COMPARATIVE STUDIES OF PHENOL OXIDASE ACTIVITY DURING PUPAL DEVELOPMENT OF THREE LOZENGE MUTANTS (lz^s, lz, lz^K) OF *DROSOPHILA MELANOGASTER*

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THE main thrust of investigations on phenol oxidases in Drosophila has focused on pigment production and thus on color mutants since the very early experiments of GRAUBARD (1933). However, the function of the phenol oxidases may prove to be equally, or more important in cuticle formation (see KARLSON and SEKERIS 1964, for proposed pathway in Diptera). The role played by phenol oxidases in the development of fixed macromolecular structures such as cuticle may possibly be clarified in mutants which have apparently normal body pigmentation, but show decreased production of melanin precursors together with structural abnormalities of the cuticle.

The lozenge pseudoalleles, first compared by GOTTSCHEWSKI (1936), have apparently normal body color. Depending on the gene effect of the particular mutant, the more than 20 known lozenge alleles have normal claws, claws with loss of color, reduced claws or complete absence of claws (CUMMINGS 1946; CHOVNICK, LEFKOWITZ and FOX 1956). Most of the homozygous lozenge females are infertile due to failure to complete development; presumably due to cessation of differentiation in early pupae, of the accessory sex organs, spermathecae and pars ovariae (ANDERSON 1945), associated with pathologies of the ovarian tissue (BENDER and GREEN 1962). An abnormality in development of the eye detected in the early pupal period causes partial loss or absence of the basement membrane of the eye resulting in disarrangement of the ommatidia with accompanying fusion of the facets and a corresponding decrease in the size of the eye (CLAYTON 1954). Brown pigment in the eye of the lozenge-alleles may be reduced to 25% of normal; the red pigment may be reduced to less than 1% of normal (OLIVER 1947; GREEN 1948). The most affected mutants are female sterile, have vestigial claws and sharply reduced eyes which lack facets and have a yellowish color and a dark rim; they may map to either the 1st, 3rd or 4th sublocus at 27.7 on the *X*-chromosome. The most normal appearing mutants map to the 1st or 2nd sublocus and in compounds with the other lozenge alleles are complementary in that the heterozygote female is phenotypically similar to wild type (GREEN and GREEN 1956; GREEN 1961).

Drosophila phenol oxidases have long been known to require activation in

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extracts (HOROWITZ and FLING 1955). MITCHELL and associates (MITCHELL and WEBER 1965; MITCHELL, WEBER and SCHAAR 1967) have recently shown that activation involves five different protein components designated A_1, A_2, A_3, P, and S. Orderly aggregation of these several different protein subunits, based on equilibrium sedimentation in sucrose density gradients, yields at least seven distinct enzyme components in different patterns at different stages in development.

As described by MITCHELL and WEBER (1965) the A components are electrophoresed to different positions in a gel system and when activated metabolize tyrosine and/or dopa, the reaction giving rise to melanin bands in the gel. The lozenge-glossy (lz^g) allele mapping to the 4th sublocus has no A_1 (monophenol oxidase) activity and reduced A_2–A_3 (diphenol oxidase) activity (PEEPLES, BARNETT and OLIVER 1968). This paper reports results obtained from a study of phenol oxidases during pupal development in the severely affected lozenge spectacle (lz^s) allele, the complementary lozenge- KRIVSHENKO (lz^K) allele, and the moderately affected lozenge (lz) allele, mapping to the 1st, 2nd and 3rd subloci, respectively.

MATERIALS AND METHODS

Stocks and culture: The Oregon-R wild stock has been used throughout these studies. The lozenge-spectacle (lz^s)mutant strain was obtained from B. H. JUDD at the University at Austin, the lozenge (lz) strain from E. B. LEWIS at the California Institute of Technology and the lozenge-Krivshenko (lz^K) strain from M. M. GREEN at the University of California, Davis. These stocks were balanced to *In (1) FM3* due to the infertility of the mutant females and in order to reduce crossing over between the *X*-chromosomes.

