

ULTRASTRUCTURE AND CELL BIOLOGY OF PIGMENT CELLS

Intracellular Dynamics and the Fine Structure of Melanocytes

**With Special Reference to the Effects of MSH¹ and Cyclic AMP
on Microtubules and 10-nm Filaments**

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INTRODUCTION

Four functions characterize the melanocyte: the synthesis of melanin granules; the growth and maintenance of dendrites; the translocation of melanin granules within the cytoplasm in response to hormones or neurotransmitters; the transfer of melanin granules to adjacent cells that have phagocytic capacities. The melanocyte of the amphibian epidermis exhibits all of these functions and thus is ideal for study. Other melanocytes may express one or the other of these functions less apparently. The mammalian epidermal melanocyte, for example, does not translocate melanin granules so rapidly; the dermal melanocyte does not transfer melanin granules; the albino melanocyte synthesizes little or no melanin; some melanoma cells do not form dendrites. The melanoma cell, on the other hand, after transplantation or growth in cell culture, has an additional capability that it shares with certain other tumor cells: it synthesizes particles that are assumed to be RNA tumor viruses.

In this report we will correlate the functions of the vertebrate melanocyte with its appearance by transmission electron microscopy. Unless specifically indicated, all electron micrographs shown are of cells fixed at room temperature in a solution consisting of 3% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.2), and 0.1% calcium chloride. The cells were refixed in 1% buffered osmium tetroxide, dehydrated in ethanol, and embedded in Epon 812 or, especially in the case of frog skin, in the less viscous epoxy mixture of Spurr (1). Thin sections were cut on an LKB Ultratome III equipped with a diamond knife. They were counter-stained with uranyl acetate and lead citrate and examined in a Hitachi HU-11B electron microscope.

¹ MSH = melanocyte stimulating hormone; cyclic AMP = adenosine-3'5'-cyclic monophosphate; DBcAMP = N²,2'-*o*-dibutyl cyclic AMP; AcCh = acetylcholine.

THE SYNTHESIS OF MELANIN GRANULES: MELANOSOMES AND TYROSINASE

Melanin granules are the secretory product of melanocytes. Like secretory granules of other cells they are bounded by a single membrane. Like many other granules they have an organized matrix with a fine structure that is typical but varies slightly among species. The fine structure of melanosomes has been discussed many times (e.g., 2-5), most recently by Klingmüller and Schmoeckel (6), Jimbow and Kukita (7), and Lutzner and Lowrie (8). Because of limitations imposed by the disproportion of the ultrathin sections (approx 80 nm) in comparison with the diameter of the granules on the one hand (approx 200 nm) and of the melanosomal filaments on the other (approx 3 nm), the disposition of the melanosomal matrix within melanosomes is not fully understood. Stereo high-voltage electron microscopy of thick sections will perhaps be more informative.

Observations made of thin sections suggest that the melanosomal filaments of the earliest recognizable stages of melanosome synthesis tend to fuse laterally (Fig. 1a) and form lattice films (Fig. 1b) of the thickness of a single filament. These thin films become folded within the confines of the limiting membrane like sheets of paper inside a mailing tube (Fig. 1c). The most elaborate lattice arrangements occur in amelanotic melanoma cells, where the process of formation of the intricate folding patterns has not been interfered with by the deposition of melanin. Onset of melanization during the filamentous stage prevents the lattice from forming and,

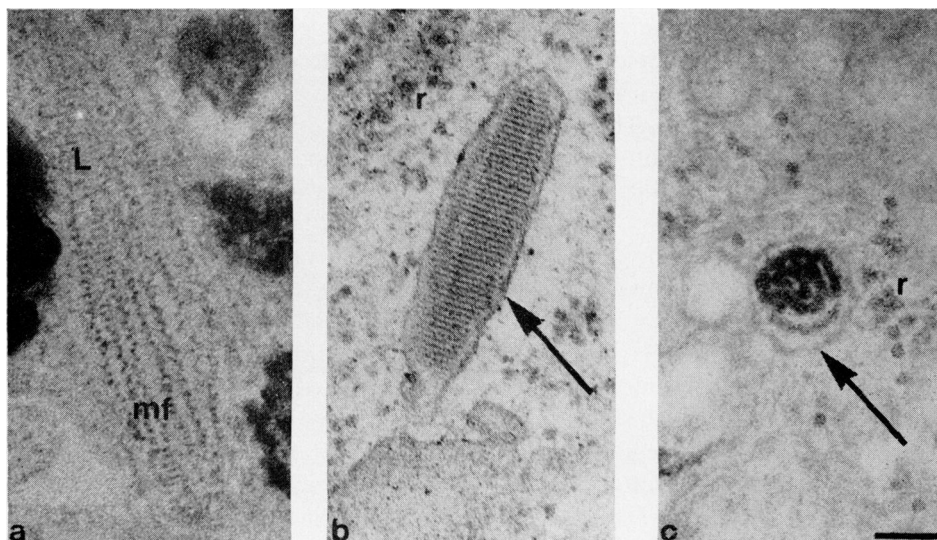


FIG. 1. Arrangement of the melanosomal matrix within melanosomes. (a) Longitudinal section of a stage-II melanosome, showing melanosomal filaments (mf) which appear to be fusing to form a lattice film (L). An alternative interpretation is that the filaments represent cross sections of lattice films that turn into the plane of the section at (L). Source: Guinea pig epidermis cultured by Dr. S. N. Klaus. (b) Longitudinal section of a stage-II melanosome, showing the lattice face-on and a well-defined limiting membrane (arrow). r, ribosomes. Source: Cloudman S91 mouse melanoma cultured by Dr. D. M. Carter. (c) Presumed cross section, stage-III melanosome. Partially melanized lattice films are folded within the confines of the limiting membrane (arrow). r, ribosomes. Source: human primary melanoma. Magnification: $\times 85,000$; scale bar = 0.1 μm .

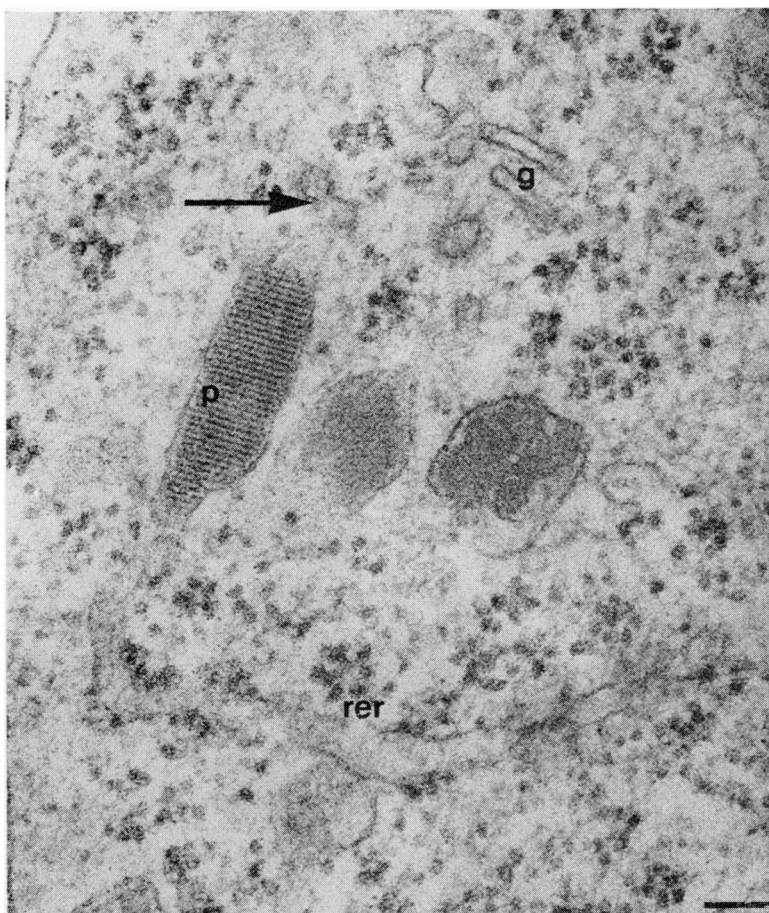


FIG. 2. Biosynthesis of melanosomes. The limiting membrane of a stage-II melanosome (p) appears to be continuous with rough-surfaced endoplasmic reticulum (rer), and with coated vesicles (arrow), presumed to be fusing with the smooth membrane. g, Golgi membranes. Source: Cloudman S91 mouse melanoma, amelanotic clone PS 4-3 (10), cultured by Dr. J. Pawelek. Magnification: $\times 85,000$; scale bar = $0.1 \mu\text{m}$.

