

## Control of Pigment Synthesis in Culture<sup>1</sup>

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Chicken embryo retinal pigment cells provide an excellent opportunity for studying a number of aspects of cell growth and synthesis of a differentiative cellular product. These cells can be removed from the eyes of chicken embryos virtually uncontaminated by other cell types, satisfactorily maintained in cell culture and produce a highly specific, morphologically and biochemically identifiable macromolecular product, the pigment melanin (1-3). Under conditions which allow proliferation, they usually stop manufacturing pigment, and previously made pigment is diluted by ensuing cell divisions (2-4). When the cultures become confluent and cell growth slows, the cells resume melanin synthesis. Whittaker attributed this to the cessation and resumption of tyrosinase synthesis (3).

Attempts to elucidate the nature and origin of the melanin granules that characterize vertebrate pigment cells relied heavily upon the electron microscope. Such studies have intimated that the Golgi complex in pigment cells of neural crest origin (skin, hair, and melanomas) are responsible for the elaboration of this organelle (5-7). An "intermediate vesicle" appears which hypothetically acquires the internal periodic structure characteristic of melanosomes. Tyrosinase is believed to constitute a major part of this protein matrix and probably becomes inactive due to the deposition upon itself of the oxidation products of L-tyrosine. Moyer thought that retinal epithelium cells have no well-developed Golgi, and may construct melanosomes directly from intracisternal dilations of the endoplasmic reticulum (8). More recently, smoothed surfaced endoplasmic reticulum closely associated with Golgi membranes of melanoma cells have been implicated in the production of melanosomes (9, 10).

The present investigation is concerned with: (a) the relationship of growth and differentiation in cultured chicken embryo retinal pigment epithelium cells as reflected both histologically and in patterns of DNA and melanin synthesis; (b) translational level controls on protein synthesis at various stages of growth and differentiation, i.e., the stability of pulse-labeled RNA, protein synthesis and melanogenesis in the presence of actinomycin D; (c) the kinetics of melanogenesis; and (d) the origin of melanosomes.

<sup>1</sup> Supported by PHS training grant No. HD-00019.

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## MATERIALS AND METHODS

**Cell culture.** Retinal epithelium pigment cells were obtained by modification of Trinkaus' procedure (11). Eyes from 8-day White Leghorn (University of Rhode Island Hatchery) chicken embryos were dissected free of mesenchyme and incubated for 30 min in Saline G (12) containing 2% trypsin (National Biochemical Co., 1:300) and 4% chicken serum (Grand Island Biological Co.) at 37°C. The scleral and choroid layers were then removed in fresh Saline G minus trypsin and serum and the pigmented epithelium was separated from the underlying neural retina and incubated in Saline G for about 30 min at 37°C. The pigment cells were dissociated by trituration and then resuspended in culture medium at  $2.5 \times 10^4$  cells/ml. Four milliliters of the final cell suspension was placed in 55-mm plastic culture plates (Falcon Plastics) which had been previously coated with 30  $\mu$ g of rat tail collagen (13). The cultures were maintained at 37°C under 5% CO<sub>2</sub> in air, and the culture medium was completely replaced every 2 days.

The culture medium consisted of 87 parts Puck's N-16 (Grand Island Biological Co.), 10 parts fetal calf serum (Grand Island Biological Co.), 2 parts chicken embryo extract (14), and 1 part distilled water containing 20% glucose and 1.2% CaCl<sub>2</sub>. The medium also contained about 10,000 IU of penicillin and 0.5 mg streptomycin per 100 ml.

**Histology.** Cultures were rinsed once with balanced saline (15) and fixed directly in the culture plates with Bouin's fluid, rinsed with distilled water, dehydrated with graded ethanols and stained with ammoniacal silver (16) and Ehrlich's hematoxylin (Eastern Scientific Co.). They were observed and photographed *in situ*.

**Biochemical procedures.** DNA<sup>3</sup> was determined by the method of Burton (17). Cultures were rinsed 3 times with cold balanced saline (15) and 0.5 ml cold 5% TCA was placed on them for 1 hr. After transfer to a 12-ml centrifuge tube, they were extracted twice with cold 5% TCA and once with 80% ethanol. The DNA was then hydrolyzed at 70°C for 30 min in 0.5 N PCA and measured colorimetrically with diphenylamine.

Melanin, present in the residue of the PCA hydrolyzate, was assayed by a modification of the turbidometric method of Foster *et al.* (18) using the extinction coefficient of synthetic dopa melanin (2). The PCA residue was extracted once with ether-ethanol (1:3) and once with absolute ether. The dry residue was dissolved in 5% KOH at 90°C for 10 min and the optical density measured at 400 nm in a Beckman DU spectrophotometer.

Protein was estimated in the 20,000 g supernatants of cell homogenates by the method of Lowry *et al.* (19).

**Radioactive precursor incorporation.** Cell cultures were incubated with 2–10  $\mu$ Ci uridine-<sup>3</sup>H (New England Nuclear; final specific activity, 4.5 Ci/mmmole) for 1 hr. Various concentrations of actinomycin D were added 10 min before the isotope when the effect of the drug on RNA synthesis was to be studied. To examine the rate of bulk RNA decay, after 45-min incubation with uridine-<sup>3</sup>H, the radioactive medium was replaced with fresh medium containing 0.1 mg/ml uridine-<sup>3</sup>H and 12.5  $\mu$ g/ml actinomycin D, which was found to inhibit RNA synthesis 98%. At appropriate times after incubation or chase, the medium was decanted, the cells were

<sup>3</sup> Abbreviations: DNA, deoxyribonucleic acid; L-dopa, L-dihydroxyphenylalanine; PCA, perchloric acid; PTU, phenylthiourea; RNA, ribonucleic acid; RNase, ribonuclease; TCA, trichloroacetic acid.

rinsed 3 times with cold balanced saline (15) and covered with 1 ml/plate of cold 5% TCA containing 0.1 mg/ml uridine-<sup>3</sup>H for 1 hr. The cells were then scraped into 12-ml centrifuge tubes, and the residue again extracted twice with cold 5% TCA. The RNA was hydrolyzed in 5% TCA at 70°C for 30 min, and 0.5 ml of the supernatant added to 10 ml Brays solution for counting in a Packard Tri-Carb scintillation spectrometer (Model 4322).

To investigate the rate of decay of protein synthesis upon inhibition of RNA synthesis, freshly fed cultures were incubated with 4–10  $\mu$ Ci L-leucine-<sup>3</sup>H (New England Nuclear Co., final sp act 5.3–13.3 mCi/mmole) and 1.25  $\mu$ g/ml actinomycin D, after various periods of incubation with actinomycin D of the same concentration. The medium was then decanted, the cells rinsed 3 times with cold saline (15), and extracted for 1 hr with cold 5% TCA containing 0.1 mg/ml L-leucine-<sup>3</sup>H. After the cells were scraped into centrifuge tubes, they were again extracted twice with 5% TCA and once with ether-ethanol (1:3). The final residues were dissolved in 0.25 ml hyamine hydroxide and quantitatively transferred to 11 ml of Brays solution for scintillation counting.

