Microtubules and Pigment Migration in the Melanophores of *Fundulus heteroclitus* L.

By

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With 11 Figures

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Introduction

The dermal melanophore is a stellate-shaped cell with processes or arms radiating out from the nucleated central region. The dense melanin granules when dispersed throughout the processes give the fish a dark color. When, on the other hand, the melanin migrates to the central region of the cell leaving the processes devoid of pigment, the fish appears pale. In this phenomenon of color adaptation, the pigment granules move rapidly in a streaming motion analogous to that observable in other types of cells. It is reasonable to assume for investigative purposes that the same or similar mechanisms might be involved in this as in other instances, i.e. that the streaming might be given direction and possibly propulsion by a population of microtubules [19].

We undertook the present study primarily to investigate microtubule distribution and involvement. Naturally there was the additional interest in observing an association between microtubules and streaming in a situation which lends itself to experimental manipulation of the streaming.

Previous Studies

Early investigators believed that the chromatophore functioned something like a muscle fiber. The mechanism of pigment dispersion in the arms of the chromatophore was likened to myofibril elongation. Hence they described the chromatophore in this condition as "expanded." Concentration of the pigment into a dense ball within the nucleated central region of the chromatophore was labeled "contraction." Though one no longer finds such a close analogy between the movements of muscle

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and the movements of pigment, the terms expansion and contraction are still used to describe the active stages of pigment migration. "Expansion of the chromatophore" refers to the centrifugal dispersion of pigment into the cell processes, and "contraction" refers to the concentration of this pigment into the central region. When neither stage of active migration is occurring, the cell is in its "resting state," and little motion of pigment is observed. The "resting state" may be used to describe a cell whether expanded, contracted, or in some intermediary state as long as the pigment is not migrating.

1. Influence of Background

The melanophores of *Fundulus* are readily induced to change their appearance. As is well known, they expand over a black background but contract over white. The time necessary to achieve such color adaptation is remarkably short, for the melanophore can disperse its pigment from the concentrated position in a matter of minutes when changed from a white to a black background. The reverse process is even faster. Such background adaptation requires light. If the fish is placed in a dark room irrespective of background color, it will always pale; that is, all melanophores will contract.

2. Neural and Hormonal Control

a) Nerves

The chief means by which the *Fundulus* controls its pigment migration is by nervous control. Each chromatophore of the dermal layer of these fish seems to be equipped with at least one expanding and one contracting nerve fiber, nerve fibers which form part of the autonomic nervous system [24]. The nerve impulse is thought to be mediated by neurohumors which have not yet been identified.

These conclusions were derived primarily from one basic experiment with modifications performed by different investigators to add to this general description of chromatophore innervation in *Fundulus*. When shallow transverse cuts are made at the base of the caudal fin of a fish maintained on a white background, some of the nerves supplying different sectors of the caudal fin are severed. As a result of this operation the chromatophores of the fin thus denervated expand, while the innervated cells remain contracted. These expanded melanophores appear as a dark band on the fin. If the fish is kept on the white background for twenty-four to forty-eight hours more, this dark band eventually disappears or "adapts." If the fish is then transferred to a black background, the cells of the innervated regions quickly expand leaving a pale band composed of the presumably denervated chromatophores. These denervated cells require considerably longer to adapt to the black background than their innervated neighbors. Mills [24] noticed that those melanophores which lagged in adaptation to a white background were not exactly the same, melanophore for melanophore, as those which lagged in black background adaptation. In other words, those melanophores whose contraction response had been retarded by the operation did not necessarily show a lag in expansion time, whereas other melanophores contracting normally did not expand in normal time. This constituted the most convincing demonstration of dual innervation at the time. Since then, however, dual innervation has been corroborated by such experiments as observing the effects on pigment migration of drugs with known effects on the autonomic nervous system [41].

The nature of the eventual color change of the denervated sector was then scrutinized. It was found that the melanophores around the lateral edges of the

denervated band are the first to approximate the pigment displacement of their innervated neighbors [11]. That is, after the original denervation, a band of the caudal fin darkens. If maintained over a white background, the expanded melanophores at the lateral edges of this strip are the first to contract; this "wave of contraction" progresses toward the center of the strip at the rate of 1 mm per fifty-two hours [32]. The analogous phenomenon is observed when the fish is then placed over a black background. From these observations, investigators such as Mills [24, 25], Parker [29–32], and Abramowitz [1] conclude that a neurohumor(s) generated in the innervated regions of the fin slowly diffuse into the denervated region and induce the appropriate migration within the chromatophore. Parker points out that since adrenalin injected into the blood causes a rapid, synchronous contraction of the denervated chromatophores, and since acetylcholine even protected by eserine causes no marked expansion, the neurohumors regulating the chromatophores are not necessarily the same as those commonly associated with the autonomic system. Parker feels that since the adaptation of the denervated chromatophores is gradual and nonsynchronous, the substance effecting this adaption is not carried in the blood and probably is not water soluble but lipid.

The picture that emerges is one of dual innervation mediated by slowly diffusing neurohumors. Therefore, in order to account for the rapid response of the chromatophores to background stimuli, one would expect some close spatial relationship between nerve endings and the chromatophore, something in the nature of a "chromato-neural junction."

b) Pituitary

Other factors also play a role in the control of pigment migration. Matthews [22] discovered that the hypophysectomized *Fundulus* still is able to adapt to background changes nearly as well as normal animals. When the melanophores are denervated, the adaptation lag for both normal and hypophysectomized fish is also about the same length of time. However, Abramowitz [2] showed that the denervated melanophores of hypophysectomized *Fundulus* are unable to expand over a black background after having contracted from the initial pigment dispersion concomitant with denervation. Both he [2] and Kleinholz [17] have isolated a melanin dispersing substance in the pituitary which is effective when the overriding nervous control is eliminated. One can conclude that in *Fundulus heteroclitus* the pituitary, presumably via a blood-borne hormone, has some subsidiary effects on adaptation to background (black, white). But it should be emphasized how subsidiary this hormonal effect is when compared, for example, to the analogous control by the sinus gland over pigment migration in the retina of Crustacea [18].

Further consideration of the controls of pigment migration within the chromatophore will be postponed until the Discussion.

Methods I

The kinetics of pigment migration within the melanophores of *Fundulus heteroclitus* were studied in isolated scales removed from the anterior dorsal region of that fish. Active migration was observed in the expanding, contracting, and pulsating cell. Expansion was induced by placing the scale into either 0.1 M. NaCl or Ringer's solution. Adrenalin (10^{-3} M.–10^{-5} M.) or 0.1 M. KCl induced contraction. Pulsations, defined as the cyclic expansion and contraction of the melanophore every few minutes, could be achieved by treating the cell first with 0.1 M. $BaCl_2$ then 0.1 M. NaCl (techniques of Spaeth [43]. Prior to such experimentation, the fish were generally kept

