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LAVENDER, A CHICK MELANOCYTE MUTANT WITH DEFECTIVE MELANOSOME TRANSLOCATION: A POSSIBLE ROLE FOR 10 nm FILAMENTS AND MICROFILAMENTS BUT NOT MICROTUBULES

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SUMMARY

Lavender is a mutation of chick neural-crest-derived melanocytes showing dilute feather pigmentation. This defect, previously attributed to a lack or attenuation of dendrites, was found to be due to a defect in melanosome translocation. The mutant phenotype, of melanin-congested perikarya and pigmentless dendrites is expressed both *in vivo* and *in vitro*. Studies with colcemid and cytochalasin B suggest that the avian melanocyte resembles a dispersing amphibian melanophore in its requirement for microfilaments but not microtubules.

Ultrastructural analysis revealed a normal complement of intracellular filaments. Microtubules, however, are scarce. Intermediate (10 nm) filaments surround and are closely associated with intracellular organelles, while microfilaments interconnect all filaments and organelles. Whole-cell centrifugation at 300 g showed that 10 nm filaments stream behind and appear to attach to mobile membrane-bound organelles including the nucleus, lipid granules and mitochondria, as well as melanosomes. It is suggested that all intracellular filaments, especially microfilaments and intermediate filaments, interconnect forming a network responsible for organelle motility.

INTRODUCTION

This study was undertaken to determine the primary cause of feather pigment dilution in the chick mutant, lavender (*lav/lav*). Pigment dilution in lavender has previously been attributed to an absence or attenuation of dendrites (Brumbaugh, Chatterjee & Hollander, 1972; Chatterjee, 1971). The present study demonstrates, using light and electron microscopy, that lavender melanocytes possess normal dendrites that lack pigment granules (melanosomes). In wild-type melanocytes, melanosomes are produced in the perinuclear region and translocated to the tips of dendrites (Brumbaugh, Bowers & Chatterjee, 1973; Brumbaugh & Zieg, 1972; Hori, 1969; Hunter, Mottaz & Zelickson, 1970; Toda & Fitzpatrick, 1972). Melanosome transfer then occurs as melanosome-containing dendritic tips pinch off and are phagocytized by adjacent epidermal cells (keratinocytes) (Billingham, 1948; Cohen & Szabo, 1968; Cruickshank & Harcourt, 1964; Klaus, 1969; Mottaz & Zelickson, 1967; Prunieras, 1969). Lavender melanocytes, both *in vivo* and *in vitro*, produce melano-

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somes normally but are defective in melanosome translocation. This defect results in a congestion of melanosomes perinuclearly and the absence of dendritic pigment. Lavender, the first mutation of intracellular organelle translocation to be described, provides a unique opportunity to study this essential but poorly understood process.

Intracellular filaments have been implicated as the primary mediators of directed intracellular organelle movements in various organisms (Buckley, 1974; Freed & Lebowitz 1970; Parpart, 1964; Rebhun, 1972). We examined the roles of microtubules and microfilaments in melanosome translocation using the drugs colcemid and cytochalasin B. These drugs were added to cultures of neural crest-derived melanocytes in an attempt to mimic the lavender defect in wild-type cells. Wild-type and lavender melanocytes were also examined ultrastructurally for the presence and distribution of the 3 major filament types of non-muscle cells: thin actin-like microfilaments (3–8 nm), intermediate filaments (10 nm) and microtubules (25 nm). Filament–organelle associations were examined using pelleted and unpelleted cells of both genotypes.

MATERIALS AND METHODS

Genetic stocks

Wild-type (*lav⁺/lav⁺*; Brumbaugh & Hollander, 1965) and lavender (*lav/lav*; Brumbaugh *et al.*, 1972) chickens and chick embryos were produced by appropriate matings of stocks maintained in the School of Life Sciences, University of Nebraska, Lincoln. Wild-type adult males are dorsally red and ventrally black, while females possess a 'salt and pepper' pattern of black and red pigment. Lavender males and females are similar to wild-type birds, but black areas are modified to a soft grey and red areas to a very pale buff.

Regenerating feather tissue

Breast feathers were plucked from adult birds. After 2 weeks, the regenerating feathers were plucked, placed in glutaraldehyde at room temperature (at 3 % in phosphate buffer, pH 7.32) and the barb ridges dissected out (Brumbaugh, 1971; Brumbaugh *et al.*, 1972). Tissue was fixed for 1 h at room temperature, then stored in fixative at 4 °C. All tissue was then osmicated, dehydrated through a graded series of ethanol and embedded in Epon.

Cell culture

Somites, neural tube and associated ectoderm, dissected from stage 16–18 chick embryos (Hamburger & Hamilton, 1951), were minced, trypsinized and plated out at 150 000 cells per 60 mm dish (Falcon Plastics, Oxnard, Ca; Brumbaugh & Lee, 1975). The plating medium was F12 (Grand Island Biological Co. [GIBCO], Grand Island, N.Y.) containing 5 % foetal calf serum (FCS) and 1 % bovine serum albumin (GIBCO). Medium was replaced after the first 24 h and every 2 to 3 days thereafter using F12 medium containing 10 % FCS (growth medium). Primary cultures gave rise to pigmented cell colonies after 7–8 days *in vitro*.

Colcemid (GIBCO) was added directly to growth medium at 1 µg/ml. Cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wisc.) dissolved in dimethylsulphoxide (DMSO) (Aldrich) or 95 % ethanol was added to growth medium at 1 µg/ml. The effects of treatment with cytochalasin B did not vary with the solvent used. DMSO alone, at the final concentration of 0.1 %, had no noticeable effect on the cultures (also see Schroeder, 1970).

Fixation and embedding

Some cells were fixed and embedded in the dish while others were treated with trypsin, rinsed and centrifuged at 300 g for 10 min, fixed and embedded in 15-ml plastic centrifuge tubes (Corning Glass Works, Corning, N.Y.). For treatment with cytochalasin B, cells were exposed for 15 min just prior to final pelleting. Cells fixed in the dish were stained with haematoxylin prior to embedding (Brumbaugh, unpublished data). All cells were fixed in phosphate buffer (pH 7.32) with 3 % glutaraldehyde, post-osmicated, dehydrated in a graded series of alcohols, infiltrated in an ethanol-Epon series, embedded in Epon, and polymerized at 60 °C for 3 days. Embedded melanocyte colonies were cut out of dishes with a razor blade and glued, cell side up, onto Epon pegs. Pellets, exposed by sawing off the end of a plastic centrifuge tube, were cut longitudinally and re-embedded.

Light and electron microscopy

All tissue was sectioned on an LKB Ultratome III. Sections (2 µm thick) were stained metachromatically with a 1:1 mixture of 1 % methylene blue, 1 % sodium citrate:1 % azure II, both in distilled water (Richardson, Jarett & Finke, 1960). Thick sections were viewed and photographed with a Wild compound microscope.

Thin sections were obtained with a diamond knife and picked up with acetone-cleaned 150-mesh parlodin-coated grids. Sections were stained for 15 min each with uranyl acetate and lead citrate. Thin sections were viewed with a Phillips 201 transmission electron microscope. Light micrographs of living, cultured melanocytes were taken with a Wild inverted microscope using phase-contrast optics.

RESULTS

Morphology of the mutant lavender

This section examines the morphology of normal and lavender mutant melanocytes both *in vivo*, using Epon thick sections of intact barb ridges, and *in vitro*, using cultured neural-crest-derived melanocytes.

In vivo melanocytes. Barb ridges are parallel columns of epidermal cells (keratinocytes) that encircle the feather shaft at its growing base. Each barb ridge contains melanocytes of neural crest origin interspersed among keratinocytes. Fig. 1 shows unstained (A, C) and stained (B, D) Epon thick sections of barb ridge tissue from regenerating adult feathers.

In wild-type melanocytes, melanosomes are uniformly distributed throughout the cell body and dendrites (Figs. 1A, B). Wild-type dendrites are visible in unstained sections due to the presence of dendritic pigment (Fig. 1A). Melanosome transfer does occur in wild type cells as demonstrated by the presence of numerous melanosome-filled dendritic tips that have been ingested by keratinocytes (Fig. 1A, arrows).

Lavender melanocytes appear to lack normal dendrites in unstained sections (Fig. 1C). Lack of dendritic pigment causes some melanocytes to appear adendritic while others appear to have short stubby dendrites. Metachromatic staining, which stains melanocyte cytoplasm relatively less than surrounding keratinocyte cytoplasm, reveals the presence of dendrites in lavender melanocytes (Fig. 1D, arrows). Serial-section analysis revealed that lavender melanocytes have pigmentless dendrites indistinguishable in size and shape from pigmented wild-type dendrites. Thus, pigment dilution in lavender chickens is not attributable to aberrant dendritic morphology, but

rather to a defect in melanosome translocation – apparently the inability to transport melanosomes dendritically. Since melanosomes are not translocated dendritically; they cannot be transferred subsequently to keratinocytes.

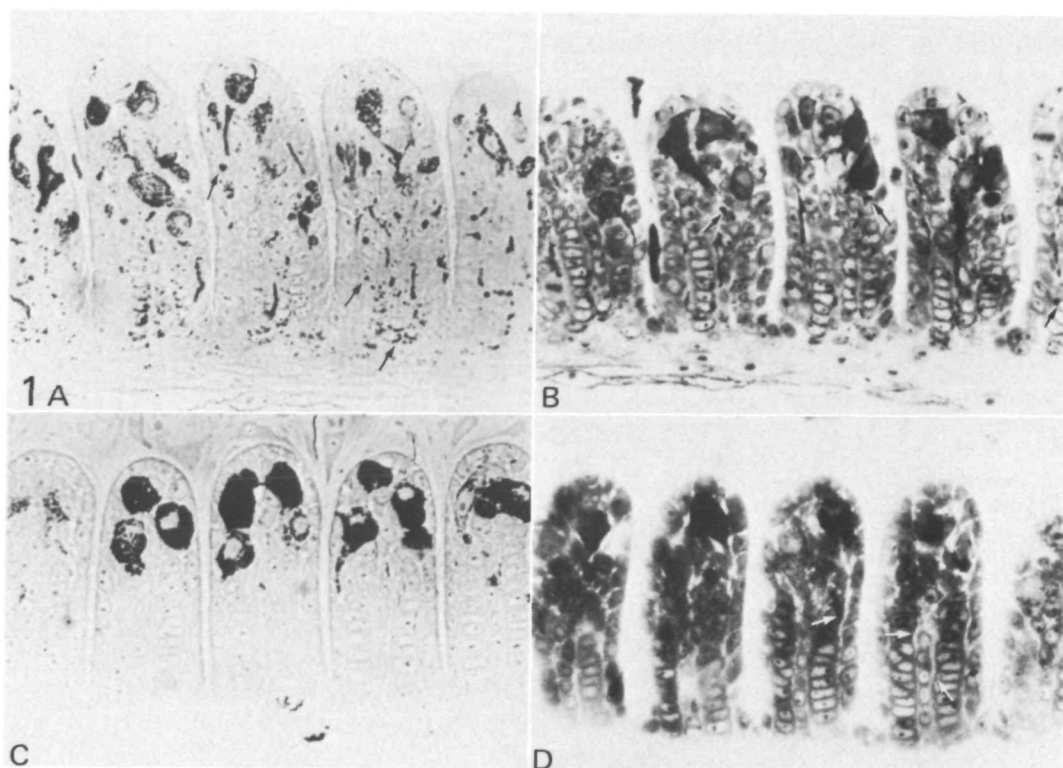


Fig. 1. Barb-ridge tissue from 2-week regenerating feathers. In the wild-type, pigment is dispersed permitting dendrite visualization in unstained sections; while in lavender, pigment is restricted to perinuclear regions making dendrite visualization possible only after metachromatic staining. A, unstained, wild-type, $\times 620$. B, stained, wild-type, $\times 620$. C, unstained, lavender, $\times 680$. D, stained, lavender, $\times 680$.

