TRANSFORMATIONS IN THE STRUCTURE OF THE CYTOPLASMIC GROUND SUBSTANCE IN ERYTHROPHORES DURING PIGMENT AGGREGATION AND DISPERSION

I. A Study using Whole-Cell Preparations in Stereo High Voltage Electron Microscopy

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ABSTRACT

Pigment migration in cultured erythrophores of the squirrel fish Holocentrus ascensionis, after manipulation with K+, epinephrine, 3',5'-dibutyryl cyclic adenosine monophosphate, theophylline, and caffeine, is essentially identical to that observed in this chromatophore in situ. For such observations, the erythrophores are dissociated from the scales with hyaluronidase and collagenase, and allowed to spread on an amorphous collagen substrate, where they resemble the discoid erythrophore in situ. In this state, they are readily fixed by glutaraldehyde and osmium tetroxide, and are then critical-point dried for whole-cell viewing in the high voltage electron microscope.

The organization and fine structure of the erythrophore cytoplasm was stereoscopically examined after fixation of the pigment granules in four experimental states: pigment dispersed, pigment aggregated, pigment aggregating, and pigment dispersing. In the dispersed cell, granules are contained in an extensive three-dimensional lattice composed of radially oriented microtubules and a network of fine filaments 3-6 nm in diameter (microtrabeculae), whereas in the aggregated cell, the microtubular system is absent, and the majority of the microtubules appear displaced into the cortices on the cytoplasmic surface of the plasma membrane. In cells fixed while aggregating, few microtrabeculae are observed, although formless thickenings are observed in the cortices, on granules, and between clumped granules. In dispersing cells, the microtubular system is reformed from material stored in the cortices and with the granules in the centrosphere.

These observations suggest that the granules are suspended in a dynamic microtubular system that withdraws during pigment aggregation and is restructured during pigment dispersion. The microtubules guide linear granule motion not by defining physical channels, but by a recognizable affinity of microtubules, microtrabeculae, and granules for one another.
The movement of pigment granules in teleost chromatophores encompasses two fundamental types of particle behavior observed in living cells: saltatory and uniform motion. The value of the chromatophore model resides in the fact that saltation occurs during the centrifugal dispersion of pigment granules, whereas the uniform velocity phase of particle motion occurs solely during the centripetal aggregation of pigment. The cytoplasmic events that lead to this difference in the inward and outward migration of pigment granules are far from understood, despite the fact that a considerable amount of work has been done on the role of specific cytoplasmic components, such as microtubules, in mediating pigment migration in general. It is the purpose of this study to elucidate the mechanism of inward and outward pigment motion.

That microtubules participate in active pigment translocation in teleost chromatophores is based on both morphological evidence and effects of antimotic agents on pigment migration. Bickle et al. (2) and Green (9) found that numerous microtubules were oriented along the direction of pigment migration in melanophores of Fundulus heteroclitus, and hence postulated a participatory role for them in mediating pigment migration. Murphy and Tilney (15) statistically proved that aligned granules reside near microtubules, thus demonstrating some type of association between granules and microtubules. However, no “arms” or “bridges” could be clearly shown to extend between the granules and microtubules. Evidence that colchicine and vinblastine inhibit pigment migration (11, 24), and that microtubules disappear after such treatment (25) further suggested an essential role for microtubules in the migration of pigment. Low temperature and high hydrostatic pressure were also shown to inhibit the migration of pigment with a concomitant disruption of microtubules in Gymnocorymbus melanophores (15).

Microfilaments have also been given considerable attention in chromatophore research. Microfilaments are numerous in amphibian melanophores (12, 13), although they appear to be few in fish melanophores (15, 19), and absent in erythrophores of Holocentrus ascensionis (18). Their role in teleost chromatophores remains obscure as their presence appears not to be essential for pigment migration.

The difference in the inward and outward motion of particles in teleost chromatophores led Green (9) to observe that granules behave as if they are embedded in a cytoplasmic continuum that “expands” during dispersion and “contracts” elastically during aggregation. The composition of this gel-like continuum was never clarified, and the relative contribution of microtubules, microfilaments, and other cytoplasmic elements to pigment translocation remains unknown. Recently, however, Schliwa and Bereiter-Hahn (19) reported evidence for a microtubule-independent contractile system in melanophores of Pterophyllum scalare. If the elastic component of the continuum does not involve microtubules, does it involve microfilaments?