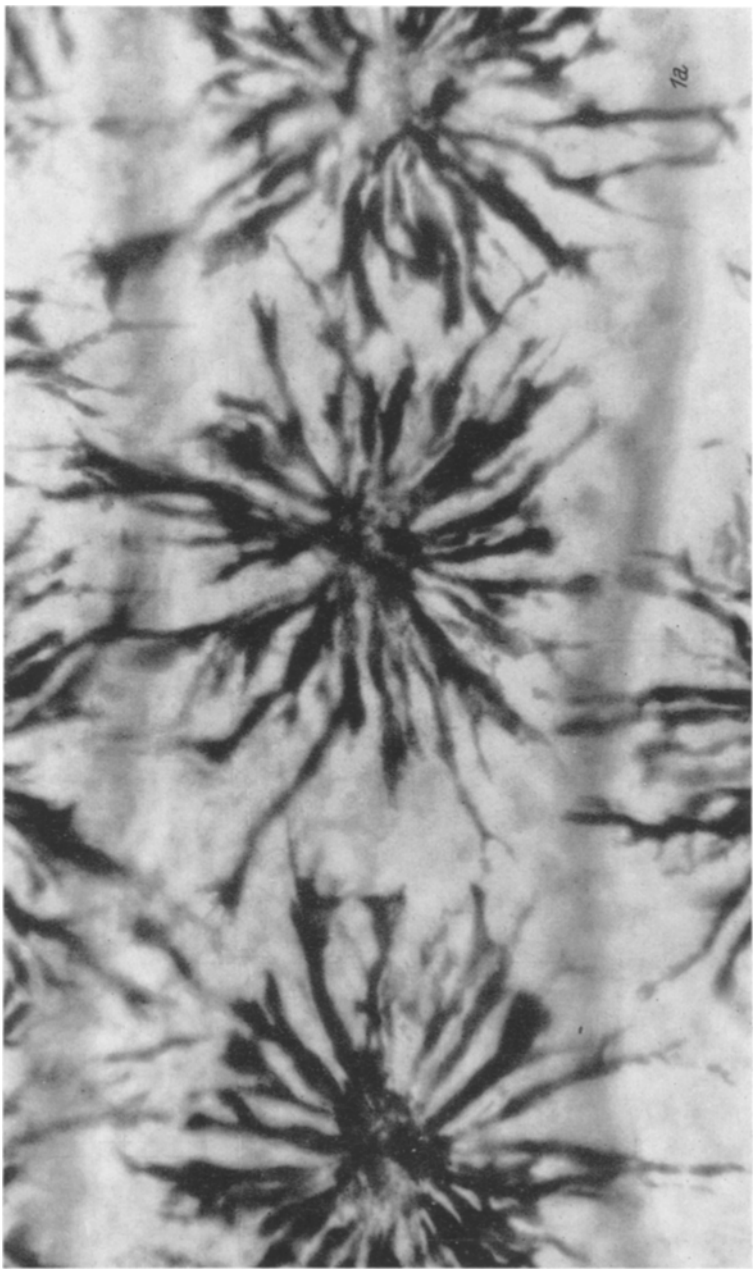

Fig. 1 a. Expanded melanophores *in vivo*. Magnification ×500.

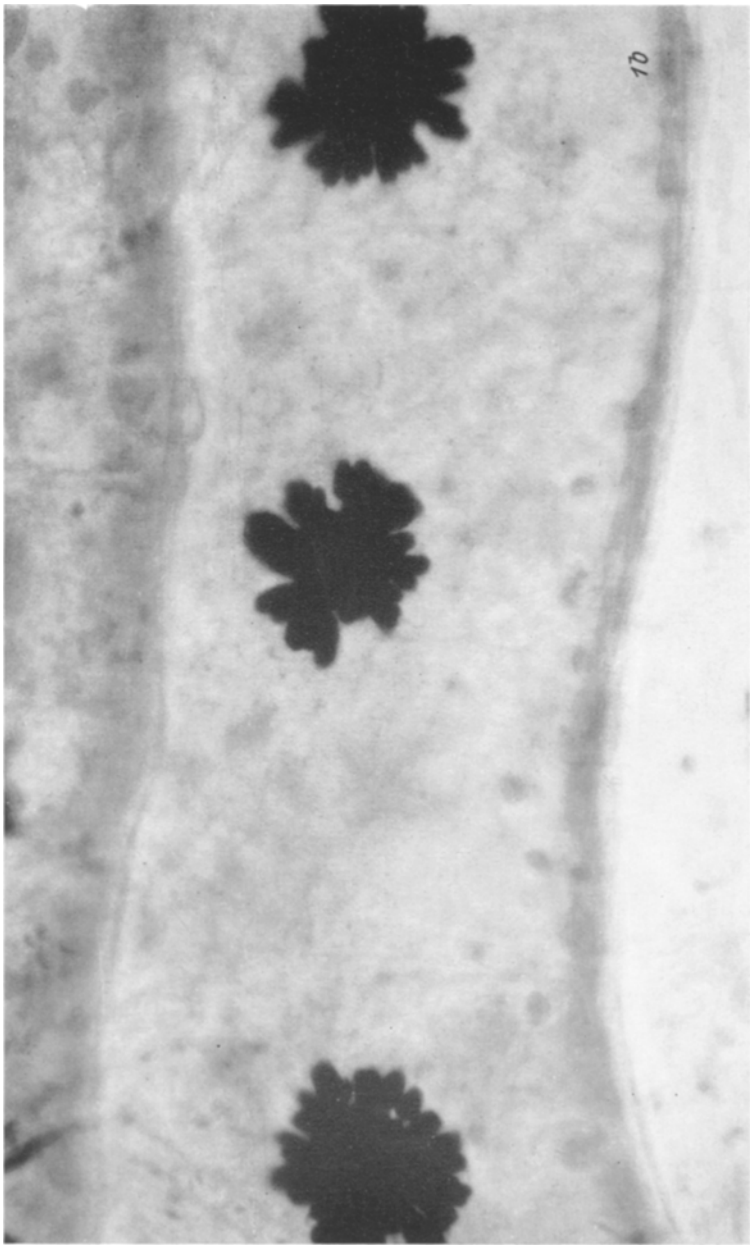

Fig. 1 *b*. Contracted melanophores *in vivo*. Magnification ×500.

overnight in an illuminated black or white tank filled with sea water. Photomicrographs were made of the expanded, contracted, and pulsating cell showing the different stages of migration (Figs. 1 *a*, 1 *b*, and 2).

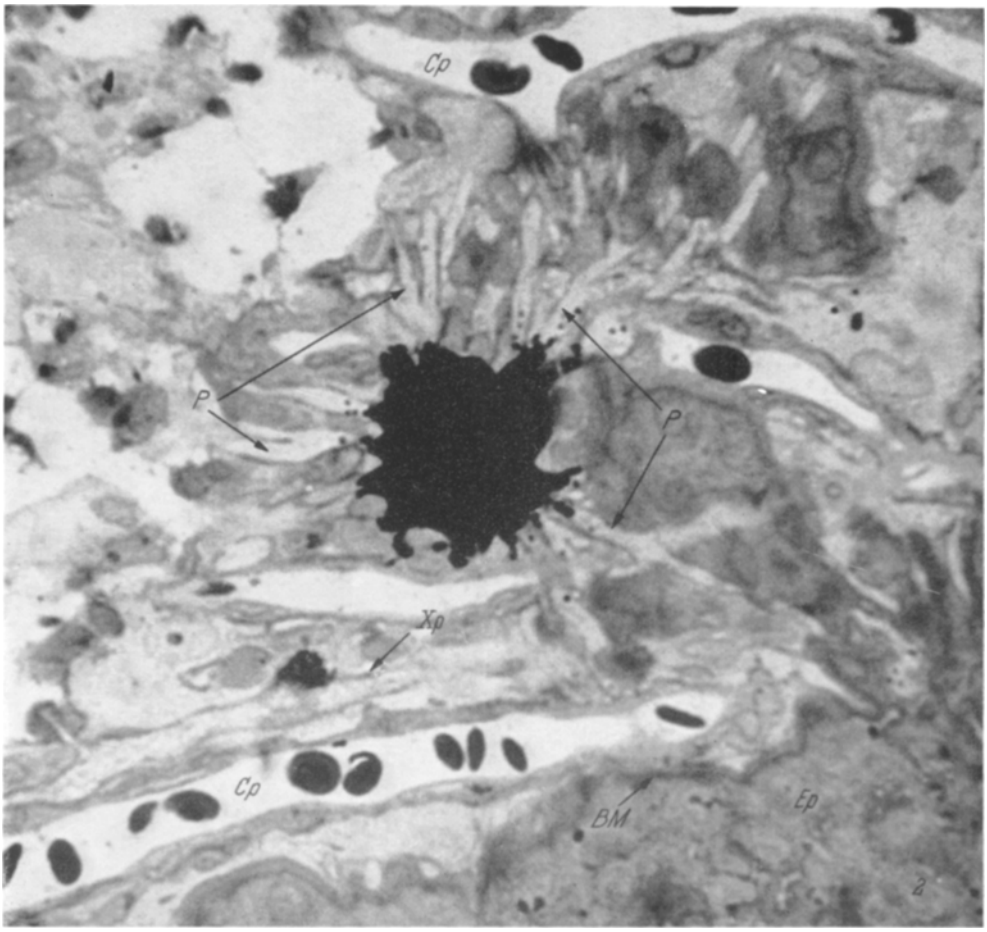

Fig. 2. Light micrograph of a horizontal section through contracted melanophore. Note the empty process (*P*) radiating into the dermis. Other structures of the dermis, such as capillaries (*Cp*) and xanthophores (*Xp*), the yellow pigment bearing chromatophore, can be seen. The basement membrane (*BM*) separating dermis from epidermis (*Ep*) is also contained in this plane of section. Magnification ×865.