if cut short, results in singly disposed melanized filaments (Fig. 4d). As melanization progresses, the gradually thickening filaments or lattice films begin to touch, leaving transitory electron-lucent channels (8).

The synthesis of melanosomes is independent of tyrosinase activity as illustrated in nature by the albino. Other examples are MSH-responsive Cloudman S91 mouse melanoma cells grown in the absence of MSH (9) (see Fig. 2a, Ref. 10) and the amelanotic clone PS 4-3 of Pawelek *et al.* (10) (Fig. 2). Early forms of melanosomes are located predominantly and in large numbers in the Golgi zone. The Golgi apparatus has therefore long been held to be the site of melanosome synthesis (e.g., 11–14). Recent evidence, however, suggests that melanosomes reach the Golgi zone via the smooth-surfaced endoplasmic reticulum (15). Tyrosinase-rich coated vesicles, originating from the Golgi apparatus, then fuse with them to form functional melanosomes (16, 17).

Since smooth endoplasmic reticulum is continuous with the rough and probably develops from it, as in liver (for recent review, see Ref. 18), it is not surprising

to find melanofilaments within rough-surfaced cisternae (19, 20). In Fig. 2, a stage-II melanosome is seen to develop as part of a focal dilatation of smooth endoplasmic reticulum that is continuous with the rough. Distally the melanosomal membrane is joined by coated vesicles near Golgi membranes. (For melanosome stages, see Refs. 14 or 27.)

During periods of rapid melanization the vesicles have been shown to become part of an extensive, continuous, tyrosinase-containing channel system that links dopa-positive Golgi membranes with melanosomes, and melanosomes with each other (21). Even preexisting melanosomes, like maternally inherited ones of amphibian pigmented retinal epithelium, become linked into this network during a new wave of melanization (22). Dopa-positive channels of rapidly melanizing melanoma cells are illustrated in Fig. 3.

The dopa reaction of Bloch and Ryhiner (23) as adapted for electron microscopy (19, 24–26) adds to the selectivity of heavy-metal staining by outlining the organelles that possess dopa oxidase or tyrosinase activity. Excessive diffusion of reaction product, however, precludes the differentiation of the tubular membrane from the tubular lumen as the site of enzyme activity. The electron-lucent centers seen in some cross sections of tubules and vesicles (Fig. 3b) suggest a membrane localization of the enzyme. After fusion with the melanosome, the enzyme should then reside in the melanosomal limiting membrane, a location favored by Toda and Fitzpatrick (14, 27). Alternatively, if the enzyme were transported in soluble form, it could settle on the melanosomal lattice, a location that has been favored traditionally and is still favored by Hearing *et al.* (28).

GROWTH AND MAINTENANCE OF MELANOCYTIC DENDRITES

Several functional similarities exist between melanocytic dendrites and neurites, but studies of melanocytic dendrites are scanty compared with investigations of axon growth and maintenance. Morphologically undifferentiated neuroblastoma and melanoma cells have been shown to differentiate into dendritic cells when incubated with cyclic AMP or phosphodiesterase inhibitors (29–31, 48). Cyclic AMP, therefore, can be used to control the outgrowth of such processes experimentally. The mechanism by which cyclic AMP influences the growth of neurites or melanocytic dendrites is not known.

The morphological differentiation of cultured hamster melanoma cells by cyclic AMP is illustrated in the phase-contrast photomicrographs of Fig. 4a,b. In the absence of theophylline or added nucleotide the cells remained spindle shaped (Fig. 4a), while cells growing in elevated concentrations of cyclic AMP became dendritic (Fig. 4b). A preliminary fine-structural investigation has shown that the newly formed melanocytic dendrites resemble neuronal dendrites (Fig. 4c). Large numbers of microtubules and some filaments lay parallel to the long axes of the processes. Axially oriented mitochondria, membrane bounded vesicles and rough-surfaced endoplasmic reticulum were also present. In addition, the processes contained melanosomes and occasional virus-like particles. The significance of the microblebs (Fig. 4c) protruding from the plasma membrane is not understood. These were not present on all dendrites examined.

Abundant tubules and some filaments are shown in cross section in a nonmalignant melanocytic process, grown in the absence of added cyclic AMP (Fig. 4d). In this section, the process resembled an axon, because it did not contain endoplas-

