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Neuroendrines in pericardial organs of the crab, *Cancer*

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BOSTON UNIVERSITY

GRADUATE SCHOOL

Thesis

NEUROENDOCRINES IN PERICARDIAL ORGANS
OF THE CRAB, CANCER.

By

N. ABBY SCHWARZ

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cop 1

Approved by

First Reader

Frank C. Belamand

Professor of Biology

Second Reader

Charles K. Leuz

Professor of Biology

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Topical Outline

Chapter	Page
I. Introduction.	1
A. Statement of purpose	1
B. The neurosecretory granule	1
C. Chemistry of neurosecretory material	2
D. Synthesis of neurosecretory material; formation of granules.	5
1. Mitochondria.	5
2. Rough endoplasmic reticulum	6
3. Nucleus	7
4. Golgi apparatus	8
E. Axonal transport of granules	10
F. Further synthesis of neurosecretory material in axon	12
G. Nature of granule membrane	14
H. Release of neurosecretory material	14
1. Method of release	14
2. Ultrastructure of neurosecretory cell wall and of capillary cell wall	17
3. Release from perikaryon	19
I. Neuronal properties of neurosecretory cell	19
1. Morphological properties.	19
2. Conduction properties	20

chapter	page
II. Methods and materials	23
A. Animals	23
B. Preparation of tissues	23
C. Method of bioassay	25
D. Preparation of media	26
E. Preparation of test fractions. . .	26
III. Results	28
IV. Discussion.	30
V. Summary and conclusions	33
VI. Bibliography.	34
VII. Abstract.	42

List of Illustrations

	Page
I. Perfusion system assembly	33A
II. Material run in <u>Homarus</u> perfusion fluid at centrifugal forces of 1000 x g, 12,000 x g, 17,000 x g and 30,000 x g.	33B
III. Material run in 0.9 M sucrose at centrifugal forces of 1000 x g, 12,000 x g, 17,000 x g and 30,000 x g	33B
IV. Material run in 0.9 M sucrose at centrifugal forces of 1000 x g, 4000 x g, 8000 x g and 12,000 x g . .	33C
V. Material run in 0.9 M sucrose at centrifugal force of 71,000 x g . . .	33C

Introduction

The pericardial organs are neurosecretory structures situated within the pericardial cavity of stomatopod and decapod crustaceans. Their location in the pericardium is such that blood approaching the heart must flow directly over them (Alexandrowicz, 1953; Maynard, 1961a). This arrangement facilitates the distribution of pericardial organ secretions to all parts of the organism. It is not known how many different neurohormones are secreted by the pericardial organs, but at least one of these has been shown to act as a strong cardioexcitor (Alexandrowicz and Carlisle, 1953; Carlisle and Knowles, 1959; Belamarich, 1963).

A review of the literature suggested that the agent responsible for cardioexcitatory activity might be localized in a subcellular granular fraction which might be isolated by differential centrifugation. Accordingly, a study was undertaken to investigate this possibility.

It is known that in neurosecretory systems the products of secretion are typically stored in axon terminations in the form of aggregated particles or granules (Scharrer and Scharrer, 1954; Welsh, 1955). Electron microscopic observation of neurohumoral organs, or aggregations of fiber terminations (neurohypophysis and urohypophysis of vertebrates; sinus glands, pericardial organs and post-comissural organs of crustaceans; corpus cardiacum of

insects) shows the constant presence of at least one class of elementary neurosecretory granules, 1000-3000 A in diameter, although their presence is not always a reliable ultrastructural criterion for true neurosecretory activity (Bern, 1962). They may be vesicular in appearance but are usually electron-dense, and may stain either Gomori-positive or negative. Small vesicles, 300-500 A in diameter, abound in the axon terminations of neurosecretory cells (Holmes and Knowles, 1960). Mitochondria are also present in these axon endings but their significance is not clearly understood.

The earlier theory that vertebrate neurosecretory materials (Schiebler, 1952), and perhaps those of invertebrates, were glycolipoprotein has been rejected due to evidence indicating that the neurosecretory material is primarily proteinaceous. In the hypothalamus of normal rats and dogs the neurosecretory material has constantly shown the presence of protein-bound disulfide groups (Barrnett, 1954). Insect neurosecretory material also contains disulfide groups (Sloper, 1957) and sulfhydryl groups (Brousse et al., 1958). The existence of disulfide groups in the neurosecretory material of certain vertebrates has been confirmed by Palay (1957) and Gabe (1960). Gabe has also shown that certain vertebrates, especially amphibians, possess hypothalamic neurosecretory material which is always PAS-positive, while others show little or no evidence of a carbohydrate moiety associated with the neurosecretory product. Sloper (1963) has demonstrated the existence of cystine,

but not methionine, in rat neurosecretory material.

The presence of indole alkylamines has been discovered in extracts of crustacean pericardial organs (Welsh, 1955; Carlisle, 1956). Carlisle and Knowles (1959) found two active components in pericardial organ extracts and suggested that one of them might be 5,6-dihydroxytryptamine and the other a precursor of the first. However, Welsh (1959) suggested that the unidentified component might be a polypeptide, unrelated to the first substance. Maynard and Welsh (1959) found an active principle in extracts of pericardial organs from brachyuran crustacea bearing a definite resemblance to 5-hydroxytryptamine. However, further study revealed that the pericardial organ extracts contained insufficient quantities of 5-hydroxytryptamine to account for their strong cardioexcitatory effects. Cooke (1962) found a similarity of cardiac response to 5-hydroxytryptamine and to pericardial organ extracts. Belamarich (1963) found two active peptides in pericardial organ extracts, differing in composition by only a small number of residues and containing more glutamine than lysine or arginine, one other negatively charged residue, and a small number of neutral amino acids. Carlisle (1964) found that half the activity of the pericardial organ extract resided in an indole fraction and half in a polypeptide fraction and presented further evidence that the active indole might be 5,6-dihydroxytryptamine.

Enzyme inactivation studies of crustacean chromatophorotropins and retinal pigment hormones, as well as of vertebrate neurosecretory materials, have shown that as with true proteins, these materials are inactivated by trypsin or chymotrypsin (Knowles and Carlisle, 1956; Peréz-González, 1957; Kleinholz et al., 1962). Maynard and Welsh (1959) found this true of the active principle in extracts of crustacean pericardial organs.

