MICROFILAMENTS AND CELL LOCOMOTION

BRIAN S. SPOONER, KENNETH M. YAMADA, and NORMAN K. WESSELLS

From the Department of Biological Sciences, Stanford University, Stanford, California 94305

ABSTRACT

The role of microfilaments in generating cell locomotion has been investigated in glial cells migrating in vitro. Such cells are found to contain two types of microfilament systems: First, a sheath of 50–70-A in diameter filaments is present in the cytoplasm at the base of the cells, just inside the plasma membrane, and in cell processes. Second, a network of 50-A in diameter filaments is found just beneath the plasma membrane at the leading edge (undulating membrane locomotory organelle) and along the sides of the cell. The drug, cytochalasin B, causes a rapid cessation of migration *and* a disruption of the microfilament network. Other organelles, including the microfilament sheath and microtubules, are unaltered by the drug, and protein synthesis is not inhibited. Removal of cytochalasin results in complete recovery of migratory capabilities, even in the absence of virtually all protein synthesis. Colchicine, at levels sufficient to disrupt all microtubules, has no effect on undulating membrane activity, on net cell movement, or on microfilament integrity. The microfilament network is, therefore, indispensable for locomotion.

INTRODUCTION

The means by which vertebrate cells migrate over a substratum has received a great deal of experimental and speculative attention. Abercrombie and his coworkers have shown that an "undulating" or "fluttering" membrane always forms at the leading edge of cells actively migrating in culture, and that the first effect of contact inhibition of migration is immobilization of this "fluttering membrane" locomotory organelle (see Abercrombie, 1961 for review). The means by which the fluttering membrane operates is unknown. Even theories proposing sol-gel transformations as the basis of locomotion include a process of contraction in the locomotor activity (Marsland, 1964). Since the contraction of muscle cells is invariably associated with filament systems, the hypothesized contractions in migratory cells may also be of a filamentous nature.

Electron microscope analyses have revealed the presence of microfilaments 40–50 A in diameter in the cytoplasm of cultured cells (Goldman and Follett, 1969), and it has been speculated that such filaments are responsible for locomotion by their contractile activity (Ambrose, 1961; Goldman and Follett, 1969; Ingram, 1969). However, there has been no direct demonstration of a relationship between locomotory behavior and cytoplasmic filaments, nor between filaments and contractile activity.

The drug cytochalasin B inhibits cytokinesis (Schroeder, 1969), salivary gland morphogenesis (Spooner and Wessells, 1970), estrogen-induced oviduct gland formation (Wrenn and Wessells, 1970), and "growth cone" function in nerve cell axon elongation (Yamada et al., 1970), apparently by altering 50 A microfilament systems in the cytoplasm. The drug has also been shown to stop cell movement in vitro (Carter, 1967). The present

study demonstrates a correlation between the effects of cytochalasin B on movement of glial cells in culture and the cytoplasmic filament systems of those cells.

MATERIALS AND METHODS

Cell Culture

Glial cell cultures were established from embryonic dorsal root ganglia. Lumbrosacral dorsal root ganglia were excised from 8-day White Leghorn chick embryos and dissociated into single-cell suspensions with 0.25% trypsin in calcium-magnesium-free salt solution by previously described procedures (Yamada et al., 1970). Cells were plated out into 35 mm in diameter plastic Petri-style tissue culture dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) at a concentration of approximately 2×10^5 cells per dish in 2 ml of culture medium.

The medium employed was that described for culturing nerve cells from the same ganglia (Yamada et al., 1970, 1971). Although some experiments were performed with nerve growth factor present, the results presented here were all done or duplicated in its absence.

Cultures were incubated at 37°C, high humidity, under an atmosphere of 5% CO_2 in air. For cultures requiring long periods of continuous microscopic observation, the bicarbonate buffer was eliminated from the medium and replaced with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid, Calbiochem, Los Angeles, Calif., modified from Fisk and Pathak, 1969). This substitution had no detectable effect on the results. All media were sterilized by pressure filtration through a type GS Millipore filter (Millipore Corp., Bedford, Mass.).

8 day embryonic chick heart was dissociated by the same procedures, and cultured under the same conditions without nerve growth factor and with HEPES. 7-day primary monolayers were dissociated with 0.25% trypsin solution and subcultured under the same conditions. All experiments were carried out with these first passage cells.

Drugs

Cytochalasin B was added to cultures at concentrations of 7 μg or 10 μg per ml of culture medium. The procedures for drug treatment, dimethyl sulfoxide (DMSO) controls, and "recovery" from cytochalasin effects were as described by Yamada et al. 1971. Cycloheximide (20 μg/ml; 7×10^{-5} M; Actidione, Upjohn Co., Kalamazoo, Mich.) was used for inhibiting protein synthesis during recovery.

Colchicine (Calbiochem) was added to both glial cell and fibroblast cultures at a concentration of 1.0 μg/ml (2.5×10^{-6} M). Stock solutions of colchicine were prepared in Hanks' balanced salt solution.

Isotope Incorporation and Radioautography

Cells were labeled with leucine-^{3}H and processed for radioautography as described by Yamada et al. (1971). Grain counts were made over single, mononucleated glial cells.

Microscopy

Living cultures were observed through phase-contrast optics on an inverted microscope in a 37°C constant temperature room. Cell movement was followed by making periodic sketches as individual cells migrated across a fixed microscopic field. Such sketches were made to scale by using a calibrated grid in the ocular.

Cells were fixed and embedded for electron microscopy in the culture dish as described by Yamada et al. (1971). Thin sections were taken parallel to the surface of the dish, beginning with the substratum side of the cell, using a Sorvall MT-2 ultramicrotome (Ivan Sorvall Inc., Norwalk, Conn.). Sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965), and examined with a Hitachi HU-11E electron microscope.

RESULTS

Normal Glial Cells In Vitro

Glial cells appear as highly spread cells on a culture dish substratum. Individual cells have irregular shapes characterized by one or occasionally several undulating membrane regions (Fig. 1). Migratory behavior traced with phase-contrast optics shows that control, DMSO-treated, and colchicine-treated (see below) cells move in the same manner: normally the long axis of the cell is parallel to the axis of movement, and one or a few regions of undulating membrane are seen at the forward tip of the cell; at unpredictable times, movement in one direction may halt, and undulating membranes may form at any other point on the cell surface and lead the cell off in a new direction. The over-all elongate shape of the cell is often adjusted to such a change in migratory direction rather slowly, the result being that a highly elongate cell can sometimes appear to move "sideways" until the shape is readjuated.

When viewed ultrastructurally, glial cells are found to possess a variety of filament systems (Figs. 2–6). Thin sections through the base of the cells, including tangentially cut portions of plasma membrane and the region just inside, reveal a

"sheath" of filaments 50–70 A in diameter arranged in a densely packed array (Figs. 5 and 6, like those in rat embryo cells [Buckley and Porter, 1967]). In places this thin sheath is thickened into bundles (see below). Individual filaments of the sheath are predominantly oriented parallel to the long axis of the cell; i.e., to the axis of cell movement. Short cross-filaments may sometimes be seen to connect long filaments of the sheath, much as sidearms connect neurofilaments in an axon (Yamada et al., 1971). In other instances, periodic densities (ranging from 6000 to 12,000 A apart) are scattered along the sheath (Fig. 6); in distribution and morphology such patches are strikingly similar to the "densities" seen in smooth muscle cell filament bundles, where they are thought to be the equivalent of the Z bands of striated muscle cells (Panner and Honig, 1970).

The thickened, bundlelike regions of the sheath are composed of 50–70-A in diameter filaments similar to those in the sheath proper. The bundles may be seen near the lateral edges of the cells (Fig. 3), or more centrally. They have been termed "stress fibers" by Buckley and Porter (1967). The bundles are recognized as thickened portions of the sheath because they can be followed in serial sections taken through the cells, beginning at the basal or substratum side, and proceeding up through the cell. These bundles can also be traced into cell processes (Fig. 2), where they are the main organelle filling the core of each process.

