THE EFFECT OF LOZENGE PSEUDOALLELES ON EYE PIGMENTATION IN DROSOPHILA MELANOGASTER. III. PIGMENT DEVELOPMENT DURING PUPAL DIFFERENTIATION

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Received February 27, 1959

One method of studying the pleiotropic actions of a gene, as expressed in various phenotypic effects in the adults, is the analysis of gene effects in a multiple allelic series. One group of such mutants which has been analyzed extensively by a number of different methods is the lozenge series of pseudoalleles in Drosophila melanogaster. One of the phenotypic effects of the lozenge genes may be observed as abnormalities in the structure and pigmentation of the compound eyes. A series of histological studies of the lozenge pseudoalleles have shown a correlation between different phenotypic effects and a single gene action on development, the failure of the cells to differentiate normally. The structure of the adult lozenge eyes and the pupal development of the mutant eyes were analyzed histologically (CLAYTON 1952, 1954b) and it was found that the failure of the ommatidial cells to differentiate normally led to the irregularities in the adult eye structure and facet appearance. A series on pigmentation in adult lozenge eyes and the structure and pigmentation in eyes of lozenge compounds (CLAYTON 1954a, 1957, 1958) indicate that a correlation exists between the structural abnormalities of the ommatidia and the distribution of the pigment granules. In the present study, the deposition of pigment during pupal development of lozenge eyes is analyzed in an attempt to correlate more closely the effects of the lozenge gene on eye structure and pigmentation.

MATERIALS AND METHODS

Pigmentation was analyzed during pupal development for Drosophila melanogaster Stephenville and eight members of the lozenge series: lozenge (Iz), lozenge glossy (Izg), lozenge 34k (Iz4k), lozenge y4 (Izy4), lozenge 3 (Iz3), lozenge spectacle (Iz5), lozenge spectacle-Bishop (Iz5B), and lozenge 36c (Iz36c). A less detailed analysis was made on lozenge Bar-Stone (IzBS). These mutants were maintained as balanced isogenic stocks using the ClB inversion (OLIVER 1947).

The age of the pupae was determined from the time of egg deposition and pupation. The techniques used for obtaining developmental stages of a particular age have been described in detail in an earlier paper (CLAYTON 1954b). Pupae were collected every five hours from the time when pigment first appeared until emergence of the adults. The heads were removed from the pupae and
unstained slides were prepared by passing sectioned material rapidly through changes of xylol, alcohol, and xylol. The unstained sections were compared with stained material from the previous study on development; the descriptions of normal development in wild type and abnormal differentiation in a group of lozenge alleles may be found in an earlier paper (CLAYTON 1954b).

RESULTS

Pigment development in the wild type eye

The first indication of pigmentation in the wild type eye appears at about 160 hours (42 hours after pupation) as a slight yellowish color. The pigment seems diffuse but this appearance may be the result of the solubility of the pigment in the fixative. The ommatidia at this time have undergone slight elongation and all ommatidial cells can be observed. The pigment is concentrated in the basal portions of the secondary pigment cells and in the basal pigment cells.

At 165 hours the pigment granules are yellowish-brown. The concentration at the basement membrane is heavier and the distribution extends peripherally in the secondary pigment cells about one third of their length. No pigment could be seen in the primary pigment cells but some brown granules were present in the postretinal pigment region. By 175 hours the brown pigment granules are present throughout the length of the secondary pigment cells and are concentrated more heavily in the basal cells (Figure 1). Very little pigment was observed in the postretinal concentration; a few scattered granules are present in the primary pigment cells. The over-all color at this time is yellowish-brown, similar to the color of the adult eyes of the brown mutant. A heavier concentration of pigment in marginal regions is dull brown, lacking the yellowish appearance of the less concentrated regions.

The color at 180 hours is light reddish-brown, confined primarily to the secondary pigment cells and basal pigment cells, although some clusters of pigment have appeared in the postretinal cells. By 185 hours the mixture of red and brown granules can be seen. The color is irregular in different regions; some ommatidia are wine red in color while others are brown toward the basal region and red peripherally. The distribution of pigment in the postretinal concentration is quite distinct, with granules appearing in rounded masses in the cells and in the processes extending upward toward the basement membrane. The pigmentation in the primary pigment cells is irregular at this time.

