FACTORS AFFECTING THE INTRACELLUALR SYNTHESIS OF KYNURENINE

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ABSTRACT

Near the time of pupation, autofluorescent kynurenine globules appear in the cells in the anterior region of the fatbody of *Drosophila melanogaster*. It has been reported previously that kynurenine synthesis may be induced in an additional group of fat cells by feeding the precursor tryptophan to *Drosophila* larvae, and that this induction of kynurenine production viewed within the fat cells is correlated with an increase in tryptophan pyrrolase activity. In the present report, conditions are outlined which result in the appearance of kynurenine in all of the fat cells. The number of cells in the fatbody which contain kynurenine is influenced by the quantity of tryptophan included in the diet, as well as by the developmental stage at the time of treatment and the duration of the feeding period on the inducer. Physical barriers modifying permeability, such as the membranous layer noted surrounding the fatbody, may be a factor in the regulation of the time and nature of the cellular induction of kynurenine synthesis. Another factor to be considered is the possibility of interference with the availability of tryptophan as a substrate or inducer for this synthesis within the cell. It is suggested that the occurrence of pteridines in some of the fat cells may modify the response of these cells to produce kynurenine, since pteridines as electron acceptors can complex with tryptophan as an electron donor. Kynurenine may be produced in the fat cells under *in vitro* conditions when they are incubated with L-tryptophan, but kynurenine is not formed when fat cells are incubated with D-tryptophan. The *in vitro* studies further demonstrate that induction of kynurenine synthesis may occur in fat cells isolated from young larvae in contrast, to *in vivo* conditions in which inducer does not effect an earlier appearance of kynurenine in the larval fatbody.

The adaptive increase in tryptophan pyrrolase activity mediated by the administration of tryptophan has been most extensively studied in mammalian liver (4, 6). Induction of kynurenine synthesis can also be achieved in *Drosophila* by feeding tryptophan to the larvae (14). In the latter instance, increased tryptophan pyrrolase activity is correlated with an increase in the number of cells in the fatbody which are producing kynurenine (12). The intracellular distribution of this metabolite in the larval fat cells can be conveniently determined by examination of this tissue with the fluorescence microscope, and it has been shown that the light blue autofluorescence characteristic of kynurenine is limited to the cells in the anterior region of the larval fatbody in the wild-type *Ore-R* strain of *D. melanogaster*. When the larvae are given tryptophan, the autofluorescence characteristic of kynurenine is extended to the fat cells located posterior to the normal site of kynurenine accumulation. Chromatographic isolation has verified an increased quantity of kynurenine in the fatbody of larvae which have been fed tryptophan.

Kynurenine appears in the fat cells of *Drosophila* larvae near the time of pupation. The present

report details the conditions influencing the regional distribution of kynurenine in the fat cells and relates the *in vivo* induction of kynurenine synthesis in these cells to the results obtained through *in vitro* incubation of cells with tryptophan.

MATERIALS AND METHODS

The *Ore-R* wild-type strain of *D. melanogaster* served as the source of normal material in this study, while several experiments utilized the mutant eye color strain, *rosy*^{1} (*ry*^{1}). The methods of collecting and raising larvae and pupae were the same as those reported previously (9). The ages of larve and pupae have been recorded from the time of their eclosion from the egg, and all experimental material has been maintained at 24°C in an incubator. For the tryptophan-feeding experiments, larvae were removed from the cream of wheat medium, washed, and transferred to dishes containing Whatman cellulose powder. Tryptophan solution was then added to this paper pulp. The control groups of larvae for these experiments were placed on paper pulp moistened with distilled water. The L-tryptophan solution (0.03 M) was prepared according to Knox (3) and stored in the refrigerator. The solution of 0.03 M D-tryptophan was prepared by the same method. Solutions of tryptophan were used within 24 hours after preparation.