The rates of melanin and protein synthesis were measured by the method of Whittaker (2, 3). Cultures were incubated for 4 or 8 hr with 1 or 2  $\mu$ Ci of uniformly labeled L-tyrosine-<sup>14</sup>C (New England Nuclear Co., final sp act 1.1–2.2 mCi/mmole). Radioactivity incorporated into acid-insoluble material in the presence and absence of phenyl thiourea (PTU), a competitive inhibitor of tyrosinase, was measured. Incorporation resistant to PTU inhibition was considered incorporation into protein, and incorporation sensitive to PTU was taken as incorporation into melanin.

For noting the effect of any particular concentration of cycloheximide or actinomycin D on melanogenesis and protein synthesis, these inhibitors were added separately to cultures from the same run, 4 plates receiving cycloheximide or 4 receiving actinomycin D. Two of the plates in each series were also treated with PTU to allow the measurement of protein synthesis and melanogenesis under these new conditions.

After the period of isotope incorporation, the cultures were extracted as for L-leucine-<sup>3</sup>H incorporation (see above) except that the rinsing and extraction solutions contained 0.1 mg/ml L-tyrosine-<sup>12</sup>C rather than L-leucine-<sup>3</sup>H. The final residues were dissolved in 0.5 ml 5% KOH, and a 0.1-ml aliquot of this solution was dispersed in 10 ml of 30% methanol/70% toluene containing 4 g of 2,5-diphenyl-oxazole and 50 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter for scintillation counting.

*Tyrosinase.* A radiometric procedure (20) was used to measure tyrosinase in total cell homogenates and various fractions of cell homogenates. After the cultured cells were rinsed with saline, they were transferred to cold 0.1 M phosphate buffer (pH 6.8) and disrupted with 10–12 strokes of a loose fitting pestle in a Dounce homogenizer. The homogenate was centrifuged at 10,000 g for 10 min at 2°C to obtain a low speed pellet. The 10,000 g supernatant was recentrifuged at 10,000 g for 10 min to minimize contamination from the rapidly sedimenting material and then centrifuged at 109,000 g for 1 hr at 5–8°C in a Model L ultracentrifuge (Spinco). The low- and high-speed pellets were each resuspended in 1.0 ml of 0.1 M phosphate buffer (pH 6.8). Then 0.2-ml aliquots of the various fractions were incubated 4 hr with 0.2 ml of 0.1 M phosphate (pH 6.8) which was 2 mM in L-tyrosine-<sup>14</sup>C (sp act 2mCi/mmole) and 0.2 mM in L-dopa.

Control tubes had all the above components but also were brought to 1.5 mM PTU. It was found that PTU gave lower control values than boiled enzyme extracts and was, therefore, preferable to the latter.

*Dihydroxyphenylalanine (dopa) oxidase.* Cultures were rinsed 3 times with cold saline, scraped into a glass homogenizing tube (Tri-R), and homogenized with a motorized teflon pestle for 30 sec in 1.0 ml of 0.1 M phosphate buffer (pH 6.8). The homogenate was centrifuged at 20,000 g for 20 min at 5°C. One-half milliliter of the supernatant was added to 0.5 ml of phosphate buffer (pH 6.8) containing 4 mg/ml L-dopa, and the optical density at 400 nm was recorded for 3 hr at room temperature (23°C) in a Beckman DU spectrophotometer. Activity was not linear during the first hour of incubation, but was estimated from the optical density changes of subsequent time periods, eg. 2–3 hr when it was linear. A control for autoxidation of L-dopa consisted of 0.5 ml L-dopa incubated with 0.5 ml of the buffer instead of an enzyme extract.

*Sucrose density gradients.* Ten or more cultures were rinsed with saline and scraped into several milliliters of cold RSB ( $10^{-2}$  M trishydroxymethylaminomethane, pH 7.4,  $10^{-2}$  M KCl,  $1.5 \times 10^{-3}$  M  $MgCl_2$ ) (21). The cells were left in this hypotonic solution for about 5 min at 0–5°C and then broken with 10–12 strokes of a loose fitting pestle in a Dounce homogenizer. Sodium deoxycholate (DOC) was added to the 10,000 g (10 min, 5°C) supernatant to a concentration of 0.2%, and it was layered on a 27 ml 15 to 30% sucrose gradient. RNase (2 µg/ml, Worthington) was added to a control series for 30 min at 0–5°C just prior to the addition of DOC. The gradients were centrifuged at 23,000 rpm in an SW 25.1 swinging bucket rotor in a Spinco model L ultracentrifuge for 135 min at 5–8°C. One-milliliter fractions were collected, and their  $OD_{260}$  was measured. Tyrosinase and DOPA oxidase activity of the fractions was determined as previously described, except that, for tyrosinase, after incubation of fractions with L-tyrosine- $^{14}C$ , aliquots were dried on small filters (Whatman No. 1) before precipitation and washing with TCA.

*Electron microscopy.* Twelve-day cultures were rinsed once with saline and fixed directly on the culture plates in cold 2.5% glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.4). The plates were kept on ice for 30 min, scraped into shell vials, and post-fixed with 2% osmium tetroxide for 2 hr at 0–5°C. The cells were dehydrated at 0–5°C with graded acetones and embedded in epon. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and viewed with a Siemens Elmiskop I electron microscope. Cultures were also fixed, dehydrated, and embedded *in situ* without propylene oxide. The plastic culture plates were then cut up, the monolayers sectioned and stained as above, and viewed with an A.E.I. 6B electron microscope.

## RESULTS

*Growth of cultures.* Most of the retinal pigment cells which attach to the collagen coated surface of the plastic culture plates do so in the first few hours. Even after 1 day in culture, the proportion of the cells which fixed to the substratum, about 50% of those inoculated, did not appreciably increase.

The homogeneity of the culture population is especially apparent during these early stages, as nonpigmented cells are rarely seen (Fig. 1a). By the second day the cell population has increased considerably and pigment distribution differs among cells, probably because of differences in division rates. Binucleate cells are