Gabe (1960) has stressed the point that there may be differences in the proteins composing neurosecretory material. The work of most investigators indicates that many kinds of materials, including simple proteins, glycoproteins, and lipoproteins, may serve as carrier substances from which the active hormonal principle may be dissociated upon release. Moses et al. (1963) found that the stainable neurosecretory material in the rat neurohypophysis was distinct from vasopressin and postulated that it might be acting as a carrier or precursor for the hormones of the neurohypophysis. However, the form in which the active hormone is stored in the neurosecretory granules before release is a subject of much controversy. Many investigators consider the stainable neurosecretory material a carrier protein of the active hormone (Acher and Fromageot, 1957; Acher, 1958; Heller and Lederis, 1961; Scharrer and Brown, 1961a), while others consider it a parent protein which is broken down to form

hormones which are active and of low molecular weights (Gabe, 1960). Ginsburg and Ireland (1963) suggest that oxytocin and vasopressin are protein-bound in the neurohypophysis and suggest that small pH changes participate in hormone release mechanisms.

The synthesis of neurosecretory material and its segregation into particles or granules has been associated with the rough endoplasmic reticulum, the nucleus, the mitochondria and the Golgi apparatus.

Some investigators have suggested that the mitochondria, which are found throughout the neurosecretory cell, are involved in the formation of the elementary neurosecretory granules. Knowles (1958 and 1961), working with Squilla mantis, indicated that the mitochondria might participate in the actual synthesis of the granules. Green and Maxwell (1959) suggested that the mitochondria might transform into granules. Challice and Lacy (1954), working with mouse pancreas, suggested that the mitochondria might fragment or bud into granules. This opinion has been held by Nishiitsutsuji-Uwo (1960 and 1961), working with several species of Lepidoptera. Holmes and Knowles (1959) found morphological alterations in mitochondria of neurosecretory cells in the pituitary stalk of the ferret after sectioning, and tentatively associated them with functional changes related to the interruption of the neurosecretory process. Hagadorn (1962) found that neurosecretory cells in leech

brain possessed giant mitochondria but was unable to elucidate their significance. Other investigators have found no evidence that the mitochondria bear any relationship to the formation of neurosecretory material or to its segregation into granules (DeRobertis, 1961; Scharrer and Brown, 1961a,b; Bern, 1962). However, Hagadorn (1962) did observe an inverse relationship between the size of mitochondria and the number of granules in neurosecretory cells of leech brain.

Most investigators agree that the rough endoplasmic reticulum, with its ribonucleoprotein granules, is probably involved in the production of the basic protein of the neurosecretory material (Scharrer and Brown, 1961a and 1962; Palay, 1958; Weiss, 1958; Bern, 1962). Nishiitsutsuji-Uwo (1960 and 1961), Scharrer and Brown (1961a) and Bern and Takasugi (1962) have observed an increase in the density and number of membranes composing the endoplasmic reticulum in the neurosecretory cell when the concentration of granules, and therefore presumably of available neurosecretory material, was sparse. However, some investigators consider a main function of the endoplasmic reticulum to be the transportation of neurosecretory material from its site of formation in the cytoplasm to the region of the Golgi apparatus (Rinehart and Farquhar, 1953; Palay, 1958 and 1960). Rinehart and Farquhar (1953), Farquhar and Wellings (1957) and Fridberg (1963) claim to have seen connections in the

cytoplasm between the endoplasmic reticulum and the Golgi apparatus. These connections have not been observed by other investigators.

When the concentration of granules in the perikaryon of the neurosecretory cell is high, the endoplasmic reticulum appears to be broken up (Afzelius and Fridberg, 1963; Scharrer and Brown, 1961b; Fridberg, 1963). Scharrer and Brown suggest that the breakdown and dispersal of the endoplasmic reticulum is probably not related to the synthesis of secretory precursors but to the invasion and storage of mature neurosecretory granules in the inter-cisternal matrix. This dispersal of the endoplasmic reticulum, they state, actually eliminates the source of secretory precursors, and it may be expected that reconstitution of the endoplasmic reticulum follows the transport and release of the neurosecretory granules.

Evidence for nuclear participation in neurosecretion has been found in fish hypothalamic neurons by Palay (1943), Enami (1955) and Ortmann (1958). However, Scharrer and Brown (1961b) found no evidence for nuclear participation in neurosecretion in the earthworm neurosecretory brain cells, nor did Bern and Takasugi (1962) in any of the vertebrate or invertebrate species they studied, nor did Follenius (1963) in the preoptic nucleus and the lateral tuberal nucleus in the trout and perch.

Participation of the Golgi apparatus in the formation of elementary neurosecretory granules, but not in the actual synthesis of the neurosecretory material, has been suggested by many investigators (Sjostrand and Hanzon, 1954; Palay, 1958 and 1960; Dalton, 1960; Nishiitsutsuji-Uwo, 1960 and 1961; Bern et al., 1961a,b; Farquhar, 1961b; Scharrer and Brown, 1961a,b and 1962). It has been proposed that the Golgi apparatus segregates the neurosecretory material into granules by removing water from it (Kirkman and Severinghaus, 1938; Wellings and DeOme, 1961), but the mechanism is unclear. Immature-appearing granules (smaller, less electron-dense) have been seen lying within the Golgi membranes (Sjostrand and Hanzon, 1954; Farquhar, 1961b; Follenius, 1963). Many investigators have suggested that "budding" is the mechanism by which the granules are manufactured; this involves the segregation of the neurosecretory material within the Golgi lamellae, enclosure by the lamellae to form the membrane of the granule, and the pinching or budding off of the formed body from the Golgi membranes (Dalton, 1960; Palay, 1960; Bern et al., 1961a,b; Wellings and DeOme, 1961; Bern, 1962; Scharrer and Brown, 1962; Lane, 1964). Finally, Farquhar and Wellings (1957) and Nishiitsutsuji-Uwo (1961) have suggested that the mechanism of granule formation may differ in different cell systems.

In the leech, cockroach and frog (Bern et al., 1961b) and in Lumbricus (Scharrer and Brown, 1961a) an inverse

relationship has been noted between the size of the Golgi apparatus and the concentration of elementary neurosecretory granules in the perikaryon of the neurosecretory neuron. Bern has suggested that cells with many granules might be storing neurosecretory material and that those with few granules but with a well-developed Golgi apparatus might be engaged in massive synthesis of the neurosecretory material. However, Rinehart and Farquhar (1953), working with mammalian anterior pituitary, observed a decrease in size and an apparent atrophy of the Golgi apparatus with a decrease in the number of granules.