In vitro incubation was accomplished by placing fatbody tissues in a mixture of Ringer's solution/0.03 M tryptophan solution (3/1). The incubation dishes remained in an O_2 atmosphere at room temperature for 45 minutes, and the tissues were then removed for microscopic examination or rinsed once with Ringer's solution and placed on chromatographic paper. The Whatman No. 1 paper was prepared prior to use by allowing the solvent to move up the paper overnight and then drying. This procedure proved advantageous for removing extraneous fluorescence material from the background. The sample solutions were applied in the form of 2-cm streaks, and the standard fluorescent compounds (kynurenine, isoxanthopterin, and 2-amino-4-hydroxypteridine) were also applied in a single streak. Chromatograms were developed for 2 hours with the solvent isopropanol: 1 per cent aqueous ammonia (2:1 v/v), dried, and examined with the HBO 200 lamp and filter system that was used for the microscopic fluorescence observations.

RESULTS

The characteristic autofluorescence of kynurenine becomes apparent in the fat cells of *Ore-R* larvae near the end of larval life when a light blue fluorescence appears in the anteriodorsal fat masses overlying the salivary glands. This fluorescence increases in intensity, and is most striking in early bubble stage prepupae when its extent and intracellular localization are best examined. For this reason, the description of the autofluorescence of the fat cells following various experimental treatments has been based on examination of pupae at this developmental stage unless indicated otherwise.

In earlier studies (10, 13) the description of the autofluorescent pattern of the fatbody designated those cells containing kynurenine as anterior cells and the remaining cells containing isoxanthopterin as posterior fat cells. For the present discussion, the region formerly labeled posterior will be subdivided, such that the area of cells extending from the normal point of termination of the anterior "kynurenine cells" to the level of the gonads will be referred to as midfatbody, and the cells posterior to the gonads including the dorsolateral extensions will be termed postgonadal fatbody.

The autofluorescence characterstic of kynurenine is restricted to the cells of the anterior fatbody in the *Ore-R* strain when the larvae are raised on cream of wheat medium seeded with yeast. This pattern of autofluorescence is also maintained when larvae are removed from food in the mid third instar and given only distilled water until they pupate. However, the number of fat cells containing kynurenine is increased when the larvae are fed tryptophan, as reported previously (12). For these earlier studies, L-tryptophan was dissolved in distilled water by slight heating. Kynurenine in the fat cells extended approximately to the level of the gonads in larvae given tryptophan solution prepared in this manner. It was later noticed that kynurenine production could be induced in the cells throughout the fatbody by feeding the larvae 0.03 M tryptophan solution made by dissolving L-tryptophan in 1 N NaOH and then restoring the solution to pH7 with 1 N HCl. The latter method of preparation given by Knox (3) was used previously in the studies on tryptophan pyrrolase activity in *Drosophila* (14) and has been used throughout the present study. The difference between the two series of feeding experiments with tryptophan suggested a quantitative response corresponding to the concentration of tryptophan in the solution offered to the larvae. Presumably, heating destroyed some of the tryptophan, so that the quantity of tryptophan

available in the solution after heating was less than calculated. A demonstration that the number of fat cells which contain kynurenine is correlated with the quantity of tryptophan fed to *Ore-R* larvae is presented in Table I. This summarization of data represents three experiments, with three male larvae examined for each dilution in the three series. When the freshly dissected larval fatbody is mounted on a slide, the tissue sometimes folds over as the coverglass is applied to the preparation. Such distortion often interferes with the observation of fluorescence of individual

TABLE I

Distribution of Kynurenine in the Larval Fatbody after Feeding Tryptophan

Treatment	Regions		
Dilution of 0.03 M Tryptophan solution	Anterior	Mid	Postgonadal
Full strength	+++	+++	++±
1/2	+++	+++	+
1/4	+++	+	−
1/8	+++	±	−
1/16	+++	−	−
1/32	+++	−	−
Water control	+++	−	−

(*Ore-R* larvae were maintained on the solutions from 65 to 68 hours of age until pupation. A visual estimate of the intensity of fluorescence is given, in which +++ indicates strong fluorescence and − indicates negative.)

cells in the folded region, and the distribution of autofluorescence in these fatbodies was, therefore, not included in the recorded data. Examination of three fatbodies for each series implies three complete dissections in which the fluorescence of all cells in the fatbodies can be viewed. For these experiments the larvae were placed on tryptophan solution at 65 to 68 hours of age, and the fatbodies of bubble stage prepupae were examined with the fluorescence microscope. A visual quantitative estimate of the intensity of light blue autofluorescence in the cells of the fatbody is presented.