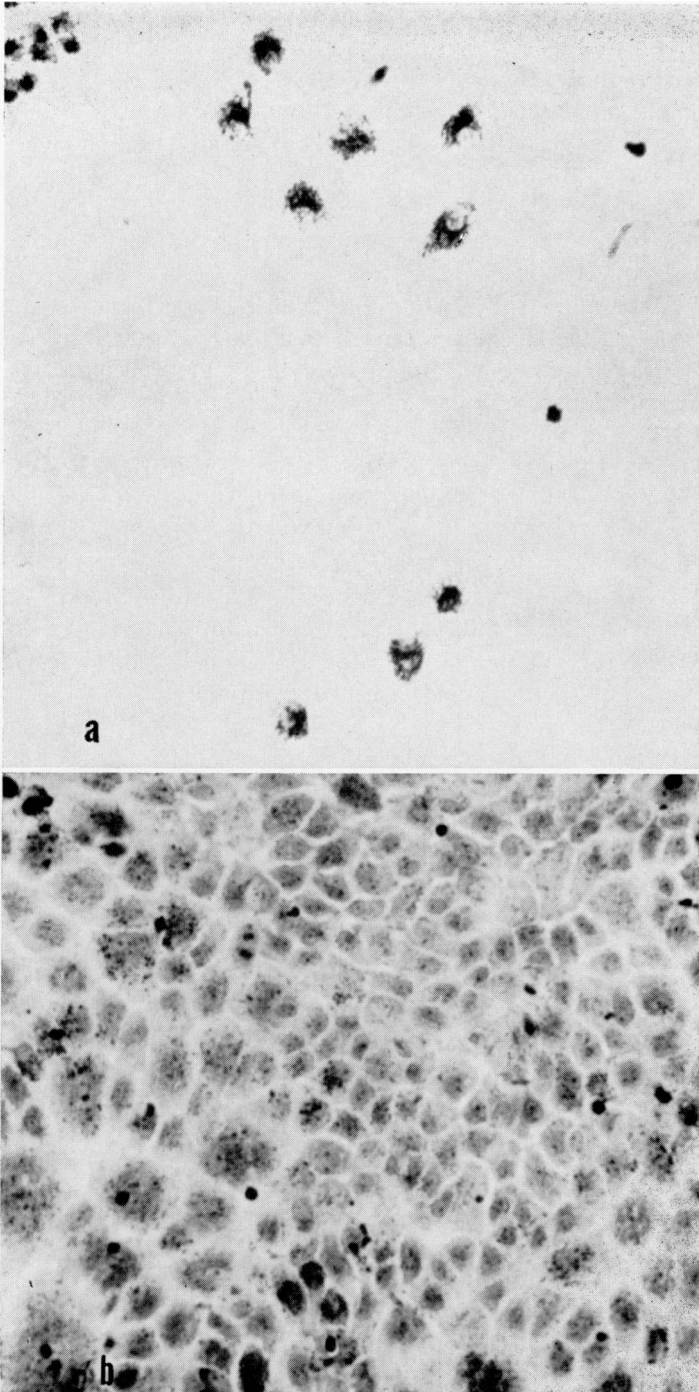


FIG. 1. Cultured 8-day embryonic chicken pigment epithelium cells. Silver and hematoxylin. (a) Cells cultured one day. They have flattened and contain large amounts of pigment. 100 $\times$ . (b) Cells cultured 4 days. Culture is confluent. Small, densely packed cells contain least pigment. 100 $\times$

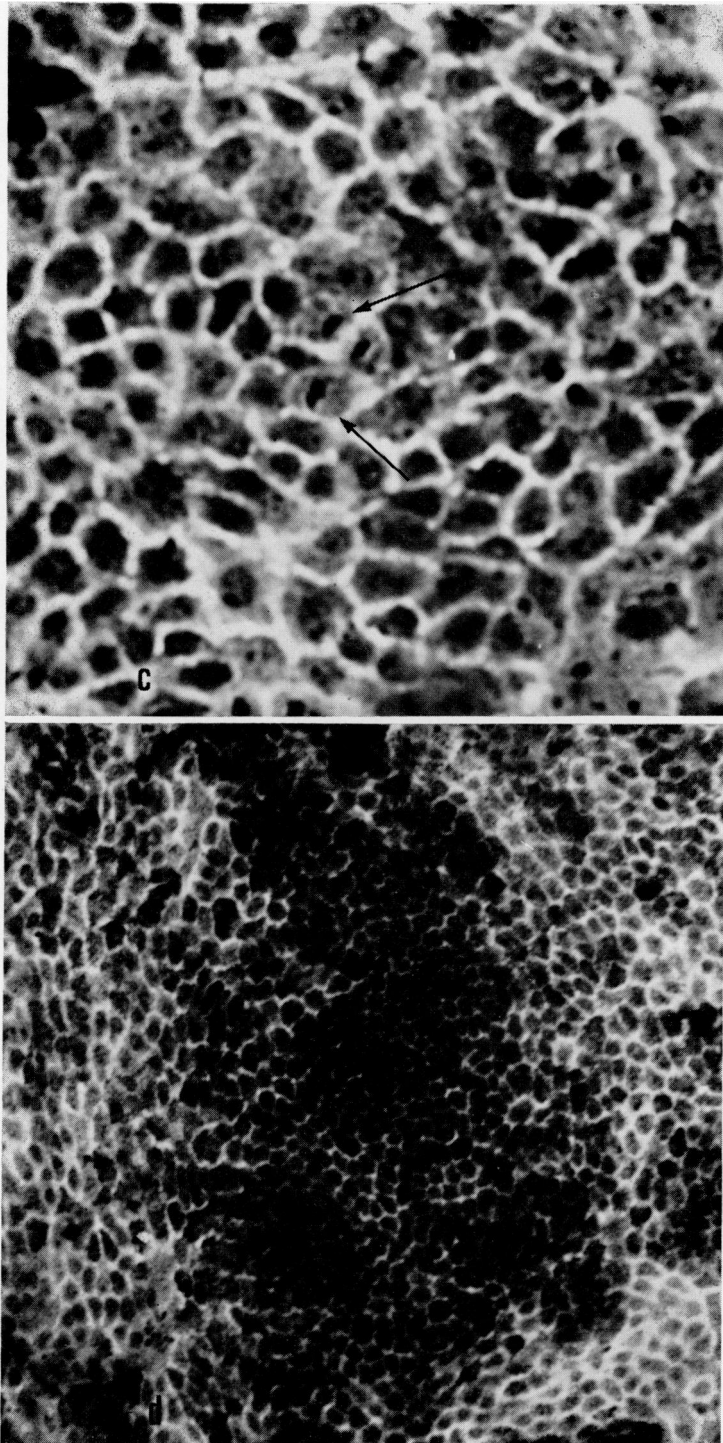


FIG. 1c and d. (c) 8-day culture at higher magnification. Arrows show cells in mitosis with newly synthesized pigment. 200 $\times$ . (d) Cells cultured 11 days. The focal nature of pigmentation is evident. 100 $\times$ .

common and appear not to divide. If the surfaces of the culture plates are not coated with collagen, fewer cells attach, and those which do grow more slowly. As a result, cultures on noncollagenized plates become confluent later and make less pigment than cultures grown on collagen.

At the end of the fourth day (Fig. 1b), the cultures are nearly confluent, but the cells are still quite flat. Where growth is most rapid, foci appear. These are groups of many small, closely packed cells, from which pigment has been diluted early. Large, flattened, often binucleate cells situated outside the foci, contain large amounts of pigment, probably because they do not divide. Pigment granules in these cells are rod shaped, like the granules in the chick retina *in situ*, whereas granules produced by cells in cultures are round [cf. Cahn and Cahn (1)].

