THE FINE STRUCTURE OF NORMAL AND NEOPLASTIC MELANOCYTES IN THE SYRIAN HAMSTER, WITH PARTICULAR REFERENCE TO CARCINOGEN-INDUCED MELANOTIC TUMORS

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ABSTRACT

The dermal melanocyte system of the Syrian hamster is particularly responsive to the melanogenetic and tumor-inducing effects of 7,12-dimethylbenz(a)anthracene (DMBA). The melanocytes of the hair follicles appear to be susceptible to the melanogenetic effect of DMBA but not to its tumor-inducing effect. The epidermal melanocytes are non-pigmented and are unresponsive to both melanogenetic and carcinogenic effects of DMBA. The pigmented granules of the dermal melanocytes of both the golden and the white hamster have an identical substructure and pattern of melanization which occurs in an orderly fashion on a delicate fibrillar component. The hair melanocytes have larger pigment granules with a more complicated fibrillar substructure. The epidermal melanocytes do not possess pigment granules but are recognized by their dendritic shape, the absence of desmosomes and tonofilaments, and the presence of racket-shaped or rod-shaped organelles. The melanin granules in neoplastic melanocytes of the golden hamster differ from corresponding normal melanocytes only in their larger size. In the white hamster, however, the melanin granules in tumors produced under identical experimental conditions are so bizarre and atypical that consideration was given to the possibility that a genetic difference in the melanization pattern between the two varieties becomes apparent in carcinogen-induced melanotic tumors. No definite conclusions could be reached as to the precise origin of the melanin granules in either normal or neoplastic melanocytes.

INTRODUCTION

A number of studies dealing with the fine structure of melanocytes have appeared in the literature in recent years (1, 2, 4, 6, 11–14). Most of these were concerned with normal melanocytes or spontaneous malignant melanomas in man and animals. The responsiveness of the dermal melanocyte system of the Syrian hamster to the melanogenetic and tumor-inducing effect of a single cutaneous application of 7,12-dimethylbenz(a)-anthracene (DMBA) has made it possible to study experimental melanogenesis under particularly favorable conditions. The response of these cells was so remarkable that a study of the ultrastructural alterations occurring in the course of carcinogen-induced neoplastic melanogenesis appeared to be of interest.
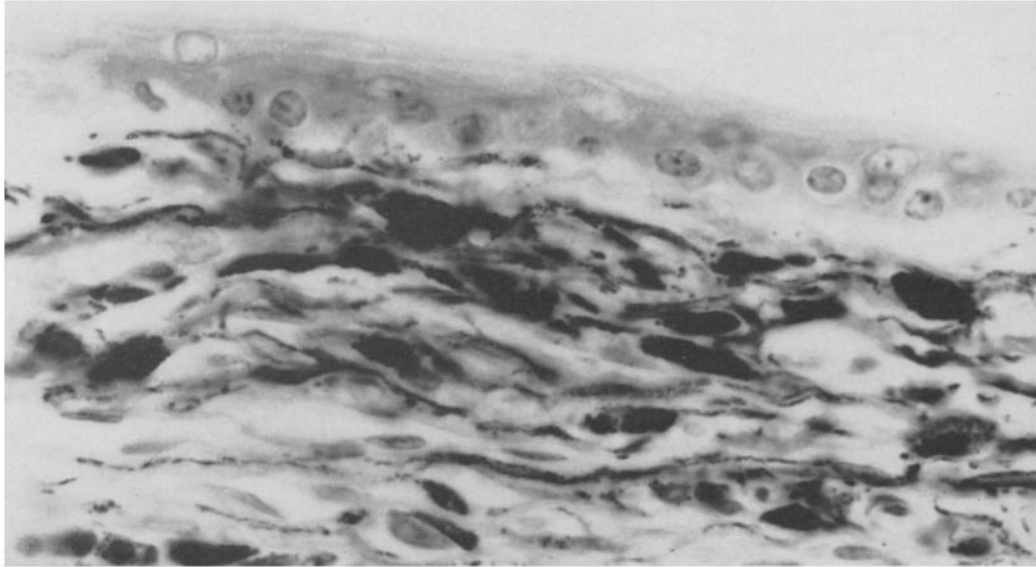
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Detailed experimental data and morphologic observations pertaining to the DMBA-induced melanotic tumors have been previously reported in the golden Syrian hamster (3) as well as in the white variety (10), which is considered a "partial albino" (8) containing melanin pigment in the ear, perineum, and retina. The following are the salient features of these experiments: (a) Multiple pigment spots and tumors are readily produced by a single application of approximately 800 µg of DMBA to the skin of the back of the Syrian golden hamster as well as of the white variety. (b) The tumors grow slowly and metastasize rarely but are readily transplantable. (c) They are composed of spindle-shaped and dendritic dermal

melanocytes showing little cytologic evidence of malignancy, thus presenting a good correlation between histologic structure and biologic behavior. The tumors in both white and golden hamsters contain melanin pigment, and thus treatment induces melanogenesis in tissues normally non-pigmented in the white animals.

