A Study of Neurotransmitters in the Asteroidea, Ophiuroidea and Crinoidea.

by

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DECLARATION

I hereby declare that the work recorded in this thesis has been carried out by myself, and it is of my own composition. I further declare that it has not been submitted in any previous application for a higher degree.

CERTIFICATE

I certify that Victor Pentreath has fulfilled the conditions laid down in the regulations for a degree of Master of Science, under Ordinance No. 51 of the University Court of the University of St. Andrews, and that he has accordingly qualified to submit this thesis for the Degree of Master of Science.

Vitae

I was educated at Christ's Hospital, Sussex and attended University at St. Andrews where I graduated in Honours Zoology in 1967. The work described in this thesis was carried out between December 1967 and March 1969.

CONTENTS

page

General Introduction

There are two well-known phrases about echinoderms. Von Uexküll called them a "republic of reflexes," and Hyman "salutes the echinoderms as a noble group especially designed to puzzle the zoologist." These statements sum up two of the problems which have in the past confronted students of echinoderms: not only do the echinoderms make use of peripheral regions which act independently of one another, but many techniques that are well tried on other groups of animals fail to produce results with members of this phylum.

Echinoderms have circular and radial nerve cords of just sufficient specialization to be called a central nervous system; they do not have a brain. As with other simple nervous systems, the bulk is intraepithelial, composed of large numbers of very small neurons. The ring and radial nerves of starfish are thickenings of this general epithelial plexus.

It is only recently that techniques have been developed which allow analysis of individual neurons. Some of these techniques have been widely applied to invertebrate nerve and nerve-muscle preparations. One would expect that the echinoderm nervous system, with its diffuse nature and small elements, could at least be studied successfully. But to date refined techniques

have only been applied to a few echinoderm species, and in still fewer cases with success.

The work described in this thesis was undertaken to extend knowledge about the nature and distribution of transmitter substances in some echinoderm nerve and nerve-muscle systems. Species were studied from the classes Asteroidea, Ophiuroidea, and Crinoidea. Histochemical techniques and assay procedures were used to determine the localisation of substances which may act as neurotransmitters in each group. At the same time the electron microscope was used to study the fine structure of certain central and peripheral nerves, and muscles of each species, in order to obtain information about the subcellular localisation of possible transmitter substances. In addition, observations were made with the electron miscoscope on neurosecretory elements in the ophiuroid nervous system.

The review by Welsh (1966) indicates the lack of data concerning neurotransmitters and neurosecretion in echinoderms. Similarly Cobb (1967) and Laverack (1968), and Cobb and Laverack (1967) have recently stressed the necessity for a reappraisal of echinoderm nervous systems with the increased resolution offered by the electron microscope.

Previous work on neurotransmitters, the anatomy, and the neurosecretory elements of echinoderm nervous systems.

(1) Neurotransmitters.

These are small, organic molecules that are involved in short-range mediation of nerve action at nerve synapses and neuromuscular junctions. Among those that are known to act in this manner in some groups of animals are acetylcholine (ACh), noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT), and gamma amino-butyric acid (GABA), or a related substance.

There is little information concerning any of these substances in echinoderms (Welsh, 1966). The evidence for a transmitter role for acetylcholine is most complete. This is described first in the brief survey below. That one or more catecholamines may also function in neurotransmission seems probable, although far from proven.

Early workers (eg. Bacq, 1935; Corteggiani, 1938) detected acetycholine-like activity when assaying extracts of both nervous and non-nervous tissues of different species. Bullock and Nachmansohn (1942) and Augustinsson (1946) showed by biochemical techniques the presence of enzymes which could hydrolyse ACh, although in these cases no data was presented

concerning the specificity of the inactivating enzyme(s). The great sensitivity of certain isolated holothurian muscle preparations to ACh has been well documented (see Welsh, 1966), and the release of an ACh-like substance from Stychopus muscle following stimulation was reported by Bacq (1939).

More recently Roberts (1964) and Cottrell (1964) detected very high levels of an ACh-like factor in the radial nerves of several starfish species. These works were amplified by Pentreath (1967) and Pentreath and Cottrell (1968), who showed, using chromatographic techniques, that the ACh-like activity in the radial cords of Asterias rubens could be accounted for by ACh alone. No evidence was obtained for any other choline ester. (In 1962 Unger eluted areas of chromatograms after separating nerve extracts of A. glacialis, and found ACh-like activity by bio-assay on the isolated Helix heart. He did not however employ techniques which exclude the presence of other cholinesters whose biological action may be very similar to ACh. see eg. Keyl, Michaelson and Whittaker, 1957). It was also shown (Pentreath and Cottrell, 1968) that ACh was not distributed evenly throughout the cord. The lateral borders of the cord, found by electron microscopy to contain

comparatively large numbers of structures resembling nerve endings with enclosed synaptic-type vesicles contained a higher level of ACh than the mid-portion of the nerve. Results also indicated that the mechanism of ACh binding in starfish nerve was similar to that in mammalian brain (Whittaker 1959). It was suggested that the synaptic vesicle which is abundant in asteroid nerve tissue, may represent the functional unit sequestering at least some of the ACh present in starfish radial cords, as is thought to be the case with mammalian brain (cf. Whittaker et al., 1964).

At the same time, histochemical techniques were applied to Asterias radial nerve to obtain information regarding cholinesterase (Che) specificity and distribution (Pentreath and Cottrell, 1968). It was found that most cholinesterase activity was due to acetylcholinesterase (AChE). The enzyme was present in regions where large numbers of vesicles were observed with the electron microscope. Furthermore the presence of AChE in nervous tissue considered to be motor suggested that at least some motor neurons could be cholinergic. It was also found that high levels of AChE were present in the outer epithelium of the nerve. The functional significance of large amounts of enzyme in this region was not explained.

Information about other possible neurohumours (namely catecholamines, 5-hydroxytryptamine, and the amino acids gamma-amino-butyric acid and glutamic acid) is less complete. Using pharmacological methods, Östlund (1954) found no evidence of an adrenaline-like substance in whole body extracts of species of brittle-stars, sea urchins or sea cucumbers, but Cottrell (1967) has obtained chromatographic evidence for the presence of dopamine and noradrenaline in the nervous tissue of both starfish and sea urchins. Some observations have also shown that adrenaline may oppose the excitor action of ACh on echinoderm body muscles (eg. Du Buy, 1936; Boltt and Ewer, 1963).

However a recent paper by Cobb (1969) describes the distribution of mono-amines in the nervous system of a starfish and a sea urchin, as determined by a fluorescent histochemical study. His work was in progress while a similar, but more extensive, study was being made for this thesis. In summary his findings, which at their time of publication were generally established in this laboratory, were as follows. First, the characteristics of the fluorescence were specific for DA and/or NA. Second, fluorescence was confined to the axons and cell bodies of nervous tissue. Third, mono-amines were not detected in sensory cells, and fourth, fluorescence in the axons and cell

bodies coincided with the distribution of small granular vesicles demonstrated during previous electron microscope studies (Cobb, 1966, 1967a). With respect to the third finding mentioned above, it was concluded that "the nerves containing monoamines represent a class of interneurons, the function of which is not yet clear". In this thesis these topics are amplified. The techniques for amine localization are applied to various species from other echinoderm classes, and attempts are made to elucidate the function of the amine-containing neurons.

There has been one report of amine oxidase, a catalyst of the biological inactivation of catecholamines and 5-HT in echinoderms. Blaschko and Hope (1957) showed by manometric methods that the digestive glands of all asteroid and echinoid species studies contained the enzyme. On the other hand neither the gut nor gonad of the sea cucumber Holothuria forskali showed any activity. They did not however undertake any measurements on purely nervous tissue.

Although 5-HT has been implicated in nerve transmission within several invertebrate phyla (Welsh and Moorhead, 1960), it would seem that this substance is unimportant in the physiology of the adult echinoderm nervous system. It has only been detected in negligible quantities in a few holothurian, sea urchin, and

starfish species (Welsh, 1966). Buznikov and Chudakova (1963) have also demonstrated that echinoderm embryos, like those of certain molluscs and polychaete worms, possessed detectable quantities of non-nervous 5-HT. They suggest that this substance plays some role in the process of cleavage of the sea-urchin egg.

Neither GABA nor glutamic acid has been detected in echinoderm nervous systems, but there is one report at hand which describes the occurrence of L-glutamic acid in the testis of Asterina pectinifera (Ikegami, Tamura and Kanatani, 1967). This is considered later.

It is apparent from this brief account that knowledge of the chemistry, physiology, and mode of action of the active substances present in echinoderm nerve tissue is very limited. There is as yet no data for the classes Ophiuroidea or Crinoidea. Within the other groups there is growing evidence for a cholinergic nerve mechanism, and it also seems likely that catecholamines may play a transmitter role.

(2) Anatomy and organization

The classical histological background to our present knowledge of the nervous system of echinoderms is attributable to Hamman (1883-1891), Christo-Apostolidès (1882), Cuénot (1887-1891),

and von Uexküll (1896-1905). Their description are thorough, but due to the extremely small size of neurons, and also to their often anomalous staining properties, they are sometimes incomplete. It should be remembered that these workers relied on use of the light microscope at its limit of resolution.

Since the end of the nineteenth century, one person has contributed significantly to our understanding of echinoderm nervous systems. Smith, in a series of papers (1937-1950) has dealt with morphological, physiological, and behavioral aspects of the Asteroidea, and has postulated a plan to explain pointing of the starfish tube-feet during walking movements (1945). The most comprehensive work to date on the structure and function of echinoderm nervous systems we owe to the same author (Smith, 1965).

With the electron microscope, it is possible to study morphology at a completely new level of resolution. There have been comparatively few published electron microscopical studies of echinoderm tissues. These studies have however shed new light on many aspects of nervous anatomy and in several cases have shown previous histological interpretations to be erroneous. Noteworthy works are those by Cobb (1966, 1967) and Cobb and Laverack (1966) on the pedicellariae and lantern of Echinus, and the nervous

system of Astropecten. One of these reports (Cobb, 1967a) serves as a good illustration of how some previous light microscope descriptions were misleading. Using methylene blue staining techniques Smith (1950) described the ampullae of Astropecten as being innervated via chains comprised specifically of four neurons. The ultimate motor neurons of each chain were swollen into flat ribbon-like forms, which stretched over the ampullae seams. He called these modified neurons "ribbon-axons". However the electron microscopic studies by Cobb (1967a) have shown that these structures are not nervous, but are muscle cells.

Other major electron microscope studies are those by Bargmann and Behrens (1963, 1968) on the ampulla and pyloric caecae of Asterias, by Bargmann et al. (1962) on the ecto-neural system of Asterias, and by Eakin and Westfall (1964), Eakin (1963) and Harnack (1963) on the starfish eyespot. The series of papers on echinoderm fine structure by Kawaguti (1964-1966) are rarely referred to, because they lack much in detail and general accuracy. Furthermore many of the micrographs he shows are of poor quality. Pentreath (1967) has described in detail the ultrastructure of the radial cords of Asterias rubens, and more recently Coleman (1969) has studied

in detail the tube foot wall and sucker of an echinoid, Diadema antillarum.

Certain other isolated fine structural studies have also been published. Millot and Coleman (1969) have described a new structure, the podial pit, in the echinoid Diadema antillarum. This pit lies at the base of the aboral podia. It is composed of a thickening of the superficial nerve felt, and is made up of a large number of small fibres. The suggestion is made that the very superficial location of the nerve tissue can be correlated with the high photosensitivity of the region. The nature of certain subcellular particles and mucous granules in the asteroid tube feet have been described by Chaet and Philpott (1964), Souza Santos (1966), and Harrison and Philpott (1966), while the fine structure of the polian vesicles of holothurians have been elegantly described by Baccetti and Rosati (1968). These last structures are outside the consideration of nervous systems and only this mention of them is made here.

There have been no detailed works on the anatomy or histology of the crinoid or ophiuroid nervous system since the classical works of the late nineteenth century.

Most echinoderm neurons are small. Rarely does a cell body exceed 15 μ at its widest point. Axon diameters are normally

0.5-3μ. Sensory cells are bipolar, and the internuncial pathways into which they discharge take the form of strands and nets woven intraepithelially within the skin and gut linings. Central pathways are contained within the radial cords and their connective circumoral ring, and consist of great numbers of closely set, longitudinally aligned fibers. These central tracts give rise to a series of bilaterally and metamerically repeated sets of nerves, some of which run to musculature and are purely motor, while others are of mixed composition.

It is generally accepted (Smith, 1965; Hyman, 1955) that the nervous tissue of each class consists of three more or less separate parts. The ectoneural system, of ectodermal origin, is chiefly sensory and comprises a large proportion of the general skin plexus and central pathways. Lange's nerve (hyponeural origin), which is always in close association with the radial cords and circumoral ring, is exclusively motor, while the apical nerve is of presumed mesodermal origin, and is also regarded as motor.

Of the three divisions, the ectoneural is the most constantly occuring, for it appears in all classes save the crinoids, where a complete circumoral ring is lacking. In this case the ectoneural fibres of the radial cords innervate the

oesophagus directly. The system is best developed in the Asteroidea (Fig. 3) where both radial nerves and circumoral ring are comprised almost wholly of ectoneural elements.

The hyponeural system (Lange's nerve in the asteroid) is not so constant in its form or degree of development as the ectoneural system. It is best developed in the ophiuroids where it consists of a closed ring from which the radial nerves to each arm emerge as a series of ganglionic swellings. At each motor ganglion, nerves arise which innervate the four invertebral muscles responsible for lateral and oro-aboral flexure of the arm (Fig 2). In the crinoids (Fig 1) the hyponeural nerve is not clearly separate, and the motor fibres innervating the arm musculature are intermingled with sensory elements. The much reduced, but separate, asteroid motor system supplies the ambulacral arm musculature.

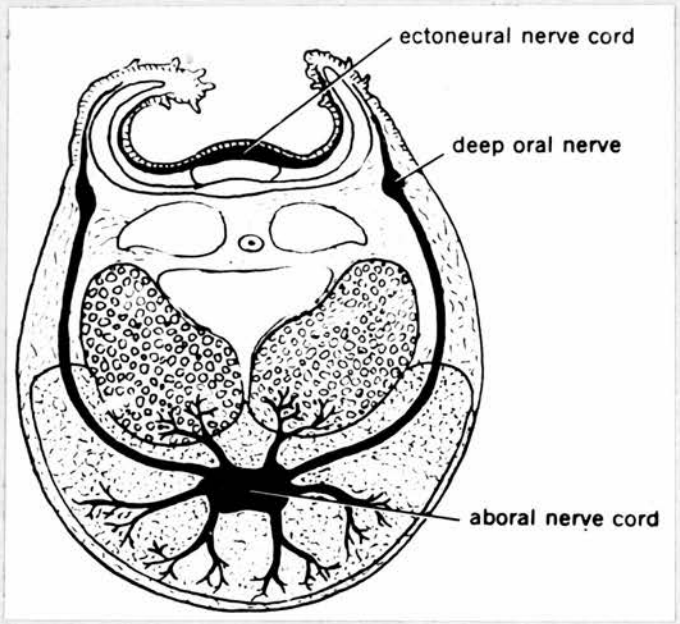
Still more variable is the apical system. In the crinoid it is greatly developed and constitutes the most important nerve centre of the body. Emanating from the nervous wall of the chambered organ are five or ten interadial nerves which branch to form a complicated plexus. Radial nerves extend distally from the plexus into the arms and pinnules. The Asteroidea are the only other echinoderms in which an apical strand is present, but it is

Fig. 1. Transverse section of an arm of Antedon.(Smith,1965).

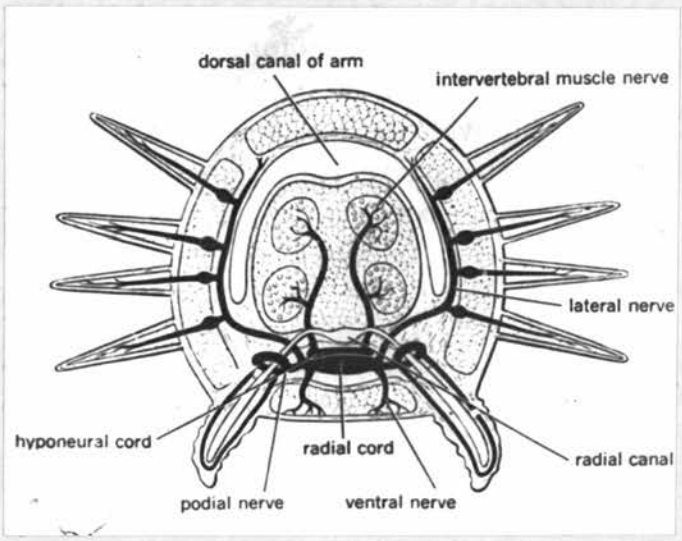
Fig.2. Transverse section of an ophiuroid arm showing the principal metameric nerves.(Lang, 1894).

Fig.3. Nerve tracts of a starfish arm. (Smith, 1965).

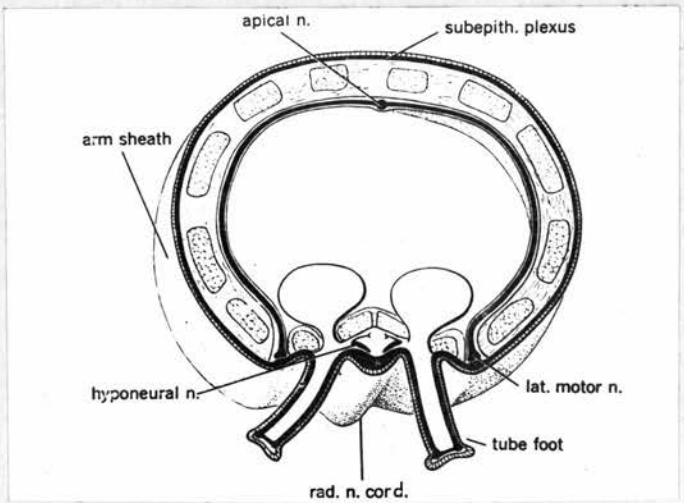
1


ectoneural nerve cord
deep oral nerve
aboral nerve cord

2


dorsal canal of arm
intervertebral muscle nerve
lateral nerve
hyponeural cord
radial cord
radial canal
podial nerve
ventral nerve

3


apical n.
subepith. plexus
arm sheath
hyponeural n.
lat. motor n.
tube foot
rad. n. cord.

doubtful whether it should be considered a discrete motor system (Nichols, 1966). It is not, however, in any way homologous with that of the crinoid (Smith 1937).

(3) Neurosecretion

Neurosecretory cells and their organization into systems responsible for the production, storage and release of hormones have been studied in a wide variety of invertebrates (see Gabe, 1966). From the few reports available it would appear that neurosecretory substances play important roles in echinoderms, as they do in other metazoans. To date, work has been carried out by four sets of investigators.

Chaet and co-workers have studied the physiological function of the "gamete-shedding substances" present in the radial nerves of many species of starfish. This substance, in synchrony with its inhibitive counterpart "shedhibin", has been shown to promote maturation of eggs and regulate the expulsion of eggs and sperm from the gonads of a population of ripe starfish (Chaet, 1964, 1966; Chaet and Smith, 1962). Preliminary data involving amino acid analyses have shown that the shedding substances contains a minimum of some forty-two amino acid moieties, implying a minimum molecular weight of around 4,800 (Chaet, 1967).

A Japanese group headed by Kanatani and Noumura has carried out similar studies in an attempt to elucidate more fully the chemical nature and cellular action of the twomfactors named above. They isolated the shedding factor and found it possessed the properties of a polypeptide (Kanatani and Noumura, 1962). More recently Ikegami, Tamura, and Kanatani (1967) have chemically identified "shedhibin" from testis of Asteria pectinifera as L-glutamic acid.

Other active substances of radial nerves, presumed to derive from neurosecretory cells, have been described by Unger (1962). Radial nerve extracts of A.glacialis can be separated by paper chromatography into two components. These act in an opposing manner on structures involved in locomotion, and on the dermal brachiae which influence color change. Apparently their action on smooth muscle is the same as that of the shedding substance, but their exact sources and chemical relationships to the shedding factor is unknown. Both Chaet and Unger propose that a substance in starfish radial nerves may be involved in osmoregulation (Chaet and McConnaughy, 1959; Unger 1960, 1962).

Neurons with the staining properties of neurosecretory cells have been found by the above-mentioned workers, and also by Fontaine (1962). Using staining methods which are selective but not specific for neurosecretory cells (ie. Gabe's paraldehyde-

fuchsin), this worker found distinct positive sites in the principal motor ganglia of several ophiuroid species. Such positive-staining sites may represent the loci of some of the physiologically active substances found in extracts of radial and ring nerves of various echinoderm species.

A Study of the distribution of catecholamines in some echinoderm nervous systems.

(1) Fluorescent histochemical studies on the nervous systems of Asterias rubens, Ophiothrix fragilis, and Antedon bifida.

This study was initially undertaken to determine whether the dopamine and noradrenaline present in the starfish nerve cord (see Cottrell, 1967) are localized in neurons, as would be expected if they serve a transmitter function. Parallel studies were then made on the nervous system of Ophiothrix and Antedon. Once a neuronal localization of the amines was established, an attempt was made to discern whether they were associated specifically with sensory or internuncial neurons, or whether they mediated activity between nerve and muscle. An attempt was also made to determine the function of the amine-containing neurons.

In order to visualize more clearly the catecholamine-containing neurons, and also to obtain information about the sub-cellular localization of amines, tissues rich in amine were studied with the electron microscope. Relevant fine-structural findings are included in this section.

Material and methods

Specimens of Asterias rubens, Ophiothrix fragilis and Antedon bifida [x] were collected locally. If not used immediately they were stored in an aquarium in continuously circulating aerated sea water.

Catecholamines were studied with the highly sensitive fluorescent method developed by Swedish workers (for general review see Falck and Owman, 1965). For the species studied difficulty was experienced in obtaining satisfactory sections of material embedded in paraffin (or ester) wax because of associated calcareous ossicles, which tended to rip the softer tissue during sectioning. Furthermore, contact with the liquid propane during freeze-drying caused distortion of the tissues attached to the ossicles. In some experiments, the calcareous material was removed beforehand by careful dissection. However, this was not always possible because of the intimate association of ossicles and nerves in certain areas of the nervous system. The problem was eventually circumvented by sectioning frozen tissue and drying the sections according to the method of Spriggs et al. (1966).

[x] There are many synonyms for these species. The names used in this thesis are those most commonly employed in the literature (see Smith, 1965; Hyman, 1955).

Material to be examined was dissected from live animals and frozen with dry ice onto a microtome chuck. Sections 10μ thick were cut on an EEG cryostat at -20°C, picked up on cover slips and then immediately placed to dry over small receptacles of fresh P_2O_5. The drying time was minimized by keeping the gap between the wet sections and the P_2O_5 as small as possible. Normally sections were left overnight to ensure complete dessication. It was found that this procedure gave very similar results to the best of those obtained with freeze dried material. Dried sections and freeze dried tissue were exposed to paraformaldehyde, which had been equilibrated to a relative humidity of 70% (Hamberger, Malmfors, and Sachs, 1965).

The specificity of the observed fluorescence was treated by immersing sections in 0.1% sodium borohydride in 90% isopropanol, a procedure known to deplete specific catecholamine fluorescence (Corrodi, Hillarp, and Jonsson, 1964). A further test of specificity was to compare the appearance of tissue prepared as above with material which had not been exposed to paraformaldehyde. In addition, tissue from reserpinized animals was examined. In these experiments, batches of animals were immersed for 1-5 days in aerated sea water containing 3.5 or 5.0 μg/ml of soluble reserpine phosphate.

The sea water was maintained in 16-17°C. It has been shown previously that reserpine depletes both DA and NA from starfish nervous tissue (Cottrell, 1967).

Sections were examined with a Leitz microscope equipped for fluorescence microscopy. The standard Aristophot-Orthulux microscope assembly was fitted with an HBO 200 mercury lamp, a dark field condenser, a BG12 excitation filter, and a 530 mμ barrier filter.

Electron microscopy

Extensive experimental fixation procedures of various echinoderm tissues (Cobb, 1967; Pentreath and Cottrell, 1968) have shown that most satisfactory results are obtained by fixing very small pieces of tissue, dissected free from ossicles, in unbuffered osmium tetroxide in sea water for short time periods. Dissection is not however always practical because of the intimate association of the tissue to be studied with skeletal material. Furthermore, the diamond knife has only limited use because conventional embedding media are softer than calcareous ossicle, consequently sections are torn and distorted. Instead, adequate preservation and cutting properties can be combined by fixation of undissected tissue areas, and subsequently decalcifying in a solution of diamino-ethane-tetra-acetic acid (E.D.T.A.) (Cobb, 1967).

Small dissected tissue pieces (Asterias stomach) or larger pieces composed of soft tissue and ossicle (pieces of arm and disc removed from live specimens of Ophiothrix and Antedon) were fixed for 1-2 hours in unbuffered 1% O_sO_4 in sea water at 4°C. Pieces not requiring decalcification were then dehydrated in a graded acetone series, and embedded in Araldite. Pieces to be de-calcified were immersed for 2-5 days in a saturated solution of E.D.T.A., and then dehydrated in acetone and embedded in Araldite. Thick sections for light-microscope orientation were stained with toluidene blue. Thin sections for electron microscopy were cut on an LKB ultramicrotome, mounted on unfilmed or Formavar coated grids, and stained for 1-3 minutes in lead citrate followed by 3 minutes in 2% uranyl acetate (Venable and Coggeshall, 1965). The sections were examined in an AEI EM6B electron microscope operating at 50 and 60 KV.

Results

Pale green fluorescence indicative of primary catecholamines was only observed in the ectoneural tissue of starfish, brittle stars, and feather stars. It could be seen both in the general subepithelial plexus (where present) and in the circumoral and radial nerves. No specific fluorescence

was observed in the hyponeural layer or in non-nervous tissue.

Fluorescence developed optimally after exposure to formaldehyde at 60°C. At higher temperatures, or longer periods of time, background autofluorescence increased and the specific pale green fluorescence was masked. The pale green fluorescence faded appreciably after ½ hour exposure to U.V. light. There was no increase in intensity of the specific fluorescence or change to deeper green colour after 3 hours exposure to formaldehyde as would be expected if adrenaline were present (see Falck and Owman, 1965). There was no yellow fluorescence indicative of 5-HT. Immersion of the sections in 0.1% sodium borohydride in 80% isopropanol caused a marked reduction of the pale green fluorescence, which to some extent could be regenerated with renewed formaldehyde treatment. Thus it appeared that the catecholamines DA and NA were responsible for this pale green fluorescence seen in the nervous system of each species.

The results obtained with each species are described separately below.

Asterias rubens

There was a high concentration of catecholamine fluorescence in the ectoneural tissue of the radial and circumoral nerves. Fluorescence was not however distributed uniformly throughout the layer. It was most intense laterally and aborally especially at, or near, the interpodial areas.

In cross-section the fluorescence had a speckled appearance, presumably due to amine-containing axons cut in cross section (Figs 4,5). In horizontal section individual, or bundles of, longitudinally running axons were seen (Figs 6-8). Frequently swellings or varicosities were observed along their lengths. The oral surface of the tissue, composed of epithelial cells with interspersed sensory neurons (Smith, 1937), did not show any specific fluorescence.

No structures in the radial cord of Asterias could be definitely identified as amine-containing cell bodies. Some fluorescent structures were seen which may possibly have been small cell bodies of the fluorescent axons in the ectoneural tissue, but such structures werefew in number compared with the large number of fluorescent axons. Occasionally, at high resolution, fluorescent processes were seen which appeared to be in connection with autofluorescing cell bodies in the ectoneural layer (Fig. 9). An electron microscopic survey (Pentreath, 1967) has shown that the ectoneural tract contains scattered cell bodies giving off axonic processes (Fig. 10). The cytoplasm of such cells is small in volume, ie. there is little space between the nuclear and cell membranes. These cells appear to be those that autofluoresce. It is possible that

Fig. 4. Amine-specific fluorescence in the radial nerve of Asterias rubens. In cross-section it can be seen that the lateral and mid-regions of the nerve fluoresce most intensely. X.40.

Fig.5. The speckled appearance of the specific fluorescence in the starfish radial nerve is presumably due to the localization of amines in axons which are cut in cross-section. The hyponeural tissue (h), and the outer nerve epithelium do not fluoesce specifically. X.100. (Kodachrome-X).

Fig.6. A horizontal section in the mid-electoneural tissue of Asterias radial nerve showing numerous amine-fluorescing axons running parallel. X.310.

Fig. 7. A section similar to that shown in Fig. 6, but at higher magnification. This colour print is included because it reproduces fairly accurately the colour of the specific fluorescence, which normally is distorted during processing of the negatives.X500. (Kodachrome-X).