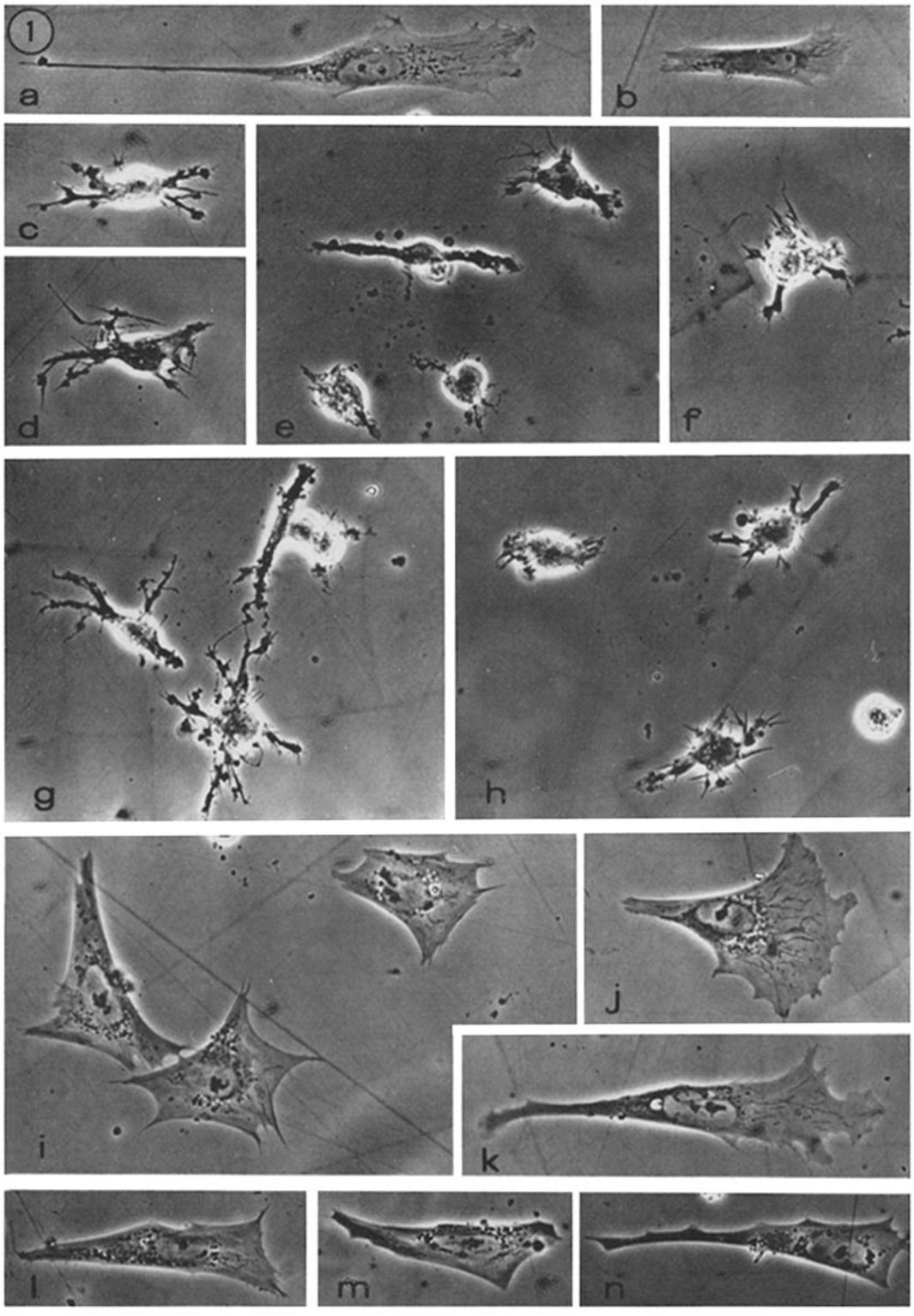
Another class of microfilaments is found immediately beneath the plasma membrane of the undulating membrane region and along the sides of the cells. In these areas, microfilaments of about 50 A diameter are arranged as a network (Figs. 4 *a*, 4 *b*, 5). This filamentous network or lattice is composed of interconnected short segments, and thereby differs from the sheath, where long straight filaments are predominant. The network appears to be arranged as irregularly shaped polygons, similar to those described for the peripheral cytoplasm of axonal growth cones (Yamada et al., 1970, 1971). Elements of the network appear to insert upon the inner surface of the plasma membrane, and in rare cases seem to be in intimate association with the edges of the filamentous sheath or its bundles (see also Buckley and Porter, 1967).

Favorable sections taken through the lower (substratum) side of the leading edge provide remarkable views of membranous structures (Figs. 7, 8). These sacs are often in the form of elongated, interconnecting channels (Fig. 7), associated in places with vesicles. Frequently, both vesicles and elongated portions of the membranous array can be traced into continuity with the plasma membrane at the leading edge of the cell. Since the set of membranous sacs is found very near the lowest surface of the cells, it has been impossible to determine beyond doubt whether they represent folds in lower plasma membrane or cytoplasmic membranes such as smooth endoplasmic reticulum. These membranous organelles have only been observed near the leading edge of cells.

Sections taken farther up in glial cells (with respect to the ventral surface) reveal other typical organelles; mitochondria and extensive rough endoplasmic reticulum, as well as free polyribosomal clusters, are present (Figs. 2 and 3). Microtubules located between the nucleus and the undulating membrane curve in a variety of directions (Fig. 2); other microtubules run parallel to the sides of the long trailing process of some cells. Finally, 100–120-A in diameter tonofilaments curve through the central cytoplasm of the cells (Figs. 2 and 3).

Effects of Cytochalasin B

The application of cytochalasin B (7–10 μg/ml) results in the complete cessation of all cell migration within 5 min, while control cells, cultured with or without 1.0% DMSO, continue normal migratory activity. In fact, more sensitive measurements with time lapse cinematography by Carter (1967) showed that inhibition of migration is "immediate." Although cell movement does not occur after 5 min, the cells remain spread and flattened on the substratum (see also Carter, 1967). After 5–15 min in cytochalasin, local cytoplasmic retractions occur at the cell periphery, resulting in a serrated appearance of the still thin and spread cell margin (see also Fig. 3 in Carter, 1967). Later (6–12 hr), extensive cytoplasmic retraction occurs and results in a ball-like shape of the central cell body from which prominent cytoplasmic processes project (Fig. 1). These processes remain in all immobilized glial cells as seemingly rigid structures for as long as 18 hr in the continuous presence of cytochalasin. During this period, migratory behavior has never been detected. In summary, the rapid inhibition of cell movement by cytochalasin, in contrast to the delayed effects on gross cell shape, shows a clear temporal separation of these two effects and establishes that cessation of locomotion is not an indirect effect of the "rounding-up" phenomenon.


1
a
b
c
d
e
f
g
h
i
j
k
l
m
n

Electron microscope observations of cytochalasin-treated cells reveal further differences between the two classes of fine filaments in glial cells. The "sheath" of filaments at the base of the cells is morphologically unaffected by the drug (Fig. 9). Similarly, the large bundles of filaments, thought to be thickened parts of the sheath, extend from the center of the cells outward into the elongate cell processes (Figs. 10 and 12). Even elevation of the drug dosage to 50 μg/ml and treatment for 12 hr does not result in disruption of these filament systems (Fig. 12). Perhaps these filament bundles are involved in maintaining the rigidity of these cell processes. If cytochalasin-treated cells possessing long processes (for example, Figs. 1 *c*, 1 *d*) are treated with colchicine (2.5×10^{-6} M; see below for ultrastructural effects of such dosage) for 24 hr no alteration in the shape of the processes takes place. Coupled with the observation that microfilament bundles are the major organelle of such processes, this result suggests that these cytochalasin-insensitive structures are capable of playing a skeletal role.

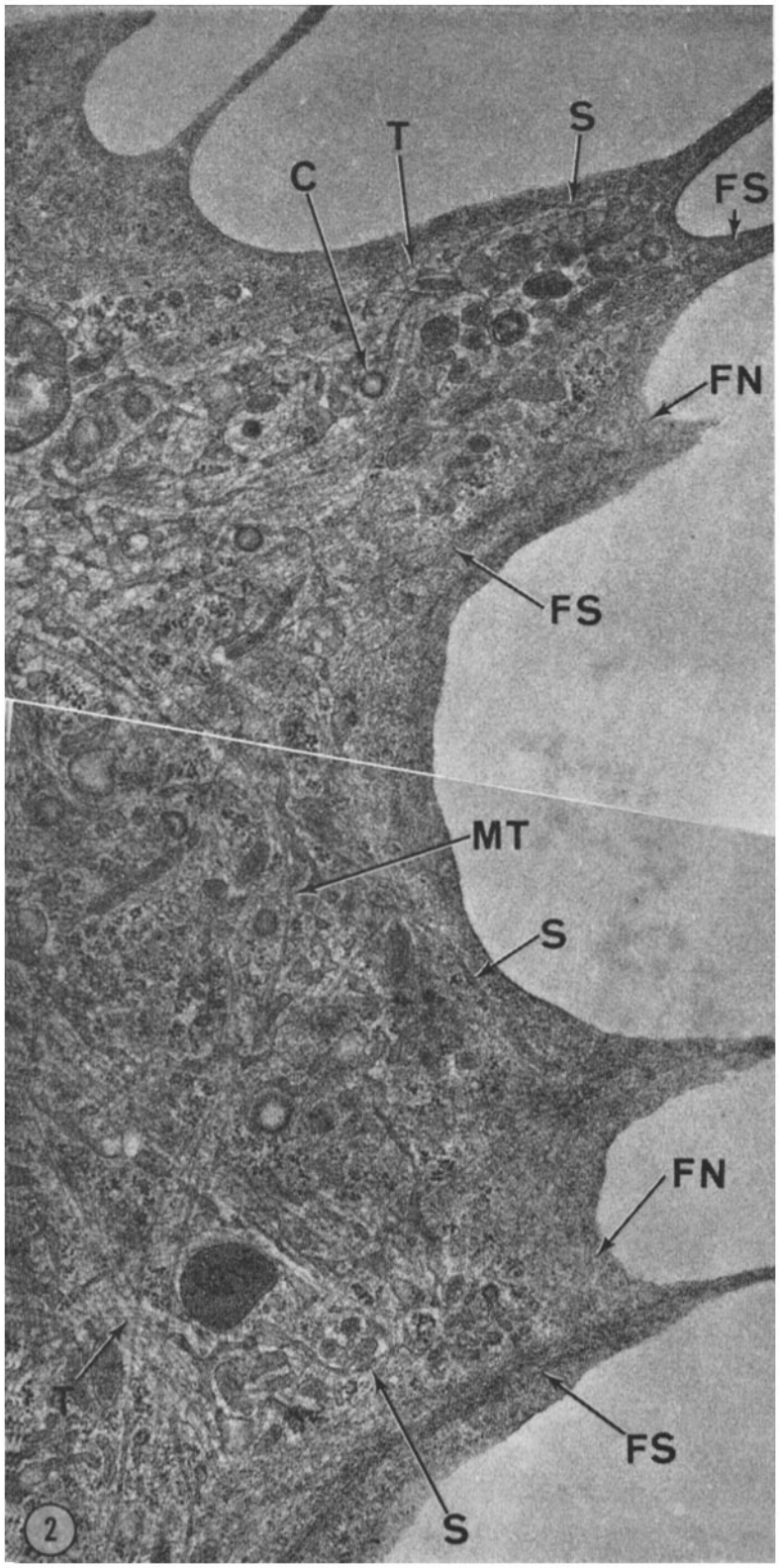
The filamentous network just beneath the plasma membrane, at both the leading edge and on the lateral sides of the cells, is altered by cytochalasin B. At places where the network is found in control cells, masses of densely packed material are present (compare Figs. 10–14 with Figs. 4 *a* and 4 *b*). Such masses are seen as early as 8 min after the drug is applied, and so their presence correlates with the rapid inhibition of movement caused by cytochalasin. The dense masses appear to be composed of closely packed short segments of filaments or of granular material (Figs. 13, 14). Even at high magnifications it is difficult to resolve individual elements in the dense masses. It is impossible to say, therefore, whether or not the dense masses merely represent a highly condensed or contracted form of the original filamentous network, or whether they are accumulations of disaggregated network material.

