Localization and Organization of Actin in Melanophores

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ABSTRACT Melanophores of the angelfish, *Pterophyllum scalare*, were studied in an attempt to demonstrate the existence of actin in these cells although microfilaments had previously not been found. By use of a variety of procedures, including immunofluorescence microscopy of intact and detergent-extracted cells, transmission electron microscopy, high voltage electron microscopy of whole-mount preparations, and labeling with heavy meromyosin-subfragment 1, the presence of a loose cortical mesh of actin filaments is demonstrated. In addition, a more parallel array of filaments is detected in microspike- and microvillus-like surface projections. There seem to be no changes in the arrangement of these filaments as a function of the state of pigment distribution. No actin filaments could be found in association with pigment granules or microtubules in more central cell portions. For reasons presently unknown, the preservation of the cortical filament network in lysed cell preparations depends strongly on the presence of an intact microtubular system. The involvement of this subplasmalemmal actin filament network in pigment granule transport remains unclear.

Pigment cells of several species of fish are generally regarded as excellent model systems for the study of intracellular transport. The value of the chromatophore model resides in the fact that a more or less uniform class of intracellular organelles (the pigment granules) is moved at relatively high velocities (several micrometers per second), and that these movements are easily observed in a light microscope. However, the exact mechanisms responsible for this rather dramatic example of intracellular motility are not entirely understood. There seems to be agreement that microtubules are intimately involved in this process (2, 3, 19–21, 30). In addition, however, there is indirect evidence indicating the presence of an elastic component in the cytoplasm of melanophores (6, 23) that apparently is able to bring about slow but measurable movements of pigment granules in the total absence of microtubules (15, 25). The biochemical nature of this second component is unknown. Morphologically, it may be identical to the fibrous elements of the cytoplasmic ground substance (19) identified as an anastomosing system of fine strands (microtrabeculae) interconnecting pigment granules, microtubules, and membranes (4, 8, 22). As originally proposed by Byers and Porter (4), granule movements may be the result of alternating cycles of contraction and expansion of this network. The composition of the microtrabecular lattice has not yet been characterized.

In general, a likely candidate for a component engaged in motility is a system based on the contractile proteins actin and myosin. So far, however, there is little conclusive evidence for the presence, not to mention involvement, of an actomyosin system in chromatophores. Filaments of 5- to 7-nm diameter have not yet been identified either in thin sections of intact cells or in extracted cells after various glycerination procedures, indicating that, if F-actin is present, it is either highly labile or in a form not recognizable with conventional techniques. Experiments using cytochalasin B gave divergent results in different systems (e.g., references 5, 7, 9, 11, 14, 16). The value of the cytochalasin B experiments is difficult to estimate, because of the multiple effects of this drug and because most of the systems studied have not been shown unequivocally to possess an actin microfilament system.

This paper describes our attempts to localize actin in melanophores by use of a variety of procedures, including indirect immunofluorescence microscopy with antibodies directed against mammalian cytoplasmic actin, and electron microscopy. Our data indicate that an actin filament system is indeed present in these cells and that it seems to be concentrated at the cell periphery close to the plasmalemma.

MATERIALS AND METHODS

Cells

Melanophores of the angelfish, *Pterophyllum scalare*, were isolated from excised scales by use of procedures described previously (24). They were cultured

THE JOURNAL OF CELL BIOLOGY · VOLUME 89 MAY 1981 267–275
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in a 1:3 mixture of Amphibian Culture Medium (Grand Island Biological Co., Grand Island, N. Y.) and Ringer's solution until they spread on the cover slip. To induce aggregation of pigment granules in the cell center, 10^{-4} M adrenalin, made up in Ringer's solution, was used. 10^{-4} M atropin in Ringer's solution served as an agent inducing pigment dispersion.

Triton X-100 Extraction

For lysis experiments, cells on cover slips were washed with stabilization buffer consisting of 0.1 M PIPES (pH 6.9), 2 mM EGTA, 4% polyethylene glycol (PEG) 6000, with or without 2 mM $MgCl_2$, and then lysed in the same buffer containing 0.1% Triton X-100 for 2-3 min.

Immunofluorescence Microscopy

Antibodies against homogeneous cytoplasmic actin isolated from bovine brain have been raised in rabbits. Antigen affinity chromatography was used to isolate the specific antiactin immunoglobulins (IgGs) (1). They were used at a concentration of 0.05 mg/ml phosphate-buffered saline (PBS). Goat anti-rabbit IgGs labeled with fluorescein, a commercial product (Miles-Yeda Ltd., Rehovot, Israel), was used at a concentration of 0.4 mg/ml of PBS. The monospecific rabbit antitubulin antibody has been described previously (18, 29). Cells were fixed for immunofluorescence microscopy by either of the following methods: (*A*) 10-min fixation was carried out at room temperature with 3.7% formalin in Ca-free PBS containing 1 mM EGTA and was followed by treatment with methanol at −20°C for 4 min. After washes in PBS, antibodies were applied. (*B*) Cells were fixed with 2% glutaraldehyde in stabilization buffer, washed with buffer, and then treated with $NaBH_4$ (0.5 mg/ml) in PBS for 2-3 min each (28). After additional washes in PBS, the cells were incubated with antibodies. Fixation procedure *B* was invariably employed on Triton-extracted cells.

