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Developmental Expression and Spatial Distribution of Dopa Decarboxylase in *Drosophila*

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Regulation of the dopa decarboxylase gene of *Drosophila* has been studied at the genetic and molecular levels. Here we report a direct assay for the tissue and temporal regulation of *Ddc*. A dopa decarboxylase (DDC) peptide was obtained by bacterial expression of a portion of the DDC gene in a pUC plasmid. Antisera raised against this biologically purified DDC peptide react specifically with Drosophila DDC in histological preparations and protein blots. The levels of DDC cross-reacting material closely parallel the levels of enzyme activity observed during development, indicating that DDC is degraded during periods of declining activity. We find that DDC is expressed in only two tissues, namely, the epidermis and the nervous system of the larva and adult. Epidermal DDC was found within the epidermal cells and was not detected in the overlying cuticle. DDC-containing neurons were observed in the central as well as in the visceral nervous system. Paired and unpaired midline neurons in the ventral ganglia are arranged in a segmental pattern. A subset of the DDC-positive neurons appears to correlate with the serotonin-positive neurons suggesting that the others are producing only dopamine. We find that the DDC activity associated with the proventriculus and ovary is due to the presence of DDC in the stomatogastric and caudal system neurons specifically associated with those structures.

INTRODUCTION

Dopa decarboxylase (DDC)[1] can catalyze the decarboxylation of dopa to dopamine and 5-hydroxytryptophan (5-HTP) to serotonin (5-HT) (Wright *et al.*, 1976; Clark *et al.*, 1978; Livingstone and Tempel, 1983). Dopamine and serotonin serve two known physiological functions in Drosophila. Dopamine metabolites serve as crosslinking agents for cuticular proteins during sclerotization while both dopamine and serotonin serve as neurotransmitters. Dopa decarboxylase is a vital enzyme which is expressed in a stage- and tissue-specific manner. Five peaks of *Ddc* expression during development are characterized by increases in both enzyme activity and mRNA accumulation (Marsh and Wright, 1980; Kraminsky *et al.*, 1980). However, because no histological assay for DDC is available, determination of tissue distribution has relied on enzyme activity measurements in dissected tissues. Enzyme activity has been detected in homogenates of four Drosophila tissues, the epidermis, the central nervous system, the proventriculus, and the ovary (e.g., Lunan and Mitchell, 1969 (epidermis); Dewhurst *et al.*, 1972 (adult thoracic ganglia); Wright and Wright, 1978 (proventriculus); Wright *et al.*, 1981 (ovary)). Enzyme assays of homogenates provide no information concerning the cellular distribution of DDC, nor do they allow one to distinguish regulation by enzyme inactivation from regulation by protein turnover.

In this communication, we document the temporal and tissue distribution of the DDC peptide during normal development using monospecific antisera raised against the cloned *Ddc* gene expressed in *Escherichia coli*. We show that DDC cross-reacting material (CRM) closely parallels DDC activity during development and we document the occurrence of DDC in epidermal cells and in a restricted set of cells of the central and visceral nervous systems. We find no evidence for *Ddc* expression in the proventriculus or the ovary but we do find DDC-containing nerve processes specifically associated with these structures.

MATERIALS AND METHODS

Plasmids containing a portion of the *Ddc* coding region inserted into all three reading frames of the *lacZ* alpha complementing gene were constructed as described (Eveleth *et al.*, 1986). Specifically, a *Xho*I-*Pst*I restriction fragment beginning near the the amino terminus of the *Ddc* coding region and extending through the translation stop codon was cloned into *Sal*I-*Pst*I cut pUC 8 and pUC 18 (Messing, 1983) and *Xho*I-*Pst*I cut pIC 19R (Marsh *et al.*, 1984; Eveleth *et al.*, 1986) and the identity of each construct was confirmed by restriction analysis. To an-

[1] Abbreviations used: DDC, dopa decarboxylase enzyme (EC 4.1.1.26; 3,4-dihydroxyl-L-phenylalanine carboxylase); *Ddc*, dopa decarboxylase gene; FITC-GAR, fluoroscein isothiocyanate-conjugated goat anti-rabbit; IPA, ^{125}I protein A; CRM, cross-reacting material; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WPP, white prepupae.

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alyze expressed proteins, transformed bacteria were grown overnight in LB medium and then diluted 10-fold with B-broth to minimize catabolite repression of *lacZ* expression. After 4 hr at 37°C, the cell density was measured and the cells were pelleted and lysed by boiling in Laemmli sample buffer (Laemmli, 1970) at a density of 40 OD_{600} units/ml. This solution (15 μl) was analyzed by SDS-PAGE and stained with Coomassie brilliant blue G-250 (Blakesley and Boezi, 1977). Two-dimensional gel electrophoresis was performed as described by O'Farrell (1975). One- and two-dimensional gels were used for protein blots as described by Burnette (1981). To quantitate the amount of ^{125}I-protein A (IPA) binding to protein blots, the region corresponding to bands on developed X-ray film was cut from the nitrocellulose and counted in a gamma counter (Beckman gamma 5000).

For protein isolation, preparative (3 mm) gels were stained, the desired band was cut out, and the protein was electroeluted from the acrylamide slice into dialysis tubing in Laemmli electrophoresis buffer. The eluate was lyophilized and SDS was removed from the pellet by ion-pair extraction (Henderson *et al.*, 1979). The resulting pellet was resuspended in saline and used as an immunogen. Approximately 100 μg was mixed with incomplete Freund's adjuvant and injected subcutaneously into several sites in rabbits. After 2 weeks, a 50-μg boost was administered and 10 days later blood was obtained via cardiac puncture and the serum was analyzed using protein blots of bacterial extracts.

To obtain developmentally staged Drosophila tissues, eggs were collected for 6 hr on dishes containing hard agar. After appropriate aging, the eggs were rinsed off the plates onto a nytex filter, dechorionated with 50% bleach, and rinsed with water. For posthatching stages, the eggs were transferred to bottles containing standard cornmeal molasses supplemented with yeast, collected at appropriate intervals, and similarly rinsed to remove adhering yeast and food. Wandering third instar larvae and later stages were selected by visual examination of morphology rather than by time. Tissue was homogenized in DDC homogenizaton buffer (Marsh and Wright, 1980) containing a mixture of protease inhibitors in the following concentrations; 1 μg/ml of each leupeptin, antipain, pepstatin, and chymostatin; 100 kallikrein units/ml aprotinin; 1 m*M* EDTA; and 0.2 m*M* PMSF. The homogenate was centrifuged for 10 min in a Brinkman centrifuge and aliquots of the supernatant were frozen at −20°C. The protein content of the supernatant was determined using the Bio-Rad protein assay following instructions provided by the manufacturer. Aliquots of the supernatant were thawed and boiled with Laemmli sample buffer prior to SDS-PAGE.

For whole-mount immunofluorescence, tissues were dissected in phosphate-buffered saline (PBS) and transferred to 10% formalin for 1–2 hr. The tissues were then transferred to methanol for 15 min and subsequently rehydrated in PBS. The tissues were placed in PBS containing the antiserum and 10% normal goat serum for 1 hr at 37°C. They were then rinsed 4× (5 min each) in PBS containing 0.1% Triton X-100 before being placed in PBS containing FITC-GAR IgG (Zymed) and 10% normal goat serum for 1 hr at 37°C. The tissues were rinsed as above and mounted in 90% glycerol for visualization. For each staining reaction parallel tissue samples were stained with preimmune serum. For frozen sections, unfixed tissues were frozen directly in OCT compound (Miles) and processed according to Fujita *et al.* (1982). Incubation with antibodies and rinsing were the same as with whole-mounts. Hemocytes were examined in smears of hemolymph fixed by either heat or formaldehyde (Arnold and Hinks, 1979).

RESULTS

Antiserum directed against bacterially expressed DDC. A subclone of *Ddc* genomic DNA, when inserted into a pUC plasmid in the *lacZ* reading frame results in the expression of a novel peptide (Fig. 1). The size of the novel peptide is as expected from a conceptual translation of the inserted *Ddc* sequence (Eveleth *et al.*, 1986). The partial DDC protein was the most abundant protein species present in the bacterial extracts. When isolated from gels and used as an antigen, this peptide elicited antiserum specific to the novel peptide (Fig. 1).

