STRUCTURE AND FUNCTION OF SPINY LOBSTER LIGAMENTAL NERVE PLEXUSES: EVIDENCE FOR SYNTHESIS AND STORAGE OF BIOGENIC AMINES AND THEIR SECRETION AS NEUROHORMONES

by

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DEDICATION

Mo Mhuirnin, Byanu:

A Righ na gile 's na greine,

A Righ nan reula runach,

Agad fein tha fios ar feuma,

A Dhe mheinnich nan dula.

Suilleachain
Imbolg
Iargalon

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STRUCTURE AND FUNCTION OF LIGAMENTAL NERVE PLEXUSES

The heart of a decapod crustacean receives innervation from three sources: the intrinsic cardiac ganglion, the extrinsic dorsal nerve, and the nerves of the arterial valves (Alexandrowicz, 1932). In addition, the pericardial organs are thought to exert hormonal influences on the cardiac network by neurosecretion into the pericardial cavity. Paired segmental nerves exit dorsally from each thoracic ganglion in macrurans (and from homologous regions of the brachyuran thoracic mass) and project to the pericardial cavity where they anastomose, forming the pericardial organs. Maynard (1961) has described numerous regions of brachyuran ganglia containing cell bodies that contribute processes to these nerves, but the central connections of these cells are virtually unknown. Segmental nerve inosculations within the pericardial cavity are referred to as trunks, bars, lamellae, and plexuses (Alexandrowicz, 1953); together they comprise the pericardial organs. These anatomical distinctions reflect the diversity of functional morphology among decapod neurosecretory structures.

In brachyuran species, the pericardial organs lie in the lateral pericardial cavity and consist of two or more longitudinal "trunks" connected by vertical "bars" near the

openings of the branchiocardiac veins (Alexandrowicz, 1953; Maynard, 1961). The anatomy of macruran pericardial organs is more complex and variable. In Panulirus they consist of paired lateral pericardial plexuses, ligamental nerve plexuses, and dorsal nerve apparati (Fig. 1A, B). The lateral plexuses are homologues of brachyuran anterior bars. The ligamental nerve plexuses and dorsal nerve apparati differ from brachyuran pericardial organs by directly abutting ligaments and myocardium near ostia.

Alexandrowicz (1932) described the "apparatus nervi dorsalis" and the ligamental nerves as "peculiar terminations of the nerves entering the pericardial cavity." They have been observed in several macruran decapods, including species of Astacus, Palinurus, Scyllarus, Homarus and Panulirus (Alexandrowicz, 1932; Maynard, 1953). After originally ascribing a sensory function to these elements, Alexandrowicz (1953) later suggested that they belong "to the system of the pericardial organs." Maynard (1960) subscribed to the latter contention, specifically referring to the ligamental nerve plexuses as pericardial organs. In Panulirus the dorsal nerve trunks carry the excitatory and inhibitory inputs to the cardiac ganglion. These extrinsic inputs originate centrally and travel to the pericardial cavity within segmental nerves 1, 2 and 3 (Maynard, 1953). Inside the pericardial cavity the segmental nerves an-

astomose to form the lateral pericardial plexuses and the dorsal nerve trunks arise from these plexuses. The dorsal nerve trunks carry numerous other fibers of segmental origin which ramify to form the dorsal nerve apparati. Only the extrinsic inputs to the cardiac ganglion actually enter the heart, where the dorsal nerve trunks sans their contributions to the dorsal nerve apparati, become the dorsal nerves proper.

Except for the brief anatomical descriptions provided by Alexandrowicz (1932, 1953) and Maynard (1953, 1960), there is little evidence in the literature to support the hypothesis that the ligamental nerve plexuses and dorsal nerve apparati function as pericardial organs. In Chapter I I report my investigations of the anatomy, ultrastructure and neurochemistry of these structures in the Pacific spiny lobster, Panulirus interruptus. These observations indicate that the ligamental nerve plexuses are indeed elaborate neurosecretory structures and should be considered pericardial organs. Plexuses rapidly synthesize octopamine, dopamine, and 5-hydroxytryptamine through established metabolic pathways. Moreover, electrical stimulation of preterminal fibers induces release of octopamine by a calcium-dependent mechanism, providing additional evidence that octopamine functions as a neurohormone in decapod Crustacea, in accord with the work of Evans, Talamo, and Kravitz (1975).

Methods

Animals

Pacific spiny lobsters (Panulirus interruptus), 0.5-1.0 kg, were purchased from Pacific Biomarine Co. (Venice, CA) and maintained in circulating oxygenated sea water at 16°C. Unless otherwise noted, dissected tissues were placed in physiological saline containing 479 mM NaCl, 12.7 mM KCl, 13.7 mM $CaCl_2 \cdot 2H_2O$, 10 mM $MgSO_4 \cdot 7H_2O$, 8.8 mM Na_2SO_4, and 11 mM Tris base titrated to pH 7.4 with maleic acid.

Light Microscopy

In order to expose cardiac ligaments, the carapace dorsal to the pericardium and the hypodermis overlying the heart were removed. Tissues were stained in situ by submersing the exposed thoracic region in oxygenated saline or sea water containing 0.001% methylene blue at 11°C. Under these conditions, the heart continued to pump the staining solution through the pericardial cavity for one to two hours. Tissues were excised, washed with saline, and photographed with an Olympus Photomicrographic System camera mounted on a Wild dissecting microscope. Dorsal nerve apparati were fixed in alcoholic Bouins solution, stained with Heidenhain's variation of Mallory's triple stain (Mallory, 1938), and 15 μ sections were examined with a Zeiss Universal microscope.

Electron Microscopy

Excised tissues were fixed immediately by the glutaraldehyde-dichromate method of Wood (1966), modified by the addition of 4% paraformaldehyde to the primary fixative and postfixation with osmium tetroxide. Friend (1976) found that this procedure optimized the number and appearance of dense core vesicles in Panulirus nervous tissue, although slightly decreasing the overall quality of tissue preservation. Preparations were dehydrated with increasing ethanol concentrations and embedded in Epon-Araldite (Mollenhouer, 1964). Thin sections were cut with a DuPont diamond knife, mounted on 100 mesh copper grids, and stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963). They were examined and photographed with a Phillips 300 electron microscope, operated at 60 Kv with 20 to 30 μ objective aperatures. Thick sections (2 μ) of the same material were cut with glass knives, mounted on slides, stained with toluidine blue (Altman and Bell, 1973), and examined with a Zeiss Universal microscope. This set of experiments was carried out with the expert technical assistance of Ms. Brenda Friend.

Neurochemistry

Ligamental nerve plexuses and dorsal nerve apparati were dissected free of the heart and incubated at 11 C for

0.3 to 6.0 hrs in 20 µl of oxygenated saline containing 13.0 mM glucose and 8-90 µM ^{3}H-tyrosine, 82 µM ^{3}H-tryptophan or 21-104 µM ^{3}H-choline chloride. Ligamental nerve plexuses were also incubated with 10-150 µM ^{3}H-tyramine. Ascorbic acid, 0.1 mM, was included in all tyrosine and tyramine incubations. Tissues were washed three times (5 min each) with saline to remove extracellular precursors and metabolites were extracted into acetone:1N formic acid (90:10) (Kariya and Aprison, 1969) containing unlabeled carrier compounds and 0.2% ascorbic acid. Precursors and metabolites were separated by high voltage electrophoresis at pH 1.9 (Hildebrand et al., 1971) and localized either by spraying with diazotized sulfanilic acid or using a Berthold radiochromatogram scanner. Positive identification of metabolites was assured by eluting radioactive compounds from the electropherogram and subjecting them to thin-layer cochromatography on EM microcrystalline cellulose plates (Brinkman Instruments Inc.) in three solvent systems: Solvent I, n-butanol--acetic acid--water (60:15:25); solvent II, n-butanol saturated with 1N HCl; and solvent III, methyl ethyl ketone--propionic acid--water (75:25:20). Dopamine synthesis in the dorsal nerve apparatus was confirmed by first collecting the catecholamine with a microalumina adsorption procedure (Sullivan, unpublished) before thin-layer chromatography in solvent III. Radioactivity was

quantified by placing 0.5 cm sections of electrophoresis paper or cellulose thin-layer in scintillation vials containing an Omnifluor (NEN), toluene, Triton X-100 (Sigma), water mixture for two days and then counting with a Beckman LS-230 liquid scintillation system at 30% efficiency.

It is worth mentioning a problem that arises in quantitating catecholamine and phenolamine synthesis by the Hildebrand et al. (1972) screening procedure. This method utilizes the diazocoupling reaction (Griess, 1858) as a method to visualize spots on electropherograms. Recently it has come to my attention that diazocoupling of catecholamines occurs in the 6' position (Venter, Arnould and Kaplan, 1975). Diazocoupling of phenolamines presumably occurs ortho to the ring hydroxyl group since the para position is substituted. Therefore one should choose the type of label carefully or consider an alternative visualization procedure because diazocoupling may lead to variable decreases in the specific activities of 3', 5'-^{3}H-phenolamines and 2', 6'-^{3}H-catecholamines depending on the extent of diazocoupling during application of the spray reagent.

Radioactive compounds were obtained from New England Nuclear (3,5-^{3}H-tyrosine, 60.1 Ci/mmol; 2,6-^{3}H-tyrosine, 49 Ci/mmol; ^{3}H-(G)-tyramine, 10.78, 6.2, or 4.93 Ci/mmol; ^{3}H-(G)-tryptophan, 7.9 Ci/mmol; ^{3}H-(N-methy)-choline chloride, 24.0 Ci/mmol or from Amersham/Searle (1, 2-^{3}H-tyramine, 1.1 Ci/mmol and ^{3}H-(N-methyl)-choline chloride, 10.1 Ci/mmol.

These were routinely repurified prior to use, tryptophan (solvent I) and tyramine (solvent III) by TLC, tyrosine and choline by high voltage electrophoresis at pH 1.9.

Octopamine Release

In the release experiments the right posterior ligamental plexus was carefully dissected in such a manner that a long piece of the motor-ligamental nerve remained intact. The plexus was pinned out in a Silgard (Dow-Corning) chamber and incubated for one to two hours in saline containing radioactive tyramine. After incubation the preparation was washed for one to two hours before initiating the stimulation paradigm. A specially designed superfusion system operated at a flow rate of 1 ml/min, effected a rapid mixing of the perfusate in the experimental chamber (Sullivan, in preparation). Samples of 0.5 ml were removed directly from the chamber at 30 sec or 1 min intervals and either placed in scintillation vials for counting or saved for chemical analysis as described above for tissue samples. This allows one to assay the "instantaneous concentration" of radioactivity in the chamber by removing a constant fraction of its volume (either 3.0 or 1.5 ml). The motor-ligamental nerve was stimulated through pin electrodes placed about 1 cm from the plexus with 0.5-1.0 msec duration square wave pulses of 3-10 V at 5-20 Hz. Current was

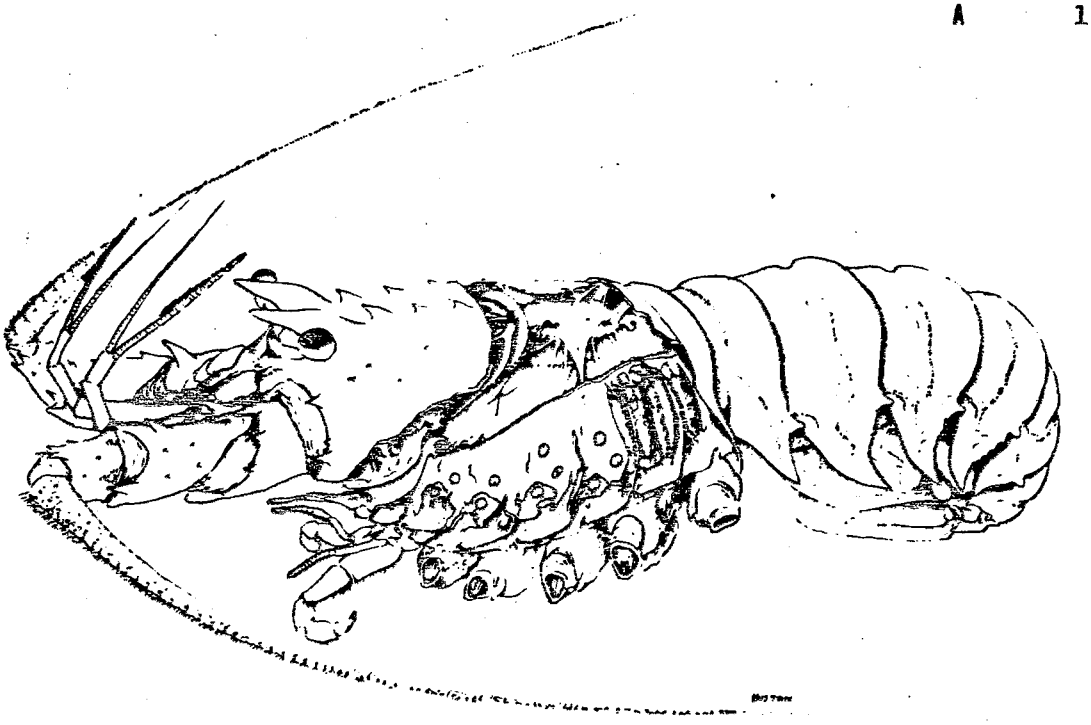
supplied by a Grass SD9 stimulator. Evoked electrical activity was monitored with stainless steel pin electrodes placed next to the posterior ligamental nerves to assure constant responses to stimuli throughout the experiment. The AC coupled signals were fed into the 5A22N differential amplifiers of a 5103N Tektronix storage scope.

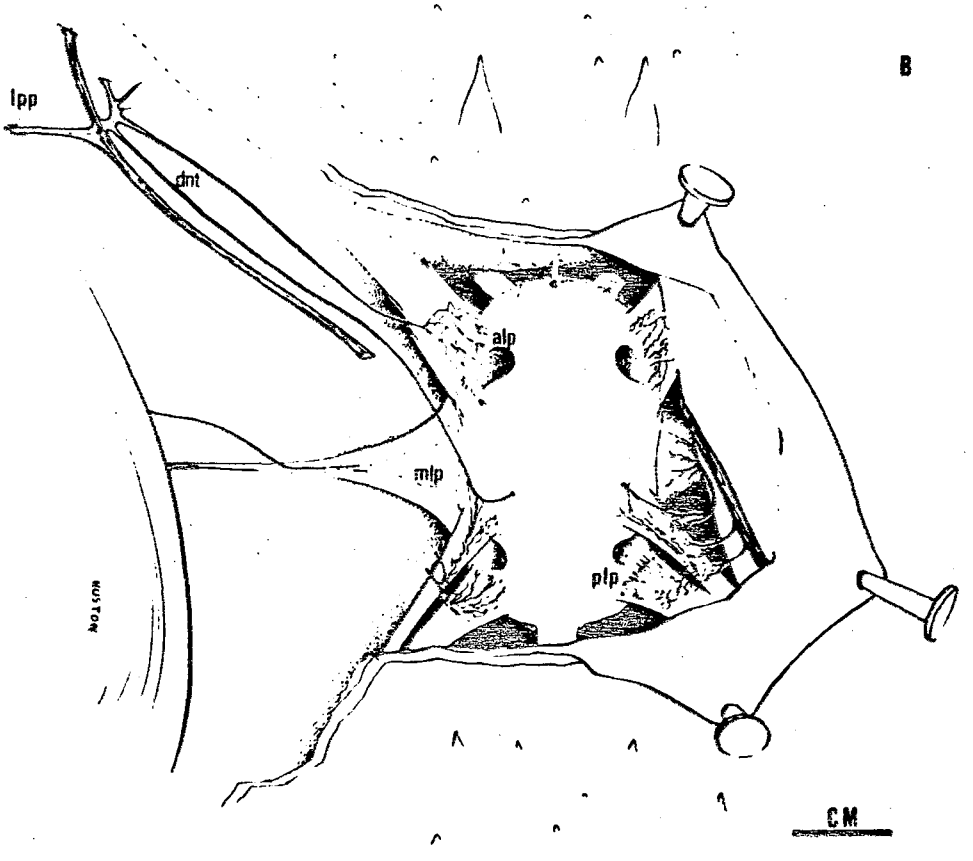
Results

Anatomy

Three pairs of ligaments suspend the heart in the pericardial cavity of P. interruptus. The anterior ligaments attach directly to the rostral pericardium, whereas the posterior ligaments are anchored to the dorsal carapace. The medial ligaments extend laterally from the heart to the epimeral (pleural) plates (Fig. 1B). Near the surface of the heart each ligament is joined by one or more nerve trunks which form the ligamental nerve plexuses as they ramify extensively and follow the diverging ligamental fibers to their cardiac attachments (Fig. 2B, 3A, E). The neural processes originate centrally in thoracic ganglia and travel to the pericardial cavity within the paired segmental nerves (SN). Antero-lateral to the heart, inosculations of segmental nerves one, two, and three form the right and left lateral pericardial plexuses (LPP) (Fig. 1B, 2A). Maynard (1953) described the LPP in P. argus; we will retain his

Fig. 1 (A) Diagram of a spiny lobster with its pericardial cavity and gill chamber exposed. The dorsal musculature was removed to provide this view. The heart is suspended in the pericardial chamber by three pairs of ligaments. The elastic ligaments aid in filling the heart during diastole.
(B) An enlarged, dorsal view of Fig. 1(A). Segmental nerves 1-3 (SN_{1-3}) anastomose in the rostral-lateral pericardial cavity forming the lateral pericardial plexus (LPP). The largest trunk leaving the plexus is the motor-ligamental nerve (ML) which was cut on the left side to expose the medial ligamental plexus and ramifications of segmental nerve 4 (SN_4). It continues caudally along the ventral surface of the dorsal musculature (shown on right) and branches forming the posterior ligamental nerve plexus (PLP). The dorsal nerve trunk (DNT) and the anterior ligamental nerve (ALN) project caudally from the LPP toward the heart. The ALN ramifies as the anterior ligamental nerve plexus (ALP).



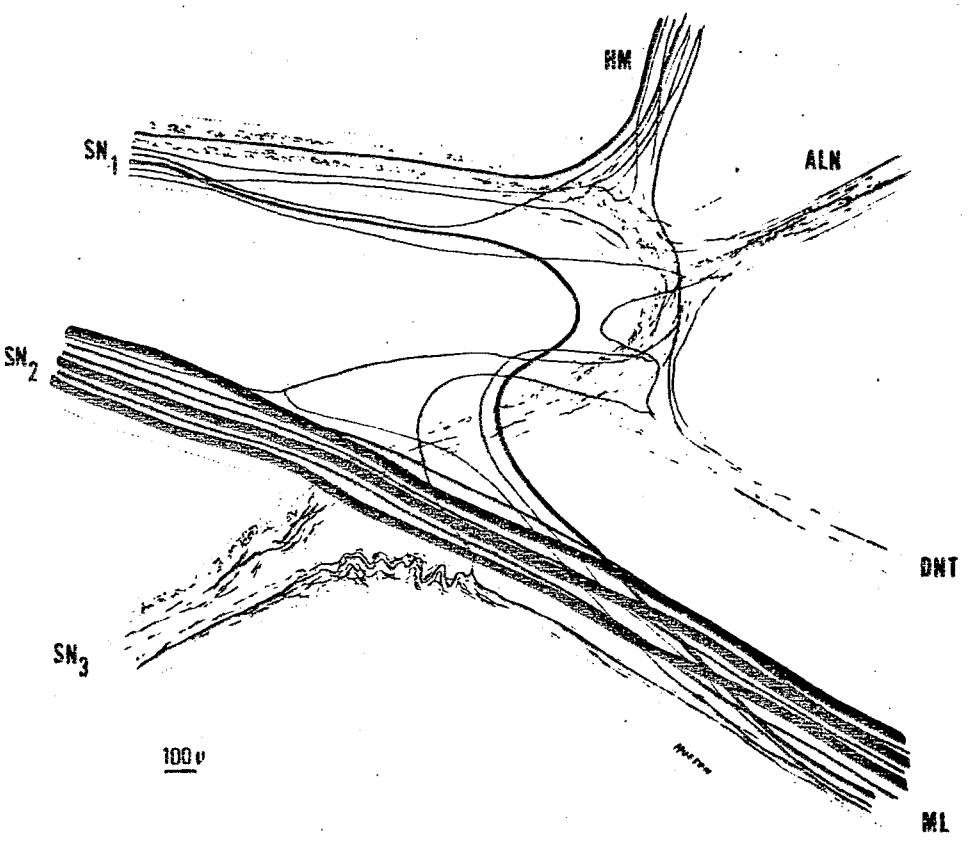

B
lpp
dmt
alp
mlp
plp
BOSTON
CM

terminology where possible. Figure 2A is a diagram of the left LPP in P. interruptus prepared from methylene blue-stained preparations. Stippled tracts represent bundles of small caliber fibers that did not stain completely. The most prominant features are six large (20-30 μ) motor axons which originate in SN_2 and continue to the dorsal musculature of the pericardial cavity in the motor-ligamental nerve trunk. Approximately ten smaller (5-10 μ) fibers enter the motor-ligamental nerve from SN_3, run pari passu with the motor axons for about one cm, and then diverge forming the posterior ligamental nerve trunks (Fig. 1B, 2B). About five bipolar cell bodies (20 x 50 u) are located in the initial segments of the posterior ligamental nerve trunks, where the trunks begin ramifying to form the posterior ligamental plexus (PLP). These are the only neuron somata that we have observed in the pericardial organs of P. interruptus. They resemble the bipolar cell bodies observed in bracyuran pericardial organs (Maynard 1961).