In erythrophores of Holocentrus, where microfilaments are absent, pigment granule velocity is approximately 10 times greater than that in melanophores (18). Supposing that aggregation is microtubule independent in erythrophores, and microfilaments are absent, the only alternative left is the “other” cytoplasmic elements for mediating the elastic phase. In the erythrophore, as in other teleost chromatophores, there is no clear morphological understanding of the other cytoplasmic elements, although Porter (18) suggested that the “flocculent” component in the cellular ground substance, as seen in sections of these cells, may participate in mediating pigment aggregation. He further deduced from micrographs of thin sections that the microtubules disassembled, at least in part, with each aggregation and reassembled during dispersion.

Preparation of the in situ chromatophore for conventional electron microscopy requires embedding and sectioning, and unfortunately this introduces two serious drawbacks for visualizing the cytoplasmic ground substance. On the one hand, thin sectioning essentially confines observations to two dimensions, which leads to difficulties in interpreting structural relationships within the entire cytoplasm. On the other hand, the embedding matrix can be expected to obscure the finer filamentous elements of the cytoplasmic ground substance (3).

The difficulties encountered in studying teleost chromatophores in situ are not limited to traditional EM methods; there are other experimental problems in working with scale preparations. One problem is that the chromatophores in the scale are beneath an epithelial barrier. It is possible that the epithelial cells dampen or delay the effects of various ions, hormones, antimotic agents, and other experimental compounds being used, or that metabolically transform them. Also,
the epithelial barrier and collagen-rich matrix surrounding the chromatophore very likely retard the rate of fixation.

The high level of innervation of *in situ* chromatophores also leads to problems in interpreting effects of experimental compounds. For example, it is unknown whether changes in external K⁺ ion concentration are acting on the nerve fibers or nerve endings on the chromatophore, or are acting directly on the chromatophore membrane.

To eliminate the inherent difficulties involved with scale preparations, the investigations reported here have isolated erythrophores of *Holocentrus* for maintenance in tissue culture. Isolation of the cell in vitro results in denervation, improved availability for treatment with experimental agents, and more rapid fixation. However, most important, a thinly spread cell in culture enables one to apply techniques developed for whole cell viewing in the high voltage electron microscope (HVEM) (8, 26). Because embedding material is not used in whole-cell preparations, the flocculent material in the cytoplasm is more faithfully depicted. By taking images at various tilt angles, visualization of the cytoplasm as a three-dimensional unit is possible, and the relationship between the pigment granules, microtubules, and the cytoplasmic ground substance can then be quickly realized without resorting to tedious reconstruction techniques from series of thin sections.

Preliminary observations on these cells, and their fine structure during various stages of aggregation and dispersion, were presented in 1976 at the First International Congress on Cell Biology, Boston, Mass. (4).

**MATERIALS AND METHODS**

**Procurment of Fish**

Live *H. ascensionis* (Osbeck), commonly known as squirrel fish, were obtained from the Bermuda Biological Station and transported to Boulder, Colo. with only a brief stopover at the Marine Biological Laboratory, Woods Hole, Mass. The fish were maintained at 23°C in a 40-gallon aquarium with seawater prepared from Instant Ocean (Aquarium Systems, Inc., Eastlake, Ohio).

**Isolation of Cells**

Scales were removed with blunt, shallow forceps, and placed in marine Ca⁺⁺ and Mg⁺⁺-free phosphate-buffered saline (M-CMF-PBS [20]). All solutions expected to be in contact with scales or cells were adjusted to a pH of 7.3. After three consecutive washings in M-CMF-PBS, the scales were placed in an enzyme solution containing 0.15% collagenase (CLS), [Worthington Biochemical Corp., Freehold, N.J.], 0.10% hyaluronidase (Sigma type II), and 5% bovine serum albumin (Sigma Fr. V [Sigma Chemical Co., St. Louis, Mo.]) in M-CMF-PBS. The enzyme solution containing the scales was incubated at 23°C for 15 min in a rocker bath set at 16 oscillations per min. Following the incubation, the cells were gently pipetted off the scales, and centrifuged at 750 g for 5 min. The enzyme solution was decanted and the pellet was washed in M-CMF-PBS. After two further washings and centrifugation steps, the cells were resuspended in ~1 ml of Eagle's basal medium with Hanks' balanced salt solution (Flow Laboratories, Rockville, Md., 1-151D), and additional NaCl to adjust the medium to 0.69 M NaCl. In addition, the medium was supplemented with 10% fetal bovine serum (Flow Laboratories, 4-055D).