By 190 hours pigment granules have appeared in the primary pigment cells and the color of the eye is wine-red (Figure 2). The appearance is very similar to the adult condition except for darkening of the color in the granules and completion of pigmentation in the primary pigment cells. The pseudocone cups have not formed at this time and the color in the primary pigment cells is irregular, varying from orange to red. The changes in pigmentation from 190 hours to emergence of the adults between 210 and 215 hours is primarily an increase in the amount of pigment in the granules and increased pigmentation in the primary pigment cells. In pupae of this age the separate postretinal pigment cells
Figures 1–8.—Photographs of unstained sections of normal and lozenge eyes at different stages of pupal differentiation. Carnoy’s fixative, initial magnification $\times 200$. Figure 1.—Normal, 172 hours. Figure 2.—Normal, 190 hours. Figure 3.—Normal, 210 hours. Figure 4.—lz, 170 hours. Figure 5.—lz, 190 hours. Figure 6.—lz, 205 hours. Figure 7.—lz?, 195 hours. Figure 8.—lz?, 210 hours.
are very distinct and more easily studied than the heavily pigmented cells of the adult eye (Figure 3). Just before emergence the structure is like the adult eye and pigmentation is similar to that of a recently emerged adult. The primary pigment cells and the distal portions of the secondary pigment cells are orange-red in color while the postretinal cells, basal cells, and the proximal portions of the secondary cells are dark red.

**Pigment development in the lozenge series**

In tracing the development of pigment in members of the lozenge series, eight of the pseudoalleles were studied in detail, from the first appearance of color in the pupal eye until emergence of the adults. The onset of pigmentation in the different regions of pigment concentration and a comparison of the pigmentation in normal ommatidia and abnormal cells are summarized in Figure 13. Detailed descriptions of the development of structural abnormalities were presented earlier (CLAYTON 1954).

The eyes of adult lozenge males are dark red in color and closely resemble, in pigmentation, the wild type eye color. The distribution of the pigment and the amount of pigment are normal except in regions of structural abnormalities. In the pupal development of the eye, pigment is first observed in the basal region of the normal secondary pigment cells and the normal basal pigment cells. At the onset of pigmentation, after 165 hours of development, the pigment color is a diffuse light yellow. By 170 hours the pigment in the basal region of the ommatidia is yellowish-brown in color. A small amount of pigment can be seen occasionally below the basement membrane but none is associated with the abnormal retinulae in the postretina at this time. These irregular retinulae, however, occasionally disrupt the pigment of the basal and secondary cell regions (Figure 4). Individuals at 175 hours have yellowish-brown pigment granules extending peripherally about one half the length of the secondary pigment cells. No pigment is present around retinulae in the postretina. Brown pigment granules are present throughout the length of the secondary pigment cells in pupae of 180 hours. In the distal portions of these cells and in some of the basal cells the color is reddish-brown, but the over-all color is brown. A few scattered granules of pigment are present in the normal primary pigment cells at this time. At 190 hours brown pigment may be observed with abnormal cells of the postretina and in the distorted primary pigment cells (Figure 5). The eye color at this time is dark red, similar to that of wild type males of the same age. By 205 hours the pigment distribution below the postretinal pigment cells and among distorted pigment cells of other regions is irregular and resembles the condition found in the adults (Figure 6). During the entire pupal development of *lz* the pigment distribution is normal except in the structurally distorted regions where the pigment cells are irregular in shape and size. The color of the granules in abnormal areas is normal and abnormalities in eye color of such regions are the result of variations in cell size and the number of granules present. Pupation in *lz* occurs slightly later than in wild type so that in both normal and *lz* eyes pigmentation begins about 40 hours after pupation.
A complete series of pupal stages was not obtained for $lz^{BB}$, but examination of those available indicates that pigmentation is similar to that of lozenge. Abnormalities in the distribution and shape of pigment cells are more common with resulting irregularities in the location of pigment granules. By 190 hours a heavy concentration of dark red granules is present in the marginal ommatidia. Some pigment occurs with abnormal postretinal retinulae and a small amount of pigment has been deposited in the primary pigment cells.