Observations

The melanophore when maximally expanded has a partially clear central region with most of the pigment found in the long, branching processes often extending from the central region as far as sixty microns. These processes are neither straight nor of uniform diameter and when filled with pigment they have a spindly appearance (Fig. 1 *a*). In the contracted cell state the pigment forms a spherical or ellipsoidal ball ranging

between twenty to forty microns in diameter and filling the central region (Fig. 1 *b*). Under phase contrast the processes, now almost devoid of pigment granules, can be resolved as clear channels within the dermis (see Fig. 2). Intermediate positions of pigment displacement can be attained by

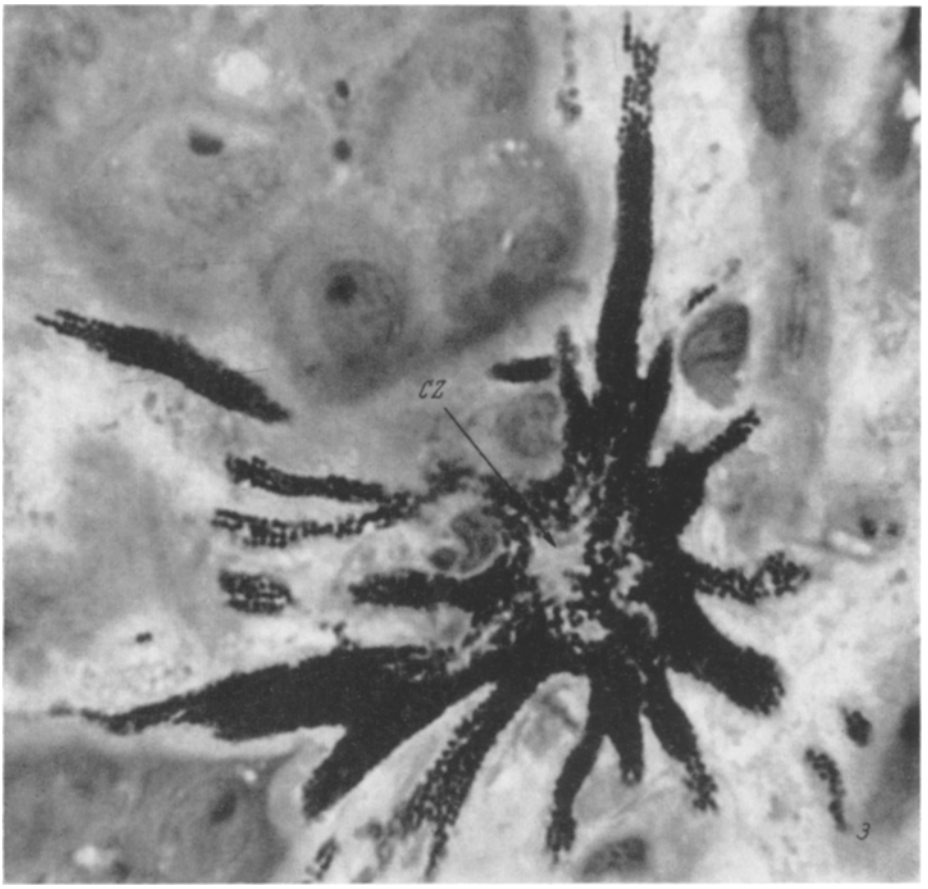

Fig. 3. Light micrograph of a horizontal section through expanded melanophore. A clear zone (*CZ*) can be seen in the central region from which the pigment is dispersed. Note the linear arrangement of the granules in the arms or processes. Magnification ×2,240.

the appropriate adjustment of adaptation time and/or background coloration.

When the scales are placed into solutions which induce migration, the pigment granules, especially those peripheral to the main body of pigment, begin a rapid vibratory movement which seems to be directed predominantly along the longitudinal axis of the cell process. Following this initial activity the pigment begins to move en masse. Migration proceeds at a uniform rate until the pigment has achieved its eventual displacement—be this complete dispersion, concentration, or an intermediary position—after which

the activity ceases. The onset of migration begins in most of the cells on the scale at the same time. Even when the melanophores have been pulsating over a period of hours this synchrony is still evident.

This togetherness is not reflected in the movements of individual granules. Small groups of granules arranged in single file (Fig. 3) along the longitudinal axis of the process are frequently observed moving counter to the mainstream of migration. It is not surprising that the amount of independence is inversely proportional to the overall rate of migration. In weaker concentrations of adrenalin (10^{-4} M., 10^{-5} M.) which induce a slower rate of contraction than 10^{-3} M., the granules show a greater tendency to move counter to the overall influx of pigment. Most of the granules are limited primarily to a linear migration along the longitudinal axis of the process. However, the more distal granules, and, hence, those least crowded by other granules, also display some Brownian motion.

Our general impression from such observations is that these granules are, in some degree, restricted to rather definite "paths" within the cytoplasm of the process. These "paths" extending from the central region run parallel to the longitudinal axis of the cell process and seem to orient the granules in their movements. The granule within such a "path" seems less affected by granules in adjacent "paths" than it does by adjacent granules of the same "path." This impression stems from the observation that small groups of granules (two to five) linearly arranged are seen migrating almost as separate units, that is, counter to the movement of granules in adjacent paths. These observations will be further discussed in conjunction with observations on the ultrastructure of the melanophores.

Methods II

To achieve further understanding of the means and control of pigment migration, we have prepared scales for observation with the light and electron microscope.

Scales were removed from the anterior dorsal region of *Fundulus*, black or white adapted overnight as previously described. These were immediately fixed in 6.5% glutaraldehyde followed by 1% OsO_4, each buffered with 0.1 M. s-collidine to a pH of 7.4. It was observed that the fixation process did not alter the initial pigment diplacement within the melanophores. The specimens were then embedded in araldite or epon. Sections were cut with a diamond knife on a Porter-Blum ultramicrotome. Those prepared for the light microscope were stained with toluidine blue and azure II. Sections cut for electron microscopy were stained with uranyl acetate and lead citrate.

Observations

1. Light Microscopy

Examination with the light microscope of sections stained with toluidine blue and azure II confirmed observations made of the chromatophores *in vivo*. When concentrated, almost all the pigment within the melanophore is found in a ball filling the central region (Fig. 2). The processes, now nearly

devoid of pigment granules, are readily visualized. The nucleus (nuclei) can often be seen projecting into the empty processes.

The expanded melanophore frequently is typified by a clear zone within the central region as most of the granules are dispersed into the processes (Fig. 3). The nucleus (nuclei) often is found to one side of the cell center. The granules appear to be arranged in columns (single file) within the processes, each column aligned parallel to the longitudinal axis of the process. This orderliness within the process is not found within the central region where the granules are scattered somewhat randomly.

These general observations of the fixed tissue at the light microscope level were followed by examination with the electron microscope.

2. Electron Microscopy

a) General description of dermis

The chromatophores examined are located in the dermal layer of the skin which overlies the scale. We can imagine the chromatophores to be sandwiched in the dermis between the supporting scale and the overlying epidermis. This epidermal covering is about five to seven cells thick. A basement membrane is found directly beneath the epidermis separating it from the dermis. Collagen fibrils (cross sectional outer diameter about 240 Å) are found directly beneath the basement membrane. This collagen in favorable sections is seen to be arranged in an orthogonal pattern and in layers of varying thicknesses. Numerous capillaries and small arterioles with nucleated erythrocytes, bundles of myelinated and unmyelinated nerve fibers, and two kinds of chromatophores (the melanophore and the yellow pigment bearing xanthophore) are the primary cell types located in the dermis.

b) Description of pigment granules and distribution of organelles in melanophores

The pigment granules of the melanophore are spherical or ellipsoidal bodies with an average diameter of about 0.5 microns. The melanin inside the granule is quite dense both to visible light and the electron beam, giving the pigment its characteristic black appearance (Fig. 4). The individual granule is surrounded by a unit membrane. The layer of cytoplasm encircling the melanin occasionally contains vesicles and dense particles. A conspicuous Golgi region is not found; nor is there any indication of an ordered arrangement of the melanin within the granule in these adult melanophores. Whether the evidence of structure observed by some investigators represents some aspect of pigment synthesis or transport is not yet determined [5, 26, 46].

Fig. 4. Electron micrograph showing a small part of a melanophore process. Each melanin granule is surrounded by a unit membrane. In what appears to be an early stage of granule development, vesicles (*Ve*) and dense particles (*Dp*) can be seen inside the unit membranes. Note the large amount of smooth *ER* (*SER*) and free ribosomes. Cortical pits (*Pt*) are present along the plasma membrane. The process is surrounded by collagen (*Co*). Magnification ×35,000.