Fig. 2 is a comparative light and electron microscopic study of melanocyte morphology in normal and lavender melanocytes. In wild-type melanocytes (Fig. 2A, B) melanosomes are uniformly distributed and melanosome transfer is normal. Melanosome-containing dendrites are found interspersed between keratinocytes in the barb ridge of wild-type chicken (Fig. 2B). However, in lavender melanocytes prominent pigmentless dendrites emanate from melanosome-congested perikarya (Fig. 2C, D). Thus, electron microscopy confirms the results of light microscopic observations, that the apparent defect in lavender melanocytes *in vivo* involves aberrant melanosome distribution.

In vitro melanocytes. Fig. 3 shows typical melanocyte morphology in wild-type (A) and lavender (B, C) melanocytes after 7 and 8 days in culture, respectively. In wild-type melanocytes, melanosomes are distributed throughout the cell body and its dendrites and accumulate at dendritic tips in preparation for melanosome transfer

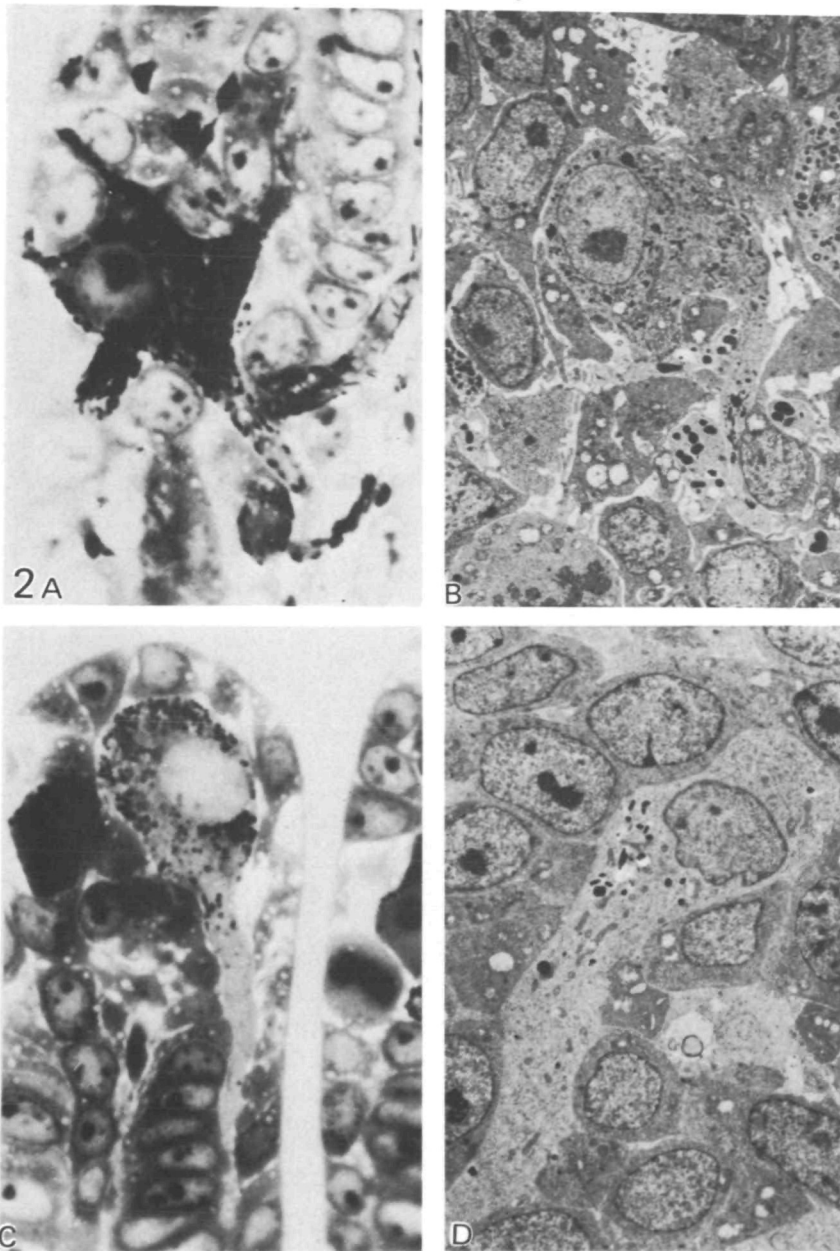


Fig. 2. Comparative light (LM) and transmission electron (TEM) microscopy of melanocyte morphology *in vivo*. In the wild-type, melanosomes are evenly dispersed. In lavender, melanosomes migrate only as far as the region of dendrite initiation resulting in melanin-congested perikarya and pigment-less dendrites. A, LM, wild-type, $\times 2400$. B, TEM, wild-type, $\times 3000$. C, LM, lavender, $\times 2400$. D, TEM, lavender, $\times 3500$.

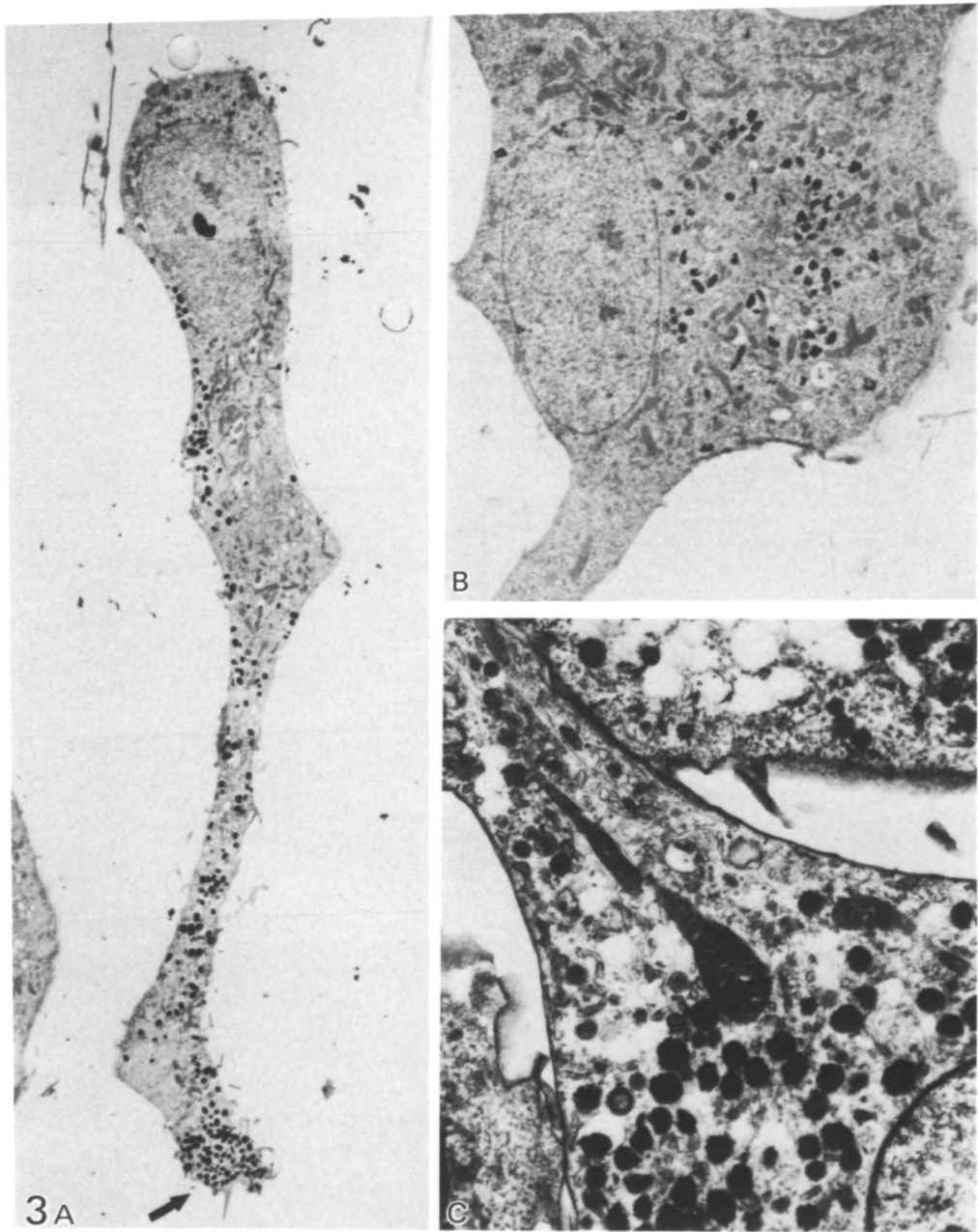


Fig. 3. Wild-type and lavender melanocyte morphology *in vitro*. A. In a wild-type melanocyte melanosomes are evenly distributed throughout the cell body and dendrite, and accumulate in the dendritic tip in preparation for melanosome transfer. $\times 3000$. B. In a lavender melanocyte melanosomes are restricted to perinuclear regions, $\times 5900$. C. The melanosomes of a lavender melanocyte migrate only as far as the region of dendrite initiation. $\times 9100$.