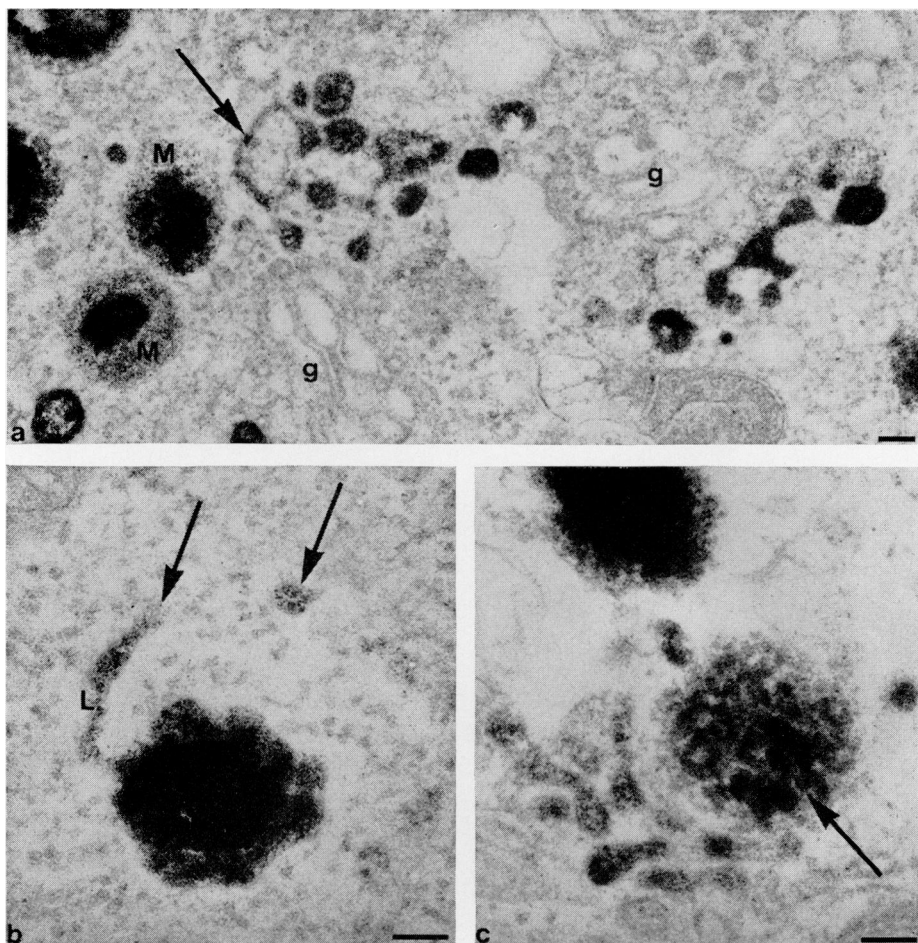


FIG. 3. Tyrosinase-containing smooth-membrane channels that link the Golgi apparatus with melanosomes. (a) Dopa-reaction product outlines vesicles and tubular channels that are presumed to be fusing with the dopa-positive limiting membrane of a stage-I melanosome (arrow). More mature melanosomes (M) have accumulated reaction product on their matrix and the surrounding space. (b) The dopa-reaction product has caused complete melanization of the melanosomal matrix, to form a stage-IV melanosome. A dopa-positive tubular channel, seen in cross section (arrows) and longitudinal section (L), appears to join with the corrugated periphery of the melanosome. (c) Grazing section through the corrugated periphery of a melanosome, into which the dopa-positive channels are presumed to insert (arrow). Source: MSH-responsive Cloudman S91 mouse melanoma cell line CCL 53 (10), grown 48 hr in MSH before being fixed in glutaraldehyde (2 hr). Cell culture of Dr. J. Pawelek. Histochemical incubation according to Rodriguez and McGavran (26); pH 6.8, 5 hr, 37°C. Magnifications: (a) $\times 53,000$; (b) and (c) $\times 85,000$; scale bars = 0.1 μm .

mic reticulum. In addition to melanosomes, tubules, and filaments, the only other formed elements present were mitochondria.

Two types of chemical used extensively in the study of nerve fibers are (1) plant alkaloids including colchicine and vinblastine, and (2) the cytochalasins, which are mold metabolites. By binding to tubulin, the 6S dimeric subunit of microtubules (33, 34), colchicine prevents assembly of subunits into tubules. Colchicine

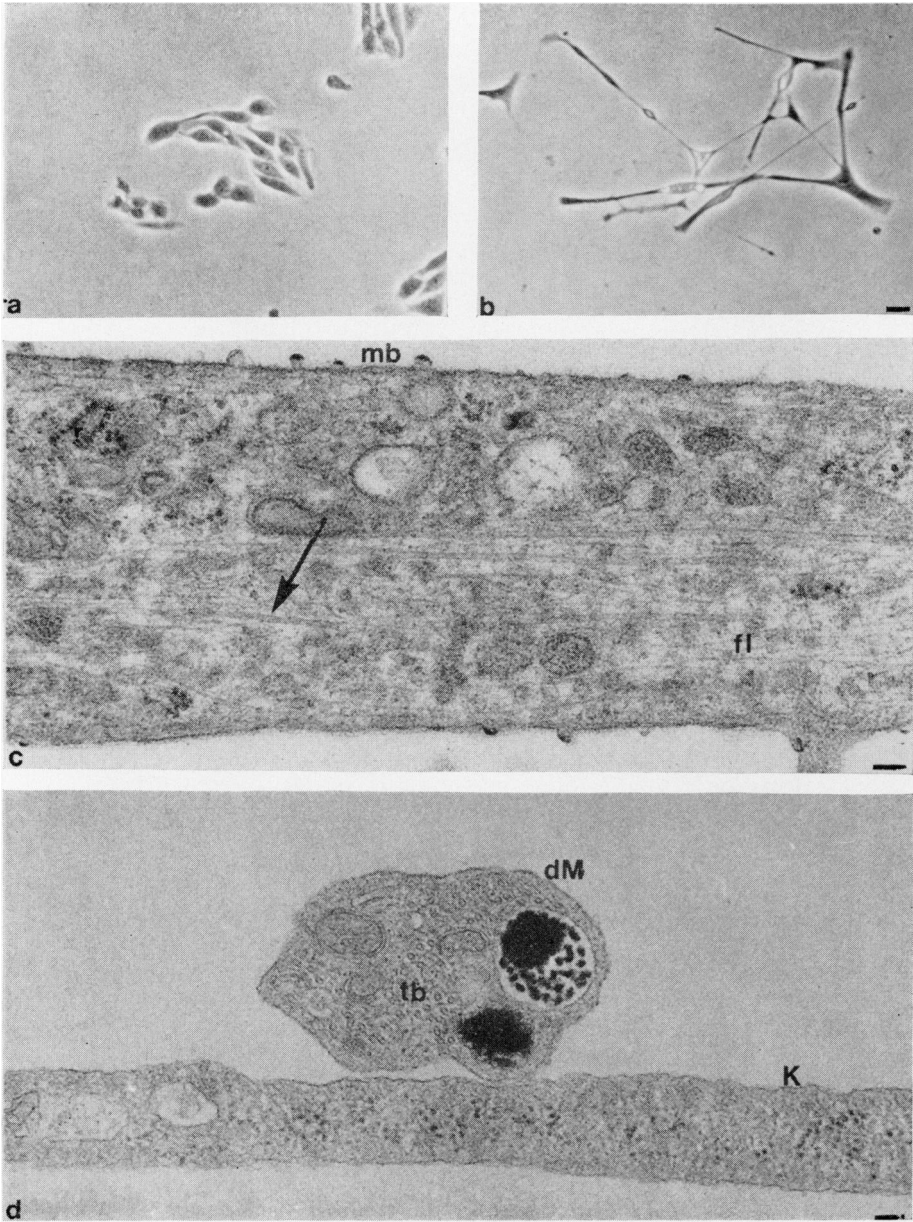


FIG. 4. Differentiation and fine structure of mammalian melanocytic dendrites in cell culture. (a) Phase-contrast photomicrograph of Greene hamster melanoma cells (32), grown on glass in minimum essential medium containing 5% fetal calf serum. The cells are spindle shaped. (b) Greene hamster melanoma cells grown 24 hr in the above medium containing DBcAMP 10^{-3} M and theophylline 6×10^{-4} M. Long processes have formed. Photomicrographs by Dr. A. DiPasquale. (c) Electron micrograph of a process of a Greene hamster melanoma cell treated as in (b) above. Arrow, microtubules; fl, filaments; r, rough-surfaced endoplasmic reticulum; mb, microblebs. (d) Cross section of a differentiated melanocytic dendrite (dM) of cultured guinea pig epidermis, overlying an attenuated portion of a keratinocyte (K). Microtubules (tb) occupy a large proportion of the dendritic cytoplasm. Note the melanized melanofilaments in the upper melanosome. Culture of Dr. S. N. Klaus. Magnifications: (a) and (b) $\times 185$; scale bar = $20 \mu\text{m}$. (c) $\times 50,000$; (d) $\times 45,000$; scale bars = $0.1 \mu\text{m}$.

could, therefore, destroy microtubules by shifting the tubule-subunit equilibrium in the direction of the subunits. The vinca alkaloids, including vinblastine, inactivate tubules by precipitating tubulin as large crystals (35, 36). The binding sites of the two agents are separate (37); colchicine can be bound into the vinblastine crystals. One of the most intriguing effects of colchicine and vinblastine on cells is the appearance of filaments, 10-nm in diameter, as the microtubules disappear (e.g., 35, 38–41, 48). The effect of colchicine stops at the formation of filaments, whereas vinblastine seems to organize the filaments into crystals. It has not yet been shown whether colchicine- and vinblastine-induced filaments contain tubulin and whether they are the same as the 10-nm “neurofilaments” that predominate in mature neurites (42).

