# THE ASSOCIATION OF A CLASS OF SALTATORY MOVEMENTS WITH MICROTUBULES IN CULTURED CELLS

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ABSTRACT

Particulate structures in the cytoplasm of HeLa and other cultured cells in interphase undergo rapid individual linear displacements (long saltatory movements, LSM). By the use of time-lapse microscopy to locate saltating particles prior to fixation and histochemical examination of the cells, structures of several kinds have been shown to move in this manner. Elements that show LSM include lysosomes, pinosomes, ingested carbon particles, lipoidal granules, and unidentified particles that appear as bright objects in positive phase contrast. The pattern of movement of the particles suggests the presence of linear guiding elements radially disposed from the cytocenter (centriole region). The participation of microtubules in these movements is inferred from the observation that LSM cease after treatment with drugs which depolymerize microtubules, i.e., colchicine, Vinblastine, and podophyllin. The directions of the microtubules in the cytoplasm of HeLa cells found by electron microscopy are consistent with the aster-like configuration predicted from study of LSM. Further support for this arrangement of cytoplasmic microtubules is provided by light microscope observations of colchicine-sensitive radial arrays of acid phosphatase granules in the cytoplasm of some cell lines.

## INTRODUCTION

Microtubules are presently associated with two functions. One is the establishment of asymmetry in the shape of the cell, as in rigid or elongated cell processes which contain groups of microtubules parallel to the long axis of the cell or cytoplasmic extension (1–3). In certain disk-shaped cells, masses of microtubules constitute marginal bands which appear to participate in producing the cell shape (4, 5). A second aspect of microtubule function is an association with the movement of intracellular formed elements parallel to the axis of microtubules. Such microtubule–associated movements have been observed in pigment cells (6), in virus-induced syncytia of cultured cells (7) and along the axopods of *Actinosphaerium* (3). The movement of chromosomes on the mitotic spindle can be interpreted as a special case of this relationship (8).

In this paper, we shall present evidence that in cultured animal cells a class of intracytoplasmic movements involving several kinds of particles is associated with microtubules. The displacements studied (LSM) have the non-Brownian, independent, and linear character of saltatory movements (9–11). As we have noted in preliminary reports (12, 13), movements of this class cease in cells treated with spindle agents. This form of particle movement is not random, but its direction depends on location within the cytoplasm; this information has been used to propose a hypothetical arrangement of the guiding elements. The model which we suggest is an aster-like radial array with its

origin at the cytocenter. Cytological observations are presented, from light and electron microscopy, which indicate that cytoplasmic microtubules form a radial array consistent with their presumed role in guiding the movements.

## MATERIALS AND METHODS

### *Cell Cultures*

Stock cultures of HeLa (wild strain), L-929 mouse fibroblasts (ex CCL-1, Cell Culture Repository), and Chinese hamster cells (pseudodiploid line C14 FAF28, Yerganian) were maintained as monolayer cultures in plastic flasks (Falcon Plastics Co., Division of Bioquest, Los Angeles, Calif.); in Eagle's minimum essential medium (14), supplemented with pyruvate and nonessential amino acids. Fetal bovine serum was employed at 20% for HeLa cultures and 10% for the other lines.

Cells were seeded in modified Sykes-Moore perfusion chambers (15), and incubated for 24 or 48 hr so as to permit cell spreading prior to treatment with drugs or to time-lapse filming. For cytochemistry or electron microscopy, aliquots of the same cells were seeded in plastic Petri dishes (Falcon Plastics Co.) or on glass coverslips placed in the dishes.

### *Cinemicrography and Analysis of Movement*

Well-spread cells were recorded on 16-mm film at 16 or 30 frames per min, using positive phase contrast and magnifications from 140 to 250. The exposure time per frame was less than 1 sec to minimize blurring of rapidly moving particles. The film records were analyzed with a stop-motion projector and quadrille-ruled screen or with a Vanguard film analyzer. A long saltatory movement (LSM) was considered to have occurred if the displacement of any particle in a single continuous movement was greater than 4 $\mu$. True Brownian movements of the particles considered in this work would probably account for displacements of less than 1 $\mu$ in any 10-sec interval (9). However, only displacements greater than 4 $\mu$ were scored in order to identify a class of clearly independent and linear movements which could readily be distinguished as discrete events. The frequency of LSM was estimated from the ratio (S) relating the total number of LSM scored to the sum of the total times of observation for all particles examined (LSM per particle-minute). Individual particles were followed throughout that portion of the film record in which they could be observed; the number of LSM was recorded, as was the total time of observation. This procedure was repeated for from 24 to 40 particles in the same cell after mapping the initial location of each particle so as to avoid confusion.

Although this procedure permits comparisons of the effects of drugs on a class of saltatory movements, it underestimates the absolute frequency of non-Brownian movements, since the most rapid displacements, those occurring in less than one frame interval, are not recorded. In addition, particles in the cytocenter may be so numerous as to preclude separate analysis of their movement. Finally, small displacements, as noted above, are excluded.

For demonstrating changes in the frequency of LSM, the experiments were carried out so that each cell served as its own control. After filming for 2 hr in stock growth medium, the chamber was perfused with fresh medium containing the agent to be tested. The same cells were then filmed for an additional 2 hr. The values of S in both film sequences were determined, and the inhibition was calculated as per cent of the control value.

As a control for any effect of the procedure in the absence of the test agent, the chamber was perfused with stock growth medium at the end of the initial 2 hr period; the value of S did not decline after prolonged exposure of the cell to the intermittent illumination of the time-lapse microscope.

When independent observers analyzed a given film, similar degrees of inhibition were estimated. The absolute value of S for a particular film record depended upon the observer, apparently because of disagreement as to whether or not a particular displacement was a continuous movement.

In some experiments, the cells were allowed to phagocytize carbon particles prior to the experimental treatment. Activated charcoal was dispersed by sonication in glass-distilled water, and a suspension of 1–10 $\mu$ particles was isolated by decantation, diluted, and sterilized by autoclaving. For use, the suspension was added to an equal volume of double-strength growth medium. Flask cultures of HeLa cells were exposed to the carbon particles for 2 hr, and then washed twice with balanced salt solution (BSS). The cells were detached with 0.5% trypsin in BSS, and twice centrifuged and resuspended in BSS. The cells, now nearly free of unincorporated carbon, were centrifuged again and resuspended in growth medium. Perfusion chambers were inoculated with 10,000 cells and incubated overnight. A minority of the cells had incorporated several carbon particles, and were selected for filming with phase-contrast or bright-field time lapse.

### *Measurement of Metaphase Accumulation*

Petri dish cultures (6 cm) containing 50,000 HeLa cells in 4.5 ml medium were incubated for 24 hr prior to use. At zero time the test agent was added in 0.5 ml BSS which contained ten times the final concentration desired. Two dishes were assayed at each concentration, as 5- or 10-fold serial dilutions. Control

dishes received 0.5 ml BSS. At 2 hr intervals, a low-power inverted phase-contrast microscope was used to count, in five randomly chosen fields, the total number of cells and the number in metaphase. In control cultures the frequency of metaphases remained near 5%. In cultures treated with concentrations of spindle agent above the threshold dose, the frequency of c-metaphases rose gradually to 30–50% over the course of 10 hr. Subthreshold concentrations produced no increase in metaphase frequency or a rise of less than 30% (incomplete blocking of spindle formation).

### *Cytochemical Procedures*

For identifying cytoplasmic particles of known pattern of movement in stained preparations, cells which had been filmed were fixed as follows. The location of suitable cells was marked by engraving a 0.5 mm circle on the coverslip with a Leitz object marker. This area was filmed for 2 hr, and the chamber was then perfused during filming with BSS followed by cold isotonic formol-sucrose (16). Thus, a given particle could be identified in the living cell and located after cytochemical reaction.

Acid phosphatase was localized by the Gomori procedure (17). After overnight storage in cold formol-sucrose, the coverslips were incubated for 2 hr in β-glycerophosphate lead nitrate substrate solution at pH 5.0. After treatment with ammonium sulfide, the preparations were counterstained in aqueous fast green, dehydrated, mounted in Permount, and photomicrographed on Kodachrome without delay. The staining observed was cytoplasmic and confined to discrete granules; no precipitate was deposited when enzyme activity was inhibited by 0.01 M NaF.

Staining of lipoidal droplets was carried out after formol-sucrose fixation. Perfusion chambers containing fixed cells were perfused with 0.06% Sudan Black B in 60% ethanol, and the staining of prominent dark granules was observed; however, the stained granules rapidly became swollen and were disrupted. Film records confirmed that ethanol concentrations greater than 20% caused dissolution of the sudanophilic droplets. When fixed chambers were perfused with aqueous 0.5% $OsO_4$, the sudanophilic droplets were stabilized and intensely stained by osmium black. Lipoidal droplets were, therefore, routinely identified by intense osmiophilia. After 30 min of exposure to 0.5% $OsO_4$, the coverslips were washed three times in water and mounted in a viscous solution of polyvinylpyrrolidone. When cells fixed in formol-sucrose were treated for 1 hr in 1:1 ethyl ether-ethanol, subsequent exposure to $OsO_4$ did not result in intensely stained cytoplasmic droplets.

### *Electron Microscopy*

Cultures for electron microscopy were grown in 6-cm plastic dishes (Falcon Plastics Co.) under 5% $CO_2$ in air. After the cells were washed with BSS, they were fixed in 5% glutaraldehyde in pH 7.0 phosphate buffer adjusted to 37°C. After addition of glutaraldehyde, the preparation was allowed to stand for 1 hr at room temperature. The cultures were washed twice with the phosphate buffer and postfixed for 1 hr in 1% $OsO_4$, made up in the same buffer. The cultures were dehydrated and embedded in Epon *in situ* (18).

Sections cut with a diamond knife were picked up on Formvar-coated grids and stained with uranyl acetate followed by lead citrate; they were examined with a Siemens Elmiskop IA (Siemens America, Inc., New York, N.Y.).

