Microinjected Fluorescent Polystyrene Beads Exhibit Saltatory Motion in Tissue Culture Cells

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ABSTRACT Microinjected 0.26-μm fluorescent, carboxylated microspheres were found to display classical saltatory motion in tissue culture cells. The movement of a given particle was characterized by a discontinuous velocity distribution and was unaffected by the activity of adjacent particles. The microspheres were translocated at velocities of up to 4.7 μm/s and sometimes exhibited path lengths >20 μm for a single saltation. The number of beads injected into a cell could range from a few to over 500 with no effect on the cell's ability to transport them. Neither covalent cross-linking nor preincubation of the polystyrene beads with various proteins inhibited the saltatory motion of the injected particles. The motion of the injected beads in cultured cells was reversibly inhibited by the microtubule poison nocodazole, under conditions in which actin-rich, nitrobenzoxadiazol-phallacidin-staining structures remain intact. Whole-cell high voltage electron microscopy of microinjected cells that were known to be moving the fluorescent microspheres revealed that the beads were embedded in the cytoplasmic matrix and did not appear to be membrane bound. The enhanced detectability of the fluorescent particles over endogenous organelles and the ability to modify the surfaces of the beads before injection may enable more detailed studies on the mechanism of saltatory particle motion.

In a variety of cell types (e.g., fibroblasts, oocytes, neurons, and chromatophores) organelles are distributed within the cytoplasm by saltatory motion. Intracellular particles can travel at speeds up to 5 μm/s over distances of 20–30 μm in a single saltation (19, 20). Such motility is ATP dependent (1, 6) and is generally inhibited by microtubule poisons (12, 23). In some cells the requisite association of motile particles with microtubules has been observed directly by high resolution light microscopic techniques (3, 15). A number of investigators have attempted to characterize further the involvement of microtubules by examining the possibility that the putative organelle–microtubule interactions are mediated by dynein. With one exception in the cases examined thus far (7), microtubule-dependent saltatory motion in a number of cell types has been shown to be sensitive to vanadate^{+5}, a potent inhibitor of dynein ATPase activity (4, 8, 10, 16, 22). Although these latter results are consistent with the hypothesis that dynein is involved in saltatory motion, the possibility that other molecules are responsible for saltations has not been eliminated. Indeed, in some cases (e.g., characean algae) the bulk of the evidence points to an actomyosin motility machine (18). And the provocative results of Edds (9) in which a glass needle has been substituted for microtubules suggests that in *Echinosphaerium*, axopodial microtubules are required only as a nonparticipatory structural framework for motility. In short, despite numerous studies on the subject, we do not fully understand the basis of intracellular particle transport.

In the work described in this paper we have used a modification of an approach introduced by Adams and Bray (2) to study saltatory particle movements in vivo. We microinjected fluorescent, polystyrene beads into tissue culture cells and found that these acellular particles are translocated in a manner indistinguishable from endogenous organelles. Use of this approach with beads having experimentally defined surfaces may allow a detailed analysis of the mechanism of saltatory motion.

MATERIALS AND METHODS

Culture of Cells: Cells from the established cell lines BS-C-1 and Pt K1 were grown on glass coverslips in an appropriate medium containing 10% fetal calf serum and 2% penicillin-streptomycin. Cells to be used for whole-mount high voltage electron microscopy (HVEM)[1] were cultured on gold finder

[1] *Abbreviations used in this paper:* HVEM, high voltage electron microscopy; NBD, nitrobenzoxadiazol.

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grids (Ernest F. Fullam, Inc., Schenectady, NY) and were fixed and critical-point dried by established procedures (25).