The Syrian golden hamster has brown hair, the follicular portion of which contains active melanocytes. The degree of pigmentation of these melanocytes is related to the hair cycle. In contrast, the white hamster, having only white hair, has no pigmented melanocytes in the hair follicles under normal conditions. In both the golden and the white Syrian hamster the dermal melanocyte
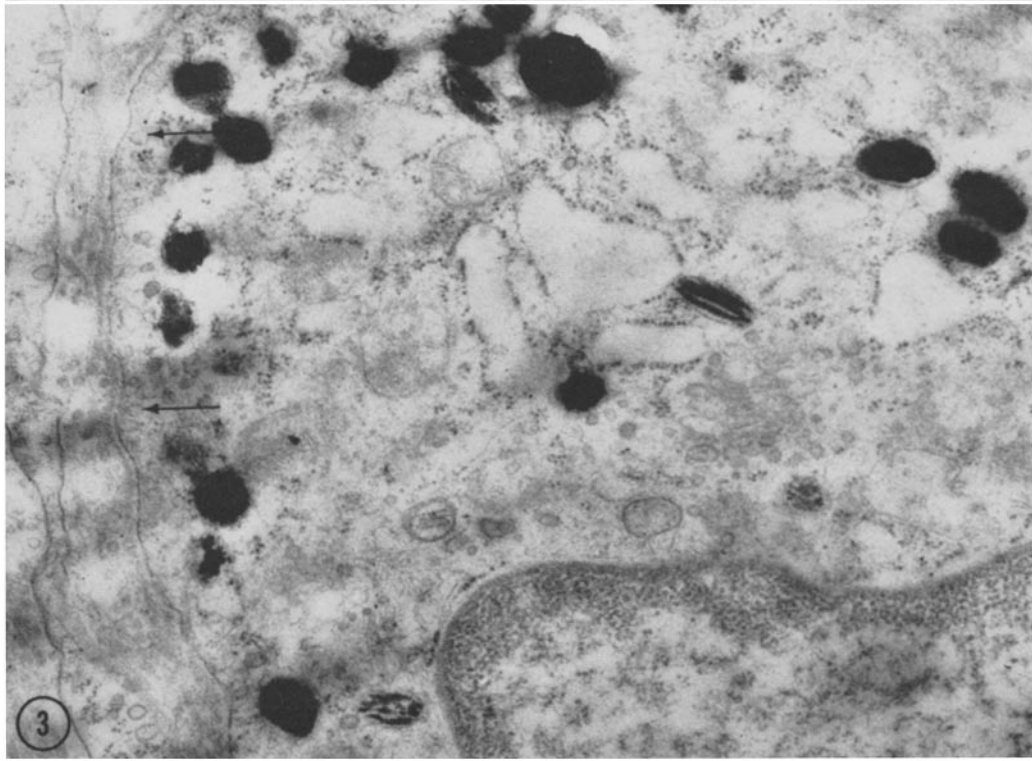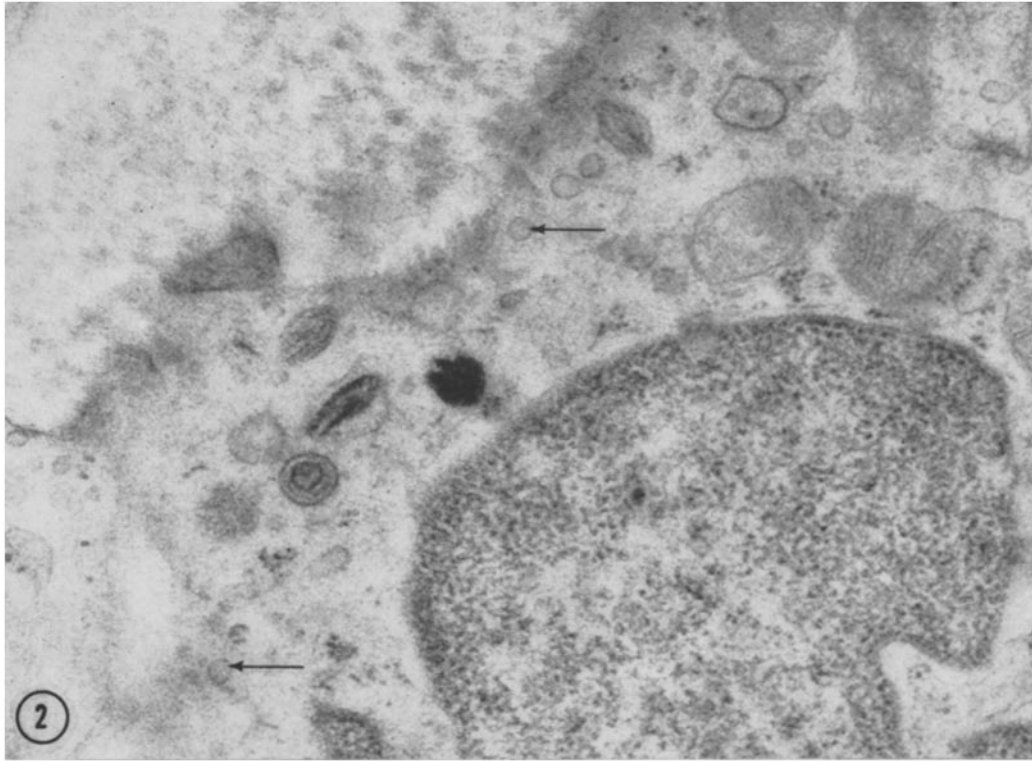
system is particularly abundant in the ears and the perineum, where a dense network of melanocytes occupies the upper dermis. This pigmentation gradually appears during the first few weeks of life. We were unable to demonstrate pigmented or non-pigmented dihydroxyphenylalanine (dopa)–positive melanocytes in the normal epidermis of either the golden or the white hamster under the light microscope, using routine staining methods as well as the dopa reaction and the Masson-Fontana silver stain, even after irradiation with ultraviolet light (9).

Study of the hair melanocytes of the golden hamster and of the normal dermal melanocytes in the young golden and white hamsters allowed us to investigate certain aspects of normal melanogenesis in comparison with melanogenesis taking place in the course of the evolution of the carcinogen-induced melanotic tumors. Some of our observations concerning the ultrastructure of normal and neoplastic melanocytes have been reported previously as part of reviews on carcinogen-induced melanotic tumors (7, 9). It is the purpose of this paper to present a detailed study of the fine structure of hamster melanocytes at varying stages of pigment formation both under normal conditions and during DMBA-induced tumor formation.

MATERIALS AND METHOD

SOURCE OF TISSUE EXAMINED : Both non-neoplastic and neoplastic tissues were obtained from Syrian golden and white hamsters, each strain bred randomly in our laboratory. For the study of normal melanogenesis the tips of the ears of golden and white hamsters were removed with sharp scissors from the living non-anesthetized animals on the 3rd, 4th, 7th, 11th, 15th, 21st, 27th, 31st, and 33rd days following birth. The study of hair melanogenesis was carried out on normal skin of the back obtained by punch biopsy from the live non-anesthesized animals. For the study of melanogenesis in tumor tissue the tumors were induced with single applications of 800 μg of DMBA by the method previously described (3). Multiple pigmented spots started to appear about the beginning of the 10th week and some of these proceeded to tumor formation. The tumor tissue was removed by simple surgical excision from the lightly anesthetized animals.

PREPARATION OF TISSUE FOR LIGHT MICROSCOPY : Tissue slices were fixed in 10 per cent neutral formalin in phosphate buffer. As routine staining procedure, hematoxylin and eosin was used for all tissues. For histochemical study of melanin formation the dopa reaction was carried out by employing either Laidlaw's frozen section method or Becker's paraffin block method.

PREPARATION OF TISSUE FOR ELECTRON MICROSCOPIC STUDIES : (a) Small pieces about 1 mm in diameter or less were immersed in freshly prepared Palade's fixative (a 1 per cent osmium tetroxide solution in acetate-veronal buffer, pH 7.6) at 0°C as quickly as possible after removal. Fixation was continued for 1 hour. The tissues were thereafter rinsed in cold distilled water for a few minutes. Dehydration was done in 50, 70, 95, and 100 per cent ethanol at 0°C, for two periods of 15 minutes in each concentration. The tissues were placed in two changes of propylene oxide for 15 minutes each, and then in a 1:1 mixture of propylene oxide and epoxy resin anhydride mixture (Epon 812) with added accelerator (DMP-30) for 1 hour and in a 1:3 mixture overnight. They were then embedded in the resin in capsules and the resin was polymerized at 60°C overnight. Sections were cut either with glass knives on a Porter-Blum microtome or with glass or diamond knives on an LKB ultrotome. (b) Staining of sections mounted on carbon grids was carried out by floating on a saturated solution of uranyl acetate in distilled water for 30 to 60 minutes. (c) The electron micrographs were taken on an RCA EMU 3C or 3F electron microscope.

FIGURE 4

Portion of normal melanocyte in hair bulb of adult golden hamster. Pigment granules at various stages of evolution are seen. Some have a distinct substructure represented by a fibrillar component which has a more irregular arrangement than that seen in the granules of the dermal melanocyte. A portion of hair papilla is illustrated in the left lower corner. X 29,000. Insert X 60,000.

