Firm Structural Associations between Migratory Pigment Granules and Microtubules in Crayfish Retinula Cells

EUGENIO FRIXIONE

Department of Biology, University of Pennsylvania, Philadelphia, Pennsylvania 19104. Dr. Frixione's present address is Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN, Apartado Postal 14-740, México 14, D.F. 07000.

ABSTRACT The morphology of associations between mobile pigment granules and microtubules of the crayfish retinula cells was examined with transmission electron microscopy. Many pigment granules were found associated with microtubules through linkages of fuzzy appearance in thin sections. The linkages were revealed as discrete strands of variable shape in rotary-shadowed replicas of freeze-fractured and deep-etched specimens. The only feature of constant morphology among these connections consisted of 2–4-nm filaments projecting laterally from the microtubules.

The firmness of the pigment granule-microtubule associations was judged by their ability to hold up during cell disruption procedures of increasing disaggregation effects in a low-Ca^{++} stabilization buffer. The results of these tests were inspected with scanning electron microscopy and with transmission electron microscopy of negatively stained preparations. Numerous pigment granules remained associated with a stable microtubule framework after the plasma membrane had been stripped away. Moreover, granule-microtubule attachments survived breakdown of this framework into free fascicles of microtubules. The pigment granules were associated with the free microtubules either individually or as clusters entangled in a fibrous material interwoven with 10-nm filaments.

These findings attest that many pigment granules are bound to microtubules through linkages that constitute effective attachments. Further, it is demonstrated that a highly cohesive substance associates the pigment granules with one another. These conclusions are discussed in terms of a pigment transport mechanism in which a network of interconnected granules would establish firm transient interactions with a supporting skeleton of microtubules.

The mechanisms that mediate vectorial transport of intracellular organelles constitute a persistently obscure question in cell biology. With few exceptions, it is generally accepted that microtubules are somehow involved in the organelle translocation process (for reviews, see references 9, 20, 41). Support for this contention derives from three main lines of evidence: (*a*) presence of microtubules, often numerous, oriented parallel to the direction of movement; (*b*) close proximity or visible connection between the transported organelles and microtubules; (*c*) disturbance of the movement by agents or conditions known to affect microtubules. Yet, the precise role of the microtubules in transport phenomena remains unknown.

The notion that microtubules could participate, even passively, in intracellular transport implies that some sort of mechanical interaction can be established either temporarily or permanently between the microtubules and the transported organelles. Consequently, increasing attention has been focused on the intimate relationship between these organelles and microtubules (1, 2, 8, 22, 28, 29, 31, 36–39, 42, 43). Nevertheless, while findings in a variety of systems suggest that certain interactions between microtubules and the transported components might be of rather general occurrence, the physical nature of such interactions has been scarcely investigated. Positive tests that observed associations constitute effective attachments have been reported only for chromosomes and fibers of the mitotic spindle (2, 8, 31), and for membrane-bounded vesicles and microtubules in *Paramecium* (1). Little is known about the mechanical resistance of organelle-microtubule associations in other well-studied examples of cytoplasmic transport.

An alternative for the analysis of intracellular transport is offered by the mobile screening pigments that assist in the

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adaptation of arthropod compound eyes to environmental illumination. Morphological and pharmacological evidence suggests that microtubules are involved in the migrations of screening pigment granules in the retinula cells of the horseshoe crab *Limulus* (26, 27) and the crayfish (14). The crayfish retinula cell is particularly interesting because the pigment migrations are extensive (~180 μm) and occur longitudinally, to some extent within the axon (3, 14, 33). Thus, the system exhibits an attractive hybrid character that shares features of two outstanding exponents of polarized intracellular motion, i.e., pigment migration in chromatophores (7, 22, 25, 29) and axonal transport in nerve cells (4, 10, 38, 40). In addition, the crayfish retinula cells are convenient as an experimental model because their general morphology is well documented (3, 24, 30, 33, 44), and pigment responses quantitatively equivalent to those observed in the intact animal can be elicited in isolated eyes directly by the presence or absence of light (14).

The purpose of this work was to inspect the morphology and the relative strength of associations between the migratory pigment granules and microtubules in crayfish retinula cells. Electron microscopy methods combined with procedures of stepped cell disruption showed that many pigment granules are firmly linked to microtubules that constitute a sturdy cytoskeletal framework in these cells. The observations confirmed also the existence of a fibrous matrix in which the pigment granules appear suspended when viewed with high-voltage electron microscopy (15, 44), and demonstrated the cohesive nature of this material. The findings are in favor of a significant interaction between the pigment granule population and the microtubule system of these cells, with direct implications for the transport mechanism. Preliminary accounts of this investigation have appeared in abstract form (11, 12).

