THE GENE ACTION AND FUNCTION OF TWO DOPA OXIDASE POSITIVE MELANOCYTE MUTANTS OF THE FOWL[1]

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ABSTRACT

Ultrastructural and autoradiographic analysis revealed the developmental genetic differences between the dopa oxidase positive *pk* and *I* mutations of the fowl. The differences were revealed by the results of five measurements involving homozygous mutant melanocytes, heterozygous melanocytes, and standard melanocytes at each of the loci. The measurements were: ultrastructural comparisons of melanosomes in pigmented epithelial (PE) and neural crest derived (NC) melanocytes, the number of ^{3}H-dopa and ^{3}H-leucine grains/μ^2 of melanosome, the ^{3}H-dopa/^{3}H-leucine ratio, and the percentage of cytoplasmic ^{3}H-leucine grains that were melanosomal. The *pk* mutation altered both PE and NC melanosomes. +/*pk* melanocytes were characterized by suppressed ^{3}H-dopa/μ^2 and ^{3}H-dopa/^{3}H-leucine values. +/*pk* cells, however, had the same percentage of melanosomal ^{3}H-leucine grains as the "pk" standard. The *I* mutation altered only NC melanosomes. +/*I* melanocytes were characterized by ^{3}H-dopa/μ^2 and ^{3}H-dopa/^{3}H-leucine values similar to the "I" standard. +/*I* cells had a lower percentage of melanosomal ^{3}H-leucine grains than the "I" standard, however. These data suggest that *pk* is a structural mutation affecting melanin binding to the premelanosome, while *I* seems to be a control gene mutation partially suppressing the production of premelanosomal components in NC melanocytes.

CELL differentiation in eukaryotes is only vaguely understood because the mechanisms causing selective gene expression have not been clearly defined. The control of gene expression in the *lac* system of *E. coli*, however, has been determined (JACOB and MONOD 1961). Controlling elements (regulator and operator) in this system were distinguished from the structural genes through the judicious use of mutations. MONOD and JACOB (1961) clearly realized how important the identification of mutations would be in providing an understanding of the mechanisms of cell differentiation. More recently, COMINGS (1972), EPHRUSSI (1972), and KAO and PUCK (1972) have reiterated the need for the characterization of eukaryotic gene mutations.

STANBURY, WYNGAARDEN and FREDRICKSON (1972) suggest that two broad functional classes of genetic mutations exist: structural mutations and control gene mutations. Even though control gene mutations are not clearly defined, they must exist (STANBURY, WYNGAARDEN and FREDRICKSON 1972).

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In this study a control gene locus is defined as any locus which does not itself produce the molecules characteristic of the differentiated state but which affects the expression of those molecules. Thus, structural mutations would affect the *quality* of such molecules, while control gene mutations would affect only their *quantity* or *distribution* (see CROW 1966).

The developing melanocyte produces a complex of molecules which form pigment granules. These organelles are composed of polymeric melanin which is laid down upon a protein framework. The enzyme tyrosinase (dopa oxidase) is responsible for catalyzing the reactions which convert tyrosine and/or dihydroxyphenylalanine (dopa) to dopa quinone. The dopa quinone becomes associated with the protein framework and is further oxidized and polymerized to melanin (BRUMBAUGH and FROILAND 1973). The immature pigment granules or premelanosomes polymerize melanin precursors until their protein matrices are obscured. These matured granules are called melanosomes (FITZPATRICK *et al.* 1966).

Pinkeye (*pk*) is a recessive mutation of the fowl, first reported by WARREN (1940), that reduces pigmentation in both eyes and feathers. Ultrastructural studies by BRUMBAUGH (1968) indicated that this mutation reduces melanin deposition upon the premelanosome. BRUMBAUGH, BOWERS and CHATTERJEE (1973) later showed that *pk/pk* melanocytes die in the presence of high concentrations of tyrosine. The mutation, however, does not eliminate dopa oxidase activity (BRUMBAUGH, BOWERS and LEE 1973).

The dominant white (*I*) mutation of the fowl whitens eumelanin (black) producing melanocytes except those of the eye. Pheomelanin (orange-red) producing melanocytes are unaffected (see HUTT 1949; BRUMBAUGH 1971). Several workers have studied the melanogenic effects of dominant white. Unfortunately, these investigations involved the White Leghorn breed rather than the single mutant stock, and the investigators were often unaware of the other pigment mutants present. HAMILTON (1940) and ZIMMERMAN *et al.* (1974) showed that White Leghorn melanocytes can produce pigment *in vitro*. HAMILTON (1940) and JIMBOW, SZABO and FITZPATRICK (1974) suggested that White Leghorn melanocytes die prematurely before pigment granules have been deposited in the feathers. BRUMBAUGH (1971), however, using a single mutant stock heterozygous for dominant white, concluded that this mutation partially suppresses the production of the melanogenic organelles.

In this study the effects of the pinkeye (*pk*) and dominant white (*I*) mutations upon melanogenesis were examined ultrastructurally. Melanocyte cell cultures of selected genotypes were assayed cytochemically and autoradiographically and the melanosomes at three locations in homozygous mutant embryos were compared to their respective standard.

MATERIALS AND METHODS

Genetic stocks

The tissues used in this study were all derived from stocks and matings of domestic fowl (*Gallus gallus*) maintained in the School of Life Sciences at the University of Nebraska-Lincoln.