FIGURE 5

Normal epidermal melanocyte of the white hamster. The cytoplasm contains some racket- and rod-shaped organelles (arrows) and a prominent Golgi zone. Note the absence of desmosomes and tonofilaments. X 28,000.
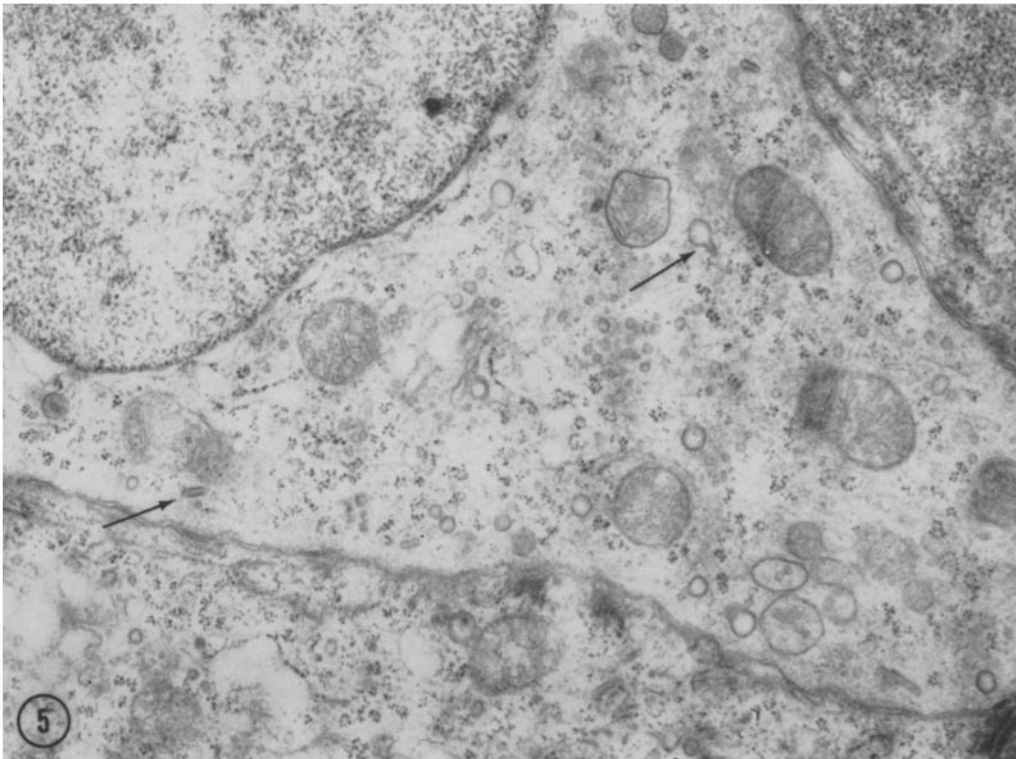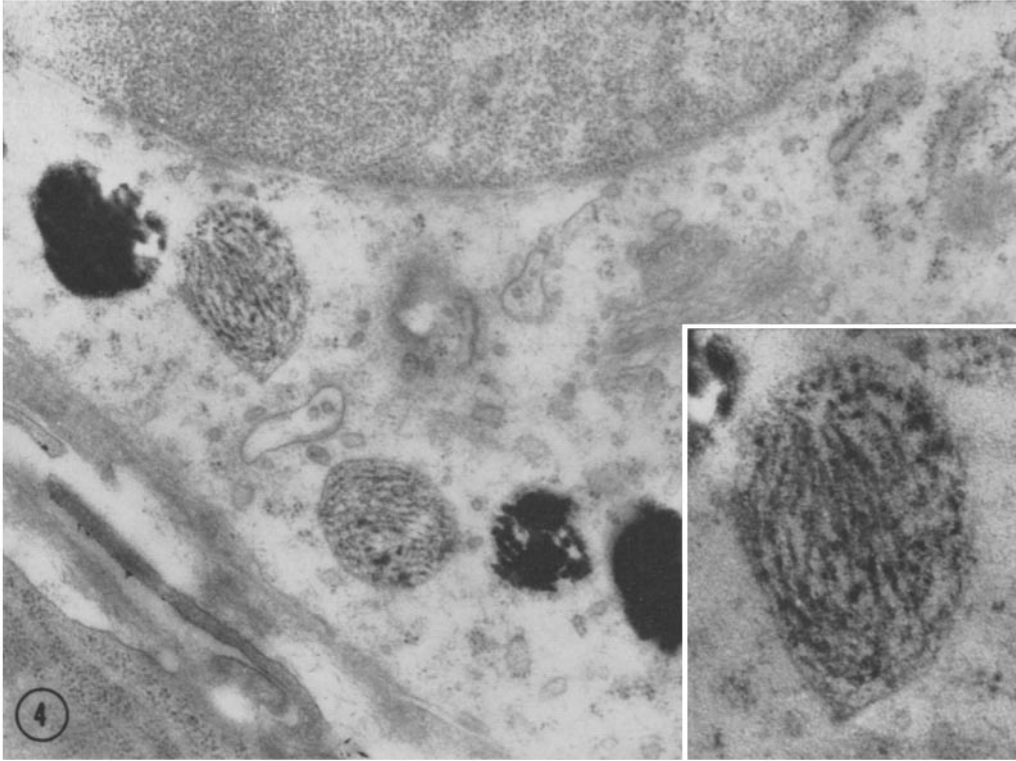
OBSERVATIONS AND RESULTS

Normal Melanogenesis

NORMAL DERMAL MELANOCYTES OF THE EAR: In the golden hamster the ear skin is not pigmented at birth even though the color of the hair is already brown. The pigmentation of the ears starts within a few days after birth and reaches maximum darkness in about 2 to 3 weeks. In the white hamster, the pigmentation of the ears starts at the age of approximately 2 weeks. It seems to progress to a stage of maximum darkness at almost the same speed as in the golden hamster.

Under the light microscope, the skin of the ears of untreated, adult golden and white hamsters shows an abundant network of dermal melanocytes interspersed with melanin-laden macrophages (Fig. 1). These melanocytes have long dendritic processes and contain fine pigment granules the color of which ranges from light to dark brown. The dopa reaction of these cells was negative, although a weak reaction might have been obscured by preexisting heavy pigmentation. The epidermis is apparently devoid of pigmented cells in both varieties.

Our initial electron microscopic studies of normal melanogenesis were carried out on biopsy specimens of the white hamster's ear, where the earliest evidence of pigmentation of the melanin granules was found at the age of 21 days (7). At this age the cytoplasm of the dermal melanocytes contained numerous smooth walled vesicles of varying sizes measuring up to about 400 mμ in the largest diameter (Fig. 2). The larger ones were elliptical in shape and contained a longitudinally oriented component made up of delicate fibrils which showed a periodicity in the order of 90 A (Fig. 2). An occasional vesicle contained concentric lamellar structures. The fibrillar component of the vesicles showed considerable variations in electron opacity. Melanization appeared to occur on the fibrillar component of the elliptical vesicles, which were regarded as early melanin granules. Occasional granules had attained such a degree of electron opacity that the filamentous substructure could no longer be discerned. By the 33rd day, melanization of most granules had progressed to a stage of complete electron opacity (Fig. 3). Other characteristics of the dermal melanocytes in the ear of the white hamster were indented nuclei, prominent Golgi zones (Fig. 3), and dendritic processes, features which they have

in common with clear cells of the epidermis of man and other animals (1).

