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# Preservation of Normal Behavior by Enucleated Cells in Culture

**(tissue culture/cytochalasin B/contact inhibition/cell locomotion)**

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**ABSTRACT** **BHK-21 fibroblasts and BSC-1 epithelial cells were enucleated with cytochalasin B. The enucleated cells were trypsinized and replated; their subsequent behavior was monitored by light optical techniques. The results demonstrate that the information necessary for attachment, spreading, shape formation, pinocytosis, contact inhibition, and cell locomotion is present in enucleated cytoplasm.**

The influence of the nucleus on the cytoplasm of eukaryotic cells has been studied primarily by surgical separation of the nucleus from the cytoplasm (1–7). There are many problems inherent in these techniques, including the possibility of irreparable mechanical damage to the cell during surgery and the limited number of enucleated cells that can be observed. Based on the original findings of Carter (8), Prescott *et al.* (9) have described a method using the drug cytochalasin B that allows mass enucleation of populations of cultured animal cells and produces little, if any, obvious cytoplasmic damage. We have modified this procedure for two cell lines, epithelial BSC-1 cells (from African Green Monkey kidney) and BHK-21 fibroblasts (from baby hamster kidney). We have also monitored the behavior of cells after enucleation, trypsinization, and replating. The results in this report demonstrate that the information necessary for normal cell-shape formation, cell locomotion, and contact inhibition is present in enucleated cells and is preserved through trypsinization and replating. Preliminary results of this work have been reported elsewhere (10, 11).

## MATERIALS AND METHODS

BHK-21 cells were grown to semiconfluence (1 to 4 $\times$ $10^4$ cells per $cm^2$) on 12-mm diameter coverslips coated with collagen in BHK-21 medium (Grand Island Biological Co.) supplemented with 10% tryptose phosphate broth, 10% calf serum, 100 units/ml of penicillin and 100 units/ml of streptomycin. The collagen was necessary to prevent all the cells from falling off the coverslip during centrifugation. BSC-1 cells were grown to monolayers (5 $\times$ $10^4$ cells per $cm^2$) on coverslips either in the same medium or in Dulbecco's modified Eagle's medium with 10% calf serum. It was not necessary to coat the BSC-1 coverslips with collagen.

In order to enucleate the cells, the coverslips were inverted (cell side down) and placed into the bottom of 15-ml Corex centrifuge tubes containing 2–5 ml of medium with 10 $\mu$g of cytochalasin B per ml. The centrifuge tubes with the coverslips were placed immediately into a Sorvall RC-2 centrifuge that had been warmed to 37° by spinning the (SS 34) rotor with the head in place for about 1 hr at 10,000 rpm (with the temperature regulator set at 37–39°). The length of time and speed of centrifugation are crucial factors for successful enucleation. BSC-1 cells were spun at 9000 rpm for 1 hr at 37 $\pm$ 2° and BHK-21 cells were spun at 6500 rpm for 50 min at 37 $\pm$ 2°.

After centrifugation, the coverslips were removed from the centrifuge and placed cell side up into 35-mm (Falcon) tissue culture dishes (Biolquest) containing 3 ml of medium without cytochalasin B. Within 30–60 min at 37°, the cells were morphologically normal and 90–99% lacked nuclei.

Enucleated cells were removed from the coverslips by treatment with trypsin–EDTA (Grand Island Biological Co.) and the cells were suspended in normal medium. The enucleated cells were then replated in small drops on 22-$mm^2$ coverslips kept in 35-mm tissue culture dishes and placed in an incubator. At time intervals after replating, the coverslips were mounted on slides (12) and observations on the enucleates were made with Zeiss phase contrast, polarized light, and Nomarski optics. Replated enucleates retain their normal morphology and behavior for at least 12–18 hr.

