Melanogenesis in the retinal pigment epithelial cell of the chick embryo. Dopa-reaction and electron microscopic autoradiography of ^{3}H-dopa

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The intracellular pathways involved in melanogenesis in the pigment epithelium of the chick embryo retina were studied by means of the dopa-reaction and electron microscopic autoradiography of ^{3}H-dopa. A small number of premelanosomes and melanosomes were identified in the pigment epithelial cells of the 4-day chick embryo. Premelanosomes, 300 to 800 nm in diameter with periodic fibrillar and helical structures, frequently were seen near the Golgi apparatus. Mature melanosomes, 300 to 800 nm in diameter of homogeneous electron-dense granular structure, were located in the apical portions of the cytoplasm. Smooth-surfaced anastomosing tubular structures also were identified around the Golgi complexes. Tyrosinase activity was first detected in the 5-day embryo in the peripheral cisternae of the Golgi lamellae by means of the dopa-reaction. Some of the small vesicles and premelanosomes also were found to be dopa-reaction positive. Electron microscopic autoradiography of ^{3}H-dopa shows that the silver grains over Golgi apparatus and smooth-surfaced endoplasmic reticulum are not changed in number for several hours after the injection of ^{3}H-dopa. In contrast, the incorporation of ^{3}H-dopa increases rapidly in premelanosomes and melanosomes following injection of the labeled compound. These observations suggest that premelanosomes are derived from the smooth endoplasmic reticulum which is continuous with the rough endoplasmic reticulum. Dopa probably passes into premelanosomes from both the Golgi apparatus and the smooth surfaced cisternae. Tyrosinase is thought to be synthesized in the rough endoplasmic reticulum and Golgi apparatus and then transferred to premelanosomes via tubular channels or small vesicles. Melanin probably is synthesized in the premelanosomes.

Key words: melanogenesis, the retinal pigment epithelium, autoradiography, ^{3}H-dopa, dopa-reaction

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The purpose of this study was to define the role of premelanosomes, melanosomes, and certain cytoplasmic fine structures in the process of melanin synthesis in retinal pigment epithelial cells.

The process of melanogenesis has been studied in the malignant melanoma of the mouse,[1-6] normal and malignant mammalian

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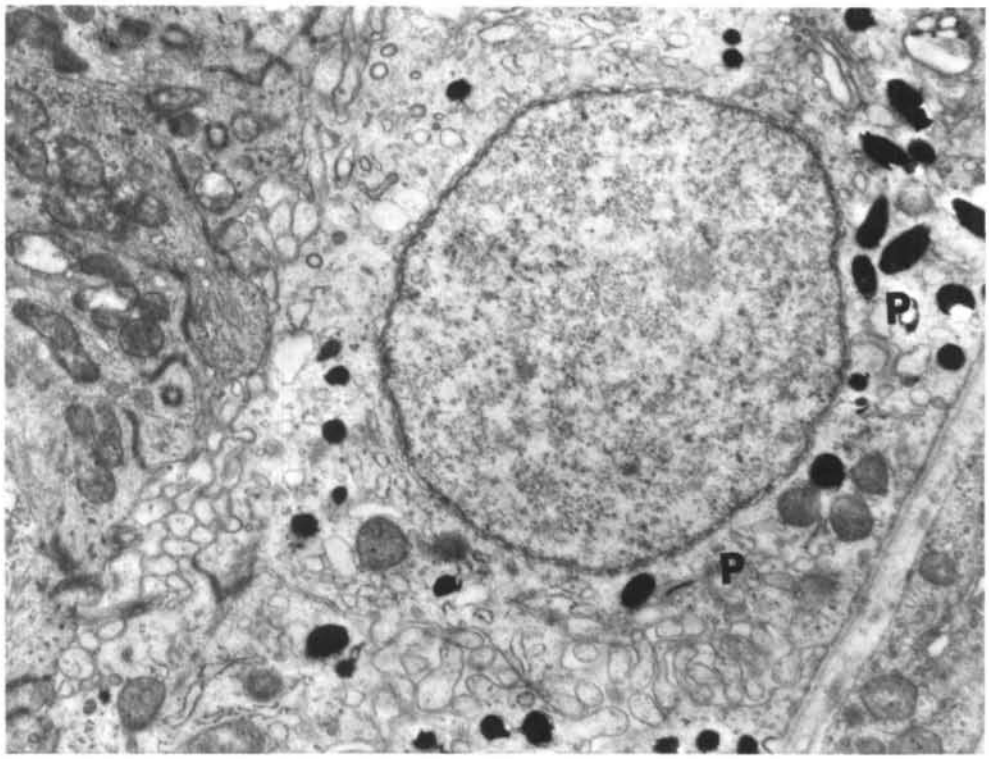