Similar observations were made in the dermal melanocytes of the golden hamster's ear. Melanization started as early as the 3rd day, and the fine structure of melanin granules was almost identical with that seen in the white hamster at 21 days of age. Most of the granules attained complete electron opacity by the 15th day (7).

The dermal melanocytes at an early stage of pigmentation often contained pinocytotic vesicles, usually located along the cytoplasmic border, in addition to melanin granules at varying stages of electron opacity. At later stages the pinocytotic vesicles were more evenly distributed and the Golgi zone became increasingly more prominent. Nothing in our observations suggested any similarity between melanin granules, or their precursors, and mitochondria.

MELANOCYTES IN THE HAIR BULB: In the light microscope, the hair melanocytes of the white hamster did not show pigmentation under normal conditions even when a silver stain was employed for the demonstration of melanin. The dopa reaction was only weakly positive but was much increased as the result of irradiation with ultraviolet light (7, 9). In the non-irradiated hair bulb of the golden variety, however, pigmented dendritic melanocytes periodically appeared at stages of anagen in the hair cycle. They were dopa positive.

Most of the hair melanocytes were found in the vicinity of the hair papillae. It was previously shown in certain genotypes of mice (5) that the tyrosinase activity and the degree of melanization vary at different stages of the hair cycle. The hair melanocytes of the golden hamster at the stage of anagen IV–V when examined under the electron microscope still contained numerous pigment granules at various stages of melanization (Fig. 4). These granules were short-elliptical in shape, measured up to approximately 1 μ in length on the average, and were about twice as large as those observed in the dermal melanocytes. The immature granules had a fibrillar component with a distinct periodicity in the order of 90 A. The fibrils were oriented predominantly lengthwise but appeared to be more numerous and more irregularly arranged than in the melanin granules of the dermal melanocytes.

EPIDERMAL MELANOCYTES: As has been pointed out above, demonstration of pigment-
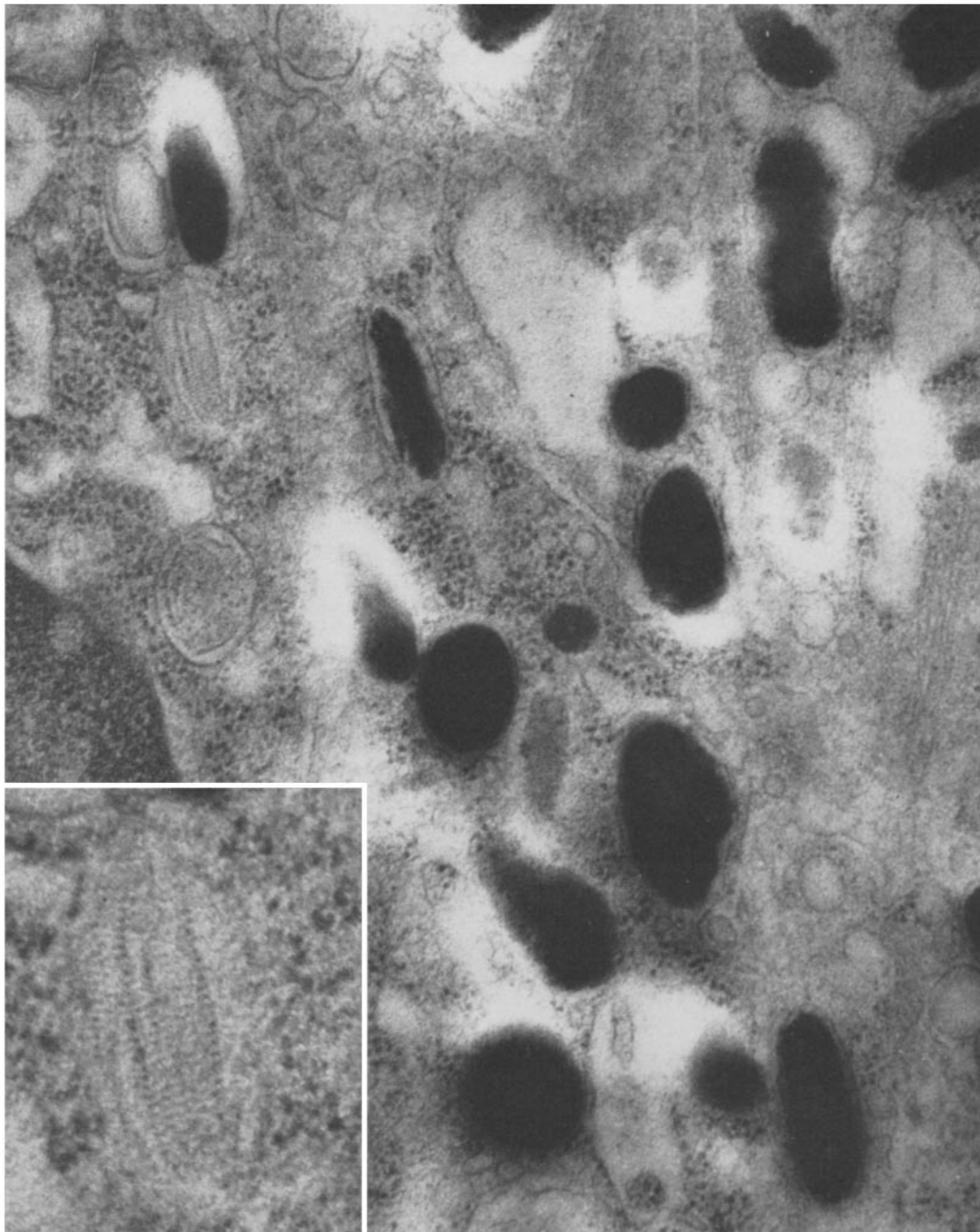
FIGURE 6

Pigmented cell of DMBA-induced melanotic tumor of golden hamster. It contains many pigment granules and also vesicles with a distinct substructure probably representing stages of pigment granule formation. Note a longitudinally oriented striated component made up of delicate fibrillar structure which shows a periodicity in the order of 90 A. X 54,000. Insert X 120,000.

ed or non-pigmented dopa-positive melanocytes in the normal epidermis of either golden or white hamster could not be achieved by means of routine staining methods, the dopa reaction, and the Masson-Fontana silver stain. However, attempts to demonstrate epidermal melanocytes in the untreated hamster with the electron microscope resulted in the finding of occasional clear cells (7, 9) which were dendritic in shape and devoid of desmosomes and tonofilaments (Fig. 5). They had a deeply indented nucleus, a feature which is also characteristic of the dermal melanocytes. The cytoplasm contained a prominent Golgi zone, numerous smooth walled vesicles, and some organelles which were racket- or rod-shaped in profile and resembled those recently described in basal melanocytes and high level clear cells of patients with vitiligo (1).

