THE ROLE OF MICROTUBULES IN THE MOVEMENT OF PIGMENT GRANULES IN TELEOST MELANOPHORES

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ABSTRACT

When microtubules in teleost melanophores are disrupted with antimitotic agents, colchicine, high hydrostatic pressure, low temperature, and vinblastine, the alignment and movement of the pigment granules in these cells disappear; during recovery, the return of alignment and movement corresponds in both time and space with the repolymerization of microtubules. Furthermore, analysis of nearest neighbor distances in untreated melanophores reveals that pigment granules are closely associated with microtubules. Other structures such as microfilaments, the endoplasmic reticulum, and the cytoplasmic matrix do not appear to be involved. Thus we conclude that microtubules determine the alignment and are essential for the selective movements of the pigment granules in these cells.

Investigations of the mechanism of movement show that microtubules are required for both centrifugal and centripetal migrations and that they do not change in number or location during redistribution of pigment. Our results further indicate that microtubules in melanophores behave as semistable organelles as determined by investigation with colchicine and hydrostatic pressure. These observations and others rule out a push-pull mechanism based on the polymerization and depolymerization of microtubules or one which distinguishes two operationally different sets of microtubules. We propose instead that particles move by sliding along a fixed array of microtubules.

INTRODUCTION

Saltatory movement—the rapid displacement of particles over long distances—characterizes many cellular activities (Rebhun, 1972). Although in some cases microfilaments have been implicated (Belanger and Rustad, 1972; McGuire and Moellmann, 1972), many other cells or cellular structures which exhibit these movements contain large numbers of microtubules. The latter include nerve processes (Smith, 1971), secretory cells (Lacy et al., 1968), cells in tissue culture (Freed and Lebowitz, 1970), the mitotic apparatus (Nicklas and Koch, 1972), and the cell extensions of suctorians and heliozoans (Bardele, 1971; Tilney and Porter, 1965). In many of these systems antimitotic agents which are known to disrupt microtubules impede particle movement. Colchicine, for example, inhibits the transport of substances in nerve axons (Kreutzberg, 1969) as well as the secretory activity of the pancreas and thyroid gland (Williams and Wolfe, 1970; Lacy et al., 1968). It also disturbs
the saltatory movement of particles in HeLa cells and amphibian leukocytes (Freed and Lebowitz, 1970; Hard and Cloney, 1971). Assuming that colchicine affects microtubules specifically, these observations and others suggest that microtubules participate in some of the streaming movements of particles within cells.

One dramatic example of intracellular particle movement is provided by teleost chromatophores. This cell is a model system for investigating the mechanism of microtubule-associated movement because its function in the organism is to cause color change by regulating the intracellular transport of tens of thousands of pigment granules (Matthews, 1931). The selective movement of these pigment granules leads to one of two possible end states—dispersion of the granule mass causes darkening, while aggregation of the granule mass has the reverse effect (see Fig. 1). Bikle et al. (1966) and Green (1968) demonstrated that the melanophores of Fundulus heteroclitus contain numerous microtubules. Others demonstrated a correlation between microtubules and granule movement by showing that the dispersion and concentration of pigment in these cells was sensitive to colchicine and vinblastine (Junquiera and Porter, 1969; Wright, 1955; Malawista, 1965, 1971a; Wikswo and Novales, 1969). Furthermore, this behavior seemed to be correlated with a reduction in the number of microtubules (Wikswo and Novales, 1972). Other mechanisms have also been postulated for granule motility in vertebrate chromatophores. These include microfilaments (Malawista, 1971b; McGuire and Moellmann, 1972), sol to gel transformations of the cellular cytoplasm (Marsland, 1944; Malawista, 1971a) and models of intracellular electrophoresis (Kinoshita, 1963).

To confirm that microtubules are required for motility, we have examined the effect of low temperature, high hydrostatic pressure, and colchicine on granule movement. We assayed the effects of these agents not by photometry of whole cells as was done previously, but by monitoring the behavior of individual particles. We have also quantitated the number of microtubules in cell processes and demonstrated by means of a statistical procedure that the pigment granules are associated with microtubules in these cells. These data and others reveal that melanophore microtubules are semistable organelles and that the mechanism of movement does not involve the reversible assembly of microtubules. We propose that the granules move by sliding along a fixed array of these organelles.

MATERIALS AND METHODS

Maintenance of Fish

Specimens of Fundulus heteroclitus (obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Mass.) were kept in 50-gallon salt water aquaria at 16°C and were fed minced frozen haddock and Tetra Min flakes (Tetra Werke, W. Germany). Specimens of Holocentrus ascensionis (marine) and Gymnocorymbus ternetzi (fresh water) were obtained from local aquarists and maintained at 22°C on a similar diet.

Preparation of Living Cells and Observation of Granule Movement

Dermal scales were removed from fish with a pair of fine forceps. The scales were mounted in teleost Ringer's solution on a microscope slide and observed with a Zeiss microscope in green light obtained with a Zeiss broadband interference filter (Carl Zeiss, Inc., New York). The scales of Fundulus were mounted in marine teleost Ringer's solution (Foster and Taggart, 1950) while the scales of Gymnocorymbus were mounted in fresh water teleost Ringer's solution (Cavanaugh, 1964). Granule movements as small as 0.5 μm could easily be detected. To induce the aggregation of pigment, potassium Ringer's (KCl substituted for NaCl in the Ringer's solution) or 10^-4 M adrenalin (Parke, Davis and Co., Detroit, Mich.) in teleost Ringer's solution was used. Adrenalin is known to act directly on the fish melanophore (Fujii, 1961) and has been used at this concentration by Spaeth and Barbour (1917) to induce pigment migration in Fundulus melanophores after pretreatment with various drugs. No toxic effects on the cells were observed and the effect of these stimuli could be reversed by rinsing the scales in the standard Ringer's solution.

Granule movements were recorded both by still photography on 35-mm film and by 16-mm cinematography. These recordings were used to measure the velocity and amplitude of individual granule movements and to determine the boundaries of the zone within which alignment and movement of the granules appeared during recovery from treatment with various agents.