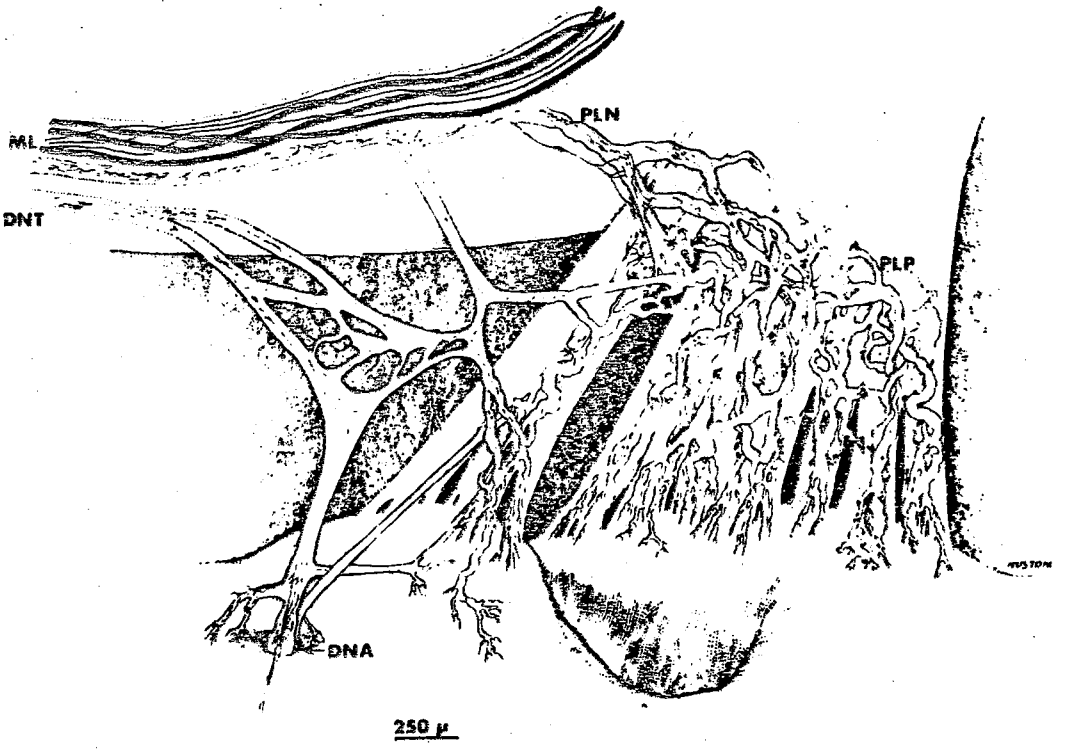
The dorsal nerve trunk and the anterior ligamental nerve trunk arise directly from the LPP and project posteriorly to the dorsal nerve apparatus and the anterior ligamental nerve plexus (ALP), respectively (Fig. 1B, 2A). Methylene blue stains 8-11 fibers in the dorsal, and at least seven in the anterior ligamental nerve trunks. Their origins from the three segmental nerves are indicated in Fig. 2A. The medial ligamental nerve plexus (MLP) arises

Fig. 2 (A) Diagram of the left lateral pericardial plexus and (B) the right posterior ligamental nerve plexus (PLP) and dorsal nerve apparatus (DNA) prepared from methylene blue fiber counts.
(A) Anastomoses of segmental nerves 1-3 (SN_{1-3}) form the lateral pericardial plexus and give rise to four nerve trunks, the motor-hypodermal nerve (HM), the anterior ligamental nerve (ALN), the dorsal nerve trunk (DNT) and the motor-ligamental nerve (ML). The DNT contributes neurosecretory processes to the DNA and carries inputs to the cardiac ganglion. ALN ramifies as the anterior ligamental nerve plexus. Same perspective as in Fig. 1(B).
(B) The ML carries the large motor fibers to the dorsal musculature and a bundle of smaller neurosecretory processes that branch to form the posterior ligamental nerves (PLN); these in turn ramify as the PLP (arrow denotes the cell body region). Branches of the DNT anastomose with ramifications of the PLN forming lamallae. The DNA lies partially hidden in a recess of the myocardium. Same perspective as in Fig. 1(A).

A



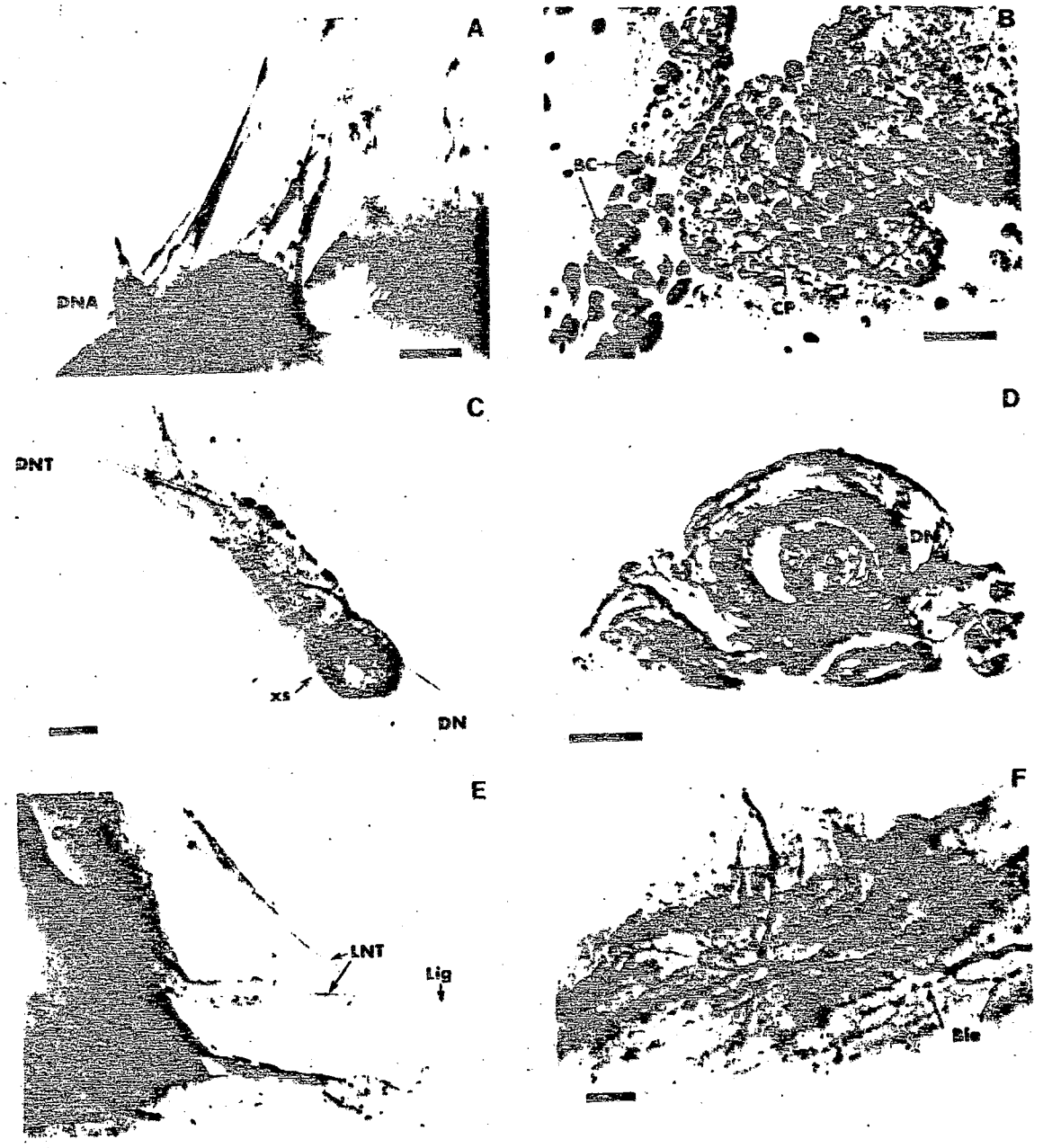
B



from a separate nerve trunk, tentatively designated SN_4 (Fig. 1B). In some preparations it appeared that ramifications of the posterior ligamental nerve trunk also contributed processes to the MLP.

The dorsal nerve trunk branches four times as it approaches the heart (Fig. 2B). The first two branches anastomose with the PLP. The other two ramify as plexuses on the myocardial surface in the vicinity of a small bulbous structure into which the dorsal nerve trunk passes. This is the dorsal nerve apparatus, and it deserves special consideration because of its unique structure and unknown function. The position of the bulb is variable. Often it is recessed in a socket but may lie exposed on the myocardial surface (Fig. 2B, 3A). Alexandrowicz (1932) and Maynard (1953) describe the bulbs as "particular swellings" and "slight swellings," respectively, of the dorsal nerve trunk at the surface of the myocardium. Histological examination has revealed that the bulb is composed of nervous, muscular, and connective tissue (Sullivan, unpublished). It has a hollow interior (Fig. 3D) with an opening on the end inserted into the myocardium. In cross section, two groups of nerve fibers can be distinguished. One group (Fig. 3D) contains three or four axons (5-10 μ) with prominent glial wrappings. These pass directly through the bulb, emerging as the dorsal nerve (Fig. 3C).

Fig. 3 Light micrographs of ligamental nerve trunks, ligamental nerve plexuses, and the dorsal nerve apparatus.
(A) Right posterior ligamental nerve plexus stained with methylene blue. Neural processes follow ligaments to their attachments around the perimeter of the posterior ostium (Ost). The dorsal nerve apparatus (DNA) lies exposed on the pericardial surface.
(B) Cross section of a major branch of a posterior ligamental nerve trunk, stained with toluidine blue. The nerve trunk, containing large, ensheathed core processes (CP), is juxtaposed with the posterior ligaments (Lig) and with hemolymph. (BC, blood cell.)
(C) A dissected dorsal nerve apparatus showing the dorsal nerve trunk (DNT) entering, and the dorsal nerve leaving, the muscular bulb. The arrow by xs indicates the level of the cross section shown in (D).
(D) Cross section of a dorsal nerve apparatus stained with Mallory's trichrome. Only the bundle of large, ensheathed axons, the dorsal nerve (DN), is prominent at this level (see text).
(E) A left anterior ligamental nerve plexus stained with methylene blue, showing ligamental nerve trunk branches (LNT) following ligaments (Lig) to their destinations around an anterior ostium (Ost).
(F) Anterior ligamental nerve trunk stained with methylene blue. Fine aborizations of a nerve process in the core of the trunk (CP) extend into the cortex and assume a bleb-like or beaded appearance (BLE) upon staining.
Calibration marks: A and E, 500 μ; B and D 30 μ; C, 200 μ; F, 100 μ.


A
DNA
B
BC
CP
C
DNT
XS
DN
D
DN
E
LNT
Lig
F
Ble

The other group (not shown) is spacially separated from the first and is composed of 30-40 smaller (2-5 μ) processes. These are continuous with fibers which also enter the last two branches of the dorsal nerve trunk. They apparently terminate at the level of the bulb as they are not represented in the dorsal nerve. Both bundles are surrounded by a thick sheath juxtaposed to the muscle fibers. In several preparations we observed the bulb contracting rhythmically. In situ, it appeared to beat 180° out of phase with the heart, but would also beat in isolation in some cases.

Ligamental nerve plexuses were examined after methylene blue staining and in cross sections stained with toluidine blue. The nerve trunks ramified extensively forming branches 50-130 μ in diameter, in close association with ligaments (Fig. 3A, E). Although usually attached to the peripheral surface of the ligaments, they completely surround ligaments in some cases. Within these branches, large central nerve fibers were observed to project processes peripherally that seemed to terminate in a series of bleb-like structures (Fig. 3F). Cross sections of individual ramifications were characterized by a central core of large fibers surrounded by a 8-20 μ cortex of smaller fibers (Fig. 3B). Core processes ranged from 4-14 μ in diameter and most were wrapped in layers of glial membrane. Cortical processes were less than 3 μ in diameter. This topological

arrangement of nervous fibers is characteristic of pericardial neurosecretory structures (Maynard and Maynard, 1961).

Ultrastructure

Thin sections of anterior and posterior ligamental plexuses were examined with the electron microscope. The ultrastructure of the plexuses was similar to that described for other crustacean neurosecretory structures (Maynard and Maynard, 1962; Bunt and Ashby, 1967; Andrews, 1973). The branches of the nerve trunks were surrounded by an acellular epineurium or sheath, 0.3-1.0 μ thick, as in brachyuran pericardial organs. As noted above, large processes (4-14 μ) formed a central core. They contained mitochondria, microtubules, and occasional electron-opaque vesicles. Glial cells and extracellular material were interspersed among the individually ensheathed fibers.

The smaller fibers, surrounding the core of large processes, ranged from 0.17 to 3 μ in diameter and contained numerous vesicles as well as mitochondria (Fig. 4). The most peripheral processes lacked glial wrappings. Many showed morphological indications of release sites, characterized by dense clusters of vesicles next to membrane thickenings that were nearly always adjacent to the epineurium (Fig. 5A-D). Some processes appeared to terminate upon another neural element, but examples were so

Fig. 4 A low power electron micrograph of a posterior ligamental nerve plexus branch exhibiting profiles with Class A, C and D vesicles, as summarized in Table 1. The epineurium (Epi) contacts the hemolymph space (clear area). Ligament tissue (Lig) is seen on the left. X 12,152. Bar:1 μ.

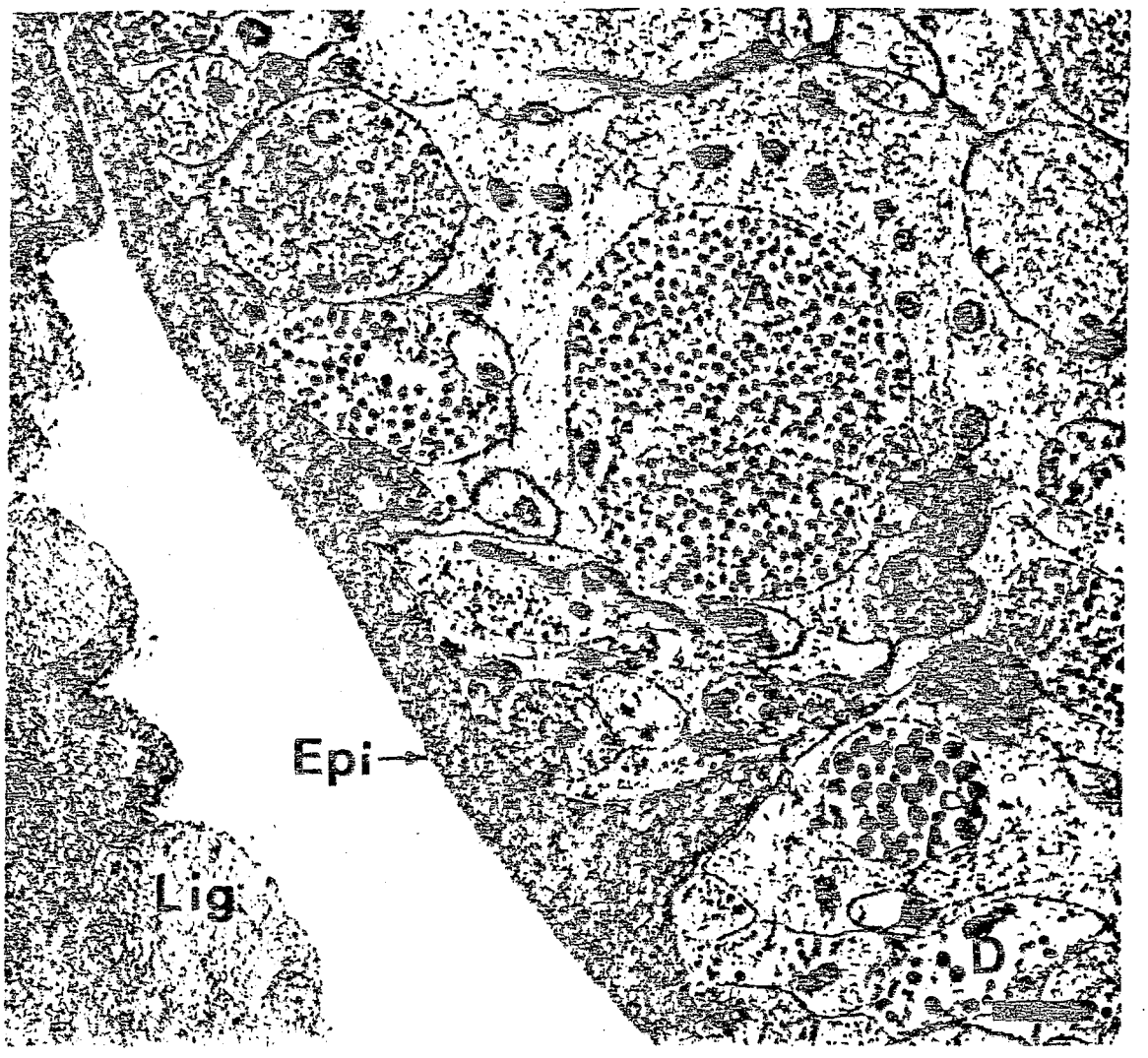
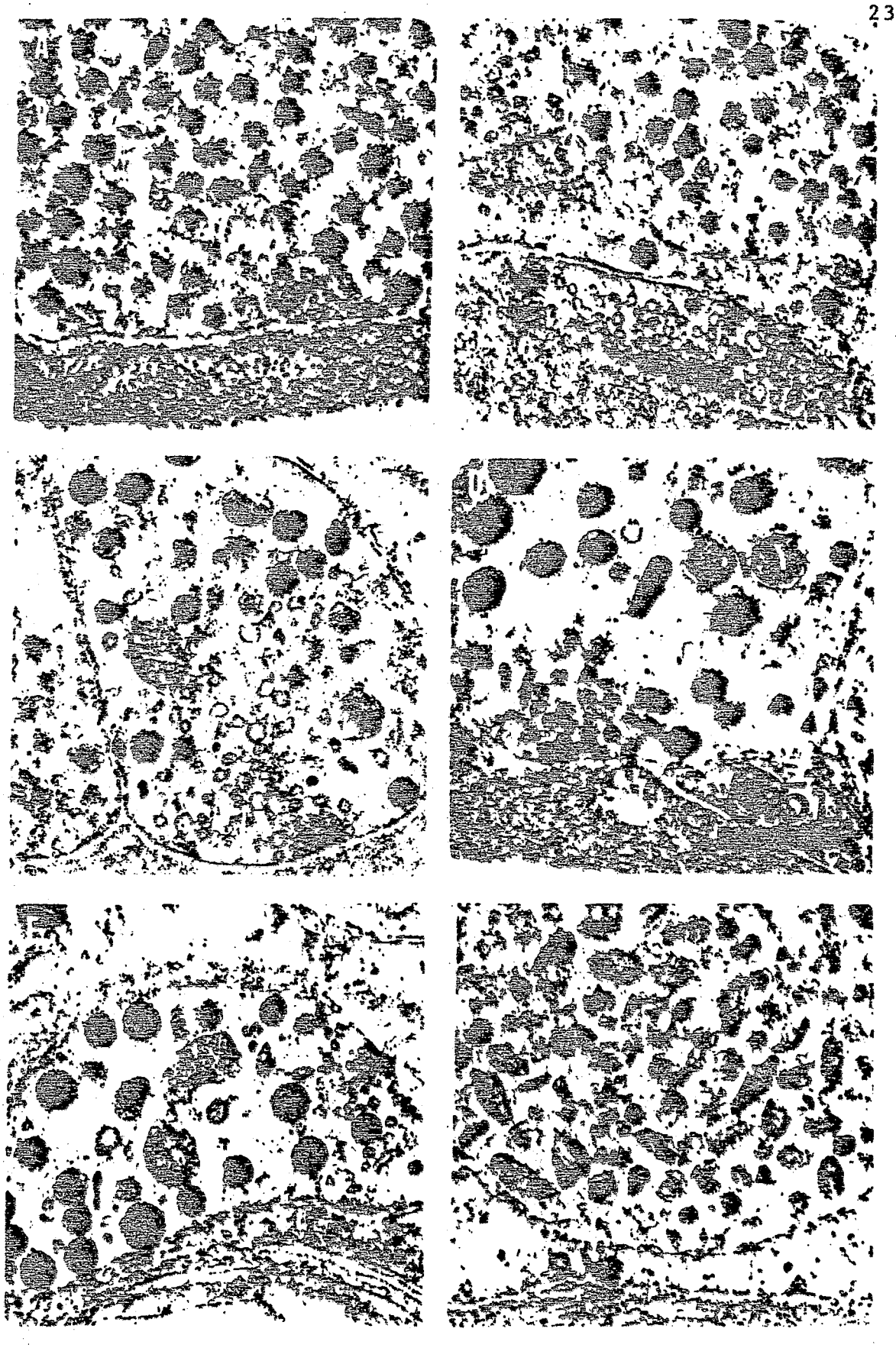

Epi
Lig
A
D

Fig. 5 Electron micrographs of vesicle classes in ligamental nerve plexuses. Letters A to F correspond to vesicle classes as described in text. Arrows indicate apparent release sites apposed to the epineurium. Scale in E applies to all. X 36,708. Bar:250 nm.



rare that one cannot be certain that pre-terminal synapses are a standard feature of the plexuses. I did not observe neuronal somata in the ligamental nerve plexuses. The only somata associated with the three pairs of plexuses seem to be the few bipolar cells near the initial branches of the posterior ligamental nerve trunks (see above).

