THE EFFECTS OF GENOTYPE AND CELL ENVIRONMENT ON MELANOBLAST DIFFERENTIATION IN THE HOUSE MOUSE[1]

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THE FOUNDATION of all embryonic development consists of the processes by which cells become differentiated from each other and from their common progenitors. These processes of cellular differentiation, though basic to an understanding of embryogeny, remain largely unknown, although two major factors are generally recognized as important in governing the course of cell development. First, the genetic makeup of cells defines the limits and the potentialities of their development, and second, the diverse cellular environments of the embryo elicit the specific developmental responses in cells which lead to their maturation into the wide variety of cell types that characterizes the adult. These local and highly specific cell environments are produced by cellular activity and are constantly changing as the responsible cells change. Thus a continually evolving dynamic interchange between embryonic cells directs their differentiation into specialized adult cells and finally into senescent cells incapable of sustaining the life of the organism.

The pigmented cells of the body—the melanocytes—have long been favored types of cells for studies of the genetic and environmental factors involved in cell differentiation. A dramatic and easily studied type of specialization characterizes these cells. Furthermore the differentiation of melanoblasts exhibits great sensitivity to changes in genetic constitution and to variations in the cellular environment in which they are transformed into melanocytes. The fact that melanoblasts can be successfully transplanted to new tissue environments has also made possible experimental analyses of the relative roles of genotype and cell environment in directing melanoblast differentiation. On the basis of previous work and that to be reported here it is apparent that within any single animal at least four distinct factors are important in melanoblast differentiation: (1) the genotype of the melanoblast, (2) the genotype of the environmental cells, (3) the environmental history of the melanoblast, and (4) the differentiated characteristics of the environmental cells—that is, whether the cells are epidermal cells, dermal cells, harderian gland cells, etc.

In this report these four factors have been considered in the differentiation of the melanocytes in house mice (*Mus musculus*) of 50 different genotypes involving genes at 15 distinct loci. The objectives of this investigation are (1) to describe the occurrence, abundance, and characteristics of melanocytes in the tissues of the nictitans, harderian gland, ear skin, hair follicle, choroid, and retina; (2) to relate these char-

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TABLE 1[1]

The occurrence of melanocytes in six tissues of the house mouse

Genotype	Retina	Choroid	Hair follicle	Ear skin	Harderian gland	Nictitans
AA	+	+	+	+	+	+
AAbb	+	+	+	+	+	+
A^va	+	+	+	−	+	+
A^vabb	+	+	+	−	+	+
A^lA^l	+	+	+	+	+	+
a^ta^t	+	NE	+	+	+	+
a^ta^tbb	+	NE	+	+	+	+
aa	+	+	+	+	+	+
$aaB^{lt}B^{lt}$	+	+	+	+	+	+
aabb	+	+	+	+	+	+
$aabbc^{ch}c^{ch}$	+	NE	+	+	+	+
AAc^cc^c	+	NE	+	+	+	+
$AAbbc^cc^c$	+	NE	+	NE	+	NE
$A^vac^cc^c$	+	NE	0	0−	+	−
aac^cc^c	+	NE	+	+	+	+
AAcc	0	0	0	0	0	0
A^vadd	+	NE	+	−	+	+−
aadd	+	NE	+	+	+	+
aabbdd	+	NE	+	+	+	+
aabbddln ln	+	NE	+	+	+	−+
aabbln ln	+	NE	+	+	+	+−
aapp	−	−	+	0	0	0
aappdd	−	NE	+	0−	0	−
aabbpppdd	−	NE	+	0−	0	0−
AASl sl	+	+	+	+	−	+
aaSl sl	+	NE	+	+	−	+
$AASl\ slW^vw$	+	+	+	+	0−	0−
$aaSl\ slW^vw$	+	+	+	+	0−	0−
aaW^vw	+	+	+	+	+	+
aaW^vW^v	+	0	0	0−	0−	0
aaW^vW	+	0	0	0	0	0
aaWW	+	0	0	0	0	0
aaWw	+	+	+	+	+	+
AAWwss	+	0	+	+	0	0
aaWwss	+	0	+	+	0	0
A^vass	+	+	+	−	+	+
A^vabbss	+	+	+	−	+	+
A^lA^lss	+	+	+	+	+	+
aass	+	+	+	+	+	+
aabbss	+	+	+	+	+	+
aaTo to	+	NE	+	+	+	+
$aaMi^{wh}\ Mi$	+	0	+	+	0	+−
$a^ta^tMi^{wh}\ Mi$	+	0	+	+	0	+−
$aaMi^{wh}\ Mi^{wh}$	−	0	0	0	0	0
$A^lA^lgl\ gl$	+	NE	+	+	+	+

TABLE 1—*Continued*

Genotype	Retina	Choroid	Hair follicle	Ear skin	Harderian gland	Nictitans
aaru ru	−	+	+	0−	−	−
aatp tp	+	NE	+	+	+	−
aaVa va	+	NE	+	+	+	0
aabbVa va	+	+	+	+	+	0
aabbddppey-1 ey-1 ey-2 ey-2			+	0	0	0

[1] The symbols used in this table have the following significance: + = substantially normal occurrence; − = greatly reduced number of melanocytes; 0 = no melanocytes; NE = tissue not examined. Spotted tissues are recorded as + when numerous melanocytes are present in the pigmented areas even though the white areas contain no melanocytes. Hair follicles are classified into only two types: + = pigmented, 0 = white. Although an entry is made for the retina of each genotype, an examination of sectioned eyes was made only for those genotypes in which a positive entry is also recorded for the choroid. Entries for the retinas of unsectioned eyes are based on gross observation alone. The $c^e c^e$ genotypes have numerous melanocytes but their granules are lighter than normal.

acteristics to the embryonic history and genotype of the melanocyte and to the genotype and differentiated characteristics of the surrounding cells; (3) to formulate an interpretation of melanocyte differentiation based upon gene action within the melanocyte and within the environmental cells.

MATERIALS AND METHODS

The mouse produces two types of melanocytes: (1) the dendritic melanocyte which arises from the neural crest and migrates into many tissues, in some of which it differentiates, and (2) the epidermal melanocyte which arises from the outer layer of the optic cup and which forms the pigmented layer of the retina. These two types of melanocytes were studied in 50 different genotypes of house mice—largely derived from inbred strains maintained at the Jackson Memorial Laboratory. The genotypes that were examined are listed in table 1. A discussion of some of the effects of most of these genes can be found in *The Genetics of the Mouse* by GRÜNEBERG (1952). The melanocytes and surrounding tissues were subjected to microscopic examination both in living preparations and in fixed preparations which were made as whole mounts or sections both stained and unstained.

Various tissues of the mouse were also dissected out and supravitally stained with brilliant cresyl blue in an effort to identify pigmentless melanocytes or melanoblasts that might be present in the tissue. The brilliant cresyl blue stain was used according to the procedure outlined by REYNOLDS (1954). These tissues were also incubated with dopa to reveal the presence of dopa positive cells. Incubation with dopa was for 24 hours in a hanging drop preparation containing 0.5 mg dopa/ml of M/10 phosphate buffer at a pH of 6.8.

Melanocytes are found in a wide variety of mouse tissues. They generally occur in the epidermal hair bulbs and in the dermis of the ear, tail, feet, scrotum, genital papilla, and eyelid; they also appear in the meninges of the brain, particularly between the olfactory and cerebral hemispheres, in the parathyroid, thymus, and

harderian glands, and in the nictitans, choroid, and retina of the eye. For the present investigation attention was centered on six tissues: hair bulbs, ear skin, harderian gland, nictitans, retina, and choroid. In many mouse genotypes these six tissues are abundantly supplied with melanocytes, and in nearly all the genotypes studied the characteristics and abundance of the melanocytes in each of these tissues showed a high order of constancy.

These six tissues were chosen not only because of the regularity of their behavior in influencing the differentiation of melanocytes but also because they appeared to represent very different kinds of differentiated tissue with little in common beyond the fact that they supported the differentiation of melanocytes.