The eyes of $lz^o$ adults are darker in color than wild type (Oliver 1940) and this difference becomes evident during pigmentation in the pupae. The first indication of pigment formation occurs in individuals of 170 hours; at this time a light yellow color is present in the normal cells of the basal region and in the proximal portions of the secondary pigment cells. Some scattered pigment granules occur in the postretinal pigment cells at 175 hours and pigmentation in distorted secondary pigment cells may be seen. The color in the basal region is dark brown at 180 hours. The granules in the secondary pigment cells are very dense at the basal portions and less concentrated in the distal regions. A few brown granules are present in normal primary pigment cells and scattered patches of brown pigment occur with abnormal cells in the postretina. By 195 hours the eye color is reddish-brown with the postretina and basal pigment a deep red color (Figure 7). The color becomes more intense just prior to the time of emergence; the cells just below the cornea are red-orange while the basal region is a very deep red (Figure 8).

Pigmentation in $lz^{ZS}$ pupae is similar to that of glossy in final distribution of pigment and intensity of eye color. However, pigment was observed at an earlier stage in this mutant than in $lz^o$. The severe distortion and abnormality of the ommatidia in $lz^{ZS}$ make the distinction of pigmentation in normal and abnormal cells difficult since only a few scattered ommatidia are normal. At 165 hours, the pigment, which is yellowish-brown in color, is limited to the region of the basement membrane. In those regions where the basement membrane is disrupted by abnormal retinulae, pigment is absent so that the basal pigment layer is not normal. During the next five hours the color becomes a deeper brown and a heavy concentration is present in the basal portion of the marginal ommatidia (Figure 9).

By 175 hours the pigment granules of the secondary pigment cells extend peripherally about one half the length of these cells. As the extension of pigment in these cells continues, the effect of the structural abnormalities becomes evident. Disrupted pigment cells show a corresponding irregularity in the distribution of the pigment. At 180 hours the pigment extends about two thirds the length of the secondary pigment cells and scattered masses of pigment have appeared in the postretinal layer of pigment cells. At this time no pigment is present around the postretinal retinulae although in individuals of 185 hours some streaks of brown pigment were observed surrounding these cells. By 190 hours the eye color is dark brown with irregularly distributed regions of reddish-brown. In those regions where pseudocones are forming some scattered pigment
Figures 9–12.—Unstained sections of lozenge eyes during pupal development. Carnoy's fixative, initial magnification × 200. Figure 9.—lz$^{z4}$, 170 hours. Figure 10.—lz$^{z4}$, 190 hours. Figure 11.—lz$^{z4}$, 210 hours. Figure 12.—lz$^{z}$, 190 hours.

Granules can be observed, but where the cornea is flattened and the pigment cells form a layer beneath the cornea no pigment is present at this time (Figure 10). The pigment in the normal primary pigment cells is yellowish-red at 200 hours, deepening to an orange-red by 210 hours, the condition present in the adult eyes. The primary pigment cells which occur in a layer beneath the cornea vary in their pigmentation from a deep red to a yellowish-red. Although the pigment in other regions is irregularly distributed as a result of structural abnormalities the color is dark red in the secondary pigment cells and in those pigment cells of the basement membrane region (Figure 11).