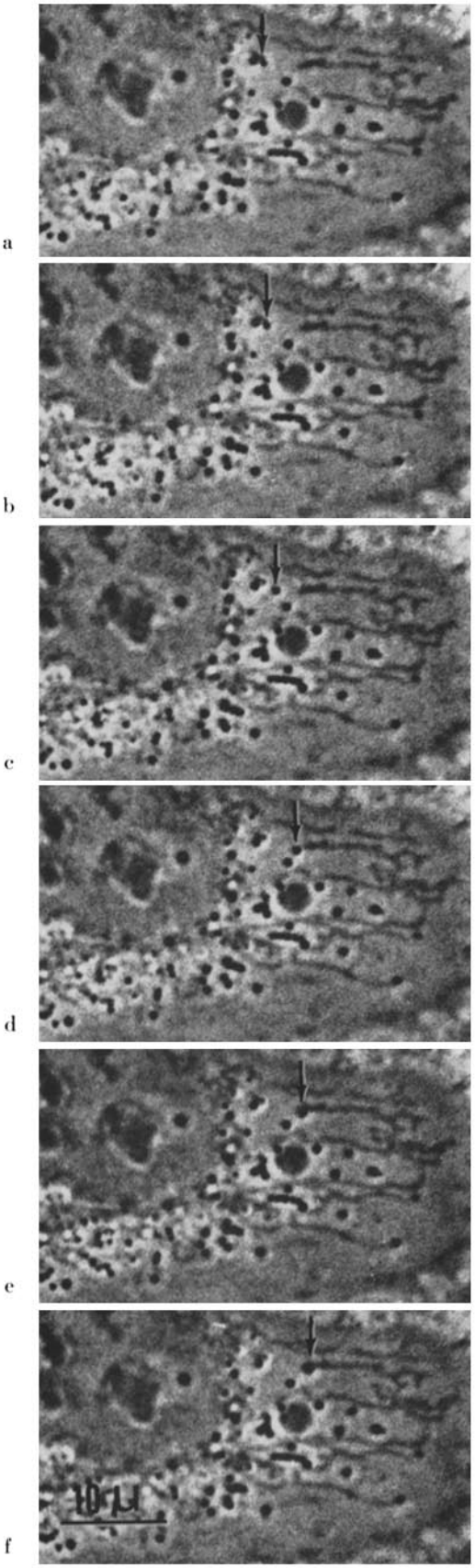
## RESULTS

### *Cytoplasmic Structures that Exhibit Saltatory Movement*

When time-lapse films of monolayer cultured cells are projected at low speed, many cytoplasmic structures are observed to make sudden jumps or saltations. An example of a displacement falling into the class of phenomena we have scored as LSM is shown in Fig. 1, which demonstrates the movement of a lipoidal granule over a distance of approximately 4 μ in less than 10 sec. Displacements of this or of greater extent appear to be independent of the flow of cytoplasmic material since adjacent particles and nearby mitochondria remain stationary during the movement. Typically, a given particle is subject to smaller displacements, probably of non-Brownian character, before and after LSM. However, a minority of particles in some cells exhibited a series of LSM closely connected in time.

We have attempted to identify the various cytoplasmic particles which show LSM, with the following results:

LIPOIDAL GRANULES: The most conspicuous cytoplasmic elements in phase-contrast images of our line of HeLa cells are nearly spherical granules which approach 1 μ in diameter and are extremely dark in positive phase contrast. They tend to aggregate near the nucleus (Fig. 1), where they undergo frequent displacements. Smaller numbers of such granules are also found in L strain and Chinese hamster cells. When cells recorded by time lapse are subsequently fixed in formol-sucrose, many granules may be recognized as those whose saltatory behavior is demonstrated in the film. When the preparation is then treated with osmium tetroxide, the conspicuous phase-dark granules are impregnated with a black deposit, indicating their lipoidal nature (Fig. 2).




LYSOSOMES: In addition to the phase-dark granules discussed above, HeLa cells contain a class of inconspicuous smaller granules of lesser phase contract. These show frequent and rapid LSM, and are thus difficult to detect in single frames from time-lapse films of living cells. They can, however, be seen when the film is projected since the granularity of the film is reduced when several frames are summed by the persistence of vision. In size and number, these small granules appear to correspond to the acid phosphatase–reactive granules which characterize HeLa cells of our line. Fig. 2 shows a series of photomicrographs of a single cell, living, after fixation and after staining for acid phosphatase. The lysosomes are smaller and more numerous than the lipoidal granules and do not correspond in position to the latter. In Chinese hamster cells, lysosomes are more conspicuous, as noted by Munro et al. (16).

PINOSOMES: In some HeLa cells, and more frequently in the other lines examined, pinocytic vesicles form in the region of membrane ruffling near the cell border and appear as circular droplets lighter than the surrounding cytoplasm in positive phase contrast. As described originally by Lewis (19) and noted by other authors (20, 21), these droplets of medium pass rapidly to the vicinity of the nucleus. This movement takes place in discrete LSM, as indicated in Fig. 3 *a*. When several pinosomes are taken in simultaneously, their movement to the center of the cell is independent and discontinuous.

CARBON PARTICLES: Time-lapse films showed that ingested particles of carbon remained capable of LSM long after endocytosis. Fig. 3 *b* shows a sequence from a bright-field film to illustrate the independent movement of a typical particle.

BRIGHT PARTICLES: Some cells contain a few small particles which appear bright in positive phase contrast; the bright appearance is observed when the objective is brought to focus. (In the case of the more common lipoidal granules, *over-*

FIGURE 1 *a–f* An example of LSM by a lipoidal granule in a HeLa cell. The dark granule shown by the arrow was displaced along a linear path in the third, fourth, and fifth frames. The independence of the movement may be appreciated from the essentially unchanged positions of other granules and of the oriented filamentous mitochondria that extend to the right. Enlarged from successive frames of a phase-contrast time-lapse film, 16 exposures per minute. × 1575.

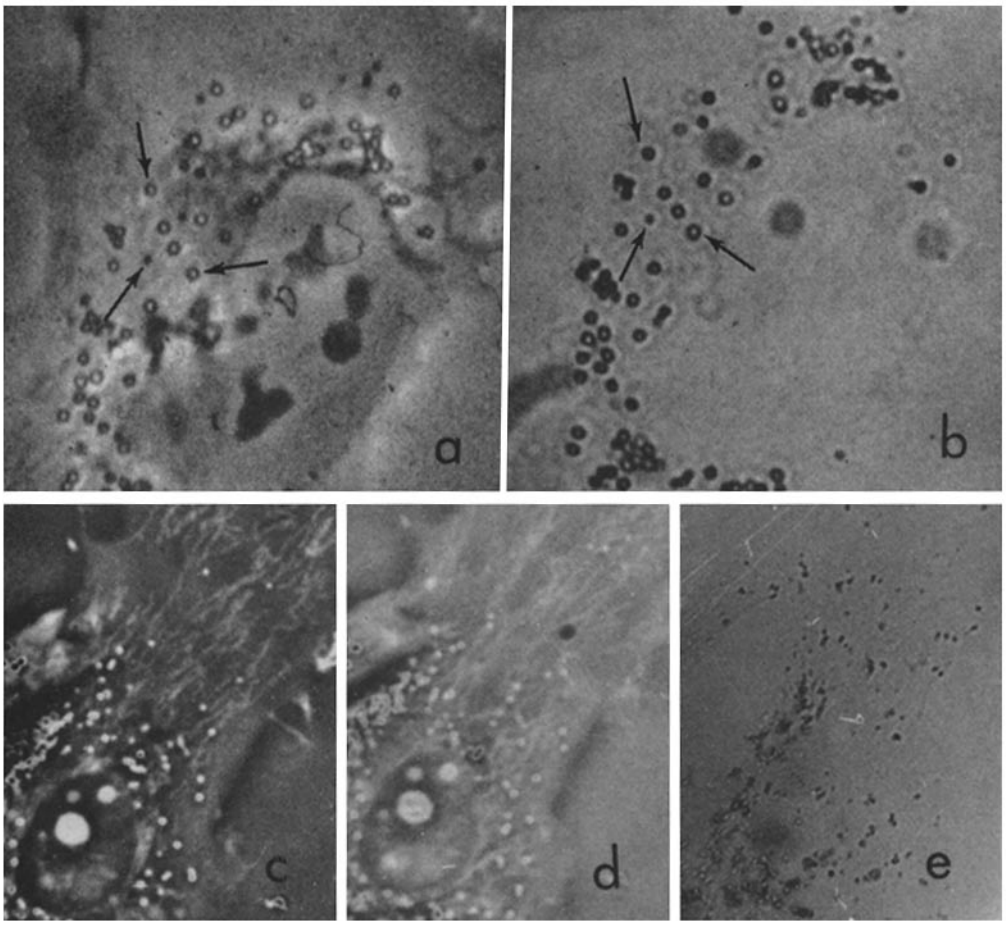



FIGURE 2 Cytochemical reactions of granules showing LSM in HeLa cells. *a*, Enlarged portion of a frame at the end of a 2-hr phase-contrast time-lapse film; approximately × 2000. The particles indicated by arrows displayed LSM in the film. *b*, The same cell after fixation and treatment with $OsO_4$; bright-field microscopy. Arrows locate the particles indicated in *a*. Other osmiophilic granules correspond in position to the phase-dark granules of the living cell. *c*, Enlarged portion of a frame from a phase-contrast time-lapse film; approximately × 1200. Printed from a reversal film original, so that the appearance corresponds to a negative-contrast phase image. *d*, The same cell, fixed with formol-sucrose 20 min after *c*; note that many dark granules have been displaced during this time. *e*, The same cell after reaction for acid phosphatase; bright-field microscopy. Note that the reactive granules are smaller and more numerous than the lipoidal granules seen in *c* and *d*, and do not correspond to the latter in position within the cell.

*focused* particles appear to be bright.) Bright particles are likely to show pronounced LSM (Fig. 3 *c*). After fixation, these elements have not been recognized, and neither osmium tetroxide nor the acid phosphatase reaction reveals particles in corresponding positions.