Fig. 1. Portion of a retinal pigment epithelial cell of a 7-day chick embryo. A few premelanosomes *(P)* containing many filamentous structures are seen near the rough-ER. (×13,000.)

pigment cells,[7–17] and the retinal pigment epithelium of the chick embryo, tadpole, mouse, and human fetus.[7–9, 17–26] A number of investigators have described the ultrastructure of normal and melanoma melanocytes during melanogenesis, but information concerning the dynamics of melanin formation in the retinal pigment epithelium is very scanty.[17–27] The excellent papers by Novikoff et al.[6] Eppig,[20] and Eppig and Dumont[21] deal with smooth-surfaced channels in the formation of melanosomes and premelanosomes of the mouse melanoma, *Rana pipiens*, and tadpole eyes.[6, 20, 21] The rapid synthesis of melanin in a melanoma probably occurs in pigmented melanocytes. The events which occur in melanoma tissue probably should not be extrapolated to normal tissue, since it is quite likely that there are differences between physiological and pathological melanogenesis.

The retinal pigment epithelium of the chick embryo was chosen for this study because its melanogenesis is rapid and uniform. Tyrosinase activity was evaluated by means of the electron microscopic dopa-reaction, and dopa utilization was studied by means of electron microscopic ^{3}H-dopa autoradiography.

Materials and methods

The retinal pigment epithelium for this study was obtained from 4-, 5-, 7-, 10-, and 12-day white leghorn chick embryos. Small pieces of the posterior segment of the retinal pigment epithelium were fixed with 2.5% glutaraldehyde solution buffered at pH 7.4 with 0.1M Millonig buffer for 2 hr, following which they were postfixed for 1 hr in 1% osmic acid solution, buffered at pH 7.4 with 0.1M Millonig buffer. The tissues then were embedded in epoxy resin. Some tissues were used for enzyme histochemistry. Dissected retinal tissues were fixed in a 4% formalin solution, which was buffered at pH 7.4 with cacodylate buffer for 20 min, and then incubated in L-3,4-dihydroxyphenylalanine (dopa) solution, buffered with cacodylate phosphate at pH 7.4, for 4 to 7 hr at 37° C. The tissues were rinsed twice with distilled water and