The development of pigment in those mutants in which there are no normal ommatidia is closely associated with the structural abnormalities that arise during pupal development. Of the five mutants in this group, only lz$^{z4}$ has a red eye color in the adult; the remaining mutants, lz$^{z}$, lz$^{z}$, lz$^{z4}$, and lz$^{z6}$, have yellowish-brown eyes with flecks of red. The first indication of pigmentation in the eyes of these mutants occurs later than in those previously described. Individuals of 170 hours show no pigment in any regions of the eye while at 175 hours yellowish-brown granules may be seen in the basal regions of the secondary pigment cells of lz$^{z4}$ and lz$^{z}$. The retinulae, which form an irregular layer beneath the pigment cells, have no pigment around them at any time during
pupal development. Each of the alleles develops a heavily pigmented rim, appearing at 180 to 185 hours and darkening with age. In \( l z^2 \) the secondary cells have brown pigment granules throughout their length by 185 hours, with the color appearing irregularly as an orange-brown after 190 hours (Figure 12). The rim is dark brown at 190 hours and becomes very dense by the time of emergence. The layer of primary pigment cells beneath the cornea is unpigmented until about 195 hours when it appears as a light brown layer, with occasional flecks of reddish brown pigment scattered irregularly.

In \( l z^4 \) the basal region of the secondary pigment cells is brown at 180 hours, and after ten hours the brown granules form more distally until they are distributed throughout the length of the cells. At 195 hours the color is reddish-brown in some regions and rust colored in others; some pigment granules may be observed in the primary pigment cells at this time also. The marginal region is dark brown at 180 hours, darkening during the next ten hours and becoming reddish brown by 205 hours. The pigmentation at 205 hours is irregular through the central portion of the eye, varying from yellowish-brown to a dark reddish-brown color.

The pigmentation is \( l z^2, l z^4 \) and \( l z^4 \) is identical as far as can be distinguished by the study of unstained sections. The secondary cells are irregularly pigmented in the basal regions at 185 hours with extension through the distal portion of these cells during the next ten hours. The cells are never regularly pigmented, and the appearance is abnormal, with scattering of brown granules throughout the secondary pigment cells. The primary pigment cells remain unpigmented until about 205 hours, when scattered brown granules appear. Pigmentation in the rim has begun by 185 hours, appearing as a brown color in the basal portion. By 190 hours the color is darker brown, but the pigmentation does not extend to the eye surface at this time. By 210 hours the rim is reddish brown, but the central regions of the eye retain in their yellowish-brown color.

**DISCUSSION**

In the development of the compound eye of *Drosophila melanogaster*, the pigment starts forming in the region of the basal pigment cells and proximal portions of the secondary pigment cells and progresses centrifugally. Elongation of the secondary cells occurs simultaneously with the outward progression of the pigment. A short time after the first appearance of pigment in the basal region, pigment granules appear in the postretinal cells and finally pigment appears in the primary cells during the period when the pseudocones are forming.

The onset of pigmentation in the different cells of the normal eye and the lozenge mutants is shown in Figure 13. By comparing the time of pigmentation it can be seen that the deposition of the pigment occurs in the same way in wild type and two of the lozenge alleles, \( l z \) and \( l z^2 \), appearing first in the secondary (S) and basal (B) pigment cells, about five hours later in the postretinal (PR) pigment cells, and finally in the primary (P) pigment cells.
Figure 13.—Onset of pigmentation in normal and abnormal ommatidia in the normal eye and eight lozenge pseudoalleles. Pigmentation in normal ommatidia is indicated by the open bars and pigment deposition in abnormal cells by the solid bars. The onset of pigmentation in normal and abnormal pigment cells is indicated in hours from egg deposition. The four regions of pigment distribution are indicated as: B—basal pigment cells; P—primary pigment cells; PR—post-retinal pigment; R—pigment in margin or rim; S—secondary pigment cells.

The difference in the time of appearance of the pigment in these different cells may be correlated with the morphological development of the ommatidial elements (CLAYTON 1954). Pupation is delayed in both $lz$ and $lz^g$; however, since differentiation of the ommatidial cells occurs at about the normal rate, the first appearance of pigment occurs at a later time but at the same relative stage in cellular differentiation. If, however, pigmentation in normal ommatidia of the mutants is compared with pigment deposition in abnormal cells, it is seen that the pigment deposits in the abnormal cells appear from five to 20 hours later than pigment in normal cells of the same region. In $lz^{zy4}$, an allele which has only a few normal ommatidia, the distinction between pigmentation in normal
and abnormal ommatidia was observed only in the primary and secondary pigment cells. The basal and postretinal regions are so severely disrupted by structural abnormalities that no normal pigmentation was observed in these regions.