4

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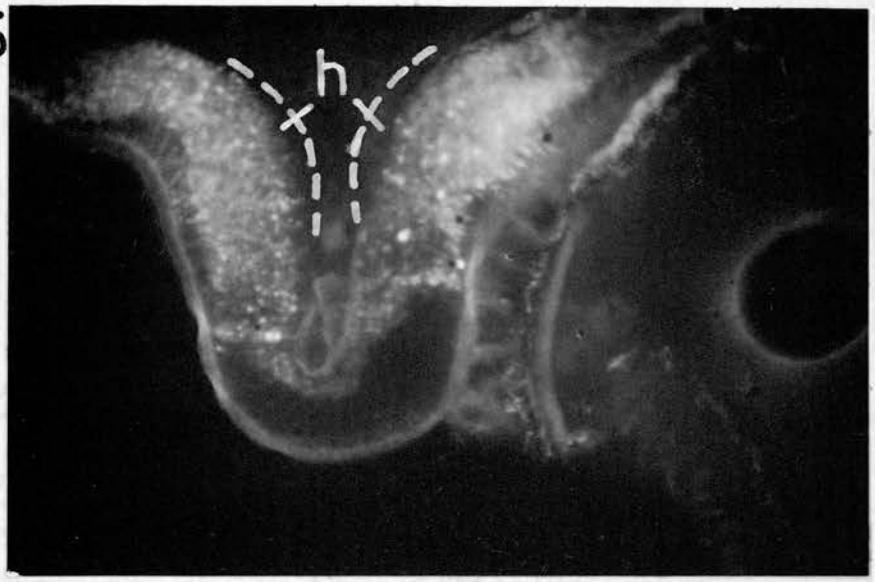

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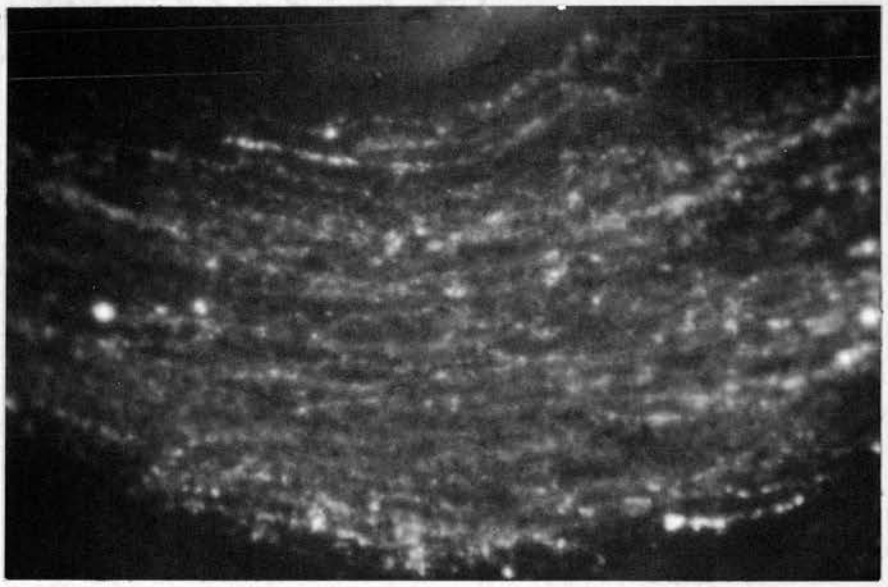
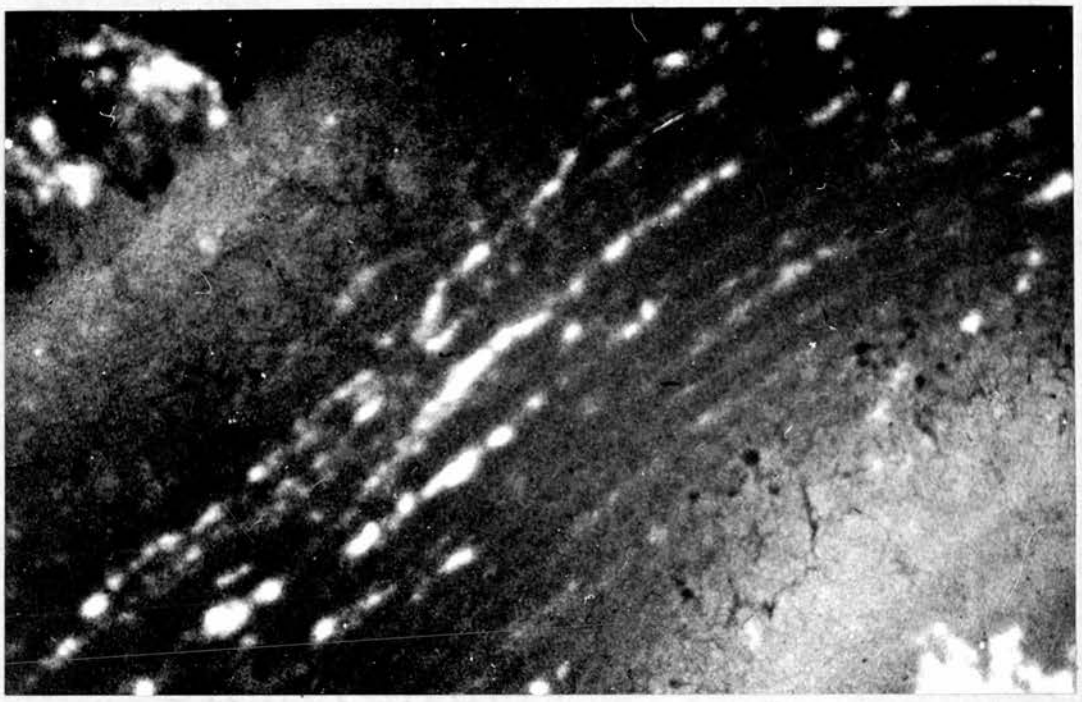


Fig.8. Most of the axons of the starfish radial cord are less than 1 μ in diameter. At high magnification it can be seen that individual axons have a beaded appearance, presumably due to swellings along their lengths which are rich in amine. Such swellings are 3-5 μ in diameter. X670.

Fig. 9. There are a few scattered bipolar and multipolar cell bodies in the deeper layers of the starfish radial cord. Such cell bodies autofluoresce. However, the axon process (arrow) which originates from this autofluorescing cell fluoresced a specific pale green colour. X820.

8



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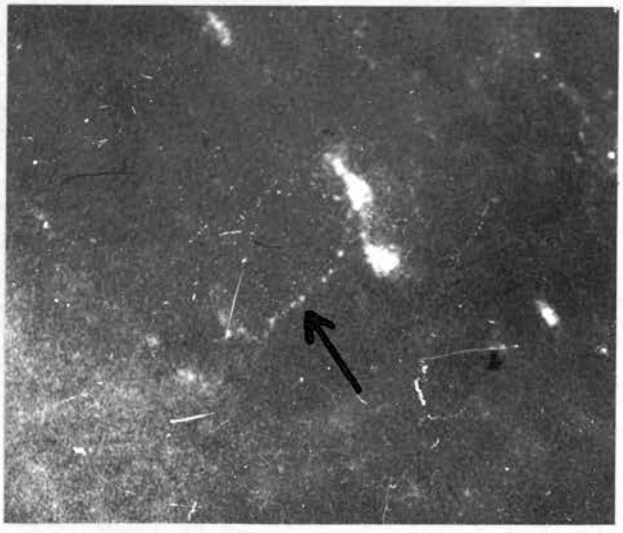

the autofluorescence seen in these cells may have masked some amine-specific fluorescence, but even if catecholamines were present in these cell bodies, their levels can be safely judged as being much lower than those of the axons. One other possibility is that the sparsity of possible fluorescent cell bodies in relation to the large number of fluorescent axons is because the cell bodies give off large numbers of processes.

Stretched preparations of the stomach (Fig.11), on the other hand, showed strongly fluorescing cell bodies which could be clearly seen to give off fluorescent processes. Electron microscopy showed that these nerve cells are situated just exterior to the thin nerve plexus which underline the stomach wall (Fig. 13). They are sparsely distributed, but they do possess an extensive cytoplasm which contains many granular inclusions (Fig. 14). Some of these granules are similar in morphology to those which have been associated with DA in other invertebrate nerves.

Contrary to the findings of Cobb (1969) the nerve layer underlying the caecal epithelium of Asterias was found to fluoresce strongly (Fig. 12).

Figs. 15-17 show parts of sections through the side wall of a podium. The thin fluorescent nerve plexus is continuous with

Fig. 10. This electron micrograph shows a small nerve cell in the ectoneural tissue of the radial cord of Asterias rubens. Two axon processes (arrows) leave the cell. The cytoplasm is restricted; most of cell body is occupied by nucleus (n). X10,000.

Fig. 11. A stretched preparation of a part of the cardiac stomach wall of Asterias showing numerous cell bodies which fluoresce specifically from primary catecholamines (some are arrowed). These cell bodies send off fluorescent axons which form a network over the stomach. X250.

Fig. 12. The nerve plexus which underlies the epithelium of the gut caecae fluoresces strongly. (L. lumen of the gut). X190.

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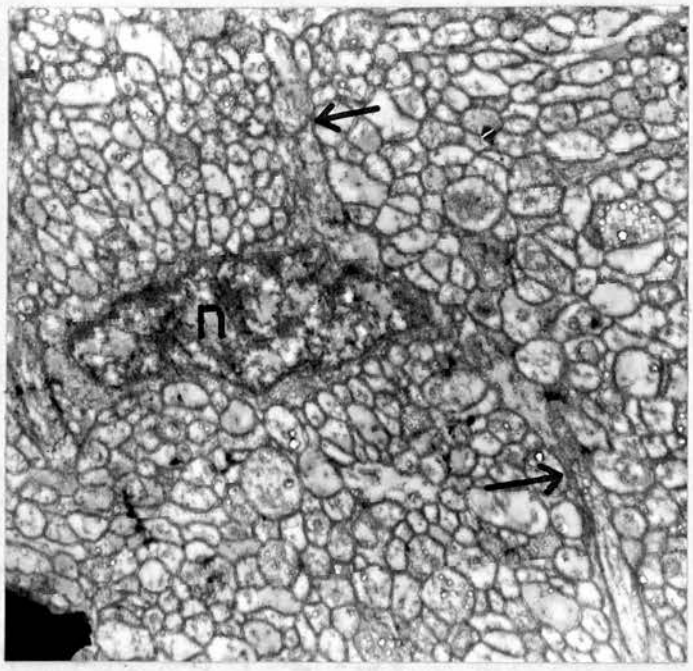

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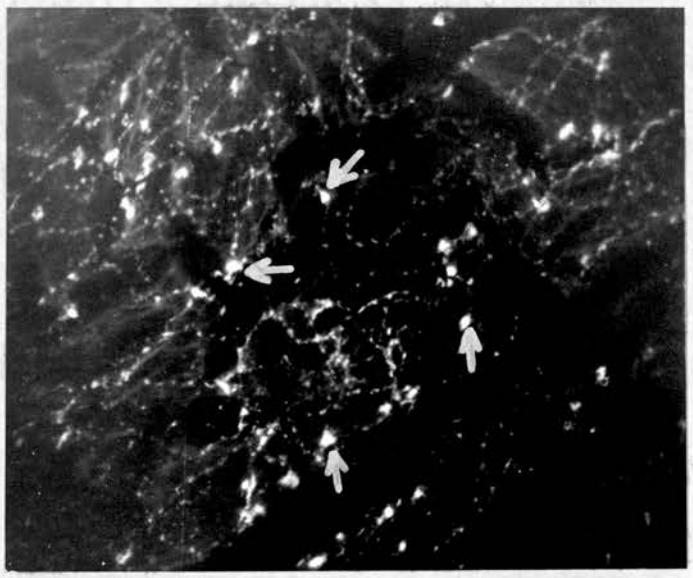

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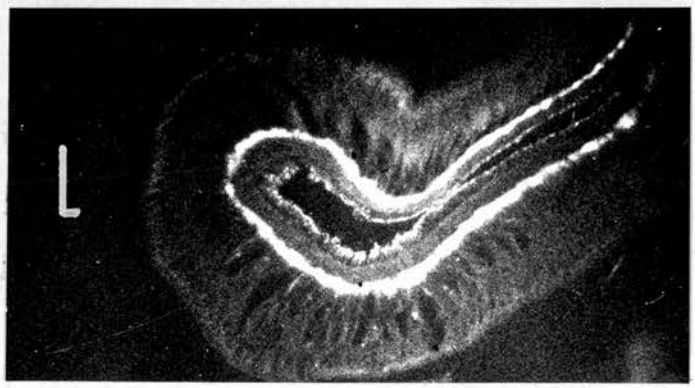

Fig. 13. Cross-section through the cardiac stomach of Asterias rubens showing from top (exterior) to bottom the heavily ciliated epithelium, the thin nerve plexus (n), the layer of supporting collagen and intermingled muscle fibres (cl), and the inner edothelial layer (en). An aggregate of mucopolysaccharide-type granules (mg) lies just beneath the ciliated border, while what appears to be a similar, but discharged group, lies above and to the left of this (arrowed). Due to the bad preservation no conclusions can be made concerning the nature of the deeper epithelial cells. The interior coelom is marked L. X5,500.

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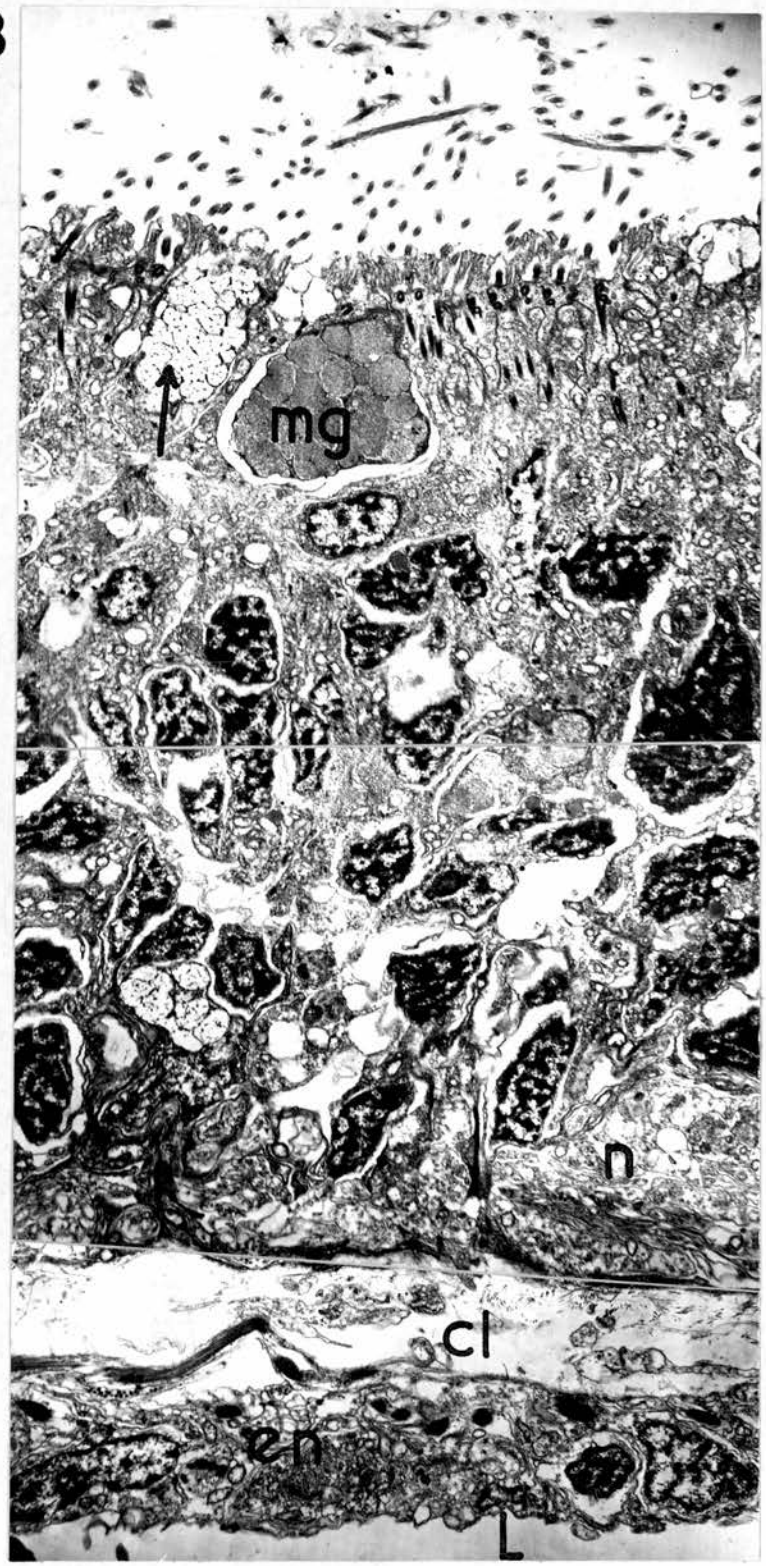

Fig.14. Unlike the nerve cell bodies in the radial nerve, those in the plexus of the cardiac stomach have a relatively extensive cytoplasm (b). These cells send off processes into the nerve plexus. c, collagen; n, nerve plexus. X9,500.

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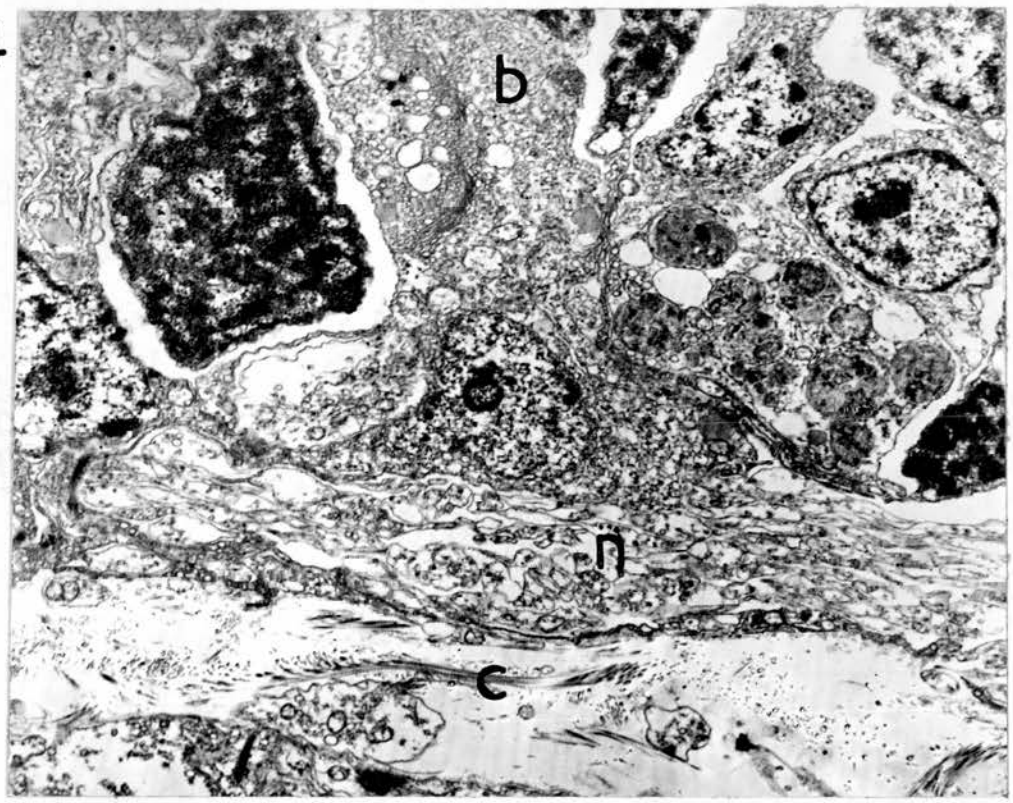

Fig.15. The thin nerve plexus underlying the epithelium of the tube-feet of Asterias is continuous with the ectoneural layer of the radial nerve. The plexus, here seen in cross-section, contains many fluorescent axons. L, lumen of tube-foot. X460.

Fig. 16. Oblique section through the wall of a tube-foot. The autofluorescing epithelium is folded since the podium is in a contracted state. n, specifically fluorescing nerve plexus. X500.

Fig. 17. The nerve plexus of each tube-foot is thickened at one point into a podial nerve. This nerve, seen here in cross-section, contains many amine-containing axons. X450.

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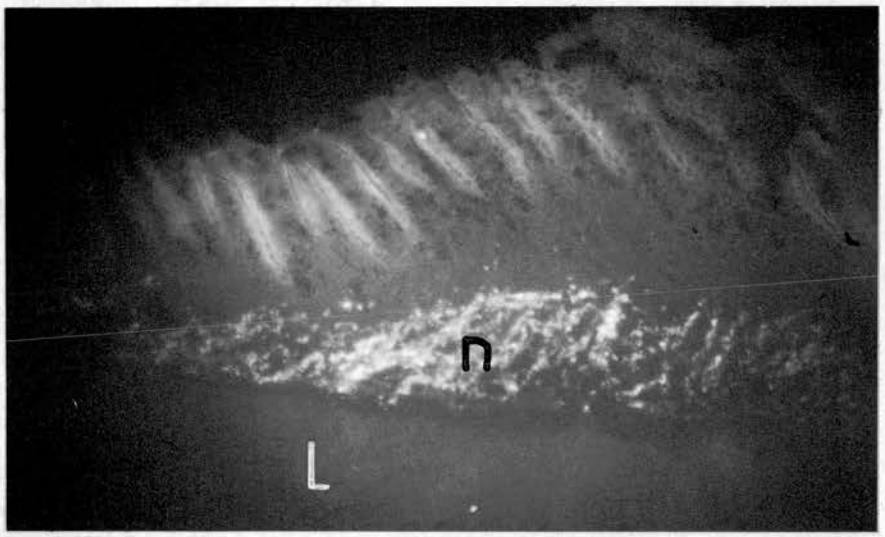

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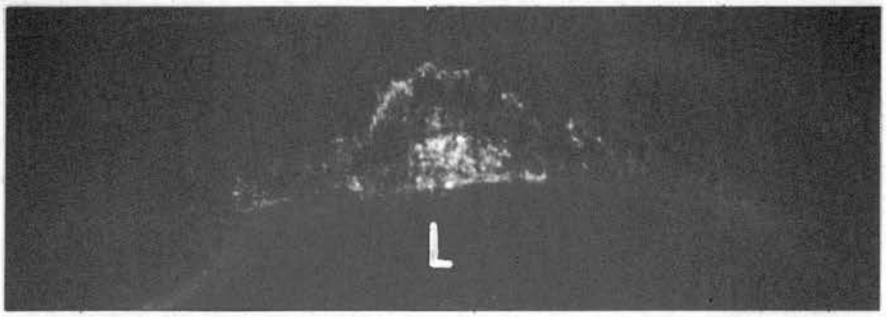

that which covers the arm sheath, and also the radial nerve. On that side of the tube foot closest to the radial nerve the plexus shows a local thickening into a longitudinal nerve which runs the length of each podium (Fig. 17). Other parts of the sensory-association plexus covering the arm and disc invariably exhibited some specific fluorescence. At its thinnest points intensity was low, but wherever the plexus thickened (ie. at the bases of pedicellariae) fluorescence was more marked. Fig. 18 shows such a thickening at the neck of an ampulla. This tissue corresponds in position to the ampulla bulb complex described by Smith (1946).

The absence of specific fluorescence in the epithelial layer covering the ectoneural layer of the radial nerves and the test is noteworthy. Smith (1937) calculated that there are at least 4,000 sensory cells per square millimetre of body surface of Marthasterias. Electron microscopic observations (Cobb, 1966; Pentreath, 1967) have indicated that many cells of the outer layer of the radial nerve give rise to nerve processes which extend into the ectoneural tract. These cells are probably the primary sense cells of the starfish, capable of perception of both tactile and chemical stimuli. The complete lack of fluorescence in the regions of these cells suggests that starfish sensory cells do not contain catecholamines. In order to test this more fully,

a discrete sensory area, the eyespot, was examined in detail. There was no specific fluorescence in the region of the photosensitive cells within the eyespot. The only specific fluorescence found near the eyespot was in the distal end of the radial nerve, upon which the eyespot lies. It was clearly separated from the sensory cells themselves (Figs. 19, 20).

Immersion of Asterias in sea water containing reserpine caused a marked reduction of the pale green fluorescence in the nervous system. This reduction was almost complete after immersion for five days in sea water containing 3.5μ grams of reserpine phosphate/ml (Fig. 21), but at higher concentrations (5μg/ml) the length of time required for the same amount of depletion was reduced to three days. This contrasts with the findings of Cobb (1969) who reports that reserpine does not reduce specific fluorescence.

In order to obtain information regarding the function of the catecholamine-containing neurons in Asterias, careful note was made of any behavioral change induced by reserpine. During reserpine treatment starfish became increasingly inactive. At four days immersion (5μg/ml) movement of the arms is slightly slowed, and so also is the righting response. There is a further increase in slowing at six days, but even at periods exceeding

Fig.18. There are aggregates of nervous tissue at the neck regions of the ampullae and tube-feet of starfish. These areas fluoresce specifically for amines, while the adjacent ambulacral ossicles (Os) autofluoresce. X190.

Fig. 19. Longitudinal section through the middle of an eyespot of Asterias rubens. The dotted line at left and top marks the outer edge of the eyespot. The positions of red pigment-containing ocelli are arrowed. A group of brightly autofluorescing cells is marked with an asterisk. The only specific amine fluorescence is at the bottom of the micrograph, and is clearly separate from the pigmented light-sensitive cells. The specific fluorescence is in the ectoneural tissue of the distal end of the radial nerve, upon which the eyespot lies. X500.

Fig.20. This micrograph shows the same section as that in Fig. 19 above, but illuminated with transmitted white light as well as u.v. light. The ocelli (arrowed) can be more clearly visualized in relation to the specifically fluorescing fibres. X500.

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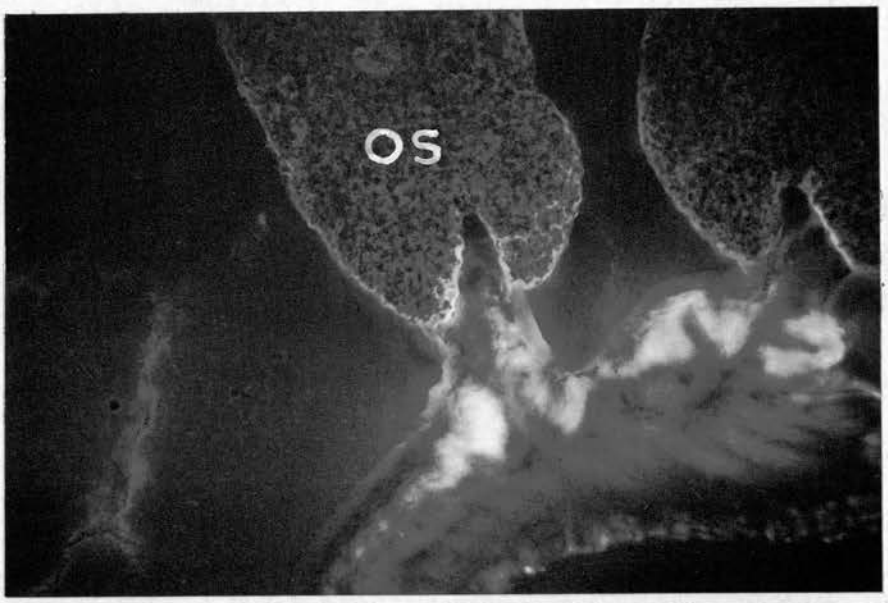

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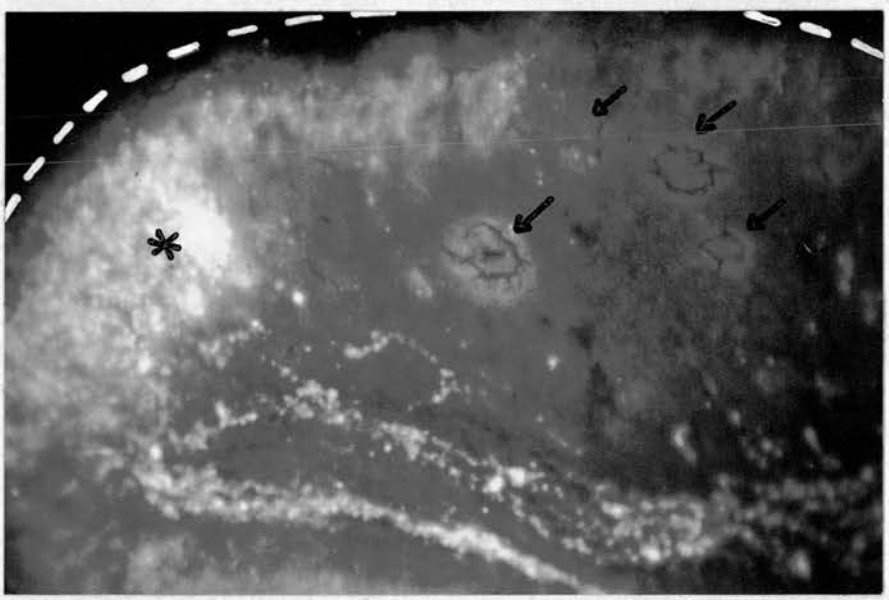

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Fig. 21. Cross-section of a radial nerve of Asterias rubens which had been immersed for five days in sea water containing soluble reserpine phosphate. Specific fluorescence has almost completely disappeared from the ectoneural tissue (cf. Fig.5). X116.

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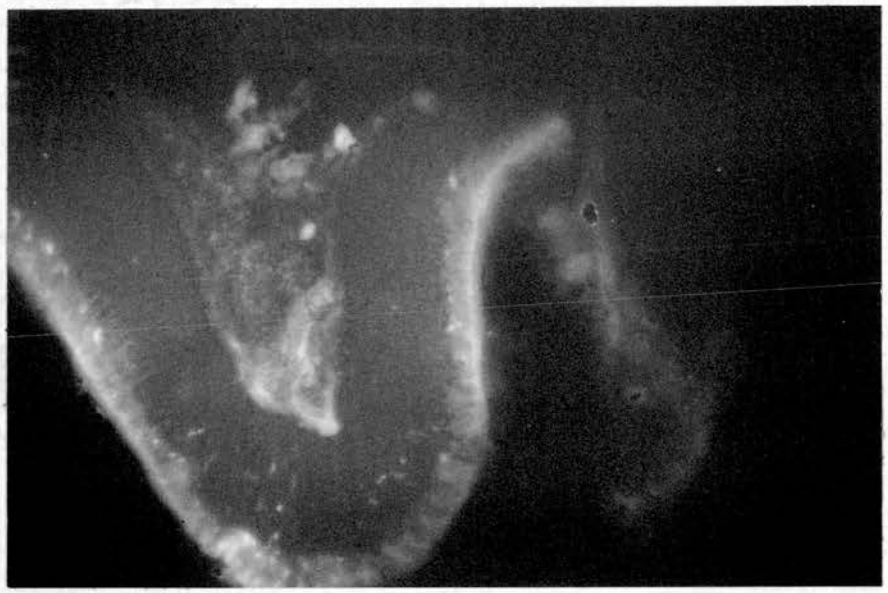


Fig.22. Diagramatic cross-section of an arm of Ophiothrix fragilis showing the general arrangement of the nervous system. The radial nerve is illustrated at a ganglion region, where both the ectoneural (ect) and hyponeural (h) elements are expanded. At interganglionic regions both parts are reduced, and the cross-sectional area of the cord would be approximately one third of that illustrated here. The ectoneural tissue is separated from the hyponeural tissue by a layer of collagen. Directly aboral to the hyponeural tissue lies the radial water vessel, r. The radial cord is bounded by an epineural sinus (es) on its oral side, and by hyponeural sinuses (hs) on its lateroaboral sides. Directly beneath the radial water vessel is the longitudinally running radial perihaemal canal (cross-hatched).

The three principal branches of the cord within an arm metamere (the latter is any segment of an arm between successive tube-feet) are as follows; first the hyponeural nerves, which are exclusively motor, second the podial nerves, which give rise to a ring ganglion at the base of a podium, and third, and largest, the lateral nerves, which ascend the arm wall giving off ganglionated branches to the spines (sg). These three nerves do not in reality leave the cord at the same point in cross-section, as is shown here for simplicity.