Experimental Materials: The microtubule inhibitor nocodazole was purchased from Aldrich Chemical Co. (Milwaukee, WI); a 10 mg/ml stock solution was prepared in DMSO and stored at 0°C until needed. Nitrobenzoxadiazol (NBD)-phallacidin was obtained from Molecular Probes (Junction City, OR). Fluorescent, carboxylated polystyrene beads (0.26 μm) were purchased from Polysciences, Inc. (Warrington, PA). Covasphere-MX fluorescent microspheres (0.5 μm) were purchased from Covalent Technology Corporation (Ann Arbor, MI). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Indirect Immunofluorescence and NBD-Phallacidin Staining: Cells grown on glass coverslips were rinsed in PBS, fixed for 20 min at 37°C in 0.1 M Na-PIPES, pH 7.3, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% glutaraldehyde, and 1.6% paraformaldehyde, and washed three times for 10 min each time in PBS. The cell membrane was permeabilized with 0.2% Triton X-100 in PBS for 2 min; the cells were then washed three times for 10 min in PBS. An affinity-purified rabbit polyclonal antibody prepared against sea urchin egg tubulin was generously provided by Keigi Fujiwara (Harvard Medical School) and was characterized as described previously (13). Rhodamine-conjugated goat anti-rabbit IgG was purchased from Miles-Yeda, Miles Laboratories (Elkhart, IN). After permeabilization the cells were labeled by established procedures for indirect immunofluorescence (24). For NBD-phallacidin staining the cells were fixed as described above but were permeabilized by plunging the coverslips into precooled (−20°C) acetone for 5 min. The coverslips were then air dried and stained with NBD-phallacidin according to the procedure recommended by the manufacturer.

Microinjection of Fluorescent Beads: The cells were pressure microinjected by a previously described modification (5) of the method of Graessmann and Graessmann (14). The 0.26-μm microspheres were diluted to a maximum concentration of 1.3×10^{11} beads/cc with the injection buffer, Dulbecco's PBS without added calcium. The micropipettes were drawn on a Brown-Flaming micropipette puller (Sutter Instruments, San Rafael, CA) from borosilicate capillaries having internal filaments (W-P Instruments, Inc., New Haven, CT). The microneedles were back-loaded by capillary action and they were then oriented for injection. If the beads were not flowing from the needle, the tip of the pipette was carefully chipped on the edge of the coverslip containing the cells. Cells within an area on the coverslip inscribed with a diamond pencil were microinjected. After injection, the coverslips were returned to the incubator for at least 6 h (usually overnight). The coverslips carrying the injected cells were then mounted in medium on a slide having glass coverslip spacers and were sealed with a 1:1:1 mixture of lanolin, petroleum jelly, and parafin. The injected cells were scored for motility of the beads with a 40× Neofluor Zeiss objective on a Zeiss epifluorescence microscope equipped with a fluorescein filter set.

For some experiments the surfaces of the beads were labeled with proteins as follows. Rhodamine-labeled BSA and cytochrome *c* were prepared by incubating the proteins with rhodamine isothiocyanate in PBS at pH 9.0 for 4 h at 4°C. After labeling, the proteins were dialyzed extensively against PBS and passed over a Sephadex G-25 column to separate labeled protein from free dye. The 0.26-μm carboxylated beads were either incubated for at least 2 h with 50% normal goat serum or purified protein (1 mg/ml) or cross-linked to proteins by use of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)(17). The EDC coupling reaction was quenched with 0.1 M glycine, pH 8.0. After carbodiimide cross-linking the beads were washed alternately with acetate buffer (0.1 M, pH 4.3) and sodium bicarbonate (0.1 M, pH 8.3), both in 0.5 M NaCl; the beads were then washed extensively in water and, finally, were suspended and sonicated in injection buffer. Covaspheres were coupled to protein as suggested by the manufacturer.

Recording of Results: Experiments were recorded with the aid of a Venus DV-2 video intensification camera (Venus Scientific Inc., Farmingdale, NY) and a Nippon Electric Company ¾-inch time-lapse video recorder equipped with a time-date generator. Images were displayed on a Conrac TV monitor where they were photographed with a 35 mm SLR camera (Nikon FM) with motordrive. Short exposure shots were made with Kodak Plus X film developed in Diafine; continuous exposures were made with Kodak Technical Pan film developed with HC 110, dilution D.

RESULTS

Microinjected Polystyrene Beads Are Translocated by Tissue Culture Cells

Fluorescent, 0.26-μm carboxylated polystyrene beads were microinjected into individual tissue culture cells. Immediately postinjection the fluorescent beads were localized near the site of needle entry and generally appeared motionless. However, after an overnight return to the 37°C incubator, the beads were distributed throughout the cytoplasm (Fig. 1) and they exhibited classical saltatory motion. The minimum time required for acquisition of motility was not precisely determined, but it is somewhere between 1 and 6 h after injection. The number of beads injected into a cell was quite variable (depending on the concentration of the beads in the needle as well as the dose) and ranged from a few to over 500; the absolute number of beads had no apparent effect on the cell's