All organelles other than the network (Figs. 10–12) appear normal in the presence of cytochalasin (even at 50 μg/ml). In particular, there is no significant or constant alteration or dislocation of microtubules or of 100–120-A tonofilaments.

A striking feature of cytochalasin-treated cells containing the dense masses is the frequent approach of ribosomes or other cellular organelles close to the lateral plasma membrane of cells (Figs. 10, 11 *a*, 11 *b*, 13). Such a condition is not seen in control cells, perhaps because such organelles are precluded from entering the peripheral region of the cells by the filamentous network that is normally there. The cytochalasin masses themselves seem to exclude ribosomes and other organelles (see Figs. 10, 13), just as the network did before its demise. Another noteworthy aspect of the cytochalasin-induced masses is their large size and distribution upward from the lower surface of the cell and inward from the periphery. Serial sections far above the lower surface reveal such masses, suggesting that large absolute quantities of material are placed in the "dense mass" form by cytochalasin.

To summarize to this point, cultured glial cells are actively migratory and contain several types of cytoplasmic microfilaments. The application of cytochalasin B results in a rapid cessation of cell migration, an alteration of the filamentous network immediately under the plasma membrane of the cells, and in later times a "retraction" from the

FIGURE 1 Control, cytochalasin-treated, and "recovered" glial cells in culture. (*a*) A highly elongate, migrating glial cell. Note the large undulating membrane at the right that characterizes the leading edge, and the long, straight extension trailing behind. Filamentous mitochondria can be seen in the thin, leading edge of the cell. (*b*) Another migratory glial cell showing the undulating membrane with filamentous mitochondria. Note the size difference between this cell and the one shown in (*a*). (*c–h*) Glial cells after 18 hr in 7 μg/ml of cytochalasin B. The central portions of the cells are "rounded," and long, often thick, processes persist. Such cells exhibit no migratory activity. (*i*) Glial cells 3 hr after removal of cytochalasin from the medium. The cells have again flattened on the substratum. (*j*) Another cell after 3 hr of recovery from cytochalasin. This cell exhibits a prominent undulating membrane and filamentous mitochondria. Such cells are actively migratory. (*k*) A migrating glial cell after 22 hr of recovery from cytochalasin. (*l–n*) Migrating glial cells after 24 hr of recovery from cytochalasin in the continuous presence of 7×10^{-5} M cycloheximide. This level of cycloheximide eliminates virtually all protein synthesis (see Table II). Living, phase-contrast microscopy. × 450.


C
T
S
FS
FN
FS
MT
S
FN
T
FS
S
2

highly spread condition to a more "rounded" one with long cell processes.

Effects of Cytochalasin on Protein Synthesis

The electron microscope results suggest that cytochalasin selectively affects microfilament systems since no other organelle, including the rough endoplasmic reticulum, is visibly altered. The possibility that the drug acts by interfering with protein synthesis was previously examined in cultures of intact dorsal root ganglia by measuring the incorporation of radioactive amino acids into hot acid-insoluble material (Yamada et al., 1970). That analysis indicated that cytochalasin causes a slight depression in protein synthesis from that seen in DMSO-treated controls. Since intact ganglia contain both nerve and glial cells in complex three-dimensional relationships, we have reinvestigated protein synthesis using cell cultures and radioautographic procedures. These techniques have allowed the analysis to be made on glial cells alone, and on nerve cells alone.

The results of the glial cell assay are shown in Table I. The average number of grains per cell is the same in control and cytochalasin-treated cultures, indicating that the drug has no effect on incorporation of leucine-^{3}H by the cells. Since such incorporation can be blocked by inhibitors of protein synthesis (see below), leucine binding is an index of protein synthesis in these cells.

Recovery of Locomotion and Protein Synthesis

The inhibitory effect of cytochalasin on cell locomotion is fully reversible. Thus, when the drug is withdrawn and the cultures are reincubated in cytochalasin-free medium, all cells again flatten on the substratum, initiate undulating membranes and begin migrating (Fig. 1). Measurable migration is detectable within 1 hr of drug removal. Furthermore, the recovery from cytochalasin appears *not* to require protein synthesis. That is, normal recovery of locomotion occurs in the presence of levels of cycloheximide (7×10^{-5} M) that eliminate 97% of protein synthesis as measured by radioautography (Table II). Such recovered cells continue to migrate actively even when cycloheximide is continuously present for 18 hr (Fig. 1). These results show that cytochalasin does not cause irreversible alterations in cellular migratory capabilities. Furthermore, the results demonstrate that continuing protein synthesis is not required for recovery from the drug nor for locomotion itself.

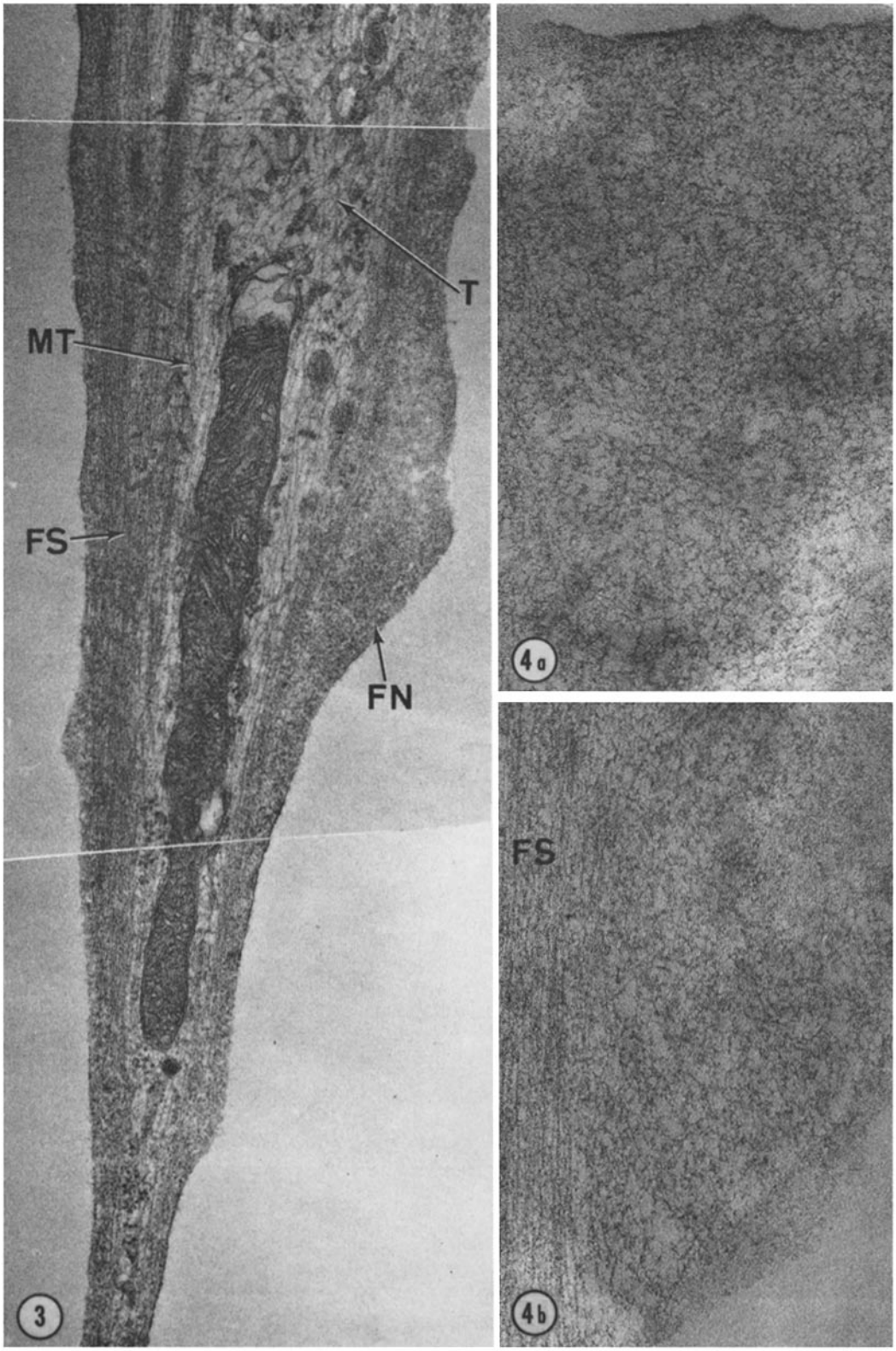
Effects of Colchicine

The possibility that microtubules play a role in cell locomotion was investigated by treating migrating cells with colchicine, since that drug is known to dissociate microtubules (Tilney and Gibbins, 1969; Robbins and Gonatas, 1964). Glial cells cultured in the presence of colchicine (1 μg/ml; 2.5×10^{-6} M) for 24 hr are capable of continued movement. That is, undulating membrane behavior continues *and* migration is still observed (Fig. 15). The drug does alter cell shape in these cultures, with the major change involving loss of long, thick processes that sometimes are seen on the cells.

