Electron Microscopic Visualization of the Filamentous Reticulum in Whole Cultured Presumptive Chick Myoblasts

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ABSTRACT This present study describes an experimental approach whereby subcellular 3-dimensional filamentous structures present within whole cells can be examined, using a conventional transmission electron microscope. This procedure uses cells which have been cultured on carbon-coated titanium grids, and treated with Triton X-100 to extract the soluble cytoplasm. Subsequent fixation and critical-point drying allows filamentous proteins to be easily visualized, due to the increase in contrast produced by removal of the ground cytoplasm. The high resolution obtainable in these preparations permitted an initial classification and description of the filamentous reticulum within cultured presumptive myoblasts. This reticulum is a continuum of filaments and cables, all elements of which appear to be interconnected. These morphological findings were then correlated with the biochemical identification of detergent-insoluble proteins, of which only actin, myosin, and, perhaps, intermediate filament and LETS protein are the major elements.

Microfilaments in cells have been described as existing as meshworks (Goldman et al., '76), networks (Spooner et al., '71; Wessells et al., '71), or as bundles of tightly packed parallel arrays of microfilaments (Buckley and Porter, '67; Goldman et al., '76) termed "stress fibers" (Lewis and Lewis, '24). Heavy meromyosin binds to microfilaments (Ishikawa et al., '69), indicating that they are largely composed of actin. The development of immunofluorescent probes has also demonstrated that "stress fibers" contain actin-like proteins (Sanger, '75). Attempts to define the spatial relationships of microfilaments by conventional transmission electron microscopic observations have been confined to two dimensions. To determine the topographical arrangement of microfilaments in cells, one would have to carry out tedious reconstructions from serial thin sections. This situation was partly alleviated by observing intact cultured cells using a 1 MeV electron microscope. This method, as described by Buckley and Porter ('75), used whole cells cultured from rat embryos, the 3-dimensional architecture of which had been preserved by critical-point drying (Anderson, '51). Subsequently, this technique was used to study the 3-dimensional fine structure of a variety of whole mounted intact cultured cells (Buckley, '75; Buckley and Raju, '76; Wolosewick and Porter, '76, '77). However, this technique is limited if the complex nature of microfilament interactions within cells is to be visualized. The poor contrast between microfilaments and surrounding cytoplasm, plus the superimposition of other cellular components, makes it difficult to determine the extent of development and disposition of microfilaments.

To overcome this problem, non-ionic detergents have been used to solubilize cytoplasmic components, leaving the insoluble filaments intact. This procedure has been applied to the study of whole cultured cells (Brown et al., '76; Osborn and Weber, '77; Small and Celis, '78; Spudich et al., '77), and has demonstrated that in different cell types, using different microscopic techniques, extraction of the cytoplasm results in a residual framework of insoluble filament bundles, plus the nucleus. These studies did not include critical-point drying of the cell preparations and thus it was

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not possible to observe the 3-dimensional configuration of the remnant filamentous structures. When, however, detergent-treated cells are critical-point dried, it has been demonstrated that spatial information on the distribution of microfilaments can be obtained either from transmission electron microscopy (Pudney and Singer, '78; Webster et al., '78) or by scanning electron microscopy (Trotter et al., '78).

The high resolving power of the transmission electron microscope is essential to study and define the supramolecular organization of filaments within cells. Furthermore, cells which have been critical-point dried retain the 3-dimensional organization of cellular organelles. Therefore, a transmission electron microscope study of the spatial orientation and 3-dimensional aspects of filaments in cells at the ultrastructural level was undertaken using cultured presumptive chick myoblasts grown on carbon-coated grids, treated with Triton X-100 and critical-point dried. Since these preparations had been critical-point dried, the scanning electron microscope was subsequently used for additional 3-dimensional visualization of filaments within the same cell. The morphological findings were then correlated with the biochemical identification of the detergent-insoluble filamentous proteins.

Removal of the cytoplasm from cells by treatment with Triton X-100 provided an experimental approach whereby the maximum resolving power of a conventional transmission electron microscope could be used to reveal spatial facets of cellular filamentous structure and organization not observed in high voltage microscopy of whole intact cells.

MATERIALS AND METHODS

Preparation of grids for tissue culture

Titanium grids (E. Fullam, Inc., Schenectady, New York) were placed on a 0.5% formvar film which had been released from a clean slide onto distilled water. The film plus the grids was picked up on a glass coverslip, dried, and lightly coated with carbon.

Culturing of cells

Cells from 11-day chicken embryo breast (pectoralis) muscle were isolated by treatment with 0.5% trypsin (Worthington) in Eagle's minimal essential medium (MEM), minus calcium and magnesium, for 20 minutes at 37°C. Single cells were separated from tissue by decanting the suspension containing the cells and centrifuging at 700 × *g* for 3 minutes. The cell pellet was resuspended in growth medium, composed of Eagle's MEM containing 10% horse serum, and 1% embryo extract (supernatant of decapitated 11-day-old chick embryos diluted 1:1 with MEM and centrifuged at 1,000 × *g* for 10 minutes to remove tissue debris). In order to remove most of the contaminating fibroblasts, cells were plated onto uncoated tissue culture dishes and fibroblasts allowed to attach for 20 minutes (Yaffe, '68). The supernatant cell suspension, enriched in presumptive myoblasts, was then transferred to collagen-coated tissue culture dishes and incubated for 24 hours. Following the 24-hour culture period, the cells were washed with MEM and then with a solution of trypsin (0.05%) and EDTA (5 mM) in Hank's balanced salt solution (GIBCO), minus calcium and magnesium, for 5 minutes in order to remove the cells from the tissue culture dish. The cell suspension was centrifuged at 700 × *g* for 3 minutes and resuspended at approximately 3×10^6 cells per ml in growth medium.

The coverslips and grids were sterilized by immersion in 70% alcohol, dried and placed in a standard Falcon plastic tissue culture dish, and a suspension of myoblasts was plated onto the grids in approximately 2 drops of medium and allowed to incubate for 1 hour. Growth medium was then added to the culture dish, so as to cover the grids. The dishes were then gently agitated to remove unattached cells from the grids, and incubated for specified intervals. At appropriate times the cells growing on the grids were viewed under a phase contrast microscope to determine their condition.

Preparation of cells for electron microscopy

The coverslips plus grids were removed from the medium, washed in hypotonic buffer, (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris HCl pH 7.4) and placed in a 1% solution of Triton X-100 in the buffer for 2 minutes at room temperature. This was followed by gently rinsing in the buffer, and then the cells were fixed by immersing the coverslips plus grids in 5% glutaraldehyde in 0.2 M S-collidine buffer, pH 7.3, for 10 minutes. The coverslips plus grids were then briefly washed in 0.2 M S-collidine buffer, followed by distilled water and 30% acetone. The cells were then treated with a saturated