By the sixth day, areas are apparent where pigment synthesis has commenced. New pigment is always visible first in closely packed, small cells within the growth foci, which by the end of the first week have expanded, so that the aggregate of cell foci form large mosaic patterns (Fig. 1d). Mitotic figures are seen most often in cells which border the pigmented areas of the foci. Deeply pigmented cells rarely, if ever, exhibit mitotic figures, but cells which have some amount of newly synthesized pigment, i.e., small, closely packed cells with round pigment granules, are often seen to be in some phase of mitosis (Fig. 1c). Although hematoxylin was used as a general cytological stain, it was clear that differences in cytoplasmic density arose primarily from contained melanin granules and not from significant differences in basophilia.

By the beginning of the second week, the cells of the foci have become very small and dense, and large, richly pigmented areas are visible (Fig. 1d).

*Accumulation of DNA and melanin.* Figure 2a shows the accumulation of DNA and melanin in two representative culture runs as a function of time in culture. Initially DNA content rises sharply while the amount of melanin increases only a little. During the second week, the rate of accumulation of DNA decreases, and the melanin content rises sharply. It is apparent that, even during early stages, melanin is increasing in the cultures and the dilution of melanin, which is evident histologically, is due to the disparity between the rates of cell division and melanin synthesis. The biochemical evidence suggests only that, statistically, cells produce melanin at a very low rate approximately until the time when their rate of growth slows.

*Rate of melanin synthesis and accumulation of DOPA oxidase.* The rate of incorporation of L-tyrosine- $^{14}\text{C}$  into pigment cells when standardized to the amount of DNA present per culture corroborates the evidence that DNA synthesis is occurring more rapidly than melanin synthesis in early cultures (Fig. 2b). At the end of the first week, the amount of L-tyrosine- $^{14}\text{C}$  incorporated into melanin per unit of DNA has dropped to a minimum. After this, it rises very rapidly. Concomitant with this increase is a sharp rise in the amount of dopa oxidase activity present in the 20,000 g supernatants from a comparable culture series. After 2 weeks of culture the rates of incorporation of L-tyrosine- $^{14}\text{C}$  into both melanin and protein decrease, as does dopa oxidase activity.

*The stability of newly synthesized RNA and of protein synthesis in the presence of actinomycin D.* When newly made bulk RNA is chased in the presence of an amount of actinomycin D which inhibits 98% of RNA synthesis (1.25  $\mu\text{g}/\text{ml}$ ), it exhibits a multiphasic pattern of decay (Fig. 3a). This pattern remained multiphasic when the data were plotted semilogarithmically. No matter what the age

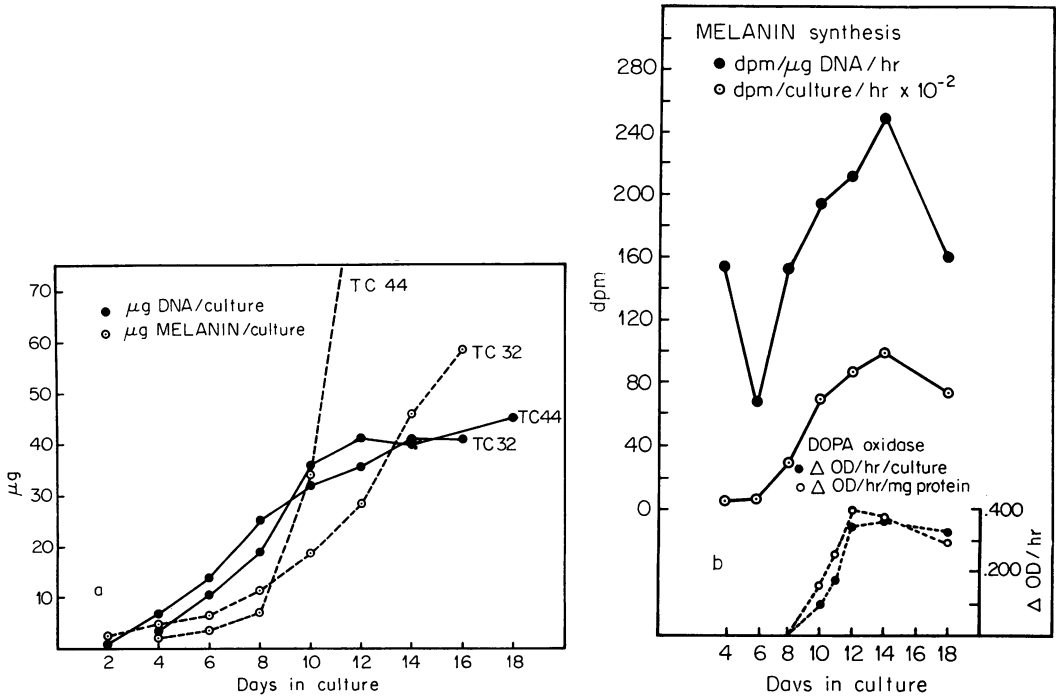


FIG. 2. (a) The accumulation of DNA and melanin in two pigment cell cultures (TC 32 and 44). (b) Rate of melanin synthesis and DOPA oxidase activity in pigment cultures.

of the culture, one-half of its newly synthesized RNA has decayed in less than an hour, a second portion disappears at a slower rate, with an average half-life of several hours and, finally, at about 6 hr, a population of stable molecules becomes evident. If we assume an average half-life of several hours for animal cell mRNA (22-24), the second phase exhibited by these pigment cells may represent primarily the decay of mRNA's, and the first may be due largely to the very rapidly turning over RNA's found by investigators in the nuclei of animal cells (25-27).

To investigate further the possibility that mRNA is represented by the nonstable but slowly decaying fraction, cultures were incubated with 1.25  $\mu\text{g/ml}$  actinomycin D for various times followed by measurement of the rate at which they incorporate radioactive precursors into protein. The rate of incorporation in both 4- and 12-day cultures decayed with a half-life of a little more than 4 hr (Fig. 3b).

The possibility exists that certain proteins of particular interest, the melanogenic proteins, tyrosinase and any others participating in melanosome structure, may depend upon long-lived messages for their synthesis during late stages when growth has slowed (see discussion). However, because they would represent a small proportion of the population of newly made protein, the presence of a small amount of stable mRNA for their synthesis might be completely masked when total RNA decay or general protein synthesis is under consideration. In order to detect such a minor stable component, a more specific assay than total RNA decay or general protein synthesis would have to be employed whereby one could measure either the synthesis of that protein directly or some functional aspect of it after RNA synthesis is blocked. Tyrosinase and any other proteins which may be necessary