The hair bulbs are composed of dense aggregations of epidermal cells that arise from invaginated thick placodes formed by the germinative stratum of the epidermis. The papilla of mesenchymal origin that pushes into the distal end of the hair bulb is important in the growth and nutrition of the hair and perhaps serves as a portal for the entrance of melanoblasts into the epidermal parts of the hair, but the papilla, itself, is unable to stimulate melanoblast differentiation. Mature melanocytes are found only among the epidermal cells in hair bulbs (fig. 1).

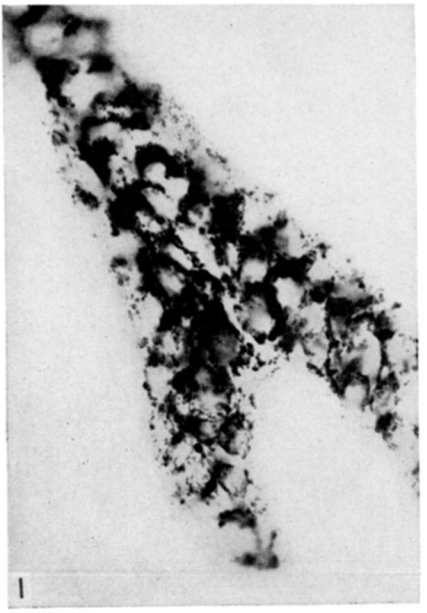
The dermis is formed early in embryonic life from loose aggregations of mesenchyme that migrate outward from the mesoderm to lie immediately beneath the epidermis. Here the mesenchyme differentiates into a dense connective tissue through which melanoblasts must migrate but in which they generally fail to differentiate. However, the dermis in a few limited areas such as in the ear and tail does provide an environment that induces melanoblast differentiation.

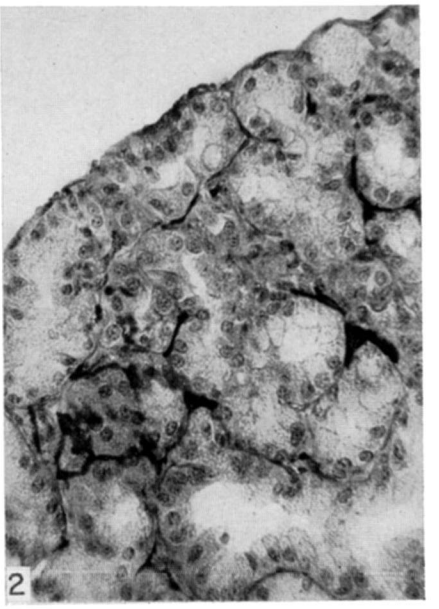
The harderian gland is of epidermal origin and arises as a solid ingrowth of cells at the inner angle of the nictitans. This gland is located in the orbit behind the eye and secretes an oily fluid that bathes the front of the eyeball. In addition the gland contains variable amounts of porphyrin depending upon the age, sex, and genotype of the mouse (STRONG 1942; BITTNER and WATSON 1946). The porphyrin characteristically occurs as reddish-brown clumps of pigment in the alveolar lumina (COHN 1955). The harderian gland in most genotypes is abundantly supplied with dendritic melanocytes distributed throughout the connective tissue that encapsulates the gland and divides it into lobules (figs. 2, 3, and 4).

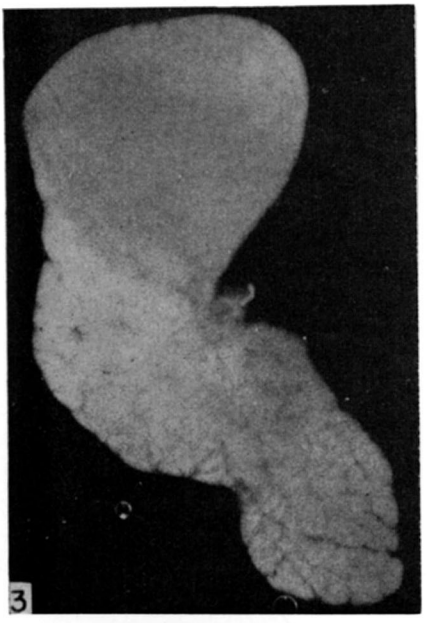
The nictitans—the rudimentary third eyelid of the mouse—arises as an epidermal fold enclosing mesenchyme. The mesenchyme develops into a central core of cartilage surrounded by dense connective tissue which lies immediately beneath the two sheets of stratified epidermis. Within this epidermis the melanocytes are packed together so as to resemble an epithelium that covers both sides of the distal edge of the nictitans (figs. 5 and 6). The concentration of melanocytes sharply diminishes proximally, and the base of the nictitans is usually devoid of pigment although melanocytes can frequently be found in the adjacent connective tissue of the orbit.

The choroid of the eye lies immediately adjacent to the pigmented retina and is derived from the mesenchymal investment of the optic cup. Melanoblasts migrate into this heavily vascularized soft connective tissue layer and there differentiate into densely packed melanocytes.

The pigmented choroidal coat of the eye can readily be distinguished from the







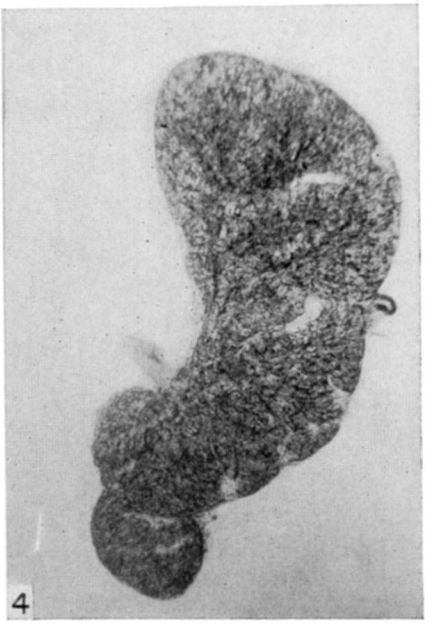
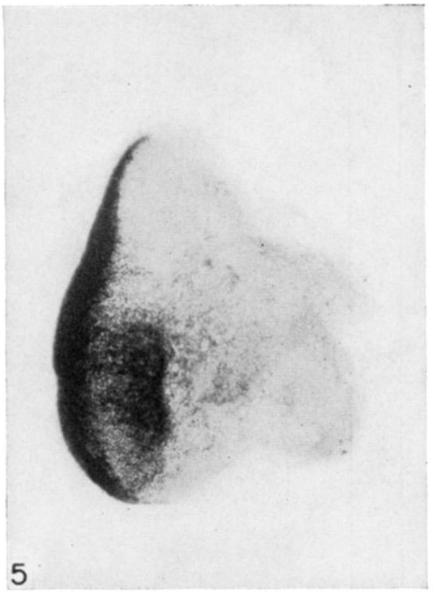


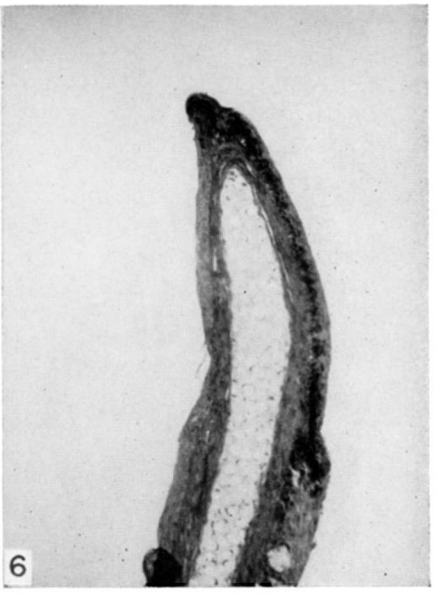
FIGURE 1.—Cleared, unstained preparation of a developing hair in the skin of a black mouse (*aa*). Note the absence of melanocytes from the region of the papilla. Melanocytes occur only among the epidermal cells of the hair. × 650.