S_1 Decoration

Heavy meromyosin-subfragment 1 (S_1) was kindly provided by Dr. Judy Snyder. It was prepared from rabbit skeletal muscle myosin according to the method of Margossian and Lowey (10). For S_1 decoration, cells were Triton-extracted as described above and then incubated for 10 min with 0.5 mg/ml S_1 in stabilization buffer. After a brief wash in stabilization buffer, cells were fixed for electron microscopy as described below.

Transmission Electron Microscopy

Intact cells were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 20 min, followed by 1% osmium tetroxide in cacodylate buffer for 10 min. Triton-extracted cytoskeletons were fixed with 2% glutaraldehyde in stabilization buffer and postfixed with 1% osmium tetroxide for 10 min. Cells were then washed, stained en bloc with 0.5% uranyl acetate and 1% phosphotungstic acid for 30 min, dehydrated, and embedded in an Epon/Araldite mixture according to Mollenhauer (12). Sections were cut on an LKB or Sorvall ultramicrotome (LKB Instruments, Inc., Rockville, Md., or DuPont Co., Sorvall Biomedical Div., Wilmington, Del.), stained with lead citrate, and viewed in either a Hitachi H 500 or JEOL 100C electron microscope.

High Voltage Electron Microscopy

For high voltage electron microscopy, cells were allowed to settle on Formvar- and carbon-coated gold grids and kept in culture medium until spread. Cells in the desired state of pigment distribution were washed with stabilization buffer and subjected to either Triton extraction followed by fixation, or fixation with 2% glutaraldehyde in stabilization buffer for 10 min. Postfixation with 1% osmium tetroxide for 2 min was followed by dehydration in ethanol and critical-point drying from ethanol by use of a Sorvall critical-point-drying system. The cells received a second thin carbon film in a Denton evaporator (Denton Vacuum Inc., Cherry Hill, N. J.) and were then viewed in a JEOL high voltage electron microscope operated at 10^6 V.