Three areas which contain neurosecretory elements are darkly shaded. These are present first, in the outer hyponeural elements, second in regions of the ventral plate bordering the epineural sinus (v), and third in the peripheral parts of the spine ganglia.

The numbered areas and lines on the tracing represent the levels of subsequent electron micrographs.

a stepping movement. An extended podium responds normally to pinching with forceps, ie. it withdraws, but this does not readily elicit a general withdrawal in all podia adjacent on the arm, as is the case in the healthy animal. Once the starfish has righted itself it does not move away; it remains quiescent. This behavioral change would appear to indicate that reserpine treatment has abolished coordination of the tube feet.

Ophiothrix fragilis

No attempt was made in this study to isolate or quantitize catecholamines chromatographically from brittle star nerves. This was due to the problems involved in obtaining sufficient nervous material, which have been mentioned above. On the other hand a careful comparison in fluorescent characteristics was made between that in Ophiothrix and that in Asterias. As they were found to be identical in colour, reducability, and fading properties, it was assumed that fluorescence in Ophiothrix was due to the catecholamines DA and/or NA.

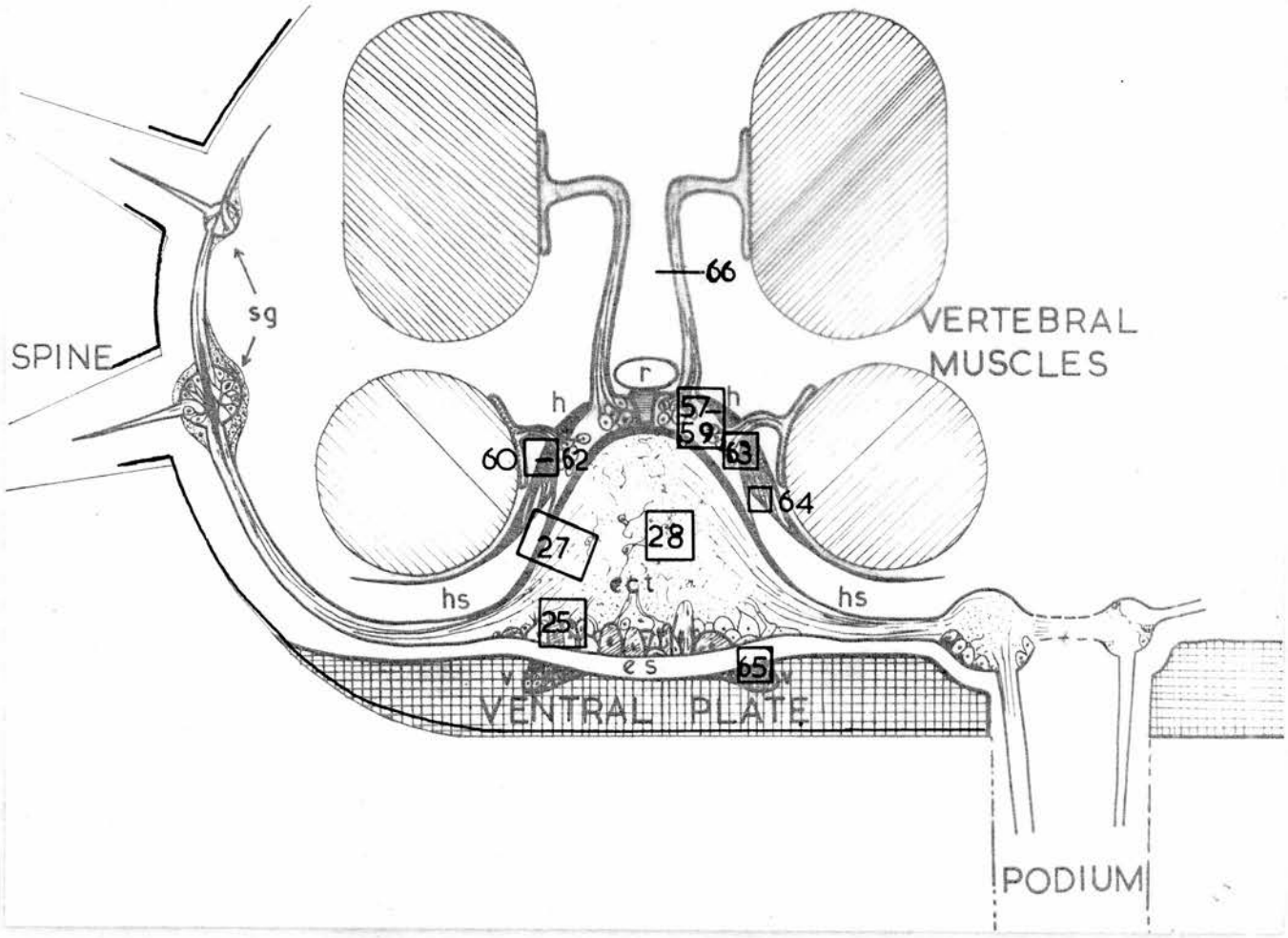
Fig. 22 shows diagrammatically the arrangement of the nervous tissues within the brittle star arm. In each experiment specific fluorescence was restricted to the ectoneural tissue of the radial nerve and lateral nerves. No specific fluorescence was present in the podia, except in their neck regions, and none was detected in the gut. The circumoral nerve was not investigated

one week, when the animal is nearing death, the righting response or arm movements are never abolished. It appears that reserpine, by depleting CA's, does not interfere significantly with behavioral responses of the animal which involve coordination of the body wall musculature.

However reserpinization caused a drastic effect on movement of the podia. If a healthy Asterias is placed on its back, or in the null position (Kerkut, 1954[X]), the arms make slow turning movements in preparation for the righting response. The podia however become immediately extended, and after "feeling" haphazardly for a short while, resume stepping in a coordinated manner. This stepping continues during the righting responses. When the starfish has resumed its normal position relative to substratum, it moves off in the direction of the dominant arm, propelled by the coordinated stepping of the tube feet.

However when a reserpine-treated animal is placed on its back the podia tend to remain withdrawn. Occasionally a few groups extend, but these do not "feel", or coordinate into

[X] In this experimental position the starfish is placed on its back on top of a small specimen tube. The tube is then placed in sea water so that the meniscus of the sea water lies well above the level of the tube feet. In this manner it is possible to study movements of the arms and tube feet, without the animal being able to right itself.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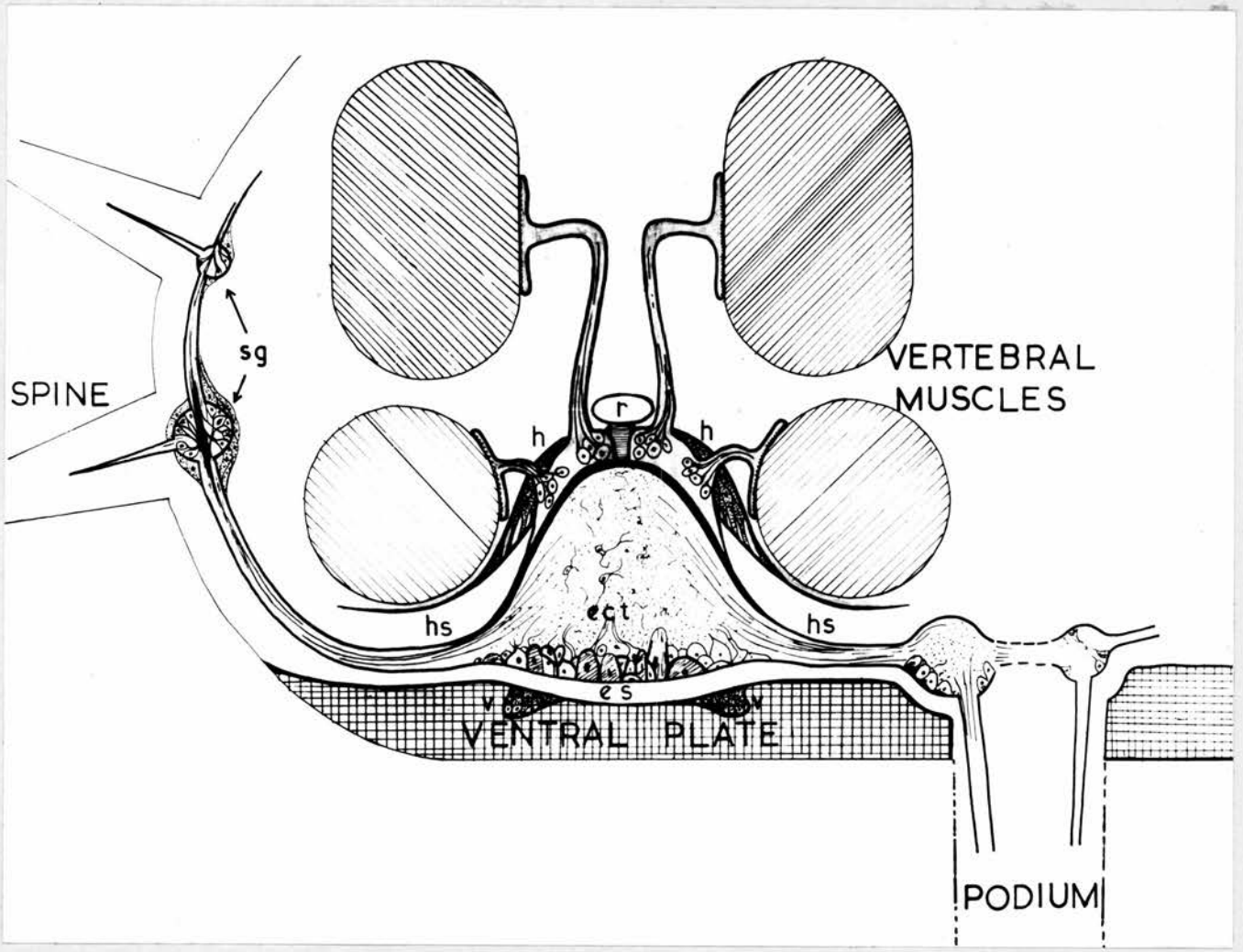
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because of practical difficulties encountered with excessive ossicle material. As might be predicted on the grounds of a restricted nervous system, there was no fluorescence at all associated with the body walls. No fluorescence was observed in ganglia which supply the spines.

In ganglion regions of the radial cord fluorescence was most extensive, but as in Asterias, it was confined to elements of the ectoneural tract (Fig. 23). No fluorescence could be detected in the hyponeural tissue. Fluorescence was sparse in inter-ganglionic parts of the cord. In these regions fluorescent fibres run parallel (Fig. 24), but in the ganglion regions no organization of fluorescent structures could be discerned.

Electron microscopy showed that the ectoneural tissue bounding the epineural sinus is composed of three cell types (Fig. 25). One of these types gives rise to a thin sheath which covers the nerve (Fig 26), while the other two send off processes into the ectoneural tract. None of these cells showed any specific fluorescence. Fine structural studies also showed that inter-ganglionic nerve areas were composed of large numbers of small diameter fibres (range 0.2-3μ) running parallel (Fig. 27), but at ganglion regions (Fig. 28) there is an interweaving of fibres

Fig. 23. A cross-section of the radial cord of Ophiothrix fragilis showing numerous specifically fluorescing structures (presumably axons in cross-section) which are confined to the ectoneural tissue. Strongly autofluorescing ossicle (os) surrounds the nerve. The hyponeural tissue which lies just beneath the radial vessel (r) does not show specific fluorescence. X150.

Fig. 24. In horizontal section varicose axons are visible in the ectoneural tissue. X350.

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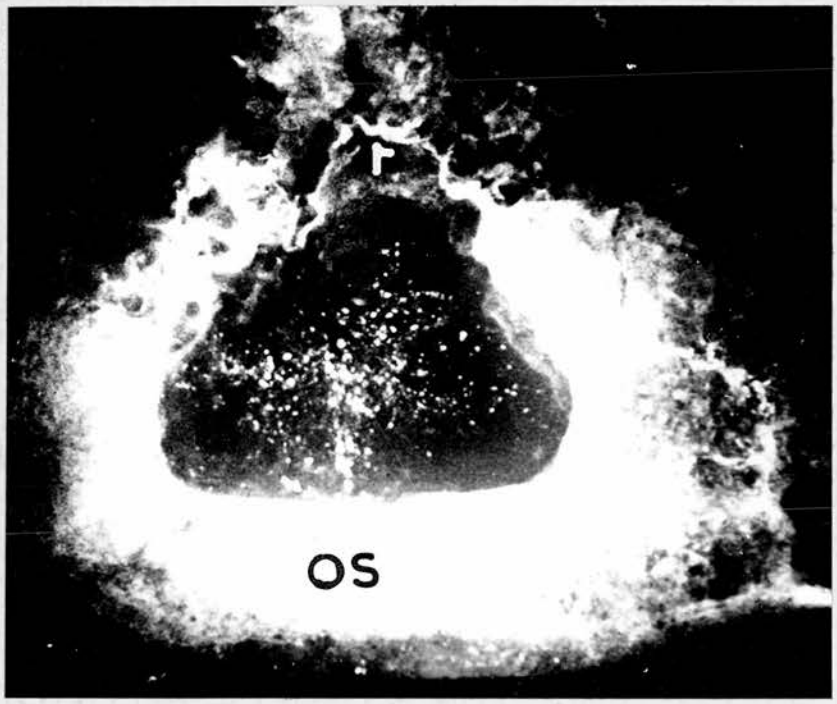

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Fig. 25. Electron micrograph of a transverse section through the oral radial cord of Ophiothrix fragilis at an inter-ganglion region. The oral layer of the ectoneural tissue is covered by cells (ecs) which support a thin basement membrane (bm). Ganglion cells (ecg) lie between these cells and the ectoneural tract (ect). m, mitochondria; es, epineural sinus. X11,000.

Fig.26. At higher magnification, the basement membrane bounding the nerve can be seen to be composed of a homogenous, lightly staining, matrix of mean thickness 0.2μ. es, epineural sinus; ecs, cytoplasm of secreting cell. Transverse section. X27,500.

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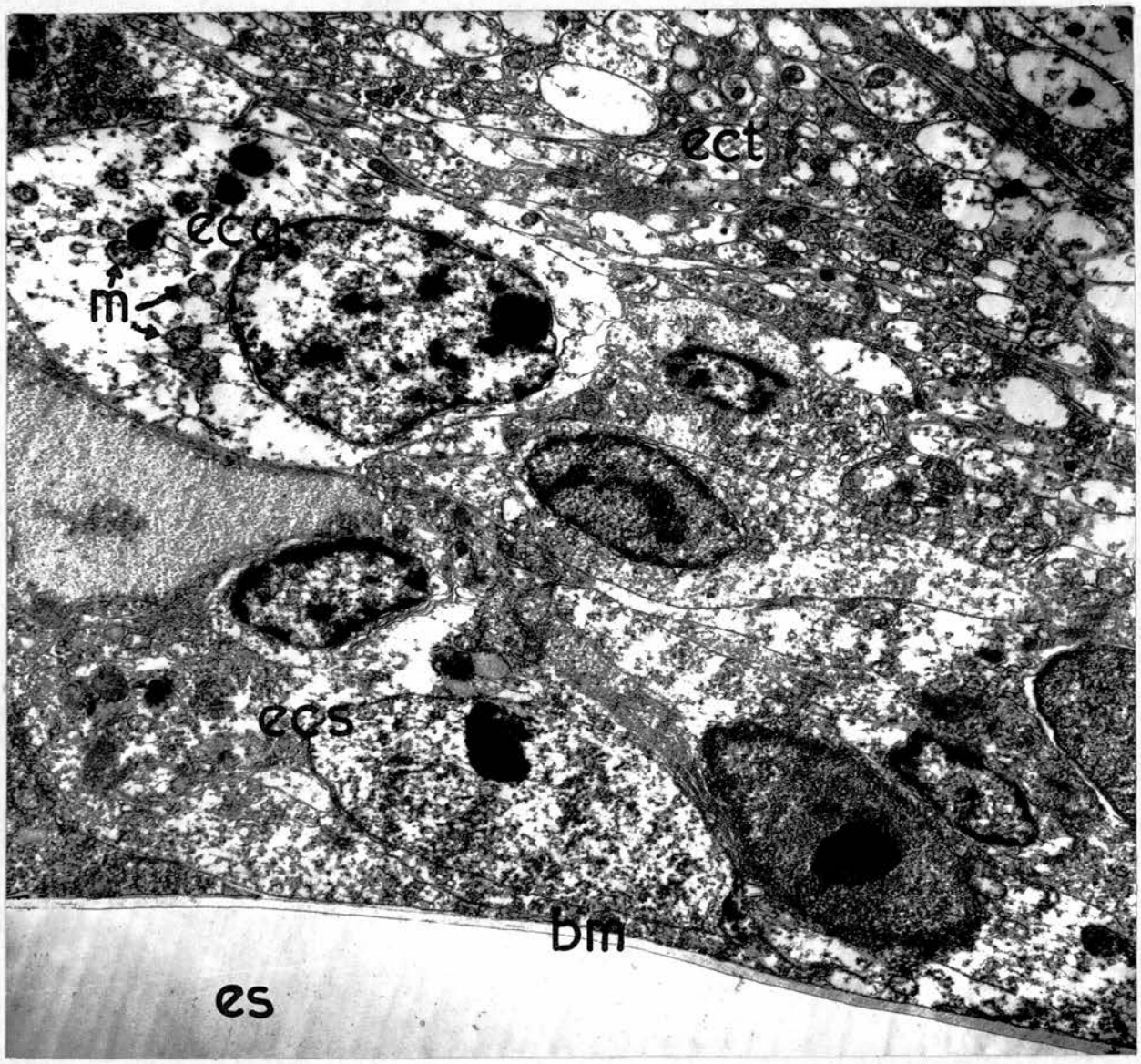

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Fig.27. Electron micrograph showing a cross-section through an aboral region of the cord of Ophiothrix fragilis at an inter-ganglion region. Axon diameters range from .2 μ to 4 μ. The section shows several supprting fibres (sf) weaving between the closely packed axons. The axoplasm contains mitochondria, clear synaptic vesicles and large numbers of neurofilaments. Glial elements appear lacking, but there are occasional aggregates of lipid droplets (lg). hs, hyponeural sinus. X10,500.

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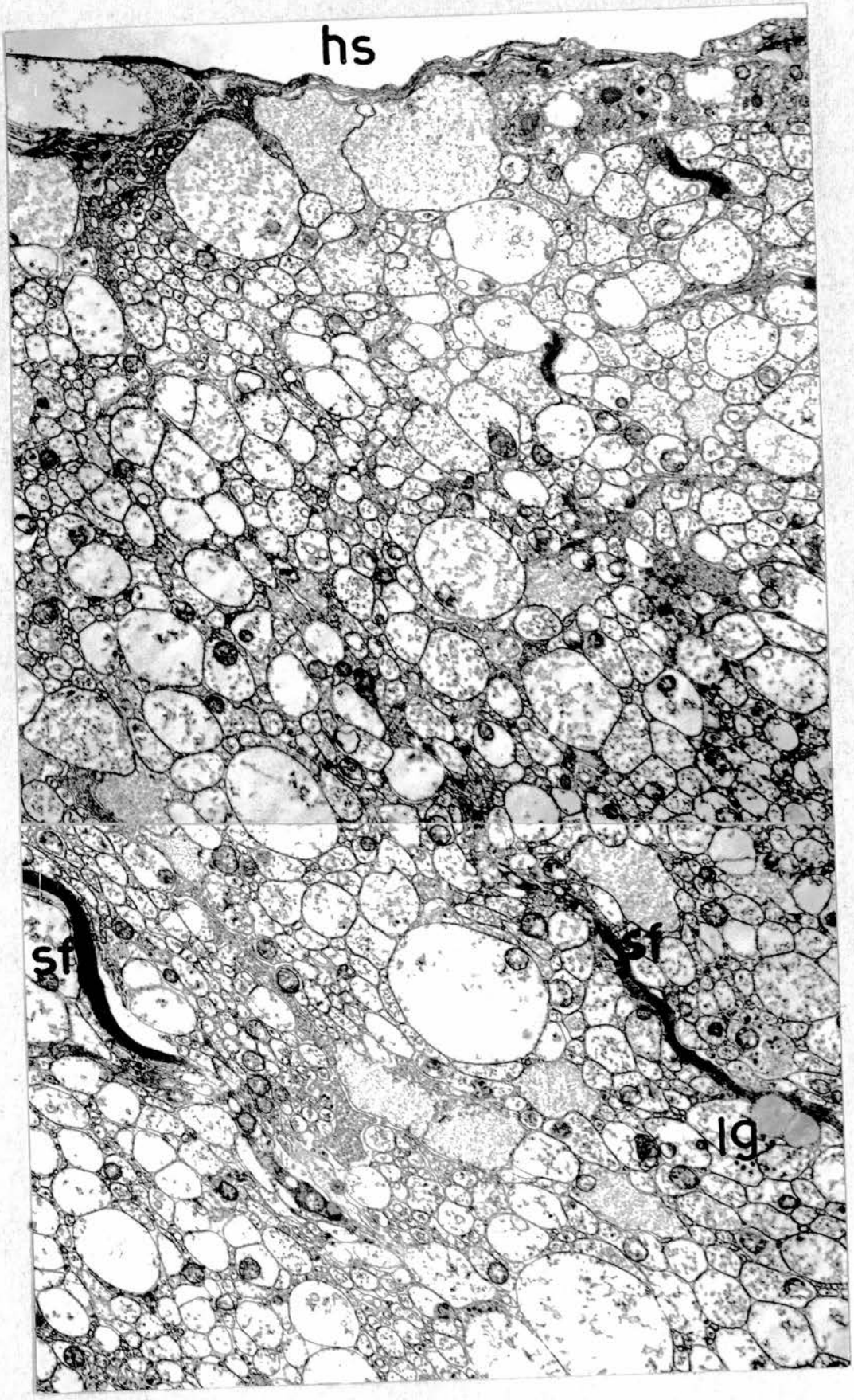

Fig.28. At mid-ganglion regions of the brittlestar radial cord there is extensive interweaving of fibres into a neuropile. Axon swellings contain large numbers of small synaptic vesicles (arrowed). There are also, but rarer, aggregates of vesicles (mean outside diameter 500 Å) which have small, electron-dense cores (dev). nf, neurofilaments. X12,000.

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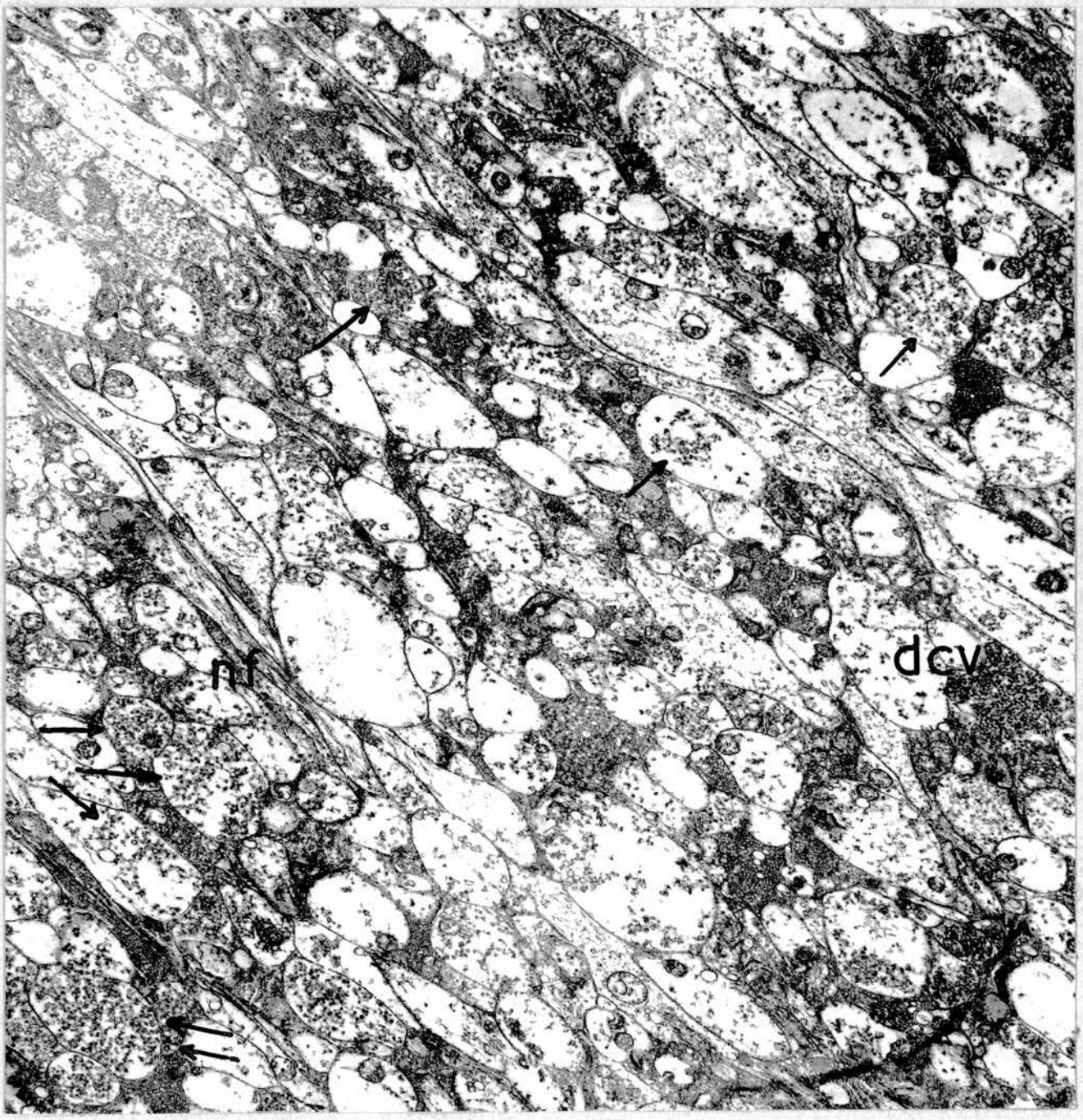

into a neuropile.

As is the case with starfish, there are a few cell bodies in the ectoneural tract. Similarly those in the brittle star have a restricted cytoplasm, but it was found impossible in the latter case to decide whether or not they fluoresced. No autofluorescent cell bodies with accompanying fluorescent processes were, however, seen. The brittle star possesses no discrete sensory areas such as the starfish eyespot. Consequently it was impossible to test whether or not catecholamines play a sensory role in brittle stars. Suffice to say that the radial cord, which is completely enclosed in Ophiothrix, and which was the only nervous structure observed to fluoresce appreciably, is very unlikely to contain sensory neurons in its deep layers.

Immersion of brittle stars for five days in sea water containing 5 µg/ml reserpine did not markedly reduce fluorescence. This might be expected since the ophiuroid nerve tracts are enclosed within the animal, and are not exposed to the environment as is the case with Asterias. After six days immersion in reserpine solution there was neither a slowing in the righting response nor in "walking" movements. However it was noticeable that the rows of podia moved more sluggishly than in the untreated animal. This change presumably results directly from the fact that

the part of the animal's nervous system least protected from the environment lies just beneath the podial epithelium. Small changes in the amine content of nerves might well not be observed by the histochemical technique.

Antedon bifida

Because of the small size of nerves and the difficulties in obtaining undistorted sections, only the arm and pinnule regions of the feather-star were studied. In both these areas specific fluorescence was limited to the ectoneural and aboral nerves (Fig. 29). No amines were present in the podia or any non-nervous tissue. It could not be resolved whether or not any of the small branches of the aboral nerve, which supply the body wall, fluoresced, because of masking by the autofluorescing brachial ossicles.

Once again there was a problem in discerning possible fluorescing cell bodies in nervous tissue. Electron microscopy showed that the ectoneural tissue was concentrated into two tracts lying on either side of the ambulacral epithelium, beneath the podia (Fig. 31). Each tract is composed of approximately one thousand small fibres. No cell bodies are present in these tracts, which fluoresced strongly.

In a similar fashion the aboral nerve is composed of large numbers of parallel-running small diameter fibres (Fig. 32). A few multipolar cell bodies are present in this nerve, but they are few in relation to the number of fibres.

Fig. 30 shows a longitudinal section through a pinnule. The fluorescing nerve is a branch of the aboral nerve.

Fig.29. Cross-section through an arm of Antedon bifida. The tissue has been distorted in sectioning, but it does show the specifically fluorescing areas. Brachial ossicle (bos) shows a faint autofluorescence, which appears brighter at the edges of the arm because of refraction properties. The strongly specific fluorescent aboral nerve lies in the middle of the picture. The ectoneural tissue lies to the top right-hand corner. Parts of this show amine fluorescence which cannot be distinguished in the micrograph because of tissue rupture. X500.

Fig.30. Longitudinal section through a pinnule showing a branch of the aboral nerve which fluoresces specifically for primary catecholamines. X490.

29

30

Fig.31. Beneath the lateral epithelium of the ambulacral groove of Antedon the ectoneural tissue is thickened into two tracts. One such tract is shown here in cross-section (ect). To the top of the picture lie the bases of epithelial cells. At the bottom is the thick sub-ectoneural collagen layer (cl). The axons of the ectoneural tract range in diameter from 0.2 to 2μ, and contain mitochondria and clear vesicles (arrows). X9,000.