In vivo, colchicine had little effect on the morphology of formed melanocytic dendrites (43, 44). This lack of a gross effect may be misleading, because these processes might be held passively within the surrounding tissues. The axopodia of *Actinosphaerium nucleofilum*, which are not attached, retracted upon disassembly of their microtubular axoneme by colchicine (45). The effects of colchicine on neurons grown *in vitro* suggest that microtubules are necessary for both growth and maintenance of neurites (46, 47). Similar observations have been made on Greene hamster melanoma cells in culture (48).

The biochemistry of the action of cytochalasin B is less clearly defined. Cytochalasin B, first investigated by Carter in 1967 (49), has at least one major effect on fine structure and function: it interrupts cellular motility and simultaneously disrupts microfilaments (50). These are thin 5–6 nm filaments of the type that bind heavy meromyosin; they are therefore thought to be actin (51). Cytochalasin B interferes with the activity of growth cones of outgrowing neurites (52). It also interferes with the activity of melanocytes growing in cell culture (53, 54). As in the case with cyclic AMP, it is not known whether the effect of cytochalasin B on structure is direct or indirect. The conflicting results and interpretations that have arisen in the recent avalanche of cytochalasin-B related literature have been reviewed by Holtzer and Sanger (55).

INTRACELLULAR TRANSLOCATION OF MELANIN GRANULES: MICROTUBULES AND FILAMENTS

Movement of melanin granules within melanocytes is most effectively displayed in lower vertebrates. In many of these animals cutaneous melanocytes play an important role during rapid changes of skin color. Matthews, in 1931, resolved an old argument by demonstrating that the presumed contraction or rounding up of melanocytes during lightening, and expansion or sprouting of dendrites during darkening, were in reality the visible results of the translocation of pigment granules within relatively fixed cellular outlines (56). This observation has been confirmed by electron microscopy (57–60) (Fig. 5).

The translocation of melanin granules in the melanocytic processes of amphibia and fish is in some ways analogous to axoplasmic flow in neurons. The speed of aggregation of melanin granules and the rate of the rapid component of axoplasmic flow are of similar orders of magnitude (cf. 61, 62). Both processes are abolished by colchicine (43, 63, 64), suggesting a dependence on microtubules. Interesting to note is that the direction of transport in these two systems is opposite: centrifugal in the axon and centripetal in melanocytic dendrites. The mechanism by which

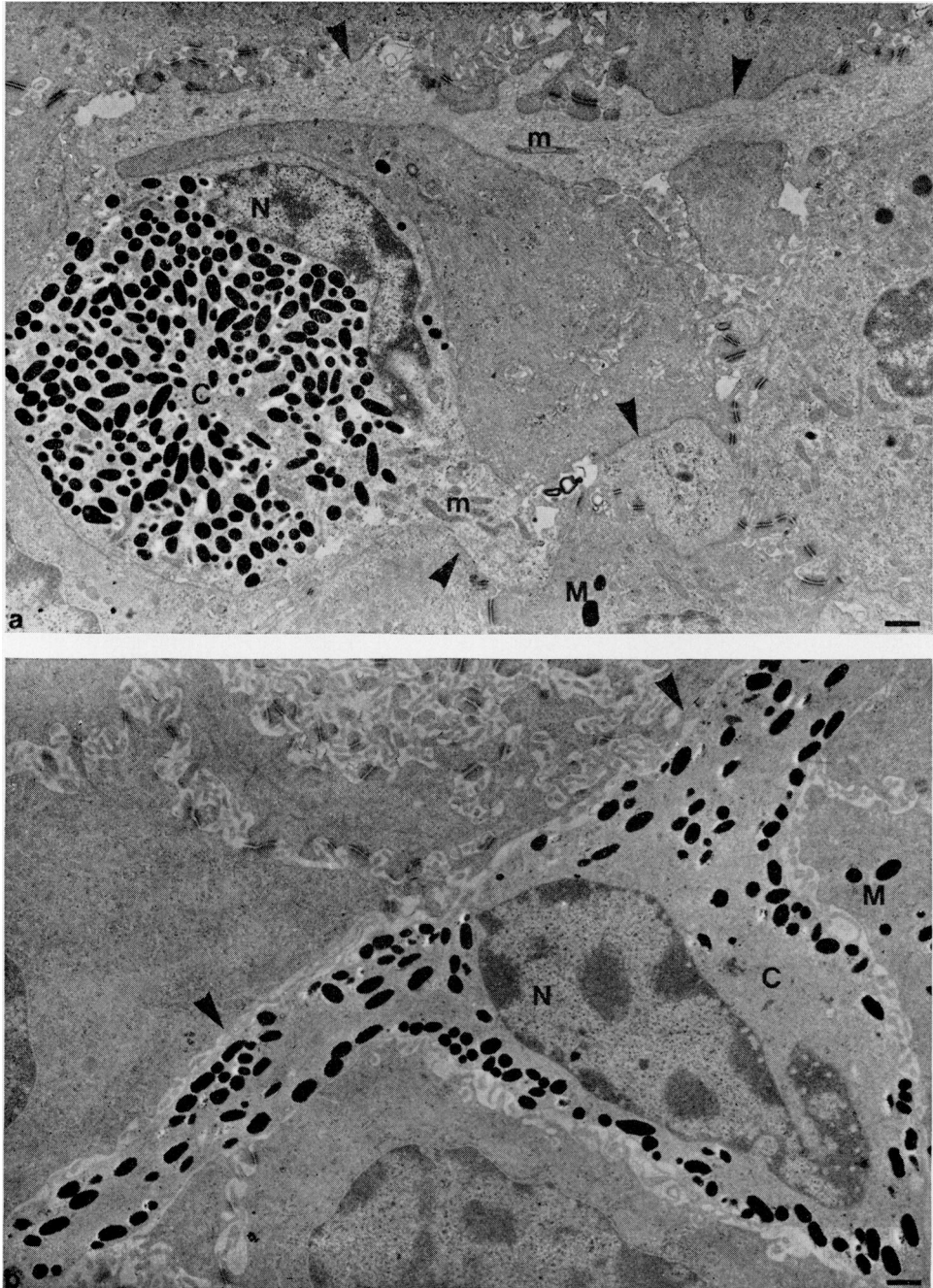


FIG. 5. Epidermal melanocytes of *Rana pipiens*. For details of incubation and fixation see legend to Fig. 6. (a) The skin was lightened by washing in Ringer's solution. The melanin granules are aggregated in the cell body; the processes are devoid of melanosomes but contain mitochondria (m). (b) The skin was redarkened with cyclic AMP through the series of reactions leading to Fig. 6d. The melanin granules are dispersed throughout the melanocytic cytoplasm, including the dendrites. Arrow heads outline the dendritic processes of melanocytes; N, nucleus of melanocyte; C, cell center of melanocyte; m, mitochondria; M, melanin granules in the cytoplasm of keratinocytes. Magnification: $\times 5000$; scale bar = 1 μm .

the direction is controlled is not known. It is known, however, that such control is exerted locally in the cell processes and not centrally, for instance, by the centrioles toward which the microtubules converge. Segments of axons and melanocytic dendrites, isolated from the cell body, have been shown to continue to transport particulate material in the appropriate directions (61, 65, 66). The autonomy and integrity of severed segments was especially well demonstrated by Kinoshita in melanocytic dendrites of *Oryzias latipes* (61). Depending on the stimulus, the melanin granules in the severed segments moved centrifugally or centripetally. They did not spill out from the cut surfaces. The mechanism of the transduction of energy from stimulus to melanin granule movement is not known.

Because microtubules were conspicuous in melanocytes of both light and dark skin, mechanisms of granule movement have been proposed that required functionally opposite microtubules (57, 67, 68), i.e., those that were responsible for aggregation and others that aided dispersion. Experiments with colchicine (43, 63, 69) and other metaphase-arresting substances (70, 71), however, indicated that microtubules were needed only in the direction of aggregation. Their disassembly aided dispersion.

