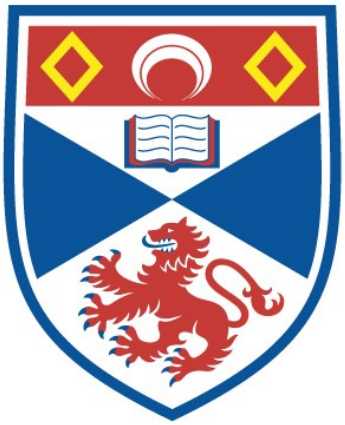
A STUDY OF THE NEUROSECRETORY SYSTEM ASSOCIATED WITH THE VENA CAVA IN THE CEPHALOPOD, ELEDONE CIRROSA (LAMARCK)

Cynthia F. Berry

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1974

This thesis describes a study of the system of nerves associated with the vena cava in the cephalopod, Eledone cirrosa (Lamarck). Alexandrowicz (1964) has proposed that this is a neurosecretory system.

The thesis is divided into seven sections. The first section is an introduction to the topic.

In the second section there is a description of the structure of the vena cava nerves. Examination of the fine structure of the system shows that the nerves contain many types of vesicle, the most numerous being electron-dense vesicles of 80 - 150 nm diameter. The vesicles are concentrated in the nerve terminals which lie adjacent to the basement membrane found on the inner side of the blood vessel wall. The appearance of the nerves is similar to that of neurosecretory neurons found in both invertebrate and vertebrate nervous systems.

The third section describes experiments undertaken to determine some of the pharmacological properties of the nerves. Extracts of the vena cava exhibit potent pharmacological activity. This activity may be due to one or more active substances. When assayed on the isolated systemic heart of E. cirrosa the active substance causes an increase in amplitude and a prolonged increase in frequency of heartbeat. The regions of the blood vessel demonstrating this activity exactly parallel the distribution of the nerve terminals within the vena cava wall. Various techniques, i.e. fluorescence histochemistry, spectrophotofluorimetry, and bioassay, reveal that the activity present in the extracts cannot be attributed to the presence of 5-hydroxytryptamine or catecholamines. Further analysis is required before the chemical nature of the substance can be determined.

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by

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A thesis submitted for the degree of

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DECLARATION

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

Cynthia F. Berry

I certify that Cynthia F. Berry has fulfilled the conditions laid down in the regulations for a degree of Doctor of Philosophy, under Ordinance no. 16 of the University Court of the University of St. Andrews and that she has accordingly qualified to submit this thesis for the degree of Doctor of Philosophy.

G. A. Cottrell

VITAE

I was educated at the County High School for Girls, Chelmsford and the Mid-Essex Technical College. I graduated in Honours Zoology from the University of St. Andrews in 1968. The work described in this thesis was carried out between October, 1968 and July, 1971.

CONTENTS

SUMMARY

1. The nervous system of cephalopod molluscs provides many unusual features which puzzle the biologist. Among these features is a system of nerves passing to the vena cava. Alexandrowicz proposed that this is a neurosecretory system.

2. Examination of the fine structure of the system in Eledone cirrosa shows that the nerves contain many types of vesicle, the most numerous being electron-dense vesicles of 80 - 150 nm diameter. The vesicles are concentrated in the nerve terminals which lie adjacent to the basement membrane found on the inner side of the blood vessel wall. The appearance of the nerves is similar to that of neurosecretory neurons found in both invertebrate and vertebrate nervous systems. Examination of the fine structure of the system in Sepia officinalis demonstrates that a similar arrangement is also present in this cephalopod.

3. Extracts of the vena cava of E. cirrosa exhibit potent pharmacological activity. This activity may be due to one or more active substances. When assayed on the isolated systemic heart of E. cirrosa the active substance causes an increase in amplitude and a prolonged increase in frequency of heartbeat. The regions of the blood vessel demonstrating this activity exactly parallel the distribution of the nerve terminals within the vena cava wall.

4. Structures within the nerve terminals may be isolated on a discontinuous sucrose gradient. It is found that the cardio-excitatory

activity is associated with the electron-dense vesicles of 80 - 150 nm diameter.

5. Gel-filtration of vena cava extracts on Sephadex columns indicates that at least two active substances are present, one with a molecular weight less than 5,000 and one with a molecular weight greater than 5,000.

6. Various techniques, i.e. fluorescence histochemistry, spectrophotofluorimetry, and bioassay, reveal that the activity present in the extracts cannot be attributed to the presence of 5-hydroxytryptamine or catechol amines.

7. The active substance resists heating at an acid or alkaline pH, is unaffected by evaporation to dryness, and is extractable in organic solvents e.g. acetone. Further analysis is required before the chemical nature of the substance can be determined.

8. Release of the active substance could not be demonstrated to occur after electrical stimulation of the nerve trunks, or by changing the ionic environment of the nerve trunks.

9. The above results support Alexandrowicz' proposal that the nerves passing to the vena cava in Eledone cirrosa form a neurosecretory system. The possible functions of this system are discussed.

SECTION 1

INTRODUCTION

The nervous system of an animal is a system of communication. It is a means by which an animal regulates processes within itself and its relationship with the environment. The nervous system is also the site of initiation of complex behaviour i.e. learning and memory. Thus study of the nervous system provides an understanding of the co-ordination of physiological processes within an animal and the co-ordination of an animal's response to its environment.

The basic unit common to all nervous systems is the nerve cell or neuron. The majority of neurons are characterised by two distinct properties: 1) the neuron maintains connections with other nerve cells or neurons, 2) the neuron has the ability to propagate nerve impulses. Other properties e.g. the ability to manufacture and release chemical substances or secretions, the presence of neurotubules, may also be present. Bullock (1965) suggests that it is not necessary that these properties be universal. The units and their properties are essentially alike in all nervous systems that have been studied. However the number of units, especially the number of inter neurons, increases greatly in higher animals and this leads to an increase in the number of connections thus allowing manipulation of more complex information. The most complex nervous systems are found in the molluscs, arthropods, and vertebrates. A study of invertebrate nervous systems demonstrates the diversity of arrangement and function that occurs; ranging from the simple nerve net as found in coelenterates to a complex brain and central nervous system as found in cephalopods (for review see Bullock and Horridge, 1965).

Cephalopod molluscs are animals characterised by fast movement, rapid colour change and complex behaviour and feeding activities. This is reflected in the organisation of their nervous system which is considered the most complex of all invertebrate nervous systems. Superficially it is very similar to that of mammals (Young, 1967) and therefore its arrangement and function have proved of great interest.

At the turn of this century the major interest in cephalopods was in anatomical descriptions of the nervous system of various species. This is shown by the work of Chéron, 1866; Pfefferkorn, 1915 and Hillig, 1912. Although this work was continued (Thore, 1936) later workers such as Ransom, 1884; Fry, 1909 and Fredericq and Bacq, 1939, started investigating the physiological effects caused by stimulation of different parts of the nervous system. More recently it has been shown that cephalopods, in particular the octopod Octopus vulgaris, are capable of learning, and attention has been focussed on the development of learning and memory processes in these animals. Much of this work has been initiated and carried out by J. Z. Young and his associates (For reviews see Young, 1961; Wells, 1962; Young, 1965; Wells and Young, 1968).

Cephalopod nervous systems exhibit some unusual features which puzzle the biologist. These are structures which are often associated with the main nerve trunks, for example the epistellar body (associated with the stellate ganglion) Young, 1936; Cazal and Bogoraze, 1949; the optic gland (associated with the optic tract) Boycott and Young, 1956; the subpedunculate body (also associated with the optic tract) Thore, 1936; Cazal and Bogoraze, 1949; and the pulsating ganglion (associated with the visceral nerve) Alexandrowicz, 1963. Although some of these structures were noted in earlier anatomical descriptions it is only recently that the possible physiological role of these structures has

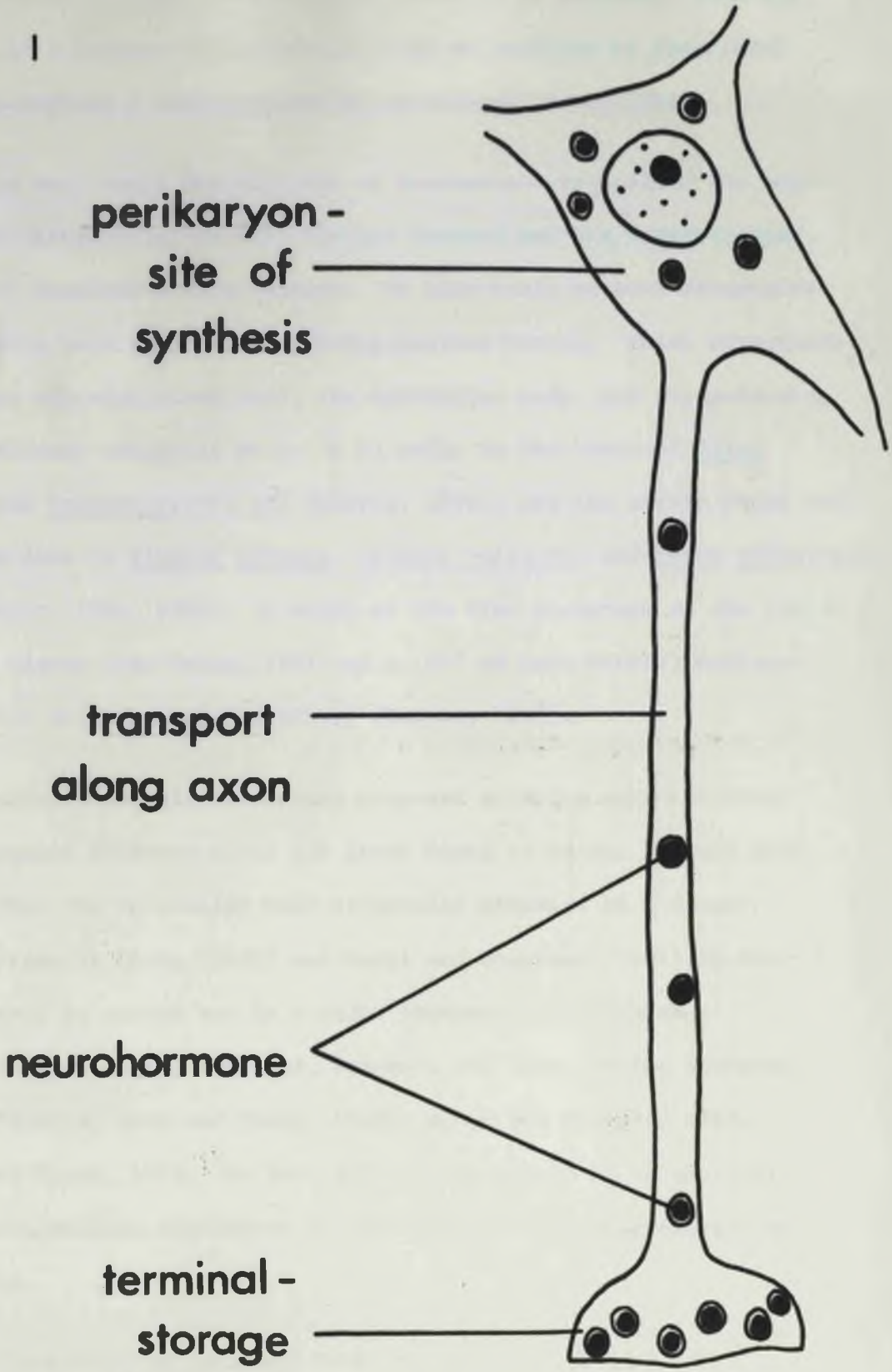
been investigated.

Another interesting feature was first noted by Chéron in 1866. This is a system of nerves that pass from the sub-oesophageal lobe of the brain to the vena cava. The unusual arrangement of these nerves was described and discussed by Alexandrowicz in 1964 and 1965. Since the nerves do not synapse with other neurons but terminate in the wall of the blood vessel, Alexandrowicz suggested that their function might be the secretion of a substance or substances into the blood stream. He therefore proposed that the system be described as the neurosecretory system of the vena cava.

While most neurons have the ability to manufacture and secrete specific chemical substances (e.g. neurotransmitters such as acetylcholine and 5-hydroxytryptamine), neurosecretory cells are neurons manufacturing and secreting hormone-like or "hormonogenic" substances (Bern, 1967b). In the generalised neurosecretory cell (Fig. 1) the neurosecretory substances or neurohormones are synthesised in the perikaryon, pass along the axon and are stored in enlarged axon terminals. In some cases the axon terminals are associated with the vascular system. Such a close association with the blood stream allows efficient distribution of the neurosecretory substances. Neurosecretory cells have been demonstrated to be present in all metazoan animal groups (Gabe, 1966). They are of great importance since they provide a link between the two major co-ordinating systems within an animal, viz. the nervous and the endocrine systems. E. Scharrer (1965) has defined neurosecretory cells as the "final common path" between these two systems.

Neurosecretion is the release from neurons of an "active" substance which affects a specific organ or physiological process within

Figure 1. Diagram of a generalised neurosecretory cell. The neurohormones are synthesised in the perikaryon, pass along the axon and are stored in enlarged axon terminals.


perikaryon - site of synthesis
transport along axon
neurohormone
terminal - storage area

an animal, often at some distance from the point of release. Thus the definition of a neurosecretory system requires evidence of functional activity as well as a consideration of morphology (Bern, 1966).

In many early descriptions of neurosecretory systems the only evidence for neurosecretion that was put forward was the demonstration of secretory droplets within neurons. On this basis several structures in cephalopods were postulated as being neurosecretory. These structures included the subpedunculate body; the epistellar body; and the pulsating ganglion (already mentioned on p. 4); cells in the brain of Illex coindetti and Ommatostrephes sp. (Martin, 1966); and the nerves which pass to the vena cava in Eledone cirrosa, Octopus vulgaris, and Sepia officinalis (Alexandrowicz, 1964, 1965). A study of the fine structure of the juxtaganglionic tissue (see Young, 1965 and p. 147 of this thesis) indicated that this too might be neurosecretory (Barber, 1967).

However not all structures proposed as being neurosecretory on morphological evidence alone are later shown to be so. Recent work indicates that the epistellar body originally proposed as a neurosecretory organ by Young (1936) and Cazal and Bogoraze (1949) is not neurosecretory in nature but is a light receptor, see Nishioka, Hagadorn and Bern, 1962; Nishioka, Yasumasu and Bern, 1966a; Nishioka, Yasumasu, Packard, Bern and Young, 1966b; Mauro and Baumann, 1968; Perrelet and Mauro, 1972. As Bern (1967a) has stated it is apparent that a neuroglandular appearance at the level of light microscopy can be deceiving.

Alexandrowicz' proposal that the nerves passing to the vena cava in Eledone cirrosa were neurosecretory was based purely on morphological evidence. The work described in this thesis was

undertaken to obtain information on the system in E. cirrosa on a morphological, pharmacological, biochemical and physiological basis in an attempt to substantiate the views expressed by Alexandrowicz and thus firmly establish an example of neurosecretion in the Cephalopoda.

SECTION 2

STRUCTURE OF THE VENA CAVA NERVES

In 1866 Chéron described some nerve branches which passed to the "parois" or lining of the vena cava of the octopods Eledone moschata and Octopus vulgaris and the decapod Sepia officinalis. The description of the system in S. officinalis was later elaborated by Hillig (1912). Pfefferkorn (1915) gave details of the system in several octopod species including Argonauta argo and described the nerves as "rami venae cavae". In 1936 Thore reexamined the system in E. moschata, A. argo and S. officinalis and investigated the origin of the nerves in E. moschata. He described them as arising "aus dem extracorticalen Neuropilem".

Although the system was first described at the end of the last century, no consideration of the possible function of these nerves was made until the work of Alexandrowicz (1964, 1965). These papers provoked new interest in the system and a similar arrangement of nerves was then described for two deep-sea cephalopod species, Illex coindetti and Ommatostrephes sp., (Martin, 1966). In 1964 Alexandrowicz described in detail the system present in Eledone cirrosa (L.). For the first time he noted that the nerves passing to the blood vessel were in separate trunks that ran alongside the "nervi viscerales" and the "nervi infundibulares posteriores". According to Alexandrowicz the system is composed of unipolar neurones which originate in a layer covering the visceral lobe of the brain and pass out posteriorly in four trunks, two trunks on each side of the vena cava. The lateral neurosecretory trunks are associated with the nervi infundibulares posteriores (posterior infundibular nerves), and the medial neurosecretory trunks are associated with the nervi viscerales (visceral nerves), which originate in the visceral lobe of the brain. In the latter part of their course the

neurosecretory trunks diverge from their respective nerves. Some axons pass to the vena cava while the remainder ramify through the muscles of the septum and appear to end blindly with no connection to any organ, see Fig. 2.

The axons of the lateral neurosecretory trunk form one nerve whereas the axons of the medial neurosecretory trunk form several nerves passing to the vessel. These nerves penetrate the muscular coat of the vena cava and form a network of fibres (described by Alexandrowicz as a "neuropile") adjacent to the inner edge of the vessel (Fig. 3). The neuropile layer extends over the cephalic end of the vena cava from the brain to the entrance of the vena hepatica. Normally the inner surface of cephalopod veins is smooth (Barber and Graziadei, 1966). However, in the neuropile region, the inner surface of the vena cava is characterised by longitudinal ridges. Alexandrowicz noted that this characteristic appears to have been observed first by Cuvier, 1817. These ridges are clearly visible in methylene blue preparations which show that the area covered by the ridges corresponds to the "neuropile" area of the neurosecretory nerves.

The nerves passing to the vena cava contain three types of axon: axons (1 μm diameter) forming the neuropile; axons (3 μm diameter) also associated with the neuropile and axons (3 μm diameter) which do not penetrate the muscular coat of the vessel but form a plexus of fibres on the outer surface, completely independent of the neuropile. The number of axons present has been estimated to be between 2 and 3 million, an unusually large number for the innervation of a blood vessel. The terminations of the nerves of the neuropile were shown to stain with paraldehyde-fuchsin (Alexandrowicz, 1964) and these findings were

Figure 2. Diagram showing the arrangement of nerves passing to the vena cava in Eledone cirrosa. Ventral view. On the right, parts of the adductor medianus muscle and the infundibularis muscle are drawn to show the pathway of the medial neurosecretory nerve trunk between these two muscles.
N.- nerve; M.- muscle; NSV - neurosecretory.
After Alexandrowicz.