Electron microscopy of colchicine-treated cells reveals that both the microfilament network (as in Figs. 4 *a* and 4 *b*) of the undulating membrane, and the sheath of microfilaments (as in Figs. 5, 6) are structurally unaltered. Of further interest is the finding that the leading edge of the cells possesses the same pattern of membranous sacs and vesicles as is found in control cells (see Fig. 7).

It is evident that the colchicine is active in these cultures because: (*a*) the nerve cells scattered throughout the cultures exhibit typical axonal retraction in response to the drug (see Yamada et al., 1970); (*b*) cells in a state of mitotic arrest are found with some frequency by electron microscopy; and (*c*) neither cytoplasmic nor spindle microtubules were observed in any of the glial cells examined (Fig. 16). Interestingly, many 100-A filaments are present in areas where microtubules

FIGURE 2 The anterior edge of a migratory glial cell. The area (*FN*) occupied by the filamentous network appears dense at this magnification, so that individual microfilaments are difficult to resolve. Some of the 50-A in diameter filaments of the sheath (*FS*) run beneath the anterior edge of the cell, and into the microspike extensions. Microtubules (*MT*) curve in a variety of directions. Elements of the smooth endoplasmic reticulum (*S*) approach close to the edge of the cell. Coated vesicle, *C*; 100-A tonofilament, *T*. × 26,000.


T
MT
FS
FN
3
4a
FS
4b

would be expected (Fig. 16), possibly representing dissociated microtubule subunit aggregates (Ishikawa et al., 1968; also as in colchicine-treated nerve cells [Yamada et al., 1971]). Clearly then, the drug affects glial cell microtubules, although net movement is not impaired.

The failure of colchicine to inhibit glial cell movement is an observation of such consequence that it is pertinent to ask if movement of other cell types is also immune to that drug. When the drug is applied to cultures of migrating chick heart fibroblasts, a cell type commonly employed in studies of cell locomotion (Abercrombie, 1961), the results are identical with those obtained with glial cells. That is, fibroblasts cultured in the continuous presence of colchicine for 24–36 hr possess undulating membranes *and* exhibit active locomotion (Fig. 17). A change in cell shape does occur in response to colchicine and involves a loss of the extremely long processes that characterize untreated cells. Shorter cell processes are maintained. Since these results indicate that both glial cells and fibroblasts continue to migrate in the presence of colchicine, it can be concluded that intact microtubules are not essential for cell locomotion.

DISCUSSION

It is becoming increasingly evident that one essential component of single-cell migration is the action of intracellular microfilaments. Such filaments, averaging 50–60 A in diameter, have been described in cells migrating in vitro and it has been suggested that they could function in locomotion by contractile activity (Ambrose, 1961; Ingram, 1969; Goldman and Follett, 1969). Although microfilaments are known to be present in the undulating membrane and processes of migrating cells, there has previously been no direct correlation between microfilament network integrity and migratory capabilities.

The current study provides such a correlation. If the filamentous network found normally beneath the plasma membrane of a migratory glial cell is altered by cytochalasin treatment, cell movement ceases. The same result applies to cytochalasin-treated nerve cells (Yamada et al., 1970, 1971) in which cessation of growth cone and microspike activity at the tip of an elongating axon correlates with a halt in axon elongation, as well as with an alteration in the morphology of the filamentous network of the growth cone and microspikes. The growth cone–microspike region can be thought of as the undulating membrane of an elongating nerve axon, so the parallel results are not surprising.

Colchicine disruption of cytoplasmic microtubules fails to halt undulating membrane activity of glial cells and heart fibroblasts, or growth cone activity of axon tips (except, in the latter case, when axon collapse finally pulls the nerve tip free of the substratum). Such findings are in congruence with the finding that epibolic cell movements in *Fundulus* continue in the presence of colchicine (Kessel, 1960). In these various cases, microtubules apparently do not play a critical role in the function of the locomotory organelle of the cells, nor in net movement over a substratum. However, since long cell processes are diminished in number following colchicine application (see Goldman and Follett, 1969, as well as our results), cytoplasmic microtubules probably do serve a skeletal function in migrating cells.

These experiments reemphasize the role of microfilaments in cell locomotion. Since involvement of those filaments is established using cytochalasin, it is crucial to understand how that drug

---

FIGURE 3 The trailing end of a migratory glial cell, as seen at the left in Fig. 1 *a*. 100-A in diameter tonofilaments (*T*) are found most centrally in the cellular process; 50-A in diameter sheath filaments (*FS*) occur more peripherally and always parallel the long axis of such a cellular extension; and the site of the filamentous network (*FN*) is indicated just internal to the lateral plasma membranes. Microtubule, *MT*. × 31,500.

FIGURES 4 *a* and 4 *b* Views of the filamentous network of the undulating membrane at the anterior (Fig. 4 *a*) and anterolateral (Fig. 4 *b*) edge of a glial cell. A portion of the filamentous sheath (*FS*) is seen on the left in Fig. 4 *b*). Note the polygonal nature of the network and its frequent approach and apparent insertion on the inner surface of the plasma membrane. As seen in Fig. 4 *b*, network and sheath filaments seem to meld together; the precise relationship between the two is not clear. Fig. 4 *a*, × 83,000; Fig. 4 *b*, × 84,000.

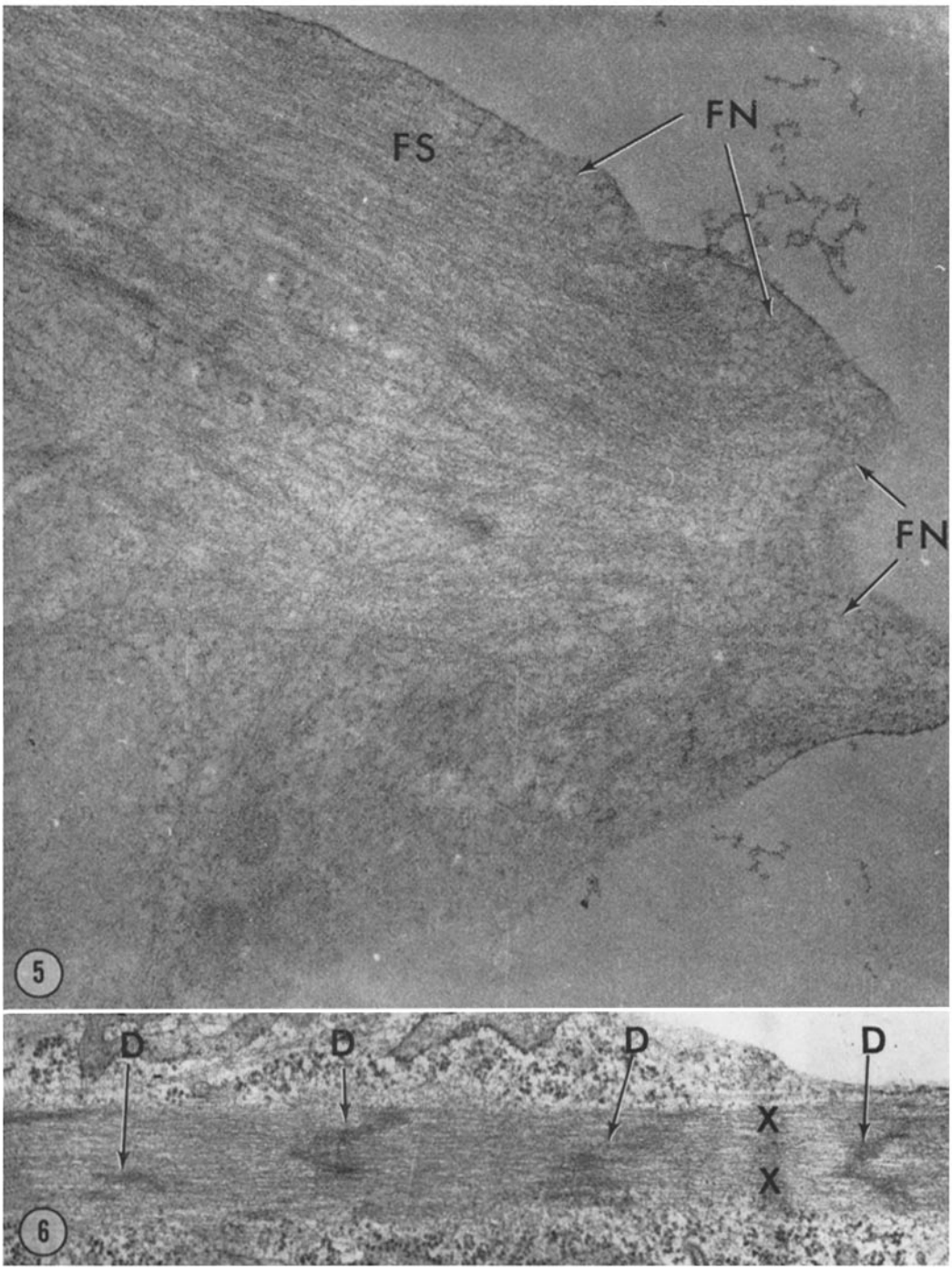


FIGURE 5 A section through the lower, anterior surface of a glial cell. Most filaments in the sheath (*FS*) tend to run parallel to the long axis of the cell (from left to right). At the top and right, the sheath grades off into the filamentous network (*FN*) that underlies the plasma membrane. × 39,500.

