Genetic Variations in the Fine Structure and Ontogeny of Mouse Melanin Granules.

FRANK H. MOVER
Department of Zoology, University of Illinois, Urbana, Illinois

SYNOPSIS. Electron microscopic observations of the fine structure and ontogeny of melanin granules in various inbred strains of mice have provided new information about the origin and structure of these organelles. The general features of granule development, consisting of the aggregation of subunits into fibers and the cross-linking of these fibers to form a sheet-like matrix on which melanin is deposited, are shared by all but one of the genotypes examined. The formation of the matrix sheet is coincident with the deposition of melanin which begins at periodic intervals along the matrix sheet. The deposition of melanin continues until the details of the underlying matrix are obscured.

The alleles at the B, C, and P loci alter the sequence of development of melanosomes in a manner suggesting that they are structural genes for different macromolecules contained in the subunits. This theory helps to integrate diverse observations on the origin of melanin granules and the genetic control of their fine structure.

The effects of alleles at the D and Ln loci indicate that these loci alter the differentiation of the melanocyte by somehow altering the mechanisms controlling the location within the cell where matrix protein begins to be synthesized. The major effect of the Ru locus is to alter the time of onset of synthesis of melanin granules; thus, it may be a regulatory gene. These observations demonstrate the importance to cellular differentiation of genetic mechanisms that alter the expression of other genes by changing time and space relationships in protein synthesis.

The A locus appears to act by altering the chemical environment in which the melanocyte of the hair follicle is differentiating. The effect of this alteration is on the aggregation of the matrix subunits which the melanocyte is producing. The altered aggregation leads to different enzymatic activity, and phaeomelanin is produced instead of eumelanin. Thus, the path along which a melanocyte from a hair follicle is differentiating may be altered at the level of aggregation of newly-synthesized protein, a fact which has interesting implications regarding embryonic induction.

INTRODUCTION

The differentiation of vertebrate melanoblasts into melanocytes has been studied extensively by developmental biologists because of the unique susceptibility of this differentiating system to experimental intervention. Likewise, the extreme malignancy of vertebrate melanomas has been the subject of extensive research by oncologists. The investigations by these two groups of biologists have provided abundant information about the biochemistry, embryology, and genetics of melanocytes and melanin production. This information has been thoroughly and frequently reviewed (DuShane, 1943, 1944; Rawles, 1948, 1953; Lerner, 1953; Fitzpatrick and Kukita, 1959; Billingham and Silvers, 1960; Silvers, 1961; Wilde, 1961; Swan, 1963).

Synthesis of melanin granules is the characteristic event in the differentiation of a melanoblast into a melanocyte. Consequently an analysis of the mechanisms controlling the origin and ontogeny of melanin granules should provide basic information on the control of melanoblast differentiation. In the last decade techniques of cell fractionation and electron microscopy have provided a means for studying these problems with a resolution heretofore unattainable in investigations of cellular differentiation. As a consequence, more than 30 papers have been published which deal in whole or in part with the fine structure and origin of melanin granules. This work, too, has
PLATE 1. Whole mounts of mouse melanocytes. All are fixed in glutaraldehyde and embedded in EPON-812. They are unstained and the pigment cells are visible because of the melanin they contain. All of these preparations were obtained from a C57 Bl/6 mouse aged 5 days post partum.

FIG. 1. Hair follicles.

FIG. 2. a. Hair bulb, longitudinal section. b. Hair bulb, transverse section.

FIG. 3. Eyelid showing two populations of melano-
Table 1. Strains of mice used as sources of material (see text).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype*</th>
<th>Origin of stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
<td>aa</td>
<td>Hopkins</td>
</tr>
<tr>
<td>C57Bl/cd</td>
<td>aabb</td>
<td>Jackson</td>
</tr>
<tr>
<td>SEC</td>
<td>aabbccc</td>
<td>Jackson</td>
</tr>
<tr>
<td>CE</td>
<td>A^bA^bCcC</td>
<td>Jackson</td>
</tr>
<tr>
<td>Himalayan albino</td>
<td>aabbCc</td>
<td>Jackson</td>
</tr>
<tr>
<td>A/Jax</td>
<td>aabbcc</td>
<td>Hopkins</td>
</tr>
<tr>
<td>Swiss albino</td>
<td>aacc</td>
<td>Hopkins</td>
</tr>
<tr>
<td>Stock 21</td>
<td>aapp</td>
<td>Jackson</td>
</tr>
<tr>
<td>BLN</td>
<td>aapp</td>
<td>Hopkins</td>
</tr>
<tr>
<td>C57 leaden</td>
<td>aabbbluu</td>
<td>Jackson</td>
</tr>
<tr>
<td>BLN</td>
<td>aabbbluu</td>
<td>Hopkins</td>
</tr>
<tr>
<td>DBA/1</td>
<td>aabbdd</td>
<td>Jackson</td>
</tr>
<tr>
<td>Black ruby</td>
<td>aaruru</td>
<td>Hopkins</td>
</tr>
<tr>
<td>Brown ruby</td>
<td>aaruru</td>
<td>Hopkins</td>
</tr>
<tr>
<td>C57Bl/6-A'</td>
<td>A'a</td>
<td>Jackson</td>
</tr>
</tbody>
</table>

* Only mutant genes are listed.

† "Hopkins" indicates that the mice were obtained from strains maintained at Johns Hopkins University. "Jackson" indicates that the mice were obtained directly or were extracted from stock maintained at the Jackson Memorial Laboratory, Bar Harbor, Maine. Some of the Jackson mice were from special pigment stocks developed and maintained by Dr. E. S. Russell under Grants E-76 and E-162 from the American Cancer Society.

Genetic Variations in Melanin

been reviewed frequently (Barnicot and Birbeck, 1958; Birbeck and Barnicot, 1959; Birbeck, 1962, 1963; Moyer, 1961, 1963; Wellings and Siegel, 1959, 1963). The majority of investigators agree upon the gross features of fine structure in melanin granules, but divergent theories of origin have been presented (Dalton, 1959; Birbeck, 1962, 1963; Seiji, et al., 1963; Moyer, 1963; Wellings and Siegel, 1963).