What about dispersion? Jande searched for microtubules in darkened epidermal melanocytes of *Rana pipiens* but found filaments which he thought were keratin (72). Intramelanocytic filaments were also seen by Fujii and Novales and tacitly suggested to play a role in the movement of melanin granules (67, 68). Recently McGuire and Moellmann have shown that dispersion of melanin granules by MSH was associated with the appearance of large numbers of intradendritic filaments. These filaments disappeared and the granules reaggregated following the addition of cytochalasin B (73). Morphologically similar filaments in keratinocytes, presumed to be keratin, were not affected. Malawista (74) and McGuire and Moellmann (73) suggested that intramelanocytic filaments were the mediators of melanin granule dispersion. This notion has since been reiterated by Novales and collaborators (75-77).

The dispersion of melanin granules by cyclic AMP or theophylline was likewise prevented and reversed by cytochalasin B (76-78). The experiments described here were designed to correlate cyclic AMP directly and indirectly with intramelanocytic filaments or microtubules.

Skins of *R. pipiens* were successively lightened and darkened by the following sequences of incubations: (1) lightening in Ringer's solution; (2) darkening with MSH; (3) relighting with AcCh added to the MSH solution; (4) redarkening with cyclic AMP. (For experimental procedure, see legend to Fig. 6). Melanocytes were examined with the electron microscope following each of the above four steps. In addition, skins lightened by washing in Ringer's solution were darkened with cyclic AMP and theophylline without prior incubations with MSH or acetylcholine.

After rinsing for several hours in Ringer's solution, when the skins had lightened and cyclic AMP levels were presumed to be low (83, 84), epidermal melanocytes appeared as in Fig. 5a. The melanin granules and some mitochondria were clustered radially around the cell center and the nucleus was pushed aside. The dendritic processes were devoid of melanin granules but contained some mitochondria. Mitochondria tended to accumulate near the base of the dendrites suggesting that during lightening, they had trailed behind the melanin granules (see lower dendrite). Fibrous components of the dendritic cytoplasm are seen in the upper process. At higher magnification these are shown to be microtubules (Fig. 6a).

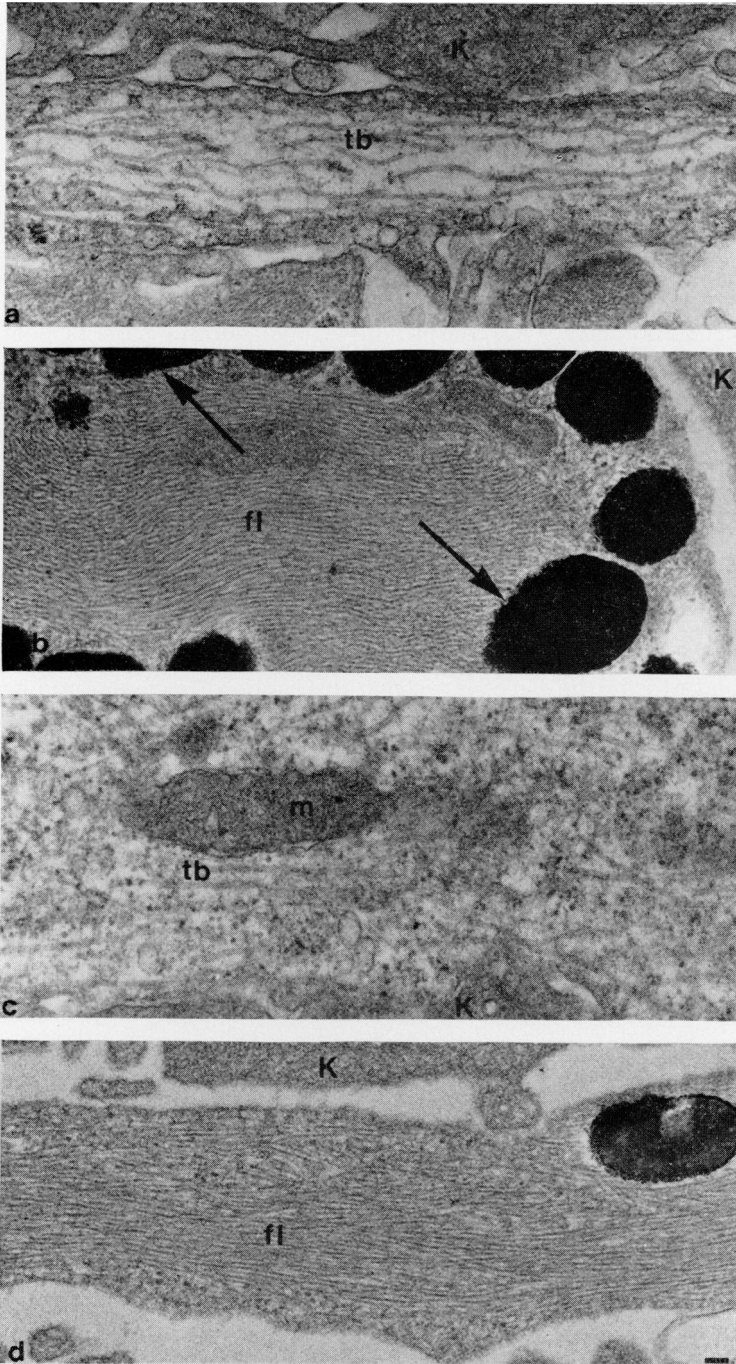


FIG. 6. Fine structure of dendritic cytoplasm of epidermal melanocytes of *Rana pipiens*. Similar to experiments reported elsewhere (79, 80), dorsal thigh skins of frogs sensitive to AcCh (81, 82) were incubated in the following sequence: (a) Ringer's solution, approximately 180 min (Fig. 6a). The skins are light and the processes devoid of melanosomes but containing many microtubules (tb). (b) Ringer's solution + MSH 1 unit/ml, 60 min (Fig. 6b). The skins became dark and the predominant cytoplasmic components were 10-nm filaments (fl). Arrows point to areas of close approximation between filaments and melanin

After incubation with MSH, when the skins had darkened and cyclic AMP levels were presumed to be high (83, 84), the melanin granules and mitochondria were dispersed throughout the cytoplasm and the nucleus had assumed a more central position. The granules that remained within the cell body were dispersed randomly; those in the dendrites were usually oriented along the long axes of the processes. At higher magnification the cytoplasm of the dendrites was shown to be packed with filaments of the 10-nm (neurofilamentous) type (Fig. 6b). Microtubules were rarely seen in these processes. Often the melanin granules were located near the plasma membrane and the filaments occupied the dendritic core. A physical attachment of filaments to melanin granules was sometimes suggested but usually the filaments appeared to sweep around the granules.

After addition of AcCh to the MSH solution, when the skins had relightened and cyclic AMP levels were presumed to be low (79, 80), the fine structure of epidermal melanocytes was essentially the same as after lightening in Ringer's solution. Although the dendritic cytoplasm was not as lucent after AcCh as after washing in Ringer's, the bundles of filaments were absent and microtubules were dominant (Fig. 6c).

Following incubation of AcCh-lightened skins with DBcAMP, the fine structural changes described for the MSH-darkened cells were accentuated (Fig. 5b). The cytoplasm was extremely dense. When resolved at higher magnification this density was again shown to be due to the presence of masses of filaments. A portion of a dendrite is shown in Fig. 6d. The cytoplasm of melanocytes of skins incubated with dibutyl cyclic AMP plus theophylline directly following the Ringer wash also had this filamentous substructure.

