GENETIC CONTROL OF CYTODIFFERENTIATION

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ABSTRACT

The cells of the anterior region of the larval fatbody of *Drosophila melanogaster* accumulate kynurenine at the end of the third larval instar, whereas the cells of the posterior region are involved in pteridine metabolism. Through a series of transplantation experiments it has been demonstrated that the anterior fat cells synthesize kynurenine. The mutant *vermilion* lacks kynurenine, and the anterior fat cells of this mutant strain lack the autofluorescence characteristic of kynurenine. When the non-allelic *suppressor* gene is combined with *vermilion*, the synthesis of kynurenine is restored in the anterior fat cells, and some of the cells of the posterior region contain kynurenine as well. A similar extension in the number of cells containing kynurenine can be induced in the normal *Ore-R* strain by feeding the precursor tryptophan. It has been concluded that the absence of a physiological process in a differentiated cell does not necessarily represent a loss of the genetic potential for that process. The normal allele at the *suppressor* locus inhibits the occurrence of kynurenine in the posterior fat cells, whereas the mutant allele su^2-*s* allows the expression of this potential. An inducer such as tryptophan can overcome this inhibition in the normal strain, and as a result the cells which are normally differentiated as "isoxanthopterin cells" may produce kynurenine as well.

At the end of the third larval instar the cells of the anterior region of the fatbody of *Drosophila melanogaster* accumulate globular cytoplasmic inclusions containing kynurenine (21). The characteristic autofluorescence of these cytoplasmic globules distinguishes the anterior fat cells from the posterior fat cells, which accumulate isoxanthopterin (22). The consistency of this pattern of cytodifferentiation among the fat cells has been verified in numerous strains of *D. melanogaster*, and only in those mutant strains with disruptions in the biosynthesis of these metabolites have alterations been noted. For example, the intense sky blue autofluorescence of the anterior fat cells is absent in the *vermilion* mutant, which lacks kynurenine, and the posterior fat cells of *rosy* prepupae display an unusual pattern of autofluorescence correlated with the absence of isoxanthopterin in this mutant strain.

The localization of these gene-controlled metabolites in the cells of the fatbody may represent the site of collection and storage of materials synthesized elsewhere in the body, or the cells of the fatbody may be the site of synthesis of these substances. Implantation of the fat cells into an environment devoid of these metabolites offers one approach to this question, which is of prime importance to further studies of chemodifferentiation among the cells of the fatbody. Anterior and posterior fat cells from the normal *Ore-R* strain have been implanted into *vermilion* flies, which lack kynurenine, in order to determine whether the normal fat cells synthesize kynurenine. The present report details these experiments.

Another question concerns the distribution of the cells with kynurenine and cells containing isoxanthopterin. The fatbody of *D. melanogaster* is a continuous tissue mass with a homogeneous population of cells when viewed by ordinary light microscopy. However, morphologically distinct lobes and convolutions permit identification of specific regions of the fatbody of the *Ore-R*

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wild type strain, and, utilizing these features, it is possible to predict which cells when examined by fluorescence microscopy will reveal kynurenine and which will contain isoxanthopterin. Does this normal pattern of cytodifferentiation of "kynurenine cells" and "isoxanthopterin cells" represent a rigid, inflexible process of regional differentiation, or are there conditions which may modify the chemosynthetic activities of these cells? The *suppressor* genes which normalize the eye pigment of the mutant *vermilion* have been useful in approaching this question with respect to the accumulation of kynurenine by the cells of the fatbody.

OBSERVATIONS

Site of Synthesis of Kynurenine

Larval tissues of *Drosophila* such as salivary glands or sections of the gut may be implanted into adult male hosts, which act as a sustentive milieu for the implanted tissue. Bodenstein (3, 4) demonstrated that these larval tissues will not grow or undergo metamorphosis in the adult male host unless larval ring glands are also implanted into the host. Larval gut cells will continue to synthesize peritrophic membrane when implanted into the adult *Drosophila* male (20), thus indicating that synthetic activities of implanted larval tissues will proceed. One means of demonstrating whether the anterior fat cells are capable of synthesizing kynurenine utilizes these techniques of implantation. Fat tissues from third instar larvae prior to any detectable accumulation of kynurenine may be implanted into mutant *vermilion* adults, which lack kynurenine. Removal of the implanted tissues after a period in the host and examination with the fluorescence microscope will reveal whether the fat cells are capable of synthesizing kynurenine.

