Functional Significance of the Crystal Cells in the Larva of *Drosophila melanogaster**

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PLATES 95 AND 96

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ABSTRACT

Cytoplasmic crystalline inclusions are found in some larval haemocytes of *Drosophila melanogaster*. Blackening can be experimentally induced in these cells, and previously it was suggested that either the substrate or enzyme for the tyrosine-tyrosinase system leading to melanin production in *Drosophila* larvae is found in these inclusions in the crystal cells. The present report is an attempt to further localize the enzyme and substrate.

Larvae have been fed on food containing α-C^{14}-tyrosine and autoradiographs of the blood cells taken from these larvae subsequently prepared. The C^{14} activity in the crystal cells is restricted to the crystal inclusions in the cells and is significantly higher than that found in the other type of haemocytes, the plasmatocytes. When samples of the blood cells are incubated in DOPA solution, the extracrystalline cytoplasm becomes blackened while the crystals themselves remain colorless. These observations are consistent with the hypothesis that the substrate is localized in the crystal inclusions whereas enzyme is found in the surrounding cytoplasm. An *in vivo* structural isolation would serve to separate enzyme and substrate rather than an inhibition by dehydrogenases as postulated by previous authors. *In vitro* examination with the phase microscope has shown that the crystal cells rupture easily and the crystals dissolve in the haemolymph. Therefore any treatment which tends to disrupt the structural integrity of the cell will allow the enzyme and substrate to come together. Humoral factors preceding metamorphosis might account for the *in vivo* release of the enzymatic reaction by initially altering the permeability of the cell.

The newly formed, soft, colorless cuticle of insects becomes hardened and pigmented by a tanning mechanism involving the entry of quinones into the cuticle where they combine with cuticular protein (12). The source of the tanning agent is the blood where the enzymatic action of tyrosinase on tyrosine leads to the production of 3,4-dihydroxyphenylalanine (DOPA). The quinone, or tanning agent, presumably arises from further oxidation products of DOPA. The latter also is a precursor of melanin. Dennell (4, 5) and Fraenkel and Rudall (8) have studied the tyrosine-tyrosinase system operating in insect blood, and the latter have shown that the reduction in the amount of blood tyrosine coincides with the increase in weight of the cuticle of *Sarcophaga* at the time of hardening and darkening. Tyrosinase activity was demonstrated in a class of haemocytes of *Sarcophaga falculata*, the oenocytoids, by Dennell, and more recently in the spherule cells of *Sarcophaga bullata* by Jones (11). Since both tyrosine and tyrosinase are found in the haemolymph, mechanisms must exist whereby the enzymatic reaction is deferred until the normal physiological moment for their activity arises. Injury, of course, would upset this mechanism, and melanin forms after the body is opened and tissues are exposed.

In 1933 Graubard (9) investigated the tyrosinase activity of some mutant strains of *Drosophila*.

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He found a loss of tyrosinase activity if larvae and pupae were ground with sand; active enzyme could be obtained by extraction with water or by grinding with sand following chloroform treatment. These results lead Graubard to suggest that an inhibitor of tyrosinase was present in the cells and that this inhibitor could be released when the cells were broken by grinding with sand. Chloroform apparently destroyed the inhibitor and active preparations of tyrosinase could be obtained. The study of inhibition of tyrosinase was extended by Dennell (4, 5) who claimed that the oxidation-reduction potential of the blood was maintained below that necessary for tyrosinase activity by the presence of dehydrogenases. This inhibitory action was removed as pupation approached with a rise of the oxidation-reduction potential of the blood. The literature concerning inhibitors of tyrosinase has been recently reviewed by Pryor (13) who has evaluated the techniques and results of previous workers and concluded that there is no evidence to support the hypothesis of inhibition of tyrosinase by dehydrogenases. Pryor suggests, therefore, that structural organization of the cytoplasm must serve to isolate enzyme from substrate *in vivo*. This return to the older theory of separation of enzyme and substrate in the cytoplasm appears feasible in view of the biochemical localization of other intracellular enzymes in the mitochondrial and microsomal fractions of cells. Of interest also is the analysis by Brunet and Kent (3) of the physiological adaptation of the right and left colleterial glands of the cockroach. The left gland secretes the substrate and the right gland secretes a β-glucosidase, thus utilizing a biochemical division of labor to separate enzyme and substrate *in vivo*.