MATERIALS AND METHODS

Epoxy-embedded Specimens: Medium-to-large crayfish *Procambarus clarki* (Girard) were obtained commercially and kept in aerated tanks until use. Excised eyes from these animals were fixed for 90 min at room temperature with 4% glutaraldehyde in physiological solution for freshwater crustaceans (17) buffered with 10 mM imidazole (pH 7.4). While in the fixative the eyes were cut in slices of 0.5–1 mm in thickness and thereafter rinsed with cold physiological solution. Postfixation was carried out for 45 min on ice with 1% osmium tetroxide in 0.1 M sodium phosphate (pH 6.2) with 0.2 M sodium chloride. The pieces were then dehydrated with ethanol and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate, and examined with a Philips 200 electron microscope.

Freeze-etched Specimens: The photoreceptor layer of the compound eye was dissected and fixed with 2.5% glutaraldehyde in imidazole buffer. The layer was then split in half, and these pieces were secured in a brass holder. The pieces were rinsed for 2–3 min in 30% methanol, which was used as a volatile cryoprotectant (18), and frozen by quick immersion in Freon 22 cooled with liquid nitrogen. The specimen holder was next transferred to liquid nitrogen and clamped on the rotary stage of a Balzers 400 freeze-fracture apparatus. The subsequent procedures were an adaptation of the method followed by Heuser and Kirschner (18) for rotary-replication of freeze-dried cytoskeletons. The pieces were fractured at liquid nitrogen temperature and etched for 5 min at −110°C under a vacuum of 10^{-6} torr. Rotary shadowing was applied with a platinum electron beam gun placed at 25° to the plane of the stage while the latter was spinning at ~60 rpm. The platinum replicas were stabilized with carbon deposition from a gun mounted at 75° above the stage. The replicas were cleaned with household bleach and distilled water, and picked up on 200-mesh grids. Electron micrographs were obtained with a Philips 200 el ~tron microscope operated at 80 kV. Prints were made from reversed-contrast negatives for a better visualization of texture and relief, and for an easier comparison with scanning electron micrographs.

Scanning Electron Microscopy: The photoreceptor layer was excised and disrupted in a modification of the medium used for skinned muscle fibers of the crayfish (34): 200 mM potassium propionate, 1 mM magnesium acetate, 10 mM EGTA, and 20 mM imidazole (pH 7.0). Free rhabdoms were obtained by gently crushing the tissue between two glass coverslips. More vigorous disruption procedures included grinding and incipient or thorough homogenization with a stainless steel pestle in a small plastic tube. Drops of the resulting suspensions were placed on gelatin-coated coverslips, allowed to settle for 2 min in a moisturized chamber, and flooded with buffer before fixation. The samples were fixed by immersion of the coverslips in 2% glutaraldehyde with 0.1 M sodium cacodylate (pH 7.4) for 15 min at room temperature. The coverslips were then rinsed with buffer and immersed in 1% osmium tetroxide in cacodylate buffer for 15 min. Preparation for scanning electron microscopy was carried out by two cycles of alternate immersion of the coverslips in a fresh saturated solution of thiocarbohydrazide and in 1% osmium tetroxide, respectively, after the method of Ip and Fischman (21). Following dehydration with acetone and critical-point drying from liquid carbon dioxide, the samples were examined and photographed with an AMR 1000A scanning electron microscope. A light coat of gold-palladium, applied with a SEM Coating Unit E5100 (Polaron Instruments Inc., Hatfield, PA), was occasionally required to minimize residual charging effects on some specimens.

Negative Staining: Drops of the above suspensions were placed on Formvar-carbon-coated grids, allowed to settle for 2 min, and the grids were drained. The preparations were stained for 10 min with 0.5% uranyl acetate and observed with a Philips 200 electron microscope. Alternatively, the sample was fixed in suspension with 2% glutaraldehyde before negative staining.

RESULTS

Observations in Intact Cells

The overall morphology of the crayfish retinula has been thoroughly described (3, 24, 30, 33) and needs only brief summation here. Eight slender retinula cells surround and contribute to form the rhabdom or actual photoreceptive element of each ommatidium in the compound eye. The rhabdom is a spindle-shaped structure of integrated layers of microvilli that project laterally from the surrounding retinula cells. The screening pigment contained in the retinula cells consists of membrane-limited spheroidal granules 0.3 μm in average diameter. In transverse thin sections of the retinula cells (Fig. 1), most of the pigment granules and other membrane-bounded organelles appear distributed around the periphery, excluded from a wide central zone mainly occupied by microtubules 25 nm in diameter. Other longitudinally oriented microtubules occur close to the plasma membrane and among the pigment granules. Thus, the microtubules constitute a massive longitudinal column that extends throughout most of the retinula cell and its axon. A fuzzy ground substance is present everywhere in the cytoplasm. Filamentous extensions of this material often connect neighboring microtubules and other organelles, thereby composing a pervading reticulation. Randomly oriented filaments ~10 nm in diameter are also observed in the cytoplasm.

