted septal zones are clearly seen in the space between apposed membranes. 8 day culture. Fig. 8 *d*, triad in a well-developed myotube showing the dense septal zone. The T-system tubule is narrow in this triad and resembles more the definitive triad of mature muscle. 13 day culture. × 50,000.

SR or any other component of the muscle cell (Fig. 10). Thus, ferritin particles may diffuse into the T-system tubules *via* connections with the sarcolemma (Fig. 11); this confirms that the lumen of the T-system tubules is in fact continuous with the extracellular fluid (see Huxley, reference 17).

DISCUSSION

We appreciate that one must use care in applying results from in vitro systems to development in vivo. It is possible that there may be differences in fine structure between muscle developing in

culture and that developing in vivo, because of the different speed of development, absence of innervation, and so forth (Okazaki and Holtzer, reference 22).

In our material, the SR has been clearly distinguished from the T-system tubules even in the early stages of development. The findings that the SR grows out from ribosome-studded membranes initially and that in some cases the tubular projections retain attached ribosomes confirm the view that the SR and ER are homologous structures. Recently, Dallner et al. (7) have studied the biogenesis of the ER membranes in the developing rat hepatocyte and have suggested that new membrane is synthesized in rough-surfaced ER and subsequently transferred to smooth ER. A similar suggestion has been proposed by Jones and Fawcett (18) in their observations on the phenobarbital-induced hypertrophy of the smooth ER in hamster liver, and by Muscatello et al. (20) and Margreth et al. (19) in their studies of the overdevelopment of the SR occurring as an early response to denervation. Our observations also suggest that rough-surfaced ER may be the site of formation of the SR membranes and that the tubular projections may extend by a "flow" of new membrane from the rough ER. The pattern of growth and budding of the tubular projection of the SR suggests that there is fusion of the new tubules to form the complicated honeycomb structures that we observed.

The organization and complexity of the SR in vivo varies with the species of animal and the type of muscle fiber. There is no information available concerning the specializations of the SR in adult chick muscle. In our material in vitro, however, we have observed a honeycomb structure and terminal cisternae. These are different from specialized differentiations such as those found, for example, in frog skeletal muscle (Peachey, reference 24).

Most profiles of T-system tubules show simple, clear, limiting membranes. Bristle-coated pits are occasionally observed formed from the T-system tubules as well as from the sarcolemma. Similar "coated" vesicles seem to be widespread in association with plasma membrane (see Roth and Porter, references 33, 34; Rosenbluth and Wissig, refer-