Near the epineurium, neuronal fibers were filled with a variety of dense and clear vesicles. The types and distribution of vesicles near apparent release sites allowed me to distinguish five different classes of neurosecretory fibers present in both anterior and posterior ligamental nerve plexuses. The characteristics of each class are summarized in Table 1, and examples are shown in Fig. 5A-E. Processes belonging to one of these five classes could usually be categorized without difficulty, even in the absence of "release" sites. At first I was not certain that class B fibers were distinct from those of class C, since the general distribution of clear vesicles within class C fibers resulted in an occasional profile that lacked the large, "crystalline" vesicles but was filled with clear ones. However, many of these profiles appeared distinctly unlike class C in that they usually lacked mitochondria and their vesicles seemed more densely packed, smaller, and more uniform in size and shape than the clear vesicles in class C processes. To establish this point, the diameters of

TABLE 1

Morphological Characteristics of Neurosecretory Processes

CLASS	VESICLE TYPES				MITOCHONDRIA
	Cytoplasm		"Release" Sites		
	$\bar{D}c$[a], nm	Distribution, Form	$\bar{D}c$, nm	Distribution, Form	
A	140	Dense core, granular matrix, closely packed, filling profile	35	Clear or small dense core, clusters only at membrane	Rare
B		(Small, clear only)	39	Clear, relatively homogeneous, usually closely packed throughout profile	Sparse or Absent
C	143	"Crystalline" or opaque matrix, some irregular shapes, matrix often not filling vesicle	54	Clear, irregular size and shape, clustered at membrane, throughout profile	Usually several, large, mixed with vesicles
D	226, 138	Two maxima in size distribution, opaque to granular, round to elongate	64	Dense core nearly fills vesicle, clustered at membrane, sparse elsewhere	Usually 0-2 near "release" site, mixed with large vesicles
E	147	Very dense to granular matrix, usually round, uniform in size	38	Clear, clustered at membrane, sparse or absent elsewhere	One or more among lg.vesicles
F	270 x 114 67	Oval to spindle shaped, irregular, closely packed Round, dense core, broken membrane may be absent, no "release" sites			

a Average diameter, corrected for section thickness of 70 nm.

several hundred vesicle profiles were measured in terminals of the two kinds. The apparent average diameters were corrected to "true" values (Froesch, 1973) using an estimated thickness of 70 nm, giving $\bar{D}_B$= 39.5 ± 7.2 nm and $\bar{D}_C$= 53.5 ± 12.6 nm. The differences between the mean diameters (Student's *t* test) and their variances (*F* test) are both highly significant ($p<0.001$), confirming the visual impression of a distinct class of fibers containing small, clear vesicles only.

A sixth fiber type, class F (Fig. 5F, Table 1), was distinguishable in sections from the PLP but was not evident in my ALP sample. I did not find a clear indication of class F release sites. It appeared that other classes were also present in the ligamental plexuses but these were either too rare or variable in preservation to be unambiguously characterized.

The large, dense granules present in class D terminals varied from 100 nm to 280 nm in diameter but were distributed between two distinct populations of mean diameter 226 nm and 138 nm. Both distributions were skewed, with maxima at 205 nm and 145 nm, respectively. Occasional elongate or dumbbell-shaped structures with identical dense appearance suggested that the larger vesicles may divide to produce the smaller ones. This notion can be supported by a simple calculation given two reasonable assumptions: 1)

the vesicles are bounded by a limiting membrane, and 2) the surface area of this membrane is conserved during the division process. The surface areas of two spheres of diameter 205 nm and 145 nm (the maxima of the size distributions of the two dense granules) are 13.2×10^4 nm^2 and 6.6×10^4 nm^2, respectively; their ratio is 2.0.

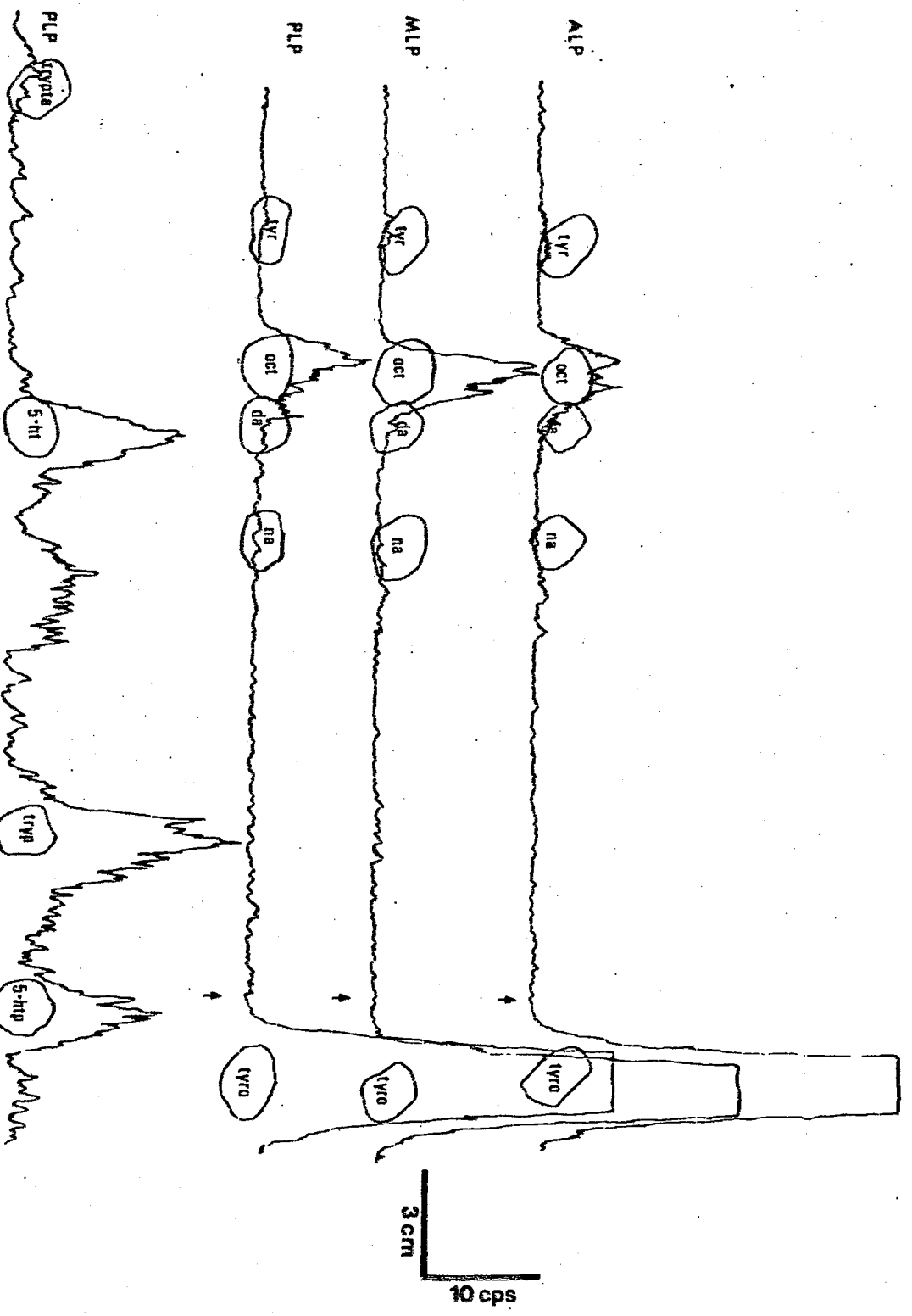
Neurochemistry

The methylene blue studies strongly suggested that the ligamental nerve plexuses and dorsal nerve apparati were neurosecretory structures. The ultrastructural observations provided further evidence and also indicated six or more different classes of neuronal elements, implying storage and release of a similar number of neurotransmitters or hormones. There was evidence that brachyuran pericardial organs contain catecholamines, phenolamines, and serotonin (5-hydroxytryptamine) (Maynard and Welsh, 1958; Clay, 1968; Barker and Hooper, 1975; Sullivan, unpublished). Consequently, I investigated the capacity of the plexuses and dorsal nerve apparatus to synthesize and accumulate these compounds and also acetylcholine.

Pericardial nervous structures were dissected from the pericardial cavity of P. interruptus and incubated with radioactive compounds known to be metabolic precursors of acetylcholine and the aromatic amines in other systems (see Methods).

In Homarus americanus, octopamine is formed by decarboxylation of tyrosine to yield tyramine, followed by B-hydroxylation of the side chain (Barker et al., 1972; Wallace, 1976). We observed ^{3}H-octopamine and ^{3}H-tyramine synthesis from ^{3}H-tyrosine in virtually all pericardial nervous tissues, including the ligamental nerve plexuses, dorsal nerve apparatus, SN_1, and the nerves innervating the valves of the ophthalmic and antennule arteries. Figure 6 (top three traces) shows radioscans of the amine region of electropherograms obtained by subjecting acetone-formic acid extracts of ^{3}H-tyrosine-incubated ligamental nerve plexuses to high voltage electrophoresis. Quantitative analysis of an electropherogram by scintillation counting of 0.5 cm sections of the paper is illustrated in Figure 7 for a dorsal nerve apparatus extract. The chemical identity of the octopamine synthesized was confirmed for all plexuses by rechromatography of material eluted from the octopamine position in three TLC solvent systems. The pattern of tyrosine metabolism was the same in all the ligamental nerve plexuses. Varying the time of incubation from 20 min to 6 hr showed that the radioactive tyramine pool reached a steady-state level in 20 min or less and remained constant or gradually declined thereafter. The labeled octopamine pool accumulated more slowly, reaching a maximum at about one hour with little change in the next five hours.

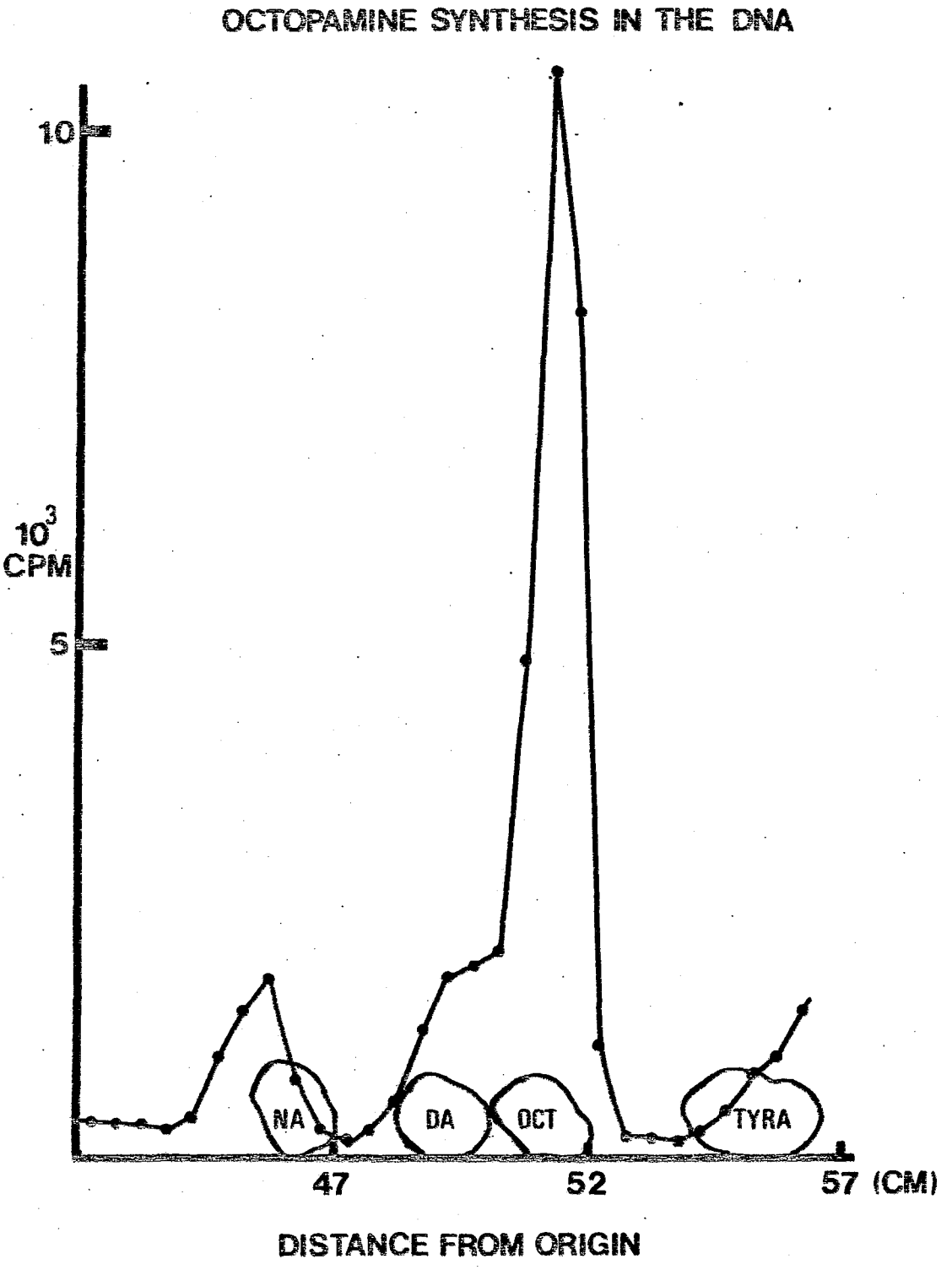
Fig. 6 Radioscans of electropherograms demonstrating the separations of ^{3}H-tyrosine and ^{3}H-tryptophan metabolites synthesized in incubation experiments (see text). Arrows indicate 30.5 cm to the left of the origin, toward the cathode. The gradient was 40v/cm and durations of runs were 2.5 hr, pH 1.9. Counting efficiency 0.5%. List of abbreviations: tyr = tyramine; oct = octopamine; da = dopamine; na = noradrenaline; tyro = tyrosine; trypta = tryptamine; 5-ht = 5-hydroxytryptamine; tryp = tryptophan; 5-htp = 5-hydroxytryptophan; others as in Fig. 1(B).


ALP
MLP
PLP
PLP
tyr
oct
da
na
tyro
5-ht
tryp
3 cm
10 cps

Octopamine was by far the major phenolamine accumulated. The ratio of ^{3}H-octopamine to ^{3}H-tyramine was about 1:1 after 20 min, and 50-80:1 at five to six hours. Maximal accumulation of the labeled metabolites is summarized in Table 2 for the various pericardial nervous structures. The ^{3}H-tyrosine concentration was varied from 8 to 100 µm, but any dependence of the rate of synthesis or maximal accumulation on precursor concentration in this range was obscured by the variability among different preparations.

Since neutral red can be a useful vital stain aiding the identification of dopamine, serotonin (Stuart et al., 1974), and octopamine (Wallace et al., 1974) neurons, I felt that it would be of interest to investigate any effects that the dye may have on octopamine synthesis. A PLP was incubated in 8 µm ^{3}H-tyrosine in the presence of 0.1 mg/ml neutral red for one hour and then washed with normal saline for five hours. The tyrosine was rapidly taken up, falling to 44% of the initial concentration in 20 min, after which it remained essentially constant. Although preterminal axons did not stain, cortical processes retained the dye after the prolonged wash. Extraction of the tissue and analysis of soluble radioactive material revealed that only ^{3}H-tyrosine and ^{3}H-octopamine remained in significant amounts. Octopamine accounted for 39% of the total radioactivity, or 1.34 pmol, within the range for similar

Fig. 7 Amine region of an electropherogram demonstrating dopamine, octopamine and tyramine synthesis from ^{3}H-tyrosine in the dorsal nerve apparatus quantified by liquid scintillation counting. Octopamine synthesis was exceptional in this experiment. Peak in NA region is "octopamine-fast metabolite" (See Evans, Kravitz, Talamo and Wallace, 1976). Abscissa is distance of cathodal migration, see text for separation details.


OCTOPAMINE SYNTHESIS IN THE DNA
10
10^3 CPM
5
NA
DA
OCT
TYRA
47
52
57 (CM)
DISTANCE FROM ORIGIN

experiments in the absence of neutral red. Since it has also been found that 0.1% neutral red has no effect on the physiological activity of leech Retzius cells (Stuart et al., 1974) or on the motor output pattern generated by the lobster stomatogastric ganglion (Barker and Kushner, unpublished), neutral red may prove to be a valuable aid in the identification of octopaminergic cells and terminal processes during both biochemical and physiological experiments.