The next series of experiments were performed to determine the most effective period for the induction of kynurenine synthesis in the posterior fat cells. For these experiments, 0.03 M L-tryptophan has been used. The entire sequence of feeding tryptophan to larvae of various ages has been repeated twice, and three specimens from each group were examined in all experiments. These observations are summarized in Table II. The intense autofluorescence of kynurenine will appear throughout the fatbody only when the feeding of tryptophan is begun by the middle (72 hours) of the third larval instar. Deprivation of tryptophan intake at any time during the ensuing period results in the restriction of kynurenine production to the anterior cells. On the other hand, kynurenine will appear in some of the cells immediately posterior to the normal site of kynurenine accumulation (that is, in the cells of the midfatbody) when tryptophan is fed to larvae after they have passed the mid third instar.

Kynurenine appears in the anterior larval fat cells near the time of pupation, and this time of appearance is not altered by feeding excessive tryptophan during the third instar. Increased tryptophan intake by the larvae influences the quantity of kynurenine produced, but does not effect an earlier appearance of kynurenine when tryptophan is fed to young larvae. *In vitro* synthesis of kynurenine by the fat cells of the midfatbody has been demonstrated previously (14). This method offers a means of testing whether the fat cells are capable of synthesizing kynurenine earlier in their development if they are removed from the body and placed in an environment containing tryptophan. The anterior regions of the fatbodies of larvae aged 68 to 70 hours were incubated for 45 minutes with L-tryptophan. Other groups were incubated for the same length of time in D-tryptophan, and others in Ringer's solution. The cells were examined with the fluorescence microscope at the completion of the incubation period. The characteristic autofluorescence of kynurenine was apparent in the cells which had been incubated in L-tryptophan, but this substance was not visible in the cells incubated with D-tryptophan, nor in the control groups of cells remaining in Ringer's solution for 45 minutes. Incubation of fat tissues in these mixtures was repeated, and in succeeding experiments the tissues were transferred to chromatographic paper after the incubation period. The occurrence of kynurenine was verified by chromatography for the tissues incubated in L-tryptophan. Photographs of the fat cells after incubation in L-tryptophan, D-tryptophan, and Ringer's solution are included in Figs. 1 to 4 together with a photograph of a chromatogram demonstrating

the isolation of kynurenine from the fatbodies incubated with L-tryptophan. Although D-tryptophan is not utilized to form kynurenine by the fat cells *in vitro*, increased kynurenine production in the fat cells is induced when larvae are fed 0.03 M D-tryptophan.

The *rosy* mutant strain lacks isoxanthopterin in its mid and postgonadal larval fat cells which accumulate 2-amino-4-hydroxypteridine, an immediate precursor of isoxanthopterin (13). In addition to the altered fluorescent color of the fat cells of the *ry* strain due to their content of 2-amino-4-hydroxypteridine, bright yellow UV-sensitive fluorescent granular material appears in these same cells. Tryptophan was fed to *ry* larvae to determine whether the variant mutant content of the mid and posterior fat cells would influence their response to an increased level of tryptophan in the larval diet. For these experiments, *Ore-R* and *ry*[1] mutant larvae were subjected to identical feeding conditions, and the fat cells of male larvae of both strains were examined at the same age for comparison of their autofluorescences. Various concentrations of tryptophan solution were used. The autofluorescence due to the presence of kynurenine was extended to the mid and postgonadal fat cells in the *ry* strain when the larvae were fed 0.03 M tryptophan. However, this fluorescence in the posterior region was not so intense as that noted in a comparable region of the *Ore-R* fatbody. An additional effect on the autofluorescence of the mid and postgonadal fat cells in the *ry* strain was noted, for the bright golden yellow autofluorescent granules were no longer visible in these cells. Comparison of the autofluorescence of the fatbodies of *ry* puparia with that of the fat bodies of *Ore-R* raised on more dilute concentrations of tryptophan confirmed this difference in response between the *ry* and *Ore-R* strains. At a concentration of 1.5×10^{-2} M L-tryptophan, as shown in Table I, the autofluorescence of kynurenine is still detectable in the mid and postgonadal fat cells of the *Ore-R* strain, whereas a weak light blue fluorescence indicating kynurenine is visible in the cells of the middle region of *ry* fatbodies at this concentration and none is detectable in the postgonadal cells. Further evidence of a differential quantitative response is apparent at a concentration of 3.8×10^{-3} M L-tryptophan in which kynurenine is limited to the anterior cells in the *ry* strain but is found in the cells of the midfatbody in *Ore-R* specimens.