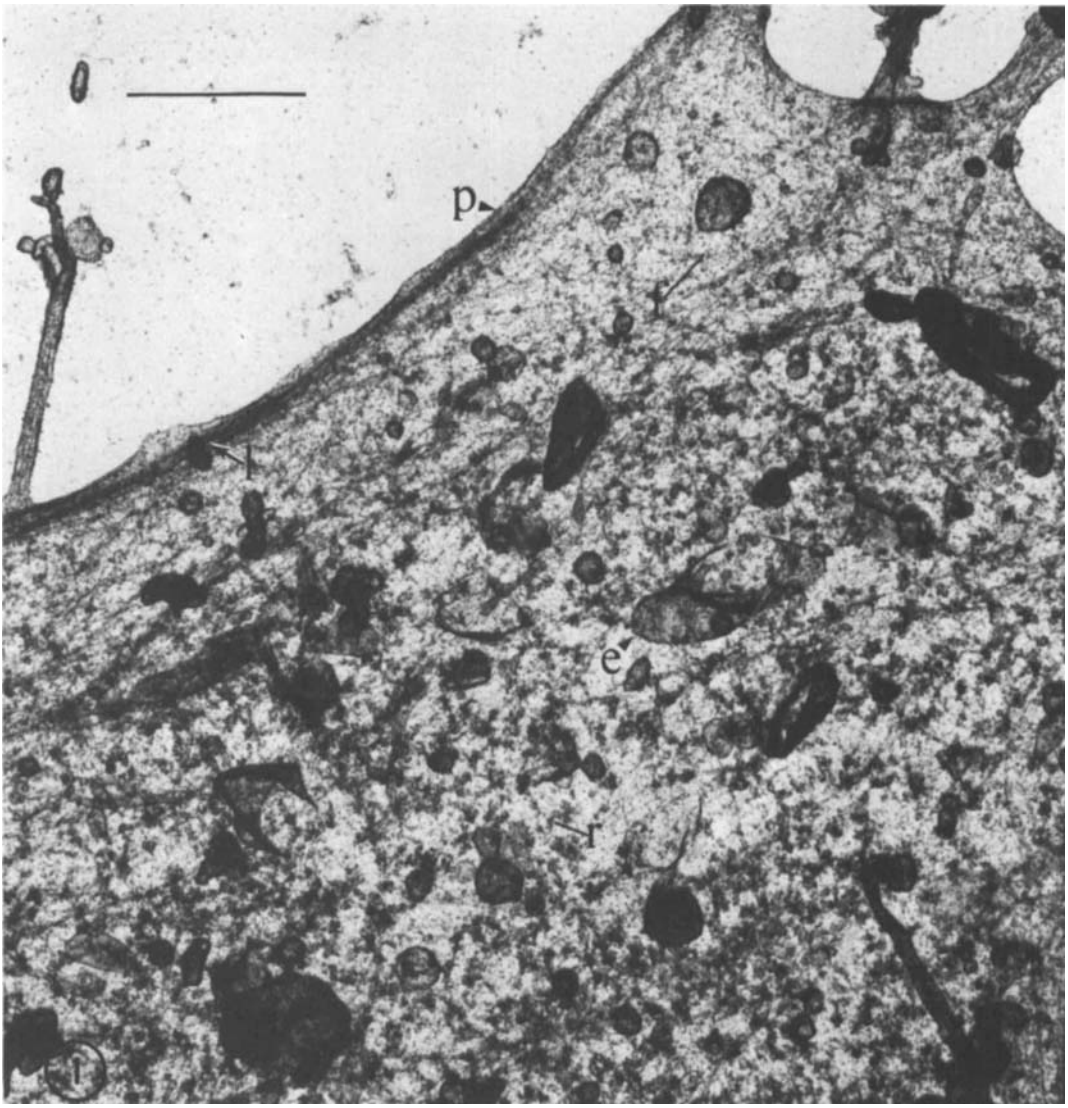


Fig. 1 The peripheral edge of a presumptive myoblast, not extracted with Triton X-100, viewed in the TEM at an accelerating voltage of 100 kv. Various cellular organelles can be seen: ribosomes (r); plasma membrane (p); and cisternae of endoplasmic reticulum (e). Filaments (f), however, are not clearly visible, except for a dense band (l) occurring beneath the plasma membrane. Bar equals 1 micrometer.

solution of uranyl acetate in 50% acetone for 3 minutes. Dehydration was continued by passing the coverslips plus grids through a graded series of acetones, 70% to 100% (2 minutes each). Finally, the grids were carefully removed from the coverslips and critical-point dried from CO_2 in a Polaron critical-point drier (Polaron Inst., Pennsylvania). Following critical-point drying, the cells were lightly coated with carbon (to provide stability in the electron microscope) and observed in a JEOL 100S electron microscope, at either 80 kv or 100 kv. Stereoscopic pairs were taken at $\pm$ 10° from the original axis. Presumptive myoblasts were also cultured for 24 hours in plastic culture dishes and processed exactly as above, except that, following fixation, the cells were rapidly dehydrated through a graded series of

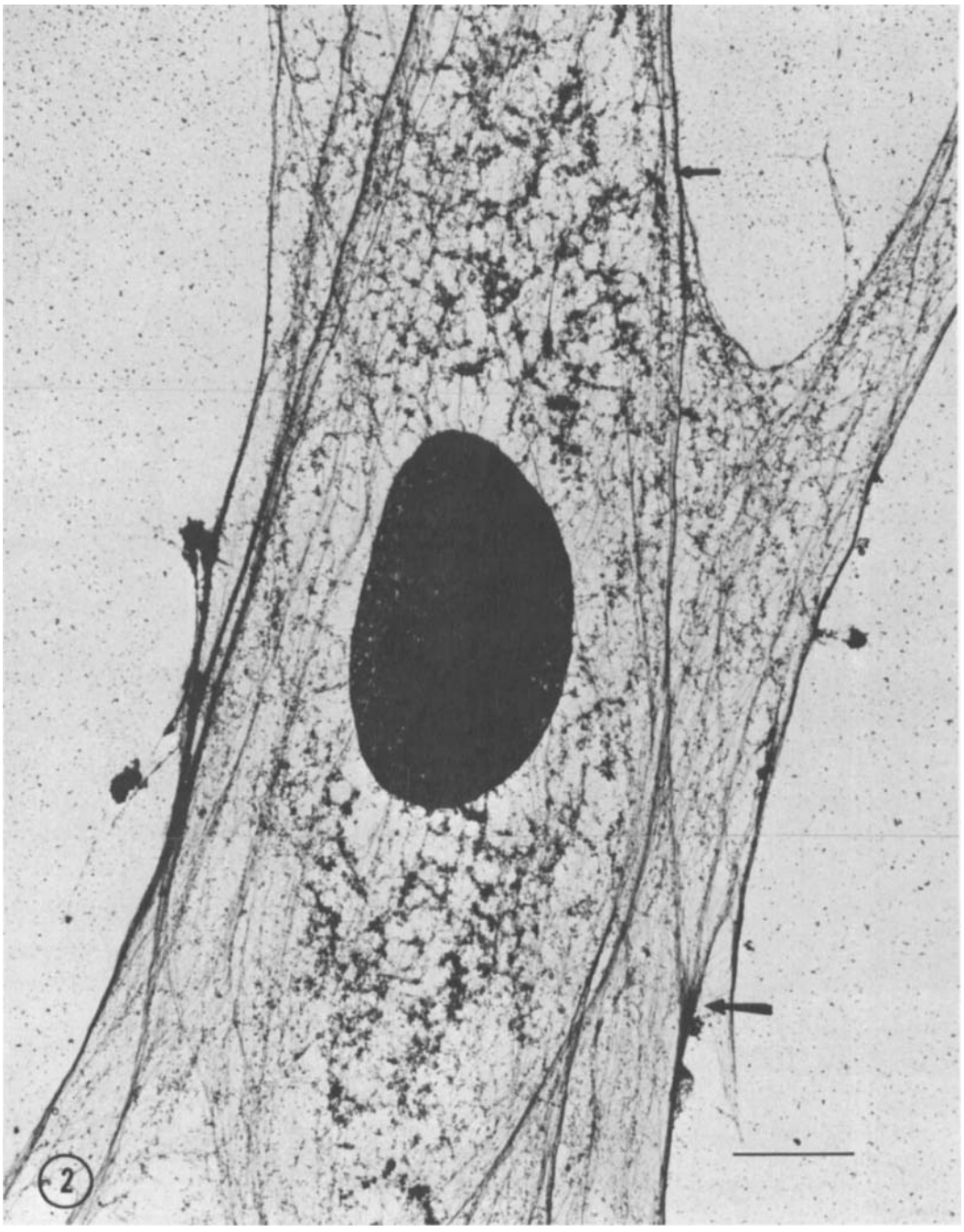


Fig. 2 Presumptive myoblast lysed with Triton X-100 and viewed in the transmission electron microscope at 80 kv. Particularly striking is the dense bundle of filaments which delineates the lateral edges of the cell. These cables often divide (large arrow) into smaller cables which then often rejoin the lateral edge cables further along the periphery of the cell (small arrow). Bar equals 4 micrometers.

