Cell, Vol. 38, 101–108, August 1984, Copyright © 1984 by MIT

0092-8674/84/080101-08 $02.00/0

Localization of Endoplasmic Reticulum in Living and Glutaraldehyde-Fixed Cells with Fluorescent Dyes

Mark Terasaki, Jindan Song, James R. Wong, Michael J. Weiss, and Lan Bo Chen
Dana-Farber Cancer Institute
and Department of Pathology
Harvard Medical School
Boston, Massachusetts 02115

Summary

Certain fluorescent dyes, previously reported to localize mitochondria, when used at higher concentrations also localize a continuous net-like structure in both living and glutaraldehyde-fixed cells. A similar reticular structure can be detected by phase-contrast microscopy and whole-mount electron microscopy in potassium permanganate-fixed cells as well. This structure is mostly tubular, with some patch-like areas, and is likely to be the endoplasmic reticulum (ER). The organization of the reticular structure is sensitive to colchicine and rotenone but not to cytochalasin B, taxol, monensin, the calcium ionophore A23187, 12-O-tetradecanoylphorbol 13-acetate, or hydrocortisone.

Introduction

Among the first observations of a reticular (net-like) structure corresponding to the endoplasmic reticulum (ER) were those of Bensley (1898), Matthews (1899), and Garnier (1911) (see reivew by Fawcett, 1981). Not until the pioneering works of Porter and coworkers was the existence of this cellular organelle firmly established (Porter et al., 1945; Porter and Thompson, 1947; Porter, 1953). The crucial discoveries regarding the function of the ER were the demonstrations that enzyme-rich microsomes are derived from the ER and that ribosomes attach to it (Palade and Siekevitz, 1956). It is now believed that the ER plays a central role in the biosynthesis of cholesterol, phospholipids, steroids, prostaglandins, and membrane and secretory proteins.

In contrast to mitochondria and lysosomes, the ER is not readily visible by light microscopy in living or fixed cells, making the cellular distribution of the ER difficult to study. Perhaps the best visualizations of the ER in a whole cell remain the first whole-mount electron micrographs of Porter and coworkers (1945). Recently, Pagano et al. (1981) localized a reticular structure which appeared to be the ER with fluorescent phosphatidic acid analogs by liposome fusion techniques at 2°C.

In the course of our studies on localizing the mitochondria with cationic fluorescent dyes, we observed that under certain conditions a reticular structure was stained in addition to mitochondria. Here we report a rapid and simple procedure for localizing a structure that appears to be the ER both in living and in glutaraldehyde-fixed cells by fluorescent microscopy. In addition, we find that this reticular structure can also be detected by phase-contrast microscopy in potassium permanganate-fixed cells.

Results

Fluorescent Staining of Reticular Structure in Living Cells

It has been reported that the lipophilic, cationic fluorescent dye $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) selectively stains mitochondria in living cells in response to the high mitochondrial membrane potential (Johnson et al., 1981). However, when a higher concentration of dye (0.5 to 2.5 μg/ml) is used, other cellular membranes become visible. Whereas very little or no staining is observed in lysosomes, endosomes, or the Golgi apparatus, the plasma membrane is dimly visible. The most striking feature is a lace-like reticular structure remarkable for its elaborate continuity throughout the cell (Figure 1). While most of the structure is net-like with a relatively uniform diameter, flat patch-like areas can also be observed. Mitochondria are brightly stained and swollen, indicating that the dye is toxic to mitochondria at this concentration.

We have achieved the best visualization of this reticular structure using the African green monkey kidney epithelial cell line, CV-1. Most of our subsequent studies were therefore carried out with this cell line.

Effects of Organic Solvents, Detergents, Glutaraldehyde, and Chaotropic Agents on Staining in Living Cells

The fluorescent staining of the reticular structure in living cells described above is completely eliminated by subsequent treatment with acetone, ethanol, methanol, Triton X-100, NP-40, or Brij 58.

However, the stained reticular structure in living cells can still be observed even if stained cells are subsequently fixed with 0.25% glutaraldehyde in cacodylate buffer. This is consistent with the fact that glutaraldehyde does not fix or remove membrane lipids. Formaldehyde also preserves the fluorescent staining, but the reticular structure becomes vesiculated in some of the cells.

Fluorescent Staining of Reticular Structure in Glutaraldehyde-Fixed Cells

The reticular structure, mitochondria, and some vesicular structures can also be stained after glutaraldehyde fixation of unstained cells. Mitochondria can be readily distinguished from the reticular structure by fluorescent intensity as well as by morphology. By use of negative printing, these two cellular components can be documented simultaneously, as shown in Figure 2. Mitochondria are indicated by the arrows. The nature of the vesicular staining remains to be determined. Some of the vesicles appear to be lysosomes.