Dopamine was the major catecholamine metabolite of tyrosine in both the ligamental nerve plexuses and the dorsal nerve apparatus. In these structures, dopamine synthesis was generally an order of magnitude less than octopamine synthesis, but was about the same in SN_1 (Table 2). As Figure 6 indicates, dopamine synthesis in the ligamental nerve plexuses could not be quantified reliably by analysis of electropherograms alone because of overlap with the large octopamine peak. When the radioactivity eluted from the dopamine position of electropherograms was analyzed by TLC in solvent II, only 29 ± 6% co-chromatographed with authentic dopamine. For ligamental nerve plexuses, the dopamine values in Table 2 are corrected for this problem. Dopamine synthesis in the dorsal nerve apparatus was confirmed by using a micro-alumina adsorption procedure followed by TLC in solvent III. Norepinephrine synthesis

TABLE 2

Accumulation of Biogenic Amines and Acetylcholine in Pericardial Nervous Tissues[a]

COMPOUND / tissue	DA	OCT	TYRA	5-HT	ACH
PLP	0.06-0.75 0.28±0.12(5)	0.87-22.6 7.37± 3.88(5)	0.02-2.64 1.09±0.49(5)	11.8-19.7 14.5± 2.62(3)	0.11-0.20 0.16±0.03(3)
MLP	0.10-0.17 0.13±0.02(4)	1.05- 9.76 3.84± 2.00(4)	0.04-0.08 0.06±0.01(4)		
ALP	0.04-0.25 0.13±0.04(4)	1.26- 3.76 2.44± 0.63(4)	0.02-0.09 0.06±0.02(4)	10.17	0.06
DNA[b]	0.06-0.15 0.12±0.03(3)	0.04- 0.79 0.30± 0.24(3)	0.02-0.10 0.05±0.02(3)		
SN_1[b]	0.02-0.06 0.04(2)	0.01- 0.05 0.03(2)	0.01-0.06 0.04(2)		

a Nerves were incubated in lobster saline containing ^{3}H-tyrosine for 1-6 hr, in ^{3}H-tryptophan for 5-6 hr or in ^{3}H-choline for 1-6 hr. Data is expressed as range and means ± S.E.M.(N), in pmol/tissue sample.

b Dopamine values are uncorrected; see text. Note equal distribution of tyrosine metabolites in segmental nerve 1 (SN_1). Other abbreviations are the same as in Fig. 6.

was not detected in any of the structures analyzed. The peak of radioactivity just preceding norepinephrine in Fig 7 was found to be a cationic metabolite of octopamine, almost certainly the same substance observed in Homarus and designated "fast product" by Evans et al. (1976). Its chemical identity is being investigated by Kravitz and co-workers (personal communication).

Tryptophan metabolism was studied in the posterior and anterior ligamental plexuses. Although it has been known for some time that brachyuran pericardial organs contain serotonin (Maynard and Welsh, 1958), its pathway of biosynthesis in crustaceans has apparently not been established previously. In a series of preliminary experiments, Barker and Hooper (unpublished) found that crab pericardial organs (Cancer magister) produced serotonin from both tryptophan and 5-hydroxytryptophan, indicating the same biosynthetic pathway used by vertebrates. This was confirmed in the present work for the PLP and ALP in P. interruptus (Table 2). The bottom trace in Fig. 6 is a radioscan of ^{3}H-tryptophan metabolites from a PLP, separated by high voltage electrophoresis, demonstrating synthesis of 5-hydroxytryptophan, serotonin and an unidentified metabolite(s) migrating between serotonin and tryptophan. Synthesis was confirmed by TLC of eluted material in three solvent systems. Tryptamine synthesis was not detected. As noted above, a

prolonged washing with normal saline after incubating with radioactive precursors is useful for indicating which metabolites are stored in neurosecretory tissue. A PLP was incubated in 12 μM ^{3}H-tryptophan for three hours, washed in normal saline for 12 hours, and then extracted metabolites were analyzed by electrophoresis. The unidentified metabolite(s) was not stored. The only tritiated compounds detected were tryptophan (2.2 pmol), 5-hydroxytryptophan (2.4 pmol), and serotonin (19.3 pmol). The relative proportions of the three compounds, 1.0:1.1:8.8, were distinctly different from the proportions found after a brief wash, where tryptophan>serotonin = 5-hydroxytryptophan (Fig. 6, bottom trace).

Only the PLP was tested in three experiments, for conversion of ^{3}H-choline to ^{3}H-acetylcholine, and acetylcholine was produced. The amount of acetylcholine accumulated was considerably less than the amounts of octopamine or serotonin (Table 2).

Octopamine Release

If the compounds synthesized and stored by the ligamental nerve plexuses serve a neurosecretory role, it should be possible to evoke their release by electrical stimulation of the appropriate preterminal axons, given sufficiently sensitive assay procedures. A convenient method follows

from the synthesis and accumulation experiments reported above, provided the radioactive substances synthesized enter the readily releasable pool of secretory material. Two methods of octopamine labeling (using either ^{3}H-tyrosine of ^{3}H-tyramine as precursor) and two methods of evoking release (pulsing with high potassium and electrical stimulation) were investigated and all proved effective. The results reported in this chapter were obtained by electrically evoked release of octopamine synthesized from ^{3}H-tyramine. The use of ^{3}H-tyramine as precursor has the advantage of rapid wash-out to a low background efflux of radioactivity (Evans et al., 1975). In preliminary experiments, MLP's were incubated in 91 μm ^{3}H-tyramine for periods of 30 min to 5 1/2 hr to determine the amount of octopamine synthesized and accumulated from its immediate precursor. Electrophoretic analysis of MLP extracts indicated that the major products were octopamine, octopamine-fast metabolite and a compound co-chromatographing with dopamine. The amount of octopamine accumulated varied from 1.91 pmol to 29.2 pmol and total synthesis (taken as the sum of octopamine and "fast metabolite") from 9.05 pmol to 31.8 pmol. The average accumulation, 11.1 pmol of octopamine, was approximately 3 times the amount accumulated when tyrosine was used as precursor.

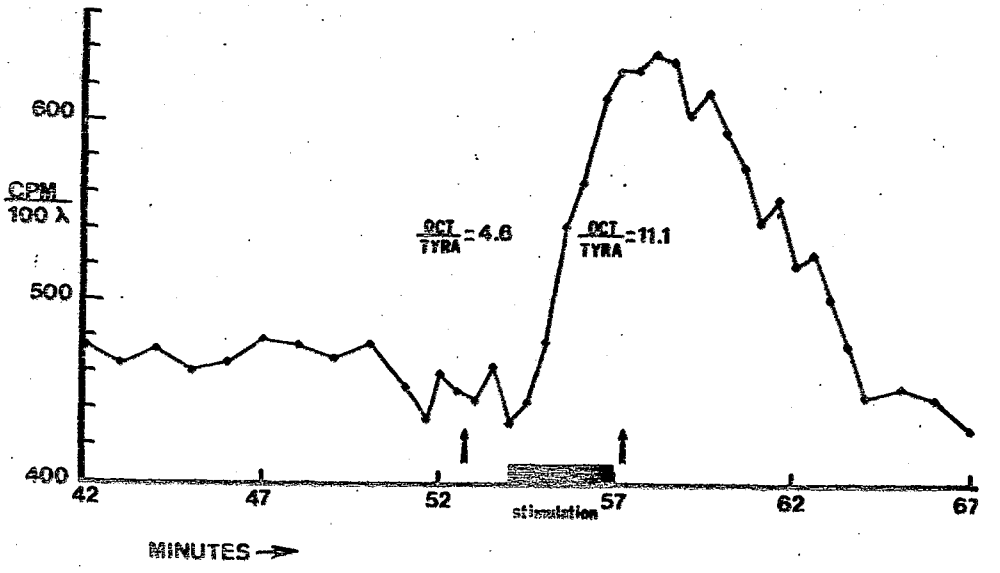
The posterior ligamental plexus was chosen for

experiments in which release was evoked by electrical stimulation of preterminal axons because the long (about 1 cm) motor-ligamental nerve trunk allowed placement of both stimulating and recording electrodes. Two experiments are presented, the first demonstrating selective release of octopamine and the second showing the dependence on calcium ion and stimulation frequency. A PLP was incubated in saline containing 150 μm ^{3}H-tyramine for five hours and then perfused with normal saline at a rate of 1 ml/min. After 40 min of washing, efflux of radioactivity had declined to a low and constant level (Fig. 8A). Stimulation of the motor-ligamental nerve for three minutes at 20 Hz caused a rapid increase in the efflux of radioactivity. Samples of perfusion fluid obtained just before and at the end of stimulation were analyzed by TLC and found to contain ^{3}H-tyramine and ^{3}H-octopamine. The ratio of radioactive octopamine to tyramine was 4.6 before, and 11.1 at the end of stimulation, indicating a selective increase in ^{3}H-octopamine induced by the stimulation (Fig. 8A).

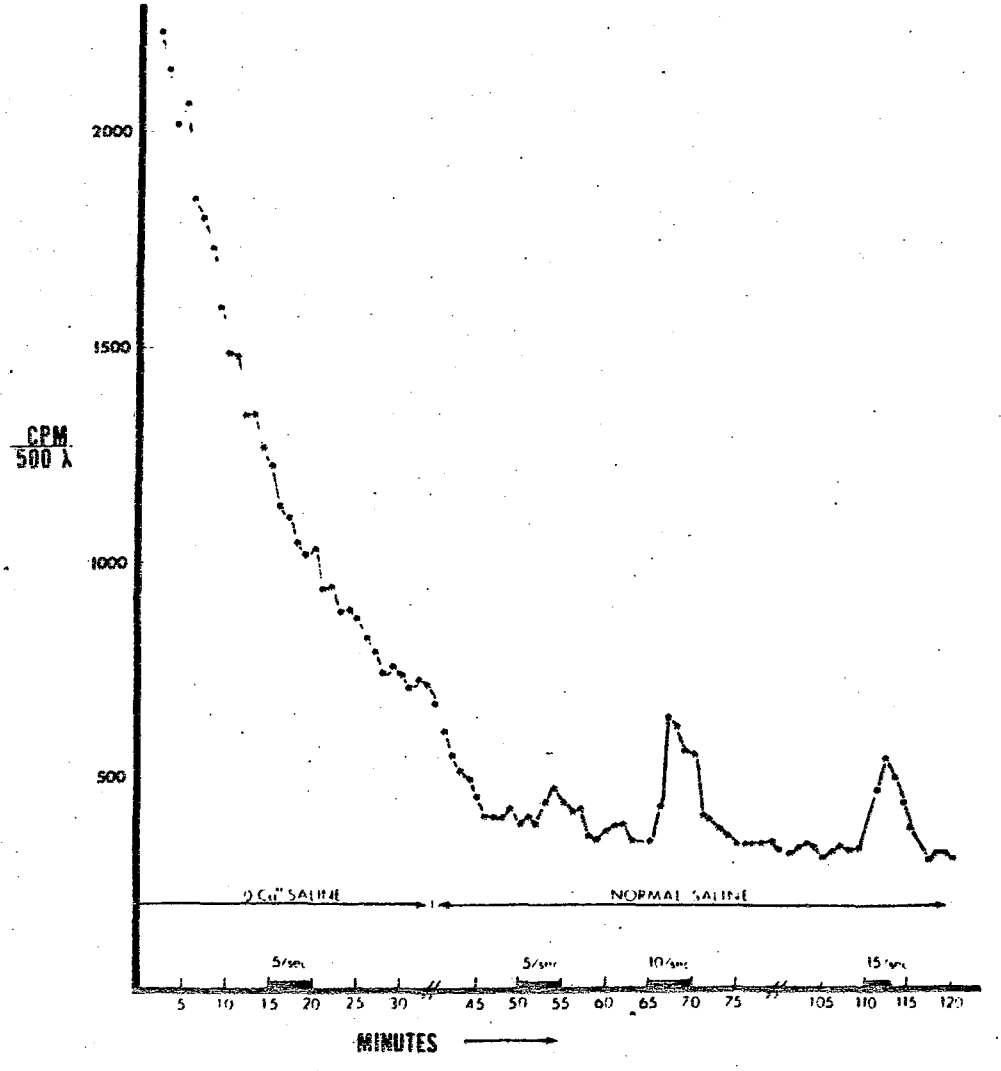
During experiments in which octopamine release was evoked by high potassium, it was found that replacing 90% of the calcium with magnesium had little effect on the amount of radioactivity released from the Panulirus PLP (data not shown). However, release was substantially inhibited when all calcium was replaced with sucrose of equal

Fig. 8 (A) Selective release of ^{3}H-octopamine from the posterior ligamental nerve plexuses by electrical stimulation. A PLP was incubated in oxygenated saline containing 150 μM ^{3}H-tyramine (6.21 Ci/mM) and 10^{-4}M ascorbate for five hr and subsequently perfused with normal saline in a continuous-flow chamber of volume 3.0 ml at a rate of 1 ml/min for an additional two hr prior to t=42. Ligamental nerve trunks were stimulated at 10V, 20/sec for 3 min. During the time interval t=52.0-62.0, 0.5 ml fractions were collected every 30 sec; other fractions (0.5 ml) were collected at 1 min intervals. A portion of each fraction, 0.1 ml was placed in a scintillation vial and counted. At t=53.0, 53.5, 58.0 and 58.5 (denoted by arrows), the remainder of the 0.5 ml samples were desalted and analyzed for their ^{3}H-octopamine and ^{3}H-tyramine contents. The averaged molar ratios are shown above the arrows.
(B) Calcium dependency of ^{3}H-octopamine release. A PLP was incubated in 10 μM ^{3}H-tyramine (10.78 ci/mM) for 1 hr and washed for 3 hr; 10 min prior to t=0 the saline was changed to 0 Ca^{++} plus an osmotic equivalent of sucrose. The chamber volume was 1.5 ml; perfusion rate was 1.0 ml/min. Aliquots (0.5 ml) were removed every minute and placed directly into scintillation vials for counting. Slash marks denote intervals when the syringe pump was turned off to change or replenish saline reservoir. Other parameters the same as in (A).

A


600
CPM
100 λ
500
400
OCT/TYRA = 4.6
OCT/TYRA = 11.1
42
47
52
stimulation
57
62
67
MINUTES

B


2000
1500
CPM
500 λ
1000
500
0 Ca++ SALINE
NORMAL SALINE
5/sec
5/sec
10/sec
15/sec
5
10
15
20
25
30
45
50
55
60
65
70
75
105
110
115
120
MINUTES

osmolarity. Berlind and Cooke (1968) had previously shown this procedure to block stimulation-induced release of cardio-acceleratory activity from the crab pericardial organ, without preventing axonal conduction of action potentials. Therefore, this method was adopted to examine the dependence of octopamine release from the PLP on calcium ion. The data illustrated in Fig. 8B were obtained from a PLP incubated for one hour in 10 μm ^{3}H-tyramine (10.2 Ci/mmol) followed by a 3 hr wash with normal saline. The perfusion medium was changed to zero calcium saline (osmolarity maintained with added sucrose) flowing at 1 ml/min. After 25 min of zero calcium perfusion, the motor-ligamental nerve was stimulated at 5 Hz for five minutes. After another 15 min, the perfusion solution was changed back to normal saline and three further stimulation periods, at 5, 10 and 15 Hz, occurred at the times indicated in Fig. 8B. The evoked compound action potential was recorded from one of the ligamental nerve trunks just after its departure from the motor-ligamental nerve (see Fig. 1B). In zero calcium saline, there was no indication that units failed. Instead, there was a significant increase in the amplitude of the compound potential and slight shifts in the conduction velocity of fiber classes, as reported by Berlind and Cooke (1968). Although the spontaneous efflux of radioactivity in this experiment had not reached a steady

background at the time of stimulation in zero calcium, there was no indication of a deviation from the exponential decline of radioactivity in the medium, suggesting effective blockade of evoked octopamine release. The subsequent stimulation periods in normal saline produced significant release detectable in the first minute of stimulation at 10 and 15 Hz. The relative proportions of evoked release per time of stimulation at 5, 10 and 15 Hz was 1.0:2.7:2.9. Repetition of the same stimulus usually evokes substantially less release, suggesting that this preparation may not have recovered completely from the effects of zero calcium at the time of the 5 Hz stimulation.

It has also been possible to demonstrate release of ^{3}H-serotonin by electrical stimulation following preincubation of the PLP in ^{3}H-tryptophan. These results and further characterization of the properties of serotonin release will be reported in Chapter II.

Discussion

Although these investigations stem directly from my Master of Arts work in Walter Morin's laboratory at Massachusetts State College at Bridgewater, the rediscovery of ligamental nerve plexuses as a form of pericardial organs developed rather serendipitously. While searching for the extrinsic inputs to the cardiac ganglion, I

noticed the impressive array of methylene blue-staining fibers associated with the ligaments supporting the heart. My attention was immediately drawn to the location of these structures; it appeared ideal for efficient delivery of neurosecretory substances into the arterial circulation. The anterior and posterior ligamental attachments nearly surround ostia so that material released from their associated neural structures would be drawn directly into the heart during diastole. It seemed probable that I was viewing elements belonging to the system of pericardial organs, but a review of the literature produced only two tentative assignments of neurosecretory function based on limited anatomical observations (Alexandrowicz, 1953; Maynard, 1960). Consequently, I initiated a study of the anatomy, ultrastructure, and neurochemistry of the ligamental nerve plexuses in Panulirus interruptus and now offer the following evidence that they are indeed components of the neurosecretory system of pericardial organs and that octopamine and serotonin are two of several secreted hormones:

1. The neural origin and gross morphology of ligamental nerve plexuses are analogous to those of brachuran pericardial organs.

2. Cortical processes exhibit what appear to be the terminal aborizations of secretory neurons. These

terminals have been characterized into six or more different classes according to their vesicle morphology.

3. The plexuses of the ligamental nerves and dorsal nerve apparatus synthesize and accumulate octopamine, serotonin, dopamine and acetylcholine.

4. Octopamine is released from the PLP by a calcium-dpenedent mechanism upon electrical stimulation of pre-terminal axons in the ligamental nerve trunk. In similar experiments (Sullivan, in preparation), we have found that serotonin is also released by this plexus. The known effects of these amines on the heart, stomatogastric ganglion, thoracic ganglia, hemocytes, and skeletal muscle argue strongly in favor of their roles as neurohumoral agents in the decapod Crustacea.

Anatomy

The ligamental nerve plexuses and dorsal nerve apparatus are continuous with segmental nerves 1-3, and thus have the same origin as other decapod pericardial organs (Maynard, 1960). At both light and electron microscopic levels, ligamental nerve plexuses resemble brachyuran pericardial neurosecretory processes (Maynard and Maynard, 1962) and the anterior ramifications of SN_1 (Andrews, 1973). The plexuses are extensive nerve trunk ramifications that contain an inner core of larger axons with glial wrappings

and an outer cortex of fine, varicose processes separated from hemolymph in the pericardial sinus by a thin epineurium.

Ultrastructure

The cortical processes contain a variety of dense and clear vesicles, generally more than one type in the same ending. We distinguished six classes of nerve fibers, five of which show apparent release sites characterized by clusters of vesicles next to membrane thickenings abutting the epineurium (Fig. 5, Table 1). Four of these latter classes (A, C, D, E) contain large, dense vesicles distributed throughout the cytoplasm and smaller, dense cored (class D) or clear (classes A, C, E) vesicles clustered at apparent release sites. The presence of apparent release sites proved essential for distinguishing certain classes as unique, because the smaller vesicles are located primarily or exclusively near membrane thickenings in classes A, D and E. In addition, profiles of classes C, D and E containing only the large, dense granules may appear quite similar and we would have been reluctant to define them separately without the criterion of obviously different "release" vesicles. (Of course, a dynamic event such as release cannot be established from static data alone; there remain other possible events at the membrane specializations, such as recapture of vesicle membrane.)

There are several risks involved in attempting to identify vesicle types observed in one study with those described in others, as different fixation and staining methods applied in the same or related species may produce spurious similarity aswell as obscure correspondence. I make these comparisons cautiously, as general indications of the homology of brachyuran pericardial organs with macruran ligamental nerve plexuses. The early papers of Knowles (1962) and Maynard and Maynard (1962) show processes in crustacean pericardial organs that are filled with vesicles similar in size and appearance to those in my class A processes. Andrews (1973) observed processes in Uca and Callinectus that may correspond to my classes A, B, C and D. He also distinguished two or more vesicle types, with smaller ones clustered at membrane specializations, in processes ocrresponding to classes A, C and D. His Group 3 granules "are characterized by a crystalline-core matrix which ranges in appearance from alternately light and dark banks to crisscrossed bands," (Andrews, 1973). These are mixed with small clear vesicles giving a striking resemblance to my class C (Fig. 5C). Similar vesicles, showing an ordered internal matrix, and possible class A fibers, are present in the second roots of thoracic ganglia in Homarus americanus (Evans et al., 1975). The same area of these roots shows synthesis of serotonin and octopamine and contains several neurons whose axons project along the lateral

branches of the roots to pericardial organs (Evans, Kravitz, Talamo and Wallace, 1976; Evans, Kravitz and Talamo, 1976). These authors do not show apparent release sites with clusters of small vesicles, so correspondence with my class A and C fibers is tentative.

