

Biochemistry and Ultrastructure of Serotonergic Nerve Endings in the Lobster: Serotonin and Octopamine are Contained in Different Nerve Endings

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SUMMARY

In this article we report that the distribution of serotonin in the lobster nervous system parallels the distribution of octopamine and that the same tissues that contain endogenous serotonin can synthesize it from tryptophan. Octopamine and serotonin are highly concentrated in a neurosecretory region of the second thoracic roots in association with a group of neurosecretory cells. The roots possess separate high-affinity uptake systems for both serotonin and tryptophan. Radioactive serotonin, accumulated in tissues during incubations with either tritiated serotonin or tritiated tryptophan, can be released, in a calcium-dependent manner, by depolarization with potassium. A detailed morphological examination of the second thoracic roots shows four distinct categories of nerve endings in the vicinity of the neurosecretory cells. Octopamine is synthesized in one of these types of endings and serotonin in another. The high-affinity uptake systems for serotonin and tryptophan are found only in association with the endings that make serotonin. These endings and all the biochemical parameters of serotonin metabolism in the roots are selectively destroyed by previous injection of animals with the neurotoxin 5,7-dihydroxytryptamine.

INTRODUCTION

There is growing evidence that in both vertebrates and invertebrates monoamines act as neurohormonal modulators of neural circuits. In the vertebrate central nervous system, monoamines are associated with nerve-ending-like varicosities, the majority of which lack the morphological specializations usually associated with classical synapses (Ajika and Hökfelt, 1973; Descarries, Beaudet, and Watkins, 1975; Descarries, Watkins, and Lapierre, 1977; Hökfelt, 1968). In the lobster, the monoamines octopamine and serotonin are both present in the central nervous system and share a parallel distribution throughout an extensive peripheral neurosecretory system. This neurosecretory system is located close to the bifurcation of each of the second roots of each thoracic ganglion and in the pericardial organ at the distal end of each second root. These two regions are characterized by a very dense superficial plexus of nerve-ending-like varicosities.

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A cluster of two to twelve neutral red-staining cell bodies is located at the bifurcation, and these cells contribute endings to the plexus of varicosities both at the bifurcation and in the pericardial organs (Evans, Talamo, and Kravitz, 1975). Both serotonin and octopamine are found in high concentration at the bifurcation and in the pericardial organ. The pericardial organ is a well-known crustacean neurosecretory organ (Alexandrowicz, 1953; Alexandrowicz and Carlisle, 1953) which contains octopamine, dopamine, serotonin, and at least one neuroactive peptide (Welsh and Moorhead, 1960; Cooke, 1964; Terwillinger, 1967; Cooke and Goldstone, 1970; Evans et al., 1976b).

In previous articles we showed that the content and synthesis of octopamine correlated closely with the number of neurosecretory cells in a root and noted that serotonin was found in the same locations as octopamine (Evans et al., 1976b). In this article we report in greater detail on the parallel distribution of serotonin and octopamine but show that they are synthesized by different types of nerve endings. This result, which suggests that serotonin and octopamine could be released independently, is relevant to observations that these two amines can have different or even opposite effects on target tissues (Battelle and Kravitz, 1978; Livingstone, Harris-Warrick, and Kravitz, 1980).

Evidence from other systems that serotonin nerve endings themselves possess a high-affinity serotonin uptake is convincing but indirect. In this article we show that serotonin synthesis and high-affinity serotonin uptake are localized to the same morphological type of nerve ending. Tryptophan uptake specifically into serotonin nerve endings has not been previously characterized. Using the serotonergic neurotoxin 5,7-dihydroxytryptamine, we determined that the serotonergic nerve endings have a high-affinity uptake system for tryptophan and that this uptake is rate limiting for serotonin synthesis.

MATERIALS AND METHODS

Tissues

The second thoracic roots (segments $T_1 - T_3$) of the ventral nerve cord of lobsters (*Homarus americanus*, 0.5 kg) were dissected in lobster saline according to the procedures described previously (Evans, Kravitz, and Talamo, 1976a, Evans et al., 1976b). Normal lobster saline had the following composition: 462 mM NaCl; 16 mM KCl; 26 mM CaCl₂; 8 mM MgCl₂; 11 mM glucose; 10 mM Tris; 10 mM maleic acid, and sufficient NaOH to adjust the pH to 7.4.

For ultrastructural studies a dissection technique was used that allowed minimal handling of the thoracic roots before fixation (Evans et al., 1976a). Abdominal portions of the lobster were removed and the animal was cut in a sagittal plane through the insertions of the legs on one side so that the ventral nerve cord remained completely enclosed in its muscular and skeletal investments. The nerve cord was then exposed by chipping away bits of the external skeleton and carefully removing attached muscle and connective tissue. The second thoracic roots on the near side of the dissection were cut by this procedure, but the roots on the far side of the cord were untouched. Fixative was poured on the preparation as soon as the roots from the first to the third segments were exposed.

Octopamine assay

Octopamine was measured by a modification of the enzyme-isotopic method of Molinoff, Landsberg, and Axelrod (1969) as described by Evans et al. (1976b). Briefly, a tritiated methyl group is added to the octopamine and the radioactive synephrine is extracted and counted.

Serotonin assays

Serotonin was initially assayed by a slight modification of the enzyme-isotopic method of Saavedra, Brownstein, and Axelrod (1973). In this assay ³H-melatonin is formed from serotonin and subsequently chromatographed on precoated Polygram Sil G thin-layer plates (Brinkmann Instruments) using the solvent system toluene-acetic acid-ethyl acetate-water (80:40:20:5). The melatonin region

of the chromatogram, identified by its fluorescence under an ultraviolet light (254 nm peak), was cut out, extracted into 0.3 ml ethanol, and counted in a liquid scintillation counter. In more recent experiments serotonin was assayed by high-performance liquid chromatography with electrochemical detection. To extract serotonin, tissues were homogenized in acidified butanol as described by Ponzio and Jonsson (1979) except that the volume of acidified butanol was varied so that at least 100 μ l acidified butanol was added for each 5 mg of tissue. This avoided the formation of a separate aqueous phase, which occurs when more than one part saline is mixed with 20 parts of acidified butanol, and into which serotonin strongly partitions. The serotonin recovery was 95–100%. We used small ground-glass homogenizers to disrupt tissues instead of an ultrasonic cell disrupter as described by Ponzio and Jonsson. The samples were analyzed using a standard liquid chromatographic procedure. We used a 250 \times 3.2 mm i.d. stainless steel column packed with Vydac strong cation exchange resin (Altex) for separation and an LC-2A electrochemical detector with a graphite paste electrode (Bioanalytical Systems). The elution solvent was 0.1M sodium acetate/citrate buffer, pH 4.6, and the flow rate was 0.3 ml/min. Both types of assays give similar results.

Incubations with radioactive precursors

Isolated second thoracic roots or pericardial organs were incubated at 12°C with shaking in saline containing ^3H -labeled precursors in small (0.5–1 ml) wells in Sylgard-coated petri dishes. The radioactive compounds 5(1,2- ^3H -N)hydroxytryptamine creatinine sulfate, 27.8 Ci/mmol; (^3H -G) L-tryptophan, 6 Ci/mole; S-(^3H -methyl)-adenosyl-L-methionine, 11 Ci/mmol; (2- ^3H -H(N)) (side chain label)-DL-octopamine, 9 Ci/mmol; (^3H -G) tyramine, 11 Ci/mmol; ^{14}C -inulin 2 mCi/g and ^3H -water, 0.25 Ci/g were all purchased from New England Nuclear Corp; (^3H -G)tryptamine, 1 Ci/mmol and (^3H -G)-DL-5-hydroxytryptophan, 3.2 Ci/mmol were purchased from Amersham & Searle. When octopamine synthesis was being measured, 1 mg/ml L-ascorbic acid, adjusted to pH 7.2, was added to the incubation medium.

At the end of each incubation the tissue was homogenized in 20–50 μ l formate/acetate buffer (0.47M formic acid, 1.4M acetic acid, pH 1.9), centrifuged, and an aliquot of the supernatant fluid was taken to determine the total amount of radioactive material extracted. A second aliquot was analyzed by high-voltage electrophoresis (6000 V at pH 1.9 as described in Hildebrand et al., 1971). After electrophoresis, the electropherograms were cut into 1-cm sections and each section was placed in a scintillation vial containing 0.3 ml 0.01M HCl for extraction for 1 hr. Four ml of Aqualos (New England Nuclear Corp.) were added, and the radioactive counts in each sample were measured in a liquid scintillation counter.

The identities of serotonin and 5-hydroxytryptophan (5HTP) synthesized from ^3H -L-tryptophan were confirmed by chromatographing the electropherograms in a second dimension. Two different solvent systems were used for chromatography: (1) butanol–acetic acid–water (120:30:50), and (2) toluene–acetic acid–ethylacetate–water (80:40:20:5). In each case all of the radioactive material comigrating with serotonin or 5HTP on electrophoresis comigrated also with the same compound in the second dimension.

In the uptake experiments with ^3H -serotonin, ^3H -tryptophan and ^3H -octopamine, ^{14}C -inulin was included in the incubation media. Double-label counting techniques were used to measure the uptake of ^{14}C -inulin and thereby to evaluate the amount of ^3H in the extracellular space of the tissue. We determined the intracellular space of the second thoracic roots as a function of the extracellular space by incubating six pairs of roots in ^3H -water and ^{14}C -inulin simultaneously and using double-label counting techniques to determine the amount of ^3H and ^{14}C in the tissue. The ratio of ^3H to ^{14}C reached a stable plateau by 5 min of incubation. We thus determined that the intracellular space of the tissues (the water space) was 40% ($\pm 12\%$) of the extracellular space (the inulin space). This value was used to estimate the intracellular space of roots when the extracellular space had been determined with ^{14}C -inulin (as in Fig. 2).

Depolarization-induced release of radioactive compounds. Tissues were incubated for 2–4 hr with radioactive compounds as above but in a smaller volume (100 μ l). Precursor was washed out by removing 95 μ l of the saline covering the tissue and replacing it with fresh saline. Next, a series of solutions with elevated K^+ (100 mM extra K^+ replacing 100 mM Na^+) with or without Ca^{2+} present were added in the same manner. From each of the wash solutions, 10- μ l aliquots were withdrawn and counted to determine their total radioactivity. To the rest of each sample, 20 volumes of ice cold 0.25% formic acid in acetone were added. Tubes were mixed and left at 4°C in the dark for 30 min to precipitate salts. The salts were removed by centrifugation and the supernatant fluid was dried under a stream of nitrogen. The residue was redissolved in 100 μ l acetate/formate buffer, and the radioactive compounds were separated by high-voltage electrophoresis. The amount of each compound was quantified as described above.