The reticular staining in glutaraldehyde-fixed cells is resistant to 4 M NaCl, 6 M urea, 4 M guanidine hydrochloride, or 5% mercaptoethanol. However, an organic solvent

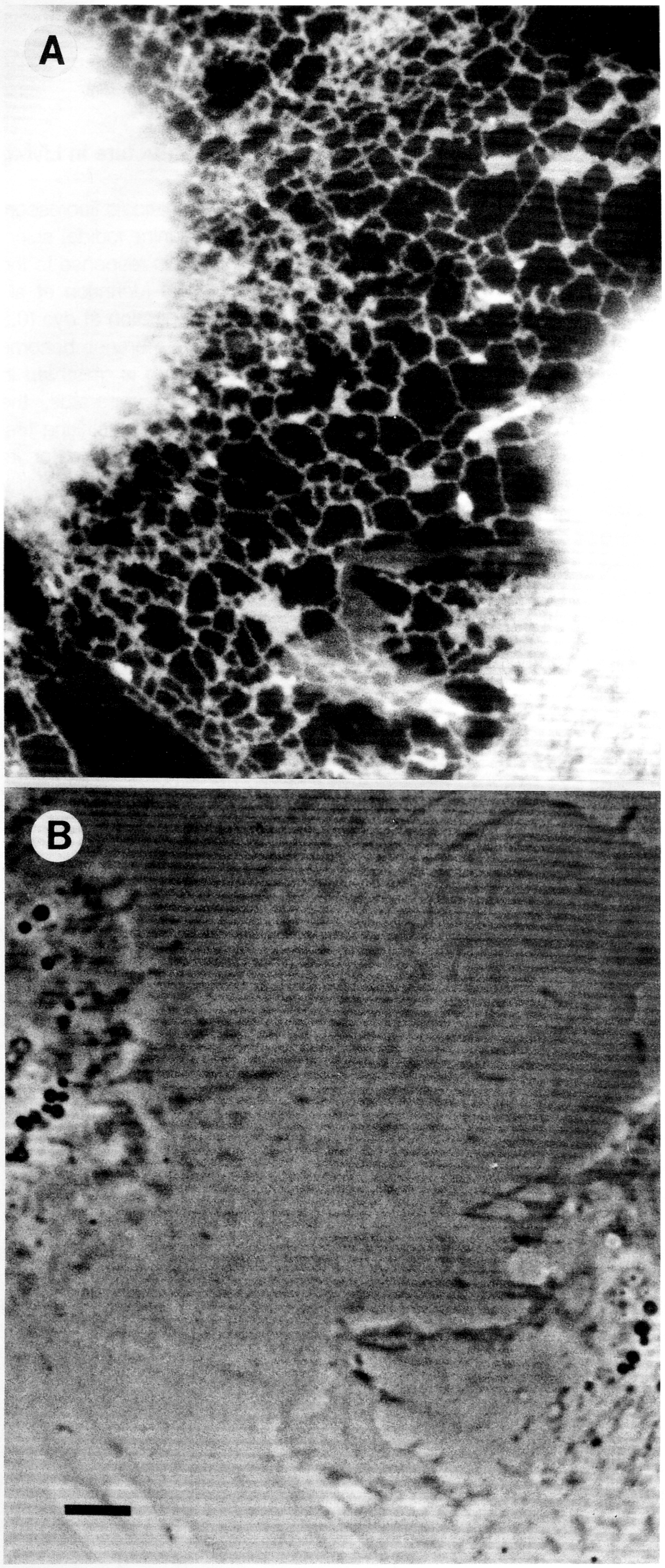

Figure 1. Localization of a Reticular Structure in Living CV-1 Cells with a Cyanine Dye, $DiOC_6(3)$, by Fluorescent Microscopy

(A) Fluorescent micrograph (B) Phase-contrast micrograph. Bar represents 5 μm.