A distinct class of globules has been found in the perikarya of neurosecretory cells by several investigators (Palay, 1960; Dalton, 1960; Bern and Takasugi, 1962; Murakami, 1962; Afzelius and Fridberg, 1963). It is of great interest, since it has been suggested that it is identical with the neurosecretory droplets described by the light microscopists (Palay, 1960). Fridberg (1963) has made a study of these droplets in the caudal neurosecretory system of Leuciscus and Phoxinus; his observations follow. The globules are 0.5 - 1.0 μ in diameter, occurring throughout the cell body but showing preference for the Golgi lamellae. They show considerable variation in their morphology, but characteristic features are the thin limiting membrane and a fine granular content of moderate electron density. Small granules or circular profiles of higher electron density can

be seen in their matrix, and rarely, areas of the size and electron density of elementary neurosecretory granules. Sometimes the globules have a lamellated organization. The surface membrane is often broken, with the globule contents enjoying direct communication within limited regions of the cytoplasm. Fridberg says, however, that it is impossible to establish whether these areas are condensed centers and precursors of the globules or actually represent a state of dissolution. The multivesicular bodies were found to be less frequent than the globules, and Fridberg did not find a genetic relation between them and the globules as maintained by Palay (1960) or Dalton (1960). In no case could he find any indications of transitional stages between the elementary neurosecretory granules and the globules, which accords with the findings of Palay (1960), Dalton (1960), Murakami (1962), and Afzelius and Fridberg (1963).

In neurosecretory systems the secretory material produced within the bodies of neurosecretory cells migrates along nerve axons to storage-release centers (terminals) composed of assembled nerve fiber endings. It is stored here until appropriate stimuli cause its release. Transport of granular neurosecretory material down the axon usually takes place as a consequence of axoplasmic flow (Weiss, 1955). The nature of axoplasmic flow is not clearly understood, but Welsh (1955) offered experimental proof of its existence by cutting the axons of neurosecretory cells and observing

a pile-up of neurosecretory material proximal to the cut. This phenomenon has since been demonstrated by other investigators. Sloper (1958) also observed axoplasmic flow from the hypothalamus to the neurohypophysis, estimating the rate of movement of neurosecretory material at 1 - 2 mm per day.

Wigglesworth (1960), in a study on ordinary neurons in the cockroach, presented some evidence that the Golgi bodies contained clear canals which became continuous with the neurofibrils of the axons. Bern et al. (1961b) found no ultrastructural evidence for the existence of such canals in the frog, leech or cockroach. Most electron microscopists have not found good evidence for the existence of special tubes or canals in the axons to guide the movements of the granules; however, Knowles (1960 and 1961), in a study on the pericardial organs of Squilla mantis, has arranged electron micrographs showing different forms of aggregate bodies in a continuous series ranging from mitochondrion-like bodies through a multilamellate form. Intermediate forms between multilamellate bodies and aggregate bodies containing vesicles were also observed. He suggested that this provided evidence of hormonal material being assembled in systems of concentric lamellae or tubules of cytomembranes which extended from the perikaryon to the axon terminal and that these systems were derived from bodies which resembled mitochondria. Neurosecretory material would therefore accumulate within systems of tubules in the axoplasm.

Knowles defined an elementary neurosecretory vesicle as an aggregation of material within a tubule, the wall of the tubule forming the bounding membrane of the particle, as opposed to the theory that the neurosecretory granules were pinched off the Golgi membranes. Nishiitsutsuji-Uwo (1960), working with several species of Lepidoptera, also observed gradations in size between elliptical mitochondrion-like bodies and more electron dense bodies that lacked cristae. DeIraldi et al. (1963) has observed neurotubules, as distinct from neurofibrils, in the axons of neurosecretory cells of the rat hypothalamus, which may serve to guide the movements of the neurosecretory granules.

Controversy exists over the question of axonal synthesis of neurosecretory material and further elaboration of elementary neurosecretory granules while they are passing down the axon. Farquhar (1961b) and others have observed small vesicular-appearing granules close to the Golgi membranes and larger ones at some distance away. In Cambarellus shufeldti (Fingerman and Aoto, 1959) and in the toad (Gerschenfeld et al., 1961) a "maturation" of the granules en route from the perikaryon to the axon terminal has been observed. On the other hand, Follenius (1963) observed no increase in size of neurosecretory granules in hypothalamic nuclei of the trout and perch, and concluded that further elaboration of material did not occur. Green and Maxwell (1959), DeRobertis (1961), Christ (1962) and

Sachs (1963) have found good evidence for further elaboration of neurosecretory material in the axon. Fridberg (1963) states that the axons of neurosecretory cells in the caudal neurosecretory system of two teleost species contained ergastoplasm and elementary granules distributed in rows and that distally the ergastoplasm was replaced by an agranular reticulum formed by membranes of the processes, which were invaginated and formed short tubules and vesicles, and whose cisterns seemed free from inclusions, containing a material of low electron density. Christ (1962) also found an endoplasmic reticulum in the axons of rabbit neurosecretory cells. However, Hydén (1960) found no evidence for the presence of RNA in the axons and concluded that no axonal synthesis of neurosecretory material was possible. Bern (1962) agreed with Hydén, finding no evidence for the presence of an endoplasmic reticulum or Golgi membranes in the axons, but he suggested that the elementary neurosecretory granules might themselves be cell organelles capable of maintaining minimal synthetic activity as they were conveyed away from the Golgi membranes and down the axon. This might explain the aforementioned observation of Farquhar (1961b) and others. Knowles (1960 and 1961) and Sloper (1963) suggested that the small neurosecretory vesicles or granules accumulated in tubules as larger ones as they passed down the axons, rather than further neurosecretory material being synthesized.