Although normally pigment appears to be heavier in the marginal cells than in other ommatidia during early pigmentation, a distinct marginal rim develops and is retained in five of the lozenge alleles. The heavy deposition of pigment in the marginal cells occurs during the developmental period when scattered pigment granules are visible in the secondary pigment cells. Although the eye colors in these five mutants, \(lz^\nu\), \(lz'\), \(lz\), \(lz^R\), and \(lz^\nu\), differ in the intensity of the red pigment, the distribution of the pigment granules is similar. No pigment was observed during pupal differentiation in the postretinal region of the eyes and although the extreme distortion of the ommatidial cells made recognition of the basal cells difficult, no pigment was definitely located in these cells.

The more striking differences in eye color appear among those alleles with severe structural irregularities of the ommatidia. In the investigation of pigmentation in the eyes of adults of the lozenge series (Clayton 1957, 1958) it was found that alleles similar in the gross structural changes in their ommatidia could vary considerably in eye color. For example, \(lz^\nu\) and \(lz'\) are indistinguishable on the basis of structural abnormalities but the eyes of \(lz^\nu\) are very dark red whereas those of \(lz^\nu\) are light brown. The distribution and number of pigment granules are similar although the amounts of red and brown pigment differ considerably in the two alleles. The development of these two mutants shows that pigmentation progresses in the same manner. The differences in eye color seem to be the result of the control of the gene over the amount of red pigment deposited in the granules during late pupal development. In \(lz'\), \(lz^R\) and \(lz^\nu\) pigmentation commences later than in the less severely affected alleles and the pigment granules remain brown until just before emergence when traces of red pigment are deposited.

The effect of the lozenge gene on the development of the compound eye seems to involve two phases, differentiation of the elements of the ommatidia and the deposition of red and brown pigments in the cells of the visual units. Differentiation occurs normally until the period of eye development in which the retinular components begin to elongate and differentiate into the various cells of the ommatidium. Structural abnormalities appear first and about ten to fifteen hours later, as pigmentation begins, variations in pigment deposition occur. In those alleles which retain some normal ommatidia, pigmentation occurs normally except for differences in the amount of red and brown pigments deposited in cells which otherwise appear to be normal. In \(lz\), one of the less severe mutants, the irregularities in eye color of the adult may be correlated with the occurrence of ommatidia which did not differentiate normally and with variations in the amount of pigment produced in the abnormal pigment cells. In the \(lz^\nu\) eye, which is darker than the wild type, the amount of pigment deposited is greater
in both proximal and distal regions of the ommatidia. Other differences in the appearance of the eye are the result of structural abnormalities.

In an histological analysis of eye development in *Drosophila pseudoobscura*, Cochran (1936) reported that changes in eye color result from an increase in the number of granules and the intensity of the color in the granules. She described two different kinds of granules in the developing pigment cells of the wild type eyes. Nolte (1950), however, analyzed the eye-pigmentary system in *Drosophila melanogaster* and found no indication of specificity of granules for a particular eye pigment; rather, he found evidence that the granules are carriers of both pigments simultaneously. This indicates that mutants affecting eye color influence the number of granules present, their size, or the amount of pigment present in the granules rather than controlling the production of granules specific for either red or brown pigment. Ephrussi (1945) studied deposition of red pigment in scarlet pupae by spectrophotometric methods. The shapes of the absorption curves change with the age of the pupae in a uniform and gradual manner that suggests that the eye colors characteristic of the different stages of development are due to changes in the composition of the pigment. The examination of both normal and lozenge eyes in pupae of different ages does not reveal a specificity of granules for either red or brown pigment, and variations in adult eye color seem to be the result of differences in the relative amounts of the two pigments deposited in the granules, the number of granules present, and the irregularities in the arrangement of the pigment cells in the mutants. An additional factor which may account for absence of pigment in some ommatidial regions is the degeneration which occurs among pigment cells in the severe alleles.

