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A Mutant Affecting the Crystal Cells in *Drosophila melanogaster*

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Summary. *Black cells* (*Bc*, 2-80.6±) mutant larvae of *Drosophila melanogaster* have pigmented cells in the hemolymph and lymph glands. In this report we present evidence that these melanized cells are a mutant form of the crystal cells, a type of larval hemocyte with characteristic paracrystalline inclusions. *Bc* larvae lack crystal cells. Furthermore, the distribution pattern of black cells in *Bc* larvae parallels that of experimentally-blackened crystal cells in normal larvae (phenocopy). In *Bc*/*Bc* zygotes black cells appear during mid embryonic development but in Bc^+/*Bc* zygotes pigmented cells are not found until late in the first larval instar. Crystal cells are present in the heterozygous larvae until this time, and paracrystalline inclusions can be seen in some of the cells undergoing melanization in these larvae. The rate of phenol oxidase activity in Bc^+/*Bc* larval cell-free extracts is less than half that of Bc^+/Bc^+ extracts whereas enzyme activity is undetectable in *Bc*/*Bc* larvae. We propose that the Bc^+ gene product is required for maintaining the integrity of the paracrystalline inclusions; in *Bc*/*Bc* larvae either the product is absent or nonfunctional so an effective contact between substrate and enzyme results in melanization of the cells. Phenol oxidase itself is either destroyed or consumed in the melanization process accounting for the absence of enzyme activity in *Bc*/*Bc* larvae. These studies confirm that the crystal cells store phenolic substrates and are the source of the hemolymph phenol oxidase activity in the larva of *D. melanogaster*.

Introduction

The characteristic phenotype of the *Black cells* (*Bc*) mutant in *Drosophila melanogaster* is the presence of pigmented cells beneath the integument in the larval, pupal and adult stages (Grell 1969). Some of the black cells circulate through the hemocoel while others are sedentary. In this report we present evidence that the pigmented cells in *Bc* are a mutant form of the crystal cells, the type of larval blood cell containing phenol oxidase (Rizki and Rizki 1959).

Approximately 10% of the circulating larval hemocytes in the *Ore-R* wild type strain of *D. melanogaster* are crystal cells (Rizki 1957a). Crystal cells contain prominent cytoplasmic paracrystalline inclusions that are absent in the plasmatocytes or their derivatives. An additional distinctive feature of the crystal cells is their sensitivity to changes in the physiological condition of the hemolymph. They rupture readily when the body wall is broken or the hemolymph is removed from the hemocoel. Such experimental manipulations that disrupt the integrity of the paracrystalline structures result in blackening of the crystal cells, and this pigment often diffuses through the surrounding hemolymph as well. Since ring-labeled ^{14}C-tyrosine was incorporated in the paracrystalline structures and phenol oxidase activity was demonstrable in the surrounding cytoplasm of the cells, it was proposed that separation of enzyme and substrate within the crystal cell is achieved by the packaging of the latter in paracrystalline form (Rizki and Rizki 1959). *Bc*, the first reported mutant gene that specifically affects the crystal cells, will be useful for analyzing the developmental characteristics and functions of this unique hemocyte type. The present report on the phenogenetics of the *Bc* mutant is the first in a series of studies on the hemolymph melanin system in *D. melanogaster*.

Materials and Methods

Strains and Culture Media

For the linkage studies the following stocks were used: *Bc* Pu^2 Pin^{Yt}, *c Bc*, *ff wi*, *L*, *M(2)S7 Bc*, nw^D, for the developmental studies the *Bc ff wt* and *Ore-R* wild-type strains were used (no-

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menclature in Lindsley and Grell 1967). Crosses for locating the *Bc* gene were maintained on a corn meal, agar, sugar, brewer's yeast medium whereas the specimens for morphological studies were grown on cream of wheat medium seeded with live yeast. Embryonic ages were timed from egg laying ($\pm 1/2$ h) and larval ages were timed from hatching (± 1 h). All experiments were conducted at 24–25° C.

Electron Microscopy

Larvae were fixed in 2.3% formaldehyde fixative buffered with phosphate at pH 7.15 and posttreated in 1% osmium fixative at room temperature. This fixation procedure preserved the cytoplasmic inclusions in the crystal cells both in the lymph glands and in the hemocoel. Some specimens were fixed in 3% glutaraldehyde-3% formaldehyde-1% acrolein in cacodylate buffer at pH 7.2. Epon sections were examined in a Philips 300 EM.

Phenol Oxidase Measurements

Phenol oxidase activity was assayed according to the method of Mitchell (1966) with the modification that the extract was passed through a P-2 gel column to remove small molecules. Larvae 94–96 h of age were homogenized in 0.1 M phosphate buffer at pH 6.3 in Tenbroeck glass homogenizers with ten strokes of a teflon pestle. The homogenate was centrifuged for 5 min at $18{,}000 \times g$ in a refrigerated Beckman J-21 centrifuge. The supernatant was passed through P-2 gel in a water-cooled column and placed in an ice bath to await enzyme activation. Approximately 1.5 h was required from the time of homogenization. Protein concentrations of the extracts were determined by both the Biuret reagent and the Lowry method using bovine serum albumin as a standard.

Assays were performed in cuvettes placed in a Zeiss PMQ spectrophotometer equipped with a 100-point automatic slit adjustment device and a cuvette holder with circulating water at 30° C. At a wavelength setting of 475mu the 100-point was adjusted against the blank cuvette containing buffer and the activated enzyme extract. To the reaction cuvette containing the activated extract in buffer, L-Dopa (0.02 M) was added and the mixture stirred rapidly. At this moment the recorder was activated to monitor the reaction rate.