In all neurosecretory systems so far studied, the elementary neurosecretory granules have been found to be bounded by a delicate semipermeable membrane. The membrane has been reported to arise from the Golgi lamellae (Scharrer and Brown, 1961b and 1962; Bern and Takasugi, 1962), or from the mitochondria (Farquhar, 1961a; Knowles, 1960 and 1961; Nishitsutsuji-Uwo, 1961). Hillarp and Nilson (1954) and Blaschko et al. (1955) found that the membrane of the granules in bovine adrenal medullary cells was of a lipoprotein nature, phospholipids and free cholesterol being essential for the structural integrity of the granules. The latter authors also suggested that the membrane shared certain properties with the cell membrane. Pérez-González (1957), working with Uca, suggested that the membranes of the neurosecretory granules in the sinus glands might be freely permeable to sodium and potassium, as isotonic solutions of NaCl, KCl and sea water caused immediate and marked release of granule contents.

The majority of experiments indicate that the hormones contained within the granules are passed across the neurosecretory cell membrane into the blood stream without concurrent passage through the cell wall of the granule itself. Palay (1955), in an ultrastructural study of the rat neurohypophysis, found that in normal and hydrated animals the axon terminations of neurosecretory cells contained many elementary neurosecretory granules and vesicles,

but that in dehydrated rats the granules and vesicles presented empty or nearly empty circular profiles. This phenomenon has been observed after stimulation of the neurosecretory cell in a number of species by many investigators, including Hartmann (1958), Knowles (1960 and 1961), Nishiitsutsuji-Uwo (1961) and Follenius and Porte (1962), and has been generally interpreted as indicating release of contents and not passage of the intact granule through the neurosecretory cell membrane. Palade (1959), studying release mechanisms of neurosecretory material in acinar cells of exocrine mouse pancreas, found that the granule membranes merged with the cell membrane, with loss of spherical shape after mergence, and suggested that this indicated that the granules released their contents without themselves being extruded through the cell membrane. Farquhar (1961a,b), working with pituitary acidophils, found a similar phenomenon, including invagination of the cell membrane to meet the granule membrane, and found no formed granules in either the perivascular connective tissue or within the vascular endothelium. Gerschenfeld et al. (1960), in an ultra-structural study of the toad neurohypophysis, suggested that upon receipt of appropriate stimulation the neurosecretory granules released their contained hormone within the axonal bulb, the liberated hormone or hormones diffusing through the cytoplasm to the cell membrane and thence through it.

Palay (1957), working with rats, noted that even if the fenestrations in the capillary endothelium were considered portals of entry into the vascular lumen, the need to account for movement of the granules across the connective tissue space and against the hydrostatic pressure within the capillary lumen would diminish the credibility of the hypothesis that the neurosecretory material is transported in granular form into the capillary lumen. Other investigators have felt similarly, but studies have been made which suggest that the possibility remains that the whole granule may be extruded through the cell membrane. Working with bovine adrenal material, Blaschko et al. (1955) showed that fresh granules injected intravenously did cause an immediate rise in arterial blood pressure of the spinal cat, although this effect was only a small part of that of the total pressor amine content. The sustained pressor effect showed that at least some of the adrenalin, not immediately available, also exerted an effect, and therefore the possibility that the granules were released intact could not be ignored. However, the presence of adrenalin in the supernatant fluids made it likely, in their opinion, that some adrenalin was present in the cytoplasmic sap. They suggested that this was the amine that was secreted when the cell was stimulated. Heller and Lederis (1961), working with neurosecretory vesicles from mammalian neural lobes, observed that probably only the contents of the granules

were released but noted that they had seen dark homogenous bodies in the neurohypophysial capillaries which looked like extruded granules or aggregates of their contents which might not just be an artefact of preparation. Scharrer and Frandson (1954), studying release of neurosecretion in the dog posterior pituitary, found that after injection of nicotine tartrate considerable amounts of neurosecretory material were seen within the greatly dilated blood vessels of the neurohypophysis, in whose tissue many hemorrhagic areas existed. They assumed that the walls of the dilated vessels had become permeable to formed particles, namely neurosecretory granules and red cells, and suggested that under normal conditions the neurosecretory substance also passed through the walls of the vessels in granular form, presumably to be dissolved in the blood. However, these were not physiological conditions, and the extent of trauma to the tissues diminishes the credibility of their conclusions.

The ultrastructure of the neurosecretory cell wall and the adjacent capillary wall is of paramount importance in studies of release of neurosecretory material. Palay (1955) described the ultrastructural picture in the neurohypophysis of rats. The work of Farquhar (1961b), which follows, presents the topography in capillaries of the mammalian anterior pituitary in detail, implementing Palay's work as well.

The basic organization of the capillary wall is

characteristic for capillaries of most endocrine glands (e.g., neurohypophysis, thyroid, adrenal cortex). The membranes of the vascular endothelial cells are in direct contact with circulating blood on one side and with the endothelial basement membrane on the other. Frequently the vascular endothelium shows cytoplasmic evaginations, devoid of cytoplasmic organelles, projecting into the perivascular connective tissue space. At these evaginations, the basement membrane is irregular. The endothelium also possesses fenestrae, 300 - 500 A in width, which in some cases are bridged by thin derivatives of the membrane of the vascular endothelial cell. Here the basement membrane is absent. The fenestrae of the vascular endothelium occur most often at those sites where large amounts of secretory material are being transported. In capillaries where the endothelium forms a continuous unfenestrated layer, the endothelium contains many small pinocytic vesicles which participate in transendothelial transport and take the place of fenestrae. The perivascular connective tissue space is bounded on one side by the irregular endothelial basement membrane and on the other by the parenchymal basement membrane. Many kinds of parenchymal projections extend into the perivascular connective tissue space, sometimes separated by the parenchymal basement membrane. In some regions of the central nervous system, the endothelial basement membrane is not separated from glial

cells by the perivascular connective tissue space, but rather, the cells abut right on the basement membrane. According to Farquhar, once the granule membrane has merged with the cell membrane the two main barriers for neurosecretory material are the endothelial basement membrane and the tenuous membrane bridging the fenestrae.

Parameswaran (1956) and Matsumoto (1958) claim to have observed discharge of neurosecretory material from the perikaryon itself in neurosecretory cells of several species of crabs. However, this phenomenon has not been observed by other investigators.

Neurosecretory neurons possess neuronal properties as well as glandular ones. Axons and dendritic processes are usually present. Nissl substance, or its equivalent, is present; a prominent endoplasmic reticulum has been demonstrated with the electron microscope. Considerable amounts of RNA have been revealed by cytochemical techniques, as would be expected in a cell actively synthesizing a protein secretory material. Silver-staining techniques have shown the presence of neurofibrils and, in some cases, of delicate neurofilaments.

