

Microtubules and the Endoplasmic Reticulum Are Highly Interdependent Structures

Mark Terasaki,* Lan Bo Chen,† and Keigi Fujiwara*

* Department of Anatomy and Cellular Biology, Harvard Medical School, and † Dana Farber Cancer Institute, Boston, Massachusetts 02115. Dr. Terasaki's present address is Marine Biological Laboratories, Woods Hole, Massachusetts 02543. Dr. Fujiwara's present address is National Cardiovascular Center, Suita, Osaka 565, Japan.

Abstract. The interrelationships of the endoplasmic reticulum (ER), microtubules, and intermediate filaments were studied in the peripheral regions of thin, spread fibroblasts, epithelial, and vascular endothelial cells in culture. We combined a fluorescent dye staining technique to localize the ER with immunofluorescence to localize microtubules or intermediate filaments in the same cell.

Microtubules and the ER are sparse in the lamellipodia, but intermediate filaments are usually completely absent. These relationships indicate that microtubules and the ER advance into the lamellipodia before intermediate filaments.

We observed that microtubules and tubules of the ER have nearly identical distributions in lamellipodia, where new extensions of both are taking place. We

perturbed microtubules by nocodazole, cold temperature, or hypotonic shock, and observed the effects on the ER distribution. On the basis of our observations in untreated cells and our experiments with microtubule perturbation, we conclude that microtubules and the ER are highly interdependent in two ways: (a) polymerization of individual microtubules and extension of individual ER tubules occur together at the level of resolution of the fluorescence microscope, and (b) depolymerization of microtubules does not disrupt the ER network in the short term (15 min), but prolonged absence of microtubules (2 h) leads to a slow retraction of the ER network towards the cell center, indicating that over longer periods of time, the extended state of the entire ER network requires the microtubule system.

THROUGH the use of morphological and biochemical techniques, it has been established that cytoplasmic filament systems are a prominent part of intracellular organization. There are three filament systems: microfilaments, microtubules, and intermediate filaments. They are often called the cytoskeleton, though it has been pointed out that their function is not analogous to that of the skeleton of animals. Instead, these systems are known to be labile, and intermediate filaments are not required for the maintenance of cell shape (Klymkowsky, 1981). Regardless of this, the filament systems are likely to play crucial structural and organizational roles.

Inside cells, there is also an enormous amount of membranes. At present, the governing principles of the organization of these membranes are not well understood. There is, however, much evidence that the membranes have a significant interaction with microtubules. For instance, the Golgi apparatus is disrupted when microtubules are depolymerized (Wehland et al., 1983; Rogalski and Singer, 1984). Microtubules have been reported to be associated with mitochondria (Heggeness et al., 1978; Wang and Goldman, 1978; Summerhayes et al., 1983) and with lysosomes (Collot et al., 1984). Recently, vesicles from the squid axon have been demonstrated to move along microtubules (Allen et al.,

1982; Brady et al., 1982; Schnapp et al., 1985; Miller and Lasek, 1985). There is also evidence for an association of tubulin with membranes (e.g., Stephens, 1985).

Close relationships of the endoplasmic reticulum (ER)¹ and microtubules have been observed in whole mount cells by electron microscopy. In critical point dried, glutaraldehyde-osmium fixed cells, microtubules and ER tubules were seen to "closely parallel one another over considerable distances, even when on curvilinear courses" (Buckley and Porter, 1975).

From electron microscopy of sectioned material, microtubule associations with cisternae type ER have been observed in plant cells (Pickett-Heaps and Northcote, 1966), tetrahymena (Franke, 1971), spermatids (Fawcett et al., 1971; Clermont and Rambourg, 1978), and also in taxol-treated dorsal root ganglion-spinal cord cultures (Masurovsky et al., 1981) and taxol-treated cultured chondroblasts (Tokunaga et al., 1983). Associations between ER tubules and microtubules are more difficult to observe because an ER tubule in thin section is difficult to distinguish from a vesicle, but an association of longitudinally sectioned ER tubules and

1. *Abbreviations used in this paper:* DiOC6(3), 3,3'-dihexyloxocarbocyanine iodide; ER, endoplasmic reticulum.

microtubules has been observed in mammalian heart muscle (Goldstein and Entman, 1979) and in the mitotic spindle of barley cells (Hepler, 1980).

A fluorescent dye method for staining the ER in whole mount cells has recently been developed (Terasaki et al., 1984). This method produces similar images of the ER as the original whole mount electron microscopic method (Porter et al., 1945; Porter, 1953), whole mount electron microscopy of glutaraldehyde-osmium fixed, critical point dried cells (Buckley and Porter, 1975), phase-contrast images (Buckley, 1964; Rose and Pomerat, 1960; see also Fawcett and Ito, 1958; Ito, 1962), a fluorescent phosphatidic acid analogue method (Pagano et al., 1981), and potassium permanganate fixation (Song et al., 1985; Terasaki et al., 1984). Although the fluorescent dye method lacks the resolution of the electron microscopic methods, it produces a very clear image of the ER and the other intracellular membranes. Staining by the fluorescent dye is also simple and rapid, which facilitates experimental manipulations.

Using the fluorescent dye method, the form of the ER was observed to be drastically altered by microtubule-depolymerizing drugs. These drugs caused many of the ER membranes to retract towards the cell center (Terasaki et al., 1984). When the drugs were washed out, microtubules and the ER returned to a distribution typical of unperturbed cells. Similar behavior was noted by Louvard et al. (1982), who used an antiserum to localize the rough ER by immunofluorescence. In intact tissues, microtubule-depolymerizing drugs have also been observed to cause drastic reorganization of the ER (e.g., Vogl et al., 1983). These effects of microtubule depolymerization are further evidence that microtubules and the ER are closely related.

In this paper, we investigate the relationships of the ER and microtubules in more detail. We used double labeling techniques to localize the distributions of these components in the same cell, and we found that the ER and microtubules are highly interdependent structures.

Materials and Methods

Cells

PTK-2 cells (derived from rat kangaroo kidney epithelium) and CV-1 cells (derived from African green monkey kidney epithelium) were obtained from the American Type Culture Collection (Rockville, MD). BALB/c 3T3 cells were a generous gift from Dr. R. Tucker (Department of Medical Oncology, Johns Hopkins Oncology Center, Baltimore, MD). Bovine aortic endothelial cell cultures were generously provided by Dr. M. Gimbrone (Harvard Medical School, Boston, MA). The cells were grown on 12 × 12-mm glass coverslips (Bradford Scientific, Epping, NH) in Dulbecco's modified Eagle's medium (DME) with 10% calf serum in an incubator at 37°C in 5% CO₂ and 95% air. Large thin cells from the goldfish scale were prepared according to the method of McBeath and Fujiwara (1984).

Reagents

3,3'-Dihexyloxycarbocyanine iodide [DiOC6(3)] has been used primarily as a potential sensitive dye (Sims et al., 1974; Scordilis et al., 1975). It has also been used to stain mitochondria in living cells (Johnson et al., 1981), sarcoplasmic reticulum in beating heart cells (Habicht and Brune, 1980), and living presynaptic nerve terminals (Yoshikami and Okun, 1984). The dye was obtained from Eastman Kodak Co. (Rochester, NY) or from Polysciences, Inc. (Warrington, PA). A stock solution was made at 0.5 mg/ml ethanol and was kept protected from light at room temperature. Working solutions were diluted on the day of the experiments, because the dye appears to break down slowly in aqueous solution.

Rabbit antisera against sea urchin cytoplasmic tubulin (Fujiwara and Pollard, 1978) was used to localize microtubules by immunofluorescence microscopy. Rabbit antisera to hamster vimentin was kindly provided by Dr. R. Goldman (Department of Cell Biology and Anatomy, Northwestern University Medical School). Rhodamine-labeled second antibodies were obtained from Cappel Laboratories (Cochranville, PA).

Nocodazole (methyl 5-[2-thienylcarbonyl]-1-h-benzimidazol-2-yl)-carbamate was obtained from Sigma Chemical Co. (St. Louis, MO). It was dissolved at 0.5 mg/ml in dimethylsulfoxide and used at 1 µg/ml media for experiments.

Fluorescent Dye Staining

All steps were done at room temperature. Cells were fixed for 3–5 min in 0.25% glutaraldehyde in a sucrose-cacodylate buffer (0.1 M sucrose, 0.1 M sodium cacodylate, pH 7.4). When cells were to be processed further for immunofluorescence staining, the fixation procedures described below were used instead. The cacodylate buffer was preferred over PBS because the ER was sometimes vesiculated when fixed in PBS.

The coverslips were stained for 10 s with 2.5 µg/ml DiOC6(3) in the cacodylate buffer. The coverslips were then washed once in the same buffer and then mounted on a cell chamber used for staining mitochondria in live cells (Johnson et al., 1980). The cell chamber was made of a small strip of silicon rubber ("Ronsil", 0.7-mm thick; North American Reiss, Bellemead, NJ) with a small hole punched in it with a cork borer. The silicon rubber strip was pressed against a microscope slide, the cacodylate buffer was used to fill the chamber, and the coverslip was mounted on the chamber. This was useful for the double labeling procedure because the coverslips were easily removable for further processing.

