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Analysis of the Role of Microtubules and Actin in Erythrophore Intracellular Motility

MARY C. BECKERLE and KEITH R. PORTER
Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80309.

ABSTRACT The *Holocentrus* erythrophore, a red pigment cell, represents a model system for the study of organized intracellular transport. We have investigated the possibility that microtubules and actin are integral components of the pigment translocating motility machine. By creating cells that have total or partial loss of the microtubule framework we have demonstrated that the presence of microtubules is essential for organized, radial transport of the pigment granules. However, in the absence of microtubules, some undirected movement of the pigment can be stimulated; this suggests that a nonmicrotubular component of the cytoplast is responsible, at least in part, for the generation of motive force. In order to test the hypothesis that this component consists of actin or actomyosin, we examined the effects of probes for these classical motility proteins. Neither microinjection of phalloidin, DNase I or *N*-ethylmaleimide-modified heavy meromyosin nor exogenous application of cytochalasin B has any effect on pigment motion, although these materials do block the actin-mediated motility of other systems in our hands. Therefore, intracellular particle transport in erythrophores does not appear to be actin or actomyosin-based.

The color changes that an organism can undergo in response to environmental stimuli are frequently mediated by the movement of pigment granules within cells called chromatophores. Erythrophores, or red chromatophores, from the squirrelfish *Holocentrus ascensionis* display such pigment granule movement in response to neural or humoral stimulation (6). This cell type represents a unique model system for the study of intracellular motility since it exhibits both saltatory and uniform velocity granule movements. Dispersion of the pigment is characteristically slow and saltatory, and by these criteria is analogous to axonal transport, intra-axopodial particle movements, and some secretory events. Aggregation, on the other hand, occurs with uniform velocity, a feature that it shares with the process of anaphase chromosome segregation, although pigment movement is much faster (16, 21). One advantage of the erythrophore lies in the fact that the saltatory and uniform velocity movements are separated temporally, thus they can be studied as mechanistically distinct processes.

Both erythrophores (6) and melanophores (4, 24, 36) contain radially arrayed microtubules that extend from the cell center to its margin, establishing channels within which the pigment granules course. Numerous investigators have attempted to define the role of these microtubules in pigment granule transport. Disruption of melanophore microtubules by a variety of means affects motility (e.g. 15, 24, 33) suggesting that these structures are required for pigment migration, but possible nonspecific (i.e., nonmicrotubule) effects of these treatments cannot be eliminated as the cause of the decline in motility noted in these studies. For example, it has been reported that both lumicolchicine *and* colchicine alter aggregative motility in melanophores (26). In this paper we present evidence that an intact microtubule framework is required for organized pigment motion in erythrophores.

Nevertheless, some slow movement of pigment granules can be stimulated in melanophores in the absence of microtubules, suggesting that nonmicrotubular components can generate motive force in these cells (26, 35). Because actin is involved in a wide variety of nonmuscle motile events (18, 25, 28, 40) it represents a prime candidate for participation in pigment granule transport. Actin microfilaments have been identified in melanophores by heavy meromyosin decoration (27, 37), but their role in pigment migration remains controversial. Obika and co-workers (27) report an increase in the number of decoratable filaments in aggregated *Fundulus* melanophores and suggest, as do other investigators (reference 1, for swordtail erythrophores) that these filaments are involved in the motility function. Other data, however, indicate that the filamentous actin present in melanophores is largely confined to the cell

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cortex and microspikes and may not be in a position in which it could facilitate granule movement (37).

In addition to the microtubules and actin filaments, erythrophores possess a lattice of fine filaments (microtrabeculae) in which the pigment granules are suspended (6). Contraction and extension of this microtrabecular network is thought to propel the pigment granules through the cytoplasm (6, 29). Although the biochemical composition of these filaments has not been determined, it is plausible that they consist of actin and associated proteins (16).

Detailed analysis of the motility mechanism(s) of erythrophores has thus far been hindered by the fact that intact biological membranes are impermeable to the probes generally used to assess the involvement of classical motility proteins such as actin and myosin. In addition to our attempts to clarify the role of microtubules in these cells, this paper describes the use of microinjection to gain access to the cytoplasm and examine the possible involvement of actin or actomyosin in the intracellular transport of pigment granules in erythrophores.

MATERIALS AND METHODS

Experimental Materials

The microtubule inhibitor nocodazole was purchased from Aldrich Chemical Co. (Milwaukee, WI). Stock solutions of colchicine and nocodazole were prepared in H_2O and DMSO respectively and stored at 0°C until use. Phalloidin was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). DNase I (essentially RNase-free, specific activity >3,000 U/mg) was from Worthington Biochemical Corp. (Freehold, NJ). N-ethyl maleimide-modified heavy meromyosin (NEM-HMM) was a generous gift of Zac Cande (University of California, Berkeley) and was handled as described previously (22). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO). A 10 mg/ml stock solution of cytochalasin B in DMSO was diluted with teleost culture medium (16) before use.

Preparation of Cells

Erythrophores from the squirrelfish, *Holocentrus ascensionis*, were cultured by slight modification of the method described by Luby and Porter (16). For light microscopy, the cells were allowed to settle on carbon-coated, glow-discharged glass coverslips. Cells to be used for whole mount high voltage electron microscopy (HVEM) were cultured on gold finder grids (Ernest F. Fullam Inc., Schenectady, NY), fixed, and critical-point-dried by the methods of Byers and Porter (6) and Wolosewick and Porter (45).

Indirect Immunofluorescence

Erythrophores grown on carbon-coated, glow-discharged coverslips were fixed in a microtubule stabilizing buffer containing 0.1 M Na-PIPES, pH 7.3, 1 mM $MgCl_2$, 1 mM EGTA, 0.1% glutaraldehyde and 1.6% paraformaldehyde for 20 min at 23°C and washed in teleost PBS (16). The cell membrane was permeabilized with 0.2% Triton-X-100 in teleost PBS for 2 min; the cells were then washed for 30 min with three changes of teleost PBS. Preimmune serum and affinity-purified antibody prepared against sea urchin egg tubulin were a generous gift of Keigi Fujiwara (Harvard Medical School, Boston, MA) and were characterized as described previously (10). Subsequent to permeabilization the cells were prepared for indirect immunofluorescence by established procedures (42). Photomicrographs were taken with a Zeiss epifluorescence microscope kindly shared with us by Peter Hepler (University of Massachusetts, Amherst, MA).