The production of melanin and the tyrosine-tyrosinase system are particularly interesting in *Drosophila* species in which numerous mutants with melanotic tumors and melanotic abnormalities are known. The existence of tyrosinase activity in *Drosophila* was first demonstrated by Graubard (9) who found differences in tyrosinase activity of various body-color mutants. Tyrosinase has also been studied in some of the melanotic tumor strains by Wilson (18), and pigmentation of the tumorous masses is the result of melanin deposition (10).

In an examination of the haemocytes of *Drosophila melanogaster*, two main classes of cells were reported (15). One type of cells, the plasmatocytes, which are spherical through most of the larval life undergo a transformation process at pupation time and eventually become extremely flattened cells. This blood cell transformation occurs in early larval life in tumorous strains, and the flattened variants aggregate either around cells in the haemocoel or around other tissues. The abnormal masses of tissue thus formed later become melanized (16). The other class of haemocytes in *D. melanogaster* can be distinguished by the presence of characteristic cytoplasmic inclusions. These crystal-like rodlets in the cells have suggested the name crystal cells. Preliminary studies with methyl alcohol and hot water revealed that these cells are correlated with the melanin-producing system of *D. melanogaster* (15), and the suggestion was made that the crystal-like inclusions may be either substrate or enzyme, thus supporting the hypothesis of structural isolation of enzyme and substrate postulated by previous authors.

These observations on the crystal cells of *Drosophila* have been extended, and further examination of the nature of the cytoplasmic inclusions with α-C^{14}-tyrosine will be the subject of the present report.

Experimental Methods

A wild type strain of *D. melanogaster*, Ore-R, has been used for all experiments. The medium contained the following ingredients: 2 per cent agar solution, 5 per cent yeast extract, 5 per cent Brewer's yeast, and 2 per cent glucose. After autoclaving, the medium was poured into sterile dishes. Eggs were collected, dechorionated, and sterilized by repeated washing in $HgCl_2$, 50 per cent alcohol, and sterile *Drosophila* Ringer solution following the method of Begg and Robertson (1). They were then transferred to the sterile medium. The first instar larvae which hatched were placed in a dish containing 5 ml. of sterile agar medium on the surface of which was added 0.2 μc. α-C^{14}-tyrosine. The larvae were allowed to feed on the medium with radioactive tyrosine for 48 hours. While they were still in the second instar they were transferred to sterile medium without α-C^{14}-tyrosine. The larvae were subsequently moved to fresh medium every hour for a 5 hour period, and finally allowed to feed without any further precaution of maintaining the medium sterile. This procedure of successive transfer was utilized to deplete the digestive tract of radioactive material.

Haemolymph samples were taken from the larvae when they were 72 to 80 hours old and the blood smears were fixed in 95 per cent ethyl alcohol. Blood samples

were also taken from larvae raised on food which did not contain α-C^{14}-tyrosine, and slides of this material served as the control specimens. All the slides were run down to water from alcohol, and Kodak permeable-base stripping film was applied following the procedure employed by Taylor and McMaster (17). An exposure period of 14 days was allowed and then the film was developed and autoradiographs of the plasmatocytes and crystal cells were examined.

Pigmentation of crystal cells was previously observed in samples of larval blood collected after *Drosophila melanogaster* larvae had been dropped intact in methyl alcohol, hot water, or hot ethanol (15). The same results can be obtained by placing a drop of methyl alcohol on a fresh sample of blood cells on a slide and allowing the preparation to dry. Under these conditions blackening is more pronounced than when whole larvae are treated with methyl alcohol.

Air-dried blood smears were incubated either in DOPA or tyrosinase. For these experiments 1 mg./ml. of either DOPA or tyrosinase in Ringer solution at pH 5.5 was used. The slides were incubated for a period of 1 hour at 25°C. Haemolymph samples placed in Ringer solution served as the control slides for these studies.