The effects of the lozenge gene on the structure and pigmentation of the compound eye have now been studied histologically in adult males, in compounds of different lozenge alleles, and in developmental stages from pupation until emergence of the adults. A direct correlation between abnormalities in the structure of the ommatidial cells and irregularities in the shape and arrangement of eye facets has been found, and the structural abnormalities of the adult eyes have been traced to irregular differentiation during pupal development of the cells which form the ommatidia. The abnormal shape and arrangement of the pigment cells are related to the distribution of pigment in adult eyes and may partially explain the amount of pigment present. Degeneration of cells in the late pupal stages among the severe alleles may result in the absence of pigment in such regions, and the irregularities in the size of the cells may account for variations in pigmentation.

Many cases of multiple phenotypic effects of a single gene have been examined in *Drosophila* and in other organisms. It has been demonstrated that pleiotropism may result from a single gene mutation that produces its primary effect early in development. However, not all pleiotropism has been satisfactorily related to a single developmental process. Grünberg (1947), on the basis of the results obtained in developmental studies on achondroplasia in the rat, con-
cluded that pleiotropism is spurious and that gene effects may be traced to a single developmental change. Schultz (1935) described the effects of a number of eye color mutants during differentiation of the compound eye in Drosophila. He considered the eye color as the result of two relatively independent aspects, the onset of pigmentation, and the increase of pigment, and observed that the fact that a given gene affects two apparently unrelated processes may indicate an unsuspected relationship or the gene may be capable of producing more than one primary effect. Nolte (1952) described the pleiotropic effect of various alleles of the white locus and suggested that alleles act on the same or a similar substrate in different regions, which may be utilized in different ways during development. If the main product of the gene action is the type of protein, it may be utilized in the eye as a protein carrier or for pigment granules and as structural protein in the spermathecae.

The series of histological studies on the lozenge pseudoalleles have not revealed any single factor which will satisfactorily explain the multiple effects of the gene. Some secondary effects have been traced to improper cell differentiation during pupal development. Anderson (1945) found that reduced fertility in lozenge females may be traced to abnormalities in the development of the female reproductive structures; the sperm stored in the ventral receptacle of the female lose their motility or viability by 72 hours after insemination (Oliver and Anderson 1945). The author has reported abnormalities in the differentiation of the cells composing the ommatidia of the lozenge eyes as the factor resulting in the abnormal facet appearance and, to some extent, irregularities in pigmentation. This series of studies, as well as many other investigations have not successfully found any one factor that could produce the complex phenotype of the lozenge gene. Studies by Green (1948), Chovnick and Fox (1953) and Clayton (1954a) have failed to show a correlation between the actions of the lozenge pseudoalleles and the loci to which they have been assigned by Green and Green (1949). Although some of the morphological changes may be traced to a single causal factor, variations in the amount of pigment deposited in eyes that are similar morphologically and differences in the interactions of the alleles when in compounds seem to indicate that the gene has more than one primary effect or that the basic relationship between the various phenotypic expressions of the gene has not yet been found.

SUMMARY

Pigmentation during the development of pupae is described for Drosophila melanogaster and eight of the lozenge pseudoalleles. The onset of pigmentation, deposition of granules in normal and abnormal pigment cells, and variations in pigment deposition resulting from structural abnormalities are given.

In lz and lz*, pigmentation in normal ommatidia progresses in the same way as in the wild type eye but irregularities in deposition of pigment occur wherever ommatidia are abnormal in shape or arrangement. Extreme distortion of ommatidial cells in lz*4, lz*, lz*, lz*8, and lz*44 pupae resulted in scattered pigment de-
posits in secondary pigment cells with most of the red and brown pigment deposited in a layer beneath the cornea. The distribution of pigment granules is correlated with the distortion of pigment cells, but the amount of pigment present in the cells may vary in alleles which histologically are similar. Abnormal cell differentiation is the primary cause of some of the irregularities in eye color, but the amount of pigment produced during pupal differentiation is either the result of some other independent effect of the lozenge gene or the relationship between these two phases of development has not yet been found.

LITERATURE CITED


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