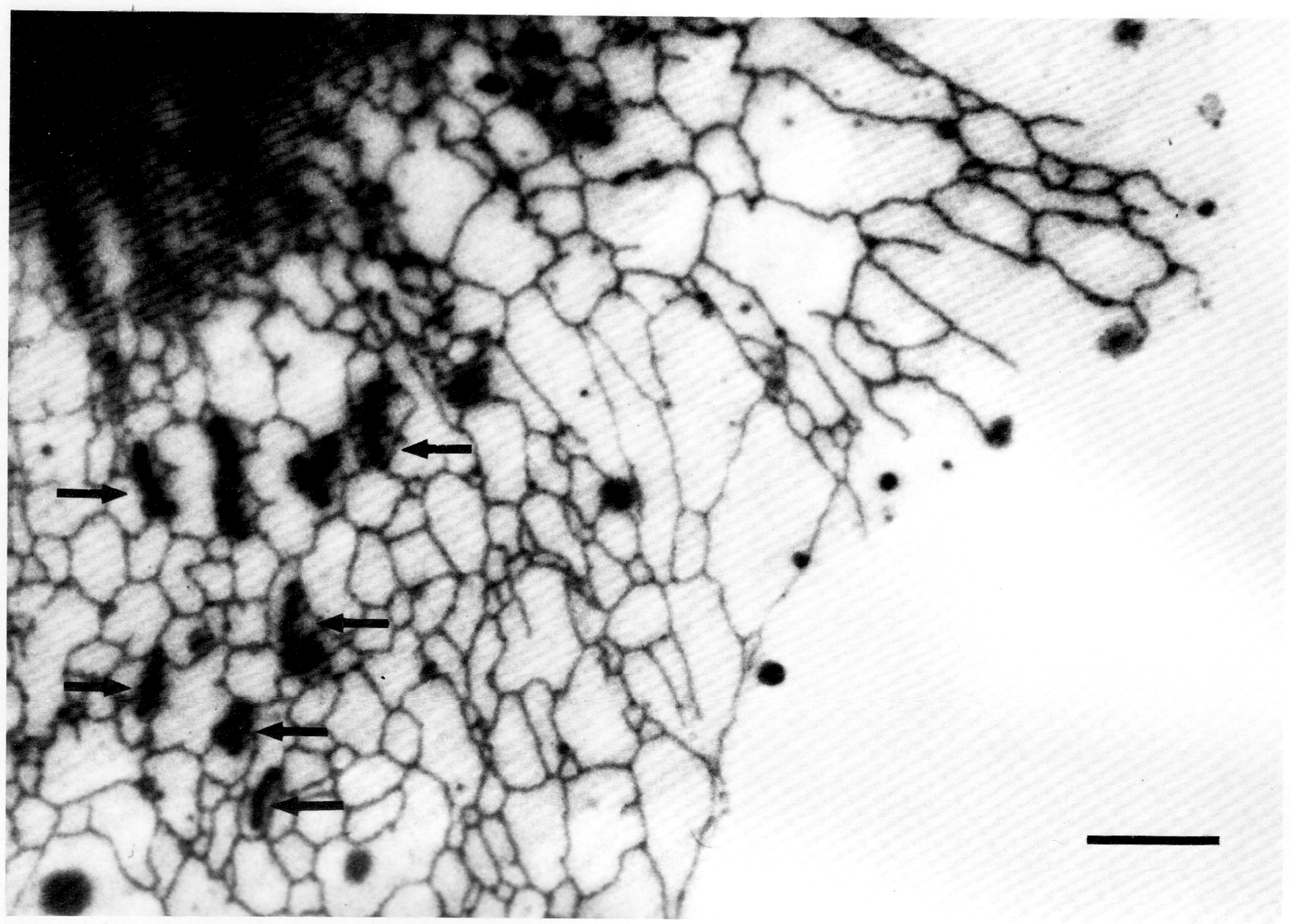

Figure 2. A Negative Print of a Glutaraldehyde-Fixed CV-1 Cell Stained with a Cyanine Dye, $DiOC_6(3)$
Mitochondria are indicated by the arrows. Bar represents 6 μm.

or a detergent eliminates this staining as it does with living cells. Significantly, if glutaraldehyde-fixed cells are treated with an organic solvent or detergent prior to dye exposure, the staining of the reticular structure is prevented. In addition, the reticular structure cannot be stained in cells treated with osmium tetroxide, a fixative for membrane lipids, whether or not the cells have been pre-fixed with glutaraldehyde. These results are consistent with the notion that the stained structure contains lipid membranes and the fluorescent dye partitions into or binds to the lipids of the reticular structure in glutaraldehyde-fixed cells.

The staining of the reticular structure in glutaraldehyde-fixed cells is more clearly visible than that of living cells because of a weaker staining of the plasma membrane. Therefore, to visualize the cellular distribution of the reticular structure the use of glutaraldehyde-fixed cells rather than living cells is recommended. In addition, mitochondria of glutaraldehyde-fixed cells have a more normal morphology, in contrast to the swollen morphology observed in stained living cells.

Detection of the Reticular Structure by Other Methods

The reticular structure observed by fluorescent microscopy can also be detected by phase-contrast microscopy in potassium permanganate-fixed cells, as shown in Figure 3B. This fixative destroys proteinaceous structure but preserves membrane lipids in organelles such as the ER and the mitochondria (Hayat, 1981). Permanganate-fixed cells were also examined by whole-amount electron microscopy (Figure 3C), and the reticular structure observed is similar to that seen in the micrographs of Porter and coworkers (1945).

When CV-1 cells were fixed in glutaraldehyde, thin sectioned, and observed by transmission EM, a corresponding network structure was shown in Figure 3D. The elements of this network structure have features classically identified as the ER by thin-section transmission electron microscopy.

Screening of Other Fluorescent Dyes

A number of other fluorescent dyes were tested on glutaraldehyde-fixed cells at 2.5 μg/ml for 2–3 min. Table 1 summarizes these results. $DiOC_6(3)$ was the dye of choice among the several dyes that stain the reticular structure. For unknown reasons the plasma membrane staining is weaker for this dye, and the staining is also more resistant to bleaching during viewing.

Visualization of the ER in Different Cell Types

A variety of cell types were stained with $DiOC_6(3)$. Figure 4 shows the reticular structure staining of a living chick embryo cell (Figure 4A), a glutaraldehyde-fixed CV-1 cell 6 hr after seeding in 0.5% serum (Figure 4B), a living rat