Microinjection Procedure

THE MICROINJECTION APPARATUS: The cells were pressure microinjected by modification of the method of Graessmann and Graessmann (12). Coverslips containing the cells were placed in an 18 mm plastic petri dish (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, CA) and positioned on the stage of a Zeiss microscope equipped with a long working distance condenser and a 40X water immersion Zeiss objective (NA 0.75). Glass micropipettes having a tip outside diameter of ~0.5 μm were drawn on a Brown-Flaming micropipette puller (Sutter Instruments, San Francisco, CA) from borosilicate capillaries having internal filaments (W-P Instruments, Inc., New Haven, CT). The micropipettes were back-loaded by inserting the blunt end into a tube containing a small aliquot of the material to be injected.

Once loaded, the micropipettes were secured in a Leitz microtool, connected to a 50-ml disposable syringe via polyethylene tubing, and oriented for injection in a Leitz mechanical micromanipulator. Gentle pressure applied to the syringe resulted in a flow of material from the tip of the micropipette.

MICROINJECTION OF CELLS: Experimental materials were injected into cells in a solution of 10% sucrose at pH 7.0. (Sucrose was used because the erythrophores did not tolerate a host of typical injection buffers including HEPES, PIPES, and K^+-citrate-glutamate). A pH of 7.0 was obtained by dialyzing the material to be injected against large volumes of 10% sucrose at pH 7.0 for 60 min with at least one change of solution. For NEM-HMM, 0.1 mM DTT was included in the injection buffer. The injection medium also contained fluorescein isothiocyanate-labeled bovine serum albumin (FITC-BSA), which enabled us to recognize unequivocally a successfully injected cell. The BSA (10 mg/ml) was labeled with FITC at pH 9.0, then dialyzed extensively against physiological saline, pH 7.0. The labeled protein was dialyzed against injection buffer immediately before use. The ability of the NEM-HMM to inhibit actomyosin was determined in part by its application to glycerinated myofibrils. This myofibril contraction assay has been described by Meeusen and Cande (23). Glycerinated rabbit psoas muscle was purchased from Carolina Biological Supply Co. (Burlington, NC).

CALIBRATION OF NEEDLES: The approximate volume of material introduced into the erythrophores by microinjection was determined by optically measuring the volume of oil droplets (assumed to be spherical) injected into the cells. The average volume injected into the cells equals 7×10^{-14} liters, or ~7% of the cell volume. Although this clearly represents an estimate of injected volume, the values obtained are comparable to those determined for injection of tissue culture cells by other techniques (38; Zavortink and McIntosh, unpublished results).

RECORDING OF EXPERIMENTS: Experiments were recorded with the aid of a Venus DV2 video intensification camera (Venus Scientific Inc., Farmingdale, NY) and a Sony one inch video tape recorder. Erythrophores injected with the "injection buffer" (10% sucrose, pH 7.0, 1–10 mg/ml FITC-BSA) retained viability and capacity to translocate pigment granules for at least 4 h. Cells injected with experimental materials were monitored for 2 h.

RESULTS

Microtubules are Essential for Organized Pigment Translocation

The radial microtubule framework of the erythrophore was visualized by indirect immunofluorescence using antitubulin antibody. The results of this labeling demonstrate the remarkable linearity and continuity of microtubules from the cell center to the cell periphery. Cells fixed in the aggregated and dispersed states show no apparent differences in magnitude or distribution of the microtubule cytoskeleton (Fig. 1 *a–d*). Exposure to 5×10^{-5} M colchicine or 10^{-6} M nocodazole induces depolymerization of the microtubules; loss of the structural integrity of the microtubule network occurs sequentially from the cell periphery toward the centrosphere. Radial saltations of the granules when the cell is in the dispersed state (shuttling), as well as aggregations and dispersions of the pigment mass, are restricted to regions of the cytoplasm where microtubules persist. After a 30 min exposure to either of the above-mentioned mitotic inhibitors the microtubules are depolymerized ~30%, and the pigment motion is coincident with the region of polymerized microtubules (Fig. 1 *e–f*). Incubation of cells with preimmune serum followed by fluorescein isothiocyanate-labeled goat anti-rabbit IgG (FITC-GAR) (Fig. 1 *g–h*), FITC-GAR only, or antitubulin followed by unlabeled GAR and finally FITC-GAR showed only background levels of staining. This work was done in collaboration with H. Randolph Byers (Harvard Medical School) and Keigi Fujiwara.

In order to examine the role of an intact microtubule framework in pigment migration, mitotic inhibitors were used to create cells with partial to total loss of the microtubule cytoskeleton. The pigment can be maintained in the dispersed state by caffeine while the microtubules are depolymerized by colchicine or nocodazole. If the microtubule framework of such