The origin of the pinkeye stock has previously been described (BRUMBAUGH 1968). Pinkeye is kept on the extended black (*E*) background to minimize the partial blindness caused by the mutation. Pinkeyed (*pk/pk/ E/E*) chicks and adults have gray feathers and pink eyes. Extended black ($+^{Pk}/+^{Pk}E/E$; the "pk" standard) chicks and adults are black with dark eyes as are the heterozygotes ($+^{Pk}/pk\ E/E$). Figure 1 shows a sample male breast feather of each genotype.

Wild-type ($+^{E}/+^{E}+^{I}+^{I}$; the "I" standard) chicks and adults have black and orange feathers and dark eyes, while homozygous dominant white ($+^{E}/+^{E}I/I$) chicks and adults have white and orange feathers and dark eyes. (The orange feathers are in the pheomelanin-producing areas which are not affected by the mutation.) The origin of the dominant white stock has previously been described (BRUMBAUGH 1971). Heterozygous ($+^{E}/+^{E}+^{I}/I$) chicks and adults are like the homozygotes except that black flecks are present in the white areas. Figure 2 shows a sample male breast feather (a normally black area) of each genotype. Particularly note the pigmented flecks in the heterozygote feather (Figure 2, center).

Melanocytes were cultured in two series. The "pk" series included pinkeye, its heterozygote, and the "pk" standard, extended black. The "I" series included dominant white, its heterozygote, and the "I" standard, wild type.

Electron cytochemistry

Seven-day-old melanocyte cultures of the three genotypes of the "pk" series and eight-day-old cultures of the three genotypes of the "I" series were tested for the presence of the enzyme dopa oxidase using the method of BRUMBAUGH and ZIEG (1972). Melanocytes were evaluated with the electron microscope for the presence or absence of electron-opaque, dopa-melanin reaction product in Golgi-related cisternae and vesicles after incubation in 5mM L-dopa (BRUMBAUGH and ZIEG 1972; NOVIKOFF, ALBALA and BIEMPICA 1968).

Embryonic-tissue comparisons

Melanocytes differentiate *in situ* in the optic cup, becoming the pigmented epithelium, and in the choroid and feathers as migratory derivatives of the neural crest. Non-iridial pieces of eye wall, including both the pigmented epithelium and choroid, and pieces of median backskin from 9-day-old extended black, pinkeye, wild-type, and dominant white embryos were fixed and processed for routine electron microscopy (BRUMBAUGH 1968). Any changes in melanogenesis at each of the three tissue locations were noted and recorded photographically using an RCA EMU3-B electron microscope.

Melanocyte cell culture

Hamburger-Hamilton stage 16–18 embryos (ca. 72 hrs incubation) were removed from their eggs under sterile conditions and placed in a balanced salt solution. The posterior two-thirds of the somites were dissected free and gently trypsinized to produce a suspension of single cells. These were plated out in Waymouth's medium (752/1; GIBCO) with 10% fetal calf serum, 1% bovine serum albumin, and antibiotics, in plastic culture dishes at a density of $5–8 \times 10^5$ cells per 60-mm dish. Many cells died during the first three culture days. On the third and fourth days small colonies of melanocytes became evident which, depending upon the genotype, began to produce pigment granules visible with the light microscope as early as days 5 and 6. Melanoblast and melanocyte colonies would account for approximately 60%–80% of the cell population by the sixth culture day. Melanocyte differentiation *in vitro* of neural crest cells obtained from somite material has been reported by HOLTZER (1968) and ZIMMERMAN *et al.* (1974).

High resolution autoradiography

Representative melanocyte cultures of each genotype were labeled with ^{3}H-dopa and ^{3}H-leucine on the seventh culture day in the "pk" series and on the eighth culture day in the "I" series. L-3 (3, 4-dihydroxyphenyl) alanine (ring-2, 5, 6-^{3}H) at a specific activity of 38.0 Ci/m mole ("pk' series) or 43.0 Ci/m mole ("I" series) and L-leucine-4, 5-^{3}H at a specific activity of 58.0 Ci/m mole ("pk" series) or 50.0 Ci/m mole ("I" series) were used. ^{3}H-dopa-treated cultures were labeled for four hours at a concentration of 0.375 mCi/ml ("pk" series) or

0.500 mCi/ml ("I" series) in regular medium. This was followed by 3 × 10 minute changes of regular medium without dopa. ^{3}H-leucine-treated cultures were labeled for one hour at a concentration of 0.250 mCi/ml ("pk" series) or 0.500 mCi/ml ("I" series) in Minimum Essential Medium (MEM; GIBCO) without leucine or serum. This was followed by 3 × 10 minute changes of medium containing unlabeled leucine (4.6 mM). The differences in specific activities between the two series reflect different batches of labeled compounds and the evalution of methods covering a 15-month period.

The cultures were fixed *in situ* for 30 minutes in three changes of 4% paraformaldehyde in 0.1 M phosphate buffer. Paraformaldehyde reduces nonspecific binding of amino acids to proteins when compared to glutaraldehyde fixation (PETERS and ASHLEY 1969). Fixation was followed by osmication and infiltration with epon using a graded series of hydroxypropyl methacrylate (HPMA)-water and HPMA-epon mixtures. At least six different colonies of melanocytes were marked from each dish. After curing, the marked areas were cut out and mounted on epon pegs for sectioning. Sections were made parallel to the plane of the dish.

