INTRACELLULAR LOCALIZATION OF KYNURENINE IN THE FATBODY OF *DROSOPHILA*

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ABSTRACT

Light blue fluorescent globules accumulate in the cells of the anterior region of the fatbody of *Drosophila* larvae near the time of pupation. This fluorescent material appears in the *Ore-R* wild type strain as well as mutant strains in which the synthesis of both the red and brown eye pigments is affected. The *vermilion* mutant, which is characterized by the absence of the brown pigment component in the eye, was the only strain among those examined which did not develop the light blue fluorescent globules. Utilizing chromatographic techniques together with the information gained by examination of the mutant strains, the fluorescent material has been identified as kynurenine. Of particular interest is the manner of appearance of the fluorescent material in the vicinity of the nuclear membrane of the fat cells.

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One aspect of tryptophan metabolism in *Drosophila* is the utilization of this amino acid as a precursor of brown eye pigment. In this biosynthetic pathway the conversion of tryptophan proceeds through formylkynurenine, kynurenine, and 3-hydroxykynurenine. The elucidation of these intermediate compounds in pigment synthesis in *Drosophila* has been accomplished with the use of eye color mutants, specifically, vermilion (*v*), cinnabar (*cn*), and scarlet (*st*) (2–4, 6, 7, 10, 13).

An abnormal appearance of pigment granules in the pupal fat cells characterizes the red cell (*rc*) mutant of *Drosophila melanogaster*. The type of pigment which accumulates in the fat cells of the *rc* mutant is related to the brown eye pigment of *Drosophila* since combination of the *rc* factor with the *v*, *cn*, or *st* factor will inhibit the development of the *rc* pigment in the fat cells (9). This pigmentation process can also be restricted by the presence of the melanotic tumor factor, tu^w (11). However, a period of larval starvation reverses this interference and allows the development of *rc* pigment in the fat cells of $tu^w rc$ flies to continue.

The abnormal formation of brown pigment in the fat cells in the *rc* strain of *Drosophila* is particularly interesting since the transplantation studies of Beadle (1) established the fatbody as a source of precursors of brown eye pigment. The *rc* mutant offers a convenient site, the large pupal fat cells, to study the formation of pigment precursors at the cellular level. Since a number of the intermediate biochemicals in the synthesis of brown eye pigment are fluorescent compounds, this reexamination of the fatbody of normal and mutant strains of *D. melanogaster* utilized the fluorescent microscope.

EXPERIMENTAL METHODS

The *Ore-R* wild type strain of D. melanogaster, the red cell (*rc*) mutant, and the following eye color mutants kindly provided by Dr. E. B. Lewis and Dr. E. Novitski have been used in this survey: vermilion (*v*), cinnabar (*cn*), scarlet (*st*), claret (*ca*), white (*w*), brown (*bw*), and the combinations *v;bw* and *cnbw*. Newly emerged larvae from each strain were collected at 2-hour intervals and transferred to stender dishes containing cream of wheat–molasses medium seeded with Fleischmann's yeast. All experimental material was maintained in an incubator at 24°C.

Larvae and pupae were dissected in Drosophila-Ringer solution, and examined immediately with the fluorescent microscope. Various mounting media for the tissues were used, but the most satisfactory method proved to be examination in Ringer solution. Observations were made with a Zeiss fluorescence microscope with mercury burner HBO 200.

RESULTS

The larval fat mass of *Drosophila* extends from the anterior end of the body (posterior to the cerebral hemisphere) to the anal segment. Utilizing relationships to various organs and topographical curvatures and spaces, this tissue mass can be conveniently subdivided into distinct regions.

There are no differences apparent in the larval fat cells from various regions of the fatbody of the normal *Ore-R* strain when freshly dissected specimens are examined with the fluorescent microscope, and the entire fatbody displays a purple autofluorescence. In larvae age 96 ± 2 hours, that is, shortly before pupation, a faint light blue fluorescence begins to appear in the anterior region of the fatbody. The accumulation of this fluorescent material continues rapidly, and within several hours the anterior fatbody is sharply differentiated from the remaining fat masses by its light blue fluorescence. Fig. 1 illustrates the approximate extent of this light blue fluorescence, and the photograph in Fig. 2 indicates the striking difference at the boundary between these regions of the fatbody.