The immune serum exhibited strong cross-reaction to a single protein migrating at 56 kDa in extracts from various stages of Drosophila development (Fig. 2). Preimmune serum showed no cross-reaction to this protein. The observed 56-kDa protein is consistent with the MW of Drosophila DDC determined empirically (Clark *et al.*, 1978) and from DNA sequence analysis (Eveleth *et al.*, 1986). In spite of the strong reaction of the antiserum to denatured DDC, enzymatically active DDC was not recognized by the antiserum, as evidenced by the failure of protein A-agarose beads to precipitate DDC activity from homogenates of WPP incubated with antiserum (not shown). Drosophila proteins separated on two-dimensional gels have also been probed with the anti-DDC antiserum. In extracts from white prepupae (WPP), the stage of maximum DDC activity, the 56-kDa protein focused to a single spot, migrating at a slightly acidic pH (Fig. 3).

Developmental expression of Drosophila DDC. Proteins extracted from developmentally staged Drosophila larvae, pupae, and adults were blotted onto nitrocellulose and probed with anti-DDC antiserum followed by IPA. The amount of radioactivity bound to the 56-kDa band from each stage was determined by gamma counting.

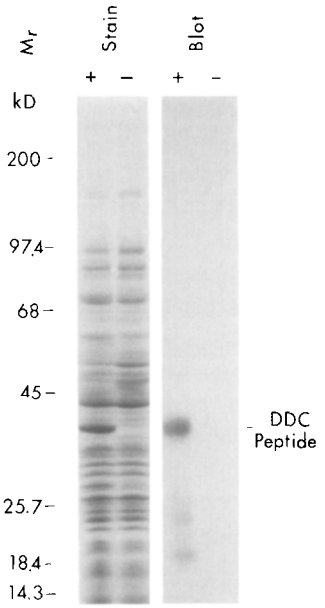

FIG. 1. Bacterial expression of a *Ddc* peptide. Protein extracts were prepared from three bacterial strains harboring pUC plasmids containing a fragment of the *Ddc* gene in each of the three reading frames relative to the *lacZ* translation initiation site (Eveleth *et al.*, 1986). The two lanes on the left show the staining pattern of extracts separated by 9–15% gradient SDS-PAGE. An abundant peptide was produced by the pUC8 plasmid, which preserved the *Ddc* reading frame (+ lanes) which was absent from the other two extracts, one of which is shown (− lanes). All extracts displayed minor quantitative variations in less abundant proteins. The right two lanes show immunoblots of duplicate gel lanes probed first with antiserum directed against the novel peptide and then with IPA. The antiserum appeared specific for the novel peptide with no reaction to comigrating bacterial proteins. The smaller peptides detected may represent degradation intermediates. The migration of MW standards is indicated to the left.

These data were compared with the developmental profile of DDC activity (Marsh and Wright, 1980; Fig. 4). This reveals that the amount of DDC activity is directly proportional to the amount of DDC protein.

Tissue distribution of DDC. DDC activity has been detected in homogenates of four *Drosophila* tissues, the epidermis (Lunan and Mitchell, 1969), the central nervous system (Dewhurst *et al.*, 1972), the proventriculus (Wright and Wright, 1978), and the ovary (Wright *et al.*, 1981). We have examined these and other tissues for the presence of DDC enzyme using indirect immunofluorescence. Staining whole-mounts of the larval nervous system with anti-DDC revealed a small subset of bilaterally symmetrical DDC-containing cells and processes both in the brain hemispheres and in the ventral ganglion (Fig. 5). Some variation occurred in the number of stained cells visible in different preparations, perhaps due to variations in antibody penetration. The following description, summarized in Fig. 7, is based on the comparison of staining patterns in more than 20 larval brains.

In the larval ventral ganglion, the DDC-containing cells displayed a reiterated pattern (Fig. 5). The ventral portion of the ventral ganglion contained three longitudinal rows of cells, one unpaired row of midline cells, and two paired lateral rows of cells. These cells appeared to be organized into 14 segmental units. The most posterior unit contained single cells in each of the lateral rows and no midline cell. The next 8 more anterior units contained a pair of cells in each lateral row and a single midline cell (Fig. 5D). Processes from the lateral cells projected toward the midline (Fig. 5F), where they were joined by a process from the midline cell. The next three more anterior units were arranged differently. Their lateral rows each contained clusters of three cells with processes projecting anteriorly and posteriorly. The midline cells in these segments tended to be offset from the lateral cells and exhibited laterally projecting processes.

The larval ventral ganglion is formed by the fusion of individual embryonic ganglia. Eight abdominal ganglia can be visualized although the embryo contains 10 abdominal segments. The two most posterior segments are small and fuse together (Turner and Mahowald, 1977). The position and differences in the organization

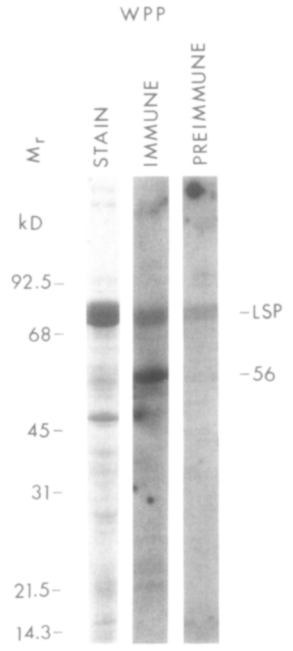

FIG. 2. Reaction of antiserum with Drosophila DDC. Proteins extracted from pupariating larvae (WPP) were separated by 9–15% SDS-PAGE. One lane was stained and duplicate lanes were immunoblotted and probed with either immune or preimmune rabbit serum followed by IPA. The immune serum reacted strongly with a 56-kDa protein, the expected MW of Drosophila DDC. Both the immune and preimmune sera displayed weak, apparently nonspecific binding to other proteins, most notably with the highly abundant larval serum proteins (LSP). The migration of MW standards is indicated at the left.

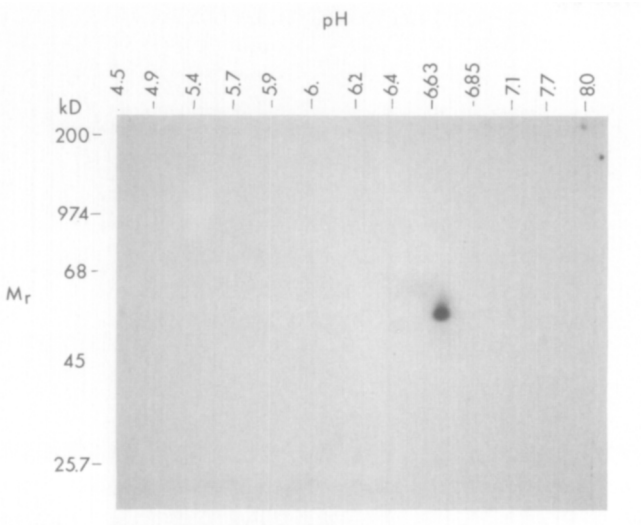

FIG. 3. Two-dimensional gel immunoblot of DDC. A protein extract from wild-type WPP was separated by isoelectric focusing in the first dimension (pH measurements from a control gel are indicated at the top) and by 9-15% SDS-PAGE in the second dimension (migration of MW standards is indicated at the left). The blot was then probed with anti-DDC serum followed by IPA.

of the DDC-containing cells suggest that the 9 most posterior units that we observe represent the ganglia of abdominal segments 1-8 and presumably a small ganglion from the 9 + 10 segment. The next three more anterior units would then represent ganglia from the three thoracic segments (see Fig. 7).

At a more dorsal level of the ventral ganglion we observed two longitudinal rows consisting of segmentally organized weakly staining pairs of cells (Fig. 5E). These cells were more lateral than the ventral cells and projected processes toward the midline. Although weak staining precluded an exact determination of their number, it appeared that 11 pairs of cells were present in both rows corresponding to each segment except the most posterior abdominal segment (Fig. 7).

Segmentally organized cells were also observed in the most anterior portion of the ventral ganglion, beneath the brain hemispheres. These were more difficult to visualize because of interference from the DDC-containing cells in the brain hemispheres, but they appeared to consist of two segmental sets with a pair of cells in each lateral row and a single midline cell (summarized in Fig. 7). These may be part of the subesophageal ganglion, which is located at the most anterior end of the ventral ganglion (Bodenstein, 1950).