Fluorescence Microscopy

A Leitz Orthomat equipped with a Ploemopak 2 containing a Leitz N-2 filter and a K-2 filter was used. Most observations and photographs were made with a Zeiss plan 100× (1.25 numerical aperture) objective lens. A fluorescein filter system (K-2) was used to observe the fluorescence of DiOC6(3)-stained cells. Observations and photographic recording of fluorescent dye staining were made within 20–30 min after staining, because the staining quality diminishes with time. The major change is that after ~10 min, vesicle staining is brighter and staining of the ER is sometimes less easy to observe in some cells. However, many cells continue to have clear staining after this time.

Photographs of fluorescent images were taken using Kodak Tri-X Pan film exposed using ASA 1000 and developed in Acufine (Acufine, Inc., Chicago, IL). Stereo micrographs were taken by the method of Osborn et al. (1978).

Fixation Procedures for Immunofluorescence

For microtubule and ER double staining, the procedure of McBeath and Fujiwara (1984) was used except that cells were fixed with 0.25% glutaraldehyde in the sucrose-cacodylate buffer instead of 2.5% glutaraldehyde in PBS.

For intermediate filament and ER double staining, cells were fixed in 0.025% glutaraldehyde in the sucrose-cacodylate buffer for 5 min at room temperature. The cells were permeabilized in 0.2% Triton X-100 in PBS for 2 min at room temperature.

"Same Cell" Technique for Double Labeling with Fluorescent Dye and Immunofluorescence

Cells were fixed as described above for the relevant antigen to be localized by immunofluorescence. During the fixation, a rubber policeman was used to scrape cells off: the policeman was drawn once vertically and horizontally, producing a four-way intersection of scraped cells in the center of the coverslip. Using a phase-contrast microscope, a map of cell positions in the vicinity of the intersection was made. After the fixation period had elapsed, the cells were stained with DiOC6(3) as described above.

In the fluorescence microscope, the scraped intersection was located. Using the map, individual cells were then located and photographed, with the sequence and position of the photographic field recorded on the map. After photography was completed, the immersion oil on the coverslip was removed and the coverslip was transferred to a PBS wash. This was followed by permeabilization and application of antibodies for immunofluorescence. After the immunofluorescence staining was completed, the cells were photographed again, using the map to locate the cells.

Cold Shock

DME with 5% serum was prepared with 25 mM Hepes buffer instead of bicarbonate. The medium was equilibrated with a water-ice mixture. Cold shock of fish scale cells was done in the same manner as McBeath and Fujiwara (1984).

Hypotonic Shock

DME prepared without buffer was diluted 1:3 with deionized water. Tris was then added to the medium to make 10 mM final concentration, and the medium was adjusted to pH 7.4. 1% serum was then added. For the experiments, the medium was equilibrated in a culture dish floating in a 37°C water bath.

Results

Morphological Characteristics of the ER and Mitochondria

To investigate relationships of the ER and microtubules by fluorescence microscopy, the morphological characteristics of the ER must be established. The fluorescent dye DiOC6(3) stains the ER and mitochondria in glutaraldehyde-fixed cells in such a way as to allow close inspection of the ER pattern. We list here some basic morphological characteristics of the ER observed by this method.

(a) The ER extends outward from the cell center and nuclear region (Fig. 1 a). Thus, like microtubules and intermediate filaments, the ER is centrally organized. The ER is more abundant in the nonmotile regions of cytoplasm than in the motile regions. This was noted in the first electron microscopic observations and led to the naming of the endoplasmic reticulum, since the network was more abundant in the nonmotile "endoplasm" than in the motile "ectoplasm" (Porter and Kallman, 1952).

(b) The ER is continuous throughout the cell (Porter, 1953). Isolated segments of ER tubules were very rarely seen. A more precise characterization may be that there is only one tubular membranous network in the peripheral regions of these cells, i.e., that all of the tubules are interconnected.

(c) The junctions of ER tubules are usually "three-way," and there is often a 120° angle between the three tubules (Fig. 1, a and b). Interesting insights can arise from an analysis of patterns (Stevens, 1974). The pattern of the ER has some similarities with the pattern of the boundary junctions of soap bubbles pressed between two glass plates. This suggests that physical or mechanical forces have some part in determining the distribution of ER tubules.

(d) Cisternae type ER is present in the cells we examined, but to a lesser degree than tubular ER. In most cells, cisternae are more abundant near the nuclear region than in the outer regions. In the cell shown in Fig. 1 b, there are large cisternae in the peripheral region, where it is easier to observe them. Different cell types have different amounts of cisternae. Approximately half of the cells in a population of growing 3T3 cells have some cisternae in their peripheral regions. A smaller percentage of CV-1, PtK-2, or bovine aortic endothelial cells have cisternae.

(e) Near the nucleus, in the thick region of the cell center, observation is difficult because many mitochondria and other membrane-bound organelles overlap each other. In the thinner peripheral regions, the ER is usually seen in one plane of focus. Detailed morphological work is limited to this region. Mitochondria in this region are usually in the same plane of focus as the ER, but by taking stereo photographs, mitochondria are found to be located above the plane of the ER in most cases (Fig. 2).

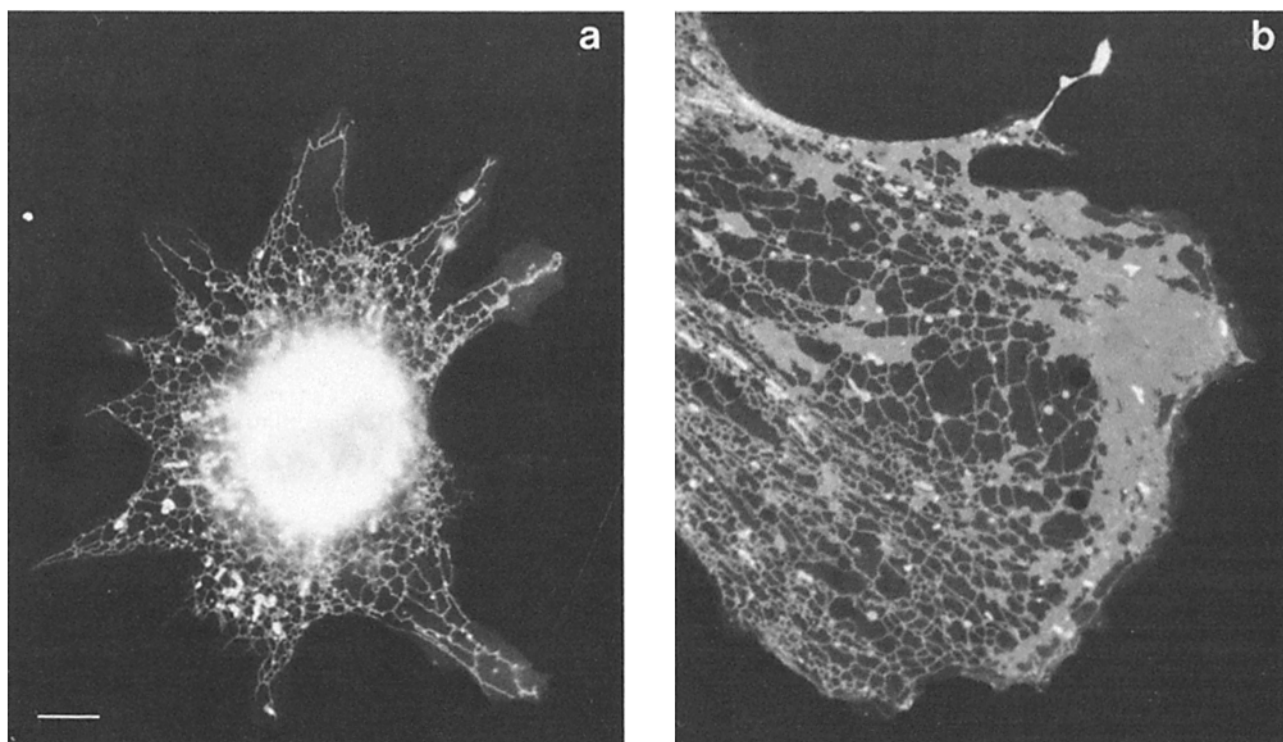


Figure 1. (a) Micrograph of an entire CV-1 cell stained with DiOC6(3). (b) Micrograph of a region of a 3T3 cell containing large amounts of both ER tubules and ER cisternae. Bar, 10 μ m.

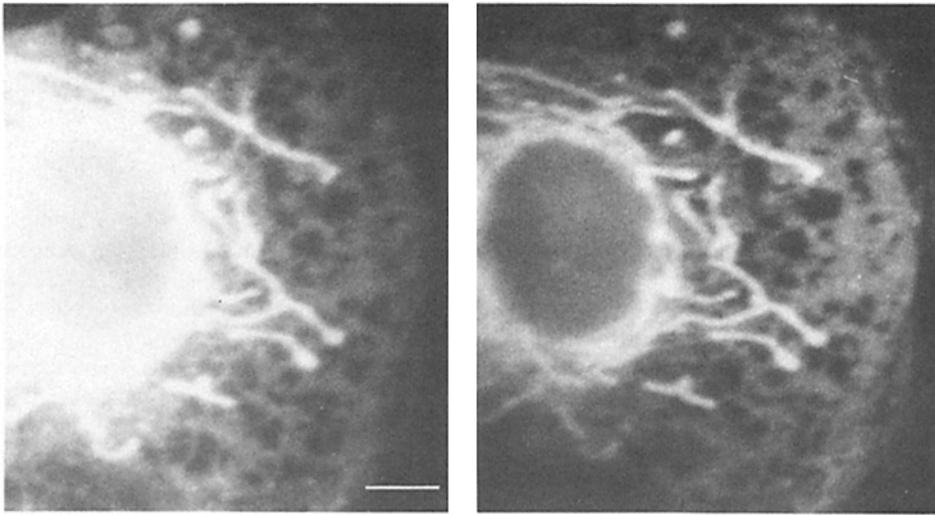


Figure 2. Stereo micrographs of a PtK-2 cell. In the peripheral region, mitochondria, which have stronger fluorescence, are seen to be located above the plane of the ER. Bar, 10 μm .