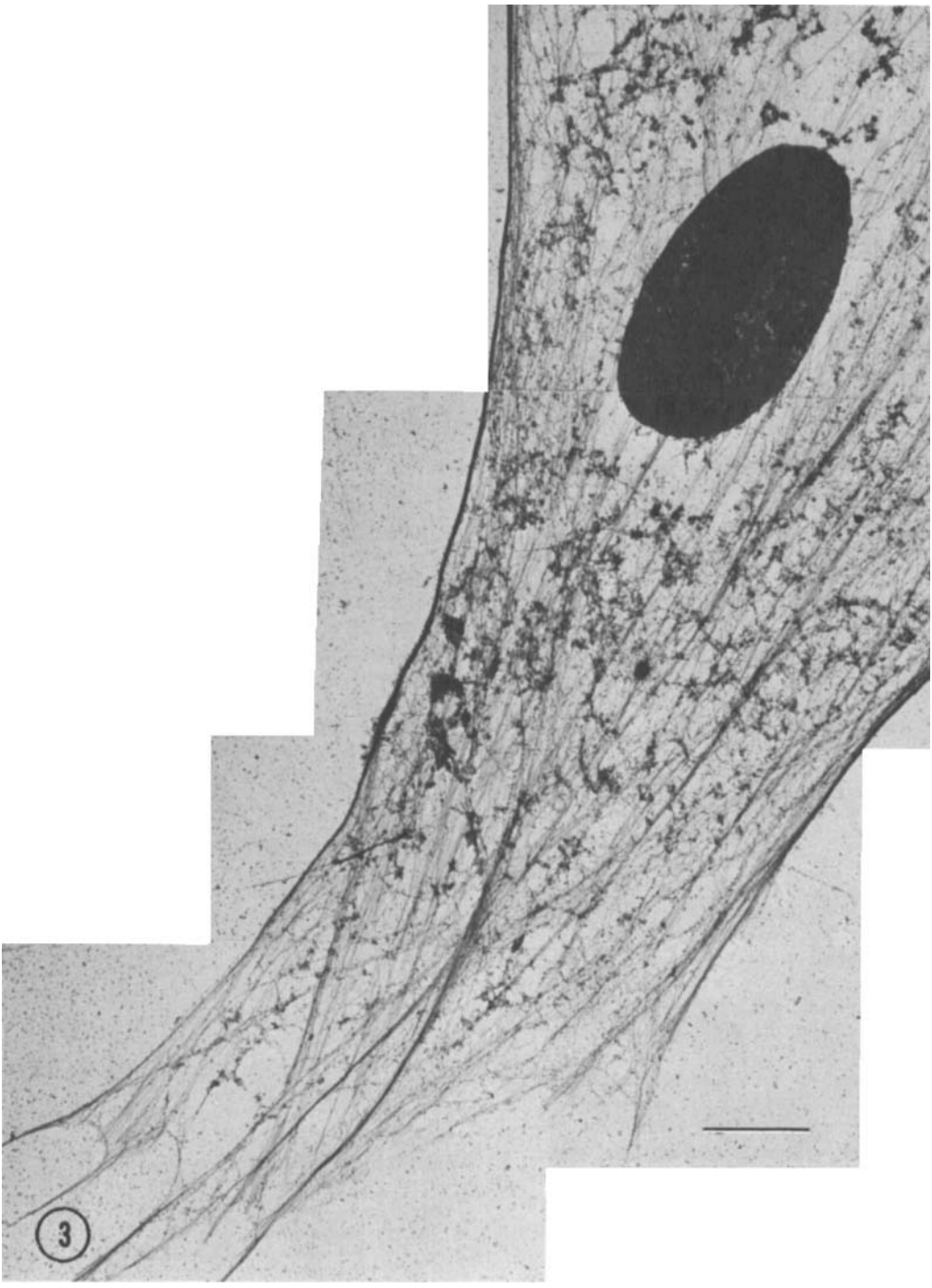


Fig. 3 This region of an extracted presumptive myoblast exhibits numerous cellular projections. As in figure 2, most noticeable are the extensive cables of filaments which course longitudinally. Also clearly evident are the dense cables of filaments present along the cell margins. Bar equals 4 micrometers.

alcohols and embedded in Epon. Thin sections were cut using a diamond knife on a Sorvall Porter-Blum Mark 2 ultrasmicrotome and contrast was enhanced in these sections by staining in a saturated solution of uranyl acetate in 50% acetone followed by lead citrate (Venable and Coggeshall, '65). Thin sections were examined in a Jeol 100S electron microscope.

Scanning electron microscopy

Following transmission electron microscopy, the grids were mounted on aluminum stubs and coated with gold/palladium in a Hummer II sputter coating apparatus (Technic, Alexandria, Virginia) and viewed in an ETEC Autoscan microscope (Hayward, California) operated at 20 kv.

Measurement of filaments

The magnification of the electron microscope was calibrated using a grating replica (E. Fullam, Schenectady, New York). Final micrographic magnifications were derived by measuring distances on the negative and corresponding print. Measurements of filaments were determined on the micrograph using a calibrated jeweler's lupe, and the diameter of these filaments was then calculated.

Electrophoresis of cellular proteins

After washing the culture plates with phosphate-buffered saline, cell cultures were treated twice, 2 minutes each, with Triton X-100 in hypotonic buffer (2 ml), and subsequently washed again with saline. The material remaining on the culture dish was freeze-dried and suspended by scraping with a rubber policeman in sample buffer for gel electrophoresis (Laemmli, '70). The Triton X-100 supernatants also were retained for analysis by gel electrophoresis. A parallel culture was washed without treatment with detergent, and scraped into sample buffer. Samples were electrophoresed on an SDS polyacrylamide slab gel (8%) with a 1% acrylamide, 0.5% agarose spacer, for 2.5 hours at 200 v. The gels were removed and stained with Coomassie brillant blue and destained in a solution of 40% methanol, 10% acetic acid.