**Culture Procedure**

Cells were plated out on a thin film of amorphous collagen prepared from rat tail tendon (10). The amount of medium per cover slip approximated 0.5 ml, and two to three drops of the cellular resuspension were added by pipette. The cultures were maintained at 23°C in an atmosphere of 2% CO₂ and 98% air.

**Experimental Procedure**

Before treatment with experimental agents, the cell medium was replaced by teleost physiological saline (TPS [6]). Cultured erythrophores were tested for their ability to respond to various agents known to disperse or aggregate pigment *in situ*. Thus, cultures were perfused with TPS containing either elevated potassium levels (15 mM KCl replacing 15 mM NaCl) or 0.1 mM epinephrine (Sigma E-4250) to induce pigment aggregation. To disperse the pigment granules, erythrophores were treated with either high NaCl (174 mM NaCl, no KCl), 5 mM theophylline (Sigma A-1755), 5 mM caffeine (Sigma -0750) or 5 mM 3',5'-dibutyryl cyclic adenosine monophosphate [dbcAMP] (Sigma D-0627, Sigma Chemical Co., St. Louis, Mo.).

**Microscopy**

The behavior of pigment granules in response to these treatments was monitored in a Zeiss Universal microscope with a ×40 water immersion lens. During observation for movements of their pigment granules, the erythrophores were fixed for electron microscopy with their pigment in four experimental conditions; dispersed, aggregated, aggregating, and dispersing. All cells were prepared for whole-cell viewing under the HVEM, using techniques developed by Gershenbaum et al. (8), and Wolosewick and Porter (26). Briefly, gold grids (London Finder honey comb) were cleaned in nitric acid and rinsed with distilled water. Five grids
were placed on a floating film of 0.7% Formvar and picked up with a round 1.5-cm cover slip. The cover slip was then coated with a thin film of carbon in the Denton evaporator (Denton Vacuum Inc., Cherry Hill, N. J.). A drop of soluble collagen was placed on the cover slip supporting the grids and spread with a small glass rod without touching the cover slip. The cover slips were then sterilized under UV radiation. The cells were cultured directly on the completed lamination consisting of a cover slip, gold grids, Formvar, carbon, and amorphous collagen.

The whole-cell fixation procedure used was that described by Wolosewick and Porter (26). The medium was replaced with TPS and, at the proper distribution of pigment, 2% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2-7.4) was dropped onto the cells. After a 15-min glutaraldehyde fixation, the cells were postfixed in 1% OsO4 in 0.2 M sodium cacodylate for 10 min. And after a distilled water rinse, the erythrophores were stained with 0.5% uranyl acetate in S-collidine (pH 4.5) for 15 min. After an S-collidine and distilled water rinse, the grids and cells were removed from the cover slip and placed in a brass grid holder. The grids and brass assembly were then taken through a rapid dehydration with acetone, and thence into liquid CO2 from which they were dried by the critical-point method (1). Grids were coated on both sides with a thin layer of carbon, and were either stored in a desiccator or transferred at once to the JEM-1000 high voltage electron microscope. The erythrophores were examined under an accelerating voltage of 1,000 kV.

RESULTS

Cell Culture

The isolation technique yielded 30-40 erythrophores on each of six cover slips when two scales were taken through the procedure. Nearly all the isolated cells adhered to the substrate within 12 h, the majority taking on in outline a stellate morphology. Some unipolar and bipolar erythrophores were also present with axonal-like processes extending two to three times the length of the stellate cell's processes. Unfortunately, a higher cell yield dictated vigorous pipetting of cells off the scales which consequently lowered their viability and lessened the degree of spreading. When the isolation was performed with utmost gentleness, fewer erythrophores were isolated (15-20 per cover slip), but the degree of spreading of the erythrophores on the substrate was greatly enhanced. Indeed, many of the cells approached the desired discoidal morphology, which most closely resembles the shape of the erythrophore in situ (Figs. 1, 2).

The erythrophores, being highly specialized and differentiated cells, do not divide in culture, though they survive up to 10 days. The large number of epithelial cells co-cultured with the erythrophores proved valuable for conditioning the small amount of medium. In addition, the degree of spreading and proliferation of the epithelial cells served as an indicator of the success of the isolation, the toxicity or non-toxicity of the medium, and the acceptability of substrate conditions.