Localization of Microtubules and the ER in Cells in Culture

Using the procedures described in the Materials and Methods section, microtubule and ER distributions were determined in the same cell. Large thin cells from the goldfish scale possess relatively few microtubules (McBeath and Fujiwara, 1984). The use of these cells offered the opportunity of clearly observing the microtubule-ER relationship throughout the cell. The distribution of microtubules and ER

was determined and an example is shown in Fig. 3. Microtubules and ER tubules are often closely associated over long distances. On the other hand, there are some microtubules without corresponding ER tubules, and some ER tubules without corresponding microtubules.

There was a difference in the geometric pattern of the ER and microtubules. As noted before, ER tubule junctions are usually three-way. Microtubules do not join together; instead, at the light microscope level, they often appear to

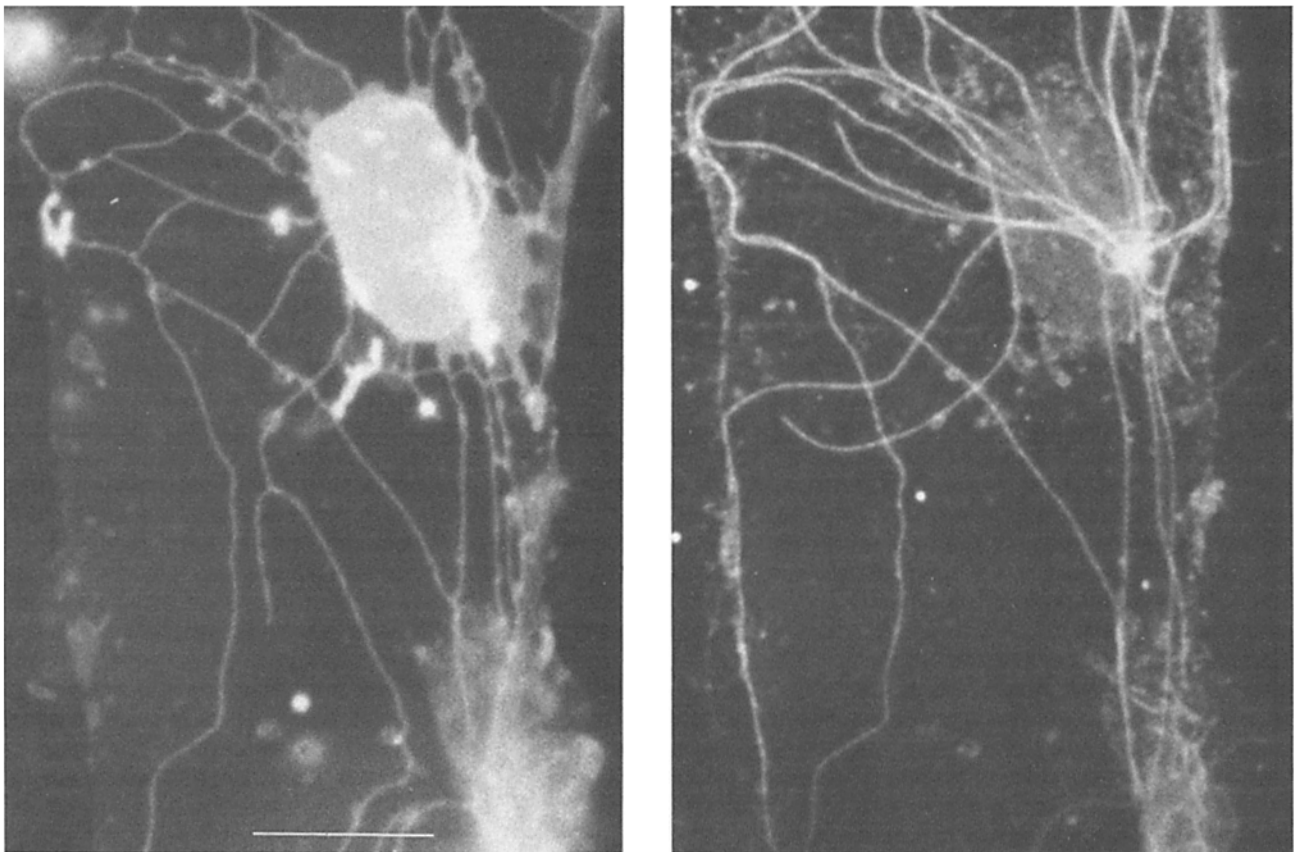


Figure 3. ER (*left*) and microtubule (*right*) distributions in a large thin cell pressed from the goldfish scale. Several ER tubules and microtubules run parallel to each other for long distances. Bar, 10 μm .

cross over each other, forming four-way "intersections." Because of this difference, microtubules and the ER cannot have a complete one-to-one correspondence.

In tissue culture cells, the number and densities per area of microtubules and ER were higher. There is a general co-distribution of the two, but it is usually difficult to observe relationships of individual elements in the central regions of the cell. There are no large regions in the cells where one is present while the other is absent.

The distributions of microtubules or intermediate filaments and the ER were determined in several different cell types. The different cell types have different patterns of distribution. At the periphery of PtK-2 cells, it is common to observe colinear ER and microtubules (Fig. 4). In the more central regions, the ER and microtubules are not as closely related. Prekeratin filaments do not extend as far out into the periphery as microtubules or the ER (not shown). Fibroblasts (3T3 and chick embryo fibroblasts) have large leading

lamellae, and the ER and microtubules in these regions are closely related, though not as closely as in PtK-2 cells. Vimentin filaments are usually absent from these spreading regions. CV-1 cells have smaller spreading regions than the fibroblasts. Microtubules and ER tubules often are strikingly coaligned, while vimentin filaments are again usually absent (Fig. 5).

ER, Microtubules, and Intermediate Filaments in Lamellipodia

To closely investigate how the distributions of these three elements are related in lamellipodia, we obtained cell cultures that included many cells with lamellipodia. CV-1 cells were made to spread by two different methods. In both of these cases, many cells with lamellipodia appeared in a predictable manner, so that it was possible to analyze the staining pattern more objectively.

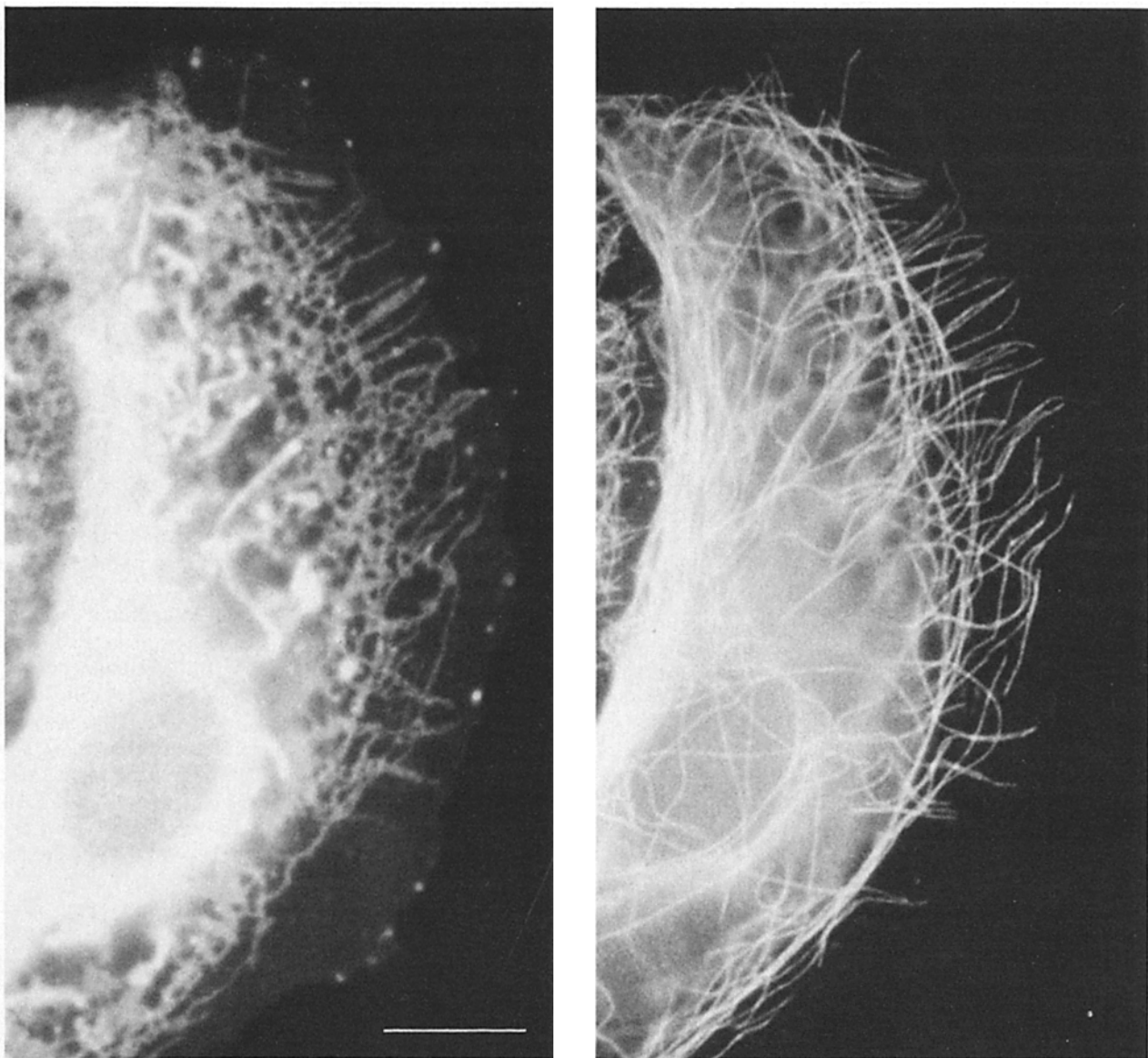


Figure 4. ER (left) and microtubule (right) distributions in a PtK-2 cell. At the periphery, there are close correlations of ER tubules and microtubules. Bar, 10 μ m.