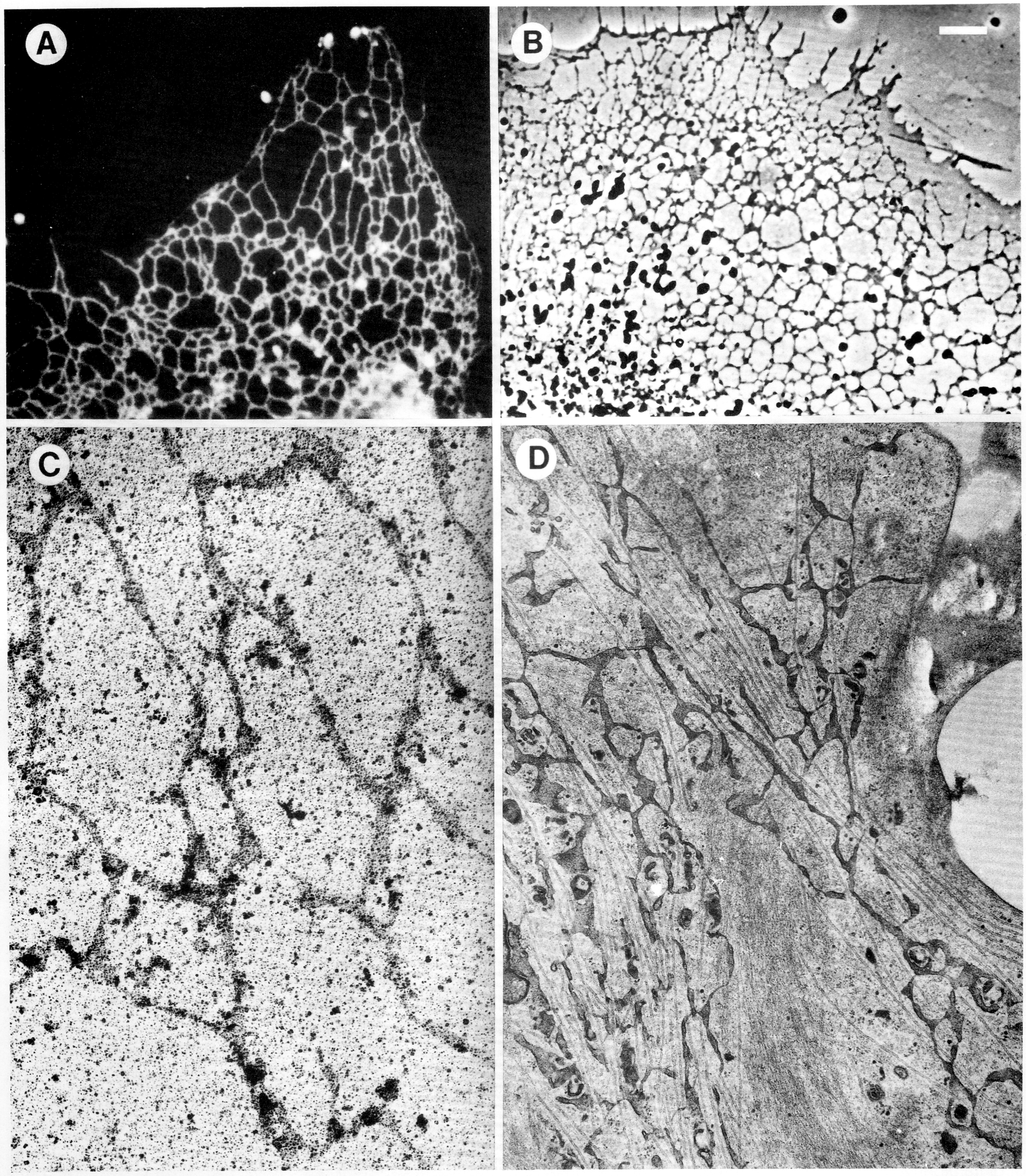

Figure 3. Fluorescent micrograph of a CV-1 cell fixed in glutaraldehyde and stained with a cyanine dye, $DiOC_6(3)$. (B) Phase-contrast micrograph of a CV-1 cell fixed in potassium permanganate. (C) Whole-mount transmission electron micrograph of a permanganate-fixed CV-1 cell. (D) Thin-section transmission electron micrograph of a glutaraldehyde-fixed CV-1 cell. Bar represents 6 μm in (A), 5 μm in (B), 0.15 μm in (C), and 0.3 μm in (D), respectively.

embryo fibroblast (Figure 4C), a living chick embryo fibroblast (Figure 4D), and a glutaraldehyde-fixed smooth muscle cell (Figure 4E). The reticular structure was difficult to observe in cell types that do not spread out on the substratum.

Effects of Colchicine, Monensin, and Other Drugs

In CV-1 cells, microtubules and microfilaments can be perturbed by colchicine and cytochalasin B, respectively. The organization of the reticular structure in CV-1 cells treated with cytochalasin B (5 μg/ml for 4 hr) is indistin-

Table 1. Compounds Screened for Staining of the Reticular Structure in Glutaraldehyde-Fixed Cells

Compound	Charge at pH 7.4	Reticular Staining	Resistance to Bleaching	Quality of Staining
Cyanines				
$DiOC_2(3)$	Positive	±	++	+
$DiOC_3(3)$	Positive	+	++	+++
$DiOC_4(3)$	Positive	+	++	+++
$DiOC_6(3)$	Positive	+	++	++++
$DiOC_{14}(3)$	Positive	–	N.A.	N.A.
$DiOC_2(5)$	Positive	+	±	++
$DiOC_6(5)$	Positive	+	±	++
$DilC_1(3)$	Positive	+	+	++
$DilC_2(3)$	Positive	+	+	++
$DilC_3(3)$	Positive	+	+	++
$DilC_4(3)$	Positive	+	+	++
$DilC_6(3)$	Positive	+	+	++
$DiSC_6(3)$	Positive	+	±	++
Rhodamines				
6G	Positive	+	±	++
3B	Positive	±	±	+
123	Positive	–	N.A.	N.A.
19	Neutral	–	N.A.	N.A.
Tetramethyl	Neutral	–	N.A.	N.A.
Fluorescein	Negative	–	N.A.	N.A.