FIGURE 6 A portion of the sheath showing periodic densities (*D*) reminiscent of Z bands in striated muscle cells (the dense region marked *X* is a sectioning artifact). Similar "densities" have been observed along the sheath of heart fibroblasts. × 35,000.

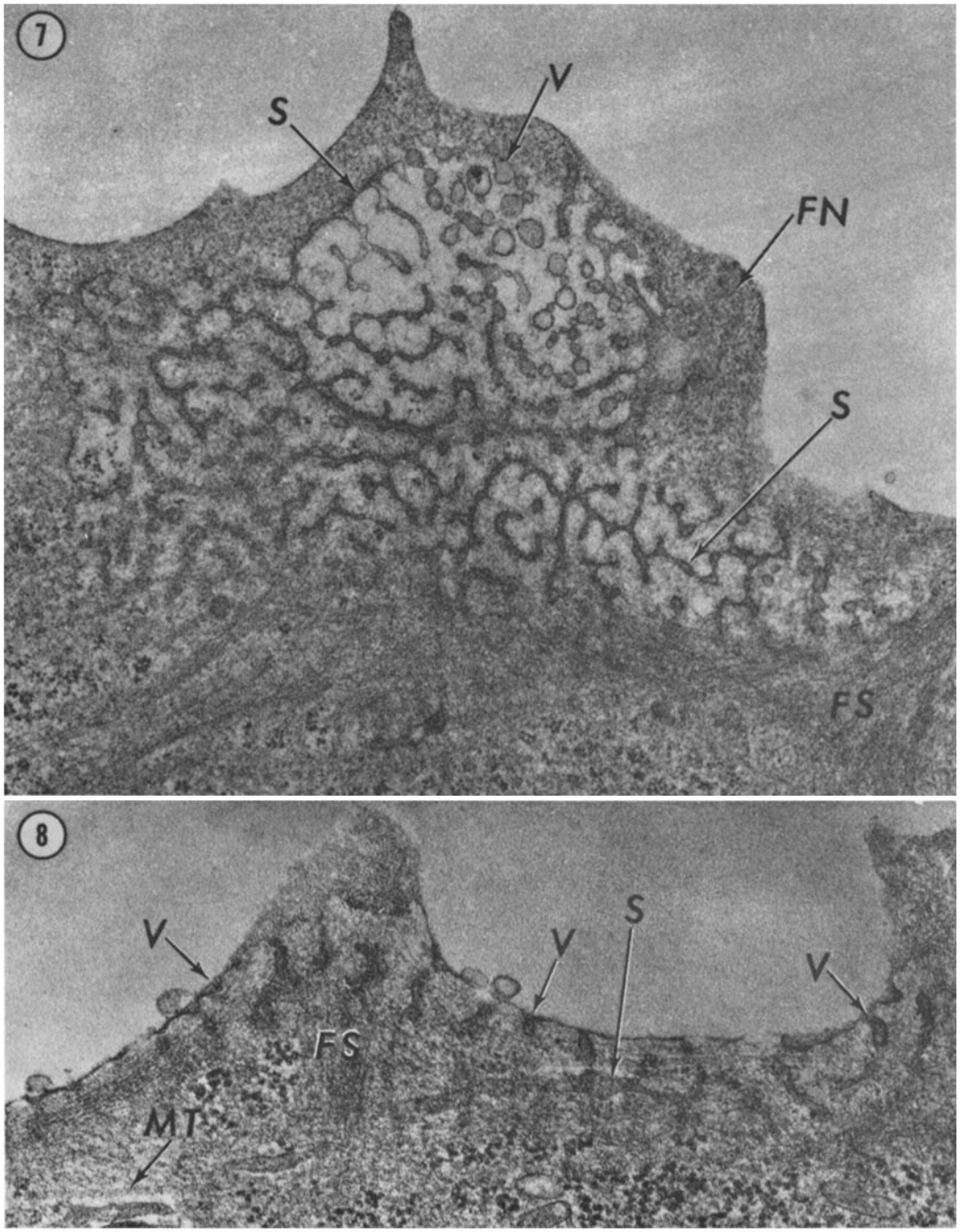


FIGURE 7 A peculiar arrangement of membrane profiles often seen near the anterior edge of a migratory glial cell (seen also in heart fibroblasts). Elongated, branching sacs (*S*) with electron-opaque material and vesicles (*V*) are the predominant structures. The site of the filamentous network (*FN*) is seen peripheral to the membranous profiles, whereas a portion of the filamentous sheath (*FS*) is present in the lower part of this photograph.

FIGURE 8 A view of apparent fusion of parts of the membraneous organelle with the anterior plasma membrane. The fact that the fusion vesicles (*V*) are not spherical suggests that these structures are not "coated vesicles" and therefore that pinocytotic activity is not the source of this phenomenon. Instead, such regions, as well as some of those in Fig. 7, may be points where new membrane is added to the anterior cell surface. *S*, smooth endoplasmic reticulum; *FS*, filamentous sheath; *MT*, microtubules. × 58,000.

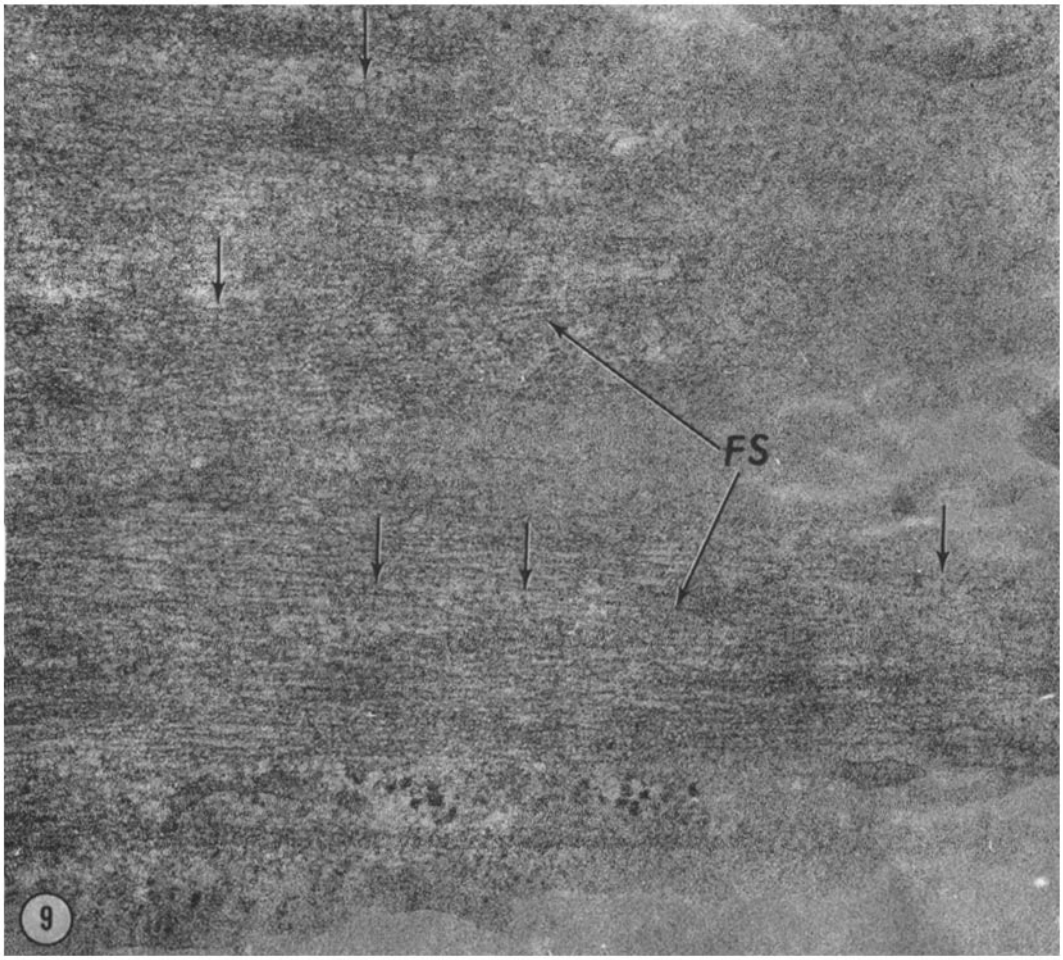


FIGURE 9 A view of the sheath (*FS*) as it approaches the anterior-most edge of a cytochalasin-treated glial cell. Hints of interconnections between individual filaments are seen (arrows), and no obvious alterations resulting from drug treatment are evident. × 80,000.

acts. Cytochalasin alters both linearly arranged sets of microfilaments (bundles) found in epithelial cells (oviduct, Wrenn and Wessells, 1970; salivary gland, Spooner and Wessells, 1970) and networks of filaments in migratory cells (nerve, glia, salivary mesoderm, heart fibroblast; summarized in Wessells et al., 1971). In both situations pools or masses of granular and short filamentous material are seen in cytochalasin-treated cells. No other organelles are visibly altered: neither the morphology nor the distribution of microtubules, nor of 100-A in diameter filaments, is changed. Furthermore, not all fine (about 50-A in diameter) filaments are altered, since both the microfilamentous sheath of migratory cells, and the core filaments of microvilli (in oviduct, Wessells et al., 1971) appear intact after prolonged treatment with high doses of cytochalasin. The continuance of protein synthesis and of normal nuclear division cycles (Carter, 1967; Ridler and Smith, 1968; Schroeder, 1969) implies that cytochalasin is not a general metabolic poison. The means by which it can act so specifically on certain classes of microfilament systems is not yet understood. Possibilities include direct action on the microfilaments, disruption of normal insertion points of microfilaments on membrane (as in junctional complexes of epithelial cells), or inhibition of processes essential