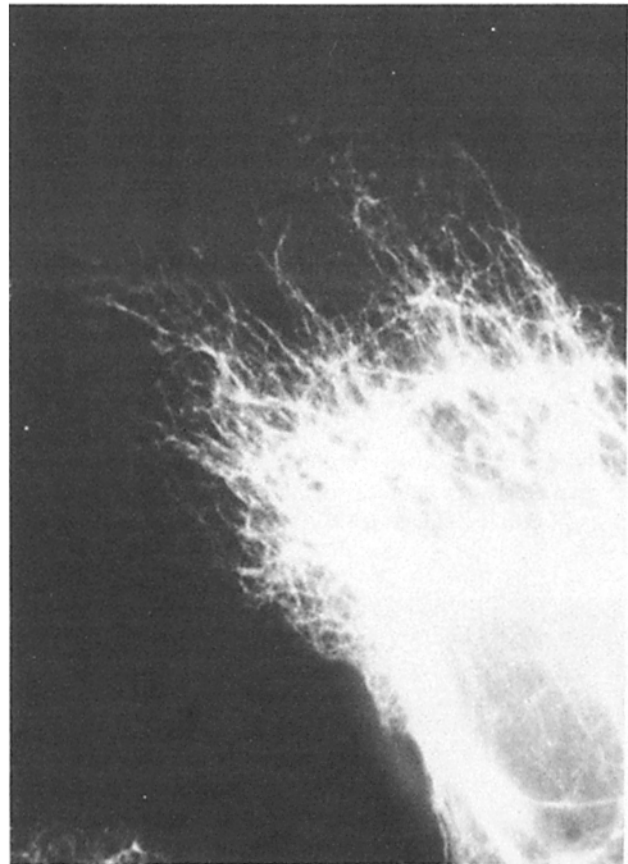
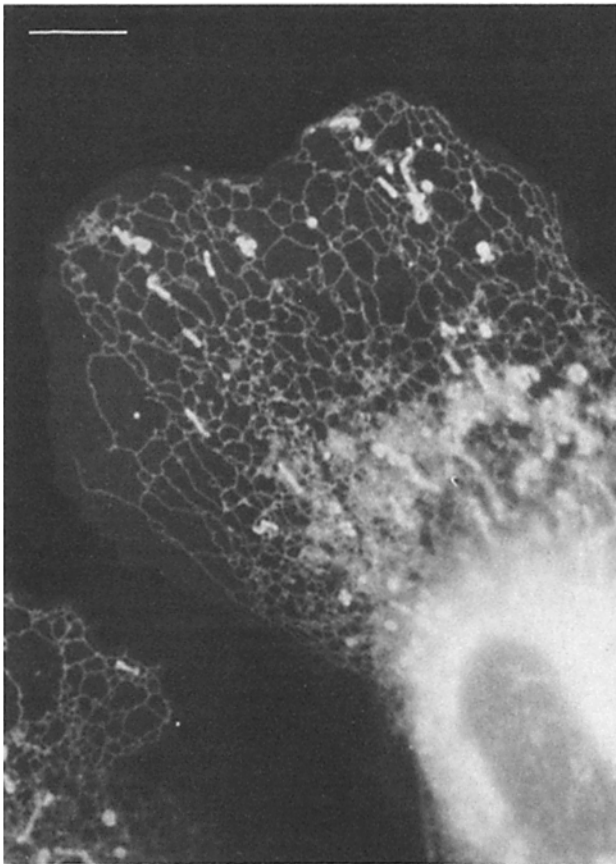


Figure 5. ER (*left*) and vimentin (*right*) distributions in a spreading region of a CV-1 cell. The ER network extends out further than the vimentin filaments. Bar, 10 μ m.

First, cells were observed during the process of attachment to the substrate after trypsinization and re-plating. CV-1 cells were plated onto glass coverslips. During initial attachment and radial spreading stages (Vasiliev and Gelfand, 1976), microtubules and the ER were uniformly distributed throughout the cell. Later, during the polarization stage, lamellipodia formed in the direction of cell polarization. Many examples of colinear microtubules and the ER were observed in these lamellipodia, while intermediate filaments were usually absent.

Another situation in which many cells with lamellipodia are observed is during the "wound healing" of a tissue culture monolayer. A confluent monolayer of CV-1 cells on coverslips were "wounded" using a rubber policeman. Cells at the edge of the "wound" migrate towards the center of the wound, producing a large number of cells moving in the same direction. The distributions of the ER, microtubules, and intermediate filaments were determined 3 h after wounding. As before, co-aligned microtubules and ER were present and had similar distributions in the spreading regions, while intermediate filaments were usually absent. These observations suggest that the distributions of the ER and microtubules are dependent on each other, particularly in the spreading regions of cultured cells.

Effects of Microtubule Depolymerization

To further investigate relationships of the ER and microtubules, the effects of reversible microtubule depolymerization

were determined. Previously, it has been observed that the ER retracts towards the central region of the cell after the addition of colcemide or colchicine, and that the effects of colcemide are reversible (Louvard et al., 1982; Terasaki et al., 1984). We tested nocodazole, another microtubule-depolymerizing drug, and found that it also caused retraction of the ER and that it was reversible. The effects of nocodazole were more rapidly reversible than colcemide, so nocodazole was used for all experiments.

The time dependence of the effects of microtubule depolymerization was observed. After brief exposure to nocodazole (30 min), the number and density of microtubules was reduced, while ER tubules remained at the same density or appeared to coalesce into cisternae (Fig. 6). There were no signs of the ER being "pulled back" towards the nucleus by individual microtubules as they depolymerize. During a 2-h period of drug treatment, the outer boundaries of the ER and microtubule distributions slowly retracted simultaneously towards the cell center. During this process, the number and density of microtubules decreased, while the density of ER tubules remained approximately the same. At the end of 2 h, the number of microtubules had greatly decreased, and most of the ER membranes had accumulated in the central region of the cell. However, in CV-1 and 3T3 cells, and to a lesser degree in PtK-2 cells, there were usually some ER tubules and cisternae remaining in the peripheral regions (Fig. 7). Longer incubation did not result in retraction of these membranes.

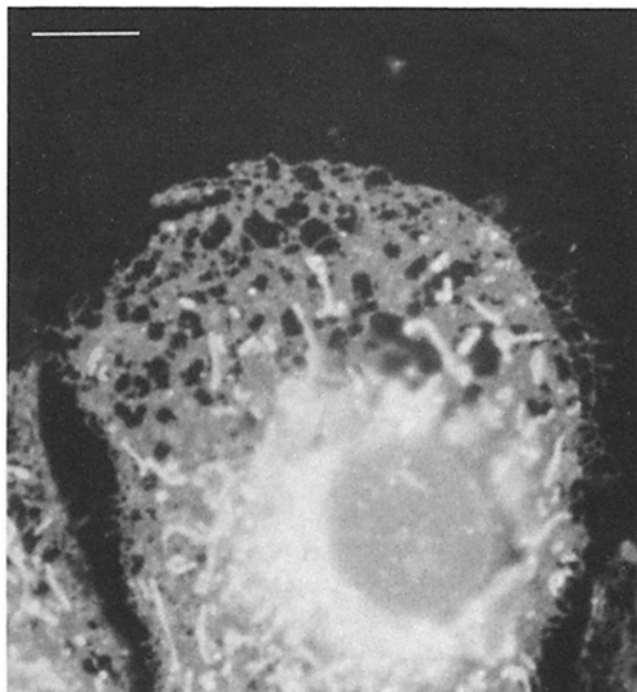


Figure 6. ER (*left*) and microtubule (*right*) distributions in a PtK-2 cell after brief exposure to nocodazole (30 min). The ER network has retracted somewhat and the density of the ER tubules has not decreased. Microtubules, however, have decreased in number and density. Bar, 10 μ m.