Pigment Behavior in Cultured Erythrophores

The physiological movements of granules in the cultured erythrophores appear to be identical to those observed in cells in whole-scale preparations. Outward movement is slower and saltatory, whereas inward motion is uniform in velocity. Most surprising is the fact that these observations were made without experimental treatment, for the isolated, denervated erythrophores pulsate as erythrophores do in whole-scale preparations, although not in unison (Fig. 1a, b). The pulsations, or the periodic centripetal and centrifugal migrations of pigment, were observed to continue up to 10 days, although the time between aggregation-dispersion cycles increases with age. A typical pulsation frequency of six times a minute was not uncommon in the first days of culturing.

Changing the relative concentrations of extracellular cations in the media affects the direction of pigment migration as it does in scale preparations, although less drastic concentration changes are necessary. TPS containing 20 mM KCl and 154 mM NaCl initiates pigment aggregation, and a return to normal TPS (5 mM KCl-169 mM NaCl) reinitiates pulsatory movements. Removal of the KCl and perfusion of the culture with 174 mM NaCl TPS halts pigment migration altogether, regardless of whether the pigment is in a dispersed, aggregated, or intermediate state.

The erythrophores in culture also respond to physiological agents that actively initiate pigment dispersion and aggregation in cells in situ. Aggregation of pigment granules occurs within 1-2 s after perfusion of the culture with 0.1 mM epinephrine in TPS (Fig. 1c, d). Dispersion of pigment granules can be brought about by treatment with either 5 mM dbcAMP or compounds that inhibit the phosphodiesterase that degrades cAMP, i.e., 5 mM theophylline or 5 mM caffeine. Complete dispersion of pigment granules was attained within 5 s after treatment with the disper-
Photomicrograph series of a living erythrophore in culture. Phase contrast with ×40 water immersion lens. × 2,800. (a) Pigment granules undergoing dispersion; (b) pigment granules maximally dispersed; (c) granules aggregating after treatment with 0.1 M epinephrine in teleost saline; (d) the erythrophore with its pigment fully aggregated. The diffraction rings around the central pigment mass suggest a cell-shape change. The acentric nucleus can be seen (to the lower right of the pigment mass) when the pigment is aggregated as well as a scattering of small granules (mitochondria) that do not migrate with the pigment granules into the cell center.

By rinsing out these agents, aggregation can be reinitiated with either KCl or epinephrine, and the application of the dispersive agents again, after a TPS wash, reintroduces dispersion. The control of aggregation and dispersion in this manner could be repeated several times before the pigment granules would refuse to disperse.

High Voltage Electron Microscopy

The overall cytoplasmic organization of the cultured erythrophore with its pigment granules...
Figure 2. High voltage electron micrograph of a whole erythrophore. The radial alignment of pigment granules and their tendency to occupy sectors coinciding with the cell’s processes are readily apparent. The nucleus resides in a position marginal to the centrosphere and does not seem to interfere with the alignment of granules. Mitochondria, mostly spherical, can be seen throughout the cytoplasm, except near the centrosphere. The areas within the rectangular outlines are enlarged in Figs. 3-5. × 4,000.

dispersed is quite similar to that observed in the in situ erythrophore. At low magnification in the HVEM (Fig. 2), a radial organization of pigment granules is apparent. The relatively dense ovoid nucleus occupies a position marginal to the cell center as also observed in thin sections (18). The mitochondria can be seen distributed throughout the cytoplasm, though they are excluded from the...
The majority of the mitochondria possess a morphology most closely resembling oblate spheroids, although more vermiform ones are also present.

Using low magnification stereomicroscopy (Fig. 3), the gross three-dimensional organization of the dispersed erythrophore cytoplast can be observed. Granules are found at all levels in the depth dimension of the cytoplasm, and those that are radially aligned appear to reside near radially oriented microtubules. The flat substrate membrane surface and the upper membrane surface can be easily identified, and thus the interior components can be readily distinguished from surface structures such as blebs and debris. besides pigment granules and the nucleus, there is little electron-dense material in the cytoplasm of the erythrophore. There appear to be very few ribosomes, and the membranous elements of the endoplasmic reticulum and the Golgi apparatus are meagerly represented. In addition, glycogen which is abundant in melanophores, is essentially absent in the erythrophore. Granules aside, the cytoplasm of the erythrophore is extraordinarily lucid in comparison to human cell cytoplasm (26).