Results

Description of the Pigmented Cells

Mutant homozygous and heterozygous third instar larvae can be easily recognized due to the presence of black cells beneath the integument. Many of the pigmented cells appear to be sedentary. A few of these are generally found in the anterior pair of the lymph glands (or blood forming organs), and others are scattered as individual cells throughout the hemocoel (Fig. 1a). If larvae are fixed by injecting formaldehyde so that the body remains stretched, the specimens can be dissected from the ventral side to determine the nature of the localization of the pigmented cells in the hemocoel. Such dissected specimens show that most of the black cells are trapped between the intersegmental muscles and the body wall, in the

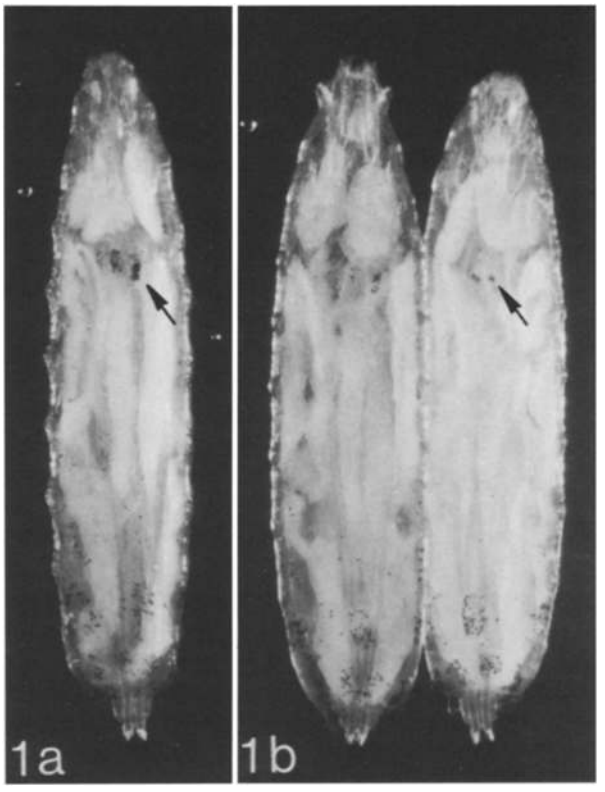

Fig. 1. a Distribution of black cells in a late third instar *Bc* larva. The *arrow* indicates the anterior pair of lymph glands; the black specks in the posterior region are melanized cells in the hemocoel. **b** Late third instar *Ore-R* (Bc^+/Bc^+) larvae treated with hot H_2O; the distribution of the blackened crystal cells in the lymph glands and hemocoel phenocopy the *Bc* mutant larva. $\times 18$

spaces between the alary muscles of the heart, and near the ostia of the heart. Numerous pigmented cells are also found in the caudal hemocoel. Some of the black cells that aggregate in the posterior segment near the caudal ostia and the two pairs of lateral ostia of the heart (Rizki 1978) can be followed in living larvae as they enter the heart, flow anteriorly, and return to the posterior hemocoel via the lateral sinuses. These free floating pigmented cells thus provide an excellent means to follow the path of hemolymph flow in the larva, a process otherwise observed with difficulty.

Freshly drawn hemolymph from third instar *Bc* larvae contains black cells, plasmatocytes, podocytes, and lamellocytes but no crystal cells. *Ore-R* larvae at this same developmental stage contain plasmatocytes and crystal cells primarily (description of cell types and frequencies in Rizki 1978). Lamellocytes, flattened derivatives of the spherical plasmatocytes, appear at the end of larval life in *Ore-R* but are abundant in third instar melanotic tumor mutant larvae where they form the multilayered walls of the melanotic capsules (Rizki 1957b). It is therefore inter-

esting that many lamellocytes are found in *Bc* hemolymph samples where they can be seen adhering to the surfaces of the black cells. Black cells are often found in clumps with other blood cells.

As noted above, care is required to preserve the integrity of the crystal cells for cytological study in the *Ore-R* strain since these cells disintegrate easily. That the absence of crystal cells in *Bc* larvae is not due to this cause is evident since disrupted cells are not found in *Bc* hemolymph samples. In view of the fact that crystal cells contain substances that may be utilized for melanin production, the next possibility to consider is that melanization of the crystal cells or their precursor cells has occurred in the mutant larvae to yield black cells.

In *Ore-R* larvae crystal cells are present in the lymph glands as well as in the hemocoel. We therefore examined epon sections of *Ore-R* and *Bc* lymph glands and caudal hemocoels with the transmission electron microscope (TEM) to compare hemocyte types in these regions of the body in both groups of larvae. Figures 2–4 illustrate the large paracrystalline structures that characterize the crystal cells in the hemocoel and in the lymph glands of *Ore-R*. Such cells were not detected in *Bc* larvae which contained melanized cells instead (Fig. 5). Some melanized cells in *Bc* lymph glands are round whereas others are irregular in shape. The melanized cell surfaces also show variation, some being smooth and others having surface extensions that are either folded membranes or filamentous projections in section. The internal structures in the black cells including the nuclei, nucleoli, chromatin and mitochondria are well preserved. Another interesting feature of the black cells in the lymph glands is the fact that they are invariably encapsulated by one or two other nonpigmented cells (Fig. 6). Presumably the latter are cells from within the lymph glands, but the possibility that hemocytes from the homocoel have infiltrated the glands cannot be excluded. Detailed ultrastructural studies on the types of cells within the lymph glands and their functions are needed to distinguish between these alternatives.