In the "pk" series, ultrathin sections were placed on parlodion-coated microscope slides. The slides were then dipped in Ilford L4 emulsion according to the method of YOUNG (1973). In the "I" series, ultrathin sections were mounted on formvar-coated grids which were then mounted on slides and emulsion-coated using a loop method (BRUMBAUGH and FROILAND 1973). The slides were exposed in sealed containers under freon gas at 4°. ^{3}H-dopa-labeled cells were exposed for 35 days, while ^{3}H-leucine-labeled cells were exposed for 8 days. At the end of the exposure period, the slides were developed for 2 minutes in Microdol-X and fixed in 20% sodium thiosulphate. After staining with uranyl acetate and lead citrate the sections were examined and photographed with the electron microscope.

At least 12 cells of each genotype and treatment were randomly selected and photographed. The negatives were viewed with a dissecting microscope and each grain was assigned to a melanogenic organelle or other cytoplasmic component by noting the type of material under the center of each grain. Background, as determined by counting the grains in areas without tissue, was less than 0.027 grains per μ^2, so no correction for background was necessary. The area occupied by the melanogenic organelles of the cytoplasm of each cell was estimated by recording the number of melanosomic "hits" underlying the dots of a grid of evenly spaced dots (0.25 cm apart) superimposed on each negative (BRUMBAUGH and FROILAND 1973).

The percent of cytoplasmic ^{3}H-leucine grains over melanogenic organelles was determined for each cell and the mean for each genotype calculated. The melanosomic concentration of each label was appraised by determining the number of grains per μ^2 of melanogenic organelle for each genotype and treatment. Confidence intervals (95%) for each mean were calculated using Student's t distribution. The mean number of ^{3}H-dopa grains per μ^2 was divided by the mean number of ^{3}H-leucine grains per μ^2 for each genotype to determine the dopa/leucine ratio. In all tabulations the raw ^{3}H-dopa data were proportionally standardized to a specific activity of 35.0 Ci/m mole and the raw ^{3}H-leucine data to a specific activity of 50.0 Ci/m mole.

RESULTS

At the gross phenotypic level the pinkeye mutation acts as a classical recessive since the heterozygote is indistinguishable from the "pk" standard (Figure 1). In contrast, dominant white appears to be a classical dominant mutation but with the heterozygote differentiated by its flecks (Figure 2).

Melanocyte cell cultures of all six genotypes possessed cytochemically detectable dopa oxidase. This is illustrated in Figure 3. Golgi-related vesicles and cisternae in wild-type (see arrow, Figure 3a), pinkeye (see arrow, Figure 3b), and homozygous dominant white (see arrow, Figure 3c) melanocytes contained deposits of electron-opaque, dopa-melanin reaction product. Melanocytes of the extended black, heterozygous *pk*, and heterozygous *I* genotypes also contained

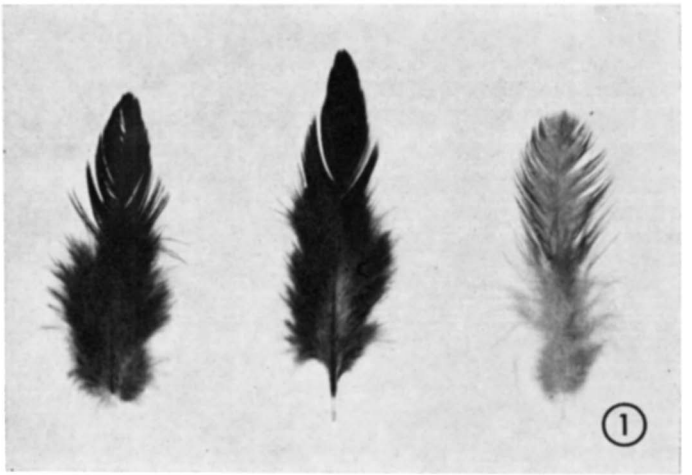

FIGURE 1.—Sample male breast feathers from the "pk" series genotypes: left—"pk" standard; center—heterozygote; right—pinkeye.

dopa-melanin reaction products. The reaction was not noticeably different in any of the genotypes examined. BRUMBAUGH, BOWERS and LEE (1973) found dopa positive pinkeye melanocytes in regenerating feathers. A positive dopa oxidase reaction in heterozygous dominant white melanocytes from regenerating feathers has also been reported previously (BRUMBAUGH 1971). White Leghorn (*I/I*) retinal melanocytes (BRUMBAUGH and ZIEG 1972) and embryonic feather melanocytes (JIMBOW, SZABO and FITZPATRICK 1974) are also positive.

The pinkeye mutation affects all melanocytes, regardless of the site of embryonic origin. This is illustrated in Figure 4. Extended black ("pk" standard) retinal melanocytes contained rod-shaped mature melanosomes, while pinkeye

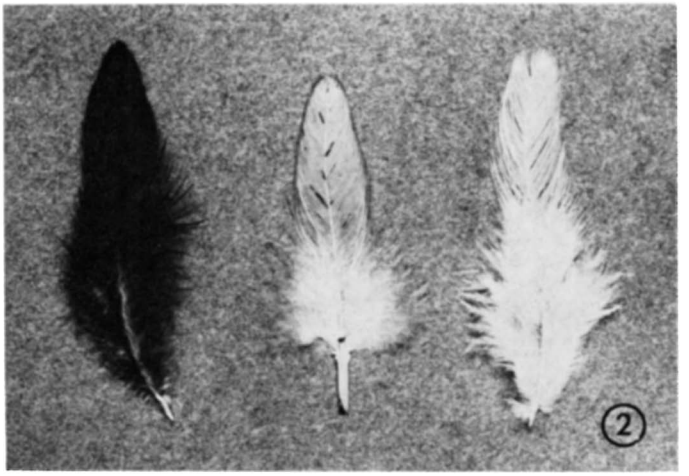

FIGURE 2.—Sample male breast feathers from the "I" series genotypes: left—"I" standard; center—heterozygote with reverted flecks; right—homozygote.