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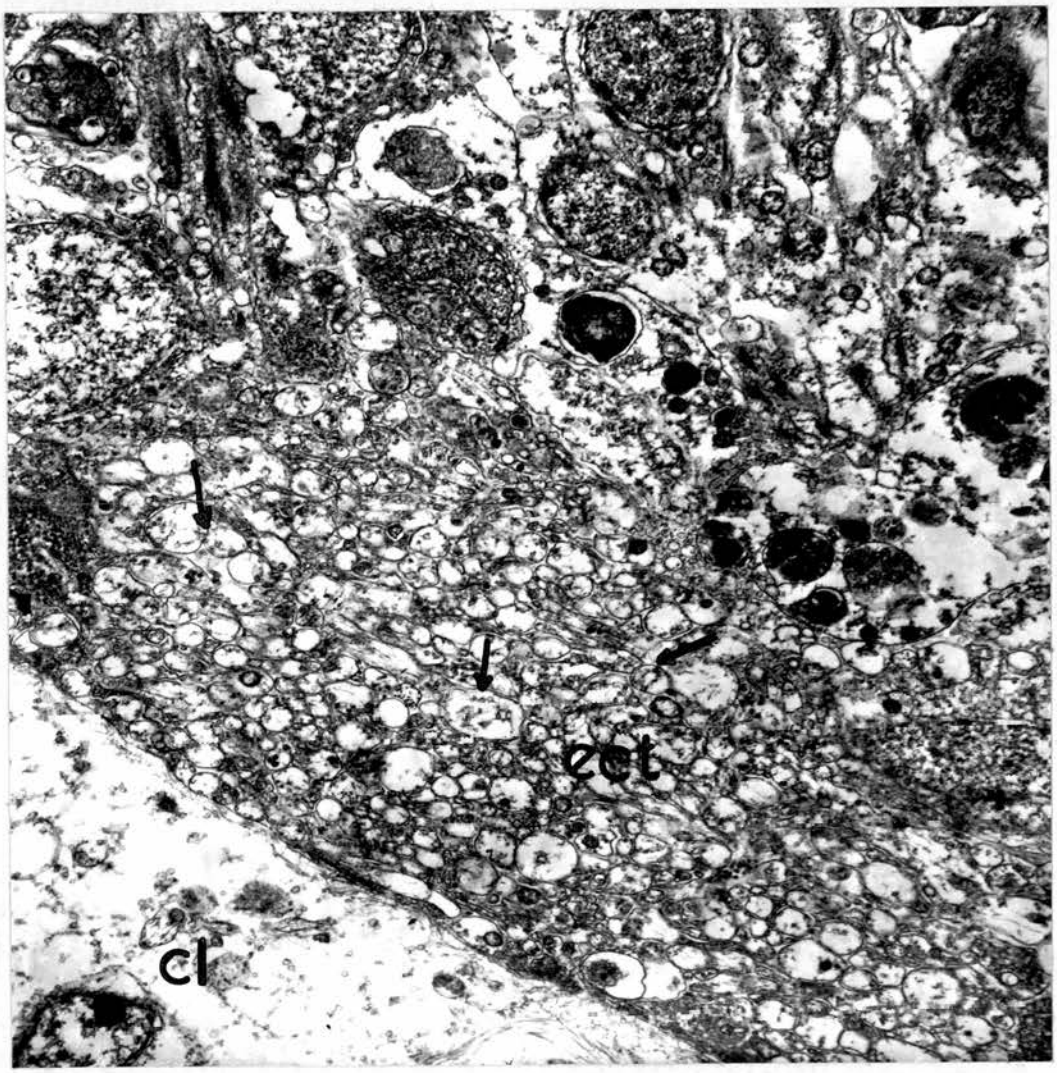

Fig.32. Electron micrograph of a cross-section through the aboral nerve of Antedon bifida. The tissue is poorly preserved as a result of de-calcification, but it serves to illustrate the tight packing of the many small axons. X11,000.

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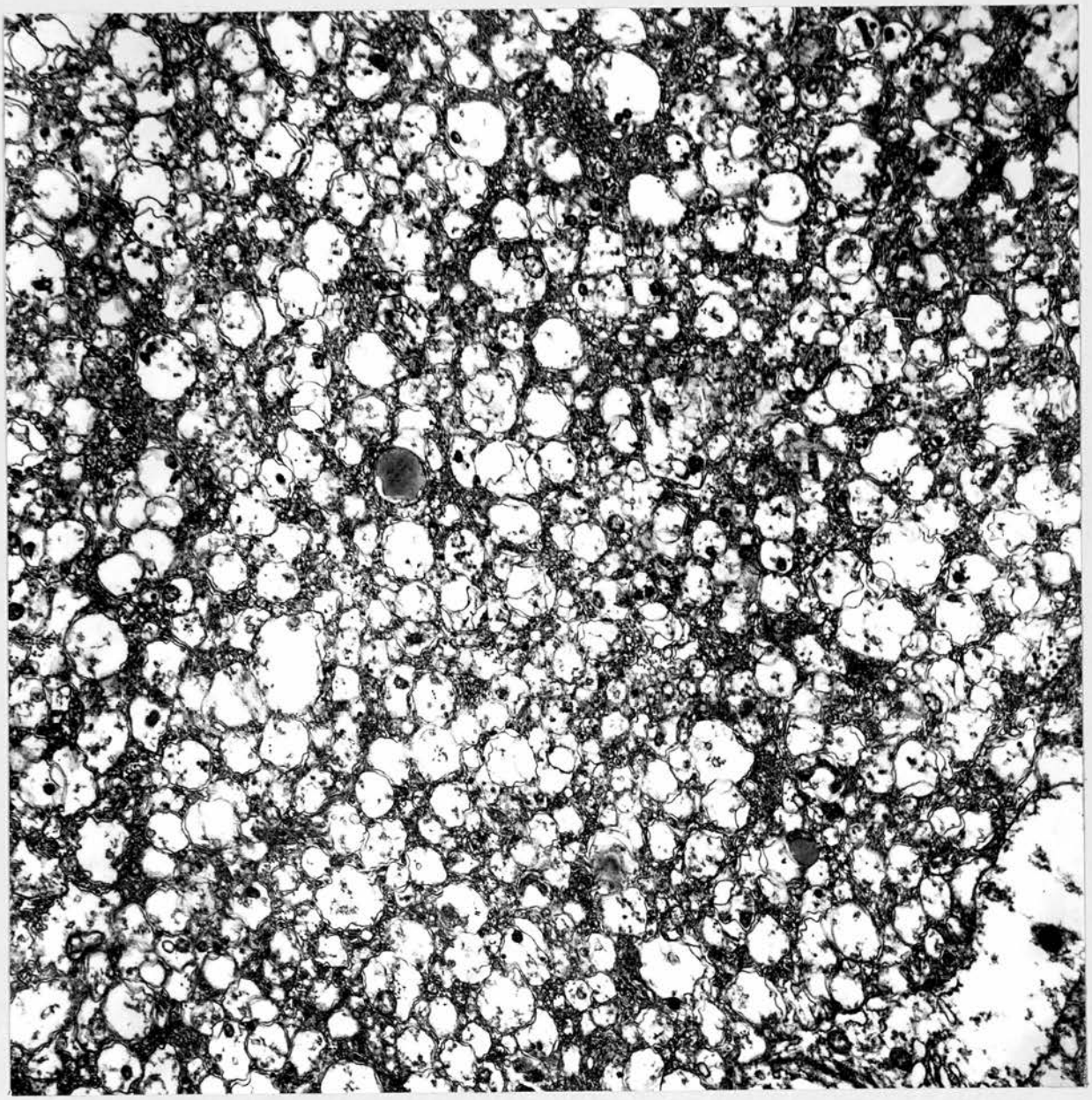


Subcellular localization of amines in Asterias radial nerve.

There is growing evidence that the small agranular or "synaptic-type" vesicles present in mammalian nervous tissue sequester ACh, while the small granular vesicles present in mammalian autonomic nerves are associated with catecholamines (see eg. Burnstock and Robinson, 1967).

Echinoderm nerves contain several different types of small vesicles, some of which are morphologically similar to those described in mammalian nerve tissues. The nature of these vesicles has been described by Cobb (1966) and Pentreath (1967). Furthermore Pentreath and Cottrell (1968) have shown that ACh in the radial nerve of Asterias rubens is particle bound, and is probably associated with small agranular vesicles.

Fig. 33 shows some of the more common vesicle types present in Asterias radial nerve. It is not known whether the different types of particles are select populations of functionally different inclusions. Any type could be a precursor or a more advanced form of another. However it would seem likely, by analogy with other tissues, that some of the particles may be important in binding the catecholamines which were shown by fluorescent studies to be present in many fibres of Asterias ectoneural tissue. An attempt was therefore made to determine the

Fig.33. Vesicular inclusions of Asterias ectoneural tissue.

(a) The most common type of vesicle has a diameter range of 250-700 Å, possess a clear core, and is normally found in aggregates. X60,000.

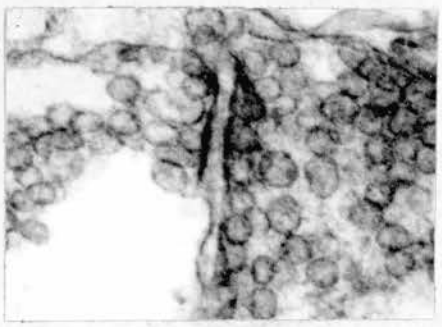
(b) This structure is interpreted as a synapse, It is common to find vesicles aligned against an axon membrane (cf. (i)), but it is relatively uncommon to find membrane thickenings as shown here. The vesicles have a mean diameter of 500 Å. Both vesicle aggregates and membrane thickenings are most frequent in the ganglion (neuropile) region of the cord. X76,400.

(c) Less frequently seen are vesicles with diameters ranging from 350-800 Å which have a small, dense core. X64,000.

(d) Still rarer are larger vesicles with a more complete electron-dense centre. Diameters range from 800-1,200 Å. Because they are found in discrete groups, they probably represent a distinct population. X100,000.

33 a

b



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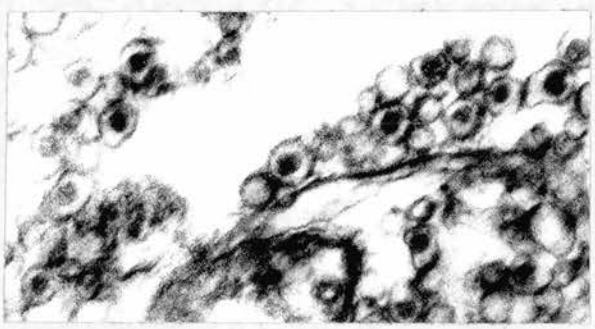

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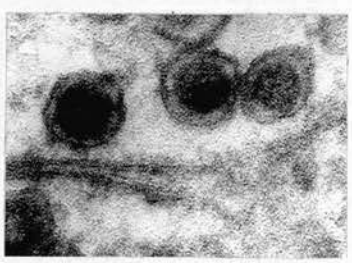

nature of the particles binding DA and NA in the radial nerve of Asterias.

Two histological stains for the demonstration of amines at the electron microscope level were employed. These methods were those described by Wood (1965, 1966), and Tramezzani et al. (1964).

Methods.

For the Wood method freshly dissected pieces of Asterias radial nerve were fixed for 3-6 hr. at room temperature in a mixture containing 3 ml 25% glutaraldehyde solution, and 20 ml of 0.2M cacodylate buffer pH 7.2. No sucrose was added to this mixture. Tissues were rinsed for 12 hr. in several changes of cacodylate buffer, and then immersed for 24-30 hr. in the following solution: 40 ml 0.2M acetate buffer pH 4.1, 1 gm $K_2Cr_2O_7$, 0.8 gm $Na_2SO_4 \cdot 10H_2O$. The tissues were then dehydrated and embedded in Araldite.

For the method of Tramezzani et al. (1964) (extended description by Cannata et al., 1968) tissue was fixed for 1-24 hr. in 6% glutaraldehyde (25% stock glutaraldehyde diluted to 12½% with distilled water and to 6% with sea water) buffered to pH 6.5 with 0.1 M phosphate buffer. After washing for 3 hr. in running

sea water tissues were treated for 1, 10, 20, 40 or 60 min. in ammoniacal silver carbonate, prepared according to Carlton and Drury, 1957. Subsequently tissues were briefly rinsed in distilled water, placed in 1% sodium thiosulphate for 5 minutes, washed again in distilled water (10 minutes), dehydrated (acetone series) and embedded in Araldite.

Results

Great difficulty was met with both techniques in adequately preserving cellular detail. This was necessarily due to the fixation of tissues in glutaraldehyde alone, and their subsequent treatment with incubating media.

(i) Method of Wood (1965).

After tissues have been fixed in glutaraldehyde and immersed in potassium dichromate, the only structures which appear electron-dense are those representing accumulations of amines (Wood, 1965, 1966).

Figs. 34-36 are electron micrographs of parts of the lateral ectoneural tissue of Asterias radial nerve. They show aggregates of electron-dense granules which appear to be located within neuron membranes.

Fig.34. Part of the neuropile of the lateral acto-neural nerve tissue of Asterias rubens. Two processes contain collections of granules which have reacted to the Wood technique, and appear electron-dense. These granules probably represent sites of amine localization. Arrows point to granules which consist of an inner area of high density surrounded by an area which is less dense. X30,000.

Fig.35. Another group of reactive granules in the mid-region of the ectoneural tract. X40,000.

Fig.36. Electron micrograph of a cross-section of the starfish radial nerve in its mid-region. Granules appear to be confined within an axon membrane. X45,000.

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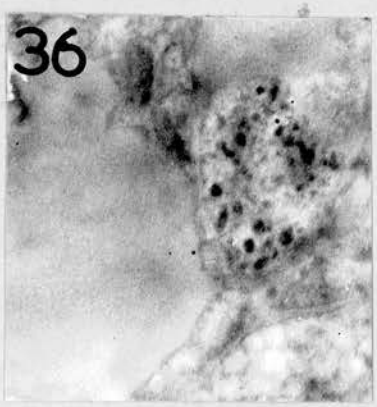


The granules range in diameter from 400-800 Å. Because the tissue is poorly preserved it is not possible to see if the granules are membrane-bounded, although there are some indications that this is so. It also appears that some granules consist of an inner area of high density surrounded by a less dense peripheral area. Some of the particles are less well stained than others. No granules were present in the hyponeural tissue.

Although it is hard to make quantitative estimates with the electron microscope, as it only allows examination of small areas, sampling indicated that there were more granule-containing processes in the lateral parts of the ectoneural tissue than in its mid-region. Previous studies (Pentreath, 1967) have shown that the lateral parts of the nerve are composed of neuropile, in which there are many processes containing either clear (diameter 250-700 Å) or dense-cored (diameter 350-600 Å) vesicles, or a mixture of both (Fig. 33). It would seem likely that some of these vesicles are those which react to the Wood technique.

It is also of note that the lateral borders of the cord are richer in amine when visualized by the fluorescent histochemical technique (Figs. 4,5).

The Wood technique was also applied to the radial nerve of Ophiothrix. Results were generally similar to those obtained with Asterias. No reactive particles were found in the hyponeural tissue, but groups of electron-dense granules were present in the ectoneural tissue of the radial cord. These granules were similar in size to those in the radial nerve of Asterias.

(ii) Method of Tramezzani, Chiocchio and Wassermann (1964).

In this technique, the final reaction product can be made selective for a particular amine by varying the periods of fixation and silver treatment (Cannata et al., 1968). After 1 hr. fixation in 5% glutaraldehyde pH 7.2, and brief (20 sec) immersion in silver carbonate, noradrenaline-containing cells show a fine precipitate which is present in cytoplasmic granules. Dopamine-containing cells, on the other hand, only react after prolonged periods of glutaraldehyde fixation (optimum approx. 24 hr), and extended silver immersion (minimum time 30 min). Adrenaline does not react under any conditions of fixation and silver treatment (Cannata et al., 1968).

It was stressed above that the technique is selective, but not specific, for catecholamines. Both melanin and

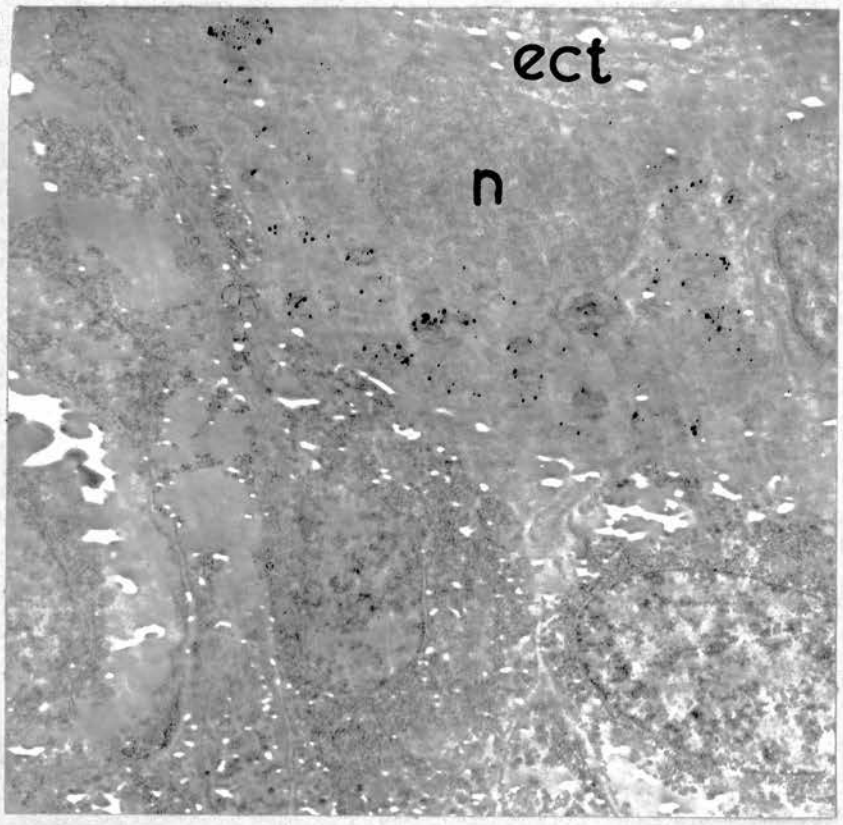
lipofuscin precipitate silver when treated for 1 hr with glutaraldehyde and 1 min with ammoniacal silver carbonate. Furthermore indolamines give a fine precipitate after 1 hr fixation and extended (40 min and upwards) silver immersion.

Because of these difficulties the following experiments were undertaken. First pieces of Asterias radial nerve were subjected to fixation periods varying from 1-24 hr, and each immersed in silver carbonate for 1 minute. These were then examined under the electron microscope, but since it was found that no reaction had taken place under these conditions, a second series of nerve pieces were fixed for 1 hr in glutaraldehyde, and treated with silver for time periods of 1, 10, 20, 40, 60 min. Examination of these tissues showed that some reaction had taken place in ectoneural elements at 40-60 min treatment with ammoniacal silver carbonate (Fig. 37). Finally, pieces of radial nerve were fixed for 24 hr in glutaraldehyde, and subsequently treated for 1 hr with silver carbonate. It was only under these conditions that appreciable deposits could be seen with the electron microscope (Figs. 38, 39). On the grounds of the methodology and specificity described by Cannata et al.(1968), these findings indicated that deposits in the radial nerve were most

Fig. 37. Electron micrograph of a cross-section through the oral ectoneural tract of Asterias. The tissue has been treated with the method of Tramezzani et al. (1964). A fine precipitate of silver is localized in lysosome-like bodies of cells which lie close to the ectoneural tract (ect). n, nucleus of ganglion cell. X12,000.

Fig.38. After longer glutaraldehyde fixation deposits are more marked, but still confined to an area of the nerve between the ectoneural fibres (ect) and the oral epithelium (ep). sf, supporting fibre. X14,000.

37



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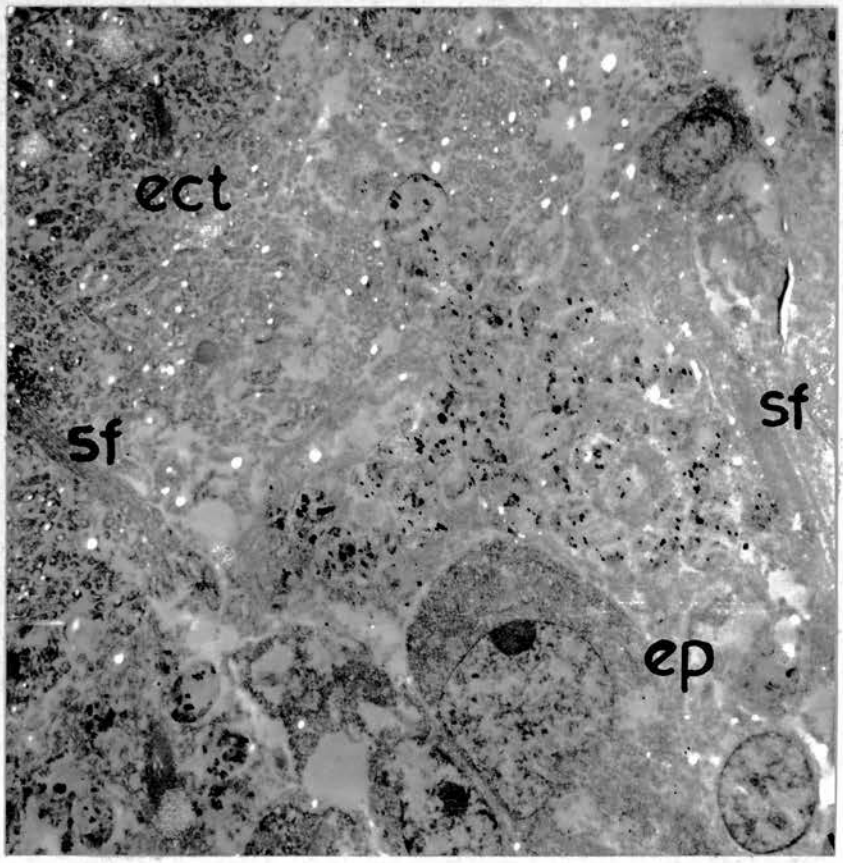

Fig.39. Certain spherical bodies present in hyponeural elements also react with the silver technique, n, nuclei of hyponeural motor cells. X15,000.

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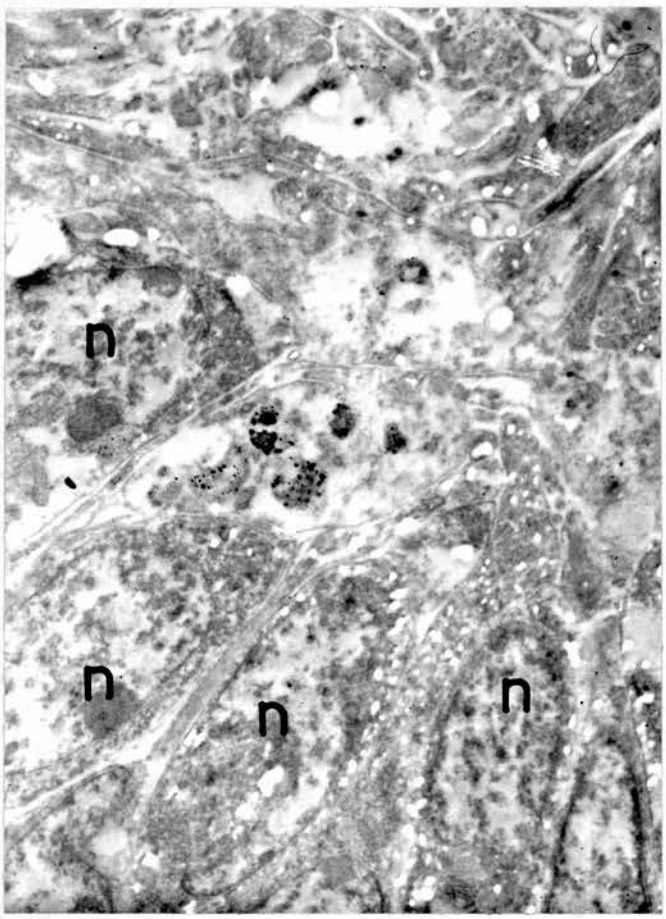


likely formed by dopamine, or less likely, 5-HT.

Fig. 37 shows a transverse section of Asterias radial nerve in the oral ectoneural region. Fine granular deposits are located in cytoplasmic granules of some ganglion cells close to the ectoneural fibre tract. These deposits are more marked after prolonged fixation (Fig. 38). Although the tissue is poorly preserved, it appears that reaction sites are aggregated within spherical, lysosome-like bodies. No reaction was observed in fibres of the ectoneural tract, or in outer regions of the epithelial cells. These results were unexpected, for as described above, amine-specific fluorescence was confined to fibres of the ectoneural tract. Moreover careful examination showed that some hyponeural elements also contained reactive sites (Fig. 39).

Arnold and Hager (1968) have recently questioned the validity of electron microscope histochemical techniques which are based on primary glutaraldehyde fixation. Their results indicate that many of the premises held by earlier workers regarding the combination of glutaraldehyde with specific compounds are untenable. Furthermore, in most techniques (including the silver method) the reaction mechanisms subsequent to fixation are postulated, not proven.

The results of the silver reaction with Asterias radial

nerve, if considered in respect to the description of Cannata et al. (1968) would indicate that dopamine was present in epithelial and hyponeural tissues. This is in disagreement with the results of the well established fluorescent histochemical technique. It is concluded here that sites in the radial nerve containing silver granules do not necessarily represent sites of dopamine.

Histochemical localization of monoamine oxidase in the radial nerve of Asterias rubens.

The importance of monoamine oxidase (MAO) in the regulation of the concentration of biologically active amines in the mammalian central nervous system has been widely reported (see Axelrod, 1959). Observations by Blaschko and Hope (1957) on the distribution of MAO in invertebrates indicated that the enzyme was present in several echinoderm species. The same workers predicted the presence of biogenic amines in asteroids and echinoids. However their studies were restricted to gut and gonad tissue; they did not study any purely nervous tissue.

As described above, the nerve plexus underlying the starfish gut caecae contains catecholamines which fluoresce when sublimated with formaldehyde gas. It is possible that the MAO detected biochemically by Blaschko and Hope (1957) is important in regulating amines in this plexus. Far greater concentrations of amines are however present in the radial nerve. For this reason a histochemical technique for the localization of MAO was applied to the radial nerve of Asterias. Special attention was paid to the distribution of MAO in relation to that of amine fluorescence.

Methods.

There have been several reported techniques for MAO demonstration (eg. Blaschko and Hellman, 1953), but some of these are unspecific (see Robinson, 1967). At present the method of Glenner, Burtner and Brown (1957), which is based on the earlier method of Francis (1953), is widely accepted as giving the most accurate localization of the enzyme. In this technique tryptamine is used in the substrate and Nitro BT (Nitrotetrazolium blue chloride : Koch-Light Ltd.) as the electron acceptor. Sites of activity are visualized by red-brown formazan deposits.

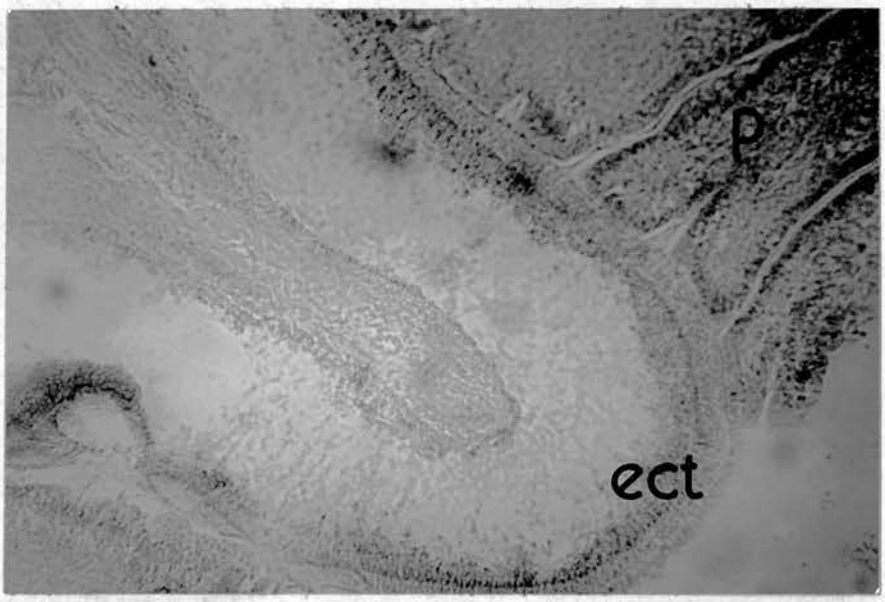
Sections of Asterias radial nerve and associated ossicles were cut on the freezing microtome. These were allowed to dry at room temperature for 5 min. Sections were incubated for 60 min at 37°C in the following medium: tryptamine hydrochloride (25mg), sodium sulphate (4 mg), Nitro BT (5 mg), 0.1M phosphate buffer, pH 7.6 (5 ml), and 15 ml distilled water; as a control alternate sections were treated in an identical way but with the omission of tryptamine hydrochloride from the incubating medium. After rinsing, sections were fixed in 10% neutral formalin for 12 hours and mounted in glycerine.

Fig.40. Cross-section of a radial nerve of Asterias rubens showing the distribution of MAO. Highest levels of enzyme activity are present in the outer epithelium of the ectoneural tissue (ect) and podia walls (p), although there are some deposits in the ectoneural tract. X110. (Kodachrome - X).

Fig.41. At higher magnification activity appears evenly distributed throughout the cells of the ectoneural nerve layer. X400.

Fig.42. Horizontal section of a radial cord of Asterias. The hyponeural tissue (h), here seen in its mid-region, contains negligible MAO activity. X100.

40



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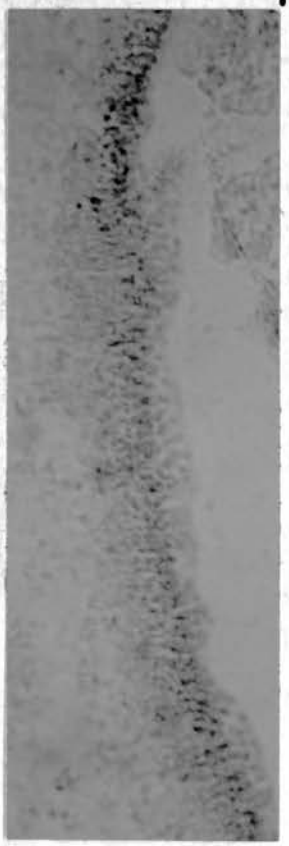

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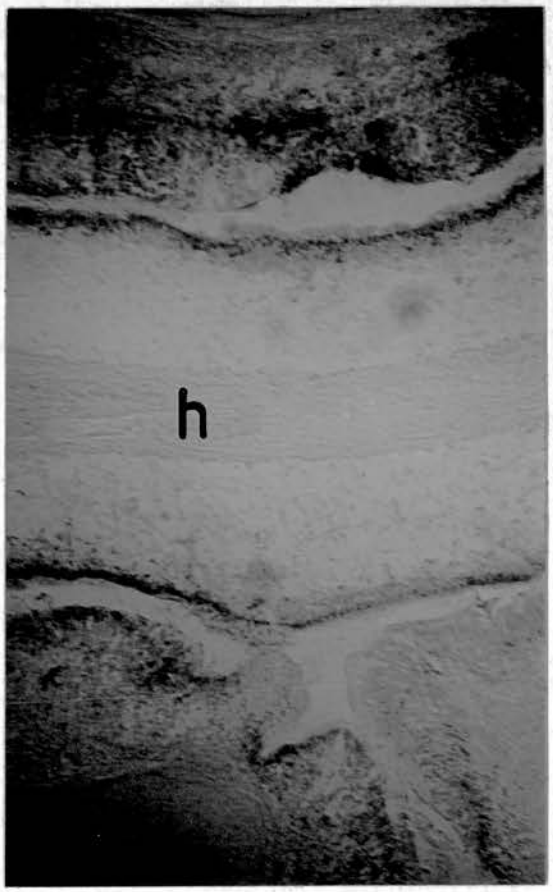

Results.