The methods used for collecting and raising *Drosophila* larvae for the present experiments as well as the preparations of cells for fluorescence microscopy were the same as those reported previously (21).[1] Ages of larvae and pupae were recorded from the time of occlusion of the larvae from the eggs. For the implantation experiments donor larvae (*Ore-R* wild type strain) 80 hours of age were dissected in *Drosophila* Ringer solution and the fatbodies were separated into sections. Since only the anterior fat cells in the normal *Ore-R* strain accumulate kynurenine, the anterodorsal region of the fatbody served as the experimental material, and the dorsolateral and the caudal regions of the fatbody were selected as control tissues. Each of the pieces of fatbody was implanted into a 2-day-old vermilion adult male utilizing a micro injection apparatus (19). An unsuppressible *vermilion* stock (v^{36f}) was used as the host strain. As an additional control v^{36f} larval fatbody was implanted into v^{36f} adults. The donor tissues were removed from the hosts approximately 16 hours after implantation and examined with the fluorescence microscope. In order to rule out the possibility of mistaking any host fat cells for the implanted donor fat cells, only those implants which were recovered intact and recognizable by their morphology as anterodorsal, dorsolateral, or caudal larval fat were recorded as successful implants and consequently examined with the fluorescence microscope. The data from the implantation experiments have been combined in Table I. These experiments demonstrate that the accumulation of kynurenine in the anterior fat cells is an autonomous property, since these cells develop their characteristic autofluorescent cytoplasmic inclusions in the *vermilion* hosts.

Distribution of Kynurenine in Suppressor Mutants

The *vermilion* eye color mutants are separable into two classes, those which are suppressible when combined with the mutant *suppressor of vermilion* (*su-v*), and those which are unsuppressible in combination with *su-v* (12, 13). Both the suppressible *vermilion* (v^s) and unsuppressible *vermilion* (v^u) mutants are blocked in the immediate utilization of tryptophan (12, 23). The consequent lack of formylkynurenine and kynurenine, precursors of brown eye pigment, leads to the phenotypic expression of vermilion eye color. The anterior larval fat cells of both v^s and v^u mutants lack the sky blue autofluorescence characteristic of kynurenine (21, and unpublished observations). The action of the *su-v* mutants in the suppression of the v^s phenotype has been recognized through the development of brown

[1] The mutant strains used in this investigation were obtained from the Drosophila Stock Center, Institute of Cancer Research, Philadelphia, through the courtesy of Dr. Irwin I. Oster. Professor Edward Novitski kindly provided the mutant alleles of *vermilion*.

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pigment in the eye. It would be expected that kynurenine will accumulate in the anterior fat cells of the combination *su-v* with *v* if the action of *su-v* is rectifying the norm created under the influence of the mutant allele of v^+, and if the occurrence of kynurenine in the fat cells is a function either correlated with, or involved in, the synthesis of eye pigment precursors.

For the present investigation the following v^s stocks and combinations were examined: v^1, v^2, v^k, su^2-*s v*, and su^3-*s cv v f/y f*:=. The v^u stocks included v^{36f}, v^{48a}, v^{51c}, and su^2-*s* v^{36f}. After a survey of the fat cells of these stocks with the fluorescence microscope, the stock su^2-*s v* was used for the description of the *suppressor* pattern.

TABLE I

Implantation Experiments

Total number of implants, 75; number of implants recovered, 64; per cent recovered, 85.

"Autofl +" indicates sky blue fluorescence characteristic of kynurenine; "Autofl −" indicates its absence. Posterior fat mass includes dorsolateral and caudal regions.