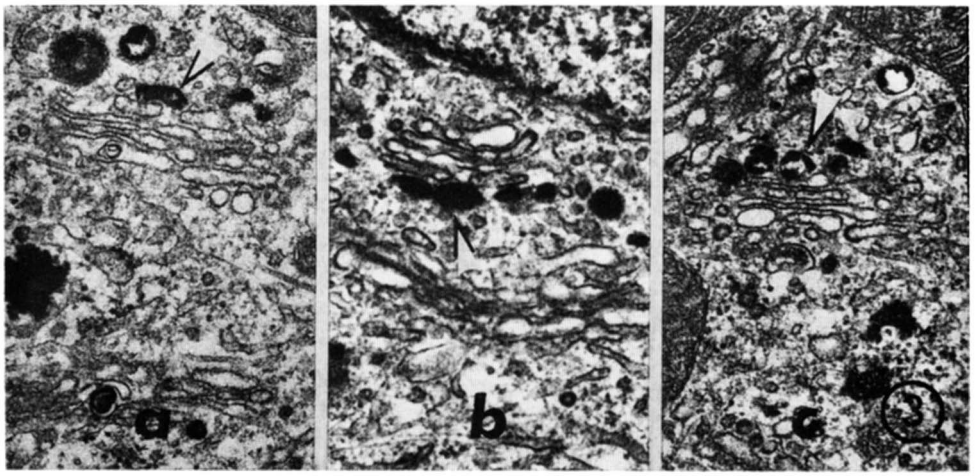

FIGURE 3.—Ultrastructural responses of cultured melanocytes to the cytochemical dopa reaction. Arrows point to dopa melanin reaction product in: a—wild-type ("I" standard); b—pinkeye; c—homozygous dominant white cells. 32,000 ×.

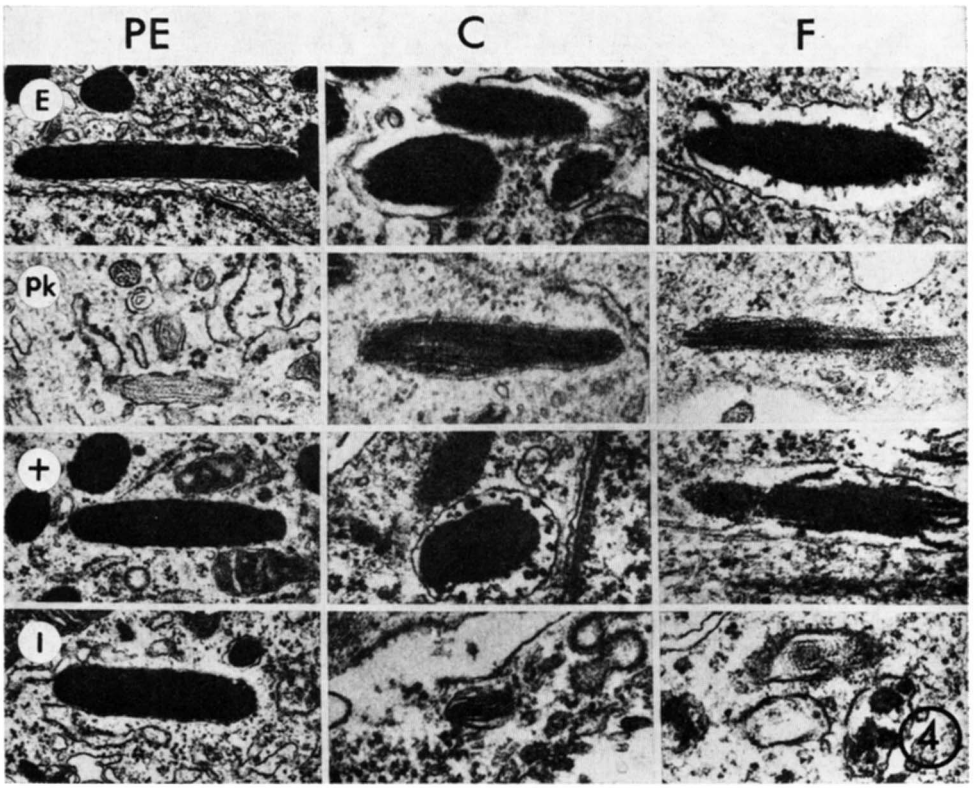

FIGURE 4.—Typical melanogenic organelles from pigmented epithelial melanocytes (PE) of the retina, choroidal melanocytes (C), and feather melanocytes (F) from extended black (*E*; "pk" standard), pinkeye (*pk*), wild-type (+; "I" standard) and homozygous dominant white (*I*) embryos. Pigmented epithelial melanocytes of *E*; +, and *I*, at 22,000 ×; all others at 35,000 ×.

retinal melanocytes possessed only small infantile premelanosomes (cf. top two in PE column, Figure 4). Extended black ("pk" standard) neural crest-derived choroidal melanocytes contained primarily melanosomes, while pinkeye choroidal melanocytes contained only premelanosomes (cf. top two in C column, Figure 4). Feather melanocytes, like choroidal melanocytes, are also derived from the neural crest. Extended black ("pk" standard) feather melanocytes contained primarily melanosomes, while the feather melanocytes of the pinkeye genotype possessed only premelanosomes (cf. top two in F column, Figure 4).