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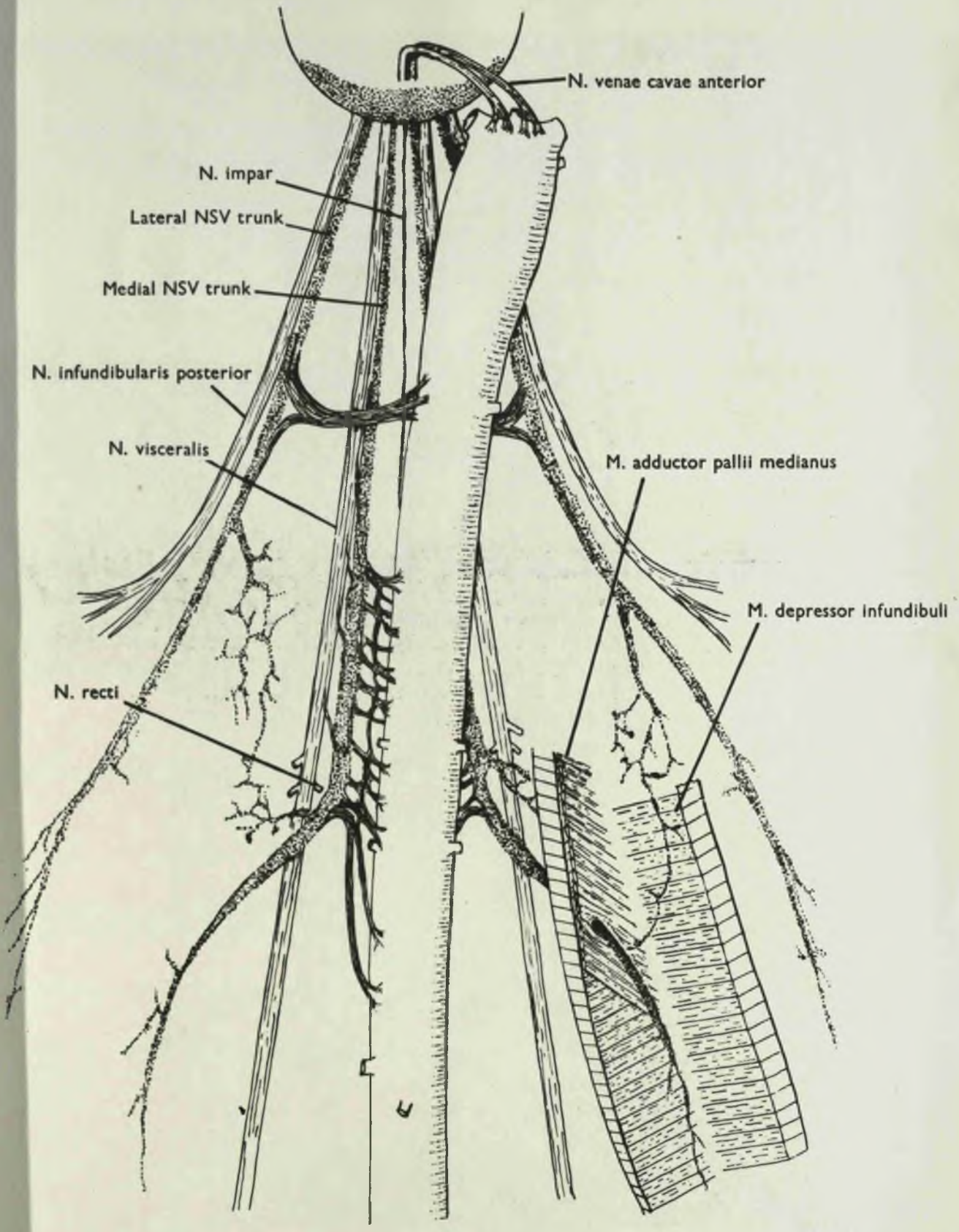
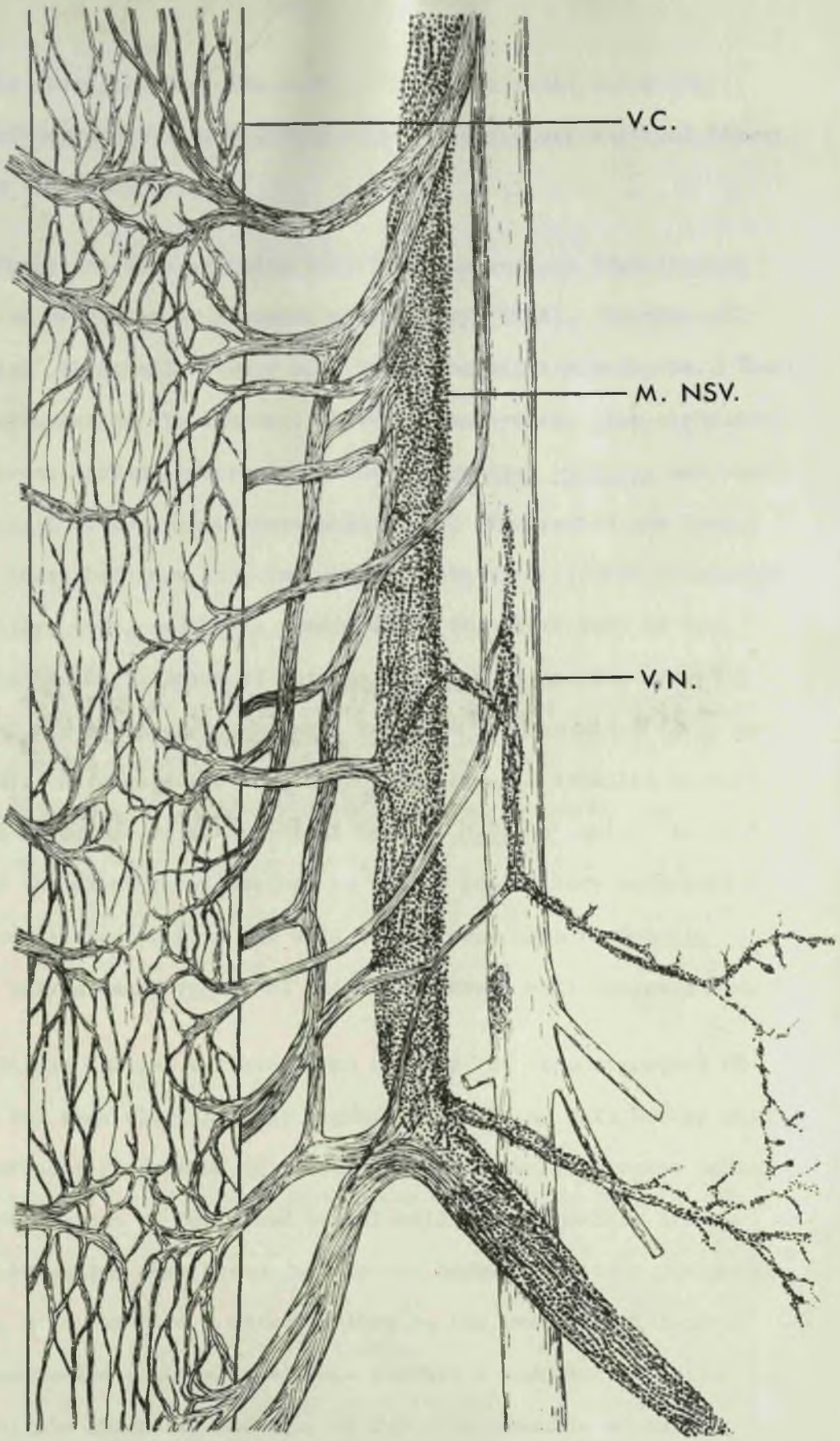

N. venae cavae anterior
N. impar
Lateral NSV trunk
Medial NSV trunk
N. infundibularis posterior
N. visceralis
M. adductor pallii medianus
M. depressor infundibuli
N. recti

Figure 3. Diagram showing fibres of the medial neurosecretory nerve trunk penetrating the vena cava and forming a neuropile in the wall of the blood vessel. V.N. - visceral nerve; M.NSV. - medial neurosecretory nerve trunk; V.C. - vena cava. After Alexandrowicz.


V.C.
M. NSV.
V. N.

confirmed by Berry (1968). The terminations also stain using the procedure of Dogra and Tandan (1964) for neurosecretory material (Berry, unpublished observations).

Methylene blue staining confirmed the general organisation of the system described by Alexandrowicz (Berry, 1968). However all Alexandrowicz' observations were made using the light microscope. There was no information on the fine structure of the system. The cephalopod central nervous system, particularly that of Octopus vulgaris was known in great detail at the light microscopic level (for review see Young, 1971), but there had been very few studies with the electron microscope. In 1963, Dilly, Gray, and Young examined the fine structure of the optic nerves and optic lobes of Octopus and Eledone and the synaptic structure of the brain of O. vulgaris has also been examined (Gray and Young, 1964). More recently Gray (1970) has given a detailed account of the fine structure of the vertical lobe of Octopus brain. In 1968 Martin used the electron microscope to make a preliminary examination of the nerve terminations in the wall of the vena cava in Octopus vulgaris. Only a small region of the blood vessel wall was examined.

It was thought worthwhile to examine the fine structure of the system and thus elucidate the organisation of the cell bodies within the nerve trunks; to examine in detail the arrangement of nerve endings in different regions of the blood vessel wall and to confirm Alexandrowicz' observation that the nerves do not terminate within the muscle coat of the vein but form a network close to its endothelial lining. Such an examination might also a) show whether a secretory material was contained within the nerve endings, b) indicate possible sites of synthesis of such material and c) reveal possible sites and methods of

release of this material. In turn, this information might also help to clarify whether the vena cava neurosecretory system is comparable to other cephalopod tissues thought by Young (1965) to be of similar type and described by him as "neurovenous tissues" (Young, 1970).

[The "neurovenous tissues" comprise 1) the subpeduculate tissue found as strands of cells running from the optic lobes to an anterior chamber organ at the back of the orbit; 2) the paravertical tissue, masses of cells at the side of the vertical lobes of the brain and especially well-developed in the decapods, both described by Boycott and Young (1956); 3) the juxtaganglionic tissue, which consists of nerve fibres accompanying the inferior mandibular nerves of the buccal mass and ending in close association with the buccal sinus (Young, 1965) and 4) the neurosecretory system of the vena cava.]

Using the electron microscope the neurosecretory system of the vena cava in Eledone cirrosa was examined in detail. Preliminary observations on the structure of the nerve terminations in the wall of the vena cava in the decapod, Sepia officinalis, were also made.

MATERIALS AND METHODS

Animals

Specimens of Eledone cirrosa, varying in size between 100 and 1,000 g, were supplied by local fishermen from Crail and Pittenweem. The animals were caught in trawl nets or were occasionally found in lobster pots. On arrival at the laboratory, they were put into a large glass tank (7' x 3' x 3') which was supplied with a continuous circulation of fresh, aerated sea-water. If not required immediately for experiments, the animals were fed on the common shore crab, Carcinus

maenas. It is interesting to note that the majority of specimens brought into the laboratory were female.

(Eledone cirrosa was particularly appropriate as an experimental animal since it is found off most parts of the Scottish coast and is normally particularly abundant in the Firth of Forth; Stephen, 1944.)

A few specimens of Sepia officinalis, caught in the English Channel, were made available to the author during a visit to the laboratory of the Marine Biological Association at Plymouth.

Isolation of the vena cava

An animal was killed by making an incision through the brain. The vena cava was then exposed. Two methods were employed to fix the tissue initially. Either the blood vessel was immediately removed from the animal and immersed in fixative or the fixative was perfused through the vena cava and the tissue then dissected. Tissues prepared by either method appeared the same when examined under the electron microscope.

Fixative

Glutaraldehyde and osmium tetroxide at various concentrations were tried as fixatives. The most satisfactory of these consisted of the following: 1 ml 25% glutaraldehyde buffered to pH 7.2 with 9 ml of 0.2 M cacodylate buffer; 0.978 g of sucrose was then added to this solution.

Fixation and embedding procedure

Pieces of vena cava measuring about 1 mm^3 were fixed for 2 1/2 hours in the buffered glutaraldehyde/sucrose solution. The tissue was washed with buffer for a further 2 1/2 hours and postfixed in a solution of 1 part 2% osmium tetroxide and 1 part cacodylate buffer for 1 1/2 hours. All processes were conducted at 20°C. The pieces of vena cava were washed in distilled water, dehydrated through a series of acetone-water solutions and placed in a 1/1 volume/volume absolute acetone - Araldite mixture for 12 - 14 hours. They were then embedded in Araldite mixture alone and left for 36 hours at 60°C.

Electron Microscopy

"Silver" sections, 60 - 80 nm thick, were cut on an LKB Ultrotome, using glass knives, and were mounted on uncoated copper grids. The sections were stained for 3 minutes with lead citrate (Reynolds, 1963) and further stained for 3 minutes with 2% uranyl acetate. The tissue was examined using an A.E.I. EM6B electron microscope operating at 60KV.

RESULTS

The neurosecretory nerve trunks run adjacent to the visceral and posterior infundibular nerves until the trunks split up and pass to the wall of the vena cava.

The axons composing the visceral and posterior infundibular nerves are similar in appearance, those of the posterior infundibular nerve being slightly larger in diameter, see Fig. 4 and Fig. 5. Axons of the visceral nerve range between 1 µm - 6 µm diameter while those

of the posterior infundibular nerve range from 3 μm - 8 μm diameter. The axoplasm contains mitochondria and some electron-lucent vesicles. The nerve trunks are enclosed by a sheath composed largely of collagen.*

The medial neurosecretory and the lateral neurosecretory nerve trunks also appear very similar, see Fig. 7 and Fig. 8. However these nerves differ markedly from the visceral and posterior infundibular nerves. Their most remarkable feature is the small size of the axons. These range from 0.1 μm to 1.8 μm diameter, with a mean diameter of 0.5 μm. Thus the axons of the neurosecretory nerves are ten times smaller in diameter than the axons composing the visceral and posterior infundibular nerves. The neurosecretory axons are packed adjacent to the "ordinary" nerve trunks with only a small sheath of connective tissue separating them, see Fig. 9.

The neurosecretory nerve trunks contain axons and cell bodies (Fig. 10). The cell bodies are characterised by the presence of many mitochondria and numerous membrane and Golgi systems which are surrounded by different types of vesicle, see Fig. 11. There is evidence that the Golgi apparatus participates in the elaboration of some of these vesicles since these systems are seen to "bleb" off electron-dense vesicles which are surrounded by a limiting membrane (Fig. 12). Stacking of membranes is also seen.

The neurosecretory axons are packed very closely together and contain several types of inclusion (Fig. 13 and Fig. 14). The types of inclusion seen are:

a) electron-dense vesicles, 80 - 150 nm diameter
b) electron-lucent vesicles, 90 - 150 nm diameter
c) electron-lucent vesicles, 30 - 50 nm diameter

*(Figure 6 has been deleted.)

d) neurofilaments, 10 - 15 nm diameter

e) mitochondria

It was found that different types of vesicle were not confinedto separate axons (Fig. 14).

Towards the latter part of their course the neurosecretory trunks leave their association with the ordinary nerves, divide into small branches and pass to the wall of the vena cava. A few branches do not go to the blood vessel but pass instead to the muscle blocks of the septum, see Fig. 15. These axons are exactly similar in appearance to those passing to the vena cava. The axons passing to the blood vessel, see Fig. 16 and Fig. 17, penetrate the muscular coat and terminate in the inner layers of the blood vessel wall. These inner layers of the vena cava are thrown up into folds (Fig. 18) giving the inner surface of the blood vessel a ridged appearance. The nerve terminations are tightly packed against one another and are found to lie adjacent to the basement membrane of the inner surface of the blood vessel (Fig. 19). The terminations contain many inclusions, see Fig. 20. These inclusions are of various types and may be divided into two categories: 1) those that are electron-opaque (electron-dense) and 2) those that are electron-lucent. The various types of inclusion are listed below:

Category 1 -	(a) electron-dense vesicles	80 - 150 nm diameter
	(b) less electron-dense vesicles	80 - 200 nm diameter
	(c) electron-dense particles	0.3 - 2 μm diameter
Category 2 -	(a) electron-lucent vesicles with bar	80 - 200 nm diameter
	(b) electron-lucent vesicles without bar	80 - 200 nm diameter

(c) electron-lucent vesicles 25 - 50 nm diameter

(d) neurofilaments 10 - 15 nm diameter

(e) mitochondria

The most numerous inclusions are the electron-dense vesicles with a diameter between 80 - 150 nm. At low magnification these vesicles appear uniformly black suggesting that their content is homogeneous. However at higher magnification they sometimes show a granular appearance, see Fig. 17, and it is seen that they possess a limiting membrane. This membrane is normally separated from the electron-dense content of the vesicle by an area of lower electron-density. In some cases the membranes are seen to have a wavy outline.

There are also vesicles of a similar diameter which appear less electron-dense, see Fig. 21, Fig. 22 and Fig. 24. These vesicles also possess a limiting membrane. Vesicles of this type are not particularly numerous and it is not clear whether they represent a population separate from the first electron-dense type of vesicle or not.

The third type of inclusion in the first category is a large electron-dense particle (with a mean diameter of 500 nm). These are found in cells which are located away from the basement membrane (Fig. 22).

There are three types of electron-lucent vesicle, shown in Fig. 20 and Fig. 21. Two of these types are of similar dimensions but one shows a central bar or disc while the other appears completely clear. The third type of electron-lucent vesicle is much smaller in diameter (25 - 50 nm).

Although each vesicle type is generally contained within

separate axon terminals this is not always so. Sometimes an assortment of vesicles is observed within axon terminals (Fig. 20). In particular, small electron-lucent vesicles are seen in terminations containing electron-dense vesicles and also in those containing the clearer type of vesicle, see Fig. 21. In some cases these small vesicles are clumped together close to the axon membrane.

Endothelial cells are occasionally seen. These are situated on the basement membrane and protrude into the lumen of the blood vessel (Fig. 22). The outer layers of the blood vessel form a muscular coat (Fig. 23).

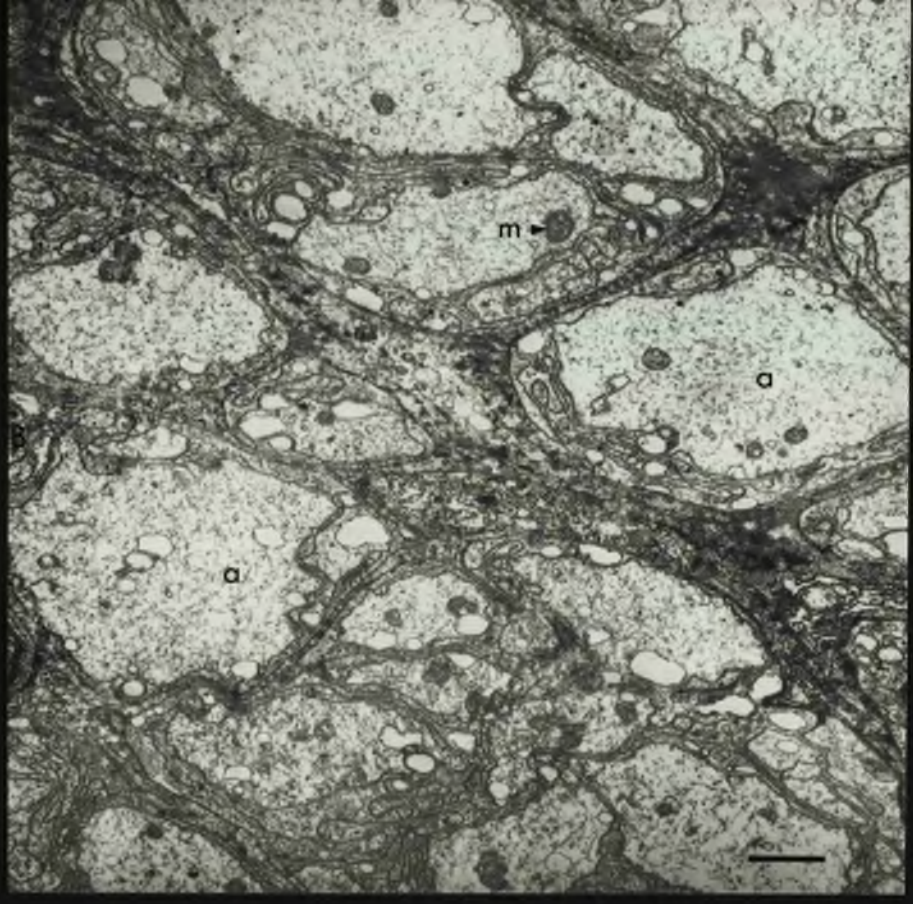
The arrangement of the neurosecretory neuropile is essentially similar wherever the nerve terminations are found in the blood vessel wall, see Fig. 24 and Fig. 25, and contrast with Fig. 21. However, in parts of the blood vessel where no ridging occurs, no nerve terminations are found adjacent to the basement membrane and the vena cava wall has the basic structure common to other cephalopod blood vessels, see Fig. 26.

There were no apparent differences in the arrangement of the neurosecretory neuropile in the young animal compared with the adult, nor between male and female members of the same species.

Examination of the nerve terminations of a similar system present in Sepia officinalis demonstrated that the organisation of the nerve terminals was exactly as found in Eledone cirrosa (Figs. 27 - 30).

Figure 4. Electron micrograph of a transverse section through the visceral nerve in the region of the neurosecretory nerve trunks. Note that the axoplasm does not contain any electron-dense vesicles.
a - axoplasm; m - mitochondrion.
Bar represents 1 µm.

Figure 5. Electron micrograph of a transverse section through the posterior infundibular nerve in the region of the neurosecretory nerve trunks.
Bar represents 1 µm.



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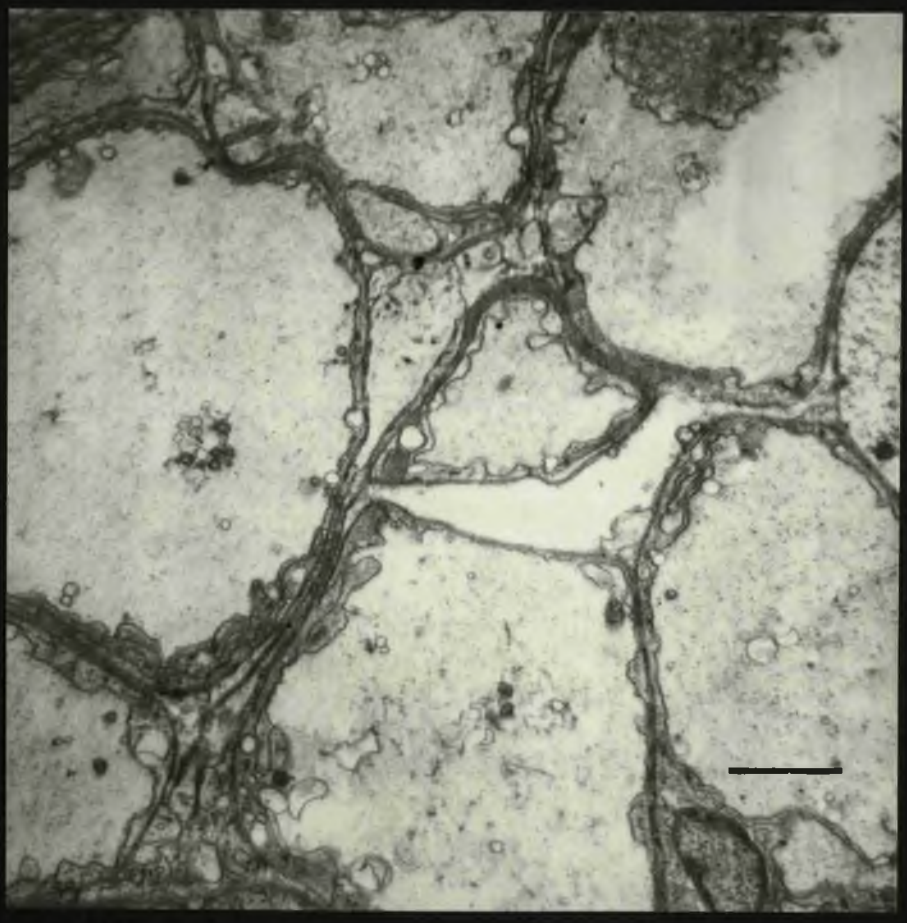
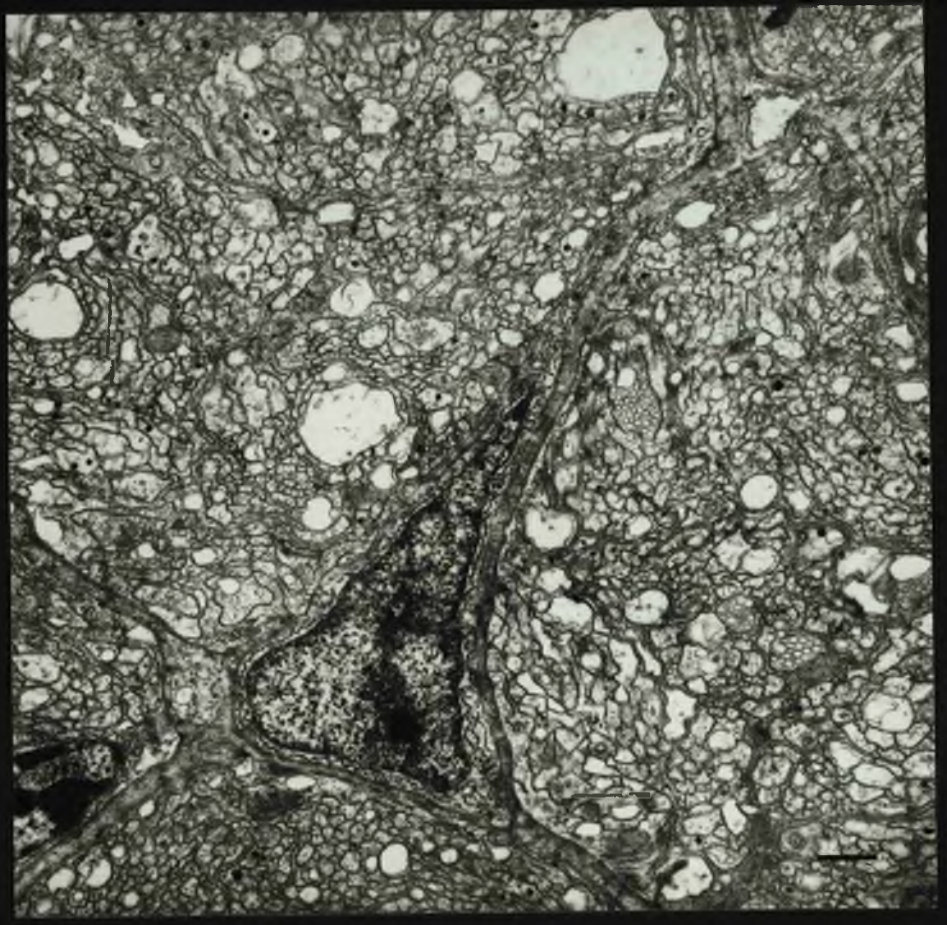


Figure 7. Electron micrograph of a transverse section through the medial neurosecretory nerve trunk. Compare the size of the axons with those found in the visceral nerve, Fig. 4.