(Fig. 3A, arrow). In lavender melanocytes, melanosomes fail to move beyond the region of dendrite initiation (Fig. 3B, C). Melanosomes are restricted to perinuclear regions in some lavender melanocytes (Fig. 3B), while in others, melanosomes migrate as far as the bases of the dendrites (Figs. 2C, 3C).

Wild-type melanocyte morphology and melanosome distribution appear unaltered by the tissue-culture environment. Lavender melanocytes possess dendrites morphologically indistinguishable from those of wild-type cells both *in vitro* and *in vivo*, but the lavender genotype is clearly distinguished by a restricted distribution of melanosomes.

The response of microtubules and microfilaments to colcemid and cytochalasin B

Non-muscle cells contain 3 major filament systems: microtubules (25 nm), thin actin-like microfilaments (3–8 nm) and intermediate filaments (10 nm). Colcemid and cytochalasin B were utilized in the present study to determine the respective roles of microtubules and microfilaments in avian melanosome translocation. Since no drug is known that specifically disrupts intermediate filaments (Shelanski, Yen & Lee, 1976), the role of this filament system must be inferred from ultrastructural studies alone.

Colcemid. Colchicine, and its analogue colcemid, bind to tubulin and cause microtubule disassembly (Wilson & Bryan, 1974). The effect of colcemid on the melanosome distribution of pre-metaphase cells was examined following up to 24 h of exposure *in vitro*. Colcemid was used at 1 µg/ml, a dose sufficient to: (1) disrupt microtubules (Dollevet, 1977), and (2) cause metaphase arrest in most colonies after 24 h of treatment.

Both wild-type and lavender melanocyte colonies were treated with colcemid before and after the appearance of visible pigment. On 2 occasions unpigmented 5-day wild-type melanocytes were treated with colcemid for 4–6 h prior to the appearance of visible pigment in an attempt to prevent subsequent melanosome translocation. In both instances melanosome production and dendritic transport were indistinguishable from that of untreated controls.

Figs. 4 and 5 show the effects of a continuous exposure of colcemid at 1 µg/ml on already pigmented wild-type and lavender melanocytes. At each time point between 0 and 24 h, a set of pictures was taken: with the phase-ring in, to demonstrate cell morphology; and with the phase ring out, to show melanosome distribution. It is evident that colcemid does not alter the distribution of melanosomes in either cell type. Even after 18–24 h of treatment with colcemid, melanosomes remain uniformly distributed in wild-type cells (Fig. 4), while melanosomes remain restricted to perinuclear regions of lavender melanocytes (Fig. 5).

Thus, treatment with colcemid does not prevent melanosome translocation in wild-type cells, nor does it alter the distribution of melanosomes in the mutant, lavender. These results strongly suggest that in the chick, microtubules are not involved in the dendritic transport of melanosomes.

Cytochalasin B. Cytochalasin B prevents microfilament-dependent processes (Carter, 1967) by disrupting actin-like, subplasmalemmal microfilaments (Bernfield & Wessells, 1970; Schroeder, 1970; Spooner & Wessells, 1970; Yamada, Spooner &

Wessells, 1971). In the present investigation cytochalasin B was used at $1 \mu\text{g}/\text{ml}$, a concentration sufficient to disrupt 3–8 nm microfilaments (Dollevoet, 1977; Schroeder, 1972).

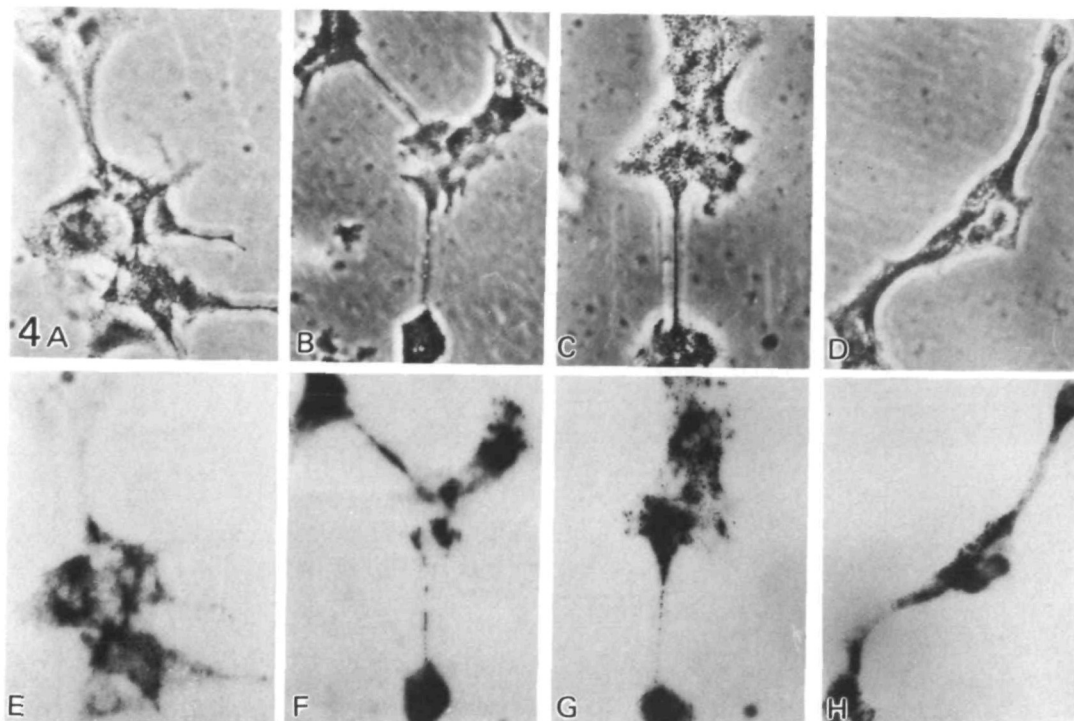


Fig. 4. Pigmented, cultured wild-type melanocytes were treated with colcemid for up to 18.5 h. At each time-point pictures were taken both with the phase ring in, to demonstrate cell morphology, and with the phase ring out, to demonstrate pigment distribution. Phase ring in: A, 0 h, control. B, 6 h, colcemid. C, 18.5 h, colcemid. D, 18.5 h, colcemid. Phase ring out: E, 0 h, control. F, 6 h, colcemid. G, 18.5 h, colcemid. H, 18.5 h, colcemid. $\times 475$.

Figs. 6 and 7 show the effects of continuous exposure to $1 \mu\text{g}/\text{ml}$ of cytochalasin B on the morphology of wild-type and lavender melanocytes, respectively. Melanosomes of wild-type cells (Fig. 6) are initially evenly dispersed but appear to clump after cytochalasin B treatment (6F–I). Melanosome clumping, observable as early as 5 min after drug addition, is well established by 10 min (Fig. 6G) and intensifies throughout the 30-min treatment period (Fig. 6F–I). Pigmentation intensified perinuclearly (Fig. 6F–I) as cell margins are pulled inward (Fig. 6A–D).

Cytochalasin B also causes dendrite attenuation and occasionally dendrite collapse in wild-type cells. In Fig. 6F (arrow) the melanosome-rich dendritic tip of one cell collapsed perinuclearly and failed to re-form after 30 min of recovery (Fig. 6E, arrow) while the dendrite of a neighbouring melanocyte was maintained proximally but appears truncated distally (Fig. 6E, double arrow). The collapse of cell margin and dendrites may in part account for cytochalasin B-induced melanosome movements.

Fig. 7 shows the effects of treatment with cytochalasin B on lavender melanocytes in

culture. Since lavender melanosomes are already perinuclear, cytochalasin B has little effect on their distribution, although perinuclear clumping does appear to intensify slightly (compare Fig. 7F and H). Cytochalasin B also causes the collapse of cell margins, the attenuation of some dendrites and the collapse of others. In Fig. 7 one dendrite became thin and broke (A, arrow), being absent from recovered cells (E). In both genotypes, cell shape and melanosome distribution returned to normal after 5 rinses with cytochalasin B-free medium and subsequent incubation at 38 °C for 30 min.

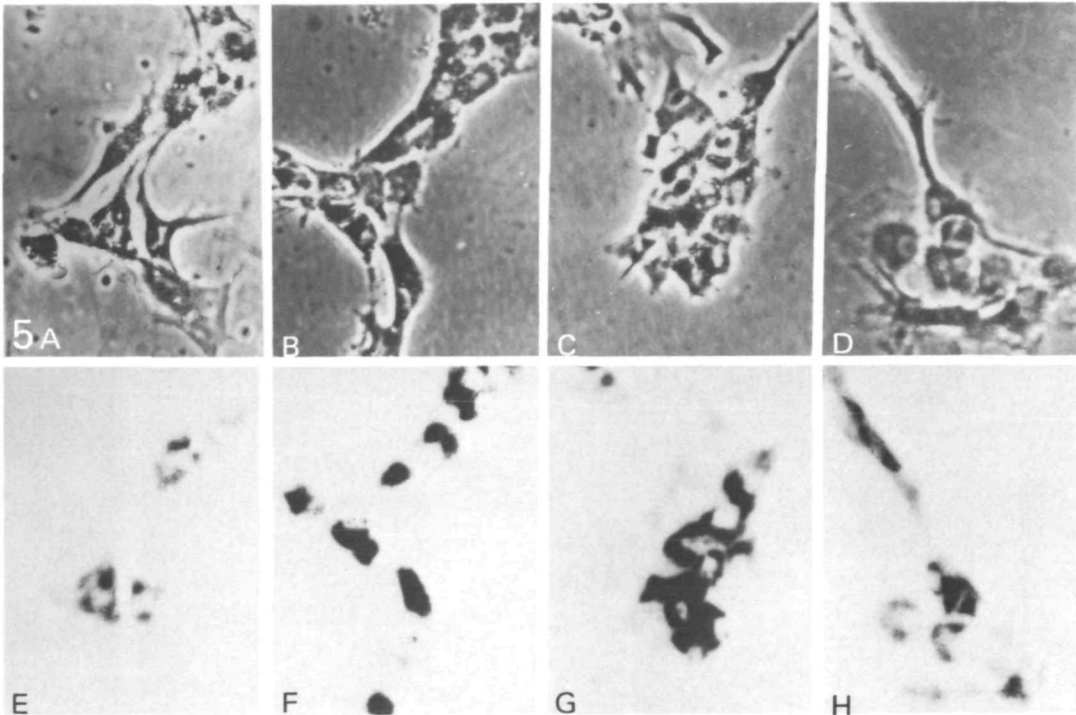


Fig. 5. Pigmented, cultured lavender melanocytes were treated with colcemid for up to 24 h. Phase ring in: A, 0 h, control. B, 6 h, colcemid. C, 24 h, colcemid. D, 24 h, colcemid. Phase ring out: E, 0 h, control. F, 6 h colcemid. G, 24 h, colcemid. H, 24 h, colcemid. $\times 465$.