Neoplastic Melanogenesis

NEOPLASTIC MELANOCYTES OF THE CARCINOGEN-INDUCED MELANOTIC TUMORS IN THE SYRIAN GOLDEN HAMSTER : The melanocytes of the DMBA-induced melanotic tumors in the Syrian golden hamster were always heavily pigmented. The pigment was dark brown, gave a positive Masson-Fontana silver stain and a negative iron reaction, and could be bleached with potassium permanganate. This indicated that the pigment was melanin. Detailed histomorphological features of this tumor were described in previous reports (3, 7, 9).

Under the electron microscope, neoplastic melanocytes of fully grown tumors were filled with melanin granules. These granules appeared round to short-oval in profile, measured up to about 800 mμ in length, and had a high electron opacity, which made a detailed analysis of the substructure impossible. At an early stage of tumor formation, however, some of the melanin granules had the appearance of smooth walled vesicles of varying

sizes and electron opacity, with a distinct substructure (7) (Fig. 6). Their fine structure was very similar to that seen in early melanin granules in the normal melanocytes. However, the size of the granules of the neoplastic melanocytes was almost two times that of their non-neoplastic dermal counterparts. Collagen (reticulin) fibers were often seen in the intercellular spaces. Occasionally, tumor cells devoid of pigment granules were also observed.

NEOPLASTIC MELANOCYTES OF THE CARCINOGEN-INDUCED MELANOTIC TUMORS IN THE SYRIAN WHITE HAMSTER : The histopathological features of the melanotic tumors in the white hamster were similar to those produced in the golden variety except for their more variable pigment content and lighter color (10). In addition to the spindle-shaped cells, pigmented or non-pigmented dendritic cells were occasionally observed. The amount and the pigment intensity of the granules in the white hamster varied greatly from one tumor to another, even in the same animal. The granules were always lighter in color than those found in the golden variety. These differences were reflected in the fine structure of the melanin granules.

In the tumors of the white hamster, the pigment granules showed great variations in size, shape, and internal structure, varying from round or oval to elongated or irregular in outline. The smaller granules usually were round and measured about 0.1 μ in diameter (Figs. 7 and 8); the larger ones were elongated or irregular in shape, measuring up to about 6 μ in length and 1.5 μ in thickness (Fig. 9). Electron-opaque material here interpreted as melanin was only sparsely distributed, and was absent entirely from many cells. When present, it occurred in irregular patches within large granules, arranged as clumped irregular material or clusters of short rods, each measuring

FIGURE 7

Non-pigmented melanocyte from white hamster tumor, showing typical convoluted nucleus, centrioles, Golgi material, and a number of smooth walled vesicles and granules of varying electron opacities. $\times$ 27,000.

FIGURE 8

Material similar to that in Fig. 7, showing centrioles, Golgi material, and a variety of vesicles and granules. Note two small elongated granules (arrows). $\times$ 26,000.
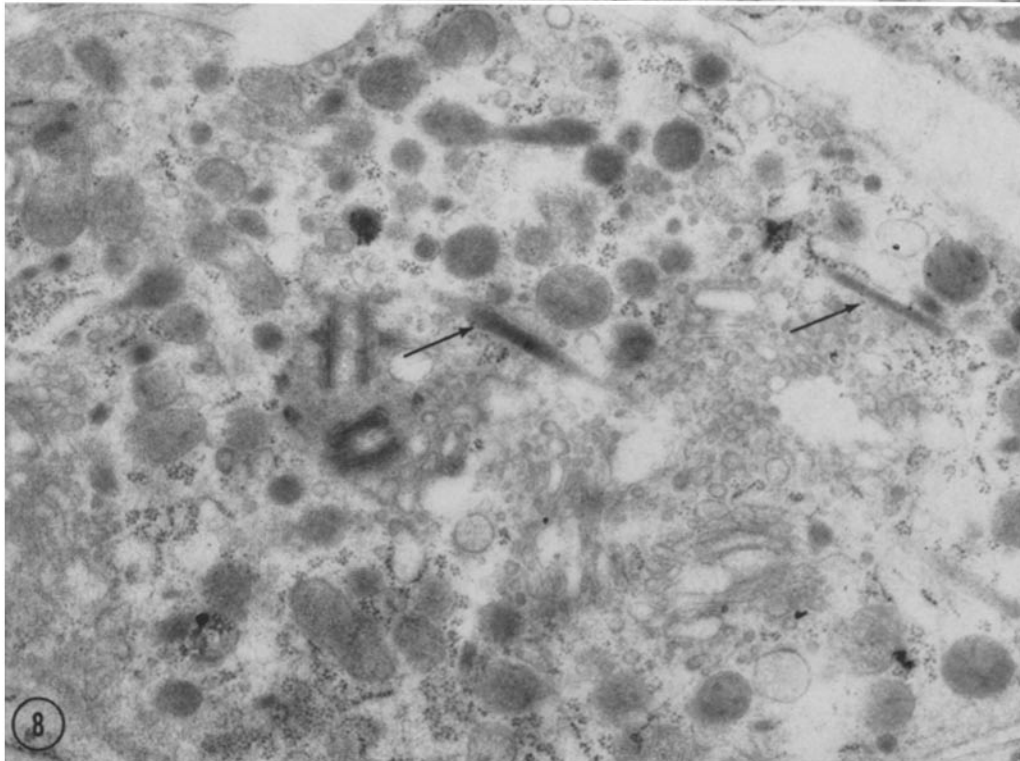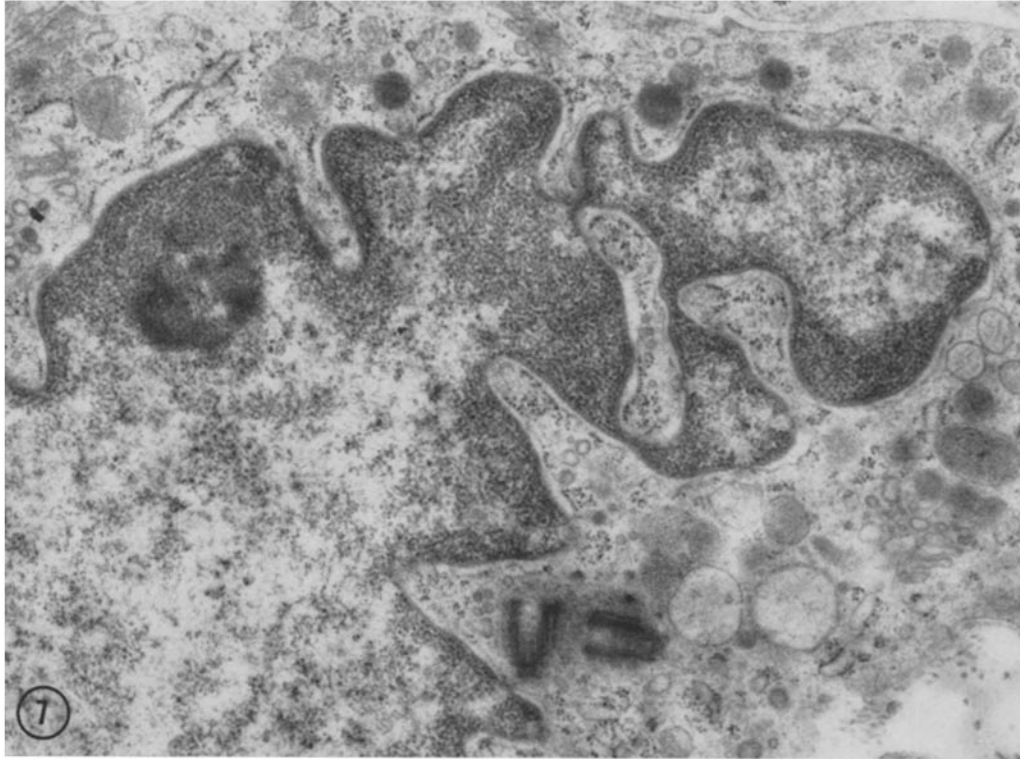
about 20 mμ in thickness and about 500 mμ in length (Fig. 10). The granules contained, in addition to melanin, at least four other components, as distinguished by their fine structure. These are: (a) amorphous granular areas of moderate electron opacity, present in inclusions with round or irregular profiles (Figs. 10 and 13); (b) elongated parallel-oriented striae often running the length of the larger elongate granules; in a few cases this material appeared to form crystalloids, filamentous in longitudinal section (Fig. 11), and hexagonal in cross-section (Fig. 12) with a spacing between centers of about 170 A; (c) between the rods, a material of lower electron opacity in which ferritin-like particles were present (Fig. 10); (d) occasional round vesicles of uniform electron opacity occurring within the boundaries of larger granules (Figs. 10, 13, and 14).