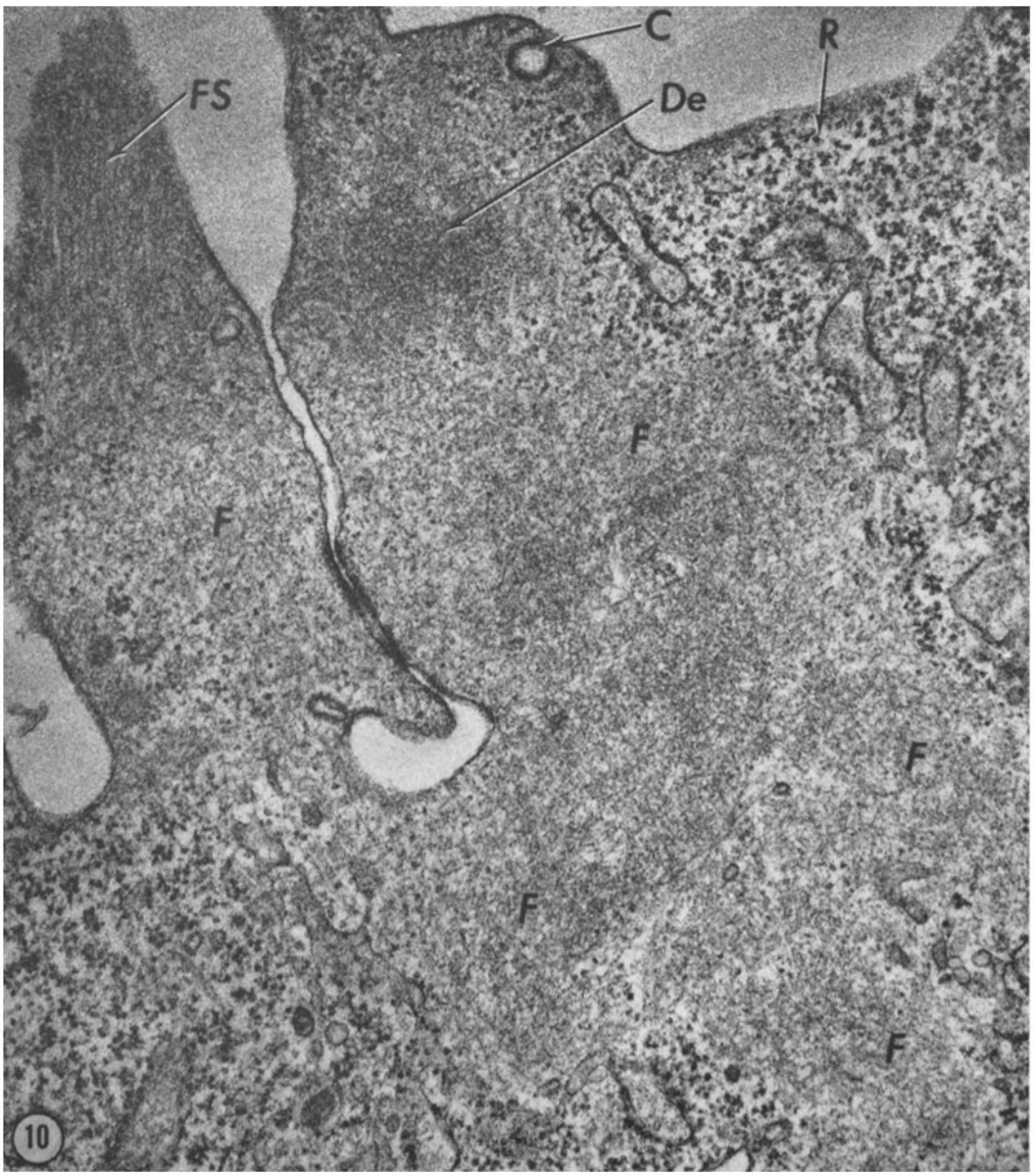
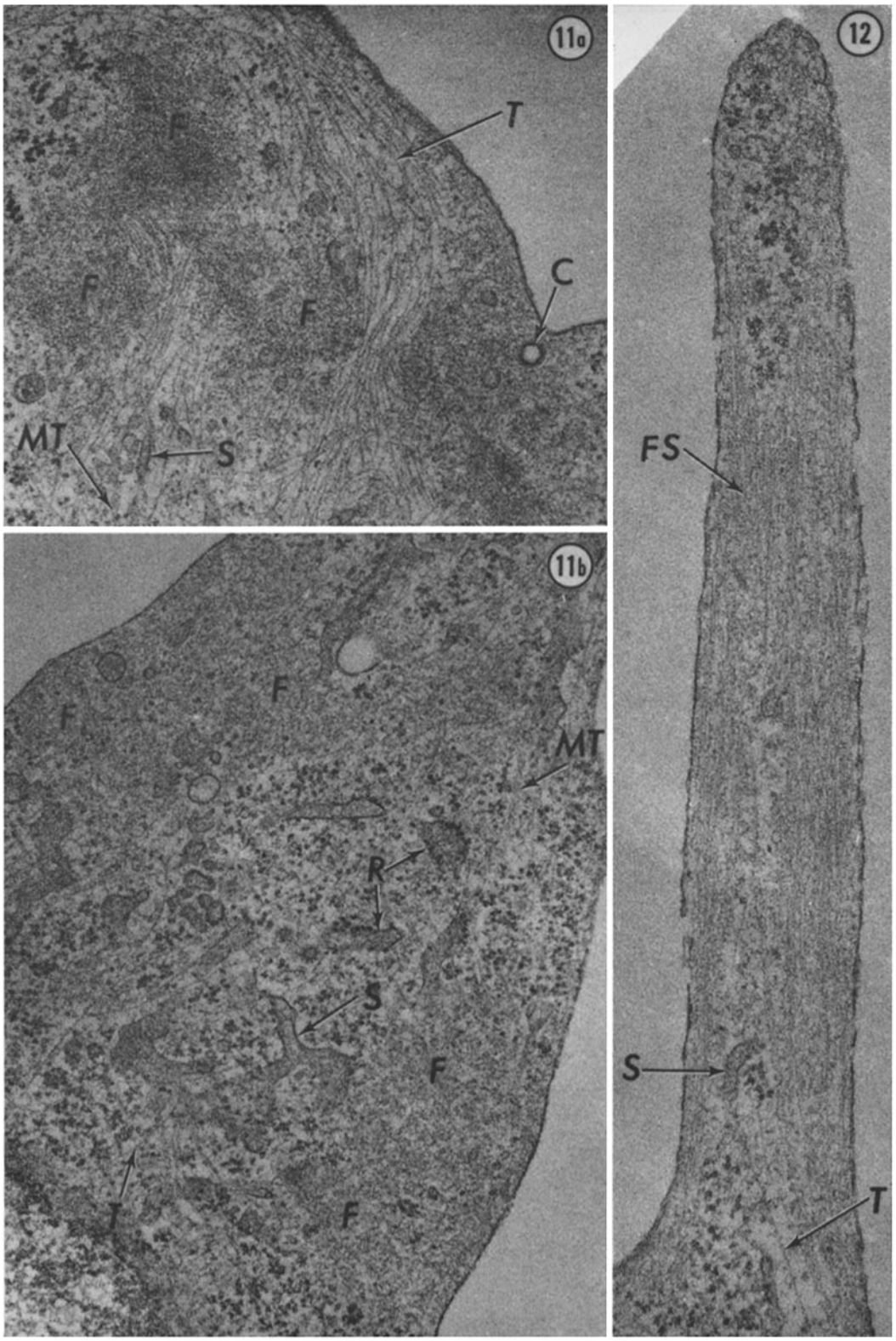


FIGURE 10 A mass of fine filamentous material (*F*) in a cytochalasin-treated glial cell. Part of the "insensitive" sheath (*FS*) is seen, as are dense regions similar to those observed in nerve growth cones (*De*) (Yamada et al., 1971). Masses like this one are indistinguishable from those found in cytochalasin-treated salivary and oviduct epithelial cells. Note that the ribosomes (*R*) approach very close to the plasma membrane, a condition not usually seen in control cells, presumably because of the presence of the filamentous network. *C*, coated vesicle. × 43,000.

for function of microfilament systems (e.g., calcium availability for a contractile apparatus; Wessells et al., 1971).

Whatever action is primary, cytochalasin does not cause irreversible loss of microfilament function, since all systems recover from drug treatment. This can take place in the presence of cycloheximide under conditions in which nearly all



FIGURES 11 *a* and 11 *b* Portions of cytochalasin-treated glial cells. Microtubules (*MT*), 100-A in diameter tonofilaments (*T*), rough (*R*) and smooth (*S*) endoplasmic reticulum, a coated vesicle (*C*), and other normal-looking organelles are present in the same cytoplasm with the cytochalasin-induced masses of filamentous material (*F*). Note here and in Fig. 10 that ribosomes and other organelles appear to be excluded from the masses formed in response to cytochalasin. Fig. 11 *a*, × 42,000. Fig. 11 *b*, × 39,000.

FIGURE 12 A cellular process on a glial cell treated with 50 μg/ml of cytochalasin. The sheath filaments (*FS*) are apparently unaffected by the drug. *S*, smooth endoplasmic reticulum; *T*, tonofilament. × 42,000.