Just dorsal and anterior to these cells was a pair of very brightly staining cell clusters of about four cells each, located at the junction of the ventral ganglia and the brain hemispheres. Large DDC-containing processes emerged from these cells and projected anteriorly to the pharynx (Figs. 5C, 6C, 7, and 8). These match the description of the maxillary nerves, which innervate the pharynx (Bodenstein, 1950). Dorsal to the brightly staining cell clusters were bilaterally symmetrical sets of cells within the brain hemispheres. Typically, there were two pairs of anterior cells, two pairs of medial cells, and two triplets of posterior cells, all of which displayed processes connecting across the commissure (Figs. 6D and 7). Clusters of three to five cells which sent processes toward the midline were also commonly observed posteriorly and laterally (Fig. 7). Several other DDC-containing cells were less consistently observed at various dorsoventral levels within the brain hemispheres. As noted above, the thickness of the brain hemispheres made the visualization of all the DDC-containing cells difficult. Additional studies using other histological techniques will be required to obtain a precise description of all the DDC-containing cells.

In preparations in which the larval brain was not removed from the surrounding tissues, several large DDC-containing processes were observed to emerge from the brain (cf. Figs. 6A and 6B and summarized in Figs. 7 and 8). The distribution of these DDC-containing processes is very similar to the distribution of nerve fibers of the stomatogastric nervous system which has been described in other insects and which contains numerous adrenergic cells (Penzlin, 1985).

One process extended posteriorly along the esophagus to the proventriculus (Figs. 6A and 6B) where it joined with a larger bulbous-shaped structure. The position of this structure suggests that it represents the ventricular or esophageal ganglion (Poulson, 1950; Campos-Ortega and Hartenstein, 1986). Emerging from the putative

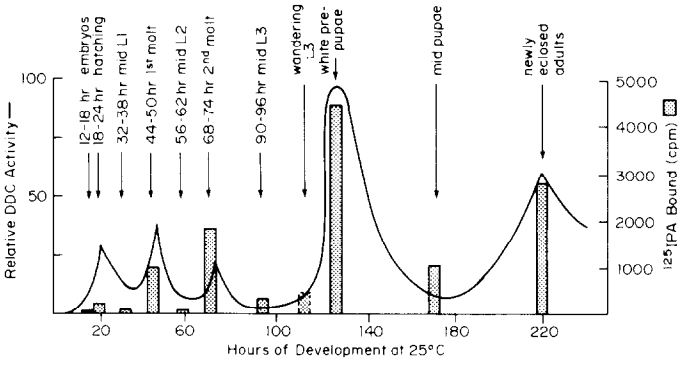

FIG. 4. Developmental expression of DDC. Protein extracts from wild-type *Drosophila* of the indicated developmental stages were separated by 9-15% SDS-PAGE and immunoblotted. The hatched bars indicate the amount of IPA bound to the 56-kDa band in each extract. The broken line around the wandering third instar bar indicates that those animals were collected on the basis of behavior rather than elapsed time. The solid line indicates the developmental profile of DDC activity relative to the peak at white prepupae (adapted from Marsh and Wright, 1980).

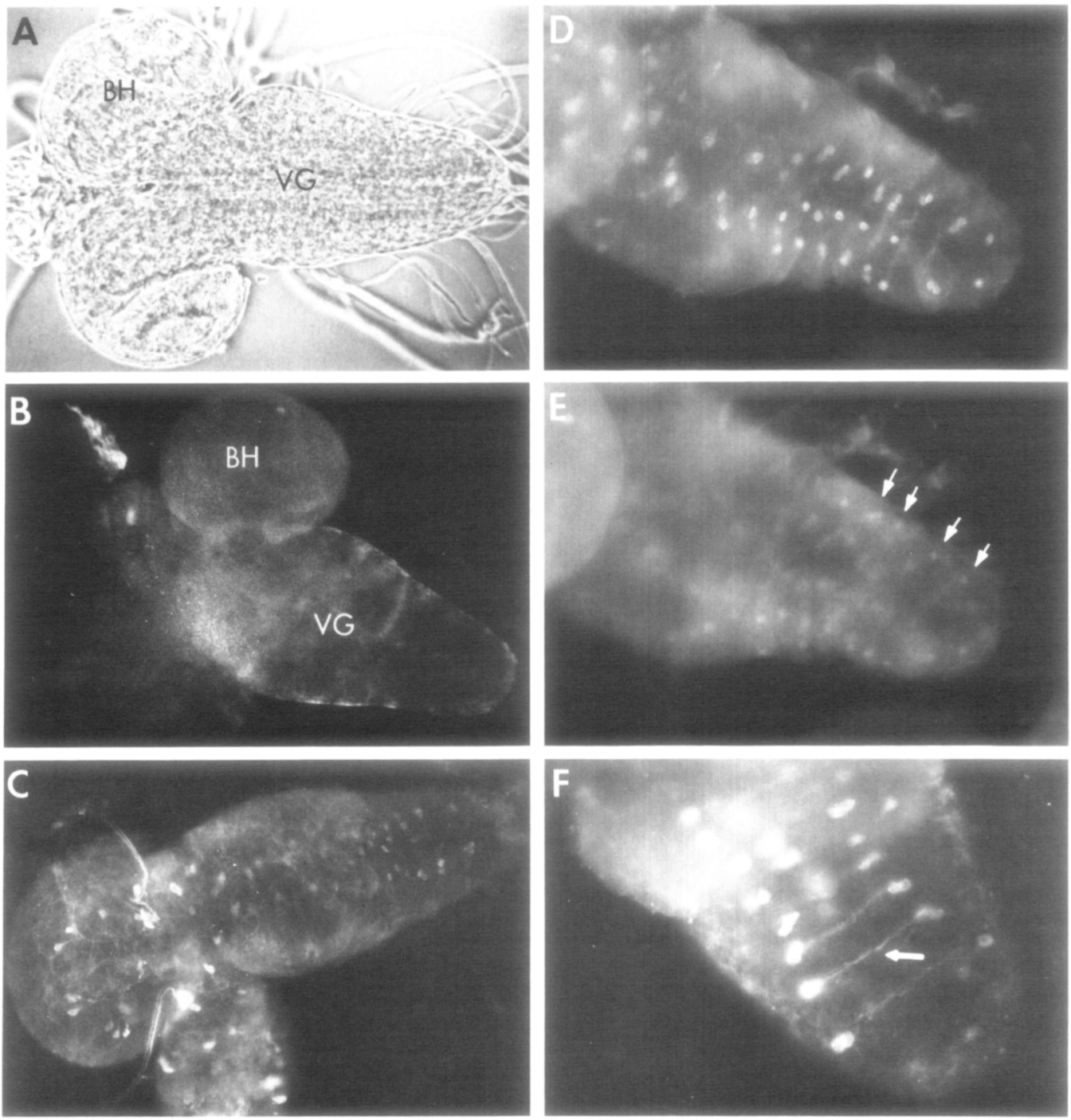

FIG. 5. DDC distribution in the wild-type larval brain. (A–C) Ventral views of third instar larval brain whole-mounts with the brain hemisphere (BH, anterior) to the left and the ventral ganglia (VG) to the right (magnification approx 160×). (A) Phase contrast image. (B) Immunofluorescence image of a similar preparation probed with preimmune rabbit serum and FITC-GAR secondary antibody. (C) Brain probed with immune rabbit serum. Note staining of cells in the ventral ganglia and the brain hemispheres, and large processes emerging from the brain hemispheres. (D) Higher magnification (ca. 250×) immunofluorescence image of a ventral ganglia whole-mount in a ventral plane of focus (brain hemispheres are dorsal). Note the segmental arrangement of stained cells corresponding to the abdominal and thoracic regions of the ventral ganglia. Not all DDC-containing cells can be visualized in this plane of focus. (E) Same preparation as in (D), but in a dorsal plane of focus. Note the segmental organization of more lateral, faintly staining cells (arrows). (F) Higher magnification (ca. 300×) immunofluorescence image of the ventral plane of focus in another ventral nerve cord. Note the processes projecting toward, and apparently joining at, the midline (arrow).