N.A.: Not applicable.

guishable from that of untreated cells. However, colchicine (20 μg/ml for 4 hr), but not lumicolchicine (20 μg/ml for 4 hr), induces a dramatic change of the organization of the reticular structure (Figure 5A), which retracts gradually towards the nucleus. Rotenone (20 μg/ml for 16 hr), which is structurally similar to colchicine and disrupts microtubules (Walsh and Chen, unpublished results), also affects the organization of the ER (Figure 5B). Colcemide (1 μg/ml) also has the same effects. In addition, if colcemide-treated cells are washed and placed in drug-free medium, the ER returns to its normal appearance within a few hours (data not shown). Taxol (20 μg/ml for 2 hr), which stabilizes microtubules, exerts little or no effect on the organization of the ER.

Monensin (1 μM for 2 hr), a sodium/proton ionophore which disrupts the Golgi apparatus and interrupts vesicular traffic (Lin and Queally, 1982), the calcium ionophore A23187 (1 μg/ml for 1 hr), phorbol ester TPA (0.1 μg/ml for 4 hr), and hydrocortisone (10 μM for 24 hr) all have no effect on the appearance of the reticular structure.

Discussion

The endoplasmic reticulum was first described by Porter et al. (1945) as a lace-like intracellular structure present in the flat periphery of well-spread tissue culture cells. Using electron microscopy to examine thin sections of fixed tissues, Palade and Porter (1954) subsequently showed the ER to be a system of membranous tubules and cisternae.

Several lines of evidence imply that the reticular structure stained by the cyanine dye $DiOC_6(3)$ is the ER. First, the same reticular structure can be observed by phase-contrast microscopy in CV-1 cells fixed with potassium permanganate. Second, the appearance of the reticular structure in permanganate-fixed cells in whole-mount electron microscopy is similar to the ER defined by Porter (1953) in the early whole mount electron micrographs of cultured cells. Third, when CV-1 cells are fixed in glutaraldehyde, treated with osmium tetroxide, and processed for thin-section transmission electron microscopy, the endoplasmic reticulum can be seen in the periphery of the cells. The structure has the same network pattern as observed in dye-stained cells or in potassium permanganate-fixed cells. Fourth, the staining is extremely sensitive to organic solvents such as acetone, methanol, or ethanol, detergents such as Triton X-100, NP-40 or Brij 58, and the membrane fixative osmium tetroxide. These agents all prevent staining if they are applied before the dye, and they all remove the staining if they are applied after the cells are stained. Fifth, treatments such as 4 M NaCl, 6 M urea, 4 M guanidine hydrochloride, 5% mercaptoethanol, which generally affect protein conformation, do not eliminate the staining in glutaraldehyde-fixed cells.

Other methods of verifying that the reticular structure is the ER remain to be tested, such as double staining with antibodies against ER proteins by fluorescent microscopy and histochemical staining on thin-sectioned cells by electron microscopy. However, the evidence presented above makes it seem probable that the reticular structure stained with $DiOC_6(3)$ dye is the ER.

The basis for the ER staining by $DiOC_6(3)$ and other dyes is still unknown. For staining of living cells, we discern two factors that need to be considered. First, the dyes that stain the mitochondria in living cells and that stain the ER are all lipophilic. The lipophilicity is probably required for the dyes to be permeable to the plasma membrane and to enter the cell, but there may also be specific dye–lipid interactions which are involved in the staining. It is known that the membranes of the various cell organelles have different lipid compositions, and dye–lipid interactions of varying degrees may account for the selective staining of organelles that we observe. Second, every dye that we have found that stains the ER carries a delocalized positive charge at physiological pH. It is possible that the ER has a significant membrane potential, negative inside, that causes these dyes to be accumulated by a mechanism similar to that proposed for mitochondria (Johnson et al., 1981). An ER membrane potential less than that of the mitochondria but higher than those of the Golgi apparatus, endosomes, and lysosomes would produce the relative staining of these organelles.