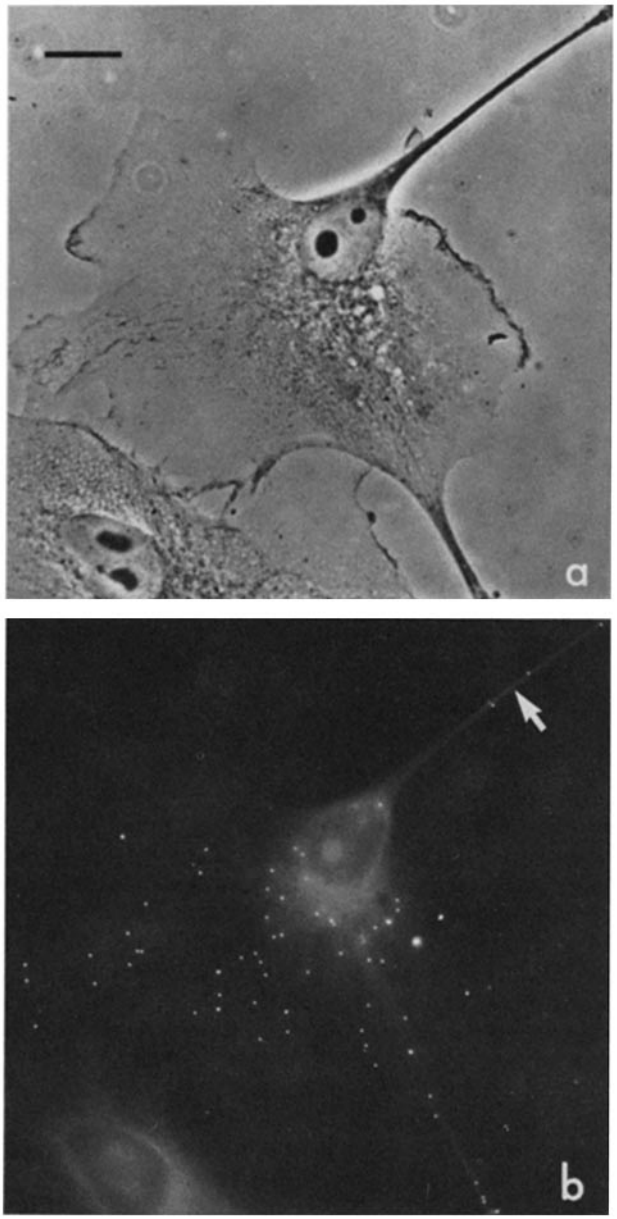

FIGURE 1 Fluorescent microspheres injected into a BS-C-1 cell. A phase-contrast image (*a*) and a fluorescent image (*b*) of a cell injected with 0.26 μm fluorescent polystyrene beads. The particles are distributed within the cell by saltatory motion. Some beads have traveled into a long cellular extension (arrow). It is clear from a comparison of the phase-contrast and fluorescence images that a dramatic increase in detectability of particles is achieved by the use of fluorescently labeled beads. Bar, 20 μm. × 500.

ability to translocate the foreign particles. Greater than 90% of all cells injected with the carboxylated beads exhibited saltatory motion of these particles (Table I). Although both BS-C-1 and Pt K1 cells transported the beads, the BS-C-1 cells were used for the remainder of the experiments because they showed the highest level of endogenous organelle activity under our culture conditions.

The movements of the microinjected beads were similar to those of cellular particles in many respects. As with endogenous organelles, the motility of a given bead was not affected by the activity of adjacent particles. In some cases, two particles situated next to each other began to move in opposite directions; alternatively, one of the two neighboring particles would move while the other remained stationary. Continuous exposures (2–4 s) of a real-time video recording of an injected cell resulted in images such as those in Fig. 2 in which the paths traveled by the fluorescent particles during the exposure period are clearly illustrated. Although the pattern of motion exhibited by a bead is generally linear (see examples in Fig. 2, *a–c*), the beads can travel much more tortuous paths (Fig. 2*c*). They can also travel paths having one or more right angle turns (Fig. 2*a*). These latter observations are somewhat difficult to reconcile with the idea that the beads are moving in intimate association with linear or curvilinear cytoskeletal elements such as microtubules.

The fluorescent particles reached velocities of 4.7 μm/s when traveling in the microinjected cells, a maximum speed comparable to those recorded for saltatory movements of endogenous organelles in other tissue culture cells (23). When the cumulative displacement of a given particle was plotted against time (Fig. 3), the stop-go, saltatory nature of the motion became apparent. The plateau regions of the curves represent stationary periods for the particle with the slope in a given region of the curve equalling the mean velocity exhibited by the particle over that time interval.