RESULTS

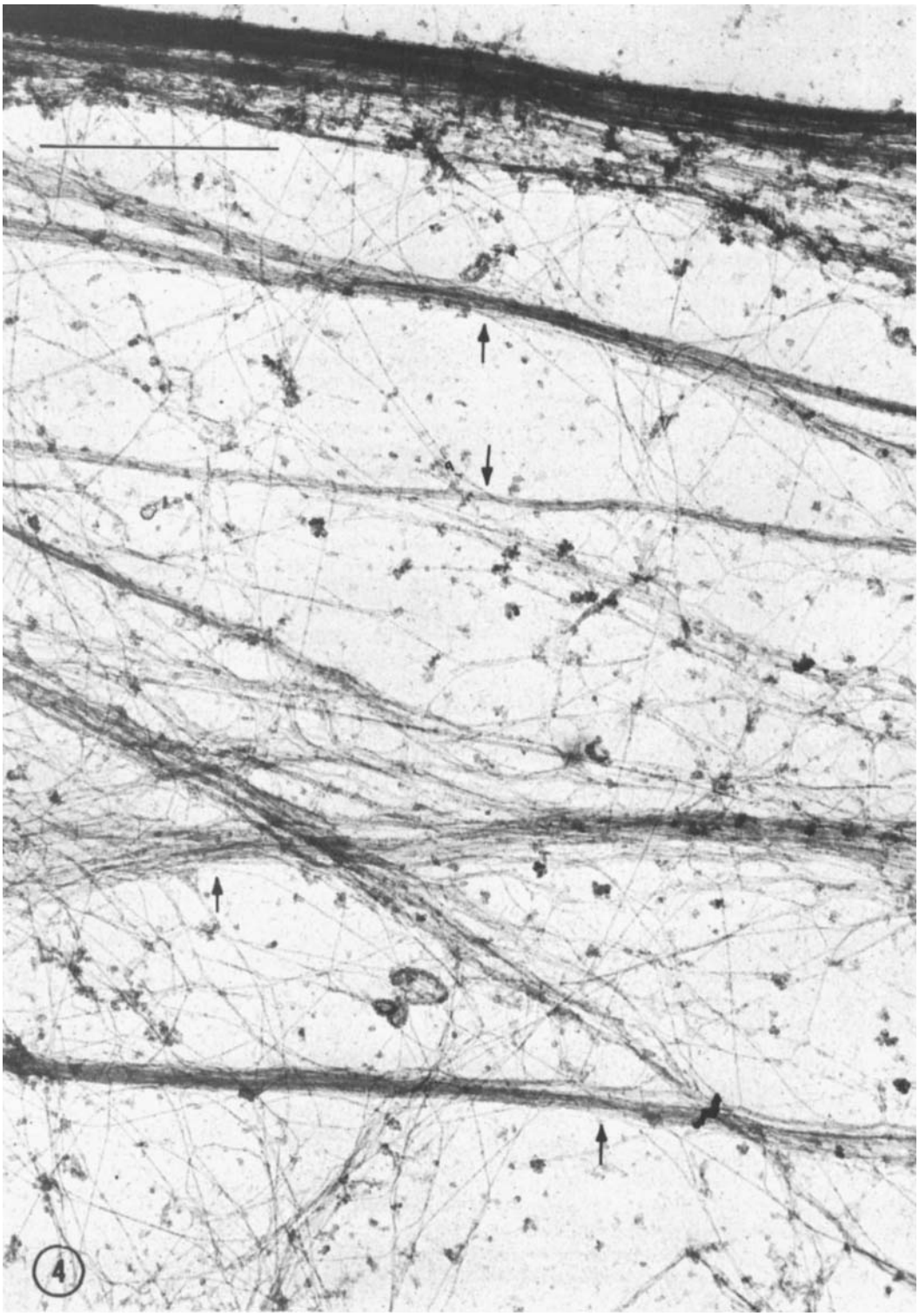
Two-dimensional observations

Cultured presumptive myoblasts, not treated with Triton X-100, but otherwise prepared exactly as extracted presumptive myoblasts, were viewed in the electron microscope at an accelerating voltage of 100 kv. At this voltage only the attenuated edges of the cells could be penetrated by the electron beam and allow observations of subcellular structures. A peripheral portion of cytoplasm of such a cell is shown in figure 1. Various organelles could be identified (endoplasmic reticulum, ribosomes, plasma membrane). The presence of microfilaments was not easily detected since the density of the surrounding cytoplasm obscured these structures, except however, for a bundle of fibers, which occurred along the lateral edge of the cell, beneath the plasma membrane.

Following extraction with Triton X-100, the highly complex extent of filament development in the presumptive myoblasts could be observed in the electron microscope. Figure 2 is a low-power micrograph illustrating the typical morphological features of a treated cell. At this magnification, the fine microfilaments could not be easily resolved. Extensive cables of filaments, however, could be observed, coursing longitudinally through the remnants of the cell body. These cables appeared to vary in their composition. They were compact and dense at the periphery where the plasma membrane would have reflected (before Triton X-100 extraction) beneath the cell body. More internal to these dense lateral edge cables, were other cables composed of loosely aggregated filaments. The organization of the cables was complex, since they were seen to branch frequently and often anastomosed with neighboring cables. The dense lateral edge cables often divided into less dense cables, and figure 2 (arrow) shows a particular case where the dense lateral edge cable had become divided into three smaller cables. One of these cables was seen to rejoin another dense lateral edge cable, further along the edge of the cell.

Aside from the cables, a light perinuclear zone was evident. Small cables and filaments traversed this area between the nucleus and the rest of the filamentous reticulum. Figure 3 is another low-power micrograph of an ex-

Fig. 4 At this magnification, the fine filaments comprising the reticulum can be clearly resolved. Different types of filamentous cables are present, varying from 60-120 nm in diameter. This micrograph shows that the dense lateral edge cable is composed of numerous tightly packed filaments. Also, the interconnections between the cables of filaments and filamentous reticulum are clearly evident (small arrows). Bar equals 1 micrometer.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tracted myoblast showing the distribution of filaments at one end of the cell body. Numerous cables running parallel to the longitudinal axis of the cell were again clearly obvious. Many of these cables could be seen to become splayed; the filaments composing these cables became disoriented and dispersed, whereas other cables continued into many of the cellular projections. In this preparation, the dense lateral edge cables delineating the remnant filamentous structure were again particularly evident. The lateral edge cables, however, became less distinct as they approached the area containing many cellular projections.

These micrographs demonstrate that the techniques did not produce any gross change in the general cellular outline and nuclear morphology. Also, the fine filaments comprising the filamentous reticulum, plus the extensive cables of filaments running through this network, were morphologically intact. The fine cellular projections produced by myoblasts during culture were also maintained.

At higher magnification (fig. 4) the composition and organization of both the fine filaments and cables of filaments were easily resolved. The topographical relationship was that of cables of filaments running through a reticulum of fine filaments. Various morphological organizations of filamentous cables are evident in this micrograph. The lateral edge cables were composed of numerous tightly packed filaments, resulting in the dense appearance of these structures. The internal cables varied from closely packed filaments to loose aggregates of filaments. The more dense cables tended to course parallel to the cell axis, while other cables, much lighter in density, coursed obliquely and, in some instances, perpendicular to the cell axis. The diameters of the different filamentous cables were found to vary from 60-120 nm. This micrograph also shows that single filaments of the filamentous reticulum interconnected with the various cables of filaments.

Figure 5 shows the fine filamentous network at high magnification (approximately 63,000 times). Small bundles of filaments may be seen which varied in diameter from approximately 15-30 nm. The fine filaments were unit structures, 7-8 nm in diameter and very often these filaments appeared beaded. Where the filaments joined or crossed over each other, there existed some dense material. Generally, the fine filaments formed vertices where five or six filaments joined. Even smaller and shorter filaments 3 nm in diameter could be seen as connecting struts between many 7- to 8-nm filaments. These small interconnecting filaments did not appear to possess any associated globules of dense material.