Neurosecretory fibers appear to be associated relatively constantly with glial elements. In many vertebrates the glial cells are especially evident as the parenchymatous pituicytes of the pars nervosa. Similar cells occur in the median eminence of the parakeet and also in the urohypophysis of the caudal neurosecretory system (Bern, 1962). Apparent

termination of neurosecretory fibers upon the glial elements themselves was seen in the rat pars nervosa by Rennels and Drager (1955), who suggested that glial cells played an important part in the release and transport of neurosecretory hormones into blood vessels. As evidence that pituicytes were essential to the normal release mechanism, they showed that after hypophysectomy the resulting accumulation of neurosecretory material in the proximal end of the infundibular stalk was accompanied by hypertrophy and hyperplasia of the remaining pituicytes. Nishiitsutsuji-Uwo (1961) observed that neurosecretory cells in Lepidopteran pars intercerebralis were enmeshed by neuroglial cell processes, among which existed canaliculi communicating with a blood sinus. The canaliculi were also in contact with the plasma membranes of the neurosecretory cells. Granules could not be seen within the canaliculi.

This shows that morphologically neurosecretory cells are good neurons. As for the possibility of conduction by neurosecretory cells, the explanation of neuroendocrine reflexes, in which neurosecretory cells participate by the rapid discharge of pre-formed secretion from their axonal bulbs, is tied up with the question of impulse conduction. Bern (1962) observed that most neurosecretory tracts and nerves investigated contained non-neurosecretion-bearing fibers along with neurosecretion-bearing ones, and that, accordingly, the recording of electrical activity from such

mixed tracts or nerves did not allow one to conclude that the neurosecretory fibers themselves were capable of conducting impulses. He noted that auxiliary neurons might innervate the axonal bulb regions; impulses conducted by them might be responsible for hormone release. However, Cooke (1964) studied electrical activity and release of neurosecretory material from pericardial organs of the crab Libinia emarginata, and presented good evidence that neurohormones were released from the terminals of neurosecretory cells as a result of action potentials propagated by the axons of the neurosecretory cells themselves. There is still a definite paucity of data to support a generalized and definitive conclusion. However, electrophysiologic experiments (Morita et al., 1961; Bennet and Fox, 1962; Ishibashi, 1962) have produced good evidence for the impulse-conducting property of Dahlgren cells and also for the synaptic transmission of impulses to them. On sections according to the Bodian method, Ishibashi (1962) interpreted nerve fibers terminating on Dahlgren cell bodies in the eel as synapses. The ultrastructure of the peripheral regions of the cells in teleosts (Fridberg, 1962) clearly showed that the cells had the bouton-like synaptic junctions described in motor neurons (DeRobertis, 1959); this also confirmed the postulation of the presence of synapses. Ultrastructural evidence of typical synaptic structures on neurosecretory neurons in the preoptic and lateral tuberal

nuclei of fishes has been presented by Follenius (1963) who stated that their presence indicated that nerve impulses may intervene in the control of secretory activity, or that these neurons may be activated and produce nerve impulses which may control release of neurosecretory material from the axon endings. Fridberg (1962) stated that the nervous control of the caudal neurosecretory system in teleosts might serve two functions: a) the release of transmitters in the synaptic vesicles of the neurosecretory cell terminals (Gerschenfeld et al., 1960; Koelle, 1961), and b) to regulate the secretory activity of the cells (Speidel, 1919). The fact that stimulation of neurosecretory cells is followed by empty synaptic vesicles in some cases (Palay, 1955) has been interpreted as meaning that the synaptic vesicles contain transmitter substance or some substance capable of causing release of neurosecretory material.

Methods and Materials

Animals

The animals used throughout this study were the sand crab Cancer irroratus, the rock crab Cancer borealis, and the common lobster Homarus americanus. All species were obtained commercially from Mill Cove Lobster Pound, Boothbay Harbor, Maine. As the animals survive best in a cold environment, they were kept at 4-8°C at all times. The medium used was either natural or synthetic sea water. The synthetic sea water was prepared by adding Rila Marine Mix (Rila Products, Teaneck, New Jersey) to deionized water in the ratio of one pound to three gallons.

Preparation of tissues

a. Dissection of crab pericardial organs

All major thoracic appendages were removed. The portion of the carapace covering the pericardial cavity was removed with scissors and scalpel to expose the heart. The heart was lifted out with forceps, care being taken not to perforate the sperm or egg ducts or the hepatopancreas. The crab was then immersed in cold synthetic sea water and the pericardial organs removed with forceps under a dissecting microscope. The pericardial organs were immediately dropped into a glass homogenizer containing the appropriate medium.

b. Preparation of lobster heart for assay

All major thoracic appendages were removed. The lobster

was cut at the thoraco-abdominal junction and the abdominal half discarded. Throughout the procedure, care was taken not to perforate the sperm or egg ducts or the hepatopancreas. The thoracic portion was bathed in cold Homarus perfusion fluid during the rest of the operation. The part of the carapace covering the pericardium was dissected away, leaving the hypodermis intact. The hypodermis was then carefully cut away, exposing the heart. The muscles surrounding the pericardium were excised. The optic and antennary arteries and some heart tissue lying directly beneath them were tied off with surgical silk, leaving a five-inch piece of the silk extending from the knot. A small incision was made at the junction of the heart and the abdominal artery and the tip of a four-inch polyethylene cannula (internal diameter 0.047") inserted and tied in place. All remaining connections to the body were severed and the heart lifted out and placed in Homarus perfusion fluid. The free end of the cannula was inserted into a rubber stopper and pulled through to a distance of about three inches. The stopper and heart were then inserted into a cylindrical lucite perfusion chamber surrounded by a water jacket. Cold perfusion fluid was immediately added to the chamber. The end of the cannula was then inserted into another piece of tubing, completing the circuit through which the perfusion fluid was to travel, and the flow was started. The optimal perfusion rate was found to be

approximately 40 ml/hour. A Grass Model 5 Polygraph, which was circuited to record impulses via a Model FT .03B strain gauge, was used to monitor the amplitude and frequency of the heartbeat. The piece of silk left attached to the heart was threaded through the strain gauge, tied in place, and adjusted so that the heart was upright and the string fairly taut (figure 1).