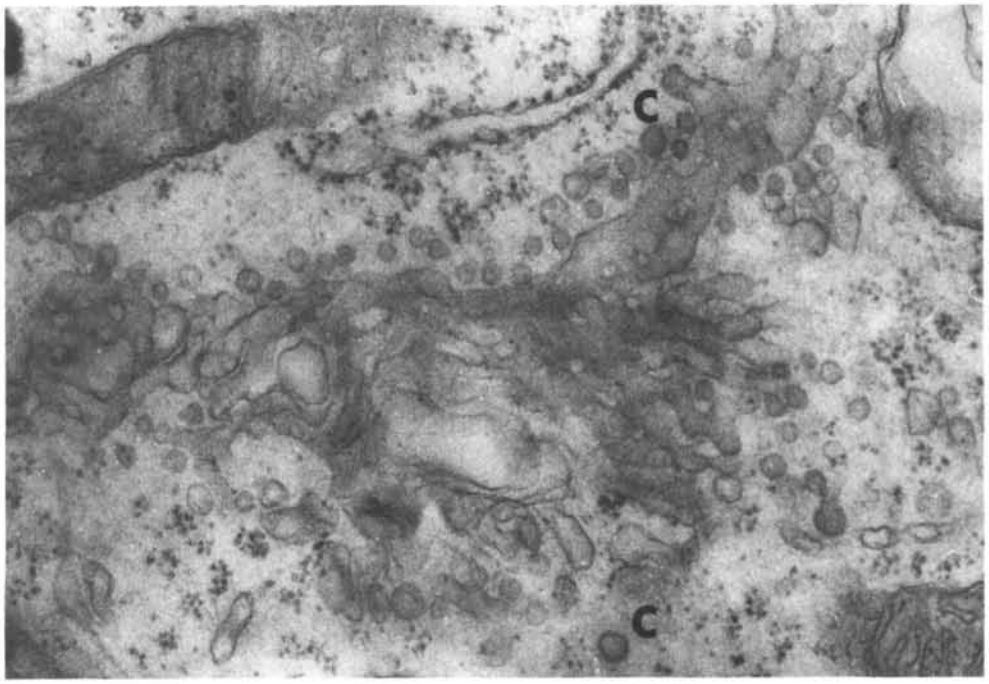

Fig. 2. High-magnification photomicrograph of the smooth-surfaced anastomosing structures which are located near the Golgi apparatus of a retinal pigment epithelial cell of a 7-day chick embryo. Some coated vesicles *(C)* are recognized in the vicinity of the tubular channels. (×42,000.)

refixed with 1% buffered isotonic osmic acid at pH 7.4 for 1 hr at 4° C. Block staining was performed with 1% solution of phosphotungstic acid for 3 hr. These tissues then were embedded in epoxy resin.

Some of the chick embryos were prepared for ^{3}H-dopa electron microscopic autoradiography. The technique involved injection of the yolk sac with 1 mCi of ^{3}H-dopa (ring-2, 5,6-^{3}H). At 1.5, 6, 12, and 24 hr after the injection, small pieces of the posterior segment of the retinal pigment epithelium were fixed in 4% formaldehyde solution at pH 7.4 with 0.1M cacodylate phosphate buffer. The tissues were postfixed for 1 hr in 1% osmic acid adjusted to a pH of 7.4 with 0.1 cacodylate phosphate buffer, following which they were embedded in the epoxy mixture. Light gold–colored sections were mounted on collodion-coated copper grids and then stained with Millonig lead solution. The sections then were coated with a thin layer of carbon and covered with Ilford L4 photographic emulsion by means of a wire loop. Following 3 months of storage, the autoradiograms were developed in either Kodak Microdol X for 5 min at 20° C or phenidone developer for 1 min at 20° C. The gelatin was removed by treatment for a few minutes with 0.05N NaOH. Each grid was stained again with Millonig lead solution and examined under an HS-12A electron microscope. The autoradiograms were photographed so that at least one entire pigment epithelial cell was seen in each field. The number of grains over each cell structure for each time interval was expressed as a percentage of the total number of grains counted over each cell structure for all time intervals combined following the injection of ^{3}H-dopa.

Results

The simple cuboidal cells of the chick embryo retinal pigment epithelium had large, oval or indented nuclei in their basal portions and many projecting microvilli along their apical borders (Fig. 1). The principal organelles observed in these cells were the mitochondria, the rough and smooth endoplasmic reticulum, the Golgi apparatus, the melanosomes, and the premelanosomes.

The Golgi area, located in the supranuclear or juxtanuclear region, consisted of 5 to 8 cisternae of Golgi lamellae and many Golgi ves-

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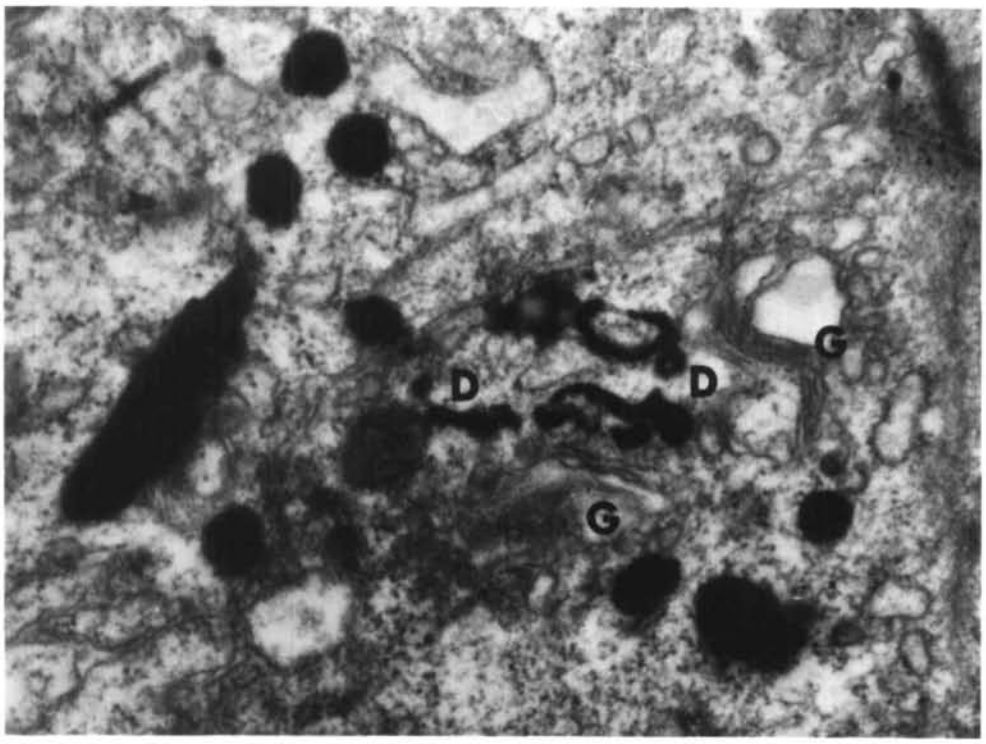