A number of the attributes of mouse melanin granules are genetically determined (Russell, 1946, 1948, 1949a, 1949b). Many of the controlling genes act within the differentiating melanoblast, but others act in cells comprising the tissue environment in which the melanoblasts are differentiating (Silvers and Russell, 1955; Markert and Silvers, 1956, 1959; Silvers, 1958a, 1958b). Therefore, an unusually refined control of differentiation in melanin granules can be obtained in mice of selected genotypes. Observation of melanin granules from mice carrying different genes affecting granule attributes reveals obvious fine structural differences. These differences in fine structure, when analyzed in the framework of current theories of biochemical genetics, lead to important conclusions concerning the origin of the granules. Moreover, a comparison of the ontogeny of melanin granules in mice carrying these different genes provides insight into the mechanisms by which genes control the differentiation of organelles and thus influence cellular differentiation.

In this paper the effects on ontogeny and fine structure of melanin granules of genes in six different allelic series known to act within the melanoblast and one allelic series known to act in the cellular environment are discussed.

Materials and Methods

Mice were raised in the colonies at the Department of Biology and the Department of Anatomy, Johns Hopkins University, and the University of Illinois, or were purchased from the Jackson Memorial Laboratory, Bar Harbor, Maine. Certain strains were the gift of Dr. Elizabeth S. Russell of Roscoe B. Jackson Memorial Laboratory. Pink-eyed blacks, black leadens, and ruby-eyed browns were obtained by appropriate breeding of available stocks. The stocks used, their genotypes, and origins are listed in Table 1.

Embryonic age was estimated from the day of the first appearance of the vaginal plug (see Snell, 1956). Since mice generally mate about midnight, the embryos were assumed to be 0.5 days old at noon on the day of first appearance of the vaginal plug in the mother.

A variety of tissues containing melanocytes of neural crest origin has been examined, including hair follicles, skin of...
PLATE 2. FIG. 9. The wall of the eye of a C57 Bl/6 mouse 1 day post partum. The retinal pigmented epithelium (RPE) is on the right, separated from the choroid coat by the basement membrane (BM). The fibroblasts (FBR) of the choroid contain a well developed endoplasmic reticulum (ER) but in the melanocytes (NGD), which are derived from neural crest, the endoplasmic reticulum is breaking down and being replaced with free polysomes (P). Mitochondria (M) and various stages of developing melanin granules (M1, M2, and M3) may be seen. The melanin granules are usually contained in vesicles in tissues prepared in this manner and the vesicles may or may not be associated with the Golgi region. Fixed in glutaraldehyde, post-fixed in osmic acid, embedded in DER, stained with uranyl acetate.

The eyelids, the harderian gland, the aortic valve of the heart, and the choroid coat of the eye. In addition, the pigmented epithelium of the eye has been investigated carefully (see Figures 1 to 8). The wall of the eye has been used most extensively, because here it is possible to obtain sections which include both neural crest-derived melanocytes and cells of the pigmented epithelium, so that the effect of
different embryonic origins on the differentiation of melanocytes may be directly compared (Figure 9). Finally, synthesis of melanin granules has been studied also in the B-16, Harding-Passey, and Cloudman S91 mouse melanomas.

Tissues were fixed in a variety of different fixatives and embedded in various methacrylates, polyesters, and epoxy resins. The most satisfactory results have been obtained by the following procedure: tissues are fixed at 0° with 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4, followed by postfixation in buffered isotonic osmic acid. This material is then dehydrated very rapidly through graded ethanols at 0°, allowed to come to room temperature, and embedded in either EPON-812 (Luft, 1961) or Dow Epoxy Resin (Winborn, 1964). These two embedding media give comparable results but the latter is much easier to section.

The blocks were sectioned on an LKB Ultratome using 40° glass knives prepared with an LKB Knifemaker. Sections were mounted on carbon-stabilized, parlodion-coated, copper grids. Contrast was enhanced by treating the sections with uranyl acetate, phosphotungstic acid, potassium permanganate, lead acetate, lead subacetate, lead citrate, and ammonium molybdate. Of these, lead citrate (Reynolds, 1963) was the most useful. The sections were examined in the RCA EMU-3f or Hitachi HU 11a electron microscopes.

For fractionation of melanin-containing tissues with sucrose density gradients, the tissues were first homogenized in 0.25 M sucrose. The homogenates were then centrifuged at 500 × g for fifteen minutes to sediment the nuclei and debris. The supernatant solution was centrifuged at 10,000 × g to sediment the mitochondria and melanin granules in the so-called “large granule fraction.” This fraction was taken up in 0.25 M sucrose and floated on a density gradient made by layering 1.5 M, 1.55 M, 1.6 M, 1.8 M, 2.0 M, 2.2 M, 2.4 M and 2.6 M solutions of sucrose in a tube and allowing it to stand in the refrigerator overnight. The gradients with sample floating on top were spun to equilibrium in 90 minutes in the Spinco Model L preparative ultracentrifuge using the SW-39L swinging bucket rotor at a force of 130,576 × g (40,000 RPM). This procedure is a modification of the technique first described by Baker, et al. (1960).

Isolated granules were assayed for melanin-synthesizing activity by incubating them for 4 hours in a mixture containing 0.5 μC/ml of UL-C14-L-tyrosine in 0.1 M phosphate buffer, pH 6.8. It was necessary to include penicillin (Squibb Penicillin G Potassium; final concentration, 2 × 10^4 units/ml) and streptomycin (Squibb Dihydrostreptomycin Sulfate; final concentration, 20 mg/ml) to prevent the growth of microorganisms in the incubation media. After incubation in C14-tyrosine the granules were washed thoroughly and incubated for 12 hours in unlabeled tyrosine. Next they were washed in 1 N hydrochloric acid and distributed to preweighed, disposable planchettes. The planchettes were dried to constant weight, weighed, and counted in a Nuclear Chicago gas flow counter.

**OBSERVATIONS**

The fine structure and ontogeny of melanin granules

The observations leading to the recognition of stages in the development of melanin granules have been discussed in detail elsewhere (Moyer, 1959, 1961, 1963). Therefore, the general features of development in melanin granules are treated only briefly here to provide a background for the interpretation of the observations of genetic variation.