Structural associations between pigment granules and microtubules can be readily found in thin sections, particularly for the innermost granules. Moreover, associations between a single granule and more than one microtubule are relatively common (Fig. 2). The associations appear as bridges of groundplasm material extending from the microtubules to the membranes of the pigment granules. The length of these bridges may vary from 30 or 40 nm to nearly 200 nm, though most of them are within the 75–100 nm range. The linking structures, of fuzzy appearance in thin sections, can be visualized with considerably better definition in freeze-fractured, deep-etched, and rotary-shadowed specimens (Fig. 3). Multiple strands of variable thickness and shape are observed spanning from single granules to single microtubules in these preparations. Despite the variability in the morphology of such structures, a uniform feature occurs repeatedly in every field inspected. It consists of very thin filaments, 2–4 nm in width, that project laterally from the microtubules and reach neighboring granules or other microtubules. Though of uniform caliber, these filaments may

have diverse linear configurations, i.e., straight, bent, or twisted. They do not show obvious periodicity along the microtubule surface. Besides connecting microtubules and pigment granules, the filaments appear structurally continuous, or partly decorated, with amorphous aggregates of globular material.

Although many pigment granules are unequivocally linked to microtubules, others are not even close to them; instead, these granules appear isolated or forming clusters of which only a few units show relationship with the microtubules. In every case, material of the groundplasm can be seen interspersed among the pigment granules and frequently bridging between them.

Observations in Isolated Cell Fragments

The relative tensile strength of the pigment granule-microtubule associations was estimated from the ability of these organelles to hold together through procedures of gradual tissue disruption in a low-Ca^{++} stabilization buffer. The dissociated components were best visualized with scanning electron microscopy. First, the permanence of the associations was inspected after the plasma membranes of the retinula cells had been stripped off. To this end, advantage was taken of the remarkable stability of the rhabdoms, which can be isolated in a relatively unaltered condition by mild mechanical treatment of the photoreceptor layer (16). Complete and virtually undistorted rhabdoms were obtained in successful preparations (Fig. 4 *a*). Apart from separating individual ommatidia, the procedure removed the plasma membranes of the retinula cells and dispersed most of their cytoplasm. However, large fragments of the microtubular columns, up to 50 μm in length, remained associated with the rhabdoms after isolation. The naked microtubule columns appear as massive bundles of long and intertwined rods (Fig. 4 *b*). Numerous particles, most of which correspond in size to the pigment granules, stayed associated with the microtubules after the plasma membranes and the rest of the cytoplasm had been dispersed away. Some of these particles could just be trapped within the microtubular framework, but many of them seem retained by means of direct attachment to the microtubules (Fig. 5). Where clusters of particles are found, they appear entangled with amorphous material that also binds to the microtubules. Other linear elements, distinguishable from the microtubules by their wavy, branched, and anastomotic character, are found randomly oriented in these preparations. They probably correspond to the 10-nm filaments observed in intact cells.

In a more drastic challenge to the stability of the pigment granule-microtubule associations, an attempt was made to break down the microtubular columns. In suspensions obtained by grinding or incipient homogenization of the photoreceptor layer in stabilizing solution, many particles remained anchored to free fascicles of microtubules (Fig. 6 *a*). Clusters of granules associated with single microtubules were common. Negative staining also provides an adequate view of these relationships (Fig. 6 *b* and *c*). The granules appear enmeshed in a fibrous material that adheres to the surfaces of the microtubules. Spinelike projections emanating from the microtubules were frequently observed. Distinct filaments of ~10 nm can be seen