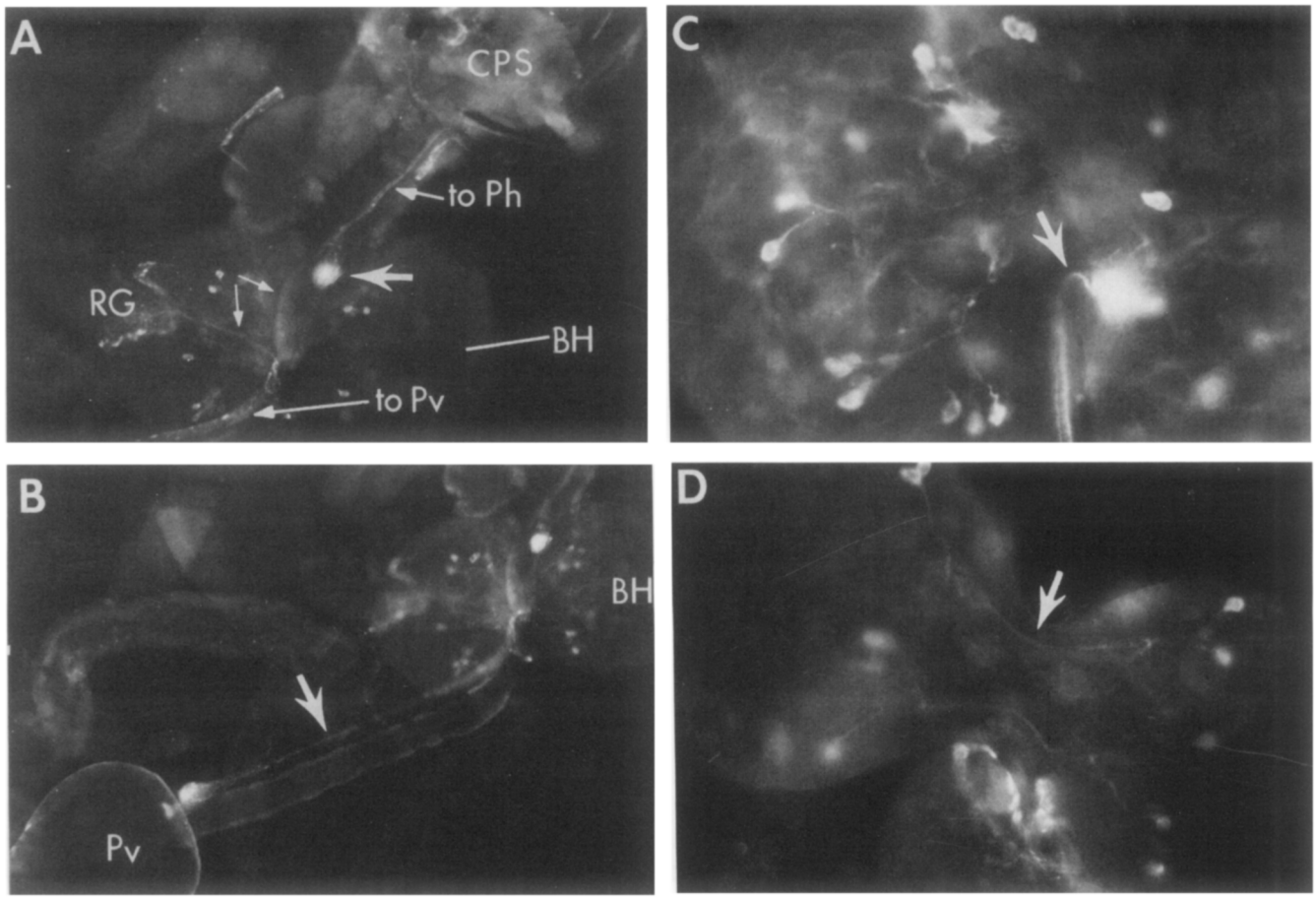

FIG. 6. DDC distribution in the larval nervous system. All panels show immunofluorescence images of tissue whole-mounts probed with immune rabbit serum and FITC-GAR secondary antibody. (A) Anterior structures dissected from a third instar larva. The brain has been left attached to the esophagus and other adjacent tissues. At midbottom of the photograph, the brain hemispheres are visible (one is labeled BH). In the upper right is a portion of the cephalopharyngeal skeleton (CPS). The sclerotized cuticle of this structure displays considerable autofluorescence (distinguishable by color from DDC fluorescence). Three DDC-containing processes emanate from between the brain hemispheres. One is directed posteriorly to the proventriculus (to Pv) and corresponds to the process labeled No. 3 in Figure 8. Two processes are directed anteriorly, the initial portions of which are indicated by small arrows. One of these extends adjacent to the esophagus (out of the plane of focus) and connects with a large, brightly staining structure, which we presume to be the frontal ganglion (large arrow), before proceeding toward the pharynx (to Ph). This process corresponds to process 2 in Fig. 8. The second anteriorly directed process connects with the ring gland (RG) where it bifurcates into an extensive lace-like network of processes. This process corresponds to process 1 in Fig. 8. During dissection, the ring gland was displaced from its normal midanterior position between the brain hemispheres (BH). The maxillary nerves were not visible in this preparation. Magnification approx 160×. (B) More posterior area of the same preparation as in (A). The posteriorly directed process (arrow) extends along the esophagus to the proventriculus (Pv) where it joins a large, brightly staining structure which may be the ventricular ganglion (magnification approx 100×). (C) Higher magnification (ca. 400×) image of brain hemispheres (ventral plane of focus) showing the origin of one of the maxillary nerves (arrow) from a cluster of brightly staining cells. Also note the staining of the surrounding cells and their processes. (D) Dorsal plane of focus from another brain showing the commissure between the brain hemispheres (arrow) which is traversed by DDC-containing processes (magnification approx 250×).

ventricular ganglion were several processes which extended posteriorly along the proventriculus to the anterior portion of the midgut and the gastric cecae (Figs. 9C and 9D). No staining was observed in similar preparations of the adult proventriculus and crop (not shown).

Three processes extended anteriorly to the pharynx, two of which were the putative maxillary nerves described above (Figs. 5C and 8). The third, central process appeared to connect with a large brightly staining structure before proceeding to the pharynx (Fig. 6A). This process and structure may correspond to the recurrent nerve and the frontal ganglion, both of which have been observed to contain dopamine in other insects (Penzlin, 1985; Brown and Nestler, 1985). In Drosophila, the recurrent nerve connects the frontal ganglion to the hypocerebral ganglion (located between the brain hemispheres, ventral to the ring gland) and also extends posteriorly from the hypocerebral ganglion to the ventricular ganglion (Poulson, 1950). Thus, the anteriorly di-

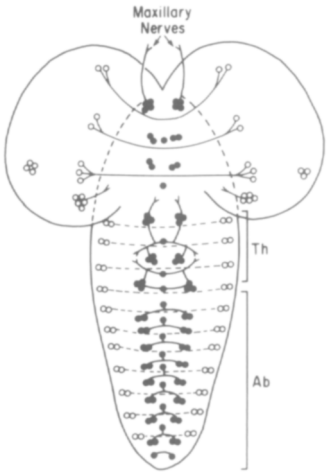

FIG. 7. Diagram of the larval brain showing the distribution of DDC-containing cells and processes. The drawing represents a composite of the most consistently observed staining patterns in over 20 larval brains. In the ventral ganglia, cells indicated in black are in a ventral plane of focus, while the cells indicated in white are in a more dorsal plane of focus. The dashed and solid lines indicate connections between the cells. The presumptive abdominal (Ab) and thoracic (Th) segments are indicated. Anterior is toward the top.

rected central process and the posteriorly directed process may both represent portions of the recurrent nerve. Both of these processes arose from a midcentral region of the brain, containing many brightly staining cells and processes. We did not observe a distinct structure which we could characterize as a hypocerebral ganglion nor were the exact points of origin distinct.

The ring gland, which is the endocrine organ in *Drosophila*, was richly endowed with a lace-like network of DDC-containing fibers with numerous varicosities. The origin of these fibers was usually indistinct, although one preparation displayed what appeared to be a fiber projecting from between the brain hemispheres to the ring gland (Fig. 6A). A pair of nerves which arises from the brain and innervates the ring gland has been described (King *et al.*, 1966).

DDC-containing cells could synthesize dopamine, serotonin, or both. The pattern of cells containing serotonin described by White and Valles (1985) and Valles and White (1986) is identical to that of a subset of the DDC-containing cells and processes which we observed. Thus it appears that some cells are synthesizing DDC but not serotonin. These include the unpaired median cells and the dorsal lateral rows of cells in the ventral ganglion. The DDC-containing maxillary nerves, putative frontal ganglion, and anterior recurrent nerve were also reported to contain serotonin. However, no serotonin staining which would correspond to the DDC-positive process projecting posteriorly to the proventriculus was reported (*ibid.*). Since the anti-serotonin antisera used above were raised in rabbits, we are currently raising DDC antibodies in rats to permit differential staining of DDC and serotonin in the same preparation.

We have attempted to stain whole embryos for DDC. No DDC was detected in 0- to 14-hr embyros, after which cuticle formation prevents antibody penetration. Serotonin has been detected in the CNS of the first instar larva (Valles and White, 1986), implying that DDC is present at this time. We have not examined larval stages prior to third instar.