The region near the center of a dispersed erythrophore contains numerous microtubules which reside at various levels of the cytoplasm (Fig. 4). The microtubules are radially oriented along the direction of pigment movement, and it appears that a greater proportion of granules and rows of granules are in regions rich in microtubules. Fine filaments, 3-6 nm in diameter, can be seen extending between granules, granules and microtubules, and cell cortices. These fine strands appear to compose the so-called lattice of the cytoplasmic ground substance, and have been termed microtrabeculae by Wolosewick and Porter (26).

The microtrabeculae are more evident if one examines a region closer to the cell margin (Fig. 5). Here the microtubules per unit volume seem fewer, and the majority tend to reside in or near the cortices of the cytoplast. The microtrabeculae, on the other hand, form an intricate three-dimensional lattice that extends between the two cell surfaces. The pigment granules are suspended in this lattice, and the aligned granules are associated

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1 A stereoscopic binocular viewer such as one manufactured by The Abrams Instrument Corp., Lansing, Mich., should be used to visualize the stereo images. Such stereo viewers magnify the images approximately two times.
with cortical microtubules via microtrabecular elements.

After a complete aggregation of its pigment granules, the erythrophore shows a dramatic change in shape (Fig. 6) as predicted by light microscopy and a recent SEM study of isolated melanophores (16). The extreme attenuation of the cell margins and the prominence of the central pigment mass are readily evident. Mitochondria do not migrate with the pigment granules, but remain outside the central region. Microtubules can be seen to extend from the cell margins and into the cell center. At a higher magnification (Fig. 7), it is clear that the space between the upper and lower membranes is free of microtrabeculae, except for small remnants extending from the cytoplasmic cortices. Microtubules lie adjacent and parallel to one another within the cortices of the cell, and when the upper and lower cortices come into close apposition near the attenuated cell margins, they appear to interdigitate and lie in one plane. The mitochondria are localized in the trabecula-free zone, and do not have connections to the cortical surfaces. Numerous tubular invaginations of the plasma membrane can be also seen throughout the trabecula-free region.

To understand better the events that lead to these dramatic transformations in the structure of the erythrophore cytoplast, a cell was fixed immediately after the initiation of pigment aggregation. Fixing the cell in the process of aggregation after treatment with epinephrine proved to be difficult to do, as the aggregation response is much more rapid with this hormone than with KCl. Thus, KCl was chosen to initiate aggregation of the pigment granules. Fixed while the pigment was in motion, the fine structural organization of the cytoplast seemed to be transitional between that of the dispersed and aggregated states. Low magnification stereopairs show that the majority of the granules have withdrawn from the cell margins; the remaining have either been left behind by the retreating pigment mass during fixation, or have been fixed in the process of migrating inward from more peripheral regions (Fig. 8). Most interesting, however, is the observation that the cytoplasmic ground substance also appears to be in a transitional structure phase. Thus, it loses its fine lacy appearance, and only the cortical microtubules persist. The microtubules are still seen extending to the cell margins. At higher magnifications, it is clear that the microtrabeculae no longer extend between the upper and lower membrane surfaces (Figs. 9, 10); they are fewer...
FIGURE 5 This is the region outlined in Fig. 2 near the margin of the dispersed erythrophore. Note that here in this thin portion of the cell, the majority of the microtubules reside in the cortices of the cell. Granules associated with the microtrabeular system are aligned with the cortical microtubules; 15° tilt. Bar, 0.5 μm. × 28,000.

FIGURE 6 Low magnification stereo images of an erythrophore that has aggregated its pigment. The central pigment mass towers above the very thin cell margins. Note should be taken of the radial orientation of the microtubules from the centrosphere and along the cortices, and into the cell processes where they lie within one plane. Several mitochondria are situated peripheral to the pigment mass. The region outlined is shown in Fig. 7; 25° tilt. Bar, 5 μm. × 3,000.
Region of the aggregated erythrophore outlined in Fig. 6. Microtubules lie adjacent and parallel to one another in the cortices, and interdigitate into one plane as the cell attenuates at its margins (lower right) during pigment aggregation. There is almost a total absence of granules and microtrabeculae in the space between the upper and lower cortices and membranes of the cell. It is to be noted that there are no microtrabecular connections between mitochondria and the cortices. The tubular structures at the lower left (arrowhead) are interpreted as invaginations of the plasma membrane, 8° tilt. Bar, 0.5 μm. × 28,000.