Bar represents 1 μm.

Figure 8. Electron micrograph of a transverse section through the lateral neurosecretory nerve trunk. Compare the size of the axons with those found in the posterior infundibular nerve, Fig. 5.

Bar represents 1 μm.



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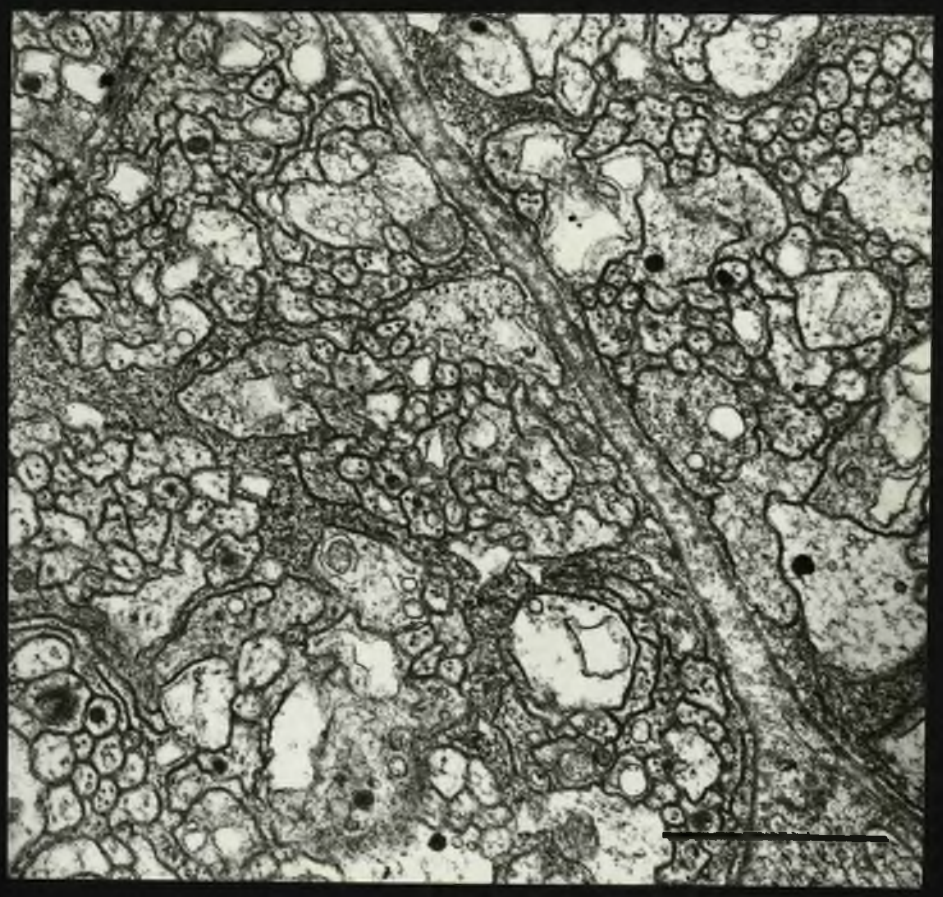


Figure 9. Electron micrograph showing the junction between the medial neurosecretory nerve trunk and the visceral nerve. Note that there is only a small sheath of connective tissue separating the two nerves.
m.nsv. - medial neurosecretory nerve trunk;
v.n. - visceral nerve.
Bar represents 1 μm.

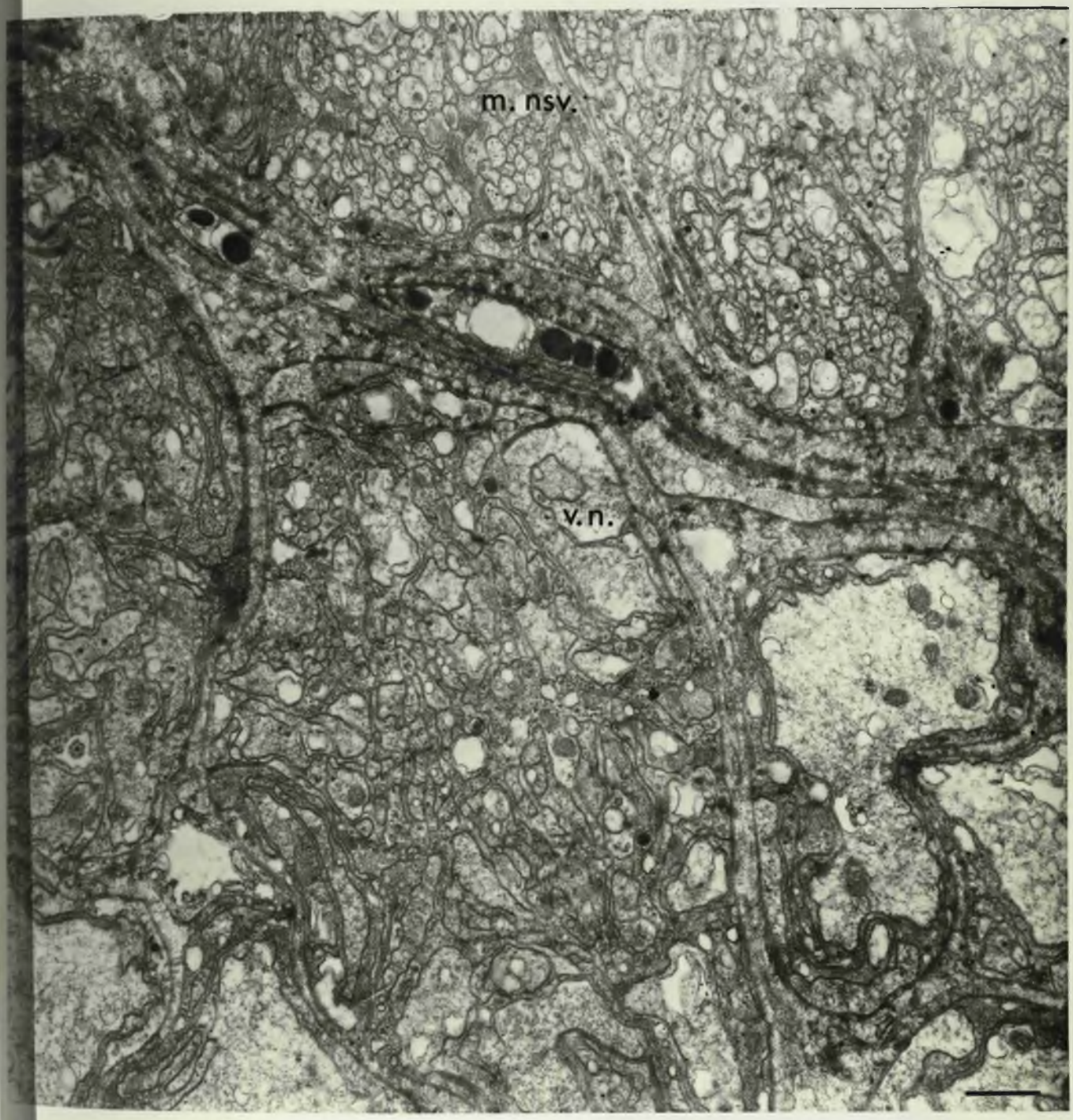
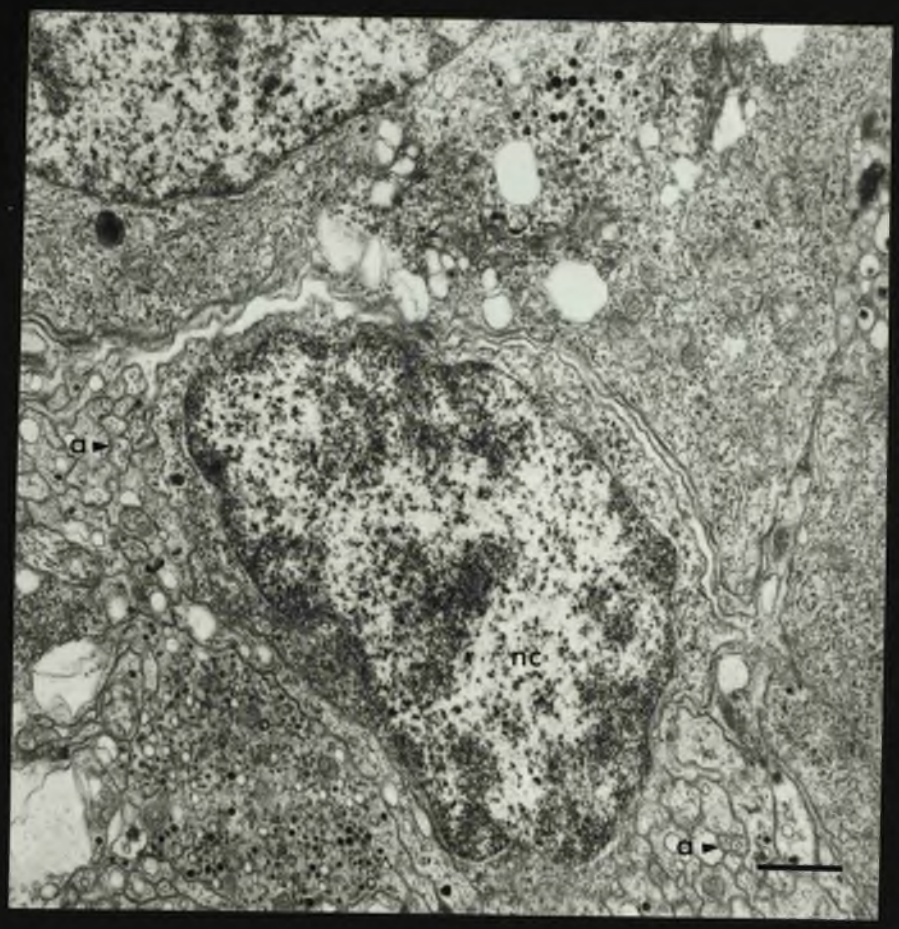

m. nsv.
v. n.

Figure 10. Electron micrograph of a transverse section through the medial neurosecretory nerve trunk demonstrating the presence of cell bodies.
a - axon; nc - nucleus.
Bar represents 1 μm.

Figure 11. Electron micrograph of a transverse section through the medial neurosecretory nerve trunk demonstrating part of a cell body. This micrograph shows some of the numerous Golgi systems present surrounded by several different types of vesicle.
g - Golgi system; m - mitochondrion; nc - nucleus; v - vesicles.
Bar represents 1 μm.

10



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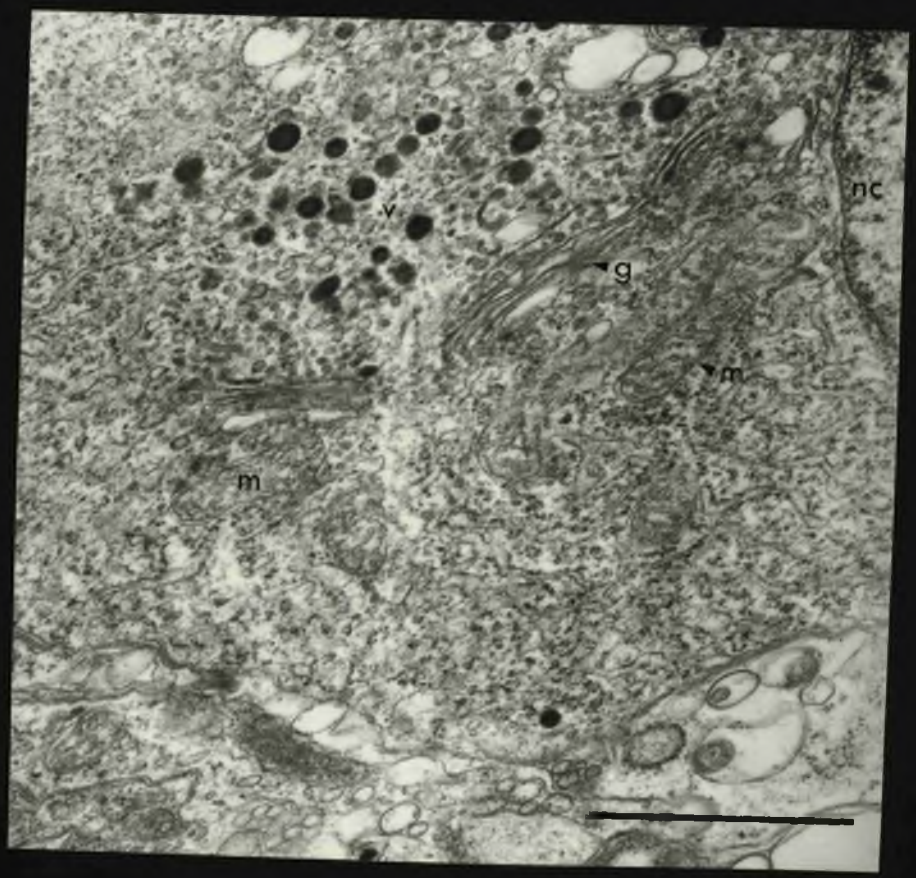


Figure 12. Electron micrograph of a transverse section through the medial neurosecretory nerve trunk demonstrating part of a cell body. Some of the Golgi systems are seen to "bleb" off electron-dense vesicles, (indicated by the arrow).
g - Golgi system; m - mitochondrion, nc - nucleus; v - vesicles.
Bar represents 1 μm.

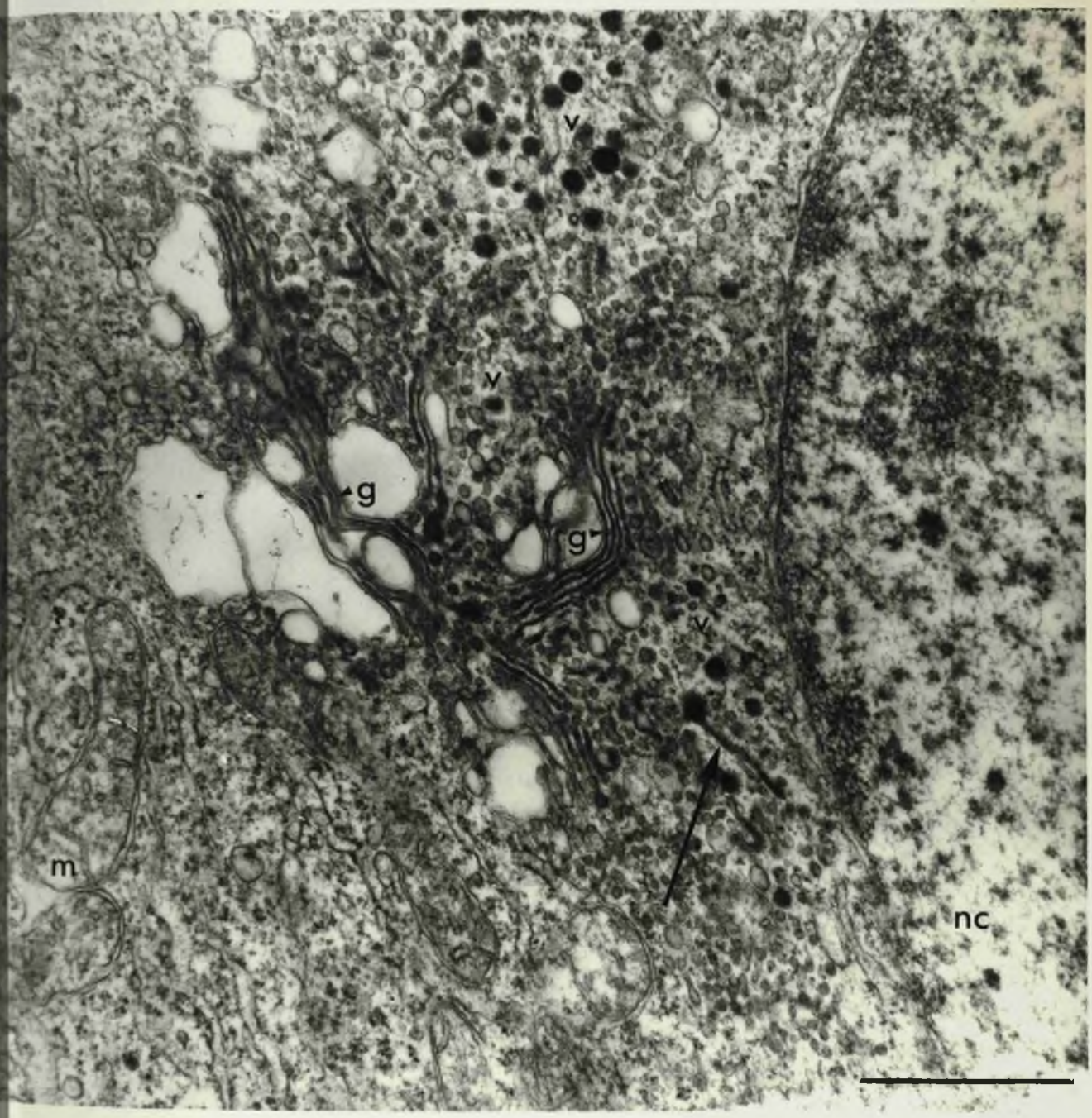
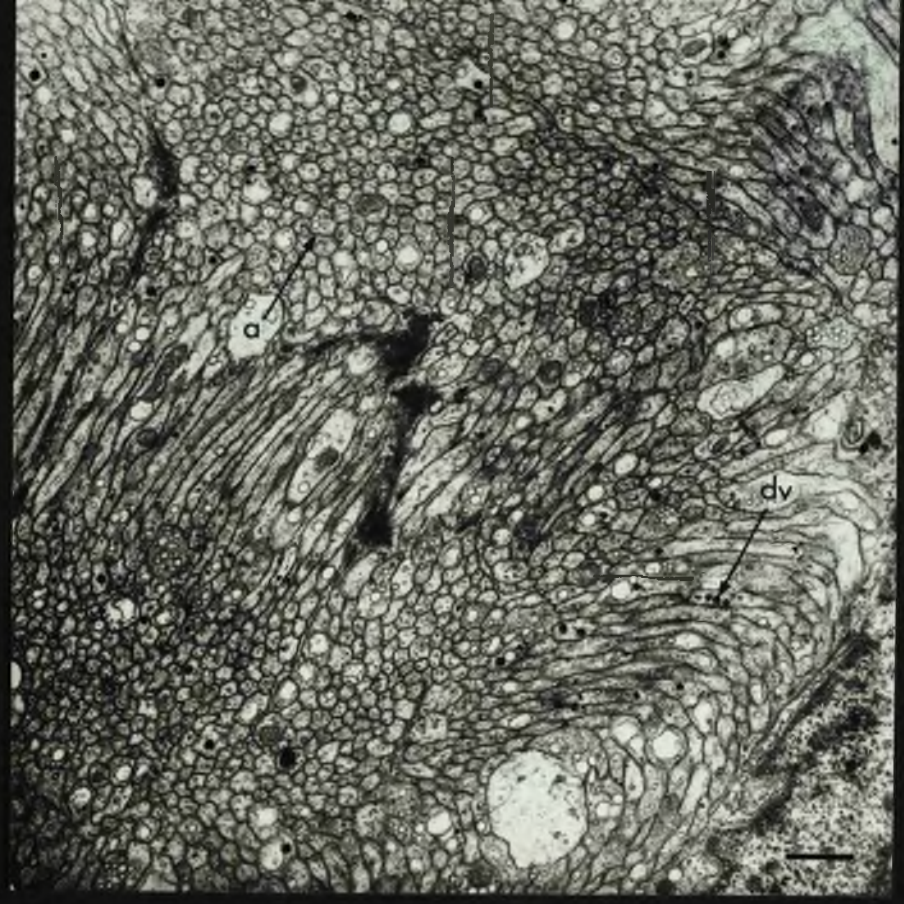