### *Kinetics*

The kinetics of saltatory movement for the several classes of particles were studied in plots of cumulative displacement as a function of time. Using the Vanguard motion analyzer (Vanguard Instrument Co., Roosevelt, L.I., N.Y.), successive frames were brought into register on a rear projection screen equipped with cross-hairs for measuring $X$ and $Y$ coordinates. The coordinates at the apparent particle center were read to 0.001 inch and recorded. The displacement of the particle ($D$) between two frames was calculated from

$$D = k\sqrt{(X_2 - X_1)^2 + (Y_2 - Y_1)^2} \qquad (1)$$

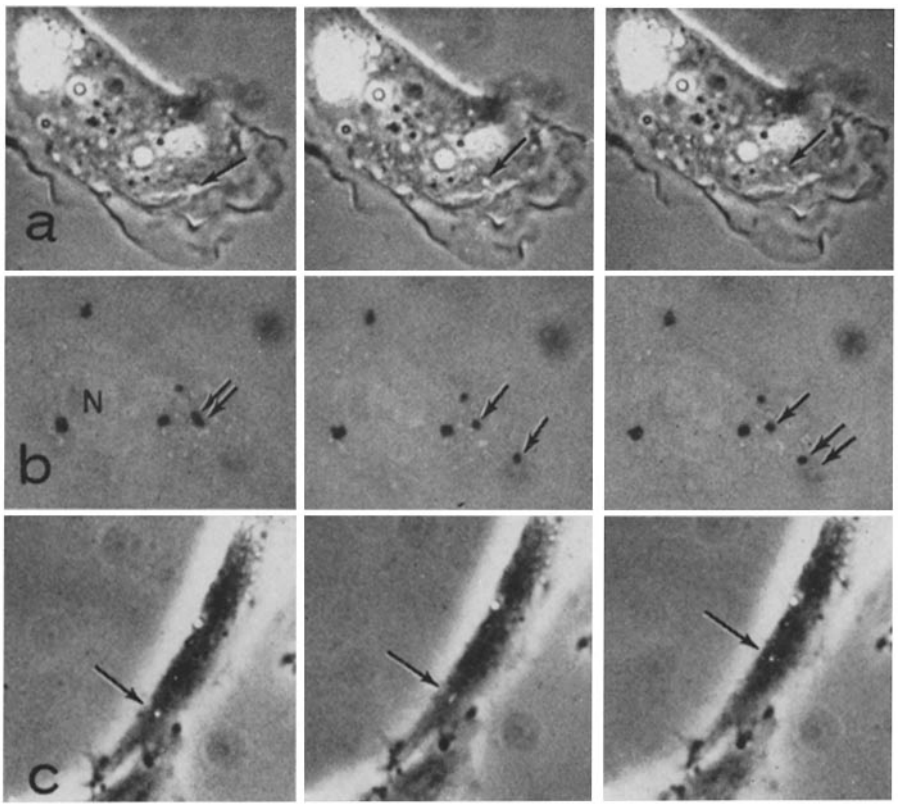



FIGURE 3 Examples of LSM by structures in the cytoplasm of cultured cells. *a*, Three successive frames from a phase-contrast time-lapse film of a Chinese hamster cell. The arrow indicates a pinosome formed in association with the ruffled membrane. In the middle photograph, the pinosome has been displaced in the direction of the nucleus; in the right hand frame, it has been further displaced and has moved closer to another pinosome which entered earlier. × 900. *b*, Three frames from a bright-field time-lapse film of a HeLa cell containing phagocytized carbon particles. The nucleus of the cell is faintly visible in the region marked *N*. In the first frame, arrows indicate two carbon particles apparently in contact. In the second frame, one particle has been displaced to the right. In the third frame, the displaced particle has recoiled slightly to the left; the third arrow shows the point of its farthest advance to the right. × 1000. *c*, Three successive frames from a phase-contrast time-lapse film of a HeLa cell, showing LSM of a bright particle in an elongated cell process. At left, the particle indicated by the arrow is shown at rest; in the middle frame, the image of the moving particle appears elongated owing to its displacement during the photographic exposure. At right, the particle is stationary in its new position. × 1150.

where the subscripts denote the frame number and $k$ is a magnification constant relating distance on the Vanguard screen to distance in the specimen. The cumulative displacement from frame to frame was plotted against frame number; the slope of such a curve measures actual particle speed, without regard to direction.

Fig. 4 shows a series of displacement-time plots for examples of the particles discussed above. LSM appear as breaks in the curve, corresponding to periods during which particles move with speeds in the range 0.4–1.2 $\mu$/sec. The regions of the curves with lesser slope correspond to smaller random displacements, many of which may be due to Brownian movement. During these periods, there is little change in net position of the particle. Following LSM, there is a tendency for the slope of the curve to decrease gradually to the speed prior to the discrete movement; this is associated with short recoil movements, which carry particles back along the previous path of saltation. Larger particles (e.g., pinosomes, carbon particles) tend to show less abrupt changes in speed than small particles (lysosomes, bright particles).

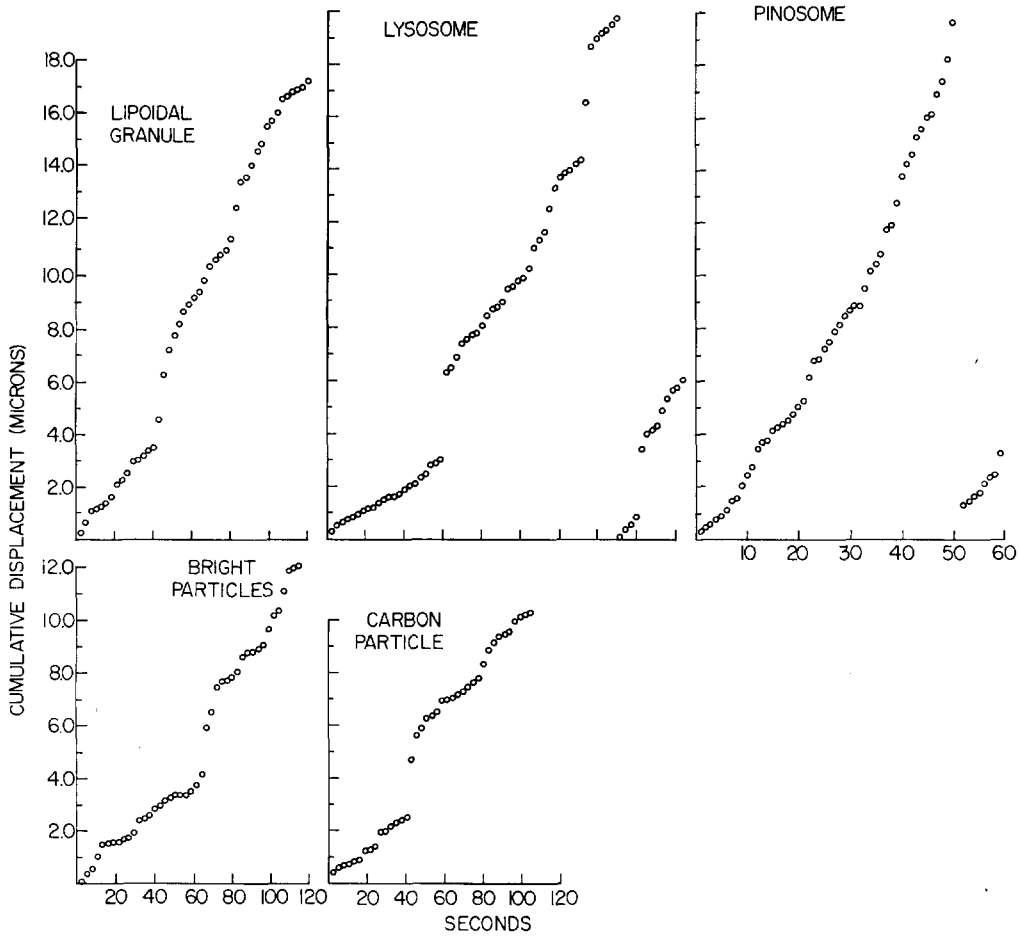



FIGURE 4 Cumulative displacement determined from successive film frames for representative saltating particles in HeLa cells. LSM appear as breaks in the curves. For total displacements greater than 20 $\mu$, the ordinate values are reduced by this amount (lysosome and pinosome curves).

### *Sensitivity of LSM to Drugs*

The effects on LSM of drugs reported to act as "spindle agents" (i.e., to produce metaphase arrest or otherwise to influence cell division) were assayed by time-lapse comparisons of the frequency of LSM before and after addition of the drug to the medium. An example of such an assay is shown in Table I, which illustrates the effect of colchicine on the movement of lipoidal granules in HeLa cells. When the cells were treated with colchicine at concentrations in the range producing metaphase arrest, the incidence of LSM declined markedly. The cells remained attached to the growth surface, with moderate retraction of the cell borders. As shown in the table, the extent of inhibition increased at higher concentrations of colchicine. The data are derived from analysis of the entire 2 hr period following introduction of colchicine, and thus are influenced by LSM that occur shortly after addition of the drug. At the end of this 2 hr period, saltatory movements are rare even at the lowest concentration tested.

Colchicine appears to inhibit LSM of all types of particles. Table II shows the effect of colchicine ($1 \times 10^{-5}$ M) on phagocytized carbon particles in a series of cells. Although the frequency of LSM varied considerably from cell to cell, in all cases it declined sharply after exposure to colchicine.

Table III summarizes the results obtained when a series of agents were tested for their effects on LSM of lipoidal granules. The concentrations of the drugs that were required for accumulation of

TABLE I

*Inhibition by Colchicine of LSM of Lipoidal Granules in HeLa Cells*

| Exp. | Colchicine concentration | S* Control period | S* After perfusion | Inhibition |
|---|---|---|---|---|
| | | | | % |
| A | $1 \times 10^{-6}$ M | 0.0264 | 0.0156 | 41 |
| B | $1 \times 10^{-6}$ M | 0.0354 | 0.0102 | 71 |
| C | $1 \times 10^{-5}$ M | 0.0470 | 0.0100 | 79 |
| D | $1 \times 10^{-5}$ M | 0.0133 | 0.0024 | 82 |
| E | $1 \times 10^{-4}$ M | 0.0330 | 0.0035 | 89 |
| F | $1 \times 10^{-4}$ M | 0.0430 | 0.0120 | 72 |
| F‡ | $1 \times 10^{-4}$ M | (0.229) | (0.050) | (78) |

* LSM per particle-minute of observation, determined by analysis of phase-contrast time-lapse films covering a 2-hr period.