In the melanocytes of all of the strains of mice examined, the sequence of developmental events in forming melanin granules follows the same general pattern. Thin “unit fibers,” often contiguous with polysomes, aggregate to form “compound fibers.” During this early aggregation a membranous boundary is often evident. The membrane is a “unit membrane” and is often incomplete even in well fixed and embedded specimens (Figures 9 and 15). The shape of the melanin granule becomes apparent when the compound fibers ag-
PLATE 3. Fine structure of melanin granules.

FIG. 10. Melanin granules in the Harding-Passey melanoma. Arrows indicate subunits aggregating to form fibers. Fixed in osmic acid, embedded in Araldite, stained with uranyl acetate.

FIG. 11. Melanin granule matrix in retinal tapetum of C57 Bl/6 mouse, 3 days post partum. Fixed in osmic acid, embedded in EPON-812, stained with uranyl acetate.

FIG. 12. Melanin granule matrix in melanocyte of hair follicle of C57 Br/cd mouse, 5 days post partum. Fixed in osmic acid, embedded in Cardolite-Maraagas, stained with lead citrate.


FIG. 14. Melanin granule matrix in retinal tapetum of C57 Bl/6 mouse, 2.5 days post partum. Fixed in potassium permanganate, embedded in methacrylate. The permanganate has removed most of the melanin, revealing the underlying matrix. Asterisk indicates area of cross-linking. Note that fibers appear hollow.

FIG. 15. Melanin granule in retinal tapetum of C57 Bl/6 mouse 3 days post partum. Asterisk indicates region where fiber appears hollow. Arrow
aggregate by cross-linking. The cross-linked fibers form a matrix such that a longitudinal section reveals their parallel orientation and a cross section shows them "end-on." These details are evident in Figures 9 to 16 and 19 to 21. When melanin granules are embedded in methacrylate their fibrous nature is very evident. However, when they are embedded in epoxy resins they show a slightly different structure. Although the fiber axis is clearly evident under these conditions, the cross-linking is more apparent and groups of parallel fibers attached by cross-links appear to form sheets of matrix material (compare Figure 21 with Figures 11 to 13). As soon as the fiber orientation is complete, melanin is deposited on the matrix, and this deposition continues until the details of the matrix are obscured by the electron-dense melanin (Figures 9 to 18).

Inspection of the fibers in melanin granules at high magnifications reveals further structural details. The unit fiber is approximately 35 Å in diameter and generally appears as a loosely coiled helix with a variable pitch of several hundred Angstrom units. The compound fibers are approximately 90 Å in diameter. In longitudinal section they appear to be hollow cylinders with a lumen approximately 35 Å in diameter and walls approximately 35 Å thick (Figures 14, 15, 16, 20 and 21). In cross section they do not appear to be hollow.

The cross-links that form between compound fibers during their aggregation into matrix sheets are fairly uniform in size. They are approximately 100 Å apart and cross-linked by fibers approximately 35 Å in diameter (Figures 11, 12, 13 and 20). Cross sections of melanin granules show that this flat lattice-like sheet is rolled up like a rug to give the mature granule its three dimensional form (see M2 in Figure 9).

Only one mutant allele, b (brown), at this locus was examined. It is recessive to the "wild type" B (black). As the names imply, the chief effect of the locus is on the color of pigment, but size and shape of the melanin granules are also affected. When viewed with the electron microscope, the brown melanin produced in animals homozygous for b is flocculent and coarsely granular and in mature bb granules, at 15 days post partum, some underlying matrix structure often may be seen. On the other hand black (BB) melanin is very finely granular, almost appearing homogeneous, and even at 3 days post partum the underlying matrix structure is often obscured (Figures 17 and 18). The finely granular black melanin gives a much denser appearance to the melanin granule than does the flocculent brown melanin, and black granules tend to shatter more readily during sectioning than do brown granules.

Two types of melanin are produced in mice. Yellow melanin, or phaeomelanin, is produced only in melanocytes of hair follicles and here only when appropriate alleles at the A (Agouti) locus are present. All other melanocytes produce eumelanin which may be either black or brown. For convenience in following gene segregation during breeding, most of the mice used in this study were selected from strains homozygous for the a (non-agouti) allele at the A locus. Mice of this type produce only traces of phaeomelanin in the hair, and thus the action of genes affecting the eumelanin is readily apparent. To study the fine structure of granules of phaeomelanin, mice of the genotype A'-'aBB were used, since nearly all of their hair follicle melanocytes produce only phaeomelanin. All of the observations may be conveniently considered according to the genetic locus concerned and are summarized in Table 2.

The B locus (Linkage group VIII)

Only one mutant allele, b (brown), at this locus was examined. It is recessive to the "wild type" B (black). As the names imply, the chief effect of the locus is on the color of pigment, but size and shape of the melanin granules are also affected. When viewed with the electron microscope, the brown melanin produced in animals homozygous for b is flocculent and coarsely granular and in mature bb granules, at 15 days post partum, some underlying matrix structure often may be seen. On the other hand black (BB) melanin is very finely granular, almost appearing homogeneous, and even at 3 days post partum the underlying matrix structure is often obscured (Figures 17 and 18). The finely granular black melanin gives a much denser appearance to the melanin granule than does the flocculent brown melanin, and black granules tend to shatter more readily during sectioning than do brown granules.
PLATE 4. FIG. 17. A typical brown (aabb) melanin granule at maturity. Note the granularity of the melanin. Methacrylate; uranyl acetate stain.

FIG. 18. Longitudinal section (LS) and cross section (CS) of typical mature black melanin granules from an aaBBbrnu mouse. Note the near homogeneity and extreme apparent density of the melanin, as well as the rod shape. In spite of the difference in magnification in Figs. 6 and 7 the black ruby-eyed granules are seen to be smaller than the brown granule. This size difference is due to the ruby-eyed gene. Ordinary black granules (aaBB) are larger than brown granules (see text). Methacrylate, very light uranyl acetate stain.
Table 2. Major effects of mutant alleles on fine structure and ontogeny of melanin granules.