Microtubules were also depolymerized by cold temperatures. Cold-induced depolymerization was faster than the drug-induced depolymerization. Almost all microtubules in the fish scale cells are depolymerized after 10 min at -2°C (McBeath and Fujiwara, 1984). The ER in these cells was often completely intact (Fig. 8), though it was also common to see cells with vesiculated ER. The effects of cold shock on tissue culture cells were similar but not as dramatic as in the fish scale cells. Depolymerization of most microtubules

resulted within 5 min at 0°C in CV-1 cells. The ER membranes remained extended, but the network was partially or completely vesiculated in approximately half of the population of cells. Prolonged treatment up to 2 h in cold did not result in retraction of the ER or in further vesiculation. It is possible that the effects of cold on the ER membrane fluidity prevents the retraction and/or causes vesiculation in some of the cells. Cold depolymerization of microtubules did result in ER retraction in the following conditions. CV-1 cells were



Figure 7. ER distribution in a CV-1 cell after long exposure to nocodazole (2 h). The ER network has retracted from the periphery, and large cisternae have formed. Arrows indicate edge of cell. Bar, 10 μ m.

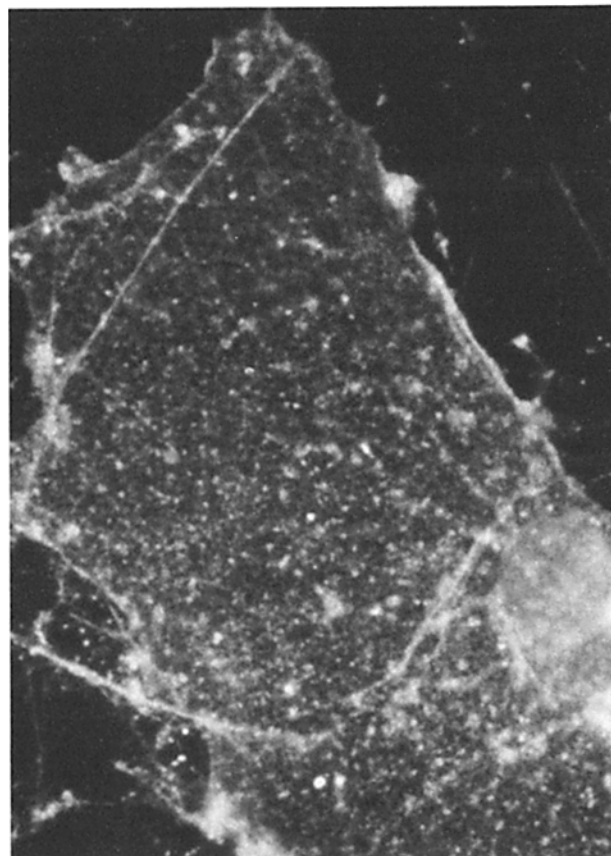
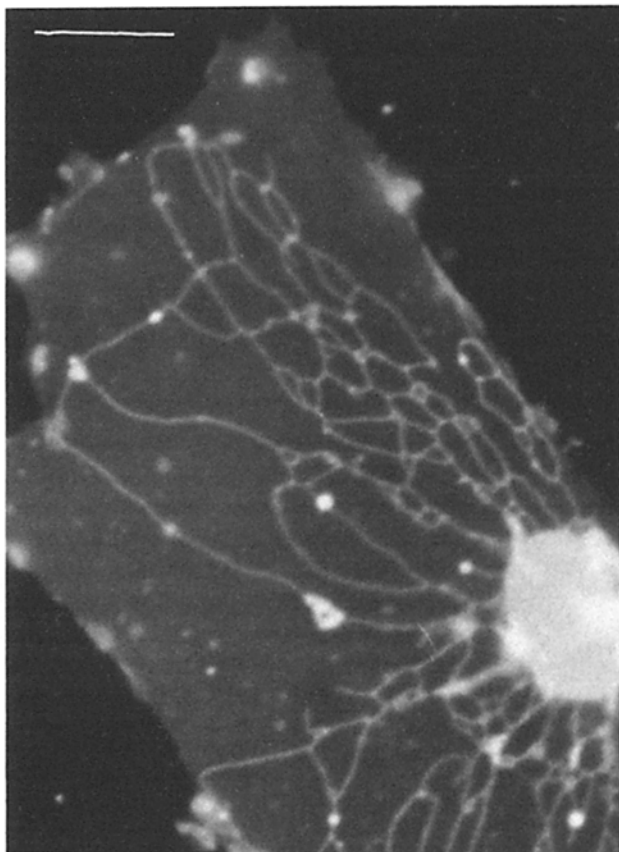


Figure 8. ER (*left*) and microtubule (*right*) distributions in a large thin cell from the goldfish scale after 10 min at -2°C . The cold temperature has resulted in microtubule depolymerization but it has left the ER network intact. Bar, 10 μm .

cooled to 0°C for 5 min and then returned to 37°C , all in the presence of 1 $\mu\text{g}/\text{ml}$ nocodazole. In cells treated in this way, most microtubules remained depolymerized at the normal temperature due to the presence of nocodazole. Within 10 min after the return to 37°C , all cells had a complete ER network, indicating that the cells with vesiculated ER had recovered. However, in all cells, the network appeared abnormal. The network appeared to lack tension on it, and there were also fewer free ends of ER tubules at the periphery (not shown). In these conditions, the ER network underwent a slow retraction towards the cell center, which was complete by ~ 2 h. This observation suggests that microtubules are necessary for the maintenance of the extended state of the ER network.

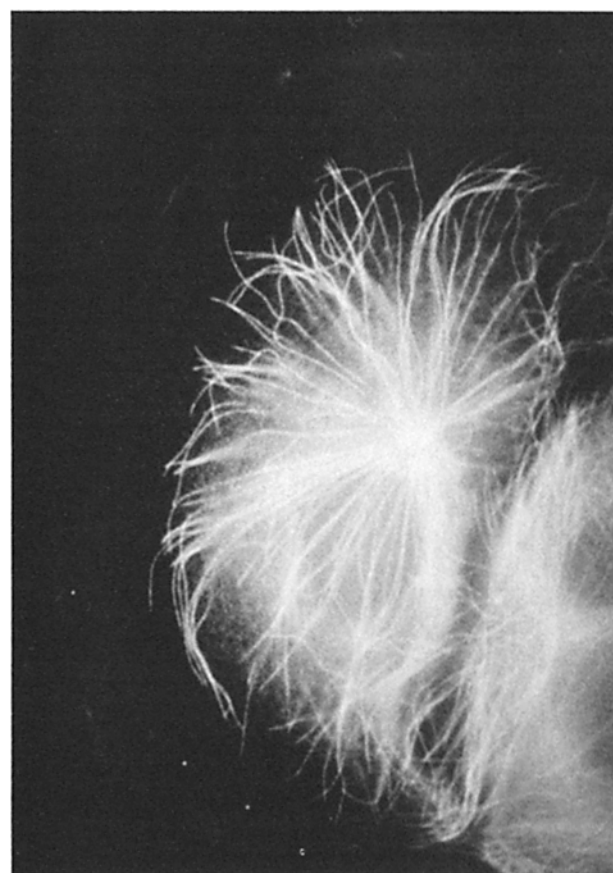
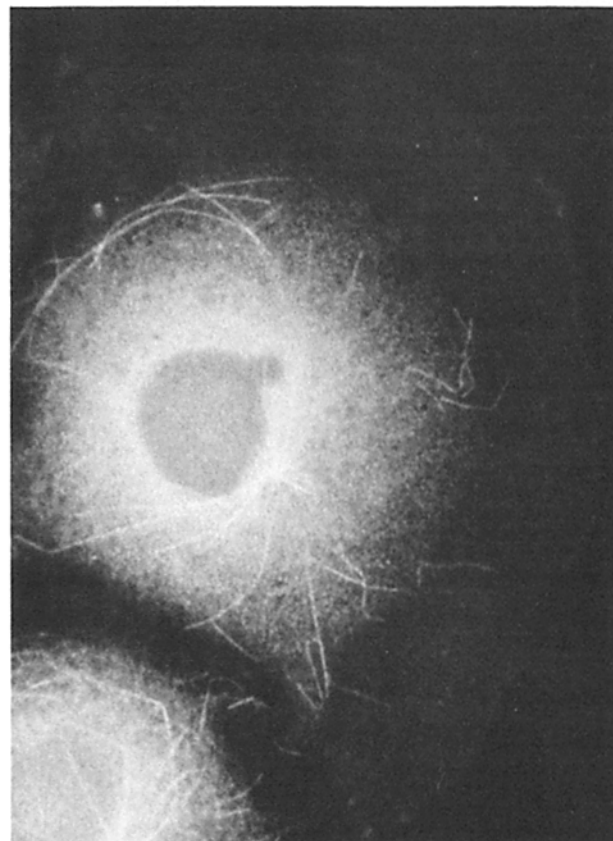
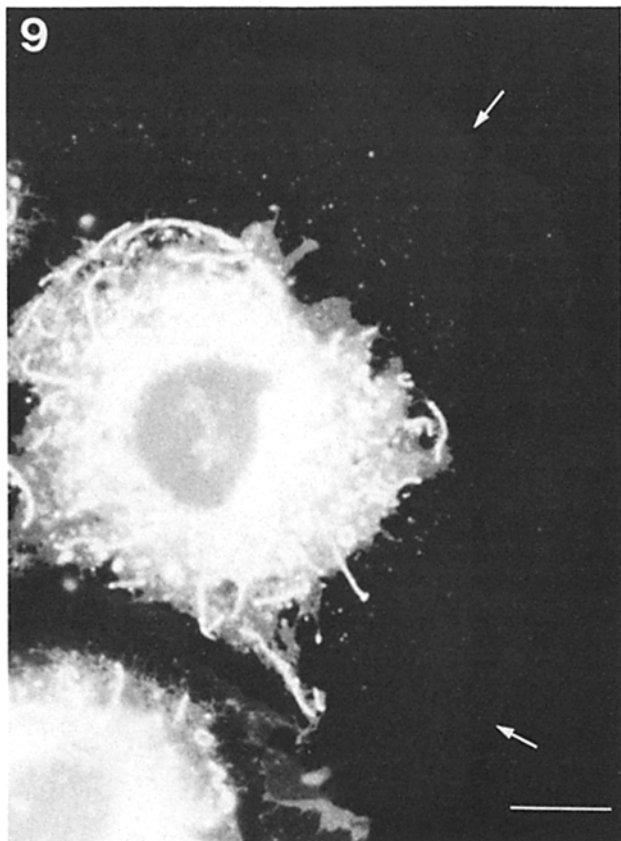
Hypotonic shock also causes a reversible depolymerization of microtubules (Brinkley et al., 1980). A 5-min treatment with culture media diluted four times with water resulted in depolymerization of most microtubules. The hypotonic shock also resulted in a vesiculation of the ER in almost all the cells. The vesiculation was rapidly reversible, with a "rumpled" appearing ER network re-forming within 5 min of return to isotonic media, and a return to normal morphology occurring by 2 h.