Physical Techniques

Low Temperature: The scales of Gymnocorymbus were mounted on a cold stage adapted for the light microscope which permitted the experimenter to change the temperature from 22°C to -5°C within several seconds. (See Murphy, 1973 for details.) The scales were kept at -5°C for 20-30 min, transferred to teleost Ringer's solution at 22°C, and examined during the
**Figure 1** Melanophores of *Fundulus heteroclitus*. The pigment in the melanophores on the right is aggregated about the center of the cell (aggregated state) while that on the left is dispersed throughout the processes of the cell (dispersed state). × 700.

**Figure 2** Detail of a *Fundulus* melanophore in the dispersed state revealing its numerous processes and a large, granule-free central zone. Each process contains hundreds of granules arranged in linear files which radiate from the cell center. Neither nuclei nor mitochondria exhibit the selective movements characteristic of the pigment granules. × 7,600.
Manuscript in preparation.

Hydrostatic pressure: The scales of *Gymnocorymbus* and *Fundulus* were placed in a small chamber which was especially designed by Edward Salmon to permit observation of living cells with the light microscope during the application of hydrostatic pressure (Salmon and Ellis, 1973). With this apparatus one could easily detect pigment granules 0.3 µm in diameter in melanoophores under pressures up to 11,000 lb/in\(^2\). For examination of chromatophores fixed during the application of hydrostatic pressure, another chamber was employed which permitted the introduction of fixative to living cells while under hydrostatic pressure. The details of this apparatus have been described by Landau and Thibodeau (1962).

Procedure for Electron Microscopy

Whole scales were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 for 1 h. Glutaraldehyde immediately arrests all granule movements and does not distort the alignment of the pigment granules or otherwise alter the appearance of melanoophores in the light microscope. After glutaraldehyde the scales were washed in buffer and postfixed in 1% OsO\(_4\) in 0.1 M cacodylate buffer at pH 7.4. The scales were rapidly dehydrated in acetone and then allowed to return to room temperature in absolute acetone. The fixation, washing, and dehydration were all performed on ice at 0°C except for the first 10 min of fixation with glutaraldehyde which was performed at room temperature. The scales were embedded in Araldite (Ladd Research Industries, Inc., Burlington, Vt.) and polymerized at 70°C for 48 h. Sections were cut on diamond knife with a Sorvall Porter-Blum MT-1 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.) and mounted on lightly carbonized, colloidin-coated grids. Supported grids helped to prevent distortion and tearing caused by the uneven expansion of the fish scale and adjacent chromatophores under the electron beam. The sections were stained in saturated alcoholic uranyl acetate (Stempak and Ward, 1964) and Reynolds' lead citrate (Reynolds, 1963) and examined with a Philips 200 electron microscope.

RESULTS

The Melanophores of *Fundulus* and *Gymnocorymbus*

The light microscopy and fine structure of melanoophores of *Fundulus* have been described in detail by Bikle, et al. (1966), Green (1968), and Wikswo and Novales (1969 and 1972) and need little further clarification here. We will mention only those observations pertinent to our experimental studies.

During aggregation or dispersion the pigment granules move in linear tracks which radiate from a common focus in the center of the cell (Figs. 2, 3, 4a). The similarity of this pattern to the astral organization of microtubules within these cells led Bikle et al. (1966) and Green (1968) to hypothesize that microtubules might govern particle movement. The focus of the aster is a well developed centrosome which contains two centrioles. It is possible, particularly in *Fundulus* melanophores, to discern two distinct distributions of microtubules in the cell; one set is found within 1,000 Å of the surface of the cell (cortical set), while the other set occupies the central portions of the cytoplasm (central set) (Figs. 18, 19).

Green (1965) has described two distinct types of movement in the melanophores of *Fundulus*, which are also observed in *Gymnocorymbus*, namely mass movement and shuttling movement. Mass movement, the coordinated cytoplasmic streaming of granules observed during dispersion and aggregation, is characterized by the uninterrupted, unidirectional movement of granules in linear files for distances up to tens of micrometers. During aggregation, granule movements are regular although constrained, while during dispersion they are more irregular and less organized. Shuttling movements, the back and forth displacements over distances of approximately 5 µm, do not result in net displacement of granules and are usually seen in cells in a partially aggregated state.

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\(^1\) Salmon, E. D., and G. W. Ellis. 1973. Miniature hydrostatic pressure chambers for observations of living cells with polarized light and phase-contrast microscopy. Manuscript in preparation.
In this report, the melanophores of two different fish were examined. The melanophores of Gymnocorymbus were used for studies of granule alignment since the cells are large and have rows of granules which can be easily traced to the very center of the cell (Fig. 4 a). The large cylindrical processes of Fundulus melanophores, on the other hand, were much better suited for determining the number of microtubules in the dispersed and aggregated states.

**Effects of Antimitotic Agents on the Intracellular Alignment and Movement of Pigment Granules**

**Low Temperature:** In the untreated melanophores of Gymnocorymbus, pigment granules are aligned into rows which form an impressive astral arrangement (Fig. 4 a). After 10 min at \(-5^\circ\text{C}\), however, the alignment of pigment granules disappeared and the granules assumed an even distribution throughout the melanophore (Fig. 4 b). With this loss in alignment of the granules, all mass motion and shuttling movements ceased and did not resume even upon the addition of adrenalin, a potent stimulus for pigment aggregation (Spaeth and Barbour, 1917), provided the melanophores were maintained at \(-5^\circ\text{C}\).

In scales fixed after 20 min at \(-5^\circ\text{C}\), microtubules (Fig. 5) were no longer observed in electron micrographs, although in rare cases a few microtubules could be seen near the centrosome. Instead a fibrous material, possible breakdown material from the microtubules was observed either in dense spherical masses up to 2 \(\mu\text{m}\) in diameter or loosely distributed along the plasma membrane of the cell. Mitochondria, vesicles, and the membranes surrounding the melanin granules appeared slightly swollen, but no differences could otherwise be detected in the morphology of cortical pits, in the endoplasmic reticulum, in the overall contour of the cell, or in the number of microfilaments.