Black Cells in Bc/Bc Embryos

Since black cells in *Bc* and their normal counterparts in *Ore-R* larvae occur in both the hemocoel and lymph glands, the question arises whether this cell type originates from the lymph glands and is discharged into the hemocoel. To answer this question we sought to determine the location of the first black cells to appear during development. Newly emerged *Bc/Bc* larvae as well as late embryos have black cells. Therefore embryogenesis of dechorionated *Bc/Bc* eggs was monitored to establish the approximate time of appearance of pigmented cells. After it was determined that pigmented cells appear about the eleventh hour of development, additional groups of eggs timed from the tenth hour of oviposition were examined at intervals of 15–20 min and the position of pigmented cells as they appeared were noted in camera lucida drawings of each egg. Several of these drawings are included to illustrate that the cells undergoing blackening are not restricted to one site in the embryo (Fig. 8). Some of the cells are dorsal to the cerebral hemisphere in the vincinity of the first pair of lymph glands; others are scattered along the furrows forming between the folds of the gut. Black cells can also be seen to accumulate around the first pair of lymph glands when viewed from the dorsal side. However, when the larva hatches and the body extends, these cells are dispersed and there is no indication that the lymph glands disintegrate. Therefore this accumulation is due to congestion of the pigmented cells and other blood cells in the hemocoel rather than localization within the blood forming organs.

Some of the eggs were selected for the presence of black cells, fixed and processed through epon, and thin and ultrathin sections were examined with phase contrast optics as well as TEM. Among this group of embryos the earliest stage of development was represented as the time when the midgut is saclike (Fig. 9).

Another interesting feature concerning pigmentation in *Bc/Bc* was found during examination of embryos. A pair of cells that had undergone blackening during cell division was discovered (Figs. 10 and 11).

Phenotype of Bc^+/Bc

Additional evidence that black cells are a mutant form of the crystal cells comes from the crystal cell phenotype of heterozygous embryos and larvae (*Ore-R* ♀ × *Bc ff wt* ♂). In this case black cells do not appear until late in the first instar and crystal cells are present in the hemolymph until this time. When black cells begin to appear in Bc^+/Bc larvae, some of the lightly melanizing cells contain distinct paracrystalline inclusions. Occasionally, in older heterozygotes one or two darker inclusions are discernible within the melanizing cells.

Phenocopy of Bc

Melanization of the crystal cells of *Ore-R* larvae is induced by treatment with methanol, ethanol, or hot

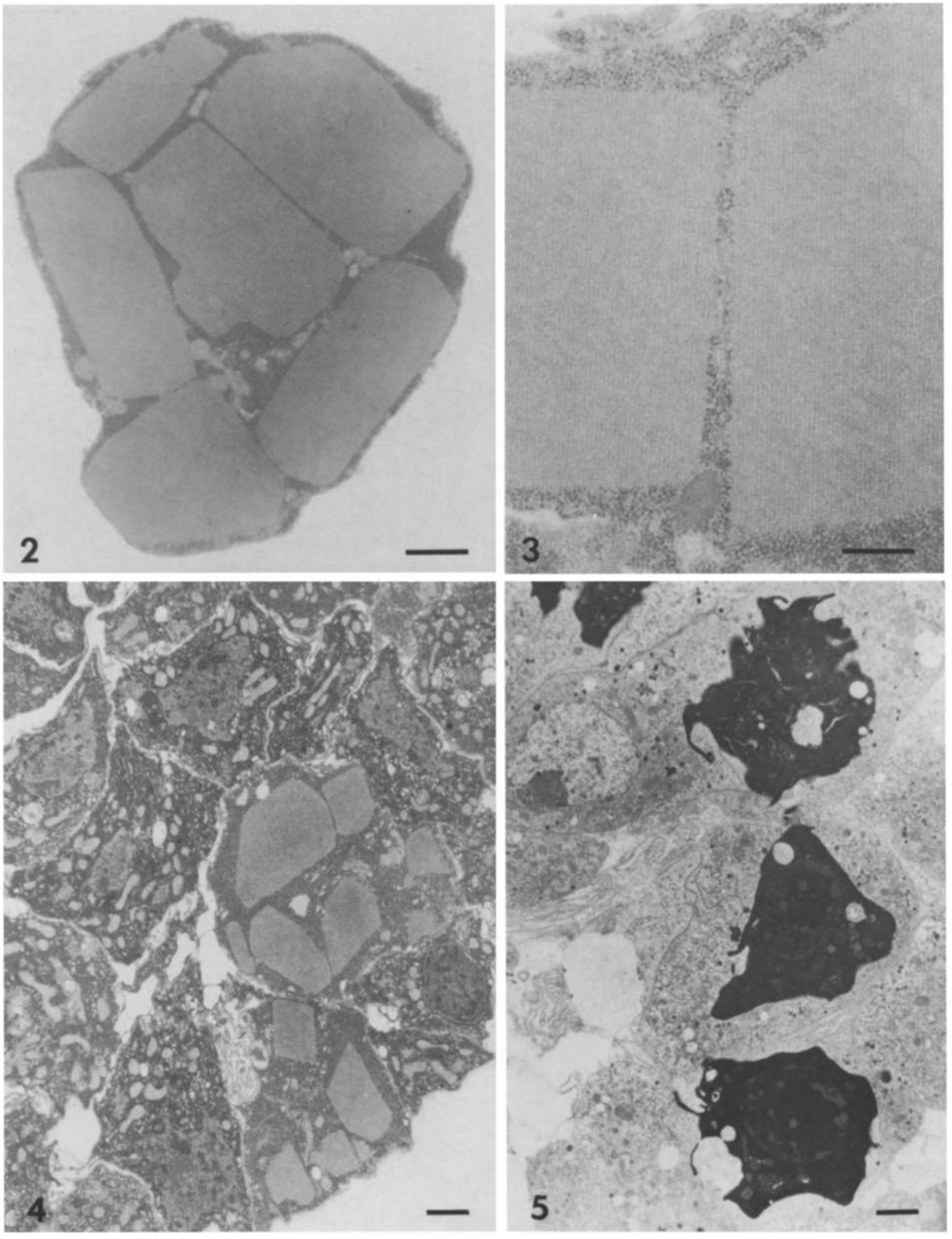

Fig. 2. Crystal cell from the hemolymph of a Bc^+/Bc^+ larva showing the paracrystalline inclusions. Scale = 1 μm