Fig. 3. Dopa-reaction products *(D)* in the retinal pigment epithelial cell of the 7-day chick embryo are seen as electron-dense reaction products in parts of the Golgi lamellae *(G)*, small vesicles, and premelanosomes. (×34,000.)

icles and vacuoles. Portions of the Golgi apparatus may form complicated anastomosing smooth-surfaced tubular structures (Fig. 2). We have observed such anastomosing structures in embryos as early as 4 days. Later, these structures developed numerous smooth surfaced channels extending in all directions. Rough endoplasmic reticulum (rough-ER) elements were distributed throughout the cytoplasm. Occasionally, smooth endoplasmic reticulum (smooth-ER) with fine fibrillar elements in its dilated cisternae were found to be continuous with the rough-ER and to be located in close proximity to premelanosomes.

Characteristic melanosomes and premelanosomes were clearly seen in the cytoplasm in the 4-day embryo. Early premelanosomes, round or oval in shape, about 300 to 800 nm in diameter, and of periodic fibrillar or helical structure, were surrounded by a definite limiting membrane. Sometimes some of the early premelanosomes showed a connection with the smooth-surfaced tubules. These premelanosomes were often observed in the vicinity of the Golgi complexes, elements of rough-ER, and anastomosing tubular structures. Round or oval melanosomes, which were almost as large as premelanosomes, were identified by their remarkably homogeneous electron densities. The limiting membrane was not detectable in these cells. A few melanosomes were recognized near the nucleus and in the basal portion of the pigment epithelium in the 4-day embryo. In time, they increased in number, and in the 10- to 15-day embryo, there was a tendency for the melanosomes to locate in the apical portion of the cell.

Sometimes coated vesicles, about 90 nm in diameter, were recognized not only near the cytoplasmic membrane but also near the premelanosomes and anastomosing tubular channels. Multivesicular bodies also were occasionally observed in the vicinity of the anastomosing tubular structures. They were approximately 400 to 500 nm in diameter and