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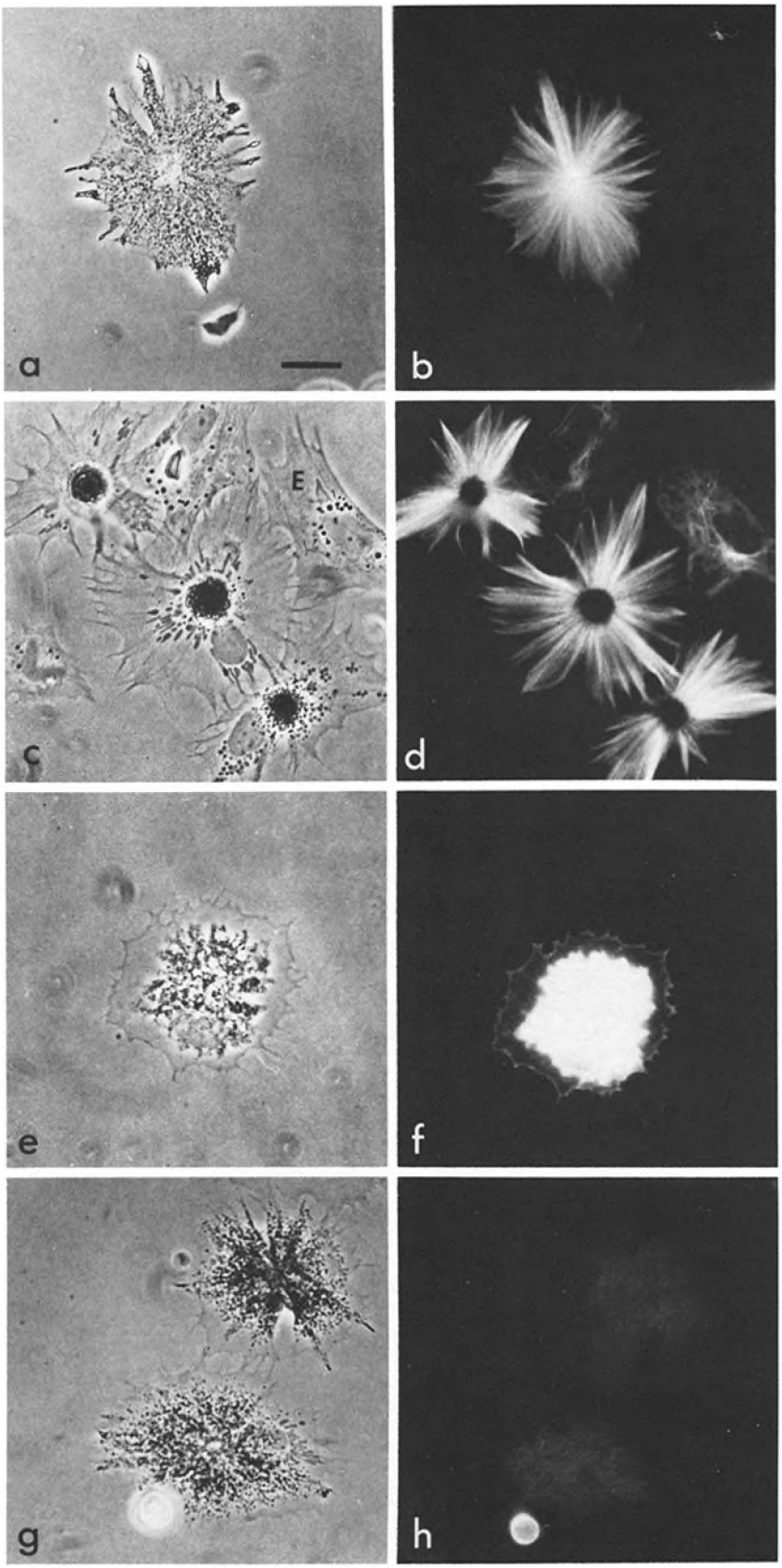

FIGURE 1 Phase contrast and indirect immunofluorescence images of cultured erythrophores stained with antitubulin antibody. In both dispersed (*a* and *b*) and aggregated (*c* and *d*) erythrophores, a prominent, radially organized array of microtubules is visible. As the microtubules are partially depolymerized by a 30-min exposure to 1 μM nocodazole (*e* and *f*), the migrations of the pigment are restricted to the central region of the cell where microtubules persist. Note the coincidence of the pigment granules seen in *e* and the fluorescently stained microtubules seen in *f*. Red autofluorescence of the pigment granules accounts for the nonfilamentous appearance of the fluorescein signal in *f*. Control cells incubated with preimmune serum (*g* and *h*) show no specific staining. *E*, epithelial cell. Bar, 10 μm. × 850.

a cell is depolymerized up to ~30%, and the cell is then stimulated to aggregate with epinephrine, the pigment granules in the central region of the cell, where microtubules persist, follow a radial course toward the cell center; a ring of pigment granules is left behind in the cell periphery where the microtubules have already disassembled (Fig. 2*a*). The microtubules that remain after such treatment with inhibitor extend from the centrosphere up to the region of the cell periphery where immobile granules are situated (Fig. 2*b*). The ability of the granules to move normally in regions of the cell where micro-