RESULTS

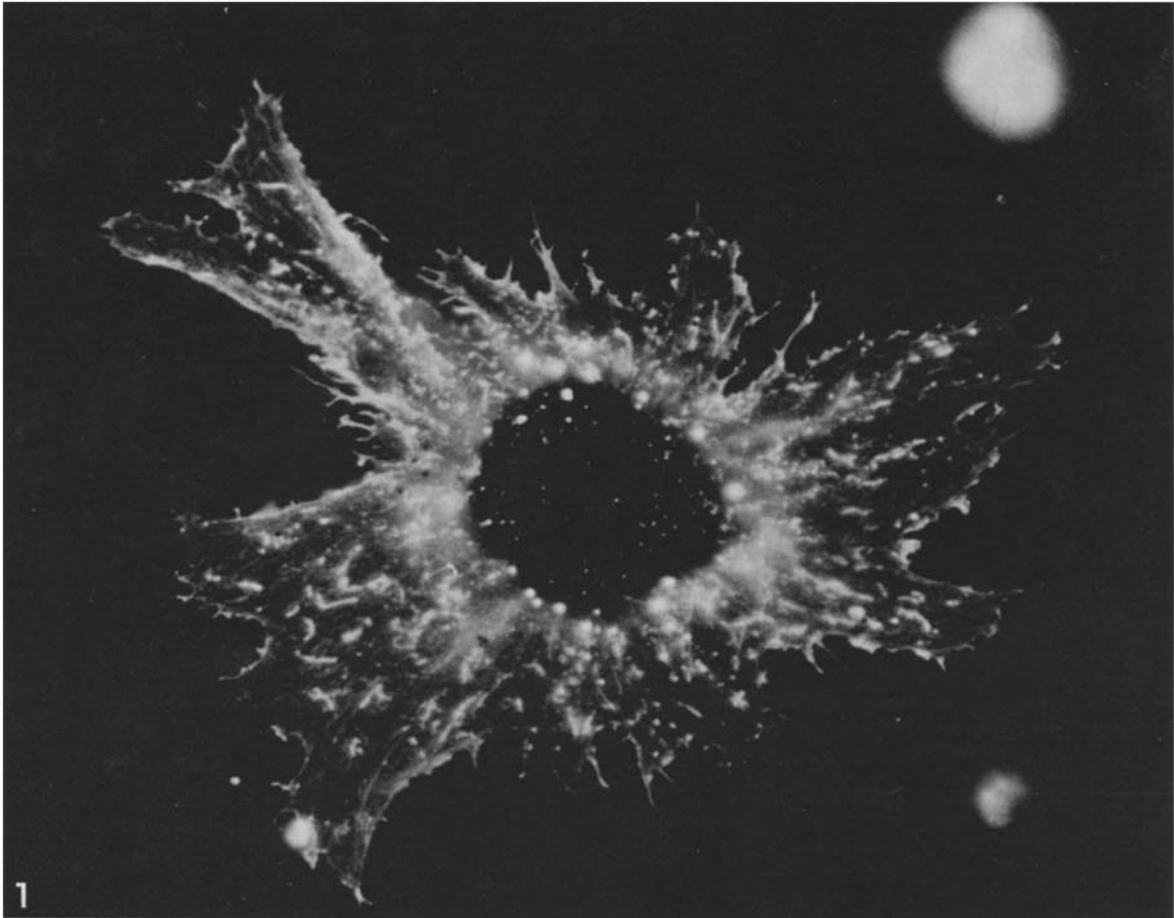
Immunofluorescence Microscopy

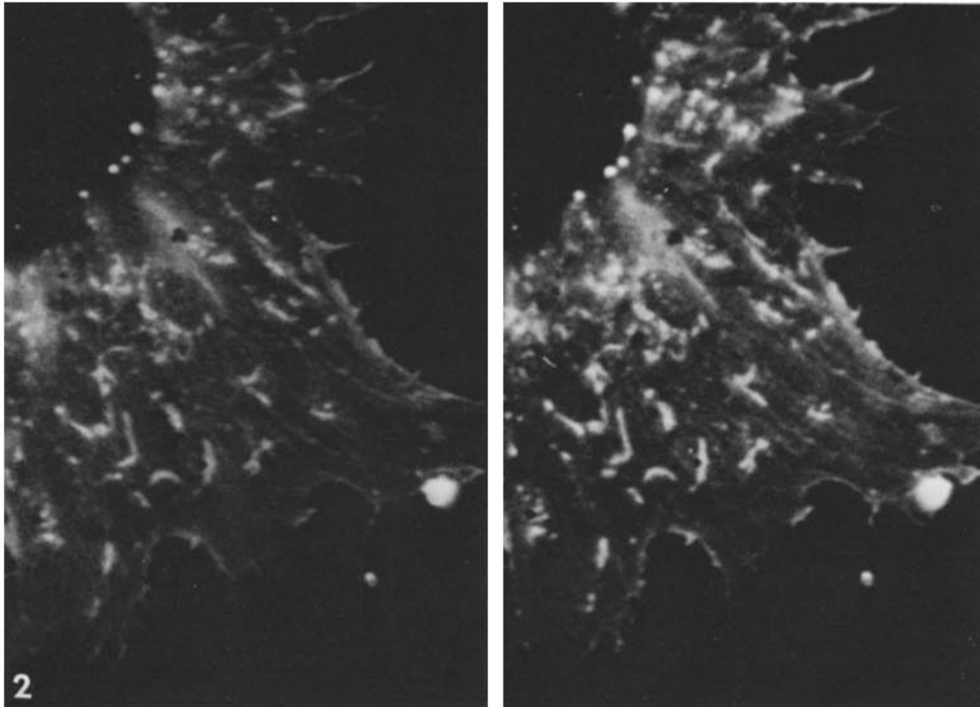
We processed melanophores for immunofluorescence microscopy in the state with pigment aggregated in order better to see details of the granule-free cell periphery. In the dispersed state, light absorption of the pigment interferes with the fluorescence signal so that details of the immunofluorescent pattern are obliterated.

The distribution of cytoplasmic actin revealed by immunofluorescence microscopy is shown in Fig. 1. The fluorescent patterns observed can be subdivided into four categories: (*a*) A distinct fluorescence along most of the outer cell margin that becomes even more intense in various surface extensions; (*b*) a bright fluorescence in vermiform or crescent-shaped structures scattered over the pigment-free peripheral cytoplasm; (*c*) a bright fluorescence in spherical structures located close to the central mass of aggregated pigment granules; (*d*) a diffuse (background) fluorescence that is somewhat more intense in the thicker area close to the pigment mass. Stereo immunofluorescence microscopy (17) was used to improve spatial resolution and to test whether the staining of the elongated structures of category (2) could perhaps be attributed to unspecific fluorescence of, e.g., mitochondria. Stereo immunofluorescence clearly demonstrates (Fig. 2) that these structures correspond to surface protrusions that extend from the generally smooth cell body into the surrounding medium. (A similar conclusion can be reached by differential focusing in the light microscope but this is more difficult to document.) The existence of such surface projections of various sizes and shapes has been confirmed by stereo high voltage electron microscopy of whole cells (Fig. 3). To further prove specificity of the staining pattern observed in the cell periphery, melanophores were detergent-extracted under conditions that preserve elements of the cytoskeleton. Preliminary experiments have shown that cells extracted by a variety of glycerination procedures using standard salt solution or modified standard salt solution (27) do not reveal any appreciable staining for the presence of actin in indirect immunofluorescence microscopy, or actin-like microfilaments in electron microscopy. However, because microtubules in those preparations were partly disassembled or almost absent, and because the preservation of filaments might in one way or another depend on an intact microtubule system, we chose lysis conditions that are equally well suited for the preservation of microtubules and filaments. Cells processed according to the method of Weber et al. (28) show basically the same staining pattern that is encountered in unlysed cells (Fig. 4, compare with Fig. 1). At the same time, microtubules are well preserved, as demonstrated by immunofluorescence microscopy using antibodies against tubulin (Fig. 5). The only difference is the total absence of the granular fluorescence of

FIGURE 1 Isolated melanophore with pigment aggregated, treated with antibody to brain cytoplasmic actin and viewed in immunofluorescence microscopy (IFM). This micrograph demonstrates the overall staining of the cells. Detailed description of the staining pattern is given in the text. Fixation with formalin, posttreatment with methanol at −20°C. × 1,200.