To study the process of granule realignment, scales were observed during rewarming to \(22^\circ\text{C}\) on the cold stage. After 1 min of recovery some reorganization was occasionally detected at the centrosome. After 3 min of recovery, however,
Sequence of photographs from a melanophore of Gymnocorymbus ternetzi prior to, during, and after treatment at -5°C. (a) Before treatment the melanin granules are aligned onto conspicuous rays which emanate from the center of the cell. (b) After a few minutes at -5°C granule alignment is obliterated as microtubules become totally disrupted. (c) 3 min recovery at room temperature after 20 min at -5°C. Short rows of granules separated by rays of clear cytoplasm are seen emanating from the centrosome. This realignment is correlated with the repolymerization of microtubules (see Fig. 10). (d) 20-min recovery. The radial alignment of granules extends from the centrosome to the outer tips of the processes. × 3,600.
rows of granules 15-20 µm long were observed near the centrosome (Fig. 4 c), but no signs of alignment could be detected either in the middle or at the tips of the melanophore processes. After 5-10 min the pattern of realignment near the centrosome intensified, but alignment was not observed in the processes. 15-20 min after removal from low temperature, rows of granules extended 30-40 µm into the processes (Fig. 4 d) and by 30 min the cells were fully recovered. The rate of centrifugal advance of this reorganization was 2-5 µm/min. A graph showing the relationship of the radius of this zone to the length of the recovery period is shown in Fig. 6.

The initiation of granule movement during recovery from low temperature was studied using adrenalin as the stimulus for aggregation of the pigment granules. The extent of movement was monitored by fixing the scales in 4% glutaraldehyde after 1 min in adrenalin Ringer's. For scales which were allowed to recover from low temperature for various periods of time, the recovery period was considered as the total time of exposure to warm (22°C) Ringer's solution. Scales that were permitted 1 min of recovery were transferred from cold Ringer's to warm adrenalin Ringer's for 1 min and then fixed. For recovery periods longer than 1 min, scales were transferred from cold Ringer's to warm Ringer's and transferred again to warm adrenalin Ringer's for the final minute of recovery and then fixed. Using this procedure, cells were observed after 0, 1, 3, 5, 10, 15, 20, and 30 min of recovery.

In control cells, granules rapidly migrate to the cell center and form a large concentrated mass during a 1-min exposure to adrenalin (Fig. 7 a). No streaming was observed, however, in cells which had been transferred to adrenalin Ringer's at -5°C (Fig. 7 b). After 1 min of recovery, granules 5 µm from the centrosome moved in response to adrenalin, leaving behind a conspicuous bald spot, while those in the peripheral cytoplasm remained immobile (Fig. 7 c). After 3 min, the radius of the zone within which granules made centripetal...

**Figure 5** Transverse section at the centrosome of a Gymnocorymbus melanophore treated with low temperature. No microtubules can be seen. Instead fibrous material often appearing as large spherical masses indicated by arrows is seen throughout the cytoplasm. Small tubular profiles of the endoplasmic reticulum are also observed. × 37,000.
Recovery Time (Min)

FIGURE 6 The realignment of granules during recovery from treatment at -5°C. Realignment is expressed as the radius of the zone within which reorganization can be seen. During recovery, this zone advances centrifugally at a rate of 2-5 µm/min. Each point represents the mean of five radii measured per cell. Four different cells are represented.

movements expanded to 10-15 µm and numerous minute aggregations of granules formed throughout the processes. After 15 min granules throughout the cell could make centripetal movements in response to adrenalin, but the patterns of granule movement during aggregation were not uniform and some clumping of the granules still occurred in the processes. After 20-30 min the response was indistinguishable from that observed in control cells (Fig. 7d). Thus the zone within which granules made centripetal movements advanced centrifugally at 5-10 µm/min, about twice the rate of the advancement of granule realignment previously discussed. The fact that the rate of centrifugal reorganization is less than the rate of advance of adrenalin response may indicate that many more microtubules are required to enforce the patterns of linear organization than are needed for movement. A graph showing the increase of the radius of this zone as a function of recovery time is shown in Fig. 8.

To observe the kinetics of repolymerization of microtubules, scales which had been cooled at -5°C for 30 min were transferred into teleost Ringer's at room temperature. After 0, 1, 3, 10, and 30 min of recovery the scales were fixed for electron microscopy. Transverse sections were cut at the centrosome and at 10-20 µm, 30-40 µm, and 70-80 µm from the centrosome. At each region, microtubules were counted and expressed as the number of microtubules observed in transsections 10 µm in width (see Fig. 9). After the first survey was completed, the entire procedure was again repeated to confirm the original findings. For each time of recovery and location within the cell ten transverse sections, representing at least two cells, were examined and tabulated. The microtubules in Gymnocrystus melanophores are not always distributed in distinct cortical and central sets as they are in the melanophores of Fundulus. During recovery it was therefore not possible to discern any differential repolymerization of the two "sets" of microtubules. Accordingly, the counts represent the total number of microtubules.

As can be seen in Fig. 10, the control cells contained approximately 100 microtubules per 10 µm wide transsection at each of the four locations. After 1 min of recovery, a few microtubules appeared near the centrosome and in the 10 to 20-µm zone, but were not found in the outer processes of the cell. Microtubules appeared to repolymerize from the centrosome, suggesting that the centrosome behaves like a nucleating center of the type described by Pickett-Heaps (1969) and Tilney (1970). After 3 min, microtubules were found throughout the melanophore, but unlike the untreated melanophore, the bulk of microtubules was present in the centrosome with a few, and those few disordered, in the processes. In fact there were more microtubules in the centrosome per unit area in the recovering melanophore than in untreated cells. After 5 min of recovery, microtubules assumed precise radial arrangement out to the 10 to 20-µm zone, but were less organized beyond this point. By 10 min, the number of microtubules in the centrosome returned to control levels, although the number in the processes was still less than in control cells. The amount of fibrous material along the plasma membrane and the number of spherical masses also decreased. Electron micrographs of cells which recovered for 30 min were indistinguishable from those of untreated cells (Fig. 3). The number of microtubules at varying distances from the centrosome is illustrated graphically in Fig. 10.