The light blue fluorescent material in the anterior fat cells is concentrated in spherical cytoplasmic inclusions. The nature of this globular material can be observed when the individual fat cells are ruptured by the application of slight pressure to the coverglass, and the cytoplasmic contents are allowed to escape. In Fig. 3, two fat cells have been displaced from the mass of fluorescing fat tissue in the vicinity of the salivary gland, and the individual globules are visible. The nuclei and cytoplasmic fat globules are non-fluorescent in comparison with these globules and are dark in the photographs. This same region of the fatbody is viewed under the phase microscope in Fig. 4. The variation in size of the fluorescent granules is demonstrated in Fig. 5. These photographs represent a late stage in the accumulation of the fluorescent material after the globules are large and scattered throughout the cytoplasm; these fat masses have been removed from the white puparial stage.

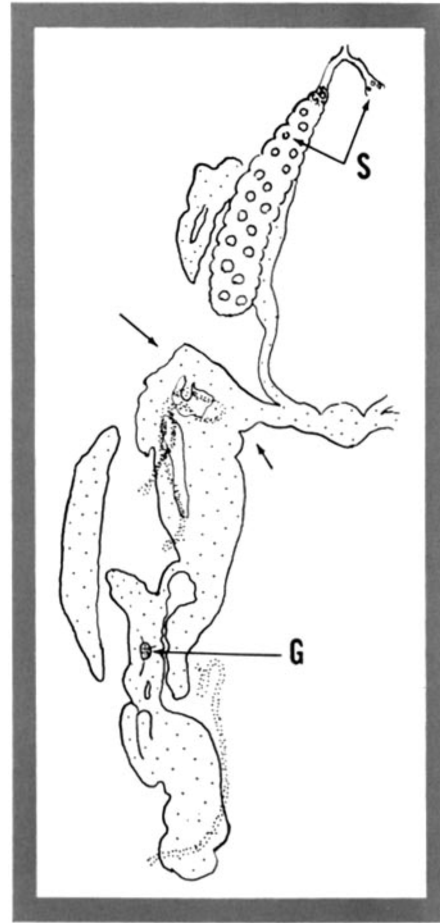

FIGURE 1

A semidiagrammatic representation of the organization of the fat mass dissected out of the left half of the larva of *D. melanogaster*. The fat mass on the right will be the mirror image. The salivary glands, *S*, are in the anterior region; associated with these glands are two fat masses which extend caudad giving rise to a commissure connecting the fat on the other side of the body and continuing to the posterior segments. The two arrows indicate the demarcation line of anterior fat mass showing blue autofluorescence due to kynurenine and the posterior fat mass. In the posterior region of the fatbody the ovary or testis, *G*, is embedded. The dotted tubular structure is the course of the Malpighian tubes.

The light blue fluorescent material begins to develop around the nucleus of each fat cell. It is first noticeable as a diffuse light blue brilliance which is distinguishable from the faint white

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autofluorescence characteristic of the nucleus. The intensity of the light blue fluorescence increases and the nucleus becomes surrounded by numerous small fluorescent spherical particles. Enlargement of the globules is apparent as the inclusions become distributed throughout the cytoplasm of the cell.

In order to determine whether this blue fluorescent material is related to the development of brown pigment of the eye, the fatbodies from eye color mutants were examined with the fluorescent microscope. In each instance, observations were begun from approximately 85 hours of larval age. Although slight differences in the exact time of appearance of the light blue fluorescence were noted, all of the following mutants developed the same pattern of intense fluorescence in the anterior fatbody as found in the normal *Ore-R* strain: *cn*, *st*, *ca*, *w*, *bw*, *cnbw*, and *rc*. However, these fluorescent granules did not appear in the fat cells of *v* larvae or pupae, and were not detectable even though observations were continued until the time that the cells of the fatbody became separated from each other in the first step of the metamorphosis of the fatbody which takes place during pupal reorganization. The light blue fluorescent material did not appear in *v;bw* pupae either.

The absence of this light blue fluorescent material in the *v* mutant suggests that its synthesis may be involved in some way with the ultimate development of brown eye pigment. The *v* gene represents a block in the conversion of tryptophan to formylkynurenine and kynurenine, whereas the biosynthesis of these substances is not interrupted in any of the other mutants examined. A characteristic feature of kynurenine is its light blue fluorescence. To confirm the identity of the fluorescent material in the anterior fat cells as kynurenine, this substance was separated by paper chromatography and the resultant R_f values compared with the R_f values of a known sample of kynurenine. Whatman No. 1 chromatography paper was used for all of the chromatograms and the procedures for solvents and color reactions followed those given by Smith (12). Anterior and posterior fat masses from the white puparial and early brown puparial stages of the *Ore-R* strain were dissected out in Ringer solution and placed directly on the chromatographic paper. Light blue fluorescent spots appeared only in the chromatograms of the anterior fat region, and in each of the three solvents used, the R_f value of the light blue fluorescent material from the anterior fatbody was the same as that of the sample of kynurenine: R_f 0.70 in IPA (isopropanol, 20: ammonia, 29 per cent, 1: water, 2); R_f 0.56 in KCl (20 per cent aqueous solution); R_f 0.58 in BA (butanol, 12: acetic acid glacial, 3: water, 5). The fluorescent spots on the chromatograms of both the known sample and the substance from the fatbody turned orange when treated with Ehrlich's reagent and gave a positive Ninhydrin test.