v
v
g
g
v
m
nc

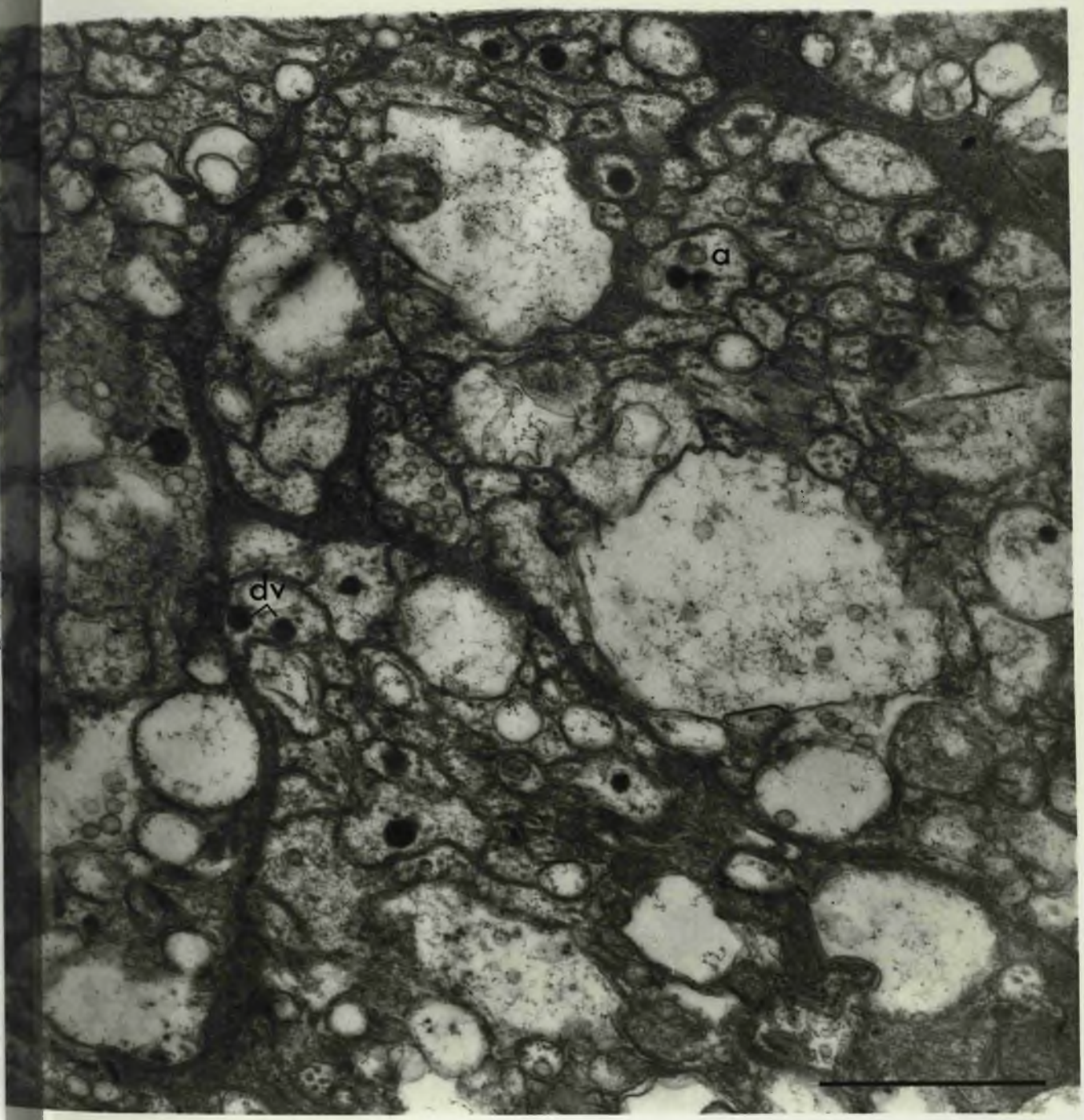
Figure 13. and
Figure 14.

Electron micrographs showing the types of inclusion found within the axons of the neurosecretory nerve trunks.
a - axon; dv - electron-dense vesicles;
lgv. - large electron-lucent vesicles;
lv - electron-lucent vesicles; mt - mitochondr
nf - neurofilaments; sm - small vesicles.
Note that in some cases different types of vesicle are contained within the same axon, (indicated by the arrow).
Bar represents 1 µm.



14

Figure 15. Electron micrograph showing the appearance of axons of the medial neurosecretory nerve passing through the muscle blocks of the septum. These axons contain electron-dense vesicles similar to those seen in the axons passing to the vena cava wall.
a - axon; dv - electron-dense vesicle.
Bar represents 1 μm.

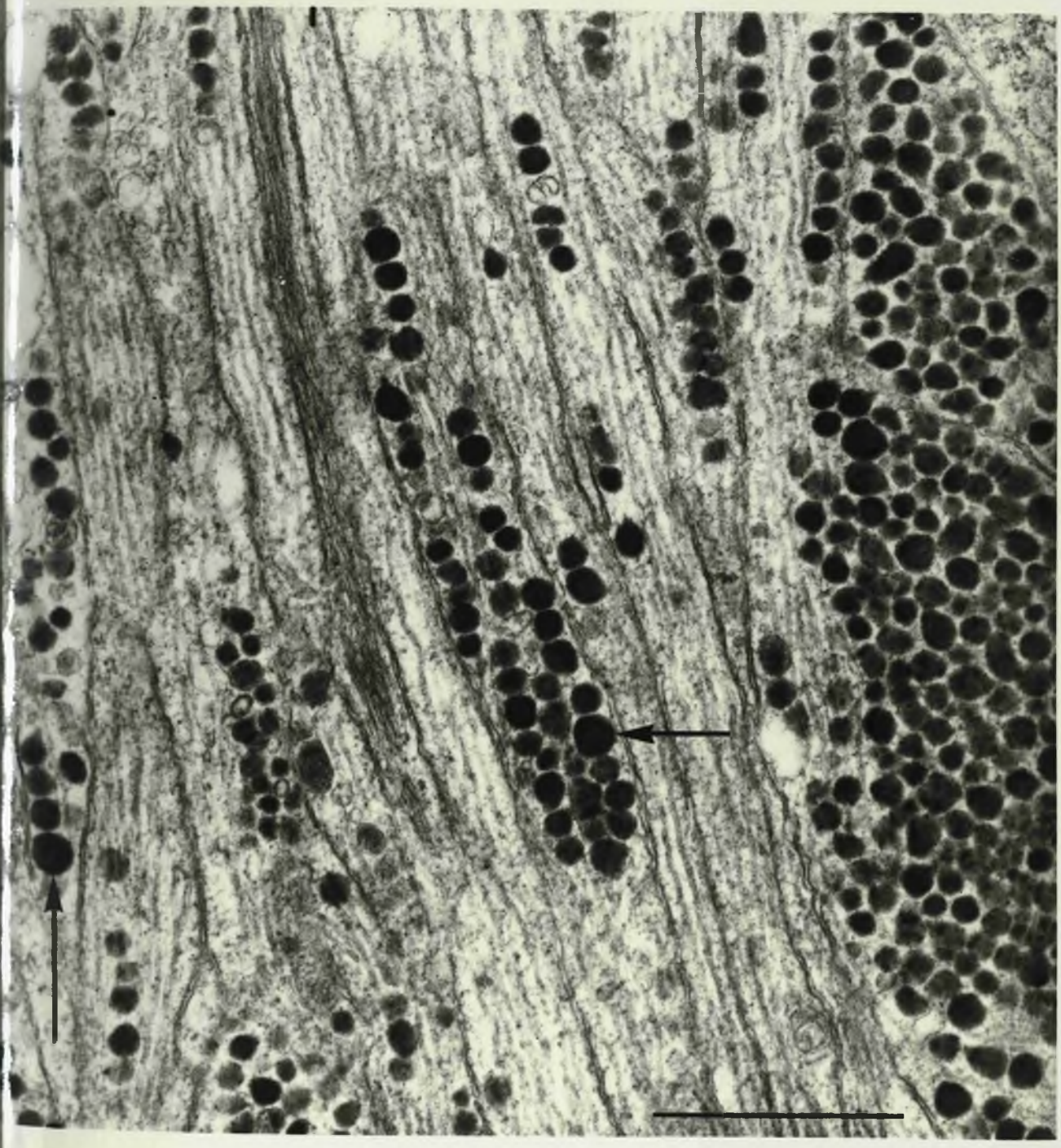


axon
rough
con-
seen

Figure 16. Electron micrograph showing the appearance of axons of the medial neurosecretory nerve as they pass through the vena cava wall. Note the numerous electron-dense vesicles (dv) and neurofilaments (nf) present.
Bar represents 1 μm.

16

Figure 17. Electron micrograph showing the appearance of axons of the medial neurosecretory nerve as they pass through the vena cava wall. Part of Figure 16 at a higher magnification. Note that the electron-dense vesicles are membrane bound. The arrows point to electron-dense vesicles whose core appears to be composed of small granular particles.
Bar represents 1 μm.



of axons
pass
16 at
iron-
ows poin
rs to be

Figure 18. Light micrograph showing a transverse section through the wall of the vena cava. The section was previously stained with toluidene blue. The inner layers of the blood vessel are thrown into folds.
b - basement membrane; l - lumen of the vena cava.
Bar represents 40μm.

b
l

Figure 19. Electron micrograph of an infolding of the wall of the vena cava. Numerous nerve endings, which contain electron-dense vesicles, lie against the basement membrane of the vessel.
bm - basement membrane; dv - electron-dense vesicl
en - endothelial cell; lu - lumen of the vena cava
ne - nerve endings.
Bar represents 1 μm.

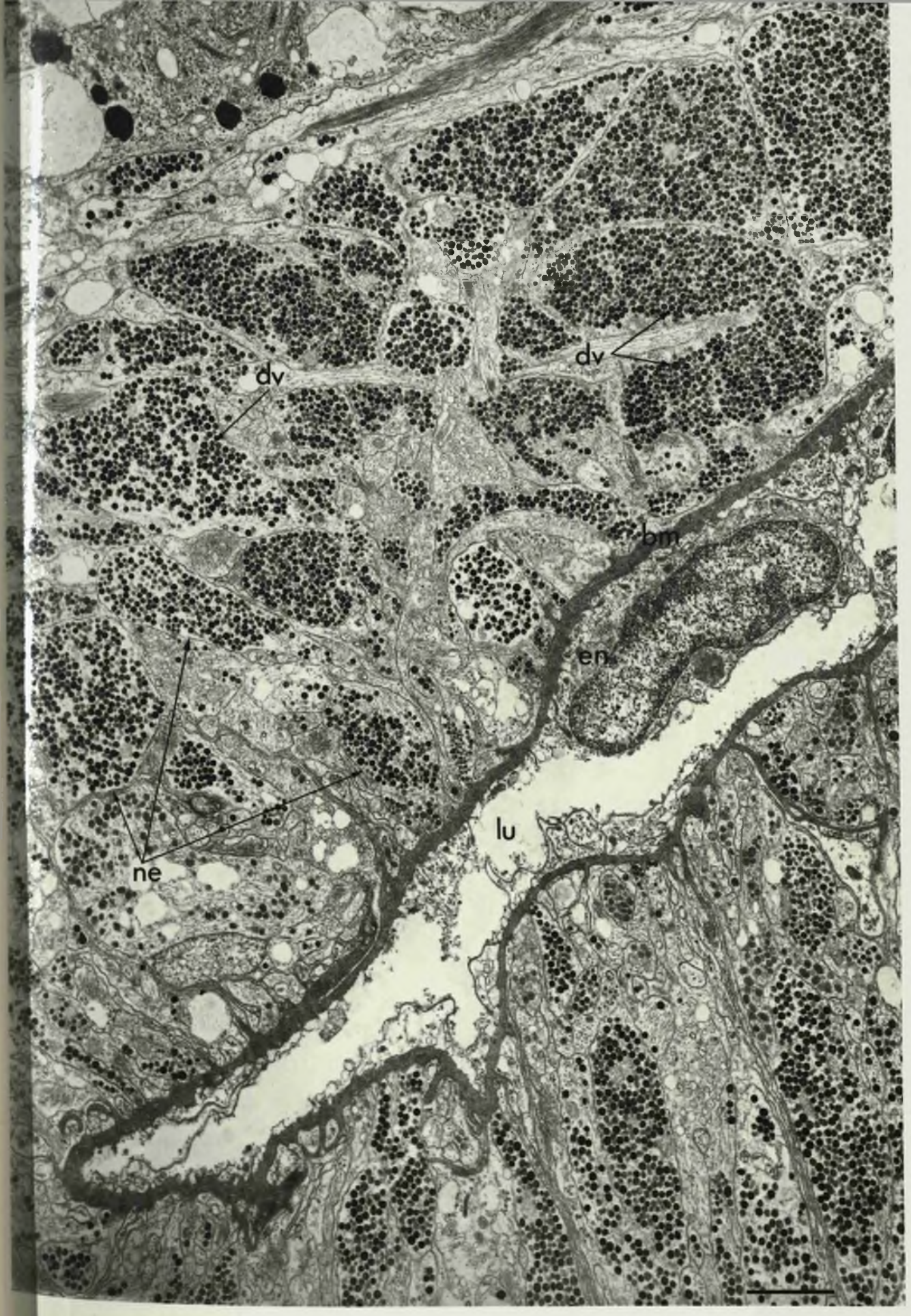

dv
dv
bm
en
lu
ne

Figure 20. Electron micrograph showing the nerve endings packed tightly against one another adjacent to the basement membrane. The nerve endings have many types of inclusion. The most numerous of these are the electron-dense vesicles. Electron-lucent vesicles are also found.
dv - electron-dense vesicle; lv - electron-lucent vesicle; lvb - electron-lucent vesicle with bar. There are some nerve endings which contain an assortment of vesicles (indicated by the arrow).
Bar represents 1 μm.

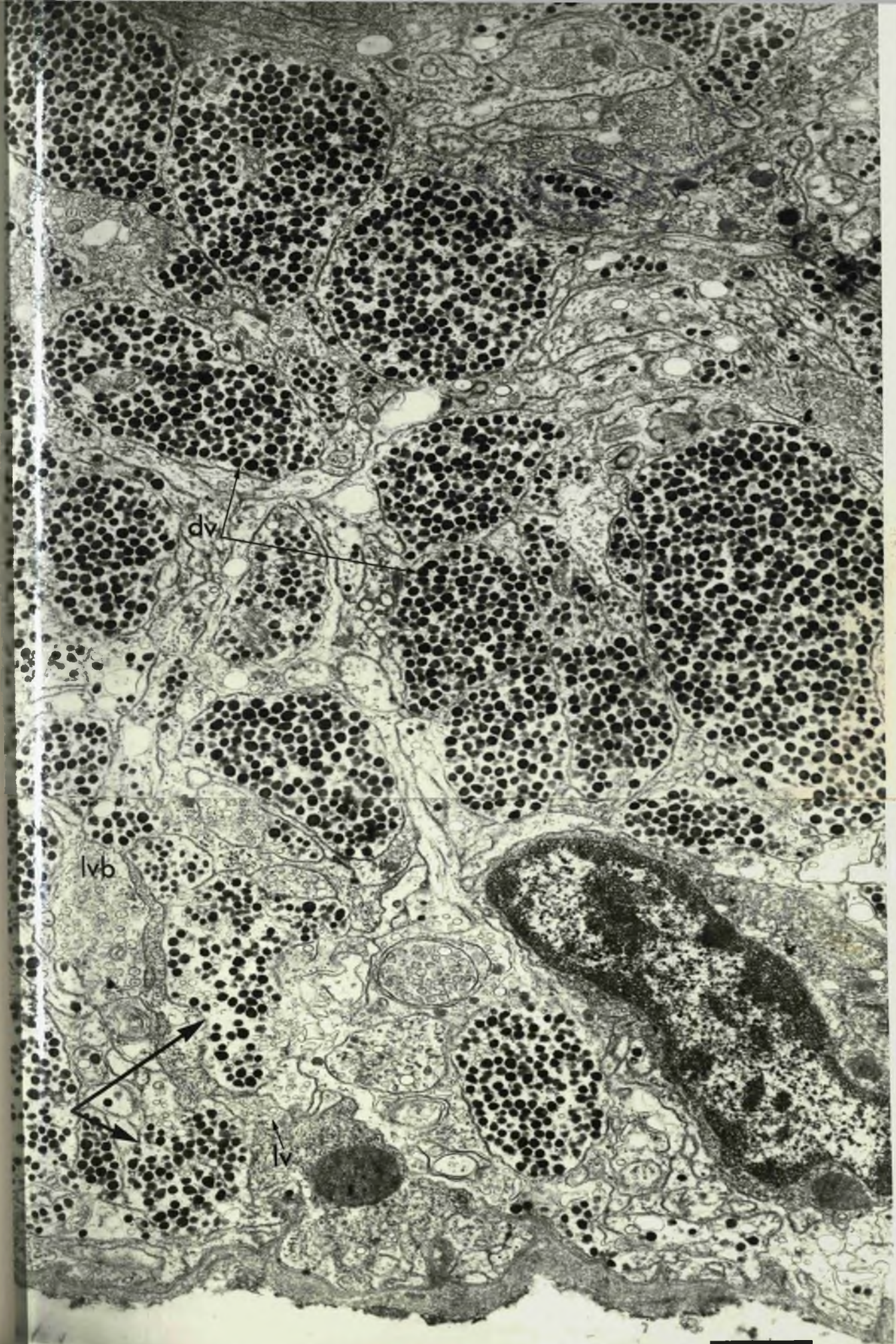

dv
lvb
lv

Figure 21. Electron micrograph showing other types of vesicle found within the nerve endings i.e. the less electr dense vesicle and the small electron-lucent vesicle Note that the small electron-lucent vesicles are found both in terminals containing electron-dense vesicles and in terminals containing electron-lucen vesicles. They are sometimes massed together close to the terminal membrane.
dv - electron-dense vesicle; en - endothelial cell; ldv - less electron-dense vesicle; lu - lumen of the vena cava; sv - small electron-lucent vesicle. Bar represents 1 μm.

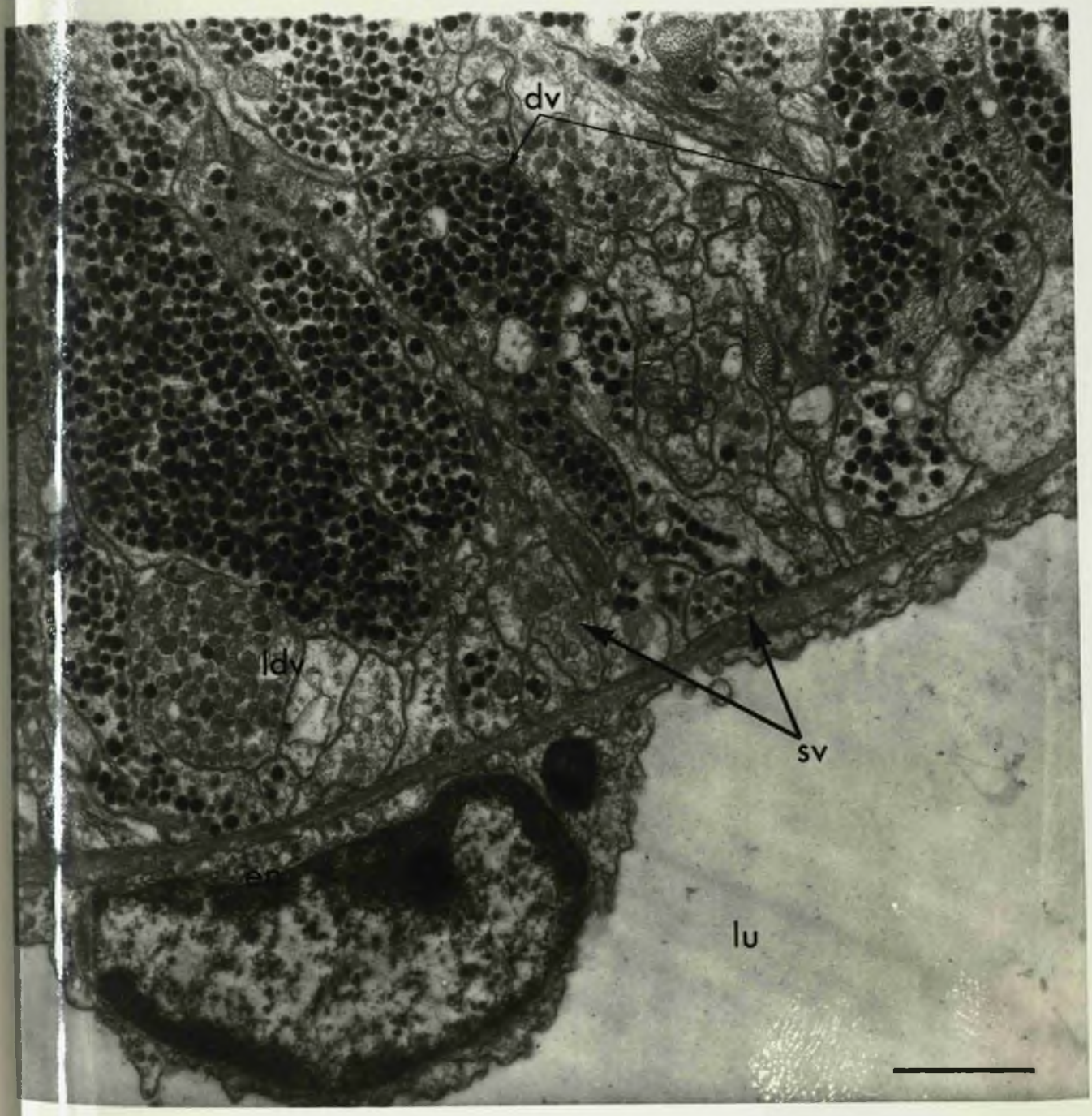

dv
ldv
sv
en
lu

Figure 22. Electron micrograph showing the large electron-dense particles which are located away from the basement membrane. As in the other micrographs large numbers of electron-dense vesicles are observed near to the lumen of the vessel.
dp - electron-dense particles; er - endoplasmic reticulum; lu - lumen of the vena cava; nf - neurofilaments.
Bar represents 1 μm.