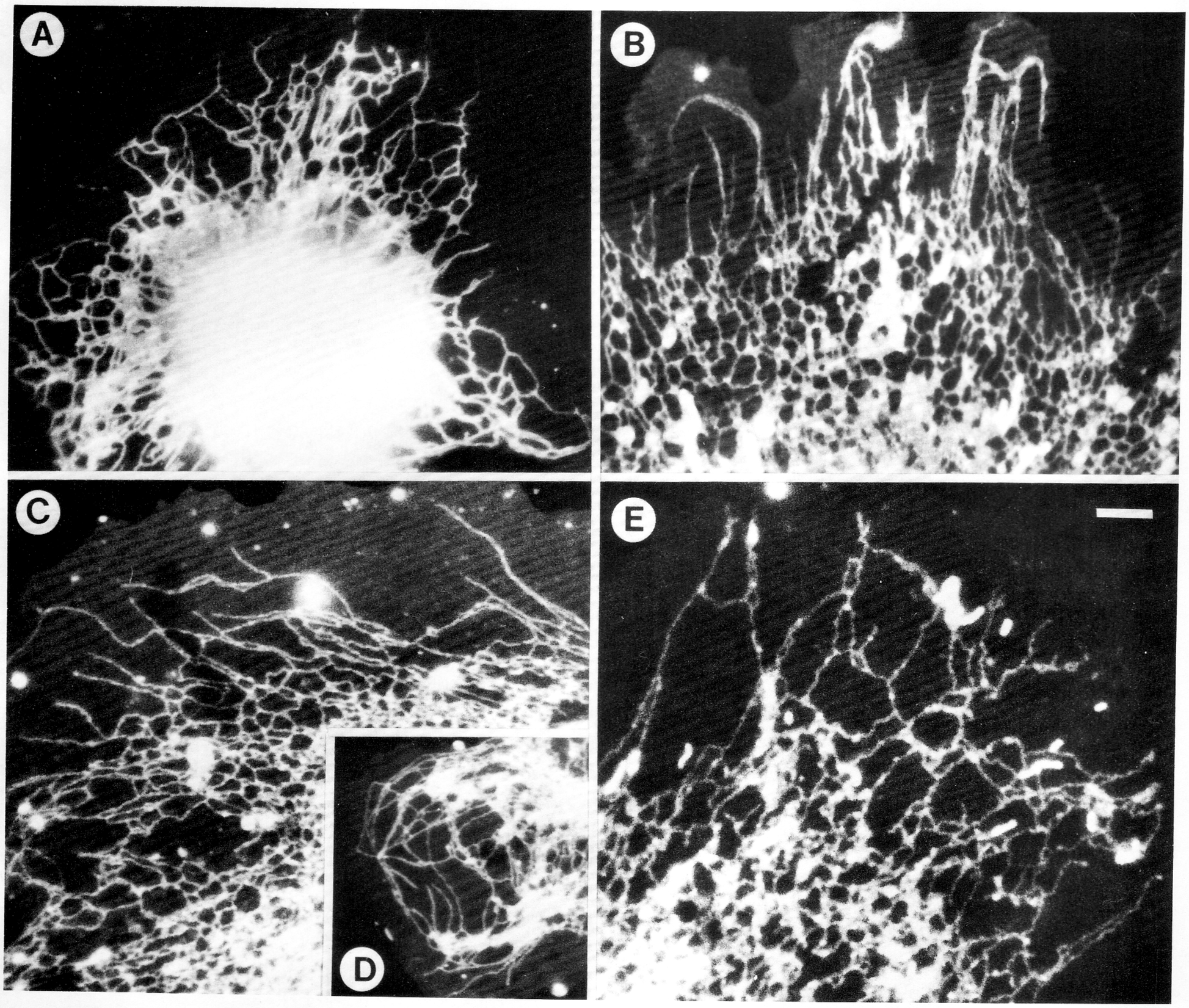

Figure 4. Fluorescent Micrograph of Cells Stained with the Cyanine Dye $DiOC_6(3)$

(A) A living chick embryo cell. (B) A glutaraldehyde pre-fixed CV-1 cell 6 hr after seeding in 0.5% calf serum. (C) A living rat embryo fibroblast. (D) A living chick embryo fibroblast. (E) A glutaraldehyde-pre-fixed smooth muscle cell. Bar represents 5 μm in (A) to (D) and 4 μm in (E).

For staining in glutaraldehyde-fixed cells, it seems less likely that a membrane potential is responsible for the observed staining although it cannot be completely ruled out. A plausible explanation is that the lipophilic dyes are partitioning into the intracellular membranes. Since all the ER staining dyes have a positive charge and lipophilic cationic dyes differ widely in their ability to stain the ER, it seems probable that a mechanism involving a combination of electric charge and lipid partitioning is at work in the staining of fixed cells.

The use of these dyes provides a new technique for investigating the relationships between the cytoskeleton and the ER. Figure 3D shows abundant microtubules in the vicinity of the ER. Colchicine and rotenone, which prevent the repolymerization of microtubules, greatly alter the distribution of the ER (Figure 5). Surprisingly, disrupting microfilaments by cytochalasin B in CV-1 cells does not grossly alter the observed structure of the ER. Microtubules may play a role in organizing the cytoplasm and in determining the location of the ER, similar to the role proposed for microtubules in the distribution of the mitochondria (Smith et al., 1975, Heggeness et al., 1978, Wang and Goldman, 1978, Summerhayes et al., 1983). Whether intermediate filaments also play a role in the organization of the ER remains to be investigated. The results reported here reinforce the notion that the cytoskeleton is crucially involved with subcellular compartmentation, including organizing the distribution of the ER. The information on the spatial arrangements of the ER provided by this fluorescent technique should expand studies of the ER, and perhaps facilitate research on the nature of the relationship between cellular organelles and the cytoskeleton.