Whole-mounts of the adult CNS also revealed DDC-containing cell bodies and processes in the brain and the thoracic ganglia (Fig. 10). Very faint staining was observed between the layers of the optic lobe which was not observed with preimmune serum (not shown). A detailed analysis of this complex pattern using frozen sections is now in progress.

DDC-containing processes were also associated with the adult oviduct (Fig. 11) and hindgut (not shown). In other insects, dopamine-containing nerves of the caudal nervous system arise in the abdominal portion of the ventral nerve cord and innervate the oviduct and hindgut (Brown and Nestler, 1985; Penzlin, 1985). Thus the posterior fibers we observed may be part of the caudal nervous system in *Drosophila*.

Frozen sections through the cuticle and epidermis of pupariating larvae revealed that epidermal cells contained DDC while the overlying cuticle failed to stain for DDC (Fig. 12). DDC staining was not observed in similar sections of third instar larvae when DDC activity was at a basal level (not shown). Antiserum-specific staining was not observed in any other tissues examined. These included imaginal discs, salivary glands, and fat body (Fig. 13) as well as muscle, the wall of the gut (see Fig. 9), and hemocytes from pupariating larvae (not shown).

DISCUSSION

Production and characterization of antisera. The advantages of using the pUC and pIC plasmids for expression of Drosophila antigens are ease of construction, the ability to express large defined fragments, and the re-

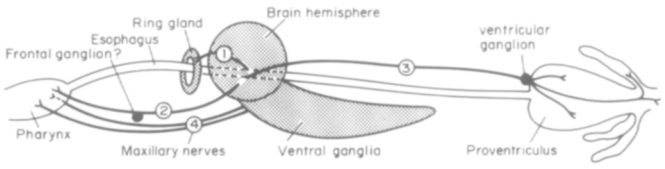

FIG. 8. Diagram of major DDC-containing processes in larvae. The larval brain is shaded, and the processes emanating from the brain hemispheres are in black, labeled 1–4. Processes 1–3 and the presumptive frontal ganglion are similar to structures containing catecholamine and indolylalkamines in the stomatogastric nervous system of other insects (cf. Brown and Nestler, 1985). Fibers 2 and 3 may represent different parts of the recurrent nerve.

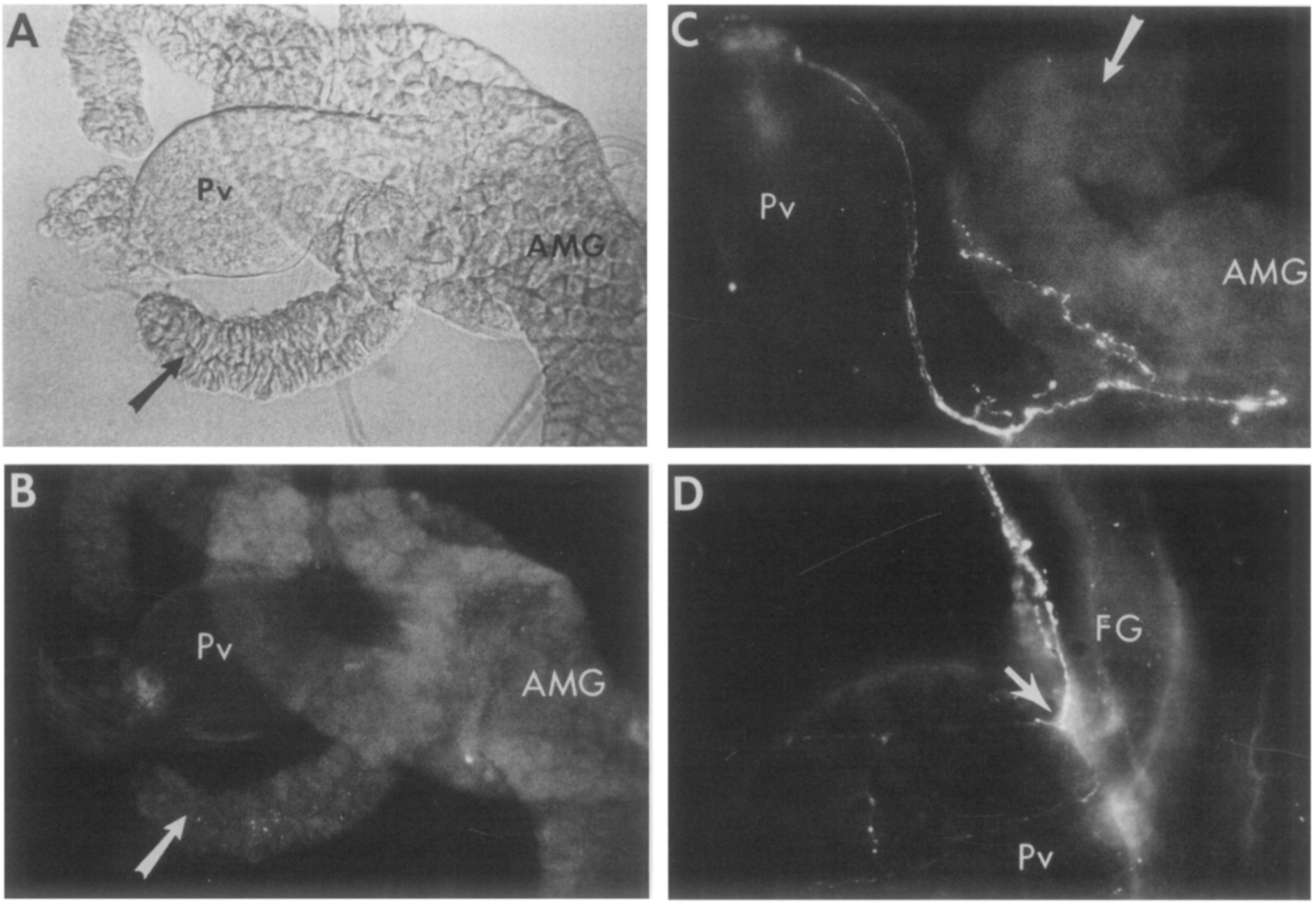

FIG. 9. DDC distribution in the larval proventriculus. (A) Phase contrast image of a wild-type proventriculus (Pv) whole-mount. The foregut was detached from the anterior (left) of the Pv during dissection. Posteriorly the Pv is attached to the anterior midgut (AMG), with its protruding arms of gastric cecae (arrow, magnification approx 160×). (B) Immunofluorescence image of the same preparation as in (A), probed with preimmune rabbit serum and FITC-GAR secondary antibody. (C) Preparation similar to that in (B), probed with anti-DDC serum. Note brightly staining processes along the surface of the proventriculus, the anterior midgut, and the gastric cecae (arrow). Also note the absence of specific staining of the gut. (D) Higher magnification image (ca. 250×) of the putative ventricular ganglion at the junction of the foregut (FG) and proventriculus (Pv). Note the continuity of the process from the brain with the processes on the proventriculus (arrow).

covery of a "fusion" protein which is complexed with as little as 5 and no more than 70 amino acids from the β-galactosidase gene. Substantial quantities of expressed protein can be obtained, presumably due to the high copy number of the plasmids (Messing, 1983; Marsh *et al.*, 1984). The large quantity of DDC peptide recovered may explain in part why our antiserum reacts specifically with the plasmid-derived peptide and very little if at all with comigrating bacterial proteins. The antigen recovered in this manner is denatured and the serum obtained reacts well with both protein blots and histological preparations.

Our observation that DDC from pupariating larvae focuses as a single 56-kDa species in two-dimensional gels contrasts with an earlier report that DDC exhibited a range of p*I* values (Hirsh and Davidson, 1981). The reason for this discrepancy is unclear. We interpret our results to indicate that the majority of DDC in this stage is not subject to extensive post-translational modification. The *Ddc* gene produces a unique primary transcript which by alternative splicing produces two species of mRNA. This results in two DDC protein isoforms which differ in their amino-terminal 35 amino acids (Beall and Hirsh, 1984; Geitz and Hodgetts, 1985; Eveleth *et al.*, 1986). Two peaks of DDC activity have been observed on ion-exchange columns, one of which is unique to the CNS (Morgan *et al.*, 1987). Although both isoforms should be present in pupariating larvae, we detected only one molecular form of DDC. This may be because the epidermis contributes over 90% of the total DDC at this stage. Alternatively, the denatured peptides may be indistinguishable on the two-dimensional gels used.