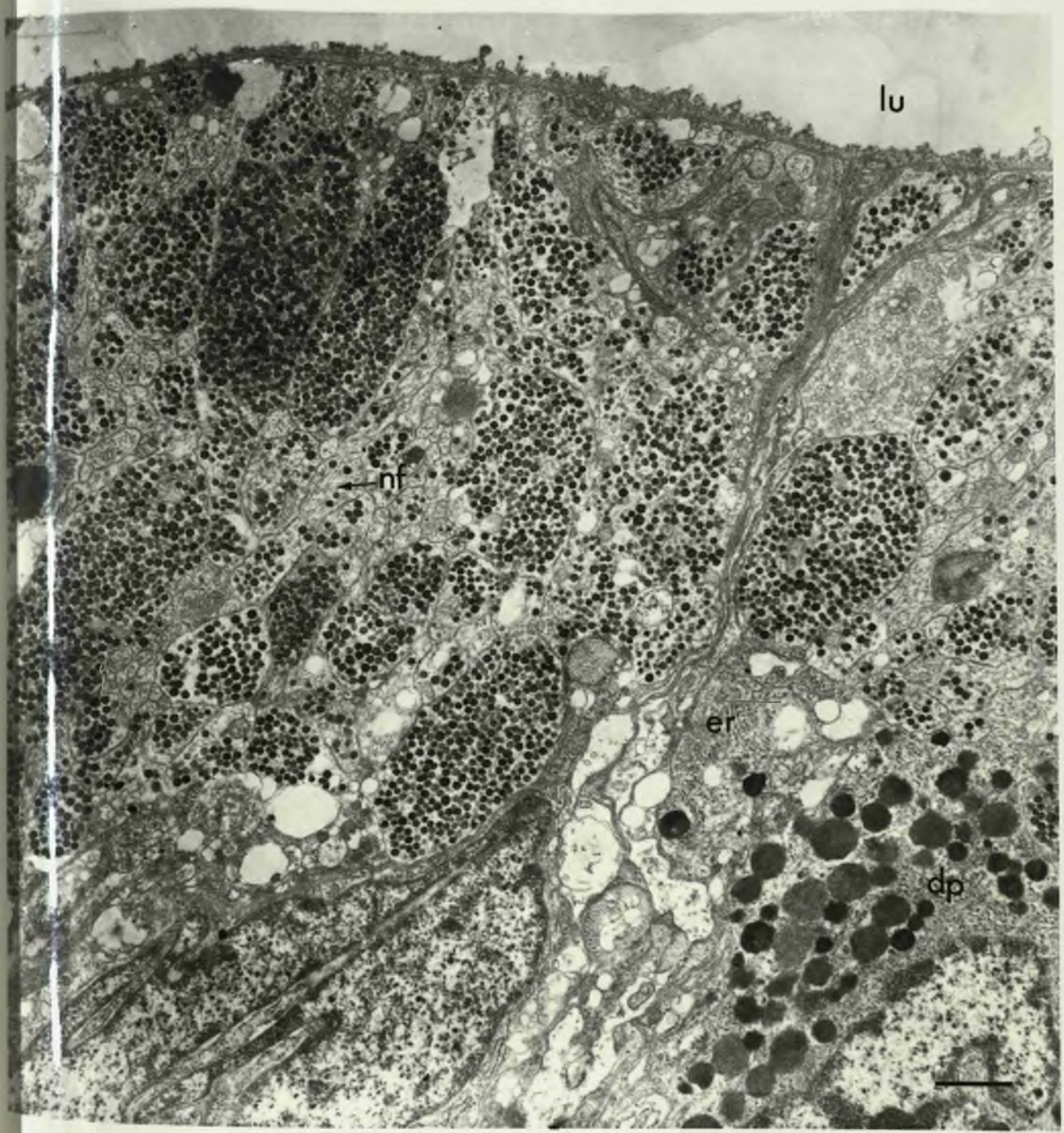

lu
nf
er
dp

Figure 23. Electron micrograph showing the muscular outer layer of the vena cava wall.
m - mitochondrion; mu - muscle.
Bar represents 1 μm.

23

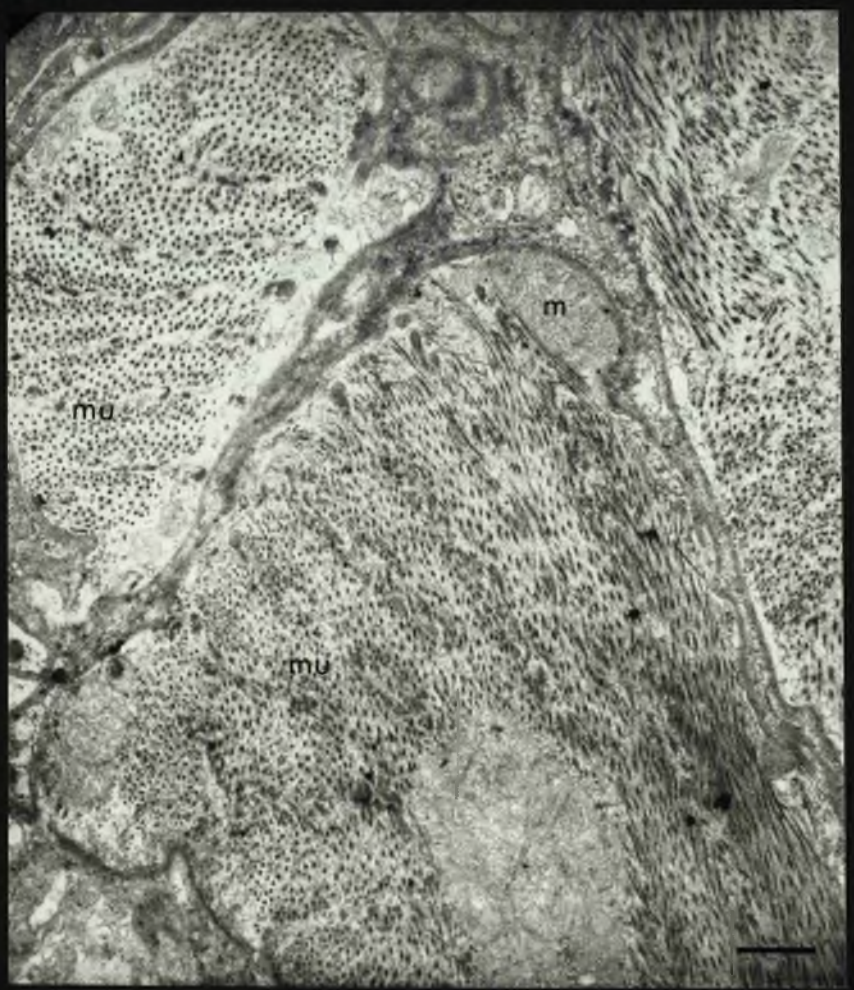


Figure 24. and
Figure 25.

Electron micrographs showing the appearance of the vena cava wall in the region of the vessel close to the brain. The most numerous inclusions are electron-dense vesicles. Other types of vesicle and electron-dense particles are also present.
dp - electron-dense particle; dv - electron-dense vesicle; ldv - less electron-dense vesicle; lv - electron-lucent vesicle; lvb - electron-lucent vesicle with bar.
Bar represents 1 μm.

25

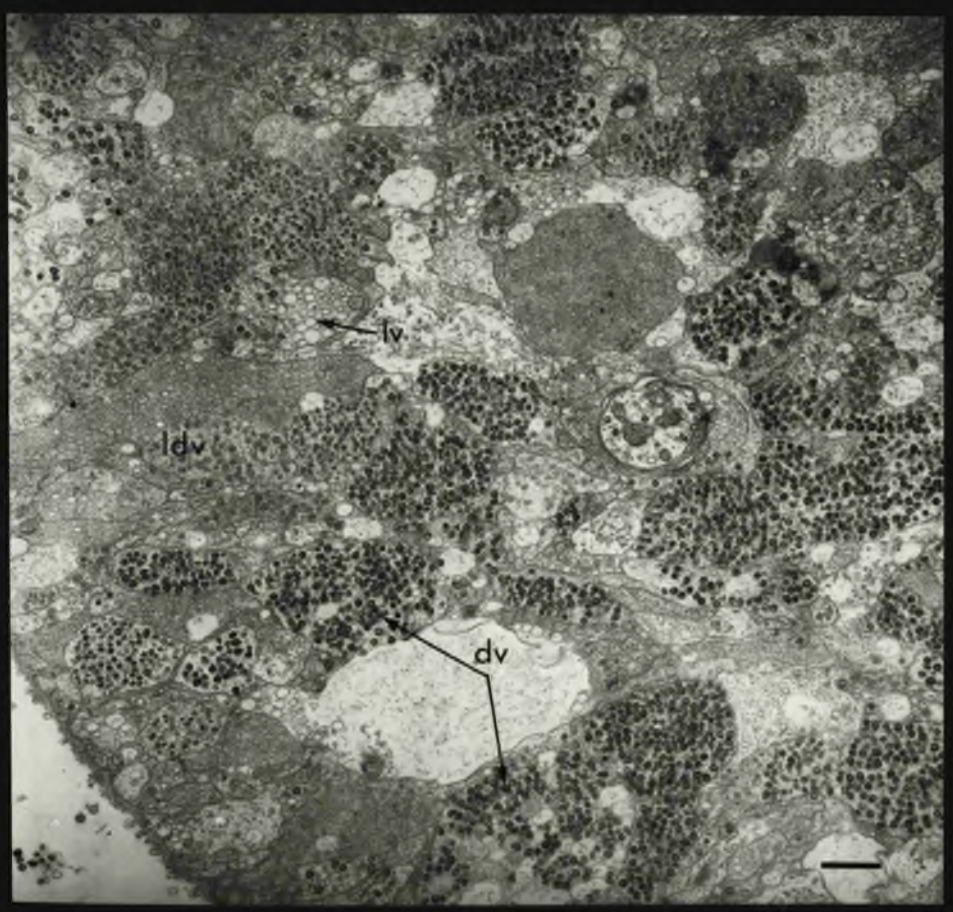
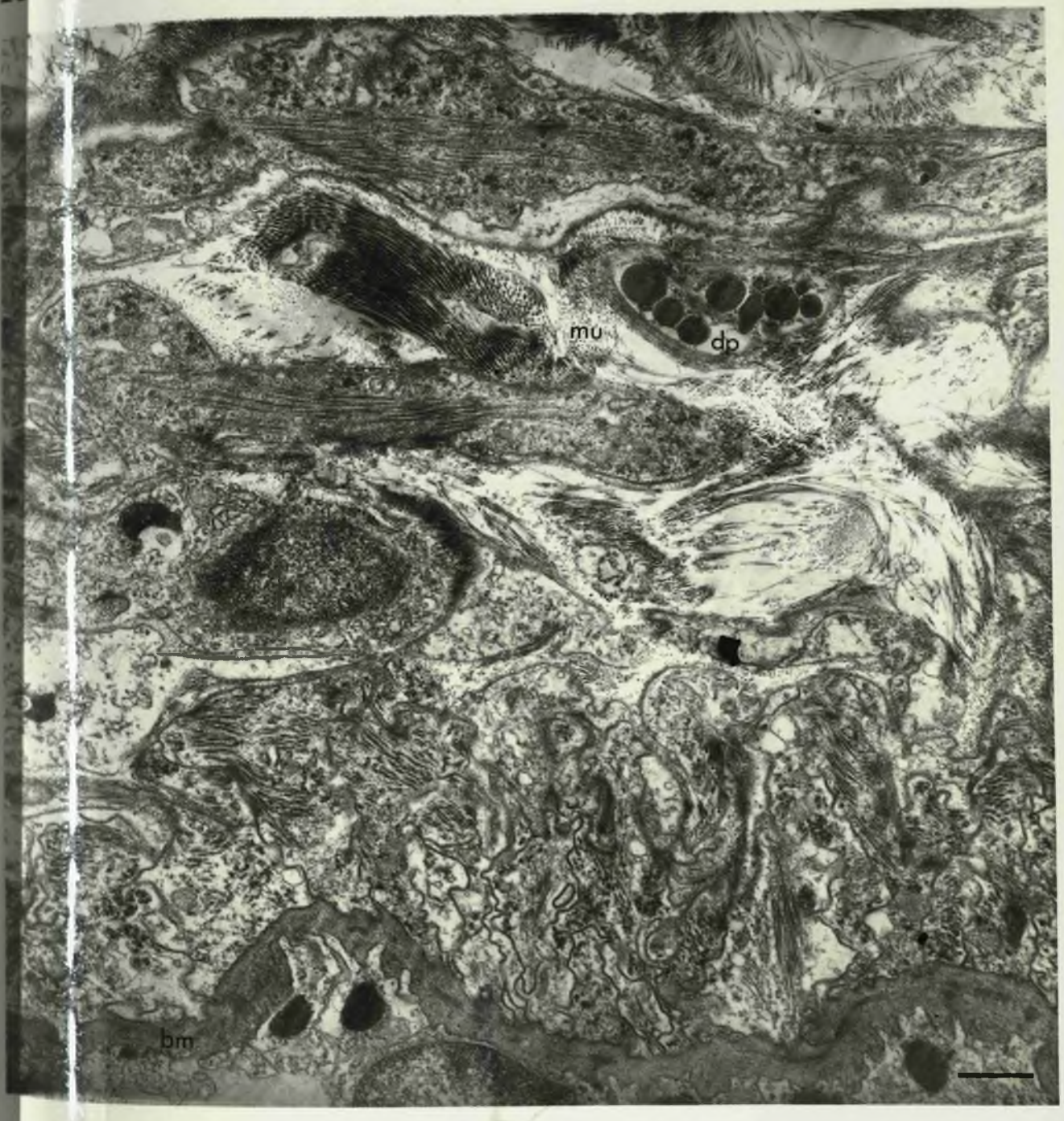


Figure 26. Electron micrograph showing the appearance of the vena cava wall in the posterior region of the vessel, below the area where ridging occurs. Note that there are no axons containing electron-dense vesicles although electron-dense particles do occ bm - basement membrane; dp - electron-dense particle; mu - muscle.
Bar represents 1 μm.

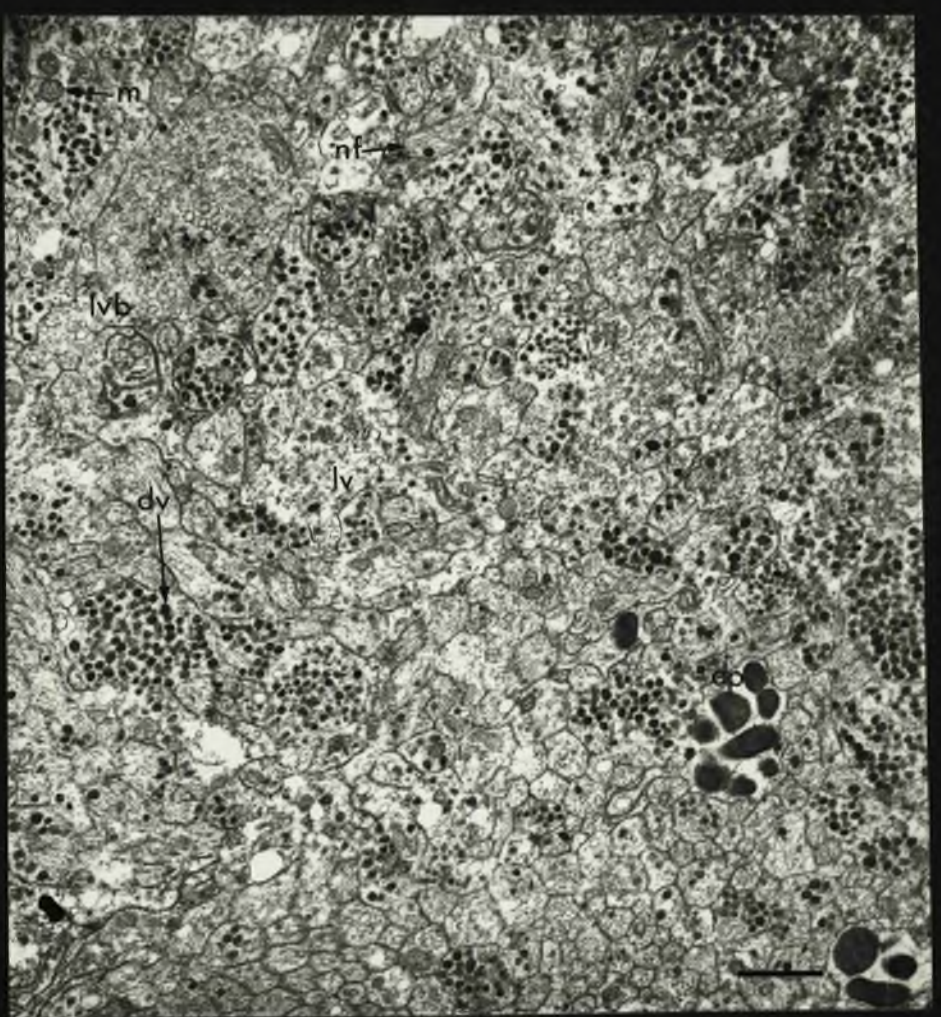

mu
dp
bm

Electron micrographs showing the organisation of the neurosecretory axons within the wall of the vena cava in the decapod *Sepia officinalis*.

Figure 27. Closely packed nerve-endings are found adjacent to the basement membrane of the blood vessel.
bm - basement membrane; ne - nerve-ending.
Bar represents 1 µm.

Figure 28. The nerve-endings contain many types of inclusion: electron-dense vesicles; electron-lucent vesicles with and without a central bar; neurofilaments and mitochondria. Electron-dense particles are also present but these are not contained within the axon.
dv - electron-dense vesicle; dp - electron-dense particle; lv - electron-lucent vesicle; lvb - electron-lucent vesicle with bar; m - mitochondrion; nf - neurofilament.
Bar represents 1 µm.

28



ISCUSSION

The present findings confirm and elaborate the observations n the system of nerves passing to the vena cava in E. cirrosa made by lexandrowicz in 1964. Neurosecretory cell bodies are present in the ayer covering the visceral lobe of the brain and in the neurosecretory runks passing out on either side of the vena cava. The majority of he neurosecretory axons pass to the vena cava and terminate adjacent o the basement membrane of the inner surface of the blood vessel. It as found that the extent of the nerve terminations corresponds with he area of ridging seen on the inner surface of the vena cava.

The neurosecretory nerves are extremely small. Their axons re less than 1 µm diameter (the size stated by Alexandrowicz) and ompare in size and general appearance with the axons of the amacrine racts of the vertical lobe of the brain of Octopus vulgaris, shown by ray (1970) to be between 0.2 µm and 0.3 µm diameter.

The most noticeable feature of the system examined in Eledone irrosa are the electron-dense vesicles. These vesicles are similar in ppearance to structures described in established neurosecretory systems oth in vertebrates and in invertebrates, for example in the neuro-ypophysis of the rat (Holmes, 1964); in the hypothalamo-hypophyseal ystem of the frog, Rana pipiens (Rodriguez and Dellman, 1970); in the orpora cardiaca of the insect, Leucophaea maderae (B. Scharrer, 1963) nd the pericardial organs of decapod crustacea (Knowles, 1962; Maynard nd Maynard, 1962). Vesicles of this type have also been described in roposed neurosecretory neurons of other molluscs, for examples see oroso, Baxter Chiquoine and Nisbet, 1964; Lane, 1964; Sakharov, rovyagin and Zs-Nagy, 1965; Simpson, Bern and Nishioka, 1966a;

Coggeshall, Kandel, Kupferman and Waziri, 1966; Nolte, 1968; Boer, Douma and Koksma, 1968; Simpson, 1969; Cottrell and Osborne, 1969 and Wendelaar-Bonga, 1970, 1971. Such structures, described by Bern (1962) as "elementary neurosecretory granules", are now considered to be present in all neurosecretory systems. However, it does not necessarily follow that all systems containing electron-dense granules are neurosecretory. In some cases it has proved difficult to distinguish neurosecretory granules from small phospholipoprotein droplets (Bern, 1966). Tombes (1970) has criticised the use of the term "granule" since he believes it carries the connotation of an inorganic inactive particle. I believe that vesicle is a more appropriate term since this may be used to describe the different types of structure normally found in neurosecretory systems. There has been much discussion about what the electron-density of these structures represents. It is generally assumed that the active substance due to be released from the neurosecretory nerve terminal is contained within the electron-dense vesicle. However in some cases the active substance is associated with a carrier substance or molecule; e.g. mammalian neurohypophyseal hormones are associated with the carrier neurophysin. Thus the electron-density of the vesicle may be due to the presence of the active substance, or to the presence of a carrier molecule, or to the presence of both.

Bargmann and Gaudecker (1969) have put forward evidence indicating that elementary neurosecretory granules from the neurohypophysis of the hedgehog are enveloped by a double membrane. They also showed that the core of the elementary granules is composed of small granular particles which they propose, represents the carrier substance (neurophysin) of the neurohypophyseal hormones. This is

interesting in view of the findings presented in this thesis. Although there was no indication that electron-dense vesicles were limited by a double membrane there was evidence that each vesicle was composed of small granular structures.

Bunt (1969) has described a "coated" vesicle, 100 nm diameter with a surface coat approximately 20 nm thick, present within the neurosecretory axon terminals of the sinus gland of the crayfish, Procambarus clarkii and Andrews, Copeland and Fingerman (1971) described "tailed" vesicles present in the sinus gland of Callinectes sapidus. No such vesicles were observed in the vena cava nerves.