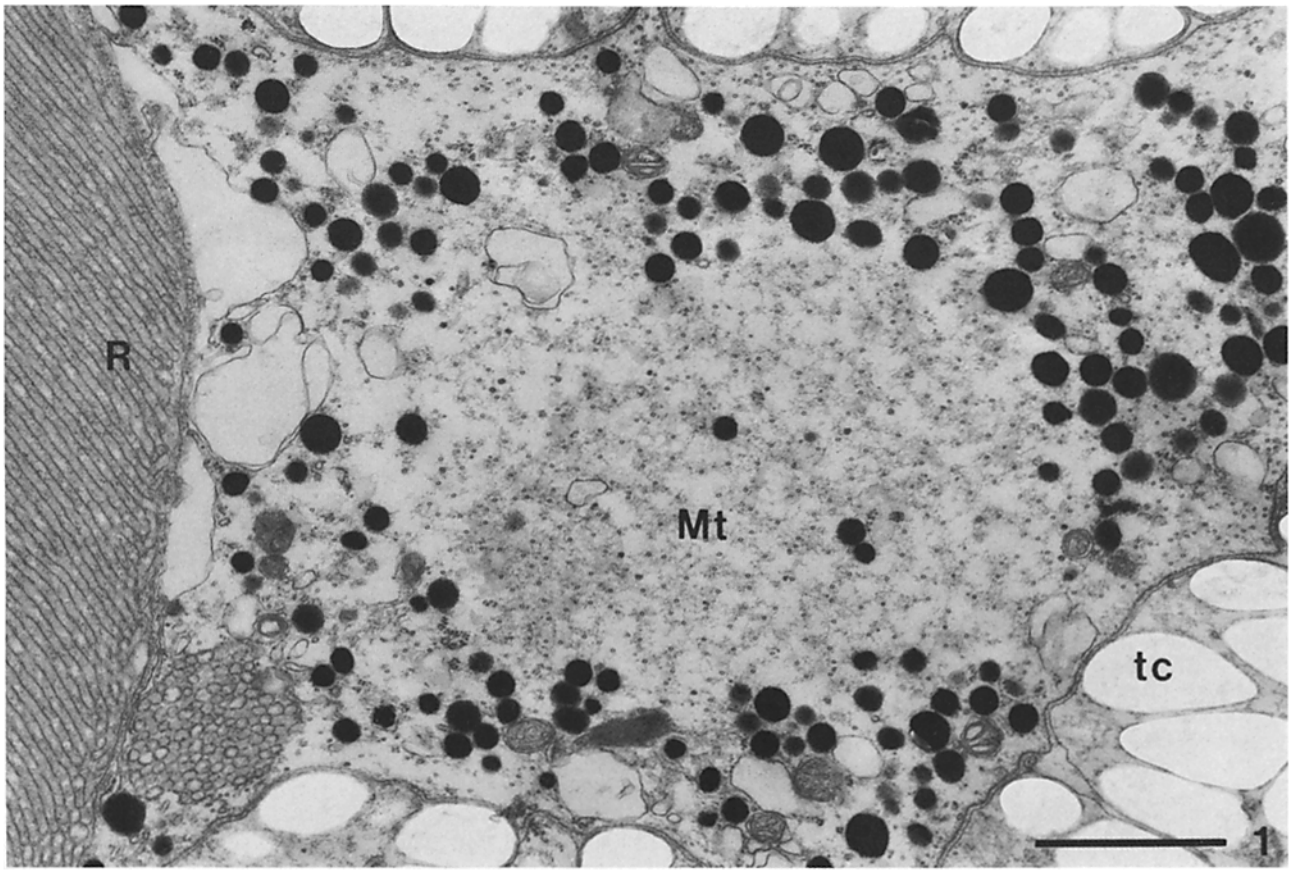


FIGURE 1 Transverse thin section of a retinula cell at the proximal third of the rhabdom (*R*). The cytoplasm contains hundreds of longitudinally oriented microtubules (*Mt*). Most of the pigment granules and other membrane-bounded organelles are peripherally distributed. The retinula cells are surrounded by tapetum cells (*tc*) characterized by large open vacuoles (24). Bar, 1 μm. × 25,000.

wriggling through the more amorphous component of this network.

Finally, in a last step of tissue disaggregation the photoreceptor layer was thoroughly homogenized in stabilizing solution. Samples of the resulting suspension showed free granules, either individual or forming small groups. In most cases the granules presented remnants of fibrous material adhered to their surfaces, but no microtubules associated with them could be found.