The wild type and mutants were raised at 27 ± 0.5° C on standard corn meal in ½ pint milk bottles for daily sample collections of white pupae (puparium formation) or cultured on a large scale (MITCHELL and MITCHELL 1964) for collection of enzyme activator material. Usually, male white pupae were processed immediately for electrophoresis or they were kept in culture vials under controlled conditions until they reached the pupal age desired. Activator material was obtained from pupae 0–48 hr after puparium formation.

As previously described (PEEPLES 1966) the Oregon-R strain was made isogenic in 1965. The 2nd and 3rd chromosomes of the lz^s strain were replaced by the 2nd and 3rd chromosomes of the Oregon-R isogenic strain late in 1965, but the lz^s gene has not been crossed out of the *In(1)dl-49* inversion. The lz and lz^K strains were not made isogenic.

In addition to the developmental study on lz^s pupae, pupae of three other lz^s-like mutants, lozenge-64j (lz^{64j}) (obtained from mutagenic studies of M. L. ALEXANDER at the University of Texas at Austin), lozenge-66c (lz^{66c}) (obtained from X-radiation studies of M. L. MCCOMBS at the University of Texas at Austin) and lozenge-y4 (lz^{y4}) [(with sn^3 and v marker genes at 21 and 33, respectively) obtained from E. B. LEWIS at the California Institute of Technology], were compared to lz^s pupae at specific stages in pupal development. Similarly, complementary lozenge-50e (lz^{50e}) mutants obtained from H. BENDER of Notre Dame University were compared to lz^K mutants at specific stages in development. Duplicated-1 (*Dp-1*) and Duplicated-2 (*Dp-2*) strains each having lz^{50e} and lz^{y4} alleles on their X-chromosomes were also obtained from H. BENDER.

Enzyme preparation and assay: The procedure for acrylamide gel electrophoresis of extracts from single pupae has been described previously (PEEPLES, BARNETT, and OLIVER 1968). The procedure for activation and substrate development of A_1 and A_2 enzymes is modified as outlined below from that of MITCHELL and WEBER (1965). A_3 is not distinguished from A_2 by our procedure.*

* A_2 is used to designate the A_2-A_3 components throughout this paper.

Proteins in the 41% $(NH_4)_2SO_4$ fraction of the lz^g (FM3) stock appear to have wild-type activator activity, but premature enzyme aggregation is reduced as is background staining of the gel following substrate application. This preparation (or wild-type activator) was routinely used for activation of all electrophoresed pro-enzymes. The 41% $(NH_4)_2SO_4$ precipitate was resuspended in 0.1 M potassium phosphate buffer, pH 6.3 (10 ml/g of pupae). This suspension was centrifuged at 34,000 $\times$ g for 10 min, to remove insoluble material. L-tyrosine and/or L-3, 4-dihydroxyphenylalanine (L-dopa) (0.4 mg/ml in 0.1 M phosphate buffer, pH 6.3), were used as substrates.

RESULTS

Melanin production from A_1 and A_2 proteins electrophoresed from extracts of single male pupae of lz^s, lz and lz^K mutants and Oregon-R wild type was determined qualitatively by the procedure outlined above. L-tyrosine and L-dopa were the substrates. In Figure 1A melanin bands produced from activated proteins from the extreme mutant, lz^s, and from Oregon-R wild-type pupae are compared at 24 hr intervals during pupal development. The lz^s channels reveal no melanin production in the A_1 band and reduced melanin production in the A_2 band as compared to the controls. The melanin deposited in the A_2 band from lz^s does, however, increase in amount at 48 hr and especially at 74 hr beyond white pupal formation (PWP) as do the melanin bands produced by the Oregon-R A_2.

Figure 1B shows the results of a similar comparison between the activities of proteins electrophoresed from extracts of the moderately-affected mutant, lz, and Oregon-R single pupae. Melanin production in the A_1 band is evident in channels containing mutant flies but the bands are less intense than those of the Oregon-R controls. This result although only qualitative is consistently found. Melanin bands in the A_2 region produced by activated proteins from pupae of the lz mutant are less intense than the control, and similar to those from the lz^s at equivalent stages in development. A_1 and A_2 components electrophoresed from extracts of single, aged pupae of the complementary lz^K allele produce melanin bands similar to those of Oregon-R wild-type control (Figure 1C) when activated and treated with tyrosine and dopa as substrates.