‡ Independent analysis of the films from Exp. F by a second observer. The values of S are higher but the degree of inhibition is similar.

metaphases were determined in sister cultures. As shown in the table, the alkaloids colchicine, Vinblastine, and podophyllin all inhibited LSM. The approximate concentration required to reduce LSM 50% was equal to or greater than that which produced complete blocking of spindle formation. Cells treated for 2 hr with colchicine ($5 \times 10^{-5}$ M) and with Vinblastine ($1 \times 10^{-7}$ M) were examined by electron microscopy; microtubules were rare in comparison with their prevalence in untreated cells.

An additional effect of Vinblastine at higher concentrations (to be described in detail elsewhere) is the induction of ameboid movement, characterized by a mass flow of cytoplasmic material not usually observed in HeLa cells.

Experiments with L strain fibroblasts and Chinese hamster cells, in which we used the agents above, have shown inhibition of LSM at drug concentrations similar to those found effective in HeLa. Cultured mouse peritoneal macrophages and cultured frog embryo cells also show inhibition of LSM after treatment with colchicine (unpublished experiments of A. N. Bhisey).

The other agents tested (Table III) did not inhibit LSM, but produced other effects on cytoplasmic motility. Adenosine triphosphate (ATP) has been reported to influence the rate of cytoplasmic streaming (22, 23). In our assay system, ATP produced no significant change in the frequency of LSM at 0.10, 0.25, and 0.50 mg/ml. At 1.0 mg/ml, LSM were unaffected, but the cytoplasmic border movement characteristic of HeLa cells ceased abruptly. This cytoplasmic inactivity continued for up to 1 hr, after which the usual membrane movements resumed.

Mercaptoethanol has been observed to modify the structure of the mitotic apparatus (24), and to interfere with the formation of flagella (25); it was therefore tested for possible interference with LSM. At the lowest concentration tested ($1 \times 10^{-3}$ M), LSM did not decline in frequency. At $5 \times 10^{-3}$ M and higher concentrations, the cells began to retract, becoming progressively less extended on the substrate. LSM continued, although movement of the cells prevented adequate assay of the frequency of saltation. The effects of mercaptoethanol appear to be distributed in the cell cycle, rather than spindle specific, since metaphases did not accumulate. On the contrary, at concentrations greater than $5 \times 10^{-3}$ M, the incidence of metaphases declined.

Puromycin was tested at a concentration (100 μg/ml) sufficient to inhibit completely the synthesis of protein in HeLa cells. In a series of films originally prepared for another study, it was observed that values of S did not change after addition of the drug, although the projected films gave an impression of increased particle motility. Further analysis showed that this was caused by an in-

TABLE II

*Inhibition by Colchicine of LSM of Phagocytized Carbon Particles in HeLa Cells*

| Cell No. | S* Control period | S* After perfusion with colchicine‡ | Inhibition |
|---|---|---|---|
| | | | % |
| 1 | 0.032 | 0 | 100 |
| 2 | 0.024 | 0.016 | 33 |
| 3 | 0.008 | 0 | 100 |
| 4 | 0.016 | 0 | 100 |
| 5 | 0.260 | 0.016 | 94 |
| 6 | 0.410 | 0.001 | 100 |
| 7 | 0.120 | 0 | 100 |
| 8 | 0.090 | 0 | 100 |

* LSM per particle-minute of observation, determined by analysis of bright-field time-lapse films covering a period of 2 hr. Each cell contained from two to eight particles.

‡ Concentration of colchicine $1 \times 10^{-5}$ M.

TABLE III

*Effects of Drugs on LSM of Lipoidal Granules in HeLa Cells*

| Drug | Range of concentrations tested | Concentration required for inhibition of LSM* | Concentration required to produce metaphase arrest |
|---|---|---|---|
| Colchicine | $10^{-4}$–$10^{-7}$ M | $10^{-6}$ M | $10^{-8}$ M |
| Podophyllin | $10^{-7}$–$10^{-9}$ g/l‡ | $10^{-7}$ g/l | $10^{-7}$ g/l |
| Vinblastine | $10^{-7}$–$10^{-9}$ M | $10^{-8}$ M | $10^{-9}$ M |
| Mercaptoethanol | $10^{-2}$–$10^{-4}$ M | Toxic | Toxic§ |
| Sodium ATP | 0.1–1.0 mg/ml | Negative | Negative‖ |
| Puromycin HCl | 100 μg/ml | Negative | Negative |

* Concentration producing an inhibition between 40 and 60%.

‡ Solution of crude resin.

§ Metaphases did not accumulate; the appearance of the cells became abnormal.

‖ Cytoplasmic movement was reduced at 1.0 mg/ml.

crease in the number and extent of small, random movements. The slope of displacement-time plots was found to be greater than during the control periods of the films, as would be expected if Brownian movement had increased.

### *Direction of LSM in the Cytoplasm*

The LSM of cytoplasmic particles are not random in direction, but are restricted in a manner that implies the presence of linear guiding elements. A series of lipoidal granules showing LSM in film records of well spread HeLa cells was selected for analysis. Particles moving close to the nuclear envelope or within narrow extended cell processes were excluded. The angles between successive LSM of the same particle were determined by projecting the film on paper, plotting the path of the movements, and measuring the angle separating successive paths with a protractor to the nearest 5°. Data for 60 pairs of LSM are shown in Fig. 5. A second movement of a given particle is most likely to follow a path parallel (0°) or opposite (180°) to the previous one. Movements at right angles to a previous movement are not common. A similar analysis of LSM of phagocytized carbon particles yielded comparable results. Thus, individual particles tend to move to and fro, as if guided by structures not resolved by light microscopy; a small reverse movement at the end of a saltatory displacement is a common occurrence.

The direction of LSM appears to be determined in part by the location of the particle in the cell. For studying this, film records of well spread and relatively stationary HeLa cells were projected frame by frame on the rear-projection screen of the Vanguard analyzer. As each LSM of a lipoidal granule occurred, the path of the particle was plotted on a transparent overlay bearing an outline of the cell at the beginning of the film record. Fig. 6 shows a group of such plots; each is a sum-

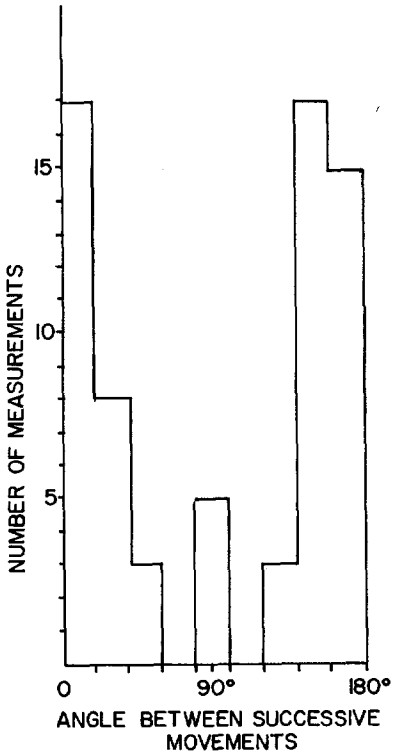



FIGURE 5 Distribution of angles between successive LSM of individual lipoidal granules of HeLa cells. 60 pairs of movements were measured from phase-contrast time-lapse films of six different cells. Movements on the same path or on a recoil path are common; movements at right angles are comparatively rare.

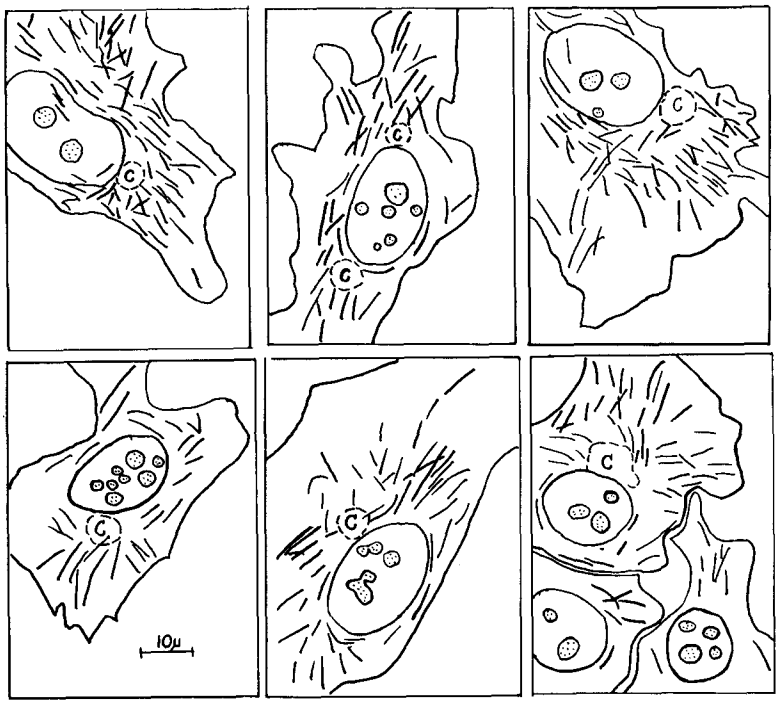



FIGURE 6 Records of the distribution of LSM of lipoidal granules in HeLa cells. Each tracing shows a reference outline of the cytoplasmic border, nuclear envelope, and nucleoli (stippled) of a filmed cell. The locations and directions of all LSM detected during 1.5–2.0 hr are indicated by inked lines marking the tracks of the moving particles. The dotted line surrounding the letter *C* indicates the apparent position of the cytocenter.

mary of the integrated LSM activity over a period of about 2 hr. The apparent location of the cytocenter, in which numerous lipoidal granules are concentrated, is indicated in each diagram although the movements in this region could not be traced with accuracy and have been omitted. The flattened condition of the cells allows the movement of most particles to be considered as if in a single plane, but in a few instances movements apparently crossed the nucleus. These are presumed to occur in the thin layer of cytoplasm above or below the nucleus.