HYDROSTATIC PRESSURE: Hydrostatic pressure was applied to Gymnocrystus melanophores which had been placed in teleost Ringer's solution to cause dispersion. Hydrostatic pressure has been previously shown to disrupt the spindle apparatus (Zimmerman and Marsland, 1964; Salmon, 1973) and depolymerize microtubules in the heliozoan axoneme (Tilney et al., 1966). Within a

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3 Salmon, E. D. 1973. Thermodynamics of spindle microtubule polymerization, an in vivo hydrostatic pressure-temperature analysis. Manuscript in preparation.
Adrenalin-induced aggregation of pigment granules in different Gymnocorymbus melanophores during recovery from treatment at -4°C. (a) Control cell. In cells not treated with low temperature granules migrate and form a large concentrated mass. (b) 0-min recovery. In the absence of microtubules, the granule mass remains dispersed, and no movements occur. (c) 1-min recovery. Granules near the centrosome move leaving behind a conspicuous bald spot. The peripheral granules remain immobile. (d) 20-min recovery. Almost all of the granules stream towards the cell center, forming a concentrated granule mass. x 2,400.

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Figure 8. Graph depicting the capacity of the pigment granules for centripetal movement after treatment with low temperature. The capacity for granule movement is expressed as the radius of the zone within which granules make centripetal excursions to the centrosome during a 1-min exposure to an adrenalin stimulus. During recovery this zone advances centrifugally at a rate of 5-10 μm/min. Each point represents the mean of 15 measurements from 5 different cells.

Figure 9. To examine the kinetics of microtubule repolymerization, microtubules were counted in cells fixed after 0, 1, 3, and 10 min of recovery at room temperature. Transverse sections were taken in these cells at the centrosome. The processes are tubular and contain microtubules which are parallel to each other and to the long axis of the process. The counts are expressed as the number of microtubules encountered in a transection 10 μm wide for each of the four zones as indicated. See Fig. 10.

Few seconds of applying hydrostatic pressure at 9,000 lb/in² most shuttling movements ceased and the granules clumped together. After 1-2 min many granules were motionless. The limited granule movements which could be observed were of such low amplitude that they could not be distinguished from the Brownian movement of granules confined within a flattened space. These movements were not present in untreated cells. The linear organization of the granules disappeared rapidly and uniformly over the entire melanophore, and after 1 min of the application of pressure, rows of granules could no longer be identified (Fig. 11).

To determine the effects of hydrostatic pressure on cell ultrastructure, melanophores of Gymnocorymbus were subjected to 9,000 lb/in² for 4 min and then fixed under pressure. In all cases, microtubules were disrupted by this treatment and were either greatly reduced in number or completely absent (Fig. 12). Large spherical masses of fibrous material, 0.5 μm in diameter appeared after this treatment. We observed slight swelling of the melanin granules and mitochondrial cristae, and the endoplasmic reticulum appeared more irregular and vesiculated; but the general ultrastructure of the pressure-treated cells remained unchanged.

The recovery of Gymnocorymbus melanophores...
Figure 11. The effect of 9,000 lb/in² hydrostatic pressure on Gymnocorymbus melanophores. Granule alignment and movement are completely disrupted by this treatment. × 2,100.

Figure 12. The effect of hydrostatic pressure on the fine structure of Gymnocorymbus melanophores. After 4 min at 9,000 lb/in² no microtubules can be seen. Fibrous material not present in control cells is seen throughout the cytoplasm as compact spherical masses. The endoplasmic reticulum is irregular. × 60,000.
from high hydrostatic pressure was also studied. Melanophores of Gymnocrumbus containing dispersed pigment were subjected to 9,000 lb/in² for 10 min. 10 s after decompression no alignment of the granules could be detected in the cell, although the granules assumed a more even distribution and the clumping of granules was reduced considerably. By 30 s most of the clumps were gone and realignment reappeared in the centrosomal region. After 40–60 s of recovery, a zone of realignment extended 5 μm. The organization of granules into rows in the processes commenced at 1.5 min and was clearly visible at the bases of the processes by 3 min. Reorganization (Fig. 13) could be seen in both the arms and the cell body by 15 min.

To observe the repolymerization of microtubules during recovery, scales of Gymnocrumbus were treated with 8,000 lb/in² for 4 min and then fixed after 40 s, 80 s, and 4 min recovery at atmospheric pressure. After 40 s, microtubules could be identified in the vicinity of the centrosome but were not seen in the processes of the cell. By this time, swelling of mitochondria and vesicles and the irregular appearance of the endoplasmic reticulum were gone. After 80 s, microtubules were found throughout the cell but did not show precise radial orientation. The masses of fibrous material induced by hydrostatic pressure were smaller in size and fewer in number after 4 min than during earlier stages of recovery.

Previous work by Marsland (1944) had shown that aggregated pigment in Fundulus melanophores promptly dispersed in response to hydrostatic pressure. To examine this effect on Gymnocrumbus, scales were placed in adrenalin Ringer’s to induce pigment aggregation and were then gradually subjected to hydrostatic pressure in increments of 1,000 lb/in² lasting 10 min each. Under pressures from 1,000–5,000 lb/in² the granule mass dispersed in a regular fashion, the granules migrating centrifugally in distinct linear files. At 6,000 lb/in² and above, however, migration and shuttling movements ceased, and the granules clumped together. Similarly, if 6,000 lb/in² or greater pressure was applied suddenly (within a few seconds) to melanophores prepared in the same way, limited migration and shuttling occurred for 20 s, but after 30–45 s, the granules clumped together and movement stopped.

These experiments were also performed on the scales of Fundulus. In contrast to the melanophores of Gymnocrumbus, however, the melanophores of Fundulus demonstrated a marked tolerance to hydrostatic pressure. When hydrostatic pressures of 11,000 lb/in² were applied to scales containing melanophores in either the dispersed or aggregated state, the shuttling movements of the granules along distinct linear tracks were reduced but were not arrested even after 24 min of treatment. Electron microscope examination of cells fixed 20 min after the application of 11,000 lb/in² revealed an intact ultrastructure complete with microtubules; only a small amount of fibrous material could be seen. After the release of 11,000 lb/in², dramatic granule streaming resumed after 2 min. Thus, although pressure at 11,000 lb/in² induced dispersion of pigment in Fundulus, it had very little effect on the retardation of granule movement or the disruption of microtubules.