The cytoplasm of dermal melanocytes was not nearly as well preserved as that of the epidermal cells. However, when the fixation was adequate, large numbers of 10-nm filaments were observed in cells in which the melanin granules had been dispersed by MSH or DBcAMP. An illustration of these filaments was published recently (85). The dendrites of dermal melanocytes in which the melanin granules had fully aggregated had collapsed in diameter to the point where no cytoplasmic components remained in them. Bundles of microtubules were seen only in a few cells that had not reached the end stage of granule aggregation.

Our evidence, as illustrated in Figs. 5 and 6, indicates that cyclic AMP induces the formation of 10-nm filaments and simultaneously reduces the number of microtubules. This reaction is readily reversible and can be repeated several times in the same specimen. Since repeated darkening by MSH and lightening in Ringer's solution proceeds in the presence of cycloheximide in concentrations high enough to prevent the incorporation of leucine into trichloroacetic acid precipitable material, we have concluded that repetitive assembly and disassembly of filaments and

granules. (c) Ringer's solution with MSH + AcCh 3×10^{-4} M, 60 min (Fig. 6c). As the skins relightened the filaments disappeared, microtubules reappeared, and melanosomes returned to the perikaryon. (d) Ringer's solution with MSH + AcCh + DBcAMP 5×10^{-3} M, 45 min (Fig. 6d). The skins redarkened, and filaments reappeared; microtubules are not seen. The incubations were terminated by aldehyde fixation (see footnote 2). In Fig. 6d, the ultrathin section was stained by immersion in 2% uranyl acetate in 95% ethanol, 30 min, 60°C, without subsequent lead staining. m, mitochondria; d, desmosomes; K, keratinocytes. Magnification: $\times 36,000$; scale bar = 0.1 μ m.

microtubules in frog epidermal melanocytes is independent of protein synthesis. Part of our evidence has appeared in abstract (86, 87).

We attribute the demonstration of filaments and the successful correlation of filaments and microtubules with the dark and light states, respectively, to the combination of preparatory procedures used.² Improvements in the preservation of morphology by fixing in warm glutaraldehyde solutions has also been reported by Peracchia and Mittler (88). The filaments are not proper "micro"-filaments as thought previously (73, 74, 78) but fall into the intermediate-size range of intracytoplasmic filaments described by Ishikawa, Bischoff, and Holtzer (89).

The equilibrium between filaments and microtubules appears to favor tubule formation. However, a pharmacologically induced switch from tubules to filaments, sometimes reversible, occurred following treatment of cells or tissues with colchicine (35, 38, 39, 90-92) or vinblastine (40, 41, 48). These agents are known to enhance dispersion of melanin granules (43, 44, 70).

In melanocytes of *R. pipiens* darkened by exposure to MSH and incubated in the presence of vinblastine, cytoplasmic areas normally occupied by 10-nm filaments contained typical vinblastine crystals (Fig. 7). These crystals are assumed to be derived from microtubular proteins (35, 36). Whether the 10-nm filaments are linear subunits of microtubules as has been suggested (57, 93-97), or whether the inverse relationship between microtubules and filaments is merely functional, is an unanswered question.

TRANSFER OF MELANIN GRANULES TO KERATINOCYTES

Evidence of melanosome transfer is ubiquitous, but knowledge of the mechanisms by which it may occur is incomplete. Injection of melanin granules, as proposed by Masson (98) can probably be ruled out. Two other mechanisms, proposed by Riehl in 1884 (99) and observed in epidermal cell cultures by Cruickshank and Harcourt in 1964 (100), entail phagocytosis on the part of keratinocytes. In the first of these, the melanin granules were said to escape from the tip of a melanocytic dendrite into the extracellular milieu before being phagocytized by the keratinocyte. In the second mechanism the entire distal portion of the melanocytic dendrite was engulfed, "probably still surrounded by a film of cytoplasm (100)."

Electron microscopic evidence that can be interpreted as being in support of the first phagocytic mechanism is the following: Keratinocytes, which are known

²The final 15 min of incubation were done at 35°C. The skins, still on their mounting rings, were then immersed in a solution containing 3% glutaraldehyde, 0.1 M sodium cacodylate buffer (pH 7.2), and 0.1% calcium chloride, temperature 35°C. The glutaraldehyde solutions were supplemented with MSH, AcCh, or DBcAMP at concentrations and in combinations in which these substances were present in the incubation media immediately prior to fixation. After 30 min in the warm glutaraldehyde mixtures and subsequent cooling to room temperature, the tissues were transferred to fresh glutaraldehyde and stored over night at 4°C. Before refixing in osmium tetroxide, small pieces of skin were selected with the light microscope to ascertain that all melanocytes included in a sample were representative of the total skin reaction as monitored by measurements of skin reflectance (cf. Ref. 80, Fig. 2a). All melanocytes in a selected area showed the same degree of dispersion or aggregation of melanin granules. Sections for electron microscopy were cut in a plane parallel to the surface of the skin.

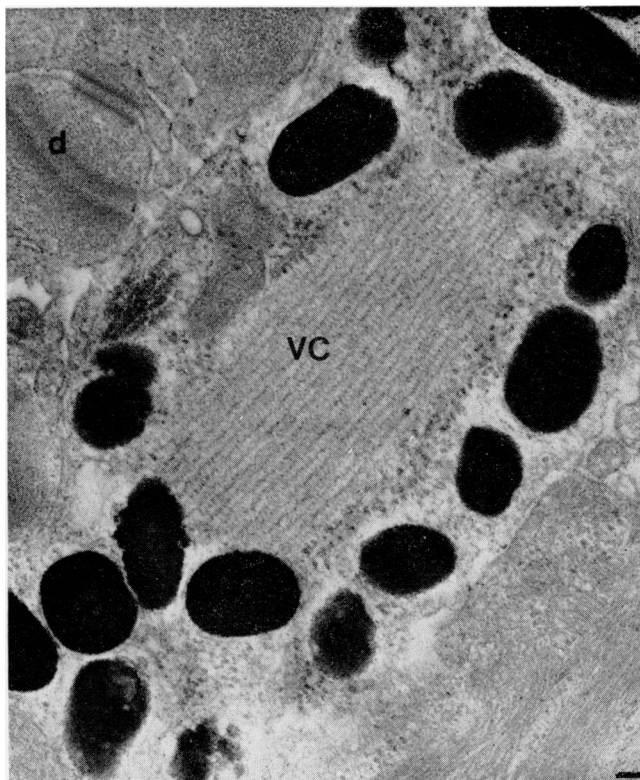


FIG. 7. Longitudinal section of a vinblastine-induced crystal (VB). Skin of *Rana pipiens*, previously lightened in Ringer's solution, was darkened with MSH 1 unit/ml in the presence of velban (vinblastine sulfate) 10^{-5} M. The warm glutaraldehyde fixative contained both MSH and velban in the above concentrations. The crystal occupies a region in the epidermal melanocytic dendrite that is normally occupied by the 10-nm filaments (cf. Fig. 6b). d, desmosomes. Magnification: $\times 36,000$; scale bar = $0.1 \mu\text{m}$.

to be avid phagocytes, phagocytize latex beads or melanin granules singly or in small groups, depending on the size of the particles (101–103). The resulting intrakeratinocytic distribution is identical to the distribution of melanin granules in normal human skin, in which large melanin granules occur singly (104), as in Negroes (105, 106), and smaller granules occur in packets (104), as in Caucasians (105, 106). An important missing fine-structural link, necessary to establish this mechanism as a functional one, is the demonstration of secretion of melanin granules into the extracellular space prior to their uptake by keratinocytes.