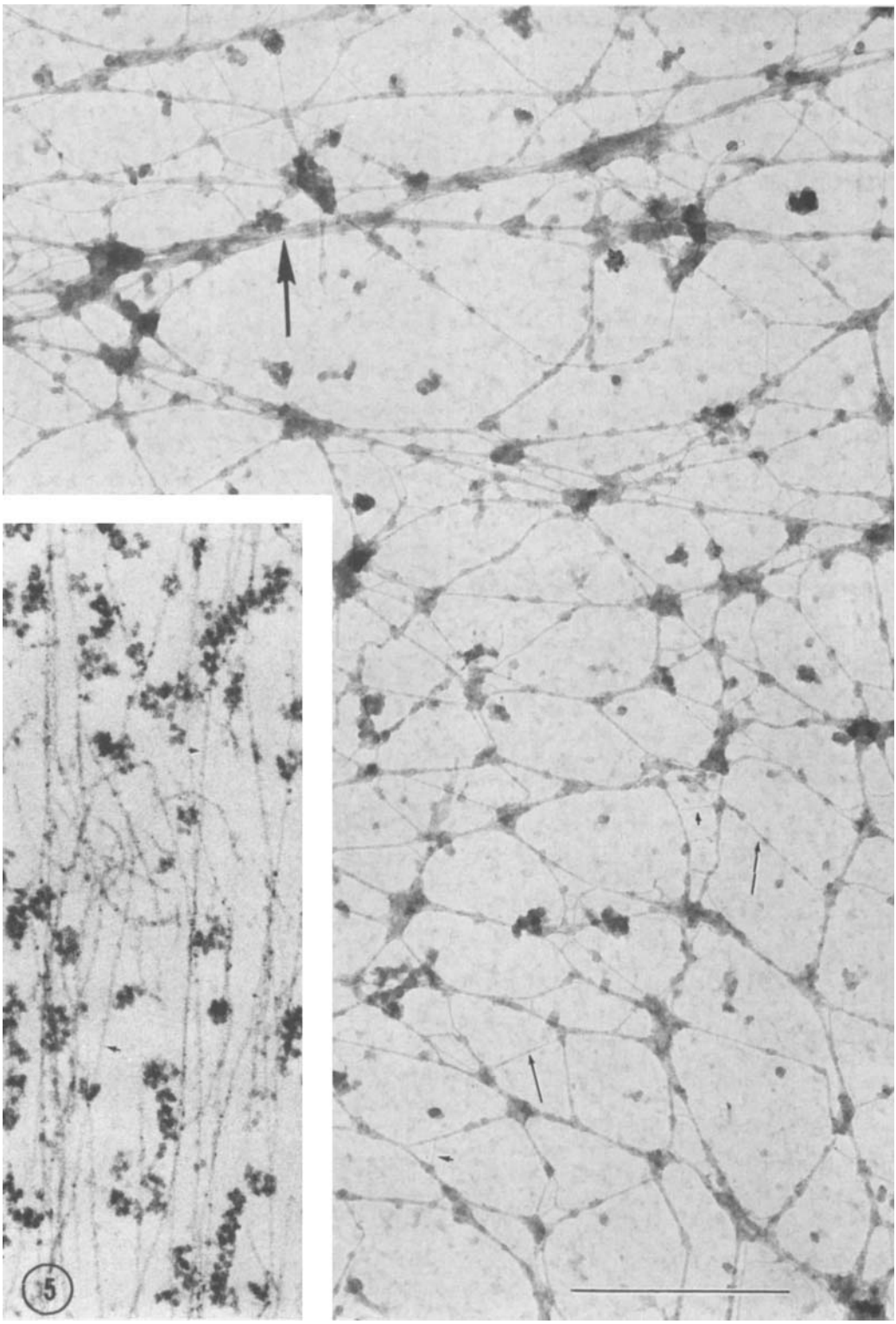
The material, left after extraction of presumptive chick myoblasts by Triton X-100, was also processed for embedding in plastic and thin-sectioned (inset to fig. 5). This procedure also demonstrated that the filamentous remnant was mainly composed of filaments 7-8 nm in diameter.

Three-dimensional observations

Figure 6 represents a stereoscopic pair of a peripheral region of a presumptive myoblast, in which the 3-dimensional distribution of the fine filaments becomes evident. The thick cables running longitudinally (to the cell axis) lie on the substrate surface with the fine filamentous network rising up toward the viewer. In this context, the reticulum could be seen to be self-supporting—the filament struts interconnecting in all directions to form a stable structure. The cables transversing the cell (perpendicular to the cell axis) were mainly situated within the filamentous reticulum, occasionally approaching the top of the cell. Thick cables from the bottom, and less dense cables on the top of the cell, often fused together near the outer margins.

Figure 7 shows two myoblasts in intimate connection (possibly in the process of fusing) observed by transmission microscopy (a) and scanning microscopy (b). The two nuclei can be seen and between them an area of close apposition of the residual filamentous reticulum. Figure 7b provides a 3-dimensional view of the same cell, viewed in the scanning electron microscope. Comparisons between figures 7a and 7b also show that the electron-opaque nuclei are not prominent in figure 7b since the gold-palladium coating accentuated the outer fibrils. The 3-dimensional aspect of the dense

Fig. 5 Most of the filaments composing the filamentous reticulum are 7-8 nm in diameter (small arrows). Some of these filaments are organized into bundles (large arrow). Short filaments 3 nm in diameter (arrowheads) also are present, connecting individual 7- to 8-nm filaments. The insert to figure 5 is a micrograph taken from a thin section of extracted presumptive myoblast embedded in Epon. Both micrographs are at the same magnification and demonstrate that the plastic-embedded filamentous reticulum is also mainly composed of filaments 7-8 nm in diameter (arrows). Some polysomes can be seen to remain with the extracted reticulum. Bar equals 0.5 micrometers.



lateral edge cables can be seen in figure 7b. In both preparations, cables coursed across the internuclear distance, with the two nuclei as an axis of orientation. Clearly evident in figure 7b is the close relationship between the cables and the edge of the nucleus. The nucleus is overlaid by filaments and cables, some of which appear (fig. 7b) to be intimately related to the nuclear membrane.

A higher magnification of a portion of this preparation (figs. 8a,b) shows the ramifying network of fine filaments in both transmission (a) and scanning electron microscopy (b). Very noticeable is the increase in apparent thickness of filaments in the scanning preparation due to the coating of these structures with gold-palladium. Many cables and filaments in figure 8a are not visible in figure 8b and must, therefore, lie within the remnant filamentous framework. At this magnification the interconnections between the dense lateral edge cable and smaller cables, in both transmission and scanning electron micrographs, can be seen.

Electrophoretic analysis of Triton-treated and non-treated myoblasts

The isolated filamentous structures derived from treatment of cells with Triton X-100 were subjected to slab gel polyacrylamide electrophoresis in the presence of sodium dodecyl sulfate, and stained with Coomassie blue. Figure 9, lane B, shows the results of the analysis of the extracted presumptive myoblasts. Only five abundant proteins could be detected, one of which has the same mobility as actin, and one the same mobility as myosin. A doublet with a higher molecular weight (250,000 daltons) than myosin was present, as well as a protein with a molecular weight of approximately 58,000 daltons. The extracted supernatant (A) and the whole unextracted cells (C) showed additional proteins not present in the filamentous structures remaining after treatment of the presumptive myoblasts with Triton X-100. All proteins represented in the residue after detergent extraction could be found in the supernatant, with the exception of the doublet band at about 250,000 daltons. This may indicate either solubilization or depolymerization of some of the filamentous proteins by this technique, their presence in the cells in a soluble form which is removed by Triton X-100, or contamination of the supernatant fraction by cells removed from the substrate by the rigorous treatment they received during extraction with the detergent.