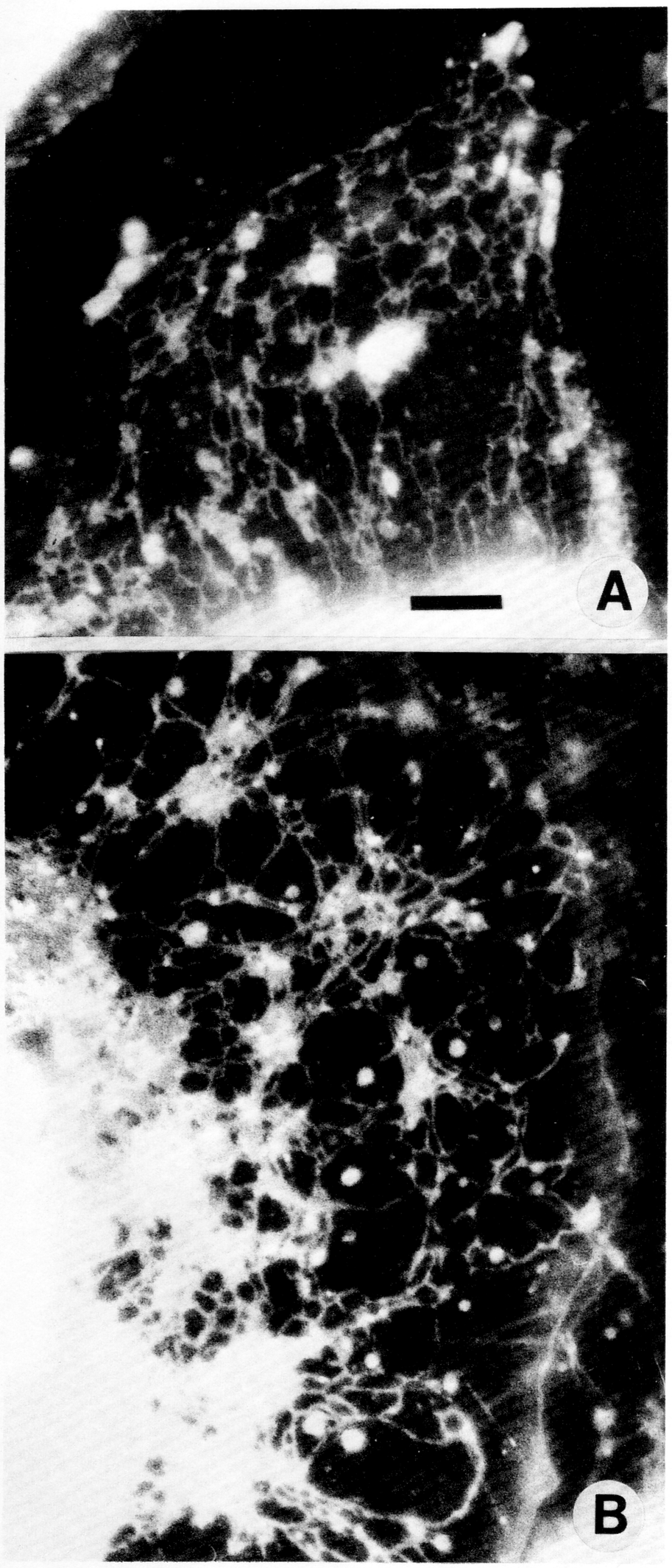

Figure 5. Fluorescent Micrographs of Colchicine-Treated (A) and Rotenone-Treated (B) CV-1 cells Pre-fixed in Glutaraldehyde and Stained with the Cyanine Dye $DiOC_6(3)$

Bar represents 6 μm in (A) and 5 μm in (B).

Experimental Procedures

Cell Culture

All cell types and cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 5% calf serum (MA Bioproducts, Walkersville, MD) at 37°C with 100% humidity and 5% CO_2. Chick embryo fibroblasts were prepared according the Rein and Rubin (1968). CV-1 (African green monkey kidney epithelial) was obtained from the American Type Culture Collection. Bovine smooth muscle cells were from Dr. B. Zetter (Harvard Medical School). Rat embryo fibroblasts were from Dr. J. G. Foulkes (Whitehead Institute).

Staining of Living Cells with Fluorescent Dyes

All cyanine and rhodamine dyes listed in Table 1 were from Eastman Organic Chemicals (Rochester, NY) and were dissolved in ethanol at 0.5 mg/ml as a stock solution.

Cells grown on 12 mm square glass coverslips (Bradford Scientific, Epping, NH) were stained in culture medium containing 0.5 μg/ml of fluorescent dye for 10 min and then mounted in a living cell chamber made of 0.7 mm thick silicon rubber (N. A. Reiss, Belle Mead, NJ) containing dye-free medium as described by Johnson et al. (1980). For short-term observations, stained cells on coverslips may be mounted on a microscope slide with a drop of dye-free medium and sealed with nail polish.

Staining of Fixed Cells

Cells grown on coverslips were fixed in 0.25% glutaraldehyde in a buffer containing 0.1 M sucrose and 0.1 M sodium cacodylate (pH 7.4) (cacodylate buffer) for 2–3 min at room temperature. Cells were stained with 2.5 μg/ml of fluorescent dye in cacodylate buffer for 30 sec at room temperature. The coverslips, after brief rinsing, were mounted in a silicon rubber chamber containing cacodylate buffer.

Fluorescent Microscopy

A Zeiss Photomicroscope III equipped with epifluorescence optics was used for visualization. Although most of the objective lenses are appropriate for detection of mitochondria, it is essential to use a 100X objective lens to see the ER. We used a Zeiss Plan 100X (NA 1.2) objective lens for this work. Commonly used filter systems for rhodamine or fluorescein are appropriate for dyes listed in Table 1. For $DiOC_6(3)$, the excitation barrier filters are those used for visualizing fluorescein. Photographs were made using Kodak Tri-X film or Ilford XP1-400 film exposed at E.I. 1600. Kodak Tri-X film was developed in Kodak HC-110 dilution B for 15 min and Ilford XP-2 in Ilford developer at 37°C for 9 min. For printing, the best results were obtained by Ilford Multigrade-2 paper (Ilford filter #5) developed in fresh Kodak Dektol.