DDC protein levels are developmentally regulated. DDC activity in whole animal homogenates exhibits five peaks

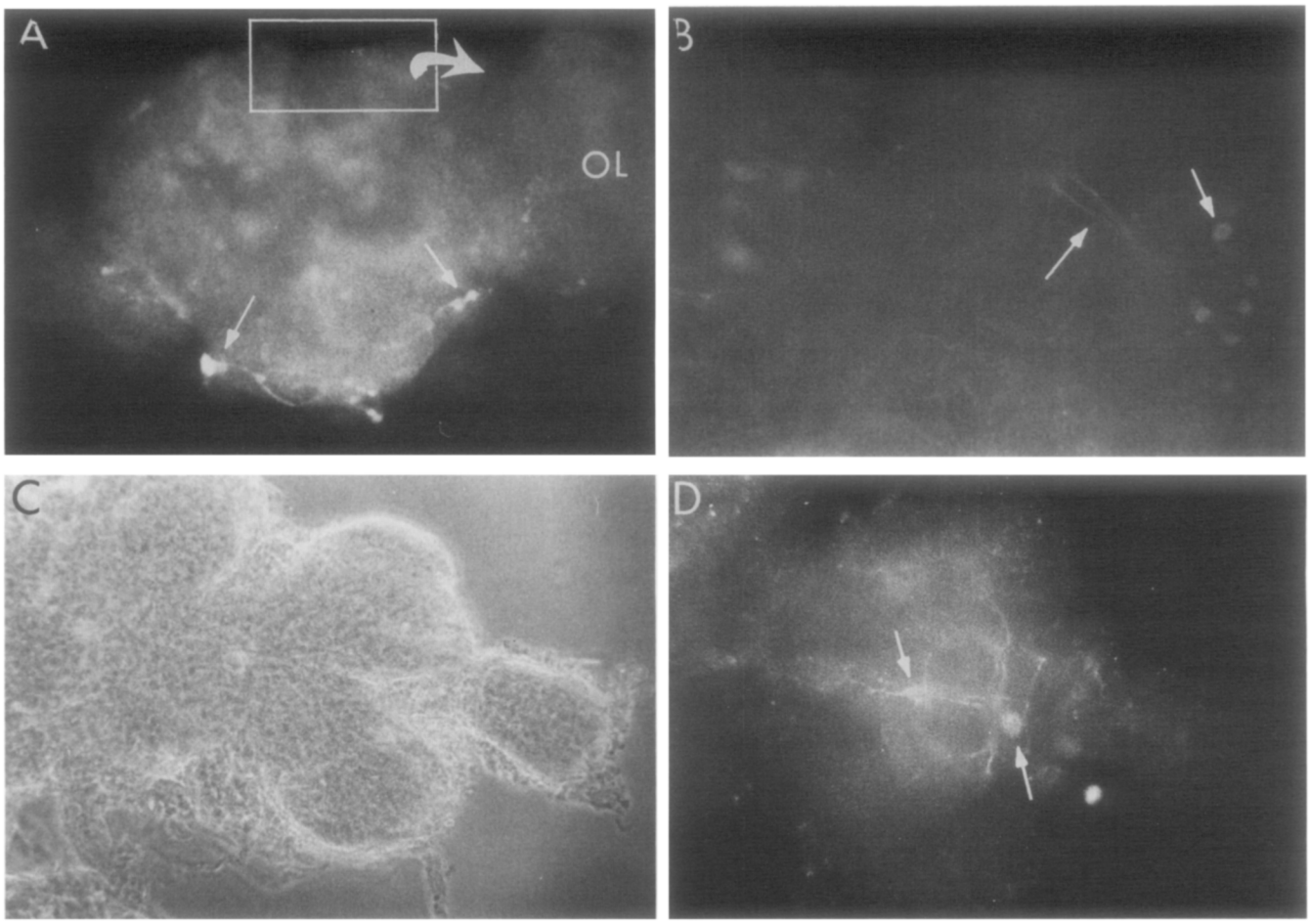

FIG. 10. DDC distribution in the adult CNS. (A, B, and D) Immunofluorescence images of adult CNS whole-mounts probed with anti-DDC serum and FITC-GAR secondary antibody. (A) An intact adult brain with anterior at the top and optic lobes (OL) left and right. The thoracic ganglia have been removed from this preparation. Note the bilateral pair of brightly staining cells and processes on the posterior margin of the brain (arrows; magnification approx 125×). Numerous other specifically staining cells and processes were visualized at other planes of focus. The relatively high background staining is due to the thickness of the preparation. The enclosed area represents the approximate region of a different brain displayed at higher magnification in (B). (B) Anterior portion of a brain showing a complex pattern of cells and processes (arrows; magnification approx 250×). (C) Phase contrast image of the posterior portion of the adult thoracic ganglia (anterior to the left; magnification 120×). (D) Same preparation as in (C), showing staining of midline cells with longitudinal and lateral processes within the third thoracic ganglion (arrows; magnification approx 160×).

during development (Kraminsky *et al.*, 1980; Marsh and Wright, 1980). We find that the amount of 56-kDa DDC CRM parallels the level of DDC activity. The loss of 56-kDa CRM during periods of declining DDC activity indicates that the DDC peptide is rapidly degraded and not held in an inactivated state during the several periods of basal DDC activity. Thus, in addition to transcriptional regulation, post-translational regulation in the form of a selective degradation system for DDC must exist. It is interesting to note that the larval epidermal cells do not divide after embryogenesis but grow by cell enlargement and endomitosis; thus, modulation of DDC activity occurs in the absence of cell division.

DDC distribution in the Drosophila nervous system. Anatomically, the insect nervous system can be divided into three interconnected parts: (1) the CNS, (2) the visceral or sympathetic nervous system, and (3) the peripheral nervous system (Penzlin, 1985). The visceral nervous system consists of three relatively independent subsystems, the stomatogastric system, the ventral visceral system, and the caudal system. The stomatogastric system functions in control of feeding and crop emptying and innervates the endocrine glands (Klemm, 1979). Catecholamines and indolalkylamines (e.g., serotonin) have been detected in the stomatogastric system of many insects (e.g., locust, cockroach, silverfish). The ventral visceral nervous system consists of unpaired median nerves in the ventral ganglion and is thought to innervate the tracheal system and some somatic muscles (Penzlin, 1985). The caudal nervous system arises from the compound terminal abdominal ganglion and supplies the reproductive organs and the proctodeum.

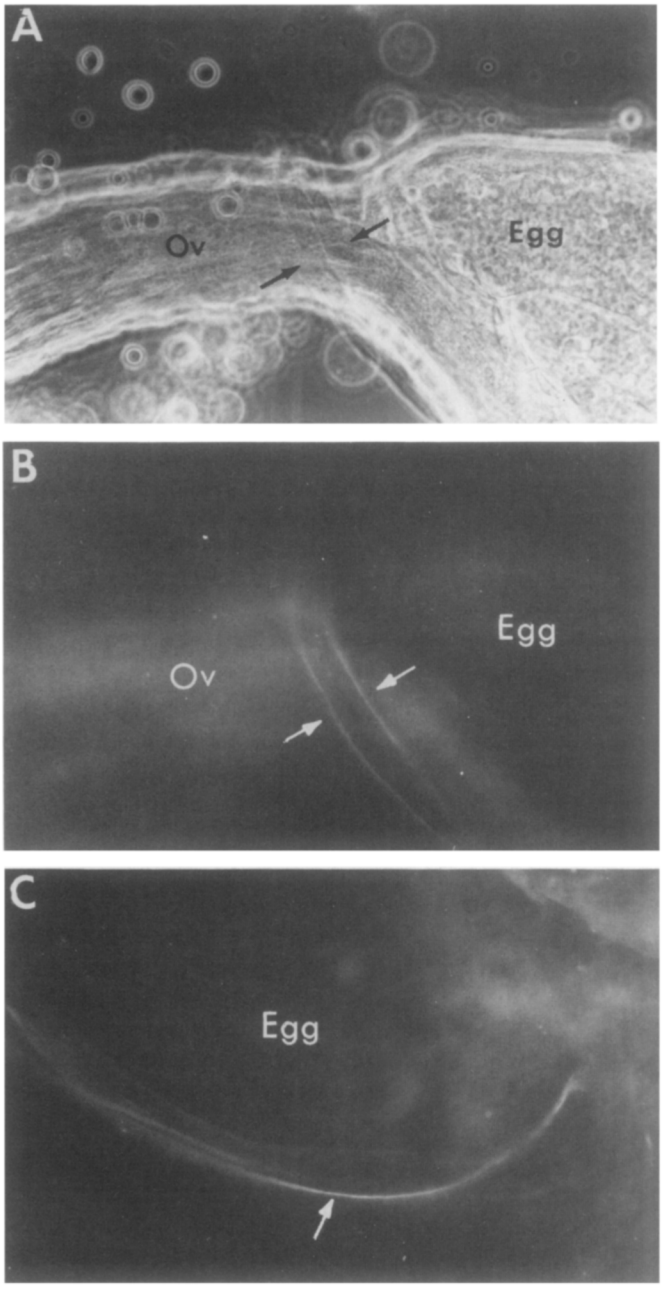

FIG. 11. DDC-containing processes in the oviduct. (A) Phase contrast image of a portion of the common oviduct (Ov) and the anterior portion of the mature egg it contains. A pair of large tubular structures can be seen looping over the oviduct (arrows; magnification approx 240×). (B) Immunofluorescence image of the same preparation as in (A), probed with anti-DDC serum and FITC-GAR secondary antibody. Note staining of a portion of each tubular structure (arrows). (C) More posterior area of the same preparation as in (B). The processes separate as they extend along the oviduct so that only one remains in the focal plane.