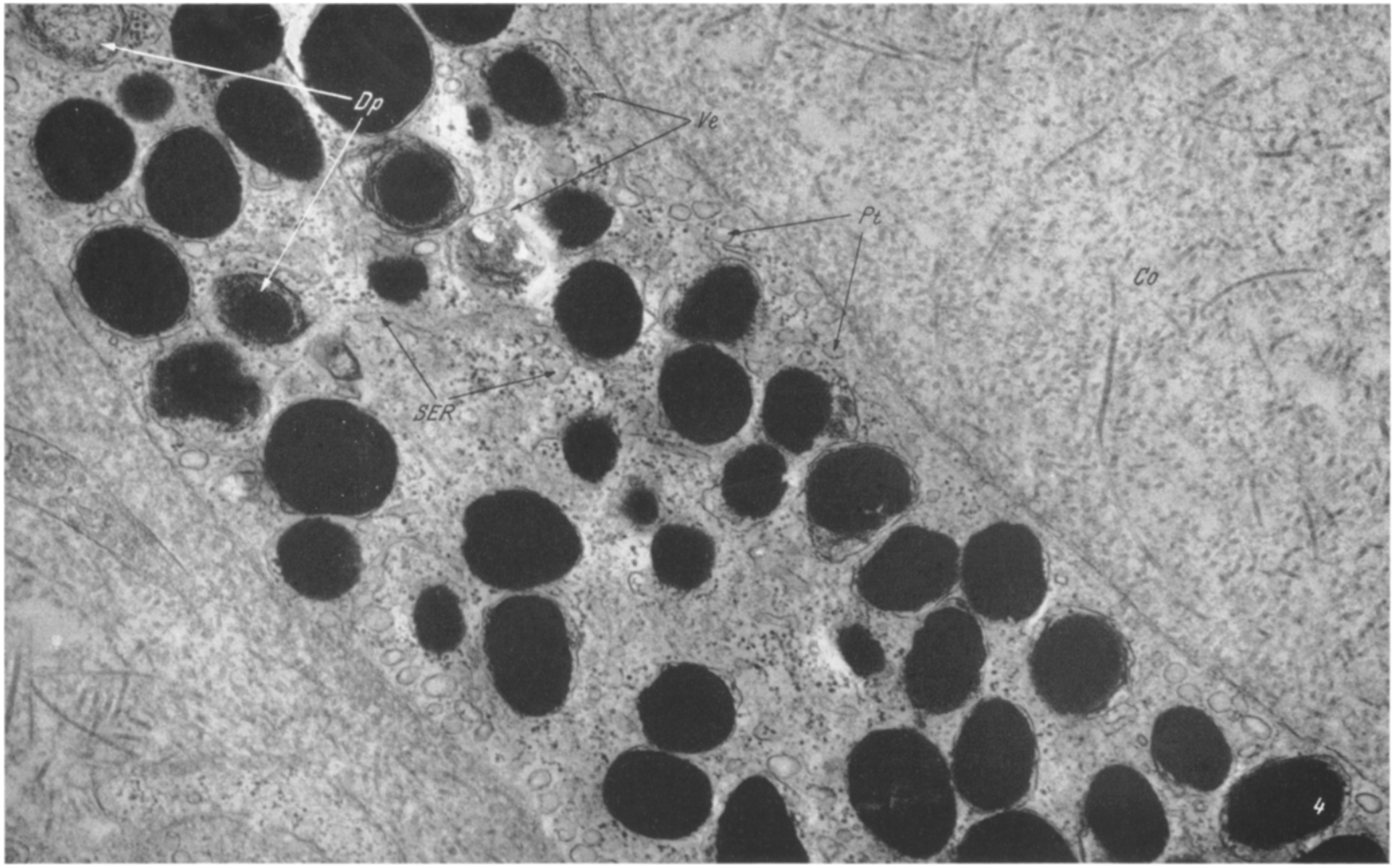

Fig. 4.

In the contracted melanophore many of the mitochondria are located distal to the mass of pigment granules concentrated in the central region (Fig. 5). Presumably they are located close to an active site of ATP hydro-

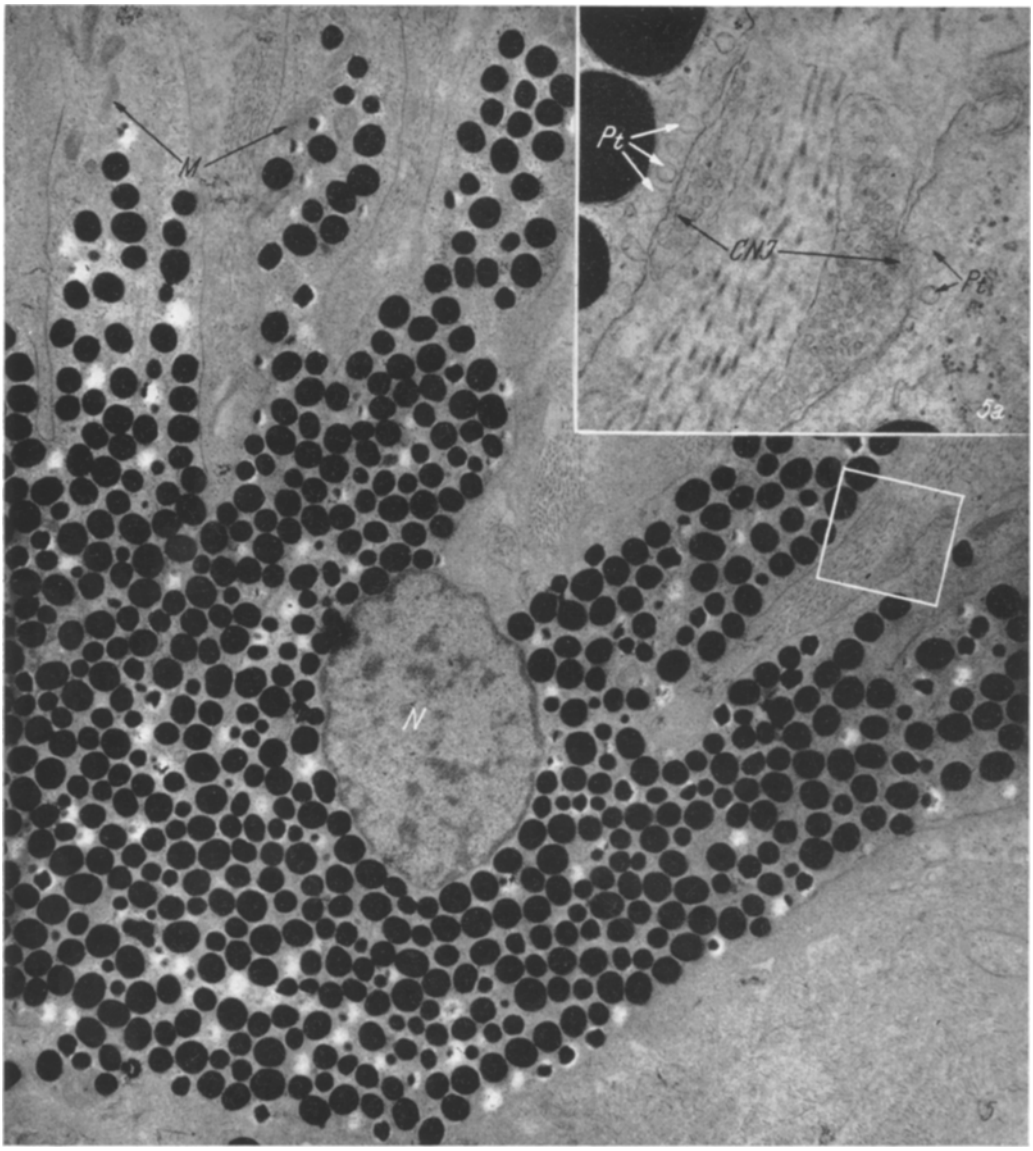

Fig. 5. Electron micrograph of a nearly horizontal section (i.e. cut in the plane in which the melanophore is extended) of a contracted melanophore. The mitochondria (*M*) are located predominantly distal to the bulk of the pigment as though not moved with the melanin. Two chromatophore-neural junctions (*CNJ*) are included within the small square and reproduced in 5 a at a higher magnification. Cortical pits (*Pt*) are numerous in the vicinity of the nerve ending. Magnification ×9,600, insert ×40,000.

lysis. When the melanophore is expanded, the distribution of mitochondria seems to be more ,generalized (Fig. 7, 9), Free ribosomes and predominantly