The larval fat cells remain in a continuous tissue mass throughout larval life. During the early pupal period, these cells become separated from each other and undergo dispersion accompanying the processes occurring during pupal development. An indication of the precise manner in which this dispersal occurs is noted in the pupal

TABLE II

Effect of Age and Tryptophan Feeding on the Distribution of Kynurenine in the Larval Fat Cells

1st treatment and age	2nd treatment and age	Fluorescence		
		Ant.	Mid	Post.
hrs.				
H_2O —66	None	+++	−	−
0.03 M Tryp.—66	None	+++	+++	++±
" —66	H_2O—70 hrs.	+++	−	−
" —66	H_2O—80 hrs.	+++	−	−
" —66	H_2O—90 hrs.	+++	−	−
" —72	None	+++	+++	++
" —72	H_2O—85 hrs.	+++	−	−
" —78	None	+++	+++	±
" —80	None	+++	+++	±
" —85	None	+++	+	−
" —90	None	+++	±	−

(+++ Indicates strong fluorescence; − none detected).

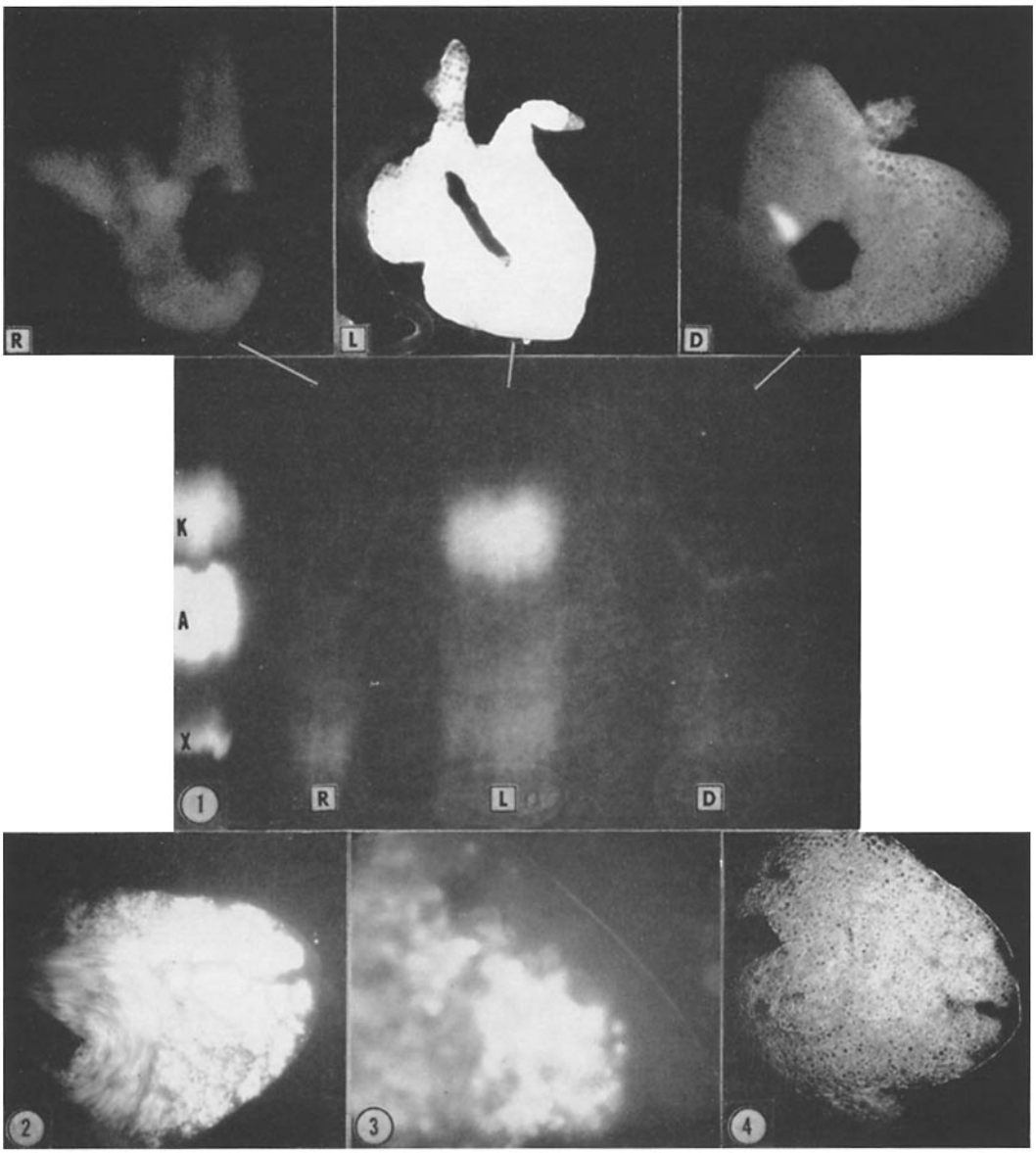

FIGURE 1 The results of *in vitro* incubation of the fatbodies from 78-hour-old larvae and a chromatogram prepared from the incubated specimens. The top row of photographs shows the fluorescence of the dorsal fat masses following incubation: *R*, incubated in Ringer's solution; *L*, incubated in Ringer's solution with L-tryptophan; *D*, incubated in Ringer's solution with D-tryptophan. The chromatogram shows the fluorescent spots separated from total fatbodies (8 in each) incubated in *R*, *L*, and *D* along with known samples of kynurenine (*K*), 2-amino-4-hydroxypteridine (*A*), and isoxanthropterin (*X*). × 55.

FIGURE 2 A region of fatbody showing "kynurenine cells" moved away from the surface layer surrounding the mass. On the left side of the specimen the cells are moved under the pressure of the coverglass during the 1-minute exposure required to record the fluorescence on film. × 55.

FIGURE 3 A region of the same specimen that appears in Fig. 2 magnified to show the internal reflection of the fluorescence caused by the "kynurenine cells" within the enveloping structure. × 352.

FIGURE 4 Same as Fig. 2. Under darkfield illumination. × 55.

and adult stages of the mutant strain *red cell* in which the anterior fat cells contain pigment inclusions (9). This reorganization of the fat cells indicates alterations in the cell surfaces or in the mechanism which binds these cells in a mass. The loss in plasticity of the intact fatbody becomes increasingly apparent as later larval and early pupal specimens are dissected. In late bubble stage specimens the fatbody is still intact within the body, but the slightest touch with a dissecting instrument destroys this integrity and free floating fat cells are obtained. Examination of the fatbody of white puparia or late larvae just beginning to show contraction of the cuticle revealed that a membranous layer envelops the fat cells. This covering was