To summarize, cytochalasin B slightly intensified the pigment clumping seen in lavender melanocytes, while in the wild-type, cytochalasin B caused an apparent perinuclear clumping of melanosomes. This effect may result from the collapse of cell margins or may indicate a role for microfilaments in avian melanosome translocation.

An ultrastructural examination of filament systems in normal and lavender melanocytes

Lavender melanocytes were examined ultrastructurally for a possible defect in 1 of the 3 major filament systems. Fig. 8 is an *in situ* longitudinal section of a cultured lavender melanocyte showing the perikaryon and the region of dendrite initiation. Within the latter region, the salient feature is an abundance of intermediate (10 nm) filaments,

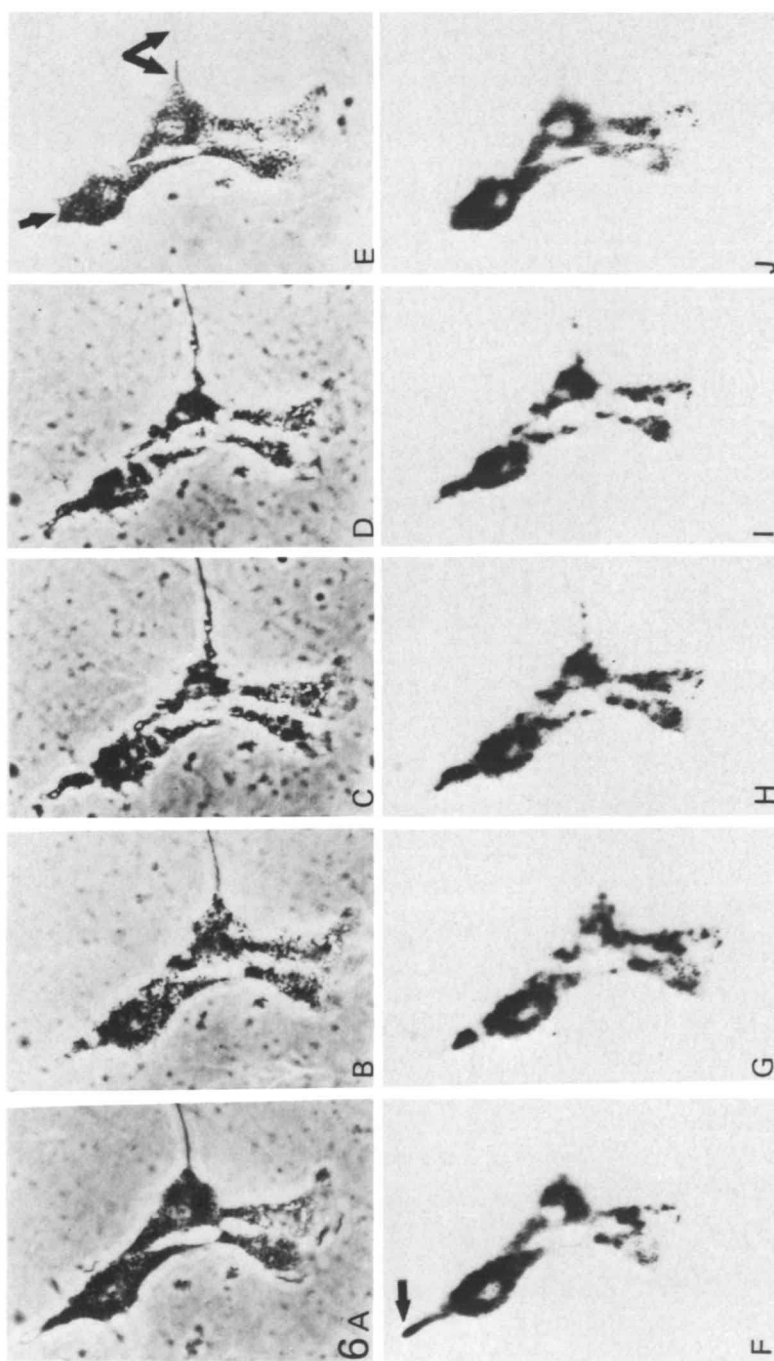


Fig. 6. Pigmented, cultured wild-type melanocytes were treated with cytochalasin B (CB) for up to 30 min. Cells were photographed every 10 min and after 30 min of recovery. Phase ring in: A, 0 h, control. B, 10 min, CB. C, 20 min, CB. D, 30 min, CB. E, 30 min, recovery. Phase ring out: F, 0 h, control. G, 10 min, CB. H, 20 min, CB. I, 30 min, CB. J, 30 min, recovery. $\times 485$.

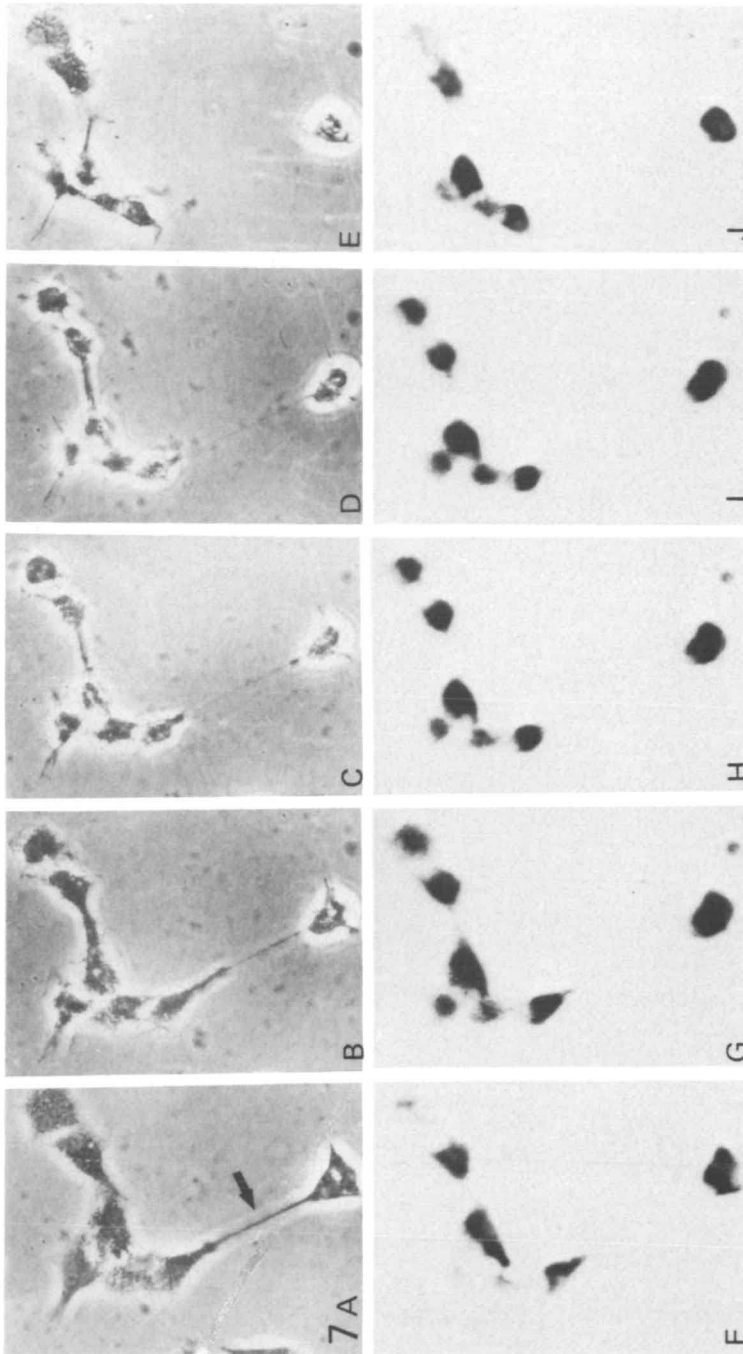
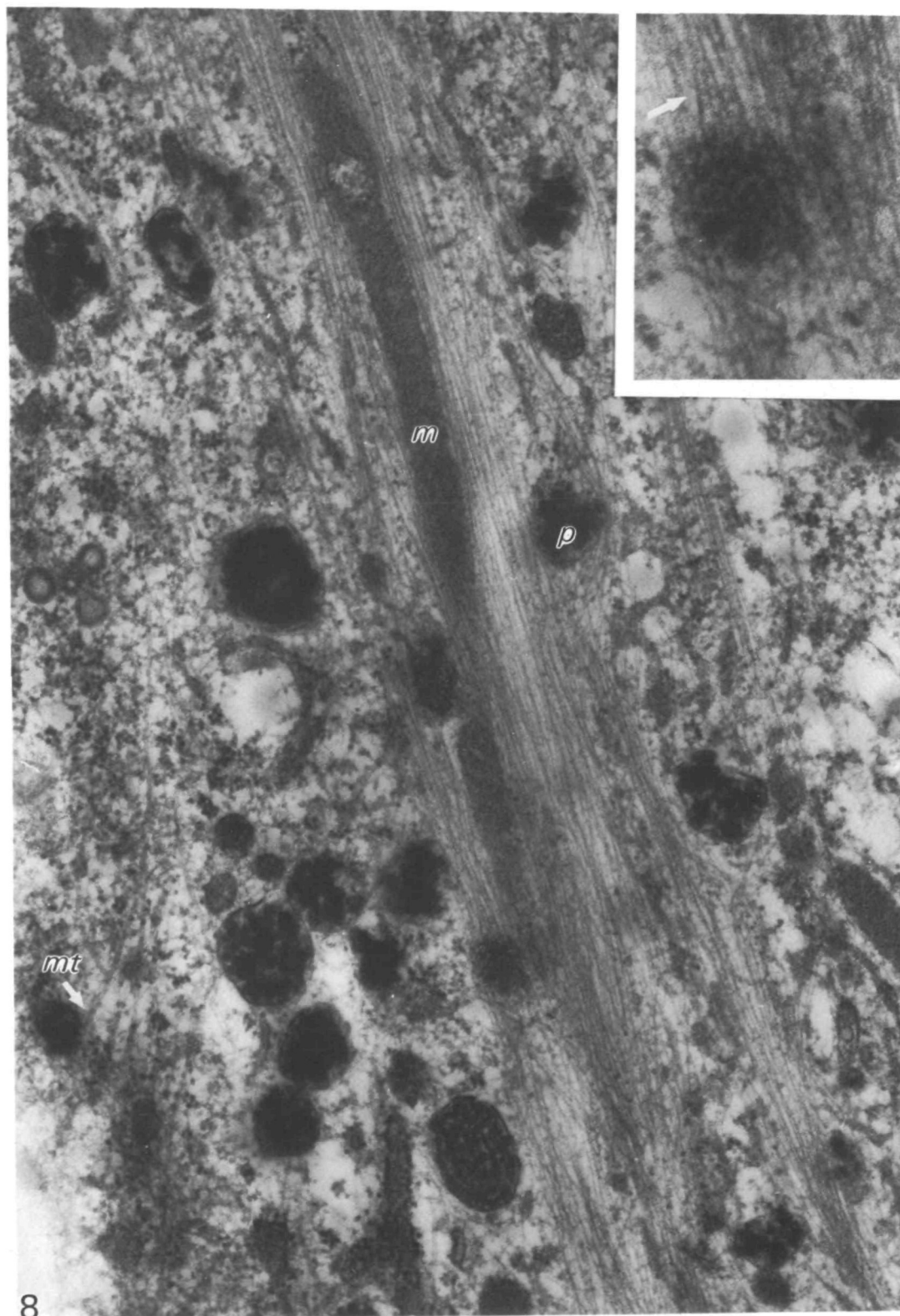