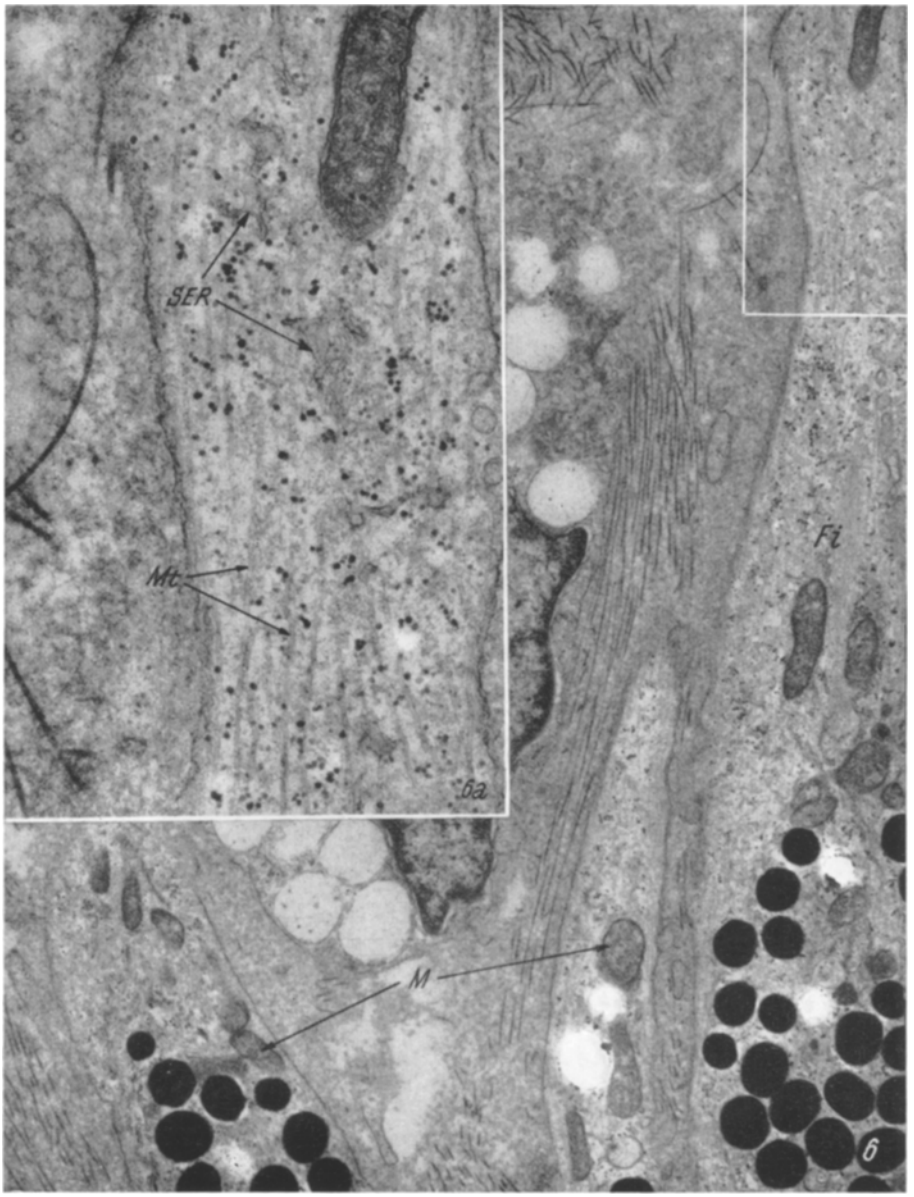

Fig. 6. This shows a section .of a process belonging to the same melanophore depicted in Fig. 5. The mitochondrion (M) again is seen just distal to the pigment. The small insert, 6 a, reproduces a part of the cytoplasm at higher magnification. It shows microtubules *(Mr)* remaining in the melanin-free process after contraction together with fine filaments *(Fi),* some smooth *ER (SER),* aad glycogen. Magni-

smooth endoplasmic reticulum (SER) are found throughout the central region and processes.

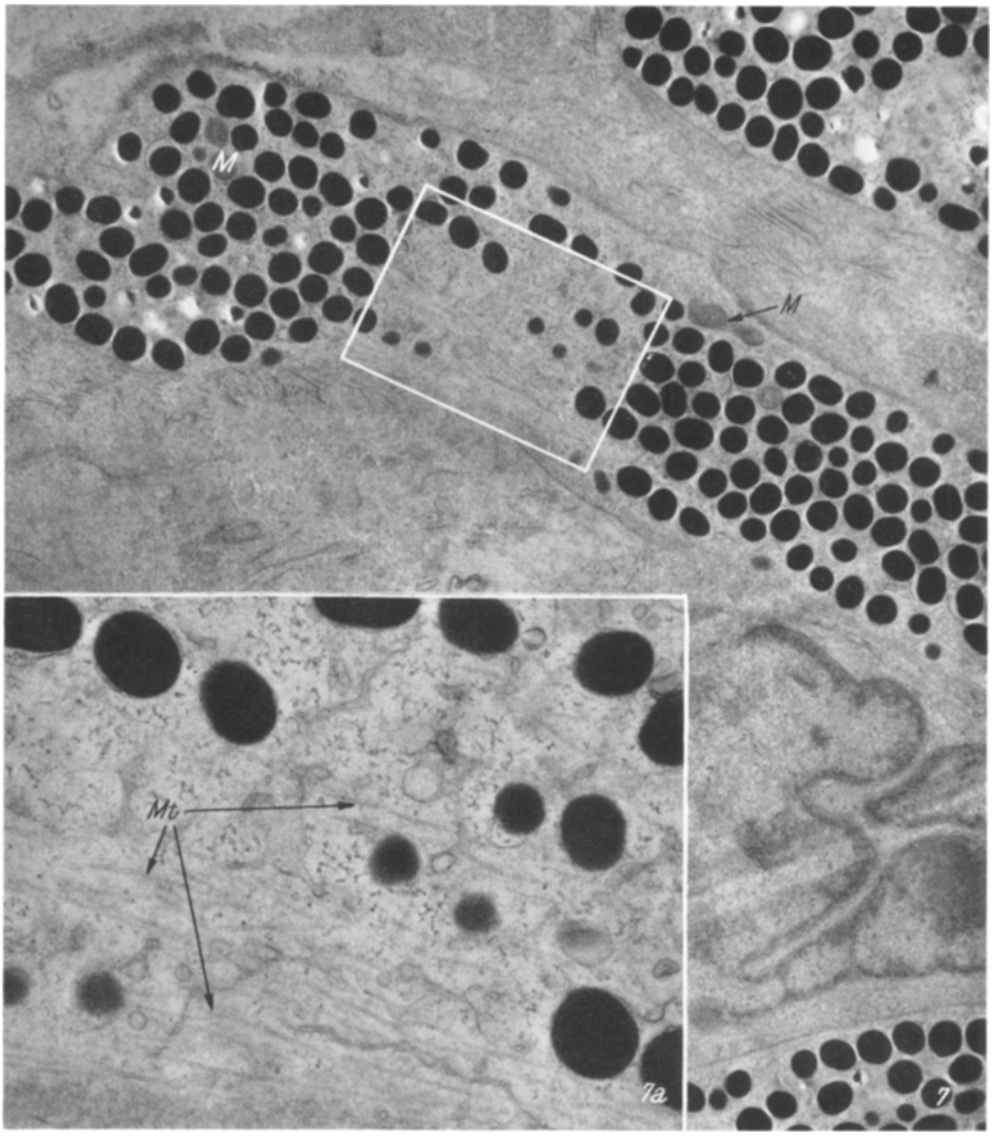

Fig. 7. Section includes a process of an expanded melanophore. There is a general distribution of mitochondria (*M*) among the melanin granules. The numerous microtubules present are oriented parallel to the long axis of the process. The insert, 7 a, shows distribution of microtubules relative to the pigment. Magnification ×9,600, insert ×32,000.

c) Cortical pits

(850 Å by 1000 Å) are just within the plasma membrane bordering the cell and often are found attached to this membrane by narrow necks (Fig. 4). The plasma membrane surrounding the central region seems to