## RESULTS

### Spreading and shape formation

Enucleated BHK-21 cells that have been trypsinized and placed in a suspension in normal medium are spherical in shape (Fig. 1*a*). The enucleated cells attach and begin to show active membrane ruffling and pinocytosis (Fig. 1*b* and *d*) within 15–30 min after replating onto glass coverslips. After 1 hr, most of the enucleates begin to look fibroblastic and many of them contain a central spherical region that excludes most cytoplasmic granules and organelles (Fig. 1*c*). This region is birefringent when observed with polarized light optics and, therefore, is identical to the birefringent spheres seen during the early stages of spreading in nucleated BHK-21 cells (13). Within 1–2 hr after replating, most of the spheres have disappeared or become smaller; this finding is coincident with the formation of typical fibroblastic cell processes (Figs. 1*d* and *e*). Fully-spread enucleated BHK-21 cells are indistinguishable from nucleated cells, except of course for the lack of a nucleus (Fig. 1*e* and *f*).

Fig. 2*a–e* demonstrate that attachment, spreading, shape formation, membrane ruffling, and pinocytosis are also normal in BSC-1 enucleates. Fig. 2*d* and *e* demonstrate that the overall shapes of fully-spread nucleated and enucleated BSC-1 cells are essentially identical.

### Localized contact inhibition of membrane ruffling

In order to observe the response of cells to mutual contact, fields of replated enucleated cells with few intercellular contacts were chosen and observed for periods of up to 2 hr. During this time interval contacts were frequently made and broken. Fig. 3*a–c* are a series of micrographs of enucleated BSC-1 cells that demonstrate that when intercellular con-