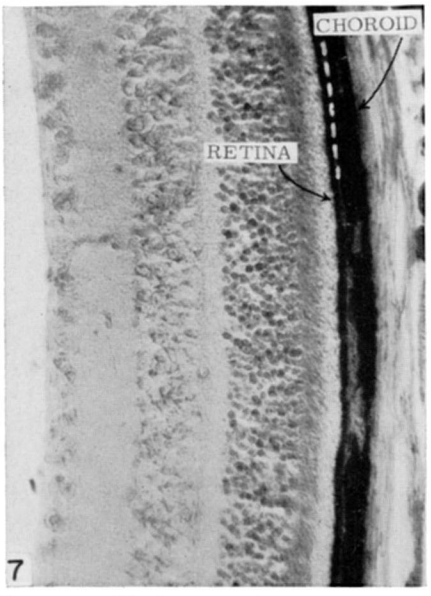
FIGURE 2.—Cross section through the harderian gland of a black mouse (*aa*). Note melanocytes in the connective tissue between the alveoli of the gland. × 290.

FIGURE 3.—Whole mount of the harderian gland of an albino mouse at ten days of age; contrast with figure 4. × 20.

FIGURE 4.—Whole mount of the harderian gland of a black mouse (*aa*) at ten days of age. Note the abundant melanocytes scattered over the surface of the gland; contrast with figure 3. × 16.







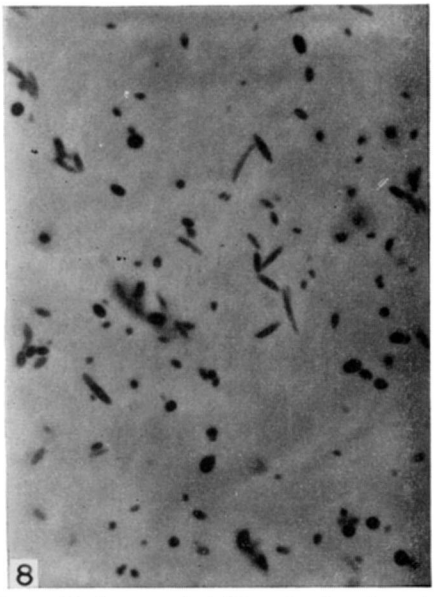


FIGURE 5.—Whole mount of unstained nictitans from a black mouse (*aa*). Note the densely packed melanocytes, particularly along the distal margin of the nictitans at the left. × 42.

FIGURE 6.—Cross section through the nictitans showing the cartilaginous core surrounded by dense connective tissue. Melanocytes are imbedded in the stratified epithelium covering the surface of the nictitans. × 120.

FIGURE 7.—Cross section through the eye of an $aaW^{v}w$ mouse. The morphology and pigmentation of this eye are normal. Note the relatively thin pigmented layer of the retina and the much thicker pigmented choroidal coat of the eye. The dotted line indicates the area of separation of the choroid from the retina. The layers of the sensory retina extend nearly to the left margin of the photograph. × 290.

FIGURE 8.—Fresh, squash preparation of the retina and choroid from a 14 day old *aa* mouse. Note the great diversity in size and shape of the melanin granules. That this diversity is due to the retina is evident in figures 18 and 19. × 1490.

adjacent pigmented layer of the retina (fig. 7). In contrast to the choroid the pigmented retina is a true epithelium only one cell thick that arises early in embryonic life from the outer wall of the optic cup. Thus the pigmented cells of the retina have an embryonic history that decisively sets them apart from all other melanocytes of the body.

RESULTS

The occurrence and abundance of melanocytes in each of the six tissues studied in 50 genotypes of mice are presented in table 1. Table 2 summarizes the data on melanocyte morphology and on the shape and color of melanin granules in various genotypes as observed in the harderian gland.

Retinal melanocytes

The epidermal melanocytes composing the pigmented layer of the retina have the same embryonic history in all mice and the morphology of the cells—cuboidal epithelium—was not noticeably different in any of the genotypes examined, except

TABLE 2

Color and shape of melanin granules and melanocyte morphology as observed in the harderian glands of twenty-four basic genotypes

Genotype	Cell morphology	Granule morphology	Granule color
AA	NF[1]	ovoid	black
AAbb	NF	spheroid	brown
aa	NF	ovoid	black
aabb	NF	spheroid	brown
aabbc^{ch}c^{ch}	NF	spheroid	brown
A^{v}a	NF	ovoid	black
A^{v}abb	NF	spheroid	brown
AAc^{e}c^{e}	NF	ovoid	black
aadd	NP[2]	ovoid	black
A^{v}add	NP	ovoid	black
aabbdd	NP	spheroid	brown
aabbln ln	NP	spheroid	brown
aabbln lndd	NP	spheroid	brown
aaSl sl	NF	ovoid	black
aaW^{v}w	NF	ovoid	black
A^{l}A^{l}gl gl	NF	ovoid	black
aaru ru	NF	spheroid	brown-black
aatp tp	NF	ovoid	black
aaVa va	NF	ovoid	black
aabbVa va	NF	spheroid	brown
aaB^{lt}B^{lt}	NF	spheroid	brown-black
aapp[3]	—	shred-like	black
aaTo to	NF	ovoid	black
aaMi^{wh} Mi[3]	—	ovoid	black

[1] NF = nucleofugal (typical melanocyte with large, extended dendrites).

[2] NP = nucleopetal (melanocyte with enlarged perikaryon and reduced dendrites).

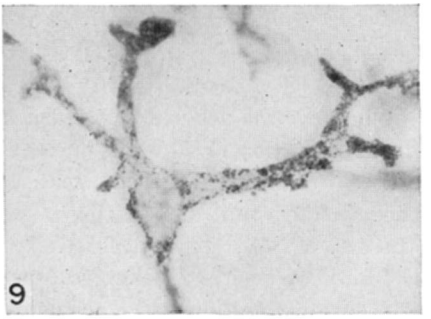
[3] Melanocytes not examined and granules observed only in hair shaft.

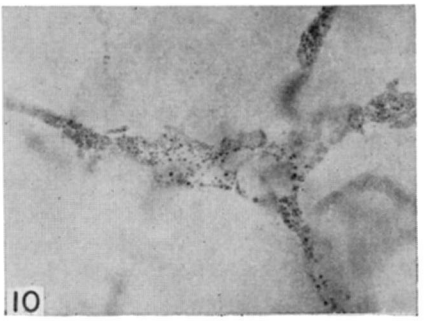
for those with morphologically altered eyes. The eyeless genotypes produced no eyes and consequently no pigmented retinas. The grossly deficient eyes of dominant white homozygotes had incomplete retinas—in which, however, the retinal cells, when present, retained a typical cuboidal morphology.

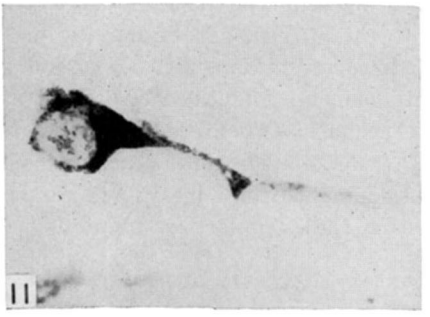
The capacity to form pigment and the characteristics of the pigment granules in the retina were greatly affected by genetic constitution. Albino animals (*cc*) of course produced no pigment even though retinal morphology was normal. The eyes of pink eyed (*pp*), dominant white ($Mi^{wh}Mi^{wh}$), and ruby eyed (*ru ru*) mice were characterized by reduced retinal pigmentation, pink eye showing the greatest reduction and ruby eye the least. Except for ruby eye all the genotypes produced either black or brown granules in accordance with the alleles present at the *B* locus (*bb* = brown, *Bb* and *BB* = black). Ruby eye granules from animals of black genotype (*aa ru ru BB*) were only slightly darker than typical brown granules and were thus intermediate between typical blacks and browns. Perhaps the most distinctive characteristic of retinal pigment granules was their striking diversity of size and shape (fig. 8) in all genotypes. Shape varied from spheres through all gradations to thin elongated rods, and in size the largest granules were at least five hundred times the volume of the smallest. This wide variation in shape and size contrasts sharply with the uniformity of granule size and morphology found in all dendritic melanocytes. In fact, the adjacent choroidal and retinal pigmented layers of the eye can readily be distinguished solely on the basis of the conspicuous differences in melanin granule morphology. These differences, occurring in adjacent cells of identical genotypes, can plausibly be attributed to the quite different embryological histories of the two types of melanocytes—one derived from the optic cup and the other from the neural crest. It is interesting to note that ruby eye genes not only reduce the number of melanin granules in the retina but also reduce both the average size and the range of diversity in granule morphology. No other gene was found that produced this effect.