In contrast, the dominant white mutation affects only those melanocytes which are derived from the neural crest. This is also illustrated in Figure 4. Both wild-type ("I" standard) and dominant white retinal melanocytes contained rod-shaped mature melanosomes (cf. bottom two in PE column, Figure 4). Wild-type ("I" standard) choroidal and feather melanocytes contained both melanosomes and premelanosomes. Dominant white choroidal and feather melanocytes, however, contained only a few, very small premelanosomes (cf., bottom two in C and F columns, Figure 4).

The autoradiographic data reported in Table 1 can only be directly compared within a given series because different genetic backgrounds, different culture ages, and different concentrations of labeled compounds were used. The "pk" series is comparable on the extended black (E/E) background, while the "I" series is comparable on the wild-type ($+^{E}/+^{E}$) background. Extended black melanocyte cultures produce melanin sooner than wild-type cultures—hence the earlier labeling period for the "pk" series.

^{3}H-leucine is not a melanogenically specific molecule and, therefore, was incorporated into all compartments of the cytoplasm and nucleus. The percentage of cytoplasmic ^{3}H-leucine incorporated into melanogenic organelles, however, indicated the proportion of newly synthesized protein molecules that were melanosomic.

Melanocytes heterozygous for pinkeye did not statistically differ from "pk" standard melanocytes in the percentage of cytoplasmic ^{3}H-leucine grains associated with melanogenic organelles (first data column, Table 1; $p>0.5$). The percentage of ^{3}H-leucine incorporated into homozygous pinkeye melanosomes, however, was only about one-half that of the "pk" standard (first data column, Table 1; $p<0.001$). The percentage of ^{3}H-leucine incorporated into heterozygous dominant white melanosomes was about four-fifths that of the "I" standard genotype (first data column, Table 1). This difference is not quite statistically significant ($0.10>p>0.05$). Homozygous dominant white melanocyte colonies possessed a variety of melanocytes, some with very few melanogenic organelles to some with moderate amounts of pigment. The mean percentage of ^{3}H-leucine incorporated into the melanosomes of this variable genotype was only one-third that of the "I" standard genotype (first data column, Table 1; $p<0.001$).

^{3}H-dopa is a melanogenically specific molecule. Figure 5 is an autoradiograph of a wild-type ("I" standard) melanocyte that has been labeled with ^{3}H-dopa. The specificity of the label is shown by the fact that all but a few of the grains are associated with melanogenic organelles. The arrows point to two labeled organelles.

TABLE 1

The effect of genotype upon the percent of ^{3}H-leucine grains melanosomic, the melanosomic concentrations of ^{3}H-dopa and ^{3}H-leucine, and the ^{3}H-dopa/^{3}H-leucine ratios

Genotype			% ^{3}H-leucine melanosomic			^{3}H-dopa melanosomic concentration‡		^{3}H-leucine melanosomic concentration§		$\frac{^3\text{H-dopa}/\mu^2}{^3\text{H-leucine}/\mu^2}$
pk locus	*E* locus	*I* locus	n*	Total grains counted	% melanosomal grains (means)	n*	Mean no. grains/μ^2 of melanosome	n*	Mean no. grains/μ^2 of melanosome	
+/+	*E/E*	+/+								
"*pk*" standard			20	2584	22.8±3.4†	18	4.9±0.6	20	1.9±0.3	2.6
+/*pk*	*E/E*	+/+								
"*pk*" heterozygote			18	2175	24.3±3.9	19	2.3±0.4	18	1.9±0.2	1.2
pk/pk	*E/E*	+/+								
pinkeye			21	3325	11.8±2.5	18	0.3±0.1	21	2.5±0.4	0.1
+/+	+/+	+/+								
"*I*" standard			49	8788	19.7±2.7	12	4.0±1.1	25	2.6±0.5	1.5
+/+	+/+	+/*I*								
"*I*" heterozygote			24	2015	15.7±2.7	15	3.4±0.8	24	1.8±0.3	1.9
+/+	+/+	*I/I*								
dominant white			19	2809	6.1±2.1	21	1.9±0.8	19	2.6±0.7	0.7

* n is the number of different cells examined.

† The expressed intervals are at the 95% confidence level.

‡ In the "*pk*" series a total of 2756 ^{3}H-dopa grains over 844 μ^2 of melanogenic organelles were examined. In the "*I*" series a total of 2113 ^{3}H-dopa grains over 534 μ^2 of melanogenic organelles were examined.

§ In the "*pk*" series a total of 1498 ^{3}H-leucine grains over 672 μ^2 of melanogenic organelles were examined. In the "*I*" series a total of 1080 ^{3}H-leucine grains over 545 μ^2 of melanogenic organelles were examined.