It may be noted in Figure 1 that the melanin in the A_2 band from one Oregon-R 96 hr sample (Figure 1A) and from one lz 96 hr sample (Figure 1B) appear darker than those of other 96 hr individuals. This darker banding appears to result from the fact that these individual animals were still pupae when processed for electrophoresis; whereas the other 96 hr individuals were freshly emerged adults.

The absence of melanin production in the A_1 band in the lz^s gel pattern implies either a reduction in activation potential of the A_1 component by wild type activator or a functional defect of the activated A_1 component. The result is an abnormality in the oxidation of dopa to dopaquinone which spontaneously polymerizes to melanin (see Figure 2). This may imply enzymatic failure in the oxidation of tyrosine to dopa and subsequent reduction in the amount of N-acetyldopamine quinone which may be utilized in cross-linking cuticular proteins.

If ethylenediaminetetraacetate (EDTA) is omitted during processing, the 10% sucrose used to increase the specific gravity of samples layered into gel slots seems also to prevent premature enzyme aggregation. Elimination of EDTA in the

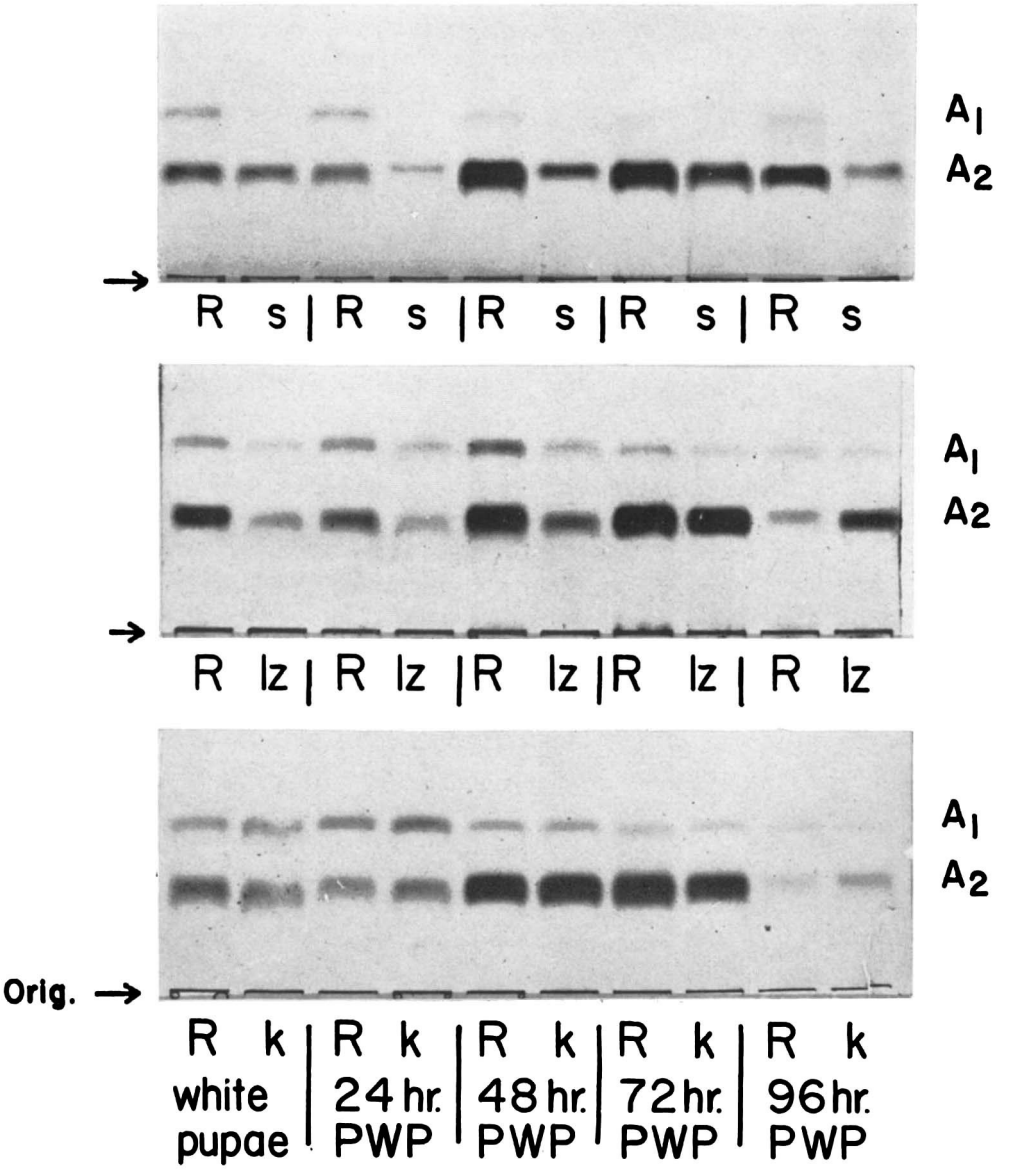