DISCUSSION

In the search for eye pigment precursors in Drosophila, Beadle (1) found the fatbody of the early pupa a source of one of the substances necessary for the biosynthesis of brown eye pigment. This v^+ substance was later identified as kynurenine when it was learned that the latter substance could serve as a precursor of brown pigment in the *v* mutant (4, 8, 13). The development of pigment related to the brown eye component in the pupal fat cells of the *rc* mutant strain of *Drosophila* further implicated this tissue as a site of accumulation of pigment precursors. Scattered red pigmented fat cells are found in the head and thorax of the late pupal and adult stages of the *rc* mutant (9). In combination with the tu^w factor and alterations imposed by a period of larval starvation, the development of *rc* pigment in the fat cells began in the early pupa prior to the reorganization of the fat cells accompanying metamorphosis (11). Under these genetic and environmental conditions it was possible to demonstrate that the formation of *rc* pigment was limited to the cells of the anterior fat masses. The cells of the larval fatbody became separated from each other in the pupal stage and the individual fat cells were redistributed in the developing pupa. The fat cells containing the *rc* pigment granules thus became located chiefly in the head and thorax of the late pupa and adult. The region of the fatbody involved in the *rc* pigment pattern is the same as that which normally accumulates light blue fluorescent material around the time of pupation. This coincidence of locale suggests that the *rc* gene has triggered a biochemical reaction that does not normally occur in this region of the body, either by removing some inhibitor or by supplying some prerequisite substance for pigmentation not usually contained in this tissue.

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Two types of pigment occur in the eye of *Drosophila*, a brown component and a red fraction. The synthesis of both substances is interrupted by mutant genes, and the use of these mutant strains has facilitated the analysis of pigment biosynthesis in *D. melanogaster*. The mutant gene, *bw*, blocks the production of the red pigment component and the eye color is consequently brown. A number of mutant factors interfere with the formation of the brown component, each at a different point in the biosynthetic pathway, and the phenotype of these mutant flies thus includes bright red eyes. The *v* gene blocks the conversion of tryptophan to formylkynurenine and kynurenine; the latter is accumulated in the *cn* mutant which is unable to form 3-hydroxykynurenine from the precursor; and the *st* mutant is a block in the biosynthesis of brown pigment at a level closer to the final pigmented product. The white eye (*w*) phenotype results when the synthesis of both the red and brown components is interrupted.

Light blue fluorescent globules in the anterior fatbody are absent from the *v* mutant but occur in the other mutants of the brown pigment series as well as in the *bw* and *w* mutants. These observations agree with the identification of the fluorescent substance as kynurenine since the latter precursor should be expected in all of the mutants tested other than *v*. Danneel and Zimmermann (5) identified kynurenine using paper chromatographic techniques in the wild type strain, and the *cn* and *bw* mutants, but obtained no kynurenine from the *v* or *v;bw* mutants. The fatbody of the white eye mutant (*w*) contained fluorescent globules; these globules were not present in the mutant combination *v;bw* which has white eyes. It is the presence of the *v* mutant factor which blocks the appearance of the fluorescent globules.

Utilizing fluorescence microscopy, the primary detectable site of kynurenine in the anterior fat cells is in the vicinity of the nucleus. Later, fluorescent globules become distributed throughout the cytoplasm. The present investigation has been restricted to examination of fat cells *in vitro* with the fluorescence microscope and phase contrast; further cytochemical studies of the nature of the fluorescing globules as well as detailed investigation of the fine structure of the cytoplasmic contents of the early period of fluorescence are needed. Biochemical genetics has established the role of the gene in the control of biochemical syntheses within the cell. The genes reside in the chromosomes in the nucleus, and the proteins and enzymes associated with cellular metabolism seem to be produced generally in the cytoplasm of the cell. The link, or the first steps, from the primary gene product to the final character in the cytoplasm remains uncovered. The present example offers desirable conditions for further study, for here a specific gene locus, *v*, is involved in the elaboration of a particular biochemical, kynurenine, which accumulates near the nuclear membranes in a group of differentiating cells during a specific period of development.