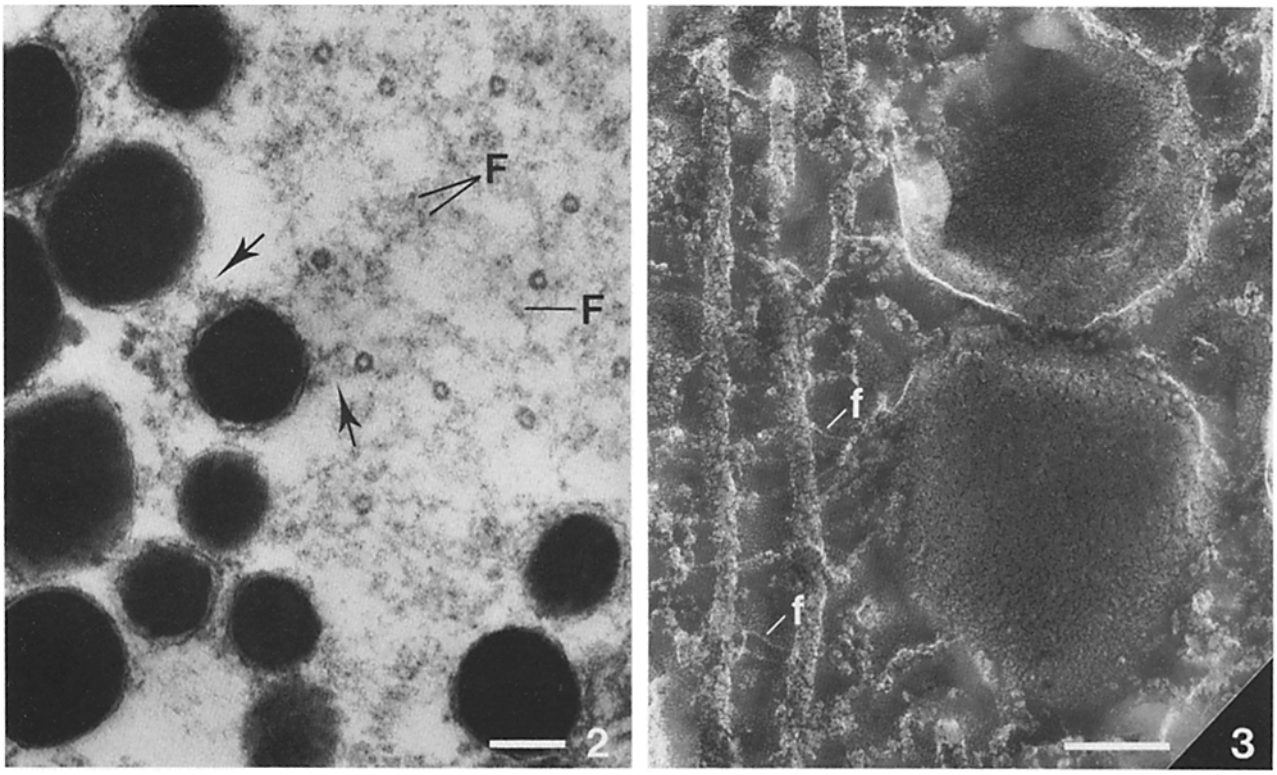
DISCUSSION

The aim of this investigation in the crayfish retinula cells was to determine whether the migratory pigment granules and microtubules are merely juxtaposed or whether there is a real physical association between these organelles. The preceding observations provide evidence that the pigment granules are structurally associated with the microtubules. Furthermore, they prove that the associations can be mechanically strong.

The actual structures linking pigment granules directly with microtubules are probably the 2–4-nm filaments observed in freeze-etched specimens. The random variability in the shape of other connecting strands could be the result of masking or partial coating of these filaments with other cytoplasmic proteins or with amorphous components such as the fibrous material. Significantly, numerous wisps of the same magnitude have been found attaching pigment granules to microtubules in two types of fish chromatophores (22). "Linker" filaments of similar caliber are also found radiating from microtubules in axons of the crayfish nerve cord (32).

A fibrous matrix in which the pigment granules appear suspended has already been visualized in the retinula cells with high voltage electron microscopy (15, 44). The matrix is now shown to be capable of holding together large clusters of pigment granules against the shearing forces applied in tissue disruption. Thus, even granules relatively distant from the microtubules can be effectively connected with these through the fibrous material. Except for the presence of the pigment granules and 10-nm filaments, this network appears similar to the microtubule-associated filamentous matrix proposed to mediate axonal transport in the crayfish nerve cord (6, 10, 32). These findings substantiate the suggestion that the cytoplasmic matrix can be endowed with the mechanical properties required to play at least a passive traction role, as implied in various models where the matrix is proposed to act as a vehicle of intracellular transport (4, 5, 7, 10).

The present results also reveal the sturdy nature of the microtubule column in the retinula cells and its firm association with the rhabdom. There is little doubt that this massive microtubule backbone could be well suited to act as a supportive framework for pigment transport, as suggested in the case of chromatophores (28, 29). It is particularly noteworthy that it was unnecessary to add to the isolation medium tubulin or agents such as glycerol, which are generally used to stabilize microtubules in vitro, in order to maintain the integrity of the microtubule columns once the plasma membranes had been stripped away. In this remarkable intrinsic stability, the microtubule columns of the crayfish retinula resemble the microtu-



FIGURES 2 and 3 High magnification micrograph of a transverse thin section similar to that in Fig. 1. The field presents the inner boundary of the pigment-rich peripheral zone. A delicate lattice of fuzzy material pervades the space among the organelles and forms connections between them (*arrows*). Linkages between pigment granules and one or more microtubules are frequent. Randomly oriented filaments (*F*) ~10 nm in diameter occur also in the cytoplasm. Bar, 0.1 μm. × 100,000. Same region as in Fig. 2 when viewed in rotary-shadowed replicas of freeze-fractured and deep-etched specimens (longitudinal view). Multiple linkages of diverse shapes interconnect microtubules and pigment granules. The only uniform feature among these linkages consists of fine filaments (*f*) 2–4 nm in caliber. Bar, 0.1 μm. × 140,000.