FIGURE 1.—Gel electrophoresis of A components obtained from male individuals of Oregon-R (R) wild stock and the lozenge mutant stocks, lozenge-spectacle (identified by s), lozenge (lz), and lozenge-Krivshenko (identified by k), of *Drosophila melanogaster*. White pupae (puparium formation) were sexed and the males were held under controlled conditions until they were processed for electrophoresis at the post white pupal (PWP) times indicated. The gels were first incubated with a 41% $(NH_4)_2SO_4$ fraction of Drosophila (see MATERIAL and METHODS), then with L-dopa and L-tyrosine. A_1 and A_2 indicate bands of melanin. The origin is marked by an arrow.

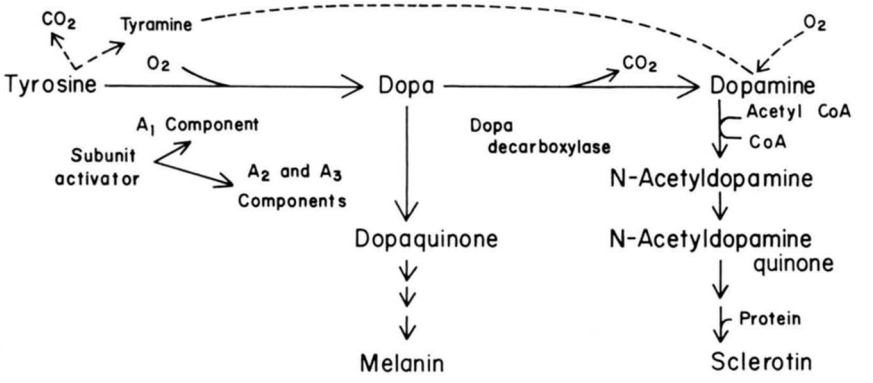

FIGURE 2.—Tyrosine metabolism related to melanin and cuticle formation in the blowfly *Calliphora erythrocephala* as proposed by KARLSON and SEKERIS (1964). The alternate pathway from tyrosine to dopamine via tyramine has been observed in mammals (see text).

preparation of the sample also serves to increase the total monophenol oxidase activity of the activated A_2 component which is primarily a dopa oxidase (MITCHELL and WEBER 1965).

Figure 3 shows the results obtained when EDTA was omitted from extracts of male white pupae and of male pupae 24 hr PWP of the several Drosophila stocks, and the electrophoresed and activated proteins were exposed for three hr to tyrosine alone (see Figure 1 for comparison). It will be noted that three Oregon-R