The actual movements of some injected beads over a 10-s period is seen in Fig. 4. Photographs were taken from a video-recorded image at 2-s intervals. Three individual particles were followed in a frame by frame analysis of the videotape and are labeled *1*, *2*, and *3* in Fig. 4. In addition to the beads that have moved during this time interval, stationary particles can also be seen.

TABLE I

Motility of Microinjected Beads in Tissue Culture Cells

Cell type	Bead type	Total cells injected	Motility status of beads			Positive for motility‡
			+	−	?*	
			No. of cells			%
BS-C-1	0.26 μm (COO^-)	489	467	15	5	96
Pt K1	0.26 μm (COO^-)	19	18	1	0	95
BS-C-1	0.26 μm/normal goat serum	98	86	11	1	88
BS-C-1	0.26 μm/Rh-cytochrome *c*	112	106	3	3	95
BS-C-1	0.26 μm/EDC-BSA	18	17	1	0	94
BS-C-1	0.5 μm Covasphere-BSA	20	5	15	0	25

* Cells in mitosis.

‡ Cells in mitosis were counted as negatives when computing the percent of motility-positive cells.

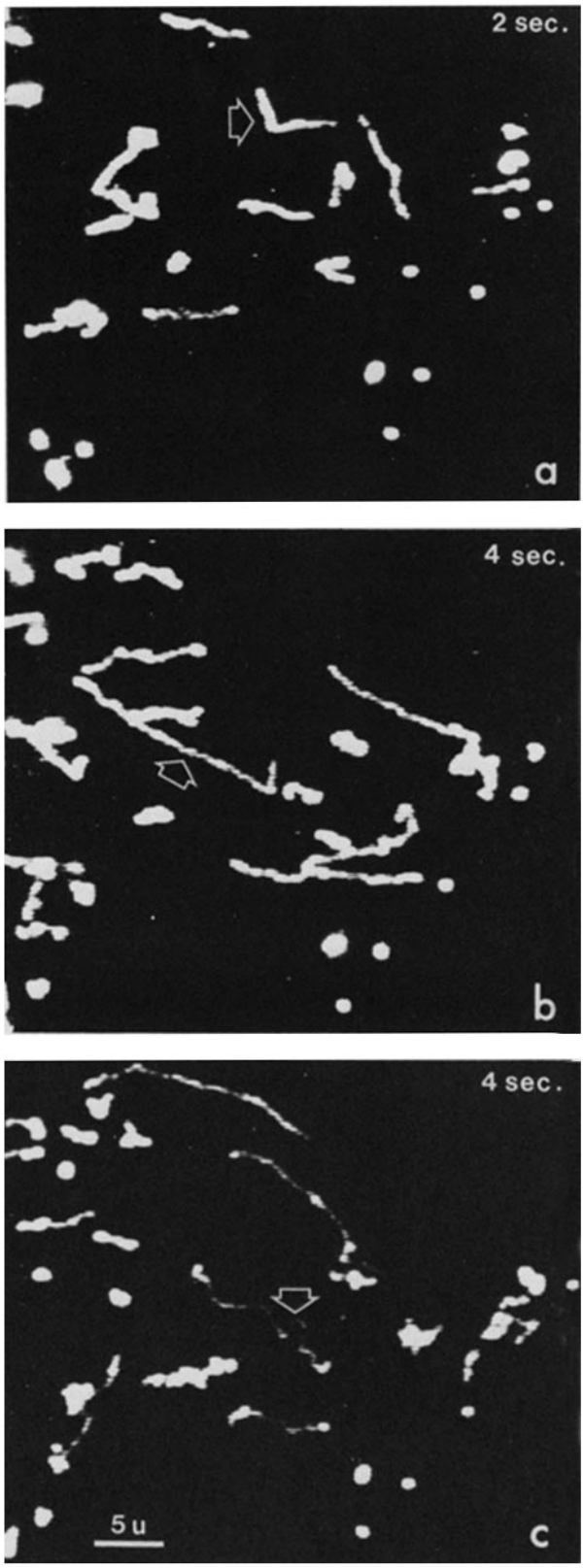

FIGURE 2 Continuous exposures (2 or 4 s) of motile 0.26-μm fluorescent beads in BS-C-1 cells illustrate the variety of paths traveled by the microspheres. The beads may make orthogonal turns (*a*, arrow), may follow extended linear trajectories (*b*, arrow), or may proceed along circuitous paths (*c*, arrow). At any given time some beads are stationary while others may travel at velocities approaching 5 μm/s. Bar, 5 μm.