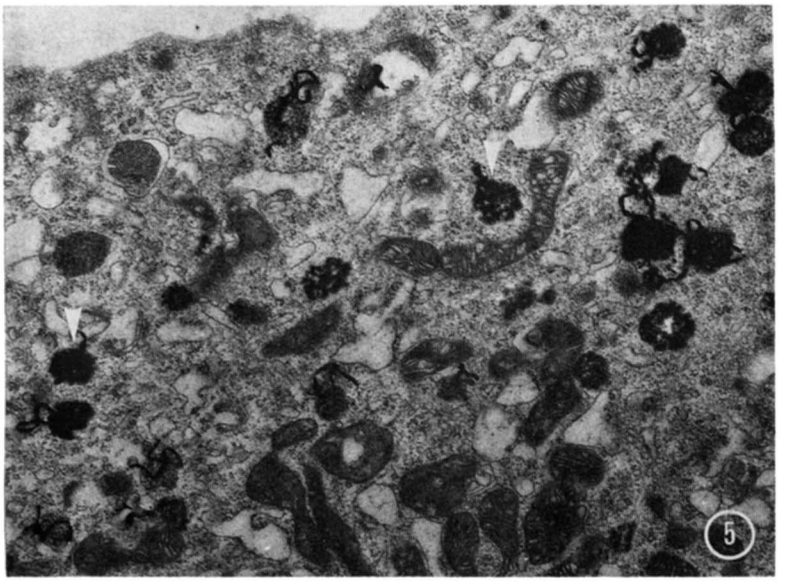

FIGURE 5.—^{3}H-dopa labeled "I" standard (wild-type) melanocyte after 8 days in culture plus 4 hours in label. The arrows point to 2 of the labeled melanogenic organelles. 18,800 ×.

The number of ^{3}H-dopa grains per μ^2 of melanosomal material (melanosomic concentration) shows the effects of the *pk* and *I* loci upon ^{3}H-dopa incorporation. Homozygous pinkeye premelanosomal material incorporated ^{3}H-dopa approximately 16 times less effectively than "pk" standard premelanosomal material (second data column, Table 1). Melanocytes heterozygous for *pk* contained melanogenic organelles which were capable of incorporating ^{3}H-dopa only about half as effectively as "pk" standard melanogenic organelles. Unlike the percent ^{3}H-leucine measurements, there is a statistically significant difference between "pk" standard and heterozygous melanosomes ($p<0.001$). Therefore, the presence of the gene product from the *pk* allele in heterozygous melanocytes definitely affects the ability of melanogenic organelles to incorporate ^{3}H-dopa.

Homozygous dominant white melanosomal material incorporates ^{3}H/dopa approximately one-half as effectively as either "I" standard or heterozygous melanogenic organelles (second data column, Table 1; $0.005>p>0.001$). Unlike the percent ^{3}H-leucine measurements, however, there is no difference between "I" standard and heterozygous melanogenic organelles with regard to ^{3}H-dopa incorporation (second data column, Table 1; $0.4>p>0.2$). Therefore, the presence of the *I* allele in heterozygous melanocytes does not seem to affect the ability of melanogenic organelles to incorporate ^{3}H-dopa.

The number ^{3}H-leucine grains per μ^2 of melanogenic organelles (melanosomic concentration) was also calculated. In this case, there was no difference between "pk" standard and heterozygous cells (third data column, Table 1; $p>0.5$). Homozygous pinkeye melanocytes, however, incorporated significantly *more* ^{3}H-leucine into their premelanosomes than the other two genotypes, which sug-

gests that on a per unit organelle basis the homozygote was synthetically more active (third data column, Table 1; $0.01>p>0.005$). Melanosomes from cells heterozygous for dominant white possessed significantly *fewer* grains that either the "I" standard (third data column, Table 1; $0.01>p>0.005$) or homozygous mutant ($0.05>p>0.025$) melanogenic organelles which did not differ from each other. This suggests that the heterozygote was synthetically less active in this case. The differences in "new" premelanosome synthesis exhibited by the homozygous pinkeye and heterozygous dominant white melanocytes as exposed by the ^{3}H-leucine measurements were probably due to differences in melanocyte maturity caused by variable culture conditions (i.e., cell density, plating efficiency, etc.). The dopa/leucine ratios described in the next paragraph tend to "level out" these maturity differences with regard to ^{3}H-dopa incorporation.

Since replicate cultures of each genotype were labeled in ^{3}H-dopa and ^{3}H-leucine at the same time, the ratio of ^{3}H-dopa grains per μ^2 of organelle to that of ^{3}H-leucine grains was calculated for each genotype. This estimates the amount of ^{3}H-dopa incorporated at that stage of differentiation per unit of "new" melanogenic protein (shown by ^{3}H-leucine incorporation) that was concurrently synthesized. The dopa/leucine ratios in the last column of Table 1 corroborate the information derived from the ^{3}H-dopa means shown in the second data column.

The homozygous pinkeye dopa/leucine ratio was approximately 24 times less than the "pk" standard ratio, while the heterozygote ratio was approximately one-half that of the "pk" standard ratio. The homozygous dominant white dopa/leucine ratio was approximately one-half that of the "I" standard and approximately two-fifths that of the heterozygote. In this case, due to decreased ^{3}H-leucine incorporation by the heterozygous melanosomes, the dopa/leucine ratio is greater in the heterozygote than in the "I" standard.