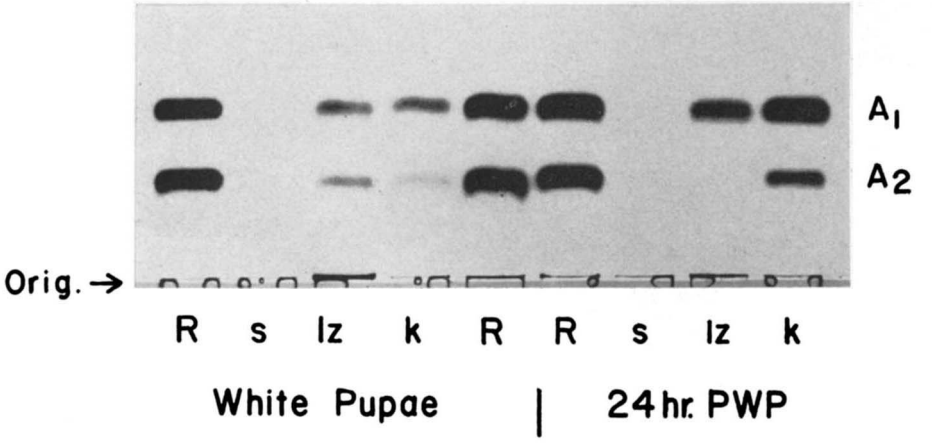

FIGURE 3.—Gel electrophoresis of samples without EDTA from single males of Oregon-R wild type and single males of the lozenge mutants s, lz, and K sexed as white pupae and either processed immediately or held under controlled conditions for 24 hr, before being processed for electrophoresis. Tyrosine was used as substrate following activation of A components. Other symbols as in Figure 1.

(R) channels show dark melanin bands in the A_2 region. The lz^s samples have no activity and the lz 24 hr PWP pattern shows no melanin production at the A_2 band. The channels containing lz and lz^K white pupae show a qualitative reduction from wild type although some caution should be exercised in the interpretation because of the possibility of natural variation. The lz^K 24 hr sample appears to have close to wild-type activity.

Two further observations are illustrated in Figure 4. First, other similarly affected lozenge mutants show gel patterns similar to either the lz^K or lz^s patterns. The gel shows results from single 48 hr male pupae when tyrosine and dopa together are the substrates. As shown previously (Figure 1C), the lz^K sample shows melanin production similar to that of the wild type. The lz^{50e} complementing allele (1st sublocus) shows slightly less melanin production from both the A_1 and A_2 activated proteins. On the contrary the melanin production of a single 48 hr pupa from the severely affected lz^{y4} stocks (4th sublocus) is similar to that of a single pupa from the severely affected lz^s stock (1st sublocus) at the same age with the melanin in the A_1 band absent and the melanin in the A_2 band reduced in amount (Figure 1A). Samples of aged single pupae from the lz^{64j} and lz^{66c} stocks, severely affected alleles like lz^s and lz^{y4}, also produce no melanin in the A_1 band and reduced amounts of melanin in the A_2 band (not shown).

The second observation from the samples in Figure 4 is the demonstration of the effect on the A components of two lozenge alleles on one X-chromosome of a *D. melanogaster* male. Duplicated-1 (*Dp-1*) and Duplicated-2 (*Dp-2*) are radiation-induced tandem duplications detected because of the complementation of the mutants lz^{50e} and lz^{y4} (BENDER 1967). The duplicated strains with the lz^{50e} and lz^{y4} mutations on the X-chromosome are nearly normal in external phenotype. As shown in Figure 4, the Dp-1 and Dp-2 samples from single 48 hr pupae give gel patterns of melanin in the A_1 and A_2 bands similar to those of the lz^{50e} allele.