Donor larvae	Host ♂♂	Autofl +	Autofl −	Total
Ore-R				
Anterior fat mass	v^{36f}	17	0	17
Posterior fat mass	v^{36f}	0	9	9
Anterior fat mass	*Ore-R*	12	0	12
Posterior fat mass	*Ore-R*	0	20	20
Total anterior		29	0	
Total posterior		0	29	
v^{36f}				
Anterior fat mass	v^{36f}	0	6	

The light blue fluorescence characteristic of kynurenine is localized in globular cytoplasmic inclusions in the wild type *Ore-R* strain. These autofluorescent inclusions are restricted to the cells of the anterior region of the fatbody, and as described previously (21, 22) there is a sharp autofluorescent boundary created by the intensity of the kynurenine autofluorescence in these cells in contrast to the autofluorescence of the adjacent cells involved in pteridine metabolism. Under the usual culture conditions no variation in the position of this boundary is noted, and the region of the fatbody containing kynurenine terminates in a row of cells proceeding ventrocaudad and forming an arc of autofluorescent material when viewed from the lateral aspect. This boundary region in the *Ore-R* fatbody is depicted in Figs. 2 and 3. The cells of the anterior region of the fatbody of su^2-*s v* white pupae contain light blue autofluorescent globules similar to those found in the anterior fat cells of the *Ore-R* strain. However, this cytoplasmic autofluorescence indicating the site of localization of kynurenine is not restricted to the anterior region of the fatbody in su^2-*s v* pupae, but extends posteriorly to the level of the gonads. There is variation in the intensity of the light blue autofluorescence among the cells in this extended region, so that the overall autofluorescence presents a mosaic appearance. Comparison of Figs. 2 and 3 with Fig. 1 illustrates the difference between the distribution pattern of the cells containing kynurenine in normal *Ore-R* pupae and that in mutant su^2-*s v* pupae. An extension of the kynurenine region also occurs in the su^3-*s v* mutant combination.

Effect of Feeding Tryptophan

Kikkawa (15) reported an increase of kynurenine in the mutant *cinnabar* as a result of feeding tryptophan. An increase in 3-OH-kynurenine was also noted in the wild type strain as a result of a diet supplemented with tryptophan. Tryptophan was therefore fed to *Drosophila* larvae in order to examine any effects on the cells of the fatbody. In some instances tryptophan was given to *Ore-R* larvae feeding on yeast, and in other experiments 65-hour-old larvae were placed on Whatman cellulose powder which was wet with a 0.1 per cent solution of L-tryptophan. The cellulose powder was prepared prior to use by washing with N HCl, rinsing with 6 changes of distilled H_2O, resuspending in H_2O, and returning the pH to 7 with NH_4OH, and finally by repeated washing with distilled H_2O. After a final rinse with acetone, the powder was dried and stored in a deep freeze. All larvae in these experiments were washed with a 1 per cent solution of NaOCl followed by repeated washing in distilled water.