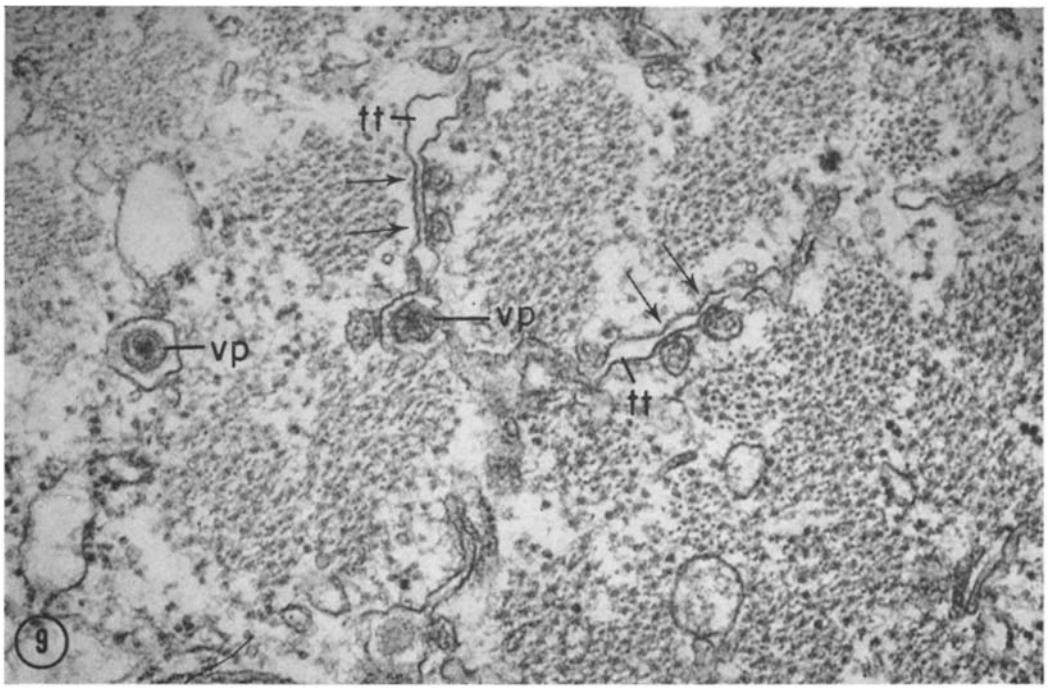

FIGURE 9 Transverse section of a myotube shows multiple dyads (arrows) formed by the association of several SR terminal cisternae with a single T-system tubule (*tt*). Some of the T-system tubules have virus-like particles (*vp*) within them. Such particles are never found in the SR. 4 day culture. × 50,000.