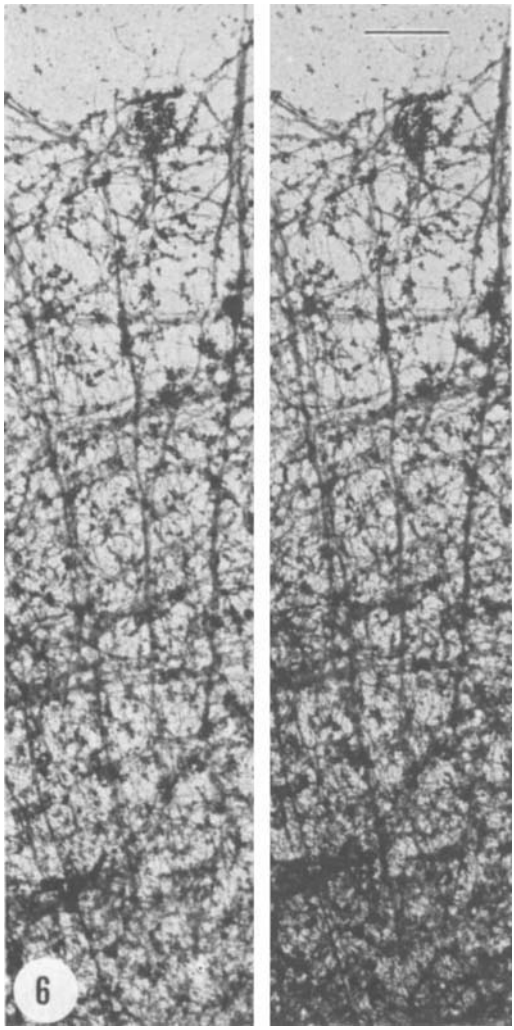


Fig. 6 A stereoscopic pair of a portion of a treated presumptive myoblast. Micrographs were taken at tilt angles of $\pm 10°$ from the horizontal axis, and demonstrate the 3-dimensional nature of the filamentous reticulum. Cables of filaments can be seen running along the lower surface of the cell. Bar equals 1 micrometer.

DISCUSSION

Triton X-100 treatment of presumptive chick myoblasts, followed by critical-point drying, demonstrates that it is possible to examine the complex spatial disposition of a network of filamentous proteins within whole mounts of cells, using conventional transmis-

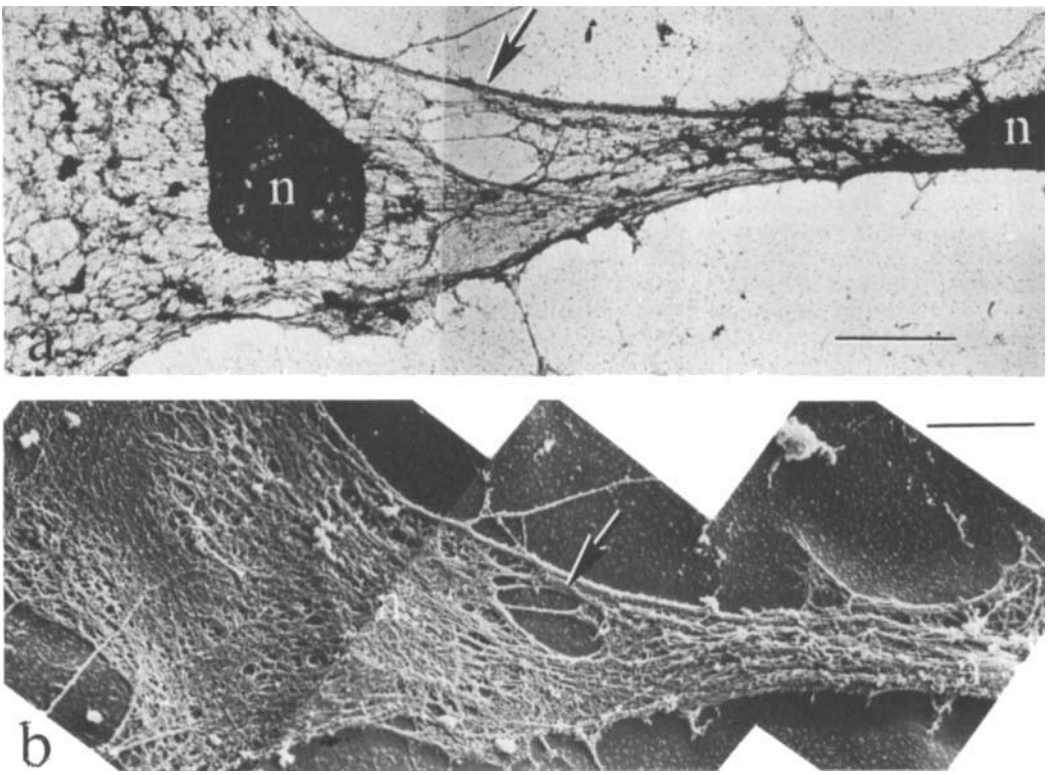


Fig. 7 Transmission (a), and scanning electron micrographs (b) of a presumptive myoblast treated with Triton. The micrographs illustrate that the same preparation can be used for transmission and then scanning microscopy in order to obtain different morphological information. The large arrows indicate an area of close apposition between two myoblasts. In both cases, the dense lateral edge cables can be distinguished. The filaments covering the nuclei (n), not easily visible by transmission microscopy (a), can be clearly seen in the scanning preparation (b). Bar equals 4 micrometers.

sion electron microscopy. The high resolving power of the electron microscope allowed us to observe single microfilaments and, by stereoscopic observation, their 3-dimensional organization. In addition, it was possible to correlate these observations with those observed in the scanning electron microscope on the same cells.

The residuum remaining after treatment of presumptive chick myoblasts with detergent was composed mainly of 7-to 8-nm filaments. The 3-dimensional observations demonstrated that these microfilaments were organized as a filamentous reticulum in which the 7- to 8-nm filaments interconnected not only with each other but also with larger arrays of filaments organized as bundles. The 7- to 8-nm filaments were seen to connect with each other at short intervals, often producing a dense reticulum. This reticulum was generally formed by five or six 7- to 8-nm filaments joined at vertices by dense material, reminiscent of the basic structure of a geodesic dome. It has been shown that filaments in cultured fibroblasts can vary from 13 nm to less than 4 nm (Ishikawa et al., '68), and those ranging from 5-7-nm bind heavy meromyosin (Ishikawa et al., '69). The study of Buckley and Raju ('76) on whole mounts of critical-point dried cultured chick embryo fibroblasts also showed that the filamentous network is composed of filaments varying in diameter (2-13 nm). Webster et al. ('78) found that in detergent-extracted critical-point dried rat kangaroo cells there was present a lattice of filaments having diameters ranging from 3-4 nm to well over 10 nm. The filaments observed in the present study could represent some or all of the lattice of "microtrabeculae" ("fine trabeculae" or "trabeculae"), averaging between 3 and 6 nm in diameter, observed by Buckley and Porter ('73), Buckley ('75), and Wolose-

wick and Porter ('77) in high-voltage studies of whole intact cultured cells. Since these "trabeculae" were continuous with bundles of microfilaments, it was suggested that they contained actin (Porter, '76).