Fig. 3. Details of the paracrystalline inclusions. There is a regular packing of the structural elements into parallel arrays and crosslinking elements with regular spacings. Note that there is no membrane surrounding these inclusions such as seen surrounding protein storage vesicles, lysosomal bodies, or mitochondria. There is little evidence of rough ER in the cytoplasm and very few mitochondria in these cells. Scale = 0.1 μm

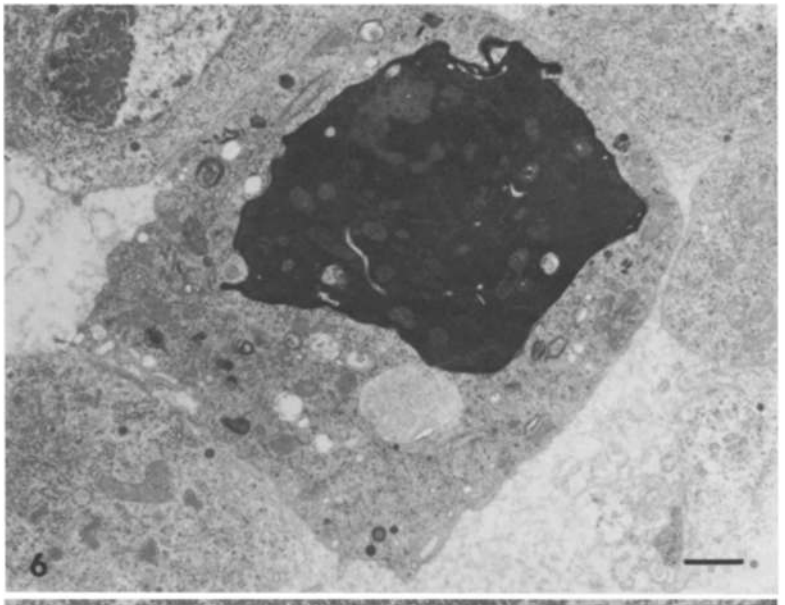

Fig. 6. A section passing through a *Bc*-cell. Note the well preserved structures in this cell, the nucleus with its nuclear membrane and nucleolus, and the other cytoplasmic organelles. The cytoplasm of the encapsulating cell contains small black inclusions which may have been extruded from the *Bc*-cell and phagocytized by the surrounding cells. Also see Fig. 11 for evidence of such material. Scale = 1 μm

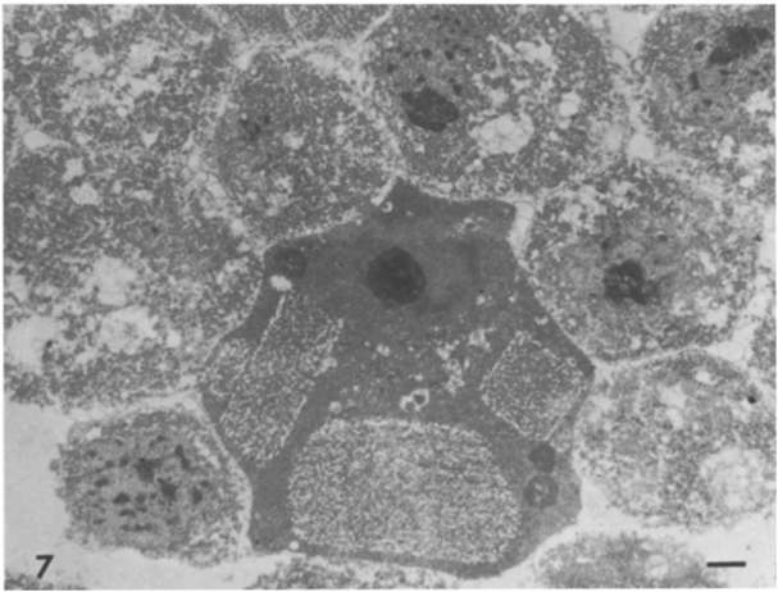

Fig. 7. A crystal cell in the lymph gland of a larva (phenocopy) treated with hot H_2O. The cytoplasm is melanized and the heat coagulation of the cytoplasm has preserved the outlines of the paracrystalline inclusions, but within the structures coagulated material still retains some disposition of parallel packing. The effect of heat coagulation on the cytoplasmic contents of the surrounding non-crystal cells shows an aggregation effect similar to that within the paracrystalline inclusions. Comparison of the size of this crystal cell with the *Bc*-cell in Fig. 6 shows that *Bc*-cells become melanized before they reach the size of the fully differentiated crystal cell. Scale = 1 μm

water (Rizki 1957a). Therefore, artificial blackening of the crystal cells in *Ore-R* (or Bc^+) larvae by these methods should mimic the distribution of the pigmented cells in *Bc* larvae if the black cells are indeed a mutant form of the crystal cells. We therefore experimented with methods to obtain a consistent blackening response of the crystal cells without disrupting them or causing rapid spreading of the induced melanin from these cells throughout the body.