Some pigmented cells contained granules of an entirely different appearance. These granules were composed of relatively large masses containing numerous clusters of pigmented rod-shaped subunits (Fig. 14). Similar formations have been described previously in macrophages of certain malignant melanomas and were presumed to represent phagocytized cytoplasmic masses (13). In our tumors, however, these masses were frequently found in the same cells with melanin granules at various stages of evolution. In a few cells, possibly representing stages in degeneration, these masses had apparently ruptured and clusters of pigmented rods were free in the cytoplasm (Fig. 15).

DISCUSSION

The precise mechanisms of pigment granule formation are still uncertain. As reported above, many melanocytes contain a spectrum of smooth walled components varying from small Golgi-like vesicles to large, dense granules. The largest of these inclusions, although still non-pigmented, bear an obvious similarity in structure to granules in which melanin is forming. These observations suggest an involvement of smooth walled components in melanogenesis as proposed by Wellings

and Siegel (13). The active pinocytosis, characteristic of both normal and neoplastic melanocytes, may also contribute to the process of pigment formation. We found no evidence for the direct participation of mitochondria. It is likely, however, that the pigment granule is a product of the cell as a whole, receiving contributions from several sources. The protein component doubtless originates from the rough endoplasmic reticulum, although direct connections with forming pigment granules were not observed.

Recently, Moyer (6) considered the possibility that melanogenesis in mouse retina may be initiated by the formation of enzymes necessary for melanin synthesis in intracisternal dilatations of the endoplasmic reticulum and also in close proximity to clumps of ribosomes floating free in the cytoplasm. According to Moyer (6), protein fibers containing melanogenetic enzymes such as tyrosinase are synthesized by these ribosomes at the sites indicated above, and are then bounded into "hollow ellipsoids" by a double membrane. The ellipsoids become filled with these fibers oriented parallel to their long axes, and melanin then polymerizes on the protein matrix thus formed.

This theory is somewhat different from that of Seiji et al. (11) for malignant melanoma cells. These authors felt that melanogenesis proceeds from smooth walled spherical Golgi-like granules, which increase in size and density, finally acquiring pigment. The differences in these two concepts partly reflect the difference in material studied, and seem to emphasize the variation in morphological details encountered in different animals and tissues.

In the hamster, both the spherical and the ellipsoid fibrillar inclusions were present in both normal and neoplastic melanocytes, although in differing amount in different cells. In the normal ear tissue, melanogenesis proceeded much as described by Moyer, except that forming pigment granules were mostly if not entirely membrane bounded, and no evidence for the production of fibrillar components of melanin granules from the ribosomes or rough endoplasmic reticulum

FIGURE 9

DMBA-induced melanotic tumor of white hamster. Portion of neoplastic melanocytes with characteristically bizarre pigment granules and many pinocytotic vesicles along the cytoplasmic borders. Most of the granules have a distinct longitudinal striation. × 35,000.
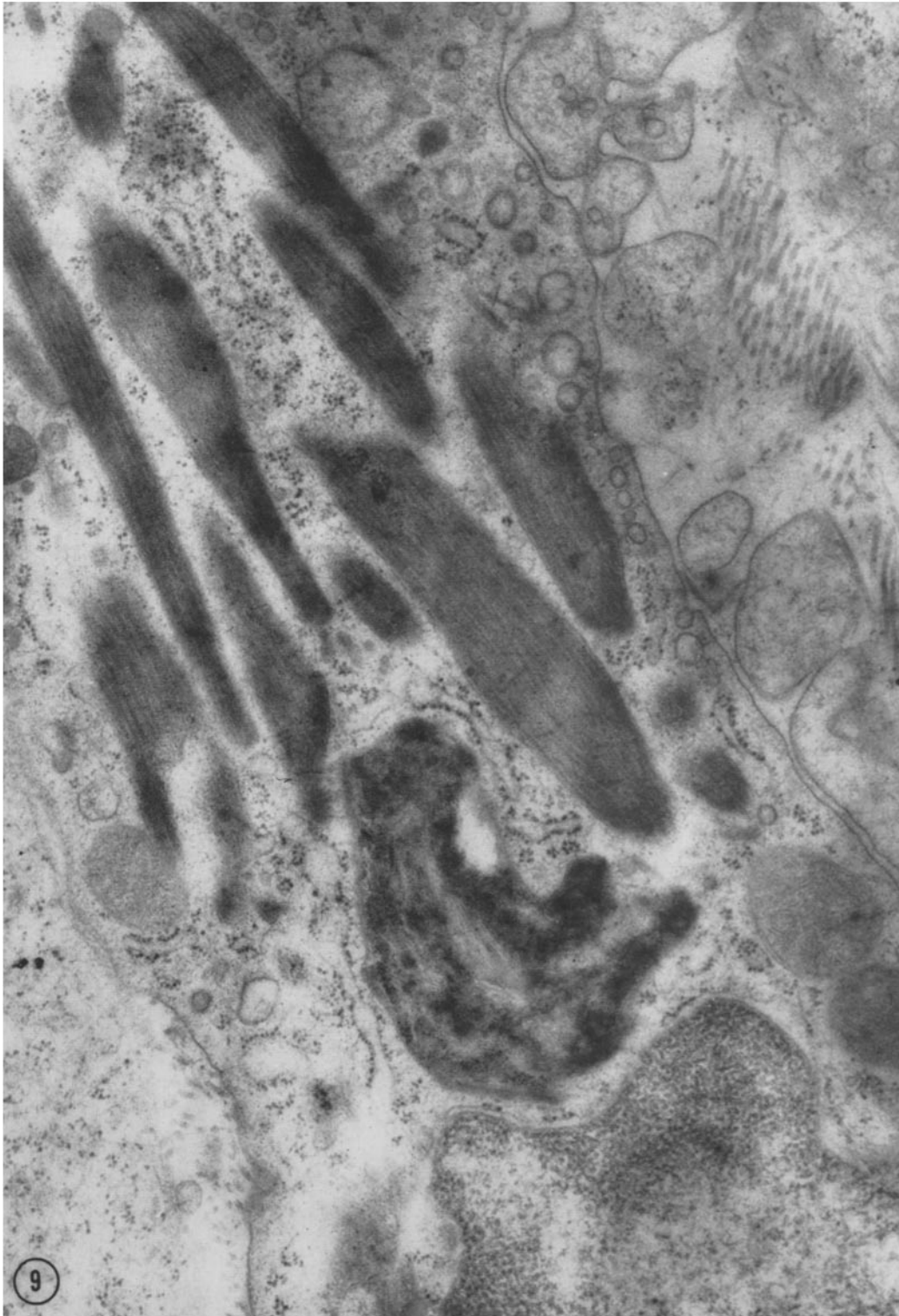
was observed. Inclusions in normal dermal melanocytes of the Syrian hamster can be arranged in a series from Golgi-like or pinocytotic smooth walled vesicles to vesicles with parallel fibrillae of increased size and elliptical profile, in which melanin is deposited apparently along the fibrillar component. As also found by Moyer, the fibrils contained a fine periodicity of about 90 A. Neoplastic melanocytes of the golden hamster followed much the same pattern, except that the mature melanin granules were slightly larger.