FIGURE 2 Stereo micrograph of part of the cell shown in Fig. 1, illustrating the immunofluorescent staining in three dimensions. The central mass of aggregated pigment granules is in the upper left-hand corner. Note that the brightly staining vermiform structures extend above the flat peripheral cytoplasm and represent a kind of microvillus. × 1,800.





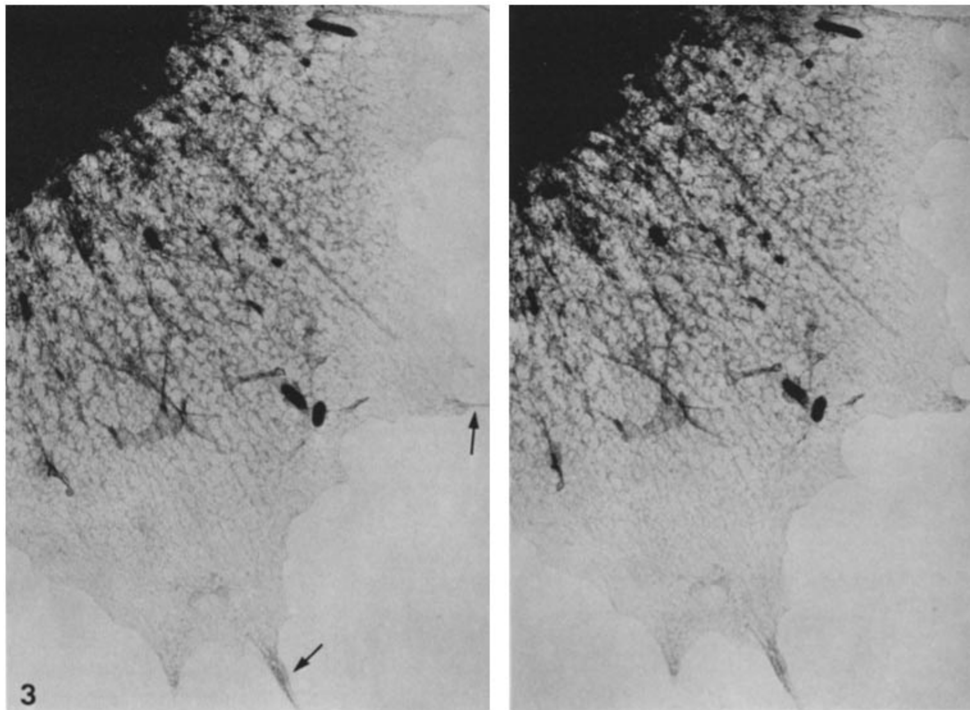


FIGURE 3 Low-magnification micrograph of a whole critical-point-dried melanophore with pigment aggregated. Pigment granules in the cell center are in upper left corner. The flat cell periphery shows radially oriented microtubules and associated elements of the cytoplasmic ground substance. Several elongated surface projections extend from the upper cell surface. Two microspike-like structures are seen at the cell periphery (arrows). × 7,500.

category *d*, indicating that this may be attributable to a class of membrane-bounded organelles whose contents are released and washed out after lysis.

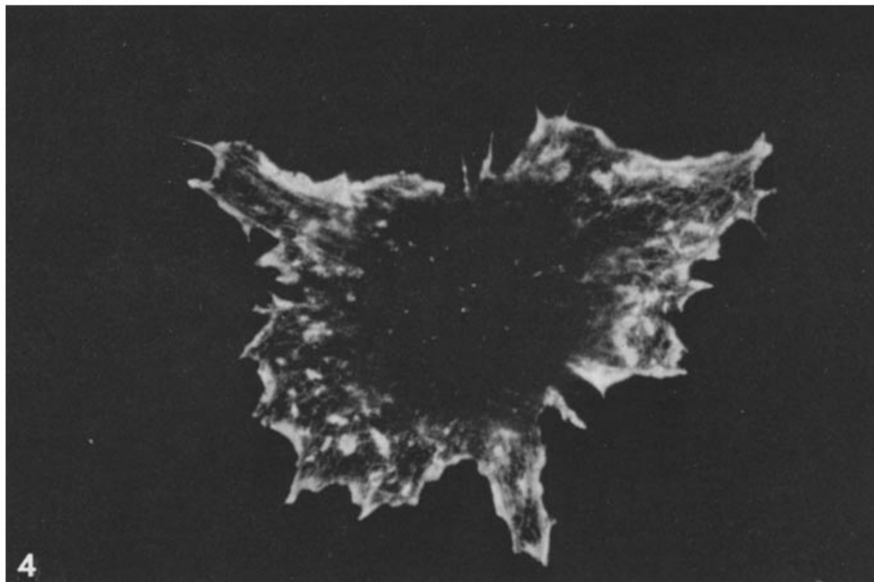
Electron Microscopy

Immunofluorescence microscopy suggests the presence of a predominantly cortical arrangement of actin in melanophores; there seems to be little fluorescence in areas where microtubules and/or melanosomes are found. However, a major pitfall in immunofluorescence microscopy with respect to melanophores is the presence of black pigment granules that, as demonstrated earlier (26), may effectively quench fluorescence signals. Therefore, the presence or absence of actin near pigment granules is not revealed. Electron microscopy has therefore been used to search for actin-like microfilaments and, if they are present, to study their spatial relationship to pigment granules and microtubules.