Anatomical methods. The standard fixation procedure of the partially dissected nerve cord (see

above) was carried out *in situ* at 22°C for 2 hr in a mixture of 3% glutaraldehyde, 20% sucrose (w/v), 0.5% CaCl₂, 1% potassium dichromate, 0.1% picric acid, and 0.1M sodium cacodylate buffer, pH 7.4 (Coggeshall, personal communication). The dissection of the roots was then completed in distilled water and the roots were postfixed in a solution of 1% osmium tetroxide in 0.1M cacodylate buffer pH 7.4. The roots were then dehydrated in a graded series of alcohols and finally embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate (Venable and Coggeshall, 1965) and examined with a Philips 400 electron microscope. Other fixatives that were used included (1) 3% glutaraldehyde-H₂O₂ solution buffered to pH 7.4 with 0.1M sodium cacodylate; (2) 2% formaldehyde, 2.5% glutaraldehyde in 0.07M sodium cacodylate buffer at pH 7.4; (3) 2% acrolein in 0.1M sodium dichromate buffer at pH 7.4; (4) primary fixation in 1% glutaraldehyde and 0.4% formaldehyde in 0.1M chromate/dichromate buffer, pH 7.2, followed by 2% osmium in the same buffer at pH 6.0.

Autoradiographic procedures. Tissues were incubated with radioactive precursors as described above. At the end of the incubation period, the roots were rinsed briefly and fixed for 6 hr in a solution of either 4% glutaraldehyde or 4% formaldehyde in 0.2M sodium cacodylate buffer pH 7.4 containing 462 mM NaCl, 26 mM CaCl₂, 16 mM KCl, and 8 mM MgCl₂. The tissues were rinsed in 0.1M sodium cacodylate buffer, pH 7.4, and then postfixed for 1 hr in cold (4°C) 1% osmium tetroxide in 0.1M sodium cacodylate buffer. The roots were subsequently rinsed for 3 min at 4°C in 0.5M maleate buffer at pH 5.2, stained *en bloc* for 1 hr at 4°C with 1% uranyl acetate in 0.5M maleate buffer at pH 6.0, and finally rinsed for an additional 30 min in maleate buffer at pH 5.2 (Karnovsky, 1967). After dehydration in a graded series of alcohols, the specimens were embedded in an Epon 812 mixture.

For light microscopic autoradiography, 2- μ m plastic sections were mounted on glass slides and dipped in a 1:1 dilution of Ilford K-5 emulsion (Polysciences, Inc., Fort Washington, NJ). The slides were exposed for two weeks at 4°C, developed for 4 min in D-19, fixed in 30% sodium thiosulfate, dried, and subsequently stained with toluidine blue.

For electron microscopic autoradiography, thin sections were transferred by a platinum loop to celloidin-coated slides and dipped in a 1:3 dilution of Ilford L-4 emulsion with a semiautomatic coating instrument (Kopriwa, 1973). The autoradiographs were exposed for three to five weeks at 4°C and developed for 1 min at 20°C in a 1:15 dilution of either D-19 or Amidol. They were then fixed in 24% sodium thiosulfate, rinsed in distilled water, transferred to copper 200-mesh grids, and post-stained with uranyl acetate and lead citrate.

5,7-Dihydroxytryptamine injections. Animals were injected in the ventral hemolymph sinus that surrounds the nerve cord on two consecutive days with 8 mg 5,7-dihydroxytryptamine (Sigma) dissolved in 0.5 ml saline containing 1 mg/ml L-ascorbate. 5,6-Dihydroxytryptamine was used in early experiments but was found to be less effective than the 5,7 isomer.

Special reagents. NSD-1055 was a gift of the Roche Research Laboratories. Parachlorophenylalanine was purchased from Sigma.

RESULTS

Biochemical Studies

Serotonin localization and synthesis in lobster tissues

A comparison of the distribution of endogenous serotonin and octopamine in the lobster nervous system is shown in Table 1. With few exceptions, there was more octopamine than serotonin in the tissues. For our biochemical and morphological studies, we used the second thoracic roots and pericardial organs because of all the tissues we measured they contain the highest concentrations of serotonin and octopamine. (The brain and subesophageal ganglion contain more total serotonin, but they are much larger tissues—the brain and subesophageal ganglion each weigh about 40–50 mg, while the second thoracic roots weigh 0.2–0.5 mg and the pericardial organs each weigh 1–2 mg.)

The tissues that contained serotonin could also synthesize it from added tritium-labeled L-tryptophan. The radioactive product was identified as serotonin by high-voltage electrophoresis and paper chromatography (Table 1; also see Methods section). In 10⁻⁶M L-tryptophan, the rate of serotonin synthesis in

TABLE 1
Octopamine Content, Serotonin Content, and Serotonin Synthesis in Segments of the Lobster Nervous System^a

Tissue	Endogenous Octopamine (pmole), n.d. less than 0.5 pmole	Endogenous serotonin control animals (pmole) n.d. less than 0.5 pmole	Endogenous serotonin 5,7-DHT-treated animals (pmole) n.d. less than 0.5 pmole	Maximum serotonin synthesis (pmole/hr.) n.d. less than 0.1 pmole/hr
Brain	22 ± 3 (5)	45.6 ± 4.5 (6)	39.0 ± 7.7 (6)	0.41 ± 0.2 (3)
Circumesophageal connective ganglion (unpaired)	3.3 ± 1.2 (5)	n.d. (3)		n.d. (3)
Subesophageal ganglion	8 ± 4.0 (3)	15.6 ± 4.1 (5)	7.5 ± 2.1 (5)	1.38 ± 0.25 (5)
Thoracic ganglia (T ₁ , T ₂ and T ₃ , averaged)	10.3 ± 1.9 (18)	5.65 ± 0.35 (3)	3.15 ± 1.15 (3)	n.d. (3)
Thoracic connective ^b	7.7 ± 1.2 (11)	n.d (3)		n.d. (3)
Second thoracic roots				
Subesophageal A	17.1 ± 3 (9)	1.25 ± 0.24 (9)		0.286 ± 0.07(3)
Subesophageal B	93.6 ± 15.7 (9)	4.1 ± 0.7 (9)		0.495 ± 0.03 (3)
Thoracic 1	80.0 ± 12 (9)	5.15 ± 0.5 (9)	0.5 ± 0.33 (8)	1.57 ± 0.28 (6)
Thoracic 2	92.2 ± 17.7 (9)	6.7 ± 0.62 (9)	0.62 ± 0.45 (8)	2.37 ± 0.45 (5)
Thoracic 3	81.1 ± 17 (9)	5.84 ± 0.8 (9)	0.68 ± 0.52 (8)	2.05 ± 0.5 (4)
Abdominal ganglia (average for first three ganglia)	5.5 ± 0.9 (3)	n.d (5)		n.d. (3)
Pericardial organ T ₂	164 ± 23 (9)	15.3 ± 2.4 (9)	2.6 ± 2.0 (11)	5.86 ± 0.88 (5)
Sinus gland		2.9 ± 0.4 (3)	1.15 ± 0.85 (3)	
Skeletal muscle (50 mg abdominal extensor muscle)	n.d.	n.d.		n.d.

^a All values are mean ± SEM. The number in parentheses denotes the number of observations.

^b Average value for unpaired connectives between each of the first three thoracic ganglia.

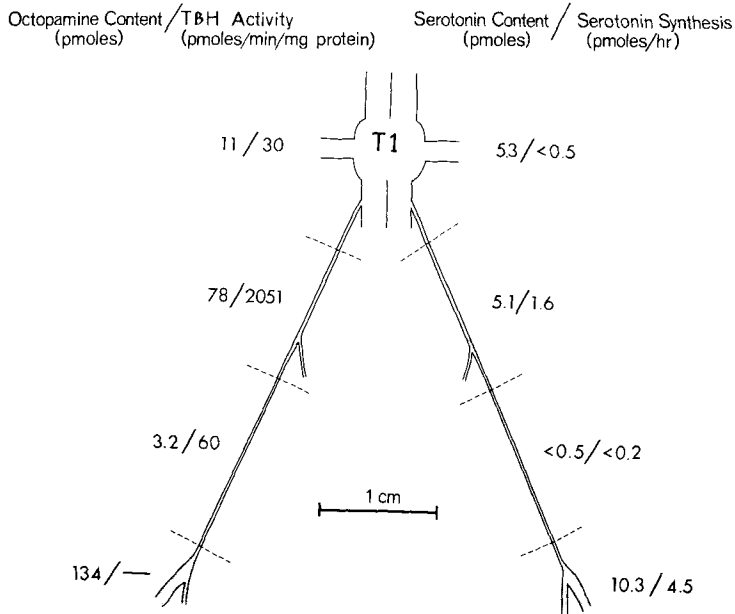


Fig. 1. Localization of endogenous octopamine and serotonin and their sites of synthesis along second thoracic roots. Thoracic ganglia and their second roots were divided as shown by the dotted lines. The stippling indicates the regions of neurosecretory endings at the bifurcation of the second thoracic root and in the pericardial organ at the end of the root. The values for octopamine content and tyramine β -hydroxylase activity (left half of figure) are from Evans et al. (1976b). Serotonin content and serotonin synthesis from ^3H -tryptophan (right half of figure) were determined as described in the Methods section.

thoracic roots and pericardial organs was maximal and the accumulation of serotonin was linear with time for 1 hr. A curve relating the rate of serotonin synthesis to the extracellular tryptophan concentration is shown in Figure 3 and explained in greater detail below.

Serotonin and octopamine distribution along thoracic roots

In Figure 1 a diagram of a first thoracic ganglion with its second roots is shown. The left half of Figure 1 illustrates some typical data on the distribution of octopamine and tyramine β -hydroxylase (Evans et al., 1976b). Endogenous serotonin and its sites of synthesis from tryptophan show an identical distribution along the roots (right half of Fig. 1). Therefore, both amines in approximately constant proportion are found in the same locations along second thoracic roots.

5,7-Dihydroxytryptamine (5,7-DHT) destruction of serotonin synthesis and storage sites

In an attempt to destroy serotonin-containing structures, lobsters were injected with 5,7-DHT into the general circulation as described in the Methods section (Baumgarten et al., 1974; Björklund, Baumgarten, and Nobin, 1974). Two to three weeks later the endogenous serotonin content of second thoracic roots was reduced 90% (see Table 1) and the capacity of the roots to synthesize serotonin from tryptophan was reduced 85%. In the pericardial organs the endogenous

serotonin content was reduced 83% and the rate of synthesis of serotonin from ^3H -tryptophan was reduced 73% (Table 1). 5,7-DHT was less effective in reducing the serotonin content of other tissues. The endogenous serotonin decreased 44% in the thoracic ganglia, 52% in the subesophageal ganglion, and only 14% in the brain (Table 1). The octopamine content and its rate of synthesis from precursor compounds (tyrosine or tyramine) were not changed by 5,7-DHT treatment. This result strongly suggests that serotonin and octopamine are localized in different compartments. We will show later that these compartments are different types of nerve endings.