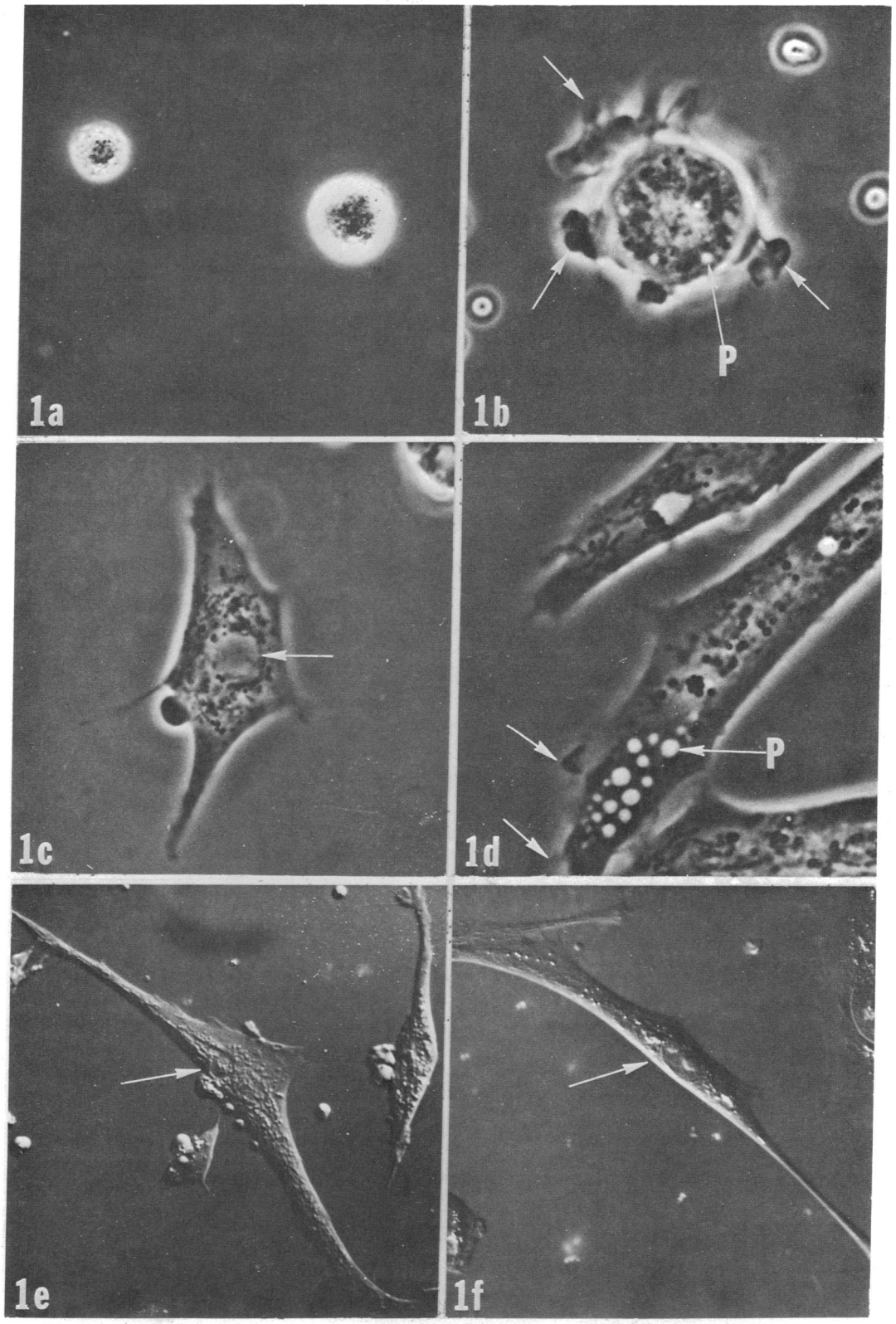


FIG. 1. Series of micrographs of spreading BHK-21 enucleates. (*a*) Enucleates observed just after trypsinization and before replating. Phase contrast, X640. (*b*) 30 min after replating, many ruffling regions are seen (*arrows*) and pinocytotic vacuoles are obvious (*P*). Phase contrast, X1800. (*c*) Enucleate with central spherical region (*arrow*) that excludes most cytoplasmic granules. Phase contrast, X1400. (*d*) The edge of an enucleate fibroblastic process (1 hr after plating). Note ruffled membrane region (*arrows*) and pinocytotic vacuoles (*P*). (*e* and *f*) Fully spread enucleated (*e*) and nucleated (*f*) cells. *Arrow* points to nucleus in *f* and nuclear region in the enucleated cell (*e*). Nomarski, X560.