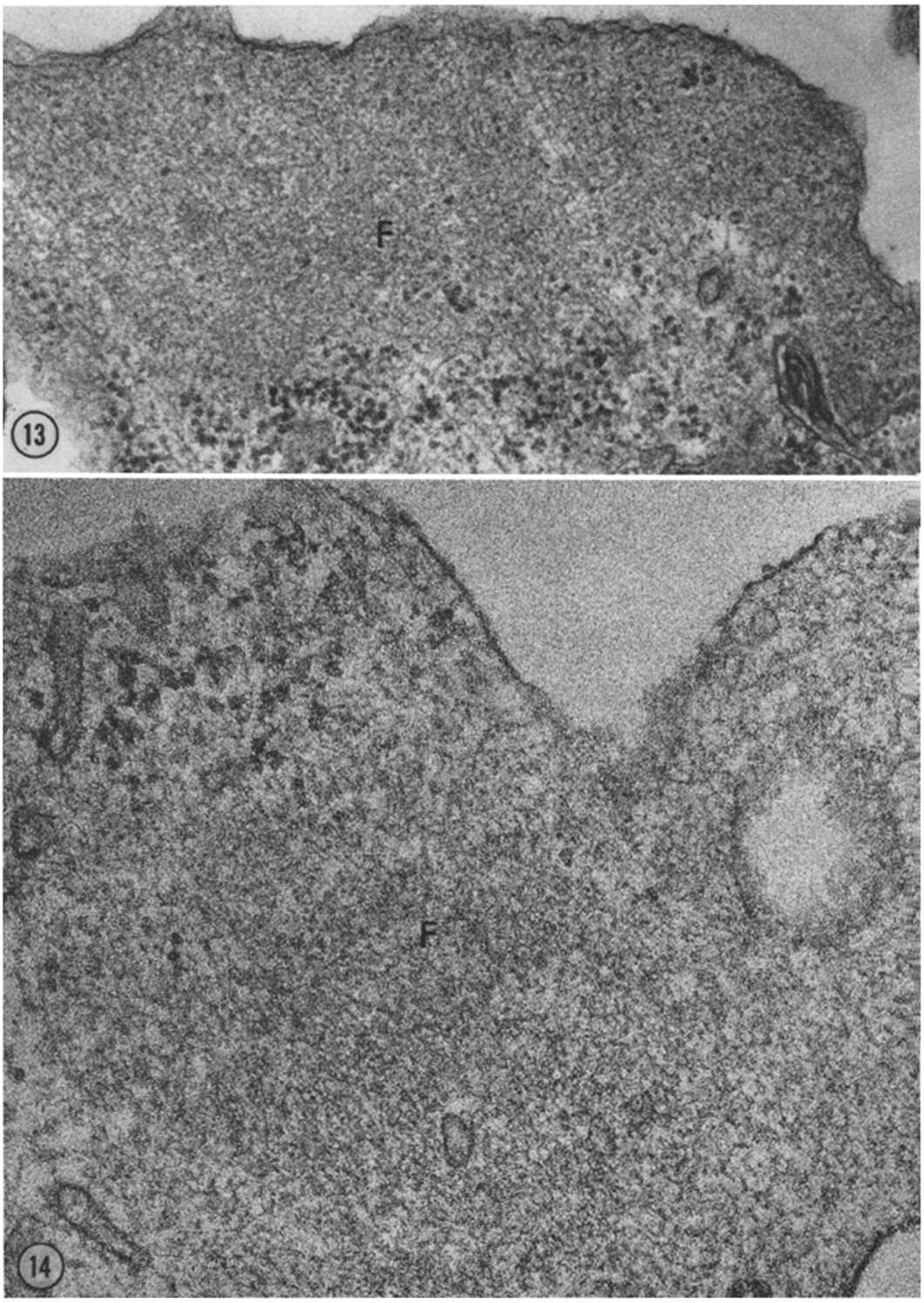


FIGURE 13 A cytochalasin-induced mass (*F*) near the surface of a glial cell after only 8 min of exposure to the drug. In comparison with Figs. 4 *a* and 4 *b*, individual elements of the network are exceedingly difficult to resolve. × 68,000.

FIGURE 14 A higher power view of a dense mass (*F*) within a cytochalasin-treated cell. It is not clear whether such electron-opaque materials represent collapsed (hypercontracted) or dissociated network components. × 103,000.

TABLE I

Radioautographic Comparison of Leucine-^3H Incorporation into Control and Cytochalasin-Treated Glial Cells

Treatment	Grains per cell ± SD
Control	76.9 ± 21.7
Cytochalasin	74.5 ± 22.4

Cells were cultured for 18 hr. At that time, half of the cultures received medium containing 7 μg/ml of cytochalasin B and half were left untouched. After 18 hr in cytochalasin, cultures received 1 μCi/ml of leucine-^3H in cytochalasin medium for 2 hr. Control cultures were labelled in parallel. Cultures were then fixed, processed for radioautography, and exposed for 8 days before being developed. Grain counts were made over 100 control and 100 cytochalasin-treated glial cells.

TABLE II

Effect of Cycloheximide on Leucine-^3H Incorporation in Glial cells during Recovery from Cytochalasin Treatment

Treatment	Grains per cell ± SD
Control	75.0 ± 22.1
Cycloheximide	2.5 ± 1.9

Cells were cultured 18 hr, treated with 7 μg/ml of cytochalasin B for an additional 18 hr, and recovered for 4 hr. Controls were rinsed and incubated in drug-free medium; experimentals were rinsed and incubated in medium containing 20 μg/ml of cycloheximide. Both media contained 1 μCi/ml of leucine-^3H. Following fixation, processing for radioautography, and exposure for 4 days, silver grain counts were made over 100 control-recovery and 200 cycloheximide-recovery glial cells.

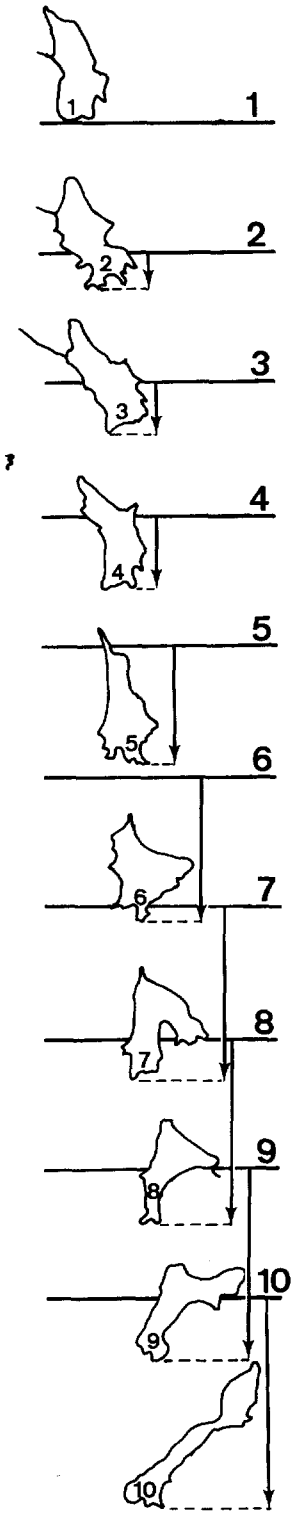


FIGURE 15 Migratory path of a colchicine-treated glial cell. These tracings were made over the last 2 hr of a continuous 48 hr incubation with 2.5×10^{-6} M colchicine. The horizontal lines represent a fixed reference mark in the microscopic field, and the tracings were made at 15-min intervals. The number near the leading edge of the cell at each time interval relates that cell to the proper reference line having the matching number. Arrows show the distance this cell has moved relative to the reference line. The distance between horizontal lines repersents 79 μ in real distance.

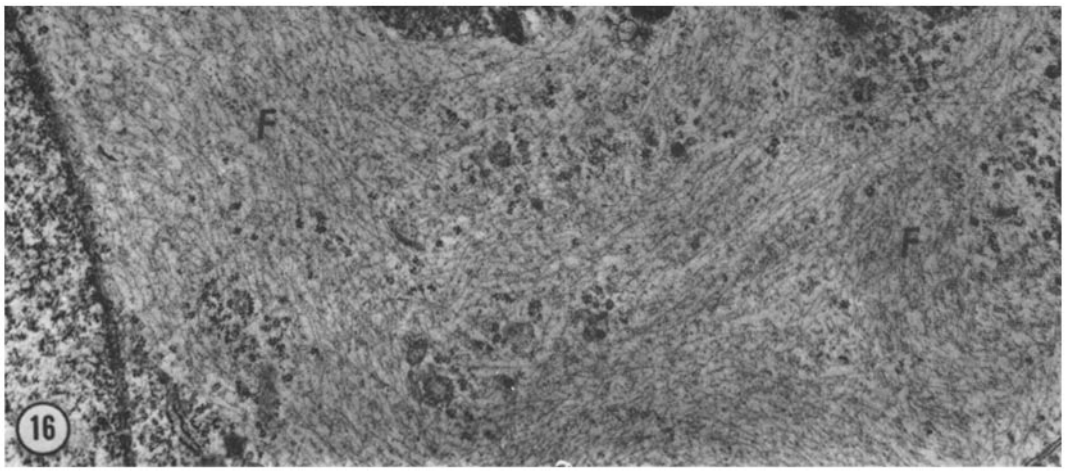


FIGURE 16 Cytoplasm in a glial cell treated with colchicine for 18 hr. The cytoplasmic microtubules are no longer present, whereas massive whorls of filamentous material (*F*) extend through the cytoplasm. Note that these filaments are quite different in morphology from the cytochalasin-induced masses of fine filamentous material. At the periphery of cells such as this one are found normal sheath filaments and a normal filamentous network. × 17,000.