Pathway of serotonin synthesis

In vertebrates, serotonin is synthesized by two consecutive reactions: the hydroxylation of tryptophan to form 5-hydroxytryptophan (5HTP) followed by decarboxylation of 5HTP to serotonin (for review see Grahame-Smith, 1967). The following experiments suggest that the same sequence of steps is involved in serotonin synthesis in lobster tissues. Second thoracic roots were incubated with different precursor compounds and the radioactive products formed were identified and quantitated using high-voltage electrophoresis (see Methods section). With incubation times of up to 4 hr in ^3H -L-tryptophan ($2 \times 10^{-6}\text{M}$), the only radioactive compounds extracted from tissues were tryptophan and serotonin (Table 2). There was no measurable accumulation of 5HTP or tryptamine, nor were any of the known lobster metabolites of serotonin or tryptamine found (see Kennedy, 1978). When an aromatic amino acid decarboxylase inhibitor (NSD-1055, 1mg/ml) was included in the incubation mixture, roots did not form any measurable radioactive serotonin, but they now accumulated ^3H -5HTP (Table 2). It can be seen that the total accumulated radioactivity is almost the same. This suggests that NSD-1055 does not limit the uptake of radioactivity by the tissue. ^3H -5HTP can serve as a precursor to serotonin, but with the decarboxylase inhibitor present, only ^3H -5HTP was found in tissues. Finally, ^3H -tryptamine was not converted to serotonin by these tissues (Table 2). Similar results were obtained with both the cell body and pericardial organ regions of roots.

High-affinity tryptophan uptake

Since serotonin can be synthesized from tryptophan without any detectable accumulation of 5HTP (Table 2), it appears that decarboxylation of 5HTP is not a rate-limiting step for serotonin synthesis. With the following experiments we explored further the nature of the rate-limiting step.

The roots were incubated with ^3H -labeled tryptophan at a low concentration [$(2 \times 10^{-8}\text{M})$ – (10^{-7}M)] for up to 2 hr. The total radioactivity taken up by tissues was measured, and the proportions of ^3H -tryptophan, ^3H -5HTP, and ^3H -serotonin were quantified. Four categories of experiments were performed: (1) control incubations, (2) incubations with NSD-1055 present, (3) incubations of tissues from animals pretreated with 5,7-DHT, and (4) incubations in the presence of NSD-1055 of tissues from animals pretreated with 5,7-DHT. The results are shown in Figure 2. At each time point the total radioactivity accumulated by the tissue was the same with or without NSD-1055 present. This is similar to the result shown in Table 2 and suggests that the uptake of radioactive tryptophan is not inhibited by NSD-1055, although serotonin synthesis is completely

TABLE 2
Radioactive Compounds Formed by Second Thoracic Roots under Various Incubation Conditions^a

Incubation conditions	# Observations	pmole of tritiated compound			
		Tryptophan	5HTP	Serotonin	
$2 \times 10^{-6}M$ ³ H-L-tryptophan 30 min	(4)	3.1 ± 1.0	n.d.	1.14 ± 0.13	n.d.
$2 \times 10^{-6}M$ ³ H-L-tryptophan + 1 mg/ml NSD-1055 30 min	(3)	4.01 ± 0.96	0.66 ± 0.11	n.d.	n.d.
$10^{-5}M$ ³ H-DL-5HTP 2 hr	(3)	n.d.	2.3 ± 0.42	0.28 ± 0.02	n.d.
$10^{-5}M$ ³ H-DL-5HTP + 1 mg/ml NSD-1055 2 hr	(3)	n.d.	3.07 ± 0.75	n.d.	n.d.
$10^{-5}M$ ³ H-tryptamine 4 hr	(3)	n.d.	n.d.	n.d.	3.5 ± 1.2

^a Incubations were for the times indicated. Products were identified by separation on high-voltage electrophoresis. Each value is mean ± SEM. Limit of detection was 0.1 pmole. In these experiments the amount of precursor in the extracellular space was not subtracted from the total.

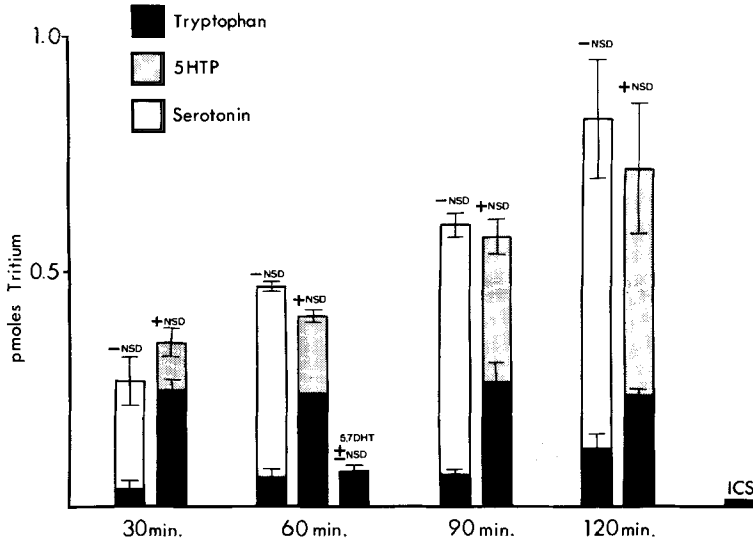


Fig. 2. ³H-Compounds found in second thoracic roots after incubation in $5 \times 10^{-8}M$ ³H-L-tryptophan. Roots were incubated for various times in $5 \times 10^{-8}M$ ³H-L-tryptophan in the presence or absence of the decarboxylase inhibitor NSD-1055 (+ or - NSD). The amount of ³H-tryptophan present in the extracellular space has been subtracted (see Methods section). The amount of ³H-tryptophan that would be present if the ³H-tryptophan in the medium had equilibrated with the intracellular space is shown by the bar on the far right (ICS). All experiments were done using roots from normal animals except for the right bar at 60 min, which shows the tryptophan uptake into roots from animals pretreated with the neurotoxin 5,7-DHT. As indicated, the uptake is the same in these roots in the presence and absence of NSD-1055. Error bars indicate SEM.

blocked. At 1 hr, there is approximately 20 times more radioactive material in the tissue than would be found by equilibration of the medium with the intracellular space of the tissue (bar on far right of Fig. 2; see Methods section for determination of intracellular space). This suggests an active uptake of tryptophan by the preparation but does not rule out the possibility that radioactivity is "trapped" in the tissue by tryptophan being converted to serotonin. Indeed, in the untreated preparation, 80-90% of the radioactivity at 1 hr is in the form of serotonin ($5 \times 10^{-8}M$ tryptophan incubation). The studies with NSD-1055 make the trapping possibility unlikely, however, since tissues incubated in the presence of the drug contain ten times as much tryptophan as would be due to passive equilibration of the intracellular space with the medium. That this large increase in accumulated tryptophan is not due to a stimulation by NSD-1055 of tryptophan uptake by nonserotonergic tissues is indicated by the following experiment (Fig. 2, 60 min). In tissues from animals pretreated with 5,7-DHT, there was a dramatic reduction in the total amount of radioactive material taken up. On the other hand, the amount of radioactive tryptophan in these tissues was the same as in control preparations; tryptophan was the only radioactive substance found, and NSD-1055 did not increase the amount of radioactive tryptophan accumulated. Taken together, the results shown in Figure 2 suggest that a special compartment in which serotonin and 5HTP can be formed has been removed by treatment with 5,7-DHT and that essentially all the radioactive tryptophan taken up into this compartment is converted to serotonin under control incubation conditions (see also Fig. 3).

In an attempt to increase the tissue accumulation of free tryptophan without producing an accumulation of 5HTP, we tried to inhibit the enzyme tryptophan

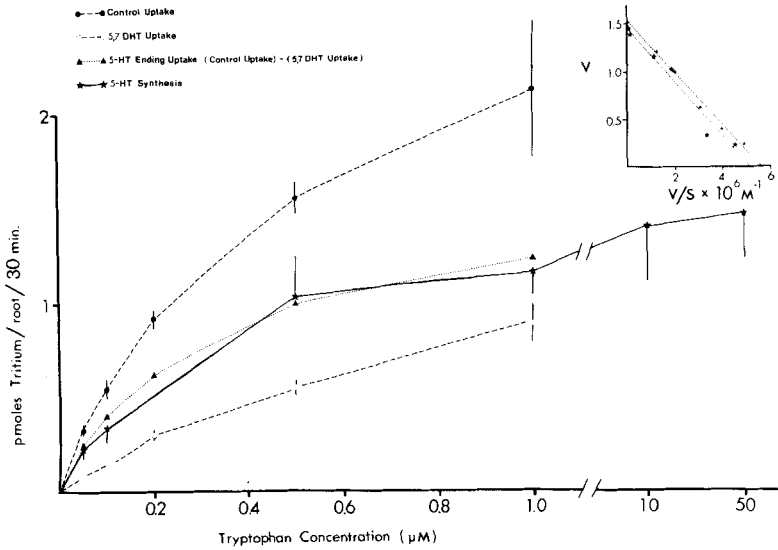


Fig. 3. A comparison of tryptophan uptake and serotonin (5-HT) synthesis as a function of tryptophan concentration. Extracellular tryptophan has been subtracted from all points. Solid circles show total uptake of tritium into roots from normal animals and open circles show uptake into roots from 5,7-DHT-treated animals. The triangles show the difference between these two graphs and represent the uptake specifically into the serotonergic endings. The stars show serotonin synthesis as a function of tryptophan uptake. The inset shows Eadie-Hofstee plots of the serotonin nerve ending uptake data and the serotonin synthesis data. Error bars indicate SEM.

hydroxylase with the drug parachlorophenylalanine (PCPA), (Koe and Weissman, 1966). When lobster second roots were incubated with PCPA, however, we saw a marked reduction of uptake of radioactive tryptophan. PCPA reduced accumulation below the levels found in 5,7-DHT-treated animals. Although we cannot tell from these studies whether PCPA is an inhibitor of lobster tryptophan hydroxylase, it does appear to be a good inhibitor of tryptophan uptake (see Knapp and Mandell, 1972a).