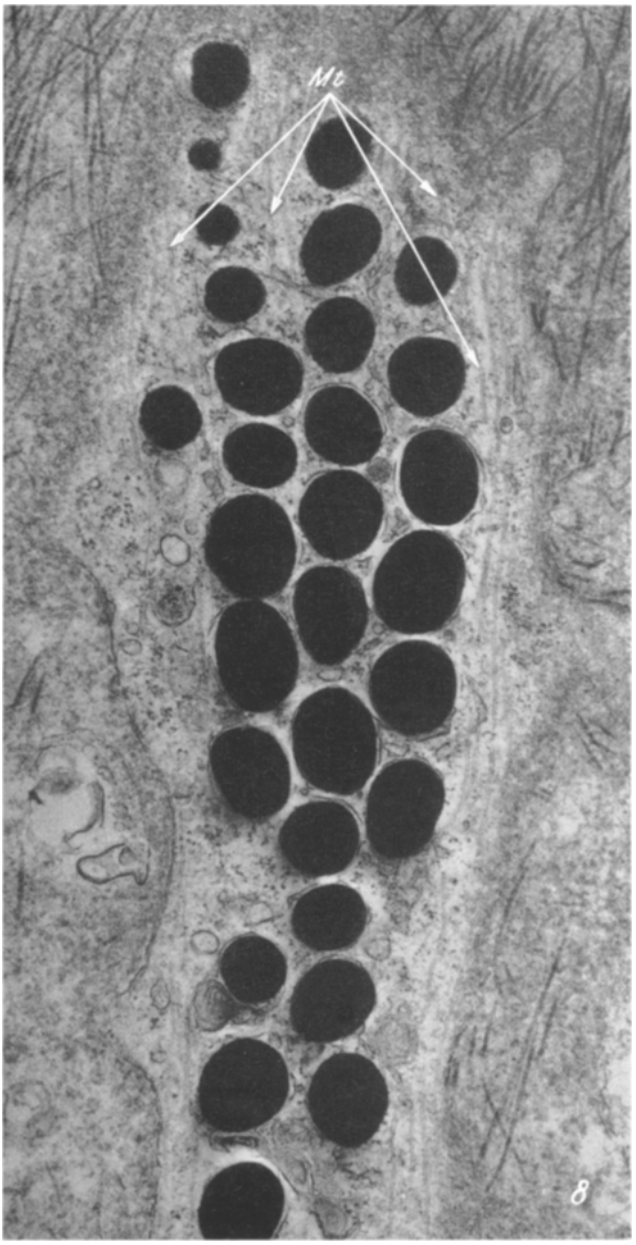

Fig. 8. Nearly longitudinal section through a chromatophore process. Granules appear ordered into linear columns or files. Microtubules (*Mt*) are present mostly near the surface of the process but they give some indication of aligning the granules. Magnification ×20,000.

have more pits per unit area than does the membrane around the processes, but other than this no direct correlation between number of pits and distance from the center of the cell has been observed.

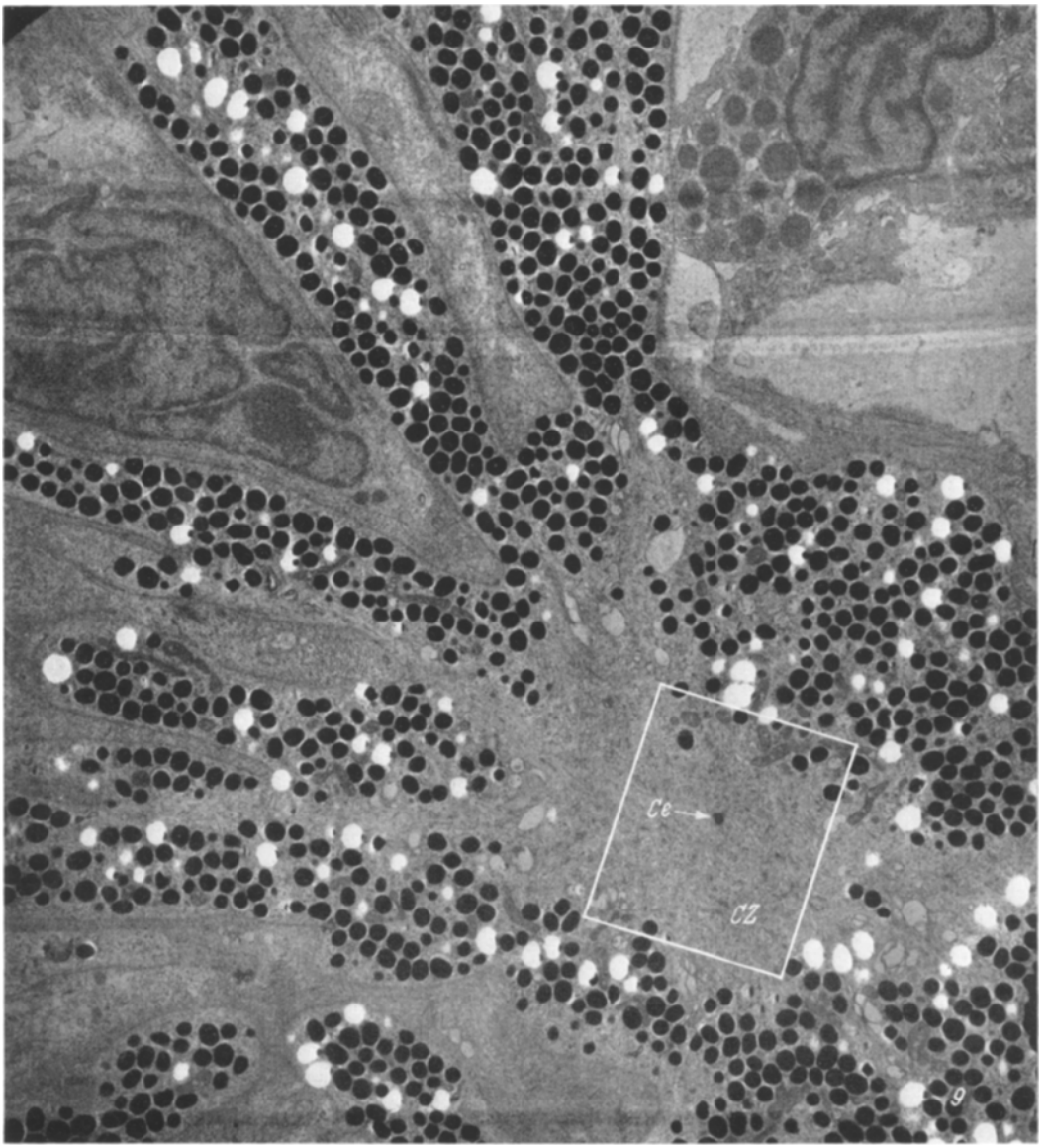

Fig. 9. Horizontal section through an expanded melanophore. Melanin granules are out in arms of cell leaving central region (*CZ*) or centrosphere relatively clear. Mitochondria can be identified around clear zone and elsewhere among pigment granules. A centriole (*Ce*) is present near the middle of the clear zone and great numbers of microtubules emanate from this region. The enclosed rectangle is reproduced at higher magnification in Fig. 10. Magnification ×6,000.