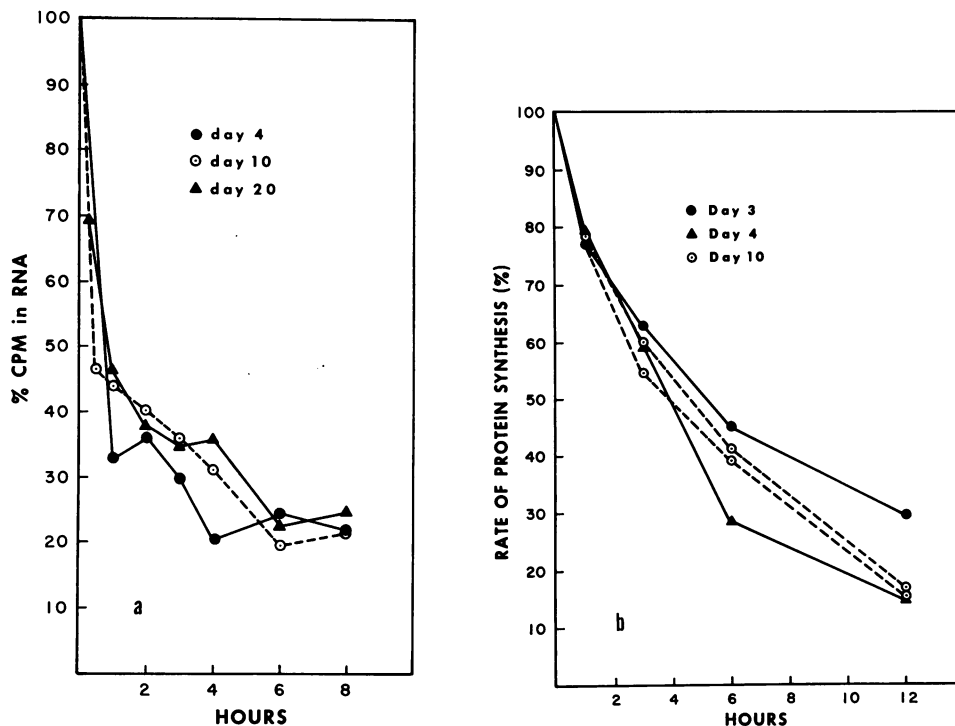


FIG. 3. The rate of decay of (a) RNA and (b) protein synthetic rates in cultured pigment cells in the presence of actinomycin D ( $1.25 \mu\text{g/ml}$ ). Control rates equal 100%.

for pigment synthesis are difficult to isolate or unknown, but can be defined by a specific and easily measurable function, melanin synthesis.

In the presence of sufficient cycloheximide, an inhibitor of protein synthesis, melanin synthesis decayed with a lifetime of about 10 hr (Fig. 4a). If the additional time required for decay of the rate of general protein synthesis in the presence of actinomycin D is also considered, the lifetime of messages specific for melanogenic proteins can be estimated. Seiji *et al.* (28) believe that tyrosinase decays *in vivo* as a direct consequence of the deposition of melanin on melanosomes.

Eighteen to twenty hours in actinomycin D, which corresponds to the time (8–10 hr) required for cycloheximide to reduce the melanogenic rate to the basal level (Figs. 4a and 4b) plus the 8–10 hr required for actinomycin D to reduce protein synthesis to the basal level (Fig. 4b) results in a melanogenic rate equal to or lower than the rate of protein synthesis (Fig. 4b). This indicates that the melanogenic proteins are similarly affected by cycloheximide and actinomycin D, i.e., that melanogenic proteins are probably not manufactured on mRNA's of any extraordinary stability, but instead that the message for melanogenic proteins and "average" cell proteins are of similar stability.

*Kinetics of melanization.* Electron micrographs (8, 29) indicate that melanosomes *in situ* attain a high level of internal organization before they become competent to commence melanization. Moyer (8) points out that the proportion of melanosomes in any one stage of melanosome maturation should be a function of the rate at which melanosomes pass through that stage. He reported that 80–85% of the melanosomes in pigmenting mouse retinal epithelial cells are in stage 3, i.e.,

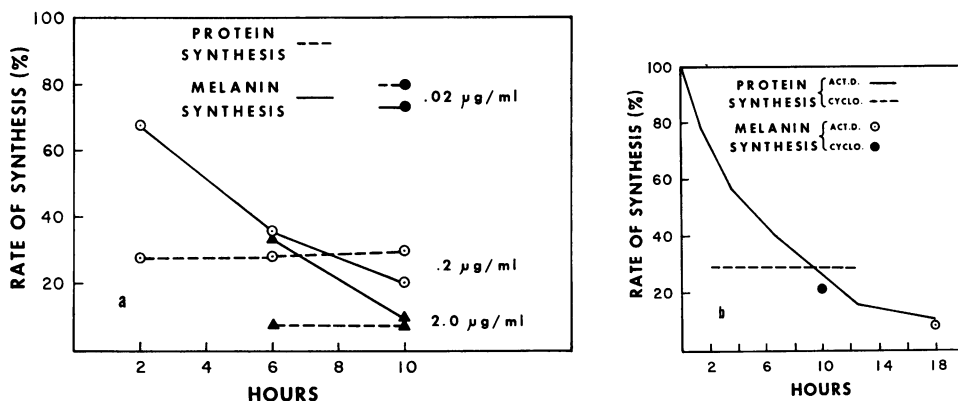


FIG. 4. The decay of protein and melanin synthetic rates in cultured pigment cells in the presence of (a) cycloheximide (.02, 0.2, and 2.0 µg/ml) and (b) cycloheximide (0.2 µg/ml) or actinomycin D (1.25 µg/ml). The points from which the lines in Fig. 4b were plotted indicating the affect of actinomycin D (—) and cycloheximide (---) on protein synthesis, are not shown for purposes of simplicity.

actively depositing melanin, while 15% or fewer are in stages 1 and 2, i.e., their matrices are still free of any melanin deposition. A knowledge of the time necessary for either melanosome construction or melanization might allow an estimate of the other.

If the assumption that the inhibition of protein synthesis by cycloheximide prevents formation of new melanosomes and prevents completion of noncompetent melanosomes is valid, then melanization in the presence of cycloheximide should reflect the maturation rate of competent melanosomes. The time required for melanosome formation may then be approximated from the ratio of premelanizing to melanizing melanosomes in the total melanosome population.

Since the time required for cycloheximide to reduce melanogenic rates to protein synthetic rates is about 10 hr (Fig. 4a), it takes about this time for a newly competent melanosome to melanize. If melanosomes of actively differentiating cells spend 15–20% of their time in the formative stages [Stages 1 and 2; Moyer (8)], it should require about 2 hr for a melanosome to pass through these formative stages. This is in good accord with the findings of Seiji *et al.* (28) that it takes about 2 hr to saturate the large particle fraction (melanosomal) tyrosinase in melanomas with leucine-<sup>14</sup>C.

When, after 10 hr, the cycloheximide-containing medium is removed from cultures and replaced with fresh medium not containing cycloheximide, the rate of protein synthesis rapidly rises to a higher level than in controls, and after about 8 hr melanogenesis reaches a comparable level.