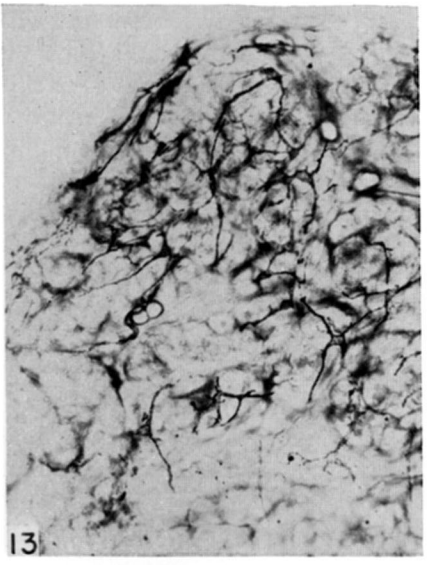
Dendritic melanocyte morphology

The dendritic melanocytes exhibit two distinct types of morphology and numerous variations in melanin granule characteristics. The commonest type of melanocyte has long relatively thick processes containing a substantial portion of the cell substance (figs. 9 and 10). The presence of the genes dilute (*dd*) or leaden (*ln ln*) results in a melanocyte (figs. 11 and 12) with fewer and thinner dendritic processes; in these cells the melanin granules are necessarily more concentrated in the main body of the cell. This type of melanocyte might be described as nucleopetal as contrasted with the commoner nucleofugal type (figs. 9 and 10). Dilute and leaden genotypes have a total volume of pigment that is at least as great as their corresponding non-dilute or non-leaden genotypes (RUSSELL 1948). The gross effect of these genes is to concentrate melanin granules into large clumps (compare figs. 13 and 14) thus reducing light absorption to give a "dilute" phenotype (RUSSELL 1946, 1948, 1949a, 1949b). These cells have been spoken of as containing "clumped" pigment granules (SILVERS 1953), but it should be noted that the granules are clumped or concentrated around the nucleus because the shape of the cell permits no other arrangement. The nucleopetal morphology probably results in an uneven release of granules from the









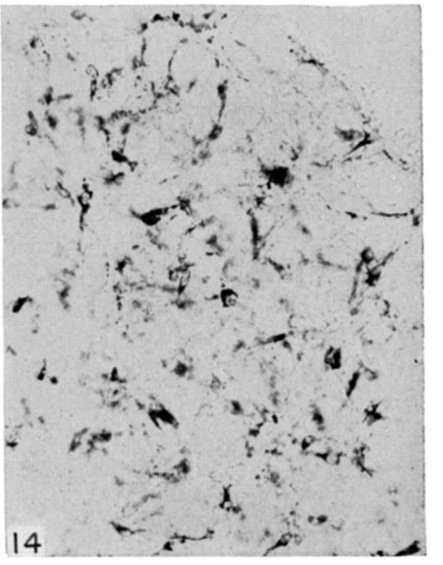


FIGURE 9.—Melanocyte from the harderian gland of a ten day old *aa* mouse. Note the distribution of melanin in thick dendritic processes. The black granules are ovoid. × 650.

FIGURE 10.—Melanocyte from the harderian gland of a ten day old *aabb* mouse. Note the thick dendritic processes and the spheroidal granules (brown). × 650.

FIGURE 11.—Melanocyte from the harderian gland of a four day old *aadd* mouse. Note the reduction in dendritic processes as compared to figures 9 and 10. The granules are ovoid and are clustered around the nucleus (nucleopetal type). × 650.

FIGURE 12.—Three melanocytes from the harderian gland of a six day old *aabbdd* mouse. Note the nucleopetal morphology and the spheroidal granules of the melanocytes. × 650.

FIGURE 13.—Melanocytes (nucleofugal) imbedded in the connective tissue capsule of the harderian gland of a ten day old *aa* mouse. Compare with figure 14. × 130.

FIGURE 14.—Melanocytes (nucleopetal) imbedded in the connective tissue capsule of the harderian gland of a four day old $A^{vy}add$ mouse. Note the dilution effect produced by the concentration of melanin around the nuclei of the melanocytes. Compare with figure 13. × 130.

melanocyte to the epidermal cells of the hair bulb. Since the dendrites are the channels through which the granules are released, the reduction in size and number of these dendrites in *dd* or *ln ln* genotypes would seem to interfere with the release of granules to the epidermal cells; such interference could account for the irregular distribution of the granules along the hair (RUSSELL 1949b). At least some of the distinctive clumping of granules may result from the eventual disintegration of melanocytes which would release large masses of melanin granules at one time.

Observation of melanocyte morphology in any one genotype can not reveal whether the particular morphology is due primarily to the intrinsic genetic makeup of the pigment cell or to the cellular environment. However, melanoblasts of *dd* genotype that invaded transplanted tissue of *DD* genetic makeup produced melanocytes with a nucleopetal (*dd*) phenotype (REED 1938; REED and HENDERSON 1940). Thus this morphological characteristic of the melanocyte is due to its intrinsic genetic makeup. The morphology and color of melanin granules is not affected by changes at the *d* and *ln* loci, and no additive effect of the genes at these two loci has been observed.

Pigment granule morphology and color; black and brown

Reference has already been made to the variability of granule morphology in the retina of all genotypes. There is also a wide range of granule size in hair although a corresponding range of granule size is not evident in the melanocytes of the hair bulbs. It must be, therefore, that the granules continue to increase in size after they have left the melanocyte and been deposited in the epidermal cells of the hair. Within the dendritic melanocytes themselves, however, granules are relatively uniform in size, shape, and color. This is particularly evident in the melanocytes of the harderian gland. All the genotypes examined, except ruby eye (*ru ru*) and light (B^{lt} B^{lt}), had distinctly brown or black colored granules in the melanocytes of the harderian gland in accord with their genetic constitution. Black granules were typically ovoid in shape and brown granules were spheres (compare figs. 9 and 10). However, ruby eye granules were spheroidal in mice of black genotype. In such mice the change in granule morphology from ovoid to spheroidal is associated with a change in color from black to dark brown. Whether color and shape are interdependent characteristics is difficult to say in this case. A valid generalization emerging from this study, however, is that no fixed relationship exists between the color and shape of melanin granules (cf. RUSSELL 1949a). Brown, black, and yellow granules exist in many different shapes and also in many sizes. This is obvious (for black and brown) in the retina (fig. 8) but it is also evident in the hair.

Yellow melanin

The differentiation of yellow granules presents a clear illustration of the importance of the cellular environment in directing the course of melanoblast differentiation. Since the formation of yellow hair is due to the gene A^y at the agouti locus, all mice carrying this gene also carry genes for black or brown at the *B* locus. An examination of the six tissues previously listed shows that in yellow mice only the melanocytes of the hair bulbs synthesize yellow pigment. Melanocytes located in the remaining five tissue environments synthesize either black or brown pigment in accord with

their genetic constitution at the *B* locus. Thus the alleles A^y, A^l, A, and a^t at the agouti locus must act through the epidermal cells of the hair bulb to establish a follicular environment that directs the differentiation of melanoblasts to become yellow pigment producing cells. When the follicular cells contain the genes, *aa*, they fail almost completely to stimulate the differentiation of yellow melanocytes, although yellow hairs are frequently present on the ears, genitalia, and around the mammae. Thus some tissues, even of *aa* genotype, can provide an environment in which melanoblasts become yellow melanocytes.