<table>
<thead>
<tr>
<th>Mutant allele*</th>
<th>Major effect of gene responsible for phenotypic characteristics</th>
<th>Major effect on fine structure and ontogeny of melanin granule</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Produces black melanin; rod-shaped melanin granules.</td>
<td>Melanin appears dense and homogeneous.</td>
</tr>
<tr>
<td><em>bb</em></td>
<td>Produces brown melanin; smaller oval-shaped melanin granules.</td>
<td>Melanin appears flocculent or coarsely granular.</td>
</tr>
<tr>
<td><em>c</em>c*bb</td>
<td>No observable effect.</td>
<td>No observable effect.</td>
</tr>
<tr>
<td><em>ch</em></td>
<td>Degree of pigmentation dependent on temperature.</td>
<td>No observable effect.</td>
</tr>
<tr>
<td><em>ce</em></td>
<td>Melanin granules smaller and fewer than <em>c</em>c*bb and <em>c</em>ch; therefore less pigment produced.</td>
<td>No observable effect.</td>
</tr>
<tr>
<td><em>cc</em></td>
<td>No detectable synthesis of melanin.</td>
<td>Ontogeny normal except melanin is not produced; melanin granules smaller and fewer than <em>cc</em>, <em>c</em>c<em>bb</em>, and <em>c</em>ch. No observable effect.</td>
</tr>
<tr>
<td><em>ln</em></td>
<td>No apparent effect on retinal pigmentation. Melanin granules clumped in neural crest-derived melanocytes.</td>
<td>Melanin granules in clumps often connected by strands.</td>
</tr>
<tr>
<td><em>dd</em></td>
<td>Clumping of melanin granules; granules smaller.</td>
<td>Delayed formation of &quot;limiting membrane&quot;; disorganization of fibers and irregular cross-linking.</td>
</tr>
<tr>
<td><em>pp</em></td>
<td>Melanin granules smaller, fewer, and irregular in shape.</td>
<td>No observable effect on structure other than size. Onset of pigmentation delayed.</td>
</tr>
<tr>
<td><em>rruv</em></td>
<td>Melanin granules smaller and fewer.</td>
<td>No compound fibers formed—no cross-linking, no apparent matrix.</td>
</tr>
<tr>
<td><em>Aa</em></td>
<td>Produces yellow melanin only in melanocytes of hair follicle; granules spherical.</td>
<td></td>
</tr>
</tbody>
</table>

* This table is a simplified presentation of the data. Only the effects of mutant alleles present in the homozygous condition are considered except for *Aa*. *A* is lethal in the homozygous condition.
† Terms such as "smaller" or "fewer" in this table are used with respect to the dominant (full color) allele at the locus under consideration.
‡ The genotype of these mice was *aaBbCcDdLnLpPpRuRu*. For simplicity in this table only mutant genes are indicated. Thus the entry *cc* indicates a genotype of *aaBbCcDdLnLpPpRuRu*.

In the retina black melanin granules vary in shape from long rods to spheres but in neural crest-derived melanocytes they are rod shaped. Brown melanin granules usually are oval or spherical. Size of melanin granules is extremely variable in the retinal tapetum, but brown granules are generally smaller than black granules in these cells. In melanocytes derived from neural crest, brown granules average about 0.5 μ × 0.8 μ while black granules average about 0.5 μ × 1.5 μ. The distribution of melanin granules within the cell is random regardless of genotype at this locus. These characteristics are sufficiently striking to indicate clearly which of these two genes is present in a homozygous condition in an animal, regardless of its genotype at the C, D, P, Ru, and Ln loci. (Since animals homozygous for albino do not make melanin, it is not possible to tell whether an albino carries genes for black or brown at the B locus by these criteria.)

2. The C locus (Linkage group I)

The C locus controls the total amount of melanin pigment produced in the animal. It affects the size and number of melanin granules produced within a given melanocyte, and one allele, at least, appears to affect the amount of melanin produced per melanin granule. The distribution of melanin granules within the melanocyte is random regardless of genotype at this locus; hence, the locus does not affect granule distribution. The alleles at this locus, in decreasing order of dominance, are: C ("wild type" or non-albino), *c*bb (chinchilla), *c*eh (extreme dilution), *c*ah (himalayan albino), and *c* (albino). Animals homozygous for each of the alleles were examined.

The sequence of developmental events in melanin granules is not altered by the alleles for chinchilla or extreme dilution present in homozygous condition. The himalayan albino mutant is tem-
perature-sensitive. When the temperature of the animal room was allowed to become quite cool in winter, the himalayan albinos reared under these conditions had dark coats and black eyes. Conversely, mice raised during the heat of a Baltimore summer were lightly pigmented (except for the skin of the nose, ears, and genital areas, which was dark), and their eyes were a ruby color. Observations of fine structure in melanin granules from the retinal tapetum of these mice suggest that temperature does not affect the amount of pigment deposited on the granule matrix; however, extending deeply between the outer segments of the rods. The vesicle labeled EPR is a profile of the tubular endoplasmic reticulum which is present at this age in the cells of the retinal tapetum. Note that the granules are outside of the vesicle. EPON-812; uranyl acetate stain.
the present data are not conclusive. The details of ontogeny of the granule matrix when this allele is present are similar to the dominant full color, regardless of temperature. Development in melanin granules of al-

PLATE 6. FIG. 20. Longitudinal sections through the matrix of the melanin granule. a. Albino (aabbcc); b. Brown (aabb); c. Black (aa). All are at the same magnification. The arrows in a. indicate the subunits. The arrows in b. indicate sites of primary deposition of melanin. The symbol, cl, indicates cross-links between adjacent fibers; m indicates places where melanin deposition has spread out along cross-linking fibers. Most of the melanin in c. has been removed by treatment with permanganate which bleaches the granules and reveals the underlying matrix.

Processing data: a. EPON-812, uranyl acetate stain; b. methacrylate, uranyl acetate stain; c. fixed in permanganate, methacrylate, unstained.
bino mice is exactly like that of non-albinos except that pigment is never deposited on the thickened compound fibers of the granules (Figure 19).