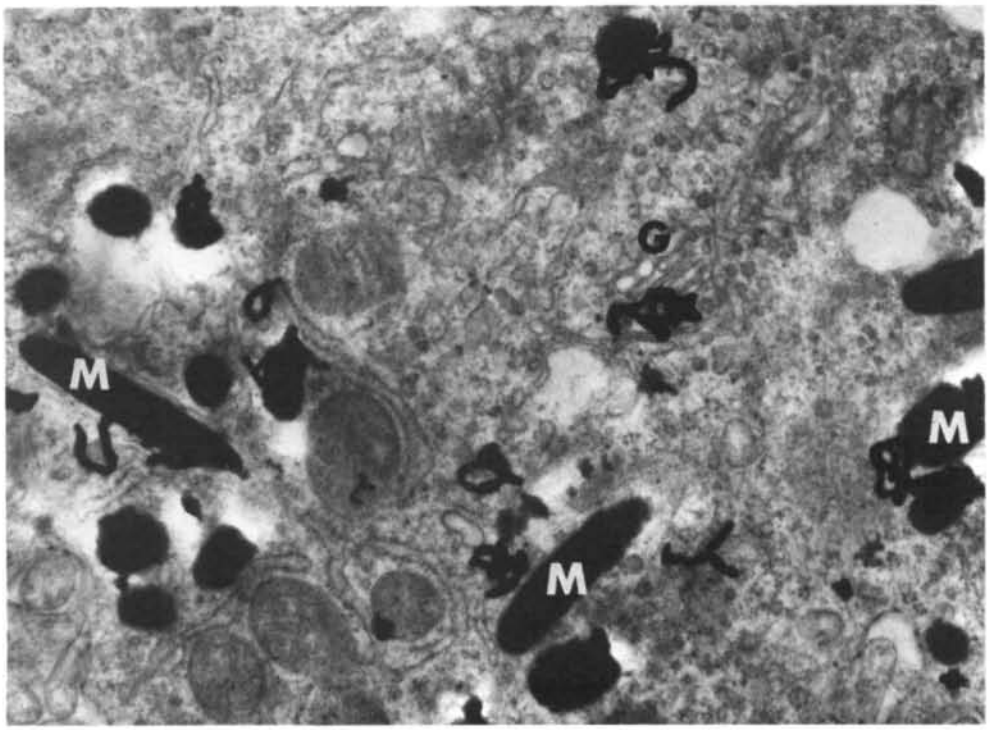

Fig. 4. Electron microscopic autoradiograph of the retinal pigment epithelium of the 7-day chick embryo, 12 hr after the injection of ^{3}H-dopa. Silver grains are recognized over the melanosomes *(M)*, premelanosomes, and the Golgi apparatus *(G)*. (×24,000.)

contained a variable number of internal vesicles.

Incubation of the pigment epithelium in the dopa-medium resulted in the production of electron-dense reaction products, which were first seen in the 5-day embryo as small vesicles in the peripheral part of the Golgi lamellae (Fig. 3). In the 7- and 10-day embryos these reaction products were markedly increased in number. Cytoplasmic reaction products rarely were observed in any cells following the eleventh day of embryonic life. No reaction products were observed in most of the early premelanosomes. Occasionally, however, small electron-dense reaction products were observed on the helical structures of some of the early premelanosomes. Dopa-reaction products were also present in some of the anastomosing tubular channels which either encircled the premelanosomes or extended out to the region of the premelanosomes. In our preparations it has been difficult to decide whether a definite dopa-reaction has occurred in most of the intermediate and old premelanosomes.

The localization of the tritium label in the various organelles within the pigment epithelium at various times following the injection of tritiated dopa is shown in Tables I and II. At 1.5 hr very few grains were present over any portion of the cells. At 6 hr after injection, silver grains were present over most of the cell organelles, but none over the rough-ER. At 12 hr following injection, about half the silver grains were located over the melanosomes and premelanosomes (Fig. 4). In contrast, silver grains over the nucleus, Golgi apparatus, and smooth-ER were decreased in number. At 24 hr after injection, the total number of grains was markedly increased. In all preparations a relatively high ratio of grains over the Golgi apparatus and smooth-ER to grains over the whole cell was maintained. The ratio of silver grains over the melanosomes and premelanosomes to grains over the whole cell reached a peak around 12

Table I. Distribution of ^{3}H-dopa in the cytoplasmic organelles of the retinal pigment epithelial cells at specific time intervals following an injection of ^{3}H-dopa

	*Distribution of silver grains (%)**			
	1.5 hr	*6 hr*	*12 hr*	*24 hr*
Nucleus	22.7	26.3	15.0	11.0
Melanosome and premelanosome	27.3	23.7	51.9	45.8
Golgi apparatus and smooth-ER	18.2	18.4	13.6	17.6
Rough-ER	0	5.3	2.9	4.5
Cytoplasmic matrix	31.8	26.3	14.2	17.6
Mitochondria	0	0	2.4	3.5
Total no. of grains per cell	4.4	7.6	12.9	25.1

*The distribution of silver grains over a cytoplasmic organelle for any particular time is expressed as a percent of the total number of grains over the entire cell at that time.