The range of vesicle type present in the Eledone system is interesting. On morphological evidence alone it is impossible to determine the significance of this. The differences described may represent different stages in the formation of the neurosecretory product; it may represent completely different products contained within separate nerve endings, or it may represent the presence or absence of carrier substances. Variety of vesicle types has been noted in other neurosecretory systems. Maddrell (1967) has expressed the hope that work with single cells will help to elucidate some of these problems. In E. cirrosa vesicles appear to arise in the perikarya of the neurosecretory cells and are then passed along the axons to the terminals. This evidence, in conjunction with the observation that different vesicle types are contained within separate nerve endings, supports the hypothesis that more than one type of secretory product is present. The electron-dense particles found in the inner layers of the blood vessel and in the sheath separating the neurosecretory tract from that of the normal nerve, but not within the neurosecretory

tract itself, do not appear to be in the same category as the other vesicles described. From the present evidence, see Figs. 9, 22 and 26, they appear to be associated with connective tissue. The finding that the vesicles in the Eledone system are exactly comparable to those found in the neurosecretory system of the vena cava in Octopus vulgaris (see Martin, 1968 and Table 1) provides further evidence that these two systems are homologous.

TABLE 1. NERVE STRUCTURE. Types of vesicle present in the neurosecretory nerves of the vena cava.

Eledone cirrosa (from this thesis)		Octopus vulgaris (fr. Martin, 1968)
electron-dense 80 - 150 nm diameter	Type I.	electron-dense 80 - 200 nm diameter
less electron-dense 80 - 200 nm diameter	Type II.	less electron-dense up to 200 nm diameter
electron-lucent 80 - 200 nm diameter	Type III.	clear 100 - 200 nm diameter
electron-lucent with dense bar or spot 80 - 200 nm diameter	Type IV.	clear, with electron-dense bar or disc 100 - 200 nm diameter
electron-dense particle 0.3 - 2 μm diameter	Type V.	electron-dense particle 0.3 - 2 μm diameter
small, electron-lucent vesicles 25 - 50 nm diameter*	Also:	small vesicles of similar size to synaptic vesicles 30 - 50 nm diameter

*As Scharrer (1963) has pointed out exact measurements of diameter

are difficult to obtain since many of the estimations are made on tangential sections through vesicles whose actual size is larger than the measured values. Sizes given in Table 1 are maximum observed diameters.

In E. cirrosa the association of the electron-dense vesicles with the Golgi apparatus in the perikarya of the neurosecretory cells indicates this either as a site of synthesis or packaging of the secretory product. However from this morphological evidence it is impossible to determine whether this product is the neurosecretory material itself or a carrier substance, or both.

There is no apparent increase in the size of the vesicles in their passage along the axons to the nerve terminals. Knowles (1964) has suggested that increase in vesicle size in the terminal regions of specific neurosecretory systems indicates that in these systems local synthesis of the neurosecretory product occurs at the terminations. No such local synthesis at the nerve terminals appears to be occurring in E. cirrosa.

The nerve terminations within the wall of the vena cava of E. cirrosa are enlarged in comparison with the size of the axons contained in the neurosecretory trunks. Such sites of storage of a neurosecretory product which are intimately associated with the blood system have been defined as neurohaemal organs (Knowles and Carlisle, 1956). Boer, Douma and Koksma (1968) and Golding (1970) have stated that the presence of a neurohaemal organ is the morphological feature whose presence conclusively establishes neurosecretory status.

Some of the Eledone neurosecretory axons terminate in between the septal muscles. These axons also contain electron-dense vesicles

similar to those present in the blood vessel terminations. The possible function of an active substance secreted into muscle tissue is obscure. Other examples of neurosecretory nerves terminating in muscle have been recorded in the aphid, Myzus persicae (Johnson, 1963; Bowers and Johnson, 1966) and in a mollusc, Helix pomatia (Scholte, 1963). It is interesting to note that Johnson (1963) remarked that in the aphid a characteristic of these muscles, which are probably influenced by the neurosecretory material, is that they undergo rhythmic contractions as part of their normal activity in the intact animal. This observation also holds for the septal muscles in Eledone which are involved in regular respiratory movements. Whether these observations have any significance is an open question.

The structure of the vena cava wall was found to be similar to that of other cephalopod blood vessels (see Barber and Graziadei, 1965, 1967a, 1967b). The very limited number of endothelial cells observed suggests that these cells form only an incomplete inner lining for the vena cava. Examination of the vena cava in Octopus vulgaris using the scanning electron microscope paralleled these observations and showed that the endothelial cells are arranged in longitudinal rows along the axis of the vessel. These rows are separated by spaces where the basement membrane of the vessel is directly exposed to the blood stream (Martin, Barber and Boyde, 1968). In contrast the basement membrane forms a continuous layer on the inside of the blood vessel. The incomplete endothelial layer may allow the passage of substances released from the nerve terminals into the blood stream since where the endothelial cells are absent the only barrier is the basement membrane which is 100 - 300 nm thick. The ridging of the inner surface of the vessel increases the surface area of the blood vessel and thus increases the area available as a site of release.

In only a small amount of the material examined was any structural evidence of release at the basement membrane noted e.g. fusion of membranes, large accumulation of microvesicles, see Fig. 21. However, at no time was there any evidence of gross changes in the appearance of the system. There were no seasonal differences to indicate the presence of cyclic activity, no differences between young and adult animals indicating the presence of a factor influencing growth, and no differences between male and female Eledone indicating the presence of a factor influenced by the sex of the animal. However the absence of evidence does not necessarily mean that a change has not taken place. When using the electron microscope to make comparisons of material it must be remembered that the sampling area is extremely small and therefore only very gross changes will be detected. As Highnam (1965) has argued the appearance of a neurosecretory system may remain the same although the secretory activity is altered, for example the rate of release may increase but if the rate of synthesis also increases so that synthesis rate equals release rate, the overall appearance of the system will remain the same. It may well be that the wrong criteria have been applied to the system for the detection of any release that has occurred.

The results obtained with Sepia officinalis indicated that the fine structure of the neurosecretory system of the vena cava in decapods is exactly comparable to that described for octopods.

The evidence obtained with the electron microscope has demonstrated that in the proposed neurosecretory system of the vena cava of Eledone cirrosa a secretory product (or products) is synthesised in the perikarya of the neurosecretory neurons, is passed along the

axons and is stored in nerve terminations which are closely associated with the blood stream. This compares directly with the "classical" concept of the organisation of a neurosecretory system. However although the morphological evidence is convincing it does not fulfill all the requirements of the definition of a neurosecretory system. It is necessary to determine the functional characteristics of the system. The experiments carried out in order to determine these characteristics are described in the succeeding sections.

Examination of the morphology of the nerves indicated that ...stance was being manufactured in the soma and passed along the ... to the nerve terminals. The position of the nerve endings indi... that the product might be released into the blood stream and ... then be distributed to affect any organ within the body. In ...icular the substance might affect structures within the central ...ular system i.e., the renal appendages, the branchial hearts, the ...dia or the systemic heart. It was important therefore to determine ... a pharmacologically active substance was associated with ... terminations. Such evidence would support the hypothesis that ... nerve endings were a site of release.

SECTION 3

Experiments were carried out to determine whether any ...macological activity was associated with the nerves that ...tona cirrosa.

The activity of extracts of various tissues and ... subsequently the specificity of the activity ... cava was determined. The main assay ... isolated systemic heart of Sepia ... used by workers as early as the beginning ... (See Fry, 1909, and others). Yonge ... action of the effects of ... central vascular system. It has also been used ... ligations (Finge and Parlund, 1954). In an ... suitable assay preparation the sensitivity ... of E. cirrosa to the active substance was ...

PHARMACOLOGICAL STUDIES

Examination of the morphology of the nerves indicated that a substance was being manufactured in the soma and passed along the axons to the nerve terminals. The position of the nerve endings indicated that the product might be released into the blood stream and would then be distributed to affect any organ within the body. In particular the substance might affect structures within the central vascular system i.e., the renal appendages, the branchial hearts, the ctenidia or the systemic heart. It was important therefore to determine whether a pharmacologically active substance was associated with the nerve terminations. Such evidence would support the hypothesis that the nerve endings were a site of release.

Experiments were carried out to determine whether any pharmacological activity was associated with the anterior vena cava of Eledone cirrosa.

The activity of extracts of various blood vessels was compared and subsequently the specificity of the activity within the wall of the vena cava was determined. The main assay preparation used was the perfused, isolated systemic heart of Eledone cirrosa. This preparation had been used by workers as early as the beginning of the present century (see Fry, 1909, and others, Fredericq & Bacq, 1939) in the investigation of the effects of electrical stimulation of various nerves on the central vascular system. It has also been used in pharmacological investigations (Fänge and Østlund, 1954). In an attempt to determine the most suitable assay preparation the sensitivity of other preparations of E. cirrosa to the active substance was also determined.

Once it was established that a specific activity was associated with the vena cava tissue it was of interest to characterise its pharmacological effects. Therefore the activity of vena cava extracts was monitored on Eledone preparations, other molluscan preparations and some mammalian preparations.

The activity of the vena cava extracts was compared with another potent pharmacological substance obtained from the tissues of Eledone sp. known as Eledoisin. This is an active endecapeptide isolated from methanol extracts of the posterior salivary glands (Anastasi and Erspamer, 1962; Erspamer and Anastasi, 1962). This substance has been shown to have a very potent stimulant action on a wide variety of pharmacological preparations (Erspamer and Erspamer, 1962).

Finally, experiments were carried out to determine whether the activity in vena cava extracts was due to 5-hydroxytryptamine or a catecholamine. These substances are known to be present in cephalopod tissues (Welsh and Moorhead, 1960; Cottrell and Laverack, 1968; Juorio, 1970) and are known to excite the isolated cephalopod heart. In 1952 Bacq, Fischer and Ghiretti determined the effect of 5-hydroxytryptamine on the isolated median ventricle of Octopus vulgaris and showed that the organ was extremely sensitive to this indolalkylamine and in 1954 Fänge and Østlund demonstrated that adrenaline, noradrenaline and dopamine all have an excitatory effect on the isolated ventricle of Eledone cirrosa. The effect of the vena cava extract was compared with the effect of standard solutions of these known substances on the isolated heart preparation of E. cirrosa. In recent years very sensitive methods for the detection of 5-hydroxytryptamine and catecholamines have been

developed. These are the spectrophotofluorimetric method of Bogdanski, Brodie and Udenfriend (1956) for detecting 5-hydroxytryptamine and the fluorescence histochemical method of Falck (see Corrodi and Jonsson, 1967) for detecting 5-hydroxytryptamine and catecholamines. It was thought pertinent to examine the vena cava tissue using these methods.

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MATERIALS

Animals

Specimens of the octopod Eledone cirrosa, the lesser octopus, were obtained and kept in the laboratory until required as described on p. 11.

Specimens of Helix aspersa, the garden snail, were collected locally. Animals were either used immediately or kept at 20°C in moist grass with slices of carrot as food.

Specimens of Mya arenaria, the clam, were also collected locally and were maintained in a supply of continuously circulating sea-water.

Specimens of albino Wistar rats were obtained from Ralph Tuck & Sons.

Specimens of New Zealand white rabbits were obtained from Ralph Tuck & Sons.

Specimens of cats, of no specific variety, were obtained from local sources.

Preparation of vena cava extracts

Extracts were normally prepared immediately after isolating the vena cava tissue. Occasionally, in order to obtain sufficient material, tissue was collected from several animals over a period of several days. In such cases the tissue was frozen and stored at -20°C before extraction. The following extraction procedure was adopted after preliminary experiments had indicated that the method was efficient

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(see Berry, 1968). In the early experiments extracts were made from the whole vessel. Later it was found that the inner ridged layer of the blood vessel contained far greater activity than the outer coat. Therefore the inner layer material only was used in the preparation of subsequent extracts.

Extraction procedure: Animals were killed by "decapitation" and the vena cava was isolated onto an ice-cold wax dish. Either the whole vessel was extracted or the inner layer was scraped off using a cooled metal spatula. A known weight of tissue was placed in a glass homogeniser containing ice-cold 50% acetone in distilled water. The tissue was homogenised (at 1000 rpm) for two one-minute periods. Extracts were then heated for 5 minutes on a boiling water bath. Insoluble material was removed by centrifugation. Supernatant solutions were evaporated to dryness under reduced pressure and the extracts were taken up in sea-water to provide the required concentration of material. Normally this was a concentration of 10 mg/ml, where the weight referred to is the weight of the original material taken for extraction. In certain cases, stated in the text, extracts at a concentration of 100 mg/ml were prepared.

Extracts of the abdominal aorta (which overlies the vena cava for part of its length), the efferent branchial vessel (which passes from the ctenidia to the systemic heart) and the visceral nerve (which lies alongside the medial neurosecretory nerve trunks for part of their length) were prepared in a similar manner and were used as controls. Extracts of specific parts of the vena cava were also prepared. All extracts were stored at -20°C until required.

I. ASSAY OF EXTRACTS ON ELEDONE PREPARATIONS

EXPERIMENTAL PROCEDURE

Assay Preparations

An attempt was made throughout to use animals of consistent size. However since the supply of animals was sometimes spasmodic this was not always possible.

a) Isolated systemic heart

The heart was isolated as described previously by Berry (1968) and Berry and Cottrell (1970). The head and tentacles of the animal were removed and the mantle and visceral sac opened from the ventral surface. The renal appendages and pericardium were carefully removed and the blood vessels to the systemic heart exposed. The gonadal artery, abdominal aorta and anterior aorta (according to Isgrove, 1909) were ligatured and cut close to the heart. The left efferent branchial vessel was cut and a piece of thread attached to one side of the vessel. This incomplete ligature was to allow passage of the perfusing fluid through the heart. A glass cannula mounted in a rubber tube, was inserted into the right efferent branchial vessel, pushed into the cavity of the ventricle and fastened in position. The dissection was carried out under sea-water and was completed within 10 - 15 minutes. The cannula was either attached to a perfusion system, as illustrated in Figure 31, or was mounted in an organ bath, containing 10 ml of sea-water, as shown in Figure 32. In both cases the heart was perfused with oxygenated sea-water at a rate of 10 ml/min and at a constant head of pressure. Isotonic recordings were made using a kymograph. Each preparation was allowed to equilibrate for a period of thirty minutes

Figure 31. Diagram of the perfusion system used for the isolated systemic heart preparation of Eledone cirrosa. a - constant pressure head; b - inlet valve for sea-water (sw); c - isolated heart; d - kymograph lever.
(Injections into the perfusion medium were made at "a")

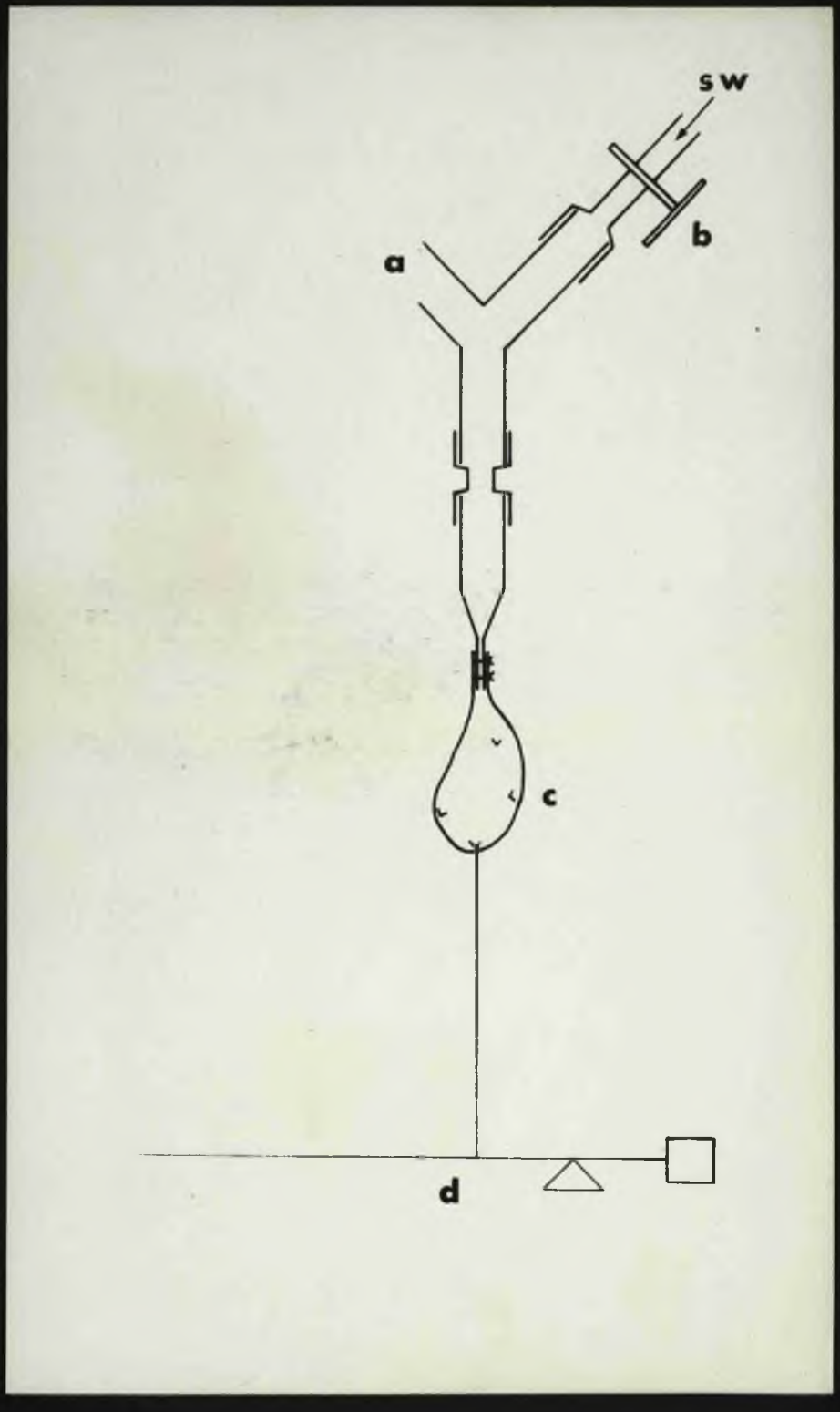
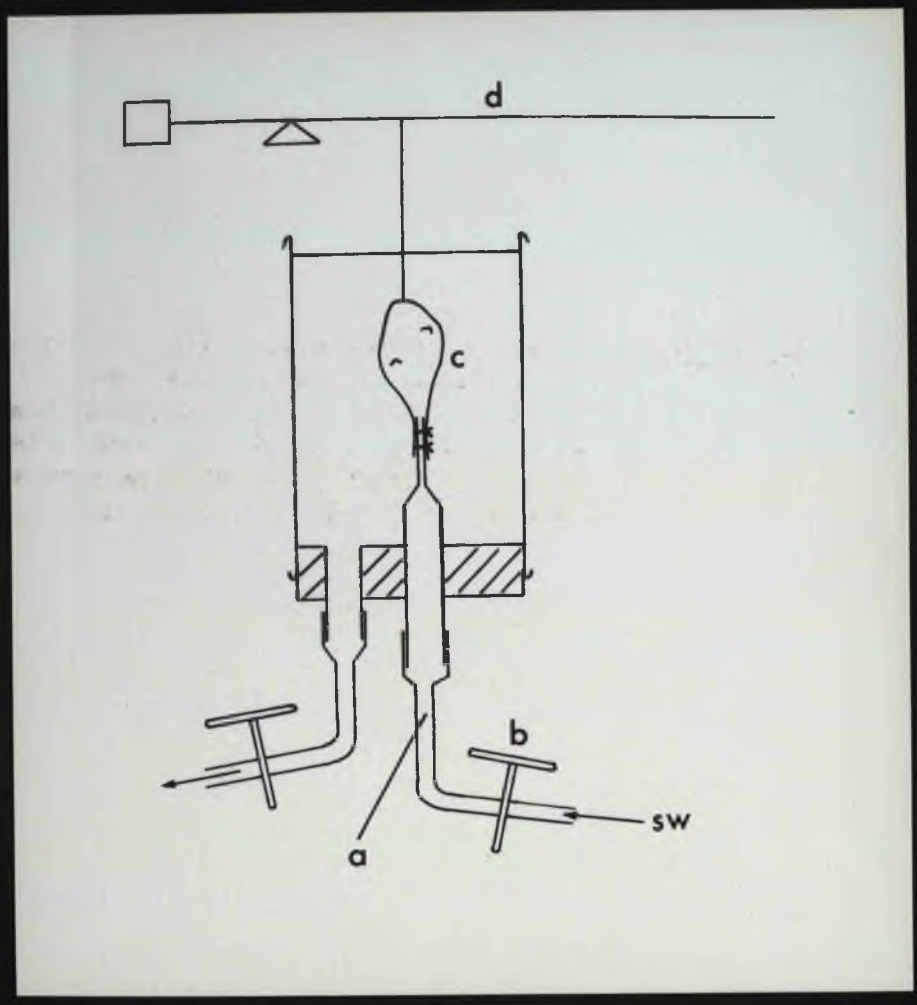

SW
a
b
c
d

Figure 32. Diagram of alternative perfusion system used for the isolated systemic heart preparation.
a - injections into perfusion medium made at this point through the rubber tubing; b - inlet valve for sea-water at constant head of pressure; c - isolated heart; d - kymograph lever.


d
c
b
a
sw

fore the start of an experiment. All experiments were carried out
room temperature (18 - 22°C).

Isolated branchial heart

The animal was killed and the contents of the visceral sac
splayed as previously described. The branchial heart and the afferent
d efferent branchial blood vessels were exposed. The afferent
anchial vessel was cut close to the heart and a piece of thread was
tached to one side of the vessel. As in the case of the isolated
stemic heart this incomplete ligature was to allow passage of the
rfusing fluid through the heart. A glass cannula mounted in a rubber
be was inserted into the efferent branchial vessel, pushed into the
vity of the heart and was tied in position. The preparation was
unted in an organ bath containing 10 ml sea-water (see Figure 32).
constant head of pressure of 5 - 10 cm water was maintained throughout.
e thread tied to the afferent branchial vessel was attached to a
art lever and isotonic recordings were made using a kymograph. The
eparation was perfused with sea-water and allowed to stabilise for
minutes before the start of an experiment. All experiments were
rried out at room temperature.