Kinetic studies of tryptophan uptake

To examine whether the uptake or the hydroxylation of tryptophan is the apparent rate-limiting step for synthesis of serotonin, we compared the kinetic parameters of tryptophan uptake and serotonin synthesis as functions of extracellular tryptophan concentration. First, we measured the difference between the total uptake of radioactivity into control roots and the uptake into roots from animals pretreated with 5,7-DHT. This difference should represent the uptake of radioactivity specifically into the serotonin compartment. Second, the amount of serotonin formed at different tryptophan concentrations was measured. These results are shown in Figure 3. The uppermost curve (filled circles) is the total tissue uptake of radioactivity. The lowest curve is the uptake into roots from 5,7-DHT-treated animals. The two virtually identical curves in the middle are the difference curve (total uptake minus uptake into roots from 5,7-DHT-treated animals; filled triangles) and the data for the synthesis of serotonin (stars). Thus, essentially all of the radioactivity taken up into the serotonin compartment is converted to serotonin under these experimental conditions,

indicating that the rate-limiting step in serotonin synthesis is tryptophan uptake into the serotonin ending rather than tryptophan hydroxylation. At ten and 100 times higher concentrations of tryptophan, there is no further synthesis of serotonin despite the fact that the total tissue accumulation of radioactivity is very large. The inset in Figure 3 is the data from this experiment graphed by the method of Eadie and Hofstee. Serotonin synthesis has an apparent K_m of $0.31 \mu M$ for tryptophan with an apparent V_{max} of 1.56 pmole/root/30 min. Tryptophan uptake into the compartment destroyed by 5,7-DHT treatment has an apparent K_m of $0.28 \mu M$ for tryptophan and an apparent V_{max} of 1.54 pmole/root/30 min. When the data for tryptophan uptake into tissues from 5,7-DHT-treated animals are graphed in the same manner (not shown), the initial portion of the graph forms a straight line, indicating an apparent K_m for tryptophan of approximately $10^{-6} M$. The rest of the graph does not form a straight line, suggesting that there is more than one additional component of uptake with lower apparent affinities for tryptophan.

The uptake of 3H -tryptophan from a medium containing a low concentration of 3H -tryptophan ($5 \times 10^{-8} M$) was not affected by a 200-fold excess of unlabeled serotonin and was not dependent on the presence of Na^+ in the bathing medium (Table 3, upper three rows).

Uptake of serotonin

When second thoracic roots or their pericardial organs were incubated with 3H -serotonin ($10^{-8} M$), tissues accumulated radioactive serotonin. After 1 hr, tissue/medium concentration ratios of 20:1 to 30:1 were seen. A curve relating the external concentration of serotonin to uptake over the range of 10^{-8} – $10^{-3} M$ revealed two components. One was nonsaturable up to a serotonin concentration of $10^{-3} M$ (not shown) and the second saturated at close to $10^{-6} M$ (Fig. 4). The inset graph shows the analysis of these data plotted by the method of Eadie and Hofstee. The apparent K_m for serotonin is $0.66 \mu M$ and the V_{max} is 6.0 pmole/root/30 min. In tissues from animals pretreated with 5,7 DHT, the saturable component is missing (Fig. 4). Replacing all of the Na^+ in the bathing medium by Li^+ dramatically reduced the uptake by roots from normal animals but did

TABLE 3
Sodium Dependence of Serotonin and Tryptophan Uptakes and Lack of Competition between the Two Uptakes^a

Incubation conditions	% Control
$5 \times 10^{-8} M$ 3H -L-tryptophan (control)	100.0
$5 \times 10^{-8} M$ 3H -L-tryptophan + $10^{-5} M$ cold serotonin	101.0
$5 \times 10^{-8} M$ 3H -L-tryptophan zero Na^+ (462 mM Li^+)	108.3
$5 \times 10^{-8} M$ 3H -serotonin (control)	100.0
$5 \times 10^{-8} M$ 3H -serotonin + $10^{-5} M$ cold tryptophan	79.9
$5 \times 10^{-8} M$ 3H -serotonin zero Na^+ (462 mM Li^+)	10.1

^a Each value represents the average of three experiments in which the intracellular tritium in a single second thoracic root was measured after incubation in tritiated serotonin for 15 min or in tritiated tryptophan for 30 min under the indicated conditions.

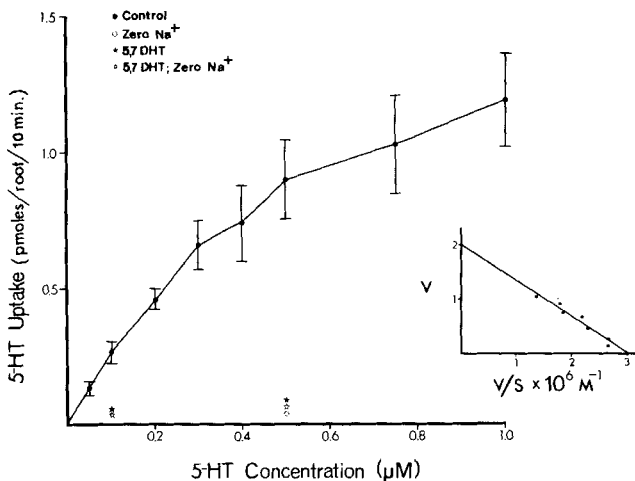


Fig. 4. Serotonin uptake as a function of serotonin concentration. Second thoracic roots were incubated in ^3H -serotonin and ^{14}C -inulin. The serotonin in the extracellular space was determined by the amount of ^{14}C present and was subtracted. A nonsaturable component of uptake was determined by extrapolating the uptake at 10^{-3}M to zero and was subtracted from the data graphed here. The inset is an Eadie-Hofstee plot of these data. The open circles show the uptake (with the nonsaturable component subtracted) into control roots when all of the Na^+ in the incubation medium was replaced by Li^+ . Similarly, the solid and open stars show the uptake into roots from 5,7-DHT-treated animals in normal and zero Na^+ .

not affect the uptake by roots from animals pretreated with 5,7 DHT (Fig. 4; Table 3, lower). As above, where serotonin did not interfere with tryptophan uptake, in these experiments tryptophan had relatively little effect on serotonin uptake (Table 3, lower half).

Release of serotonin

When roots were incubated in low concentrations (10^{-8}M) of serotonin for 2 hr and then washed until the efflux of radioactive material reached a steady level, much of the radioactive material accumulated by tissues could be released by changing the normal bathing medium to one containing high K^+ (100 mM higher K^+ and 100 mM lower Na^+ [Fig. 5(a)]. The release of radioactive material was reversibly blocked by replacing the Ca^{2+} in the bathing medium by Mg^{2+} [Fig. 5(a)]. Roots that have been incubated in ^3H -tryptophan (10^{-6}M for 2–4 hr) also can release radioactive substances on depolarization with K^+ , and this release is also Ca^{2+} dependent [Fig. 5(b)]. The radioactive materials released from tissues incubated with ^3H -tryptophan were analyzed by high-voltage electrophoresis. It was found that K^+ treatment caused an approximately 50-fold increase in efflux of radioactive serotonin, while the efflux of radioactive tryptophan was unchanged [Fig. 5(b)]. Roots incubated in ^3H -serotonin released 1.05 ± 0.19 pmole of ^3H -serotonin during a 5-min depolarization with K^+ . Roots incubated in ^3H -tryptophan released 1.6 ± 0.3 pmole of ^3H -serotonin from a total of 3.0 ± 0.55 pmole of serotonin estimated to have been present in the tissue at the end of the incubation. Finally, roots that were incubated with ^3H -tryptophan in the presence of the decarboxylase inhibitor NSD-1055 accumulated ^3H -5HTP but did not release any radioactive materials above the background release upon depolarization with K^+ [Fig. 5(c)].

Morphological Studies

Rationale

In following morphological studies, we have attempted to localize the sites of synthesis of serotonin and octopamine in the second thoracic roots. We took advantage of our observations that under specific conditions the radioactive precursors tryptophan and tyramine taken up by tissues could be essentially completely converted to their respective radioactive products serotonin (see Fig. 3) and octopamine (see Evans et al., 1976a). Aldehyde fixatives were used to immobilize octopamine and serotonin in tissues (Livingstone, unpublished; see also Fischman and Gershon, 1964) in order to visualize the sites of synthesis of the amines by electron microscopic autoradiography.

General morphology

The bifurcation region of the second thoracic roots consists of two distinct regions: (1) a thin outer sheath containing the cell bodies and axonal processes of the thoracic root neurons, and (2) an inner core consisting of through axons. The through axons are wrapped by neuroglial cells and connective tissue fibers and hemolymph percolates through these elements in narrow thin-walled sinuses. The cell bodies of the root neurons vary in their number and location along a root. They are usually found within 1–2 mm of the central ganglia and often form tight clusters close to the site where the roots divide into medial and lateral branches. When individual root cells are injected with horseradish peroxidase or procion yellow, an extensive branching pattern of associated processes and endings is clearly seen (Evans et al., 1975). Each cell gives rise to several 7–10 μm axonal processes whose course and termination are highly variable. One of these processes goes out the lateral branch of the root toward the pericardial organ, while most of the other processes end locally, giving rise to an extensive array of varicosities that ramify in the most superficial portion of the root close to the group of cell bodies. The expanded varicose portions of the terminal processes are 3–10 μm in length and 1–3 μm in diameter and are connected by narrower processes of 0.1–0.2 μm diameter. Some of this morphology has been described previously (Evans et al., 1975).