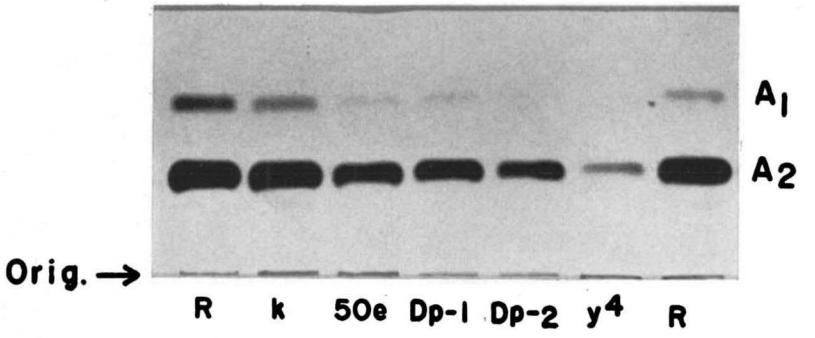

FIGURE 4.—Gel electrophoresis of samples from single male pupae of Oregon-R wild type and lozenge mutants, k, lozenge-50e (identified by 50e), Duplicated-1 (Dp-1), Duplicated-2 (Dp-2), and lozenge-y4 (identified by y4), sexed as white pupae and held under controlled conditions for 48 hr before being processed for electrophoresis. Tyrosine and dopa were used as substrates for melanin production following activation of the A components. Other symbols as in Figure 1.

DISCUSSION

The apparent total absence of melanin production in the A_1 band accompanied by a reduction of melanin production in the A_2 band found in the lz^g mutant (PEEPLES *et al.* 1968) and in the severely affected mutants lz^s, lz^{64j}, lz^{66c}, and lz^{y4} as reported in this paper, as well as the partial absence of melanin production in the A_1 and A_2 bands of the moderately affected *lz* mutant, would tend to associate the lozenge pseudoalleles with phenol oxidase activity. These results are all the more interesting in that the lz^g allele and the more extreme alleles have a total absence of melanin production in the A_1 band during the four days of pupal development and not simply reduced amounts of melanin or a shift in the time during development when melanin production is most active (MITCHELL, WEBER, and SCHAAR 1967; LEWIS and LEWIS 1963).

The reduction or apparent absence of activity of the activated A components may only indicate an abnormality in oxidizing dopa to dopaquinone as mentioned above (see Figure 2). However, in the case of lz^s where there is reduced production of melanin in the A_2 band with dopa as substrate (Figure 1A) and no observable production of melanin in the A_2 band with tyrosine as substrate (Figure 3), it appears that there is little oxidation of tyrosine to dopa by the activated A_2 component. This same argument would apply to the case of the *lz* mutant at 24 hr PWP (Figures 1B and 3) when the A_2 region of *lz* has activity with dopa but not with tyrosine.

Also, as reported previously (PEEPLES *et al.* 1968) lz^g extracts do not produce any observable melanin when tyrosine is the substrate in a conventional spectrophotometric assay. Yet, lz^g extracts can produce melanin from exogenous dopa under the same conditions. This suggests again that little tyrosine is oxidized to dopa by lz^g in the assay system. Preliminary studies also suggest that the lozenge mutants unable to oxidize tyrosine to dopa are also unable to oxidize tyramine to dopamine. The wild-type fly metabolizes tyramine to a melanin-like product in the A_1 and A_2 bands, presumably by first oxidizing the tyramine to the diphenol dopamine (DALY 1967) (Figure 2).

On the basis, then, that lz^g and other lozenge mutants are unable to produce dopa, it is reasonable to postulate a lack of dopa in these mutants for conversion into either dopaquinone and then melanin or into the N-acetyldopamine quinone which may be used for cross-linking cuticular proteins causing tanning and hardening of the cuticle (SEKERIS and KARLSON 1962).

The absence or reduction in size of claws in some of the lozenge alleles (CHOVNICK *et al.* 1956; and CUMMINGS 1946), may be due to a deficiency of N-acetyldopamine quinone needed to cross-link and thereby harden the claw proteins. Alternatively, the claw proteins may simply not be formed. The absence of color in fully formed claws may then be due to limited quinone and consequent failure in quinone polymerization (MASON 1955).

The failure of the spermathecae to complete development may be related to an abnormality in the development of their capsular structure. This capsular structure is shaped by a brown and hardened cuticulin shell evident in microscopic

views of Drosophila spermathecae. CLEMENTS and POTTER (1967) have demonstrated with the electron microscope the presence of a similar cuticular shell in the spermathecae of the mosquito *Aedes aegypti*. The pars ovariae, on the other hand appears to have no hardened structure.