RESULTS

There is some variability in the proportions of the cell types in haemolymph samples, but approximately 5 to 10 per cent of the haemocytes of the larval stages of *D. melanogaster* are crystal cells. The number and size of these rectangular cytoplasmic inclusions varies, but each cell generally contains 4 or 5. The crystals appear refractile when examined with the phase microscope in haemolymph samples. These *in vitro* examinations have been extremely useful in revealing the ease with which the crystal cells rupture and the manner in which the crystals disappear in the haemolymph. The body of the larva is opened and as soon as the haemolymph flows on the slide, the preparation is covered and examined immediately with the phase microscope. The crystals dissolve very quickly in the haemolymph; or if the cell becomes swollen and does not rupture, as happens occasionally, the crystals can be seen dissolving within the cell. As the crystals dissolve, they appear to split longitudinally into smaller rod-shaped fragments and within the disintegrating crystal fragments a minute spherical structure is seen which then also quickly disappears. This internal element has been pictured in camera lucida drawings previously (reference 15, Figs. 16 and 28) when it was observed, but the extent of its occurrence was not realized. After the crystal cell ruptures and the crystals disappear, an empty vesicle or "ghost" containing a nucleus remains. Carnoy, hot water, or hot fixatives preserve the crystal structure within the cell.

Blackening of the crystal cells occurs when methyl alcohol is added to a freshly prepared haemolymph sample. Figs. 1 and 2 illustrate the blackening of the crystals as well as the cytoplasm of the cells. There appears to be a gradation in the reaction; *i.e.*, blackening in the smaller cells is more restricted to the crystals while larger cells show blackening of the cytoplasm. This photograph also demonstrates very clearly the diffusion of the pigment from the crystal cells to the other haemocytes. Similar results are obtained when intact larvae are treated with methanol: the crystal cells blacken, some crystal cells are ruptured as revealed by the presence of empty vesicles, and the surrounding tissues in the body become pigmented. When intact larvae are immersed in hot 85 per cent ethanol or hot water (80°–85°C.), blackening is limited to the crystal cells. No evidence of broken cells or empty vesicles is found in this case.

Haemolymph samples, incubated in DOPA solution (Fig. 3), showed a darkening of the cytoplasm of crystal cells while the crystals remained intact and appeared colorless. Incubation in tyrosinase yielded inconclusive results. When DOPA and tyrosinase were used in previous studies of melanization of cuticle and hereditary melanotic tumors of *Drosophila* (13, 15), it was noted that incubation in DOPA resulted in intense melanosis whereas only a slight blackening was produced in these tissues after incubation in tyrosinase. A weak reaction after incubation in tyrosinase would be difficult to evaluate in the case of the crystal cells where examination is limited to air dried blood smears. No staining of the crystals has ever been observed in sectioned material, but the cytoplasm stains intensely with both acid and basic dyes (14).

Autoradiographs of the crystal cells reveal that the reaction foci in these cells are almost exclusively limited to the crystals (Figs. 4 to 8). Photomicrographs of the same crystal cells have also been made with the phase microscope to show the position of the crystals in the cells. The mean number of grain counts per crystal cell with standard deviation was 12.6 ± 2.98 (N = 12

cells) whereas for each plasmatocyte the mean grain count was 2.4 ± 1.5. (N = 50 cells). The crystal cell shown in Figs. 4 to 6 had an exceptionally high grain count of 36 which placed it so far above the range found in the other cells that it was not included in the sample used for obtaining the average count. Ruptured crystal cells showed positive activity in their vicinity, and intact crystals outside the cells also had reaction foci. The plasma which surrounded the intact crystal cells forming the background in the autoradiographs showed no incorporation of C^{14} (Figs. 7 and 8).