Colchicine and Colcemid: Colchicine and Colcemid at concentrations of 2 x 10⁻³ M slightly perturbed, but did not arrest granule movement in the melanophores of Gymnocrumbus and Fundulus after scales had been exposed to the drugs for 1 h at room temperature. The alignment of melanin granules, although disturbed, could still be detected. In addition, the granules exhibited active shuttling movements and aggregated in response to an adrenalin stimulus. Even with modifications to promote entry of these alkaloids, granule movement was not affected; these included partial digestion of fish scales in collagenase and incubation in 1% dimethylsulfoxide plus colchicine. After collagenase treatment, pieces of the dermis could be isolated from the scale so that the melanophores were directly exposed to 10⁻⁴ M Colcemid for 2 h. The pigment
in isolated melanophores of Fundulus cultured individually on cover slips exhibited shuttling movements after incubation in Colcemid for 70 min and aggregated rapidly in response to adrenalin. Microtubules were partially disrupted by this treatment as was earlier shown by Wikswo and Novales (1972), but considerable numbers of microtubules remained in the melanophores of Gymnocorymbus and Fundulus whose scales had been incubated for 30 min in $2.5 \times 10^{-8}$ M colchicine.

In contrast to the above results, normal granule movements in Gymnocorymbus melanophores were not observed if scales were incubated in $10^{-5}$ M colchicine or Colcemid for 30 min at $-5^\circ$C, and then returned to room temperature. The cells contained a nonaligned, uniformly dispersed population of melanin granules (Fig. 14). Shuttling and mass motions were arrested and could not be restored by the addition of adrenalin. If the concentration of Colcemid was reduced to $10^{-7}$ M, granule movement was impaired but not inhibited.

For electron microscopy, scales were treated with $10^{-5}$ M colchicine or Colcemid at $-5^\circ$C for 30 min, warmed to room temperature for an additional 30 min and then fixed. Microtubules were never observed. Mitochondria, endoplasmic reticulum, cortical pits, and melanin granules all appeared normal, and some fibrous material was also observed (Fig. 15). It was difficult to reverse the effect of these drugs, since granule movements only partially recovered after rinsing for 6 h.

**VINBLASTINE:** When Gymnocorymbus scales were incubated in $10^{-8}$ M vinblastine sulfate in teleost Ringer's, granule alignment began to disappear after 25 min. During aggregation in response to adrenalin, the granules did not move in linear files but swerved and moved in undulating patterns during migration to the cell center. After 45 min many cells showed conspicuous vinblastine crystals (Fig. 16), and no mass movements occurred in response to adrenalin. Electron microscope examination of cells treated with $10^{-8}$ M vinblastine revealed the presence of typical vinblastine crystals as described by Bensch and Malawista (1969): hexagonally packed tubular profiles in cross section with a parallel lattice structure in longitudinal section (Fig. 17). No intact microtubules were found in cells containing vinblastine crystals. The cell cytoplasm was dense, and the smooth endoplasmic reticulum, cortical pits, and melanin granules showed little or no distortion. The number and size of the crystals increased with time so that after

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**FIGURE 14** The effect of $10^{-8}$ M Colcemid on granule alignment in Gymnocorymbus melanophores. Realignment does not occur when cells are returned to room temperature in the presence of Colcemid. Colcemid alone causes only partial disruption of microtubules and does not completely arrest granule movement. $\times 2,400$. 

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FIGURE 15. The effect of Colcemid \((10^{-4} \text{ M})\) on fine structure in *Gymnocorymbus* melanophores. Scales are incubated in Colcemid at \(-5^\circ\text{C}\) for 30 min and then returned to room temperature for additional treatment for 30 min. Microtubules are never seen, but fibrous material not present in control cells is observed. \(\times 97,000\).

2.5 h many cells were filled with large rectangular crystals.

**Comparison of the Number of Microtubules in Melanophores in the Dispersed and Aggregated States**

From the previous section we concluded that microtubules are essential for granule alignment and movement. To distinguish between the various possible mechanisms of movement, it is necessary to compare in a quantitative fashion the relative numbers of microtubules in melanophore processes which contain pigment in the aggregated and dispersed states. It is also important to determine if changes in distribution of microtubules occur, that is, changes in the number of “cortical” and “central” microtubules in a process. For these purposes transverse sections of *Fundulus* scales were made 20-30 \(\mu\text{m}\) from the centrosome from approximately five cells each in the dispersed and aggregated states. Microtubules were counted in 17 empty processes (aggregated state) and 12 full processes (dispersed state) and classified according to their distribution (Figs. 18, 19). They were designated “cortical” if they were found within the limits of the cortical pits, invaginations of the plasma membrane, or “central” if they were beyond this limit. There was usually no difficulty in classifying the microtubules. Since the diameters of the processes, and so the number of microtubules per process, vary, the microtubule counts were expressed as the number of microtubules per \(\mu\text{m}^2\) of transectional area.

As shown in Table I, no changes were noted in the distribution of microtubules in these processes. Of the 953 microtubules counted in the processes of melanophores in the dispersed state, approximately half were found along the cortex and half in the central portions of the cell; a similar distribution was observed for 1,393 microtubules counted in cells in the aggregated state. Furthermore, the overall density of microtubules was found to remain constant for both states: \(1.52 \pm 0.31\) microtubules per \(\mu\text{m}^2\) in the dispersed state and \(1.46 \pm 0.11\) microtubules per \(\mu\text{m}^2\) in the aggregated state. Student’s \(t\) test showed no significant difference between the two means (Sokal and Rohlf, 1969).