Apart from the 7- to 8-nm filaments which formed most of the complex reticulum, there were distinctly shorter, smaller filaments, 3 nm in diameter, which were seen to connect individual 7- to 8-nm filaments. The identity and function of the 3-nm filaments is not known. They could represent an accessory molecule to actin, such as tropomyosin, or alpha-actinin, or could possibly represent an early point in the polymerization of G to F actin.

The 7- to 8-nm filaments are also continuous with and coalesce into extensive cables composed of parallel aggregations of filaments. These cables course through the cell body over long distances (micrometers) and presumably correspond to the so-called "stress-fibers" originally observed in living cultured cells (Lewis and Lewis, '24; Buckley and Porter, '67). In our study, the diameter of these cables varied from 60-120 nm and they were heterogeneous in their fine structure, containing filaments of varied thicknesses, possibly myosin or 10-nm filaments, in addition to 7- to 8-nm filaments. While Lazarides and Weber ('74) have shown that stress fibers contain actin, it has been demonstrated that other proteins may be present as well, such as myosin (Weber and Groeschel-Stewart, '74), tropomyosin, and alpha-actinin (Lazarides, '76). The cables of filaments present in Triton X-100 treated myoblasts were often seen to branch and anastamose with neighboring cables. Since these cables were also connected to the 7- to 8-nm filament reticulum it appears as if the whole system of filamentous structures is, morphologically, an integrated one.

An extremely dense cable occurs along the lateral edge of the Triton X-100 extracted myoblasts at the point where the plasmalemmal surface would have reflected underneath the cell. We have termed these structures dense lateral edge cables. The presence of subplasmalemmal filaments has been described in numerous cell types (Buckley and Porter, '67; Spooner et al., '71; Taylor, '66). In presumptive chick myoblasts the cortical layer of filaments does not form a sheath enveloping the entire cell; instead they are restricted to the lateral edges of the cell. Similarly located bundles of microfilaments have been described in glial cells (Luduena and Wessels, '73). Since it was observed that the bundles were associated with the immobile lateral surface of glial cells, a skeletal function for these bundles was suggested. In the present study the dense lateral edge cables appeared thicker in the nuclear region of the remnant cell body and became noticeably thinner and less dense when they approached the ends of the cell where surface activity was occurring. Thus, the dense layer of subplasmalemmal filaments in the immobile nuclear area could represent a rigid system, supplying support and stability for the cell as it extends filopodia.

A study on detergent-treated cells followed by critical-point drying and examination in a transmission electron microscope was recently carried out by Webster et al. ('78). They also demonstrated that in extracted rat kangaroo PtK2 and mouse 3T3 cells, there exists a 3-dimensional organization of filaments and microfilamentous bundles. When these cells were reacted with an antibody to actin, using an indirect immunoferritin procedure, it was shown that in addition to microfilament bundles, actin was contained in a lattice-like structure present in these cells.

The use of critical-point dried cells allows microscopic examination, not only by transmission but also scanning electron microscopy. Thus, morphological information obtained with the transmission electron microscope can be conveniently correlated with that seen with the scanning electron microscope on the same cell. However, due to the diminished resolving power of the scanning microscope and the coating of gold-palladium the cells receive during preparation for scanning microscopy, morphological correlations are restricted. Trotter et al. ('78) observed mouse 3T3 cells extracted with Triton X-100 using the scanning electron microscope. This study was limited to bundles of filaments and also demonstrated that they formed a complex system which branched repeatedly and exhibited extensions which connected these bundles with

Fig. 8 Detailed area of figure 7: (a) transmission, and (b) scanning electron microscopy. The increased thickness of the filaments due to gold-palladium coating (about 10 μm) can be readily seen in the scanning picture. Filaments visible in the transmission micrograph (a) and not visible in the scanning picture must lie within the filamentous reticulum. Also noticeable in figure 7a is unextracted dense material, presumably retained by the dense filamentous reticulum. Bar equals 1 micrometer.