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Explanation of Figures

Photomicrographs 2, 3, and 5 were taken with Zeiss fluorescence equipment with principal excitation λ360 mu with barrier filters GG4 and BG23; darkfield condenser with glycerin between the slide and the front lens of the condenser.

FIGURE 3

Autofluorescence of the cytoplasmic globules of the fat mass associated with the salivary glands. Note the absence of fluorescence from the nuclei, *N*, and also the fat globules, *F*. × 240.

FIGURE 4

Same preparation as Fig. 3 under phase contrast; comparable regions in this photograph and Fig. 3 are indicated by arrows. × 240.

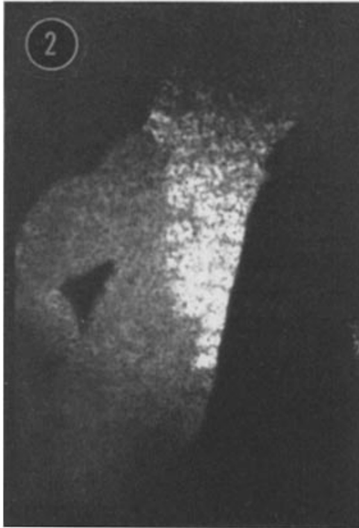


Figure 2

Photomicrograph showing the chemodifferentiation among the cells of the anterior fat mass removed from the early brown pupa of the *Ore-R* strain of *D. melanogaster*. The bright autofluorescence in the anterior fat cells is due to the presence of kynurenine in the cytoplasmic granules. × 38.

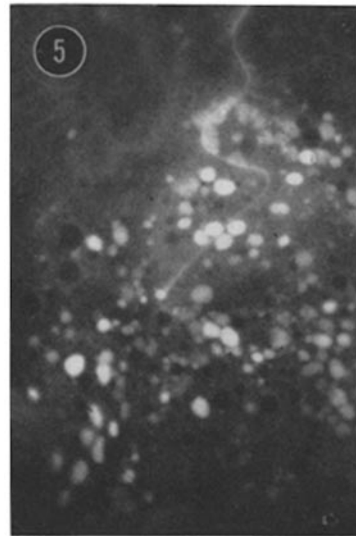
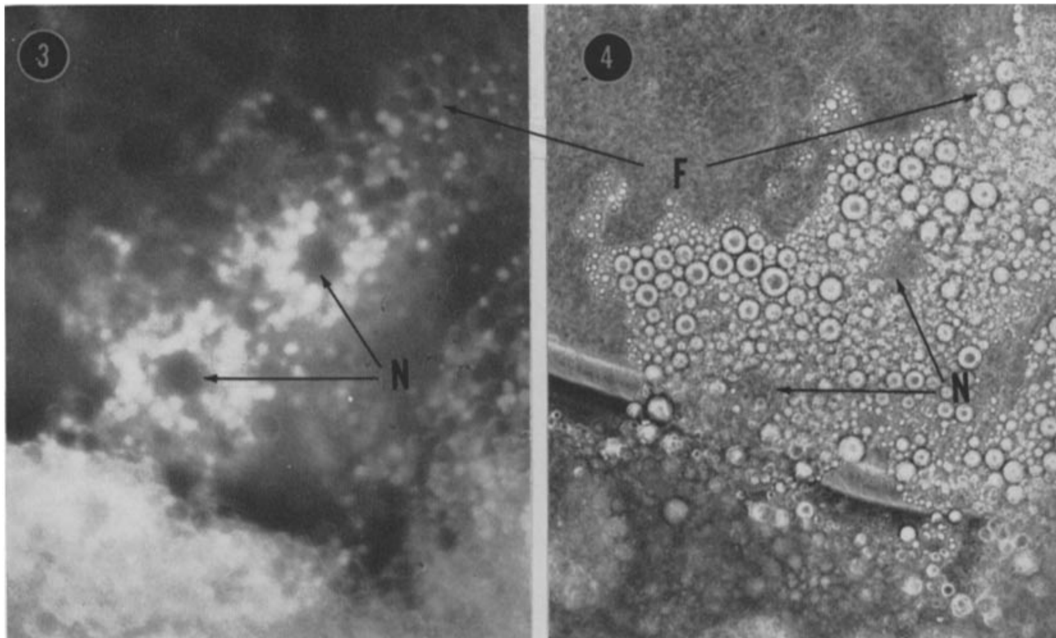


Figure 5

Autofluorescent cytoplasmic globules of the anterior fat cells spread out by gentle pressure over the cell. The meandering white line in the same field is a trachea. × 240.



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