The fat cells of the white pupae and early

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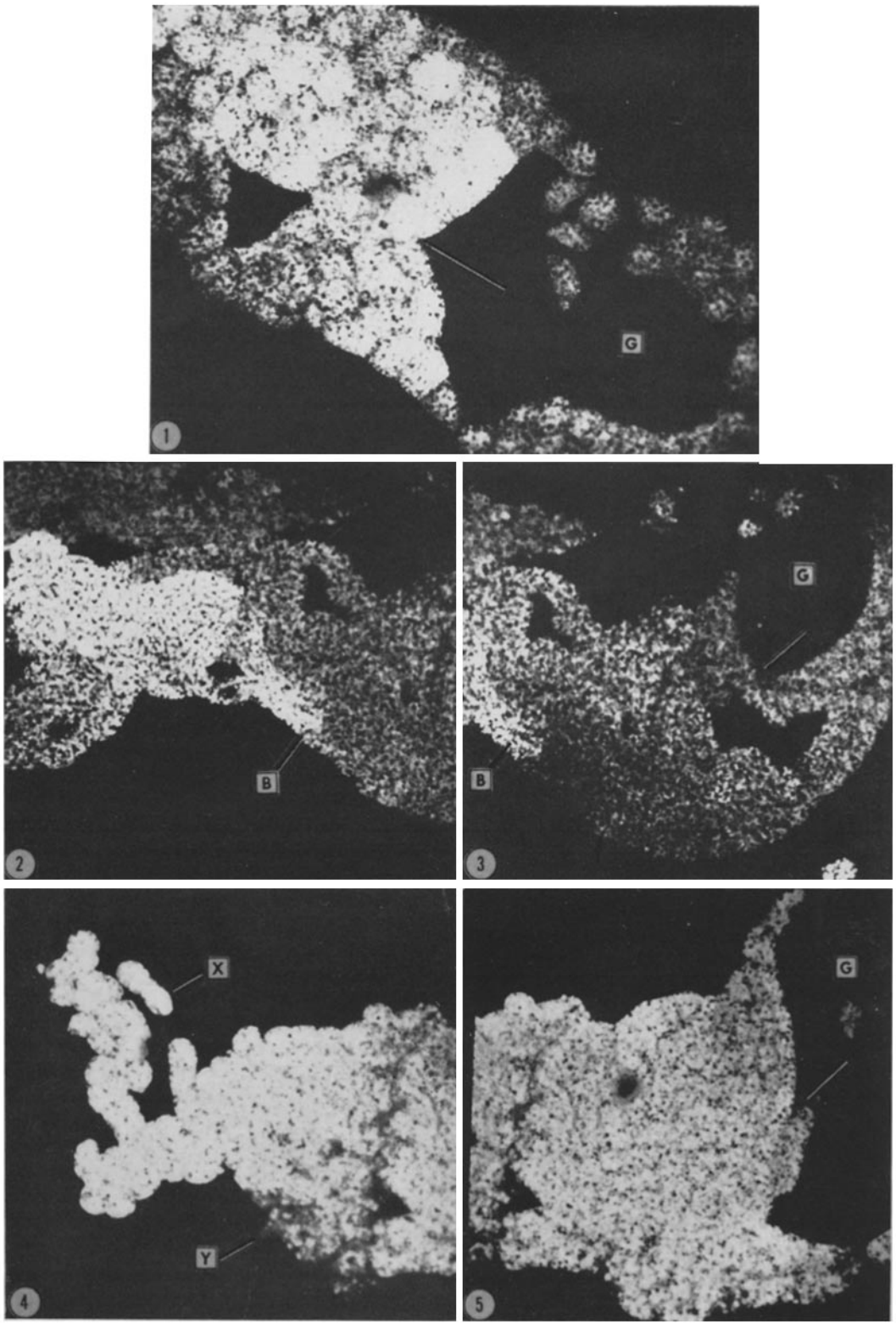

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brown pupae (bubble stage) were examined with the fluorescence microscope. The anterior "kynurenine cells" displayed an intense light blue autofluorescence, and a similar autofluorescence was also apparent in the cells posterior to this region. The distribution of cells with kynurenine after tryptophan feeding resembled the pattern of the *su*^{2}-*s v* strain, but the autofluorescence of tryptophan-fed *Ore-R* larvae was more intense and included a greater number of fat cells (Figs. 4 and 5). The cells which under normal feeding conditions contain 2-amino-4-hydroxypteridine and isoxanthopterin now contained kynurenine as well as the former components. The nature of the globular cytoplasmic contents is clearly observed when these cells are ruptured during observation (Figs. 6 and 7).

Chromatograms of the fatbody of tryptophan-fed larvae and control larvae verified that the autofluorescent material visible in the cells of tryptophan-fed larvae is the same as that autofluorescent material which is normally restricted to the anterior cells, and that this light blue intracellular autofluorescence indicates kynurenine localization. For each chromatogram, fatbodies from 12 early bubble stage pupae were used. This amount of material from larvae fed on yeast or from control larvae placed on H_2O produces only a barely detectable fluorescent spot of kynurenine on the chromatograms, and a clear-cut increase in kynurenine is thus apparent in the larvae which have been fed tryptophan (Figs. 8 and 9). Adhering tissues including salivary glands and gonads were dissected free of the fatbodies and removed, so the fatbodies are the sole source of fluorescent materials visible in these chromatograms. In order to rule out the possibility of microbial contamination in the tryptophan solution on the cellulose powder, this medium was streaked on chromatographic paper

Explanation of Figures

All specimens were mounted in freshly prepared Ringer solution on a microscope slide made of a Corning no. CS 5860 filter. The specimens were slightly flattened during observation and held in this position by clamping. Since the fatbody of *Drosophila* is a single cell layer, proper spreading allows viewing of each cell in the entire fatbody. These photographs were prepared from original High Speed Ektachrome 35 mm transparencies. Zeiss fluorescence equipment was used with a darkfield condenser and mercury burner HBO 200, principal excitation λ365 mμ, barrier filters to exclude below λ410 mμ and beyond 650 mμ. The chromatograms were photographed with the same light source.