DISCUSSION

The presence of cytochemically detectable dopa oxidase in pinkeye melanocytes shows that the *pk* mutation does not eliminate this enzyme. SEIJI and IWASHITA (1965) have shown that melanin deposition and dopa incorporation occur almost exclusively at the premelanosome, even though the enzyme may be located elsewhere. Thus, the presence of tyrosinase (dopa oxidase) does not necessarily assure melanin deposition. The autoradiographic data indicate that the product of the *pk* allele does not incorporate ^{3}H-dopa as well as the normal gene product. This reduced incorporation is not directly due to a reduction in tyrosinase (dopa oxidase) activity. Homogenates of homozygous pinkeye retinal melanocytes possessed 76% of the dopa oxidase activity of "pk" standard retinal melanocytes as measured spectrophotometrically (KENNEDY and BRUMBAUGH, unpublished data). This reduction is not statistically significant and not nearly as severe as the 16-fold reduction in ^{3}H-dopa incorporation reported in this study. BRUMBAUGH and FROILAND (1973) have presented evidence that strongly suggests that cysteines are involved in linking melanin intermediates to premelanosomal proteins. Thus, we conclude that the *pk* mutation, which does not appear to

affect dopa oxidase significantly, does appear to affect the ability of the premelanosomes to bind dopa and/or its melanin intermediates.

The presence of cytochemically detectable dopa oxidase in homozygous dominant white melanocytes shows that the *I* mutation does not eliminate this enzyme either. JIMBOW, SZABO and FITZPATRICK (1974), however, found reduced cytochemical dopa oxidase activity in White Leghorn (*I/I*) melanocytes. Such a reduction in enzymatic activity was not noted in the present study. This discrepancy is probably due to the genetic difference between White Leghorns and the single mutant stock used in this study (see discussion below).

Compared to its standard, there are fewer melanogenic organelles in *I/I* melanocytes. This shows that the mutant either prevents the assembly of melanosome components into recognizable structures, reduces their synthesis, or allows premature degradation of these organelles. This correlates with the results of BRUMBAUGH (1971), who found a reduced number of melanogenic organelles in heterozygous melanocytes from regenerating feathers. HAMILTON (1940) and JIMBOW, SZABO and FITZPATRICK (1974), however, using White Leghorn melanocytes, concluded that the hypomelanosis was due to lower viability and early cell death. JIMBOW, SZABO and FITZPATRICK (1974) noted large autophagosomes in White Leghorn melanocytes that were not present in our dominant white melanocytes. The difference is probably due to the complex genetic nature of the White Leghorn breed. HUTT (1949) has stated that White Leghorns possess, in addition to the *I* mutation, the sex-linked barring (*B*) mutation. Some White Leghorns may also carry blue (*Bl*) (personal observation). Since both barring and blue produce hypomelanosis as single mutants (HUTT 1949), it is logical to conclude that they also exert an influence when combined with dominant white. The early cell death in the melanocytes of the White Leghorn breed may not necessarily be due to the gene action of the *I* mutation.

One purpose of this study was to provisionally characterize each locus as either a structural or control gene locus. Such classifications are based on the definitions (given in the beginning of this paper) which emphasized qualitative changes for structural mutations and quantitative changes for control gene mutations. The embryological, autoradiographic, and genetic data from this report will be integrated into control gene and structural gene hypotheses for each locus.

If *pk* is a control gene mutation, it must alter a process which is common to both retinal and neural crest melanocytes since both embryonic cell types were affected. It could not be responsible for initiating melanogenesis since dopa oxidase and premelanosomes are evident in the melanocytes. It would have to affect some process subsequent to the initiation of pigment synthesis in two cell lineages which begin synthesis at distinctly different times during development.

By definition a control gene mutation must affect the quantity of at least one of the melanogenic molecules. In this case, it must be an important melanin-binding molecule, as the autoradiographic data indicate. The reduced number of premelanosomes in *pk/pk* cells would support such a hypothesis. At the same time, however, the molecule must be so small as not to affect the amount of melanogenic protein synthesized in the heterozygote, since the percent of ^{3}H-

leucine grains over melanogenic organelles was the same in standard and heterozygous cells.

If *pk* is a structural mutation, it must affect a melanogenic molecule that is common to both retinal melanocytes and neural crest melanocytes. This would mean that only one set of melanogenic structural genes is present in each haploid genome. Most animal structural genes occur as single copies, the most notable exception being the sequences coding for histones (DAVIDSON *et al.* 1975; DAVIDSON and BRITTEN 1973). Specific molecules known to have only one copy of structural DNA per haploid genome include: mouse globin (HARRISON *et al.* 1973), silkworm fibroin (LIZARDI and BROWN 1975; SUZUKI, GAGE and BROWN 1972), and chicken ovalbumin (SULLIVAN *et al.* 1973). Although the number of tyrosinase and related melanogenic genes per haploid genome has not been directly determined, it is consistent with the general evidence to hypothesize a single copy of each.

Like the *pk* locus of the fowl, mutations at the *c* locus of the mouse affect both retinal and neural crest melanocytes. The products of the mutant *c* alleles are more thermolabile (COLEMAN 1962) and have different electrophoretic enzyme patterns (WOLFE and COLEMAN 1966) than the product of the normal allele and are usually classified as structural alterations. HEARING (1973), however, has reported that albino (*c/c*) extract inhibits melanogenesis. He concludes that this shows that the *c* locus is "regulatory" rather than structural. His reported data do not rule out a possible extract dilution effect, however. Therefore, based on the similarity of tissue types affected, it is logical to hypothesize that the *pk* mutation of the fowl is structural like the *c* mutation of the mouse.