SILVERS and RUSSELL (1955) have shown that melanoblasts of *aa* genotype produce yellow pigment when they invade $A^yac^ec^e$ (white) hair follicles. Likewise A^ya melanoblasts produce black pigment when incorporated into *aa* hair follicles. Therefore, the agouti locus constitution of a melanoblast is not decisive in the differentiation of either black or yellow melanin. Further evidence of this is the occurrence of black (or brown in *bb* animals) pigment in A^ya melanoblasts occurring in those tissue environments, other than the hair follicle, which stimulate melanogenesis. The intrinsic capacity of the melanoblast of, for example, A^yaBB genotype is apparently to produce black pigment as shown by the fact that such mouse melanoblasts transplanted to the coelom of white leghorn chicks there develop into black melanocytes (SILVERS unpublished).

Evidence has also been obtained demonstrating that the agouti locus plays a role within the melanoblast in determining the response of the pigment cell to local tract differences and general tract differences (REED and HENDERSON 1940; SILVERS and RUSSELL 1955). Further evidence for this intrinsic effect of the agouti locus may be deduced from the fact that the population of black pigment cells in *aaBB* ear skin is more numerous than in A^yaBB ear skin.

In addition to the A^y gene the recessive alleles at the gray lethal locus (*gl gl*) also appear to control the synthesis of yellow melanin through the mediation of the epidermal follicular cells. Agouti gray lethal animals produce hairs with obviously reduced pigment in the subterminal yellow band but with black pigment present in normal amounts in other regions of the hair. Furthermore, a large part of the yellow pigment that is present is distributed in clumps somewhat like the distribution found in dilute leaden animals. Melanocytes are unable to carry on a high level of yellow pigment production and are unable to release their pigment in the same manner as melanocytes producing black pigment. Gray lethal melanocytes in all pigmented tissues except the hair bulbs produce characteristic black granules only. Thus the gray lethal gene appears to act within the epidermal cells of the hair bulb in the presence of the A^y, A^l, A, and a^t genes to weaken and render abnormal the stimulus for yellow melanin production.

Genotypes without dendritic melanocytes

Of the 50 genotypes listed in table 1, 35 can induce the differentiation of melanocytes in each of the six selected tissues. Except for a few genotypes (indicated in table 1 by double symbols) there is remarkably little variation between animals of the same genotype in the pigment characteristics of these six tissues. In other tissues such as the meninges, thymus, etc., melanocytes were observed to be present irregu-

larly even in inbred strains of mice. This variability may indicate extreme sensitivity to minor fluctuations in embryonic history or to residual genetic differences in the inbred lines. In any event, melanogenesis occurs in at least one of the selected six tissues in all of the genotypes studied except albino (*cc*). Albino animals lack the enzymatic mechanism required for melanin synthesis. In three genotypes ($Mi^{wh}Mi^{wh}$, WW, WW^v) melanin is restricted to the retina and in a fourth, W^vW^v, pigmentation is usually restricted to the retina. These genotypes, particularly the first three, therefore do not differentiate any dendritic melanocytes. This absence of dendritic melanocytes cannot be due to any genetic incapacity to synthesize melanin for melanogenesis occurs in the retina of these animals. Since W^vW^v animals do occasionally produce dendritic melanocytes in ear skin or in the eyelid or in the harderian gland, it seems most plausible to attribute the lack of melanocytes in the tissues to the tissues themselves—that is, melanoblasts probably migrate out in normal fashion from the neural crest but except for occasional areas in W^vW^v animals the melanoblasts never find a cellular environment in which they can differentiate into mature melanocytes. Definite conclusions must await experimental transfer of presumptive melanoblasts of these genotypes to tissue environments that are known to be genetically favorable for melanoblast differentiation.

Melanoblast differentiation and the cellular environment

In twelve genotypes melanocytes regularly differentiate in at least one tissue in addition to the retina, but fail to differentiate in one or more of the remaining tissues. Melanoblasts of these genotypes obviously have the capacity to become fully mature melanocytes. However, some of the tissue environments are rendered unsuitable by their genetic makeup for melanocyte differentiation. The fact that in a single animal some of these five tissues provide a suitable environment for melanoblast differentiation while others do not emphasizes the fact that both the intrinsic genetic constitution and the right embryonic history are essential to a tissue in elaborating an environment that will stimulate melanoblasts to become melanocytes.

Alleles at the *C* locus provide a good example of action within the melanoblast. Melanoblasts of *cc* genotype produce no tyrosinase (FOSTER 1951) and therefore no melanin in any tissue environment. The extreme dilution alleles c^ec^e diminish the amount of pigment deposited on each granule—the gross effect being therefore to produce more lightly pigmented melanocytes in every tissue in which they differentiate. However, in hair bulbs of A^y genotype melanoblasts containing the genes c^ec^e are unable to synthesize yellow pigment granules and consequently the hair of $A^yac^ec^e$ animals is white, although the retina, choroid, harderian gland, etc. contain melanocytes with light black granules.

The genes *Sl* and W^v apparently interacted to depress the differentiation of melanoblasts in the nictitans and in the harderian gland as well. The combined effects of these genes can be regarded as an enhancement of spotting since about half of the harderian glands examined (10) contained no melanocytes and those containing pigment were frequently spotted.

The nictitans appears to provide the least favorable environment (of the six selected tissues) for pigment formation since it is the most sensitive to adverse genetic

changes. Two genotypes reduce or prevent pigmentation of the nictitans alone. These are varitint (*Va va*) which prevents, and taupe (*tp tp*) which drastically reduces, melanoblast differentiation in the nictitans. Leaden (*ln ln*) is also associated with a noticeable reduction in nictitans pigmentation, and dilute (*dd*) may likewise have a specific effect on the nictitans in combination with A^v or with *ln ln*.

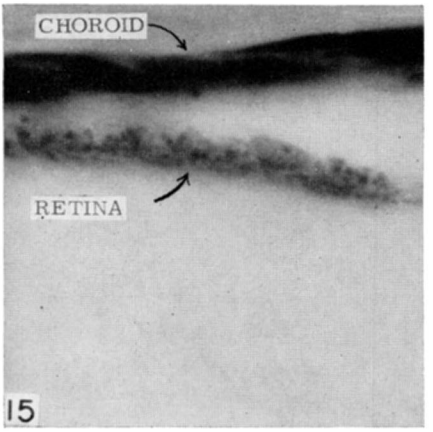
Four loci affected the pigmentation of the choroid—Mi^{wh}, *W*, *p*, and *s*. Of these Mi^{wh} was the most potent since even in heterozygous condition it rendered the choroid completely unsuitable for melanoblast differentiation. In homozygous condition the *W* or W^v alleles also prevented all choroidal pigment. The genes for piebald spotting have previously been reported as affecting choroidal pigmentation (GATES 1926; DUNN and MOHR 1952). In the present investigation the effects of piebald spotting on the choroid were found to be exceedingly variable. Some eyes exhibited almost normal choroids with only an occasional white spot while others contained an almost pigmentless choroid. Genotypes of *Ww ss* constitution produced eyes with no detectable choroidal pigment; apparently this effect is due to an interaction between *ss* and *Ww* that enhances spotting in the choroid as it does in the hair. Pink eye (*pp*) reduced both retinal and choroidal pigmentation; choroidal pigment was restricted to the region of the iris and only small amounts were found there. A comparison of these various genic effects on eye pigmentation can readily be appreciated by examining sections through the eyes as shown in figures 15, 16, 17, 18, 19, and 20.