Treatment of larvae with distilled water at 70° C gave the desired results. Figure 1b shows the heat-induced 'phenocopy' of *Bc*. The term phenocopy is used in a descriptive sense only; the high temperature kills the larvae but the distribution of the melanized crystal cells in the body segments, along the heart, and in the lymph glands is precisely that seen in *Bc* larvae. An electron micrograph of a melanized crystal cell in the lymph gland of a phenocopy shows the

Fig. 4. Crystal cells in the lymph gland of the Bc^+/Bc^+ strain. Scale = 1 μm

Fig. 5. A section of *Bc/Bc* lymph gland showing three black cells. These cells are encapsulated by one or two surrounding cells. The boundaries of the encapsulating cells are visible at the 5 and 10 o'clock positions of the black cell in the middle of the frame. Comparison of the black cells with the crystal cells in figure 4 indicates that Bc-cells are somewhat smaller than the normal crystal cells; this suggests that melanization occurs at an early stage of cell growth. Scale = 1 μm

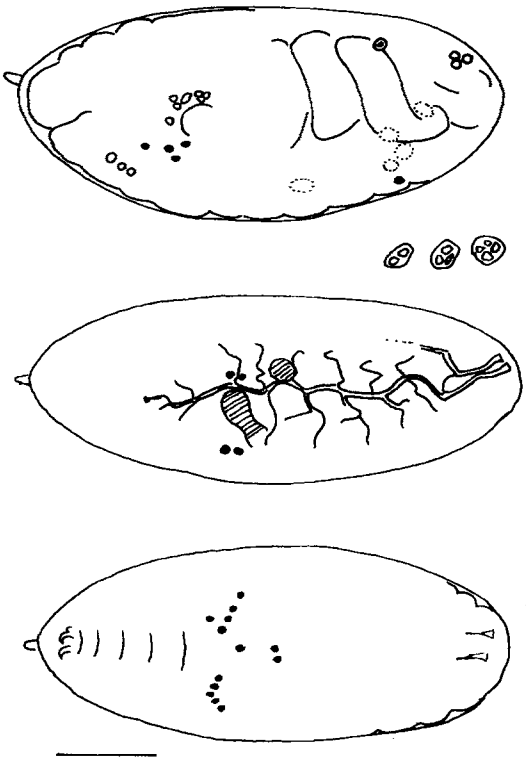

Fig. 8. The distribution of black cells in *Bc/Bc* embryos during early and late embryonic stages of development. The top drawing is a composite of two different embryos which were followed from the 10-14th h of development at 15 min intervals (lateral view). The position of each *Bc*-cell was recorded as it first appeared. When the first *Bc*-cell appeared, the folds of the gut indicated were not present. The *solid circles* are black cells from one specimen and *open circles* from another specimen; the *dotted circles* are yolk spheres which do not blacken. The shape and appearance of yolk inclusions in the three yolk spheres outside the embryo are diagrammatic. The second drawing (lateral view) shows the fully developed tracheal system at the gas filled stage. The *solid circles* are the *Bc*-cells and the crosshatched structures are the fat body. The third diagram is a fully mature embryo (ventral view) showing the disposition of *Bc*-cells in the mid ventral hemocoel. All drawings are camera lucida sketches; cells were scored by optical focusing on the lateral or ventral half of the embryos. The anterior of the embryos is on the left. Scale = 100 μm

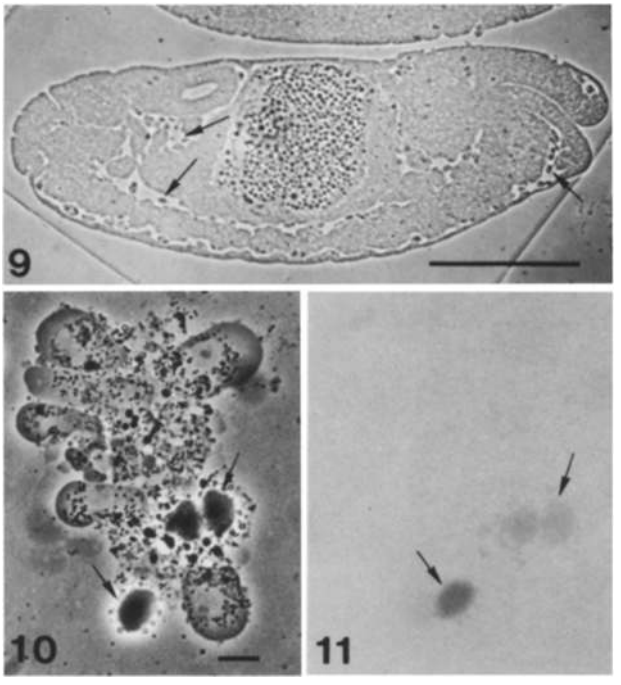

Fig. 9. This section represents the youngest stage of embryo in which *Bc*-cells were detected. It corresponds to Fig. 56 in Poulson (1950) – after completion of shortening of the embryo but before the frontal sac invagination. The section is parasagittal. The scale bar (100 μm) is located to the anterior-ventral region of the specimen. Some of the hemocytes (*arrows*) are clearly visible between the spaces occupied by the various tissues. Phase contrast, thick epon section

Fig. 10. A group of blood cells with black cells (*arrows*) from a *Bc/Bc* embryo. One of the cells has recently divided and the two daughter cells are still attached. Phase contrast, scale = 10 μm

Fig. 11. The same preparation as in Fig. 10, but photographed with transmitted light. The blackened cells are clearly visible and fine melanin droplets surrounding these cells can also be seen

heat-coagulated material of the paracrystalline structures and their outlines in the melanized cell (Fig. 7). The induced blackening appears in the cells within minutes of treatment, but the melanin from these cells will gradually diffuse through the body if the specimens remain immersed in H_2O for an extended period.