Isolated tube of vena cava

A length of vena cava 2 1/2 cm long was removed from a position
mediately anterior to where the vessel divides and passes to the renal
pendages. (This is a region of the vessel where there is no "neuro-
cretory" neuropile present.) A glass cannula mounted in a rubber
be was inserted into one end of the vena cava tube and tied into
sition. A piece of thread was attached to the other end of the

vena cava tube. The preparation was mounted in a similar manner to that described for the isolated branchial heart preparation and isotonic recordings were made using a kymograph. The preparation was perfused with sea-water and allowed to equilibrate over a period of 30 minutes. All experiments were carried out at room temperature.

Perfusing medium

Well-oxygenated, filtered sea-water was used as the perfusing fluid (after Fredericq and Bacq, 1939; Erspamer and Ghiretti, 1951; Fange and Østlund, 1954). (Fry (1909) mentions use of a modified Ringer solution but this caused abnormal acceleration of the isolated heart. He eventually used a mixture of this Ringer and sea-water. Other workers had used sea-water alone satisfactorily and this method was adopted for the present experiments.)

Assay

Usually extracts were applied to assay preparations in 0.1 ml doses using a hypodermic syringe. Each dose was injected into the perfusion system, a short distance from the heart, at the point indicated in Figs. 31 and 32. The injected solutions were therefore diluted before reaching the heart and were rapidly washed through the preparation. Such a procedure did not allow the determination of an absolute threshold for a given substance. However it did permit a comparison of the effects caused by different extracts.

The effect of extracts of the vena cava on the isolated systemic heart was compared with the effect of extracts of other blood vessels. The effect of extracts of different regions from the same vena cava was also ascertained. The activity of vena cava extracts on each of

the following preparations: the isolated systemic heart, the isolated branchial heart, and the isolated vena cava, was compared in an attempt to determine the most sensitive and thus the most suitable assay preparation. All experiments were repeated on several preparations.

RESULTS

In these experiments the normal frequency of the beat of the isolated systemic heart of Eledone cirrosa was found to range between 14 and 22 beats per minute. The preparation was found to be very sensitive to any changes in pressure.

The vena cava extract, whether prepared from the whole vessel or the inner layer scrapings, was found to have a pronounced cardio-accelleratory property, see Fig. 33. This response was normally accompanied by an increase in the amplitude of heartbeat and was particularly prolonged, frequently lasting 10 to 20 minutes. Sometimes the response appeared to consist of two parts, an initial transient increase in frequency and amplitude followed by a longer lasting increase in activity. These changes were sometimes accompanied by an increase in tone. It was noted that if extracts were not boiled during the extraction procedure the excitatory activity was not retained. This occurred even if the non-boiled extracts were frozen although loss of activity was accelerated at room temperature. Storage of boiled extracts at -20°C for periods of several weeks did not affect the activity of the extracts.

Greatest activity was shown by extracts prepared from the region of the vena cava which was most densely innervated. In one series of experiments entire anterior venae cavae were removed and cut into four

pieces of equal lengths: an anterior length (closest to the brain), two mid-portions and a posterior length (closest to the heart). Extracts were prepared from each portion and the activities assayed on the isolated heart (Figure 34). It was found that in every case greatest activity was associated with extracts of the anterior region, i.e. the portion of the vessel where methylene blue staining revealed the most extensive supply of nerves. The region of the vessel nearest the heart showed no activity whereas the middle regions of the vessel only showed slight activity. In addition, extracts from the inner layer of the anterior region of the vena cava were found to have ten times the activity of the outer layers of the vessel.

On some occasions the isolated systemic heart would not beat. However the addition of vena cava extract would initiate beating, the amplitude and frequency of beat increasing with the concentration of the extract, see Figure 35.

It was found that repeated additions of the extract led to loss of sensitivity of the heart preparation and therefore it was not possible to obtain a dose-response relationship. Also, concentrated extracts were found to cause arrest of the heart, see p. 63.

The branchial heart preparation was more difficult to set up and maintain than that of the systemic heart. Two different patterns of beating were observed (Figure 36). One was irregular in the amplitude of beat although the frequency of beat was regular while the other was regular both in amplitude and frequency of beat. The vena cava extract caused a pronounced increase in tone of both patterns of beating but did not appear to affect the frequency of heart beat. Comparison of the same extract applied successively to a branchial heart preparation

and a systemic heart preparation of the same specimen of E. cirrosa indicated that the latter was a much more sensitive preparation, see Figure 37.

The vena cava is normally pulsatile. Addition of vena cava extract increased the amplitude and frequency of these pulsations, see Figure 38.

Extracts of the abdominal aorta and efferent branchial vessel caused an increase in amplitude of beat of the isolated systemic heart. This increase was not as marked as that shown by the vena cava extract and was much shorter in duration. There was no attendant increase in frequency of heart beat (Figure 39).

COMMENT

In this study the frequency of beat of the isolated systemic heart of Eledone cirrosa was found to be lower than that recorded for a similar preparation by Fänge and Østlund, (1954) i.e. 14 - 22 beats per minute compared with 24 - 26 beats per minute. However the present observations did correspond to observations made by Fry (1909). Such differences are probably due to changes in experimental conditions e.g. temperature.

The shape of the response given by the systemic heart preparation to the addition of vena cava extracts - an initial transient increase in amplitude followed by a more prolonged increase in frequency of beat - suggests that there may be more than one active substance present in the extracts.

The activity associated with vena cava extracts was shown to be very specific. Activity was only present in extracts of tissue

which had been shown to contain terminals of the "neurosecretory" nerves.

The observation that vena cava extracts initiated activity in quiescent preparations resembles results obtained with other molluscan tissue extracts. As quoted in Agarwal, Ligon and Greenberg (1972) Jullien, Ripplinger and Joly (1956) and Meng (1960) found that substances extracted from the brain and heart of Helix pomatia initiated activity in hypodynamic hearts of the same species. Also, Agarwal and Greenberg (1969) reported that a cardioactive substance from ganglion extracts of the bivalve Amblema neisleri caused quiescent hearts of another bivalve species, Megalonias bokyiniana to beat.

The finding that activity was not retained by non-boiled extracts suggests that without heating enzymatic breakdown of the active substance occurs. Similar observations were made by Maynard and Welsh (1959) on extracts from crustacean pericardial organs.

Figure 33. The effect of vena cava extract (VC) on the isolated systemic heart of Eledone cirrosa. The arrow indicates the time of injection of the extract into the perfusion medium. The trace has been split into two and the lower trace follows on immediately from the upper trace.

Normal beating frequency: 15 beats per minute; beating frequency 1 minute after addition of extract: 28 beats per minute.
a) 30 min. after injection of extract.
b) 120 min. after injection of extract.

The blue arrow indicates the direction of contraction.

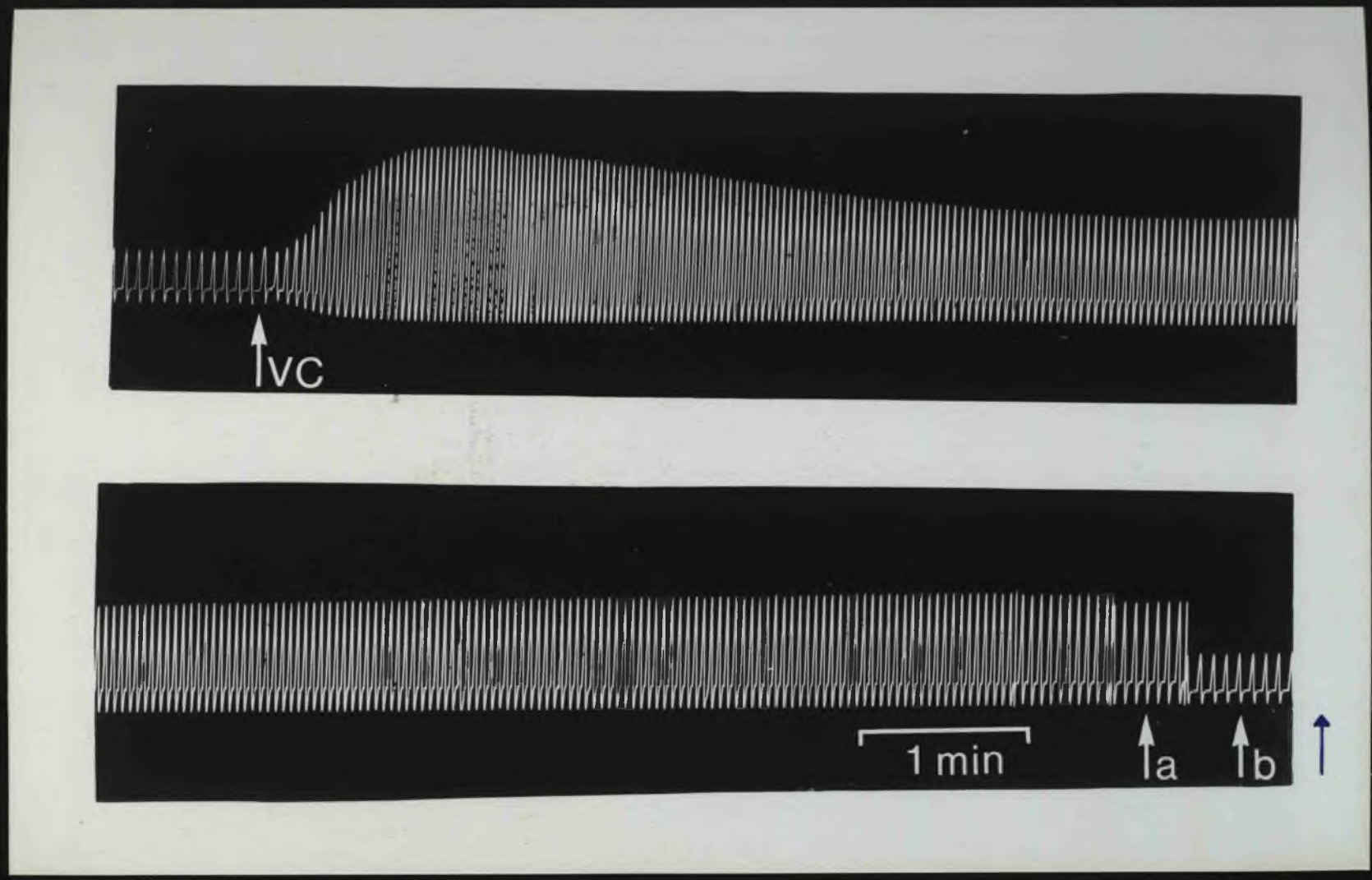

vc
1 min
a
b

Figure 34. The activity of extracts of different regions of the anterior vena cava assayed on the isolated systemic heart of Eledone cirrosa. The two regions of the blood vessel closest to the heart (H_I and H_{II}) showed no activity whereas the middle region of the vessel showed a slight increase in amplitude and frequency (H_{III}). Greatest activity was always shown by the anterior region of the blood vessel (B). The line indicates the time of injection of the extract into the perfusing medium.

The blue arrow indicates the direction of contracti

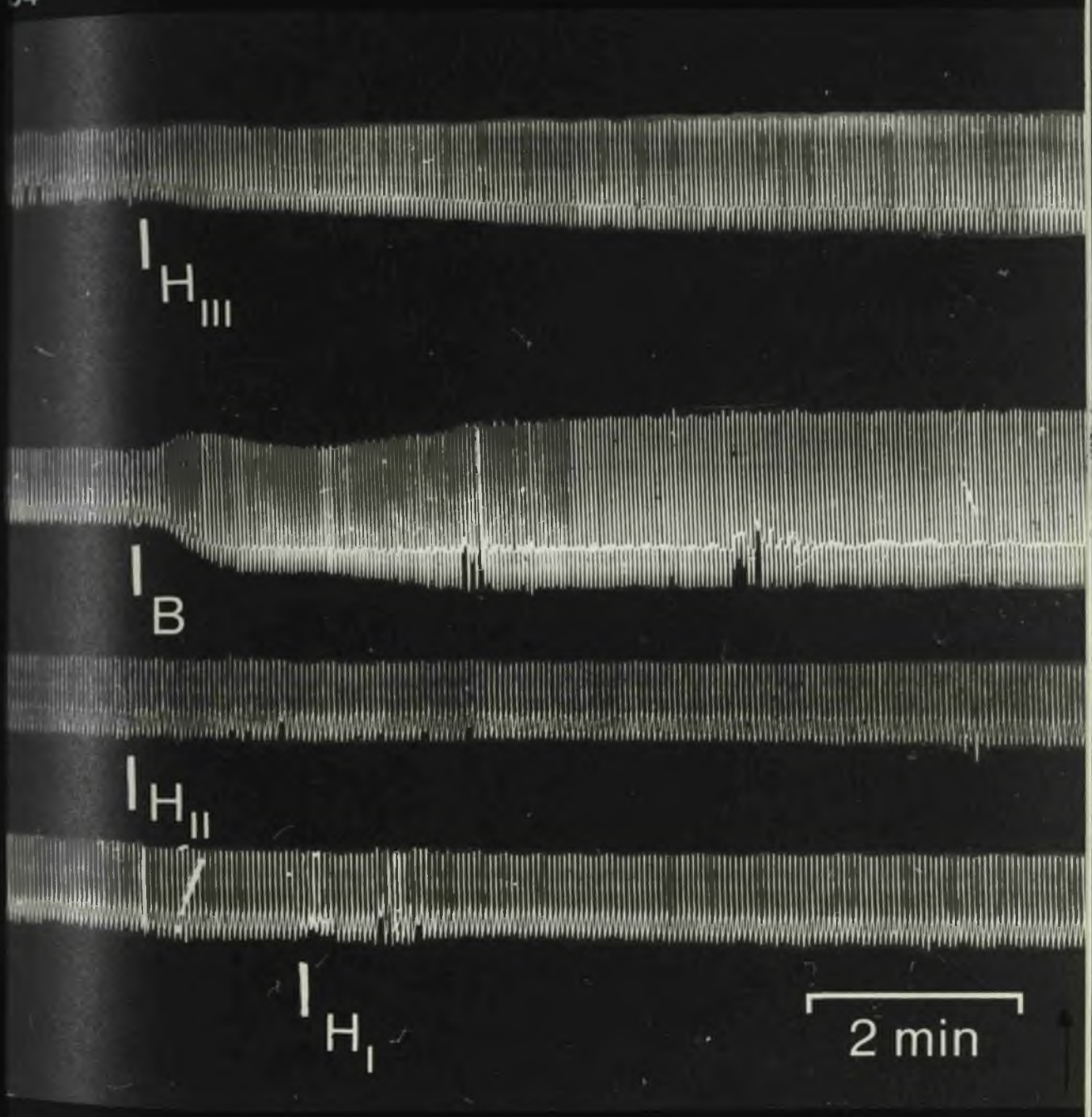

$I_{H_{III}}$
I_B
$I_{H_{II}}$
I_{H_I}
2 min

Figure 35. The effect of vena cava extract on a non-beating isolated systemic heart of Eledone cirrosa. Note that the sensitivity of the assay preparation decreases with time.

A. Extract concentration is 1/10 standard vena cava extract.
B. Extract concentration is 1/10 standard.
C. Extract concentration is 1/5 standard.
D. Extract concentration is 1/2 standard.
E. Extract concentration is 1/5 standard.
F. Extract concentration is 1/10 standard.
G. Extract concentration is 1/5 standard.

The arrows indicate the time of injection of the extract into the perfusing medium. All injections were in 0.1 ml aliquots. Time mark represents 1 minute.

The blue arrow indicates the direction of contracti

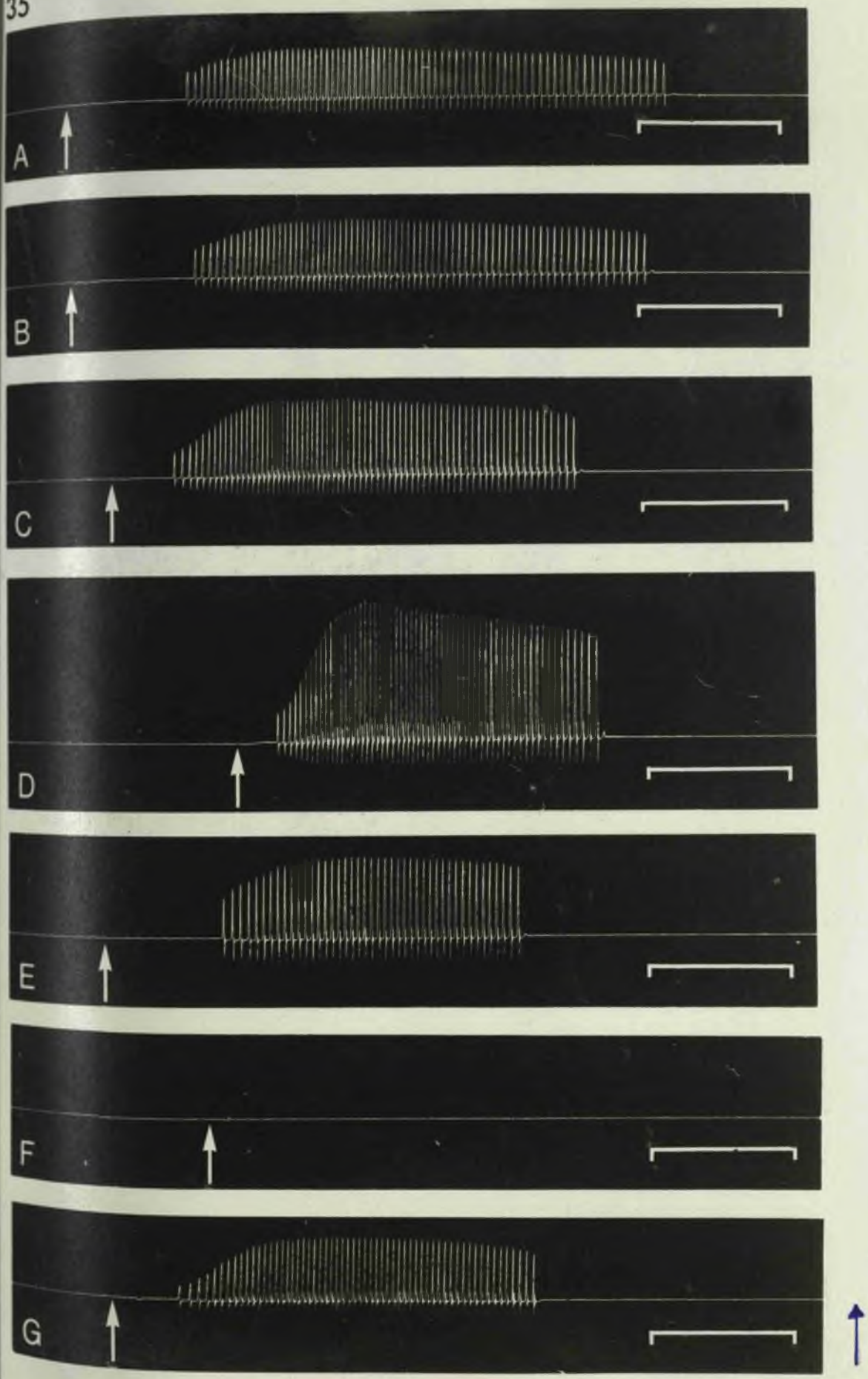

A
B
C
D
E
F
G

Figure 36. Two different rhythms of heartbeat were noted in the resting condition of the isolated branchial heart preparation of Eledone cirrosa; an irregular rhythm as shown in the top trace and a regular rhythm as shown in the bottom trace. Addition of vena cava extract to the perfusing medium, indicated by the arrowheads, caused a pronounced increase in tone but had little effect on the amplitude or frequency of heartbeat. Time mark represents 1 minute.

The blue arrow indicates the direction of contraction.

36

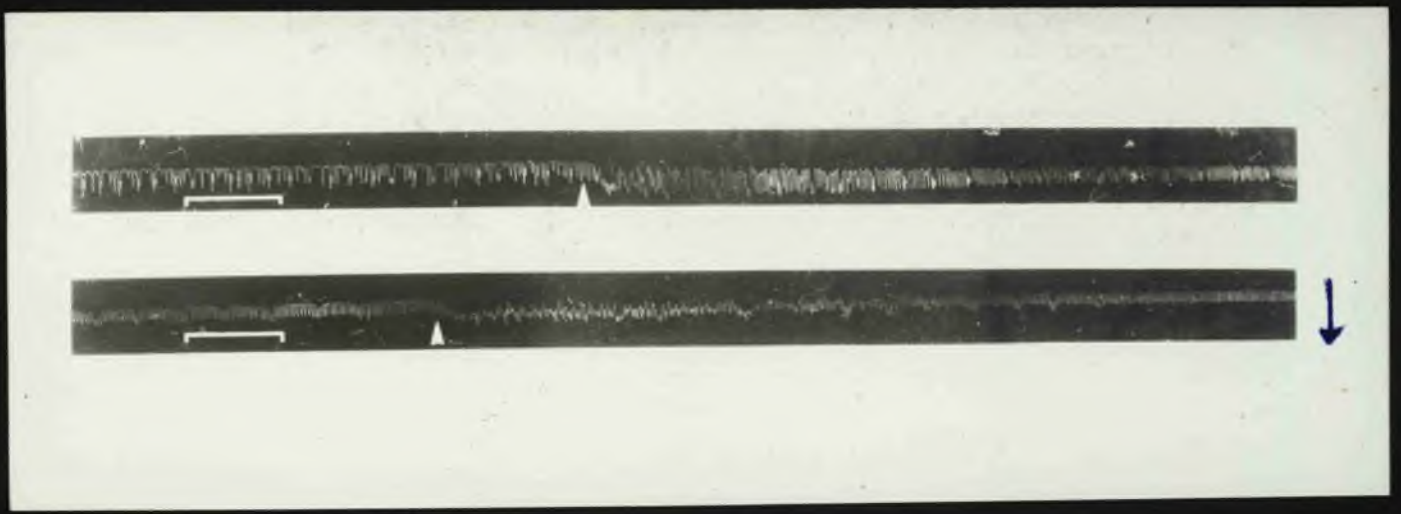


Figure 37. The effect of vena cava extract on the isolated branchial heart of Eledone cirrosa compared with the effect on the isolated systemic heart of the same animal.