Microfilaments have not been detected in situ in melanophores prepared for conventional thin-section electron microscopy, with one exception. We have observed oriented fine filaments only close to the base of microspike-like surface protrusions in cells fixed shortly after isolation from the scale and attachment to a glass substrate (Fig. 6). These filaments are of 5- to 6-nm diameter and extend into the base of the microspike as a parallel bundle.

Although, in general, microfilaments are not readily detectable in thin-sectioned material (with the exception just described), this does not necessarily mean that they are not present in other cell areas. Filaments might be hidden in, or be part of, the trabecular lattice in critical-point-dried cells, or otherwise obscured in resin-embedded cells. We therefore employed detergent extraction under various conditions in conjunction with either sectioning of resin-embedded material or whole-mount electron microscopy to better visualize the microfilaments (if present).

When isolated cells are Triton-extracted in stabilization buffer, microtubules and intermediate-sized filaments are well preserved, but most of the matrix components of the cells are extracted. In fact, these cells appear rather "empty." Only at the cell periphery are fibrous components other than microtubules or intermediate-sized filaments observed. Here, thin filaments (5- to 6-nm diameter) form a loose meshwork as demonstrated in the stereo pair of Fig. 7. In some places, several filaments assume a more parallel orientation; presumably, they have been part of surface protrusions or small microspikes in the intact cell (Fig. 8). The cortical filaments are clearly different from intermediate-sized filaments (10-nm diameter), which are relatively straight and oriented parallel to the radially arranged microtubules. Thin filaments are almost exclusively observed in the cell periphery, and not in association with pigment granules. By virtue of their decoration with heavy meromyosin-S_1, they can be identified as F-actin (Fig. 8*c*). The extent of extraction achieved with Triton X-100 is documented in whole-mount, critical-point-dried cells (Figs. 9 and 10). The extensive microtrabecular lattice associated with pigment granules in intact cells (4, 21) has been almost completely removed. Although there are some fine filaments preserved at the upper cortex of the cell (which presumably are part of the cortical filament web), the space between the upper and lower cortex appears virtually empty. Actin filaments remain associated with the cell cortex regardless of the distribution of pigment granules within the cell body.



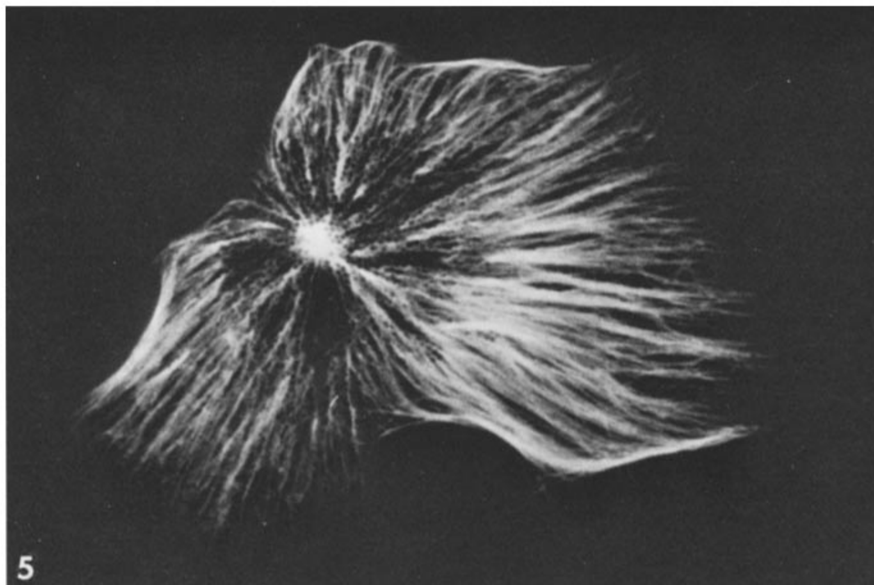


FIGURE 4 Melanophore lysed into stabilization buffer without Mg ions, fixed with glutaraldehyde, reduced with $NaBH_4$, and treated with antibody against actin. The overall staining pattern is the same as in unlysed, formalin-fixed cells (see Fig. 1), except for the absence of the staining in spherical granules close to the aggregated pigment mass. × 1,200.

FIGURE 5 Cell treated as in Fig. 3 but stained with antibody against tubulin. The microtubule system appears completely intact. × 1,200.

DISCUSSION

It has been noted in several studies of vertebrate chromatophores with the electron microscope that these cells apparently do not contain actin-like filaments (e.g., references 13, 19, 20). Not only do they lack the highly organized filament bundles (stress fibers) characteristic of a wide variety of epithelial and fibroblastic cell types, but also filaments arranged in a three-dimensional network, as in ruffling regions of many cultured cells, are not demonstrable in thin sections of various chromatophores. However, it has not been clear whether this is attributable to a general lack or paucity of this structural component, different physical-chemical properties, or an arrangement unlike that encountered in various other cell types. In fact, the filaments shown in Fig. 6 seem to be the first demonstration of F-actin in conventionally embedded and sectioned chromatophores. Because the pigment cell system is not yet amenable to biochemical studies that might demonstrate the presence or absence of certain classes of proteins, including actin, we had to choose other approaches to obtain information about the possible presence of actin in this cell type.