first viewed with the fluorescence microscope by observing the internal reflection of the autofluorescence of the fat cells from this surface layer. Figs. 2 to 4 illustrate this means of viewing the membrane surrounding the fat cells. (Electron micrographs of this structure have been prepared by Mr. Richard Hays during an examination of the ultrastructure of the cells of the fatbody.) Alterations in the surface layer are also apparent after regions of the fatbody have been incubated in either Ringer's solution or Ringer's solution containing tryptophan. A gelatinous consistency is assumed by this material following such incubation procedures.

DISCUSSION

The production of kynurenine by fat cells *in vitro* does not parallel the *in vivo* appearance of kynurenine in the fat cells with respect to two factors. Stearic specificity is exhibited under *in vitro* conditions, for only L-tryptophan and not D-tryptophan is utilized in the production of kynurenine. This difference, however, need not reflect a variation in behavior of the fat cells. Conversion of D-tryptophan to L-tryptophan has been suggested in the mammalian system (5), and a similar mechanism located outside the fatbody in the larva of *Drosophila* would explain the induction of kynurenine production when D-tryptophan is fed to the larvae. An alternative explanation, of course, may be improper *in vitro* conditions to effect this inversion should it normally occur in the fat cells. The second difference between the *in vitro* and the feeding experiments is the time of appearance of kynurenine in the fat cells. Isolated fat cells accumulate kynurenine much earlier than this substance becomes apparent in these cells when they remain in the larval body. A number of factors may account for such a difference, one of which is the availability of substrate within the fat cells during larval development. The matrix surrounding the fat cells, as well as the surface of the fat cells themselves, must be included in the consideration of permeability. During, and preceding, the period of dispersion of the fat cells in the pupal stage, modifications in the surface properties of the fat cells might be suspected as well as the alterations which have been observed in the layer covering the fatbody. It has been reported that extensive changes in the surfaces of the hemocytes occur near the beginning of pupation (11) and factors in the hemolymph associated with the processes of pupation may be influencing surface properties of other cells of the body as well. The present studies do not exclude the possibility that kynurenine is continually being synthesized by the fat cells and removed from this site during larval life; detection of kynurenine fluorescence under these conditions would represent the period of accumulation.