Disorganization of the ommatidial layer of the eyes of the lozenge mutants, as well as possibly the smaller size of the eyes of some of the mutants may be related to the partial or complete absence of the basement membrane of the eye. NOLTE (1961) observed ommochrome in granules larger than in the pigment cells in the bulbous bases of the retinulae of the normal Drosophila ommatidia. These bulbous bases block the openings in the basement membrane according to NOLTE. They may thereby keep the normal ommatidia from being forced through the basement membrane as they elongate. Even the least affected lozenge fly has some ommatidia in the posterior part of the eye displaced below the normal ommatidial layer. When these misplaced ommatidia elongate disruption of the normal ommatidial layer results, leading to pseudocone displacement and the appearance of fused facets on the surface of the eye. The most severely affected lozenge flies have no basement membrane in the eye, no normal ommatidial layer, a glassy surface of the eye and an eye reduced in size (CLAYTON 1954). However, there is at this time no evidence relating the structure of the basement membrane of the Drosophila eye to phenol oxidase structure or function.

In the severely affected lozenge flies there is almost total loss of red pigment and more than 75% decrease in brown pigment (OLIVER 1947; GREEN 1948). This simultaneous reduction in the products of both pteridine metabolism and tryptophan metabolism may be due to disruption of the granule in which both pigments are presumed to be synthesized.

MOYER (1966) describes pre-melanin proteins in the house mouse which form cross-linked fibrils within the pigment granules to give a regular matrix. Melanin appears in a non-random manner within the matrix. Mutations in the mouse which affect the kind and quantity of melanin produced also affect the cross-linking of the fibrils and the integrity of the fibrils.

Melanin is not known to be produced during the formation of the Drosophila eye pigments. However, the Drosophila pigment granule structure may be similar to that of the pigment granules in the mouse. Thus, an abnormality in phenol oxidase structure might be reflected in low pigment production associated with abnormal pigment granules.

Reduction or loss of the melanin-producing function of activated A_1 and reduction of the melanin-producing function of activated A_2 from lozenge mutants indicate possible aberrations of these phenol oxidases which would have an effect on the amount of N-acetyldopamine quinone available for tanning and hardening cuticular proteins (KARLSON and SEKERIS 1964), especially in those parts of the animal where the chitin structure is presumably limited or absent. Thus the pleiotropic effects of this group of mutants may be correlated, and simply explained as due to aberrations in phenol oxidase activity which may be observed as decreased production of melanin and this in turn may be related to decreased production of the cuticular tanning and hardening agent, N-acetyldopamine

quinone. Further investigations are planned to test these relationships between phenol oxidases and lozenge mutants.

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SUMMARY

Activated A_1 (monophenol oxidase) melanin production is absent in extracts from aged single pupae of the severely affected lozenge-spectacle (lz^s) mutant, reduced in extracts from aged single pupae of the moderately affected lozenge (lz) mutants and is equivalent to wild type in extracts from aged single pupae of the complementing lozenge-Krivshenko (lz^K) mutant. Lozenge-64j (lz^{64j}), lozenge-66c (lz^{66c}) and lozenge-y4 (lz^{y4}) which are similar in phenotype to the lz^s mutant also lack A_1 melanin production. Lozenge-50e (lz^{50e}) which is almost wild type phenotypically, and a complementing allele like lz^K, also has apparently normal A_1 melanin production when tyrosine and dopa are used as substrate for the enzyme-substrate reaction.—Activated A_2–A_3 (primarily diphenol oxidase) melanin production is reduced in extracts from single pupae of the severely affected lz^s-like mutants and in extracts from lz single pupae at all stages in pupal development when tyrosine and dopa are the substrates. When tyrosine alone is the substrate lz^s extracts produce no observable melanin and lz extracts produce no observable melanin by the activated A_2–A_3 at 24 hr beyond puparium formation.—Extracts from Duplicated-1 *(DP-1)* and Duplicated-2 *(DP-2)* single male pupae with lz^{50e} and lz^{y4} alleles on each *X*-chromosome give melanin production similar to that from extracts of the mutant lz^{50e} when single pupae of the same age are examined separately and tyrosine and dopa are the substrates.—The possible relationship between phenol oxidase activity and the lozenge pleiotropic pattern of defects is discussed.

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