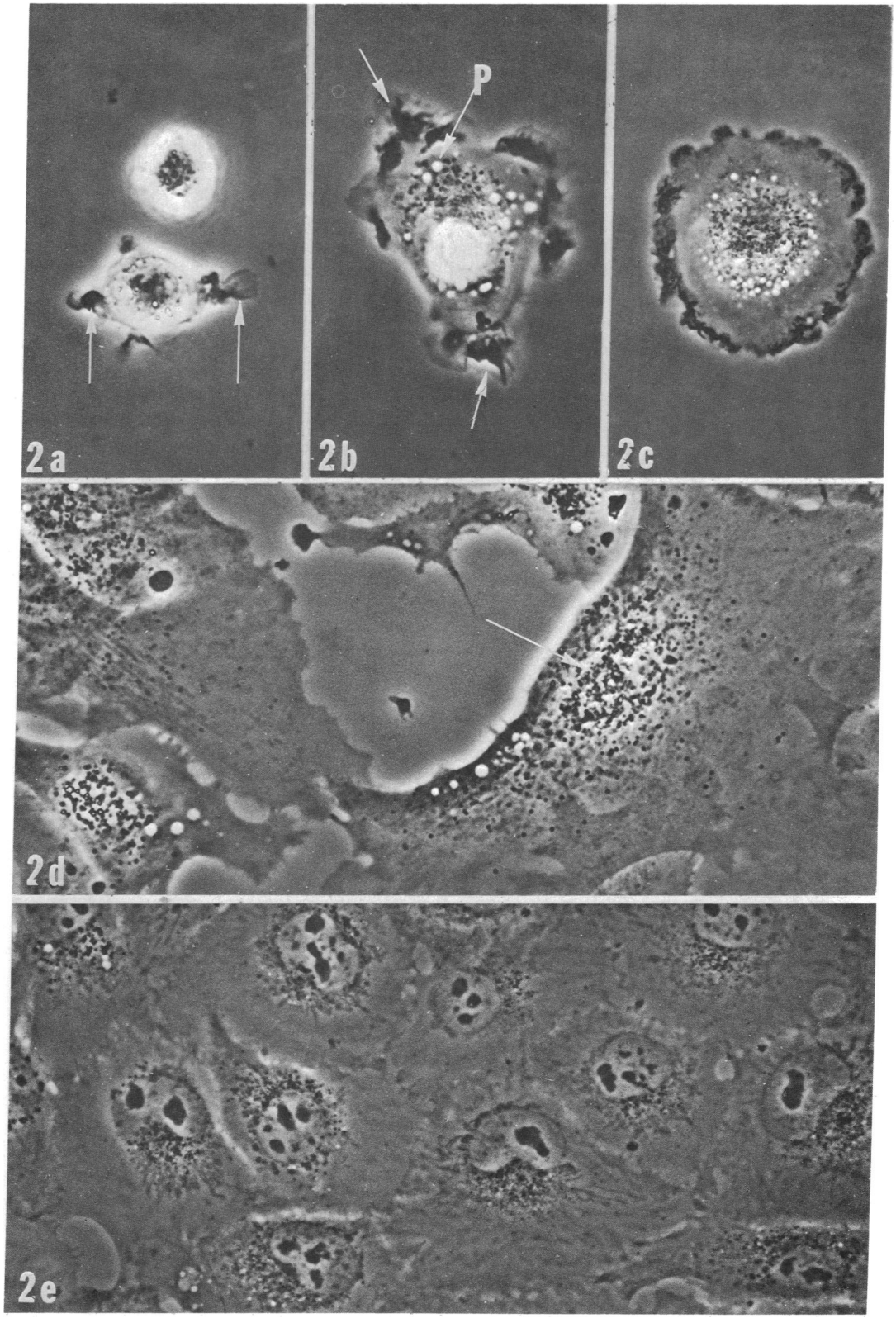


FIG. 2. Series of micrographs of spreading BSC-1 enucleates. (*a*) Enucleates at 30 min after replating. One of the cells is still spherical and the other is just beginning to spread. Note ruffling regions (*arrows*). Phase contrast, X880. (*b*) 45 min after replating, the enucleates continue to spread and ruffling regions (*arrows*) with pinocytotic vacuoles (*P*) are seen. Phase contrast, X880. (*c*) 75 min after replating, the cells begin to look epithelial and there is continued membrane ruffling and pinocytosis at the edge. Phase contrast, X640. (*d* and *e*). Fully spread enucleated (*d*) and nucleated (*e*) BSC-1 cells. *Arrow* points to the region in an enucleate where the nucleus normally resides. Phase contrast, (*d*) X840; (*e*) X550.