In the white hamster, neoplastic melanocytes presented a much more complex picture. Many cells contained a wide spectrum of smooth walled components, varying from Golgi and pinocytotic vesicles to granules of increasing size and electron opacity. Many of these contained a finely granular component of medium density, resembling the "pre-melanosomes" of Seiji et al. (11). Some of these granular inclusions contained bundles of fibers or melanin rods, closely resembling those formed in pigment granules of normal tissue, except that several bundles frequently occurred within a single granule. Interestingly enough, many of these bundles still maintained a roughly elliptical profile, even though they were not closely associated with the limiting membrane of the granule. Other pigment granules had rod-like profiles, in which the major component was a series of parallel striae. As in the granular inclusions, these rod-shaped pigment granules possessed irregular areas of melanization. Again, as

in normal dermal melanocytes, melanin was arranged in clusters of short rods, and these were often elliptical in outline. These bundles were occasionally oriented parallel to the long axis of the inclusion, but were more frequently arranged in a random fashion. The striated component was absent from pigmented areas, suggesting that the striae are dissolved or disrupted during the formation of the pigment (7). In the largest and most heavily pigmented inclusions, the striae were completely lacking. Although it is at present impossible to determine the evolution of these bizarre pigment granules with any degree of certainty, we should like to suggest the following sequence: Small smooth walled vesicles with granular contents increase in size to form large spherical or irregular inclusions of medium electron opacity. In some cases elongated striae form, giving the inclusion a rod-like profile. In either granular or striated inclusions, melanization can occur. It does so, much as in the normal granule, first by the formation of fibrils arranged in elliptical bundles upon which the melanin later accumulates. This process involves the disappearance of the striae in rod-shaped granules, so that they eventually contain only haphazardly arranged bundles of melanin rods. In some cells such granules apparently burst, and distribute the melanin bundles throughout the cytoplasm.

Initially, we attributed these pronounced and bizarre structural aberrations of the melanin granules in the tumors of the white hamster to


FIGURE 10

Material similar to that in Fig. 9. The large elongated granules contain parallel striae separated by material of lower electron opacity. Note cross-section of elongated granule at arrow. Small dense ferritin-like particles occur between striae, and also free in the cytoplasm. Some granules lack elongated striae but instead contain dense granular material of similar electron opacity (g; see also Fig. 13). Most granules contain small patches of electron-opaque material sometimes arranged in clusters of short rods, here interpreted as melanin (m). In a few places granules also contain homogeneous spherical vesicles within their structure (v). Note also mitochondria and pinocytotic vesicles at cell margin. × 37,000.

FIGURE 11

Rod-shaped granule, showing crystalloid structure. × 66,000.