DISCUSSION

Fraenkel and Rudall (8) imitated the natural darkening process of the cuticle of *Sarcophaga* by immersing the mature living larvae in methyl alcohol. This darkening by methyl alcohol could not be produced in isolated cuticles. These authors suggested that methyl alcohol may destroy inhibitors of the enzymes involved in the production of cuticular darkening, or it may allow diffusion of the chromagen from the blood of the larva to the cuticle by changing the permeability of the cuticle. Dennell (4) studied the effect of methyl alcohol on blood samples taken from *Sarcophaga* larvae. He likewise suggested that methyl alcohol destroyed inhibitors of the tyrosinase reaction, since this treatment revealed the presence of tyrosinase activity in a class of haemocytes called the oenocytoids. Dennell also noted that exposure to the air before fixation of the blood sample caused the oenocytoids to become brown. The oenocytoids were not found in young larvae, but appeared in full grown larvae and increased in number until the time of pupation when they disappeared from the blood. Tyrosine also increases in the haemolymph in late larval life, and Dennell explained the inhibition of the enzymatic reaction of tyrosinase in the oenocytoids on the tyrosine in the blood by the presence of a dehydrogenase system which maintained the oxidation-reduction potential of the blood below that necessary for the reaction. By a series of ligature experiments he showed that an increase in the oxidation-reduction potential of the blood occurred at the time that the pupation hormone was released from Weismann's ring. With the rising potential, the enzymatic oxidation of tyrosine in the blood would begin, and passage of these oxidation products to the cuticle is correlated with the eventual formation of the hardened and darkened puparium.

The cuticle of *D. melanogaster* may be darkened by immersing intact larvae in methyl alcohol and the latter will also blacken one particular class of blood cells, the crystal cells. The crystal cells of *D. melanogaster* may be considered analogous to the oenocytoids described by Dennell as the source of tyrosinase in *Sarcophaga* larvae. The crystal cells are differentiated from the plasmatocytes of *D. melanogaster* by their crystal-like cytoplasmic inclusions which have been observed to disappear very quickly when they are exposed to the larval haemolymph. The blackening produced by methyl alcohol treatment is most intense in the crystals of the smaller crystal cells, whereas the larger crystal cells show darkening of the cytoplasm and often diffusion to the surrounding cells. This apparent larger size of some crystal cells after treatment may be due to swelling which, in turn, would allow leakage of pigment from the cell.

Further localization of substrate and enzyme within the crystal cells themselves can be demonstrated. Incubation of crystal cells with DOPA clearly shows darkening of the cytoplasm with no alteration of the crystal inclusions. This observation leads to the conclusion that tyrosinase is present in the cytoplasm, possibly associated with mitochondrial or microsomal structures since the darkening is granular in appearance. On the other hand, the incorporation of C^{14} from α-C^{14}-tyrosine in the crystal structure is an indication that this is the site of accumulation of the substrate. In a recent study of the degree of adsorption of various impurities during the crystallization of L(−)tyrosine, Fels (7) has shown that the amount of any impurity incorporated is related to similarity of structure to the L(−)tyrosine. These crystal-like inclusions may represent a relatively pure reservoir of substrate for the tyrosinase reaction; or, they may be bundles of substrates for tyrosinase as well as other systems involving phenolase-substrate complexes.

Induced darkening of the crystal cells of *Drosophila melanogaster* by agents such as methyl alcohol, acetone, and ether indicates that lipide solvents are especially valuable in triggering off the induced pigmentation process. The structural isolation of enzyme from substrate in the crystal cells can serve to prevent the *in vivo* oxidation of the substrate to melanin. The release of the enzymatic reaction by experimental means utilizing lipide solvents suggests that disruption of cytoplasmic membranes with lipide components may

be involved. An insoluble lipoprotein fraction forming the framework of the cell as described in the "ghost cell" preparations of Bruemmer and Thomas (2) may be postulated as the anchoring mechanism of the enzyme-substrate separation in the crystal cells. Treatment of the crystal cells in blood samples with minute quantities of methyl alcohol will dislocate the enzyme and substrate and allow the biochemical reaction to proceed with the result that blackening becomes visible in the crystal inclusions or the entire cytoplasm depending upon the degree of disruption of the cellular organization. The *in vivo* release of the enzymatic oxidation in late larval life may be effected by the alterations in the humoral factors in the haemolymph which are correlated with metamorphosis. At this time the plasmatocytes undergo extensive morphological changes and a decrease in the frequency of the crystal cells occurs (15). The present status of the problem of phenols and their relation to hardening and darkening of insect cuticle has recently been reviewed by Dennell (6).