Although the description of the visceral nervous system is based primarily on larger hemimetabolous insects, the parallels with our observations of DDC-containing cells in both the larval and adult nervous systems of Drosophila are striking. For example, in the larva, DDC-containing neurons innervate the pharynx, ring gland, proventriculus, and foregut (a pattern similar to that described for the stomatogastric system in other insects). The staining of unpaired ventral midline cells in the larval ventral ganglion is also noteworthy although it is not known whether these cells represent part of the ventral visceral system. In the adult, DDC-positive processes are associated with the oviduct and hindgut (a pattern similar to that described for the caudal system). Interestingly, we do not find DDC-containing neurons associated with the proventriculus or crop of the adult, indicating changes in the distribution of DDC during metamorphosis. The staining patterns we observed in the stomatogastric and caudal nerves suggest that a subset of fibers within large nerve tracts contains DDC (cf. Figs. 6C, 11A, and 11B). Thus, the DDC-containing cells may be involved in only some of the functions of these nerves and these functions may change during development. The physiological role of the DDC-containing neurons in these elements of the Drosophila nervous system is unknown. Nevertheless, it appears that catecholamines and/or indolalkylamines are widely distributed in the Drosophila visceral nervous system.

White and Valles (1985) describe a laterally symmetrical set of serotonin-positive cells in the ventral ganglion and larval brain of Drosophila. A subset of the DDC-positive cells appears to be identical to the serotonin-containing cells. This is consistent with the proposed role of DDC in decarboxylation of 5-hydroxytryptophan to serotonin (Livingstone and Tempel, 1983). The antibody labeling methods used here cannot distinguish whether these cells utilize serotonin exclusively or synthesize dopamine as well. In contrast, the cells which contain DDC but not serotonin appear to utilize dopamine exclusively.

DDC-containing cells and processes are also present in the adult CNS. Although the function of monoamine neurotransmitters in the Drosophila brain is not known, DDC has been shown to be required in adults during associative learning (Tempel *et al.*, 1984). We observed very faint staining between neuropil layers in the compound eye. This may reflect a role for monoamine neurotransmitters in vision as has been suggested in other insects (Pitman, 1985; Brown and Nestler, 1985).

Drosophila DDC is axonally transported. It is possible that DDC is restricted to the neuronal cell body and that dopamine or serotonin is transported to the nerve terminals. Alternatively, DDC itself could be transported. DDC is known to be transported in vertebrate axons (Grafstein and Forman, 1980). The staining of neuronal processes which we observed indicates that Drosophila DDC is also axonally transported.

DDC is associated with epidermal cells during pupar-

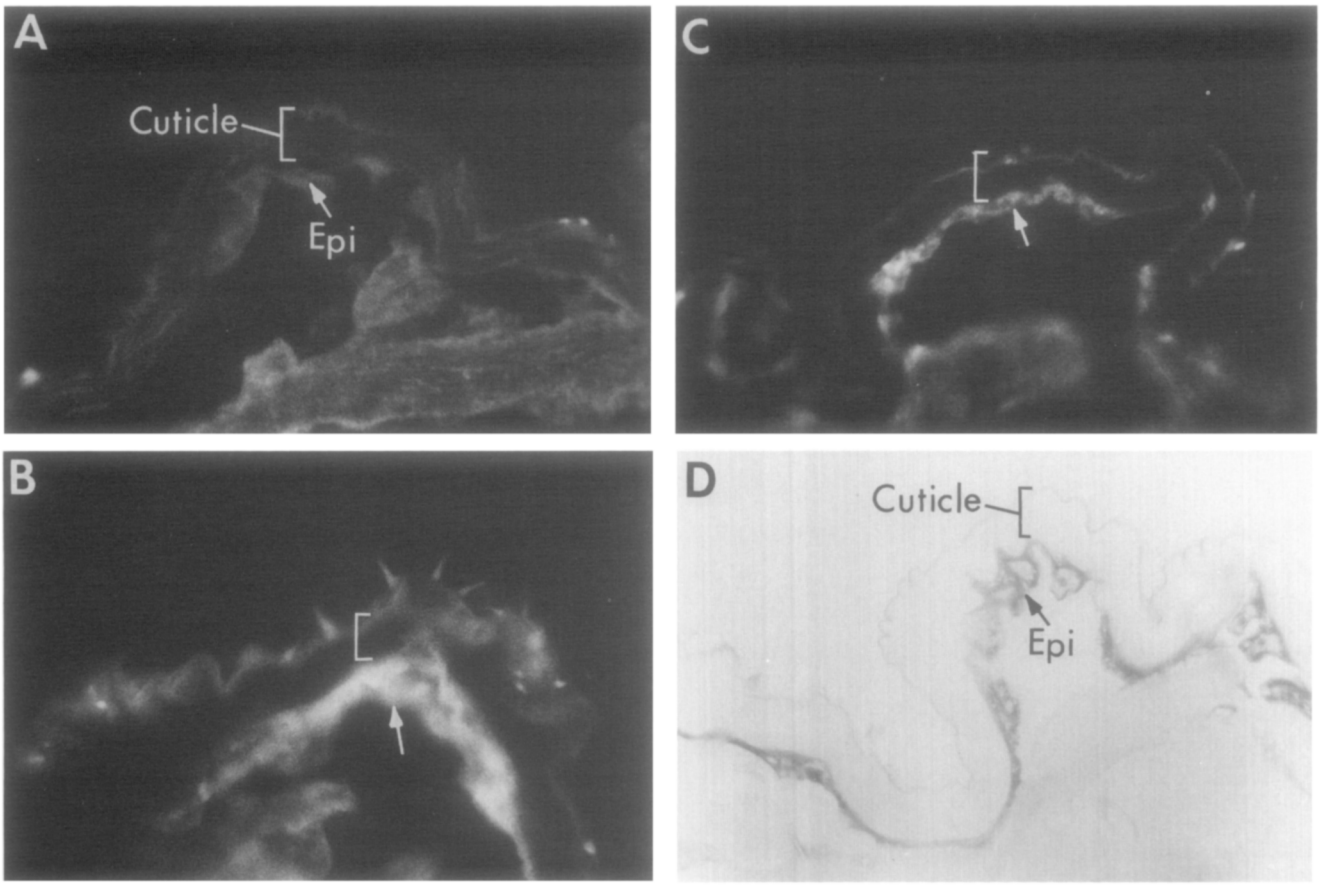

FIG. 12. DDC in the pupariating larval epidermis. (A) A one-segment-wide portion of a frozen section of a wild-type WPP, probed with preimmune rabbit serum and FITC-GAR secondary antibody. The cuticle is bracketed and the underlying epidermis (Epi) is indicated by an arrow (A–D; magnification approx 100×). (B) Preparation similar to that in (A), probed with anti-DDC serum. Note staining of cells beneath the cuticle and the autofluorescence of cuticle hooks. (C) Preparation similar to that in (B), also showing staining of cells beneath the cuticle. (D) Stained, plastic section through tissue similar to that shown in (A–C), showing the outlines and nuclei of the epidermal cells (Epi) underlying the cuticle (bracketed).

iation. Some of the enzymes implicated in cuticle hardening are located in the cuticle (e.g., polyphenol oxidase, Wigglesworth, 1972). We see strong DDC staining in the epidermal cells of pupariating larvae and no staining of adjacent cuticular structures nor any staining in the intermolt cuticle. Thus DDC is found within the epidermal cells and does not appear to be exported to the cuticle. This is consistent with a model in which catecholamines are synthesized in the epidermis and then transferred to the cuticle where they participate in crosslinking cuticular proteins (Wigglesworth, 1972). However, we have not yet examined the epidermis during larval molts and the degree of crosslinking in the larval cuticle is controversial. Although the cuticle has been described as being "incapable of growth" (Wigglesworth, 1972), larval growth is continuous (Williams, 1980) and there is increasing evidence that growth of the larval cuticle is also continuous (Kaznowski *et al.*, 1985). Thus the outermost cuticular layers, which are formed in association with the peaks of DDC activity, must be flexible enough to allow for growth. Cuticle added during periods of basal DDC activity (i.e., intermolt) may be assembled differently than the outer layers. This is consistent with claims that the outer cuticular layers of some insects are hardened and not the inner layers (Wigglesworth, 1972).