This image was obtained from an erythrophore fixed in the process of pigment aggregation. It is observed that the fine and lacy appearance of the cytoplasm in Fig. 3 has vanished. The majority of pigment granules have withdrawn from the margins, although a few small aggregates persist in the peripheral cytoplasm. The numerous microtubules are now in the cortices of the cytoplasm, and presumably help to maintain the cell's shape during aggregation; 15° tilt. Bar, 2 μm. × 7,200.
FIGURE 9  This stereo image shows the deformation of microtrabeculae in the erythrophore fixed during the aggregation of pigment; it illustrates the reason for the loss of the lacy appearance of the cytoplasm in Fig. 8. One observes that a considerable number of formless thickenings are now part of the cortices (arrow, long tail), and are also applied to the surfaces of granules and vesicles. The largest clumps of granules to the right are near the cell center. Several cortical microtubules are visible extending from this region to the left towards the cell margin. Vesicles (arrow, short tail) and tubular invaginations (arrowhead) can be seen; 12° tilt. Bar, 0.5 μm. × 56,000.

FIGURE 10  Higher magnification of a region in Fig. 9. Greatly thickened trabeculae can be seen connecting the closely associated granules (arrowhead). Note as well the dense formless thickenings in the cortices (arrow); 9° tilt. Bar, 0.2 μm. × 56,000.
in number, and those that remain either run between granules or protrude blindly into the space between the upper and lower surfaces. There appear to be very few connections between the pigment granules and the cortical microtubules (Figs. 8, 9). A considerable amount of the amorphous material of the trabeculae seems to have withdrawn into the cortices (Fig. 10). The microtrabeculae that connect adjacent granules are generally shorter and thicker (20-30 nm) than the microtrabeculae observed between granules in dispersed cells; they seem to have contracted.

When the erythrophore is fixed immediately after the initiation of pigment dispersion (Fig. 11), the organization of the more central cytoplasmic ground substance has the appearance of that found in dispersed cells. Even though only a few granules have had time to leave the central region, the cytoplasm beyond the pigment seems already to have achieved a fine lacy organization. At higher magnifications, this lacy structure can be shown to comprise the microtrabecular lattice (Fig. 12). Near the centrosphere, where granules are just beginning to migrate outward, the microtrabeculae that are present are particularly evident. The granules lie supported in the microtrabeculae, and are again preferably distributed along the microtubules.

Evidence of the transition in the structural state of the microtrabeculae during dispersion can be observed in the marginal regions where the cell remains attenuated and relatively free of pigment granules (Fig. 13). Here it is clear that the microtrabeculae are restructured before the arrival of

Figure 11 Whole cell image of an erythrophore fixed soon after the onset of dispersion. The cytoplasmic ground substance has again taken on the lacy appearance of the dispersed cell in Fig. 3. Only a few granules have left the cell center (C). The nucleus (N), as usual, seems not to get in the way of the migrating granules. Mitochondria (m) are observed at various distances from the cell center. Numerous microtubules extend from the pigment mass into the cell margins. The two outlined regions are shown in stereo in Figs. 12 and 13. × 8,000.
FIGURE 12 Region immediately adjacent to the pigment mass outlined in Fig. 11, showing the extensive microtrabecular system between the cortices of the cell. Pigment granules associated with the trabeculae are distributed along cortical and central microtubules; 10° tilt. Bar, 0.5 μm. x 28,000.

FIGURE 13 Marginal area outlined in Fig. 11 of an erythrophore fixed while undergoing pigment dispersion. Note the parallel cortical microtubules (arrowheads) and the association between them and the microtrabeculae. The microtrabecular system seems to be reformed or restructured in part from the amorphous material (arrow) sequestered within the cortices and along the microtubules during pigment aggregation; 9° tilt. Bar, 0.5 μm. x 40,000.
the advancing pigment granules. And evidence for any microtrabecula-free zone has vanished as the microtrabeculae extend between the cortices all the way up to the flattened cell margin. In the regions of attenuation, the microtrabeculae can also be seen, though in such regions they form only a two-dimensional lattice that appears fragmented. However, these flattened microtrabeculae are continuous with a dense amorphous material (the cortical layers) that is sequestered between the opposing membranes. And at the boundary, where the two opposing membranes delaminate, this amorphous material seems to contribute to the formation of the three-dimensional microtrabecular system.