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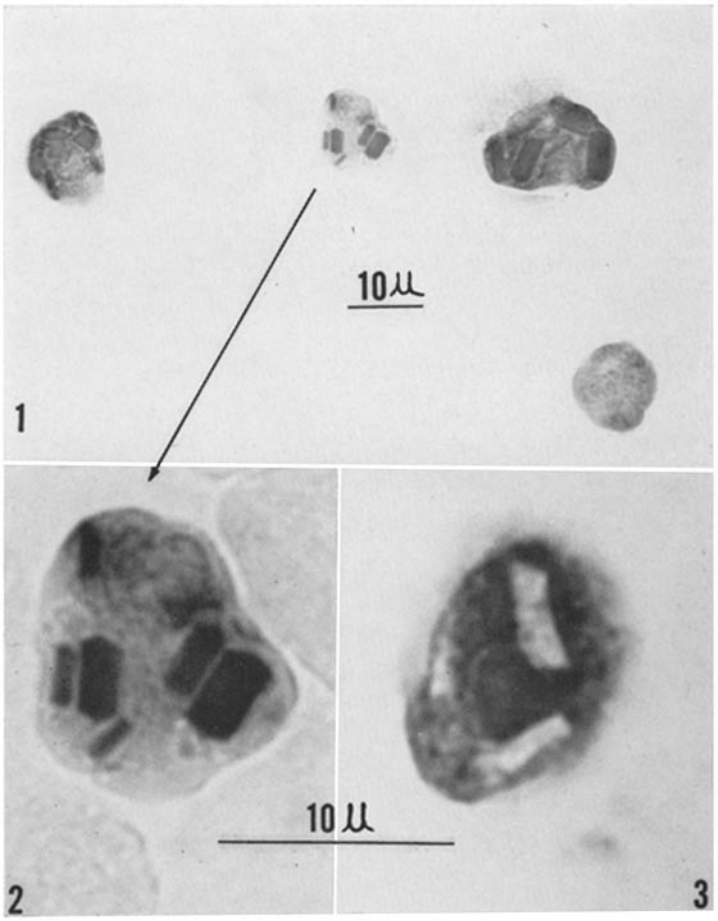
Explanation of Plates

Plate 95

Fig. 1. Blood smear which has been treated with methyl alcohol. The photograph shows four crystal cells with varying degrees of darkening depending upon the size of the cell. There is also diffusion to the surrounding plasmatocytes from the largest crystal cell. × 1000.

Fig. 2. An enlargement of one of the crystal cells shown in Fig. 1 to illustrate the geometric form of the crystals. Melanization is more heavily localized in the crystals than the surrounding cytoplasm. × 3200.

Fig. 3. A crystal cell which has been incubated in DOPA. There is a localization of DOPA-melanin in the cytoplasm while the crystals remain colorless. The specks on the crystal structures are overlying cytoplasmic particles. × 3200.



(Rizki and Rizki· Functional significance of crystal cells)