The second phagocytic mechanism was termed "apocoptation" by Klaus (107). Its occurrence in cell culture has been confirmed numerous times by phase-contrast time-lapse cinemicrography (107–110) (Fig. 8). Electron microscopic evidence in support of apocoptation has been derived from the systematic observation of developing epidermal melanin units *in vivo* (111, 112) and *in vitro* (109, 113, 114). Still outstanding is electron microscopic confirmation of the complete separation of observed intrakeratinocytic portions of melanocytic cytoplasm from the mother dendrite. This requires serial cross-sectioning through the terminal region of a dendrite, into the keratinocyte, on through the apocoptated dendritic tip. The

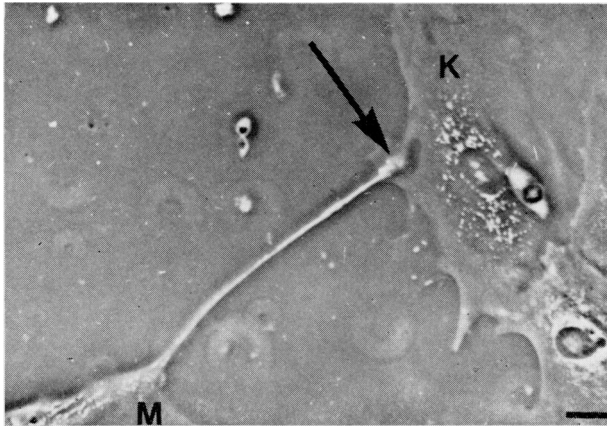


FIG. 8. Phase-contrast photomicrograph (negative), taken at or near the time of transfer of melanin granules from a melanocyte (M) to a keratinocyte (K). Arrow points to the site of transfer. Source: black rabbit epidermal cells grown in monolayer culture. Magnification: $\times 270$; scale bar = $20 \mu\text{m}$.

electron micrograph of Fig. 4d was obtained during a vertical approach to a transfer site (113).

VIRUS-LIKE PARTICLES OF TRANSPLANTED MELANOMA CELLS

The growth of tumor after transplantation of tumor tissue into a new host animal may occur through two distinctly different mechanisms. In the first, the new host animal serves merely as a culture vehicle. The proliferating tumor cells are descendants of the donor. In the second mechanism, healthy cells of the new host become transformed into tumor cells by a process akin to infection. The newly proliferating cells are descendants of the new host. It is in relation to the second mechanism that the virus-like particles found in transplanted animal tumors may play an important role. For example, in instances where cell-free ultrafiltrates of tumors, or virus-like particles shed by tumors, have induced new tumors, or have transformed normal cell lines into malignant ones, viruses have been implicated to be the infective agent (115–118).

Virus-like particles in melanoma tissue were discovered early in the history of electron microscopy of tumor cells. In fact, the only qualitative fine structural difference observed between normal and neoplastic cells was the “presence of extracellular and intracytoplasmic particles of the size range of virus in some, but not all, tumors examined (119).” Two major classes of tumor virus are now recognized, the DNA and the RNA tumor viruses. Comparative evidence suggests that the melanoma particles are RNA tumor viruses.

The terminology used to describe RNA tumor viruses is confused, as pointed out not long ago by Dalton (120). The original designations A-, B-, and C-particles by Bernhard (121–123) were redefined by others a decade later. A-, B-, and C-designations linked criteria of fine structure with those of location (124). The A-particle was intracellular, appeared doughnut-shaped in thin section and measured roughly two-thirds of the diameter of the extracellular particles. B- and C-particles were extracellular. During and shortly after budding from the plasma

membrane the cores of B- and C-particles were electron lucent, the B-particle having four distinct shells and the C-particle three. Some time after release from the cells, their cores became electron dense, that of the B-particle shifting eccentrically and that of C-particles remaining centrally located. Meanwhile, particles with the fine structure of C-particles have been found intracisternally, i.e., in an intracellular location, typically occupied by A-particles. De Harven calls immature particles budding from plasma membranes "enveloped A-particles" (125). To add to the confusion, A-particles are often incomplete and appear in thin section like the letter "C."

The original classification and its variants were based on particles of murine origin. Recently, Dalton demonstrated ultrafine staining differences between C-particles of murine, feline, and human origin (126, 127). These findings suggested that, in addition to species specific reverse transcriptases (128), RNA viruses may possess species specific fine structure. The most strikingly different particle found to date is the intracisternal virus-like particle of hamster tumors. The hamster particle has been designated "H"-particle because of its host animal (129, 130), or "R"-particle because of the radially disposed filaments that connect the central dense portion with the limiting membrane (131, 132).

As proposed by Temin (133), the designation "RNA tumor virus" is preferable to the commonly used "C-type particle" because the latter disregards the equally oncogenic B-particles of mouse mammary tumors. In the majority of cases, however, especially in the case of the intracellular particles, neither the presence of RNA nor the viral identity of the particles have been established.

In transplanted mouse melanoma cells, abundant A-particles have been found within and near the endoplasmic reticulum (19, 134, 135). The biosynthetic machinery geared to produce these particles occupies a large portion of the cytoplasm and is separate from the centers of melanosome synthesis. An intracisternal particle of Cloudman S91 mouse melanoma, approximately 100 nm in diameter, with morphological features of a C-particle, is shown in Fig. 9a. It rests in a pocket of smooth membrane which is continuous with the rough-surfaced endoplasmic reticulum. In addition to the numerous intracellular particles, large expanses of smooth membrane are present that have thickened into electron dense sheets (Fig. 9b). These sheets, sectioned tangentially at (tg), are as wide (approx 15 nm) and as electron dense as the outer shell of the virus-like particles. Typical C-particles of a cultured S91 mouse melanoma cell are seen budding into the culture medium from the exposed surface of the plasma membrane in Fig. 9c.

The abundant intracisternal particles of transplanted hamster melanoma cells are found most frequently in pockets of rough-surfaced endoplasmic reticulum including the perinuclear cisternae. The particles appear to bud into the lumen (Fig. 10a), either singly, or fused into chains (116, 136-138). The chains may be several microns long and look filamentous at low magnification. The delicate fine structure of the hamster melanoma particle is shared by intracisternal particles of other tumors of hamster origin (129, 130, 139, 140). A thorough description has been given by Thomas *et al.* (129). The particles measure approximately 100 nm in diameter. They contain a relatively small (40 nm), sometimes hollow, central bilaminar core, the external surface of which is scalloped by the presence of spikes (Fig. 10b). Each spike gives rise to a fine filament, about 2.5 nm thick, that radiates toward a clearly delineated limiting membrane. Twelve to thirteen of these may be visible in a cross section of a single particle. Extracellular particles are