FIGURE 12

Granule showing regular packing of dense material, probably a cross-section of rod-shaped crystalloid. × 122,000.
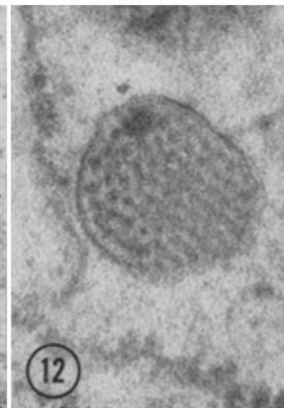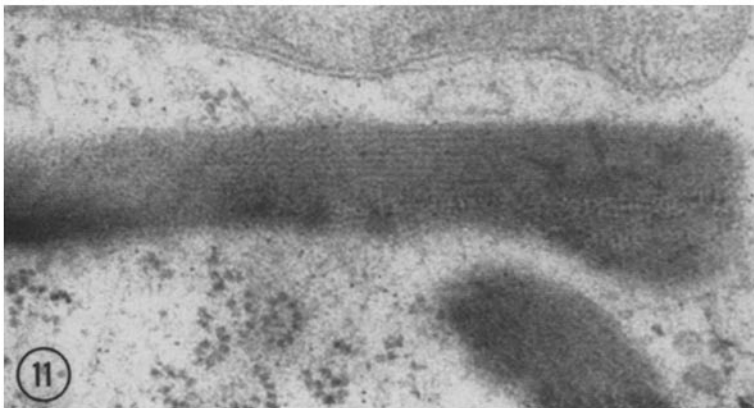
some unknown effect of the carcinogen upon the precursors of melanin granules (9). However, identical tumors in the golden hamster show only a slight deviation from the normal morphology, consisting primarily in the larger size of the granules. The differences in response are almost certainly attributable to variation in the genetic constitution of the two strains. These tumors can readily be transplanted between white and golden hamsters. Thus, whether or not these differences in melanogenesis are due to the genotype of the melanocyte itself or to some systemic factors should be amenable to study.
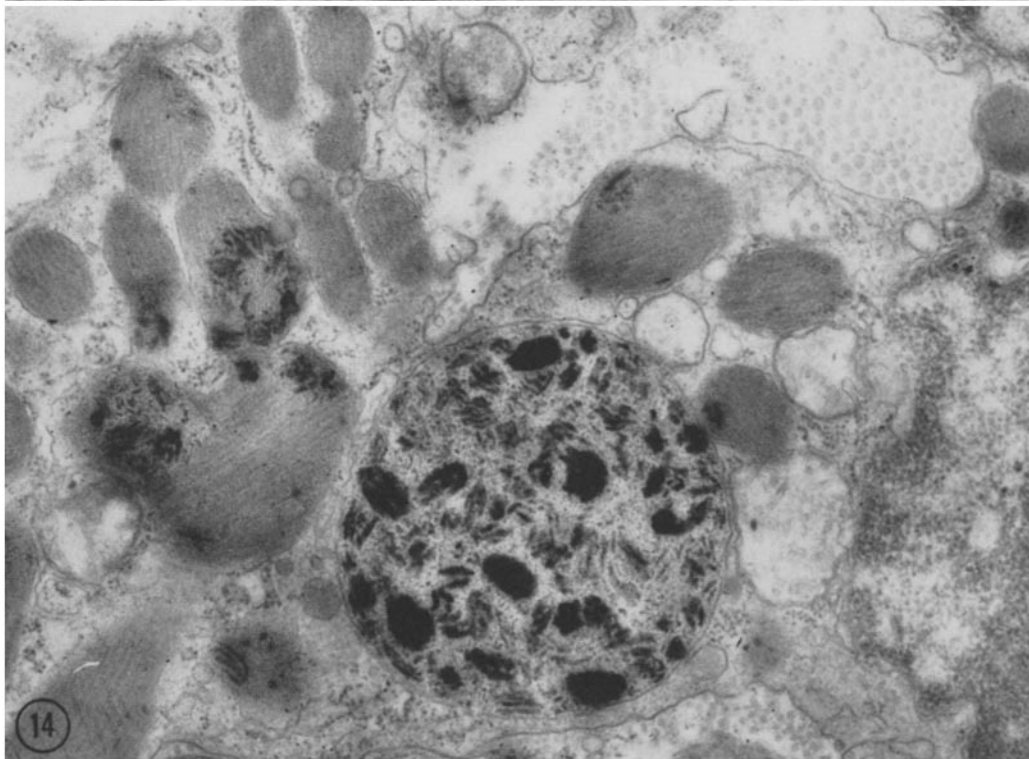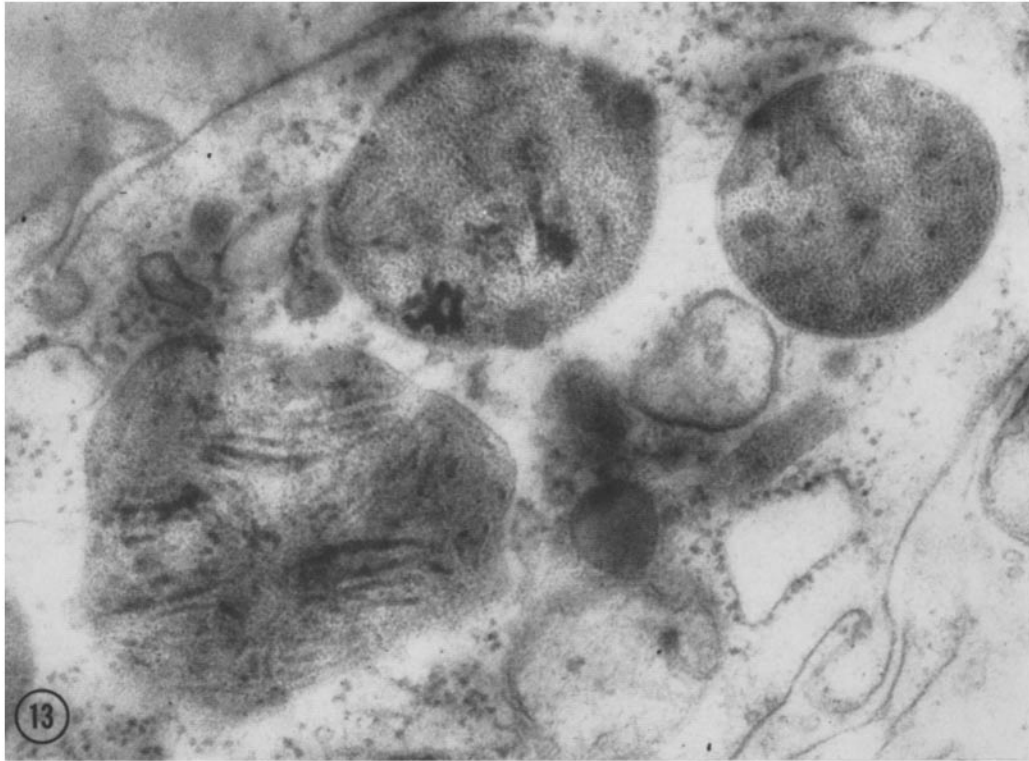
FIGURE 13

Material similar to that in Figs. 9 and 10, showing large granules with round or irregular profiles, containing dense granular material. Rods of differing electron opacities in the largest granule apparently represent stages in melanin formation. × 58,000.

FIGURE 14

Portion of pigmented tumor cell in white hamster. Within the cytoplasm are large granules and striated inclusions, containing numerous disoriented clusters of electron-opaque rods representing melanin. Oriented striated components of the particles are absent from melanized areas, and are completely lacking in the large central pigmented granule. It seems possible that such heavily pigmented granules arise through the dissolution of the striated components, although it is also possible that they are phagocytized portions of adjacent necrotic cells. × 28,000.
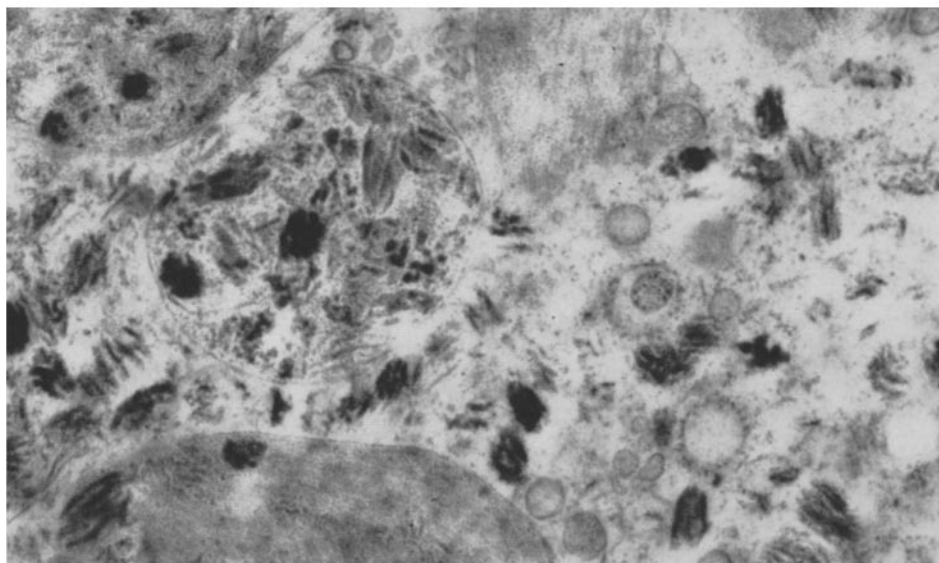
FIGURE 15

Same material as in Fig. 14. Clusters of pigmented rods are free in the cytoplasm, probably through rupture of pigment granule membrane. X 31,000.

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