Fig. 40 is a cross-section of a radial nerve of Asterias. It is apparent that no close similarity exists between areas of enzyme distribution and areas rich in amine (cf. Fig. 5). There are a few scattered deposits in the ectoneural tract, but greatest activity is present in the outer cell body layer of the nerve. Electron microscopical observations (Pentreath, 1967) have shown that there are both nervous and non-nervous cells in this layer, but even at higher magnification (Fig. 41) it is impossible to ascribe activity to a particular cell type(s). There does not appear to be any appreciable activity in the hyponeural tissue (Fig. 42). The walls of the tube-feet are also rich in enzyme. Control sections showed no deposits.

Summary of catecholamine studies.

The radial nerves of Asterias rubens contain appreciable quantities of dopamine and noradrenaline (Cottrell, 1967). The results of the present work are consistent with this observation and, at the same time, argue for a relative lack of adrenaline and 5-HT. The nervous systems of Ophiothrix and Antedon

also contain primary catecholamines.

In Asterias and Ophiothrix specific fluorescence is located exclusively in the ectoneural tissue. This tissue layer is most extensive in the starfish. Where it is thin (podia and caecae) fluorescence was confined to a few deeper fibres of the plexus, but wherever thickenings were observed (ampullae bulbs, radial and circumoral nerves), fluorescence was more extensive. In brittle stars the ectoneural tissues of the arms are restricted to the radial and lateral nerves; no specific fluorescence was seen in the arm sheath.

In Antedon specific fluorescence was limited to the ectoneural tracts and aboral nerves. Again no fluorescence was seen in the arm sheath.

No specific fluorescence was detected in known sensory or motor tissues of any species. This would indicate that DA and/or NA are associated with an internuncial class of neurons in each animal.

Reserpine greatly reduced the intensity of amine specific fluorescence in the Asterias nervous system. This substance also brought about a behavioral change by affecting coordination of the podia.

At the fine structural level the Wood technique indicated that amines present in the ectoneural tissue of Asterias were bound

to small granular vesicles of diameter ranging from 400-800 Å. These granules appeared to be membrane-bounded, and be intra-neuronally located in aggregates. A similar situation was found in the ectoneural tissue of Ophiothrix. A second method (Tramezzani et al., 1964) for the sub-cellular localization of catecholamines indicated reactive sites in Asterias radial nerve, but results could not be relied upon because of their unspecificity.

The distribution of monoamine oxidase in the radial nerve of Asterias was determined histochemically. Highest levels of MAO were found in the oral layer of the nerve cord, and in the tube feet walls.

Studies of cholinergic systems in starfish and brittle stars.

The radial nerve of Asterias rubens contains large amounts of ACh (60µg/g wet weight) (Pentreath and Cottrell, 1968). This substance is not distributed evenly throughout the nerve. The lateral borders of the cord, shown by electron microscopy to contain comparatively large numbers of nerve endings with enclosed "synaptic vesicles", contained higher levels of ACh than the mid-portion of the nerve. It was also shown (Pentreath and Cottrell, 1968) that a specific cholinesterase, acetylcholinesterase (AChE), was present in the hyponeural (motor) tissue, and in the outer epithelium of the cord.

The present study follows on from this work. Firstly a comparative study was made on the nervous system of Ophiothrix fragilis. Assay procedures were used to find out what amounts of ACh, if any, were present in brittle star radial nerve, and the distribution and specificity of cholinesterase within the cord was determined by light microscope histochemistry. Secondly an electron microscope histochemical technique for the localization of AChE was applied to starfish radial nerve. This study was made to find out the precise localization of AChE within the tissues shown by light microscope histochemistry to be rich in the enzyme.

Thirdly, an impregnation technique was applied to Asterias in relation to possible cholinergic systems. This was the Zinc Iodide-Osmium (ZIO) method, originally employed by Champy (1913) as a selective stain for nerve terminals, but which recently has been used to stain nerve tissues at the electron microscope level. It appears that the technique stains a distinct population of intraneuronal inclusions, which some authors (Akert and Sandri, 1968) have suggested are specific to cholinergic neurons.

Measurement of acetylcholine in the radial nerves of Ophiothrix fragilis.

Methods

In three experiments the radial nerves from 10 Ophiothrix fragilis were dissected and placed into a homogenizer containing 1 ml 10^{-4}M eserine in distilled water adjusted to pH 3.0 with HCl. After thorough homogenization nerve suspensions were heated at 100°C for 5 min. and centrifuged to remove solid material.

The ACh activity of the supernatant was assayed on the isolated Mya heart (Cottrell, Pentreath and Powell, 1968).

Results

The results of three bio-assays of radial nerve extracts on the clam heart preparation showed that appreciable quantities of ACh were present. Each value was in the range 20-30 µg ACh/gm wet tissue. This is less than the value of 60 µg/gm for Asterias. It would also be reasonable to expect similar binding properties of ACh in Ophiothrix radial nerve, as electron microscopy showed that there were large numbers of synaptic vesicles identical to those in Asterias.

Histochemical localization of cholinesterase in the radial nerves of Ophiothrix fragilis.

In this study a quantitative estimation of enzyme activity was not attempted. This was due to the difficulties in obtaining sufficient material for analysis. Instead histochemical techniques were used to determine whether or not the cholinesterase present was specific to ACh, and a visual comparison of the density and localization of staining in nerve sections was made at the histological level.

Methods

Cholinesterases were detected in Ophiothrix by the method of Gerebtzoff (1953) as described by Barka and Anderson (1963). Pieces of arm and disc containing the nerve tissue to be studied were dissected from live animals and frozen with dry-ice onto a microtome chuck. Sections of 5-15μ thickness were cut on a EEG cryostat at -20°C and picked up on cover slips. Sections were fixed by flooding the cover slips with 10% formalin in sea water (acetate buffer added to adjust to pH 5.5) for 5-10 min. After brief washing, sections were placed in the incubating medium.

The substrates employed were acetylthiocholine iodide and butyrylthiocholine iodide (Koch-Light). Controls were made by employing the following esterase inhibitors: eserine

sulphate, which inhibits both AChE and ChE; ethopropazine hydrochloride, which specifically inhibits ChE activity (Naik, 1963); and B.W. 284, which specifically blocks AChE. These inhibitors were used at 10^{-4}M concentrations. Incubation time was varied between 1 and 30 hr.

Results.

Results obtained by the described technique were similar to those obtained previously for Asterias.

Both acetylthiocholine (ACTh) and butyrylthiocholine (BuTh) were hydrolysed slowly. Optimal staining took place at 15 hr for acetylthiocholine but was longer for butyrylthiocholine. ACTh + 10^{-4}M BW284 gave no staining at 15 hr. Eserinized controls were also negative.

In some cases BuTh gave an anomolous black deposit. However the correct reaction colour is specifically brown, so this was considered artifactual. In the radial nerve reaction with ACTh was confined to the most oral layer of the ectoneural tissue (Fig. 43). No reaction in the nerve was obtained with BuTh. There was also slight deposition in the mid-ectoneural tract, and in the hyponeural tissue. Activity was high in the vertebral muscles. This activity was not abolished

Fig.43. Cross-section through an arm of Ophiothrix at the level of the radial nerve. The tissue has been incubated for 12 hr in acetylthiocholine substrate. Greatest AChE activity is present in the most oral layer of the cord, the edge of which is marked. In this area there are large numbers of ectoneural ganglion cells. A lateral nerve leaves the bottom left of the cord. There are appreciable deposits in this area. Other areas of activity are present in the hyponeural elements (h), and the adjacent vertebral muscles (vm). r, radial water vessel; es, epineural sinus; os, ossicle. X180.

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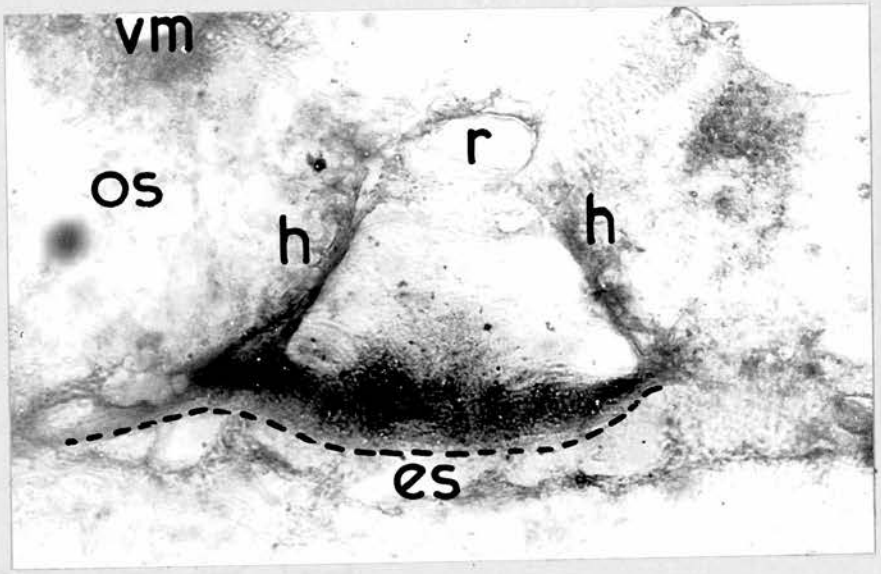

completely by BW284. Furthermore, unlike the radial nerve BuTh gave an appreciable deposit in the muscles at 12 hr. This was inhibited by parallel incubation with ethopropazine hydrochloride. It can be concluded that activity in the radial nerve is due to a specific esterase, AChE, whereas that in the vertebral muscles is due to both AChE and ChE.

Electron microscopical localization of cholinesterase in the radial nerve of Asterias rubens.

To gain more insight into the precise localization of cholinesterase in the starfish radial nerve, an electron microscope histochemical survey was made on Asterias nerve cord. Previous results have shown that starfish radial nerve contains only the specific cholinesterase, AChE (Pentreath and Cottrell, 1968). For this reason the nerve would seem very suitable for the fine structural study of the enzyme, since the possibility of interfering reaction products caused by reacting non-specific enzyme is eliminated.

Methods.

The method employed was that described by Karnovsky (1964). In this technique tissues are fixed in formalin, or in a mixture of formalin and glutaraldehyde. Some difficulty was met in adequately preserving tissue structure with these fixatives.

A variety of both formalin and formalin-glutaraldehyde fixatives, each of different concentrations, pH and molarity were employed. Some success was obtained by fixing very small

tissue pieces of Asterias radial nerve in 10% formalin in sea water without buffer (pH 5.5) containing 8 gm sucrose per 100 ml (ie. 0.25 M). This procedure gave good final localization, but was not generally used because sites of localization were only limited to the outer few microns of the tissue. At any depth exceeding 10μ or so sites of activity could be seen, but the tissue was too poorly preserved for any conclusions to be drawn about their structural localization. An alternative was to cut cryostat sections (5-10 μ) and formalin-fix these, but it was found that this procedure immediately resulted in loss of structure due to freezing. However, formalin-fixation was of some value in studying the outer epithelium of the cord.

For studying the deeper layers of the nerve and the tube feet it was found that best results were obtained by primary fixation in glutaraldehyde. Careful comparison with formalin/ sucrose fixed material (it is under these conditions that cholinesterase is optimally preserved for histological purposes - Frost et al., 1967) showed that there was some loss of enzyme activity with this method, but there was a marked improvement in fine structure.

Very small pieces (less than 1 mm^3) of nerve were fixed

for 1-4 hr in 5% glutaraldehyde (25% stock glutaraldehyde diluted to 12½% with distilled water and to 5% with sea water) containing phosphate buffer at pH 6.9. No sucrose was added to this mixture.

After fixation, whether in formalin or glutaraldehyde, tissue pieces were washed for 1-3 hr. in running sea water. They were then incubated in the medium of Karnovsky (1964), buffered to pH 6.0 with 0.1 M sodium hydrogen maleate buffer. Pieces were incubated for 15, 20, 40, 60, 90, 120 min. in each experiment, because enzyme activity varied from animal to animal. The incubations were made in the cold (0 to 4°C) to reduce both spontaneous hydrolysis and the rate of enzyme activity. The substrates used were acetylthiocholine iodide and butyrylthiocholine iodide (both approx. 0.2mM), while controls were similar incubating mediums containing the specific AChE inhibitor BW284 (Burroughs and Wellcome 284C51 dibromide) (Naik, 1963), or the general cholinesterase inhibitor eserine (both at 10^{-4}M).

Subsequently tissues were washed for 10 min in running sea water, and post fixed in 1% osmium tetroxide in sea water for 1 hr. Following this they were processed for

electron microscopy in the same manner as routine material (page 20).

Results.

The overall picture of enzyme distribution in the radial nerve was the same as that given by light microscope methods. Granular deposits were distributed first amongst the cells of the ventral nerve layer; second, sparsely throughout the fibres of the ectoneural tract; and third, in the cells and fibres of the hyponeural tissue. All reaction was prevented by 10^{-4}M BW284 and 10^{-4}M eserine.

A striking feature was the uniform distribution of activity in the microvilli which cover all the outer cells of the nerve (Fig. 44). The initial locus of deposition is on the single unit membrane which is the wall of a single microvilli. All sites of the membrane show equal activity, thus at a low magnification the outer layer of the nerve appears bound by an electron-dense layer. At high magnification individual microvilli in longitudinal section (length 3 μ) are presented as weakly defined membranes with a speckling of electron opaque granules along their length.

This activity is extended to the membranes of the ectoneural cells. Where two cells make contact there are extensive foldings of the cell walls which are linked by septate desmosomes. Such regions show considerable enzyme deposits. Multipolar and bipolar cells are situated in deeper areas of the epithelium. These cells project processes between neighbouring supporting cells (Pentreath, 1967). Each process gives rise to a cilium which in turn extends between microvilli to the exterior. A single cilium is covered with sites of activity. The basal body and associated root filaments are especially rich in specific enzyme.

All cell types of the ectoneural tissue have scattered deposits in their cytoplasm and on their outer membranes (Fig. 45-47). The precise localization cannot always be resolved due to the poor preservation, which is a necessary limitation of the technique. However, it can be seen that AChE is located chiefly in two places. First in the reticulum of membranes and irregular vesicles which surround the nucleus (Figs. 45, 46), and second on the neuron membranes (Fig. 47). It also appears that deposits in both these regions

Fig.44. Electron micrograph of a cross-section through the oral epithelium of the ectoneural nerve cord of Asterias. The tissue has been fixed in formalin and incubated for 20 min at 0°C in acetylthiocholine substrate. The microvilli border of the nerve is rich in enzyme activity. X14,000.

Fig.45. Section through the oral ectoneural tissue of Asterias (glutaraldehyde fixed, incubated acetylthiocholine for 40 min at 2°C). Deposits of AChE are located chiefly in areas close to epithelial cell nuclei (n). In these areas there is endoplasmic reticulum and large numbers of irregular vesicular bodies (unpublished observation with routine fixed material). ec, fibre region of the ectoneural tract. X10,000.

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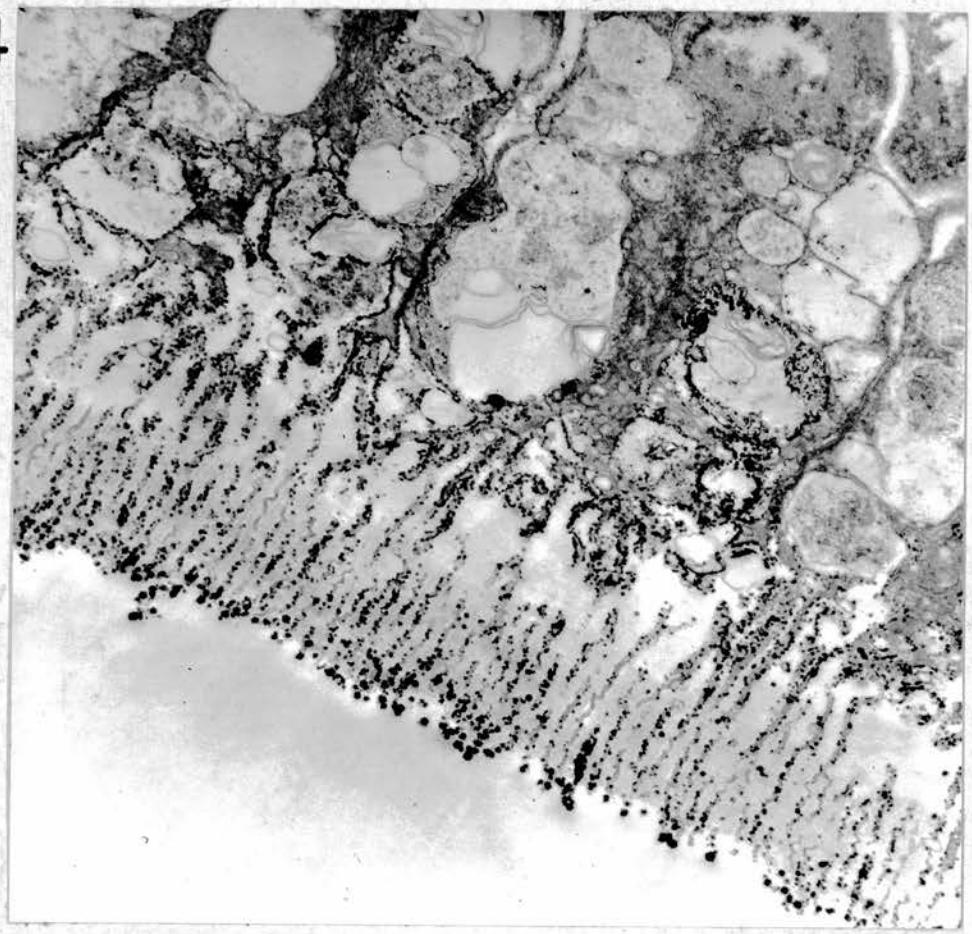

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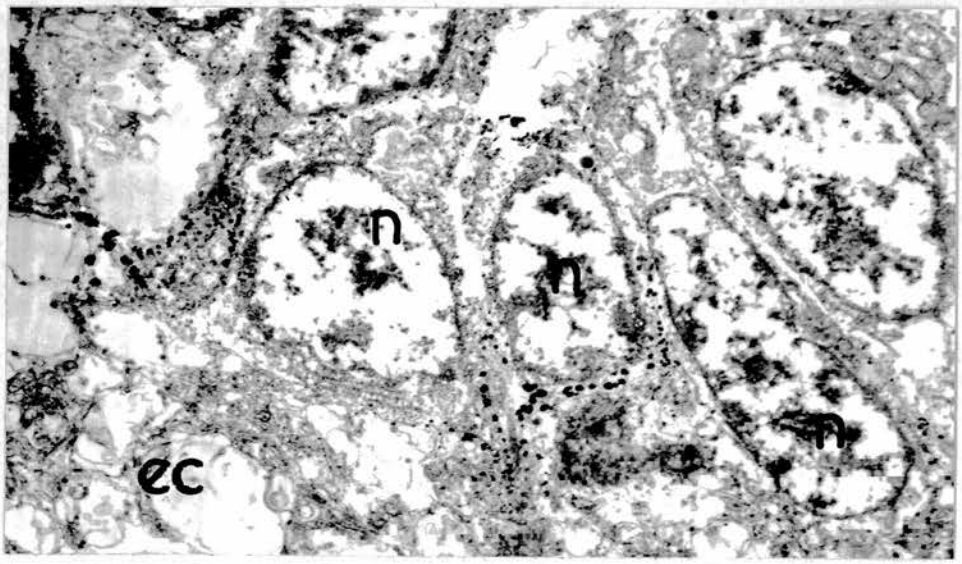

Fig.46. Micrograph of similar area to that shown in Fig.45. Where adjacent ectoneural ganglion cells lie close together, as indicated by their nuclei (n) deposits are heavy. Fixation loss due to the technique does not allow interpretation regarding processes leaving such cells, but it is clear that enzyme activity is high where the ectoneural cells border the tract (ect). m, mitochondria; sf, supporting fibres. X15,000.

Fig.47. There are scattered sites of AChE activity on the outer membranes of the ectoneural cells (arrows). Treatment as in Fig. 45. n, nucleus. X15,000.

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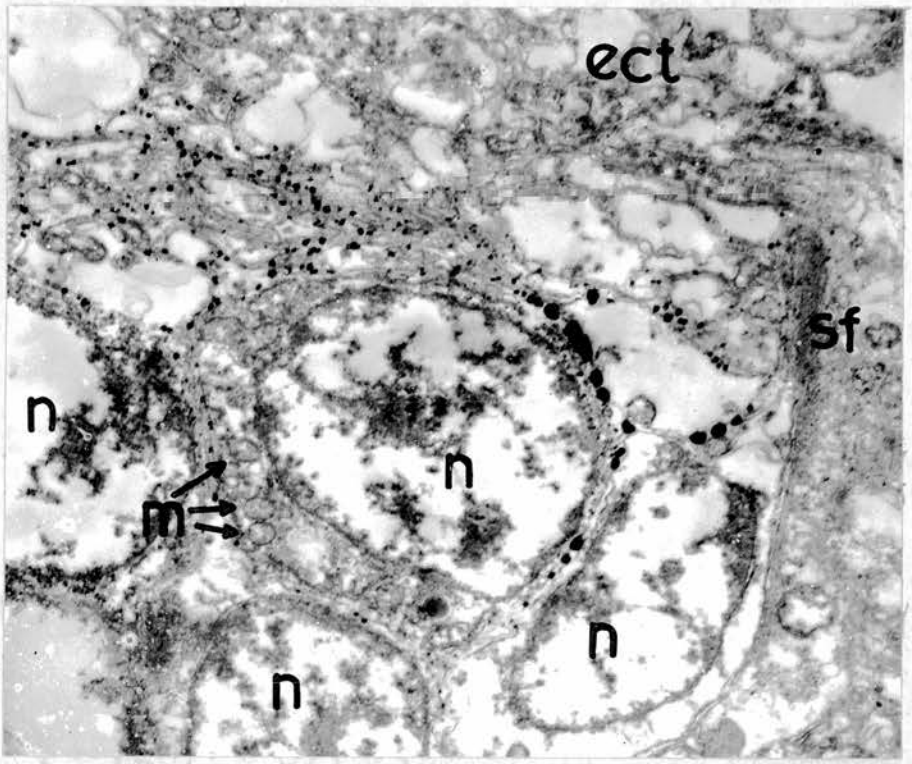

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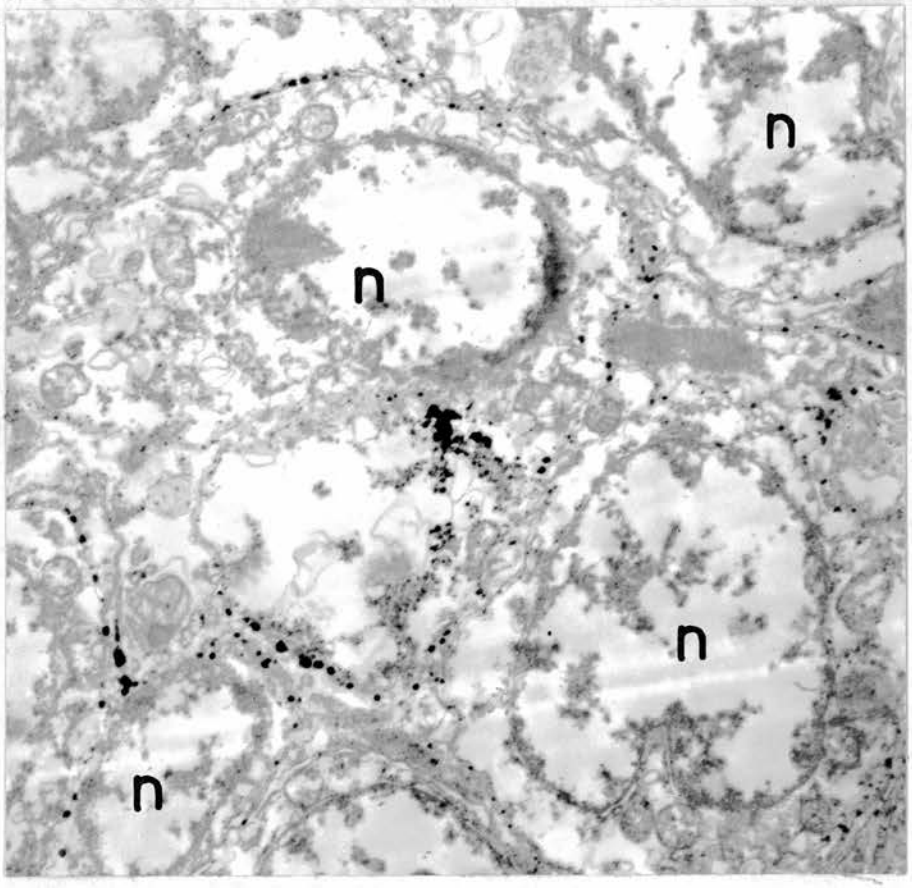


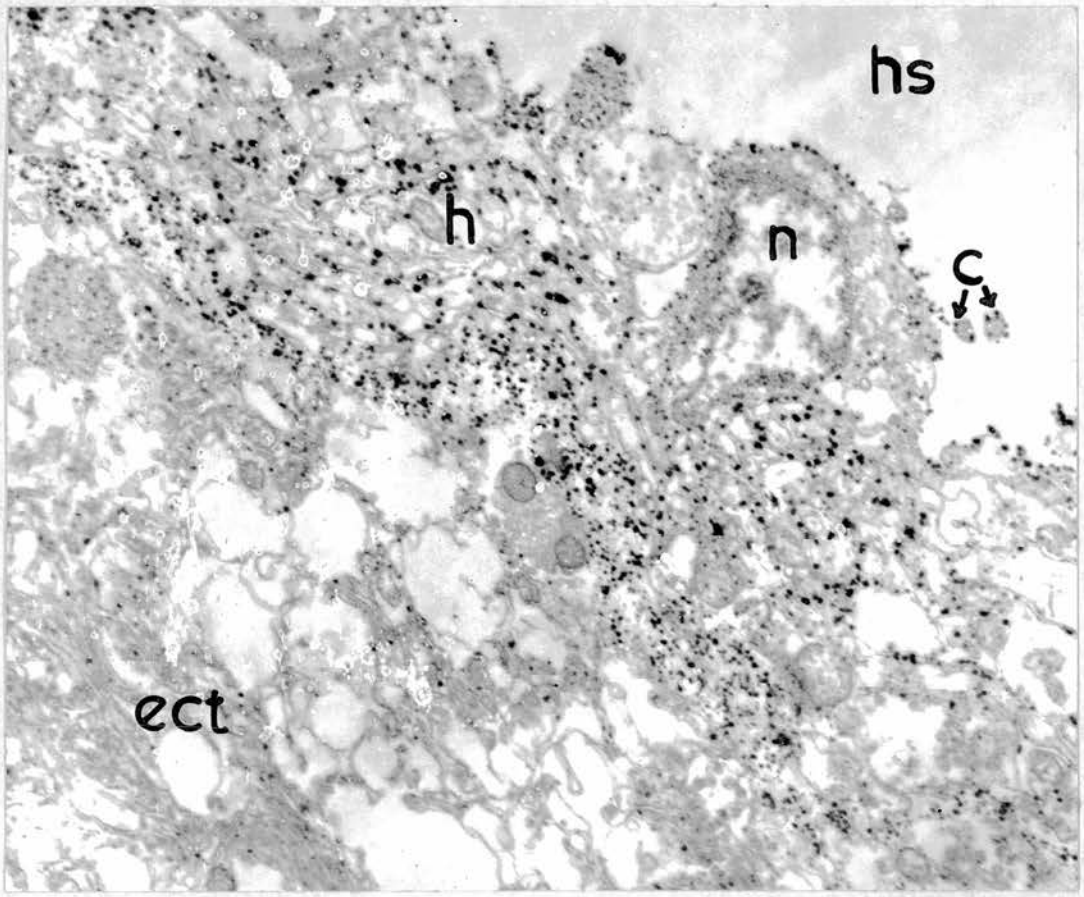
are most extensive where a cell process leaves a cell to enter the general sub-epithelial plexus (or, on the other hand, where a fibre from the tract enters the epithelium).

Within the ectoneural tract there is an even but relatively sparse distribution of deposits. It appears that infrequent intraneuronal deposits are loci of clear "synaptic-type" vesicles. Other enzyme sites are seen on axon membranes. However, it has been pointed out above that the ide tification of either a nerve-nerve or nerve-muscle synapse is not easy even in well fixed echinoderm material. Accepted criteria for the identification of a synapse have been modified (Cobb, 1966) to include points of axonal abutment with or without membrane thickenings, but which necessarily possess aggregates of vesicles close to the membrane. Many such structures are present in the ectoneural tract. Undoubtedly some sites of AChE activity in the radial nerve represent synaptic areas, for the number of enzyme deposits in a sample area of tissue agrees with the number of synaptic areas in a similar area of osmium-fixed material. It is also of note that no activity was observed in the proximity of axons or varicosities containing

Fig.48. Micrograph of a transverse section through an aboral region of the radial nerve of Asterias. Tissue fixed in 10% formalin and incubated in acetylthiocholine iodide for 30 min at 2°C. The ectoneural tract (ect) lies to the bottom left. There are only a few scattered sites of activity in this tissue. Hyponeural elements (h) stain more extensively. n, nucleus of motor cell; c, cilium cut in t.s. (arrows). hs, lumen of the hyponeural sinus. X15,000.

Fig.49. Cross-section of a podial wall of Asterias. Formalin fixed, acetylthiocholine incubated for 30 min at 2°C. mv, microvilli border; c, cilia; d, areas of desmosome joining between neighbouring epithelial cells. X10,000.