*Localization of tyrosinase activity in cell fractions.* If melanogenic proteins are synthesized on polyribosomes and are transformed by the interaction of nascent protein chains into incipient melanosomes, as Moyer (8) implies, polyribosomes isolated from pigment cells might exhibit dopa oxidase and/or tyrosinase activity. Furthermore, if these polypeptides interacted to initiate melanosome formation while still bound to ribosomes by sRNA, these ribosome complexes might be prevented from disaggregating into single ribosomes upon RNase treatment. Consequently, dopa oxidase and tyrosinase assays were performed on fractions of pigment cell homogenates isolated from sucrose density gradients with the expectation

TABLE 1  
DISTRIBUTION OF TYROSINASE IN CENTRIFUGAL FRACTIONS OF PIGMENT CELLS

(a) Distribution of tyrosinase in cell fractions <sup>a</sup>		
Fraction	Tyrosinase activity (cpm)	% Tyrosinase activity
LSP	14959	93.2
HSP	1093	6.8
HSS	0	0
(b) Distribution of tyrosinase. <sup>b</sup>		
Fraction	Tyrosinase activity (cpm)	% Tyrosinase activity
LSP	20383	94.9
LSS	1102	5.1

<sup>a</sup> LSP, low speed pellet (10,000g for 10 min); HSP, high speed pellet (109,000g for 1 hr); HSS, high speed supernatant (109,000g for 1 hr).

<sup>b</sup> LSS, low speed supernatant which after 10,000g for 10 min, was respun at 15,000g for 10 min, and 20,000g for 20 min to reduce contamination from LSP. LSS is the equivalent of HSP + HSS of the previous experiment (Part a).

that if any such activity could be demonstrated, particularly in a specific polysome fraction, the homogenates could then be treated with low concentrations of RNase to investigate the association of ribosome bound activity. Kretsinger *et al.* (30) found that ribosomes synthesizing collagen did in fact demonstrate resistance to RNase.

Initially 3 fractions from pigment cells of an advanced culture age were obtained by differential centrifugation and assayed for tyrosinase activity (Table 1a). Most of the activity was located in the low-speed pellet (10,000 g, 60 min) which presumably contains most of the melanosomes, and no activity was found in the high speed supernatant (109,000 g, 60 min). The high-speed pellet (109,000 g, 60 min) which should consist primarily of ribosomes and polyribosomes contained about 7% of the total tyrosinase. Even if the low speed supernatant was centrifuged several more times (Table 1b), it retained most of its activity. This could be due either to intrinsic ribosomal tyrosinase or to contamination from the melanosome fraction.

When the low-speed supernatant was fractionated by sucrose density gradient centrifugation, it displayed the typical ribosomal pattern: a large well-resolved, slow moving peak, presumably single ribosomes, and a faster moving heterogeneous zone, which was sensitive to RNase. Dihydroxyphenylalanine oxidase or tyrosinase activity could not be unequivocally demonstrated in specific fractions from any region of the gradient, but a low level of PTU-sensitive incorporation occurred in all parts of the gradient, indicating perhaps that very large polysomes containing tyrosinase had been degraded to polysome fragments of random sizes. No L-tyrosine-<sup>14</sup>C was incorporated into the postribosomal fractions.

*Electron microscopy.* Cultured retinal pigment cells display a well developed Golgi apparatus, profiles of rough surfaced endoplasmic reticulum, free ribosomes and ribosomal clusters, dense mitochondria of different sizes and shapes, numerous vesicles, polymorphic nuclei, and pigment granules in various states of development (Figs. 5 and 6).

The pigment granules are bounded by membranes even when fully melanized (Fig. 5). Partially melanized granules demonstrate a subgranularity or nonamorphous nature, as if the melanin had been deposited upon them at particular sites, giving rise to melanin "units" [Drochmans (31)]. Premelanosomes or non-



FIG. 5. Electron micrograph of pigment cell cultured 12 days. Note membrane bounded melanin granules (m), Golgi (g), and incompletely melanized granules (im). 10,000 $\times$ .

melanized melanosomes demonstrate the typical substructure (Fig. 6): closely packed fibers of a periodic or helical nature, bounded by a membrane.

Most salient to the considerations of the present study, however, is the nature of the rough surfaced endoplasmic reticulum. The cisternae of this structure contain a substrate of granular or periodic nature and higher electron density than the cytoplasm. Forms exist that may represent intermediates in the transition of endoplasmic reticulum to melanosomes (Figs. 6 and 7), i.e., the cisternae are more dilated, the dense material within them is more ordered, and the ribosomal content of the membranes is diminished.

## DISCUSSION

*Morphological and biochemical aspects of growth.* As in other culture systems (32–37), the pigment cells in the present investigation exhibited a degree of independence of the growth and differentiative processes. As with cartilage (38), the processes are not totally exclusive in individual cells, since cells with newly made pigment could be seen in mitosis or preparing for division (incorporating thymidine-<sup>3</sup>H into their nuclei). Whittaker (2, 3) also describes the separation of growth and differentiation in cultured retinal pigment cells, and there are numerous *in vivo* examples of pigment cells of the retinal epithelium (39, 40) and of the iris (41) losing their melanotic characteristics during regeneration of the sensory retina and lens, respectively.

Because of the manner in which the isolated cells attach to the tissue culture plates, i.e., as randomly distributed single cells or small clumps of cells, the growing culture population in a short time becomes heterogeneous with respect to various aspects of differentiation and remains so for the extent of the culture period. Cells which are located at the center of a growing focus cease division and begin to differentiate early, while cells near the periphery of the focus do so later.

The biochemical data representing the accumulation of DNA and melanin also demonstrate the incomplete separation of growth and differentiation or, in this case, cell division and the attainment of epithelial appearance and pigment production. After the rate of accumulation of DNA has begun to decrease at about 8 days, melanin accumulation and the rate of L-tyrosine-<sup>14</sup>C incorporation into melanin increases dramatically with a concomitant increase in dopa oxidase activity. The morphological evidence shows that the two processes, which overlap in the total cell population, also occur simultaneously in individual cells. Growth, as represented by cell division and the accumulation of a cell specific macromolecular product, are not necessarily exclusive but probably are separated in time and space owing to their dependence on quite different environmental conditions, such as cell population density.

The close relationship between cellular morphology and differentiated cell function is particularly striking in these cells. Only cells which are epithelioid, i.e., are small and adhere closely to each other, eventually produce pigment. Cahn and Cahn (1) claimed to be able to maintain cells in a pigmented state during rapid growth, perhaps because their culture conditions kept cells small and closely adherent, a condition which is apparent in their photographs and which they stress.

*Translational level controls of protein synthesis.* Considering the similarity of the rate of decay of newly synthesized RNA and the decrease in protein synthetic rates in the presence of actinomycin D in both rapidly and slowly growing cells, the average stability of mRNA's for general proteins appears to be independent of the proliferative condition of the cells. Regardless of whether or not these cells are rapidly dividing, i.e., independent of their physiological state, the half-life of their mRNA ranges between 4 and 4½ hr, a figure consistent with measurements made for other kinds of animal cells (22, 23).