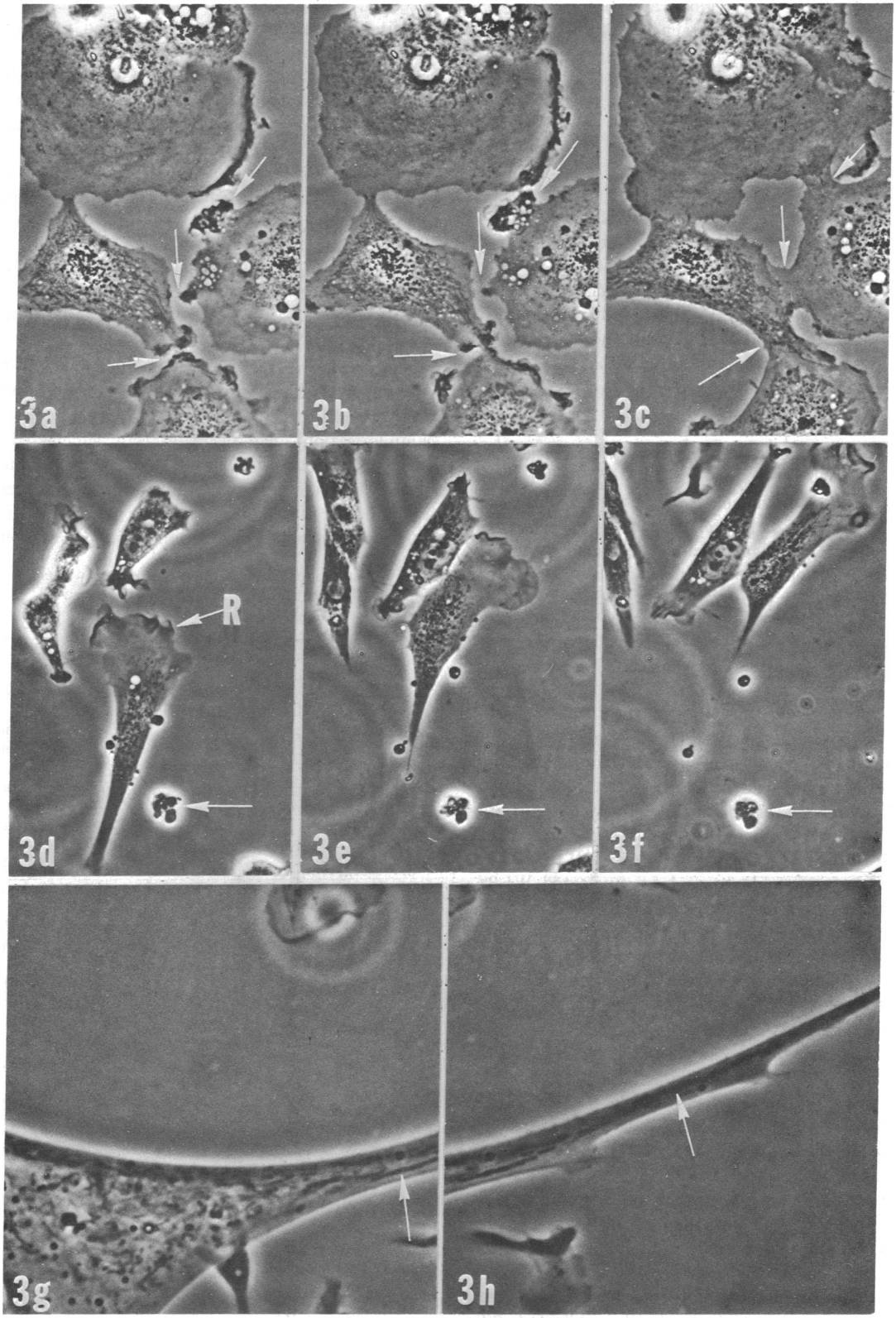


FIG. 3. (*a–c*) Series of micrographs taken of BSC-1 enucleates during a 30-min period of observation, beginning 3 hr after replating. *Arrows* point to ruffling regions before (*a*) and after establishment of contacts (*b* and *c*). Phase contrast, ×520. (*d–f*) Series of micrographs taken of a locomoting BHK-21 enucleate over a period of 1.5 hr, beginning 3 hr after replating. Note fan-shaped pseudopodium and the ruffling front edge of the cell (*R* in *d*). A piece of debris in the microscope field (*arrows*) acts as a reference point. Phase contrast, ×560. (*g–h*) Two micrographs of the same BHK-21 enucleate demonstrating that normal organelle distribution is seen. Note the filamentous mitochondria in the cell process (*arrows*). Phase contrast, ×1600.

tacts are established, obvious membrane ruffling and pinocytosis adjacent to the ruffling regions are inhibited, the typical response expected if cells contact-inhibit each other (14, 15).