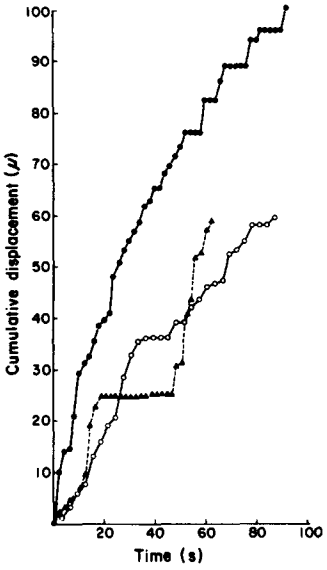

FIGURE 3 Movement of microinjected particles (0.26 μm) as a function of time. The distances traveled by three injected beads are plotted with datapoints at 2-s intervals. Horizontal regions of the curves represent periods of time in which no net displacement of the particle occurred.

Modification of the Surfaces of the Beads Does Not Impair Motility

In an attempt to block nonspecific protein binding sites on the surfaces of the microspheres, the beads were treated in the following ways. The 0.26-μm carboxylated beads could be covalently cross-linked to BSA by use of carbodiimide. The conditions that produced effective cross-linking were determined in pilot experiments using a rhodamine-labeled protein in which the uptake of the protein by the polystyrene beads was monitored by fluorescence microscopy, and the association of the protein with the beads was stable to low- and high-ionic strength washes. Alternatively, the beads were simply incubated with either 50% normal goat serum or rhodamine-conjugated cytochrome *c*. As determined by observation of the beads with fluorescence optics, the particles did adsorb the rhodamine-labeled protein and we assume that the unlabeled serum proteins likewise stuck to the surface of the beads. The labeling of the polystyrene beads with proteins did not perturb their ability to be transported by the tissue culture cells (Table II).

Larger beads (0.5 μm Covaspheres) cross-linked to BSA have also been microinjected into BS-C-1 cells. Covasphere product information indicates that the particles covalently bind protein in a reaction that goes rapidly to completion thereby eliminating nonspecific binding after termination of the reaction. These particles were observed to move in a smaller percentage of the injected cells than were the smaller particles; however, 25% of the cells did translocate the larger beads. The reason for the reduction in motility seen for the 0.5-μm particles is not clear. It is possible that the large size of the beads restricts their motion, but this seems unlikely since aggregates of 0.26-μm beads are frequently seen to be moving in injected cells. Or perhaps the Covasphere surface reduces (without eliminating) the ability of the beads to adsorb