The autoradiographic data support the structural mutant model, if *pk* alters melanin-binding ability as has been discussed previously. The homozygous mutant condition severely restricts the ability of premelanosomes to bind dopa, as indicated by the reduced number of ^{3}H-dopa grains per unit area of premelanosome when compared to the "*pk*" standard. This is further substantiated by an even greater reduction in the dopa/leucine ratio, which is due to an increased incorporation of ^{3}H-leucine by homozygous *pk* premelanosomes. Even though *pk/pk* premelanosomes contained more new protein that either the "pk" standard or heterozygous melanogenic organelles, they bound very little ^{3}H-dopa. The heterozygote data also fit the structural mutation hypothesis. The ability to bind dopa is approximately one-half that of the "pk" standard. This shows that the products of both the normal and mutant alleles are being assembled into melanogenic organelles. This is verified by the fact that premelanosome synthesis, as measured by the percent melanosomal ^{3}H-leucine grains, was the same for heterozygous and "pk" standard cells. The reduced amount of recognizable premelanosomal material in *pk/pk* homozygotes may not necessarily reflect a deficiency of production. It is possible that the mutated molecules, due to their genetic modification, were not as readily assembled into recognizable organelles or were prematurely degraded.

If *I* is a structural mutation, it must be of such a nature that it affects only neural crest melanocytes and not retinal melanocytes. Either separate sets of structural genes are activated for neural crest melanocytes as opposed to retinal

melanocytes, or the structural mutant lesion is not expressed in the different environment of the retinal melanocytes. It has already been hypothesized (see previous discussion) that multiple copies of structural genes do not occur. It has also been determined that melanogenesis in crude extracts of *I/I* retinal melanocytes has the same heat inactivation curve as the "I" standard, suggesting at least some structural similarity between the products of these two alleles (KENNEDY and BRUMBAUGH, unpublished data).

The reduction in the percent of cytoplasmic ^{3}H-leucine incorporated into melanogenic organelles in both heterozygous and *I/I* melanocytes suggests a diminution in the synthesis of some melanosomal component. If *I* is a structural mutant, however, this reduction must be interpreted as poor assembly of mutant molecules or their premature degradation. The reduced number of ^{3}H-dopa grains per μ^2 of melanogenic organelle and the reduced dopa/leucine ratio found for *I/I* melanosomes suggests a qualitative change and is consistent with a structural gene hypothesis.

As a control gene mutation *I* would affect only melanocytes derived from the neural crest. Although both retinal and neural crest melanocytes probably share the same structural genes (see previous discussion), they are apparently activated by different genetic mechanisms. BRUMBAUGH and LEE (1972, 1975) have shown that the blue mutation of the fowl, like the dominant white mutation, also affects only neural crest derived melanocytes.

The *I* locus data encompassing the percent of cytoplasmic ^{3}H-leucine grains associated with melanogenic organelles fit the control gene hypothesis. The *I* allele reduced the number of melanogenic molecules in both the heterozygous and homozygous mutant cells. This is unlike the pinkeye mutation, where similar measurements indicated no difference between "pk" standard and heterozygous melanocytes. The fact that the number of ^{3}H-dopa grains per μ^2 of melanogenic organelle and the dopa/leucine ratio were not reduced in heterozygotes when compared to the "I" standard shows that the presence of the *I* allele does not qualitatively affect the melanosomes by altering dopa binding ability. This is in contradistinction to the *pk* mutation, which caused lowered heterozygous values. If a control gene mutation does not alter the quality of melanogenic molecules, then the *I/I* melanosomes should not have a reduced ^{3}H-dopa/μ^2 value and a reduced dopa/leucine ratio. It should be noted, however, that the reductions were only by approximately one-half and were not nearly as great as the *pk/pk* reduction. It is possible that the small size of *I/I* melanosomes makes determining the source of the ^{3}H-dopa grains less accurate, thus lowering the *I/I* value.

The unstable nature and the dominance of the *I* mutation also suggest that it is a control gene mutation. Control gene mutations can often be expected to have dominant rather than recessive expressions (PATTERSON, KAO and PUCK 1974). Although the frequency of reverted clones in feathers of dominant white heterozygotes has not been determined, it appears high enough to support explanations other than back mutation. No flecks or "ticks" were visible in pinkeye birds. The reverted cells in dominant white heterozygotes may represent melanocytes that have "escaped" from the melanogenic suppression caused by the mutation.

Although the data do not completely eliminate other interpretations, they do conveniently support the hypotheses that pinkeye is a structural premelanosomal mutation and that dominant white is a control gene mutation suppressing premelanosomal production. As previously discussed, other interpretations have inherent difficulties. For example, if both mutations were structural, then the embryological observations would require two haploid sets of melanogenic structural genes. The neural crest set could be mutated in dominant white, but both sets would have to be mutated to produce the pinkeye condition. The latter situation is rather unlikely. Until the components of the melanogenic system can be isolated and their primary structures determined, it will be necessary to draw tentative conclusions based on indirect evidence (see PATTERSON, KAO and PUCK 1974).

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