Hormone and Transmitter Candidates

The anterior, medial, and posterior ligamental nerve plexuses, the dorsal nerve apparatus and SN_1 all synthesize dopamine and octopamine. The ALP and PLP were shown to synthesize serotonin and acetylcholine as well and the MLP contains amounts similar to the other plexuses. Tyramine and 5-hydroxytryptophan are intermediates in the production of octopamine and serotonin, respectively, but are not accumulated in a relatively stable storage pool in the absence of their amino acid precursors. A similar synthetic capacity exists in the pericardial organs of Cancer (Barker and Hooper, 1975 and unpublished data).

The amount of octopamine synthesized and retained by the ligamental nerve plexuses from radioactive tyrosine increases for about one hour and levels off, changing little for the next five hours. Comparing the amount of octopamine accumulated in this "rapidly labeled" pool with the total content in the three plexuses (Sullivan et al., 1976) reveals that only 2-3% of the entire octopamine pool is readily labeled under these experimental conditions. For

the endogeneous levels of dopamine and acetylcholine in ligamental nerve plexuses see Sullivan et al., 1976.) The situation is similar for dopamine, where the percent of total content synthesized in my experiments was 0.9-1.2% for the three plexuses. For acetylcholine, I observed synthesis of only 0.5% of the PLP content. A larger fraction of the serotonin pool, 15%, was labeled in the PLP. At least some of the newly synthesized octopamine must enter the pool available for release by action potentials. Determining if the 2-3% of total octopamine readily labeled is preferentially released, or perhaps a measure of the releasable fraction, requires further work.

The potential neurosecretory or transmitter substances now demonstrated in pericardial organs, besides the four investigated here, include one or perhaps two similar peptides (Belamarich, 1963; Belamarich and Terwilliger, 1966). Others may exist as well, and I would not be surprised if more classes of nerve fibers than the six I have indentified are eventually established. Unfortunately, none of the potential neurosecretory substances has been localized to a particular type of terminal. This may be a difficult task, as the specific reuptake systems that have aided identification of catecholamine- and serotonin-containing synaptic endings by autoradiography would seem inappropriate for a neurosecretory organ.

With regard to the four compounds studied in this work,

the relative content (Sullivan et al., 1976) and synthetic capacity (Table 2) of the three pairs of ligamental nerve plexuses are essentially the same, except for acetylcholine in the PLP. Its presence at about twice the relative level contained in the anterior and medial ligamental plexuses might be explained by the few bipolar neurons that I observed only in the PLP. If these are sensory, as their appearance suggests, they may well be cholinergic like many other crustacean sensory neurons (Barker et al., 1972; Hildebrand et al., 1974).

The dorsal nerve apparatus appears to differ from the ligamental nerve plexuses in that the former accumulates nearly half as much dopamine as octopamine whereas in the latter the range is from 3.4% to 5.3% (Table 2). The conspicuous association of dopamine with the dorsal nerve apparatus may be a clue to its function, which remains an intriguing question. It may well be a neurosecretory organ, but its unique features suggest a role not shared by the ligamental nerve plexuses. Since it forms a chamber, likely to contain neurosecretory terminals, through which the dorsal nerve passes, it may serve to modulate the activity of inputs to the cardiac ganglion. The fact that it beats out of phase with the heart raises the possibility that it may be a vestigial valve, a notion supported by the presence in stomatopods of arterial valves innervated by segmental nerves in analogous locations (Alexandrowicz, 1934). Or

the hollow bulb, whatever its evolutionary origin, may now serve to store, and then deliver into the heart at critical intervals, secretions of the dorsal nerve trunk processes that I observed to enter the apparatus but which failed to exit with the dorsal nerve. Interestingly, a similar bulb lies on the heart of Cancer magister but appears devoid of innervation (Sullivan, unpublished observations).

Although this report leaves little doubt that ligamental nerve plexuses are pericardial organs, they may not be present in all macrurans. They have been described in a limited number of genera, Palinurus, Scyllarus, Homarus (Alexandrowicz, 1932), and Panulirus (Maynard, 1953; this paper). (Alexandrowicz examined H. vulgaris; however, I did not find ligamental plexuses in limited examinations of H. americanus.) The elaborate anatomical and biochemical properties of these structures, and the fact that all six panuliran ligamental nerve plexuses can be dissected in less than five minutes, should make them attractive for future investigations.

Release and Actions of Octopamine and Serotonin

Selective, calcium-dependent release of octopamine can be evoked by a six-fold increase in potassium from proximal segments of second thoracic ganglion roots of Homarus americanus (Evans et al., 1975), and from trunk regions of pericardial organs near the distal ends of the lateral

branches of second roots (Evans, Kravitz, and Talamo, 1976). These regions are highly enriched in octopamine, containing more than an order of magnitude more octopamine than the lateral second root branches that interconnect them (Evans, Kravitz, Talamo, and Wallace, 1976). The proximal segments of the roots contain up to a dozen cell bodies that stain with neutral red and send axons out the lateral branches as shown by cobalt back-filling (Evans, Kravitz, Talamo, and Wallace, 1976). At least some of these axons reach the pericardial organs since the cell bodies can be fired antidromically and synaptically by electrical stimulation at the pericardial region (Evans, Talamo, and Kravitz, 1976). However, despite both antidromic and synaptic excitation of the root cells, it has not been possible to demonstrate octopamine release upon electrical stimulation of lateral second root branches (Evans, Talamo, and Kravitz, 1976).

These studies demonstrate that electrical stimulation of axons leading to the posterior ligamental nerve plexus of Panulirus does evoke selective, calcium-dependent release of octopamine. It is not immediately clear why I was able to obtain release by the normal physiological mechanism when this is not successful in the Homarus system. Because serotonin has been found associated with the proximal root segments also (Evans, Kravitz, Talamo, and Wallace, 1976), and since serotonin neurons stain well with neutral red (Stuart et al., 1974), it may be that both serotonergic

and octopaminergic neurons exist in the proximal roots, but send their axons to different destinations.

In the panuliran PLP, about 15% of the serotonin pool can be readily labeled, compared to 2-3% for octopamine. Stimulation of the ligamental trunks similarly induces release of several times more radioactive serotonin than the amount of octopamine released (see Chapter III).

Are octopamine and serotonin crustacean neurohormones? In the few years since the discovery that octopamine is localized in crustacean nervous tissue (Barker, Molinoff, and Kravitz, 1972), there have been reports of its presence and possible function in a number of invertebrate preparations (Kehoe and Marder, 1976). Recently, Axelrod and Saavedra (1977) reviewed investigations dealing with both vertebrates and invertebrates with the aim of building a case for octopamine as a neurotransmitter. However, there is little direct support for this hypothesis, and in Crustacea, the evidence increasingly points to hormonal, modulatory functions rather than to a role as an effector of rapid synaptic communication at discrete junctions between neurons. Hormonal functions for serotonin have been suspected since it was first reported to be present in brachyuran pericardial organs (Maynard and Welsh, 1958). It is now apparent that both octopamine and serotonin are produced and stored in considerable quantities in the

pericardial organs of spiny lobsters and in the initial portion of the thoracic segmental nerves (dorsal roots) giving rise to the pericardial organs of Homarus. Octopamine is present in greater amounts, being about ten times as concentrated in the proximal portion of dorsal roots (Evans, Kravitz, Talamo, and Wallace, 1976), and thrice more in ligamental nerve plexuses (Sullivan et al., 1976) than serotonin. Compared to acetylcholine or dopamine, serotonin and octopamine are five and fourteen times as concentrated in the ligamental nerve plexuses, and the synthetic activity of pericardial organs of Panulirus and Cancer show similar differences. The magnitude alone of the production and storage of serotonin and octopamine in comparison with other neuroeffectors suggests a neurohormonal function.

The pericardial organs are but one terminus of a larger neurosecretory system that includes the anterior ramifications, postcommissural organs, and eyestalk glands in decapods (Maynard, 1961) and the system of median connectives and dorsal trunks in stomatopods, ostracods and isopods (Alexandrowicz, 1953). Panuliran eyestalks also synthesize octopamine (A. Auerbach, personal communication) and contain large amounts of serotonin (Fingerman et al., 1974). This widespread system of trunks, neurosecretory plexuses, and groups of peripheral cell bodies receiving

cholinergic input (Wallace et al., 1974) is remisiscent of the vertebrate sympathetic nervous system (Wallace et al., 1974).

If released into the arterial circulation in vivo, would octopamine achieve a broad distribution? Preliminary experiments (R. Sullivan, unpublished) suggest that 90% of octopamine remains unchanged after 20 min of incubation in Panulirus hemolymph. Evans, Talamo and Kravitz (1976) report the same situation exists in Homarus. Thus all tissues are possible target sites. However, octopamine is rapidly metabolized as it passes through the hepatopancreas into three products. Two of these products correspond to the "fast" and "slow" metabolites reported by Evans et al., (1976) and there is one other unidentified metabolite which is negatively charged at pH 1.9 suggesting a sulfonated or phosphorylated derivative.

A number of physiological effects of octopamine and serotonin on crustacean tissues have been described. DL-octopamine, 10^{-7}M, (Lingle, personal communication) and serotonin, 10^{-7}M (Dudel, 1965; Wheal and Kerkut, 1975) are known to potentiate synaptic transmission at the crustacean neuromuscular junction by a presynaptic mechanism. Evans et al. (1975) have shown that 10^{-5}M DL-octopamine effects a long lasting contracture of Homarus cheliped opener muscle. We have found that 10^{-6} to 10^{-5}M DL-octopamine excites the isolated perfused hearts of Panulirus, Homarus and Cancer

(Sullivan and Barker, 1975; see Chapter III). Grega and Sherman (1976) reported similar effects on a variety of neurogenic arthropod hearts.

Of interest is the fact that the physiological effects of octopamine and serotonin on crustacean tissues are associated with increased tissue levels of cyclic AMP. Sullivan and Barker (1975) reported four and elevenfold increases in cyclic AMP levels in ganglia and hearts, respectively, of Panulirus and Cancer after 15 min exposure to 10^{-4}M DL-octopamine. Batelle and Kravitz (1976) reported that both serotonin at 10^{-7}M, and octopamine at 10^{-6}M, produce sevenfold and twofold increases, respectively, in cyclic AMP levels of the lobster opener muscle. In addition, these authors have demonstrated sterospecific receptors on hematocytes and peripheral muscle fibers which activate the adenylate cyclase of intact cells at 10^{-8} to 10^{-7}M D(-) octopamine. The correlations between cyclic AMP increases and the physiological effects of octopamine and serotonin remain open questions. The diversity of these physiological effects and their apparent association with increases in adenylate cyclase activity also support the neurohormonal hypothesis.

The many lines of evidence supporting a neurohumoral role for octopamine and serotonin in Crustacea leave little room for doubt, but the crucial test remains. The compelling

demonstration would be to correlate a physiological or behavioral response, to a natural stimulus, with changes in blood concentration of serotonin or octopamine in an intact animal. The response should be linked to a causal chain of events established in isolated systems, leading to primary hormone-receptor interactions. Likely target tissues, about which considerable information is available, lie conveniently near the pericardial organs. In particular, the cardiac ganglion, the neural network controlling the scaphognathites, and the stomatogastric ganglion offer relatively simple, rhythmically active motor output systems known to be modulated by biogenic amines.

STIMULUS-COUPLED SEROTONIN RELEASE FROM PLEXUSES

Presently there are at least three possible physiological roles for serotonin (5-hydroxytryptamine) in invertebrate and vertebrate nervous systems. Serotonin may be released at synaptic loci and serve as a neurotransmitter, or it may be released into the circulating body fluids as a neurohormone modulating the activities of local and distant target tissues, or it may simply be an intermediate in the biosynthesis of other indole compounds.

Recently it has come to my attention that, despite numerous physiological (Paupardin-Tritsch and Gerschenfeld, 1973; Gerschenfeld and Paupardin-Tritsch, 1974), biochemical (Carlson, Kehr and Lindqvist, 1976; Chase, et al., 1968; Cottrell and Powell, 1971; Wienreich, et al., 1973; Sullivan, Friend and Barker, 1977) and pharmacological (Aghajanian, Fotte and Sheard, 1970; Anden, Corrodi and Fuxe, 1971; Gerschenfeld and Paupardin-Tritsch, 1974; Paupardin-Tritch and Gerschenfeld, 1973; Loveland, 1963) investigations supporting the transmitter and hormone hypotheses, the question of calcium-dependent, stimulus-coupled serotonin released by normal physiological mechanisms has never properly been addressed. Since the role of serotonin as an intermediate in the synthesis of the pineal hormone,

melatonin, is clearly established (Axelrod, 1970), calcium-dependent, stimulus-coupled serotonin release should be regarded as the primum criterium in the assignment of transmitter and hormonal roles. In this regard calcium-dependent, stimulus-coupled release means that the serotonin release is quantitatively related to firing frequency during a constant period of stimulation and requires extracellular calcium ions.

A number of investigators have observed serotonin release from vertebrate synaptosomes and brain slices with either high potassium salines or field stimulation (Mulder, VanDenBerg and Stoof, 1975; Chase, Katz and Kopin, 1969; Katz and Kopin, 1969; McIlwain and Synder, 1970; Saldate and Orrego, 1975). However, it is doubtful whether either method approximates in vivo release. In fact, field stimulation leads to conflicting results concerning the effects of calcium and lithium on ^{3}H-serotonin release (Chase, et al., 1969; Katz, et al., 1969; Saldate, et al., 1975). Stimulation of the optic tract resulted in the release of ^{3}H-serotonin from slices of guinea pig superior colliculus but the release was not calcium dependent (Kawai, 1970). However, high potassium indiced release from synaptosomes is specific and calcium dependent (Mulder, et al., 1975). A preliminary yet promising report suggests that stimulation of the molluscan giant cerebral neurons,

which are known to synthesize and store serotonin (Wienreich, et al., 1973), effects serotonin release in the presence of chlorimipramine (Gerschendfeld, Hamon and Paupardin-Tritsch, 1976). Although field stimulation of molluscan connectives and ganglia effects an increased efflux of radiolabel from ganglia and hearts preincubated in ^{3}H-serotonin (Chase, et al., 1968; Ascher et al., 1968), the ^{3}H-compounds released were not identified and calcium and frequency dependency were not demonstrated for the same preparation. Moreover, it is doubtful that ^{3}H-serotonin is taken up by molluscan nerve cells (Ascher, et al., 1968). Similar reservations apply to the stimulus-induced release of serotonin from the extracardial nerve of Helix (-Rozsa and Perenyi, 1966). Thus, in toto, The evidence to date for calcium-dependent, stimulus-coupled serotonin release from nervous tissues is incomplete and by no means compelling.

In the course of my studies concerning the physiological role of serotonin found in crustacean neurosecretory tissues (Sullivan, et al., 1977), I have observed that large quantities of serotonin are synthesized (10-20 pmol ^{3}H-serotonin/plexus) and stored (50-100 pmol/plexus) in the ligamental nerve plexuses of the spiny lobster, Panulirus interruptus (Sullivan, et al., 1977; Sullivan, Friend and McCaman, 1976). Ligamental nerve plexuses are an anatomical variation of the decapod pericardial organs (Sullivan, et

al., 1977 and Alexandrowicz, 1953) and one probable role for serotonin in the lobster is that of a neurohormone (Sullivan, et al., 1977). In order to gain an understanding of the quantitative relationships between circulating serotonin levels and receptor activation at target sites we have been investigating the physiological parameters governing the secretion of serotonin from ligamental nerve plexuses induced by high potassium pulses and electrical stimulation. These studies support the hypothesis that serotonin is a neurohormone.

Methods

Spiny lobsters, Panulirus interruptus, 0.5-1.0 Kg were obtained from Pacific Biomarine Co. (Venice, CA) and maintained in circulating oxygenated sea water at 16 C. In each experiment the right posterior ligamental nerve plexus and the efferent motor-ligamental nerve were dissected free of the pericardial chamber, pinned out in a specially designed, Sylgard (Dow-Corning) perfusion chamber and perfused at a rate of 1.0 ml/min (by means of a syringe pump) with oxygenated physiological saline. The saline contained 479 mM NaCl, 12.7 mM KCl, 13.7 mM $CaCl_2$-$2H_2O$, 10 mM $MgSO_4$-$7H_2O$, 8.8 mM Na_2SO_4, and 11 mM Tris base, titrated to pH 7.45 with maleic acid. Details of the anatomy are described elsewhere (Sullivan, et al., 1977).

The design of the perfusion chamber effects a rapid mixing of the perfusate producing a homogeneous solution in less than 15 sec, and allows for the measuring of the "instantaneous concentration" within the chamber by simply removing a constant fraction (1/3) of its total volume (1.5 ml) once every minute with an Eppendorf pipette. Sample aliquots were either saved for chemical analysis or assayed directly for radioactivity by liquid scintillation spectroscopy using a fluor consisting of Triton X-100 (30% V/V)-toluene (61%)-H_2O (9%)-Omnifluor (0.5% W/V). The counting effeciency was 30%.

Stainless steel stimulating and recording electrodes were attached to the appropriate nerves (see below) prior to incubations in radioactive precursor. The stimulating current (4-10 V, 0.16-0.50 msec duration square wave pulses supplied by a Grass SD-9 stimulator) was applied to the motor-ligamental nerve approximately 1 cm from the branching points of the posterior ligamental nerves (Fig. 9A). The polarity was chosen to yield a minimum threshold. The recording electrodes were placed on both branches of the posterior ligamental nerve and AC-coupled electrical signals were fed into a Textronix 5013 N storage scope via two 5A22N differential amplifiers.

After obtaining good electrical contacts and insulating the electrodes with Vaseline, the preparations were incubated

Fig. 9 (A) In situ diagram of the posterior ligamental nerve plexus (PLP). Arrows denote the positions of the stimulating electrodes (S) on the motor-ligamental nerve (ML) and the recording electrodes (R) on the ligamental nerves.
(B) Specific release of ^{3}H-serotonin due to supramaximal stimulation of ligamental nerves at 10/sec (t=15-18). Arrows denote sample aliquots subjected to radiochemical analysis for determining molar efflux ratios. See text. The molar ratios above the arrows indicate that ^{3}H-serotonin accounts for greater than 97% of the increase in radiolabeled efflux.

A
ML
S
PLP
B
CPM / 500 λ
1000
500
MINUTES
10
20
30
5HT / TRYP+5HTP = 10.8
5HT / TRYP+5HTP = 2.2
10 / SEC

in 500 µl of 5-13 µM ^{3}H-(G)-tryptophan, 7.0 Ci/mM (NEN) for 2.25-4.5 hrs and then washed with normal saline for 2 to 14 hrs.

In order to determine whether serotonin was released specifically, 0.5 ml aliquots of the perfusate were placed in silanized conical test tubes; 200 µl were subjected to liquid scintillation counting directly and the remaining 300 µl were evaporated to dryness in an EVAPO-MIX (Buchler inst.) at 40-50 C. The residues were extracted overnight at 4C with 100 µl of acetone-N formate (90:10) containing 0.5 µg serotonin and tryptophan as cold carriers and 50 µg ascorbate as an antioxidant. The extracts were subsequently concentrated under a stream of nitrogen and analysed by high voltage electrophoresis (Hildebrand, et al., 1971). Under these conditions the average recovery of ^{3}H-serotonin is 39 ± 2% (N=3).

All incubations and release experiments were performed in a cold room at 12-15 C.

Results

Previously I reported that serotonin is the only tryptophan metabolite stored in quantity in the plexuses after a prolonged wash (Sullivan, et al., 1977). In one experiment I isolated and characterized the radioactive materials which contributed to the resting and stimulus-

induced efflux of label. (See Methods.) The results indicate that serotonin is also the only tryptophan metabolite released upon nerve stimulation. In this experiment (Fig. 9B), the preparation was incubated for 3 hrs and subsequently washed with normal saline for 14 hrs prior to time zero. Aliquots of the perfusate prior to and during release were subjected to radiochemical analysis and the molar ratio of ^{3}H-serotonin/(^{3}H-tryptophan + ^{3}H-5-OH-tryptophan) was determined for each aliquot.