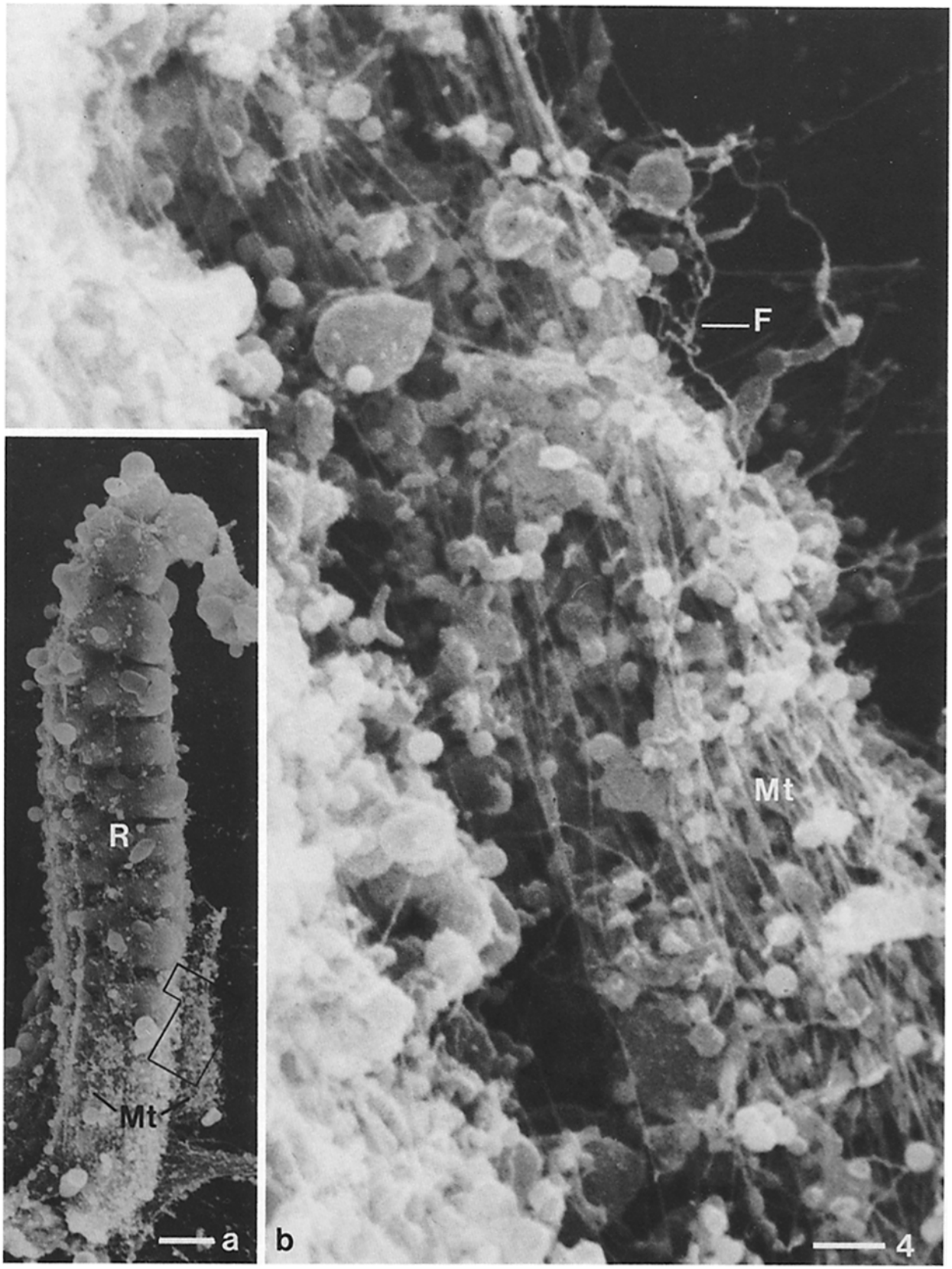


FIGURE 4 (*a*) Scanning electron micrograph of an isolated rhabdom (*R*). The isolation procedure has removed the plasma membranes of the retinula cells and dispersed most of their cytoplasm. However, large fragments of the microtubule columns (*Mt*) remain associated with the rhabdom. The small rectangle on the right shows the field presented at higher magnification in (*b*). Numerous particles stay associated with the naked columns of long and intertwined microtubules after the plasma membranes have been stripped off. Branched and sinuous filaments (*F*) are also associated with particles and microtubules. *a*: Bar, 10 μm. × 950. *b*: Bar, 1 μm. × 12,500.