**Locomotion of enucleates**

Enucleated BHK-21 cells were monitored for locomotory behavior. Enucleated cells were found with the general configuration of a locomoting fibroblast. They possessed a typical fan-shaped pseudopodium [as described by Ambrose (16)] and translocated with the ruffling margin of the pseudopod as the leading edge of the cell. Fig. 3*d–f* are a series of micrographs of a single BHK-21 enucleate moving across the microscope field. This series also demonstrates that enucleates show normal contact inhibition of cell locomotion, due to the fact that the nucleated cell and the enucleated cell (see in Fig. 3*d–f*) successfully avoid each other and move off in oblique directions after making contact.

**The distribution of organelles in enucleates**

As normal nucleated cells spread over a substrate after trypsinization, organelles such as mitochondria are redistributed from the center of the cell towards the periphery of the spreading regions. In addition, these organelles show saltatory movements (17). The enucleated spread cells contain identical distributions of organelles, which also show saltatory movements. This distribution is especially evident in the long cell processes of replated BHK-21 enucleates, where filamentous mitochondria are seen along the long axis of cell processes (Fig. 3*g* and *h*).

## DISCUSSION

It is obvious from this study, as well as the studies of others (9, 18, 19), that enucleation with cytochalasin B does not result in irreversible damage to cultured cells. The viability of enucleated cytoplasm is further demonstrated by its ability to survive trypsinization and replating. This latter finding is in disagreement with the findings of Wright and Hayflick (18), who were able to replate enucleated WI-38 cells only by their removal from the substratum with EDTA, and not with trypsin. Based on their finding, these authors concluded that enucleated cytoplasm is incapable of resynthesizing the proteins necessary for respreading of WI-38 cells without the direction of the nucleus. Our results suggest that either the information for resynthesis of the surface proteins implicated in normal attachment and spreading of cells is available in the cytoplasm, or that these materials are somehow preserved by the enucleate during trypsinization. Since many enucleated cells attach and spread in the presence of cycloheximide, which inhibits over 95% of the protein synthesis in nucleated cells (ref. 20, and unpublished observations), it is likely that surface proteins that are removed or destroyed by trypsinization are also stored in the cytoplasm, and that these proteins can be reinserted into the cell surface during spreading.

Our observations demonstrate that replated epithelial and fibroblast cytoplasm behave identically to nucleated cells (10, 13, 20) with regard to normal attachment, spreading, shape formation, pinocytosis, contact inhibition, and cell locomotion. Goldstein *et al.* (3) also demonstrated that cell locomotion, membrane ruffling, and pinocytosis occurred in enucleate fragments prepared by microdissection of HeLa cells. These combined results suggest that the information necessary for many aspects of cultured cell behavior is present in the cytoplasm, probably in the form of preformed subunits of essential molecules involved in these processes and in the form of stable messenger RNA molecules. In support of the former idea, it has been demonstrated that the assembly of preformed subunits of microtubules and microfilaments appears to be directly involved in nucleated cell spreading and shape formation (20). Therefore, it is likely that identical fiber assembly processes take place in spreading enucleated cells. Preliminary electron microscope observations have confirmed that such is the case (unpublished results). Furthermore, the observation that birefringent spheres arise in enucleates in the same position as in nucleated BHK-21 cells, and that they become smaller as enucleated cells spread, indicates that 100-Å filaments are also distributed normally in enucleates (13–20).

The findings of this and other studies demonstrate that a wide range of physiological activities, including virus replication (19) and protein synthesis (Dr. Peter Jeppesen, personal communication), can occur in cells enucleated with cytochalasin B. The viability of enucleates produced by this method is, therefore, well established and the means are now available for attempting a broad spectrum of studies on nuclear–cytoplasmic interactions in eukaryotic cells.

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