Fig. 7. Pigmented, cultured lavender melanocytes were treated with cytochalasin B (CB) for up to 30 min. Phase ring in: A, 0 h, control. B, 10 min, CB. C, 20 min, CB. D, 30 min, CB. E, 30 min, recovery. Phase ring out: F, 0 h, control. G, 10 min, CB. H, 20 min, CB. I, 30 min, CB. J, 30 min, recovery. $\times 485$.



which course longitudinally along the dendrite and envelop mitochondria and melanosomes. Microfilaments are also abundant, forming a network that appears to interconnect all intracellular structures. At a higher magnification (inset) microfilaments appear to link intermediate filaments to each other and to the melanosome that they envelop. Microtubules are scarce and located primarily at the cell periphery.

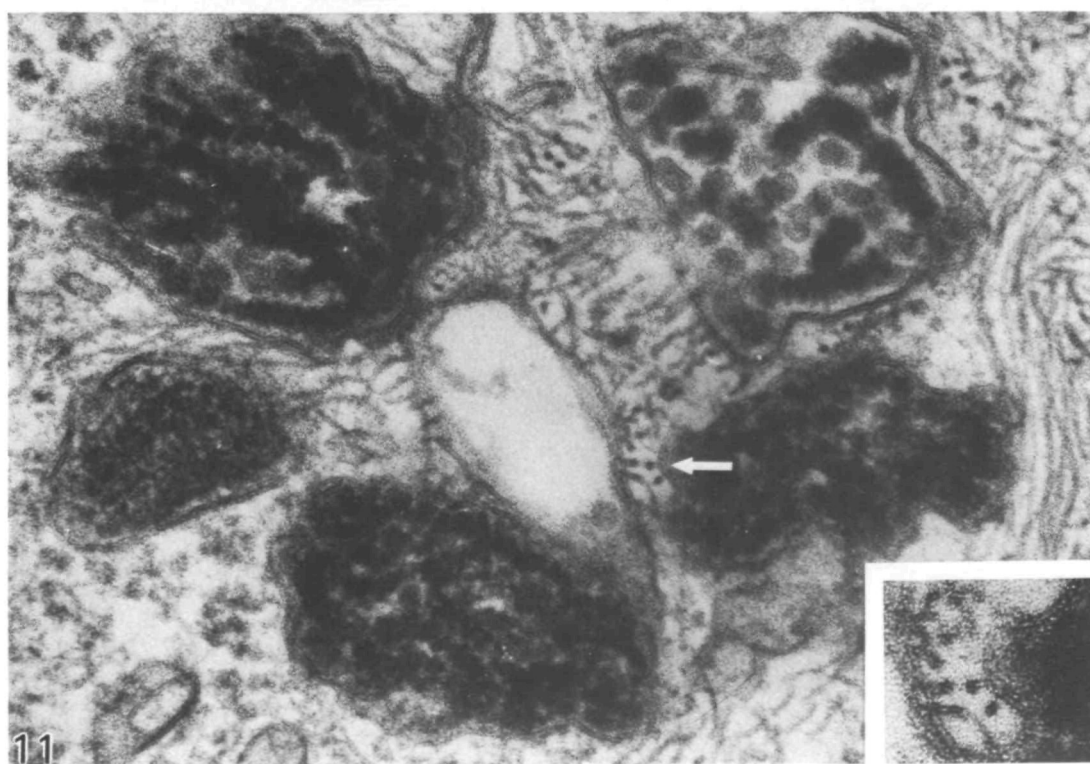
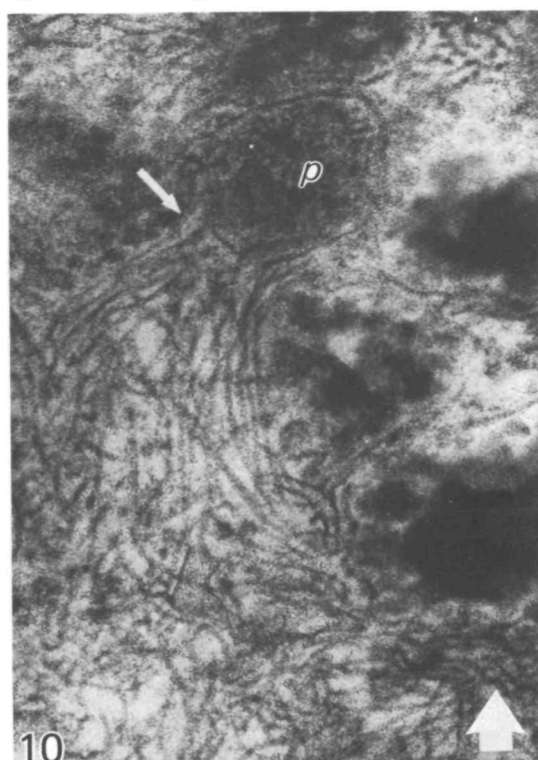
Fig. 9 is a longitudinal section through the dendrite of a cultured wild-type melanocyte. Bundles of intermediate filaments surround and appear closely associated with a melanosome. Analysis of many sections revealed that the amount and distribution of filament types does not appear to differ between wild-type (Fig. 9) and lavender (Fig. 8) melanocytes. In both genotypes microtubules are scarce; intermediate filaments surround and envelop intracellular organelles and microfilaments form a network that appears to interconnect intracellular structures. Thus, lavender melanocytes contain a normal complement of the 3 major filament types and these filaments are distributed in an apparently normal fashion.

To investigate the nature of filament-organelle associations in lavender and wild-type melanocytes, whole cells were trypsinized and pelleted at 300 g, an acceleration sufficient to cause organelle displacement without cytolysis. Centrifugation of intact cells resulted in the downwards displacement of nuclei, melanosomes, mitochondria and other intracellular organelles. Thus, intracellular material was subjected to an upward cytoplasmic streaming, caused by nuclear displacement, and a downward acceleration. It was reasoned that intracellular organelles that are sufficiently well attached to filaments should remain attached following organelle displacement. The intracellular network of microfilaments, if firmly attached to organelles, would be expected to move with its associated organelles, or be torn apart as the structures that attach to it pull away from each other.

The results of serial-section analysis of electron micrographs suggest that intermediate filaments are firmly attached to the membranes surrounding various intracellular organelles in both the wild-type and lavender genotypes. In pelleted whole cells, intermediate filaments stream behind (Fig. 10) and are closely associated with the melanosome membranes of both wild-type (Fig. 11, arrow and inset) and lavender (Fig. 17) melanocytes. Lavender melanocytes show intermediate filament-melanosome membrane associations as distinct and as numerous as those of wild-type cells.

Intermediate filaments in both genotypes appear to be attached to the outer membranes of mitochondria. Fig. 12 shows that bundles of intermediate filaments stream behind the displaced mitochondrion of a pelleted wild-type melanocyte. Higher magnification (Fig. 14) shows that intermediate filaments surrounding a wild-type mitochondrion appear to be inserted into the outer mitochondrial membrane. Similar

Fig. 8. Bundles of intermediate filaments fill the dendritic cytoplasm of a cultured lavender melanocyte, enveloping mitochondria (*m*) and melanosomes (*p*). Microtubules (*mt*) are found at the cell periphery. $\times 37\,500$. Inset: intermediate filaments are closely associated with both ends of a melanosome, which they ensheath. These filaments themselves appear interconnected by microfilament crossbridges (arrow). $\times 89\,000$.



intermediate filament attachments to mitochondria are seen in pelleted lavender melanocytes (Fig. 13).

Intermediate filaments appear to be attached to, and extend cytoplasmically from the nuclear envelope in both genotypes. In Fig. 15, abundant intermediate filaments stream cytoplasmically from the nuclear envelope in a pelleted wild-type melanocyte. Intermediate filaments appear to be inserted between 2 nuclear pores (Fig. 15 inset, arrows) in this melanocyte. A tangential section of the nuclear envelope of a lavender melanocyte (Fig. 16) also suggests intermediate filament insertion into the outer nuclear membrane. Thus, intermediate filament attachments to mitochondria, melanosomes and the nuclear envelope are indistinguishable in wild-type and lavender melanocytes.

Intermediate filaments are not only attached to, but appear to interconnect, intracellular organelles. This is true for both genotypes. Fig. 17 shows intermediate filaments interconnecting melanosomes, mitochondria, lipid granules and the nuclear envelope of a lavender melanocyte. Intermediate filament attachments to membrane-bound lipid granules also occur in both genotypes (Figs. 17, 18). A close association between intermediate filaments and Golgi vesicles is also found in both wild-type (Fig. 15) and lavender (Fig. 19) melanocytes. Thus, analysis of cell pellets reveals that intermediate filaments closely associate with, appear attached to and interconnect, various motile organelles in both genotypes including: melanosomes, mitochondria, lipid granules and the nuclear envelope.