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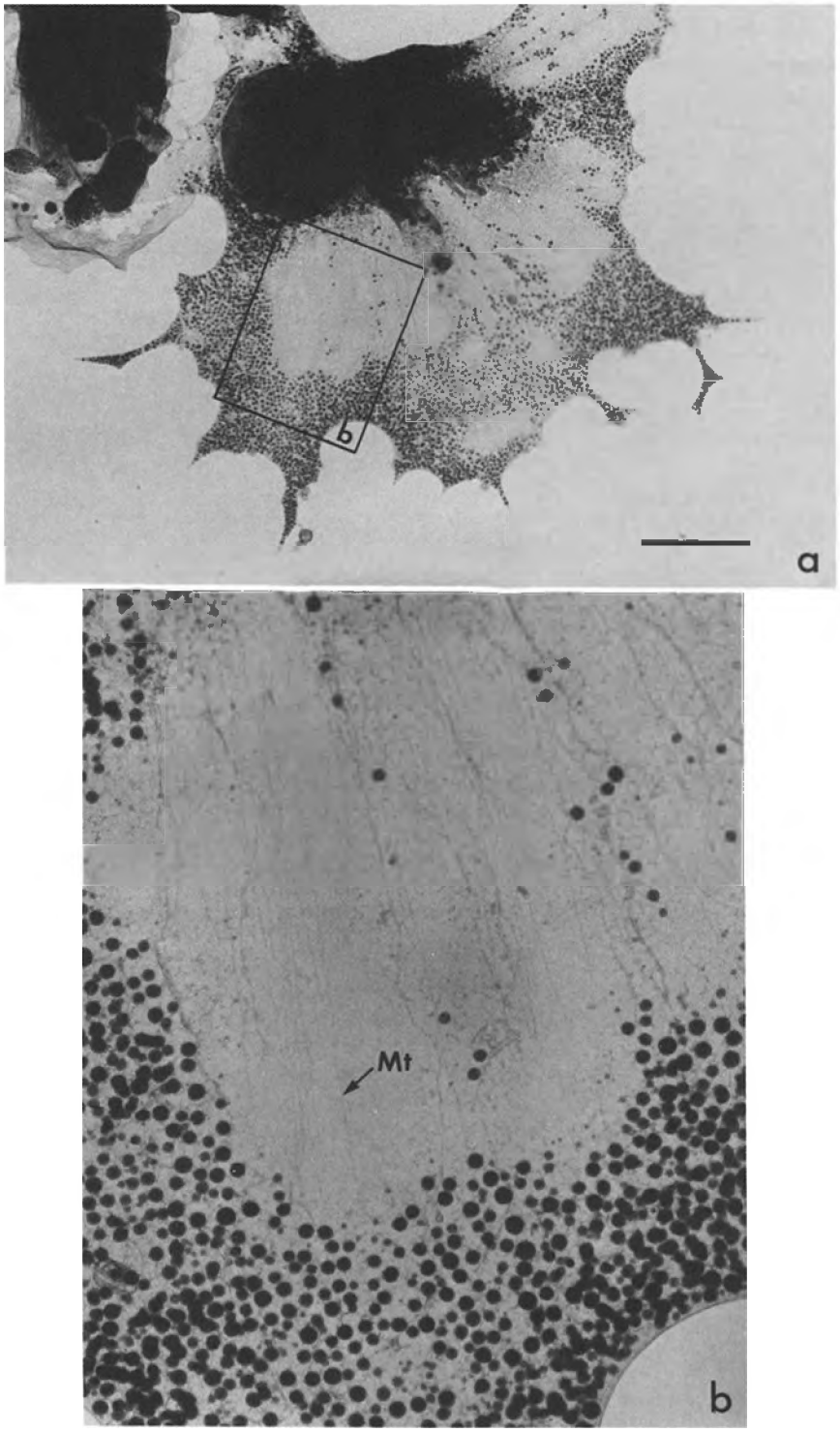

FIGURE 2 (*a*) A low magnification high voltage electron micrograph of a whole cell incubated with 1 μM nocodazole plus 5 mM caffeine for 30 min and then stimulated to aggregate its pigment with epinephrine. Only the centrally located granules aggregate, leaving a band of randomly distributed granules in the periphery. (*b*) A high magnification view of the blocked region in *a*, illustrates that numerous microtubules (*Mt*) extend up to, but not into, the region where immobile granules remain. The pigment granules are capable of normal radial movement only in the region where microtubules remain assembled. Bar, 6 μm. *a*, × 2,500. *b*, × 11,000.

tubules have not yet depolymerized serves as an internal control for any nonspecific or toxic effects of the colchicine or nocodazole.

Without the dispersive stimulation of caffeine the granules continue to aggregate and disperse during the treatment with a microtubule inhibitor; however, the extent of the excursions of the granules from the cell center declines as the microtubules depolymerize. Light microscopic analysis of single erythrophores processed subsequently for electron microscopy clearly demonstrates that granule transport occurs only where the microtubule framework remains intact.

Microtubule-independent Pigment Movement

The microtubule framework of an erythrophore can be depolymerized completely by exposure to 10^{-6} M nocodazole for 6 h, or by exposure to this same concentration of drug for 90 min, the first 15–20 of which are at 4°C. All microtubules are disassembled as determined by immunofluorescence and HVEM. Under these circumstances, the cells retain their shape while the pigment granules lose their radial alignment and appear to be randomly distributed throughout the cytoplasm (Fig. 3*b*). The normal radial distribution of pigment is seen in

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Fig. 3*a*. Stimulation of microtubule-depleted cells with epinephrine for 1 min has no apparent effect on the distribution of the pigment granules, whereas control cells aggregate immediately. However a 15-min exposure induces local clumping of the granules in the nocodazole-treated cells (Fig. 3*c–d*; *c* represents 95% of the cells, *d* the remaining 5%). This redistribution of the pigment is not seen in unstimulated cells, nor in cells that have intact microtubules. Our interpretation of the images shown in Fig. 3*c* and *d* is that at least some of the motive force required for aggregation is effective in the absence of microtubules, but the cell has lost its sense of direction. The cell in Fig. 3*d*, like the one seen in *c*, was discoid in shape before exposure to epinephrine. The morphology induced by the stimulus to aggregate seems to result from many randomly directed, regional contractions of the cytoplast.

Although this experiment illustrates the occurrence of some microtubule-independent movement of the pigment, no normal, centripetal translocation of the pigment granules takes place in the absence of the microtubules. Epinephrine has not been observed to induce complete aggregation in cells depleted of microtubules.

Is Actin or Actomyosin Involved in Pigment Migration?

As described above, when the microtubule framework of an erythrophore is depolymerized by treatment with nocodazole, the cell retains the capacity to move the pigment granules in response to epinephrine although this movement is not radially organized. Similar microtubule-independent movement of pigment has been described in melanophores (26, 35). These results suggest that some nonmicrotubular cytoskeletal element is responsible, at least in part, for the development of the motive force. Because of the widely held view that actin and myosin are involved in nonmuscle motile systems and the evidence linking these molecules to chromatophore motility, we examined the possibility that these proteins are important in erythrophore pigment granule translocation.