When a hypotonic shock and recovery was performed in the presence of nocodazole, the ER network re-formed in the absence of microtubules. The appearance of the ER was similar to that of cells recovering from cold shock in the presence of nocodazole. As before, the ER underwent a slow retraction towards the cell center.

Effects of Microtubule Repolymerization

PtK-2 cells were plated in tissue culture medium containing 1 $\mu\text{g}/\text{ml}$ nocodazole and allowed to spread overnight. In these cells, the peripheral regions were completely free of ER (Fig. 9). Nocodazole was washed out of these cells and the behavior of microtubules and ER was observed. The ER appeared to recover more slowly than the microtubules. Microtubules and the ER of a cell after 7 min recovery is shown in Fig. 10. There were many microtubules with no accompanying ER tubules, but almost all ER tubules were associated with a microtubule. After 20 min, the recovery was complete in most cells, with very close correlations of microtubules and the ER at the periphery (not shown).

Recovery from nocodazole was slightly different when previously attached CV-1 cells were treated with nocodazole (1 $\mu\text{g}/\text{ml}$) for 2 h. Most of the ER retracted after this treatment, but there were some tubules and cisternae remaining in the peripheral regions (Fig. 7). At 5 and 10 min after removal of nocodazole, many microtubule fragments were observed in the periphery. ER membranes were not associated with these fragments. Instead, the ER network gradually extended outwards with time. At 15 min, these microtubule fragments were no longer abundant and instead there were approximately the normal number of microtubules associated with the microtubule organizing center. As with PtK-2 cells, the ER seemed to recover somewhat more slowly than the microtubules. At 20–25 min, the appearance of the microtubule and ER distribution was normal.



Figures 9 and 10. (Fig. 9) ER (*left*) and microtubule (*right*) distributions in a PtK-2 cell that was plated and allowed to attach for 17 h in the presence of nocodazole. The cell has spread but the ER has not advanced outwards from the central region of the cell. Arrows indicate edge of cell. (Fig. 10) ER (*left*) and microtubule (*right*) distributions in a PtK-2 cell, treated as in Fig. 9, and then placed in drug-free media for 7 min. Microtubules and the ER have begun to advance outwards. There are more microtubules than ER at this time. The ER tubules are aligned with microtubules. Bar, 10 μ m.

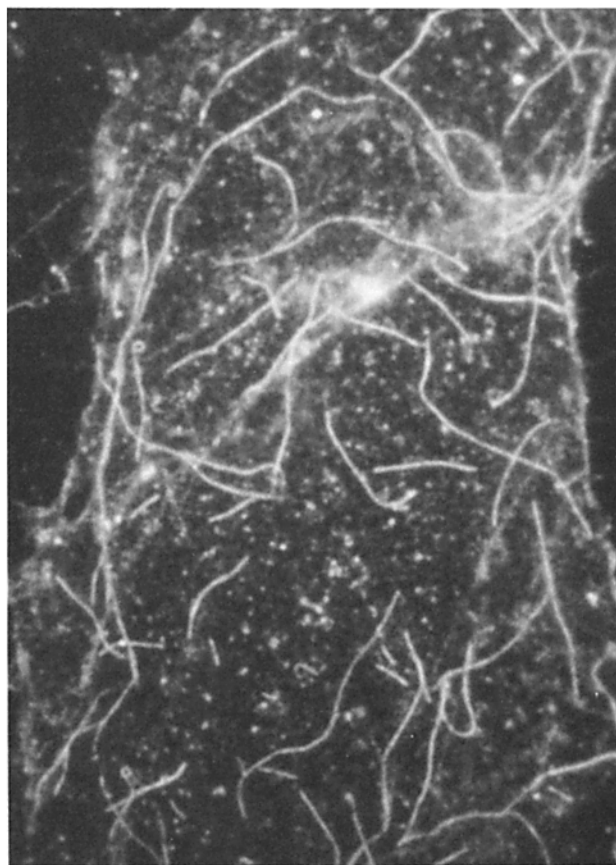
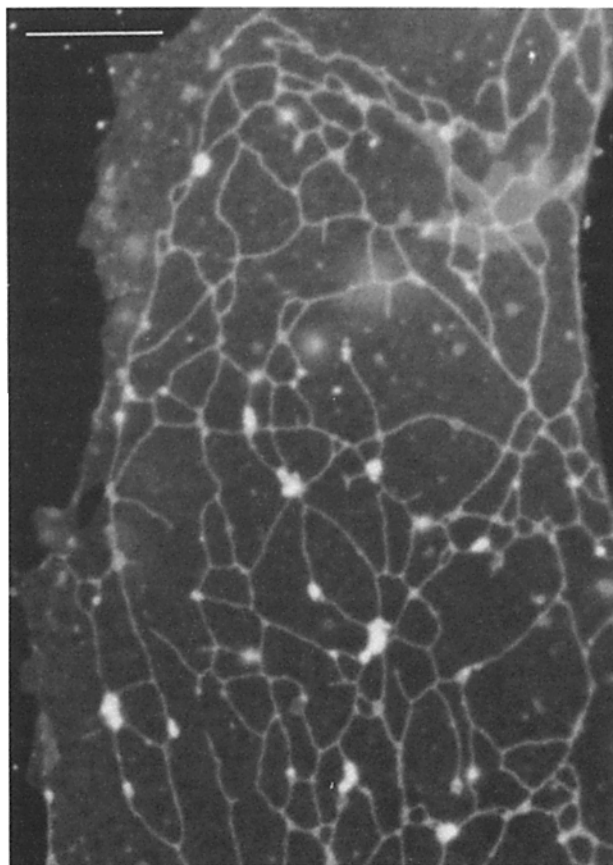


Figure 11. ER (left) and microtubule (right) distributions in a large thin cell, from a goldfish scale, that has recovered for 5 min from a cold shock (10 min at -2°C). At this time, there are many microtubule fragments, and approximately half of them are aligned with ER tubules. Bar, 10 μm .

When microtubules in the fish scale cells were depolymerized by a 10-min cold shock, the ER of many cells remained extended and intact (Fig. 8). The recovery of microtubules was observed when such cold shocked cells were returned to normal temperatures. At 5 min, many microtubule fragments were observed and approximately half of them were coaligned with ER tubules (Fig. 11). By 20 min, the number of microtubule fragments had decreased and the bound microtubules were associated with the ER to the same degree as control cells. The observations suggest that microtubules can polymerize along pre-existing ER tubules, though it is not known if the ER tubules direct the polymerization, if a surrounding organization causes the microtubules to polymerize along the ER tubules (Porter et al., 1983), or if microtubule fragments can bind to the ER.

Behavior of Intermediate Filaments during Microtubule Depolymerization

Intermediate filaments also retract towards the cell center when microtubules are depolymerized (Ishikawa et al., 1968; Goldman, 1971). In nocodazole-treated cells, ER and intermediate filaments were observed to collapse in a similar manner in that the intermediate filaments did not precede or lag behind the ER (not shown). When CV-1 or 3T3 cells were treated with 1 $\mu\text{g}/\text{ml}$ nocodazole for 2 h, a majority of the cells had some ER tubules and cisternae remaining in the peripheral regions (Fig. 7), while in some cells, the peripheral regions were completely free of ER. Mitochondria were of-

ten present in these "islands" of membranes. When the "islands" of ER were composed only of cisternae, it was common for them to be unassociated with either microtubules or intermediate filaments (not shown). When, however, the "islands" had tubular ER in them, there were usually microtubules and vimentin in the region (not shown).

Discussion

The internal membrane network of the ER was first clearly observed in the thin peripheral regions of tissue culture cells by electron microscopy (Porter et al., 1945; Porter, 1953). With the development of thin sectioning methods, the internal membranes of many cells have been observed. Although vesicular membranes are abundant and are clearly important in shuttling materials between different membrane compartments, it is remarkable that most intracellular membranes are tubular in form (Weibel et al., 1969; Bolender, 1974). Thin section electron microscopy (Porter and Blum, 1953; Palade and Porter, 1954), cell fractionation techniques (Claude, 1941; Hogeboom, 1949; Palade and Siekevitz, 1956), and histochemical techniques (Sheldon et al., 1955) have established that the ER performs many important functions (Palade, 1956; Porter, 1961; Fawcett, 1964), including synthesis of extracellular-bound and membrane proteins (Palade and Siekevitz, 1956; Palade, 1975), synthesis of membrane lipids (Wilgram and Kennedy, 1963), and calcium sequestration (reviewed by Somlyo, 1984).