LSM tend to be parallel to the nuclear envelope when the particles are close to it, except in the region of the cytocenter where the distribution is radial. In regions more remote from the nucleus, the predominant pattern of movement is also radial. In extended cell processes, particles saltate in the longitudinal direction; movements at right angles to the axis are rare, even when the processes are relatively broad.

LSM appear to be more common in some cytoplasmic areas than in others containing similar particles. Particles lying close to the nuclear envelope or within extended cell processes are likely to show many LSM. Thus, the tracings in Fig. 6 show a large number of trajectories in these locations.

## *Probable Arrangement of Microtubules as Guiding Elements: A Model*

Our observations are consistent with the hypothetical arrangement of guiding structures shown in Fig. 7. Since drugs that depolymerize microtubules inhibit saltatory movements of the type we are considering, it is reasonable to attribute to the microtubules some role in determining the direction of the movements. If this is the case, the actual arrangement of the cytoplasmic microtubule array should reflect the preferred paths of LSM in different regions of the cell. Our model envisions a radial array of long microtubules with its origin in the vicinity of the centrioles, close to the nuclear

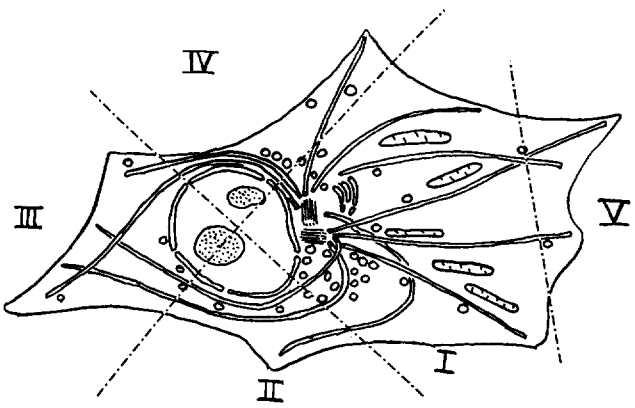



FIGURE 7 Proposed arrangement of cytoplasmic microtubules in a monolayer cultured animal cell, e.g., strain HeLa. Microtubules (double black lines) are indicated as originating from the vicinity of the centriole pair and radiating throughout the cytoplasm. Circular lipoidal granules are associated with the tubules; elongated mitochondria are oriented with their axes parallel to the tubules. The dotted lines and Roman numerals indicate the scheme for noting the locations of electron micrographs used to determine microtubule directions in the various regions of the cell.

envelope. In this region, microtubules are presumed to converge; here lipoidal granules are concentrated. From the cytocenter, the radiating microtubules pass to all parts of the cell, so that microtubules (and consequently LSM) pass parallel to the nuclear envelope. In cytoplasmic regions distant from the nucleus, the microtubules are presumed to lie parallel to the axes of extended cell processes.

The diagram (Fig. 7) indicates that individual tubules might pass unbroken from cytocenter to cell border. However, this assumption is supported only by the failure to find ends of microtubules by electron microscopy, as described below. Individual LSM are of limited length (Fig. 6).

Evidence that the array of cytoplasmic microtubules has the configuration suggested by this model is considered in the next two sections.

### *Radial Arrays of Acid Phosphatase–Reactive Granules*

The existence of an array of microtubules like that postulated in Fig. 7 is supported by the colchicine sensitivity of radial-stranded structures found in the cytoplasm of cultured cells. In dense coverslip cultures of L strain fibroblasts and Chinese hamster cells stained for acid phosphatase, the reactive granules (lysosomes) often occurred in striking radial arrays (Figs. 8 *a* and *d*). Distinct radial configurations were present in 10–15% of the cells, individual strands in up to 50%. Cultures at lower cell densities did not show the phenomenon, but the reactive granules tended to be polarized in the cytoplasm and concentrated in one or two locations close to the nucleus (presumably the cytocenter). HeLa cultures never showed strands; reactive granules were concentrated in the cytocenter with numerous small granules distributed throughout the peripheral cytoplasm.

The strands appear to be intrinsic cytoplasmic structures, elaborated as the cells multiply more slowly in crowded cultures. When samples were fixed at intervals during growth of the cultures, the fraction of cells possessing strands gradually increased after the cultures became confluent. The possibility that they might represent intracellular infection by microorganisms was tested by adding cells from postconfluent cultures to thioglycollate broth medium; no growth resulted. Cultures grown in the antibiotic kanamycin (Grand Island Biological Co., Grand Island, N.Y.) (500 pg/ml), to inhibit the growth of mycoplasma, showed no change in the occurrence of the strands. Further, the strands have not been observed in mitotic cells found in the cultures in which strands do occur in the interphase cells.

Thus, we interpret the strands as a variant distribution of lysosomes reflecting association of the granules with an aster-like array of microtubules having its origin at the cytocenter.

This interpretation was tested by application of colchicine to cultures maintained for 24 hr after the cells became confluent. If the arrays depend on microtubules as structural elements, depolymerization of the microtubules would be expected to disperse the strands. As shown in Fig. 8, exposure of the cultures to colchicine ($1 \times 10^{-5}$ M) resulted in rapid disappearance of the stranded granules. Sister cultures fixed without exposure to colchicine showed the normal frequency of radial figures. L strain fibroblasts responded somewhat more rapidly than Chinese hamster cells, but after 60 min

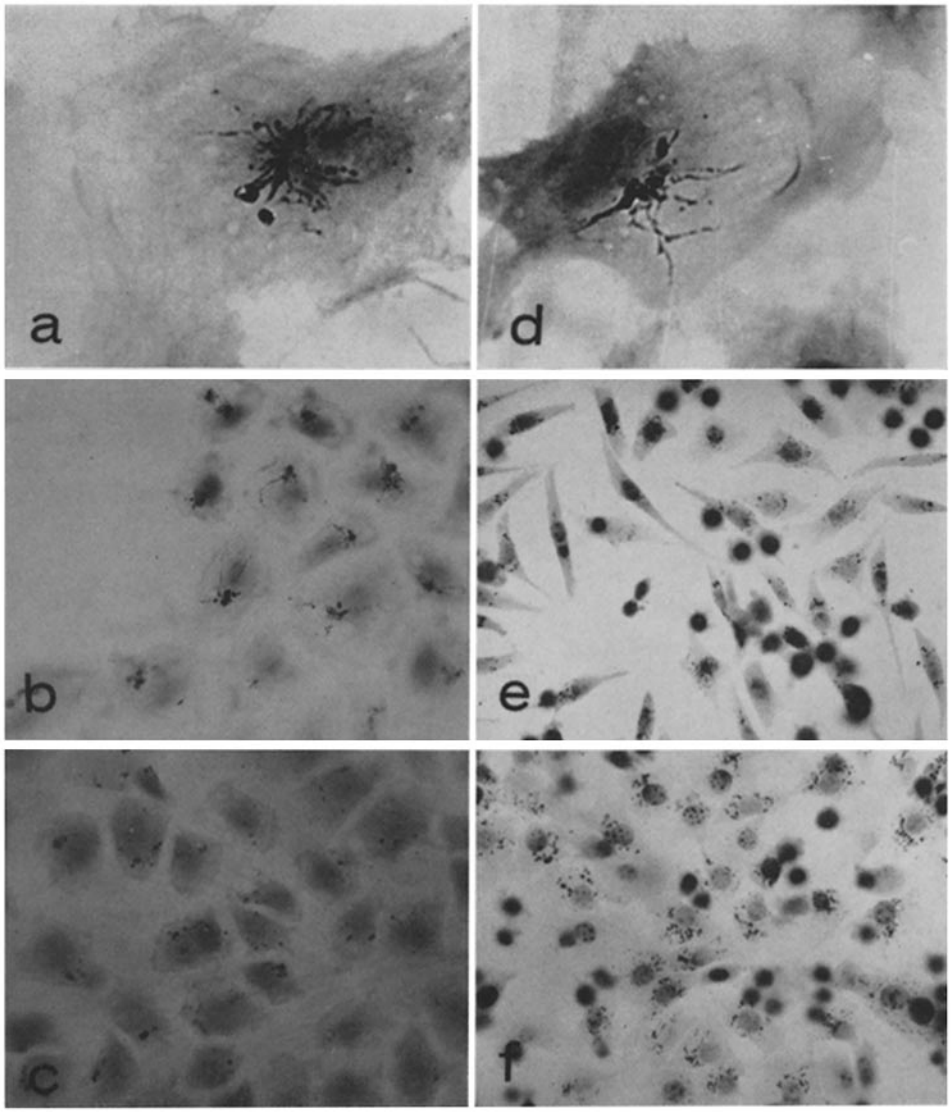



FIGURE 8 Colchicine-sensitive radial arrays of acid phosphatase–reactive granules in cultured cells. Gomori's acid-phosphatase reaction with fast green counterstain. *a*, Chinese hamster cell from a high-density culture at high magnification. Strands of granules radiate from a point adjacent to the nucleus, an appearance found in 10–15% of the cells. × 1200. *b*, Chinese hamster cells fixed 10 min after addition of colchicine ($1 \times 10^{-5}$ M). Strands are still present in some cells. × 200. *c*, Chinese hamster cells fixed 60 min after addition of colchicine ($1 \times 10^{-5}$ M). No strands are found, and the acid-phosphatase granules are in many cases no longer polarized in their distribution relative to the nucleus. Later samples show further depolarization. *d*, L strain fibroblast from a high-density culture, at high magnification. Radiating strands of granules originate from a point near the nucleus. × 1200. *e*, L strain fibroblasts 10 min after addition of colchicine ($1 \times 10^{-5}$ M). No strands remain, but the granules in some cells still show polarization at one side of the nucleus. × 200. *f*, L strain fibroblasts 60 min after addition of colchicine ($1 \times 10^{-5}$ M). Reactive granules are randomly distributed in the cytoplasm of most cells. × 200.