Table II. Relative concentration index of ^{3}H-dopa grains per unit area of subcellular compartment*

	1.5 hr	*6 hr*	*12 hr*	*24 hr*
Nucleus	0.66	0.77	0.44	0.32
Melanosome and premelanosome	8.53	7.41	16.22	14.31
Golgi apparatus and smooth-ER	15.17	15.33	11.33	14.67
Rough-ER	0	0.35	0.19	0.30
Cytoplasmic matrix	0.75	0.62	0.33	0.41
Mitochondria	0	0	0.75	1.09

*Relative concentration index = $\frac{\%\text{ grain count}}{\%\text{ volume}}$.

hr following the injection. These results indicate that the greatest melanin biosynthetic activity occurs in the smooth-ER and Golgi apparatus and not in the rough-ER.

Discussion

There are four major theories concerning the origin of melanin granules, namely, those of nuclear, lipochondrial, mitochondrial, and Golgi origins. The predominant belief is that the Golgi apparatus is exclusively involved in granule formation.[14, 15, 28, 29] Moyer[7, 8] however, has suggested that smooth-ER is the source of melanosomes, on the basis of his studies of the mouse embryo eye. Novikoff et al.[6] have proposed that premelanosomes arise from the dilated cisternae of smooth-ER in a specialized region near the Golgi apparatus. Their observations are based on the cytochemistry of tyrosinase and acid phosphatase. Maul,[12] using cultured human melanin cells, also has suggested that premelanosomes develop within the tubular smooth-ER, which is connected with the Golgi apparatus. Earlier biochemical and ultrastructural studies by Breathnach and Wyllie[17] and Seiji[2] provided evidence that premelanosomes are formed directly from Golgi complex–derived, smooth-surfaced, tyrosinase-containing vesicles.

Most of the round or oval premelanosomes in our study of the early chick embryonal pigment epithelium were located around the rough-ER, Golgi area, and anastomosing tubular structures. Occasionally they showed an attachment of smooth-surfaced structures to their outer membranes. Portions of the rough-ER near premelanosomes had numerous attachments with the smooth tubules. Welling and Siegel[15] have observed that continuity between the "rough-surfaced" membrane of ergastoplasm and the "smooth-surfaced" membrane of the Golgi sac frequently is demonstrable in the mammalian melanocyte. Many other investigators have confirmed these findings and have postulated that the smooth profiles of ER are formed from the rough elements in adrenocortical cells.[30, 31] In our study, the dilated cisternae of smooth-ER which is continuous with the rough-ER was observed to contain some fine fibrillar material. Welling and Siegel,[15] Maul,[12] and Stanka[24] also have observed the fine fibrillar materials in the dilated cisternae of the rough-ER. Palade et al.[32] maintain that the rough-ER synthesizes the protein component of the membrane of the smooth-ER. On the basis of our studies, it seems likely that the premelanosomes of the retinal pigment epithelium of the chick embryo originate from the dilated smooth-ER continuous with the rough-ER.

Anastomosing tubular structures which are present in the perinuclear region of the 4-day embryo become well developed during early embryonic life but are relatively sparse just prior to hatching. This reduction in the num-

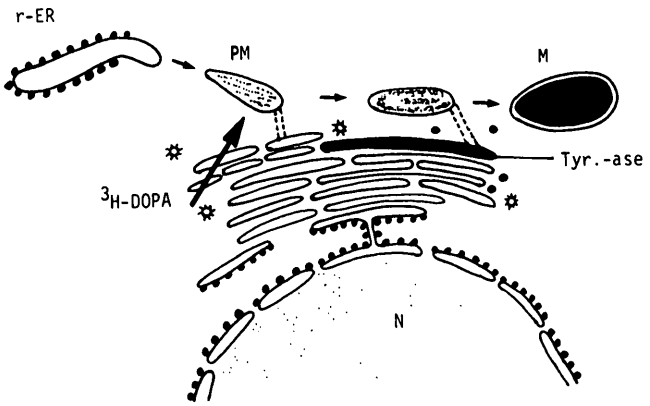

Fig. 5. Schematic diagram of the histogenesis of melanin granules. The premelanosome *(PM)* is shown to be derived from the smooth-ER which is continuous with the rough-ER *(r-ER)*. Dopa is transported into premelanosomes from the Golgi apparatus as well as from smooth-surfaced cisternae. Tyrosinase *(Tyr.-ase)* is transferred to premelanosomes via tubular channels or small vesicles. Melanin may be synthesized in premelanosomes. *M*, Melanosome; *N*, nucleus.

ber of anastomosing tubules in older cells has been particularly emphasized by Eppig and Dumont.[21] It seems most likely that these changes are due to attachment and subsequent separation of the tubular structures to premelanosomes or other cytoplasmic components. Maul[12] has observed the presence of an anastomosing network of membranous tubules in obliquely cut sections of human melanoma cells and has concluded that these structures are part of the Golgi apparatus. Thus consideration now must be given to the possibility that these anastomosing tubular structures consist of both Golgi apparatus and smooth-surfaced tubules.