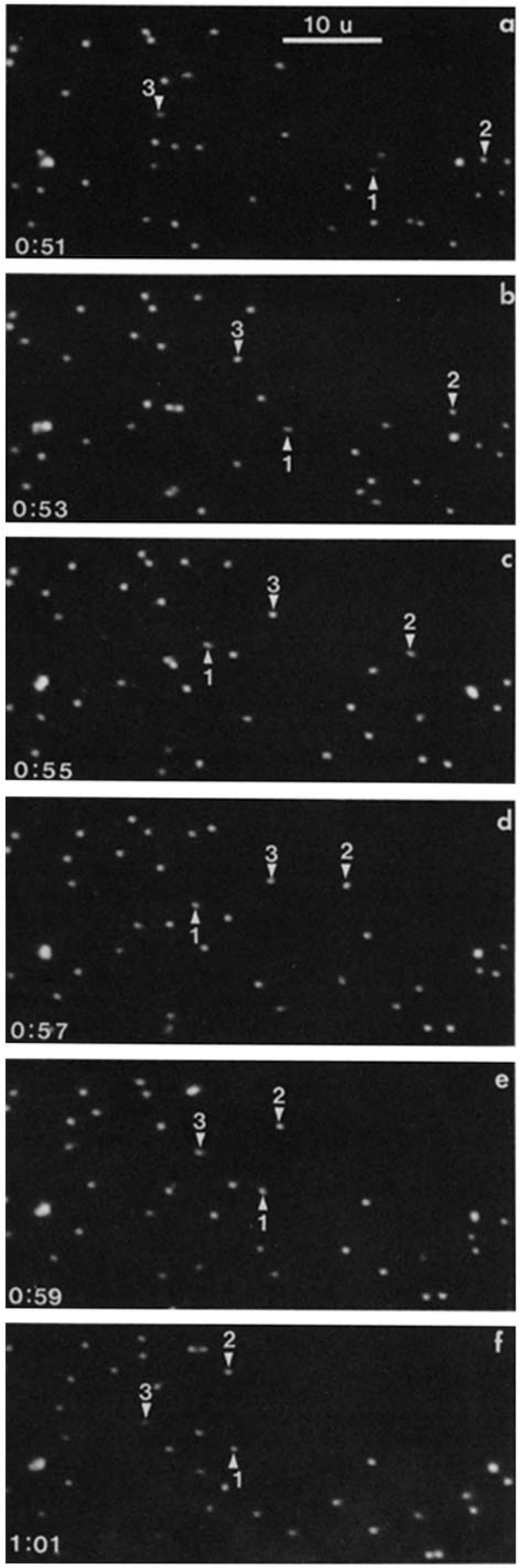

FIGURE 4 Movement of microinjected 0.26-μm beads. Sequential photographs taken from a videotape at 2-s intervals illustrate the motion of the fluorescent particles. Three beads were followed on successive video frames and are labeled with arrowheads for the 10-s period presented here. Note that some of the beads in the field have not moved during this time. Bar, 10 μm.

TABLE II

Nocodazole Reversibly Inhibits the Saltatory Motion of Injected Particles

	Control				Treatment with nocodazole				Recovery from nocodazole			
	No. of cells			Positive for motility‡	No. of cells			Positive for motility	No. of cells			Positive for motility
	+	−	?*		+	−	?		+	−	?	
				%				%				%
Exp 1	82	0	1	99			NA				NA	
Exp 2	157	2	8	94	0	161	10	0			NA	
Exp 3	296	1	3	99	0	302	2	0	239§	3	11	94

NA, not applicable.

* Cells in mitosis.

‡ Cells in mitosis were counted as negatives in determining the percent of motility-positive cells.

§ Only 253 total cells were scored at this stage because one coverslip was lost.

force-generating molecules from the cytoplasm. Alternatively, it is possible that the reduced percentage of motility-positive cells merely reflects the fact that in our hands very few 0.5-μm beads could be introduced into a single cell, thus reducing the probability of detecting bead motion during the observation period. Because of the technical difficulties involved in microinjecting these large particles into cells, 0.26-μm spheres were used routinely.

A Mitotic Inhibitor Blocks the Saltatory Motion of Microinjected Particles

BS-C-1 cells were microinjected with 0.26-μm fluorescent microspheres and were returned to the 37°C incubator. The next day the microinjected cells were identified by fluorescence microscopy and the percentage of cells having motile beads was determined. As shown in Table II, a high percentage (94 or 99%) of the microinjected cells contained motile particles. These cells were then transferred into medium containing 1 μg/ml nocodazole in order to disassemble microtubule arrays. After a 6-h exposure to the drug, the same cells were again scored for bead motility. All saltatory motion had ceased (Table II). Finally, the nocodazole-containing medium was replaced with drug-free medium and the cells were allowed to recover from the microtubule inhibitor. The next day these cells were again observed and most had regained the capacity for saltatory motion (Table II).

Sample coverslips containing microinjected cells were fixed for light microscopy at each of the above steps, that is, after the initial motility of the beads was established, after exposure to nocodazole, and after recovery from the microtubule poison. Cells from each of these categories were prepared for immunofluorescence with antitubulin antibody or stained with NBD-phallacidin in order to visualize the status of microtubules and microfilaments during the experimental manipulations (data not shown). It was clear from the fluorescence images and the data on bead motility for the same cells that with the loss of microtubules came the loss of saltatory motion. Moreover, when the microtubule arrays were restored, particle motion recommenced. Filamentous actin, as visualized with NBD-phallacidin, was not perturbed by the nocodazole treatment. These results provide evidence that nocodazole treatment induces disassembly of microtubules without destroying the integrity of actin filament bundles. However, since this staining technique is probably not adequate to resolve single actin filaments, the possibility that actin is participating in bead motion cannot be eliminated.