The evidence that the lifetime of messages for proteins specific to the differentiated cell, in this case tyrosinase and any other proteins necessary for melanogenesis, is similar to that for general proteins in these cells is interesting in view of what has been described for other differentiating cells. In reticulocytes (42, 43), lens fibers (44–46), and keratinizing epidermal cells (47), proteins seem to be

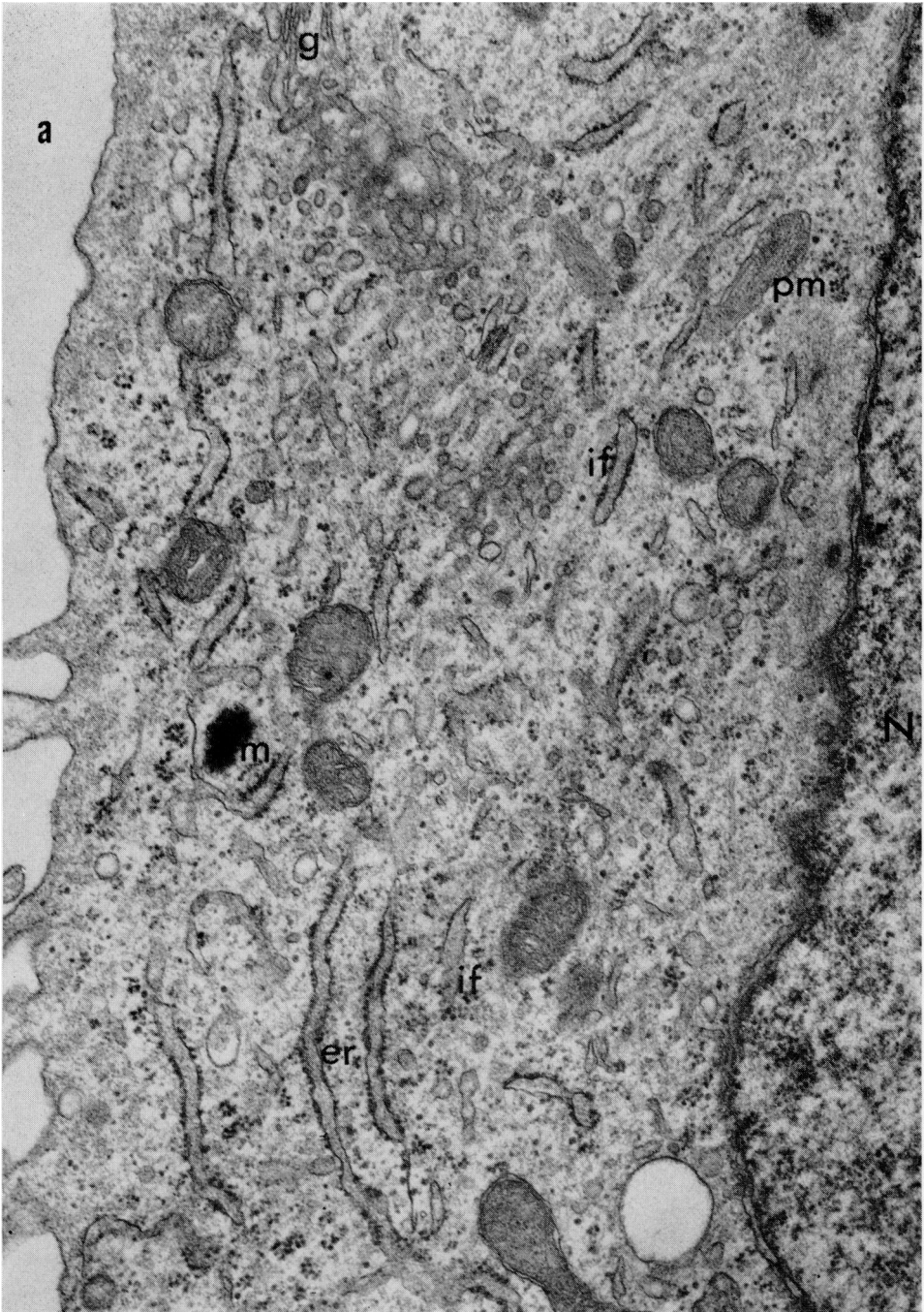


FIG. 6a. Electron micrograph of 12-day cultured pigment cell at earlier stage of development than Fig. 5. It contains more rough endoplasmic reticulum (er) and fewer melanin granules (m). Note "intermediate forms" (if) which may represent a stage in premelanosome (pm) development from cisternae of the endoplasmic reticulum. Golgi (g). 33,000 $\times$ .

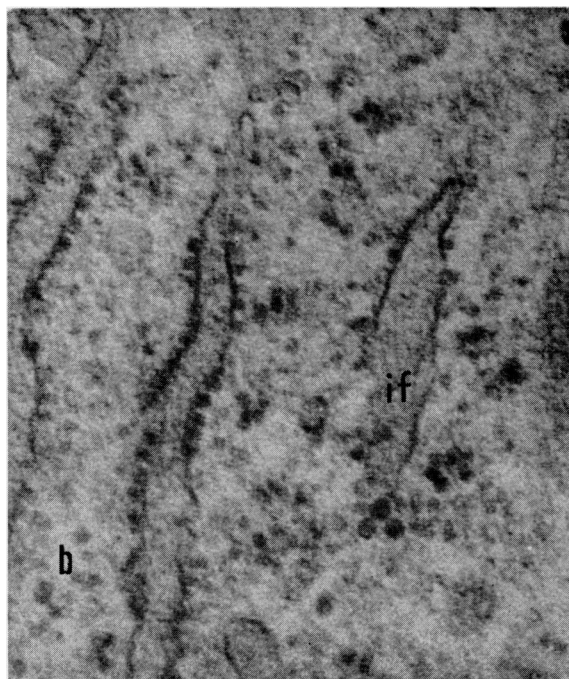


FIG. 6b. Intermediate form (if) from Fig. 6a at higher magnification. 99,000 $\times$ .

manufactured on relatively long-lived mRNA's (greater than 24 hr). In these cases, however, the cells are terminal; either their nuclei or the cells themselves curtail metabolic activity, e.g., RNA synthesis and oxidative metabolism, while their major protein components are being synthesized. Pigment cells, however, are nonterminal, actively metabolic, and totally viable as evidence by their ability to regenerate rapidly *in situ* or in cell culture. It is not surprising, therefore, to find that, allowing for the time of decay of melanogenic capability, actinomycin D and cycloheximide affect melanogenesis to the same degree. It may be inferred that melanogenic proteins are made on mRNA's with an average half-life of about 4 hr.

Considering other nonterminal cell types, Bloom *et al.* (22) found collagen synthesis in cultured fibroblasts to depend on a relatively short lived mRNA with a half life of about 3 hr, while Wilson *et al.* (48) claimed that rat liver albumin is synthesized on a mRNA of 32 hr half-life. The criteria for the identification of melanogenic capacity (melanin synthesis) and collagen (hydroxyproline) synthesis, however, is probably more rigorous than that for albumin synthesis, the latter being radioactivity that migrates with albumin upon acrylamide gel electrophoresis.