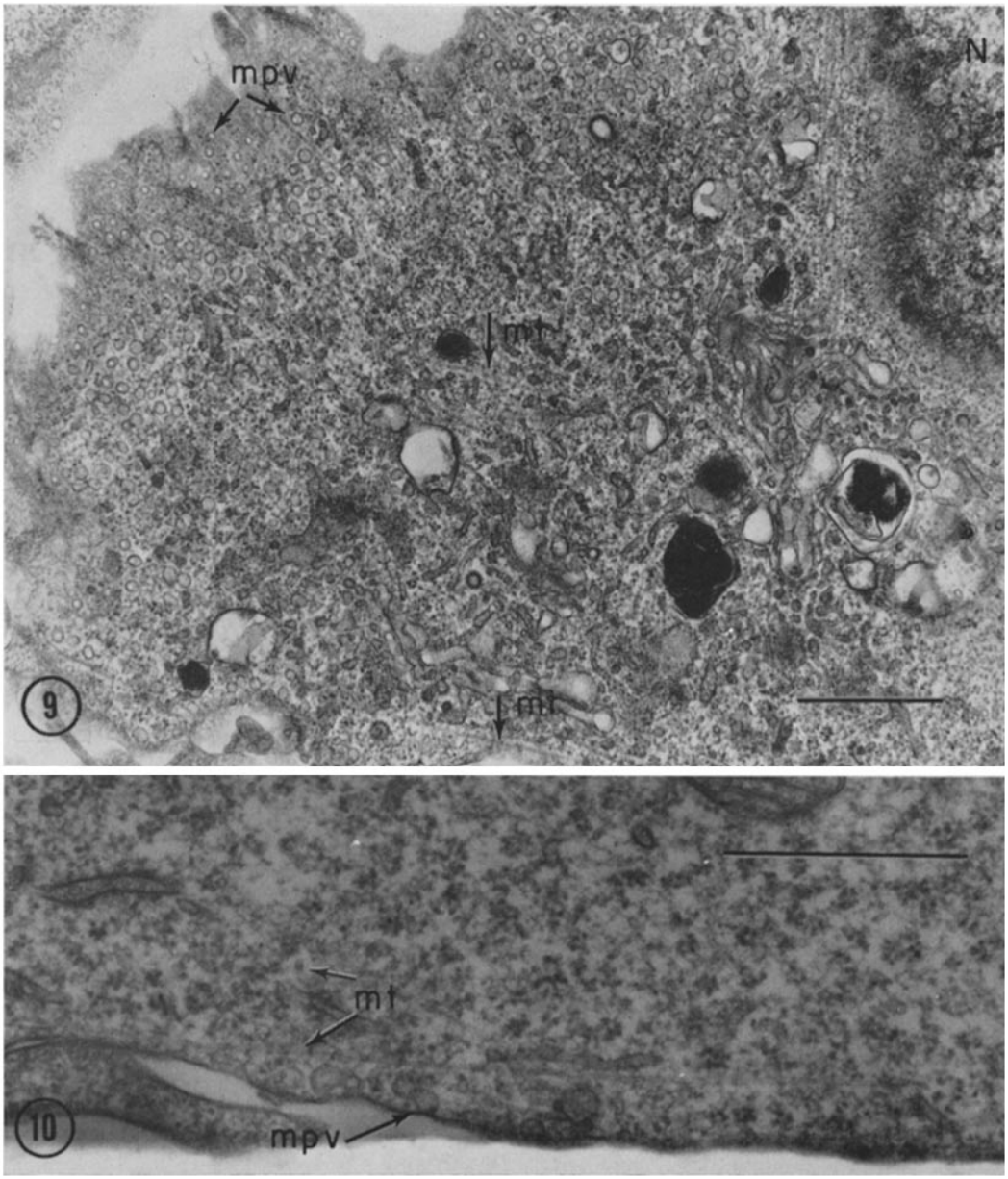



FIGURE 9 HeLa cell sectioned in a plane slightly oblique to the growth surface; the section lies close to the attached surface of the cell. At upper right is a portion of the nucleus (*N*) with obliquely sectioned nuclear pores. From right to left, the section comes progressively closer to the cell surface. Microtubules (*mt*) occur most commonly at a level just deeper in the cell than flasklike micropinocytic vesicles (*mpv*) seen at the extreme left. Many of the latter show pale circular openings to the subcellular space. × 20,000.

FIGURE 10 HeLa cell sectioned perpendicular to the growth surface. At the bottom is seen the attached surface of the cell. The position of oblique and longitudinal sections of microtubules (*mt*) is just deeper than that of the micropinocytic vesicles (*mpv*). × 34,000.

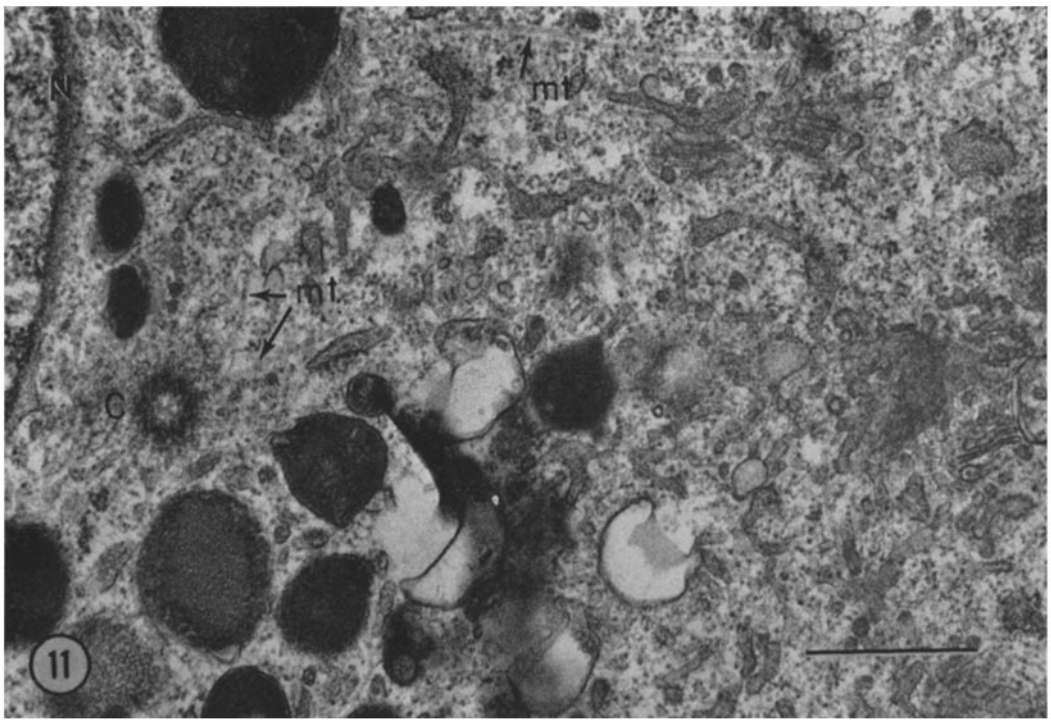



FIGURE 11 Cytocenter region of a HeLa cell, sectioned parallel to the growth surface and through the level of a centriole (*C*). Microtubules (*mt*) in longitudinal view converge on flocculent material adjacent to the centriole and elsewhere appear in generally radial relation to this point. *N*, nucleus. × 24,000.

no strands were found in either cell line. With longer colchicine treatment, the reactive granules became randomly distributed in the cytoplasm as described by Robbins and Gonatas (26).

### *The Arrangement of Cytoplasmic Microtubules in HeLa Cells*

The apparent abundance of microtubules in electron micrographs of cultured cells sectioned parallel to the growth surface varies strongly with the depth at which the section passes through the cell. Microtubules appear to be preferentially located in the region near the attached surface. In sections slightly oblique to the attached cell surface (Fig. 9), microtubules are most frequently recognized in that part of the section just internal to the flasklike invaginations of the cell membrane (micropinocytic vesicles) which stud this surface of the cell. Fig. 10 shows a comparable region in a section cut perpendicular to the plane of the culture dish. Microtubules also occur near the free surface of the cell, while in the midregion they are relatively rare.

The length of individual tubules remains uncertain. The cytoplasmic microtubules of HeLa cells extend uninterrupted over distances at least as great as 5 $\mu$, as judged from their extent in favorable single sections and their continuity in superimposed micrographs of serial sections examined by the stereo technique of Ashton and Schultz (27). Long individual tubules may also be followed in stereo electron micrographs of 1,000 A sections. However, we have not been able to reconstruct a sufficient thickness of the cell by these methods, to determine the complete course of individual tubules.