FIGURE 1

The larval fat mass from an early bubble stage ♂ puparium of *su*^{2}-*s v*. *G* indicates the gonad. The autofluorescence anterior to the gonad is of the intense sky blue color characteristic of kynurenine, while the cells around the gonad and extending posteriorly fluoresce deep blue with their content of isoxanthopterin. The position of the bar indicates identical regions in Figs. 1, 3, and 5. × 80.

FIGURES 2 AND 3

The larval fat mass from an early bubble stage ♂ puparium of the *Ore-R* wild type strain. Fig. 3 is a continuation of Fig. 2 extending posteriorly, as is evident from the repetition of the overlapping areas in the photographs. Specimen transferred to cellulose powder wet with distilled H_2O at 65 hours of age. The intense autofluorescence of kynurenine in the anterior fat terminates in a row of cells extending ventrocaudad (*B*). This distribution of cells with kynurenine is found in wild type larvae under normal feeding conditions; this specimen has been placed on H_2O from age 65 hours as a control specimen for the tryptophan feeding experiments. The extension of "kynurenine cells" in the *suppressor* strain is illustrated by comparing Figs. 1 and 3. × 50.

FIGURES 4 AND 5

Larval fat mass from an early bubble stage ♂ puparium of the *Ore-R* strain. Larvae placed on cellulose powder with L-tryptophan solution at 65 hours of age. The cells containing kynurenine now extend to *G*, and the adaptive increase in the number of "kynurenine cells" is illustrated by comparison of Figs. 3 and 5. The region labeled *X* is enlarged in Fig. 6, and Fig. 7 is an enlargement of area *Y*. × 50.

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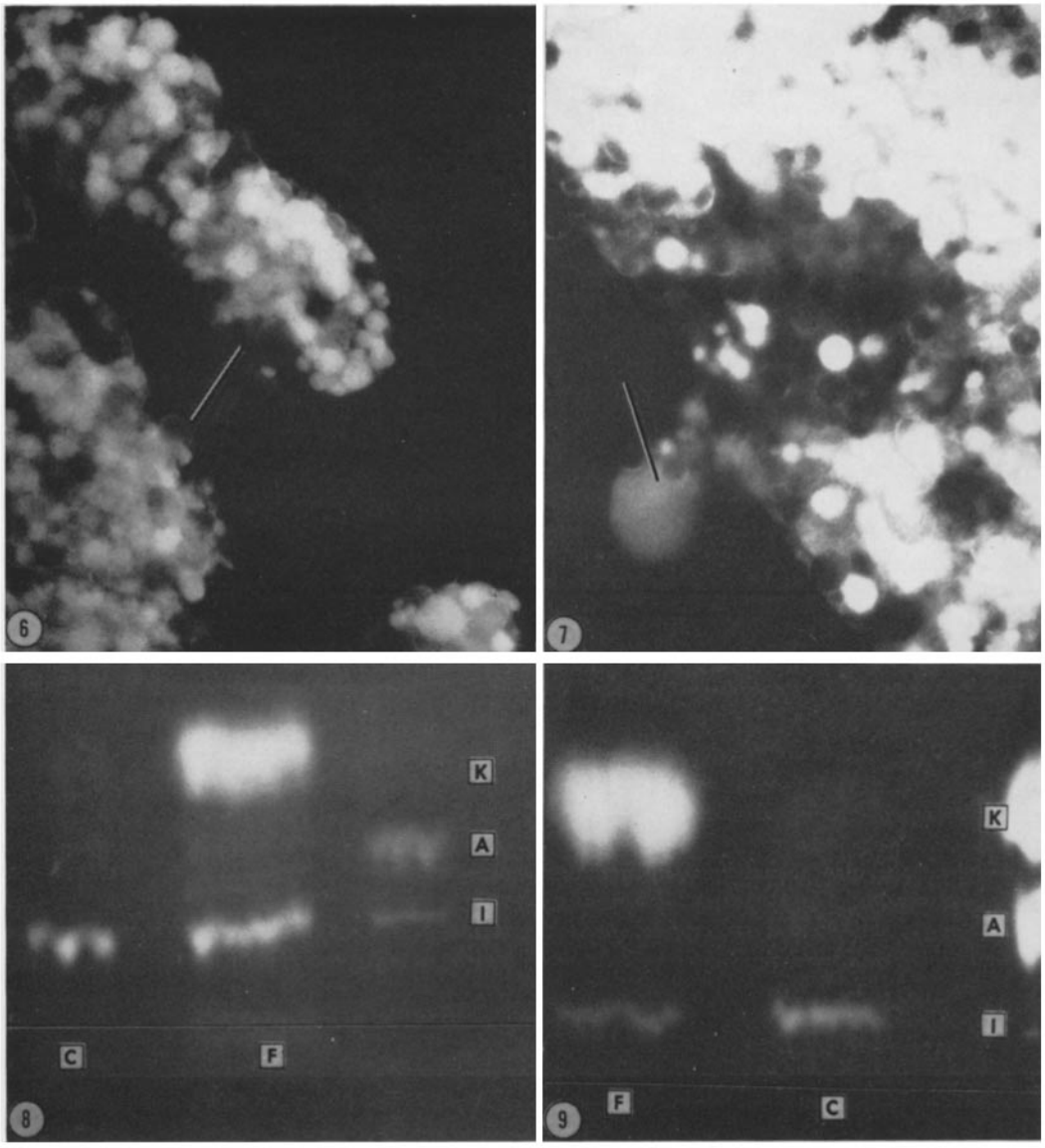