Prior to stimulation the mean molar ratio ± S.D. (N) was 2.2 ± 1.2(3) and during release the ratio increased to 10.8 ± 2.9(3). This increase is significant ($p<0.01$) and indicates that greater than 97% of the radiolabel in the release peak was due to ^{3}H-serotonin. At the end of this experiment the molar ratio of labeled compounds remaining in the tissue was determined to be 4.23 (data not shown).

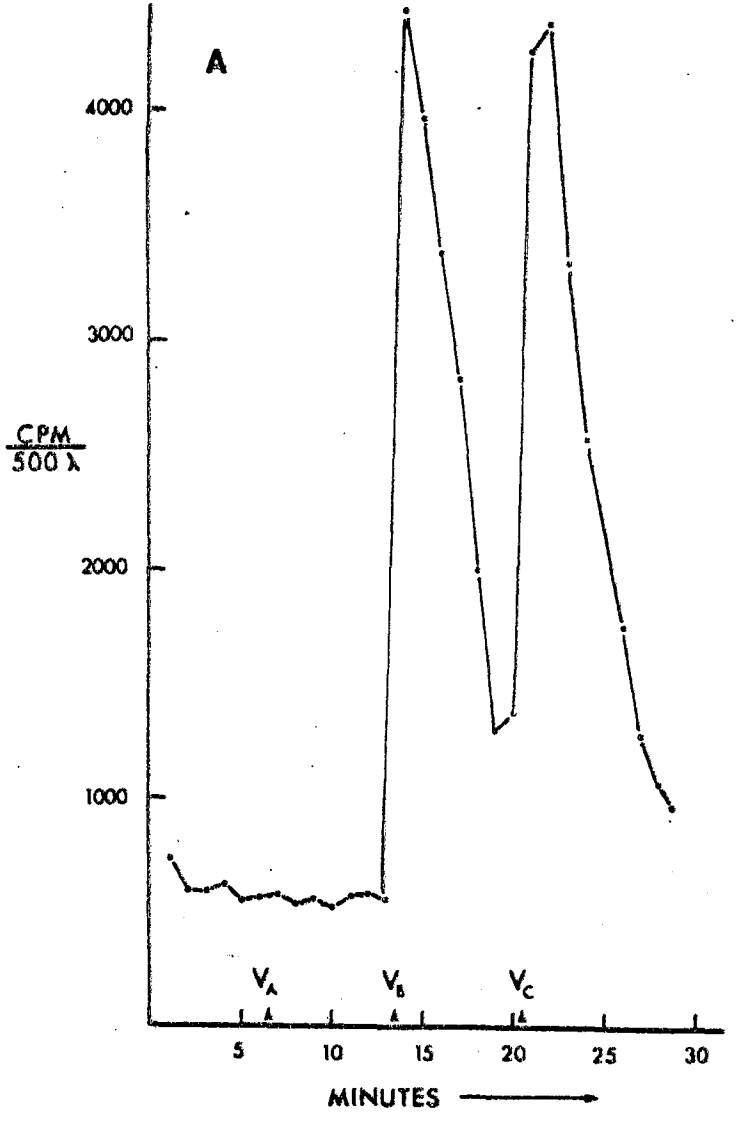
The rather slow decay of ^{3}H-serotonin release (Fig. 9A) is in part due to the time constant of the perfusion chamber ($t_{1/2}$ = 1.2 minutes at 1 ml/min) and partially due to the topological distribution of nerve endings in the cortical (surface) layer of the plexus where release occurs. Although release sites are usually juxtaposed to an acellular epineurium which separates the cortical nerve processes from the hemolymph (Sullivan, et al., 1977), fingerlike projections of the epineurium extend 8-20 μm into the

cortical layer forming tortuous diffusion channels which probably retard the efflux of radiolabel.

Supramaximal stimulation of the posterior ligamental nerves elicites a large compound action potential approximately 40-50 msec in duration (Fig. 10B). Similar recordings have been obtained from crab pericardial organs (Cooke, 1964). The compound action potential is composed of at least three distinct components, as distinguished by their conduction velocities and stimulus thresholds. The A component of the action potential exhibits the lowest threshold and fastest conduction velocity (approx. 6 m/sec). The B component is elicited by intermediate levels of stimulating current and is characterized by conduction velocities in the range of 0.8-1.0 m/sec. In addition, this component exhibits an absolute refractory period of 20 msec and is relatively refractory up to 50 msec suggesting a maximum rate of normal firing to be approximately 20/sec (Sullivan, unpublished observations). The C component is slowest, exhibiting a conduction velocity of 0.4 m/sec and is elicited by the highest levels of stimulating current. It should be noted that the actual form of the compound action potential is somewhat variable depending on the electrode configuration.

Thus by increasing the stimulus voltage, component A along, components A + B or A + B + C can be elicited.

Fig. 10 Correlation of ^{3}H-serotonin release with a specific component of the compound action potential.
(A) The release of ^{3}H-serotonin in response to various stimulating currents, 7.5/sec for 30 sec. V_A = 4.3 V recruited only the A component; V_B = 6.0 V recruits both the A and B components, whereas, V_C = 10 V recruits all three components, A, B and C. Release of ^{3}H-serotonin is associated with the B component of the compound action potential.
(B) Compound action potential recorded from the first branch of the posterior ligamental nerve illustrating the three major components A, B and C. Calibrations equal 10 msec and 0.02 mV.


A
4000
3000
2000
1000
CPM
500 λ
V_A
V_B
V_C
5
10
15
20
25
30
MINUTES

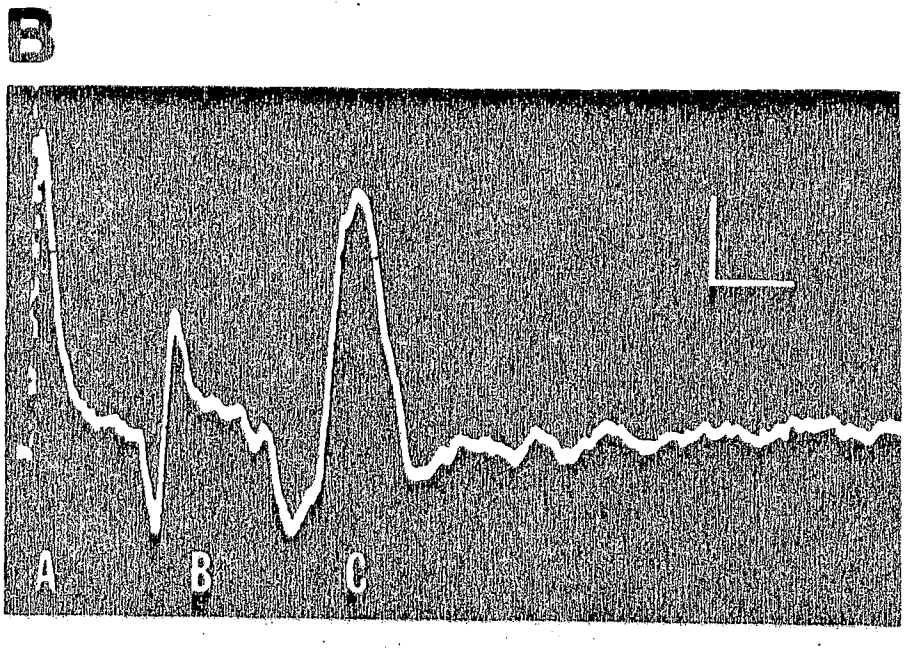
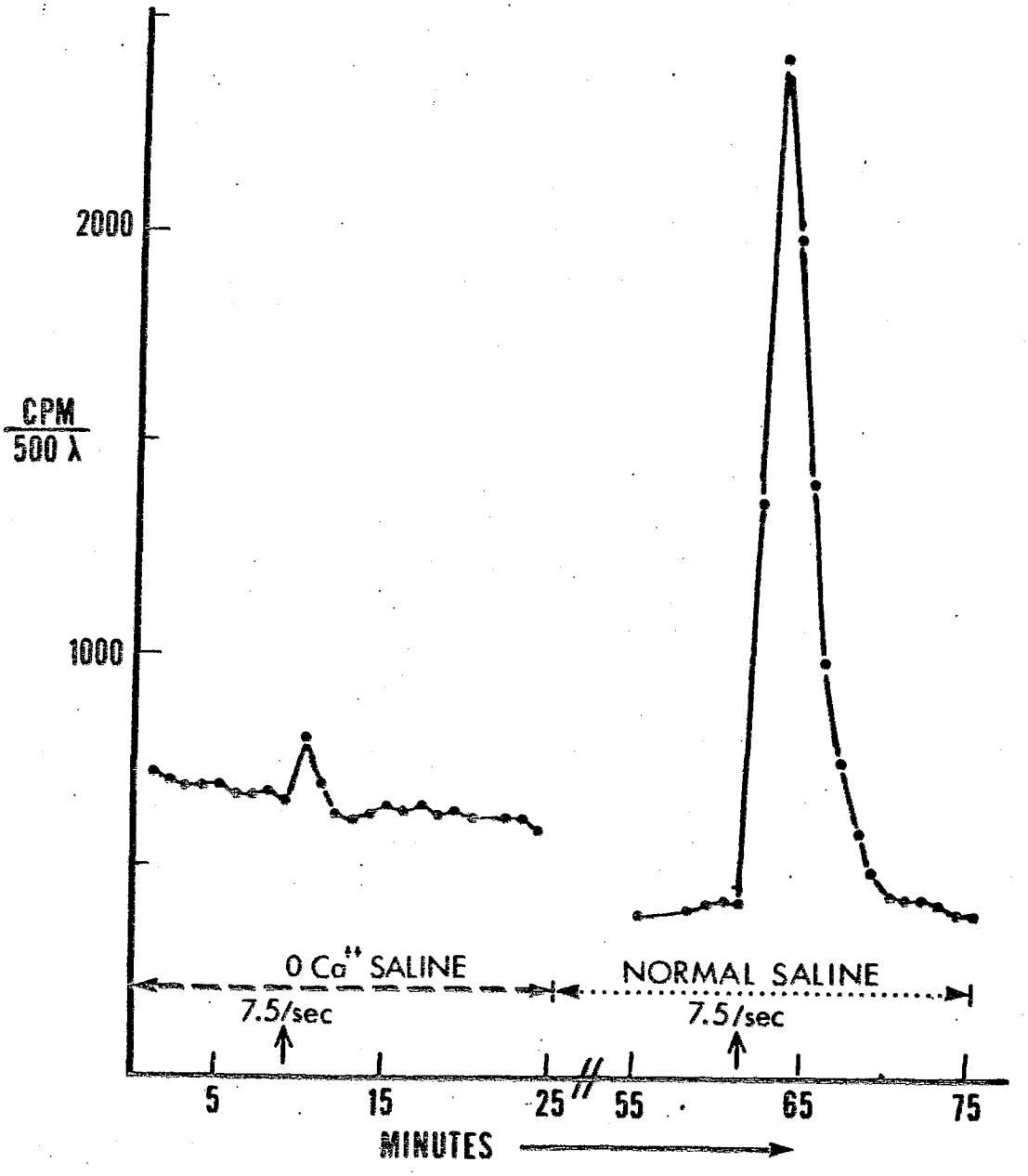

B
A
B
C

Fig. 10A shows that currents (at 4.3 V) sufficient to excite only the A components, do not result in serotonin release. Increasing the stimulation current to the level of the A + B components (at 6 V) produces a large release of serotonin. However, a further increase in the current to a level (at 10 V) sufficient to recruit the C component fails to increase serotonin release. In four tests on three experimental preparations, serotonin release was found to be associated with the B component. Therefore, it is likely that the B component is selectively associated with serotonin release.

Calcium mediation of "stimulus-secretion coupling" (Douglas and Rubin, 1963) is now a widely accepted phenomenon and often used as a criterion to indicate that release is occurring from nerve terminals (Hammerschlag and Roberts, 1976). A similar calcium dependency has been demonstrated for the release of peptide hormones (Berlind and Cooke, 1968) and octopamine (Sullivan, et al., 1977) from decapod pericardial organs whether release is neurally evoked or induced by high potassium pulses (Sullivan, et al., 1977; Evans, Kravitz and Talamo, 1976). Serotonin release was also blocked in the absence of calcium.

The data in Fig. 11 were obtained from a posterior ligamental nerve plexus which was incubated in 4.8 μM ^{3}H-tryptophan for 4.5 hrs and then washed for an additional 2

Fig. 11 Inhibition by 0 Ca^{++} saline of ^{3}H-serotonin release due to supramaximal stimulation at 7.5/sec for 2 min. Arrows indicate beginning of stimulus period. Slash marks denote 30 min wash in normal saline.


2000
CPM
500 λ
1000
0 Ca++ SALINE
NORMAL SALINE
7.5/sec
7.5/sec
5
15
25
55
65
75
MINUTES

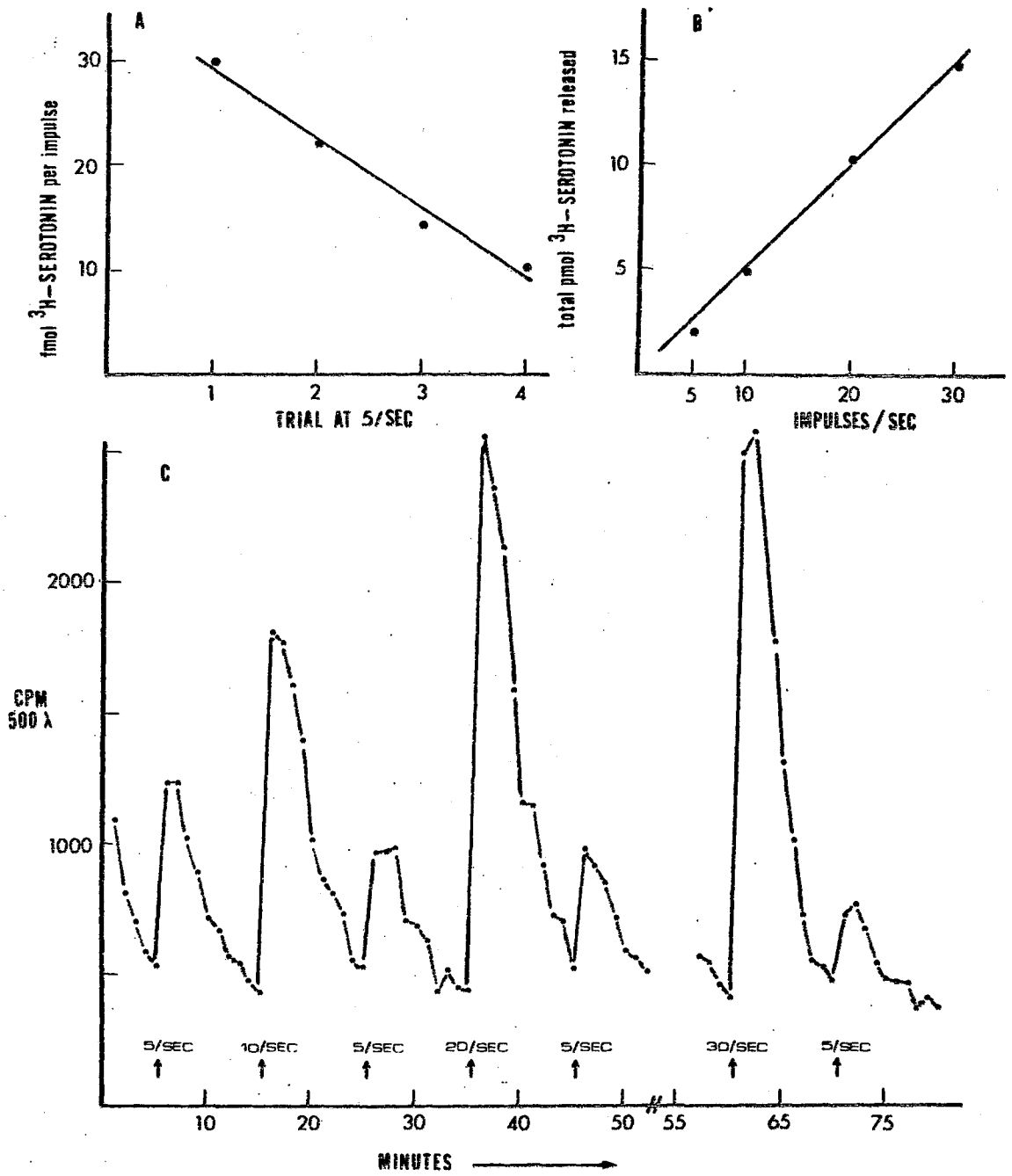
hrs in normal saline. Fifteen minutes prior to the first time point on this graph the saline wash was switched to 0 Ca^{++} saline. The osmolarity of this saline was maintained by the addition of sucrose.

These data (Fig. 11) clearly illustrate the calcium dependency of serotonin release from ligamental nerve plexuses; thus, another neurohumoral system provides additional support for the "Ca^{++} hypothesis" (Douglas, et al., 1963). In this particular experiment, release in 0 Ca^{++} was reduced to 3% that in normal saline. In two additional experiments with Mg^{++}-substituted 0 Ca^{++} saline, serotonin release was totally abolished.

Several experiments with this system (data not shown) have revealed that 20 minutes or more exposure to 0 Ca^{++} is necessary to maximize the inhibition of release mechanisms and a similar time (note break in the time axis of Fig. 11) in normal saline is needed to fully reverse this inhibition. Zero-calcium saline also inhibits high potassium-induced ^{3}H-serotonin release, but it does not significantly affect the unstimulated efflux of radiolabel nor does it inhibit compound action potentials elicited by nerve stimulation.

Fig. 12 illustrates that ^{3}H-serotonin release is quantitatively related to the stimulus frequency. Since the amount of ^{3}H-serotonin release is reduced upon

Fig. 12 Stimulus-coupled ^{3}H-serotonin release from the posterior ligamental nerve plexus. (A) The total ^{3}H-serotonin release/impulse in four 5/sec trials indicated in 4C. Release decreases linearly. (B) Frequency dependence of total ^{3}H-serotonin release corrected for decrease shown in 4A. The mean of the first two 5/sec values was chosen as a standard 5/sec value. The release values at higher frequencies were obtained by multiplying the standard 5/sec value by the ratio, observed release at each higher frequency divided by the mean of the prior and subsequent 5/sec values. (C) Time course of ^{3}H-serotonin release. Ligamental nerves were stimulated at each frequency for a constant time interval of 15 sec. See text for experimental details. Arrows denote the time of stimulation.


A
fmol ^{3}H–SEROTONIN per impulse
30
20
10
1
2
3
4
TRIAL AT 5/SEC
B
total pmol ^{3}H–SEROTONIN released
15
10
5
5
10
20
30
IMPULSES / SEC
C
CPM
500 λ
2000
1000
5/SEC
10/SEC
5/SEC
20/SEC
5/SEC
30/SEC
5/SEC
10
20
30
40
50
55
65
75
MINUTES

successive stimulus trials (Sullivan, unpublished observations), a stimulus paradigm was designed in order to provide the internal controls necessary to determine whether release was proportional to the total number of stimuli delivered at varying frequencies. A train of supramaximal stimuli was applied for 15 seconds to the motor-ligamental nerve at frequencies of 5, 10, 20 and 30 Hz with a 5 Hz control before and after stimulation at higher frequencies.

The data (Fig. 12A-C) demonstrate that ^{3}H-serotonin release is stimulus-coupled. Total release values were quantified by integrating each peak, subtracting background, and correcting for the volume fraction assayed. In Fig. 12A the total release values at 5/sec were divided by the total number of impulses to yield release per impulse values. A comparison of the 5/sec peaks (Fig. 12C) reveals the diminution of release upon repeated stimulus trials. This is graphically illustrated in Fig. 12A where the total femtomoles of ^{3}H-serotonin released per impulse are plotted against the trial number. When the total amount of ^{3}H-serotonin released at each frequency of stimulation greater than 5/sec was normalized to account for the reduction upon successive trials (see legend, Fig. 12), and plotted versus frequency, Fig. 12B results. A near linear relationship was found between the stimulus frequency and release in this experiment. Under these conditions (constant stimulus

duration and varying frequencies), a linear relationship between stimulus frequency and total release suggests non-facilitated, stimulus-coupled release, but does not rule out the possibility of facilitation that develops rapidly at all frequencies used.