Method of bioassay

When the heart was beating at a steady frequency and amplitude, 1 ml of the test sample was introduced into the system and the valves adjusted so that perfusion fluid would flow through the system to the heart, carrying the sample with it (figure 1). Activity, if present, was usually apparent within a minute after the sample was introduced into the system. Once maximal activity was recorded, the chamber was drained, fresh perfusion fluid was added to it, and the heart was allowed to restabilize at the original frequency and amplitude. The next sample was introduced approximately five minutes after restabilization. Samples and perfusion fluid were kept at the same temperature throughout the procedure (10-15°C). The lobster heart was capable of beating regularly for three to four hours, while crab hearts were found to be undependable both in regularity of beat and in survival time. When testing fractions on the heart, one fraction known to exhibit activity was tested at

the beginning and at the end of the period during which the heartbeat was monitored. This was done to check constancy of cardiac response. In some cases the heart exhibited weaker responses to fractions after approximately one hour of recording.

Preparation of media

Preliminary experiments indicated that an 0.9 M sucrose solution made up with glass-distilled water was adequate for maintaining isosmotic conditions. A 1.0 M sucrose solution was also found to be useful.

Homarus perfusion fluid was prepared using glass-distilled water, according to Cole (1941):

<u>compound</u>	<u>gms/liter</u>
MgCl ₂	1.68
NaCl	27.08
Na ₂ SO ₄	2.69
KCl	1.17
CaCl ₂	5.68

To the liter of salt solution was added 17.6 mls H₃BO₃ (31.4 gms/l) and 0.96 ml NaOH (20.3 gms/l); final pH 7.4 - 7.6.

Preparation of test fractions

All preparation of homogenates and fractions was carried out in the cold (4-8°C). The pericardial organ homogenate in its final form contained five pairs of pericardial organs in two mls of the appropriate medium. For comparison studies, ten pairs of pericardial organs were divided contralaterally between Homarus perfusion fluid and 0.9 M sucrose. After homogenizing, the preparations were

decanted into separate 8-ml polyethylene centrifuge tubes. Differential centrifugation was carried out using an International High-Speed Refrigerated Centrifuge: Model HR-1. For centrifugation at 71,000 x g, a Spinco Model L Preparative Ultracentrifuge with a 40,000 rpm angle head was used.

Each fraction was centrifuged for 30 minutes. The supernatant was decanted into another 8-ml polyethylene centrifuge tube for further centrifugation. The sediment was recentrifuged for 20 minutes with 2 mls of the appropriate medium, and the supernatant which was obtained was added to the original supernatant. The single exception to this procedure was the 71,000 x g fraction, which was centrifuged for 60 minutes and not washed.

Upon completion of centrifugation, the sediments were resuspended in 3 mls of perfusion fluid and decanted into 5-ml pyrex tubes, placed in a boiling water bath for 5 minutes, and cooled to 10°C. Fractions were usually tested immediately after cooling, but it was found that keeping them in the cold overnight did not affect the results.

It would have been desirable to test for cardioexcitatory activity in the supernatant resulting from the highest-speed centrifugation in each experiment. However, this was not possible where pericardial organs were treated in 0.9 M sucrose because this medium affected the heartbeat when tested alone, causing a slight decrease in frequency and

increase in amplitude. Therefore, only the final supernatant from pericardial organs which had been homogenized and centrifuged in perfusion fluid was tested. Because of the effect of the sucrose solution on the heart, all sediments had to be resuspended in perfusion fluid to be tested.

Results

A survey was first made to determine at what centrifugal forces sediments exhibiting cardioexcitatory activity could be obtained. The centrifugal forces used were 1000 x g, 12,000 x g, 17,000 x g and 30,000 x g; these were taken in part from the literature (Hogeboom et al., 1948; Blaschko et al., 1955; Evans, 1962). Comparison runs were made using 0.9 M sucrose and Homarus perfusion fluid. The only supernatant tested was that from the 30,000 x g fraction run in perfusion fluid.

Figure 2a shows the relative activities of fractions run in Homarus perfusion fluid. Material sedimenting at 12,000 x g consistently exhibited greatest activity. The second highest response was evoked by the supernatant from the material centrifuged at 30,000 x g. Material sedimenting at 17,000 x g and 30,000 x g always exhibited negligible or no activity.

Figure 2b shows the relative activities of fractions run in 0.9 M sucrose. Material sedimenting at 12,000 x g consistently exhibited greatest activity. Less but marked

activity was present in the 17,000 x g sediment. The 30,000 x g sediment always exhibited the least activity and occasionally none.

Material brought down at 1000 x g always caused a strong cardiac response. However, while it is included in the figures, it is excluded from comparison as it was assumed to contain mainly unbroken cells and various subcellular components. The 1000 x g sediment in sucrose always exhibited activity less than or equal to the corresponding sediment in perfusion fluid. It also exhibited activity less than or equal to the material run in sucrose which sedimented at 12,000 x g.

The marked cardioexcitatory effect of the supernatant from the material centrifuged in perfusion fluid at 30,000 x g suggested that a higher-speed centrifugation of the corresponding sucrose supernatant be attempted. Accordingly, the supernatant was centrifuged at 71,000 x g and the resulting sediment resuspended in perfusion fluid, boiled, cooled and tested. On two occasions it was found to elicit a strong cardiac response and on two others it exhibited no activity whatsoever (figure 4a and 4b).

An attempt was made to further localize cardioexcitatory activity in the 1000 x g - 12,000 x g range. All material was run in sucrose. The additional centrifugal forces used were 4000 x g and 8000 x g. The attempt was unsuccessful, as the sediments exhibited decreasing activity in the order: 1000 x g, 4000 x g, 8000 x g, 12,000 x g (figure 3).

Discussion

The present data does not permit a definitive statement regarding the nature of the hormone-containing particle, as sediments were not examined with the electron microscope. However, the literature supports the assumption that the cardioexcitor is contained within at least one type of neurosecretory granule. Where other types of neurosecretory materials were isolated and identified, they were found to be associated with electron-dense granules approximately 1500 A in diameter, sedimenting below 17,000 x g (Blaschko et al., 1955). The results of this study show that the greatest part of the cardioexcitatory material sedimented below 17,000 x g. In addition, electron microscopic examination of Cancer pericardial organs (Maynard and Maynard, 1962) revealed the presence of one class of electron-dense neurosecretory granules, approximately 1500 A in diameter. Mitochondria and neurosecretory granules may share properties of density and membrane permeability, so that when subjected to differential centrifugation or density gradient centrifugation they may sediment together (Blaschko et al., 1955; Heller and Lederis, 1961). However, neurosecretory material from every species so far examined has been found to exist within granules and not within mitochondria.