The microfilament network, so striking in unpelleted, untrypsinized melanocytes (Figs. 8, 9), is not apparent and is probably fragmented by viscous shear in pelleted cells of both genotypes (Figs 10–15). Treatment of melanocytes, prior to pelleting, with cytochalasin B (an agent known to disrupt microfilaments but not intermediate filaments or microtubules; Spooner, 1973) did not alter distribution of filament of filament–organelle associations seen after pelleting.

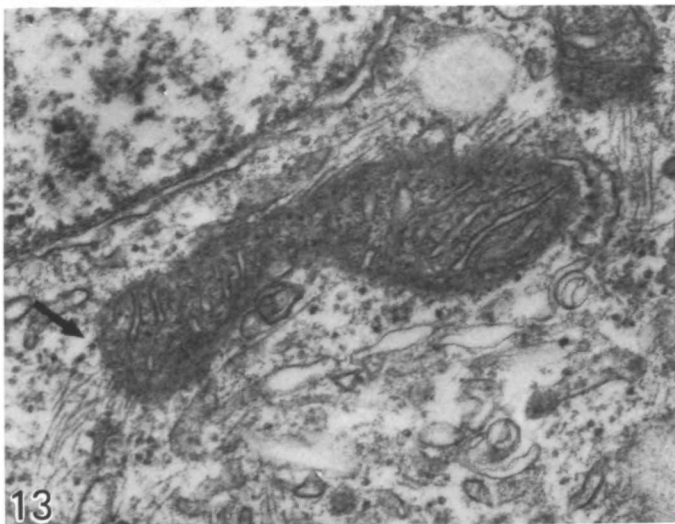
DISCUSSION

Lavender is an avian pigment-cell mutant with an aberrant melanosome distribution: melanosomes fail to migrate into the dendrites, giving the appearance that dendrites are attenuated or absent. Examination of lavender melanocytes by light and transmission electron microscopy revealed normal cell morphology with dendrites

Fig. 9. A bundle of intermediate filaments, coursing longitudinally through the dendrite of a cultured wild-type melanocyte, making close contact (arrow) with a melanosome (*p*). *t.* $\times 60000$.

Fig. 10. Intermediate filaments stream behind a melanosome (*p*) to which they appear attached (small arrow) in a pelleted, wild-type melanocyte. The large arrow indicates the direction of centrifugal force. $\times 108000$.

Fig. 11. Intermediate filaments appear to be inserted (arrow) directly into melanosome membranes in a pelleted, wild-type melanocyte that has been treated with cytochalasin B prior to pelleting. Intermediate filaments appear unaffected by the drug. $\times 112000$. Inset, $\times 187500$.



present. The production of melanosomes was also normal although their distribution was not. Thus, the dilute pigmentation of lavender birds is attributable to a lack of melanosomes within dendrites, which in turn is due to defective melanosome translocation. Because melanosomes are absent from the dendrites, they are not transferred to neighbouring feather keratinocytes, hence the faded appearance of the feathers.

The mouse mutants, dilute and leaden, are described in the literature as adendritic (Gerson & Szabo, 1969; Market & Silvers, 1956; McGrath & Quevedo, 1964; Straile, 1964). In these mouse mutants, as in lavender, pigment is restricted to perinuclear regions and dendrites are not visible. This striking similarity to lavender suggests that these mutants may possess dendrites that could be visualized by the techniques used in this study.

The dilution mutant, lavender, like the mouse mutants, dilute and leaden, appears to be expressed autonomously. Reed (1938) and Reed & Henderson (1940) found, by reciprocal grafting experiments with mice, that dilute melanocytes produce dilute pigmentation and non-dilute melanocytes produce non-dilute pigmentation, regardless of the genotype of the hair follicle. Mutant morphology in the chick mutant, lavender, is expressed *in vivo* in the barb ridge, as well as *in vitro* in the absence of keratinocyte cell contact. Thus, autonomy is strongly suggested for lavender melanocytes in culture, but not absolutely proven, since a few epidermal cells are present in melanocyte cultures.

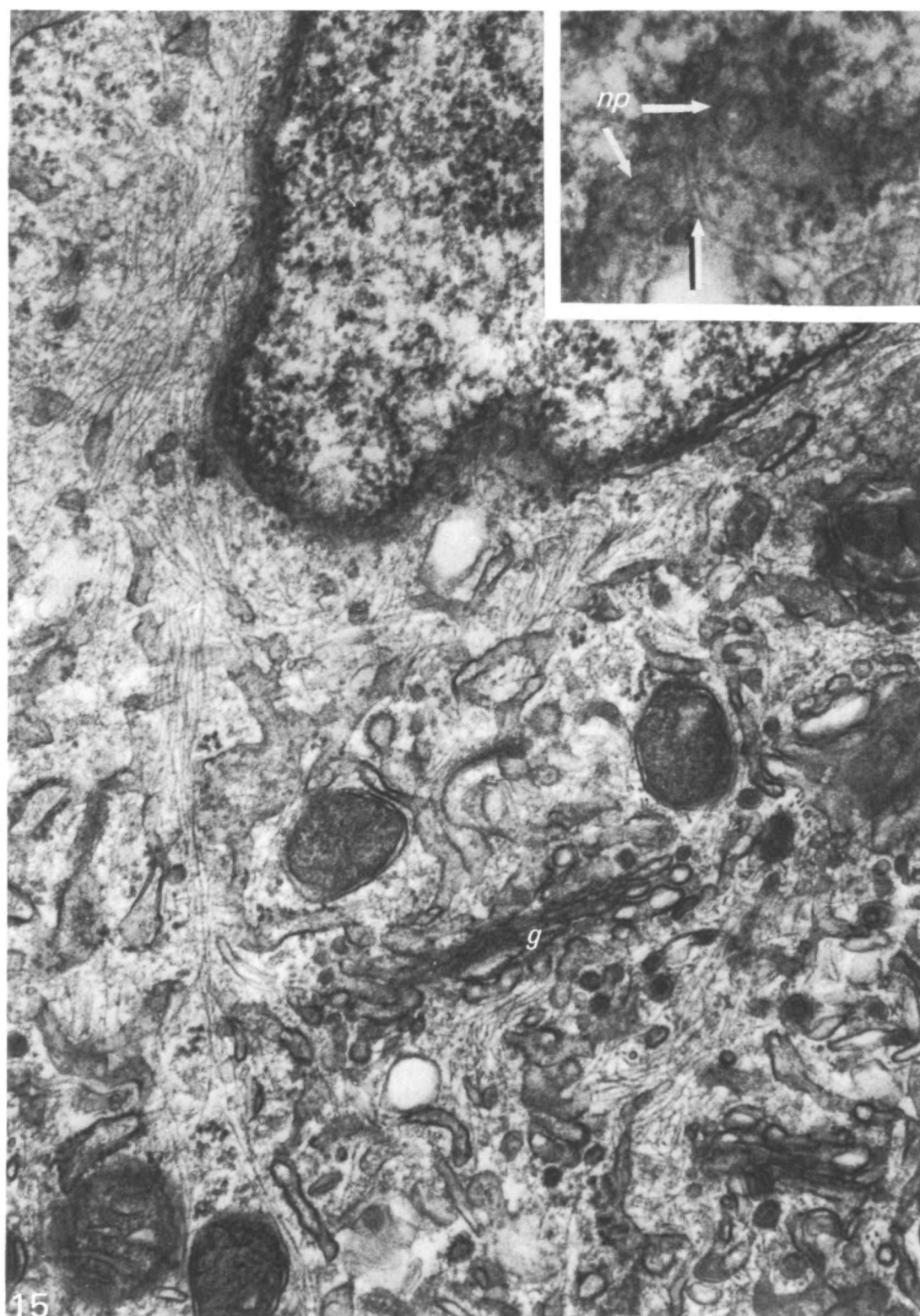
While very little is known about the mechanism of melanosome translocation in avian and mammalian melanocytes, the melanosome translocation of amphibian and teleost pigment cells (melanophores) is well studied. The latter move rapidly and reversibly in response to changes in lighting. Aggregation (lightening) occurs when melanosomes move out of the dendrites into the cell centre; while dispersion (darkening) occurs when melanosomes move into the dendrites (Fingerman, Fingerman & Lambert, 1975). In amphibian and teleost melanophores, microtubules appear to be required for aggregation, as colchicine inhibits aggregation and enhances dispersion (Lyerla & Novales, 1972; Malawista, 1975; Wikswo & Novales, 1969). In amphibian melanophores, microfilaments appear to mediate melanosome dispersion, as cytochalasin B inhibits dispersion and promotes aggregation (Fisher & Lyerla, 1974; McGuire & Moellmann, 1972).

Avian and mammalian melanosomes move slowly and irreversibly into the dendrites, dispersing but not, under normal conditions, aggregating (Fitzpatrick, Miyamoto & Ishikawa, 1966). In the present study, ultrastructural analysis revealed that the avian melanocyte, like its mammalian counterpart, contains very few microtubules (Jimbow

Fig. 12. Intermediate filaments (small arrow) stream behind a mitochondrion in a pelleted, wild-type melanocyte. The large arrow indicates the direction of centrifugal force. $\times 108000$.

Fig. 13. Intermediate filaments appear attached (arrow) to the mitochondrial membrane of a pelleted lavender melanocyte. $\times 49000$.

Fig. 14. Intermediate filaments surround and appear to be inserted (arrows) into the outer mitochondrial membrane of a pelleted wild-type melanocyte. $\times 109800$.