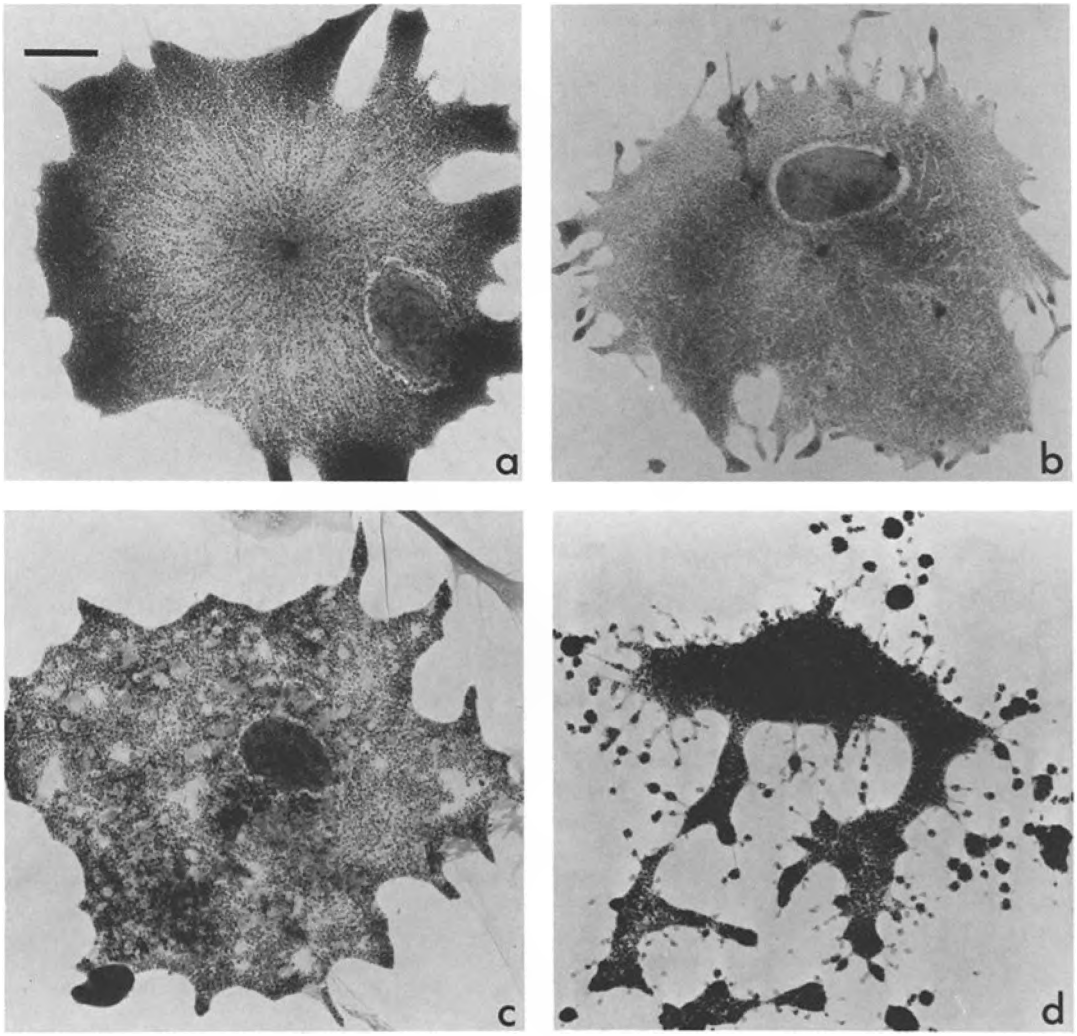

FIGURE 3 (*a*) A high voltage electron micrograph of an erythrophore in the dispersed state. Note the radial organization of the pigment granules. (*b–d*) These erythrophores were treated with nocodazole and 5 mM caffeine to depolymerize the microtubules while leaving the pigment in the dispersed state. After such a treatment, the pigment granules lose the radial arrangement seen in *a* and are randomly distributed within the cell (*b*). When such a microtubule-depleted cell is stimulated to aggregate its pigment with epinephrine (*c–d*), the granules are redistributed and appear to be clumped. The cells contain pigment-rich and pigment-poor regions. ~5% of the cells exhibit the morphology seen in *d*, the remainder that in *c*; before exposure to epinephrine all cells monitored were approximately discoid in shape. In the absence of microtubules, the granules can move to some extent when stimulated to do so, but there appears to be no directional information. Bar, 4 μm. × 2,250.

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CYTOCHALASIN B: Cytochalasins have been shown to inhibit a variety of actin-based motility events (41). Treatment of erythrophores with cytochalasin B at 10 μg/ml in teleost culture medium results in a characteristic and dramatic cytoplasmic retraction; however, the microtubules remain intact and pigment granules are competent to aggregate and disperse (Fig. 4 *a* and *b*). After 1 h of exposure to the drug, the cell is highly arborized, but recovers its discoid shape if the cytochalasin solution is replaced with fresh medium. The morphological changes evident with cytochalasin B treatment indicate that the agent is biologically active, however no effect on intracellular motility was noted.

Although cytochalasin B affects numerous actin-mediated events in vivo (41), the possibility remained that a class of motility-related erythrophore actin exists in a form inaccessible to the drug. Therefore, the effects of a variety of other probes for actin were examined.

There are two additional probes that specifically perturb actin structure and function: DNase I, which disrupts filamentous actin (13), and phalloidin, which stabilizes it (44). Because these do not readily penetrate biological membranes, their effects on pigment movement were examined by microinjecting the erythrophores. The possibility that actin and myosin, acting in concert, are the basis of granule motility was examined by microinjection of the specific actomyosin contraction inhibitor, *N*-ethylmaleimide-modified heavy meromyosin (NEM-HMM). The results of these microinjection studies as well as control experiments are described in what follows.

THE RESPONSE OF ERYTHROPHORES TO CONTROL INJECTIONS: Erythrophores microinjected with $\sim 7 \times 10^{-14}$ l of injection buffer (that is, FITC-BSA in 10% sucrose at pH 7) aggregate their pigment granules in response to the microinjection needle, but redisperse the pigment and regain normal granule movements within 3 min (Fig. 5 *a*). Within 10 s of the injection the entire cell is brightly fluorescent, illustrating that the injected material is distributed uniformly in the cells. To test a cell's ability to undergo a complete aggregation and redispersion, the injected erythrophore was touched with a microneedle. This manipulation stimulates the cell to aggregate its pigment and the granules then redisperse. The ability of the cells to shuttle granules, that is, to move them in short radial saltations while the cell is in the dispersed state, was also monitored. Cells competent to aggregate, disperse, and shuttle pigment after injection were scored as "recovered." The buffer-injected erythrophores were observed for up to 4 h; neither a decrement in pigment granule movement nor a change in cell shape was detected during this time.