Observations on the Hemolymph of Bc^+/Bc^+, Bc^+/Bc and Bc/Bc Larvae

It is well known that insect larval hemolymph blackens when it is exposed to air. What is not clear is the source of this melanization. To study this property of *Drosophila* hemolymph, single larvae were opened on small disks of filter paper soaked with 0.05 M phosphate buffer (one set at pH 6.3, another at pH 8.4) and the hemolymph was allowed to diffuse through the paper. The rest of the carcass was removed quickly without rupturing any internal organs. The papers with the hemolymph samples were allowed to stand, taking care that they did not dry.

In both sets the papers with Bc^+/Bc^+ hemolymph turned black, *Bc/Bc* gave no reaction, and Bc^+/Bc gave blackening that was visibly less than that of Bc^+/Bc^+ (Fig. 12). In another series of experiments up to five *Bc/Bc* larvae were opened on the same spot of paper, but no perceptible blackening was noted. When hemolymph from larvae of different genotypes was applied to a single spot, the presence of

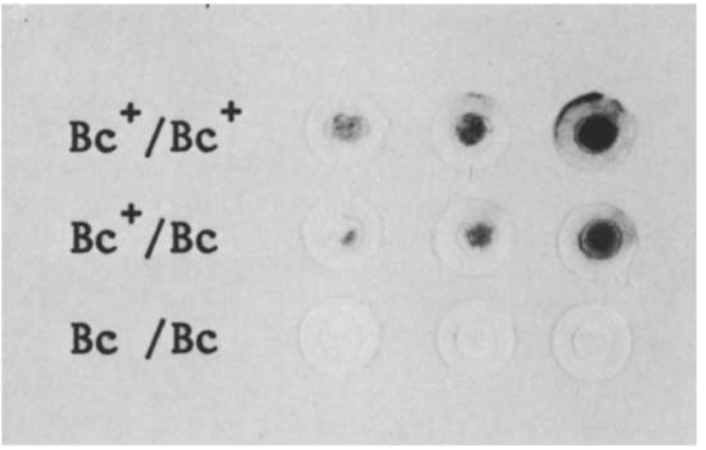

Fig. 12. Action of larval hemolymph phenol oxidase on endogenous substrate. The Whatman #4 filter paper disks soaked with pH 6.3 phosphate buffer and larval hemolymph were allowed to develop blackening for 15–20 min. The rows are samples of the three genotypes. Columns from left to right: hemolymph from 1 larva, 2 larvae, 4 larvae. Quantitative differences between the normal and heterozygotes are apparent; the blackening reaction is absent in the mutant homozygotes. The samples were applied to the inner filter paper disks, diameter 3 mm, resting on outer disks, diameter 6 mm; the latter were periodically moistened to prevent drying of the samples

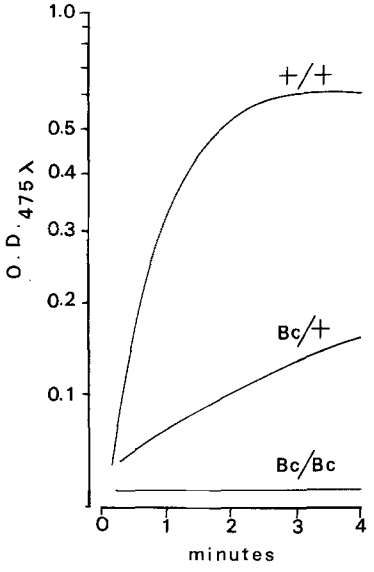

Fig. 13. Recordings of rate curves of phenol oxidase activity. Incubation mixtures contained 0.9 ml of 0.02M DOPA in buffer and 0.1 ml cell-free extract (mg protein: Bc^+/Bc^+, 0.22; Bc^+/Bc, 0.20; Bc/Bc, 0.25)

Table 1. Results of crossover experiments to locate *Bc*

Genotype of females	Order of recombining loci	Recombination frequency	Sample size
$c+Bc/+M(2)S7+$	$c-M(2)S7$	0.0074	860
	$M(2)S7-Bc$	0.020	–
$+nw^D+/M(2)S7-Bc$	$M(2)S7-nw^D$	0.021	870
	nw^D-Bc	0.010	–
$Bc++Pu^2\ Pin^{Yt}/$ $+fj\ wt++$	$Bc-fj$	0.01	199
	$wt-Pu^2$	0.12	(Count incomplete)
	Pu^2-Pin^{Yt}	0.11	–

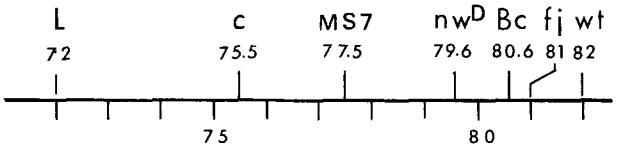

Fig. 14. Map of the region in the right arm of the second chromosome which contains *Bc*. The numbers indicate map positions

Bc/Bc hemolymph did not interfere with blackening of Bc^+/Bc^+ hemolymph.