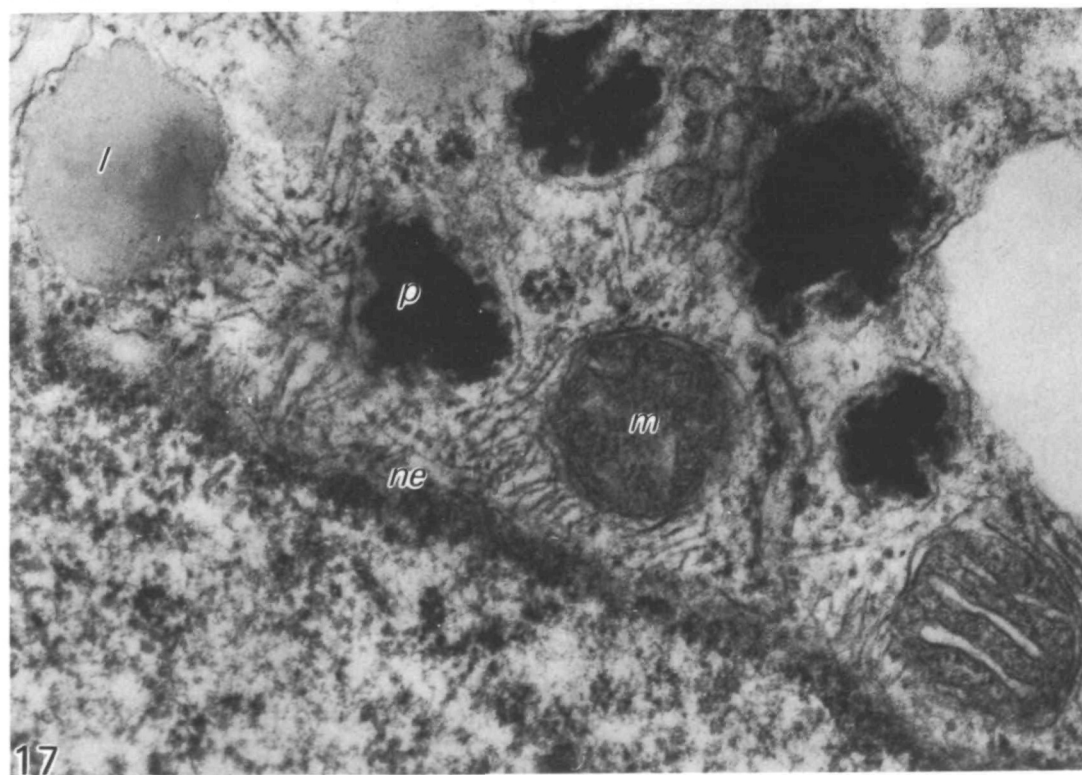
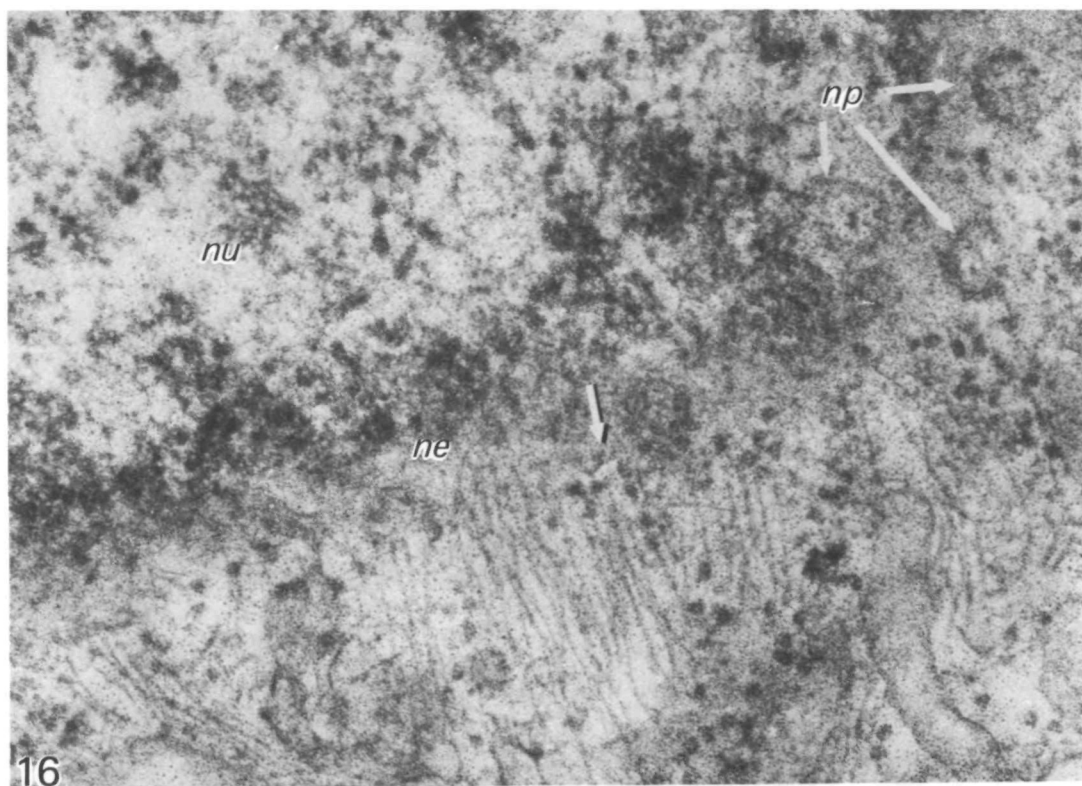


& Fitzpatrick, 1975), while abundant microfilaments and intermediate filaments are found throughout the cell and its dendrites (Jimbow & Fitzpatrick, 1975; Sauk, White & Witkop, 1975; Wikswo & Szabo, 1973). Studies using colcemid and cytochasin B suggest that microfilaments, but not microtubules, may be involved in directing avian melanosome movements. Avian melanosome dispersion does not appear to require or be mediated by microtubules, as $1 \mu\text{g/ml}$ of colcemid, a concentration sufficient to cause metaphase arrest, neither alters the distribution of melanosomes in mature melanocytes nor prevents the appearance of phenotypes characteristic of each genotype. Microfilaments do appear to be involved in avian melanosome dispersion, as $1 \mu\text{g/ml}$ of cytochalasin B disrupts cell morphology and alters melanosome distribution in both normal and mutant melanocytes. In wild-type cells, cytochalasin B partially mimics the lavender defect, while in lavender it intensifies the already perinuclear concentration of pigment. Thus, our results suggest an analogous mechanism for melanosome translocation in amphibian and avian systems. The avian melanocyte resembles a dispersing amphibian melanophore in that melanosome dispersion is disrupted by cytochalasin B but unaffected by colcemid.

The present study failed to reveal the cause of the translocation defect in lavender. No ultrastructural differences were found between wild-type and lavender melanocytes in their distribution or in their interconnexions with intracellular filaments. Both genotypes showed a normal complement of filaments. Microtubules were scarce, intermediate filaments filled dendrites and enveloped intracellular organelles, and microfilaments connected intermediate filaments to each other. Intermediate filaments were connected, directly or via microfilament attachments, to various mobile intracellular organelles including the nucleus, mitochondria, melanosomes and lipid granules. Microtubules probably do not play any significant role in avian melanosome translocation because lavender melanocytes are not defective in microtubule content. Microtubules are minor constituents of avian melanocytes and do not differ in their distribution or content in either genotype. Colcemid does not alter the distribution of melanosomes of either genotype whether in mature melanocytes or in melanocytes treated at the onset of melanogenesis.

The abundance of both microfilaments and intermediate filaments in avian melanocytes and the interconnexions of these filament types to each other and to various intracellular organelles suggest that both filament types may be involved in organelle motility. Microfilaments are likely candidates for mediators of organelle motility (Cloney, 1966) for they have been shown to contain actin (Lazarides, 1975; Palevitz & Hepler, 1975) and to exhibit contractile properties in non-muscle cells (Rodewald, Newman & Karnovsky, 1976; Schroeder, 1972; Spooner, 1973; Wessells *et al.*, 1971).

Fig. 15. This wild-type melanocyte has been treated with cytochalasin B prior to pelleting. Intermediate filaments appear unaffected by the drug and extend cytoplasmically from their apparent site of attachment at the nuclear envelope or are closely associated with the Golgi system (g). $\times 48000$. Inset: two 10 nm filaments are inserted (arrow) between nuclear pores (np) in an *en face* section of the nuclear envelope. $\times 84800$.



Attachment has been suggested between thin (3–8 nm) microfilaments and mitochondria, chloroplasts, endoplasmic reticulum and nuclei (Buckley & Porter, 1967; Crawford & Castle, 1975; Palevitz & Hepler, 1975). Actin-like microfilaments also form a contractile network, which is found just beneath the plasmalemma and may be inserted into it (Bernfield & Wessells, 1970; LeBeux & Willemot, 1975; Yamada *et al.* 1970). This network is thought to be responsible for plasmalemmal motility such as ruffled membranes, growth cones and microspikes (Spooner, Yamada & Wessells, 1971).

Lavender melanocytes showed an extensive and apparently normal microfilament network interconnecting all intracellular structures. Cell pelleting failed to enhance visualization of these microfilament associations. Thus, while there is evidence to suggest that the lavender mutant may represent a defect in microfilament function, lavender melanocytes appear to be normal in content, distribution and associations of microfilaments with other intracellular structures. Despite the lack of any apparent morphological defect in microfilaments, the abundance and extensive interconnexions of microfilaments found in both genotypes suggest that microfilaments may play some role in directing organelle movements. These ultrastructural studies do not rule out the possibility that lavender may be a defect of microfilament function, perhaps preventing microfilament-mediated contractions necessary for normal melanosome movements. The defect might also be one that affects microfilament associations with each other, with other filament types, or with melanosomes themselves.

Although intermediate filaments are ubiquitous elements of both muscle and non-muscle cells and have been assumed to be primarily structural, their function is unknown (Izant & Lazarides, 1974; Eriksson & Thornell, 1979; Lazarides, 1980; Small & Celis, 1978; Small & Sobieszek, 1977). Other investigators have shown intermediate filaments to be in close physical association with the nucleus (Lehto, Virtanen & Kurki, 1978; Metuzals & Mushynski, 1974; Small & Celis, 1978), with melanosomes of amphibian melanophores (Moellmann, McGuire & Lerner, 1973), and with mitochondria and endoplasmic reticulum (Buckley & Porter, 1967). Intermediate filaments have also been reported to be one of the main constituents of the perinuclear region (Lehto *et al.* 1978; Small & Celis, 1978; Starger & Goldman, 1977).

While the present study provided no evidence for an intermediate filament defect in lavender melanocytes, the abundance and close associations of these filaments with all motile intracellular organelles in both genotypes suggests that they may participate, either actively or passively, in directed organelle translocations (Buckley & Raju, 1976; Schliwa & Euteneuer, 1978; Starger & Goldman, 1977).

In the present investigation, intermediate filaments fill the cytoplasm of avian melanocyte dendrites. Whole-cell pelleting revealed that intermediate filaments appear

Fig. 16. Intermediate filaments appear to be inserted (arrow) into the outer nuclear membrane in a tangential section of the nuclear envelope (*ne*) from a pelleted lavender melanocyte. Nuclear pores (*np*); nucleus (*nu*). $\times 108000$.

Fig. 17. Intermediate filaments appear to connect melanosomes (*p*) mitochondria (*m*), and lipid granules (*l*) to the nuclear envelope (*ne*) in a pelleted lavender melanocyte. $\times 72900$.