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at the time the pupae were removed for chromatography. Tryptophan was readily demonstrated on the chromatograms, but no kynurenine was detected.

The fluorescent spots of kynurenine from control larvae, tryptophan-fed larvae, and a known sample of kynurenine were cut from the chromatographic paper and eluted with 0.2 ml distilled H_2O. This material was transferred to micro cuvettes and curves were run in a Zeiss PMQ II spectrophotometer. The reference cuvette contained the elute from a blank section of the same chromatogram at an Rf corresponding to that of kynurenine. Correspondence of the maxima at wavelengths 226, 258, and 356 mμ confirmed the identity of the fluorescent spot from the tryptophan-fed larvae as kynurenine. The quantity of kynurenine obtained from the control larvae was insufficient for detection under these conditions.

DISCUSSION

It has been demonstrated in several instances that morphogenetic processes are under genic control. The classical studies of Poulson (17, 18) on the chromosomal deficiencies of *Drosophila* indicated that the early events in embryogenesis are influenced by genetic factors. The early cleavage of the egg in *Drosophila* is likewise related to genetic determinants (7, 8). These studies demonstrate a causal relationship between a gene and a specific morphogenetic process at a given moment, but the status of the residual genetic information in the nuclei of the cells of the developing organism is not clear. Utilizing the technique of implantation of nuclei from developing amphibian embryos into enucleated eggs, Briggs and King (6) have demonstrated that nuclei undergo restrictions in morphogenetic potential as differentiation proceeds. From the present study of cytodifferentiation it becomes clear that the absence of a cellular process need not imply a loss in the genetic potential controlling this specific process.

The fatbody of *Drosophila melanogaster* is composed of a morphologically homogeneous population of cells, but the utilization of fluorescence microscopy reveals a functional differentiation among these cells. Some of the fat cells synthesize kynurenine and the others are engaged in pteridine metabolism. Implantation of the larval fat cells into the adult *vermilion* male shows that the anterior fat cells can synthesize kynurenine whereas the posterior fat cells with their content of pteridine products do not synthesize kynurenine under these same conditions. The question whether this inability of the posterior fat cells to synthesize kynurenine represents a loss in genetic potential is answered when the genetic material of these

FIGURE 6

Cells of the anterior fatbody with globules containing kynurenine. The bar indicates extruded fat globules which do not fluoresce. × 320.