MICROINJECTION OF DNASE I: DNase I binds monomeric actin stoichiometrically and causes depolymerization of f-actin (13). When it is injected into erythrophores at either 10 or 40 mg/ml, it produces a characteristic change in the morphology of the erythrophore (Fig. 5 *b*). When introduced into the cells at 10 mg/ml, the cells begin to retract within 60 min; the cells injected with DNase I at 40 mg/ml acquire altered morphology within 30 min. Despite this DNase I-induced change in cell shape, there is no effect on the cells' ability to translocate pigment even after 2 h (Table I). The effect of DNase I on erythrophore morphology is not due to the high protein concentration of the injected material since comparable concentrations of FITC-BSA or peroxidase do not alter cell shape.

Injection of the 10 or 40 mg/ml solutions of DNase I into motile *A. proteus* does induce an alteration in the streaming pattern (data not shown; 43). The cytoplasm of the amoeba flows into the region where the cell was injected, and eventually streaming ceases as the cell detaches from the substrate. After injection of a lower concentration of DNase I (1 mg/ml), a brief alteration in streaming is noted, but normal movement is

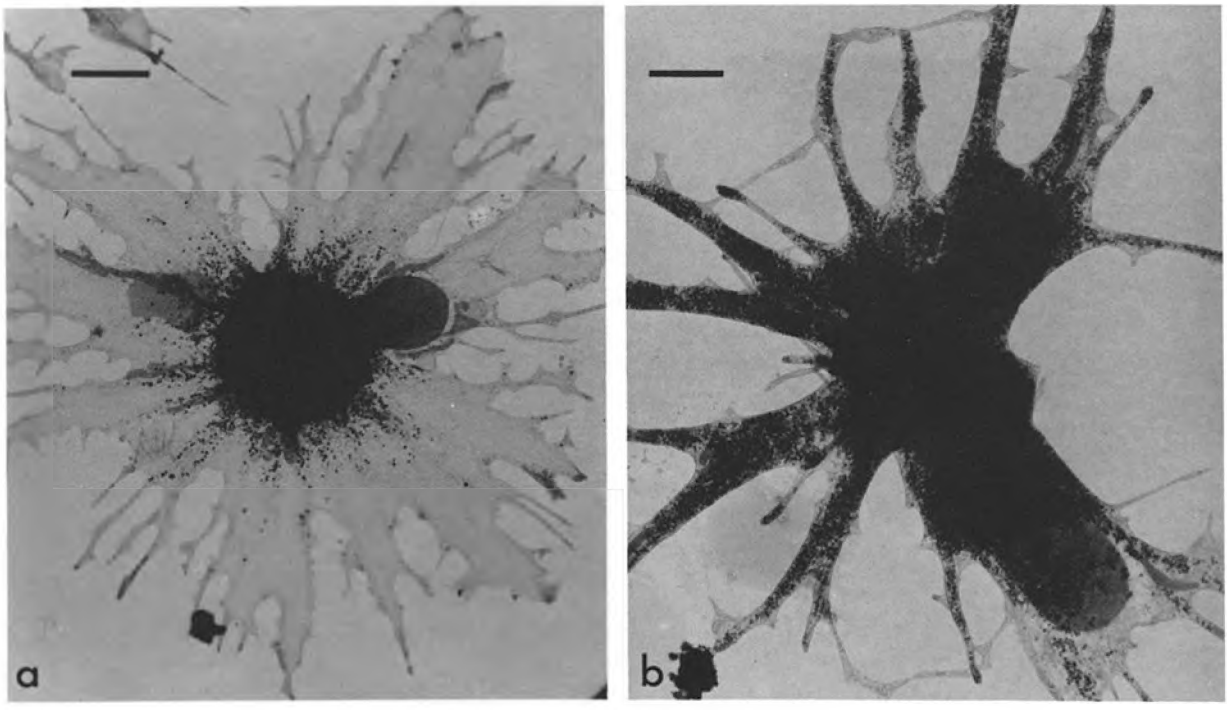

FIGURE 4 High voltage electron micrographs of erythrophores treated with the actin disrupting agent cytochalasin B at 10 μg/ml for 60 min. This treatment induces dramatic retraction of the cell, but the pigment remains competent to aggregate (*a*) and disperse (*b*) within the cytoplasmic arborizations. The cells recover their normal shape when returned to cytochalasin-free medium. Bars: *a*, 4 μm; *b*, 3 μm. *a*, × 2,500. *b*, × 3,300.