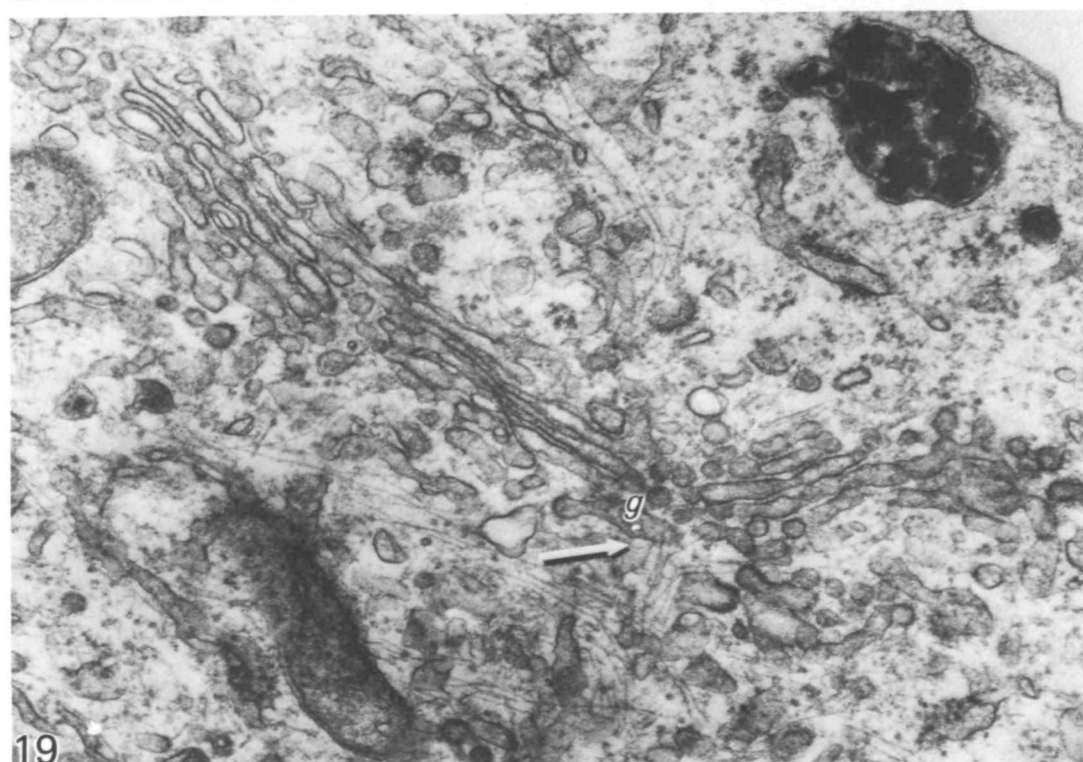
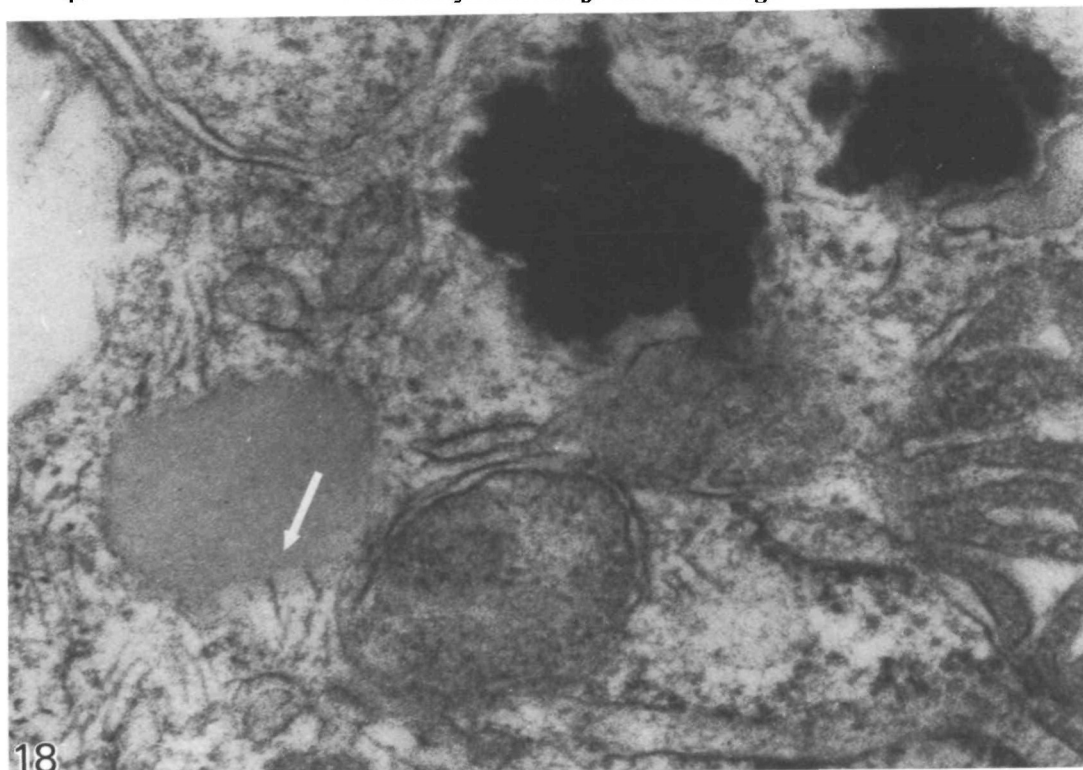


Fig. 18. Intermediate filaments appear to be inserted (arrow) into a lipid granule in a pelleted lavender melanocyte. $\times 108\,000$.

Fig. 19. Intermediate filaments are closely associated (arrow) with Golgi vesicles (g) in a pelleted lavender melanocyte. $\times 54\,600$.

to be attached to melanosomes and other membrane-bound organelles in both genotypes. After centrifugation, intermediate filaments of both genotypes stream behind displaced organelles and appear to be inserted into the outer membrane surrounding melanosomes, mitochondria, lipid granules and the nucleus. Although attachment has not been proven unequivocally, the following observations strongly support filament-organelle attachment: (1) pelleting greatly enhanced the number and clarity of

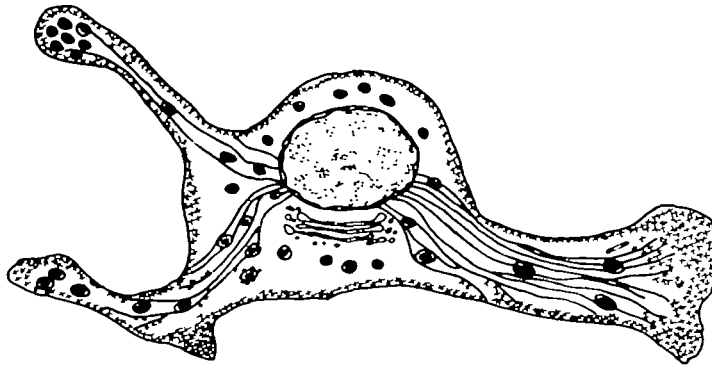


Fig. 20. A model for filament-melanosome associations. Intermediate filaments (—) attached to melanosomes are connected at one end to the nuclear envelope and at the other to the subplasmalemmal microfilament network. Actin-like microfilaments (x) are attached along the length of intermediate filaments.

filament-organelle associations seen; (2) intermediate filaments trailed behind displaced organelles after centrifugation of intact cells; and (3) intermediate filaments appear to terminate on, and be inserted into, organelle membranes. This close association of intermediate filaments with organelle membranes suggests that these filaments may play some role in normal organelle positioning and/or motility.

The present investigation provides evidence that microfilaments and intermediate filaments are closely associated with each other as well as with various intracellular organelles. Studies on a variety of cell types in different organisms suggest that all 3 major filament types interconnect to form a 3-dimensional filament meshwork (Buckley, 1975; Buckley & Porter, 1967; Burton & Fernandez, 1973; Lazarides & Hubbard, 1976; Schliwa & Euteneuer, 1978; Yamada *et al.* 1970), which is closely associated with various intracellular organelles (Buckley & Porter, 1967; Buckley & Raju, 1976; Cloney, 1966; Crawford & Castle, 1975; LeBeux & Willemont, 1975; Metuzals & Mushynski, 1974) and is continuous with (Buckley, 1975) the contractile (Wessells *et al.* 1971) subplasmalemmal microfilament network. It has been suggested that the filaments within the network are responsible for organelle motility (Buckley & Raju, 1976; Goldman, 1971; Starger & Goldman, 1977). Evidence for the existence of myosin and tropomyosin (Hitchcock, 1977) associated with the intracellular filament meshwork of non-muscle cells (Buckley & Raju, 1976; Lazarides, 1975; Weber & Groeschel-Steward, 1974) suggests that actin-myosin-like contractions of microfilaments may direct organelle motility (Buckley & Raju, 1976). Because all filaments of the intracellular filament meshwork are interconnected, localized actin-myosin-directed contractions could pull intermediate filaments, and the organelles to which

they are attached, towards the side of contraction. Thus, intermediate filaments might act as cables, anchored to organelles at one end and pulled by microfilaments at the other. Movement of melanosomes into the dendrites would require only that the magnitude and/or frequency of microfilament-mediated contractions be greatest at dendritic tips.

Fig. 20 illustrates the filament-filament and filament-organelle associations within a normal melanocyte. An intracellular meshwork of intermediate filaments and microfilaments is shown to interconnect the nucleus and plasmalemma with intracellular organelles (Metuzals & Mushynski, 1974). This filament meshwork may direct the translocation of various intracellular organelles including melanosomes. The meshwork may also be structural, maintaining cell shape and ensuring normal organelle positioning.

Lavender melanocytes, although defective in melanosome translocation, show no apparent ultrastructural defect. Although filament systems are most probably involved in and essential to organelle translocation, the nature of the lavender defect was not resolved by ultrastructural analysis. Our results show that the lavender mutation is more complex than previously envisaged and suggest that a microfilament-related defect may be involved.

We would like to thank the following for their comments and criticism of the manuscript: Charles Harvey, Robert Hausmann, Lawrence Karr, Loren Knapp, Aron Moscona and Bonnie Sedlak. We would also like to thank Shieko Kanno and Jackie Godsey for typing this manuscript. This study was supported in part by N.I.H. grant GM18969.

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(Received 18 March 1980 - Revised 10 March 1981)