48



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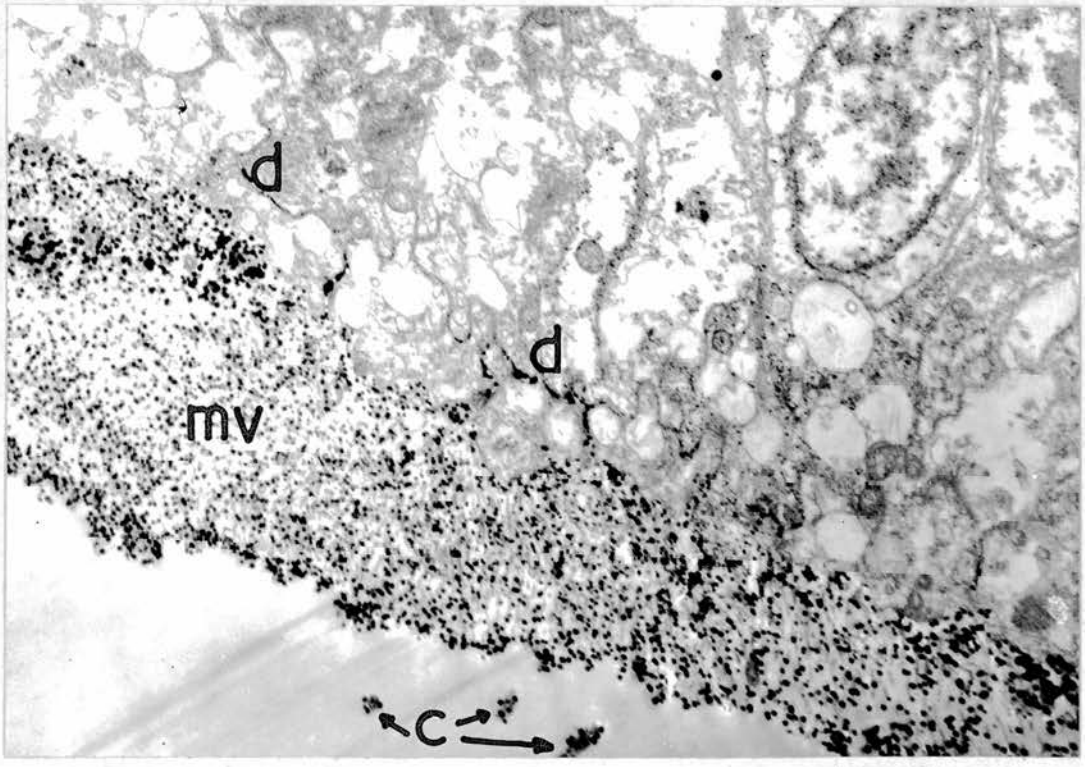


dense-cored vesicles.

Both cell bodies and fibres of the hyponeural system showed extensive deposits (Fig. 48). Cilia projecting into the lumen of the hyponeural sinus were also stained.

The epithelial cells of the tube-feet, which were shown by light microscope histochemistry to be rich in enzyme, had deposits which were distributed similarly to those described for the epithelium of the radial nerve. Fig. 49 shows heavy deposits on the microvilli border of a podium.

An electron-microscopical study of zinc-iodide osmium impregnation of neurons in Asterias radial nerve.

Starfish radial nerve contains appreciable quantities of ACh and catecholamines. Fluorescent histochemistry showed clearly that the latter were contained with neurons, while electron microscopy techniques indicated that the intra-neuronal location was in the form of small granules. It would also seem that ACh is particle-bound, probably to clear "synaptic-type" vesicles (Pentreath and Cottrell, 1968).

Several workers (Akert and Sandri, 1968) have studied the zinc-iodide osmium (ZIO) method at the electron microscope level. Their results have indicated that the stain is specific for certain subcellular neuronal inclusions. Furthermore these inclusions may be specific for the cholinergic neuron.

In order to obtain information regarding the relation of amine-containing neurons to those which may contain ACh in the radial nerve of Asterias, the ZIO technique was studied in this tissue. Since, however, there is at present some disagreement on the precise reactivity of the stain, a brief survey of the method is given here.

Originally it was thought that adrenergic structures were specifically responsive to ZIO impregnation on the basis of their reducing properties (Coujard, 1943, 1950; Champy, Coujard, and Cáujard-Champy, 1946), but this hypothesis has since been disproved by Hillarp (1959), who demonstrated that the basic histochemical reaction was unchanged after the administration of reserpine.

More recently Akert and Sandri (1968) used the ZIO technique at the electron microscope level and described the selective staining of synaptic vesicles in the subfornical organ and the myoneural junction. They state that the "structural affinity of the zinc iodide-osmium stain to nerve terminals is based on the selective staining of synaptic vesicles, whose content form an electron-opaque reaction product". Other synaptic structures, the junctional folds, membranes and mitochondria did not react. Dense-cored or large agranular vesicles also did not stain. In both tissues studied (cat subfornical organ, mouse diaphragm) "active sites were limited to the content of synaptic vesicles, rather than the coating of their surface". In conclusion, they advanced the hypothesis that the reactive sites depicted cholinergic elements.

However, Maillet (1962), with the light microscope, found a positive reaction in the Golgi region of different cells and tissues, and concluded that the stain was in fact nonspecific. This interpretation was supported to some extent by Pellegrino de Iraldi and Gueudet (1968) who found that in sympathetic neurons, lysosomes as well as vesicles took up the stain. But the same workers also found by electron microscopy that " practically all the clear synaptic vesicles" of rat pineal nerves "reacted intensely with the ZlO mixture". It was also known that dense-cored or granulated vesicles of rat pineal nerves stored appreciable quantities of catecholamine and serotonin (Pellegrino de Iraldi and Gueudet, 1968). This latter class of vesicles reacted positively with the histochemical technique of Wood (1965, 1966) for catecholamines and indoleamines. An added finding in their work was that reserpine, previously shown by several workers (see eg. Hökfelt, 1968) to have a long lasting blocking action on the storage mechanism of adrenergic granules, abolished both the ZlO reactive sites in clear synaptic vesicles, and the Wood positive sites in dense-cored vesicles. The apparent contradiction with the results of Hillarp (1959) on the action of

reserpine on the Z10 reactive material could not be explained, but it was pointed out that Hillarp made his study at the light microscope level. Pellegrino de Iraldi and Gueudet concluded the Z10 stain depicts a component present in the synaptic vesicles of pineal nerves which can be depleted by reserpine but which is neither a catecholaime nor 5-HT.

Despite these conflicting reports it would seem that the technique has great potential in the study of nervous systems. It is now generally assumed that a pre-synaptic ending has three main structural elements: mitochondria, neurofilaments, and vesicles. It is of interest that the Z10 stain depicts the latter, whereas silver impregnation methods select neurofibrils, and the "synaptic stain" of Rasmussen (1957) and Armstrong et al. (1956) is mainly associated with mitochondria. Vesicles are more specific synaptic organelles than are neurofilaments and mitochondria, thus the Z10 stain would appear to be the most specific stain for nerve terminals. Moreover, much of the recent published data on the technique can not be relied upon because of the great sampling errors encountered when dealing with vesicle

determinations at the electron microscope level.

Echinoderm axons contain few mitochondria or neurofilaments, but contain large numbers of vesicles. For this reason alone nerve tissues from members of the phylum would provide good material for a histological study of the stain.

However, it was mentioned earlier that one of the aims of this work has been to find a possible means of distinguishing the relation between amine and ACh-containing neurons in echinoderms. Electro-physiological studies (Cobb, 1964) have clearly shown that there are several separate conducting pathways in the circumoral ring of Echinus. Similarly Sandeman (1965) has identified what appears to be two classes of fibres in the radial cord of Strongylocentrotus. But previous studies by Pentreath (1967) had shown that, in the case of Asterias, after examination of many hundreds of micrographs it was impossible to categorize neurons of the central pathways into morphologically differing groups. The Z10 technique was applied to the radial nerves of Asterias in the hope that any differences between neuron types with respect to vesicle content would be made apparent.

Furthermore, if the stain has specificity for the cholinergic neuron it would be valuable in the study of echinoderm nervous systems.

Methods.

The Z10 mixture was prepared in the same way as that described by Akert and Sandri (1968): 15 gm of zinc dust, and 5 gm of iodine (crystalline) were added to 200 ml of distilled water. 5 ml of the filtered solution was added to 2 ml of 2% $O_s O_4$ shortly before use. Pieces of freshly dissected Asterias nerve were immersed in the mixture for periods of 1-20 hr. at room temp. Because some parts of the nerve reacted quickly with the stain, some impregnations were carried out at 0°C (Akert, personal communication). At varying time periods (normally between 2-10 hr.) pieces of tissue were removed from the Z10 mixture, briefly rinsed in distilled water, and dehydrated and embedded in the normal fashion.

Results.

The stain was applied with some success to asteroid radial nerve. Difficulties were at first encountered in regulating impregnation. Vesicles took up the stain within half

an hour of fixation, but subsequent to this, deposition increased steadily around the original locus, until at five hours the whole tract appeared totally blackened, with nerve terminals and vesicles completely shattered. The critical period for staining of vesicles varied throughout the tissue, consequently at one hour some nerves would be just beginning to stain, while others were completely masked. This rendered a comparative study of neurons impossible. On the suggestions of Akert (personal communication) both pH and temperature of fixation were lowered (pH 5.4, 4°C). The resulting picture was considerably improved.

It was found that deposition was confined almost exclusively to axons of the ectoneural tract. After one hour impregnation many intraneuronal clear vesicles in this nerve layer possessed partially or completely electron-opaque centres (Fig. 50). Dense-cored vesicles or other axoplasmic inclusions were never stained. If impregnation was extended to 8 hr deposits were increased around the initial locus (Fig. 51) until eventually (15 hr and upwards) an axon which previously contained clear vesicles would be almost black in cross-section (Fig. 52). Such areas of deposition did not extend for any distance along an axon, ie. a fibre containing vesicles

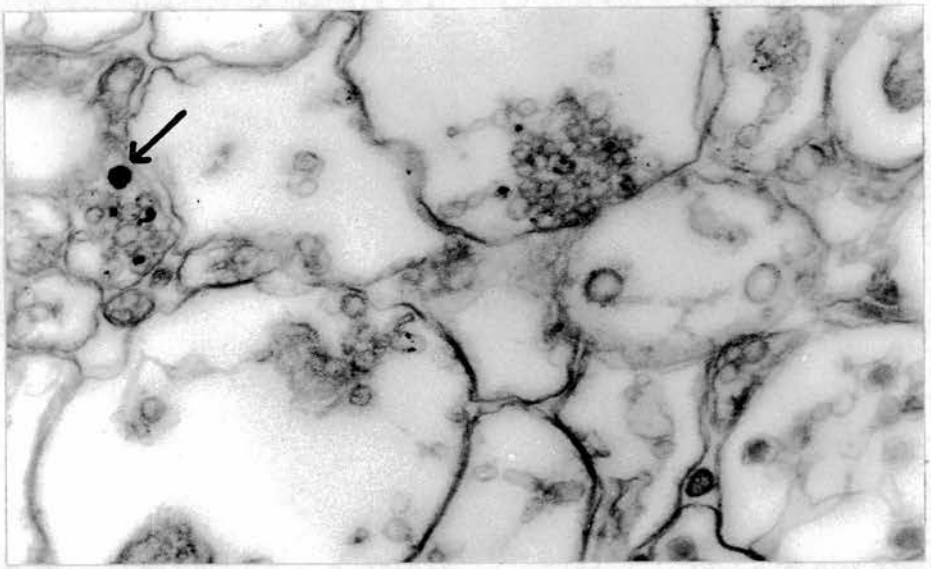
was not completely impregnated. Rather sites were limited to points within an axon where vesicles were aggregated. A longitudinal section through the ectoneural tract showed large numbers of fibres with such a periodic staining along their length (Fig. 53). These points of deposition may represent synaptic areas. Other axons in the tract did not react at all. It would naturally have been rewarding if it were found that unreactive fibres did not contain clear vesicles, but dense-cored granules. However, as has been described above, fibres of the ectoneural tract cannot be safely classified in terms of vesicular content. Certainly in any cross-section fibres are present which contain only clear or dense-cored vesicles, but there are also many which contain both. In the latter case it was found that clear vesicles stained while dense-cored vesicles adjacent to these did not (Fig. 54).

It was also observed that not all areas of the ectoneural tract contained the same number of reactive fibres. This finding was enhanced by subjecting the radial nerve to prolonged periods of incubation. In these circumstances the stain continues to be deposited around the initial vesicle loci, so that at 30 hr. the extent of staining can be visualized with the light microscope. Fig. 55 is a cross section

Fig.50. Cross-section through the ectoneural tract of Asterias. After 1 hr impregnation many synaptic vesicles possess partially or completely (arrow) electron opaque centres. X55,000.

Fig.51. At 8 hr staining with the Z10 stain aggregates in the ectoneural tissue are more extensively stained. X30,000.

50



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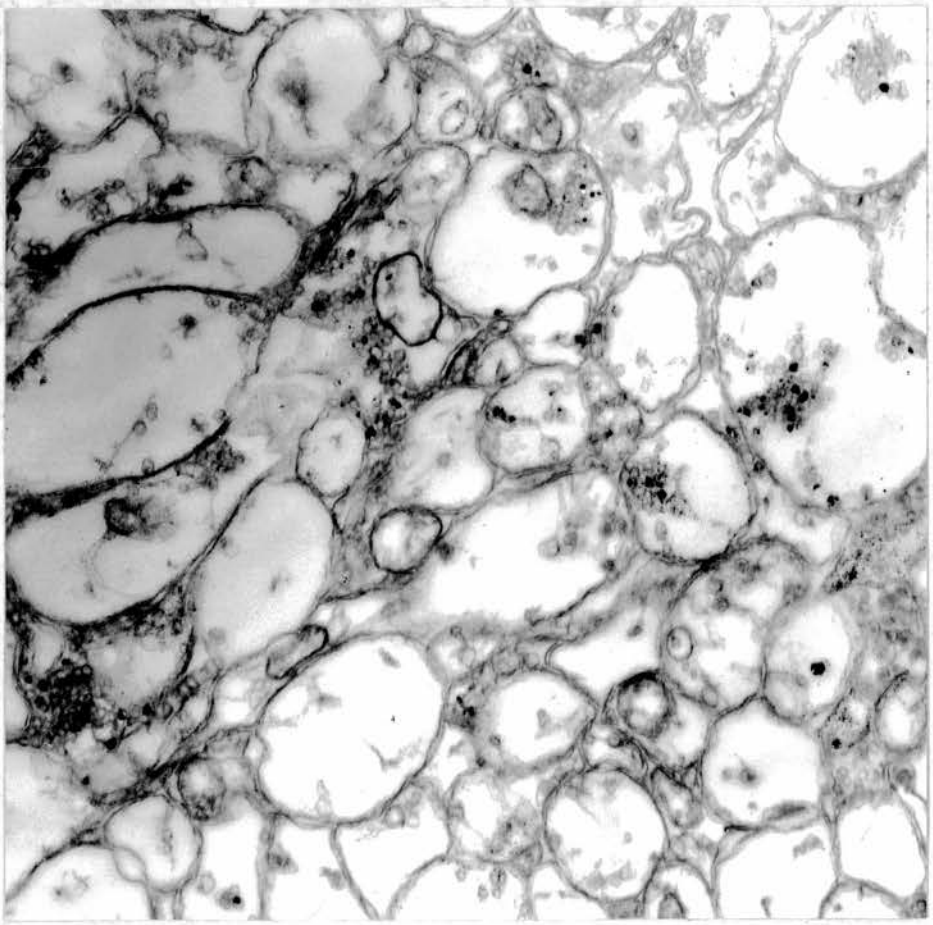
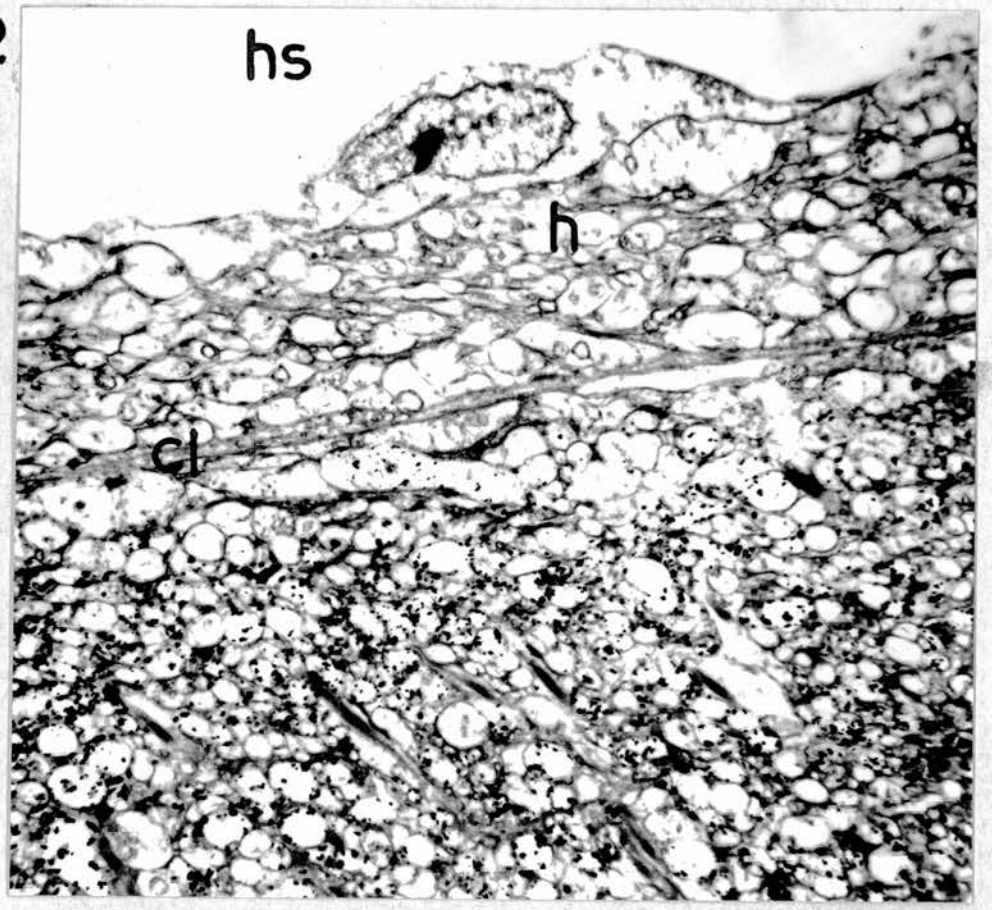


Fig.52. After further impregnation (15 hr) a large number of ectoneural axons are partially or completely blackened in cross-section. Hyponeural elements (h) do not take up the stain. cl, collagen layer dividing the ectoneural and hyponeural tissues; hs, hyponeural sinus. X6,000.

Fig.53. Longitudinal section through the ectoneural tract (15 hr impregnation). Axons do not stain uniformly along their length. The dark granules mark the sites of extended deposition around initial locii which are aggregates of clear, synaptic-type vesicles. X15,000.

52



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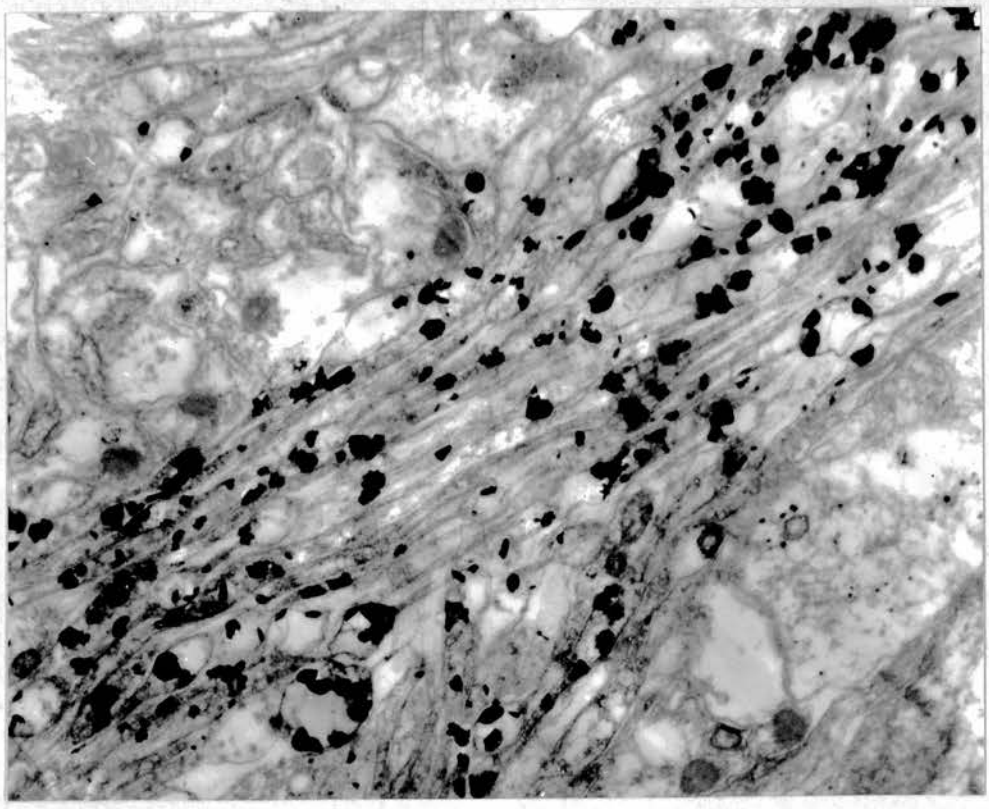
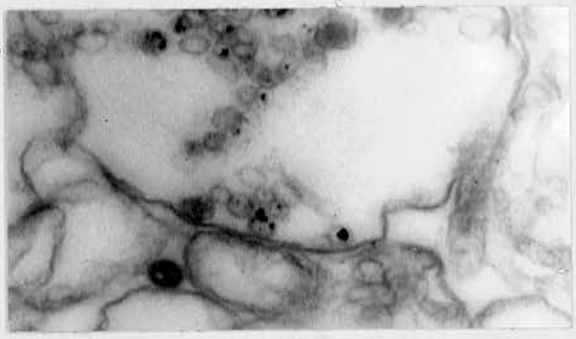


Fig.54. Dense-cored vesicles do not take up the stain, but neighbouring clear vesicles do (2 hr impregnation). Subsequently however deposits increase around the clear vesicles, and cover the dense vesicles. X60,000.

Fig. 55. Light micrograph of a cross-section of Asterias radial nerve after extended impregnation (30 hr). Neither hyponeural tissue not the general ectoneural epithelium has reacted with the stain. Within the ectoneural tract the most active areas lie orally and laterally (compare Fig.54). h, hyponeural tissue; ep, ectoneural epithelium. X100.

54



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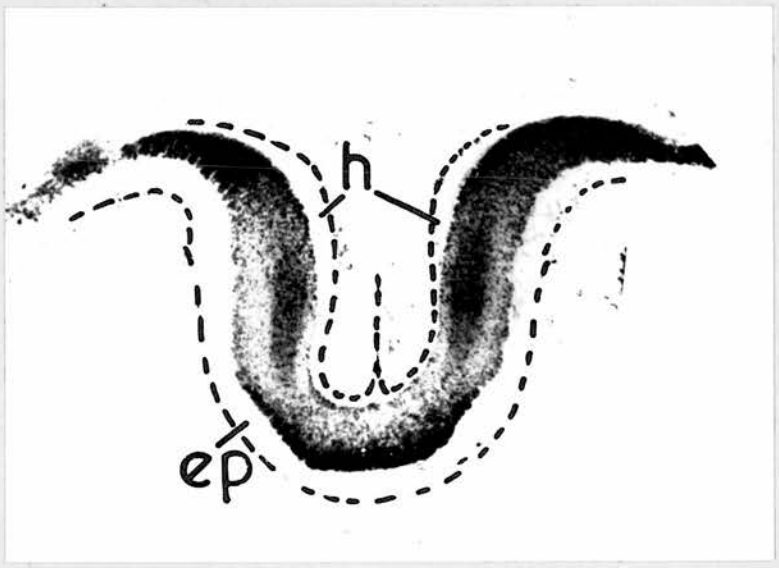


of the cord subsequent to such procedure. The most oral and lateral areas of the tract contain a much larger proportion of impregnated axons than deeper regions. It is of interest to compare this picture with that given by fluorescent microscopy (Figs 4,5). When the radial nerve of Asterias is seen in cross-section it is apparent that the most oral and lateral parts of the nerve are not so rich in amine as the mid-region. This result indicates that the Z10 stain reacts preferentially with nerves which do not contain catecholamines.

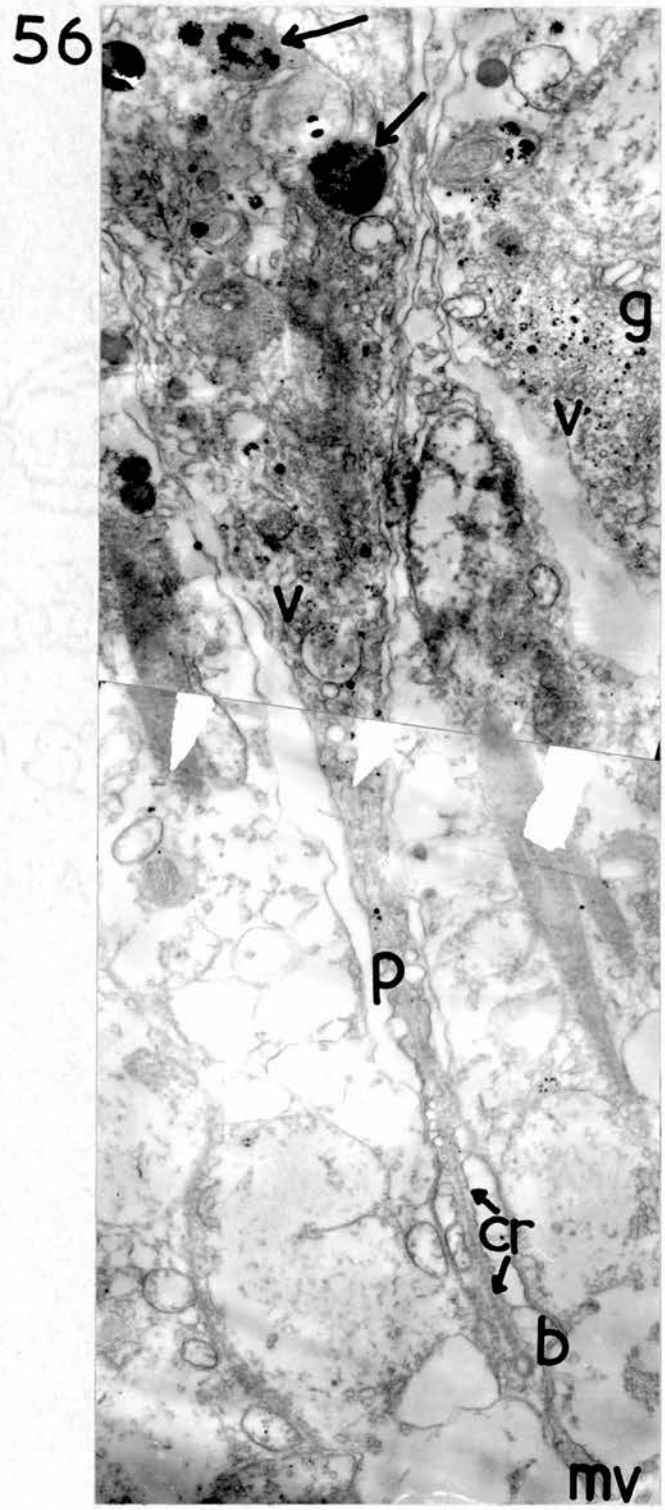
Pellegrino de Iraldi and Gueudet (1968) reported that reserpine abolished Z10 activity in clear synaptic vesicles. This was not however found to be the case with Asterias radial nerve. After animals had been immersed for 4 days in sea water containing 5 µg/ml soluble reserpine phosphate there was no marked reduction in staining. Similar extended impregnation to that described above following reserpinization produced the same result as illustrated in Fig. 55.

The above description has been restricted to tissue composed almost exclusively of neuron fibres. Other areas of the nerve, namely the epithelium of the ectoneural tissue and the hyponeural system, possess structures which also reacted with the Z10 stain. Such sites of reaction were rare. It was

mentioned above that in the ectoneural tissue impregnation was specific for the synaptic vesicle. Akert and Sandri (1968) made similar deductions in their studies of mouse diaphragm and cat subfornical gland. Moreover, sites of deposition in the radial nerve epithelium tissue signify that the stain is not in all tissues specific for the synaptic vesicle. Fig. 56 shows a cell containing lysosome-like bodies and a whole variety of vesicles which have reacted with the Z10 stain.

Akert (personal communication) has confirmed that organelles other than the synaptic vesicle are susceptible to the stain in some tissues. Furthermore, he suggested the likelihood that the stain is specific for the choline molecule. Such a postulate would explain why choline containing phospholipids are stained in the Golgi apparatus of liver cells and neurons. An attempt to test this was made by immersing starfish whose general body surface is known to take up free exogenous amino acids (Ferguson, 1967), from the surrounding medium, in solutions of choline chloride in sea water. A comparison was made with the Z10 stain between treated and untreated animals. Some cells of the nerve epithelium did in fact show a slight increase in staining after immersion in

Fig.56. Occasionally organelles within same epithelial cells react. Two such cells are shown here. The cell shown most completely is nervous and gives rise via a process (p) to a cilium (rootlet filaments, cr) which projects at right angles from its basal body (b), beyond the microvilli border (mv). Many vesicles (v) within the cytoplasm take up the stain at 2 hr. These are associated with Golgi structures (g). Other, larger lysosome-like bodies (arrows) also stain strongly. X15,750.


56
g
v
v
p
cr
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mv

varying concentrations (10^{-3}M to 0.5M) of choline chloride in sea water for one to five days. Impregnation was more extensive in vesicular structures within these cells. This lends some support to Akert's suggestion.

However increased staining after such treatment cannot be considered as direct evidence for a ZlO-choline affinity. First it is impossible to rule out individual variations in the degree of epithelial impregnation. Since increased deposition was but slight, the experiment would necessarily have to be repeated many times. Second, it is possible that the choline molecule may, after absorption, be immediately employed in metabolism and not stored. In this case increase in staining may be due to substances other than choline.

Cells of the hyponeural tissue were generally unresponsive to ZlO impregnation. Fibres of this tissue differ from that of the ectoneural tract in two respects. First they have a larger average diameter (mean 1.5μ; cf. 0.8μ in the ectoneural tissue), and second, they do not possess large numbers of clear vesicles. On grounds of the latter, it would be expected that they would not react significantly to ZlO.

At this point it may be concluded that, within the

ectoneural tract, the Z10 stain specifies two neuron types: those which contain clear vesicles and those which contain clear vesicles plus dense-cored vesicles. Previous results (Pentreath and Cottrell, 1968) are consistent with the view that ACh is sequestered within small clear vesicles in the starfish radial nerve. The distribution of Z10 reactive sites suggests the possibility that the stain may react with cholinergic neurons in the ectoneural tissue, but only at their synaptic sites. If vesicles are not present in an axon it will not be impregnated. The occasional organelles within epithelial cells which take up the stain may be part of the cholinergic system in the cell perikaryon, related to synaptic vesicles present in the cell's axon which extend into the tract. Experiments undertaken to test Akert's suggestion of specificity for the choline molecule produced results which cannot be safely interpreted.