Detection of the ER by Phase-Contrast Microscopy

Cells grown on coverslips were fixed in potassium permanganate (0.5% [pH 7.0]) for 30 min at room temperature. Microscopy was carried out with a Zeiss phase-contrast objective lens, Plan 100X. Photographs were made with Kodak 2415 film exposed at E.I. 200 and developed in Kodak Technidol.

Electron Microscopy

For whole-mount electron microscopy, cells were grown in Formvar-coated gold grinds, fixed in potassium permanganate (0.5%, [pH 7.0]) for 15 min, washed in water, dehydrated in 25%, 50%, 75%, and 100% ethanol and examined by a Zeiss Electron Microscope OM-10. For thin sections, cells were grown in culture dishes, fixed in 2.5% glutaraldehyde in phosphate buffered saline, embedded in Epon, and sectioned by a LKB microtome. Thin sections were prepared and examined by Elizabeth Beaumont in Dr. Henry Slater's laboratory at this Institute with a Phillips EM 300 electron microscope.

Various Reagents

Cytochalasin B, monensin, A23187, rotenone, and hydrocortisone were obtained from Sigma Chemical Company. 12-O-tetradecanoylphorbol 13-acetate (TPA) was obtained from Dr. Paul Fisher at Columbia University. Taxol was obtained from Dr. Frank Solomon at the Massachusetts Institute of Technology.

Acknowledgments

This work was supported by grants from the Council for Tobacco Research and the National Cancer Institute. M. T. was supported by an NCI postdoctoral training grant to Dr. A. B. Pardee. J. D. S., supported by the China Medical Board, is a Visiting Professor from China Medical College, Shenyang. M. J. W. was supported by a fellowship from the Muscular Dystrophy

Association. L. B. C. is a recipient of an American Cancer Society Faculty Research Award.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 7, 1984; revised May 4, 1984

References

Bensley, R. R. (1911). Studies on the pancreas of the guinea pig. Am. J. Anat. *12,* 297–389.

Fawcett, D. W. (1981). The Cell, Second Edition (Philadelphia, Pennsylvania: W. B. Saunders Co.), pp. 303–368.

Garnier, C. (1899). Contribution a l'etude de la structure et du functionnement des cellule glandulaires sereuses. Du role de l'ergastroplasme dans la secretion. Thesis, Nancy, No. 50. In The Cell, Second Edition (Philadelphia, Pennsylvania: W. B. Saunders Co.), p. 366.

Hayat, M. A. (1981). Fixation for Electron Microscopy (New York: Academic Press), pp. 1–501.

Heggeness, M. H., Simon, M., and Singer, S. J. (1978). Association of mitochondria with microtubules in cultured cells. Proc. Nat. Acad. Sci. *75,* 3863–3866.

Johnson, L. V., Walsh, M. L., and Chen, L. B. (1980). Localization of mitochondria in living cells with rhodamine 123. Proc. Nat. Acad. Sci. *77,* 990–994.

Johnson, L. V., Walsh, M. L., Bockus, B. J., and Chen, L. B. (1981). Monitoring of relative mitochondrial membrane potential in living cells by fluorescent microscopy. J. Cell Biol. *88,* 526–535.

Lin, J. J. C., and Queally, S. A. (1982). A monoclonal antibody that recognizes Golgi-associated protein of cultured fibroblast cells. J. Cell Biol. *92,* 108–112.

Mathews, A. (1899). The changes in structure of the pancreas cell. A consideration of some aspects of cell metabolism. J. Morphol. (Suppl.) *15,* 171–222.

Pagano, R. E., Longmuir, K. J., Martin, O. C., and Struck, D. K. (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., and Porter, K. R. (1954). Studies on the endoplasmic reticulum. I. Its identification in cells in situ. J. Exp. Med. *100,* 641–656.

Porter, K. R. (1953). Observations on a submicroscopic basophilic component of the cytoplasm. J. Exp. Med. *97,* 727–750.

Porter, K. R., and Thompson, H. P. (1947). Some morphological features of cultured rat sarcoma cells as revealed by the electron microscope. Cancer Res., *7,* 431–438.

Porter, K. R., Claude, A., and Fullam, E. (1945). A study of tissue culture cells by electron microscopy. Methods and preliminary observations. J. Exp. Med. *81,* 233–241.

Rein, A., and Rubin, H. (1968). Effects of local cell concentrations upon the growth of chick embryo cells in tissue culture. Exp. Cell Res. *49,* 666–678.

Smith, D. S., Jarlfors, U., and Cameron, B. F. (1975). Morphological evidence for the participation of microtubules in axonal transport. Ann. NY Acad. Sci. *253,* 472–506.

Summerhayes, I. C., Wong, D., and Chen, L. B. (1983). Effect of microtubules and intermediate filaments on mitochondrial distribution. J. Cell Sci. *61,* 87–105.

Wang, E., and Goldman, R. D. (1978). Functions of cytoplasmic fibers in intracellular movements in BHK-21 cells. J. Cell Biol. *79,* 708–726.

Note Added in Proof

Fluorescent (Figure 3A) and phase-contrast (Figure 3B) micrographs of the same cell have recently been prepared. The reticular structures are exactly superimposable.