Phenol Oxidase Activity

The preceeding experiments with hemolymph samples at pH 6.3 suggest that blackening in Bc^+ samples results from endogenous phenol oxidase and substrate in the hemolymph. These experiments do not reveal whether the lack of response with *Bc* hemolymph samples is due to the absence of phenol oxidase or the substrate. Therefore phenol oxidase activity in cell-free extracts of Bc^+/Bc^+, Bc^+/Bc and Bc/Bc larvae was assayed by the method of Mitchell (1966), and three representative rate curves are given in Fig. 13. Mitchell noted that the linear portion of the rate curve is shortened when the concentration of extract is increased, and this result was apparent with increasing concentrations of Bc^+ extracts. However, no phenol oxidase activity was recorded in *Bc/Bc* larval extracts even with 2- to 4-fold increases in protein concentrations. When *Bc/Bc* extract was added to Bc^+/Bc^+ extract, the rate curve paralleled that for an equivalent concentration of Bc^+/Bc alone.

Genetic Mapping of Bc

Initial experiments demonstrated that *Bc* assorts independently of the first, third and fourth chromosomes,

however, linkage to the second chromosome was observed. Offspring of *Bc/Cy* males are either *Bc* or *Cy*, but not both nor neither. Other preliminary data showed that *Bc* is located on the right arm of the second chromosome.

Results of crossover experiments to locate *Bc* with respect to nearby marker loci are presented in Table 1. *Bc* is given a map location of 80.6 based on its being one map unit to the right of nw^D. The locus of *fj* is unambiguously to the right of *Bc*, but the count was incomplete and we do not believe that the standard position of *fj* should be adjusted on the basis of this result. In Fig. 14 the map of the region of the chromosome in the vicinity of *Bc* is diagrammed.

Discussion

A minimum of six protein components participate in phenol oxidase activity of *Drosophila* (Seybold et al. 1975). This complex enzyme system has been studied in a number of *Drosophila* mutants (Mitchell 1966; Lewis 1960; Yamazaki and Ohnishi 1968; Peeples et al. 1969; Warner et al. 1974), and there is extensive literature on phenol oxidases of other insects as well (reviews by Hackman 1974; Richards 1978). Although phenol oxidase is involved in the hardening and darkening of the insect cuticle, this enzyme activity is found in the hemolymph as well and it has generally been assumed that components of the hemolymph phenol oxidase system participate in cuticular sclerotization. A distinction between hemolymph and cuticular phenol oxidases was first noted by Ohnishi (1954); additional differences between the enzymes from the two sources were later reported (Yamazaki 1969). *Bc* is the first reported mutant whose phenotypic characteristics distinguish the phenol oxidase activity in the larval hemolymph.

Earlier studies on *Drosophila* hemocytes suggested that the melanization system in the larval hemolymph is confined to the crystal cells (Rizki 1957a; Rizki and Rizki 1959). The distribution of the heat-induced melanized cells in *Ore-R* larvae parallels that of the black cells in *Bc* larvae, indicating that the potential for melanization in the *Bc* crystal cells or their precursor cells has been realized. Since no phenol oxidase activity remains in *Bc/Bc* larval hemolymph, the phenol oxidase activity detected in the larval stage of Bc^+/Bc^+ individuals must represent that of the crystal cells. It is obvious that the *Bc* mutant will be a useful tool for studying developmental profiles and functional relationships of the phenol oxidase activity in the hemolymph as distinct from other phenol oxidases, such as cuticular. Since the pigmentation of the puparium and adult cuticle in *Bc/Bc* individuals appear normal, the larval hemolymph phenol oxidase may not be required for these processes. Studies on these interrelationships as well as the role of the crystal cell function in wound healing and melanization of melanotic tumors are in progress.

The copy of *Bc* larvae achieved by heat coagulation is a good representation of the mutant phenotype, but it is obvious that the processes involved in cellular melanization under this condition differ from those occurring in *Bc* individuals in vivo. Blackening in *Bc* preserves the ultrastructural details of the cells; heat coagulation of the crystal cells forms a diffusible melanin product and the coagulated cytoplasm surrounding the paracrystalline structures preserves the outline of these inclusions while the empty space contains coagulated material. What is the nature of the melanin precursor in the paracrystalline inclusions? It was suggested earlier that the storage form in the crystal cells may be DOPA (Rizki and Rizki 1959). Lunan and Mitchell (1969) demonstrated that the storage form of tyrosine in *Drosophila* is tyrosine-0-phosphate, and Harper and Armstrong (1974) subsequently showed the existence of larval alkaline phosphatase for conversion of this storage form to tyrosine. The latter will then be available for phenol oxidase activity. Heat coagulation and melanization of the crystal cell raises the question whether the storage form of the substrate for melanization in these cells is tyrosine-0-phosphate. If so, then the latter can be blackened nonenzymically, or the intracellular environment is such that the enzymes are not denatured by hot water (70° C).

Although *Bc/Bc* larvae lack detectable phenol oxidase activity, it is unlikely that the *Bc* mutant gene blocks hemolymph phenol oxidase activity. If the latter were true, there should be no melanized crystal cells in these larvae. It is well known that phenol oxidases are destroyed during the discharge of their function (Mitchell 1966; Richards 1978). Therefore, the absence of phenol oxidase activity together with black cells in *Bc/Bc* larvae signifies self-destruction of the enzyme with the melanin formation in the crystal cells. This process of cross-linking during melanization will also account for the well preserved ultrastructure of the melanized cells. In line with previous studies on the compartmentalization of enzyme and substrate in the crystal cells (Rizki and Rizki 1959), we propose that the primary defect in *Bc* involves the loss of the physical barrier between phenol oxidase and its substrate bound in the paracrystalline structures. We further suggest that the Bc^+ product is a protein required for binding the substrate in the paracrystalline structures so it becomes inaccessible to the enzyme(s). The expression of the *Bc* phene in heterozygotes can be explained by this view. In