Miyamoto and Fitzpatrick,[27] by means of biochemical techniques, have demonstrated tyrosinase activity in the retinal pigment epithelium of the 6- to 14-day check embryo. Their data indicated that tyrosinase activity was first detectable in the 6-day embryo, following which it gradually increased and reached a maximum in the 10-day embryo. The results of our histochemical studies are quite consistent with these biochemical observations. No tyrosinase activity could be detected after 14 days of embryonic life. We first detected tyrosinase activity in some parts of the Golgi complex in the 5-day embryo, following which activity increased up to 10 days.

El-Hifnawi and Hinrichsen[26] recently have detected tyrosinase activity in some of the rough-ER and small vesicles of the iris pigment epithelium in the 4-day embryo by means of histochemical techniques. Palade and co-workers[33–35] have indicated that secretory proteins are synthesized by ribosomes, following which they are released into the cisternae of the ER through which they are transported to the region of the Golgi apparatus where they are concentrated and packed into granules. The production of tyrosinase in the retinal pigment epithelium probably is handled in a similar fashion. The enzyme is synthesized in the rough-ER, following which it passes through tubules which connect tubular anastomosing structures and the premelanosomes or it is transported in small dopa-reaction–positive vesicles to the premelanosomes.

Hirsch et al.[5] have described melanin synthesis in the pigment cells of the Cloudman S-91 mouse melanoma by means of high-resolution autoradiography with ^{3}H-labeled dopa. Initial melanin biosynthesis occurred predominantly in the ER and associated ribonucleoprotein particles. Then the tritium label moved to the melanosomes and mature melanin granules. According to Blois and Kallman,[36] ^{14}C-dopa is incorporated into the melanin of pigmented mouse melanomas at a

rate which is proportional to the rate of melanin synthesis of a given tumor at the time of administration. Nakai and Shubik[3] have demonstrated by means of electron microscopic autoradiography that an appreciable amount of ^{14}C-dopa is incorporated into the melanosomes of Harding-Passey melanoma cells at both early and advanced stages of melanization. They also have shown that the melanosome is a site of melanin synthesis as well as of tyrosinase localization.

It should be emphasized that most of the studies of melanin synthesis have been performed in various pigmented melanomas in which melanin synthesis is extremely rapid. It also is important to stress that the rates of melanin synthesis and melanin pigment transportation may vary from tumor to tumor. In contrast, our studies were performed in a physiological system in which normal melanin synthesis occurred at a rapid rate. Our ^{3}H-dopa electron microscopic autoradiography studies demonstrated little uptake in the 5-day embryo but appreciable label of the 10-day embryos, with continual increase in tissue uptake during the first 24 hr following the injection of ^{3}H-dopa. The ratio of the number of the silver grains over the Golgi apparatus and the smooth-ER to the number of the total grains over the whole cell remained almost constant from 1.5 to 24 hr following the injection. On the other hand, the labeling of premelanosomes and melanosomes increased rapidly during the first 12 hr after the injection and continued up to 24 hr after injection. These observations suggest that much of the label migrated from the Golgi apparatus and the smooth-ER into the premelanosomes and melanosomes. Kobayashi,[37] in his studies of amine synthesis, has reported that dopa migrates directly into secretory granules, some of which may be in transit to the Golgi apparatus. Our studies lead us to conclude, however, that much of the dopa first is incorporated directly into the Golgi apparatus and not into the Golgi–rough-ER system. It then is transported from the Golgi apparatus to premelanosomes and melanosomes by the smooth-surfaced tubular channels or small vesicles (Fig. 5). Melanin is relatively insoluble and is thought to be metabolically quite inert. Hirsch et al.[5] have reported that there is no evidence that melanin undergoes metabolic turnover. Our studies, however, do suggest some metabolic activity for melanin. Some of the mature melanin granules labeled with ^{3}H-dopa. This raises the possibility that melanin may undergo slow metabolic turnover, similar to that of bone and cartilage.

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