Electron microscopy

The superficial regions of the thoracic roots contain many small axons and granule-filled endings wrapped in a matrix of collagen fibers (Fig. 6). The axons contain scattered granules, small mitochondria, tubules of the endoplasmic reticulum, and occasional vacuoles. They are usually 1–2 μm in diameter but frequently taper to 0.1–0.2 μm as they course through the connective tissue fibers. By far the most conspicuous feature of the superficial regions of the roots is the presence of numerous granule-filled enlargements that resemble nerve endings. On the basis of the granules they contain, the endings can be classified into four groups (see below). These endings are usually separated from the surface of the root by as little as 5 μm of connective tissue fibers. Occasionally they border on the small hemolymph channels that course through the root. In cross-sectioned profiles, the endings occur either singly, partially wrapped by glial and connective tissue elements, or in groups consisting of one or more of the different types of endings in close apposition inside a common glial sheath (Figs. 6 and

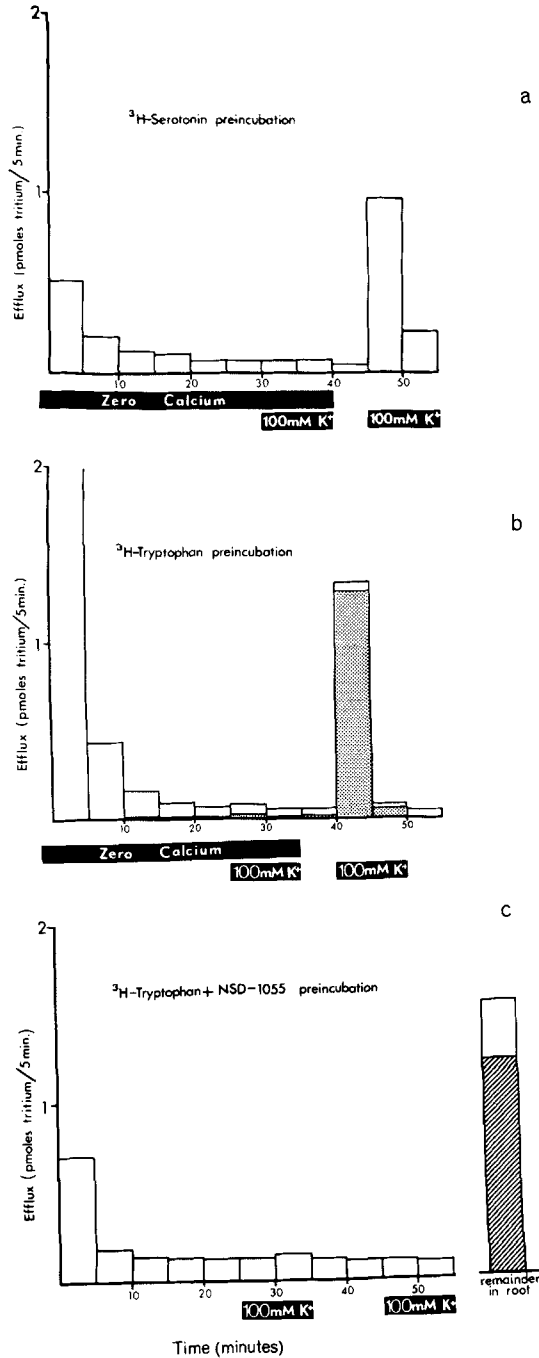


Fig. 5. Depolarization-induced release of serotonin. (a) A second thoracic root was incubated in $10^{-8}M$ ^3H -serotonin for 2 hr and then washed for 45 min by repeated changes of the medium. The medium was changed to one in which all of the Ca^{2+} had been replaced by Mg^{2+} (0–40 min). When the tissue was exposed to a high K^+ (100 mM higher K^+ 100 mM lower Na^+) zero Ca^{2+} medium, there was no change in tritium efflux. When the medium was changed to one containing a normal Ca^{2+} concentration, elevated K^+ resulted in an increased efflux of tritium. (b) A second thoracic root was incubated in $10^{-6}M$ ^3H -tryptophan for 3 hr and then washed for 30 min. At times between 10 and 50 min, the tritium was analyzed by high-voltage electrophoresis. The stippled regions represent serotonin and the white regions represent tryptophan. Depolarization with K^+ resulted in a Ca^{2+} -dependent release of serotonin but produced no change in the efflux of tryptophan. (c) A

7). In the latter case, the membranes of adjacent endings are always separated by a 25-nm space. No obvious membrane specializations characterize the regions where endings lie in close apposition to each other.

Type 1 endings. These occur as expanded varicosities that are 1–3 μm in diameter and up to 10 μm in length and run longitudinally along the surface of the root. They contain numerous large (150–250 nm) round or elliptical dense granules and clusters of small (45–55 nm) round vesicles [Fig. 8(a)]. The large granules have an electron-dense homogeneous matrix that is closely molded to the smooth enclosing granule membrane. We have not observed any clustering of dense granules in close proximity to the plasma membrane, nor have we seen any interactions of the granule membrane with the plasma membrane. There are two types of small vesicles: one type is clear and occurs in clusters that are closely associated with the plasmalemma of the ending, while the other type is moderately dense and is found scattered throughout the ending. The clusters of clear vesicles are not associated with any obvious specializations of the plasmalemma of the ending. In addition to granules and vesicles, the endings also contain scattered small mitochondria, microtubules, a few vacuoles, and some profiles of agranular reticulum.

Type 2 endings. Type 2 endings are similar in size to the type 1 endings. They are the most numerous of the four types of endings. They contain a heterogeneous population of granules that vary in size from 60–250 nm and may be round, elliptical, or dumbbell in shape [Fig. 8(b)]. The density of the granule matrix is lower than in type 1 endings, but on occasion a few granules with very dense cores are observed. The cores of the low density granules are textured in appearance but lack the highly organized substructure of the type 3 endings (see below). Granule clusters and interactions of granule membranes with the plasma membrane have not been observed in these endings. Mitochondria, microtubules, and tubular profiles of agranular reticulum are also present.

Type 3 endings. Type 3 endings contain large granules and small, clear vesicles. The large granules are not as closely packed as in the type 1 and type 2 endings, and the small, clear vesicles may outnumber them. The granule matrix is less dense than that of the type 1 granules but more dense than that of type 2 granules; however, it has a distinct crystalline substructure that is observed regardless of the fixative used [Fig. 8(c)]. The subunits of this matrix are arranged hexagonally and consist of hollow cylinders 7.5 nm in diameter and 300 nm in length [Fig. 8(c), inset]. The granules are elongated with an average length of 300 nm and an average width of 200 nm, and the long axis of the granule is parallel to the axis of the cylinders forming the matrix. Generally, the membrane lining the granule is irregular and the matrix is usually separated from it by a narrow space. The granules occasionally have a stalklike, matrix-free projection which is closely apposed to the plasmalemma of the ending. In addition to the crystalline granules, the endings also contain numerous small (45–55 nm) clear vesicles that have either round or slightly irregular profiles and occur either interspersed among the dense granules or in clusters along the length of the ending.

second thoracic root was incubated in $10^{-6}M$ ^3H -tryptophan for 3 hr in the presence of 1 mg/ml NSD-1055. All solutions in this experiment contained 1 mg/ml NSD-1055. The root was washed for 30 min before sampling of the medium began. Pulsing with elevated K^+ did not release any tritium from the root. At the end of the experiment the root was homogenized in acetate/formate buffer and the radioactive materials were analyzed by high-voltage electrophoresis. The bar at the right shows that at this time the root contained both tryptophan (white area) and 5-hydroxytryptophan (hatched area) but no serotonin.

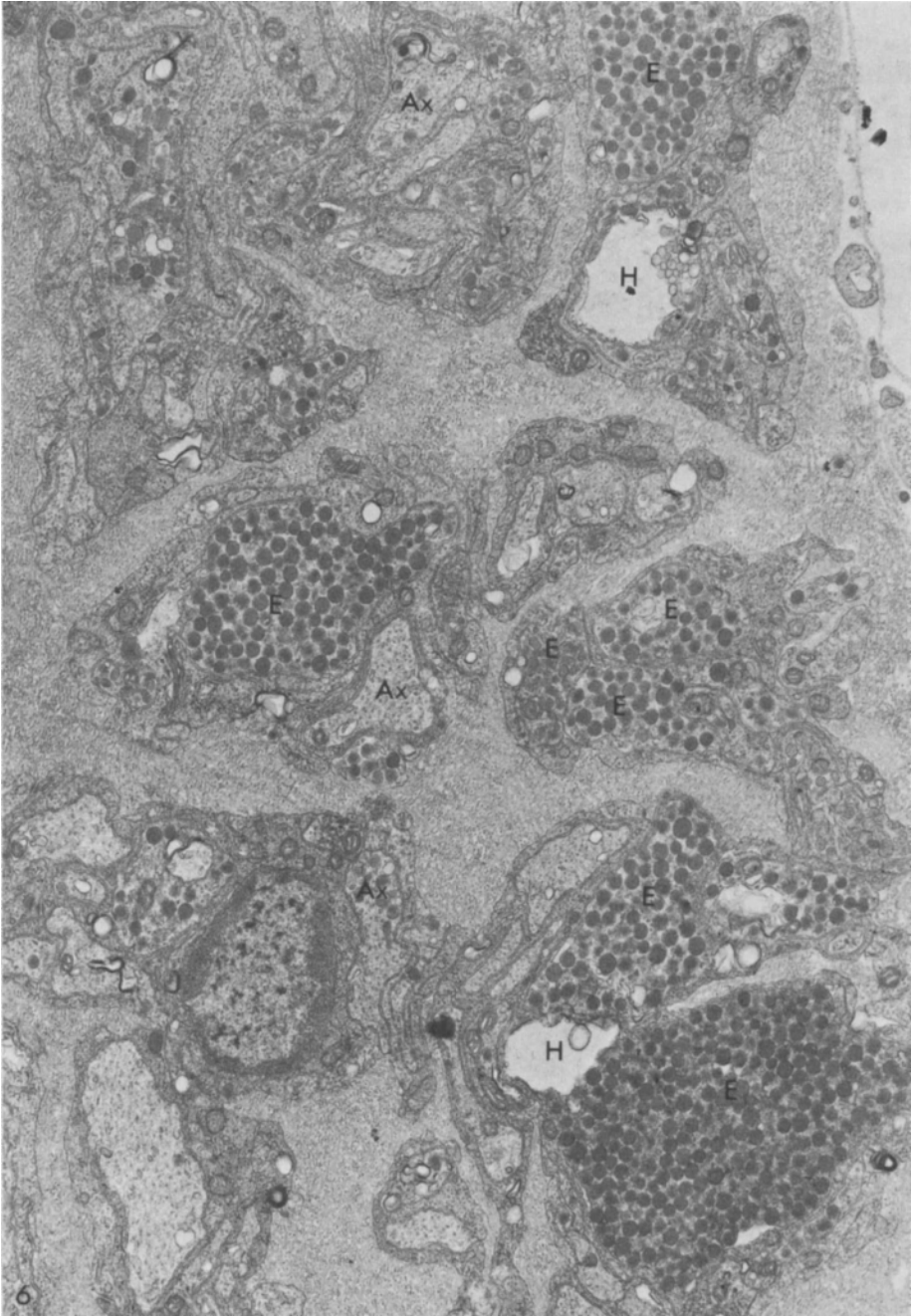


Fig. 6. Electron micrograph of a cross section of the peripheral region of a second thoracic root. Many small diameter axons (Ax) are found wrapped in a matrix of connective tissue fibers. The axons contain scattered granules, small mitochondria, vacuoles and profiles of the endoplasmic reticulum. The most conspicuous feature of the region is the numerous granule-filled enlargements that resemble nerve endings (E). Some of these endings occur in close proximity to hemolymph sinuses (H). Blood cells are not visible in these two sinuses because of the plane of section. $\times 7300$.