Bc^+/Bc individuals crystal cells with paracrystalline inclusions are present during the major part of the first larval instar and pigmented cells only appear toward the end of this instar. Some of the cells developing color at this transitional stage show the presence of typical paracrystalline inclusions. We interpret these observations as follows. In the early first instar the amount of Bc^+ product is sufficient to bind the available phenolic substrate. As the larvae continue to feed the influx of phenolic catabolites in some of the crystal cells will exceed the level of the Bc^+ product thereby allowing enzyme-substrate contact. When this point is reached, melanization will begin. Only a small population of crystal cells in their early stages of this differentiation sequence in which substrate has not exceeded the binding capacity of the Bc^+ product will remain unmelanized and serve as the source of the phenol oxidase that is detectable in Bc^+/Bc larvae. This reasoning also suggests that the appearance of black cells in Bc/Bc zygotes should coincide with the first accumulation of phenolic substrates in the hemocoel of the developing embryo.

During extensive studies on the hemocytes of *D. melanogaster* (review in Rizki 1978), crystal cells have not been observed undergoing mitosis. Positive identification of a crystal cell is based on the presence of the paracrystalline inclusions and the well-differentiated crystal cell packed with many inclusions may no longer undergo cell division. The melanization of a cell in division in the Bc/Bc embryo is therefore an interesting phenomenon. Assuming that Bc/Bc lacks the ability to bind substrate in an inaccessible state, it would seem that one of the early steps in the determination of the crystal cell is the appearance of phenol oxidase. This determined cell can continue to divide. The subsequent step, i.e., entry of substrate into the crystal cells, in the presence of Bc^+ product will form paracrystalline inclusions; in the absence of Bc^+ product, the cell will melanize.

Poulson (1950) described the origin of the first pair of lymph glands from the dorsolateral mesoderm in embryos at 11–12 h of age, and agreed with earlier workers that the blood cells are probably derived from the thin midventral part of the mesoderm. Equally interesting is his observation that some of the embryonic blood cells arise from yolk cells that are not enclosed in the midgut, remaining in the anterior and posterior regions of the embryo. On the basis of these observations Poulson (1950) concluded that the lymph gland cells and the early embryonic blood cells are distinct populations. The distribution pattern of the first group of black cells to appear in Bc/Bc embryos agrees with this conclusion.

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References

Grell EH (1969) *Bc: Black cells.* Drosophila Inform Serv 44:46

Hackman RH (1974) Chemistry of the insect cuticle. In: Rockstein M (ed) The Physiology of insecta, 2nd edn, vol VI. Academic Press, New York, London

Harper RA, Armstrong FB (1974) Alkaline phosphatase of *Drosophila melanogaster.* III. Tyrosine-0-phosphate as substrate. Biochem Genet 11:177–180

Lewis HW (1960) Genetic control of dopa oxidase activity in *Drosophila melanogaster.* I. Analysis of wild type, sable, suppressor-of-sable, and suppressed sable strains. Genetics 45:1217–1231

Lindsley DL, Grell EH (1967) Genetic variations of *Drosophila melanogaster.* Carnegie Inst Washington Publ No 627

Lunan KD, Mitchell HK (1969) The metabolism of tyrosine-0-phosphate in *Drosophila.* Arch Biochem Biophys 132:450

Mitchell HK (1966) Phenol oxidases and *Drosophila* development. J Insect Physiol 12:755–765

Ohnishi E (1954) Tyrosinase in *Drosophila virilis.* Annot Zool Jpn 27:33–39

Peeples EE, Geisler A, Whitcraft CJ, Oliver CP (1969) Comparative studies of phenol oxidase activity during pupal development of three lozenge mutants (lz^s, lz, lz^k) of *Drosophila melanogaster.* Genetics 62:161–170

Poulson DF (1950) Histogenesis, organogenesis, and differentiation in the embryo of *Drosophila melanogaster* meigen. In: Demerec M (ed) Biology of *Drosophila.* John Wiley & Sons, Inc., New York

Richards AG (1978) The chemistry of insect cuticle. In: Rockstein M (ed) Biochemistry of insects. Academic Press, New York, London

Rizki TM (1957a) Alterations in the hemocyte population of *Drosophila melanogaster.* J Morphol 100:437–458

Rizki TM (1957b) Tumor formation in relation to metamorphosis in *Drosophila melanogaster.* J Morphol 100:459–472

Rizki TM (1978) The circulatory system and associated cells and tissues. In: Ashburner M, Wright TRF (eds) The genetics and biology of Drosophila, vol 2b. Academic Press, New York, London

Rizki TM, Rizki RM (1959) Functional significance of the crystal cells in the larva of *Drosophila melanogaster.* J Biophys Biochem Cytol 5:235–240

Seybold WD, Meltzer PS, Mitchell HK (1975) Phenol oxidase activation in *Drosophila:* A cascade of reactions. Biochem Genet 13:85–108

Warner CK, Grell EH, Jacobson KB (1974) Phenol oxidase activity and the lozenge locus of *Drosophila melanogaster.* Biochem Genet 11:359–365

Yamazaki HI (1969) The cuticular phenoloxidase in *Drosophila virilis.* J Insect Physiol 15:2203–2211

Yamazaki HI, Ohnishi E (1968) Phenol oxidase activity and phenotypic expression of the melanotic tumor strain tu^s in *Drosophila melanogaster.* Genetics 59:237–243

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