As described by Alexandrowicz (1953) and Carlisle and Knowles (1953), the branches of the nerves composing the

pericardial organs of Cancer break up into fine fibrils forming neuropile-like networks. Secretory material is assumed to be released from the terminations of these fibrils. It was hoped that homogenization would rupture these fibrils, releasing their contents. This was apparently only partly accomplished. In addition to free neurosecretory granules, the homogenate probably contained various subcellular components and a wide size range of pieces of neurosecretory terminals. This is indicated by the results of differential centrifugation within the 1000 x g - 12,000 x g range. Therefore, even though material sedimenting at 12,000 x g consistently exhibited activity greater than or equal to that shown by material sedimenting at 1000 x g, it cannot be assumed that the 12,000 x g sediment contained a large percentage of free neurosecretory granules. Nor can it be assumed that it contained the greatest amounts of cardioexcitor.

However, it can be seen that the greatest part of the active material sedimented below 17,000 x g and that on two occasions another highly active sediment was brought down at 71,000 x g, while intermediate centrifugation at 30,000 x g yielded material which demonstrated only little activity. These findings afford presumptive evidence for two classes of hormone-containing particles or granules. This assumption is supported by the literature. Using electron microscopy, Knowles (1960 and 1964) found two types of neurosecretory

fibers in pericardial organs of Squilla mantis, and two classes of neurosecretory granules differing in size and internal structure. In several species of decapod crustaceans, Maynard (1961b) found three types of neurosecretory cells sending fibers to the pericardial organ-anterior ramification complex. Maynard and Maynard (1962) found that Cancer pericardial organs contained neurosecretory granules of only one size but observed smaller vesicles in the same nerve endings, having a diameter of approximately 500 A. Carlisle (1956) found two areas of activity after chromatography of Cancer pericardial organ extracts. Belamarich (1963), using paper chromatography, found two active compounds in extracts of Cancer pericardial organs.

With reference to the isotonicity of the sucrose solution used, the 1000 x g sucrose sediment usually exhibited less activity than the corresponding sediment in perfusion fluid, and the 12,000 x g sucrose sediment usually showed greater activity than its perfusion fluid analogue. Perfusion fluid, while hypertonic, is apparently not strongly so, as the 12,000 x g sediment in perfusion fluid exhibited great activity. If perfusion fluid had caused total rupture of nerve endings and of neurosecretory granules, little or no activity would be expected in any perfusion fluid sediment. However, a large part of the cardioexcitatory material was apparently released into the supernatant, as may be seen by the marked activity of the perfusion fluid supernatant from material centrifuged at 30,000 x g.

Summary and Conclusions

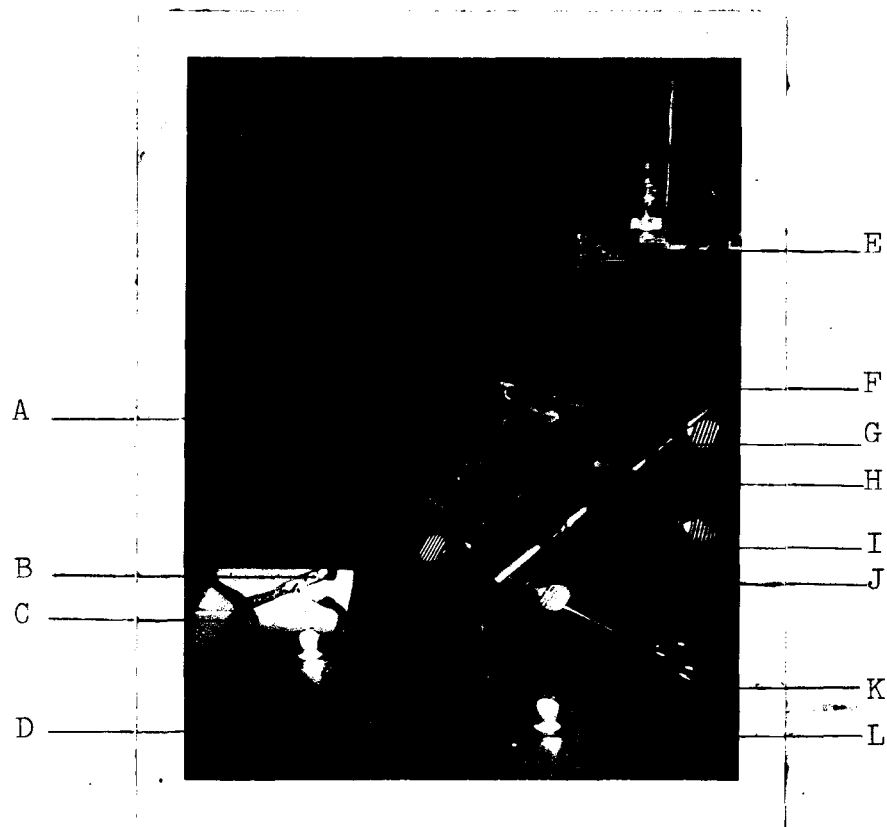
The method of differential centrifugation was applied to crude 0.9 M sucrose homogenates of pericardial organs of two species of the crab, Cancer, in an attempt to isolate cardioexcitatory material elaborated and secreted by these organs. Materials resulting from centrifugations were tested for the presence of the cardioexcitor by assay on live Homarus heart.

1. Active material sedimented below 17,000 x g. Maximum activity was exhibited by material which sedimented at 12,000 x g.

2. Some active material sedimented at 71,000 x g.

These results support the assumption that hormonally active material is contained within at least one type of granule. Presumptive evidence is offered for the existence of a second type of neurosecretory granule.