No membrane specialization is seen where clusters of vesicles lie close to the plasmalemma. In addition to the granules and vesicles, the endings also contain

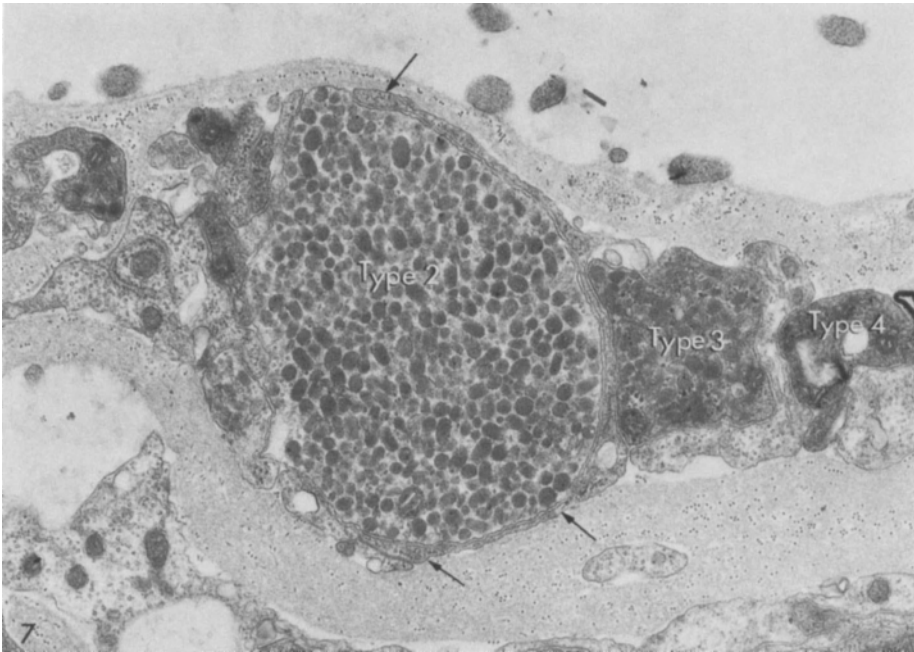


Fig. 7. Three different types of nerve endings occur in close proximity to each other near the surface of the root. The endings are separated from each other by processes of glial cells (arrows). $\times 23,000$.

long filamentous mitochondria, scattered glycogen granules, and irregularly shaped vacuoles.

Type 4 endings. These endings contain a population of vesicles whose size, shape, and density varies. Some of these pleomorphic vesicles are round or elliptical 45–70 nm profiles with transparent contents, while the predominant kind is tubular or flattened with a moderately dense content [Fig. 8(d)]. The vesicles are distributed uniformly throughout the ending and do not cluster preferentially near the membrane. Frequently the endings contain dense accumulations of glycogen and long filamentous mitochondria. Type 4 endings are not always distinct varicosities along axons but are often meandering ribbonlike processes that run longitudinally along the surface of the root. They are the least numerous of the four types of endings and the most difficult to identify because of their dense accumulations of glycogen. Treatment with the serotonergic neurotoxin 5,7 DHT caused the almost complete disappearance of the biochemical parameters of serotonin neurons. Parallel morphological studies showed that only the type 4 endings were missing, apparently replaced by degenerating profiles. This suggests that the type 4 endings are serotonergic and this suggestion is confirmed by the autoradiographic findings described below.

The approximate proportions of the different types of endings in a typical root are the following: type 1, 27%; type 2, 33%; type 3, 24%; type 4, 16%. These four types of endings are always present in approximately the same proportions regardless of the fixative used.

Cell bodies. Cell bodies contain large dense granules resembling those seen in type 1 endings. We attempted to trace the processes of endings back to cell

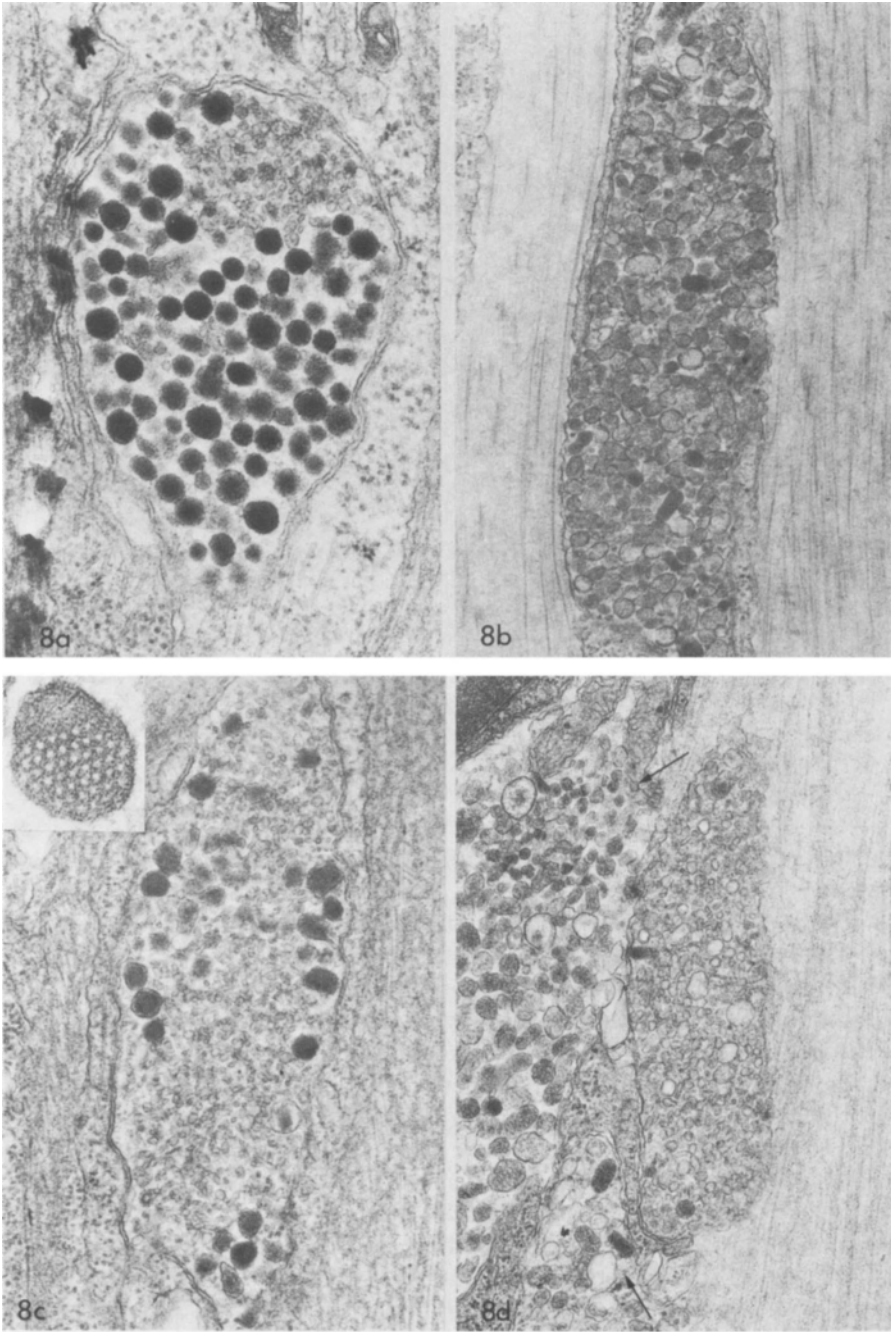


Fig. 8. Electron micrographs of the four types of nerve endings. (a) Type 1 ending contains large (150–250 nm) round, dense granules and clusters of small (45–55 nm) round vesicles. The large granules have an electron-dense, homogeneous matrix that is closely molded to the smooth enclosing granule membrane. There are two types of small vesicles: round clear vesicles commonly found in clusters associated with the plasmalemma of the ending and dense vesicles which are scattered throughout the ending. $\times 26,000$. (b) Type 2 ending contains a heterogeneous population of granules that vary in size from 60 to 250 nm and may be round, elliptical, or dumbbell in shape. The density of the granule matrix is lower than in the type 1 endings, but a few granules with very dense cores may occur. The cores of the low-density granules are textured in appearance but lack the highly organized substructure of the type 3 endings. $\times 29,000$. (c) Type 3 ending contains large granules

bodies using serial sections, but the tortuous axonal processes were so long and thin that we were able to connect only one ending (a type 1) to a cell body. Intracellular injections of horseradish peroxidase were also done in hopes of filling a cell and its processes and endings. Unfortunately, the morphology of the endings was insufficiently preserved to permit identification of the granules and vesicles.

Autoradiographic studies

Light microscopy. Roots were incubated for 2 hr in radioactive precursor compounds ($2 \mu\text{M}$ ^3H -tyramine, $0.2 \mu\text{M}$ ^3H -L-tryptophan, or $0.1 \mu\text{M}$ ^3H -serotonin), fixed, processed, and subsequently exposed for one week for light microscopic autoradiography (see Methods section). With all three radioactive compounds a similar pattern was seen. Punctate clusters of silver grains were observed over the entire surface of the root (Fig. 9). These clusters occurred in greatest number in the vicinity of the cell bodies and decreased in density proximal and distal to this region. Silver grains were not found directly over root cell bodies in any of the roots examined. There were some differences, however, in the labeling pattern seen with each of the isotopes.

With tyramine, it was difficult to find incubation conditions in which the amount of label in the tissue in the form of octopamine was consistently greater than 80%. When electrophoresis of one member of a pair of roots (see Methods section) indicated the presence of a high percentage of the metabolic products of tyramine and octopamine, the other member of the pair showed diffuse labeling over the entire tissue. It is possible that connective tissue and/or glial cells produce these metabolites (Kennedy, 1978). When a high percentage of the radioactivity in the tissue was found to be octopamine, the autoradiographs usually showed punctate grain clusters on the surface of the root.

In the incubations with radioactive tryptophan and serotonin (but not with tyramine), in addition to the clusters of grains at the root surface, two or three small axons with a diameter of $2\text{--}4 \mu\text{m}$ were also labeled (Fig. 9). These could be traced through the root in association with axons arising from the root cell bodies, but they never contacted or joined root cell bodies. In one root incubated with ^3H -serotonin, a detailed examination of cross sections of the root proximal and distal to the cell body region showed three lightly labeled axons where the root first leaves the ventral nerve cord and one labeled axon where the root expands to join the pericardial organ.