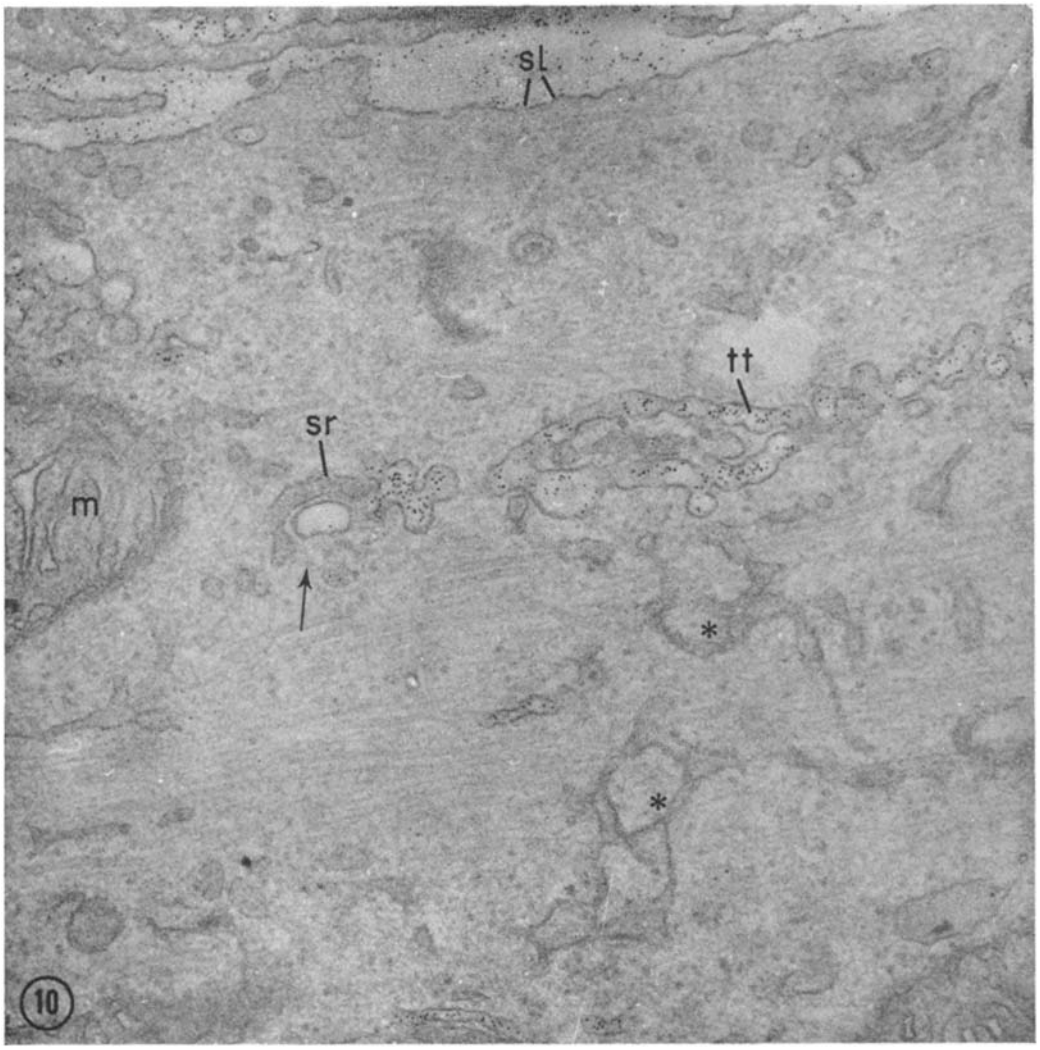

FIGURE 10 Ferritin diffusion. Longitudinal section of a myotube shows ferritin particles, about 110 A in diameter, within the T-system tubules (*tt*) only. Ferritin is not seen within the SR honeycomb (*) profiles nor in other components of the myotube. *m*, mitochondria; arrow, close connection of T-system tubule and SR; *sl*, sarcolemma, weakly stained with lead citrate; *sr*, sarcoplasmic reticulum. × 56,000.

ence 32) and Golgi apparatus (Bruni and Porter, reference 6). Since the T system can be considered to be part of the plasma membrane, it is not surprising that coated inpocketings may be formed from the T-system tubules as well as from the sarcolemma. What association, if any, the bristle coat has with the proliferation of the T-system tubule remains to be discovered.

The first indication of identifiable T-system tubules is found in early myotubes. Many inpocketings of the sarcolemma similar to what are commonly referred to as micropinocytotic vesicles can also be seen. In view of the subsequent appearance of tubules penetrating deeply into the interior of the myotube, it would be of interest to learn what, if any, relationship obtains between the initiation of pinocytosis in some cells and the initiation of the T-system tubules in muscle cells.

As in adult muscle, T-system tubules can form specialized connections with the SR: the triads. At