Morphologically, the membrane network of the ER is pervasively distributed throughout most cells. At present, it is not known how this distribution is established and maintained, or indeed why the membranes have this distribution. There is also a seeming paradox of function and morphology of the ER: the ER performs many different and apparently unrelated functions, yet the tubular ER is apparently a completely interconnected structure. An additional issue is how vesicles are related to the ER.

In view of previous evidence for microtubule-membrane interactions, it is possible that the organization of the intracellular membranes is interrelated with that of the protein filament systems. We have used the immunofluorescence method for localizing microtubules and intermediate filaments, and a fluorescent dye method for localizing the ER in thin, spread cultured cells. We observed that microtubules and ER membranes have similar, though not identical, distributions. Our observations rule out several types of possible relationships of microtubules and the ER. Since microtubules and the ER do not have identical distributions, they clearly do not have a one-to-one relationship. Furthermore, neither have a strict, moment to moment requirement for the other. In other words, each ER strand does not require an adjacent microtubule to maintain its form, and each microtubule does not require an adjacent ER tubule to remain polymerized.

Our observations suggest, however, that ER extension and microtubule polymerization are closely related. Colinear microtubules and ER were commonly observed in the lamellipodia (Fig. 4). Since lamellipodia are regions where many new extensions of both occur, these observations strongly suggest that the two often, or always, extend outwards together. This is supported by observations made during repolymerization of microtubules after drug-induced depolymerization. During this process, microtubules and the ER were closely related (Fig. 10). In addition, when cells were plated in nocodazole, the ER did not extend into the cell periphery (Fig. 9). Thus, coaligned microtubules and ER in untreated cells probably arise because the two were made together. An important issue is whether microtubules precede the ER, the ER precedes microtubules, or both advance together. Due to the relatively low resolution of the fluorescence microscope (~ 200 nm), this could not be determined and awaits studies at the ultrastructural level.

If microtubules and the ER are made together, the question arises why the two systems do not have identical distributions throughout the whole cell. An explanation may be provided by the experiments on microtubule depolymerization. Once an ER tubule and microtubule have been extended, depolymerization of the microtubule apparently has no immediate effect on the accompanying ER tubule (Figs. 6 and 8). We suggest then that extension of a free-ended ER tubule requires an accompanying microtubule, but that microtubule depolymerization (Mitchison and Kirschner, 1984; Schulze and Kirschner, 1986) has no effect after the ER tubule has become part of the ER network.

Once the ER has been extended into the peripheral regions, the network as a whole can be retracted by prolonged treatment with microtubule depolymerizing drugs (Fig. 7). Microtubule depolymerization precedes retraction of the ER (Fig. 6), so that microtubules do not pull in the ER as they depolymerize. Instead, the absence of micro-

tubules seems to create a condition that results in the slow retraction of the membranes. These results suggest that the entire extended network of the ER is an unstable, or metastable, configuration of membranes that requires a continued interaction with the microtubule system to remain extended.

Cell spreading and translocation are prominent activities of the thin spread cells we have used (Rubin, 1974), and it is interesting to consider our morphological observations in terms of these cellular functions. The lamellipodia are sites of cytoplasmic spreading and translocation (Abercrombie, 1961). It seems clear that the few ER tubules present in the lamellipodia are the first extensions of the ER, and that more ER membranes are extended into this region as the cell spreads or translocates further. We have observed that microtubules and the ER are closely associated in these regions. Intermediate filaments, on the other hand, are often completely absent from the spreading regions (Hynes and Destree, 1978; also Fig. 5, this paper). These relationships in the lamellipodia indicate that microtubules and the ER advance into the lamellipodia before the intermediate filaments.

Regulation of motile activities in the lamellipodia is not well understood at present, but there is evidence that calcium is involved (Taylor et al., 1980). We suggest that newly extended ER membranes in the lamellipodia play a role in the regulation of motile activities by serving as a sink for calcium. As more and more ER membranes are extended into the advancing lamellipodia, the membranes are proposed to lower the cytosolic calcium concentration, thereby stabilizing the regions of cytoplasm that have already been advanced.

This proposal can explain the effects of microtubule-depolymerizing drugs on the formation of lamellipodia. Treatment with these drugs results in the development of lamellipodia around the entire periphery of the cell, so that the cell is effectively attempting to move in all directions (Vasiliev et al., 1970; Vasiliev and Gelfand, 1976; Gail and Boone, 1971; Goldman 1971). We have observed that these drugs also prevent the ER from extending into the periphery (Fig. 9), and we suggest that the absence of calcium sequestering activity in the periphery contributes to the abnormal distribution of lamellipodia.

In summary, we have combined a fluorescent dye staining technique with immunofluorescence to study the organization of the ER, microtubules, and intermediate filaments in thin, spread cells in culture. We have found that microtubules and the ER are very closely related in the lamellipodia of these cells, and we have considered their relationships in terms of the processes of cell spreading and translocation.

We thank Dr. Elena McBeath for advice on using her fixation technique and on using the large thin cells from the fish scale. We also thank Dr. Karen Hedberg and Micheal Aronow for discussions and work done in the early stages of this work.

This research was supported by National Institutes of Health grant HL-32544 and National Science Foundation grant PCM-81-19171 to K. Fujiwara, and by National Institutes of Health grant CA-22659 to L. B. Chen.

Received for publication 30 January 1986, and in revised form 12 June 1986.

References

- Abercrombie, M. 1961. The bases of the locomotory behavior of fibroblasts. *Exp. Cell Res. Suppl.* 8:188-198.
- Allen, R. D., J. Metzals, I. Tasaki, S. T. Brady, and S. P. Gilbert. 1982. Fast axonal transport in squid giant axon. *Science (Wash. DC)*. 218:1127-1128.