To determine the distribution of microtubules for comparison with the directions predicted from LSM, we examined the courses followed by microtubule segments in micrographs of various regions of the cell. Low-power micrographs of entire cells were not employed, since short segments of microtubules were difficult to recognize. A series of micrographs at an original magnification of 10,000 was therefore prepared, each keyed to the schematic regions illustrated in Fig. 7, and including

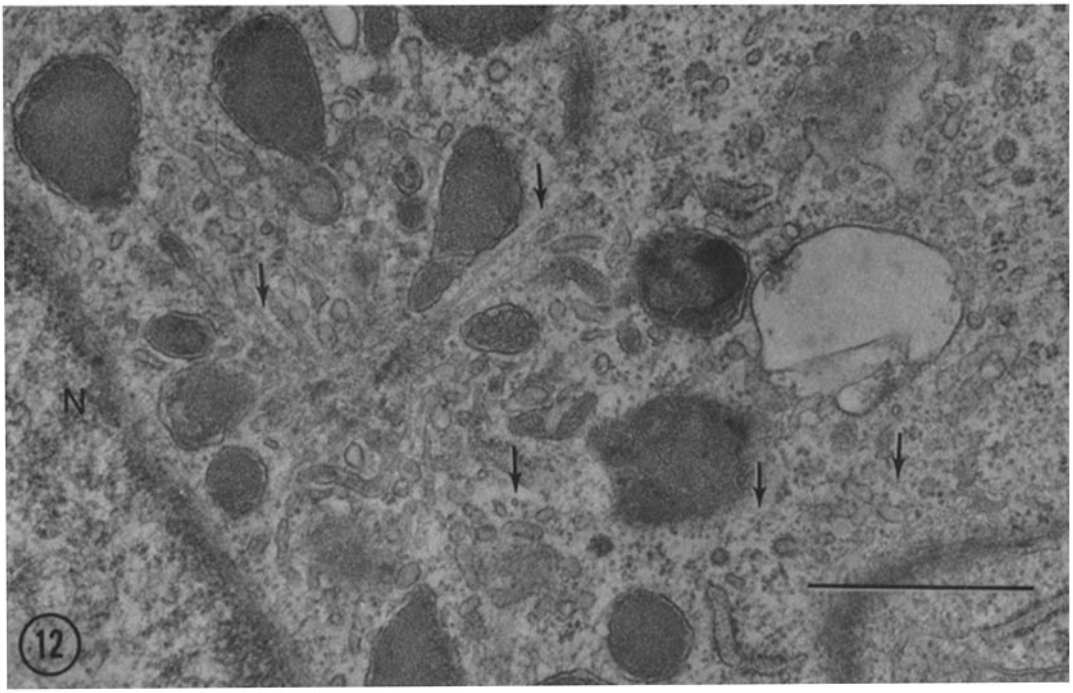



FIGURE 12 Cytocenter region of another HeLa cell, the section passing closer to the growth surface than Fig. 11. Microtubules (arrows) radiate from flocculent material like that associated with the centriole. *N*, nucleus. × 30,000.

samples from 28 different HeLa cells. Many cells were used for several micrographs. The reference system was as follows:

*Region I:* The quadrant that contains the cytocenter. It is recognized by the presence of centrioles when these are included in the plane of section, or by lysosomes, Golgi structures, and a relatively scanty population of ribosomes when the centrioles are absent.

*Regions II and IV:* The quadrants adjacent to Region I ( See Fig. 7).

*Region III:* The quadrant opposite the cytocenter.

*Region V:* Any extended cell process, without regard to its directional relation to the cytocenter.

There was a prevalent or majority pattern which characterized the microtubule segments in a given micrograph, although the directions of microtubule segments were not completely uniform. Most microtubules lay nearly enough in the plane of section to allow recognition of their direction. Since the tubules do not appear to form bends of small radius, the direction of an observed segment was taken as an approximation of the path of the tubule as a whole. Only in the cytocenter region do any appreciable number of microtubules pass perpendicular to the growth surface and appear as cross-sections. (An example may be noted in Fig. 12.)

Micrographs of Region I suggested a radial distribution of microtubule segments. As shown in Fig. 11, sections that contain a centriole usually lie relatively far from the attached cell surface and contain few microtubules. The microtubules present appear to radiate from flocculent material associated with the centrioles themselves. Sections passing between the centrioles and the growth surface show other tubules radiating from the flocculent material that lies below the centriole (Fig. 12). Sections which pass close enough to the growth surface to include the zone of greatest microtubule abundance (Fig. 13) show quite numerous microtubules radially disposed in the cytocenter region. Microfilaments are also common at this level in the cytoplasm.

Micrographs from Regions II and IV shows that the predominant paths taken by microtubules through this part of the cytoplasm lie parallel to

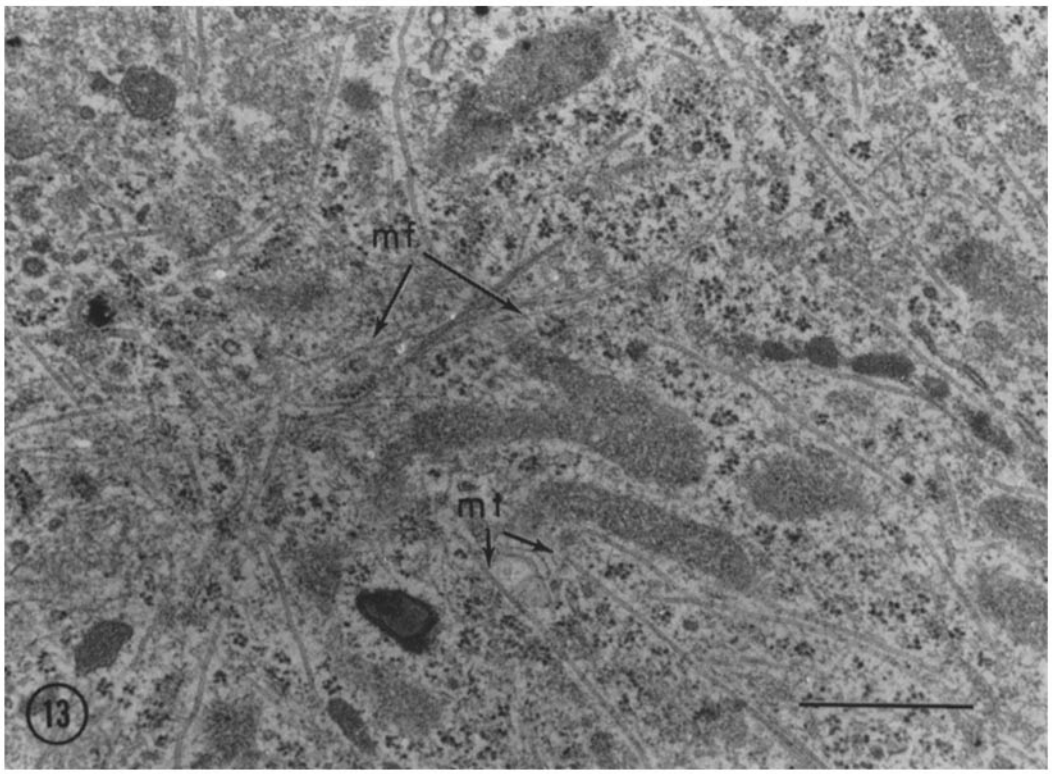



FIGURE 13 Cytocenter region of another HeLa cell, sectioned even closer to the growth surface than Fig. 12, so that the section passes through the zone of greatest microtubule abundance. Longitudinal profiles of microtubules (*mt*) are numerous and radially disposed. Microfilaments (*mf*) are also common at this level. × 24,000.

the nuclear envelope (Fig. 14). This is the direction expected from the model, and would account for the frequent saltatory movements of granules along the nuclear margin.

Micrographs from Region III, opposite the cytocenter, show a less uniform distribution of microtubule directions (Fig. 15). A frequent finding, as illustrated in the figure, is that many tubules diverge as if to pass around the nucleus on either side. In sections close to the attached surface, some microtubules appear to pass between the cell membrane and the nuclear envelope.

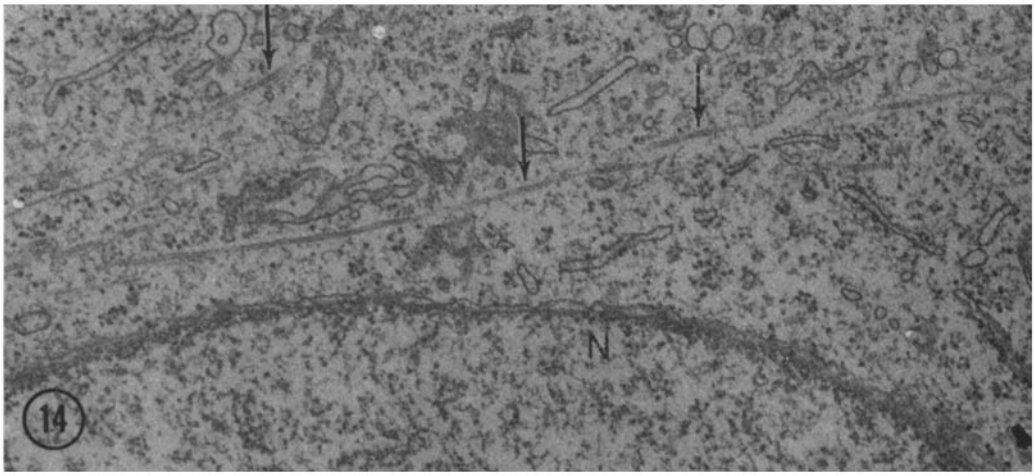
In extended cell process, the prevalent direction of the microtubules is along the axis of extension (Fig. 16). Micrographs of the region at the base of narrow cell processes showed that the uniformity of microtubule direction was greater in the extension than in the cytoplasm closer to the nucleus.

Thus, the information from the micrographs is consistent with the scheme shown in Fig. 7.