PLATE 96

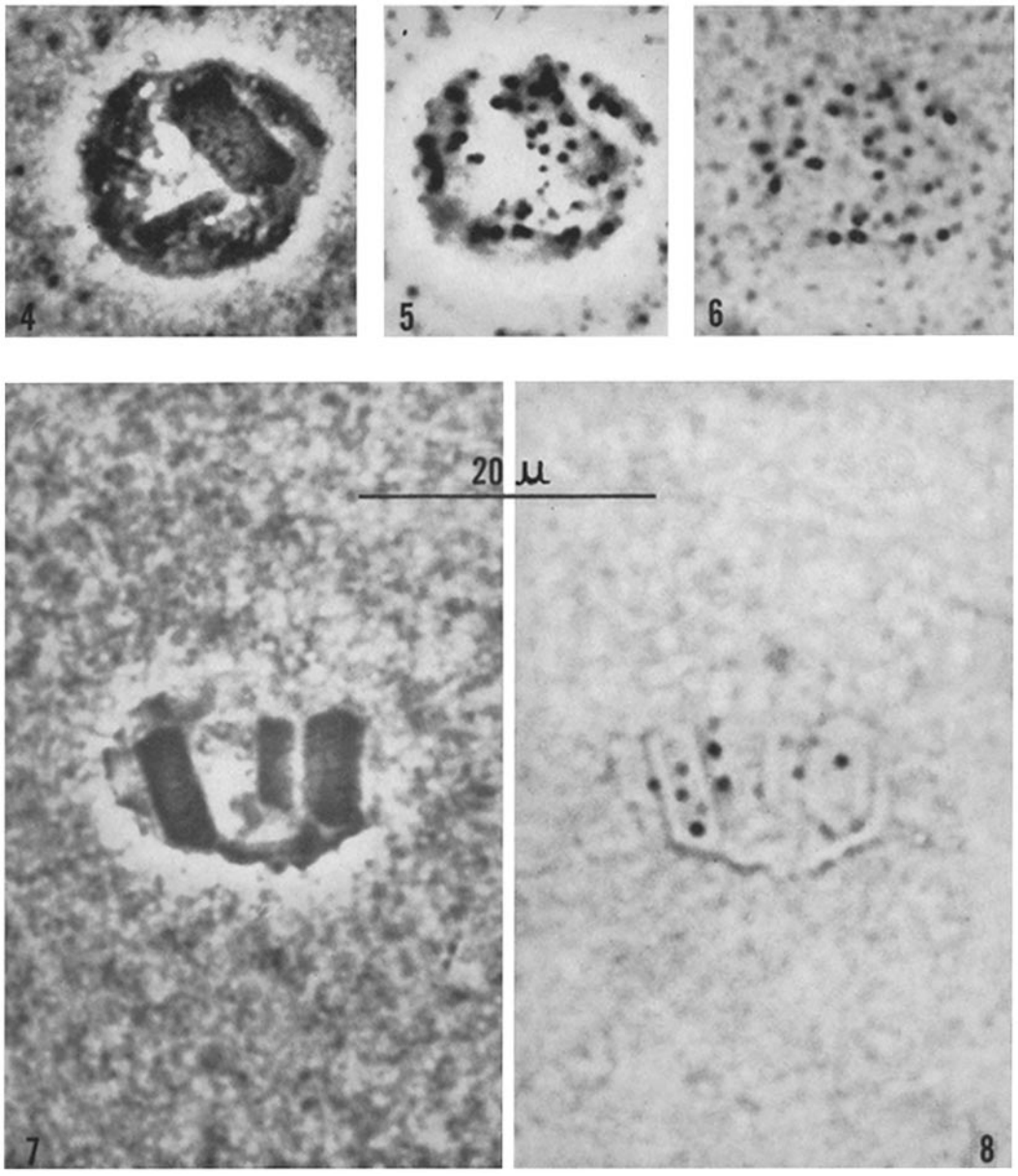
FIG. 4. Optical section of a crystal cell under the phase microscope to show the position of the crystals in the cell. The sample was taken from a larva which has been fed on food containing α-C^{14}-tyrosine. × 2100.

FIG. 5. Same cell as in Fig. 4 at an upper focal level showing some of the crystals and the reaction foci on the emulsion. Phase photomicrograph. × 2100.

FIG. 6. Autoradiograph of the same cell as shown in Fig. 4. Photograph taken at the focal level of the emulsion. × 2100.

FIG. 7. Phase photomicrograph of another crystal cell from a larva which has been fed food containing α-C^{14}-tyrosine. × 2100.

FIG. 8. Photograph of the same cell as Fig. 7 at the focal level of the emulsion. × 2100.



(Rizki and Rizki: Functional significance of crystal cells)