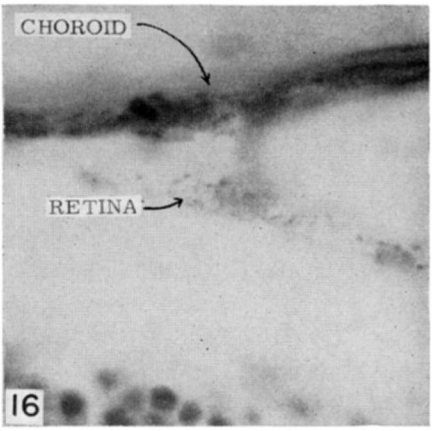
Vital staining

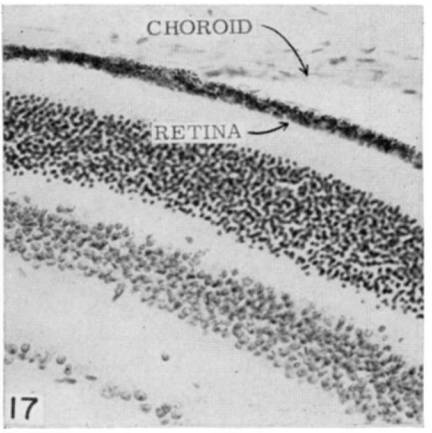
In the presentation of the results of this investigation the point of view has been preferred that in all the genotypes examined (except *cc*) melanoblasts capable of forming melanin were present in each of the six selected tissues. Whether the melanoblasts differentiated into pigmented melanocytes then depended upon the kind of environment provided by the surrounding cells. Alternative views are possible and a common one is that melanoblasts are not present in the tissues that fail to become pigmented. To test these points of view efforts were made to stain melanoblasts or pigmentless melanocytes supravitally with brilliant cresyl blue and also with dopa. The harderian gland and ear skin of albino, black, and of spotted animals of various ages were stained with brilliant cresyl blue according to the procedure outlined by REYNOLDS (1954).

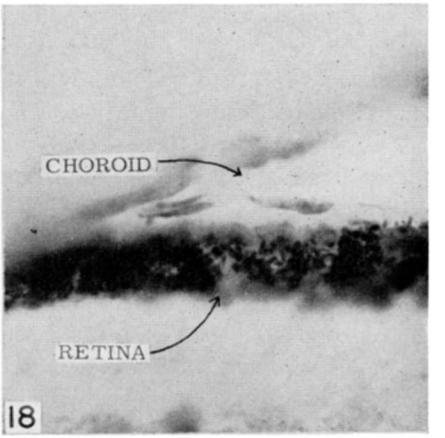
The dopa stained only those cells that had already begun to synthesize melanin and thus failed to reveal the presence of melanoblasts in any of the tissues examined. The results with brilliant cresyl blue were more variable and more difficult to interpret.

The ear skin of *aaBB* mice (C57 BL/6) at birth and at one, two, three, and four days of age was stained with brilliant cresyl blue. The ears of these mice at birth should have contained approximately equal concentrations of pigment cells either as melanoblasts or melanocytes. With increasing age the melanoblasts should have changed into melanocytes. Thus a melanoblast stain would reveal larger numbers of cells in the younger mice and in older animals the decrease in melanoblasts would be paralleled by a proportionate increase in visible melanocytes. Although melano-


CHOROID
RETINA
15


CHOROID
RETINA
16


CHOROID
RETINA
17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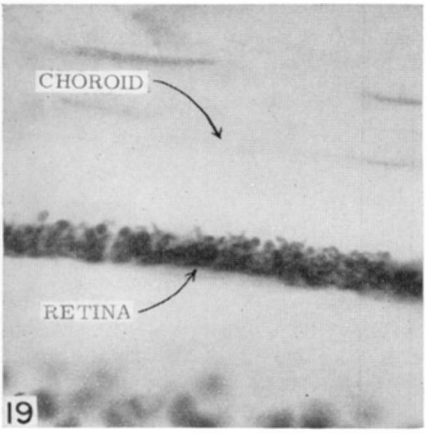
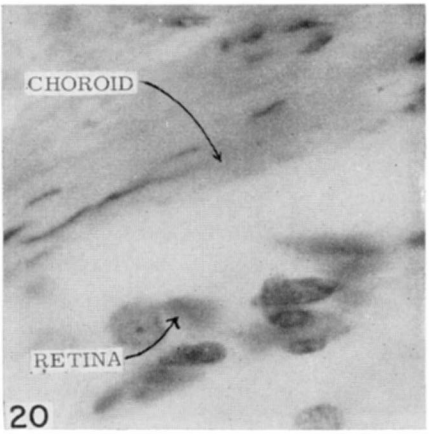
RETINA
18


CHOROID
RETINA
19


CHOROID
RETINA
20

FIGS. 15–20

cytes with only a few melanin granules did stain selectively with the brilliant cresyl blue the younger melanoblasts which must have been present in younger ears did not stain. In the ear skin of older animals (albino, black, or spotted) a variety of cell types took up the brilliant cresyl blue stain. A common type of cell revealed by the stain so closely resembled melanocytes in morphology and in the size and distribution of their blue stained granules that, except for the color of their granules, they could not be distinguished in appearance from mature melanocytes. However, because of the results obtained with the skin of newborn mice, these cells can scarcely be embryonic melanoblasts. Such cells may, however, be pigmentless melanocytes—that is, melanoblasts that have differentiated into mature type cells without the formation of melanin granules (see BILLINGHAM and MEDAWAR 1953). The lack of specificity of the stain makes the results merely suggestive, and tentative conclusions based on staining reactions cannot be accepted without support from other evidence. With these limitations in mind the evidence from staining lends tenuous support to the belief that melanoblasts of all genotypes invade these six tissues. Where the cellular environment proves unfavorable for their normal maturation their differentiation is diverted towards a type of maturity that does not involve melanogenesis—that is, a pigmentless melanocyte which stains with brilliant cresyl blue. The truly embryonic melanoblast will not however stain with the brilliant cresyl blue.

DISCUSSION

Tissue environments

The hypothesis of melanoblast differentiation that is favored in this investigation places the primary responsibility for the differentiation directly upon the cellular environment of the melanoblast. This hypothesis is preferred because it is the most economical and because some evidence directly supports it. On the basis of present evidence it may be assumed that a melanoblast possesses at most two metabolic systems by which it responds to melanogenic stimuli from the cell environment. Hair follicles of A^y genotype activate the reaction system in melanoblasts that leads to yellow melanin. A^l, A, and a^t genotypes result in the production of some yellow

FIGURE 15.—Cross section through the normal retina and choroid of an *aabbdd* mouse. The photograph was taken of an area in which the choroid and retina became partly separated during sectioning. This figure serves as a standard for comparing with figures 16–20. × 1215.

FIGURE 16.–Cross section through retina and choroid of an *aaru ru* mouse. Note the great reduction in the pigmentation of the retina. × 1215.

FIGURE 17.—Cross section through retina and choroid of an $Mi^{wh}Mi$ mouse. Note the absence of choroidal pigmentation. × 220.

FIGURE 18.—Cross section through retina and choroid of an Mi^{wh} *Mi* mouse. This photograph was taken of the preparation shown in figure 17 to show in higher magnification the retina and choroid in the area where they have been partly separated. No choroidal pigmentation is present. × 1215.

FIGURE 19.—Cross section through retina and choroid of an $aaWW^v$ mouse. Note the normal retina and the absence of choroidal pigmentation. Variation in granule size and shape in the retina is also evident. × 1215.