Summary of studies on cholinergic systems.

The radial nerves of the brittle star, Ophiothrix fragilis, contain 20-30 μg ACh/gm fresh tissue. This value is approximately half that reported for starfish.

The distribution of AChE and non-specific cholinesterase in the brittle-star nervous system was determined histochemically. Highest levels of AChE were found in the most oral layer of the nerve cord. The vertebral muscles contain both AChE and ChE.

The distribution of AChE in the radial nerves of Asterias was determined at the electron microscope level. The overall distribution of enzyme was consistent with an earlier report made using light microscope techniques (Pentreath and Cottrell, 1968). With the increased resolution activity was seen in the following regions: (1) in the outer membranes of the microvilli and ciliated cells which cover the nerve and tube-feet; (2) in the cytoplasm and cell membranes of the neurons which underlie the nerve epithelium; (3) in some of the synaptic-type vesicles and axon membranes of the ectoneural tract: and (4) in parts of the cell bodies and fibres of the hyponeural tissue.

The ZIO staining technique was applied to the radial nerve of Asterias for two reasons. First, to obtain information about the relation of amine-containing neurons to those which may contain ACh and second, to attempt to resolve the conflicting reports regarding the nature of the reacting elements.

With regard to the former it was found that in the absence of cell bodies, ie. in the deep ectoneural tract, the stain was specific for the small, clear synaptic vesicle. Thus only those neurons which contained small, clear vesicles or a mixture of small, clear vesicles with dense-cored vesicles were stained. Axons containing only dense-cored granules were not stained. Because however organelles of some ecto-neural cell bodies also occasionally took up the stain, the technique was not specific for the clear synaptic vesicle in all Asterias nerve tissues. A comparison of the gross distribution of Z10-stained structures with that of amine-containing elements in the radial nerve would indicate that the stain does not show affinity to amine-containing elements.

Regarding the second reason it was found that reserpine treatment did not affect the susceptability of Asterias radial nerve tissue to the Z10 stain, whereas such treatment depleted catecholamines. It is stressed here that the Z10 technique appears to have a much closer affinity to possible ACh-containing neurons than to amine-containing neurons.

The effect of drugs on ciliary movement in Asterias rubens.

Many of the cilia covering the starfish body surface and coelomic spaces are organized into tracts which beat in a co-ordinated manner. The arrangement of such tracts and the directions of the currents which they set up have been described by Budington (1942). Two such tracts are situated as follows: First, on the surface of the radial nerves which lie in the mid-ambulacral groove, and second, on the aboral coelomic epithelium between the mesenteries supporting the pyloric diverticula. The former sets up a ciliary flow towards the mouth (ie. centripetal), and the latter sets up a current towards the arm tip. The nature of the ciliated cells of the radial nerve have been described by Pentreath (1967).

Pentreath and Cottrell (1968) suggested the possibility that the high levels of the AChE in the epithelial layer of the radial cord might be involved in a mechanism controlling ciliary movement. The work of Bülbring, Burn and Shelley (1953) is relevant to this. These workers proposed that ACh may act as a local hormone regulating ciliary beating in the gill plates of Mytilus.

In order to gain more information concerning the control of ciliary movement in Asterias, nerve and aboral arm preparations were exposed to varying concentrations of metabolic drugs.

Methods.

Pieces of test of Asterias rubens were extirpated and pinned in small dishes of sea water. The following substances were added to the sea water: acetylcholine iodide (10^{-8} - 10^{-3} gm/ml), eserine sulphate (10^{-8} - 10^{-2} gm/ml), atropine (10^{-6} - 10^{-2} gm/ml), adrenaline, noradrenaline and dopamine (10^{-6} - 10^{-3} gm/ml). Ciliary flow was followed by use of a carmine suspension in sea water, introduced to the area under observation from a small-bore pipette. Rate of movement was measured by timing the passage of individual particles across the units of a calibrated binocular eye-piece with a stop-watch. Drugs were administered to the sea water after ciliary flow had reached a constant rate.

Results.

It very soon became apparent that ciliary flow was generally unresponsive to the drugs added, even at high con-

centrations. The normal rate of particle passage of both tracts was 4-5 cm/minute. Of the compounds tested only ACh produced a possible reaction. At very high concentrations (10^{-3} - 10^{-2} gm/ml sea water) this substance caused a slight decrease in particle movement, but at such concentrations the significance is doubtful. None of the other drugs caused any noticeable modification in ciliary beat.

Mechanical responses of the isolated apical arm muscle of Solaster endeca to postulated neurohumours and related substances.

The great sensitivity of holothurian longitudinal muscles to ACh, and the opposing action to ACh of catecholamines is well-known. However this echinoderm preparation would seem to be exceptional for two reaons. First, the available reports regarding preparations from other classes indicate general insensitivity to drugs (see Welsh, 1966), and second there are very few muscles in the other groups which are of sufficient size to be isolated easily.

During the course of this work it was noticed that the aboral arm musculature of the common sunstar, Solaster endeca, was particularly well developed. Subsequently it was found possible to dissect the muscle from the arm, providing a nerve-muscle preparation some 5 cm in length and 3 mm in thickness.

Because of the unusual size and easy dissection of this asteroid tissue, it was found to be a suitable preparation for study in conventional organ baths.

Methods.

The apical muscle of Solaster endeca was dissected from the live animal and suspended vertically in a small organ bath. One end of the muscle was attached to the bottom of the bath, while the free end was joined by cotton thread to a kymograph lever, weighted to 1 gm. Well aerated sea water flowed through the bath. The flow was stopped prior to the addition of a drug, but was resumed as soon as the maximum effect of the drug was registered at the kymograph drum. The following drugs were added in final bath concentrations ranging from 10^{-8} - 10^{-3} gm/ml : dopamine, nor-adrenaline, 5-HT (creatinine sulphate), L-glutamic acid, ACh bromide, physostigmine sulphate.

Results.

As was found with the cilia tract preparations, most of these drugs were unaffective, even at high concentrations. Dopamine, NA, adrenaline and 5-HT caused no significant affect on the apical muscle at bath concentrations of $10^{-4}/10^{-3}$ gm/ml. ACh however produced a marked contracture at 10^{-6} gm/ml. Threshold contraction was obtained in several experiments with concentrations of 0.2 - 0.5 X 10^{-7} gm/ml. With a bath concentration of 0.5 X 10^{-5} gm/ml ACh the muscle was maximally

contracted. The effects of ACh contractions were removed by 5-10 min washing with sea water. Eserine treatment (10^{-5} gm/ml for 20 min) lowered the threshold to ACh to approx. .5 X 10^{-8} gm/ml, and also potentiated above-threshold contractions.

Glutamic acid was the only other substance among those tested which gave a reaction. At 10^{-6} gm/ml bath concentration a contraction was obtained which was equivalent to that with the same concentration of ACh. Higher concentrations gave increased contraction. Furthermore, it was found that 10^{-6} gm/ml glutamic acid lowered the threshold to the ACh response.

In addition to these experiments the effect of drugs was tested on the isolated digestive glands of Asterias rubens. ACh gave slight contractions (threshold) at 10^{-6} gm/ml bath concentration. Dopamine, in the same concentration, caused a slight relaxation. Other catechol and indoleamines were without effect.

Spectrophotofluorometric determination of 5-HT in the radial nerve of Asterias rubens.

An indole amine with the fluorescing characteristics (excitation peak near 300 mμ and peak of fluorescence in 3 N HCl at 540 mμ) was found by Welsh and Moorhead (1960) in extracts of whole or nearly whole Asterias forbesi, Strongylocentrotus drobachiensis, and Synapta inhaerens.

The amounts were small, but it was concluded that if 5-HT were restricted to nerve tissue low levels would be expected. To investigate the possibility that purely nervous tissue would yeild larger amounts of serotonin, Welsh and Cottrell (1964, unpublished) extracted and assayed by the fluorescence method and by use of the Mercenaria heart, radial nerve tissue of Asterias forbesi. As values obtained were low (in the order of a few tenths of a μg/gm wet nerve) it was decided that the radial nerves of Asterias forbesi did not contain significant quantities of the amine (see Welsh, 1966).

To ensure that 5-HT could be excluded from playing a significant role in the nervous system of Asterias rubens, which was the species principally studied for this thesis, quantities of radial nerve from this animal were assayed for 5-HT.

Methods.

1 gm quantities of radial nerve and gut caecae material of Asterias rubens were extracted and fluorometrically determined for 5-HT by the method of Quay (1963), which is based on the earlier work of Bogdanski et al. (1956). Activation and fluorescence peaks were read in an Aminco-Bowman spectrophotofluorimeter, and recorded by means of a Bryans model 22020 auto plotter.

Results

The results of several experiments indicated that only negligible quantities of the amine were present. In one estimation the radial nerve from 5 animals (total wet weight of tissue approx. .75 gm) gave a value of less than 1 µg. In a second estimation 10 µg of synthetic 5-HT was added to the final extracted sample from the radial nerves of five animals. All the 5-HT added to the nerve extract was recovered. A third experiment, which employed nerve material from a dozen starfish (approx. 1.5 gm weight wet nerve), gave values of a few tenths of a microgram per gram of wet nerve.

It would appear, therefore, that the radial nerves of Asterias rubens, like those of other starfish (Welsh, 1966) contain insignificant quantities of 5-HT.

The fine structure of the nervous system and neurosecretory cells of Ophiothrix fragilis.

Although neurosecretory cells have been demonstrated to occur in a wide range of invertebrate phyla, the echinoderms have been investigated scarcely at all in this respect (Bullock and Horridge, 1965; Welsh, 1966). The published works deal chiefly with the gamete-shedding substances of starfish (Chaet, 1967), and with products associated with osmoregulation and pigment physiology in the radial nerves of some asteroids (Unger, 1962: see introduction).

However one worker has demonstrated neurocrine activity in the Ophiuroidea. Fontaine (1962) using staining methods which are selective, but not specific, for neurosecretory cells (ie. Gabe's paraldehyde-fuschin) showed that all of the motor ganglia of the disc and arm regions of Ophiopholis aculeata contained aggregates of neurosecretory cells. On the grounds of shape, size and staining properties the same person concluded that within each ganglia these neurosecretory cells fell into three morphologically distinct groups.

During the course of fine-structure studies on the nervous tissue of Ophiothrix fragilis, which were made to help interpretation of fluorescent studies, it was found that many

hyponeural cells contained large numbers of electron-dense granules. The diameter of these granules ranged between 500 Å and 1,800 Å. None of the cells in the hyponeural tissue of Ophiothrix fluoresced specifically after paraformaldehyde treatment, neither did they react to the glutaraldehyde-dichromate technique of Wood (1966). Thus it seemed that the granule-containing cells did not contain an amine. Moreover the granules present in these cells had the same appearance as those found in other known or proposed neurosecretory systems in other situations, eg. in neurohypophyseal nerve endings (Gerschenfeld, Tramezzani and De Robertis, 1960).

Another interesting finding in the radial nerve motor ganglia of Ophiothrix, though not related to neurosecretion, was that some neurons were very large. As mentioned earlier, most echinoderm neurons are very small, with axon diameters rarely exceeding 3 μ. But certain fibres in the hyponeural tissue of Ophiothrix were seen to have diameters of 10 μ and upwards. With regard to this it will be remembered that Smith (1965) proposed that "giant" fibres may effect the arm movements of brittle stars.

In the following section these two important features of the nervous system of Ophiothrix are described more fully.

Methods.

Whole arms of Ophiothrix fragilis were processed for electron microscopy as described previously (page 20).

Results.

The granule-containing cells are situated chiefly in areas just lateral to the hyponeural tissue, where the latter is expanded into ganglia which supply the vertebral muscles (Fig. 22). The cells sometimes contain lipid deposits, and often lie close to the hyponeural sinus (Figs 57, 64). Unlike the smaller dense-cored vesicles which are found in axons of the ectoneural tract of Asterias and Ophiothrix (see Fig.33), the whole of the content of the proposed neurosecretory granules is electron dense.

As far as a comparison can be made between the light micrographs published by Fontaine (1962), and the electron microscope observations reported here, it would seem that the cells in Ophiothrix hyponeural tissue which contain dense

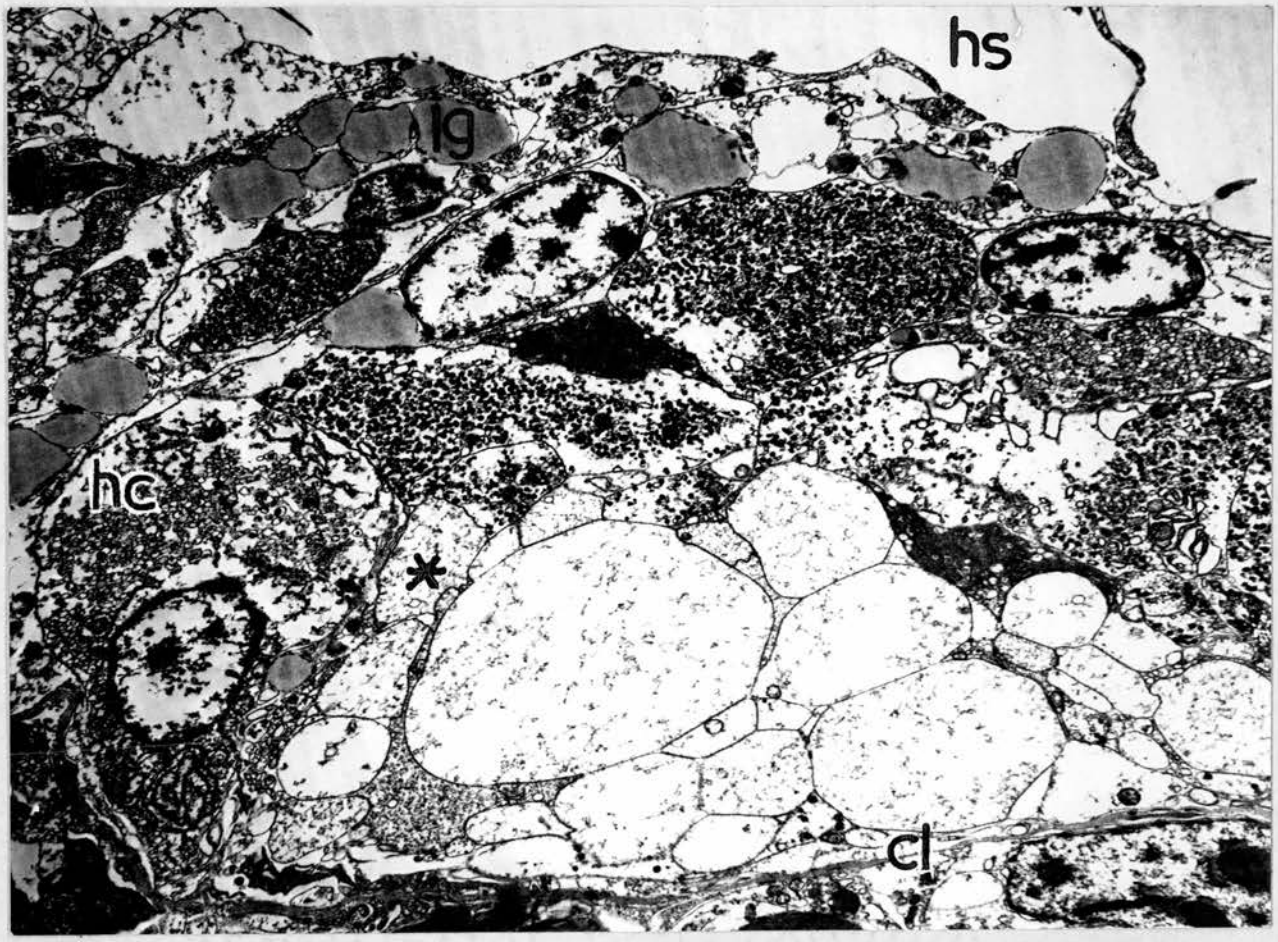
granules, correspond in position to those in Ophiopholis which are reactive to neurosecretory stains. However in this study no difference in the fine structure of individual granule-containing cells were found, which could be correlated with the three distinct types of cell found by Fontaine (1962).

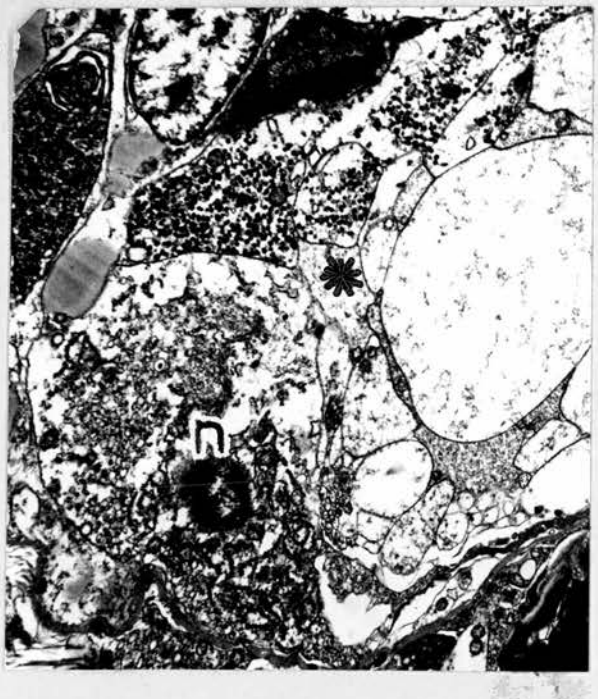
A distinguishing feature of the granule-filled cells are large whorls of reticulum which lie close to their nuclei (Figs 61, 62). Sometimes the membranes of the structures are loosely connected (Fig 61), but often they are tightly packed (Fig. 62). Granules are present between the membranes of the circular reticulum which are identical to the granules in the surrounding cytoplasm. In the same tissue section there are both loosely woven and tightly packed whorls, so it would seem unlikely that the former type are an artifact of bad fixation. Furthermore, a comparison between Fig. 61 and Fig. 62 shows that when the whorl is expanded, the surrounding cytoplasm is filled with granules to a greater extent than when it is contracted. Palay and Palade (1955) have described very similar structures (termed "onion-like corpuscles", "onion-whorls") in the mammalian central nervous system. The same workers associates these structures with active protein manufacture. It is tempting to say that in the brittle star,

Fig. 57. Cross-section through a hyponeural ganglion of Ophiothrix. A motor cell body (hc) lies to the left of the picture. It's cytoplasm contains a variety of vesicular bodies. The process marked with the asterisk belongs to this cell (see Figs. 58,59). A thin layer of collagen (cl) separates the large hyponeural axons from the ectoneural tissue. Aboral and lateral to the nerve processes lie cells whose cytoplasm is rich in electron-dense granules. Lipid droplets (lg) are commonly associated with these cells. hs, hyponeural sinus. X7,500.

Fig. 58. Electron micrograph of a section some 3 μ distal to that shown in Fig. 57. The tip of the nucleus (n) is included in the section. The asterisk indicates the same process as above. X7,500.

Fig. 59. A section again approximately 3 μ distal to Fig. 58. The nucleus is not present, while the asterisk here marks the axon hillock of the motor cell body. X7,500.


57
hs
lg
hc
cl


58
n

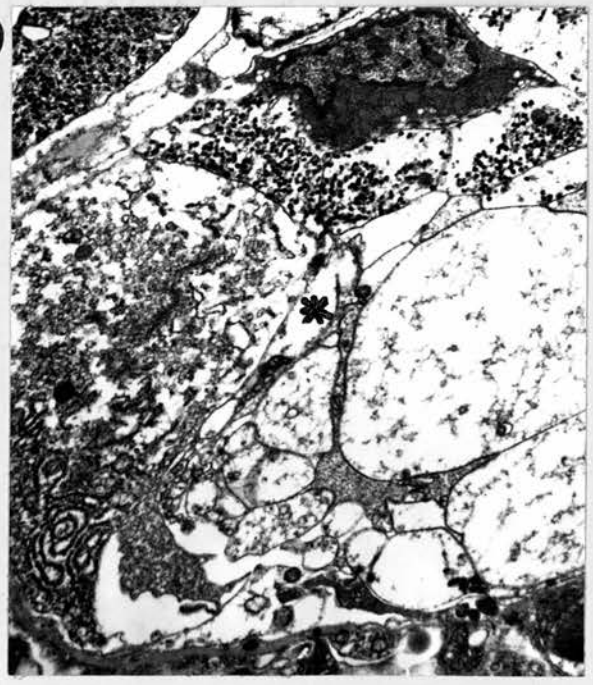
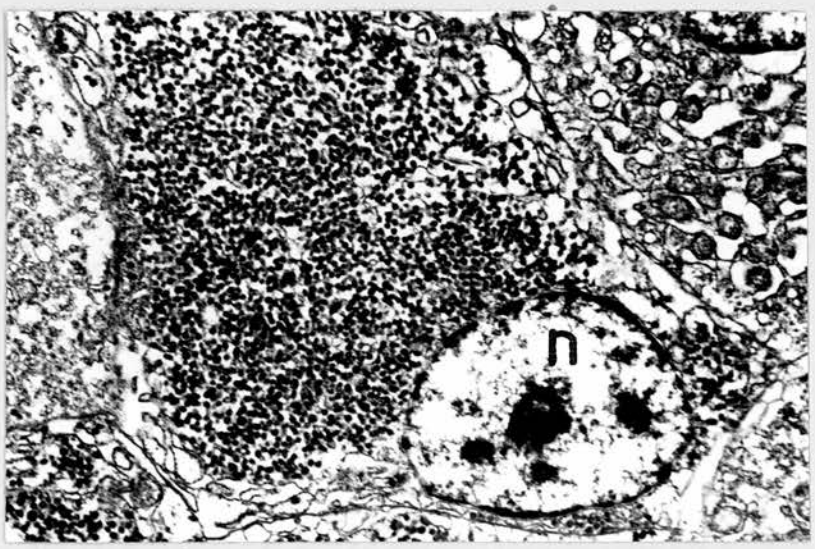

59

Fig. 60. A cell in the lateral hyponeural tissue of the radial cord which is packed with elementary neurosecretory granules. n, nucleus. X10,000.

Fig. 61. Electron micrograph of a whorl-like structure in the cytoplasm of a neurosecretory cell. This structure, which is commonly found amongst the elementary granules consists of loosely packed, concentric membrane foldings. X15,000.

Fig. 62. In other instances the cisternae are tightly packed. Granules are present in the middle and in the outer rings of the body (arrows). l, lipid substance. X14,000.

60



61

62

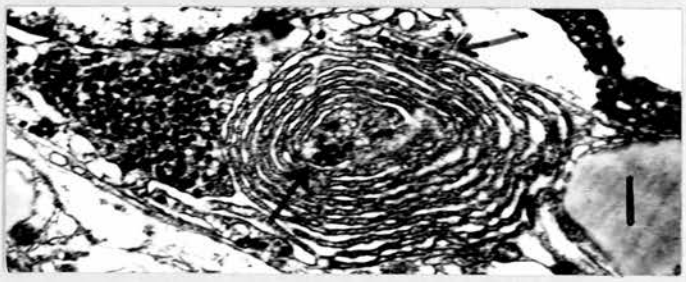
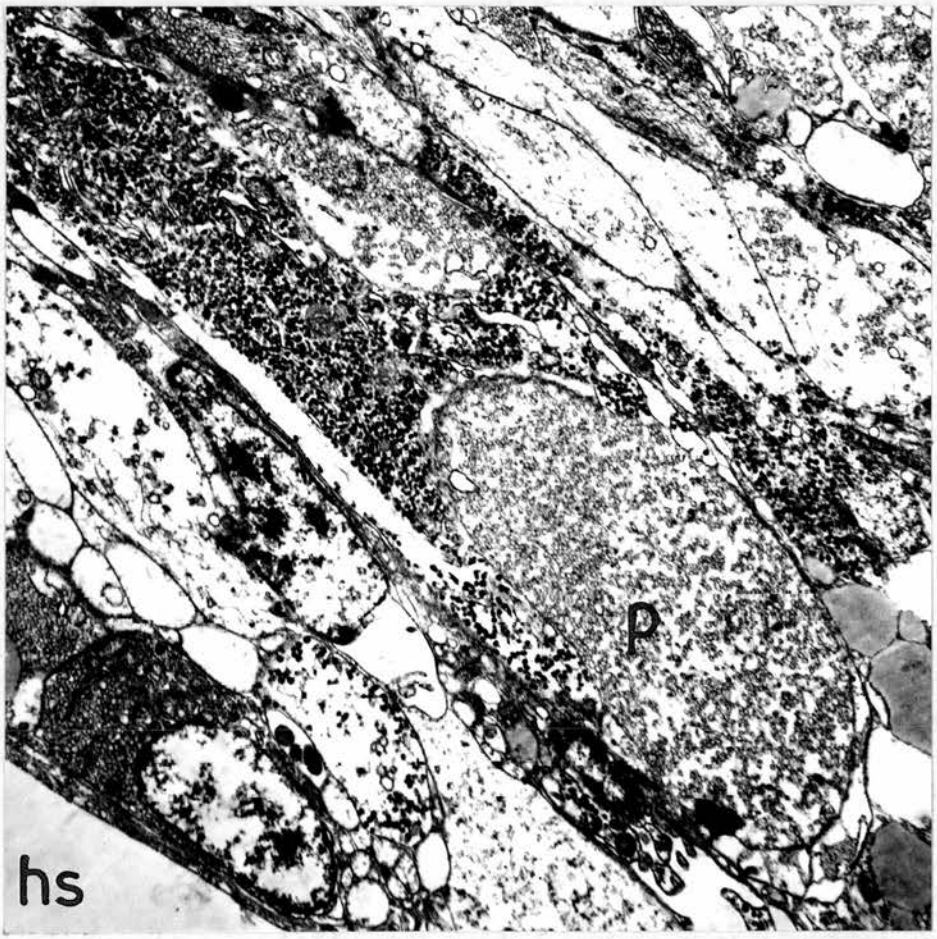


Fig. 63. Micrograph of an oblique section of Ophiothrix. hyponeural tissue showing a large process (p) which is full of clear vesicles. The diameters of these vesicles range from 500-1,300 Å. hs, hyponeural sinus. X7,500.

Fig. 64. Granule-filled processes from the hyponeural tissue extend to the hyponeural sinus (hs). The fixation is not of sufficient quality to allow conclusions regarding the precise arrangement of the membranes, but it is clear that many granules lie very close to the hyponeural sinus. X9,100.

63



64

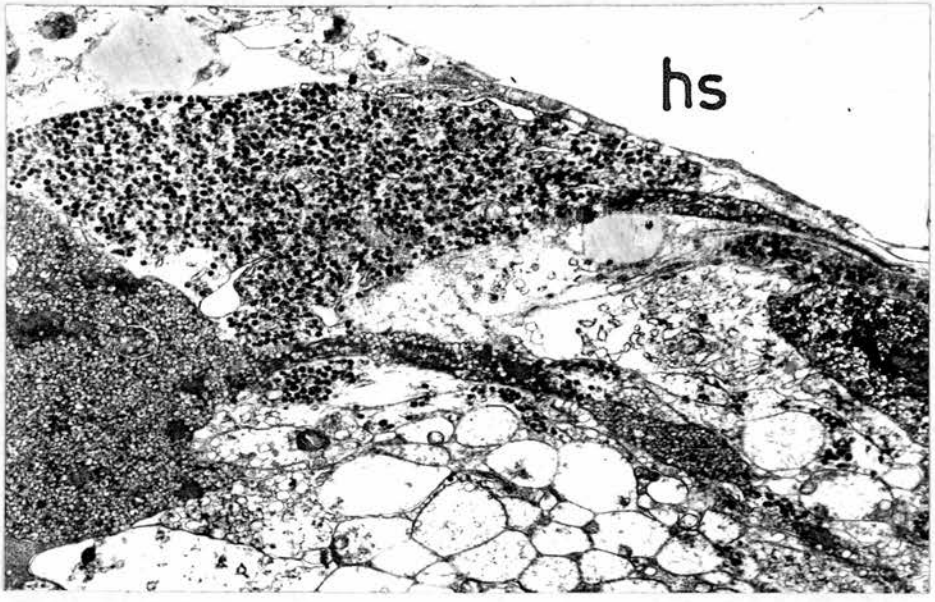


Fig. 65. Cells containing elementary neurosecretory granules are also present in regions of the ventral plate. The cell shown here is separated from the epineural sinus (es) by collagen strands (cl). The cell (ec) is very closely associated with, and is perhaps responsible for laying down the collagen. A small group of nerve fibres (nt), cut in cross-section, lies beneath the neurosecretory cell. n, nucleus of neurosecretory cell. X10,000.