**Nearest Neighbor Analysis of Granule-Microtubule Distances**

We have demonstrated that alignment and movement of pigment granules in melanophores require the presence of microtubules. One would therefore expect to observe a close physical association between pigment granules and microtubules; however, cursory examination of electron micro-
graphs does not reveal any obvious association. This is due largely to the fact that thin sections do not always include the regions of closest proximity between the granules and microtubules. To determine if this association could be statistically demonstrated, we therefore used a modification of the nearest neighbor analysis of Clark and Evans (1954). For this analysis, we used the erythro-

**Figure 16** The effect of $10^{-5}$ M vinblastine on granule alignment in *Gymnocorymbus* melanophores. After 30-45 min at room temperature, crystals form which appear as rectangular regions from which pigment granules have been excluded. Granule movements are completely arrested. $\times 1,700$.

**Figure 17** The effect of vinblastine on the fine structure in *Gymnocorymbus* melanophores. $10^{-5}$ M vinblastine sulfate induces the crystallization of microtubule protein into characteristic honeycomb lattices. No free microtubules are observed in the cytoplasm. $\times 82,000$. 

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FIGURE 18 Transverse section of a process of a *Fundulus* melanophore in the aggregated state. Although pigment granules are absent, the cytoplasm contains numerous microtubules (arrows). × 70,000.

FIGURE 19 Transverse section through a process of a *Fundulus* melanophore in the dispersed state. The processes contain pigment granules and microtubules (arrows). The density and distribution of the microtubules are the same as in the aggregated state (see Fig. 18 above). These data are summarized in Table 1. × 70,000.
phores of the squirrelfish, *Holocentrus ascensionis*. Unlike the melanophores examined above, erythrophores contain pigment granules which are spherical rather than ellipsoidal and have only a small number of membraneous structures which interfere with the calculation of the density of microtubules in the free cytoplasm (Fig. 20). The ultrastructure of erythrophores is not presented here as Porter has already described it elegantly (Porter, 1973), but from the arrangement of pigment and the distribution of microtubules they appear nearly identical to the teleost melanophores described above.

For the general case in determining the nearest

### Table 1

Comparison of the Density and Distribution of Microtubules in the Processes of *Fundulus* Melanophores in the Dispersed and Aggregated States

|                        | Dispersed state | Aggregated state |
|------------------------|-----------------|------------------|
| **Central microtubules** |                 |                  |
| microtubules/µm²*      | 0.80 ± 0.18     | 0.75% ± 0.07     |
| % total microtubules   | 52.5%           | 51.3%            |
| microtubules counted   | 470             | 708              |
| **Cortical microtubules** |               |                  |
| microtubules/µm²*      | 0.72 ± 0.15     | 0.71 ± 0.06      |
| % total microtubules   | 47.5%           | 48.7%            |
| microtubules counted   | 483             | 685              |
| **Total**              |                 |                  |
| microtubules/µm²*      | 1.52 ± 0.31     | 1.46 ± 0.11      |
| microtubules counted   | 953             | 1393             |
| area measured (µm²)    | 647             | 959              |

* Expressed as microtubules per µm² of the entire process.

**Figure 20** Transverse section through a *Holocentrus* erythrophore. The cytoplasm consists almost entirely of pigment granules and microtubules. The cytoplasm of this cell appears extremely hydrated. Nearest neighbor measurements of granule-microtubules distances in this cell show that the granules are closely associated with the microtubules. (See Table II.) × 60,000.
neighbor distance between individual points (Pielou, 1969), one computes the quantity $2n\lambda^2$, where $\lambda^2$ is the mean squared distance between individual points and their nearest neighbors, $\lambda$ is the density of the population (the mean number of individuals per circle of unit radius) and $n$ is the sample size. $2n\lambda^2$ has a chi square ($\chi^2$) distribution with $2n$ degrees of freedom under the assumption that a random pattern prevails, and thus any observed value of $2n\lambda^2$ may be tested for significance by using the $\chi^2$ distribution with $2n$ degrees of freedom.

In the case where one is interested in the distance between pigment granules and their nearest neighboring microtubules, it becomes necessary to modify the expression since the relatively large pigment granules cannot be treated as points (Ewens, 1973, personal communication). The basic procedure is as follows. From electron micrographs of transverse sections of erythrophores one measures the density of the microtubules in the cytoplasm ($\rho$), the radius of the pigment granule ($r_0$) and the distances from the center of each pigment granule to the nearest neighboring microtubule ($r$) (see Fig. 21). A $\chi^2$ value is computed according to the formula:

$$\chi^2_{\text{obs}} = 2n\rho \sum_{i=1}^{n} (r_i^2 - r_{i0}^2).$$

Ewens' derivation of this expression is treated in detail elsewhere (Murphy, 1973). We now compare this computed value ($\chi^2_{\text{obs}}$) with an expected value (read directly from a table of the percentage points of the chi square distribution) with $2n$ degrees of freedom at the 95% significance level. As the observed $\chi^2$ value becomes smaller than this tabulated value, the deviation becomes increasingly significant, indicating that the pigment granules are physically associated with the microtubules. In practice, we calculate the probability that the expected $\chi^2$ value for a random distribution is less than the observed value. We reject the hypothesis of a random distribution if this probability is less than some conventionally chosen value (5%). This calculation takes into account the fact that the granules have different diameters. If the granules had the same diameter, it would be necessary to modify the expression, but this is not the case here. The fact that we are examining sections of granules rather than whole granules makes the observed nearest neighbor distance a conservative one, the real distance being, if anything, smaller than the apparent measured distance. Finally, differences in the distribution of the microtubules themselves do not appreciably affect the calculation. For example, if the microtubules were clumped, then the deviation between the observed $\chi^2$ and the expected $\chi^2$ for a random distribution becomes even more significant.

Electron micrographs of transverse sections of Holocentrus erythrophores were prepared at a total magnification of 54,350. The area of the free cytoplasm (needed to calculate the density of the microtubules, $\rho$) was measured by comparing the weight of the photograph (the regions occupied by granules and vesicles are cut out) with the weight of a similar piece of paper of known area. The results obtained from seven different cells are presented in Table II. In all cases but one, where fixation artefacts were suspected, the observed $\chi^2$ values were much less than the $\chi^2$ values expected for a random distribution. The deviation may be considered highly significant at better than 99.0%, indicating that the pigment granules are indeed closely associated with microtubules.