In a second preparation, the 15 sec stimulus period was applied in a 5 sec "on", 5 sec "off" manner and the relative total release per impulse at 5, 10 and 20 Hz was 1.0:2.2:1.6 respectively. Both experiments indicate that ^{3}H-serotonin release is stimulus-coupled, but these limited data do not permit conclusions about the frequency dependence of facilitation.

Discussion

In another report I demonstrated that serotonin is synthesized and stored in the ligamental nerve plexuses of the spiny lobster and suggested that serotonin is released as a neurohormone from these tissues (Sullivan, et al., 1977). The results of the present experiments clearly demonstrate the release of ^{3}H-serotonin upon electrical stimulation of the efferent motor-ligamental nerve. Neurally evoked release is specific for this tryptophan metabolite, accounting for more than 97% of the radiolabel released upon stimulation. Release is stimulus-coupled to the activity of a group of neurosecretory processes exhibit-

ing conduction velocities of 0.8-1.0 m/sec. In addition, a 20 minute exposure to 0 Ca^{++} saline blocks all but a small per cent of stimulation-induced release. This appears to be the first report of calcium-dependent, stimulus-coupled serotonin release from identified neurosecretory fibers.

These studies provide quantitative evidence supporting the hypothesis that serotonin serves a neurohormonal role in Crustacea. Considering that there are three pairs of ligamental nerve plexuses in the pericardial cavity and taking into account the total amount of ^{3}H-serotonin released by 15 sec of stimulation at 30 Hz i.e., approximately 15 pmol over a 10 min time interval (Fig. 12C), one can calculate that in vivo, similar stimulation of all plexuses would result in the release of 90 pmol of serotonin into the total blood volume (75 ml for a 0.5 kg lobster). I will assume that considerable mixing occurs since the heart would beat some 500 times during this 10 min interval with a stroke volume of 1 ml (Maynard, 1960) and that there is negligible degradation. All things considered, a hemolymph concentration of 1.2 nM would obtain. This concentration is a minimum estimate since unlabeled serotonin is not included, but is in fact near threshold for serotonin-induced cardioacceleration (Berlind and Cooke, 1970; Sullivan and Barker, in prep.) and neuromuscular facilitation

(Lingle, 1976). In view of the fact that ligamental nerve plexuses directly abut the myocardial surface, released serotonin would be drawn immediately into the heart during diastolic filling, thus subjecting the cardiac network to considerably higher concentrations of serotonin. These calculations, based on serotonin release by normal physiological mechanisms, provide additional evidence that circulating serotonin levels may exceed the threshold for excitation of several crustacean neuromuscular systems.

ENDOGENOUS LEVELS AND METABOLISM OF BIOGENIC AMINES

Octopamine and serotonin are the major amine metabolites of tyrosine and tryptophan in crustacean nervous tissues (Barker, Molinoff and Kravitz, 1972; Barker and Hooper, 1975; Evans, Talamo and Kravitz, 1976 and Sullivan, Friend and Barker, in press). In the spiny lobster, Panulirus interruptus, the highest levels of biogenic amine synthesis and accumulation occur in the ligamental nerve plexuses of the pericardial cavity (Sullivan et al., in press); moreover, both amines are known to be released from the ligamental plexuses upon stimulation of the preterminal nerve trunks. In vivo, serotonin and octopamine would be released into the hemolymph, but the metabolic fate of biogenic amines in this tissue is presently unknown.

Kennedy (1977) has investigated the metabolism of dopamine, octopamine and serotonin in lobster nervous tissues and discovered a rather unique metabolic pathway which includes sulfonation of ring hydroxyls and/or beta-alanylation of the amine moieties. Thus, three metabolites are possible for each amine. For example, the metabolites of octopamine are: octopamine-p-sulfate, beta-alanyloctopamine and beta-alanyloctopamine-p-sulfate. Beta-alanylation of biogenic amines in crustaceans was first reported by Arnould and

Frentz (1975); these investigators found high levels of carcinine (beta-alanylhistamine) in extracts of Carcinus hearts. Furthermore, they demonstrated that this compound was released from rhythmically beating hearts maintained in vitro.

If octopamine and serotonin serve a general hormonal role in the decapods, they would have to be released into the hemolymph in sufficient quantities to effect a physiological response, and persist in an unmetabolized form for a sufficient period of time in order to be carried to their target sites by bulk flow. Thus it seemed of interest to determine the endogenous levels of biogenic amines in the ligamental nerve plexuses and their metabolic fate in hemolymph. The former would place an upper limit on the size of the releasable amine pools and the latter may reflect the extent to which amines are distributed in an unmetabolized form.

The results of these experiments provide additional evidence supporting the notion that biogenic amines play a neurohormonal role in the decapod crustaceans. Both serotonin and octopamine are stored in sufficient quantities within the plexuses to exert their effect via release into the general circulation, and biogenic amine metabolism in hemolymph is extremely slow indicating that this tissue may be an appropriate vehicle for their distribution. Additional

studies have revealed that octopamine is rapidly metabolized by the hepatopancreas, suggesting that this organ plays an important role in monoamine metabolism in the Crustacea.

Methods

Animals

Panulirus interrupti were maintained in tanks of circulating sea water at 16 C.

Radioenzymatic Assays

The endogenous levels of octopamine and 5-hydroxytryptamine in ligamental nerve plexuses and dorsal nerve apparati were determined by the radioenzymatic assays of Molinoff et al. (1969) and Saavedra et al. (1973). These assays were carried out in the laboratory of Richard McCaman at City of Hope Medical Center, Duarte, CA. Tissue samples were washed with physiological saline (as noted in Chapter I), placed in either 100 µl of 95% ethanol or acetone: 1N formic acid (95:5) and extracted by soaking for 1-2 hrs. Aliquots of these extracts were evaporated to dryness in a vacuum centrifuge and taken up in the appropriate assay buffer. The assay procedures involved: 1) N-methylation of octopamine with phenlyethanolamine-N-methytransferase (PNMT) and radiolabeled S-adenosyl-L-methionine as a methyl donor (Molinoff et al., 1969), and 2) N-acetylation of 5-hydroxytryptamine with

N-acetyltransferase and acetyl coenzyme A, and subsequent O-methylation of the N-acetyl adduct with pineal hydroxyindole-O-methyltransferase using radiolabeled S-adenosyl-L-methionine (Saavedra et al., 1973). The reactions were stopped by the addition of 0.5 M sodium borate, pH 10, and radiolabeled adducts were extracted into an organic phase (toluene or a toluene-isoamylalcohol mixture, 3:2 v/v). Aliquots of the organic phase were placed in scintillation vials and evaporated to dryness. After the addition of counting-fluor to each vial, the radioactivity in each aliquot was determined by liquid scintillation spectroscopy.

Extraction and Derivatization of Phenylethylamines for Gas Chromatography

In order to control for the nonspecificity of the PNMT radioenzymatic assay for octopamine, it was necessary to confirm these results by an independent analytical procedure. This was accomplished by gas-liquid chromatography of the pentafluropropionic acid anhydride derivative and electron capture detection (see Anggard and Sedvall, 1969).

Ligamental nerve plexuses were placed in 100 µl of buffer (0.47 M formic acid and 1.4 M acetic acid, pH 1.9), frozen, thawed and extracted by a 1 hr soak. Aliquots of the extracts were washed three times with an ether-benzene (5:2 v/v) mixture. The organic phase was aspirated and 10 µl

aliquots of the buffer phase were removed, placed in 0.5 ml Reacti Vials (Alltech Assoc.) and dried under a stream of anhydrous N_2. The residues were redissolved in an ethylacetate-pentafluoropropionic acid anhydride (Pierce Chem. Co.) mixture (50:50 v/v). The vials were tightly capped and the derivatization reaction was allowed to proceed for 1/2 hr in a 60 C sandbath. Reagent and volatile by-products were removed by a stream of anhydrous N_2 and the oily derivatives were taken up in an appropriate volume of heptane (20-100 µl) for injection (0.5 µl volume) into the gas chromatograph.

Gas Chromatography

The pentafluoropropionyl adducts were chromatographed with a Beckman GC-5 gas chromatograph equipped with an electron capture detector of cylindrical design (Uthe and Solomon, 1974) and a Beckman 567100 electrometer. The column was a 1.0 m X 2.0 mm I.D. glass tube packed with 1% SE-60 on acid washed DMCS chromosorb W (Alltech. Assoc.). Chromatographic conditions were as follows: carrier gas, argon-methane (95:5); velocity of the mobile phase, 1.0 m/sec; column and detector temperature, 175 C; injection port temperature, 190 C; Range, 100; Attenuation, 64. The detector was operated in the pulse mode. Square wave pulses (30V, 1 µs duration) were applied every 100 µs and produced

a standing current of approximately 1 nA at 175 C. The amplified signals were recorded by a Speedomax G recorder (Leeds and Northrup Co.).

Lobster Hemolymph and Hepatopancreas Incubations

Hemolymph (100 µl) was removed from the lobster's pericardial cavity by means of a plastic tuberculin syringe and transferred to a 0.5 ml polypropylene micro test tube (BIO·RAD) containing sufficient (2-^{3}H(N))-DL-octopamine (side chain label, 9.5 Ci/mmol) acetate (NEN) to effect a total concentration of 11 µM. The tubes were quickly capped and the contents mixed on a vortex mixer for 15 seconds. The use of plastic containers prevented the clotting reaction. The incubations were allowed to proceed for 5-20 minutes at 11-15 C and were stopped at various times by the addition of 100 µl of 1.5 N HCl in 50% ethanol. The tubes were centrifuged to compact precipitated proteins and 10 µl of the supernatants were analyzed by high voltage electrophoresis and thin-layer chromatography. (See Methods section Chapter I for analytical details.)

Hepatopancreas tissue was dissected free of the cephalothorax and placed in a dish of physiological saline (as noted in Chapter I). For each incubation, 5-10 ceca, 1-3 mg wet weight, were dissected free, thoroughly washed with saline, and placed in conical plastic vials containing 20 µl

of 5-20 μM ^{3}H-octopamine in physiological saline. Tissues were incubated at 11-12 C for 1-60 minutes. After varying periods of time, the incubation medium was removed and the tissues were washed with 0.5 ml of normal saline for 5 minutes. Tissue samples were subsequently removed, blotted dry and placed in aother vial containing 20 μl of electrophoresis buffer (see above). Tissues were extracted by an additional one hour soak in buffer.

Results

Endogenous Levels of Octopamine and Serotonin

When the endogenous levels of octopamine and serotonin in ligamental nerve plexuses were determined by the radioenzymatic assays, octopamine was found to be the major biogenic amine. Previously serotonin was thought to be the predominant biogenic amine in decapod pericardial organs (Maynard and Welsh, 1959) but these data (Table 3) demonstrate that the total number of picomoles of octopamine (1262) in panuliran ligamental nerve plexuses is approximately three times higher than those of serotonin (429). In contrast, octopamine and serotonin are 5 and 14 times more concentrated than acetylcholine (Sullivan et al., 1976) in the plexuses.

In general there were no significant differences in the concentrations of biogenic amines between paired plexuses.

TABLE 3

COMPOUND	OCTOPAMINE	SEROTONIN
tissue	pmols/tissue	pmols/tissue
RALP	60-147 101±39(4)	35-66 45±13(4)
LALP	147-229 180±32(4)	14-80 50±24(4)
RMLP	105-316 206±82(4)	38-128 68±36(4)
LMLP	93-250 180±56(4)	42-117 66±30(4)
RPLP	122-413 272±104(4)	20-129 80±42(4)
LPLP	170-335 241±60(4)	38-118 84±29(4)
LDNA	23.7(2)	8-20 12±6(3)
RDNA	58.6(2)	5-37 24±14(3)
TOTAL	1262	429
mole ratio	3	1

The endogenous levels of octopamine and serotonin (5-HT) in the pericardial organs of P. interruptus. List of abbreviations: RALP and LALP, right and left anterior ligamental nerve plexuses; RMLP and LMLP, right and left medial ligamental nerve plexuses; RPLP and LPLP, right and left posterior ligamental nerve plexuses; RDNA and LDNA, right and left dorsal nerve apparatus. See text. Data expressed as mean ± S.D.(N).

The only exception to be noted was the small but significant ($p<.025$) difference in the octopamine concentrations of right and left anterior ligamental nerve plexuses (see Table 3). Interestingly this difference was not reflected in the serotonin concentrations.

When average concentrations of biogenic amines in pairs of plexuses are compared i.e., posterior vs medial vs anterior, the posterior ligamental nerve plexuses consistently contained the highest levels of amines. This observation may be due to the slight differences in the actual size of the plexuses and not a result of concentration differences. In general, the posterior ligamental plexuses are largest and the anterior plexuses are smallest.

Since the octopamine radioenzymatic assay (Molinoff et al., 1969) utilized the enzyme PNMT to catalyze the N-methlyation of octopamine, the possibility of having measured a combination of octopamine, phenylethenolamine and norepinephrine values had to be considered. The synthesis data in Chapter I tend to eliminate the possibility of significant levels of noradrenaline accumulating in these tissues; however, the possibility of endogeneous phenylethanolamine levels could not be overlooked since a number of investigators have reported the presence of this amine in vertebrate nervous tissues (Saavedra and Axelrod, 1977). Thus, it was necessary to use gas-liquid chromatography as an alternative

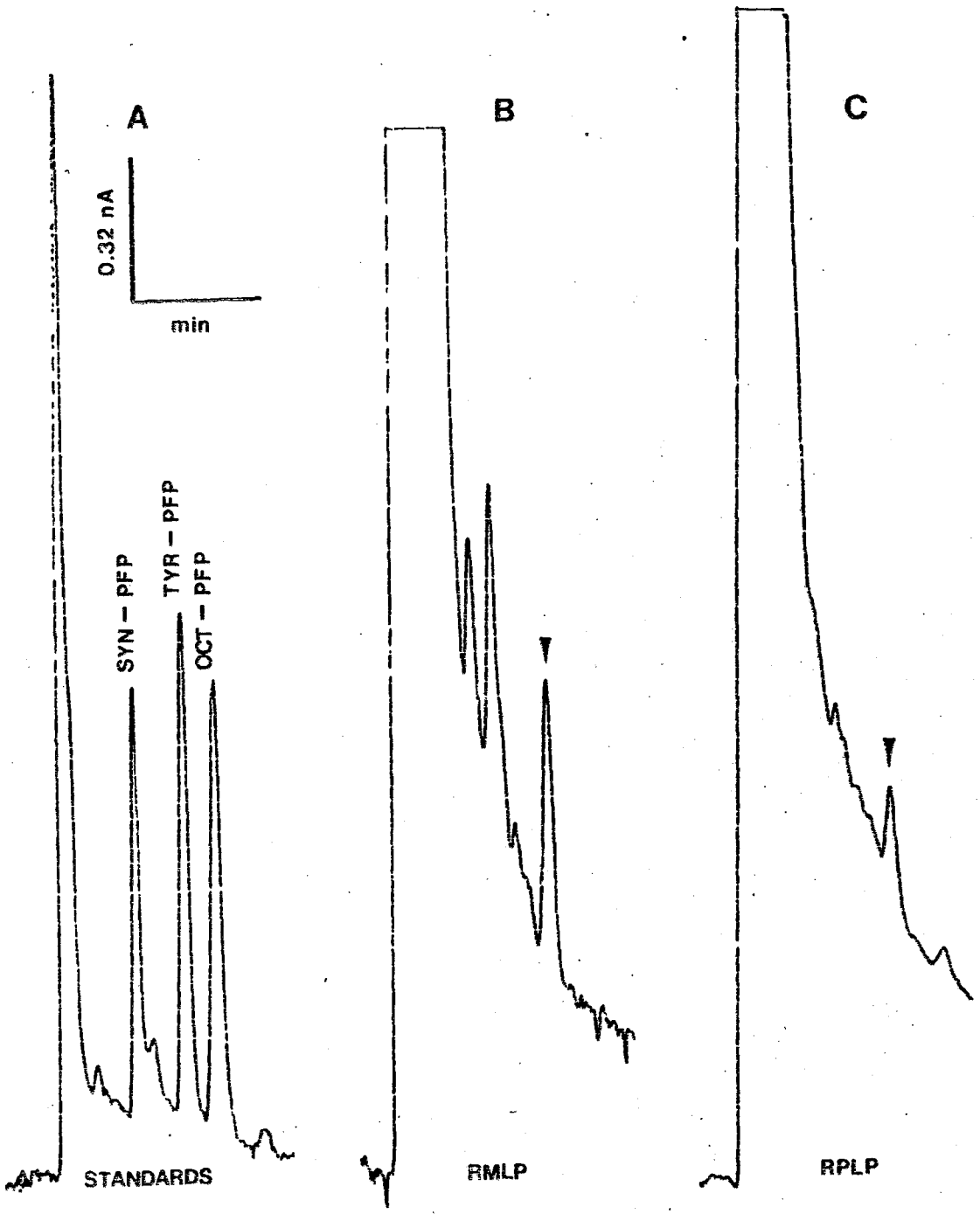
technique to confirm the octopamine levels.

Fig. 13A is the response of the electron capture detector to 37.8 pg of standards, tyramine, octopamine and synephrine. I might point out that conditions noted in Methods optimize resolution/time (see Karger et al., 1973) yielding a base line separation within 1 minute.

Fig. 13B and C are representations of the chromatograms obtained upon injection of tissue extracts. The arrows denote the elution volume of the octopamine present in each extract. The elution volume of octopamine contained in tissue extracts was confirmed by co-chromatographing standard octopamine with tissue extracts. Note also that a compound from the tissue extracts co-elutes in the same column volume as does synephrine. However, since synephrine synthesis does not occur in the ligamental nerve plexuses, I am reluctant to identify this compound as synephrine.

Extracts of ligamental nerve plexuses from two lobsters were analyzed by this technique. Although the quantitative data varied (up to 10 fold), the averaged values of 153.4 pmol/RPLP and 350 pmol/RMLP compare well with the 272 pmol/RPLP and 206 pmol/RMLP values obtained using the radio-enzymatic procedure (see Table 3). Thus we can be relatively confident of the octopamine values obtained by the radio-enzymatic procedure.

Fig. 13 Chromatographic analysis of phenolamines in extracts of pericardial organs from P. interruptus.
(A) Standard separation of 37.8 pg each of synephrine (SYN-PFP), tyramine (TYR-PFP) and octopamine (OCT-PFP) as their pentafluoropriopionyl adducts by gas-liquid chromatography.
(B) and (C) Separation of endogenous components obtained from extracts of the right medial ligamental nerve plexus (RMLP) and right posterior ligamental nerve plexus (RPLP) respectively. Arrows denote the octopamine peaks. Co-chromatography of standard octopamine confirmed the presence of octopamine in the extracts. Quantitation was based on a standard response curve (not shown) prepared from triplicate injections of reference standards. The detector response was linear in the range of 8-40 pg of amine. Conditions: 1% SE-60, 175 C, ^{3}H electron capture detector.