We have demonstrated here through the use of a variety of procedures that an actin-like component is indeed present in melanophores. The evidence obtained so far indicates that: (*a*) F-actin can easily be detected in the cell periphery. (*b*) Actin

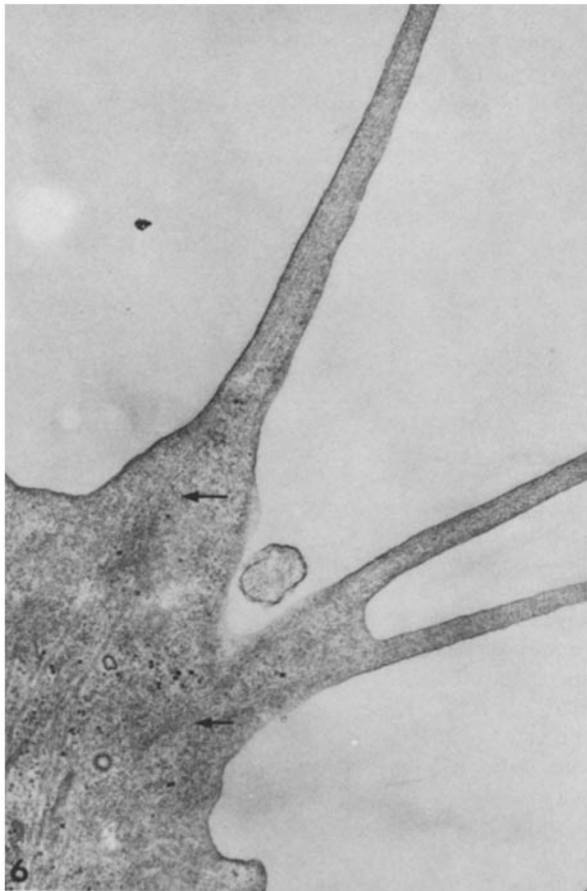


FIGURE 6 Transmission electron micrograph of marginal part of a melanophore fixed immediately after attachment to the cover slip; pigment is aggregated. The three microspikes show parallel arrays of fine filaments (arrows; ~6 nm diameter) that extend into the bases of the surface projections. × 3,100.

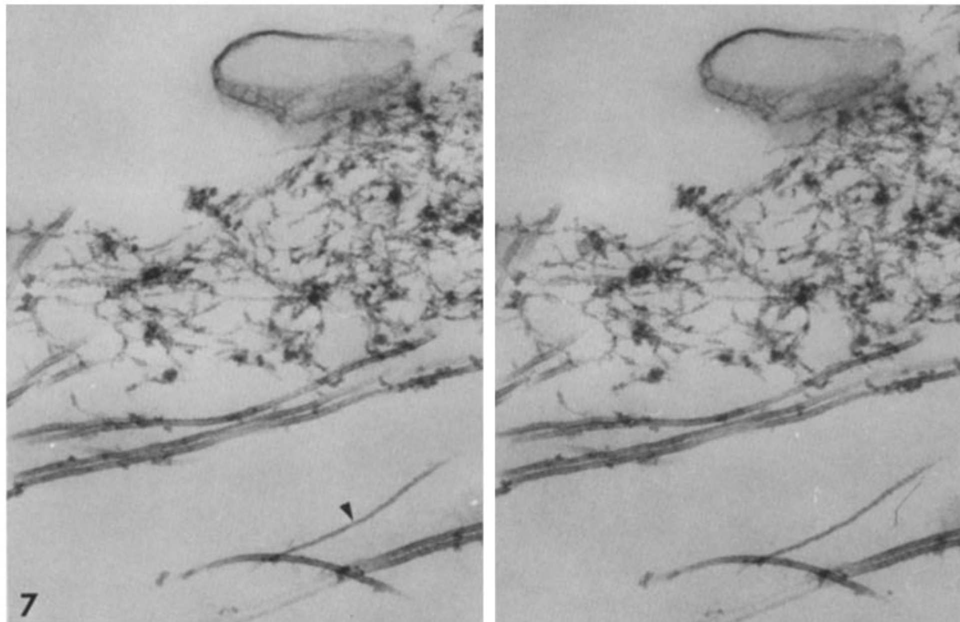


FIGURE 7 Stereo electron micrograph of a 0.25-μm-thick section of a melanophore lysed into stabilization buffer, fixed with glutaraldehyde plus 0.4% tannic acid, and then processed further using routine procedures. This cortical portion of a cell with pigment dispersed demonstrates a loose meshwork of thin filaments. Electron-dense particles (probably ribosomes and glycogen granules) appear trapped in the filament net. Arrowhead indicates intermediate-sized filament running parallel to microtubules. × 58,000.