In several experiments light microscopic autoradiography was performed on roots incubated with $0.2 \mu\text{M}$ ^3H -octopamine. In all cases, silver grains were found scattered over the entire tissue. This diffuse pattern of labeling probably

and small, clear vesicles. The matrix of the granules is intermediate in its density between granules of the type 1 and type 2 endings, but it has a distinct crystalline substructure. The numerous, small ($45\text{--}55 \text{ nm}$) clear vesicles have either round or slightly irregular profiles and are scattered throughout the ending. Inset: The subunits of the granule matrix are hexagonally arranged and consist of hollow cylinders 7.5 nm in diameter and 300 nm in length. The granules are elongated with an average length of 300 nm and an average width of 200 nm ; the long axis of the granule is parallel to the axis of the cylinders forming the matrix. $\times 26,000$. Inset: $\times 106,000$. (d) Type 4 ending contains a population of pleomorphic vesicles. Some are round or elliptical with transparent contents, while others are tubular or flattened with a moderately dense content. The vesicles are uniformly distributed throughout the ending and do not cluster near the membrane. A type 2 ending is present at the left (arrows). $\times 28,000$.

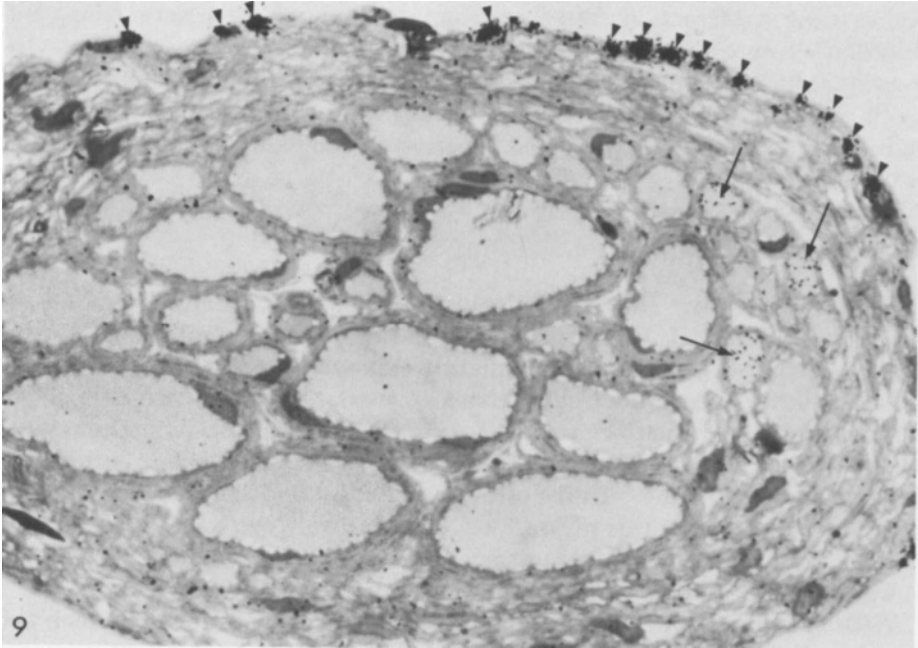


Fig. 9. Light microscopic autoradiograph of a cross section of a second thoracic root after incubation with ^3H -serotonin. Punctate clusters of silver grains (arrowheads) are observed along the periphery of the root. In addition, three small axons (arrows) are labeled. $\times 1600$.

represents the nonspecific uptake of octopamine by connective tissue cells and the formation of the three metabolites of octopamine (Evans et al., 1976b). Electron microscopic autoradiography was not attempted with tissues incubated in ^3H -octopamine.

Electron microscopy. Thin sections were prepared from roots incubated with radioactive compounds, coated with emulsion and exposed 10–21 days.

^3H -Tyramine incubations

In incubations with ^3H -tyramine silver grains were found exclusively over type 3 endings [Fig. 10(a)]. These endings were found close to the surface of the roots; therefore their location correlated well with the locations of the clusters of grains observed with the light microscope. Unfortunately, because of the size of the grains, it could not be determined whether the label was associated with the crystalline granules or with the small, clear vesicles found in the endings. Axons and cell bodies did not have a label associated with them.

^3H -Tryptophan and ^3H -serotonin incubations

With ^3H -tryptophan and ^3H -serotonin incubations, the clusters of silver grains were found exclusively over the type 4 endings [containing pleomorphic vesicles, see Figs. 10(b) and 10(c)]. No silver grains were observed over cell bodies or the other three types of endings, but occasionally a lightly labeled axon was found.

^3H -Tryptophan and NSD-1055. In the incubations with radioactive tryptophan almost all the radioactivity in the tissue at the time of fixation is actually

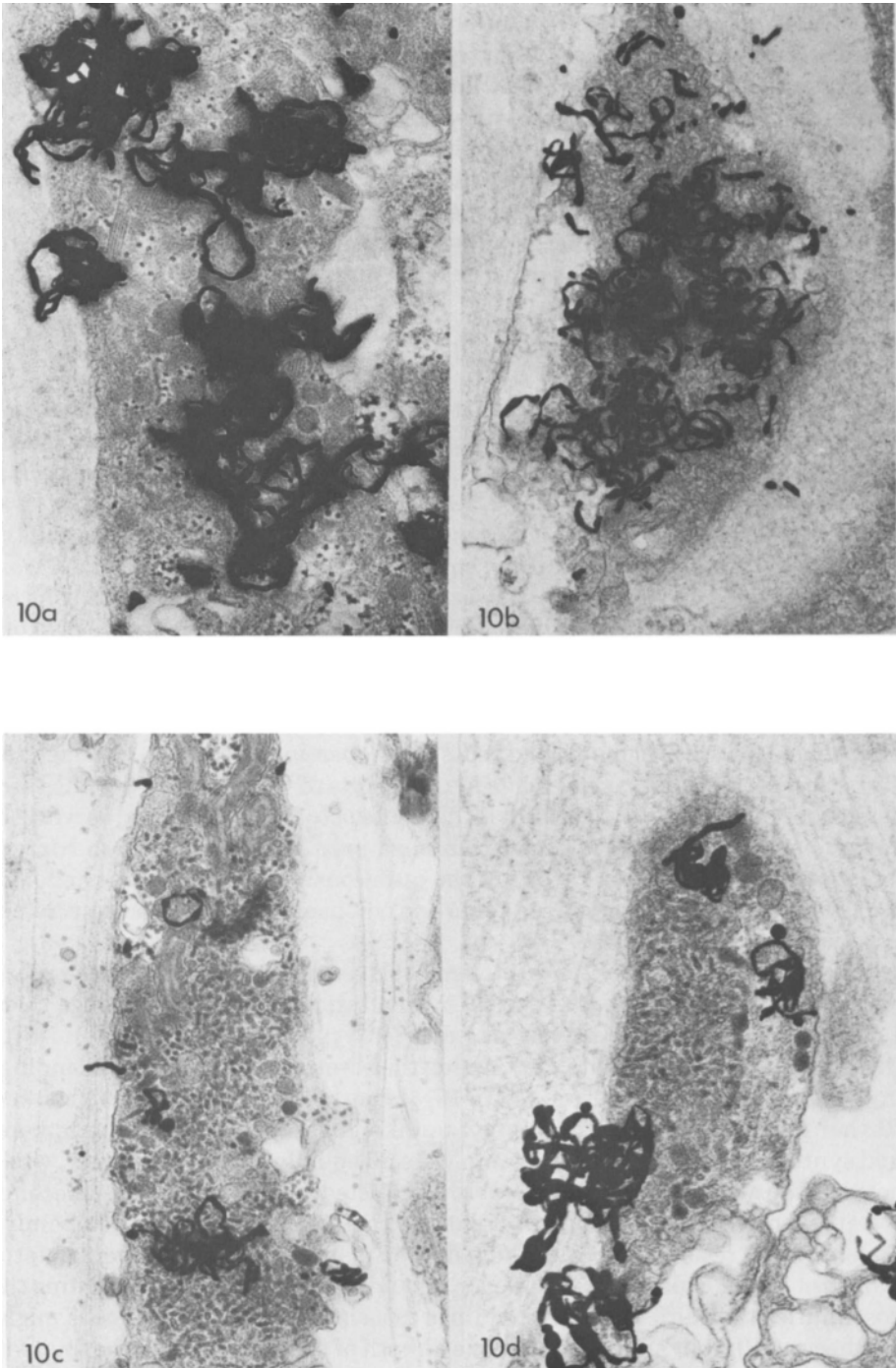


Fig. 10. Electron microscopic autoradiograph of labeled nerve endings. (a) ^3H -tyramine; silver grains are found exclusively over type 3 endings containing crystalline granules and small, clear vesicles; $\times 34,000$. (b) ^3H -tryptophan; silver grains are associated exclusively with type 4 endings; $\times 36,000$. (c) ^3H -serotonin; silver grains again are associated with type 4 endings; $\times 36,000$. (d) ^3H -tryptophan and NSD-1055; type 4 endings are labeled; Thus, the distribution of silver grains is identical to the results of tryptophan incubations without the decarboxylase inhibitor. $\times 44,000$.

in the form of serotonin (see above). It is possible therefore that the same endings were labeled after incubation with ^3H -tryptophan and ^3H -serotonin because serotonin synthesized from tryptophan was released from one location and then taken up and localized at the site of the high-affinity uptake system for serotonin. To eliminate this possibility, roots were incubated for 2 hr in medium containing $0.2\ \mu\text{M}$ ^3H -tryptophan and 1 mg/ml NSD-1055. Under these conditions, tryptophan and 5-HTP are found in tissues, but no serotonin is formed (see above). In these experiments, the distribution of silver grains seen in both light and electron micrographs was identical to the results with tryptophan incubations without the decarboxylase inhibitor [Fig. 10(d)].

DISCUSSION

In our earlier studies we showed that octopamine was found, synthesized, and released at two separate locations along lobster second thoracic roots in association with a group of neurosecretory neurons and their proximal and distal processes (Evans et al., 1975, 1976a, 1976b). A strong positive correlation was found between the amounts of endogenous octopamine present in a root and the numbers of cells seen. In addition, small groups of cell bodies dissected from a root contained the enzyme tryamine- β -hydroxylase (the enzyme that forms octopamine), while no detectable activity was found in the other types of axons found in a root (Evans et al., 1976b). Thus, the conclusion was reached that octopamine was likely to be found within and synthesized by the root neurons. In this article we demonstrate that serotonin is found, synthesized, and released at the same locations along second roots as octopamine. Moreover, although the statistical correlation was not made in the results of this article, since the serotonin content of roots is a constant 6–7% of the octopamine content, as with octopamine, a strong positive correlation must exist between serotonin content and cell number. Therefore, one of the questions we address in this article is the nature of the association of serotonin and octopamine with the neurosecretory neurons and their processes.