DISCUSSION

Control of Pigment Migration

Smith and Smith (22) demonstrated that erythrophores aggregate their pigment when the scale is immersed in a solution of 0.1 M KCl. This elevation of the external potassium concentration tends to depolarize any excitable membrane in the scale. Thus, it is conceivable that pigment aggregation in whole scale preparations might be the result of the depolarization of innervating fibers ending on erythrophores, rather than the result of direct erythrophore membrane depolarization. The finding that erythrophores in culture aggregate their pigment in response to elevated levels of KCl proves that depolarization of the erythrophore membrane alone can directly initiate the aggregation of pigment. Although this finding might not seem too surprising, it becomes more interesting when considered in conjunction with the fact that periodic translocations of pigment (pulsations) are also not innervation dependent. Although the synchrony of erythrophore pulsations in situ would suggest some kind of electrical coupling, the observations on isolated cells indicate that pulsations are driven by some intrinsic mechanism of the cell.

Oscillations in the membrane potential or forced depolarization of the membrane by potassium is likely to affect the permeability of the membrane to various ions, particularly Ca++. It is difficult to demonstrate, however, that an influx of Ca++ causes aggregation, or to reliably interpret experiments that manipulate external Ca++ ions. One problem is that in varying the external Ca++ levels, the membrane’s permeability to sodium is changed, thereby affecting the excitability of the membrane. In addition, Ca++ chelators or ionophores may have unknown effects on the cell. Nevertheless, the surface invaginations seen in thin section (18) and in the whole-cell preparations may provide a specialized system for the delivery of external ions into the interior of the cell. It is possible that the surface invaginations mediate the aggregation and dispersion of granules by controlling the release and sequestration of Ca++ by internal vesicles, similar to what is achieved by the T-tubule system in skeletal muscle. On the other hand, this system could deliver the relatively high external Ca++ concentration directly into the cell, as in smooth muscle.

Although the influx and pathway of Ca++ into the erythrophore cytoplasm is uncertain, Ca++ is a likely choice for mediating pigment migration. One obvious reason for this is that calcium plays a crucial role in the most studied mechanisms of intracellular motility. For example, the elastic character of pigment aggregation in the erythrophore resembles the calcium-induced contraction of the spasmoneme in the stalk of peritrich ciliates (23). Withdrawal of the calcium causes the spasmoneme to re-extend and it may be that lowering of internal calcium levels in the erythrophore may permit the redispersion of granules. It was reported in the same paper by Weis-Fogh and Amos (23) that the presence of adenosine triphosphate (ATP) is not a requirement for the contraction or extension of the spasmoneme, although ATP may be the indirect energy source for extension by way of an ATP-requiring Ca++ pump that would lower the intracellular Ca++ concentration. While, as just noted, the aggregation of pigment also appears to be an elastic release of stored energy that does not require ATP, the analogy to the spasmoneme does not go further, for pigment dispersion is directly dependent on oxidative phosphorylation (11). In addition, Miyashita (14) reported that in fish melanophores the pigment dispersion is dependent on ATP and Mg++. Thus, the energy requirements for the dispersion of granules simulate the more familiar intracellular motility mechanisms that are dependent on the cyclic hydrolysis of ATP. The ATP-dependent dispersion, followed by an elastic release of stored energy on aggregation, fits with the simple observation that cultured erythrophores invariably die with their pigment aggregated, or are always “exhausted” in the aggregated state after successive treatments for
dispersion and aggregation. Further study is needed to determine whether the participation of ATP during dispersion is through adenylate cyclase to cAMP or through the direct activation of a motile gel, or a combination of these events.

**The Role of the Microtubule in Pigment Migration**

As in many types of cells, the microtubules in the erythrophore provide both a structural and an orienting framework. The observations on whole dispersed cells demonstrate that the microtubules extend from the centrosphere, and fuse with the cell cortex at various distances from the center. When the pigment granules aggregate, the more interior positioned microtubules are displaced, at least in part, to the cell cortices. Thereafter, the only structural component remaining is the extensive cortical microtubule framework, and it is assumed that this prevents the withdrawal of the cell processes during the sudden aggregation response. Some central microtubules may disassemble, but this needs examination by morphometric methods.