FIGURE 7

Cells which contain isoxanthopterin as well as kynurenine after tryptophan feeding. Some of the fat globules from these cells have been extruded by slight pressure on the coverglass in a manner comparable to Fig. 6 (bar). Together with the extruded fat globules, the deep blue autofluorescent cytoplasm characteristic of "isoxanthopterin cells" is released. The intense globules contain kynurenine. × 320.

FIGURES 8 AND 9

Chromatograms of the isolated fatbodies from early bubble stage *Ore-R* pupae. *C*, control larvae placed on H_2O at 65 hours of age; *F*, larvae placed on L-tryptophan at 65 hours of age. Known samples: *K*, kynurenine; *A*, 2-amino-4-hydroxypteridine; *I*, isoxanthopterin. Ascending chromatography was performed on Whatman no. 1 paper with isopropanol: 1 per cent ammonia (2:1 v/v) as the solvent for 2 hours at room temperature. The increase in kynurenine of the tryptophan-fed larvae is clearly demonstrated since each sample includes 12 fatbodies from control pupae and 12 fatbodies from tryptophan-fed pupae. The intact fatbodies in the chromatogram in Fig. 8 were placed directly on the chromatographic paper, whereas in Fig. 9 the samples of fatbodies were transferred to the chromatographic paper after grinding in Ringer. In Fig. 9 the background fluorescence has been reduced by allowing the chromatographic paper to develop in the solvent overnight and drying before use.

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cells is subjected to manipulation or the external milieu of these cells is changed.

The *vermilion* mutant, which lacks kynurenine synthesis, shows no apparent alteration in the cells which are involved in pteridine synthesis. The mutant *v* gene blocks kynurenine synthesis and hence the light blue autofluorescence characteristic of kynurenine does not appear in the cells of the anterior fat mass. When the non-allelic gene mutation *suppressor of vermilion* is introduced into the *vermilion* genotype, not only do the anterior fat cells recover kynurenine synthesis, but also cells which are involved in pteridine metabolism begin to show kynurenine accumulation. On the basis of these observations it may be postulated that the maintenance of physiological differentiation of pteridine-producing cells and the absence of kynurenine in these same cells in the normal genotype does not represent a loss of genetic potential to synthesize kynurenine, but that this differentiation is genetically controlled by the normal allele at the *suppressor* locus. The exact physiological mechanism involved in the prohibition of kynurenine formation in the posterior fat cells of the wild type larva is not clear, and reference should be made to the fact that the mutant allele at the *suppressor of vermilion* locus also acts as a suppressor of the mutant body color *sable* (5).

The alteration that appears in the cells of the posterior region when the extracellular environment is changed by feeding excessive tryptophan indicates the specific nature of the system operating in these cells. Tryptophan metabolism in *Drosophila* involves a pathway from tryptophan ⟶ formylkynurenine ⟶ kynurenine ⟶ 3-hydroxykynurenine ⟶ brown pigment (9). *Vermilion* larvae will synthesize brown pigment if they are fed formylkynurenine or kynurenine. Glassman (11) has demonstrated the presence of the enzyme kynurenine formamidase in the wild type as well as in the *vermilion* mutant. More recently Baglioni (1, 2) has demonstrated the presence of tryptophan pyrrolase in *Drosophila*. The latter enzyme, which catalyzes the conversion of tryptophan to formylkynurenine, has been extensively studied in mammalian liver, where the inducible nature of this enzyme has been demonstrated (10, 14, 16, 24). The increased level of kynurenine in wild type *Drosophila* larvae fed on tryptophan, as indicated by chromatographic analysis as well as by the extended number of fat cells showing kynurenine autofluorescence, is presumably correlated with an increased level of the inducible enzyme tryptophan pyrrolase. The appearance of kynurenine in the posterior fat cells of normal larvae when given an inducer such as tryptophan, as well as the release of kynurenine synthesis in these cells under the influence of the *suppressor* gene, offers an ideal system for the study of genetic control of an inducible enzyme system *in situ* and its relation to cytodifferentiation.

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