Our biochemical studies characterized the serotonin system in these roots. These studies revealed that at the sites of endogenous serotonin storage there were independent high-affinity uptake sites for tryptophan and serotonin. With the drug 5,7-dihydroxytryptamine, a drug that destroys serotonin nerve endings in other animals (Baumgarten et al., 1974; Björklund et al., 1974) we showed further that high-affinity tryptophan uptake, high-affinity serotonin uptake, and synthesis and storage of serotonin were all equally labile to this drug, while octopamine synthesis and storage were unaffected. Thus, the entire serotonin system seems likely to be found in a separate compartment from the octopamine system. This result could be explained if the neurosecretory cells were heterogeneous. Other explanations, however, should also be considered, including the possibilities that different nerve endings associated with the same cells might synthesize different amines or that one or both of the amines might be found in endings close to the neurosecretory cells but have their cell bodies at some completely different location. To explore the matter further, we undertook our morphological studies. At the thoracic root bifurcations we found a complex tangle of granule-filled processes and four morphologically distinguishable types of nerve endings. The tissue, therefore, was closer in appearance to a typical crustacean neurohemal organ (Andrews, 1973; Bunt and Ashby, 1967; Maynard and Maynard, 1962) than the relatively simple structure we expected. Moreover,

autoradiographic studies demonstrated no labeling of cell bodies with any of the isotopic precursors used. The electron microscopic autoradiographic studies did demonstrate clearly that only two types of nerve endings are involved in the synthesis and storage of amines. Type 3 endings synthesize octopamine from tyramine and type 4 endings synthesize serotonin from tryptophan. We do not know the nature of the substances found within the other two kinds of nerve endings, nor can we tell whether the octopamine within a type 3 ending is associated with the large granules with crystalline cores or with the small clear vesicles.

We believe that these studies show the first clear demonstration of the morphological features of identified octopaminergic nerve endings. Hoyle et al. (1974) have reported that endings identified as processes of the dorsal unpaired median neuron of the locust contain large dense core vesicles, and it has recently been reported that the dorsal unpaired neurons are probably octopaminergic (Evans and O'Shea, 1978; Hoyle, 1975). Endings containing crystalline granules and small vesicles have been described by Sullivan, Friend, and Barker (1977) in the analog of the pericardial organ of the Pacific lobster and in the pericardial organs of the crab (Andrews, 1973). It was thought, however, that the crystalline granules represented a condensation of the matrix of one of the other types of granules (Andrews, 1973).

Serotonin-containing axons and nerve endings have been reported before in both vertebrate and invertebrate species. The lobster serotonin endings (type 4) containing a heterogeneous population of clear vesicles are more similar in their morphological features to serotonin endings in the vertebrate central nervous system (Chan-Palay, 1976; Descarries et al., 1977) than to other invertebrate serotonin nerve endings or vertebrate gut serotonin endings, which contain a mixture of granular and agranular vesicles (Coggeshall, 1971; Cottrell and Osborne, 1970; Dreyfus, Sherman, and Gershon, 1977; Goldman, Kim, and Schwartz, 1976; Rude, Coggeshall, and van Orden, 1969; Weinrich et al., 1973). The majority of the catecholamine and serotonin endings in the vertebrate brain lack the morphological features of classical synapses, namely close membrane apposition and pre- or postsynaptic densities (Ajika and Hökfelt, 1973; Chan-Palay, 1976; Descarries et al., 1975, 1977). Similarly, we did not see any such morphological specialization in the lobster endings, and there is no obvious association of these endings with any particular postsynaptic structure.

While our results show clearly that serotonin is made in one kind of nerve ending and octopamine in another, we have not been able to associate the endings with cell bodies. Part of this difficulty is that the root cell bodies are not labeled with isotopic precursors. It is possible that the cell bodies do not have uptake mechanisms for the precursor compounds or that activities of synthetic enzymes are too low to allow accumulation of transmitter products. Both of these are reasonable possibilities on the basis of information available in the literature. For example, Pentreath and Cottrell (1975), using autoradiography, found that nerve endings and axons but not cell bodies of the giant serotonin-containing neurons of the snail *Helix pomatia* take up exogenous serotonin.

On the other hand, it is possible that the root cells are neither octopaminergic nor serotonergic and the cell bodies of the octopamine and/or serotonin endings are at some remote, as yet unidentified site, with only their endings associated with the root neurons. For serotonin, the hypothesis that the cell bodies are elsewhere may be further supported by the autoradiographic labeling of three axons where they enter or exit from the ventral nerve cord (see Fig. 9). Daly,

Fuxe, and Jonsson, (1973) have found that serotonin axons and nerve endings are much more susceptible to destruction by 5,6-DHT than are serotonin cell bodies. The differential reduction of endogenous serotonin caused by 5,7-DHT treatment in various lobster tissues (see Table 1) might suggest that the serotonin cell bodies are in the brain and/or in the subesophageal ganglion, while the second thoracic roots and pericardial organs contain only the nerve endings. The differential reduction in serotonin could as easily be explained, however, by different accessibility of the toxin to the various tissues. Cooke and Goldstone (1970), using histofluorescence, identified both catecholaminergic (green) fibers and serotonergic (yellow) processes in crab pericardial organs. These fluorescent processes were clearly distinguishable from the methylene blue-staining fibers of the neurosecretory processes. Cooke and Goldstone were able to trace the catecholaminergic fibers back to a pair of large green fluorescent cell bodies in the commissural ganglion but were not able to find the cell bodies of origin of the serotonergic processes. In a histofluorescence study of the central nervous system of the crayfish, Elofsson et al., (1966) found only one pair of yellow fluorescent cell bodies in the brain and only a few cells in the subesophageal ganglion.

Another point of this article is the demonstration that the sites of synthesis and storage of serotonin and the high-affinity uptake system for serotonin itself both reside within a single type of nerve ending. It is reasonable that this entire machinery should be a part of a serotonergic ending, but evidence from other systems supporting this notion has been indirect. In mammals, serotonin uptake, as shown by autoradiography, has been used to identify presumed serotonergic nerve endings (Aghajanian and Bloom, 1967; Chan-Palay, 1976; Descarries et al., 1975). The evidence supporting the assumption that serotonin is taken up by the same endings that synthesize and release it is of two types. First, the distribution of the uptake system closely parallels the distribution of endogenous serotonin and the enzyme tryptophan hydroxylase (Aghajanian and Bloom, 1967; Saavedra, 1977). Second, the destruction of serotonin nerve endings by brain lesions in the midline raphe nuclei or by treatment with 5,6- or 5,7-dihydroxytryptamine results in parallel decreases in serotonin, tryptophan hydroxylase activity, and serotonin uptake (Björklund et al., 1974; Kuhar, Roth, and Aghajanian, 1972). In our studies we also find parallel distributions of endogenous serotonin, serotonin synthesis, and high-affinity serotonin uptake. Our studies show further the existence of a high-affinity tryptophan uptake mechanism that is localized morphologically to the same structures that take up serotonin (type 4 endings). We showed that this pattern of localization did not result from synthesis and release of serotonin from one site and subsequent uptake and localization to the type 4 endings, but rather that the two mechanisms, high-affinity tryptophan and serotonin uptake, are confined to the same structure. A further demonstration that all biochemical parameters of serotonin metabolism are associated with a single structure comes from experiments with animals pretreated with the drug 5,7-DHT. This treatment causes simultaneous decreases in serotonin content, serotonin synthesis, high-affinity tryptophan uptake, and high-affinity serotonin uptake, and eliminates only the type 4 endings from the tissue without affecting octopamine content or synthesis. A final point concerns the evidence that the high-affinity serotonin and tryptophan uptake sites are two distinct molecular entities. The two compounds do not compete with each other for uptake, and serotonin uptake is sodium dependent while tryptophan uptake does not require sodium in the bathing medium.

The kinetics of tryptophan uptake specifically into serotonin nerve endings have not been well studied elsewhere. In brain synaptosomes from areas of the brain rich in serotonin endings, there are at least two components of tryptophan uptake with the higher affinity component having a K_m for tryptophan of about $10^{-5}M$ (Bauman et al., 1974; Belin and Pujol, 1972; Knapp and Mandell, 1972a). However, tryptophan uptake is not reduced in synaptosomes from animals with midline raphé lesions (Kuhar et al., 1972). Moreover, various clearly nonserotonergic tissues also show more than one component of tryptophan uptake with K_m values as low as $10^{-5}M$ for tryptophan (Bauman et al., 1974; Hamon and Glowinski, 1974). Although we did not attempt to resolve the different components of uptake in lobsters, we also saw active accumulation of tryptophan by tissues from 5,7-DHT-treated animals. The highest affinity component we observed in such experiments has an apparent K_m of $1 \mu M$ for tryptophan. What we have eliminated in the experiments with 5,7-DHT, however, is a single, saturable high-affinity uptake for tryptophan, and it is this component whose kinetic properties we studied. We have shown further that tryptophan uptake by this mechanism is the rate-limiting step for serotonin synthesis since the rate of tryptophan accumulation by the serotonin endings is no greater than the rate of synthesis of serotonin from tryptophan. Gershon, Baldessarini, and Wheeler (1974) have suggested that there is likely to be a high-affinity uptake of tryptophan into serotonin nerve endings in rat spinal cord. Their suggestion is based on studies of tryptophan uptake in synaptosomes prepared from rat spinal cords from control and 5,6 DHT-treated animals. They observed that at concentrations of 10^{-7} and $10^{-6}M$ tryptophan, tryptophan uptake was reduced by about 17% in synaptosomes from lesioned rats while it was only reduced by 7% in $10^{-3}M$ tryptophan. Knapp and Mandell (1972b) have also suggested that tryptophan uptake into serotonin endings may be rate limiting for serotonin synthesis since both serotonin synthesis and the higher affinity component of tryptophan uptake are inhibited 50% by $10^{-3}M$ cocaine.

The entire situation is less clear with octopamine and the type 3 endings. Preliminary biochemical studies (unpublished observations) have failed to demonstrate the existence of high-affinity octopamine uptake. The autoradiographic studies with octopamine confirm this suggestion. Tyramine, on the other hand, will selectively label the octopamine endings. Whether this is due to a high-affinity uptake mechanism for tyramine or tyrosine remains to be established. It is also unclear why an avid uptake mechanism should exist for serotonin and not for octopamine.

Finally, the radioactive serotonin accumulated by these tissues could be released by depolarization with potassium when either tryptophan or serotonin was the precursor compound and release was a calcium-dependent process. In experiments with lobster pericardial organs Sullivan (1978) has demonstrated Ca^{2+} -dependent, selective release of 3H -serotonin formed from 3H -tryptophan using electrical stimulation. In our studies we show further that with an amino acid decarboxylase inhibitor present, tissues accumulate as much radioactive material in the form of tryptophan and 5-hydroxytryptophan as they would normally accumulate radioactive serotonin, yet neither of the amino acids are released by pulsing the tissues with potassium. This suggests that the likely release vehicle, the nerve ending vesicles or granules, can only accumulate and release the amine product serotonin and not the precursor amino acids.

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