FIGURE 20.—Cross section through retina and choroid of an *aapp* mouse. A very few melanin granules can be found in the retina and choroid of these animals although they are difficult to find and none are evident in this photograph. × 1215.

melanin in hair follicles. All other tissues (and follicles of *aa* genotype) that provide a melanogenic stimulus activate the metabolic system that leads to the formation of black or brown melanin in accord with melanoblast genotype. The simplest assumption is that all tissues that provide melanogenic environments (except follicles inducing yellow melanin formation) activate melanoblasts by essentially the same metabolic devices. For if these devices are not alike then melanoblasts must be equipped with a variety of reaction mechanisms—one for each type of environment in which they may reside. This is implausible in view of the large variety of tissue environments in which melanocytes occur. Thus we may assume (yellow melanin aside) that all melanoblasts are exposed to the same differentiating stimuli in all melanogenic tissue environments and that they respond by means of a single reaction system. The different tissues of the mouse either elaborate these stimuli or they do not. The capacity of a tissue to elicit melanogenesis depends upon the embryonic history of the tissue (that is, what kind of tissue it has become—nictitans, harderian gland, etc.) and upon the genetic composition of the tissue. For example, as the primordium of the harderian gland gradually matures into the adult tissue it simultaneously establishes (like an intrinsic by-product) a tissue environment in which melanoblasts will become melanocytes. However, if the harderian gland cells happen to contain the genes Mi^{wh} Mi then they do not elaborate an environment suitable for melanoblast differentiation, even though in other respects the harderian gland cells are normal. It is not just the presence of Mi^{wh} Mi genes that prevents formation of a melanogenic environment, because melanogenic environments are established by cells of this identical genotype in the hair bulb and in the ear skin of Mi^{wh} Mi mice. Thus the expression of genic activity is completely dependent upon the type of cell in which the gene activity occurs. Furthermore, cells of the same general type (follicular cells, for example) but located in different regions of the body may exhibit striking differences in their effects on adjacent cells. This is clearly shown by the work of REED (1938). This investigator transplanted skin from embryonic albino mice (a^ta^tcc) to the dorsal and ventral regions of black-and-tan mice (a^ta^t) and of dilute black-and-tan mice (dd a^ta^t). Melanoblasts from the host invaded the transplant and produced tan hairs or black hairs in conformity with the dorsal or ventral origin of the grafted skin and regardless of the location of the graft on the host. Ventral epidermis of albino genotypes provides an environment that will activate responsive melanoblasts (a^ta^t) to produce yellow ("tan") melanin. Grafted dorsal albino ($aacc$) epidermis stimulates differentiation of black melanocytes from either dilute or non-dilute hosts, but the morphology of the pigment cell remains autonomous—that is, melanocytes of dilute (nucleopetal) genotype impart a dilute type of pigmentation to hairs composed of epidermal albino cells of non-dilute genotype. Thus the expression of the black-and-tan gene in a melanoblast is dependent upon the particular tissue (dorsal or ventral epidermis) in which the melanoblast resides, while the expression of the gene for dilution is intrinsic to melanoblasts and autonomous with regard to the cellular environment.

Spotted pigmentation patterns

Spotted pigmentation patterns, though common in many kinds of animals, are among the most perplexing to interpret. RAWLES (1947) demonstrated that mouse

pigment cells arise from the neural crest. These migratory melanoblasts invade many tissues in some of which they synthesize melanin. It can be assumed therefore, that a white spot is due either to the absence of melanoblasts or to their failure to differentiate in that area. Absence of melanoblasts might be attributed either to failure to migrate into the area or to their destruction after arrival and before the synthesis of pigment occurred. Several considerations can be brought to bear in evaluating these possibilities. First, the existence of islands of pigmented hairs surrounded by white areas seems inconsistent with any effective block to melanoblast migration. In this connection BILLINGHAM and MEDAWAR (1953) have identified cells in white spotted areas which appear to be derivatives of melanoblasts as judged by their distribution, morphology, and staining reactions. Secondly, skin from a white spot when transplanted to a pigmented area is invaded by host melanoblasts that deposit pigment in the previously white area (RAWLES 1953, 1955; SILVERS and RUSSELL 1955). If spotting is due to an exclusion or killing of melanoblasts by hostile tissues then such mechanisms must be transitory. Nevertheless, responsibility for white spotting does seem to reside within the skin (or other spotted tissue such as choroid or harderian gland) rather than within the melanoblast. Since melanoblasts throughout the body are probably alike and certainly so genetically, their failure to synthesize pigment must be due to lack of suitable stimuli. In passing it may be noted that any mechanism intrinsic to the melanoblasts that prevented on the average a certain fraction of them from maturing into melanocytes would result in a dilution effect rather than a spot. The mutant silver (*si si*) may indeed involve such a mechanism. According to DUNN and THIGPEN (1930) silvering is the result of a generalized reduction in the number of pigment granules. White, black, and mosaic hairs are intermingled all over the body to produce a silvering effect similar to that obtained by CHASE (1951) with X-radiation. Since a random destruction of melanoblasts probably forms the basis of the silvering effects of X-radiation, it seems plausible to ascribe hereditary silvering (*si si*) to a corresponding random genetic destruction of melanoblasts. In support of this hypothesis is the fact that a genetic destruction of melanoblasts is responsible for the white plumage of white leghorn chickens (HAMILTON 1940). Melanoblasts differentiate in white leghorn feather follicles but die before depositing noticeable quantities of melanin in the epidermal cells of the feather—hence the feathers are white.

White spotting in contrast to generalized loss of pigment is probably due to localized failures of the tissue environment to promote melanoblast maturation. Some evidence from tissue cultures of chick skin suggests this viewpoint. MARKERT (1948) found that in tissue culture chick dermis inhibits melanoblast differentiation while the epidermis is at least neutral and may promote melanoblast differentiation. The increased pigmentation of mouse skin after painting with turpentine and acetone (REYNOLDS 1954) may likewise be attributed to a disturbance of normal tissue activity resulting in the destruction of the inhibiting properties of the skin. The fact that migrating melanoblasts can pigment hair follicles of transplanted white spotted skin (SILVERS and RUSSELL 1955) suggests that disruption of the tissue at the margins of the graft might remove normal inhibiting properties of the follicles. However, the careful work of RAWLES (1955) disproves this hypothesis for the hooded rat. She demonstrated that transplantation alone does not result in pigmentation of white

spotted skin. An alternative, and more plausible hypothesis, attributes the absence of melanocytes in a white spot to the lack of an essential step in a sequence of differentiating stimuli. Melanoblasts that had developed past this blocked step in adjacent areas before migrating into the spotted follicles could then respond to the succeeding stimuli present in spotted skin and thus mature into melanocytes.

On the other hand, the analysis of a spotted mutant (splotch) of the mouse by AUERBACH (1954) tends to fix responsibility for pigment failure on the neural crest rather than upon the cell environment. AUERBACH implanted presumptive neural crest of *Sp Sp* embryos into the coelom of chick embryos. These implants yielded no pigment cells although corresponding transplants from splotch heterozygotes did produce pigment. Mice heterozygous for splotch have a white belly spot and white extremities; the homozygote dies at approximately 14 days in utero. By this time the retina is pigmented but the existence of dendritic melanoblasts can only be determined from transplants all of which have so far failed to produce pigment, even in a favorable cell environment. In many respects *Sp* resembles Mi^{wh}, *W*, and W^{v} in its effect on pigmentation. The heterozygotes all have white belly spots and white extremities; the homozygotes are all essentially one big white spot except for the pigmented retina. Except for splotch, however, the neural crest development of these mutants is not grossly abnormal but subtle abnormalities of the neural crest in the homozygotes could prevent the origin of melanoblasts. Alternatively, homozygous tissues may not produce the stimuli required to bring about the maturation of melanoblasts. Since