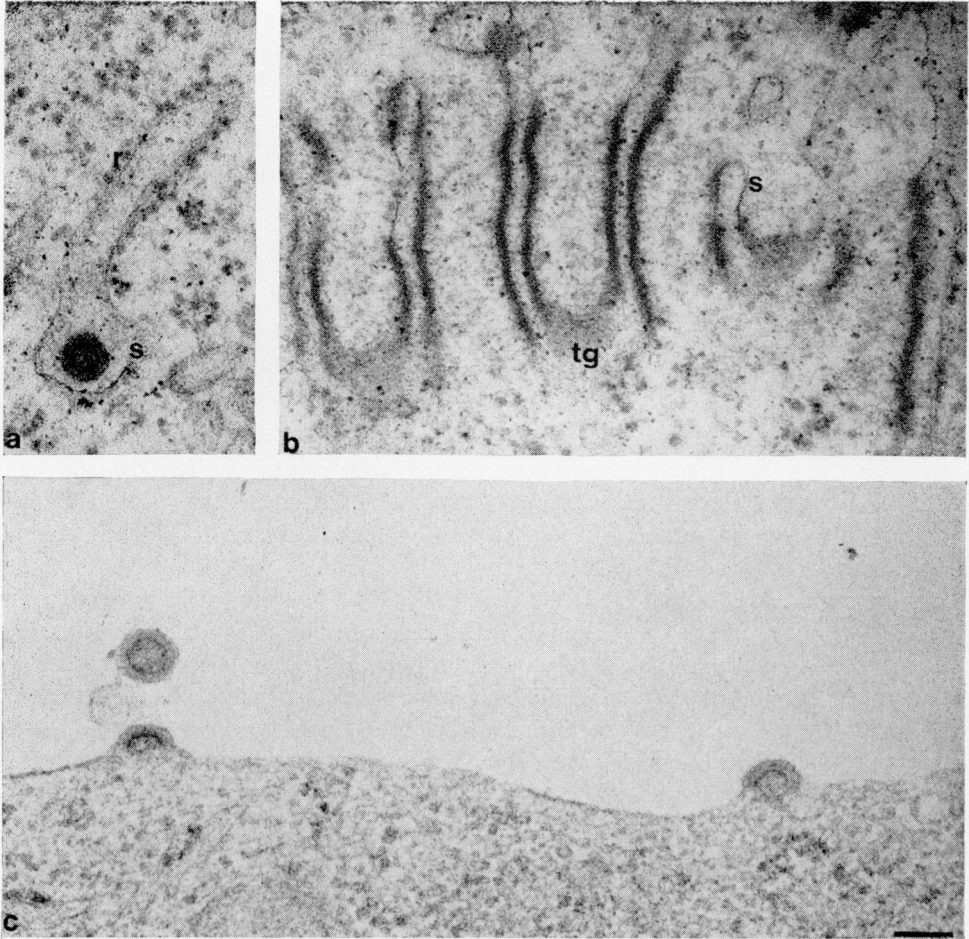


FIG. 9. Virus-like particles of cultured Cloudman S91 mouse melanoma cells. (a) An intracisternal C-type particle rests in a pocket of smooth (s) endoplasmic reticulum which is continuous with the rough endoplasmic reticulum. r, for rough and ribosomes. (b) Dense sheets, continuous with smooth endoplasmic reticulum (s), are found in intracytoplasmic regions of active particle synthesis. Note that the smallest diameter of these thickenings is equal to the width of the particle's capsule. tg, tangential section. (c) Vertical section through a melanoma cell grown in monolayer culture. C-particles are shown in progressive stages of budding and release from the exposed plasma membrane. Only few particles were found budding from the membrane facing the glass substrate. Magnification: $\times 85,000$; scale bar = $0.1 \mu\text{m}$.

sparse in hamster tumors and have been seen only near cells grown *in vitro*. The few extracellular hamster particles seen by us were morphologically similar to mature murine C-particles.

The origin of RNA tumor viruses is obscure. At the time of their discovery they were presumed to have been acquired some time during the many generations of tumor transplants (119). To this day, reports of RNA-type viruses in primary tumors are rare. Two hypotheses have been formulated to explain the origin and function of the RNA tumor viruses. The viral oncogene hypothesis of Huebner and Todaro (141, 142) states that normal vertebrate cells contain the genetic in-

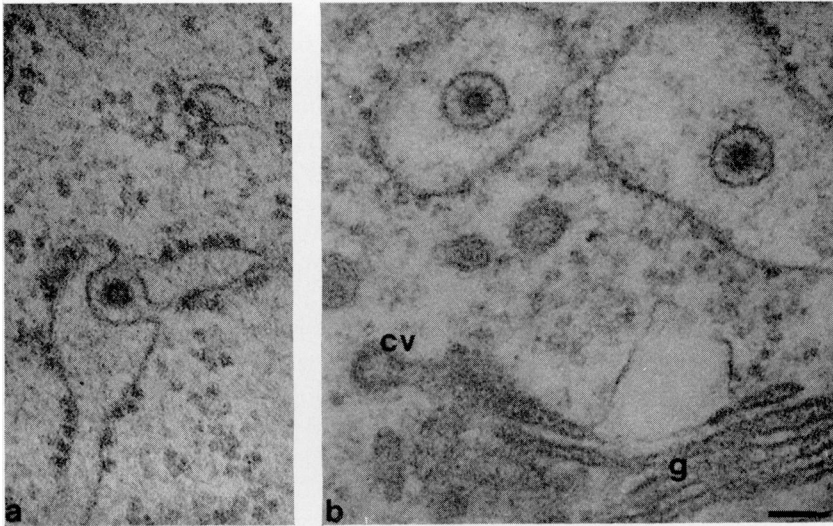


FIG. 10. Virus-like particles of transplanted Greene hamster melanoma cells (solid tumor). The hamster tumor particles are almost exclusively intracisternal. (a) A particle appears to bud into a cisterna from opposite faces of rough-surfaced endoplasmic reticulum. (b) Dilated rough endoplasmic reticulum, located near stacks of Golgi membranes (g) and budding coated vesicles (cv), contains virus-like particles. Note the delicate architecture of the particle on the left. Typically, not a full complement of spokes is visible, suggesting that the alignment of the spokes is skewed. Magnification: $\times 85,000$; scale bar = $0.1 \mu\text{m}$.

formation of the virus in the form of an inactive “virogene.” The virogene is transmitted vertically from parent to offspring. The “oncogene” is a portion of the virogene, responsible for transforming a normal cell into a tumor cell. Carcinogens, radiation, or exogenous virus exert their primary effects by activating the oncogene.

In the protovirus hypothesis of Temin (133, 143) the synthesis of viruses and cellular transformation depend on an initial infection by an RNA protovirus. The viral information is transferred to the cellular genome—where it is stored as a DNA “provirus”—by means of an RNA-directed DNA polymerase (reverse transcriptase) (144, 145). In the protovirus hypothesis the mechanism of initiation of spontaneous tumors, or tumors induced by carcinogens or radiation, seems to be explained less well than in the oncogene hypothesis. However, if the latency between the formation of the provirus and its expression were unlimited, the protovirus hypothesis would be remarkably similar to the oncogene hypothesis.

EPILOG

The tendency is to regard electron micrographs as representations of the sum total of fine structure compressed into two dimensions. More realistically, electron microscopy is a method of ultrafine dissection, not because of the thinness of the tissue slices but because of the selectiveness inherent in fixation, embedding, and heavy-metal staining. In order to detect fine structure with present-day electron microscopic techniques, molecules have to be immobilized (fixed), dehydrated, and tagged (stained) with heavy metals. Routinely, they are immobilized by aldehydes, dehydrated in fat solvents, and tagged with osmium, uranium, and lead. Many types of molecules are lost in the procedure, or, if retained, fail to bind heavy metals.

To detect the fine-structural counterparts of a biological function, the microscopist has to choose a system that executes this function to best advantage. He has to immobilize the *functionally relevant* molecules, and, if possible, eliminate others that would interfere by superimposition. This effort requires different methods of fixation, embedding, and staining for different classes of molecules. One needs to remember only the history of the electron microscopy of cytoplasmic microtubules (146, 147), or of myosin filaments of smooth muscle cells (148, 149). More recently, the awakening interest in the role of lipids in cellular functions has spurred research into embedding or sectioning procedures that eliminate dehydration in lipid solvents (150–156).

The time has come in pigment cell biology when the look-and-see approach to electron microscopy has to give way to the controlled experimental approach. Such an approach includes the adaptation of methodologies to permit the detection of specific anticipated answers. Strictly speaking, the final incubating medium in experiments that include electron microscopy is the emulsion of the photographic paper!

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