HVEM of Microinjected Cells

BS-C-1 cells were cultured on Formvar-coated gold finder grids and were microinjected with 0.26-μm beads. The cells that contained motile beads were identified the next day by fluorescence microscopy and their locations were recorded so they could be identified in the electron microscope. Whole cell HVEM of the injected cells revealed that the particles were enmeshed in fine filaments and they did not appear to be membrane bound. They were sometimes seen in association with microtubules, as in Fig. 5, sometimes with actin cables. We have no way of knowing whether a particular bead was moving at the time of fixation, so an association with a particular cytoskeletal element does not necessarily imply a functional relationship.

DISCUSSION

In this paper we have presented evidence that fluorescent polystyrene beads exhibit saltatory motion when microinjected into cultured cells. The movements of the beads, like those of endogenous organelles, are rapid and are sensitive to the microtubule inhibitor nocodazole. The polystyrene beads are transported continuously and do not appear to have any final destination for at least 6 d after injection (as late as we have looked.) Perhaps this point represents a major distinction between the fate of microinjected nonbiological particles and functional organelles. Whereas an endogenous particle such as a phagosome ultimately fuses with a lysosome and is processed, the polystyrene beads have no targeting information. The behavior of the injected beads in BS-C-1 cells suggests to us that these cells move anything that is not specifically anchored. Moreover, it appears that cytoplasmic order is conferred not at the motility step but at a subsequent step; that is, the particles are moved at random and will continue to be so moved until they reach their specific intracellular receptor. Since no intracellular destination exists for the polystyrene beads, they remain in constant motion.

At first glance it seems that a likely explanation for the constitutive and extensive translocation of injected particles is that the cytoplasm is in constant turmoil and its churning propels the intracellular particles. However, as observed by Rebhun for endogenous organelles (19) and here demonstrated for microinjected polystyrene beads, adjacent particles are independent with respect to their saltatory motion. If intracellular currents were responsible for moving the particles, one would not expect that two immediately adjacent particles would be able to accelerate in opposite directions.

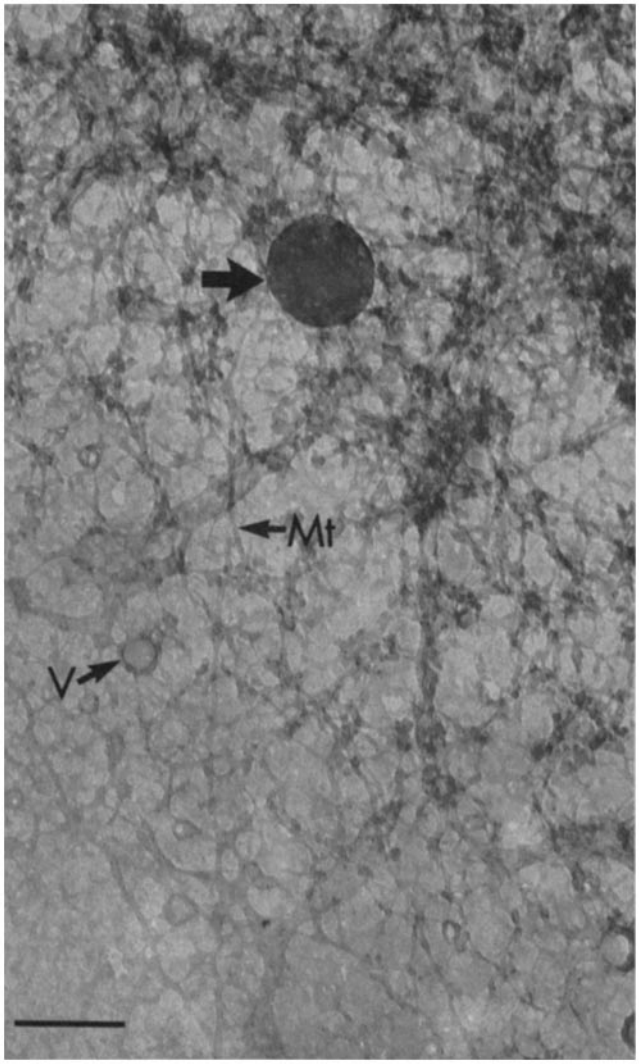

FIGURE 5 A whole-cell HVEM of a region of a microinjected BS-C-1 cell. This cell was observed to be moving the 0.26-μm beads before fixation. A single polystyrene bead is visible in the field (unlabeled arrow). In this case there is a microtubule (*Mt*) running alongside the bead. The polystyrene bead does not appear to be membrane bound. *V*, small endogenous vesicle. Bar, 0.25 μm. × 55,700.