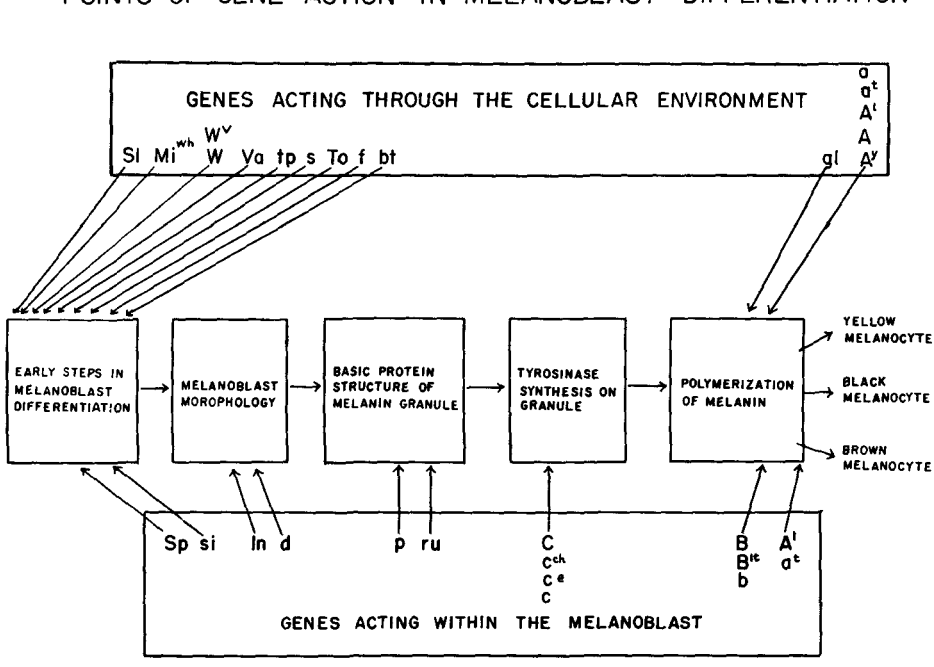


FIGURE 21.—Probable times and places of gene action during melanoblast differentiation in the house mouse.

the cells of these mutants do have the genetic capacity to produce melanin as shown in the retina, the failure to do so in other tissues of the body must stem from abnormal embryonic relationships at some step in melanoblast differentiation. The problem is to discover the time and place during development at which the genes *Sp*, *Mi*^{wh}, and *W* act to prevent pigmentation.

Similar problems are presented by the mutants flexed tail (*ff*) and belted (*bt bt*) each of which is characterized by a variable spotted pigmentation pattern. Since the flexed tail genes, in particular, exert other significant effects on metabolism such as siderocytic anemia and tail flexure (KAMENOFF 1935; GRÜNEBERG 1942a, 1942b) it is probable that they at least act through the tissue environment in affecting melanoblast differentiation.

Place of gene action

It seems reasonable to consider melanoblast differentiation as proceeding through an orderly sequence of steps each of which is dependent upon the successful completion of the previous step. This stepwise differentiation offers many opportunities for genic intervention either by acting directly within the melanoblast or by altering the behavior of the cells composing the tissue environment.

In figure 21 an attempt is made to summarize the probable principal points of gene action on the sequence of steps leading to melanoblast differentiation. (A key to the gene symbols used is provided in table 3.) A similar attempt has been made by WRIGHT (1941) for the genes affecting pigmentation in the guinea pig. In the present scheme for the mouse the spotting genes—*Mi*^{wh}, *W*, *Va*, *s*, *f*, *bt* (and also *To*, *tp* and *Sl*)—are all represented as affecting the early steps in melanoblast differentiation by controlling the tissue environment. The genes at the agouti (*A*) and gray lethal (*gl*) loci likewise appear to act via the tissue environment but in this case on the terminal step of melanogenesis—that is on the step responsible for the

TABLE 3

A key to the gene symbols used in figure 21 and elsewhere in this report

Gene symbol	Name of gene	Gene symbol	Name of gene
a	non-agouti	*gl*	grey-lethal
A	agouti	*ln*	leaden
A^{l}	light-bellied agouti	*Mi*^{wh}	dominant white
a^{t}	black-and-tan	*p*	pink eye
A^{y}	yellow	*ru*	ruby
b	brown	*s*	piebald
B	black	*si*	silver
B^{lt}	light	*Sl*	steel
bt	belted	*Sp*	splotch
c	albino	*To*	tortoise shell
C	colored	*tp*	taupe
c^{ch}	chinchilla	*Va*	varitint waddler
c^{e}	extreme dilution	*W*	dominant spotting
d	dilution	*W*^{v}	viable dominant spotting
f	flexed-tail		

polymerization of melanin to yield the final color—yellow, brown, or black. The action of the genes at the remaining six loci can be interpreted as occurring within the melanoblasts themselves. Dilute (*dd*) and probably leaden (*ln ln*) control melanoblast morphology independently of the tissue environment (REED 1938). Pink eye (*pp*) (RUSSELL 1949a) and ruby eye (*ru ru*) both alter the shape of melanin granules and presumably therefore change the protein matrix of the granule within the melanocyte. The albino locus (*cc*) controls the enzymatic activity essential for melanogenesis and this enzyme, when present, is located within the melanoblasts themselves. Furthermore, albino tissue can support the differentiation of non-albino melanoblasts (REED 1938). Finally genes at the *B* locus affect melanin polymerization to determine the brown or black color of the granules. In contrast to yellow the brown or black color of any mouse appears to be independent of tissue environment and therefore is probably autonomously determined by the melanocyte. The fact that genes exist which independently affect black (e.g. *pp*) and yellow (e.g. *gl gl*) pigmentation makes improbable any linear sequence in the production of these pigments and strengthens the hypothesis that the alleles at the *A* and *B* loci control alternative paths in the polymerization of melanin.

Although these genes have been discussed as if they acted at only one time and place, it should be realized that many of them probably exert significant influences on the course of development by their metabolic activity in a variety of tissues at frequent intervals in development. In fact, the agouti locus appears to act both within the melanoblast and in the tissue environment to direct differentiation. Hence in figure 21 the alleles A^t and a^t are listed in two positions. Other genes may likewise deserve a double listing. Nevertheless, in this discussion an effort has been made to focus attention on what is believed to be the major contribution of the gene to melanoblast development.

The scheme of figure 21 is of course hypothetical but seems to be consistent with all the major facts known about the effects these genes have on pigment formation in the mouse. No such interpretation can be convincingly established by observation alone; experimental reassortment of melanoblasts and tissue environments will be necessary for a critical testing of the scheme. Fortunately such experimental interchanges are feasible and constitute the next step in gathering evidence with which to delineate the relative roles of genotype and embryonic history in the control of cellular differentiation.

SUMMARY

1. The occurrence, abundance, and characteristics of melanocytes in the tissues of the house mouse have been described. These characteristics have been related to the genotype and embryonic history of the melanocyte and to the genotype and differentiated characteristics of the following six tissues: nictitans, harderian gland, ear skin, hair follicle, choroid, and retina.

2. Melanoblast differentiation requires a favorable genotypic constitution and embryonic history in both the pigment cell and in the cells composing the tissue environment.

3. In general the color of melanin granules is related to their morphology—brown

granules are spheres and black granules are ovoids. However, since retinal melanocytes (either brown or black) produce granules of extremely diverse sizes and shapes, no necessary dependence can exist between granule shape and color.

4. Yellow melanin is formed by melanocytes only in the cellular environment of a hair follicle of appropriate genotype. All other melanogenic tissue environments induce the synthesis of either black or brown melanin in accord with the genes present in the melanocyte at the *B* locus.

5. Genes exist that affect the synthesis exclusively of black, brown, or yellow melanin, or of all three colors; these genetic effects render improbable any linear sequence among these pigments in the synthesis of any of them.

6. The nucleopetal (*dd*—dilute, or *ln ln*—leaden) or nucleofugal (*D-Ln-*) morphology of melanocytes is determined exclusively by their own genetic makeup independently of the genetic constitution of the cells in the tissue environment.

7. White spotting on pigmented animals may be interpreted as a failure of the tissue environment to elicit an essential step in melanoblast differentiation.

8. Neither brilliant cresyl blue nor dopa selectively stain melanoblasts although dendritic cells similar in appearance to melanocytes do stain with the brilliant cresyl blue.

9. A scheme is presented which summarizes the probable principal points of gene action (for 19 loci) on the sequence of steps leading to the differentiation of melanocytes.

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