According to Scott and Bell (45), only terminal cell types, those which produce some specific product at a time when they are becoming metabolically inert, produce long-lived mRNA. Kafatos (49) suggests that mRNA stability lightens the transcriptional load that genes must carry. Kinetics dictates that a single-copy gene can only carry a large proportion of total cellular protein synthesis by producing long-lived mRNA. This appears to be the situation in gland cells of the moth *Galea*, where cocoonase zymogen at times constitutes over 50% of cell protein synthesis and depends on messages of about 100 hr half-life compared to other proteins, whose synthetic rate diminishes to one-half after actinomycin D treatment of 2½



hr. This might also be important in hemoglobin-producing reticulocytes, keratinizing skin, and crystallin-producing corneal cells.

*Kinetics of melanization.* Because of the asynchronous nature of melanosome formation and melanin deposition in cell populations, and even within individual cells, it is difficult to ascertain morphologically the length of time required for these events. Electron micrographs of various kinds of pigmenting cells show melanosomes in various phases of formation and pigmentation (5, 6, 3, 29, 50). If protein synthesis is required for melanosome formation but not for melanization, then cycloheximide might very well isolate the processes of melanization from those of melanosome formation. Since melanin synthesis drops to the basal level of protein synthesis after 8–12 hr in the drug, all melanosomes which were competent for melanization at the time of addition of cycloheximide have been exhausted during the period, assuming that no new melanosomes were formed.

From the electron microscopic evidence of Moyer (8) concerning the proportion of premelanosomes (no pigment) to pigmenting melanosomes, we might estimate that several hours are required for the construction of the competent melanosome.

The fact that cycloheximide is not toxic either to cells or to their melanizing activity is supported by the reversibility of protein synthesis and melanogenesis after cycloheximide treatment.

*Melanosome origin.* Various sites have been proposed as the precursor of the melanosome, among them the nuclear membrane, mitochondria, and Golgi vesicles [reviewed by Moyer (8)]. Electron microscopic evidence (5–7, 9, 10) has added much weight to the argument for Golgi origin, particularly in pigment cells of neural crest origin. This does not preclude the probability that melanosomal proteins are synthesized on ribosomes and then transferred to the Golgi zone for packaging into vesicular units [Caro (51)].

Alternatively, Moyer suggested that the retinal pigment epithelium may accomplish this packaging within intracisternal dilations of the endoplasmic reticulum with which ribosomes are associated. Later he published electron micrographs which intimated that the melanosomal matrix of retinal pigment cells may form free in their cytoplasm after release of their protein "building blocks" from ribosomes which were not membrane-attached.

Most of the evidence for the Golgi origin of melanosomes or packaging of melanosomal proteins is dependent upon the existence of young melanosomes in Golgi areas. The electron micrographs in the present study demonstrate structures which appear to be intermediate between rough surfaced endoplasmic reticulum and premelanosomes in cells which also contain a well developed Golgi apparatus. The intermediate forms (see results) begin to resemble melanosomes within the same cell. One might postulate that the dense network of ribosomes that line the endoplasmic reticulum produce the structural melanosomal proteins which enter the cisternae and interact to form the definitive matrix. As the matrix grows by accretion due to the entry of more protein, the cisternae of the reticulum may swell and lose their ribosomes as their membranes become the bounding membranes of the maturing melanosomes.

The present study and Moyer's (8) utilize retinal pigment cells rather than neural-crest-derived pigment cells used in most investigations. Perhaps they have arrived at different means of constructing melanosomes during phylogeny. It is noteworthy that retinal pigment cells do not normally secrete pigment granules as do pigment cells of the skin.



In any event, Seiji *et al.* (28) have shown that the kinetics of incorporation of L-leucine- $^{14}\text{C}$  into tyrosinase derived from the small- and large-granule fractions of mouse melanoma cells is consistent with the hypothesis of transfer of tyrosinase from ribosomes to melanosomes.

The complete absence of tyrosinase activity in the postribosomal supernatant from homogenates of cultured retinal pigment cells could be due to either the absence of the protein from the soluble portion of the cellular cytoplasm or its presence there in an inhibited form. The existence of inactive protoxyrosinase has been firmly established (52, 53). Most of the small amount of ribosomal tyrosinase activity disappears when the ribosomes are further purified by sucrose density gradient fractionation. Although the role of inactivation cannot be ruled out, it is likely that some tyrosinase activity may have been released to the ribosomal (109,000 g) pellet by the disruption of active melanosomes, despite the gentle homogenization procedure used. None was solubilized.

Since intracellular melanization occurs only on a large structural element, the melanosome, it is not unexpected that ribosomal tyrosinase activity could not be seen in cell-free fractions. The small amount of L-tyrosine- $^{14}\text{C}$  incorporating activity dispersed heterogeneously throughout the density profile and the high ratio of  $\text{OD}_{260}$  in the monosome peak to  $\text{OD}_{260}$  in the polysome areas of the density profile suggest that polysomes were broken. More careful polysome preparation coupled with means of identifying tyrosinase other than by enzyme activity, e.g., immunochemistry, might allow us to question experimentally the manner in which the enzyme and any other proteins with which it becomes associated enter the melanosomal complex.

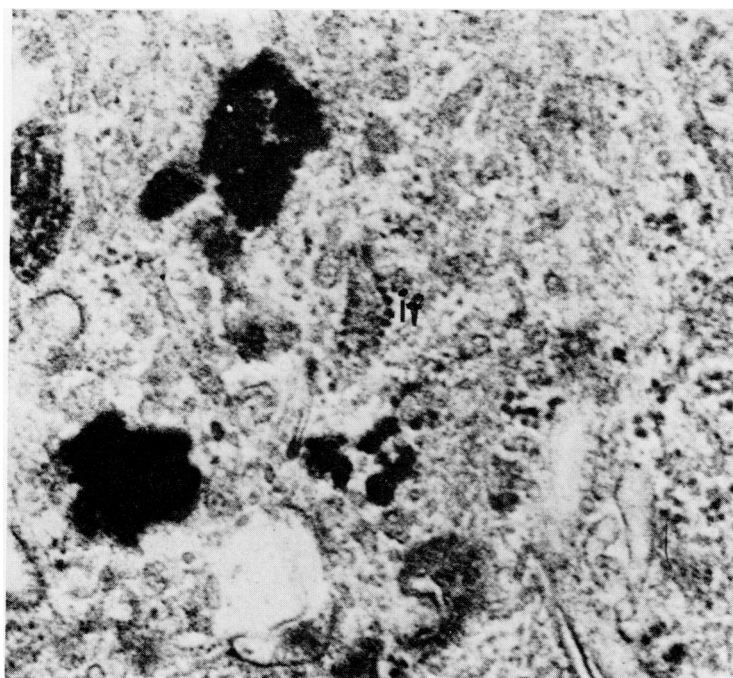


FIG. 7. High magnification of area in a cell showing intermediate form (if) with thicker fibrils.  $60,000\times$ .

## ACKNOWLEDGMENT

I thank Dr. John R. Coleman and Dr. Annette W. Coleman for their assistance and encouragement.

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