In the retina there is no obvious difference in size and shape of himalayan albino melanin granules or those of chinchilla or non-albino mice; however, granules in mice homozygous for extreme dilution are smaller and fewer than in full color, chinchilla, or himalayan albino mice. Again, there is no obvious difference in size or number of granules in mice homozygous for extreme dilution or albino.

Alleles at the albino locus which allow the deposition of melanin on the melanin granule matrix do not affect the appearance of the pigment as determined by the genotype at the B locus.

3. The P locus (Linkage group I)
The one mutant allele, p (pink-eyed dilution), at this locus was examined. It is recessive to the full color, P. Pink-eyed homozygotes show a considerably diluted coat color which can be correlated with the fact that less melanin is produced and the melanin granules are smaller in size and irregular in shape. The appearance of the granules when observed with the light microscope may be likened to the shreds of rubber produced by the use of a gum eraser.

The membranous boundary of the early fiber aggregates is apparently slower to form in pink-eyed mice so that a greater percentage of melanin granules may be found without this structure than when the dominant allele is present. This fact makes pink-eyed mice particularly suitable for the study of early fiber origin (Figure 21).

The fibers of the early pink-eyed melanin granules do not become arranged in the orderly semi-parallel arrays characteristic of the full-color allele. Instead they are often oriented in several different directions in the same plane of sectioning. Furthermore, even when adjacent fibers are more or less parallel, the distance between them is quite variable in contrast to the situation when the dominant allele is present. Moreover less cross-linking between fibers is evident in granules from pink-eyed homozygotes (Figure 22).

The lack of orientation of the fiber matrix of the melanin granule in pink-eyed mice makes it difficult to assess the granularity of the melanin which is being deposited. Occasional granules are found in which part of the matrix is fairly well oriented, however, and examination of the melanin deposited on these areas shows that the P locus does not affect the appearance of melanin as determined by the B locus. The distribution of melanin granules within the cell is not altered by the P locus.

4. The D locus (Linkage group II)
Only one mutant allele, d (maltese dilution), at this locus was examined. It is recessive to the full color D. Dilute melanin granules are smaller than those of the dominant genotype, and in hair they occur in clumps rather than being evenly distributed. Furthermore, melanocytes of neural crest origin in dilute mice tend to be rounded rather than dendritic in morphology. Dilute melanin granules vary in shape from spheres to rods and are slightly smaller than the granules of full color melanocytes. They occur very close together in the cell in clumps and often are attached to one another and to the nuclear membrane by fibrous material (Figure 23). The developmental sequence of dilute melanin granules is identical to that of non-dilute granules in every other particular, and the quality of the melanin deposited on them is consistent with the genotype at the B locus.

5. The Ln locus (Linkage group XIII)
The mutant allele, ln (leaden), at this locus is recessive to the full-color allele. In the hair, Inln melanin granules are clumped, causing a dilution in coat color. However, retinal melanin granules are randomly distributed, and eye color is not noticeably affected. The size, shape, and ontogeny of melanin granules are unaffected by the ln allele. Moreover, the quality of melanin, as determined by the
B locus, is not influenced by the Ln locus.

6. The Ru locus (Linkage group XII)
   Ruby eye (ru), the mutant allele at this locus, is recessive to the full-color allele. When homozygous, it causes dilution of hair color and dilution of eye color. The melanin granules are smaller and there

PLATE 7. FIG. 21. Early stages of development of melanin granules in a pink-eyed black mouse (app). The symbol R designates clumps of ribosomes and the arrows show small fibers extending from them into the forming granule matrices. Two mitochondria (M) are also seen. Methacrylate; uranyl acetate stain.
are fewer per cell in the mutant homozygote than when the dominant allele is present. Intracellular distribution of the granules is random; hence, the locus does not affect distribution. The quality of pigment deposited on ruby eye melanin granules is characteristic of the genotype at the B locus. The developmental sequence of ruby eye melanin granules is the same as in the "wild type" instead of the neatly parallel array seen in other genotypes. Note reduction in cross-linking. Methacrylate; uranyl acetate stain.
except that the onset of synthesis of melanin granules in the retina is delayed until parturition in ruby-eyed homozygotes (Figure 18).

7. The A locus (Linkage group V)

The mutant allele $A^i$ (yellow lethal) at the Agouti locus is lethal in the homozygous condition, the embryos dying shortly after birth. This allele is responsible for a yellowish coloration in the retina of mice.

PLATE 9. FIG. 23. Melanin granules in the retina of a dilute mouse (aabbdd). Arrows indicate strands of material connecting the granules to each other and to the nuclear envelope. In other respects the granules are typical brown granules. Methacrylate; unstained (OsO₄ fixation).
after implantation. Hence, it is recessive with respect to lethality. It is dominant with respect to coat color, however, and mice heterozygous for \( A^y \) have yellow coats. Melanocytes of the hair follicle make phaeomelanin instead of eumelanin although all other melanocytes in the body make eumelanin in accordance with their genotype at the B locus. The melanin granules in the hair follicle melanocytes of \( A^y \) heterozygotes are spherical in shape and yellow in color. They are slightly smaller than \( bb \) melanin granules. The ontogeny of these granules is quite different from that of granules producing eumelanin. No organized matrix is formed and the phaeomelanin is deposited in discrete but randomly distributed areas on a tangled mat of extremely fine fibers that are almost translucent to the electron beam.