- Bolender, R. P. 1974. Stereological analysis of the guinea pig pancreas. *J. Cell Biol.* 61:269-287.
- Brady, S. T., R. J. Lasek, and R. D. Allen. 1982. Fast axonal transport in extruded axoplasm from squid giant axon. *Science (Wash. DC)*. 218:1129-1131.
- Brinkley, B. R., S. M. Cox, and D. A. Pepper. 1980. Microtubule-plasma membrane interactions: analysis by tubulin immunofluorescence and electron microscopy. In *Testicular Development, Structure and Function*. A. Steinberger and E. Steinberger, editors. Raven Press, New York. 305-314.
- Buckley, I. K. 1964. Phase contrast observations on the endoplasmic reticulum of living cells in culture. *Protoplasma*. 59:569-588.
- Buckley, I. K., and K. R. Porter. 1975. Electron microscopy of critical point dried whole cultured cells. *J. Microsc.* 104:107-120.
- Claude, A. 1941. Particulate components of cytoplasm. *Cold Spring Harbor Symp. Quant. Biol.* 9:263-272.
- Clermont, Y., and A. Rambourg. 1978. Evolution of the endoplasmic reticulum during rat spermiogenesis. *Am. J. Anat.* 151:191-212.
- Collot, M., D. Louvard, and S. J. Singer. 1984. Lysosomes are associated with microtubules and not with intermediate filaments in cultured fibroblasts. *Proc. Natl. Acad. Sci. USA*. 81:788-792.
- Fawcett, D. W. 1964. Structural and functional variations in the membranes of the cytoplasm. In *Intracellular Membrane Structure*. S. Seno and E. V. Cowdry, editors. Chugoku Press, Ltd., Okayama. 15-40.
- Fawcett, D. W., W. A. Anderson, and D. M. Phillips. 1971. Morphogenetic factors influencing the shape of the sperm head. *Dev. Biol.* 26:220-251.
- Fawcett, D. W., and S. Ito. 1958. Observations on the cytoplasmic membranes of testicular cells, examined by phase contrast and electron microscopy. *J. Biophys. Biochem. Cytol.* 4:135-152.
- Franke, W. W. 1971. Cytoplasmic microtubules linked to endoplasmic reticulum with cross-bridges. *Exp. Cell Res.* 66:486-489.
- Fujiwara, K., and T. D. Pollard. 1978. Simultaneous localization of myosin and tubulin in human tissue culture cells by double antibody staining. *J. Cell Biol.* 77:182-195.
- Gail, M. H., and C. W. Boone. 1971. Effect of colcemid on fibroblast motility. *Exp. Cell Res.* 65:221-227.
- Goldman, R. D. 1971. The role of three cytoplasmic fibers in BHK-21 cell motility. I. Microtubules and the effects of colchicine. *J. Cell Biol.* 51:752-762.
- Goldstein, M. A., and M. L. Entman. 1980. Microtubules in mammalian heart muscle. *J. Cell Biol.* 80:183-195.
- Habicht, J., and K. Brune. 1980. Carbocyanine dyes stain the sarcoplasmic reticulum of beating heart cells. *Exp. Cell Res.* 125:514-518.
- Heggeness, M. H., M. Simon, and S. J. Singer. 1978. Association of mitochondria with microtubules in cultured cells. *Proc. Natl. Acad. Sci. USA*. 75:3863-3866.
- Hepler, P. K. 1980. Membranes in the mitotic apparatus of barley cells. *J. Cell Biol.* 86:490-499.
- Hogeboom, G. H. 1949. Cytochemical studies of mammalian tissues II. The distribution of diphosphopyridine nucleotide-cytochrome c reductase in rat liver functions. *J. Biol. Chem.* 177:847-858.
- Hynes, R. O., and A. T. Destree. 1978. 10 nm filaments in normal and transformed cells. *Cell*. 13:151-163.
- Ishikawa, H., R. Bischoff, and H. Holtzer. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. *J. Cell Biol.* 38:535-555.
- Ito, S. 1962. Light and electron microscopic study of membranous cytoplasmic organelles. In *The Interpretation of Ultrastructure*. R. J. C. Harris, editor. Academic Press, New York. 129-148.
- Johnson, L. V., M. L. Walsh, B. J. Bockus, and L. B. Chen. 1981. Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. *J. Cell Biol.* 88:526-535.
- Johnson, L. V., M. L. Walsh, and L. B. Chen. 1980. Localization of mitochondria in living cells with rhodamine 123. *Proc. Natl. Acad. Sci. USA*. 77:990-994.
- Klymkowsky, M. W. 1981. Intermediate filaments in 3T3 cells collapse after intracellular injection of a monoclonal anti-intermediate filament antibody. *Nature (Lond.)*. 291:249-251.
- Louvard, D., H. Reggio, and G. Warren. 1982. Antibodies to the Golgi complex and rough endoplasmic reticulum. *J. Cell Biol.* 92:92-107.
- Masurovsky, E., E. Peterson, S. Crain, and S. Horwitz. 1981. Microtubule arrays in taxol-treated mouse dorsal root ganglion-spinal cord cultures. *Brain Res.* 217:392-398.
- McBeath, E., and K. Fujiwara. 1984. Microtubule kinetics in cells studied by immunofluorescence. *International Cell Biology 1984*. S. Seno and Y. Okada, editors. Abstr. 4210.
- Miller, R. H., and R. J. Lasek. 1985. Cross-bridges mediate anterograde and retrograde vesicle transport along microtubules in squid axoplasm. *J. Cell Biol.* 101:2181-2193.
- Mitchison, T., and M. Kirschner. 1984. Dynamic instability of microtubule growth. *Nature (Lond.)*. 312:237-242.
- Osborn, M., T. Born, H. Koitsch, and K. Weber. 1978. Stereo immunofluorescence microscopy: I. Three-dimensional arrangement of microfilaments, microtubules and tonofilaments. *Cell*. 14:477-488.
- Pagano, R. E., K. J. Longmuir, O. C. Martin, and D. K. Struck. 1981. Metabolism and intracellular localization of a fluorescently labeled intermediate in lipid biosynthesis within cultured fibroblasts. *J. Cell Biol.* 91:872-877.
- Palade, G. E. 1956. The endoplasmic reticulum. *J. Biophys. Biochem. Cytol.* 2:85-98.
- Palade, G. E. 1975. Intracellular aspects of the process of protein secretion. *Science (Wash. DC)*. 189:347-358.
- Palade, G. E., and K. R. Porter. 1954. Studies on the endoplasmic reticulum. I. Its identification in cells in situ. *J. Exp. Med.* 100:641-656.
- Palade, G. E., and P. Siekevitz. 1956. Liver microsomes. An integrated morphological and biochemical study. *J. Biophys. Biochem. Cytol.* 2:171-200.
- Pickett-Heaps, J., and D. Northcote. 1966. Organization of microtubules and endoplasmic reticulum during mitosis and cytokinesis in wheat meristems. *J. Cell Sci.* 1:109-120.
- Porter, K. R. 1953. Observations on a submicroscopic basophilic component of the cytoplasm. *J. Exp. Med.* 97:727-750.
- Porter, K. R. 1961. The ground substance; observations from electron microscopy. In *The Cell*. D. J. Brachet and A. E. Mirsky, editors. Vol. II, Chapter 9. Academic Press, New York. 621-675.
- Porter, K. R., M. Beckerle, and M. McNiven. 1983. The cytoplasmic matrix. In *Modern Cell Biology*. Vol. 2. B. Satir, editor. Alan R. Liss, Inc., New York. 259-305.
- Porter, K. R., and J. Blum. 1953. A study in microtomy for electron microscopy. *Anat. Rec.* 117:685-710.
- Porter, K. R., A. Claude, and E. Fullam. 1945. A study of tissue culture cells by electron microscopy. *J. Exp. Med.* 81:233-241.
- Porter, K. R., and F. L. Kallman. 1952. Significance of cell particulates as seen by electron microscopy. *Ann. NY Acad. Sci.* 54:882-891.
- Rogalski, A., and S. J. Singer. 1984. Associations of elements of the Golgi apparatus with microtubules. *J. Cell Biol.* 99:1092-1100.
- Rose, G. C., and C. M. Pomerat. 1960. Phase contrast observations of the endoplasmic reticulum in living tissue cultures. *J. Biophys. Biochem. Cytol.* 8:423-430.
- Rubin, H. 1974. Regulation of animal cell growth. In *Cell Communication*. R. P. Cox, editor. John Wiley & Sons, Inc., New York. 127-146.
- Schnapp, B. J., R. D. Vale, M. P. Sheetz, and T. S. Reese. 1985. The structure of cytoplasmic filaments involved in organelle transport in the squid giant axon. *Cell*. 40:455-462.
- Schulze, E., and M. Kirschner. 1986. Microtubule dynamics in interphase cells. *J. Cell Biol.* 102:1020-1031.
- Scordilis, S. P., H. Tedeschi, and C. Edwards. 1975. Donnan potential of rabbit skeletal muscle myofibrils I: electrofluorochromometric detection of potential. *Proc. Natl. Acad. Sci. USA*. 72:1325-1329.
- Sheldon, H., H. Zetterqvist, and D. Brandes. 1955. Histochemical reactions for electron microscopy: acid phosphatase. *Exp. Cell Res.* 9:592-596.
- Sims, P. J., A. S. Waggoner, C. H. Wang, and J. F. Hoffman. 1974. Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. *Biochemistry*. 13:3315-3330.
- Somlyo, A. P. 1984. Cellular site of calcium regulation. *Nature (Lond.)*. 309:516-517.
- Song, J., M. Terasaki, and L. B. Chen. 1985. Whole-mount electron microscopy and phase-contrast microscopy of endoplasmic reticulum in potassium permanganate treated cultured cells. *J. Cell Biol.* 101(5, Pt. 2):57a. (Abstr.)
- Stephens, R. E. 1985. Evidence for a tubulin-containing lipid-protein structural complex in ciliary membranes. *J. Cell Biol.* 100:1082-1090.
- Stevens, P. 1974. Patterns in Nature. Little, Brown & Co., Boston. 240 pp.
- Summerhayes, I. C., D. Wong, and L. B. Chen. 1983. Effect of microtubules and intermediate filaments on mitochondrial distribution. *J. Cell Sci.* 61:87-105.
- Taylor, D. L., J. R. Blinks, and G. Reynolds. 1980. Contractile basis of amoeboid movement. VIII. Aequorin luminescence during amoeboid movement, endocytosis and capping. *J. Cell Biol.* 86:599-607.
- Terasaki, M., J. D. Song, J. R. Wong, M. J. Weiss, and L. B. Chen. 1984. Localization of endoplasmic reticulum in living and glutaraldehyde fixed cells with fluorescent dyes. *Cell*. 38:101-108.
- Tokunaga, S., T. Friedman, Y. Toyama, M. Pacifici, and H. Holtzer. 1983. Taxol induces microtubule-rough endoplasmic reticulum complexes and microtubule-bundles in cultured chondroblasts. *Differentiation*. 24:39-47.
- Vasiliev, J. M., and I. M. Gelfand. 1976. Effects of colcemid on morphogenetic processes and locomotion of fibroblasts. In *Cell Motility*. R. Goldman, T. Pollard, and J. Rosenbaum, editors. Vol. A. Cold Spring Harbor Laboratory, New York. 279-304.
- Vasiliev, J. M., M. Gelfand, L. V. Domnina, O. Y. Ivanova, S. G. Komm, and L. V. Olshevskaia. 1970. Effect of the colcemid on the locomotory behaviour of fibroblasts. *J. Embryol. Exp. Morphol.* 24:625-640.
- Vogl, A. W., R. W. Linck, and M. Dym. 1983. Colchicine-induced changes in the cytoskeleton of the golden mantled ground squirrel (spermophilus lateralis) Sertoli cells. *Am. J. Anat.* 168:99-108.
- Wang, E., and R. D. Goldman. 1978. Functions of cytoplasmic fibers in intracellular movements in BHK-21 cells. *J. Cell Biol.* 79:708-726.
- Wehland, J., M. Henkart, R. Klausner, and I. V. Sandoval. 1983. Role of microtubules in the distribution of the Golgi apparatus: effect of taxol and microinjected anti-alpha tubulin antibodies. *Proc. Natl. Acad. Sci. USA*. 80:4286-4290.
- Weibel, E. R., W. Staubli, H. R. Gnagi, and F. A. Hess. 1969. Correlated morphometric and biochemical studies on the liver cell. *J. Cell Biol.* 42:68-91.
- Wilgram, G. F., and E. P. Kennedy. 1963. Intracellular distribution of some enzymes catalyzing reactions in the biosynthesis of complex lipids. *J. Biol. Chem.* 238:2615-2619.
- Yoshikami, D., and L. M. Okun. 1984. Staining of living presynaptic nerve terminals with selective fluorescent dyes. *Nature (Lond.)*. 310:53-56.