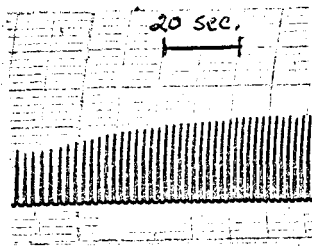
Figure 1
Perfusion System Assembly



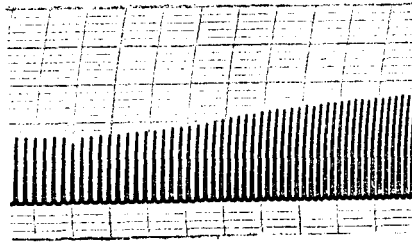
Legend

- A - perfusion fluid outlet
- B - needle valve for adjusting flow rate
- C - tubing to perfusion fluid reservoir
- D - point at which sample is introduced
- E - strain gauge
- F - water outlet
- G - heart
- H - water inlet
- I - tubing for chamber drain
- J - cannula and tubing
- K - bypass
- L - main flow path

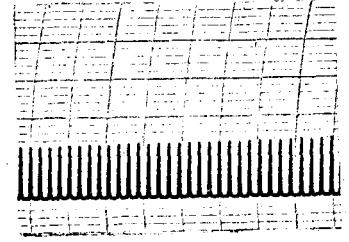
Figure 2 a

Homarus Perfusion Fluid

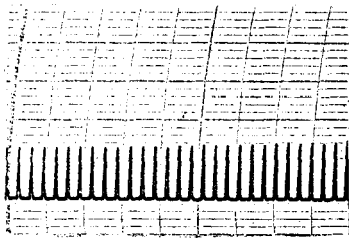
1000 x g



12,000 x g



17,000 x g



30,000 x g

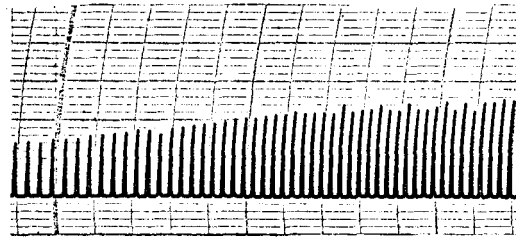
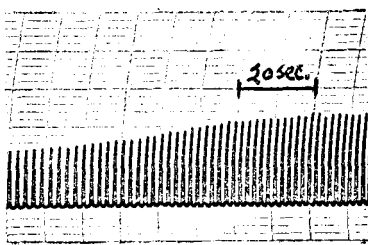
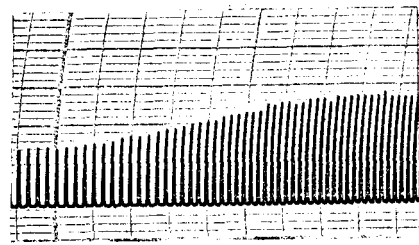
supernatant from material
centrifuged at 30,000 x g

Figure 2 b

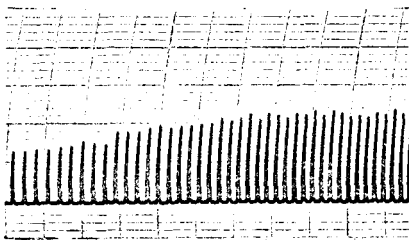
0.9 M Sucrose



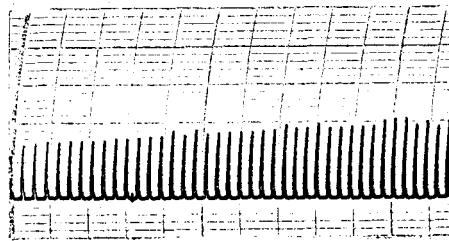
1000 x g



12,000 x g



17,000 x g



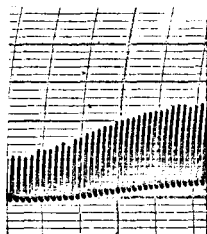
30,000 x g

Figure 3

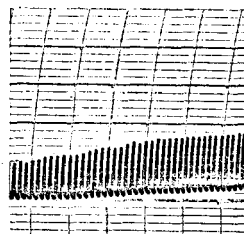
0.9 M Sucrose



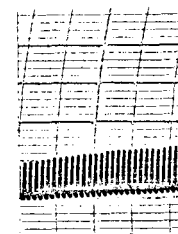
1000 x g



4000 x g



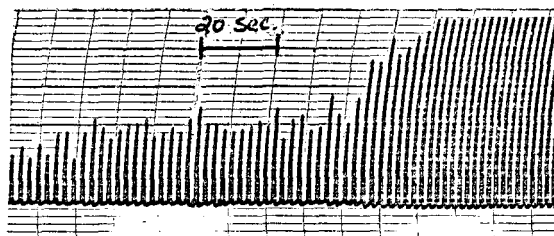
8000 x g



12,000 x g

Figure 4 a

0.9 M Sucrose

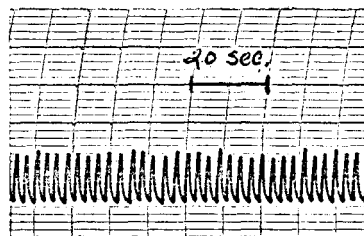


71,000 x g

Maximal activity was greater than indicated. The sensitivity of the recorder was set too high, so the maximum shown here represents the limit of the range of the pen.

Figure 4 b

0.9 M Sucrose



71,000 x g

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Abstract

The pericardial organs are neurosecretory structures situated within the pericardial cavity of stomatopod and decapod crustaceans. Their location in the pericardium is such that blood approaching the heart must flow directly over them. This arrangement facilitates the distribution of pericardial organ secretions to all parts of the organism. It is not known how many different neurohormones are secreted by the pericardial organs, but at least one of these has been shown to act as a strong cardioexcitor. Electron microscopy of pericardial organs has revealed the presence of membrane-bounded granules, approximately 1500 A in diameter, which are believed to contain the neurosecretory material. An attempt was made here to isolate the cardioexcitor by means of differential centrifugation of crude 0.9 M sucrose homogenates of pericardial organs from two species of Cancer. Live Homarus heart served as bioassay material. Cardioexcitatory effects were found in material which sedimented below 17,000 x g. On two occasions another active sediment was found at 71,000 x g. The present data is in agreement with the hypothesis that the cardioexcitor is contained within neurosecretory granules. However, a definitive statement to this effect cannot be made, as sediments were not checked microscopically. Presumptive evidence is offered for the existence of a second type of neurosecretory granule.