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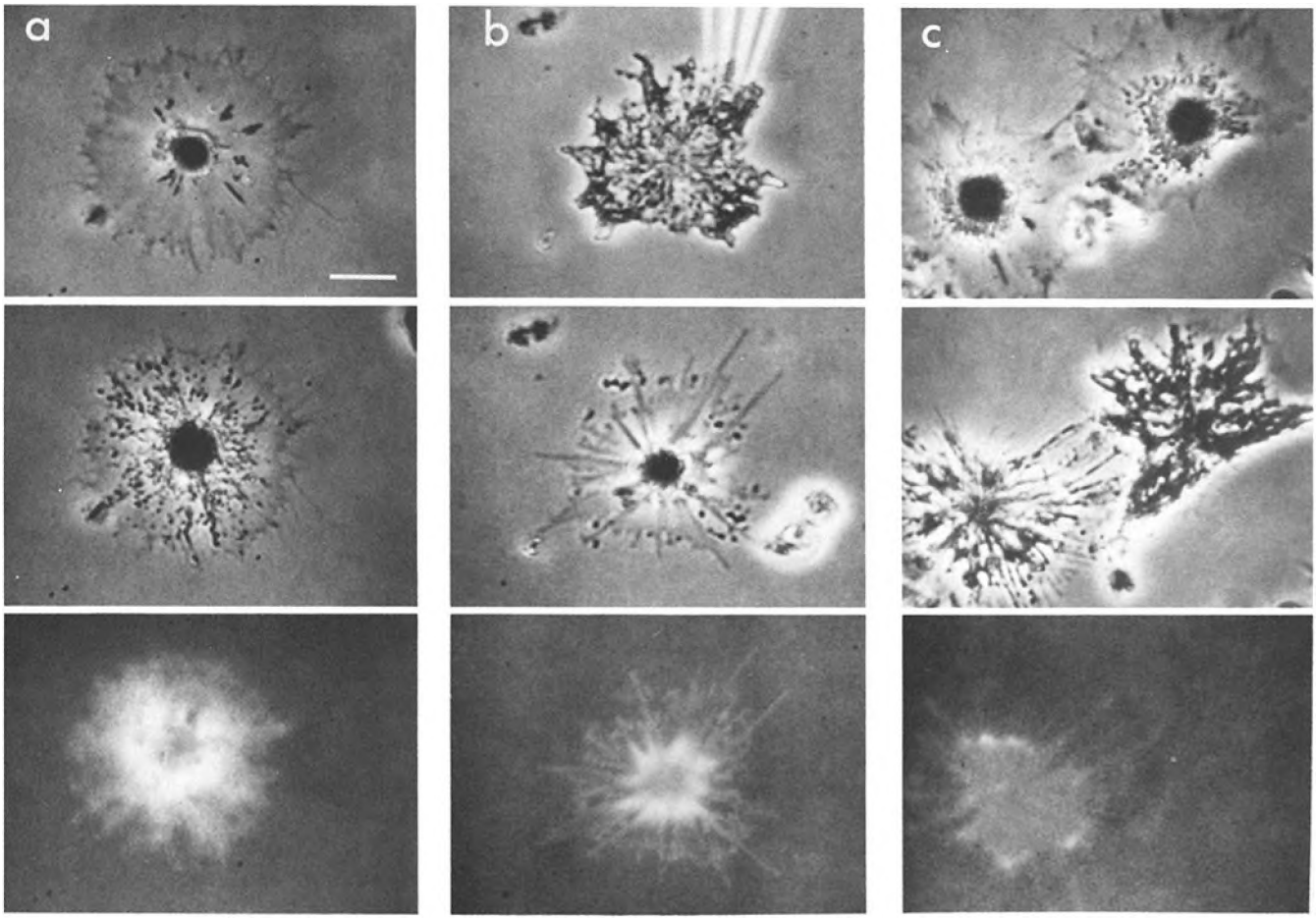

FIGURE 5 The effects of microinjection of actin perturbing agents. Panel (*a*) Phase image of a control cell before injection (*top*), 30 min after injection with injection buffer (*middle*), and a fluorescence image demonstrating that the cell has been successfully injected (*bottom*). There is no change in cell shape and the pigment granule motility is unaffected. Panel (*b*) Phase image of an erythrophore before injection with DNase I (40 mg/ml) (*top*). The micropipette is seen at the top of the field. Within 30 min postinjection (*middle*), the cell has retracted, although it translocates pigment normally. A fluorescence image (*bottom*) shows that the cell contains FITC-BSA and therefore has been microinjected. Panel (*c*) Phase image of two uninjected erythrophores (*top*). The middle micrograph shows the same cells 15 min after the cell on the left was injected with 1 mM phalloidin. Note the numerous thin cellular extensions from the periphery. Compare this cell with the uninjected erythrophore on the right. The uninjected cell retained its shape as seen in the top (preinjection) micrograph. A fluorescence image (*bottom*) demonstrates that the cell on the left was injected; the cell on the right was not injected. Bar, 9 μm. × 1,000.

TABLE I

Microinjection of Probes for Actin and Actomyosin

	Concentration in the micropipette	No. of cells injected	No. of cells recovered
	mg/ml		
DNase I	10	35	34
	40	16	15
Phalloidin	0.8 (1 mM)	25	24
NEM-HMM	2.5	59	58

restored within a few minutes. Injection of the erythrophore injection buffer alone only transiently disrupts normal streaming and cell attachment.

The fact that the DNase I at 10 mg/ml perturbs the actin-based motility of *A. proteus* demonstrates that it is functional in the erythrophore injection buffer. Yet it fails to affect pigment granule transport in the erythrophore even at a four-fold higher concentration.

MICROINJECTION OF PHALLOIDIN: Phalloidin, an actin-specific phallotoxin, binds f-actin, promotes polymerization of actin filaments, and stabilizes these filaments against depolymerization by cytochalasin B and DNase I (44). Erythrophores injected with 1 mM phalloidin do not lose the ability to translocate pigment granules (Table I). However, within 10 min of the injection, the injected cells begin to display an altered morphology. Although still actively translocating pigment granules, the cell margins retract and many long filapodia extend from the cell periphery (Fig. 5*c*). Within 1 h post injection, these surface protrusions reach a length approximately equivalent to ½ the original cell radius.

As previously described (39), injections of 1 mM phalloidin into *A. proteus* induce rapid retraction of cytoplasm into a central mass with a concomitant and permanent separation of hyalo- and granuloplasm and cessation of streaming (data not shown). With somewhat lower concentrations (0.1 mM), the morphological change accompanying the injection is not so dramatic, and the amoebae recover normal streaming capability within ~10 min.

MICROINJECTION OF NEM-HMM: NEM-HMM competes with native myosin for binding sites on actin filaments and is

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not released in the presence of MgATP (23). As a result of this specific association with actin, NEM-HMM serves as a high fidelity probe for actomyosin-based motility events. It has been shown to inhibit teleost retinal cone shortening (28) as well as cytokinesis in vivo (22).