Since no membrane coating of the polystyrene beads was detected by whole-cell HVEM, it is tempting to question the importance of membrane-encoded information in intracellular motility. However, the possibility remains that the polystyrene beads are able to move by virtue of an association with endogenous membrane-bound organelles via a so-called "piggyback" mechanism (2). Although we did not see any consistent association of the beads with vesicles, (neither those resolvable by light microscopy nor those resolvable by HVEM), a detailed electron microscopic analysis would be required to rule out this hypothesis.

An alternative explanation for the motility is that the injected beads adsorb force-transducing molecules to their surfaces and thus obtain motility competency. The idea that cytoplasmic particles could be coated with dynein or myosin enabling them to move along microtubule or actin tracks, respectively, has been proposed. And recent experiments (21) in which myosin-coated polystyrene beads have been shown in vitro to move along the actin cables of *Nitella* internodal cells have demonstrated the feasability of such a model for motility. The likelihood that adsorption of cellular proteins is prerequisite to bead motility is diminished somewhat by the fact that particles precoated with a variety of proteins are motile; but the possibility that a force-transducing molecule is associating with the surfaces of the microinjected beads cannot be eliminated in our experiments. If adsorption of cellular proteins were required before the beads were able to move, this might partly explain the lag time after injection before motion of a high percentage of beads is observed. It should be possible to test the role of specific cytoplasmic proteins (e.g., myosin) in the bead motion by precoating the beads with a purified protein and monitoring the time interval required for acquisition of motility and the average velocities attained by pretreated versus untreated beads.

In the microinjected tissue culture cells, the polystyrene beads, although exhibiting a preference for certain tracks or regions within the cytoplasm, were seen to move in a variety of directions. Certainly they moved freely both toward and away from the cell center. In contrast, polystyrene beads injected into crayfish axons (2) were observed to move only in an anterograde direction. This result suggests that the axon contains a motility system for transport from the cell body toward the axon terminus with which the injected particles are compatible. Further, it may be inferred from the inability of the beads to move toward the nerve cell body that the retrograde translocation system in these axons is somehow distinct from systems in which the polystyrene beads can be assimilated, e.g., the anterograde system of these same axons and the saltatory motility machine of BS-C-1 and Pt K1 cells. From this perspective it is intriguing that microinjected 0.26-μm polystyrene beads do not appear to be translocated with the pigment granules in melanophores from the angelfish *Pterophyllum scalare* (our unpublished observations). In preliminary studies neither the massive aggregation nor dispersion of melanosomes caused significant displacement of the polystyrene beads within the melanophores 1 d postinjection. Although pigment granule dispersion, anterograde and retrograde fast axonal transport, and particle motion in cultured cells can all be described as saltatory, the ability or nonability of polystyrene beads to be carried in each of these cases distinguishes them. One interpretation of these results is that despite very similar appearances, a common molecular motor to drive saltatory motion does not exist. Experimental corroboration of the concept of saltatory movements having distinct mechanisms is provided by work in which classes of saltatory movements in eggs (20) and neurons (11) have been shown to exhibit differential sensitivities to pharmacological agents such as colchicine, cytochalasin B, and erythro-9-(3-[2-hydroxynonyl])adenine.

Although we cannot say at this point how the beads are moving within cells, the fact that they move at all is interesting in itself and provides a novel way to study intracellular transport. The vast majority of studies on intracellular transport have sought to examine the mechanism of motility by looking at the structural supports for motion, the cytoskeletal elements. By perturbation or disruption of these cytoplasmic railroad tracks, the role of various cytoskeletal components in intracellular transport has been probed. With the use of injected polystyrene beads, one can now ask what characteristics define a functional cellular train, that is, what features of a particle confer capacity for motility.

In addition to providing a new perspective for studies on

the mechanism of motility, the system possesses three major advantages. First, no perturbation of normal cytoarchitecture nor cellular events occurs during the experiments. Second, the beads exhibit high intensity fluorescence and they do not photobleach; thus single particles can be followed unambiguously for long periods of time. Finally, the surfaces of the polystyrene particles can be defined experimentally before microinjection. We think that this experimental system complements currently popular approaches for studying intracellular transport.

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