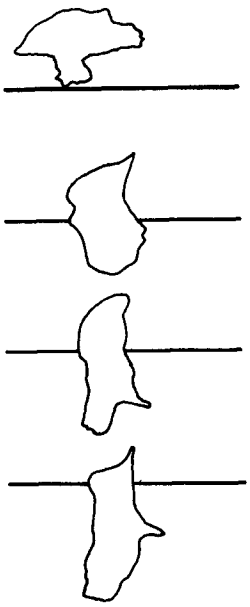


FIGURE 17 Migratory path of a colchicine-treated chick heart fibroblast. Colchicine (2.5×10^{-6} M) was continuously present for 36 hr. The tracings were made at 30-min intervals. The distance between horizontal lines represents 79 μ.

protein synthesis is inhibited (Yamada et al., 1971; Spooner and Wessells, 1970). Interestingly, reappearance of filaments can occur in oviducal epithelial cells in the absence of estradiol in the medium (the latter compound causes initial appearance of oviduct microfilaments; Wrenn and Wessells, 1970). It is likely, therefore, that recovery from cytochalasin involves reassembly of functional microfilament systems from pre-existing precursors.

An interpretation of the role played by cytochalasin-sensitive microfilaments, or certain other organelles, in movement can best be discussed within the framework of current models for cell locomotion.

Ingram (1969) has proposed that the leading edge of a migrating cell extends and adheres to the substratum. Then, a contraction takes place in the region between that anterior point of adhesion and some more posterior point of adhesion of the cell to its substratum. The latter point of adhesion, being "weaker," breaks free from the substratum because of the contractile force, and the posterior part of the cell is pulled forward. By repeating this sequence, the cell would move forward, much as an earthworm does. Our observations are compatible with Ingram's model. Significant points are (*a*) the microfilament network and the plasma membrane are the only organelles found in the cortex of the undulating membrane; (*b*) the network appears to insert on the plasma membrane; (*c*) cytochalasin treatment alters the network and halts net cell movement; (*d*) cytochalasin inhibits contractions such as occur in smooth and cardiac muscle cells,

in metamorphosing ascidian tadpole tail cells, and in blood platelets (Wessells et al., 1971); (*e*) the microfilament network is a three-dimensional lattice which, if capable of contracting in any plane, could generate extension, retraction, or "fluttering" of the cell periphery; (*f*) finally, the presence of the network just beneath the entire plasma membrane of the cell correlates with the fact that an undulating membrane can form at any point on the cell periphery. Although in sum these arguments imply that the structural basis for contraction in Ingram's model is the microfilament network, a direct demonstration of contractility has not yet been achieved.

The role of cytochalasin-*insensitive* filamentous organelles in movement is not clear. On the one hand, the filamentous sheath of migratory cells could be contractile. If so, such contraction alone is not sufficient to generate cell movement, since cytochalasin-treated cells with intact sheaths do not move. On the other hand, the sheath might serve as a skeletal system. In this case, the filaments would stabilize changes in cell shape, a role consistent with the careful observations on "stress fibers" in rat cells (Buckley and Porter, 1967). The presence of the sheath filaments, but not microtubules, as the major organelles within the long processes of cytochalasin-treated cells may be indirect evidence for such a skeletal function.

The remaining feature of locomotion, critical to the model, concerns the structural basis for the differentially strong adhesions that the leading edge of the cell must make with its substratum. If the series of membranous structures found in sections through the lower surface of the undulating membrane (see Figs. 7, 8) represent convolutions in the plasma membrane, that structure could function as an "adhesive organelle." Alternatively, the peculiar organelle may be a focal point for addition of new membrane at the leading edge of the cell. That such a process is a normal component of cell movement is suggested by two experiments. Bray (1970) provides evidence that new surface materials of elongating axons are added at the advancing tip. Ingram (1969) reports that carbon particles initially adhering to the anterior-most part of the plasma membrane of fibroblasts pass backward as a cell advances, and accumulate in a region over the nucleus. The observations on both cell types can be explained if new membrane is deposited at the leading edge of the cells. Furthermore, for fibroblasts, membrane must be resorbed at some more posterior position, to compensate for that added at the front (see Abercrombie and Ambrose, 1962).

We wish to thank S. B. Carter for cytochalasin, Susan Sleeper for invaluable aid with electron microscopy, and Belen Sosa for technical assistance.

This work was supported principally by National Science Foundation Research Grant GB-15666, and also by National Institutes of Health Grants GM-16530 and HD-04708. Dr. Spooner is supported by an NIH postdoctoral fellowship, and Dr. Yamada by an American Cancer Society Institutional Cancer Grant award.

Received for publication 10 August 1970, and in revised form 17 November 1970.

Note Added in Proof: M. Abercrombie, J. E. M. Heaysman, and S. M. Pegrum (1970. *Exp. Cell Res.* **62**:389.) have also concluded that new "surface" is added at the front of migratory heart cells. J. M. Vasiliev, I. M. Gelfand, L. V. Domnina, O. Y. Ivanova, S. G. Komm, and L. V. Olshevskaja (1970. *J. Embryol. Exp. Morphol.* **24**:625.) show that "directional translocation" of fibroblasts does not take place in cells lacking microtubules. Those data, in combination with that reported above, suggest that the locomotory system of the cell functions in the absence of microtubules, but that the ability to orient with respect to certain asymmetries in the environment is dependent upon those organelles.

REFERENCES

ABERCROMBIE, M. 1961. The bases of the locomotory behavior of fibroblasts. *Exp. Cell Res. Suppl.* **8**:188.

ABERCROMBIE, M., and E. J. AMBROSE. 1962. The surface properties of cancer cells: a review. *Cancer Res.* **22**:525.

AMBROSE, E. J. 1961. The movements of fibrocytes. *Exp. Cell Res. Suppl.* **8**:54.

BRAY, D. 1970. Surface movements during the growth of a single explanted neuron. *Proc. Nat. Acad. Sci. U.S.A.* **65**:905.

BUCKLEY, I. K., and K. R. PORTER. 1967. Cytoplasmic fibrils in living cultured cells. A light and electron microscope study. *Protoplasma.* **64**:349.

CARTER, S. B. 1967. Effects of cytochalasins on mammalian cells. *Nature (London).* **213**:261.

FISK, A., and S. PATHAK. 1969. HEPES-buffered medium for organ culture. *Nature (London).* **224**:1030.

GOLDMAN, R. D., and E. A. C. FOLLETT. 1969. The structure of the major cell processes of isolated BHK-21 fibroblasts. *Exp. Cell Res.* **57**:263.

GOLDMAN, R. D., and E. A. C. FOLLETT. 1970. Birefringent filamentous organelle in BHK-21 cells

and its possible role in cell spreading and motility. *Science* (*Washington*). **169**:286.

INGRAM, V. M. 1969. A side view of moving fibroblasts. *Nature* (*London*). **222**:641.

ISHIKAWA, H., R. BISHOFF. and H. HOLTZER. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. *J. Cell Biol.* **38**:538.

KESSEL, R. G. 1960. The role of cell division in gastrulation of *Fundulus heteroclitus*. *Exp. Cell Res.* **20**:277.

MARSLAND, D. 1964. Pressure-temperature studies on ameboid movement and related phenomena: An analysis of the effects of heavy water (D_2O) on the form, movement and gel structure of *Amoeba proteus*. *In* Primitive Motile Systems in Cell Biology. R. D. Allen and N. Kamiya, editors. Academic Press Inc., New York. 173.

PANNER, B. J., and C. R. HONIG. 1970. Locus and state of aggregation of myosin in tissue sections of vertebrate smooth muscle. *J. Cell Biol.* **44**:52.

RIDLER, M. A. C., and G. F. SMITH. 1968. The response of human cultured lymphocytes to cytochalasin B. *J. Cell Sci.* **3**:595.

ROBBINS, E., and N. K. GONATAS. 1964. Histochemical and ultrastructural studies on HeLa cell cultures exposed to spindle inhibitors with special reference to the interphase cell. *J. Histochem. Cytochem.* **12**:704.

SCHROEDER, T. E. 1969. The role of "contractile ring" filaments in dividing *Arbacia* egg. *Biol. Bull'* **137**:413.

SPOONER, B. S., and N. K. WESSELLS. 1970. Effects of cytochalasin B upon microfilaments involved in morphogenesis of salivary epithelium. *Proc. Nat. Acad. Sci. U.S.A.* **66**:360.

TILNEY, L. G., and J. R. GIBBINS. 1969. Microtubules in the formation and development of the primary mesenchyme in *Arbacia punctulata*. II. An experimental analysis of their role in development and maintenance of cell shape. *J. Cell Biol.* **41**:227.

VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.

WESSELLS, N. K., B. S. SPOONER, J. F. ASH, M. O. BRADLEY, M. A. LUDUENA, E. TAYLOR, J. T. WRENN, and K. M. YAMADA. 1970. Microfilaments in cellular and developmental processes. *Science* (*Washington*). **171**:135.

WRENN, J. T., and N. K. WESSELLS. 1970. Cytochalasin B: Effects upon microfilaments involved in morphogenesis of estrogen-induced glands of oviduct. *Proc. Nat. Acad. Sci. U.S.A.* **66**:904.

YAMADA, K. M., B. S. SPOONER, and N. K. WESSELLS. 1970. Axon growth: Roles of microfilaments and microtubules. *Proc. Nat. Acad. Sci. U.S.A.* **66**:1206.

YAMADA, K. M., B. S. SPOONER, and N. K. WESSELLS. 1971. Ultrastructure and function of growth cones and axons of cultured nerve cells. *J. Cell Biol.* **49**:000.