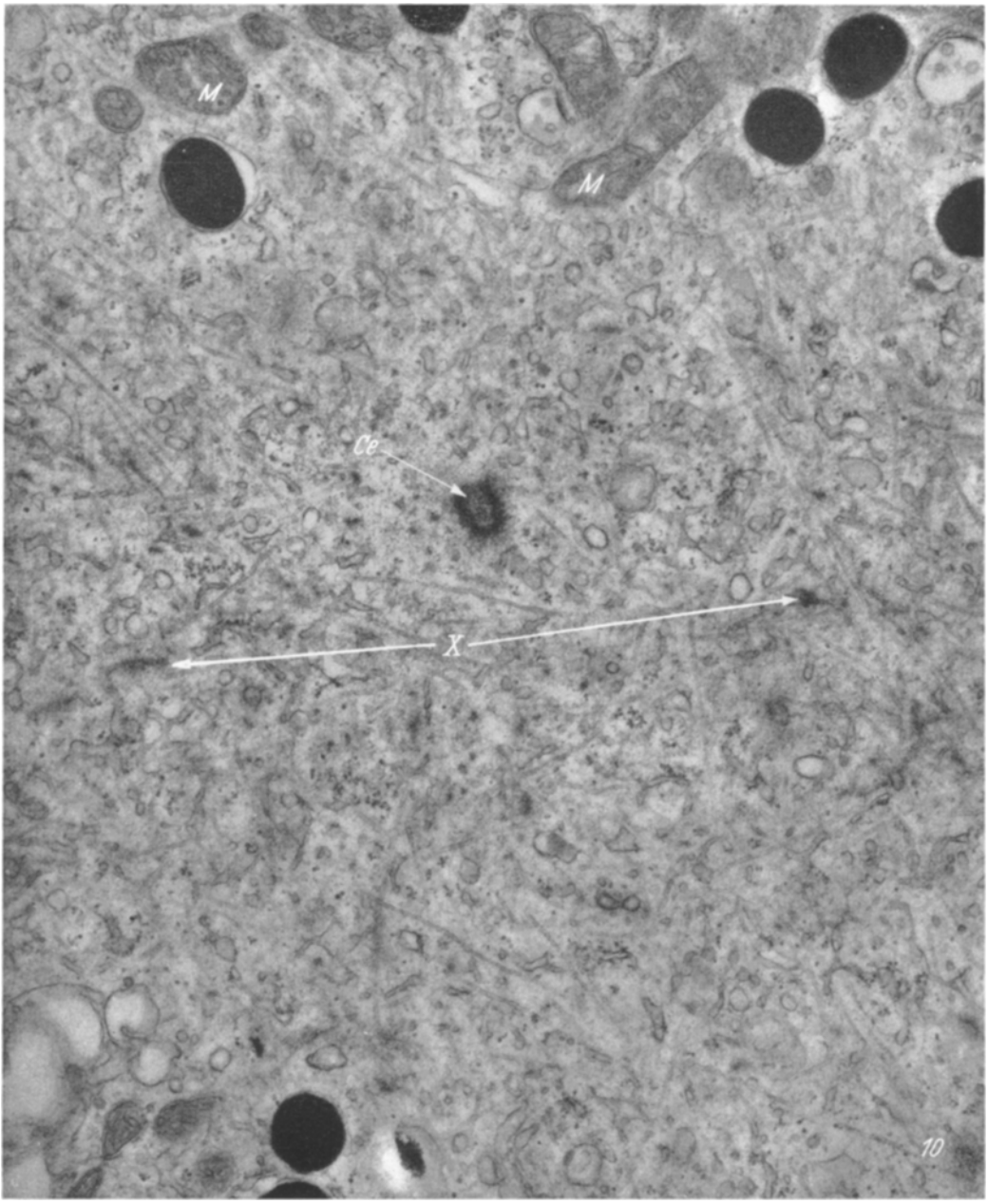

Fig. 10. Here the clear zone or centrosphere area of Fig. 9 is enlarged. The microtubules are numerous and apparently random in orientation. The material of the centriole is very finely filamentous, and material of similar texture can be recognized at points marked *X* as well as elsewhere. Profiles of vesicles, presumably part of the endoplasmic reticulum, are common. Mitochondria (*M*) mingle with pigment at margin of clear zone. Magnification ×35,000.

d) Nerves and nerve endings

Nerves containing both myelinated and unmyelinated fibers traverse the dermal layer. These primarily autonomic nerve processes frequently are found adjacent to the chromatophores. Structures morphologically similar to nerve endings found in nerve-nerve junctions are seen adjacent to the

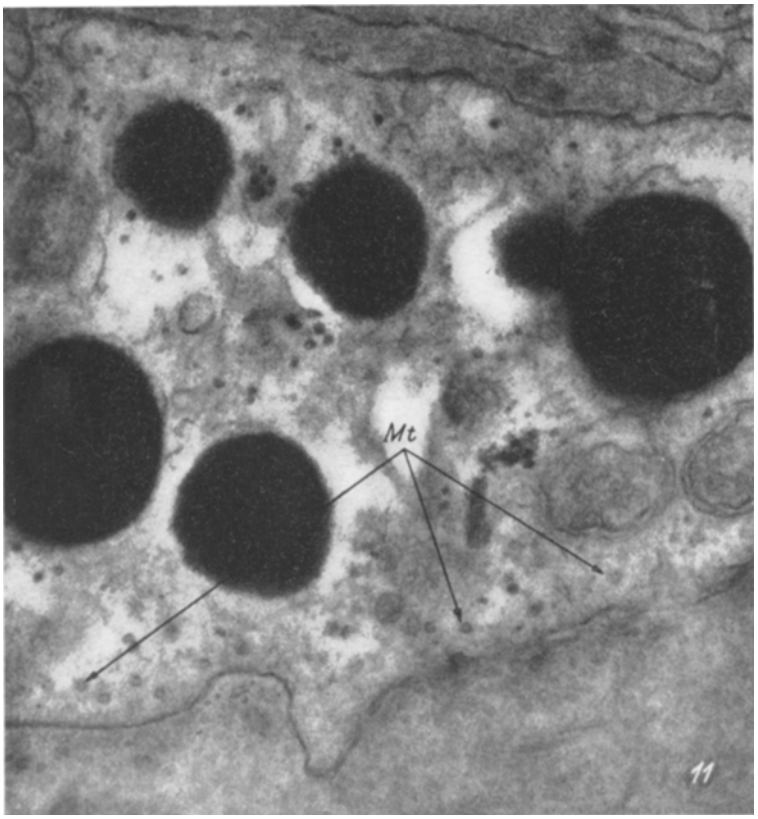

Fig. 11. Transverse section through the process of a melanophore. Cross sections of microtubules (*Mt*) are seen in the cortical region of the cell just beneath the limiting plasma membrane. Magnification ×41,600.

plasma membrane of the chromatophores (Figs. 5, 5 *a*). These "chromatoneural junctions" contain typical synaptic vesicles ranging in diameter from 350 Å to 650 Å. Sometimes a mitochondrion is observed within the nerve ending. Other presumed neural endings are found not in contact with the chromatophores but instead completely surrounded by collagen.

e) Microtubules

Microtubules are common in the melanophores. These long, slender, seemingly hollow cylinders have an average cross sectional diameter of 225 Å.

Their length is uncertain, but in favorable sections they can be traced for several microns. It is conceivable, but not yet shown, that one micro-

tubule extends the entire length of a cell process. The microtubules, as a rule, are straight, unbranching structures, but they seem to be able to bend slightly around granules and conform to the curves of the processes. This suggests a flexibility in a structure which tends elastically to be straight.

Microtubules can be found throughout the melanophore whether the cell is contracted or expanded (Figs. 6, 6 *a*, 7, 7 *a*). They are arranged parallel to the longitudinal axis of the cell process and are found in higher numbers nearer the plasma membrane than nearer the central axis of the process as revealed by tangential and cross sections (Figs. 8 and 11).

In the cell process the microtubules seem to align the granules into rather well defined columns parallel to the longitudinal axis of the process (Fig. 8). Within the central region where their number per unit volume is higher than in the processes, large groups of microtubules are seen passing in between the granules. Their influence in aligning the granules seems less obvious in this region than in the processes.

f) Centriole

The microtubules of the melanophore emanate into the processes from a point within the central region of the cell. At this point is a centriole (Figs. 9, 10). Attachment of the microtubules directly to the centriole has not been demonstrated. Rather they seem excluded from a region with a radius of about 0.25 microns, devoid of SER vesicles and pigment, which surrounds the centriole. Finely filamentous material comprising a centriolar matrix is observed radiating from the centriole into this zone. Other dense aggregations of similar material can be observed nearby (Fig. 10). Though this filamentous material has not yet been identified, one cannot help but interpret it as also involved in the microtubule-centriole relationship.

The spherical distribution of the pigment granules about the centriole indicates its central position in pigment dispersion. In sum, the centriole seems to be the focal point for the emanation of microtubules and the migration of pigment.

Discussion

1. Neural and Hormonal Control

As noted above, control over pigment displacement within the chromatophore is exercised in the fish by the autonomic nervous system and the pituitary gland. Domination by the nervous system of hormone regulation is evident in *Fundulus* chromatophores, but nevertheless, the pituitary does have some influence. The identification of the pituitary substance has not been made to our knowledge, but presumably it functions in a fashion analogous to the influence of MSH on melanophores of amphibians. The means by which the nervous influence is transmitted are equally vague. Parker, for reasons briefly mentioned earlier, argues against the neurohumors adrenalin and acetylcholine usually associated with the autonomic nervous system. He favors slowly diffusing "lipohumors" that will not dissolve in the blood and thus be transported quickly to the denervated chromatophores. Speculation on the nature of the neurohumors originates

from observations on the gradual adaptation of the denervated chromatophores in the caudal band, an adaptation beginning with those cells closest to the innervated chromatophores and slowly spreading to the central axis of the caudal band. Such gradual spreading of the adaptation influence from the innervated area to the denervated area is in direct contrast to the rapid, synchronous contraction of the denervated melanophores induced by injection of adrenalin into the blood. However, to our knowledge it has not yet been established that the neurohumor directly mediating the nervous influence over the innervated chromatophore is the same substance that presumably diffuses into the denervated region.

Examination of the chromatophores with the electron microscope revealed some structures that may play a significant role in mediating the influence of pituitary and nerves over pigment migration. Nerve endings were found, some in junction with the melanophore and others seemingly isolated by the collagen. It may turn out that these "isolated" nerve endings actually do form a connection with the chromatophore, but that this junction was not contained within the plane of section. A less plausible explanation for these "isolated" nerve endings is that they form no actual junction with the chromatophore, but rather transmit their influence through the intercellular matrix, say by a "lipohumor."

Cortical pits projecting into the chromatophore from their points of continuity with the plasma membrane were found. Similar structures have been observed in endothelial and other cells. Their role in the endothelium has been interpreted as a submicroscopic form of pinocytosis, possibly related to the transport of material from the blood through the capillary wall [6]. Perhaps the pits within the chromatophore provide a similar function; perhaps such substances transported into the cell via the pits include neurohumors or pituitary hormones. Elucidation of the role of the pits in chromatophores has not been accomplished, but their distribution throughout the cell and their obvious connection to the plasma membrane make them likely candidates for the reception of substances from without the cell. We would suggest that this reception may be specific for certain hormones.