## DISCUSSION

### *The Association of LSM with Microtubules*

The disappearance of LSM in cells treated with drugs able to destroy microtubules provides the main evidence that microtubules are involved in this form of motility. Colchicine, the most extensively studied of these microtubule agents, has been shown by Taylor and his coworkers (28, 29) to bind specifically to the characteristic protein of the microtubule subunit; when applied to cultured cells, it causes the disappearance of microtubules as formed structures (26, 30). Vinblastine, which interacts with the microtubule protein in a different fashion (31), also depolymerizes microtubules (26, 31). Podophyllin, although its interaction with microtubule protein has not been studied, appears from earlier work (32) and from our own study to be an authentic "spindle agent," i.e., it



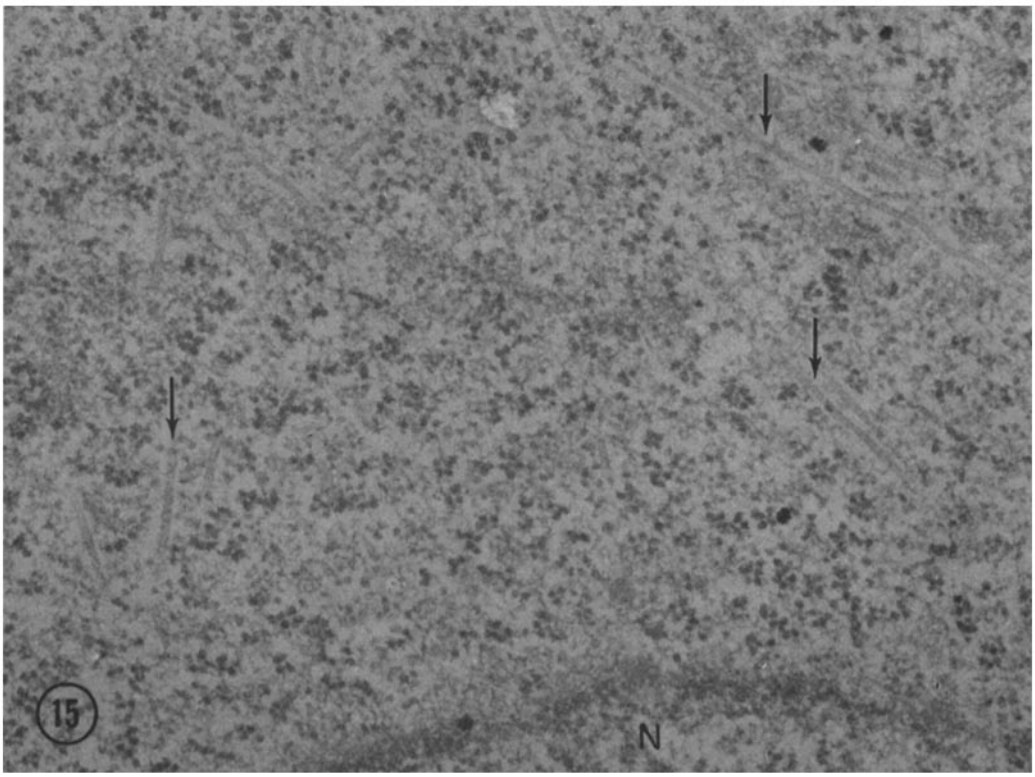




FIGURE 14 Cytoplasm of a HeLa cell in the quadrant adjacent to the cytocenter (Region II). A portion of the nucleus (*N*) is at the bottom of the figure; the cytocenter lies in the adjacent quadrant to the left. Microtubules (arrows) pass peripheral to the nuclear envelope. × 24,000.

FIGURE 15 Cytoplasm of a HeLa cell in the quadrant opposite the cytocenter (Region III). Microtubules (arrows) appear to diverge as if to pass on either side of the nucleus (*N*). × 28,000.

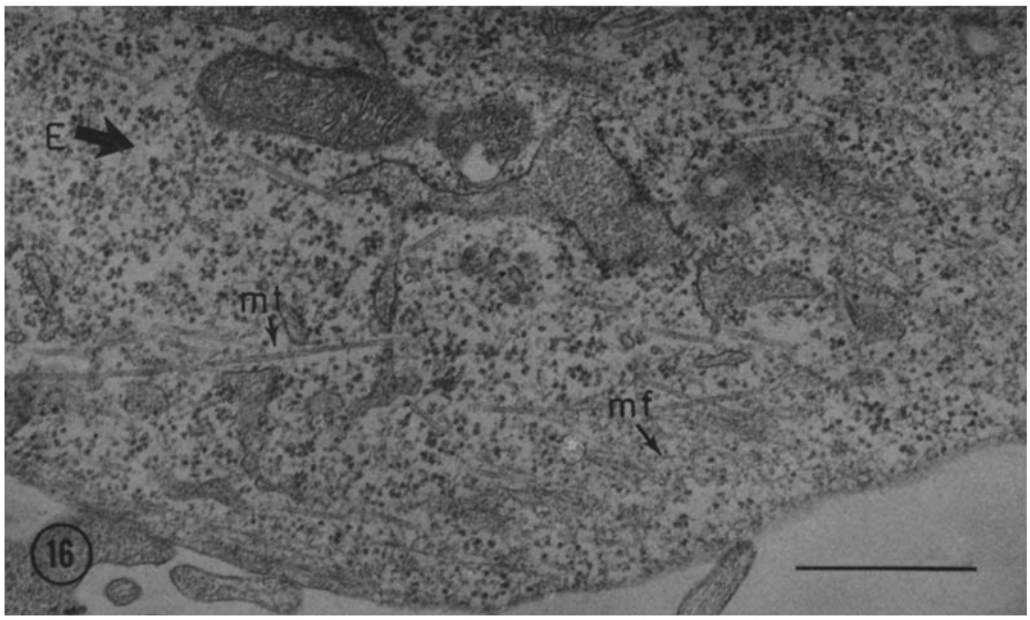



FIGURE 16 Cytoplasm of an extended process of a HeLa cell (Region V). The approximate direction of extension of the process as a whole is indicated by the heavy arrow (*E*). Microtubules (*mt*) are generally parallel to the axis of extension. Microfilaments (*mf*) are found in the area close to the edge of the cell. × 24,000.

leads to accumulation of c-metaphases (mitoclasic effect) (33). Three structurally dissimilar agents thus share common properties: they destroy microtubules and prevent the conspicuous movements of the LSM class.

Observations on other cell culture systems relate saltatory movement to microtubules. Klaus (34) observed particles moving along stress lines in cultured carcinoma cells; after treatment with colchicine, the lines faded and movements ceased. DuPraw (35) reported saltatory particle movements in cultured cells of honeybee embryo, and presented electron micrographs of shadow-cast preparations that show the association of granules with linear elements interpreted as microtubules. Other instances of linear movement of intracellular elements have been found to be sensitive to colchicine. In the movement of pigment granules of chromatophores (6), the movement of mitochondria and other particles along the axopods of *Actinosphaerium* (3), and the transport of nuclei to the center of virus-induced syncytia (7), microtubules are structurally associated with the moving elements and movements cease when the microtubules are destroyed.

Negative results with colchicine were reported by Rebhun (11), who found that saltatory movements of echinochrome granules persisted in sea urchin eggs exposed to $10^{-3}$ M colchicine. This treatment did, however, prevent the appearance of the division spindle, even when the eggs were rinsed prior to fertilization. He rejects microtubules as the important elements in his system. However, depolymerization of existing microtubules may require a higher concentration of colchicine than is required to prevent formation of the spindle, as in our own experiments (Table III). Ciliary microtubules are stable to colchicine, although the drug prevents formation of new cilia, presumably by binding to new protein subunits as they are formed (36). It may be noted that Tilney and Gibbins (37) found that immediate effects on motility in the sea urchin gastrula required concentrations of colchicine as high as $10^{-2}$ M, which suggest that the drug might permeate these cells more slowly than mammalian cells.

The possibility that an interaction of microtubules and microfilaments plays a role in saltatory movements has been discussed by Buckley and Porter (38). Microfilaments with diameters rang-

ing from 50 to 150 A are prominent cytoplasmic structures in cultured cells, particularly near the attached surface; they are often associated with microtubules. However, these filaments are not colchicine sensitive; rather they may be greatly increased in abundance when the microtubules have been destroyed (26, 30). Thus, while microfilaments may be force-generating structures, as Rebhun (11) has proposed, their presence does not appear to be a sufficient condition for the class of saltatory movements with which we are concerned.

The mechanism of the association between microtubules and saltating cytoplasmic elements remains obscure. Colchicine and similar drugs may produce effects on cytoplasmic viscosity or on unrecognized cytoplasmic structural elements in addition to depolymerizing the microtubules. Further, non-Brownian movements smaller than the class which we have selected for study may have different characteristics, and may not be related to colchicine-sensitive structures. Thus, at present we can only conclude that the *direction* of LSM is related to microtubule direction; the question of how the force is generated to produce the movement remains open.

### *The Significance of Saltatory Movement*

Saltatory movements may provide a mechanism by which a cell can bring particulate materials into spatial relations favorable for their interaction. The cytoplasmic microtubule system may constitute an intracellular transport system of widespread occurrence. As examples, the microtubule-dependent movement of pigment granules in melanocytes (6), and the movement of ingested materials along the axopods of *Actinosphaerium* (3) have apparent functional roles. Bidirectional streaming in neurons appears to involve microtubules, and may play a part in the rapid transport of material along nerve processes (39, 40).

Our observation that materials incorporated by phagocytosis and pinocytosis are transported by LSM suggests that saltation might facilitate the addition of lysosomal contents to phagosomes. The interference of colchicine with the activity of phagocytic cells has been well documented (41); although the absence of microtubules does not prevent endocytosis, it interferes with other aspects of phagocytosis.

Thus, the saltatory motility observed in cultured cells may reflect a basic form of intracellular organization. In epithelial-like cultured cells, the main consequence of saltatory movement may be the retention of lysosomes and phagocytized materials within the confines of the cytocenter, in proximity to the Golgi apparatus and other structures of the smooth endoplasmic reticulum. As others have noted, in colchicine-treated cells this polarization is lost (26).

### *The Cytoplasmic Microtubule Array as a Persisting Aster*

A radial array relative to the centrioles appears to be the major form of microtubule distribution in cultured cells; this organization may be a persisting form of the more elaborate aster found in dividing cells. Saltatory particle movements are conspicuous along the rays that form the asters of cleaving embryos (42). Prominent radiating structures in the ground cytoplasm of amphibian leukocytes were found to be associated with the centrioles by Pollister (43), who concluded almost two decades ago that oriented "micelles" of structural protein were likely to compose such structures and to determine an imposed orientation of mitochondria and other cytoplasmic organelles. Rebhun (42) examined amphibian leukocytes in the living state and noted that saltatory movements of particles follow these radial paths. In most cells, conspicuous astral rays are not found in interphase, but recent studies make it clear that interphase centrioles are often associated with radially disposed microtubules. For example, in HeLa cells the centrioles are a focus of microtubules at all stages of the cell cycle except $G_1$ (44).

It is not clear whether all microtubules in cultured cells are associated with the centrioles, or whether some form an independent system. Microtubules in blood platelets and cells of higher plants are obviously independent of centrioles. In cilia and flagella, however, the centriole (basal body) is a universal structural feature. The function of centrioles in organizing the microtubule array in cultured cells and in saltatory movements remains a subject for further experiments.

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