All of the cells in the fatbody of the *Ore-R* normal strain will show the characteristic light blue fluorescence of kynurenine if the larvae are placed on freshly prepared 0.03 M L-tryptophan solution at 65 hours of age and allowed to continue feeding on this solution until pupation. The brilliance of this fluorescence surpasses the intense autofluorescence occurring in the anterior cells of *Ore-R* larvae, raised on normal medium, or of any of the mutant strains of *D. melanogaster* which have been examined. The results of a series of feeding experiments with L-tryptophan indicate that the latest period at which a concentration of 0.03 M L-tryptophan can be effective is the middle of the third larval instar (72 hours). After this age there is a progressive loss of effectiveness in obtaining kynurenine in the fat cells by feeding tryptophan. This loss first becomes evident in the postgonadal cells and later in the pregonadal cells. An interruption in the feeding of tryptophan prior to the period when the larvae are preparing to pupate will result in a restriction of kynurenine to the anterior cells of the fatbody; that is, kynurenine is limited to those cells in which it occurs under normal feeding conditions. It appears that tryptophan is effectively being removed from the system and a

physiological balance is maintained in the body. Kynurenine production in the postgonadal cells is achieved only with continued intake of tryptophan when presumably there is a high level of this substance in the body.

When *Ore-R* wild-type larvae are maintained on their normal diet of yeast, the fat cells which contain isoxanthopterin do not accumulate kynurenine globules, and the latter remain restricted to the anterior fat cells. However, a period of feeding on tryptophan will result in the appearance of kynurenine and isoxanthopterin in the same cell. It is possible that the presence of pteridines, or other biochemical processes unique to the mid and posterior regions of the fatbody, may be a factor in determining whether the potential to produce kynurenine is expressed. Isenberg and Szent-Györgi (2) have demonstrated that the behavior of tryptophan as an electron donor leads to the formation of a complex between tryptophan and riboflavin. Pteridines behave as electron acceptors, and complexing between tryptophan and pteridines has more recently been shown (1, 7). It is interesting to speculate whether the exclusion of kynurenine from the mid and posterior fat cells of *D. melanogaster* might represent an example in the living cell where such a complexing mechanism may occur. Should identical quantities of tryptophan reach an anterior cell and a posterior pteridine cell, then any tendency for tryptophan to complex with pteridines in the latter cells may decrease the availability of tryptophan for enzyme induction and kynurenine production. It has been demonstrated that a high 2-amino-4-hydroxypteridine content in the *ry* mutant is correlated with a change in the quantity of tryptophan necessary for kynurenine production in the posterior cells. On the other hand, there is a differential response to the quantity of dietary tryptophan among the cells containing isoxanthopterin in the *Ore-R* strain and among the posterior fat cells in the *ry* strain. A higher concentration of tryptophan is required to effect kynurenine production in the posteriormost cells (postgonadal cells) than in the cells immediately anterior to the gonad. Quantitative data on the pteridine content of individual fat cells in these different regions is lacking, and such information will be valuable for the further evaluation of the relation of pteridine content to the production of kynurenine within the same cell. Other differences may also exist in the subcellular content of the cells within the posterior region of the fatbody, and some of these differences might account for the differential response to kynurenine production that has been observed. Previous studies on the tu^w (*tumor-w*) mutant strain indicate the possibility of regional differences among the posterior fat cells (8), for some of the cells within the postgonadal area in this strain become encapsulated and melanized while the remaining cells in the postgonadal region do not.

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