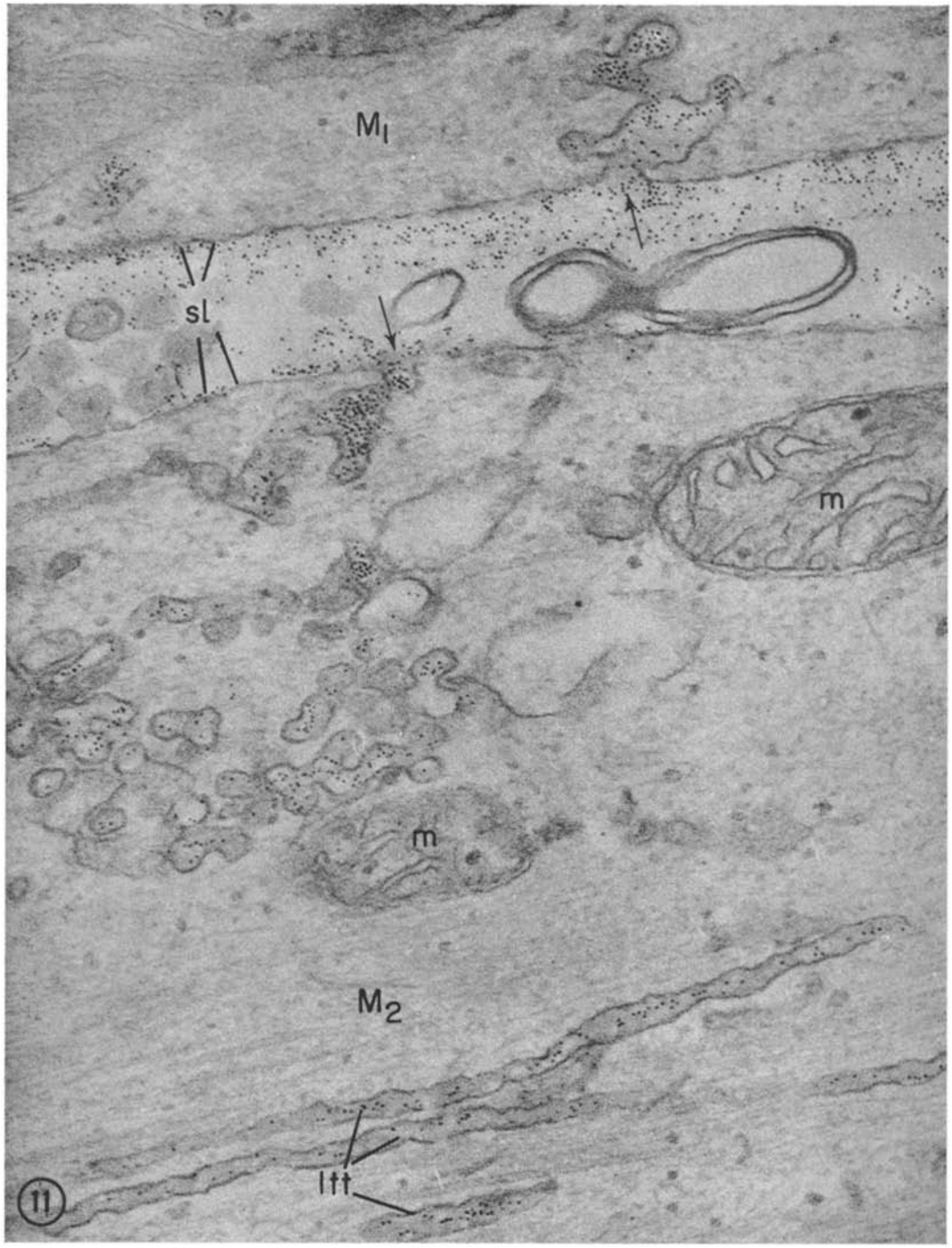

FIGURE 11 Ferritin diffusion. Longitudinal section of two myotubes (M_1, M_2) shows ferritin particles, about 110 A in diameter, within the T-system tubules. Invaginations of the T system are seen to be continuous with the sarcolemma (arrows). Some T-system tubules are found to run longitudinally beside the myofibrils for considerable distances (*ltt*). *sl*, sarcolemma; *m*, mitochondria; weakly stained with lead citrate. 6 day culture. × 76,000.

first, the triadic structures in early myotubes are formed independently of the growing myofibrils. Later a somewhat closer morphological association of triads and myofibrils is seen. Some of these early connections may be broken during the development of a definitive triad. Mature triads in our material are recognized by their dense or septate zones of electron opacity between the apposed membranes of the T system and SR, as reported in triads of mature muscle fibers by several investigators. The terminal cisternae of the SR are filled with a diffuse dense material, as has also been described for mature muscle fibers. Allen and Pepe (1), who studied myogenesis in the chick embryo, noted that the "tubular system" is the first indication of a banding pattern of the myofibrils. From their figures, the tubular system they described seems to correspond to the tubular components of the SR in our material.

Veratti (38, 39) brilliantly demonstrated with light microscopy a reticular apparatus in a variety of muscle fibers, with silver-impregnation methods (reviewed by Smith, reference 35). The work has recently been highlighted by electron microscopists, e.g. Bennett (3), one of the first to observe by electron microscopy the elaborate network of the interfibrillar membranous systems in striated muscle fibers. The metal-impregnated structure of Veratti is now widely accepted as the SR and T system (Porter, reference 27). Veratti illustrated the irregularity of the network in the muscles of embryonic, larval, and some newborn animals. It is interesting to note the similarity between the irregular pattern of the reticular apparatus in the younger forms in Veratti's material and that of the SR and T system in our material. Likewise, such irregularity in the developing SR and T system can explain the unusual course of the T tubules and anomalous connections between the T system and SR, such as dyads or pentads, occasionally seen in mature muscle.

A problem of considerable interest to students of muscle development is the positioning of the sarcomeres of adjacent fibrils in register in mature muscle. Nothing is known, either, of the events which order the Z line and/or I band of the growing myofibril into their characteristic relationship with the SR or T-system tubules. Perhaps the SR and/or T system play some role in positioning the myofibrils.

Within 4 days of culture, spontaneous contractions can be seen in some muscle cells. Shortly after myofibrils can be detected by fluorescent antibody techniques, muscle cells in vitro are able to contract (Holtzer and Abbott, reference 14). Although it has been shown here that proliferation of the SR and T system starts almost simultaneously with the formation of myofibrils, the participation of the SR and T system in spontaneous contraction is quite unknown.

Mononucleated cells, i.e. presumptive myoblasts, in early cultures contain neither myofibrils nor SR and T-system tubules. Myotubes, even in the earliest stages after formation, have all these structures present. We have not found evidence that the formation of one group of structures is initiated significantly before that of another. It is interesting that these spatially separated events of quite different nature, i.e. membrane proliferation and myofibril formation, occur simultaneously in these differentiating cells.

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