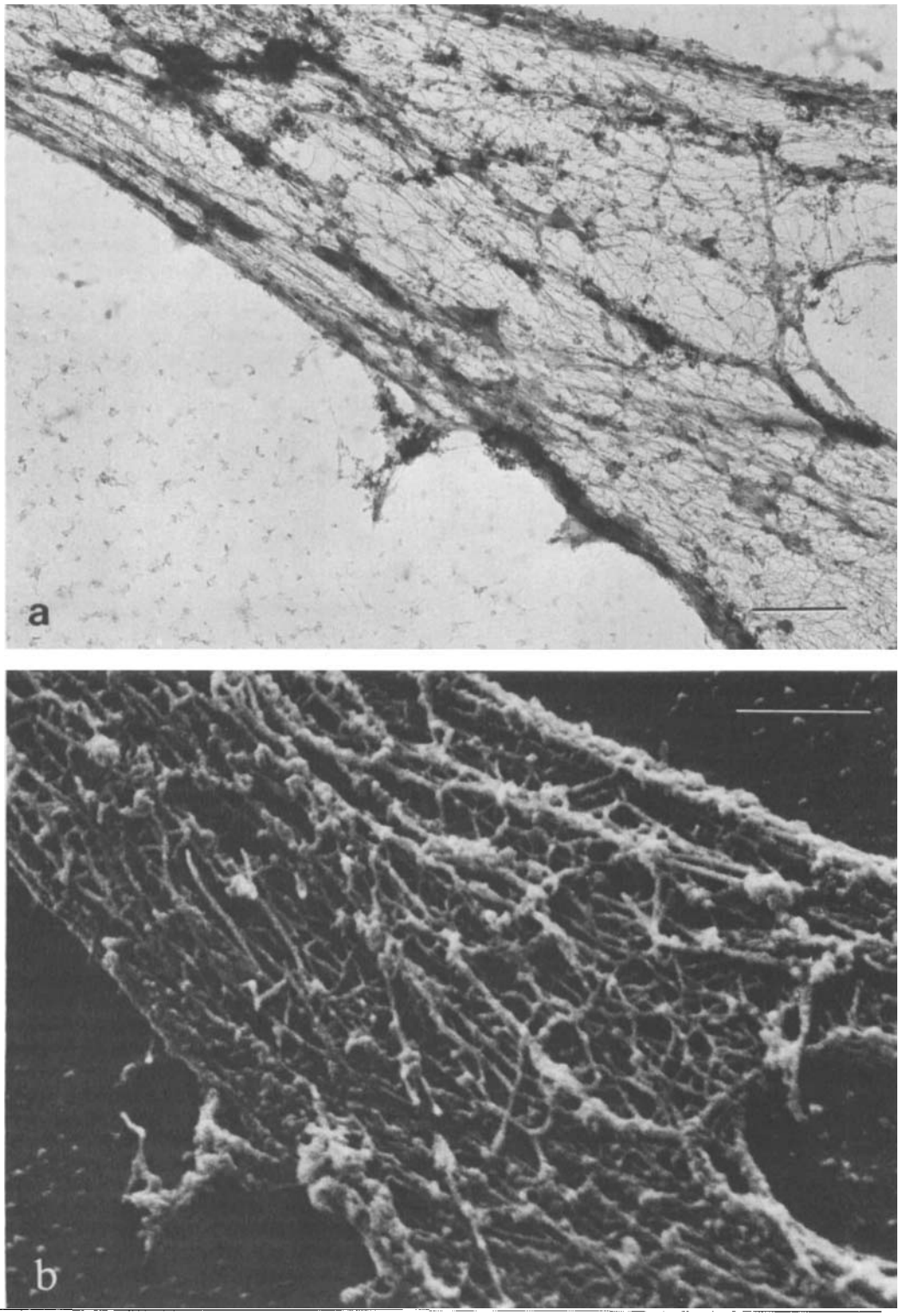


Figure 8

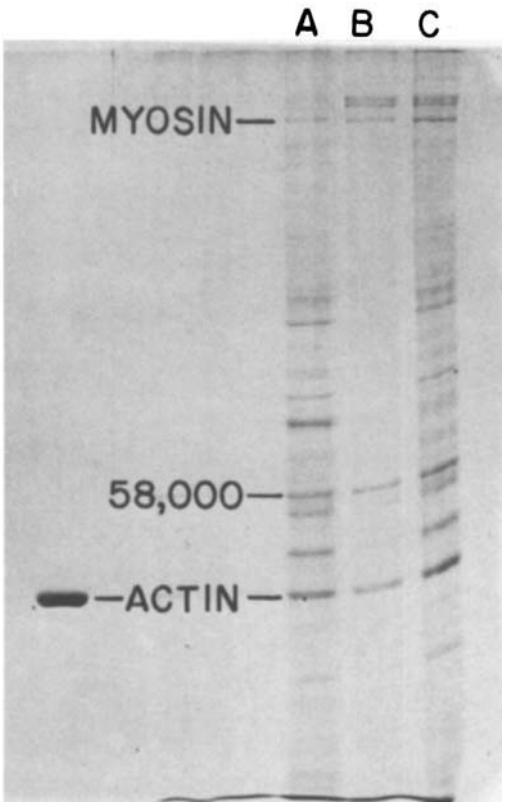


Fig. 9 Gel electrophoresis of presumptive myoblasts. Coomassie strain of equivalent amounts of (C) whole control cells, (B) filamentous structures remaining after cells were treated with Triton X-100 and washed in buffer, and (A) the supernatant remaining after cells had been treated with Triton X-100, and the filamentous residue had been removed.

each other. Our scanning electron microscope observations demonstrated that the nucleus was overlaid with microfilaments and cables of filaments, some of which appeared to be intimately associated with the nuclear membrane. Similar findings were also reported by Trotter et al. ('78). The close association of nucleus and microfilament bundles is intriguing since cytochalasin B, which affects filament integrity, causes the nucleus to be extruded from the cell (Shay et al., '74). Since the nucleus represents a morphologically stable structure within cells, it could serve as a natural anchorage for filaments, allowing the nucleus to be suspended at a central point within the cytoplasm rather than to be continually displaced during cell movement.

As also noted by Trotter et al. ('78), Triton X-100 treatment of cultured cells does not lead to detachment of the filamentous remnant from the substratum. Thus, removal of much of the surface membrane material of cultured presumptive chick myoblasts does not affect their ability to remain firmly anchored even during the treatment they receive for preparation for electron microscopy. This would appear to suggest some kind of filamentous interaction with the substratum to allow for such adhesion, perhaps through an intermediate molecule such as LETS protein.

The characterization of the residual filamentous structures remaining after the detergent treatment of presumptive chick myoblasts, by gel electrophoresis, demonstrated that they were composed of a few major proteins. Most of the filamentous protein consisted of a 43,000-dalton component which possessed the same mobility as actin. This correlated well with the morphological findings since most of the remnant filamentous reticulum was composed of 7- to 8-nm filaments, a diameter consistent with that formed by the polymerization of actin (Huxley, '63). These findings suggest that the filamentous reticulum in the presumptive myoblasts is comprised mostly of actin. However, confirmation of this can only be obtained by reacting the extracted cells with HMM or a suitably labelled antibody to actin (work in progress). Other abundant proteins occurred. One had the same mobility as myosin (220,000 daltons). A doublet of higher molecular weight (250,000 daltons) as well as a protein of molecular weight approximately 58,000 daltons were also present. The high-molecular-weight doublet protein of 250,000 daltons most likely corresponds to LETS protein, since this is unextracted by non-ionic detergents (Hynes et al., '76), and has been found to occur in chick myoblasts (Chen, '77), with identical molecular weights to those observed here. However, it should be emphasized that, while abundant proteins in the extracted reticulum can be characterized, other molecular components, such as RNA, appear to be present also, since polysomes can be seen intimately associated with this matrix.

Fellini et al. ('78) have reported that in presumptive chick myoblasts 10-nm filaments migrate at a molecular weight of 58,000 daltons. Since it has also been demonstrated that 10-nm filaments are insoluble in Triton X-100 (Small and Sobieszek, '77), it is likely that the 58,000-dalton protein seen in the

present study corresponds to 10-nm filaments. However, these filaments were not particularly evident as a discrete species in the morphological studies. It is possible that 10-nm filaments could be components of the large cables of filaments seen coursing through the remnant cell body, particularly as these cables appeared to be morphologically heterogeneous in their fine structure. Finally, the 10-nm filaments may be so intimately attached to the microfilaments, as to create a composite filament which varies in thickness throughout its length. Such heterogeneous filaments were seen in figure 5. The potential shrinkage that can occur during critical-point drying lends to the possibility that the filamentous reticulum could be mainly composed of 10-nm filaments which have been reduced to a diameter of 7-8 nm during the preparation of these cells. In fact, observation by phase-contrast microscopy carried out on the same cell during culture, Triton X-100 extraction, fixation, and following critical-point drying does demonstrate that the cells do undergo a shrinkage in toto due to the preparative techniques used (Pudney and Singer, in preparation). Other investigations have also demonstrated that significant changes in size occur in isolated lymphocytes (Billings-Gagliardi et al., '78) and isolated nuclei (Kirschner et al., '77) when these preparations undergo critical-point drying. Comparison of filament diameters, measured from Triton X-100 extracted critical-point dried chick presumptive myoblasts, with filaments measured from plastic-embedded thin-sectioned material demonstrated that the values obtained from the two differing techniques were essentially the same. This would indicate that, although overall shrinkage of the total filamentous reticulum does occur during critical-point drying, the diameters of the filamentous components remain virtually unaffected.

Although filaments have been studied intensively in a wide variety of cell types, their supramolecular organization has not been fully appreciated due to the difficulty in observing these structures using previously established microscopic techniques. Thus, the terms "filament net" (Spooner et al., '71) or "filament lattice" (Yamada et al., '71) have been used to describe the organization of filaments in various cell types. However, only after removal of the ground cytoplasm and critical-point drying can the 3-dimensional complexity of this system be seen using the superior resolving power of the transmission electron microscope.

While the biochemical and morphological properties of the filamentous reticulum can be described precisely, the functional aspects remain to be elucidated. The filaments found within a cell are usually termed the cytoskeleton. This term implies a rigid structural role for the filamentous components, even though the cytoskeleton may be composed, in part, of contractile proteins. The proteins comprising our filamentous reticulum are sufficient to provide motility. Furthermore, this term lacks the flexibility necessary to account for the diversity of the filamentous elements and their plasticity during cell movement. "Filamentous reticulum" better describes a system of filaments which can possess both structural and contractile properties, thus allowing a dual role within the cell: a morphological scaffold in some areas of the cell and a mechanism for motility in other areas.

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Note added in proof: Using different techniques, similar observations have been reported by Ip and Fishman in the October 1979 issue of the Journal of Cell Biology (*83:* 249-255).