---

PLATE 10. FIG. 24. Melanin granules in a melanocyte from a hair follicle of a yellow mouse (C57 Bl/6-Ay). Note the lack of an organized matrix and the incomplete “unit membranes” bounding the granules. The arrow indicates the thin, barely-visible fibers on which the phaeomelanin is deposited. The asterisk is in an area where this deposition is just beginning. The nucleus of the melanocyte is indicated by N. The circles enclose desmosomes linking the melanocyte to an adjoining hair follicle cell. Fixed in osmic acid, embedded in Epon-812, stained with uranyl acetate.
GENETIC VARIATIONS IN MELANIN

There is no ordered aggregation of fibers nor is there any organized cross-linking. As the phaeomelanin accumulates, the areas of deposition spread and fuse until finally the granule assumes a very dense homogeneous appearance (Figure 24). Very occasionally the areas of melanin deposition in these granules are found in linear arrays reminiscent of intermediate stages in the ontogeny of eumelanin granules, but even then no organized matrix is visible. Infrequently, hair follicle melanocytes in $A^v$ heterozygotes make eumelanin, and in these cases the ontogeny and structure of the granules are identical with what was seen in non-agouti cells and depend on the genotype at the B locus. With the exception of the hair follicle melanocytes, all the melanocytes of $A^v$ heterozygotes make normal eumelanin, and the structure and ontogeny of their granules are identical with what is seen in non-agouti mice.

Experiments with melanin granules isolated on sucrose density gradients

When the large granule fraction (consisting of melanin granules and mitochondria) obtained from B-16 melanomas is sedimented to equilibrium through a sucrose density gradient, four zones are obtained. The lightest of these (density 1.205 to 1.220) consists of mitochondria. The melanin granules are distributed in three heavier zones designated M1 (average density 1.245), M2 (average density 1.281), and M3 (average density 1.324). The M1 fraction consists almost entirely of unpigmented or very lightly pigmented melanin granule matrices. The M2 fraction consists of melanin granules in intermediate stages of pigmentation. The M3 fraction contains melanin granules in terminal stages of pigmentation and mature granules. All three fractions incorporate C\textsuperscript{14}-tyrosine into melanin and the M3 fraction has the highest specific activity expressed as counts per minute per mg dry weight.

Similar experiments have been carried out with melanin granules isolated from the S-91 melanoma and from the skins of 5-day-old C57 Bl/6 mice. Some of these data are presented in Table 3. These results must be considered preliminary and subject to modification depending on the outcome of work now in progress in my laboratory.

Granules isolated from B-16 and S-91 melanomas may be dissociated by treatment with 6 M guanidine hydrochloride or 0.26% sodium deoxycholate (DOC). When these suspensions are centrifuged, intensely-pigmented pellets are obtained. The supernatant solutions, when centrifuged in the analytical ultracentrifuge show multiple small peaks, suggesting that they contain a variety of different macromolecular pieces (Figure 25). If the supernatant solutions are dialyzed to remove the dispersing agent, reaggregation occurs. When the granules are dissociated with guanidine hydrochloride and reaggregated by dialysis, their ability to incorporate C\textsuperscript{14}-tyrosine into melanin is abolished. When the dispersing agent is DOC, however, the reaggregated material will incorporate tyrosine into melanin. Again, these results are preliminary and subject to modification. At the present time the dissociation and reaggregation process is being studied with the electron microscope in my laboratory.
The molecular basis of melanin granule structure

Analyses of naturally occurring melanin pigments (Serra, 1946) have shown that they consist of melanoproteins in which the melanin polymer is firmly bound to a protein moiety. Moreover, C¹⁴-leucine is incorporated first into the microsomal fraction and then into the melanin granules of mouse melanomas (Seiji, et al., 1963). Furthermore, the unit fibers of early melanin granules are frequently in contact with ribosomes (Moyer, 1960, 1963, and Figure 21). These findings suggest that protein forms an integral part of the melanin granule. Finally, the granule matrix has tyrosinase activity, and the sites of this activity are blocked by the progressive deposition of melanin upon the matrix. In other words the tyrosinase activity of melanin granules is inversely proportional to the amount of melanin they have produced, presumably because the newly-formed melanin is combining with the active sites on the matrix (Seiji and Fitzpatrick, 1961).

The alterations in fine structure of melanin granules resulting from allelic substitution at the B, C, and P loci show that three different structural features of the melanin granule are controlled by mutants at three different genetic loci. Clearly, the appearance of the melanin, the ability to make melanin, and the orientation of the fibers are independently mutable characteristics of the granules. These characteristics could all result from gene-induced changes in the primary amino acid sequences of matrix protein subunits. Changes in amino acid sequence might alter the binding of the melanin polymer to its protein moiety, thus changing the appearance of the melanin produced. Similarly, changes in amino acid sequence could alter the configuration of the active site of tyrosinase, thus affecting the production of melanin; and finally, such changes might alter the cross-linking sites on the compound fibers, thus interfering with the orderly aggregation of the matrix. Alterations of this kind in matrix protein subunits might be expected to produce just those subtle changes in matrix fine structure that have been reported here (Table 2).

Electron micrographs taken at high magnification show a very pronounced structural subunit in the matrix of the melanin granule (Figures 10 to 16 and 19 and 20). A plausible hypothesis is that these subunits are macromolecular aggregates similar in nature to the elementary particles of mitochondria (Green, 1959; Fernandez-Moran, 1962; Parsons, 1963) or the quantasomes of chloroplasts (Park and Pon, 1963; Park and Biggins, 1964). The melanin granules may be dissociated by treatment with DOC, so it is likely that the matrix of the melanin granule is made up of these subunits arranged in a regular pattern upon some sort of membranous sheet. Perhaps the B, C, and P loci control the structure of three proteins (B, C, and P proteins) contained in the subunits.
Melanin granules from brown (bb) mice apparently incorporate twice as much C\textsubscript{14}-tyrosine as BB granules (Coleman, 1962). This finding, together with the difference in appearance of black and brown melanin (Figures 6 and 7), suggests that in addition to providing a structural framework for the attachment of tyrosinase molecules, the B protein somehow influences the activity of the enzyme and the molecular structure of the melanin. Similar evidence on the interaction of the p allele with the c\textsuperscript{e} allele suggests that the P protein is involved in more than simple cross-linking since the amount of C\textsubscript{14}-tyrosine incorporated by c'c'pp granules is greater than would be expected on the basis of the size and number of the granules (compare the data of Russell, 1949b, to those of Coleman, 1962). This unexpectedly high incorporation of C\textsubscript{14}-tyrosine in CCpp granules is similar to the complementation phenomena seen in microorganisms. These considerations indicate that the B and P proteins are intimately related to the C protein. They may serve to stabilize the C protein in its active configuration.