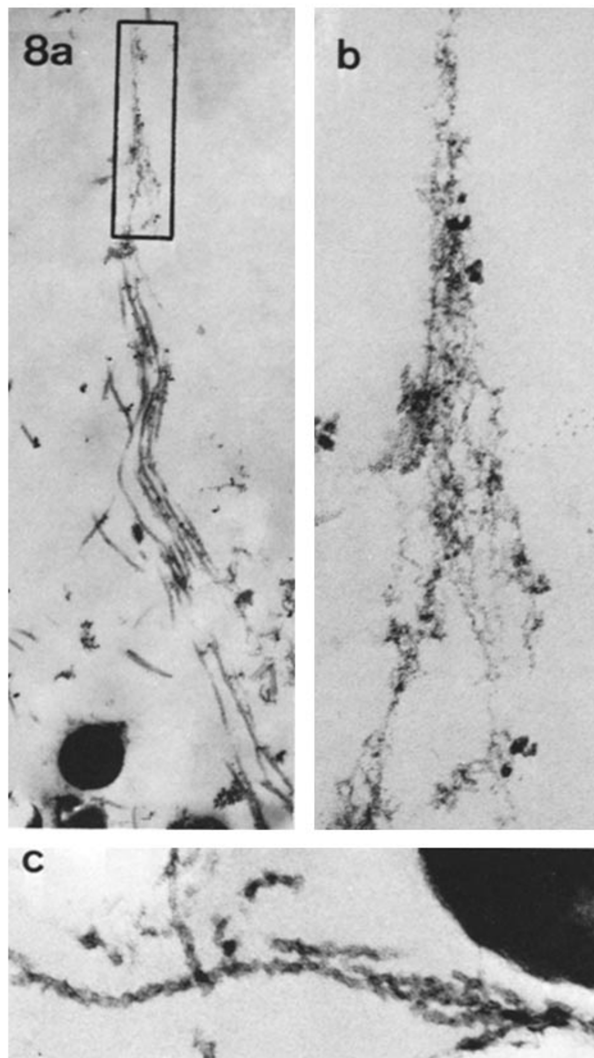


FIGURE 8 Melanophore lysed as in Fig. 7 but fixed without tannic acid. (*a*) Surface projection presumably corresponding to a microspike in overview. × 18,200. (*b*) Higher magnification of the meshwork of filaments encountered in the peripheral region of this structure. Note the more or less parallel alignment of filaments. × 76,000. (*c*) Melanophore lysed as in Fig. 7 but subsequently treated with HMM-S_1 for 10 min. Filaments in the cell periphery are decorated with an arrowhead pattern. × 106,000.

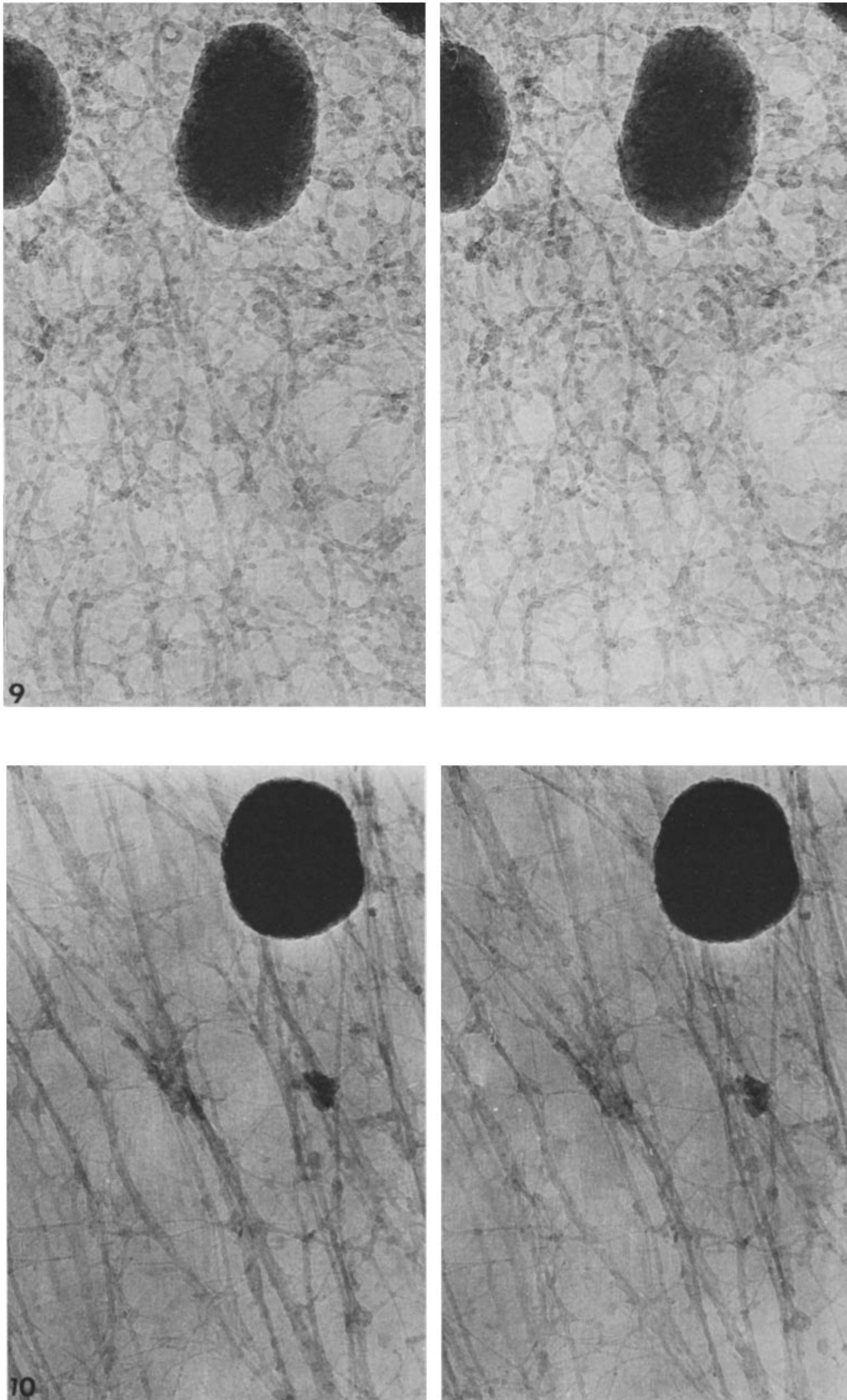
filaments are very rarely found in more central portions of the cell commonly occupied by pigment granules, microtubules, and the microtrabecular lattice. (*c*) Actin filaments appear in two forms of arrangement: (*i*) as a loose mesh or network that, in the intact cell, is probably located directly underneath the cell membrane, as in many other cells of various origin, and (*ii*) as a roughly parallel aligned bundle in various kinds of surface projections, similar to those previously documented in other cell types. (*d*) Actin filaments are clearly different from intermediate-sized filaments in morphology, arrangement, and localization within the melanophore. (*e*) For reasons presently unknown, F-actin in detergent-opened or glycerinated melanophores can only be demonstrated under conditions that also result in a good preservation of the prominent microtubular apparatus of these cells. (*f*) As judged by electron microscopy of Triton-extracted cells, there is little or no change in the arrangement or distribution of thin filaments as a function of the state of pigment distribution.