The microtubules clearly provide direction for the migration of pigment. It appears from observations on the whole dispersed cells that the granules do not necessarily move in structural “channels” in which, at minimum, three microtubules or two microtubules and a membrane surface might define a physically linear channel. Although granules are occasionally found in such channels near the cell center, where there are more microtubules per unit volume, granules are also found aligned along one or two cortical microtubules.

The proximity of linearly distributed granules and microtubules reflects an affinity between the granules and microtubules. This has led several investigators to propose a sliding mechanism for granules along microtubules (7, 14). However, even though the frequently noted “arms” or “bridges” between granules and microtubules can be visualized in the HVEM as microtubeculae, their presence does not necessarily suggest a sliding mechanism where the motive force is provided by an interaction between granules and microtubules.

**Role of the Microtubeculae in Pigment Migration**

The HVEM images of whole-cell erythrophore preparations demonstrate that there is a dramatic difference between the microtubeculae in the dispersed and aggregated states (Fig. 14). During aggregation, it appears that part of the microtubeculae shorten and translocate with the pigment granules while another part is left behind, attached to the upper and lower cortices of the cell. The microtubeculae that were once components in a fine three-dimensional lattice suddenly transform or become formless blobs on the inner surfaces of the cell cortex. On the other hand, as dispersion initiated, there is a lengthening and restructuring of the microtubeculae out of the cortices and from the centrosphere, and the embedded granules seem to follow. Much of this continuum, or lattice substance, is reconstructed before the granules arrive and the fact that pigment dispersion is relatively slow and is saltatory in character may reflect complexities involved in the reconstruction process. The higher degree of organization observed in the dispersing cell also fits well with evidence that dispersion is energy requiring and energy storing (11, 14).

Recent work by Pollard (17) has demonstrated the gelation of actin-containing extracts of *Acanthamoeba* is dependent on ATP and Mg++. In addition, contraction of the gelled extract is brought about by the addition of low Ca++ concentrations. With slightly higher levels of Ca++, the gel contracted so violently that it tore apart. It is conceivable that the energy-dependent lengthening and restructuring of the microtubeculae during pigment dispersion is analogous to the gelation of the amoeba extract. Also, the shearing forces generated in the contracting extract may be similar to the extremely rapid aggregation of pigment in erythrophores.

It is possible that the material of the microtubeculae represents, in part, the filamentous coating on microtubules (MAPs) that Dentler et al. (5) have observed on in vitro polymerized brain microtubules. When their microtubule-associated proteins (MAPs) are left out of the assembly procedure, the filamentous coating on the microtubules is lost. Of particular interest, Sloboda et al. (21) have found that one of the two MAPs that co-purifies with microtubules appears to be a cAMP-dependent protein kinase. The significance of these MAPs is uncertain, yet the investigators suggest that the protein may be involved as a motile protein component for axoplasmic transport. Similarly, as cAMP mediates pigment dis-
During pigment dispersion (Fig. 14 A), a dynamic three-dimensional lattice displaces the pigment granules as it expands and contracts along the microtubules and cortices of the cell. A preferred association of granules with microtubules mediated by the microtrabecular elements results in the linearly directed dispersion of granules. During pigment aggregation (Fig. 14 B), the continuum containing the granules suddenly contracts, and part of the microtrabecular system withdraws into the cortices for storage during aggregation, while another portion aggregates with the pigment granules into the centrosphere. As the pigment granules aggregate, the central microtubules are displaced into the cortices, and the upper and lower membranes are drawn together in the cell margins. Later, during pigment dispersion, the lattice is restructured out of the material stored in the cortices and along microtubules and around the granules.

It appears that the microtrabeculae are intimately involved in mediating pigment migration, yet the molecular events which bring about the transformation of the microtrabeculae, i.e. their...
shortening and lengthening, is entirely unknown. Clearly, the structural changes observed are compatible with Green's hypothesis (9) that there is an expansion and contraction of a continuum in which the granules are supported. The fact that the lattice-like continuum dramatically changes its structure during pigment aggregation suggests that the microtubules are not artifacts of fixation, but rather exist in the living cell. Whether they, in their deformation and their reformation, provide the motive force for pigment motion is less clear, but it is a possibility that lends itself to further investigation.

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