That the chromatophores are directly innervated has been shown physiologically and morphologically. But the nature of the "diffusing neurohumors" and the transport of the pituitary hormones into the chromatophore are still unclear.

2. The Role of the Microtubules

When one examines the internal morphology of the melanophore for a plausible mechanism by which conceivably the cell could disperse or concentrate its pigment, one's attention is drawn to the microtubules because of their distribution and orientation. Here, as in several other types of cells, these long slender structures are arranged in such a way as a) to give support for the asymmetries in cell form, b) to guide intracellular movement of cytoplasmic particles, and c) to provide, by some device, a propulsive force [36].

The plasma membrane is clearly not a rigid form-supporting surface, and cells whose permanent shape deviates as radically from the spherical form as does that of the melanophore are generally thought to possess some internal "cytoskeleton." In many instances of asymmetry such as cilia [14], the discoidal erythrocytes of some fish [10], the caudal sheath of developing sperm [7], the extended arms of protozoans [44], there exists an associated population of microtubules. Not only have microtubules been shown in these and other instances, but their arrangement relative to the asymmetries makes them logical candidates for the tasks of form maintenance. Thus in cilia the well-known 9 + 2 complex of microtubules forms the longitudinal axis of the cilium; in the slender axopods of *Actinosphaerium* a double array of parallel microtubules comprises the axial component. It seems, indeed, to be a general rule that microtubules in asymmetric cells and cell extensions are aligned parallel to the long axis of the asymmetry and are situated predominantly near or within the cell cortex.

Microtubules also appear to be involved in the intracellular movement of cytoplasmic particles a) in defining the channels and direction of movement and b) possibly also in providing the motive force. Examples of this apparent involvement include the streaming in nerve cell processes [45] where microtubules are universally present, the cytoplasmic movements in plant cells [19], and the migration of particles up and down the axopods of heliozoans [44] to mention only a few. In all these cited cases, as in the chromatophore, the direction of motion and the orientation of the microtubules parallel each other. Likewise it is evident that the motion of the melanin granules does not depend on any withdrawal or change in shape of the melanophore arms.

This latter fact was suggested first by Matthews who reported in 1931 that he was able to visualize clear channels remaining in the dermis after the melanophore had apparently contracted [21]. Either the cell retained its form and only the melanin granules moved, or the cell in contracting left behind a space which outlined its uncontracted shape. The former is easy to demonstrate. In a manner suggested by Spaeth [42] we photographed various stages of pigment migration in a pulsating cell over the course of an hour to corroborate Matthews' observations and our own made on preserved material with phase-contrast optics (Fig. 2). The successive expansions of the melanin content, separated by at least one contraction, into precisely the same pattern each time, suggests not only that the processes hold their form between contractions but also that the granules return to the same spaces within the process. These conclusions are borne out now by the electron micrographs of contracted melanophores in which it is clearly evident that the melanin-free pseudopod persists during "contraction" and that it is richly populated with microtubules. These latter run parallel to the long axis of the cell extension and are long and remarkably straight. They have been observed to curve around a melanin granule as though accommodating to its presence. Hence they would seem to be flexible or elastic structures showing a tendency to return to the straight form after distortion. Obviously their distribution and their phy-

sical characteristics make them ideal for a skeletal role, though of course their involvement in form maintenance needs supplemental support. It is pertinent to point out that no other structure appears in the cytoplasm of the melanophore process which could as reasonably be assigned this role.

In addition, as suggested earlier, the microtubules would appear to bear some relationship to pigment migration. Our observations on the kinetics of migration indicate that some organizing influence exists in the cytoplasm of the cell processes to channel the pigment along rather fixed paths parallel to the long axis of the process. Spaeth [43] noted also that migration seems to take place along fixed axes in the cytoplasm. Further to this point, Ballowitz [3] observed that the movement of the individual granules seemed occasionally to be independent of the overall cytoplasmic streaming, that is, cytoplasmic granules were sometimes seen to move antiparallel to the general direction of pigment migration. Similar erratic and independent movement of cytoplasmic particles has been noted in the tubule-rich axopods of *Actinosphaerium* [44]. If one supposes that the microtubules merely define channels within the cytoplasm in which the pigment granules roll passively with the cytoplasmic stream, then such "independent" movements as these would be surprising. If, on the other hand, one supposes that the microtubules exert a positive influence (a motive force) on pigment migration, then one could probably design a model which would account for this erratic or "independent" motion as well as other phenomena of pigment concentration and expansion.

The two-directional motion of the pigment granules along these "fixed channels" is, for example, another aspect of pigment behaviour that must be explained by a postulated mechanism controlling migration. The means by which the pigment is dispersed seems to be the same as the means by which it is concentrated, only in reverse. The same sort of granule orientation and movement is evident in both cases. Two observations should perhaps be emphasized in this regard: a) the microtubules remain in the pseudopods whether the granules are dispersed or concentrated, and b) the microtubules have not been observed to terminate on the granules. These facts seem to rule out a "push-pull" model for pigment movement, a model analogous to that proposed for the movement of chromosomes within the spindle during mitosis (review in 16, 23). One can imagine the granules migrating along "fixed channels" within the cytoplasm perhaps like toy trains along electrically charged tracks. By adjusting the current flowing through the tracks the operator can regulate the speed and direction the engine moves along the tracks. Somehow in the melanophore a 0.1 M. NaCl or Ringer's solution "throws the switch" one way and adrenalin or 0.1 M. KCl solution "throws the switch" the other way. To extend this simple analogy one might say that the erratic movements of the granules during migration indicate either that the granules "jump the track" or that the "tracks" are individually controlled. That the pigment granules show little or no activity during the resting state implies that induction of pigment migration is not just a matter of "throwing the switch" from "disperse" to "concentrate" but that the controls also have an "off" position. Perhaps the controls reside in two

systems of tubules, one based on the cortex and the other based on structures within the central region, each system regulating one directional aspect of the migration.

As of now the mechanism by which the microtubules exert a motive force, if any, on pigment granules is not evident. Ledbetter and Porter [19] imply that cytoplasmic streaming may be either a result of an undulating motion of the microtubules or a product of some kind of interaction between the stationary tubules and the streaming particles. Weiss et al. [45] see in the neuron a peristaltic motion associated with axoplasm flow, a motion which may in fact be due to undulating tubules. However, no such undulations have been observed in melanophore pseudopods during pigment migration. We prefer, therefore, for this phenomenon, a model based on some interaction between the surfaces of the pigment granules and the relatively stationary microtubules.

If we accept the hypothesis that microtubules exert a motive force on pigment granules, then we might look for the control center which turns the force on or off. The centriole seems an obvious structure to choose, at least for the system of microtubules emanating from it and the migration these tubules seem to direct. However, its choice as the single controlling factor or even the need for a single controlling factor is not at all clear. Matthews [21] blocked off a melanophore process trapping the granules distal to the block. This indicated to Matthews that the control of migration is not necessarily located solely in the central region. Possibly the morphological evidence that microtubules may emanate from sites in the cell cortex [7] as well as from centrioles and the satellites of centrioles should suggest that in the melanophore there may be two sets of microtubules, one based on the cell center, the other on the pseudopodial tips coinciding thus with the double innervation. One system would interdigitate with the other and when separately activated would move the granules toward one or the other of these sites.

Summary

The dermal melanophores of *Fundulus heteroclitus* L. have been investigated by light and electron microscopy with the purpose of revealing the mechanisms controlling pigment migration. As predicted by earlier studies, the nerve endings of a double innervation were found adjacent to and in synaptic relation to the melanophore surface. Not expected were the large number of small pits or invaginations present in the cell surface. These appear identical to the so-called micropinocytotic vesicles found generally in cells of the vascular endothelium and smooth muscle. In chromatophores they are more reasonably interpreted as receptor sites for neurohormones than as uptake and transport mechanisms.

Observations made on the kinetics of pigment migration within the processes of these melanophores indicate that the granules move along relatively fixed channels arranged parallel to the long axes of the processes. Examined at fine structure levels, the zones of cytoplasm around these channels are found to be populated by microtubules about 225 Å in diameter

aligned parallel to the direction of pigment movement. These long slender elements are present in the processes regardless of whether the melanin is concentrated in the cell center or dispersed. It is reasoned from these and other observations that the microtubules function as cytoskeletal elements which help maintain the extended form of the melanophore arms and at the same time define the channels in which the pigment moves. The possible role of the tubule in generating the motive force for pigment migration is discussed.

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