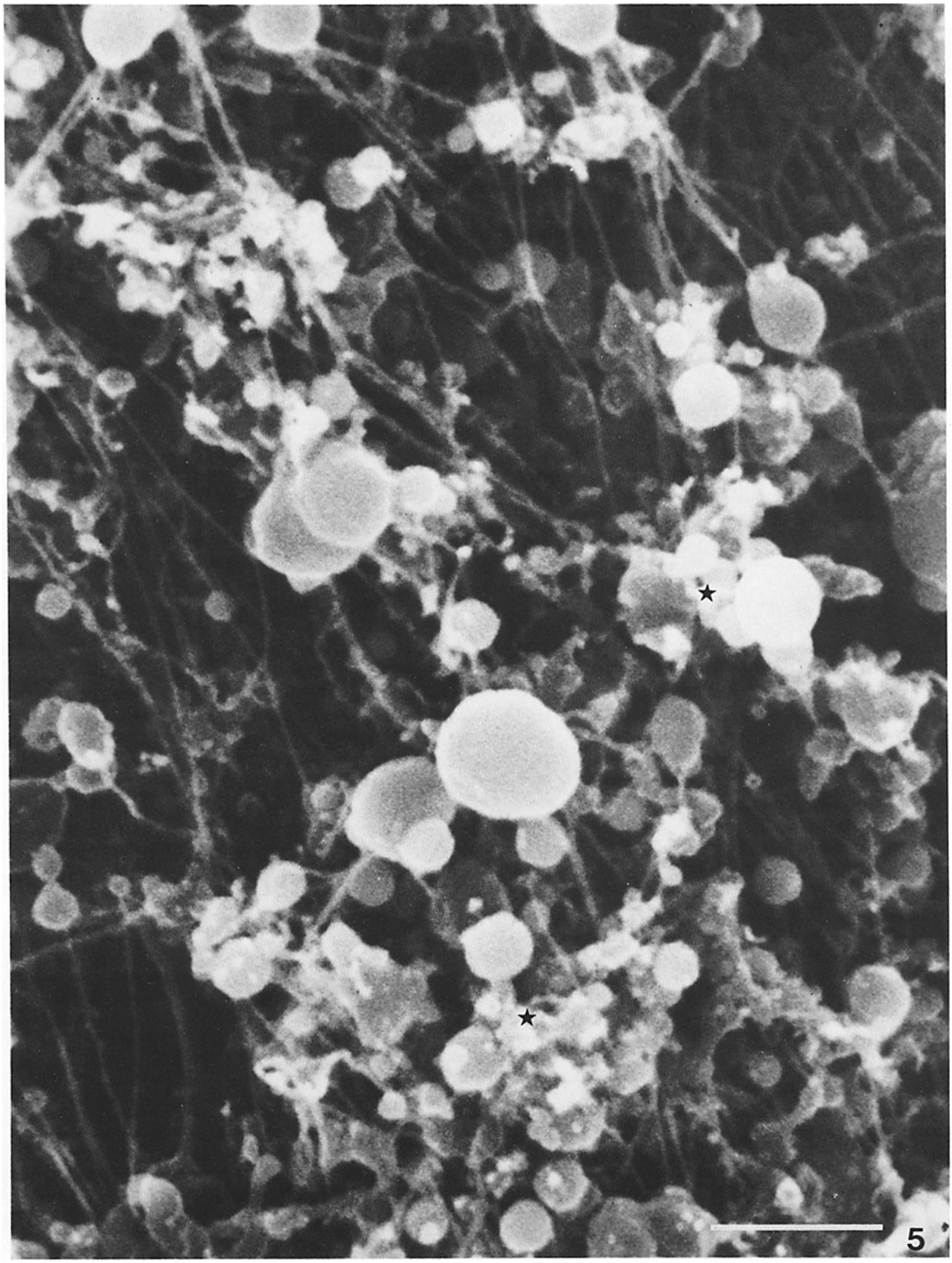


FIGURE 5 High magnification scanning electron micrograph of a microtubule column associated with an isolated rhabdom. The microtubules appear slightly thicker (30–35 nm) than usual due to the osmium-thiocarbohydrazide coating. They tend to maintain their longitudinal orientation (*top-left* to *bottom-right* in the micrograph) despite the mechanical treatment applied to remove the plasma membrane of the cells. Either individual particles or clusters entangled with amorphous material (*stars*) can be seen attached to the microtubules. Bar, 1 μm. × 30,000.