DDC is absent from other cell types. DDC activity has been measured in homogenates of the proventriculus where it has been hypothesized to participate in synthesis of the peritrophic membrane (Wright and Wright, 1978; Rizki, 1956). We do not detect any DDC in the proventriculus itself but we do find strong staining in the putative esophageal ganglion and in nerve processes surrounding the proventriculus. We suggest that the activity associated with the proventriculus is due to the codissection of these neurons and that DDC activity is not required for maturation of the peritrophic membrane.

In some insects, the eggshell is tanned and DDC activity is present in the ovary (e.g., mosquito, Schlaeger

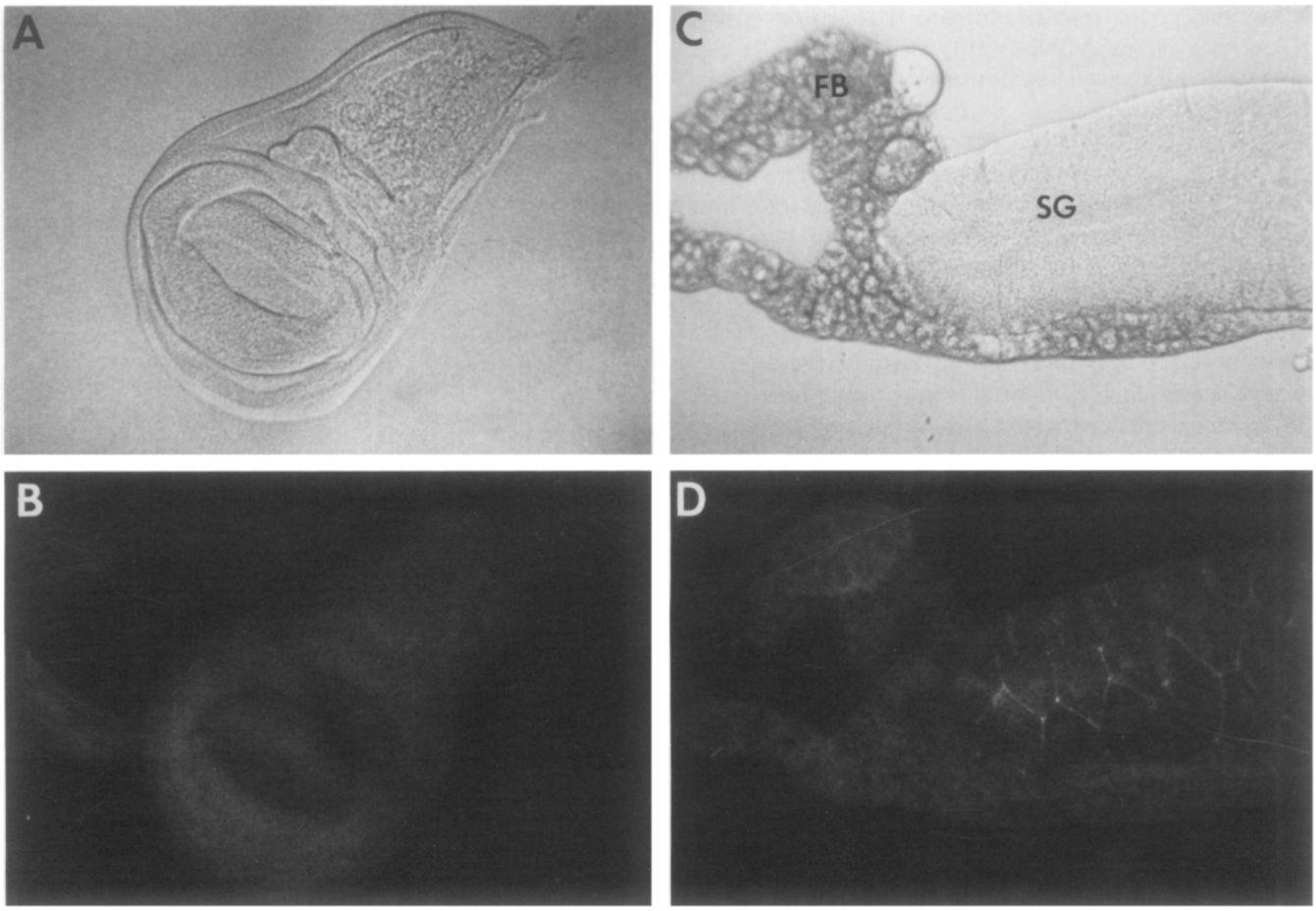

FIG. 13. Absence of DDC in other tissues. (A) Phase contrast image of a wing disc whole-mount (magnification approx 100×). (B) Same preparation as in (A), probed with anti-DDC serum and FITC-GAR secondary antibody. Note the lack of staining. (C) Phase contrast image of a portion of a salivary gland (SG) whole-mount with associated fat body (FB; magnification approx, 100×). (D) Same preparation as in (C), probed with anti-DDC serum and FITC-GAR secondary antibody. The nonspecific staining between salivary gland cells was also seen with preimmune serum (not shown).

and Fuchs, 1974). Although the Drosophila egg shell is not tanned, it has also been reported that DDC activity is associated with the ovary (Wright *et al.*, 1981). We find no evidence for any DDC within the ovary, including the egg chambers. We suggest that the DDC activity associated with the ovary is the result of DDC-positive neural processes running along the oviduct and associated structures (see above). Thus, it seems unlikely that maternally contributed DDC can account for the ability of *Ddc* mutants to develop to late larval stages (Valles and White, 1986). The function of the neurons associated with the oviduct is not known since Ddc^{ts2}, which effectively abolishes DDC activity, is fertile at restrictive temperatures (Wright *et al.*, 1981). The female sterility originally associated with the Ddc^{ts1} chromosome (*ibid.*) has now been separated from the *Ddc* locus, (Wright, 1987) and no other female sterile alleles of the *Ddc* locus have been recovered.

We have stained a variety of other tissues for DDC and not found any detectable staining in the gut, salivary glands, fat body, malphigian tubules, or discs. We have also tested for DDC-positive blood cells, since it has been proposed that hemocytes are the target organ for ecdysone induction of *Ddc* during sclerotization (Gillot, 1980). We have not observed DDC-positive blood cells in pupariating larvae although the epidermal cells clearly contain DDC. This would appear to rule out hemocytes as the site of DDC activity during tanning of the puparium. However, the insect defense mechanism involves blood cells encapsulating foreign substances with melanin. It is possible that DDC-positive blood cells will be found in animals responding to foreign material.

During metamorphosis, the larval brain tissue (which is diploid) is retained and resculptured (Bodenstein, 1950) to give rise to the central nervous system of the adult. The observation that a very small number of neurons specific for each segment are positive for DDC in both the larval and the adult nervous systems provides a convenient cell marker to follow the respecification of the nervous system during morphogenesis.

In summary, our results indicate that expression of DDC activity is restricted to only two tissues, the epidermis and the nervous system (both central and visceral). Clearly, however, the regulation in these two tissues differs (e.g., DDC is present in the brain during the third instar but absent from the epidermis). The developmental peaks of DDC expression detected in whole animal homogenates occur in response to ecdysone (Marsh and Wright, 1980; Kraminsky *et al.*, 1980). However, these peaks represent primarily epidermal DDC activity. Our observations suggest that DDC expression in nervous tissue may be relatively insensitive to ecdysone levels and developmental stages. Scholnick *et al.* (1986) have recently reported closely linked regulatory elements which are necessary for *Ddc* expression in the central nervous system. What factors serve to distinguish DDC-producing neurons from their immediate nonproducing neighbors remains to be established.

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