A
B
C
0.32 nA
min
SYN – PFP
TYR – PFP
OCT – PFP
STANDARDS
RMLP
RPLP

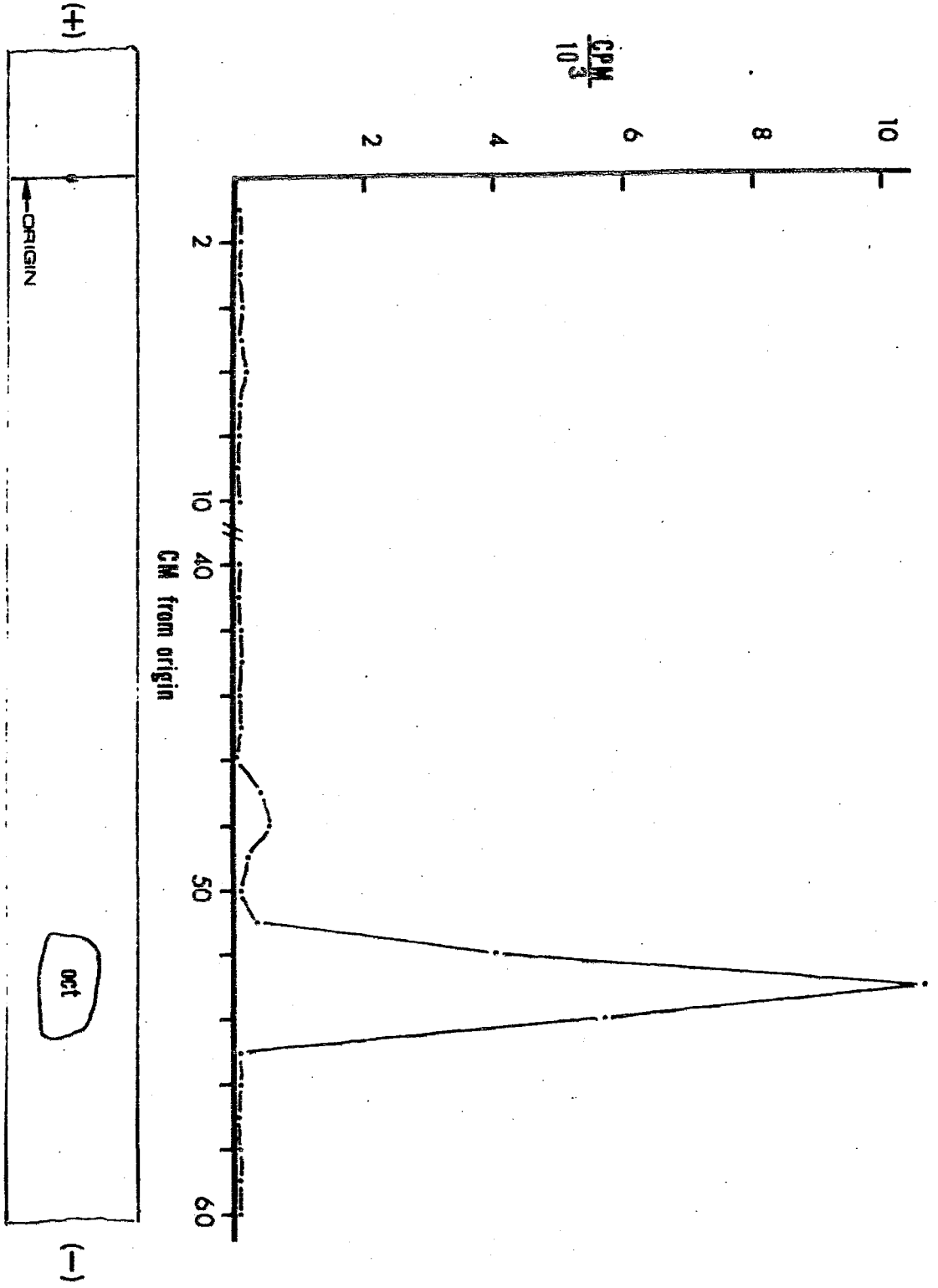
Metabolism of ^{3}H-Octopamine by Hemolymph and Hepatopancreas Tissues

When octopamine was incubated with hemolymph for up to 20 min, it was found that hemolymph exhibited virtually no capacity to metabolize this amine. Figure 14 represents an electropherogram obtained after a 20 min incubation in 11 μM ^{3}H-octopamine. Note that greater than 98% of the radioactivity co-chromatographed with authentic octopamine. (A small peak of radioactivity (1.5% of total) appeared at the arigin (6 cm toward the cathode, Fig. 14) suggesting that hemolymph may exhibit a slight capability of sulfonating octopamine.) The peak running just before octopamine was an unidentified radioactive impurity which amounted to 6% of the total counts.

When hemolymph was extracted by the method noted above only 38% of the total radioactivity was recovered in the supernatant. However, reextraction of the precepitate with an additional 100 μl of acid-ethanol increased the total recovery to 72%, suggesting that the missing octopamine was trapped within the rather large pellet of precipitated protein rather than covalently bound.

When octopamine was stored in the acid-ethanol solution for extended periods of time (weeks), it apparently underwent a chemical reaction forming an additional product.

Fig. 14 ^{3}H-octopamine metabolism by hemolymph of P. interruptus. The cathodic distribution of radioactivity on an electropherogram obtained from an extract of lobster hemolymph previously incubated 11 μM ^{3}H-octopamine for 20 min. These data demonstrate that greater than 98% of the octopamine remained unmetabolized. (The peak just before octopamine represents an unidentified radioactive impurity.) Note break in the abscissa, 10-30. In this region there were no significant radioactive peaks. See text for further explanation.


(+)
ORIGIN
oct
(-)
CM from origin
2
10
40
50
60
2
4
6
8
10
CPM
10³

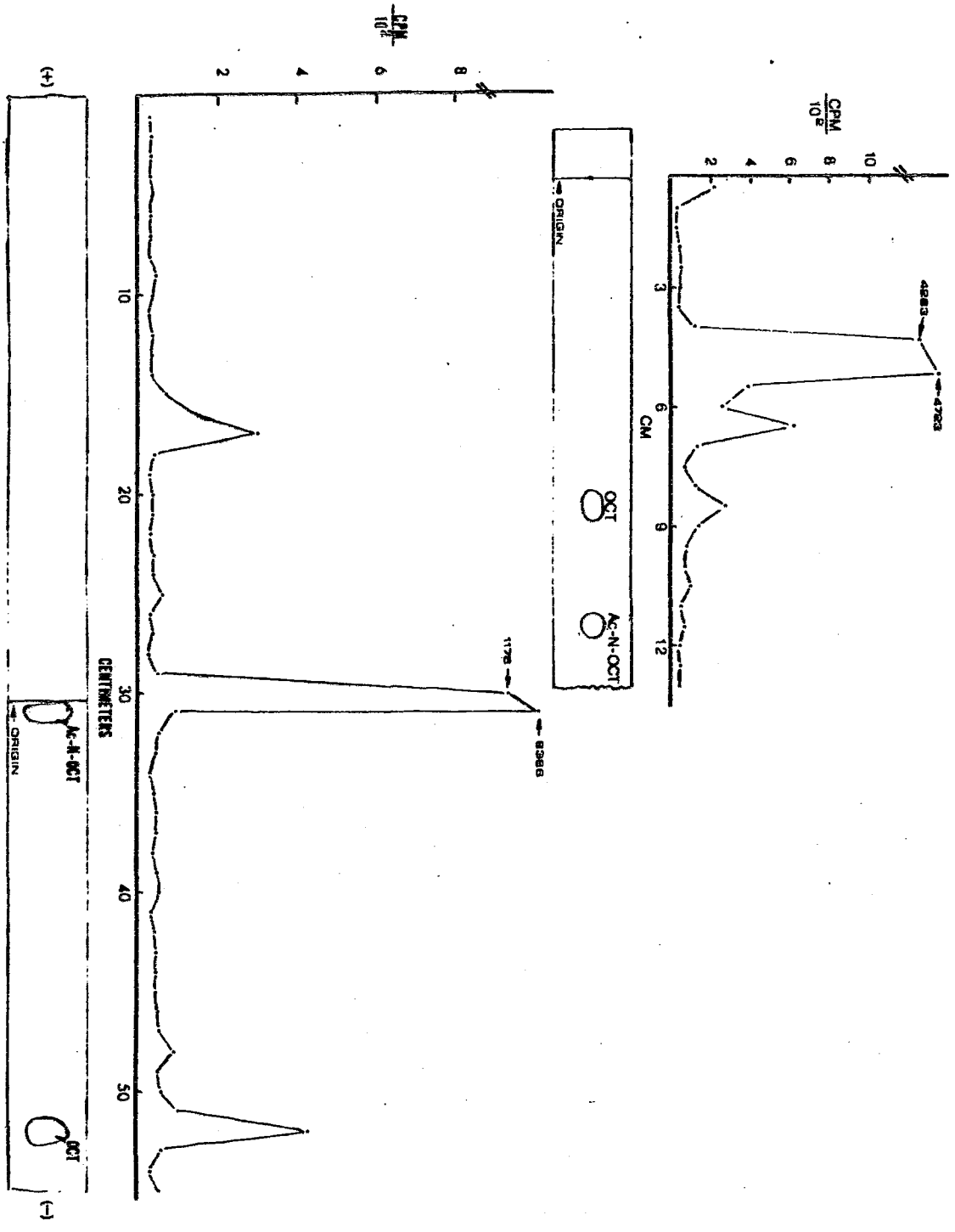
The reaction may possibly involve the dehydration of the aliphatic side chain since 1-phenylethanols are noted for this reaction (Morrison and Boyd, 1968). Thus, the use of strong mineral acids as extraction agents is to be avoided.

The major metabolite of octopamine found in extracts of incubated ceca run at the origin in the electrophoretic separation system (Fig. 15A). This neutral metabolite (i.e., no net charge at pH 1.9), most likely represents either octopamine-p-sulfate or beta-alanyloctopamine-p-sulfate as described by Kennedy (1977). The former may account for the bulk of radioactivity at the origin since ceca exhibit a low capacity to synthesize and accumulate beta-alanyloctopamine (Table 4 and Fig. 15A).

In addition to the neutral and cationic metabolites identified by Kennedy (1977), these experiments demonstrate the accumulation of an additional, as yet unidentified, anionic metabolite in hepatopancreas ceca (Fig. 15A). This metabolite begins to accumulate in substantial quantities (1% of total cpm taken up) after 20 min of incubation (Table 4). The negative charge at this pH probably indicates that a sulfonation or phosphorylation reaction had occurred. Considering its late appearance, it most likely is a secondary metabolite (either deaminated or conjugated) of the major neutral metabolite mentioned above.

When ceca were incubated for varying periods of time

Fig. 15 ^{3}H-octopamine metabolism by lobster hepatopancreas.
(A) The distribution of radioactivity on an electropherogram obtained from an extract of ceca that were incubated in 10 μM ^{3}H-octopamine for 30 min. (In the hepatopancreas incubations, the ^{3}H-octopamine was repurified before use.) The remaining counts are approximately equally distributed between authentic octopamine (OCT) and the unidentified, negatively charged metabolite. Octopamine and N-acetyloctopamine (Ac-N-OCT) were used as reference standards. The sulfonated metabolites, octopamine-p-sulfate and beta-alanyloctopamine-p-sulfate, co-chromatograph with n-acetyl-octopamine and represent 94% of the radioactivity. Beta-alanyloctopamine has an R_f = 0.81 X R_f (OCT) under these conditions. See Methods.
(B) Analysis of the same extract by thin-layer chromatography, But/AcOH/H_2O (60:15:25) demonstrating that N-acetyl-octopamine is not an octopamine metabolite in these tissues. See text for further explanation.


CPM / 10²
ORIGIN
OCT
Ac-N-OCT
CM
4283
4763
CENTIMETERS
1178
8388
(+)
(-)

(1-60 min) in 20 μM ^{3}H-octopamine, it was found that 60-90% of ^{3}H-octopamine which remained in the tissue after the wash-out period had been metabolized. Table 4 summarizes the results of one of these experiments and lists the distribution of radioactivity (at the various times as % of total cpm taken up) amongst the various classes of metabolites. The data demonstrate that the synthesis of neutral metabolite predominates throughout the whole time course, ranging from 50% in the first minute to 80% after one hour. Note that the percentage of cpm in the beta-alanyloctopamine region decreases throughout this time course suggesting that at least some of the origin counts represent beta-alanyl-octopamine-p-sulfate.

Figure 15A represents the distribution of radioactivity on an electropherogram obtained from an extract of ceca that were incubated in 10 μM ^{3}H-octopamine for 30 minutes. It demonstrates that 94% of the label remaining in the tissue after a 5 min wash had been converted to the neutral metabolite(s) with an additional 2% of the label metabolized to the anionic product. In this particular experiment the ceca took up 12% of the total radioactivity in the incubation medium. However, 2% of the medium counts ran as the neutral metabolite(s), and 4% as beta-alanyloctopamine, suggesting that these metabolites may ultimately regain access to the hemolymph and be excreted. The remaining medium counts

TABLE 4

Incubation Time (min)	Total CPM*	%Octopamine	%B-alanyloctopamine	% Sulfonated (neutral)	% Anionic
1	4.0×10^4	36.5	14.0	49.5	0.1
3	10.3×10^4	33.3	14.3	52.3	0.1
5	8.0×10^4	33.3	15.5	50.3	0.1
10	13.5×10^4	39.7	15.3	44.6	0.4
20	12.6×10^4	8.7	11.3	78.9	1.0
60	20.2×10^4	8.0	8.7	80.0	3.3

* Total radioactivity taken up by ceca

The distributions of ^{3}H-octopamine metabolites in lobster hepatopancreas tissue after varying periods of incubation. Ceca were incubated in saline containing 20 µM ^{3}H-octopamine for 1-60 minutes. Radiolabeled compounds were extracted into an acedic buffer and extracts were analyzed by high-voltage electrophoresis. (See Fig. 15A for a representative electropherogram.) The distributions are expressed as the percent of the total radioactivity taken up by the ceca. See text.

co-chromatographed with octopamine.

In these experiments N-acetyloctopamine was used as a reference standard because a number of investigators have reported that insect nervous tissues (Dewhurst et al., 1972; Evans and Fox, 1975 and Vaughan and Neuhoff, 1976) and crustacean epidermis (Summers, 1968) metabolize biogenic amines by N-acetylation. My first observations suggested that a similar metabolic pathway might exist in the hepatopancreas tissues since the major metabolite co-chromatographed with the N-acetyl adduct in the electrophoretic separation system (Fig. 15A). However, re-chromatography of the extracts on cellulose thin-layers with butanol-acetic acid-water (60:15:25) revealed that the neutral metabolite(s) was something other than N-acetyl-octopamine (Fig. 15B), notably, a mixture of octopamine-p-sulfate and beta-alanyloctopamine-p-sulfate.

Discussion

The endogenous levels of octopamine reported here indicate that octopamine is the major biogenic amine in the macruran pericardial organ system. Octopamine is three times more concentrated than serotonin and approximately fifteen times more concentrated than either dopamine or acetylcholine (see Sullivan et al., 1976).

The magnitudes of the endogenous stores of octopamine

and serotonin in this neurosecretory organ provide additional support to the hypothesis that they serve as neurohormones. If the endogenous levels of amines represent the releasable pools (as suggested by the serotonin release experiments in Chapter II), it seems reasonable to estimate that nanomolar concentrations of octopamine and serotonin obtain in the lobster hemolymph.

Could the endogenous octopamine content of pericardial organs account for some of the observed cardioexcititory activity exhibited by extracts of pericardial organs? In one series of experiments the ligamental nerve plexuses from approximately ten lobsters were extracted with acidified ethanol and the deproteinized extracts were subjected to high voltage electrophoresis (see Methods Chapter I). The entire biogenic amine region (from serotonin to tyramine) was cut into strips, eluted into physiological saline and assayed for cardioexcititory activity. One large peak of cardio-excititory material was detected; it co-chromatographed with reference standard ^{3}H-octopamine. When the eluted material from this peak was subjected to thin-layer chromatography (Solvent 1, see Chapter I), the cardioexcititory material again co-chromatographed with the standard ^{3}H-octopamine. Thus it seems likely that at least some of the cardio-excititory activity exhibited by crude extracts of peri-cardial organs may be due to the endogenous levels of

octopamine.

The major conclusion to be drawn from the octopamine metabolism experiments is that the enzymes necessary to metabolize biogenic amines are virtually absent in hemolymph. Considering that both octopamine and serotonin are metabolized by the same metabolic pathways (Kennedy, 1977), my results suggest that biogenic amines released from the ligamental nerve plexuses obtain a wide distribution throughout the animal. Excepting the possibility of effective removal by high affinity binding sites, it would seem reasonable to assume that any tissue or organ in the animal may serve as a target site.

I might point out that the first tissues encountered by biogenic amines released from the plexuses would be the other neural elements within the plexuses and the hemolymph itself. In fact the hemolymph is a target tissue for released octopamine. Battelle and Kravitz (1976) reported that 10^{-7} M DL-octopamine in Homarus americanus hemolymph is sufficient to increase cyclic-AMP levels in hematocytes. It is also possible that octopamine and serotonin play an intraplexus role, either controlling or modulating the release of other pericardial hormones. However, these roles seem dubious since peptide synthesis most likely occurs in specific cell body regions of the thoracic ganglia; (see Maynard, 1961 and Terwilliger, 1967) and neither dopamine nor serotonin

effects the release of pericardial "excititory peptide" (Berlind, Cooke and Goldstone, 1970).

The observations concerning the rapid metabolism of octopamine by hepatopancreas ceca confirm the earlier reports of Marmaras *et al*. (1971), who noted that the hepatopancreas had a greater capacity to metabolize ^{14}C-dopamine than did "whole heads" from the decapod, *Upogebia littoralis*. It is probable, however, that these authors were in error in identifying the dopamine metabolites as noradrenaline and adrenaline, but one cannot rule out the possibility of alternative metabolic pathways *in vivo*.

The cationic and neutral metabolites observed in these studies exhibit the same electrophoretic properties as the sulfonated and beta-alanylated metabolites described by Kennedy (1977). Kennedy has partially characterized the enzymes involved in both metabolic pathways. Sulfonation requires an additional substrate, 3'-phosphoadenosine-5'-phosphosulfate, and beta-alanylation requires both beta-alanine and ATP.

Evans *et al*. (1976) have noted that sulfonated metabolites are the major products found in hemolymph 15 minutes after injection of radiolabeled phenolamine into live *Homarus*. The data reported here also indicates sulfonation to be the

major step in the metabolism of octopamine by the hepatopancreas (see Table 4). This predominance of neutral metabolites may reflect the extent of hepatopancreas activity in the metabolism of circulating biogenic amines.

If one considers that after 1 minute of incubation, 5-10 ceca (approximately 0.01% of the total wet weight of the hepatopancreas) metabolize 4.1 pmol of octopamine (Table 4), then one can calculate that the entire hepatopancreas can metabolize octopamine at a rate of 40 nmol/min, the major products being the neutral (sulfonated) metabolites. Taking into account that the hepatopancreas receives approximately 3% of each stroke volume (based on a comparison of the diameters of the cardiac arteries and the Poiseuille-Hagen equation, i.e., flow$\propto r^4$) via the hepatic arteries, and considering the hepatopancreas as a "metabolic sink" for biogenic amines, greater than 60% of the sulfonation observed by Evans et al. (1976) may be accounted for by hepatopancreas metabolism.

It is difficult to assess the extent of beta-alanylation occurring in hepatopancreas tissues. Table 4 suggests that beta-alanyloctopamine pools equilibrate rapidly and then decrease with time. However, this is in contrast to increases with time for both the sulfonated and anionic metabolites. It is also possible that beta-alanylated products are rapidly sulfonated and the bulk of the radio-

activity accounted for by the neutral metabolites (Table 4) represents B-alanyloctopamine-p-sulfate rather than octopamine-p-sulfate. Further chromatographic experiments requiring the appropriate reference standards are necessary to resolve this matter.

Presently, the physiological significance of beta-alanylated biogenic amines is unknown. Arnould and Frentz (1975) found no effect of beta-alanylhistamine on the heart beat of Carcinus. Similarly, I have been unable to detect any cardioexcititory materials in the electrophoretic region corresponding to beta-alanyloctopamine (unpublished observations). Its synthesis in the hepatopancreas may suggest that this amine serves only as a metabolite of octopamine. Moreover, molecular models of beta-alanyloctopamine indicate that the protonated amine is capable of forming an intramolecular hydrogen bond with the side chain hydroxyl group. Such a bond may reduce the ionic interactions of this molecule and facilitate its diffusion from tissues.

In summary, these experiments show that both octopamine and serotonin are stored in substantial quantities in the pericardial organs of P. interruptus. The released amines would reach their target sites via the hemolymph which exhibits little capacity to metabolize biogenic amines. Both observations provide additional evidence to support the general hypothesis that octopamine and serotonin serve as neurohormones in the decapod Crustacea.

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