Figure 20b shows sites of deposition of melanin on the matrix of a bb melanin granule. The number of these sites per granule can be counted and the weight of the granule can be estimated from its density and its average volume. When this is done for granules of the B-16 melanoma it is found that the average weight of a B-16 melanin granule is about $1.8 \times 10^{-14}$ g. The average B-16 granule contains about 1000 sites of melanin deposition, so each site must weigh about $2.0 \times 10^{-15}$ g. When this value is multiplied by Avogadro's number a “molecular weight” of about $1.0 \times 10^6$ is obtained. This value is high, probably because it also includes the weight of the melanin deposited on the granule. Nevertheless, it supports the idea that the subunit of the granule is a complex aggregate of molecules containing the enzymatic activities necessary to produce melanin.

In light of the evidence discussed here, it seems likely that the matrix of the melanin granule is a complex aggregate of chemically functional protein subunits bound to some sort of lipid component.

**Genes that act indirectly on the structure of the melanin granule**


The allele d at the D locus and the allele ln at the Ln locus cause clumping of granules in melanocytes derived from neural crest (Russell, 1949a; Markert and Silvers, 1956, 1959). Neither allele alters the incorporation of tyrosine (Coleman, 1962). The present observations show that the melanin granules in mice homozygous for either of these alleles are not altered structurally, although the d allele alters size. Hence, the D and Ln loci control differentiation of the melanocyte indirectly by restricting synthesis of melanin granules to discrete areas within the cell. Perhaps these loci somehow alter the interactions of messenger ribonucleic acid and ribosomes. If the basic mechanisms of protein synthesis were altered, “pleiotropic effects” might be expected. The alleles at the D locus exhibit “pleiotropism” since they profoundly alter the production of the enzyme, phenylalanine hydroxylase. However, the locus “is not directly responsible for the production of this . . . enzyme” (Coleman, 1960). The fact that animals homozygous for d exhibit clumping of retinal melanin granules, while those homozygous for ln do not, suggests that the two loci act by different means.

2. Control of the onset of synthesis of granules. The Ru locus.

Since the ontogeny, shape, and fine structure of melanin granules are unaltered in ru ru mice, it seems likely that the major effect of the gene is to delay the onset of granule synthesis. During the course of the delay, the concentrations of substrates or co-factors for protein synthesis might become altered in a way that would affect the size and number of granules produced. The skins of newborn ru ru mice incorporate 14% less tyrosine than the skins of dominant homozygotes (Coleman, 1962).
This reduction in activity can be accounted for by the reduction in number of granules produced by the mutant homozygote, so ru probably does not alter the structure of matrix protein. Perhaps the Ru locus controls the activation of loci that direct the synthesis of matrix protein.

Further research on the action of the D, Ln, and Ru loci may provide information on the mechanisms by which certain genes indirectly alter the direct expression of other genes. Obviously, such mechanisms are extremely important in cytodifferentiation.


Observations of the effects of A alleles on different genetic backgrounds together with grafting experiments show that this locus acts in the cells of the hair follicle rather than in the melanocytes themselves (Silvers and Russell, 1955; Markert and Silvers, 1956; Silvers, 1958a, 1958b; Galbraith, 1964). Furthermore, when skin of newborn mice is grown in organ culture the pigment that is produced is black regardless of genotype at the A locus. The hair follicle melanocytes of the cultured skin may be induced to form yellow pigment, however, by the addition of reduced glutathione (Cleffmann, 1963). This effect of reduced glutathione on the melanocytes of cultured skin is not blocked by actinomycin D (Moyer, Campbell, and Petrovich, unpublished data) so it probably does not represent an alteration in the active genome of the melanocyte. The electron micrographs of yellow granules show that no organized matrix is formed during the development of the granules. Thus, these data suggest that the A locus acts in hair follicle cells in some unspecified way and that, as a result of this action, the redox potential within the melanocytes in the hair follicle is changed. This change apparently interferes with orderly aggregation of the protein subunits and causes an altered enzymatic activity leading to the production of phaeomelanin instead of eumelanin. It has not yet been possible to isolate yellow granules on sucrose gradients but further work on these granules may be expected to improve our understanding of the aggregative process involved in the production of melanin granules.

The origin of melanin granules

Edmund Güttes (1953) reviewed the theories of origin of melanin granules based on investigations with the light microscope. He listed four theories: nuclear origin, lipo-chondrial origin, mitochondrial origin, and Golgi origin. The observations of electron microscopists have shown clearly that the first three theories are incorrect but that the Golgi apparatus is involved in the synthesis of granules in certain cases (Dalton and Felix, 1953; Dalton, 1959; Birbeck, 1962, 1963; Moyer, 1963; Seiji, et al., 1963; Wellings and Siegel, 1963).

The role of the Golgi apparatus in the synthesis of melanin granules is not clear, however. Weissensfoles (1956) used phase contrast microscopy and electron microscopy to study differentiating melanoblasts in tissue culture. He found that melanin granules were formed in "pigment-forming centers" which were distinct from the Golgi apparatus though near it in the cytoplasm. Stäubli and Loustalot (1962), studying hamster melanomas and normal melanin-producing tissues, found that fibers showing axial periodicity were the "immediate precursors of the melanosome (melanin granule)," but found nothing to relate them to the Golgi apparatus nor to ribosomes. Rappaport, et al. (1963a, 1963b), studying carcinogen-induced tumors in the hamster, were also unable to relate early melanin granules to the Golgi apparatus. Zelickson, et al. (1964) showed by means of autoradiography with the electron microscope that tyrosinase activity first appeared in the endoplasmic reticulum and later was concentrated in the melanin granules of S-91 melanoma. In mouse melanomas the early melanin granules are associated with "free-floating" ribosomes, endoplasmic reticulum, and Golgi apparatus. However, melanin granules are synthesized in the retinal tapetum of the mouse in the total absence of any structure resembling...
the Golgi apparatus (Moyer, 1963). Therefore, it is clear that Golgi apparatus is not necessary for the formation of melanin granules. The fact that Golgi apparatus occurs in melanocytes derived from neural crest may merely reflect metabolic differences between these cells and the cells of the retinal tapetum. The similarity between melanocytes of the hair follicle and secretory cells has been pointed out (Moyer, 1961; Birbeck, 1962). The points of similarity are the presence of Golgi apparatus and roughsurfaced endoplasmic reticulum, and the extrusion of the products of synthesis in both types of cells. Nevertheless, cells lacking these two morphological features certainly synthesize protein.