The procedures employed here seem to indicate a predominantly cortical arrangement of F-actin. In many cell types, subplasmalemmal microfilaments provide a shape-stabilizing element for certain peripheral cellular structures (such as microvilli and microspikes) or even the entire cell. The subcortical filament network in melanophores might have a similar cytoskeletal role. Support for this contention comes from microinjection experiments employing DNase I (Schliwa, Wehland, and Weber, unpublished observations), which caused characteristic shape changes of melanophores. If DNase I acts to disassemble F-actin after injection, then the shape changes might be attributed to DNase I–induced breakdown of the subcortical actin filament network. Whether cells in the scale display a similar cortical actin organization can not be decided at the moment. However, because isolated cells retain the remarkable characteristics of the microtubule apparatus as well as the cell's ability to move pigment granules, and because only a few hours elapsed between isolation of the cells and in vitro observation, it is probable that major features of the in situ cell are retained under our in vitro conditions. That cortical microfilaments have not been observed in cells in situ seems understandable in view of the relatively high degree of irregularity of the loose filament weave. This would be very difficult to recognize in thin sections because of overlap with other components of the peripheral cytoplasmic matrix of melanophores.

Our experiments also indicate some interrelationship between microtubules and actin filaments in detergent-treated cells. If microtubules are not preserved well, the actin filament component cannot be detected by either immunofluorescence or electron microscopy. This would be understandable on the basis that the cortical disposition of actin filaments is dependent for its integrity on the coexistence of intact microtubules.

Previous high voltage electron microscope studies of whole cells have demonstrated the presence of a complex three-dimensional system of filamentous strands (microtrabeculae) that apparently undergoes profound structural transformations as it mediates the centripetal and centrifugal transport of granules (4, 8, 22). These observations lead to the conclusion (4) that microtrabeculae are an important part of the motile machinery responsible for intracellular transport. Given this importance, it would be of particular interest to assess whether or not actin filaments are part of it. The lysis experiments show that very little of the trabecular lattice is retained using the current procedure. This would indicate that its molecular composition and/or structural properties are probably different from that of the cortical filamentous layer, which is preserved under the same conditions. Although the lysis procedure used here retains cortical F-actin while dissolving the microtrabecular mesh, this does not rule out the possibility that a finely divided actin network is present in the trabecular system but that we are unable as yet to preserve and fix it. Nevertheless, the apparent difference in Triton-extractability between the cortical actin mesh and the granule-associated microtrabecular lattice probably indicates a physiologically significant difference, elucidation of which will be of particular importance in the future.

We thank George Wray for keeping the High Voltage Electron Microscope (HVEM) facility in excellent working condition throughout this study. The expert secretarial assistance of Joyce Albersheim is also gratefully acknowledged.



FIGURES 9 and 10 Comparison of unextracted and extracted melanophores at high magnification. Whole-mount preparations of cells fixed in the process of pigment aggregation and viewed in the high voltage electron microscope. Both images show granules from the margin of the aggregating pigment mass. Cell center is beyond the top. Fig. 9 shows an unextracted cell. Note the three-dimensional system of microtrabeculae associated with microtubules and pigment granules. Fig. 10 shows an extracted cell. Microtubules are well preserved. Some filaments are associated with the microtubules of the upper cortex. Microtrabeculae have been removed by the extraction. × 60,000.

This study was supported in part by grant no. 1R01 GM 27324 from the National Institute of General Medical Sciences, National Institutes of Health (NIH), to K. R. Porter and by a Heisenberg fellowship from the Deutsche Forschungsgemeinschaft to M. Schliwa. The HVEM facility at the University of Colorado at Boulder was supported by grant no. NIH 5P41 RR00592 from the Division of Research Resources of NIH.

Received for publication 28 April 1980, and in revised form 1 December 1980.

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