65

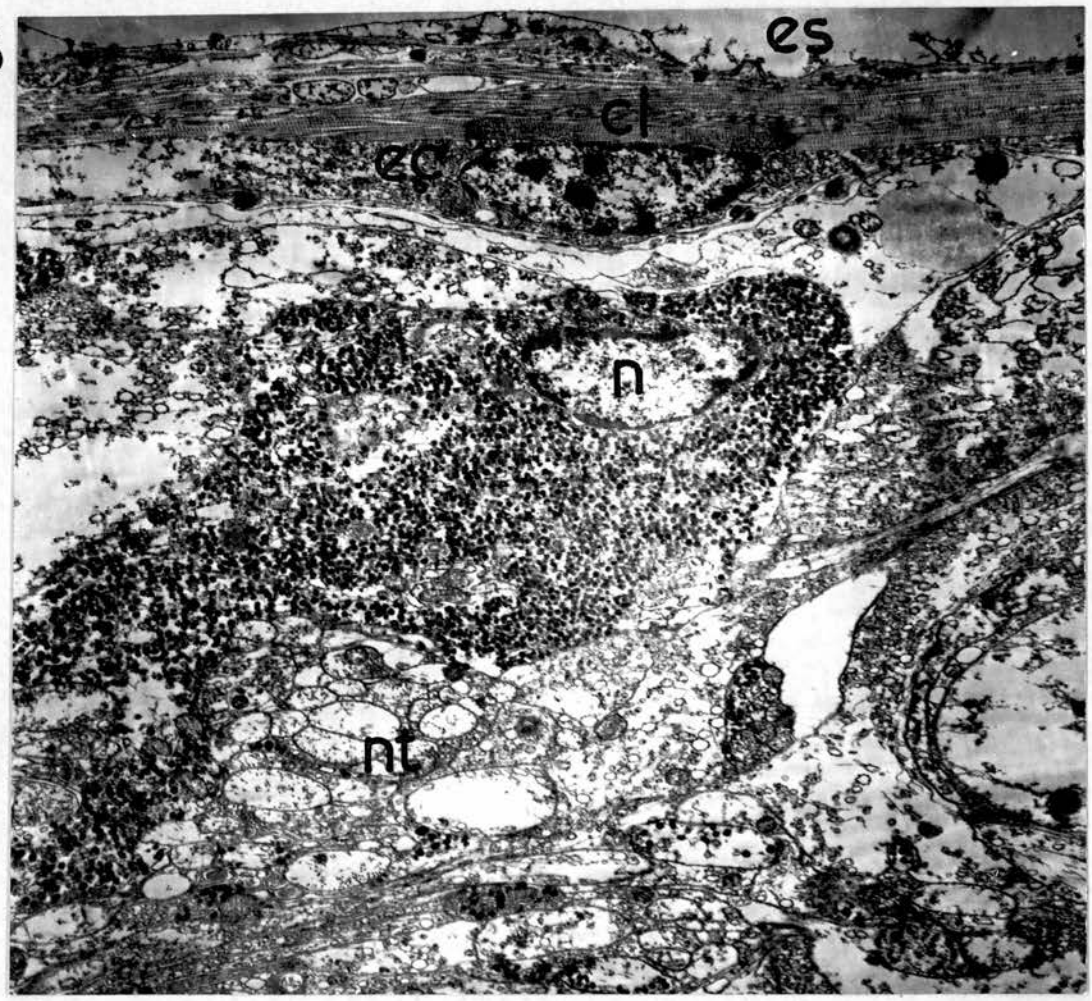
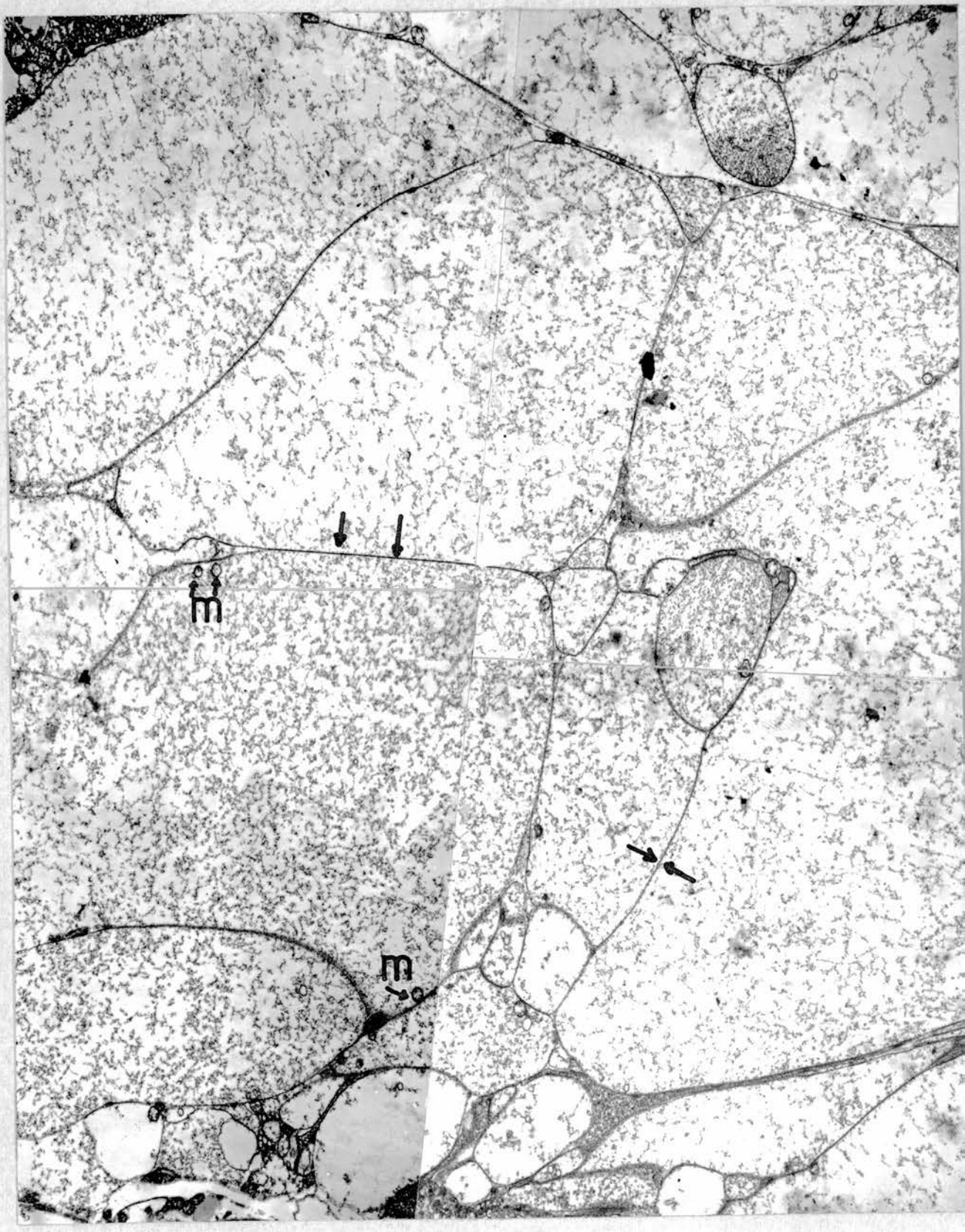


Fig. 66. Transverse section through the middle of a hyponeural nerve process (see Fig. 22). The axons contain a few mitochondria (m) and diffuse filaments, but do not contain vesicles. Adjacent axon membranes are sometimes tightly opposed (arrows). X8,300.

6



such structures when tightly packed are actively manufacturing granules, but there is no proof because the fate of the granules is unknown.

Intermingled with the granule-filled cells are processes which contain large numbers of smaller, clear vesicles with diameters ranging from 500-1,300 Å (Fig. 63). The function of these nerves is not known, but they do not appear to be final motor neurons, since they do not extend beyond the hyponeural ganglia. It is possible that these vesicles are analogous to the dense granules, but are at a stage in the secretory cycle where the dense core is not present.

In areas of the ventral plate bordering the epineural sinus there are more cells which contain electron dense granules (Figs. 22, 65). Such cells are morphologically identical to those of the hyponeural tissue. Normally they are separated from the sinus by collagen fibres, but in some instances granule-filled processes penetrate between the collagen layers.

The motor neurons of the hyponeural tissue are of the largest recorded in echinoderms. Fibres have a cross-sectional diameter of up to 10 μ (Figs. 57, 66). The tissue is aggregated

in ganglionic regions either side of the radial haemal canal. At these points in the order of forty cell bodies and/or their processes may be seen in cross-section.

Figs. 57-59 are sections taken at 3 μ intervals to show the origin of an axon process from a hyponeural cell body. The latter is typically 15-30 μ in outside diameter, with a nucleus 5-15 μ in diameter. The cytoplasm contains a reduced reticulum and many irregular membrane-bounded inclusions.

The hyponeural motor neurons innervate the ventral vertebral muscles directly, and the dorsal by a pair of vertically ascending nerve trunks (Fig. 22). Each of these is composed of approximately 20 processes from the hyponeural tissue. Fig. 66 is part of a horizontal section through the arm just aboral to the hyponeural tissue. It shows the mid-region of a dorsal muscle nerve in cross-section. The fibres have diameters of up to 15 μ. In many places the membranes of adjacent neurons are tightly opposed. These regions are reminiscent of the "tight" or electrical transmitting junctions described in other phyla (see Dewey and Barr, 1962), but even at high power the exact relations between membranes could not be

resolved, because fixation was not sufficiently good. The necessary steps to elucidate this point, ie. careful dissection of the nerve prior to fixation to eliminate de-calcification, and more extensive microscopy to ensure that membranes were not sectioned obliquely, were not undertaken. The axoplasm of the hyponeural nerve processes contains a few mitochondria and many diffuse filaments, but does not contain any vesicles.

On the basis of the observations made above, in particular the large number of granules contained with the outer cells of the hyponeural tissue, it appears that neurosecretion is extensively associated with motor neurons in the radial nerve of Ophiothrix. The target sites and functions of the neurosecretory products is unknown, but since the cells are invariably close to adjacent coelomic spaces, it is likely that transport takes place within these heavily ciliated sinuses.

Discussion

There is growing evidence that there are two distinct populations of neurons in the central nervous system of echinoderms. One of these contains acetylcholine, the other dopamine and/or noradrenaline. Other substances which have been implicated in neuroeffector mechanisms in other animal groups are either absent or present in very small quantities (Welsh, 1966). The results of the present work are consistent with this concept and, at the same time, argue for a lack of adrenaline and 5-HT.

The distribution of catecholamines in Asterias, Ophiothrix, and Antedon is generally similar to that reported by Cobb, (1969) for other echinoderm species, the starfish Patiriella calcar and the sea urchin Heliocidaris erythrogramma. In each animal, amine specific fluorescence appears to be located exclusively in the ectoneural tissue (a possible exception to this is the specific fluorescence detected in the aboral nerves of Antedon. Since however there is no data available about the embryological origin of this nerve, and its relationship to other possibly homologous nerve tissues in the other classes is doubtful (Smith, 1937), this finding is not

considered significant). This tissue layer is most extensive in starfish. Where it is thin (podia or gut caecae) fluorescence was confined to a few deeper fibres of the plexus, but wherever thickenings were observed (ampullae bulbs, radial and circumoral ring nerve) fluorescence was more extensive, but still restricted to the deeper parts of the nerves.

The ectoneural tissue of brittle stars is restricted to the radial and lateral nerves, and the circumoral ring. In Ophiothrix specific fluorescence was not seen anywhere within the arm sheath. Furthermore no fluorescent fibres were observed in the mid or tip regions of podia. This may be because the ectoneural layer is so thin and sparsely distributed that amines are not present in sufficient levels to be detected. Appreciable fluorescence however was present in the neck regions of podia. In Antedon the thin ectoneural layers underlying the oral grooves again fluoresced strongly.

Cobb (1969) has reported the presence of cell bodies showing specific amine fluorescence in the radial nerve of Patiriella calcar and he concluded that the amines are present in internuncial neurons of asteroids. In the present work no specific fluorescence was observed in sensory neuron cell bodies,

or in the hyponeural tissue, which is thought to be predominantly motor in function, of any species studied. If amines were associated with sensory ectoneural elements one would have expected to have seen at least some amine-fluorescence in cells of the outer nerve epithelium of Asterias, because of the known localization of sensory neurons in the epithelial layer of this species (Smith, 1937). Furthermore no amine fluorescence was detected in the discrete sensory eyespot. Thus extra evidence is provided for Cobb's suggestion of an association of the amines with internuncial neurons. This certainly seems most likely. However, the possibility cannot be completely ruled out that sensory neurons within the radial cords of starfish may also, or even perhaps alternatively, contain the amines. It could be argued that the fluorescent axons contain higher levels of amines than the cell bodies, which may in fact contain too little to be detected. There is the added difficulty in Asterias that although some structures were observed in the ectoneural layer which might conceivably have been cell bodies of interneurons, they were few in number in relation to the large number of fluorescent axons. Thus, either the cell bodies which give rise to the fluorescent axons

are indeed few and give off large numbers of processes, or they are relatively inconspicuous because of their small size and/or relative lack of amine. Two pieces of evidence point to the second alternative. First, electron microscopy showed that the few cell bodies present in the mid-ectoneural tract did in fact have a very restricted cytoplasm (Fig. 10), often there being only ½ μ or so between the cell and nuclear membrane. This value is considerably less than the diameters of many amine-containing varicosities (2-3 μ). Second, on several occasions small autofluorescing cell bodies were seen in the nerve which appeared to give rise to specifically fluorescing axons (Fig. 9). Finally there is one indirect piece of evidence in that the stomach contained abundant fluorescent cell bodies of presumed interneurons which were shown by electron microscopy to have extensive cytoplasms containing many granular inclusions (Fig. 14). Neither brittle nor feather stars possess discrete sensory areas such as the starfish eyespot. Consequently it was impossible to test whether or not catecholamines play a sensory role in these animals. But this would not seem likely in Ophiothrix, for the radial cord, which was the only nervous structure observed to fluoresce appreciably, is completely enclosed by ossicle material, and probably does not contain

sensory elements. Thus, on balance, it would seem that the dopamine and noradrenaline present in echinoderm nervous systems are localized in interneurons.

One other problem is whether or not the dopamine and noradrenaline are localized in the same cells, or in different cells which are in close association. This question however is universally applicable to animal neurons which are classified as amine-containing on the grounds of present day techniques. The problem will remain until a method is devised which distinguishes individual catecholamines at the fine structural level. Suffice to say here that it is generally assumed that dopamine and noradrenaline are present in the same cells, and there is no reason why echinoderm neurons should be assumed otherwise.

Reserpine greatly reduced the intensity of the amine specific fluorescence in the Asterias nervous system. This observation contrasts with the studies on Patiriella, where the drug appeared to be without effect (Cobb, 1969). The reason for the discrepancy is unknown, as is that between Ophiothrix

and Asterias reported here. However, in the case of Ophiothrix it is assumed that permeability barriers play a part in minimizing the effect of reserpine, but this assumption has not yet been tested by injecting reserpine solution into live brittlestars.

Concomitant with the reduction in fluorescence marked behavioral changes were brought about in starfish, and to some extent in brittle stars. Such changes increasedslowly over a period of one to six days. Ferguson (1967) has shown that the body surfaces of starfish are capable of absorbing amino acids from the surrounding medium. Presumably reserpine exerts its effect via the same absorptive routes. The most extensive changes take place in Asterias, where the radial cords and circumoral ring are in direct contact with the exterior. In this animal tube-foot movement and coordination is progressively destroyed during the reserpine treatment. These changes indicate that neurons in the radial cord of Asterias which contain catecholamines are of importance in the control of tube-foot movement. By analogy it is possible other catecholamine-containing elements in the ectoneural tissue of the gut and at spine bases are important in controlling movements of these organs. These implied

roles for catecholamine-containing neurons in starfish will be discussed later.

As might be expected from the internal nature of their nervous system, the behaviour of brittle stars is little affected. At six days neither their righting responses nor "walking" movements are altered. The only noticeable difference is that the rows of podia (which lack suckers) move more sluggishly than in the untreated animal. If a healthy brittlestar is placed on its back, both the arms and the podia move rapidly, attempting to right the animal. When the reserpinized animal is upturned, the arms thrash as normal, but the podia move sluggishly. These changes presumably result directly from the fact that the only part of the animal's nervous system unprotected from the environment lies just beneath the podial epithelium.

There is growing evidence that in vertebrates catecholamines are associated with granulated vesicles, while clear vesicles contain acetylcholine or certain amino acids (cf. introduction). Both vesicle types are present in large numbers in echinoderm nerve tissue. Cobb (1969) correlates fluorescence distribution with that of nerve elements containing electron-dense granules (up to 700 Å diameter). His assessments are

probably correct. However, the inadequacies of a classification of echinoderm neurons in terms of vesicle populations have been stressed above. Furthermore it is impossible to assess under the electron microscope the number of varicosities present, and compare this to fluorescent areas under the light microscope. Such a correlation would only be valid with the results of extensive serial sectioning.

In this work two techniques were applied to the radial nerve of Asterias in an attempt to determine the nature of particles binding DA and NA. Both methods showed positive intracellular amine-rich sites, but for reasons described earlier, only the results of one technique (Wood, 1966) were relied upon. In that case results indicated that amines were sequestered in granules whose diameters ranged from 200 Å - 800 Å. Furthermore such granules were usually encountered in large intra-neuronal aggregates. Although bad fixation was a necessary limitation of the technique, it appears that such groups are contained within axon varicosities some 1-3 μ in diameter. This size range agrees well with high power objective fluorescent measurements, when some allowance is made for diffusion of amine during the processing for fluorescent

microscopy. Thus it would appear that DA and NA are bound in echinoderm nerve tissue to particles similar to those reported in other invertebrate nervous tissue (Cottrell, 1967). It is also likely that some granules in the radial cord of Asterias which stain with the technique of Wood (1966) are of the same morphological type as those described by Cobb (1969).

Blaschko and Hope (1957) showed that monoamine oxidase was present in the gut of several starfish, sea urchin, and holothurian species. In this thesis MAO was shown histochemically to be present in the central nervous system of starfish, where its presence might be predicted on the grounds of its importance in the regulation of active amines in other nervous systems (Axelrod, 1959). However, in the radial nerve no close similarity existed between areas of enzyme distribution and areas rich in amine. A few scattered deposits were found in the ectoneural tract, but greatest activity was present in the outer cell body layer of the nerve. It was not found possible to ascribe activity to any particular cell type(s) in this layer. There was also no enzyme activity in the hyponeural tissue. With the exception of the latter, the distribution of MAO in the radial nerve of Asterias was reminiscent of the distribution of acetylcholinesterase (Pentreath and Cottrell, 1968). It has

not been found possible to account for the preponderance of MAO in the nerve epithelium.

Acetylcholine and its specific esterase have been studied in the radial nerves of Asteras rubens (Pentreath and Cottrell, 1968). In this work it was found that the radial cords of brittle stars are very similar to those of starfish in terms of ACh content and AChE distribution. Ophiothrix nerve cord contains about 30 µg ACh/gm fresh tissue. Experiments were not undertaken to determine the binding properties of this substance, but it is probably similar to that in Asterias, since electron microscopy showed that there were large numbers of synaptic-type vesicles identical to those implicated with ACh binding in the starfish.

Within the radial nerve of Ophiothrix AChE activity was confined chiefly to the oral cell body layer. This is the same situation as that in Asterias. A comparison of the morphology of this region in starfish and brittle stars allows some conclusions to be drawn regarding the role of AChE. The oral layer of the asteroid nerve supports numerous cilia. Bülbring, Burn and Shelley (1953) proposed that ACh may act as a local hormone regulating ciliary beating in the gill plates of Mytilus. In

consequence it was suggested (Pentreath and Cottrell, 1968) that AChE in the epithelial layer of the starfish cord may be similarly involved in a mechanism controlling ciliary beat. But there are no cilia or microvilli on the radial nerve of brittle stars; thus indirectly it would seem that the high AChE levels in the nerve epithelium of both classes are not related to a cholinergic cilia control mechanism. Further evidence for a non-ciliary association of AChE in Asterias has been obtained by the failure of cholinergic drugs to modify currents maintained over the nerve by ciliary movement. It was also postulated that the enzyme could be involved in the regulation of ionic movement through the outer nerve layer of Asterias. This was prompted by the work of Kock (1954). However, such a mechanism would seem infeasible in brittle stars. The epineural sinus bounding the ventral radial nerve of Ophiothrix forms a closed system, which has no openings to the exterior. It would seem unlikely that the ionic composition of this fluid system is identical to sea water. Furthermore, the oral layer of Ophiothrix nerve cord is completely covered by a non-cellular layer of appreciable thickness.

One other possible explanation given to account for the

high AChE levels in Asterias ventral cord was that a cholinergic mechanism releases the gamete-shedding substances of starfish shown by Chaet (1966, 1967) to be localized orally in the cord (Pentreath and Cottrell, 1968). ACh has been implicated in the release of neurohypophyseal hormones (Daniel and Lederis, 1966). There is no available information regarding a gamete-releasing factor in brittle stars, but it is pointed out here that the presence of such a factor is unlikely because there is first, no direct way in which any substance liberated from Ophiothrix nerve cord could reach the exterior, and second, there are no gonads in the brittle star arm.

Thus it may be said that the cells of the oral ectoneural tissue of Ophiothrix are cholinergic, and that the intense staining in the regions simply represents AChE activity in the cell bodies of these neurons. It is likely that a similar situation is true for Asterias

The hyponeural tissue of both classes is presumed to be motor in function. This would certainly appear to be the case in Ophiothrix, for in this species processes from the hyponeural ganglia are the only nervous elements in contact with the vertebral muscles. The light-microscope localization of AChE

in the hyponeural tissue of starfish and brittle stars suggests that at least some of the motor neurons are cholinergic.

Evidence for cholinergic transmission at neuromuscular junctions in echinoderms has been summarized by Welsh (1966). The observation that the vertebral muscles of brittle stars contain appreciable quantities of AChE and ChE suggests further that ACh may act as a transmitter in this group. Extra evidence for the involvement of ACh was obtained by the marked contracture caused by this substance in low concentrations (10^{-6} gm/ml) on the isolated apical muscle of Solaster. Eserine treatment lowered the threshold of contraction to ACh to 10^{-8} gm/ml, and also potentiated above-threshold contractions. Furthermore, with the exception of glutamic acid, none of the active substances tested produced a noticeable affect on the muscle. The contraction effected by glutamic acid may itself be significant, since another report (Ferguson, 1966) has shown that the digestive glands of the starfish Echinaster spinulosus respond to low concentrations of this substance. However steps to elucidate this point (ie. by attempting to isolate and quantitize the substance from purely nervous tissue) were not undertaken.

The studies of AChE localization in Asterias radial nerve at the fine-structural level were consistent with, and amplified previous light-microscope findings. Within the sensory-association plexus the enzyme is located chiefly in cell bodies and in the cilia and microvilli which they give rise to. It has not been found possible to account for the areas of high activity on the microvilli membranes, but it is feasible that the enzyme is associated with permeability barriers. The surface of the cord has been reported to contain large numbers of sensory elements (Smith, 1965). Cilia located on the epithelium of the cords set up currents which direct particulate material to the disc (Budington, 1942). Some of these presumably fulfill sensory functions, because if noxious stimuli (chemical or mechanical) are applied to the exposed surface of the cord, the starfish reacts away from the stimulus. Since all the ciliated cells are rich in AChE, it is likely that sensory elements are cholinergic. Many other cells of the oral nerve layer, including bipolar and multipolar ganglion cells (Pentreath, 1967), have sites of activity in their cytoplasm and on their outer membranes. It is possible that these too are cholinergic, but because roles of AChE other than the

inactivation of nerve transmitter, which have been dealt with above, cannot be ruled out, a final conclusion cannot be drawn in Asterias. Many axons in the ectoneural tract showed scattered sites of activity along their membranes, but it is not known if such axons have their cell bodies in the outer nerve epithelium. This would however seem likely, and is discussed more fully later. On the basis of the distribution of enzyme deposits and their relation to the abundance of clear vesicles which have been implicated with ACh binding, the ectoneural tissue of Asterias may well contain cholinergic synapses.

The Z10 impregnation technique produced some interesting results regarding the specificity of the stain, and also showed that some previous reports may be misleading. It is emphasized here that little is known concerning the structure of the active complex. The original stain of Champy (1913) employed the cation K^+. More recently Maillet (1962), showed that all soluble iodides, monovalent or divalent, can be used to depict peripheral nerve fibres. He obtained consistently reliable results with zinc iodide. Subsequently workers have used this salt in preparations of the

complex. No attempt has been made by any worker to explain reaction mechanisms. Suffice to say here that the complex reacts with a limited number of substances is nerve tissue during the fixation process.

The starfish ectoneural tract does not contain an even distribution of catecholamine-containing neurons. They are most frequently found in mid- and lateral regions of the nerve. There are very few fluorescent fibres in the most oral mid region of the nerve. It is interesting to compare this picture with that given by the Z10 stain. When the radial nerve of Asterias is seen in cross section it is apparent that those areas which fluoresce strongly generally react lightly with the Z10 stain, and vice-versa. In the ectoneural tract the Z10 stain specifies those axons which contain either clear vesicles or a mixed population of vesicles. The Z10 complex does not impregnate axons which contain only dense-cored vesicles.

Outside the ectoneural tract certain sub-cellular organelles other than synaptic vesicles react to the stain. These are situated in the cytoplasm of nerve cell bodies in the oral layer of the starfish nerve. It has been shown that such cells give off axon processes into the ectoneural tract (Pentreath, 1967), and it is possible that the organelles within the epith-

elial cells which stain are associated with vesicles.

The evidence which indicates a close association between the clear synaptic vesicle and ACh in the starfish nerve has been mentioned above. The susceptability of these particles to the Z10 stain, and the differentiation between amine-rich areas and Z10 susceptible areas, indicate that in Asterias radial nerve the stain reacts preferentially with cholinergic elements. Reserpine treatment does not affect the degree of impregnation in starfish. This contrasts to the findings of Pellegrino de Iraldi and Gueudet (1968). The axons of the hyponeural tissue, which are considered to be cholinergic, do not react with the stain, but they also contain very few synaptic-type vesicles.

The electron microscope is of great help in elucidating the arrangement of echinoderm nervous systems, but is limited because it only allows examination of small areas, and cannot give details of ramification along the length of fibres. Cobb (1966) has described in detail, and contributed much to our present knowledge of fine structural anatomy in the classes Echinoidea and Asteroidea. The general features of the nervous systems of these groups are first, that axons are

tightly packed, with few supporting fibres and glial cells, second that motor neurons are separated from other elements in the central neurons by a layer of collagen, and third, that the motor elements are slightly larger than those of the ectoneural tissue. With one exception the fine-structural studies made in this thesis indicate that the same rules apply to the nervous systems of feather stars and brittle stars. The exception is that the motor elements of the latter class are very much larger than those in the ectoneural tissue.

In echinoderms many second order motoneurons are supposed to occur near muscles and to receive excitation from central (ie. first order) motorneurons (cf. vertebrate autonomic system) (Smith, 1965). Serial sectioning of the hyponeural-vertebral muscle system of Ophiothrix was not undertaken, and in consequence the possibility of such a chain of innervation in Ophiothrix cannot be ruled out. It is stressed however that it is unlikely. Despite this the hyponeural elements of brittle stars are by far the largest recorded in echinoderms.

There is some difficulty in interpreting the precise relationship of these cells with the vertebral muscles. Cobb (1966, 1967) has shown that in several structures (eg. the

ampulla seam of Astropecten) muscles send long clear processes or "muscle tails" into nerve tissue, in which latter region transmission is effected. A similar situation appears to be true in Amphioxus (Flood, 1966). In connection with this Cobb has also forwarded the idea that such echinoderm muscles are singly innervated at their distal end and only at this point. In this thesis semi-serial sectioning indicated that the very large hyponeural elements had their origins in motor cells and not in the vertebral muscles. This contrasts slightly to the reports of Cobb regarding motor innervation in the other classes, and thus it cannot be stated with certainty until serial sectioning is undertaken to elucidate this point. It was also postulated in this work that the close apposition of the cell membranes of the hyponeural elements might indicate electrical transmission. Bargmann and Behrens (1963) have implied that tight junctions exist in the ampullae of Asterias, as does Prosser et al. (1965) in their description of the electrical activity in the gut of various echinoderms. They did not however supply any structural evidence in these cases. Moreover it is generally accepted that the criteria for

identification of a "tight" junction or nexus is repeated finding of opposed membranes with different fixation procedures and at very high magnification (eg. Dewey and Barr, 1964). Such studies were not undertaken with Ophiothrix, and would not be practical because of loss in preservation due to necessary de-calcification. Consequently it is here proposed, not proven, that the hyponeural elements of Ophiothrix oppose "tightly".

One important outcome of the finding that the brittle star hyponeural elements are large is that further experimentation is made possible. Cobb (1966) has stressed the limitations regarding an intracellular electrophysiological approach toward an understanding of the physiology of echinoderms. This has been chiefly due to the small size of all neurons. Such work may now be possible with brittle stars.

The granule containing cells associated with the hyponeural tissue and other motor ganglia of Ophiothrix have been termed neurosecretory for three reasons. First, they correspond exactly in position to the positive neurosecretory staining cells in the related species Ophiopholis aculeata

(Fontaine, 1962). Second they are very similar in appearance to proposed neurosecretory cells in some other invertebrates (eg. Berry and Cottrell, 1970; in press), and third, the cells are very closely situated to the fluid filled sinuses which extend along the brittle star arm. Nothing is known concerning the nature or target sites of the neurosecretory product.

The fluid-filled sinus systems within the ophiuroid arm have been reported to serve a cushioning function on the radial nerve (Smith, 1965), but it would now seem likely that they also serve as a transport system. Similar granule-filled cells have been observed in the hyponeural tissue of the starfish (Pentreath, unpublished observation). These may be homologous to those in the brittle star.

One unresolved question is the relation of cell bodies to axons in the ectoneural tissue of starfish. The cells of the oral epithelium give off axonic processes into the cord, but in Asterias the number of cells is considerably less than the number of axons. The few scattered bipolar and multipolar cells present in the mid-ectoneural tissue are likewise insufficient to account for the large number of axons.

The alternative is that many cell bodies are located peripherally. This idea has been developed by Cobb (1966) into a hypothesis to account for the co-ordination of echinoderms. Briefly this is as follows. Sensory information from the surface of the animal is passed to ganglia. Each ganglia principally serves one particular organ. The areas of neuropile in the lateral radial cord of Asterias which control the movement of individual tube feet are one such type of ganglia. Adjacent ganglia are in contact with each other, and each contains a pacemaker system. Depending on the degree of sensory input, or activity within a ganglia, nerve activity can pass from one ganglia to another, or many ganglia. Transmission between ganglia is passed chiefly by way of the central ring and radial cords. In any region of the body, groups of ganglia may be subject to dominance from other ganglia, or in some cases organs all over the body may be subject to a single pacemaker. In this latter case dominance can change from one pacemaker to another, eg. the starfish undergoes changes in arm dominance during walking movements.

The conclusions to this thesis relate closely to this

theory. In the starfish most catecholamine-containing elements lie lateral in the nerve, where there is an interweaving of axons into a neuropile. Such areas of neuropile are the ganglia which control tubefoot movement. Other amine-rich areas lie in thickenings of the general body ectoneural plexus which are other ganglia supplying the spines and pedicellariae. In brittle stars likewise most fluorescent elements are located in the principal cord ganglia which supply the metamerically arranged spines and tube-feet. Amine-containing fibres run between the various ganglia of each species.

The radial cords of starfish and sea urchins contain far greater numbers of amine-containing elements than brittle stars. At the same time they possess tube feet which are more highly developed than those of ophiuroids. In starfish reserpine depletes catecholamines, with the result that the tube feet are unable to coordinate. This indicates that both the pacemaker and the dominant properties of their controlling ganglia are interfered with.

Thus the available data indicates that echinoderm

sensory information passes via cholinergic neurons to local ganglia. At this point some, or all of this input is integrated by neurons which contain catecholamines while the final path from the ganglia to the muscle is again cholinergic.

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