**DISCUSSION**

The Alignment and Movement of Granules

We have demonstrated that the alignment and movement of pigment granules in melanophores is dependent on the presence of microtubules. Antimitotic agents such as low temperature and high
hydrostatic pressure completely disrupt microtubules in these cells and also obliterate alignment and arrest the movement of pigment granules in both centrifugal and centripetal directions. Granule movement is totally inhibited in treated cells and cannot be induced by the addition of adrenalin which normally stimulates granule aggregation. During the earliest stages of recovery from treatment with low temperature, granule movement and alignment could be found only near the centrosome, the region where microtubules also initially reappear. Granule movement at the periphery reappears concomitantly with the reappearance of microtubules in the same regions. Thus we conclude that microtubules determine the alignment and are essential for the movement of pigment granules in these cells.

Finally, our observations confirm and extend the finding made by Junquiera and Porter (1969) and by Wikswo and Novales (1969, 1972) that the movement of pigment in teleost chromatophores is retarded by colchicine. If we treat melanophores under similar conditions the same results are obtained—granule movement although retarded is not arrested and microtubules are not completely disrupted. If, however, we treat the melanophores with colchicine at \(-4^\circ\text{C}\) for 30 min before warming to 22\(^\circ\text{C}\), all the microtubules depolymerize and remain so and concomitantly no granule motion occurs. Thus our experiments with colchicine are completely consistent with the observations with vinblastine, low temperature, and hydrostatic pressure.

Other cytoplasmic elements do not appear to be related to the movement of pigment granules. The microfilaments, endoplasmic reticulum and sol to gel transformations of the cytoplasm, which have been implicated by others, were investigated, but no relationship between these elements and granule movement could be found. The details of these investigations are given elsewhere (Murphy, 1973).

We should mention that the amphibian melanophore is different from the teleost melanophore both in speed of granule movement and in the number of microtubules and filaments present. There is no a priori reason to suspect that the mechanism for granule movement need be the same in the melanophores from these two types of organisms. In fact, there are adequate reasons to suspect differences.

**Mechanisms of Movement**

Two general mechanisms may be considered which could provide the motive force needed for granule movement: (a) the microtubules could push or pull the granules through the cytoplasm by polymerization and depolymerization or (b) the granules could actively glide along fixed microtubules.

**Polymerization and Depolymerization of Microtubules:** Franz (1939) proposed that there were two operationally distinct sets of fibers in teleost chromatophores—a fixed set of "exoskeletal fibers" and a labile central set of "endoskeletal fibers", which moved the granules by means of polymerization and depolymerization. Green (1965, p. 26) subsequently reported that two sets of microtubules could be discerned in *Fundulus* melanophores in the aggregated state; one set appeared to move with the granules and the other did not.

### Table II

*The Nearest Neighbor Distances between Pigment Granules and Microtubules in Holocentrus Erythrophores*

| Cell number | Number of granules measured \((n)\) | Density of microtubules \((\rho)\) | Observed \(x^1\) | Random expected \(x^1\) \((2n)\) | Probability that \(x^1_{\text{exp}}\) is less than \(x^1_{\text{exp}}\) |
|-------------|-----------------------------------|---------------------------------|-----------------|-----------------------------|----------------------------------|
| 1           | 115                               | .023                            | 182.3           | 230                         | <.01                             |
| 2           | 49                                | .034                            | 36.7            | 98                          | <.01                             |
| 3           | 82                                | .017                            | 72.4            | 164                         | <.01                             |
| 4           | 120                               | .015                            | 106.5           | 240                         | <.01                             |
| 5           | 29                                | .011                            | 26.3            | 58                          | <.01                             |
| 6\*         | 52                                | .047                            | 69.7            | 104                         | <.01                             |
| 7\*         | 127                               | .016                            | 268.7           | 254                         | .25                              |

* The unusually high density of microtubles \((\rho)\) required that the nearest neighbor distances be measured more accurately than to the nearest 0.5 mm.

† Cell poorly preserved.
other remained associated with the surface. More recently, Porter\textsuperscript{1} (1973) has suggested that granule movement is associated with the labile properties of microtubules in the erythrophores of a different teleost, \textit{Holocentrus}.

Counts of microtubules in the processes of \textit{Fundulus} melanophores, however, show that the density and distribution of microtubules remain unchanged in both the dispersed and aggregated states. If the central microtubules were labile organelles which depolymerized upon pigment aggregation, the number, density, and relative proportion of central microtubules would be expected to decrease, but this is not observed. Let us suppose, however, that the distribution of a given microtubule could change over the length of a cell process so that microtubules which were centrally located in one segment of a process could be associated with the cortex at some other point. In this case, as the central microtubules depolymerized during aggregation, some microtubules would remain in the central cytoplasm which were actually part of the fixed cortical set. The data conflict with this interpretation in two important respects. In the longitudinal sections of cell processes with and without pigment, microtubules were always parallel to the long axis of the process. In addition, the proposed model would result in a dramatic decrease in the density of microtubules upon pigment aggregation, whereas the data indicate the density in the two states remains the same.

Alternatively, one could hypothesize that during aggregation cortical microtubules assemble toward the cell center as the central microtubules disassemble at their distal ends; the number of microtubules at many levels of sectioning might therefore appear the same. We observed, however, that the distribution of microtubules does not change during aggregation. Examination of sections obtained at various distances from the cell center shows no change in the density or distribution of cortical and central microtubules. Furthermore, longitudinal sections of processes of cells fixed during the process of pigment aggregation do not reveal changes in distribution of microtubules near the margin of the granule mass. Thus, contrary to the work of others, these data indicate that the microtubules in \textit{Fundulus} melanophores are relatively stable organelles and do not depolymerize upon aggregation of pigment. Although it is difficult to reconcile Porter's (1973) observations on erythrophores with the present data, it is conceivable that the pulsation and surging of pigment within these cells is due to a mechanism unrelated to the aggregation of pigment in melanophores.