NEM-HMM in the erythrophore injection buffer inhibits contraction of glycerinated myofibrils, and when injected into PTK-2 mitotic cells at ~2.5 mg/ml, blocks cytokinesis and thus increases the percentage of binucleate daughter cells (46). However, when this same biologically active NEM-HMM is microinjected into erythrophores, no effect on the intracellular transport of pigment granules was noted in 58 out of 59 cells (Table I). After all injections had been completed, the NEM-HMM was again assayed for ability to inhibit myofibril contraction, and in all cases the NEM-modified protein had remained active in the injection buffer.

DISCUSSION

The Role of Microtubules in Pigment Granule Motility

As demonstrated above, the radially organized transport of pigment granules requires the presence of intact microtubules. It has been postulated that chromatophore microtubules generate motive force by undergoing cycles of depolymerization and repolymerization (32, 34), however our data and those of Murphy and Tilney (24) conflict with such a model. If cycles of microtubule disassembly and assembly were required for normal pigment motion, we would not expect the cells to be able to aggregate and disperse the pigment repeatedly in the presence of colchicine or nocodazole (see Results) since microtubule reassembly presumably could not occur.

Further evidence that an alteration in the polymerization state of tubulin is not prerequisite to pigment motion is provided by our recent experiments with the membrane-permeable, microtubule-stabilizing agent, taxol (20, 31). When erythrophores are incubated with taxol (30 μg/ml) for 48 h, the cells remain competent to shuttle, aggregate and disperse their pigment granules (our preliminary results). Thus, even if microtubules do disassemble with the aggregation of pigment (34), such disassembly is not prerequisite to motility in erythrophores.

The question remains—do the microtubules participate directly in motive force generation or do they merely provide passive structural support for the motion? Edds (9) has reported that an artificial axopod made with a glass needle can support normal particle motion in *Echinosphaerium*, suggesting that microtubules, *per se*, are not essential for intra-axopodial transport. In chromatophores, however, where microtubules are not required for maintenance of cell shape, these structures may play a more direct role in motility. Near neighbor analysis has demonstrated that melanophore pigment granules exhibit a strong tendency to reside adjacent to microtubules (24), thus providing circumstantial evidence for the direct involvement of microtubules in transport. Moreover, the fact that two inhibitors of dynein ATPase activity block saltatory pigment granule motion in erythrophores (3) and melanophores (8) suggests that dynein-microtubule interactions may be important in motive force generation in chromatophores.

The Role of Actin or Actomyosin in Pigment Granule Motility

None of the probes used in our experiments to perturb actin or actomyosin function had any effect on erythrophore pigment movement when microinjected or, in the case of cytochalasin B, when applied exogenously. Because there can be numerous uninteresting explanations for a negative result, we have been cautious to insure that the injected probes were indeed biologically active in the injection buffer and that the concentrations used would have been adequate to yield inhibition of motility if actin or myosin were involved in granule movement. In the cases of cytochalasin B, phalloidin, and DNase I, the erythrophores underwent predictable changes in cell shape as a consequence of exposure to the actin-perturbing agents, an indication that the probes were active when injected.

Moreover, when either phalloidin or DNase I in erythrophore injection buffer was injected into actively streaming *A. proteus*, the actin-dependent cytoplasmic streaming and locomotion of the amoebae were inhibited, whereas neither had any effect on pigment granule movement when injected into erythrophores. Clearly, then, any lack of inhibition of granule movement observed in these experiments is not due to inactivation of the probes in the injection buffer or to some nonspecific adsorption of the phalloidin or DNase I to the FITC-BSA. Both probes remained biologically active—yet even at a concentration fourfold higher than that required to stop amoeboid movement, the DNase I failed to affect pigment migration. Similarly, in the erythrophore injection buffer, the NEM-HMM inhibited both contraction of glycerinated myofibrils and cytokinesis in PTK mitotic cells (46), whereas it had no effect on pigment motion.

It could be postulated that these probes are not affecting actin-mediated granule movement because the erythrophore actin is not present in the classical g or f forms, or because it is masked by other proteins and is thus inaccessible to the probes. Admittedly, one cannot eliminate the possibility that erythrophores utilize a unique form of actin in translocating their granules. However, among the numerous nonmuscle cells that have been shown to require actin for motility, there is certainly no precedent for such a nontraditional actin.

The possibility that actin is masked by proteins and is therefore inaccessible to probes is diminished by the fact that cytochalasin B, phalloidin and DNase I presumably bind to different sites on actin (2, 5, 19); it seems unlikely that all of these sites are masked by associated proteins. Certainly, this would not be a factor in the NEM-HMM experiments since actin and myosin normally go through cycles of dissociation and reassociation to generate force. The pigment granules continue to move normally after NEM-HMM injection, presumably providing ample opportunity for the injected actomyosin inhibitor to locate its binding site on actin. These probes for actin and actomyosin have been shown to inhibit a diverse set of motility events including cytokinesis (7, 22), some forms of axoplasmic transport (11, 14), and cytoplasmic streaming (25, 39, 43). It seems improbable to us that none of them would be successful in affecting pigment transport if it were actin- or actomyosin-based.

The level of our analysis of cells after injection does not enable us to detect subtle changes in the pigment granule movement, such as slight enhancement or retardation of the speed of translocation. However, it is clear that the pigment cell remains competent to aggregate, disperse, and shuttle granules even after lengthy exposure to probes for actin and myosin involvement in the transport mechanism.

How Do the Pigment Granules Move?

Microtubules are required for organized radial transport of erythrophore pigment granules and the mechanism by which

microtubules facilitate transport may involve a dyneinlike molecule (3). Nevertheless, some motility machinery apparently persists in the absence of microtubules. Since neither actin nor actomyosin appears to be required for intracellular transport, the molecular identity of the machine becomes more obscure. At present, the only other well-characterized motility-related molecule that remains a candidate is the calcium-sensitive, contractile protein of the *Vorticellid* spasmoneme (30). Although there is no direct evidence implicating spasmin in granule movement, the fact that the erythrophore motility machinery is calcium sensitive (17) and apparently contractile (6) is consistent with such a proposal.

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