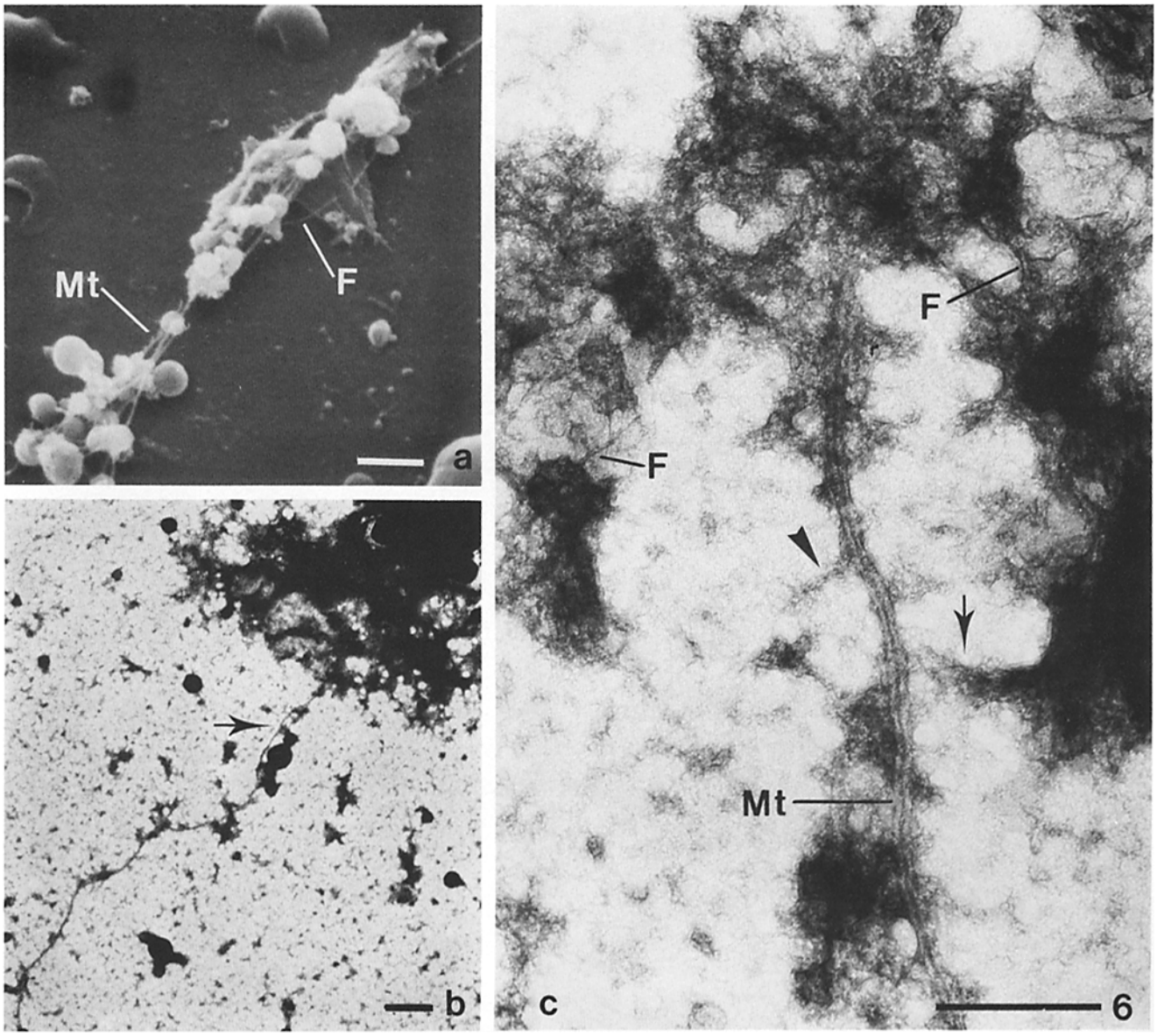


FIGURE 6 (*a*) Scanning electron micrograph of cytoplasm suspension obtained by incipient homogenization of the retinula cells. Many particles remain attached to free fascicles of microtubules (*Mt*) after the cytoskeleton has been broken to pieces. Spinelike projections (*Mt* pointer) can be seen on some microtubules. Filaments (*F*) appear frequently associated with these fragments. (*b* and *c*) same cytoplasm suspension when viewed with transmission electron microscopy of negatively stained samples. Numerous pigment granules can be associated with single microtubules (*Mt*) either by direct linkages (*arrows*) or through a fibrous material in which large clusters of particles appear enmeshed. Isolated granules, probably torn away from the clusters, lie scattered. The fibrous material adheres to the surface of the microtubules forming spinelike projections (*arrowhead*). Distinct filaments (*F*) of ~10 nm are seen through the more amorphous component of this network. *a*: Bar, 1 μm. × 10,000. *b*: Bar, 1 μm. × 7,000. *c*: Bar, 0.25 μm. × 100,000.

bule cytoskeletons of the nutritive tubes of telotrophic insect ovaries (19).

There are two possibilities to account for changes in pigment position, considering the firm associations between the pigment granules and microtubules: (*a*) that the attachments be fixed, in which case at least those microtubules linked to pigment granules should travel jointly with these granules along the cell; (*b*) that the attachments be detachable, either by mere dissociation or coupled to microtubule turnover, so that the granules would be set free to advance alongside a relatively immobile microtubule column. A recent morphometry study (reference 11, and manuscript in preparation), which shows a basically similar longitudinal distribution of microtubules for cells with the screening pigment in opposite extreme positions, does not support the first possibility. Also, the intertwined arrangement of the microtubules in the column would hardly be favorable for the displacement of a subset of tubules loaded with permanently attached clusters of pigment granules.

A variant of the first possibility is that the whole microtubule column would move along the retinula cell together with the screening pigment. However, the velocity of pigment migration (0.3–0.4 μm/s [14]) exceeds by far the values reported for the rate of tubulin progress along nerve cells (0.25 mm/d [4]). Therefore, it is unlikely that the microtubules move with the

pigment, and the associations between granules and microtubules are probably transient in nature. For axonal transport in ganglion cells of the guinea pig visual system (4), transient interactions between a peripheral filamentous network and a central axis of microtubules and neurofilaments have also been concluded, from biochemical and other evidences. An analogous model could be envisaged for the screening pigment migration along the crayfish retinula cells, where a sleeve of pigment granules contained in a fibrous matrix apparently slides over a relatively stationary column of microtubules.

If the associations between microtubules and pigment granules are detachable, they must be under physiological control. It is possible that the intracellular levels of free Ca^{++}, which seem to have a direct influence on the screening pigment position (13, 23), could be of significance for the regulation of these interactions. Interestingly, Ca^{++}-dependent structural changes have been described in the microtrabeculae supposed to mediate axonal transport in nerve cells (40) and pigment migration in fish chromatophores (25). The permanence of intermicrotubule bridges in heliozoan axonemes is also affected by Ca^{++} (35). In this regard, the very low concentration of free Ca^{++} used for the present experiments (with 10 mM EGTA) possibly favored paralyzation of the linkages in a "locked" state. Work in progress will attempt to elucidate whether the microtubule-pigment granule interactions are subject to regulation in the crayfish retinula cells.

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Reprint requests should be addressed to Dr. Frixione at his present address.

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