Our results with colchicine also show that the microtubules in melanophores behave as semistable organelles. The data show that a large number of microtubules remain in the presence of colchicine, and also demonstrate that pigment may be repeatedly induced to disperse and aggregate after 2-h incubation in \(10^{-4}\) M colchicine. Electron microscope examination shows that numerous microtubules remain in these cells. These observations alone argue against a depolymerization hypothesis since we have also shown that once depolymerized, microtubules cannot reform in the presence of colchicine.

It seems unlikely that the cells are simply impermeable to colchicine. Taylor (1965) has shown that the penetration of \(5 \times 10^{-7}\) M colchicine into cultured KB cells is complete within 10–15 min and blocks cells in metaphase within 5 min. Furthermore, other cultured cells, including HeLa cells, fibroblasts, and leukocytes are easily affected by this alkaloid (Freed and Lebowitz, 1970; Brinkley, et al., 1967; Hard and Cloney, 1971), and vinblastine, an even larger molecule than colchicine, penetrates melanophores without difficulty. Olmsted and Borisy (1973) report similar resistance to colchicine by microtubules obtained from porcine brain in an in vitro system. If colchicine is added to microtubule protein which has been depolymerized at 0°C, no microtubules reappear upon subsequent rewarming to 37°C. If, however, colchicine is added to polymerized microtubules at 37°C, the microtubules are partially disrupted, but substantial fragments of microtubules remain after 60 min. These results are in agreement with our observations on melanophores. Chang (1972) has also reported that microtubules in cultured nerve cells are resistant to treatment with colchicine. In cultured nerve cells, particle movement continues even after incubation in \(10^{-4}\) M colchicine for several hours. These results indicate that chromatophore microtubules are disrupted more easily by some antimitotic agents (hydrostatic pressure, low temperature) than by others (colchicine). It is possible that these

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\textsuperscript{1} Porter, K. R. 1970. Report of granule movement in \textit{Holocentrus} erythrophores. Presented at the Annual Meeting of the Society for General Physiology.
agents are differentially effective in removing stabilizing factors which may be attached to the cytoplasmic surfaces of the microtubules, but this apparent paradox can also be explained if we compare the mechanisms of action of these different agents.

It has been well documented that colchicine binds to unpolymerized but not to polymerized microtubule protein (Wilson and Meza, 1972) and that colchicine-bound monomers are incapable of polymerization (Borisy and Taylor, 1967a, 1967b; Rosenbaum and Carlson, 1969; Wilson and Friedkin, 1967). Labile microtubules such as those in the spindle apparatus and in heliozoan axonemes are known to be sensitive to colchicine (Brinkley et al., 1967; Inoué, 1952; Inoué and Sato, 1967; Tilney, 1968) and are thought to exist in an equilibrium with the monomeric units which comprise them (Taylor, 1965; Inoué and Sato, 1967). Colchicine theoretically disrupts spindle microtubules by binding the free monomers as they become available, thus reducing the effective pool of monomers available for repolymerization. Through a mass action effect, the microtubules would continue to depolymerize in order to maintain the equilibrium. Low temperature and hydrostatic pressure, however, cause the disruption of microtubules by shifting the equilibrium constant of the polymerization reaction (Inoué and Sato, 1967; Salmon, 1973). It is reasonable to assume, therefore, that microtubules could be disrupted by an increase in hydrostatic pressure or a decrease in temperature but could be only slightly perturbed by colchicine if the microtubules were under conditions which strongly favored the polymerized state. When scales were incubated in colchicine at low temperature, and then allowed to return to room temperature, mass movements, shuttling movements, and microtubules were absent. Thus low temperature caused the depolymerization of a relatively stable array of microtubules and colchicine prevented repolymerization.

From our observations of both the resistance of microtubules to colchicine and hydrostatic pressure and the counts of microtubules in cell processes, we conclude that the microtubules of melanophores are relatively stable structures. A mechanism for granule movement based on the reversible assembly of microtubules in these cells is therefore unlikely, and the further distinction of two operationally distinguishable sets of microtubules made by some workers is unnecessary.

**MICROTUBULE-GRANULE INTERACTION:**

An alternative mechanism for intracellular movement is that pigment granules undergo displacement in relationship to fixed microtubules. Although one can demonstrate statistically by nearest neighbor analysis that granules are closely affiliated with microtubules, it has not yet been possible to determine the nature of this association. Bundles of microtubules are known to undulate in the axonemes of cilia and flagella and the contractile axostyle. If similar motion occurred in melanophores, it is conceivable that granules could be passively transported. It is now clear, however, that motion is accomplished in axonemes through the interaction of adjacent microtubules by means of an ATPase bridge, dynein (Gibbons and Rowe, 1965; Mooseker and Tilney, 1973; Summers and Gibbons, 1971). Since there is no known case where cytoplasmic tubules undulate, it seems unlikely that granule movement could be accounted for by this mechanism.

Alternatively, pigment granules could move by actively associating with microtubules through interconnecting bridges. McIntosh et al. (1969) have postulated a model for chromosomal movement reminiscent of the sliding filament model for the contraction of muscle fibers. According to this model, bridges studded along polarized microtubules interact with neighboring microtubules of opposite polarity to generate force. Spindle fibers displaced with respect to one another in this way could partially account for the movement of chromosomes during anaphase. The bridge hypothesis has been subsequently discussed in reference to axonal transport of particles in neurons (Smith, 1971), cellular elongation (Burnside, 1971), and particle movement in heliozoans (Tilney, 1970). Microtubules could also act like a scaffold to bind and orient other elements such as actin which generate force. The microfilaments in Fundulus melanophores, however, are few in number and do not appear to be actin-like (Murphy, 1973).

We have demonstrated that microtubules are essential for granule movement in melanophores. Since it is unlikely that granules are passively moved by the polymerization and depolymerization of microtubules, we propose that the granules glide along the microtubules through active interaction. Since dynein, a force-generating enzyme, selectively binds to microtubules and takes the form of arms on axonemal outer doublets, it is...
tempting to speculate that the "cross-bridges" on microtubules in chromatophores may also be an ATPase. The fact that antimitotic drugs impair granule movements in nervous and secretory tissue as well as chromatophores suggests that cytoplasmic microtubules may be involved in a common mechanism for particle movement found in a wide variety of cells.

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