At the time of synthesis of the granule matrix, the cells of the retinal tapetum in mice contain large numbers of ribosomes and polyribsomes floating free in the cytoplasm but no rough-surfaced endoplasmic reticulum. Retinal melanin granules are not extruded but simply remain in the cell where they were formed. Moreover, the pigmentation cycle in mouse hair extends over a period of 5 to 10 days, while in the retinal cells it lasts approximately 21 days. Therefore, the synthesis is much slower in the retina. Possibly the differing morphologies of these cells simply reflect differences in the rate at which the cells are synthesizing protein.

In the exocrine cells of the pancreas, newly-synthesized protein is concentrated in the Golgi vesicles (Palade, 1959). If Golgi apparatus functions the same way in melanocytes derived from neural crest, its role in the production of melanin granules is clear. In cells where matrix protein is being produced at a rapid rate, some of the protein must be concentrated in the Golgi region. According to the subunit hypothesis most granules would be expected to form in the region where the protein subunits were most concentrated. However, if aggregation of subunits is indeed the primary event in the synthesis of melanin granules, then granules would form wherever the proteins could aggregate, and these areas of aggregation need not be limited to the Golgi region even in cells where the Golgi apparatus is an outstanding structural feature. Thus, the subunit hypothesis provides a basis for reconciling the last remaining controversy over the origin of melanin granules.

**Problems of nomenclature**

1. Fuscin and lipofuscin.

Many authors have used the terms "fuscin" or "lipofuscin" to describe the pigment of the retinal tapetum. In fact, a popular textbook of histology states that "... fuscin, the pigment of the retina, is slightly different from melanin..." (Ham, 1957). The terms have been freely used by electron microscopists also (Porter, 1956; Yamada, et al., 1958; Porter and Yamada, 1960; Bernstein, 1961; Fernandez-Moran, 1961; Yamada, 1961). This usage is based upon staining reactions of the pigment. However, lack of detailed information on the chemical nature of these reactions makes the distinction between melanins and fuscins difficult, if not open to question (Pearse, 1953). Actually, the retinal tapetum contains the enzyme tyrosinase, and the activity of this enzyme is correlated with pigment production (Coulombre, 1955; Fitzpatrick and Kukita, 1959). Moreover, many electron microscopic observations show that the melanin granules in the retina are structurally identical, except for size, with those of pigment cells in the choroid coat (Moyer, 1959, 1961, 1963; Porter and Yamada, 1960; Bernstein, 1961; Yamada, 1961). The pronounced similarity of structure, ontogeny, and genetic variation of retinal and choroidal melanin granules, when considered with the biochemical evidence cited above, is convincing proof that the pigment produced in the retinal tapetum is melanin. Therefore, the terms "fuscin" and "lipofuscin" no longer should be used when referring to retinal pigment.

2. Melanosome, melanin granule, melanoblast, and melanocyte.

The Fifth International Congress of Pigment Cell Biologists, meeting at the New York Academy of Sciences in 1961, agreed
to adopt the term "melanosome" to describe melanin pigment inclusions. The term was first used by Braunsteiner, et al. (1958), and generally is considered to be appropriate because of the intricate structural, developmental, and genetic features of these inclusions. However, I believe the term should be used in a slightly different context. The observations discussed above suggest that a repeating unit composed of various proteins with the enzymatic capabilities for making melanin is the building block from which the melanin granule matrix is assembled. Such a small repeating functional unit is reminiscent of the ribosome, the elementary particle or "oxysome" of mitochondria, and the "quantaosome" of chloroplasts. In fact it appears to be an elementary particle of melanin production; hence I submit that it should be called a "melanosome" to emphasize its similarity to the other "fundamental particles of biology" (Calvin and Calvin, 1964).

It seems necessary to have a name for the assembled matrix but this name should not imply pigmentation because the matrix does not become pigmented in albinos. For this purpose I suggest "melanogen" body or granule. This usage would do away with ambiguous terms like "unpigmented pigment granule" and "amelanotic melanosome." The terms "pigment granule" and "melanin granule" should be reserved for the mature, fully-pigmented structure.

Melanoblasts have been defined as cells with the potential to become melanocytes. As soon as melanin appears within a melanoblast, it becomes a melanocyte. The present observations suggest that the term "melanocyte" be extended to include all cells which are producing, or have produced melanogen bodies, whether these melanogen bodies become pigmented or not. Thus, it is possible to have unpigmented melanocytes. Fortunately, the unpigmented melanocytes of albinos can be detected with the light microscope (Silvers, 1958c) so this terminology, though etymologically inappropriate, is not unreasonable.

ACKNOWLEDGMENTS

It is a very real pleasure to acknowledge the invaluable discussions and criticisms provided by Doctors Michael Beer, Clement Markert, Elizabeth Russell, and Douglas Coleman. I am grateful to Doctors Russell and Coleman for providing laboratory facilities at Jackson Laboratories in the summer of 1963. I also wish to thank Dr. Catherine Verhey, Willie A. Campbell, Alan Cohen, Jerilyn Petrovich, and Richard Terry for their contributions to these investigations when they were students in my laboratory.

I am indebted to Mary Middaugh, Ursula Ursprung, Pamela Younkin, and Phyllis Mannerling for their patience, loyalty, and technical skill. Finally I wish to thank Beverly Wright for expert assistance with the manuscript.

REFERENCES


Russel, E. S. 1948. A quantitative histological study of the pigment found in the coat-color mutants of the house mouse. II. Estimates of the total volume of pigment. Genetics 33:228-256.


Russel, E. S. 1949b. A quantitative histological study of the pigment found in the coat-color mutants of the house mouse. IV. The nature of the effects of genetic substitution in five major allelic series. Genetics 34:146-166.