A. Addition of 0.1 ml VC extract to perfusing medium of branchial heart preparation.

B. Addition of 0.3 ml VC extract to perfusing medium of branchial heart preparation.

C. Addition of 0.1 ml VC extract to perfusing medium of systemic heart preparation.

Arrows indicate the time of addition of extracts to the perfusing medium. Time mark represents 2 minutes.

The blue arrow indicates the direction of contract

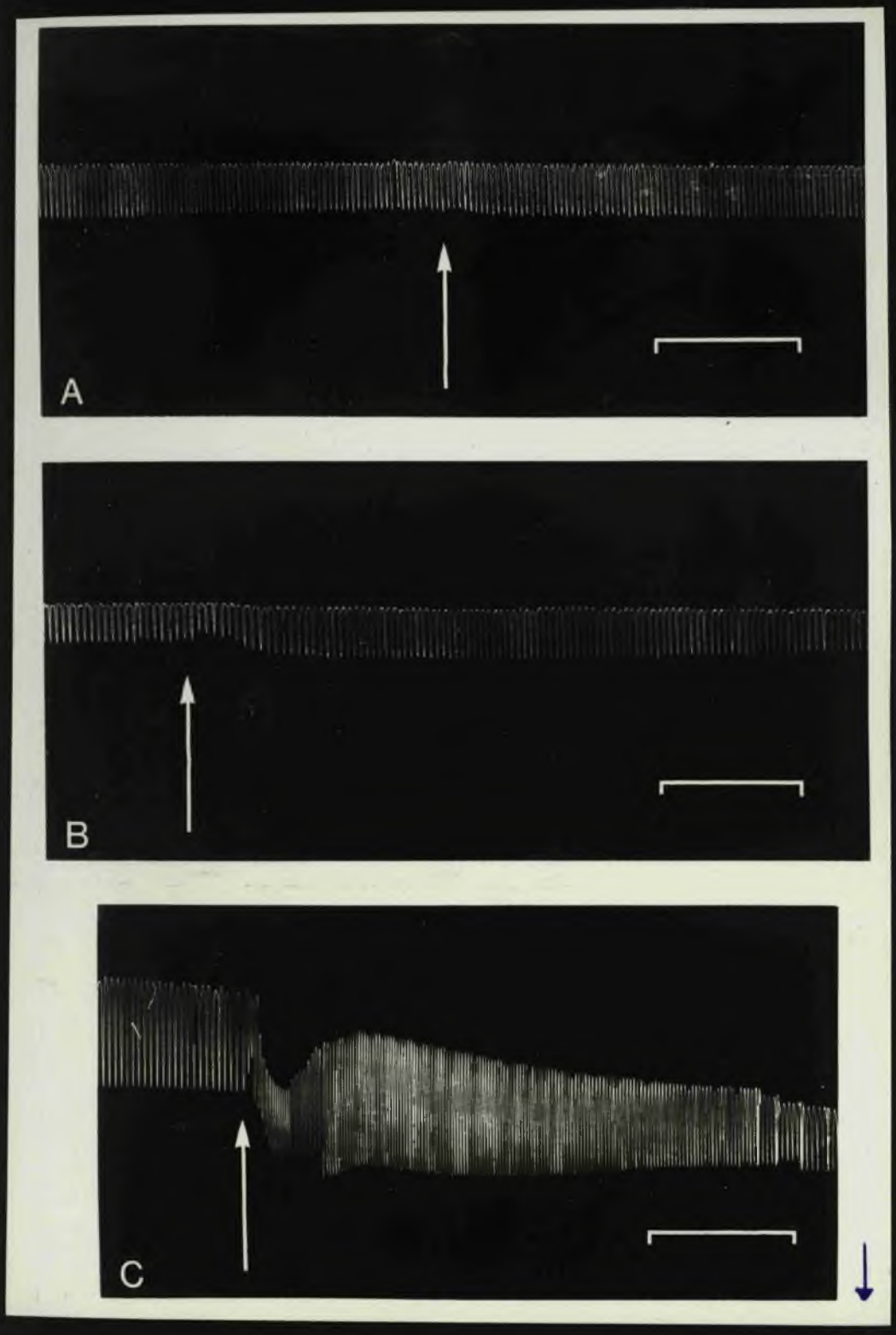

A
B
C

Figure 38. The effect of vena cava extract on an isolated tube of vena cava of Eledone cirrosa. The arrowhead indicates the time of addition of VC extract to the perfusing medium. Time mark represents 1 minute.

The blue arrow indicates the direction of contraction.

38

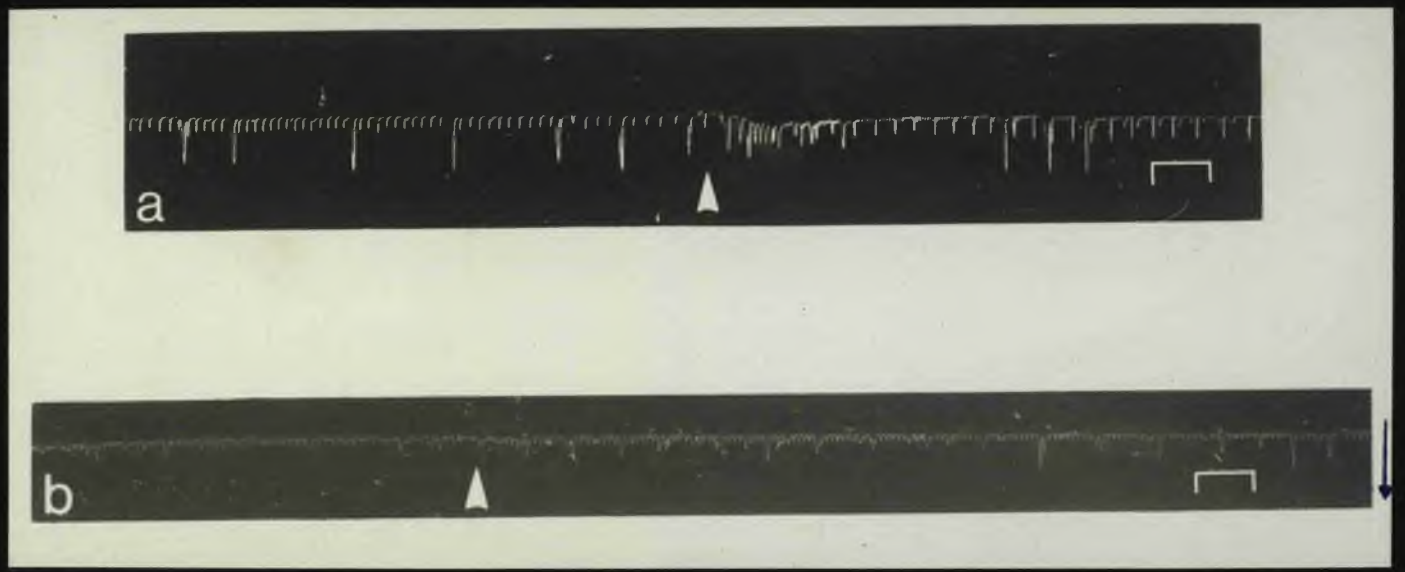
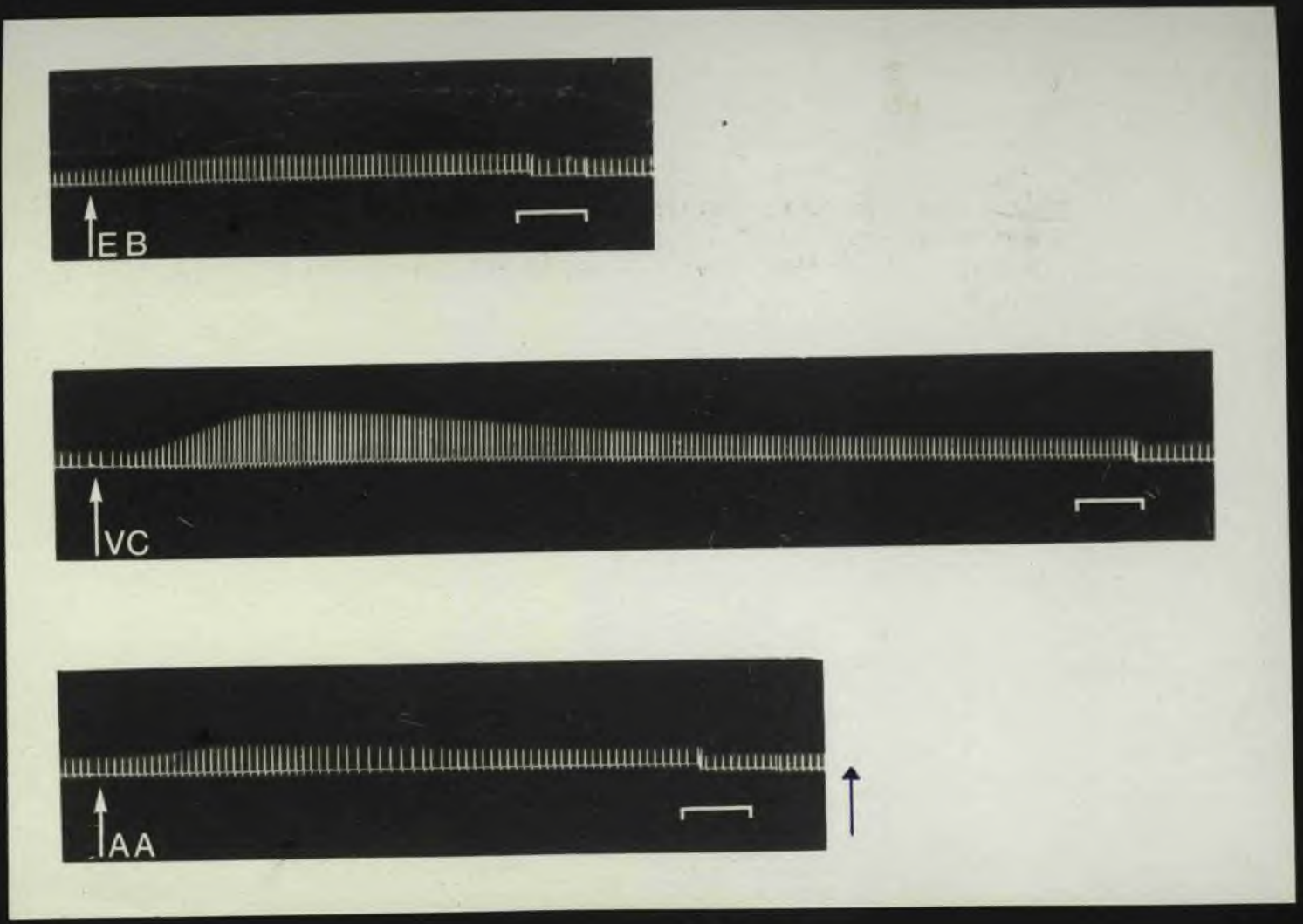
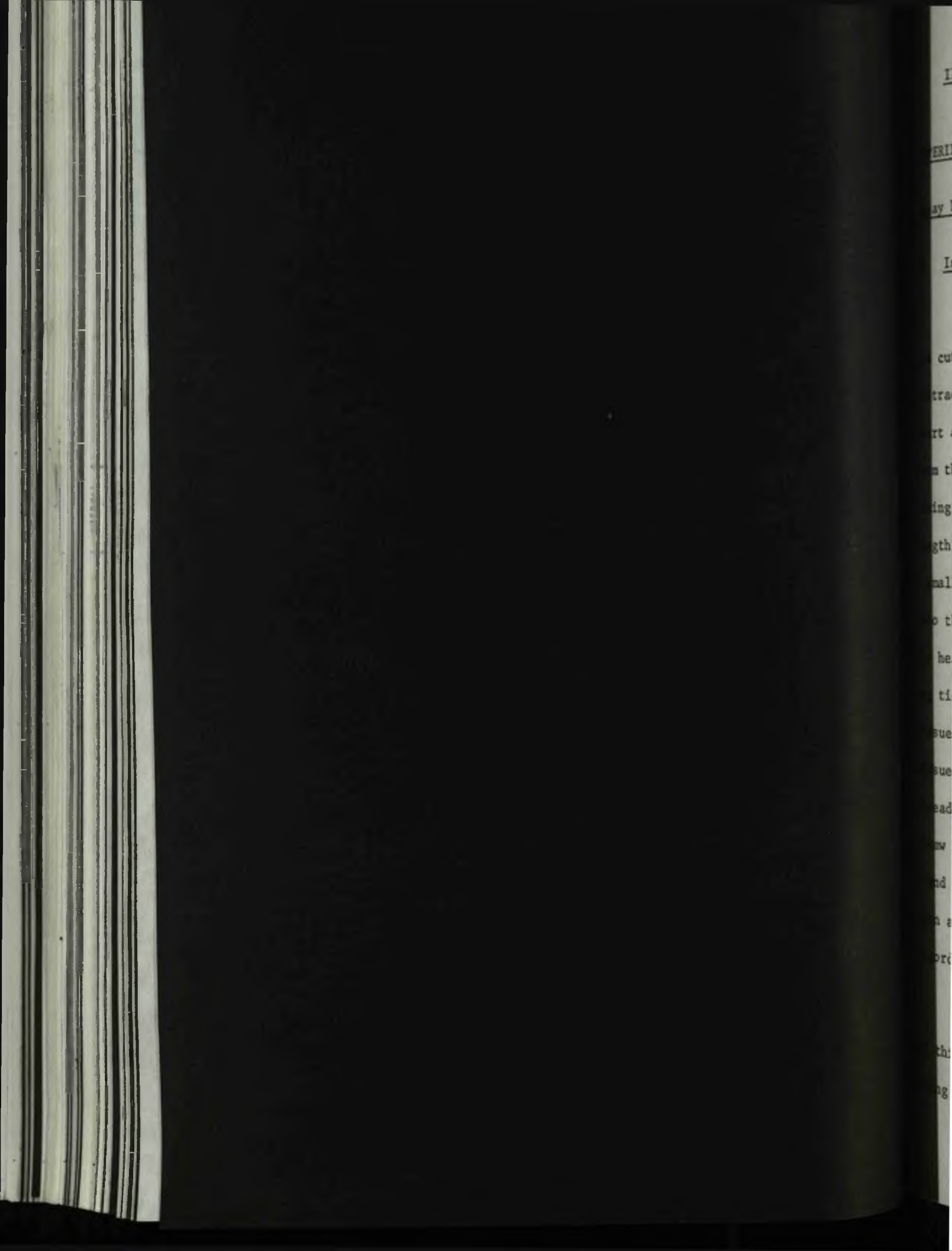


Figure 39. The successive effect of extracts of the efferent branchial vessel (EB), the anterior vena cava (VC) and the abdominal aorta (AA) on the isolated systemic heart of Eledone cirrosa. Arrows indicate the time of addition of extracts to the perfusing medium. Time mark represents 1 minute.

The blue arrow indicates the direction of contraction.


EB
VC
AA



II. ASSAY OF EXTRACTS ON OTHER MOLLUSCAN PREPARATIONS

ERIMENTAL PROCEDURE

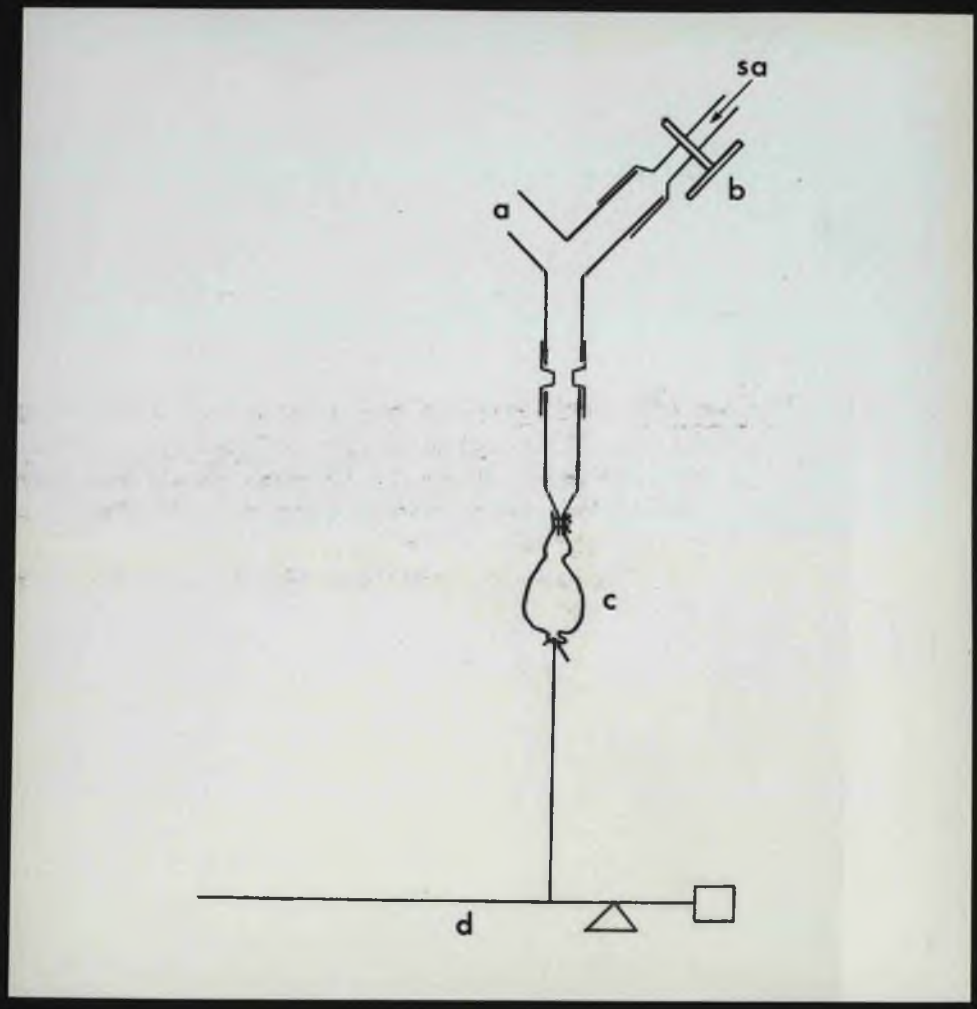
ay Preparations

Isolated Helix heart

The heart of Helix aspersa was isolated as follows. The shell cut away from the animal and the columellar muscle was cut to prevent traction of the specimen. The collar muscle was pinned down and the rt and kidney, (lying within the pulmonary chamber), were visible n the dorsal surface. The heart was exposed by cutting the mantle ing of the pulmonary chamber. The pericardium was removed, and a gth of thread was passed underneath the auriculo-ventricular junction. mall opening was made in the auricle, a glass cannula was inserted o the auricular chamber and was fastened in place with the thread. heart was lifted by means of the cannula and freed from the surround- tissue, care being taken to ensure that a small piece of kidney ue was left surrounding the base of the ventricle. This kidney ue provided a site for anchoring an insect pin which carried a ad from a heart lever. In this case the lever used was a drinking w (after Powell, personal communication) since a metal lever was d to put too much strain on the heart tissue. The preparation was n attached to a perfusion system as shown in Figure 40. Isotonic rdings were made using a kymograph.

The heart was perfused with Meng's solution (for composition his saline see the Appendix, p. 175), a constant pressure head g maintained throughout. The preparation was allowed to equilibrate

Figure 40. Diagram of the perfusion system used for the isol heart preparation of Helix aspersa.
a - constant pressure head; b - inlet valve for Meng's saline (sa); c - isolated heart; d - kymo- graph lever.
(Injections into the perfusion medium were made at "a").


sa
a
b
c
d

ver a period of 30 minutes. Extracts were injected into the perfusing edium via the side arm of the cannula, see Figure 40. All experiments ere carried out at room temperature.

) Isolated Mya heart

The heart of Mya arenaria was isolated after the method of elsh and Twarog (1960). Care was taken to ensure that the ligatures ere tied onto the ventricle as recommended by Cottrell, Pentreath and owell (1968). The heart was mounted in a perfusion bath with a volume f 20 ml. The preparation was washed several times with sea-water and as allowed to stabilise over a period of 60 minutes. Maintenance of he specimen and the method of applying test extracts and solutions was imilar to the procedure described by Cottrell, Pentreath and Powell 1968). All experiments were carried out at room temperature.

reparation of extracts and standard solutions

Extracts of the vena cava tissue of Eledone cirrosa were repared as described on p. 29.

Standard solutions of the following substances, of known concentration, were made up in distilled water.

5-hydroxytryptamine: as serotonin creatinine sulphate
(BDH Chemicals Ltd., Poole, England.)

Acetylcholine: as acetylcholine chloride
(BDH Chemicals Ltd., Poole, England.)

say

The effect of vena cava extract on the isolated Helix heart as compared with the effect of the indolalkylamine, 5-hydroxytryptamine.

The effect of the vena cava extract on the isolated Mya heart was compared with the effect of acetylcholine.

In all experiments the final dilutions of the known drugs were made in the appropriate perfusing medium. The stated doses of drugs administered refer to the amount of drug added to the perfusing medium and not to the concentration of drug within or surrounding the assay preparation.

RESULTS

It was found that the extracts of the vena cava of Eledone cirrosa had a positive chronotropic and inotropic effect on the isolated heart of Helix aspersa. At low concentrations the extract caused an increase in amplitude of beat but at higher concentrations it caused a prolonged increase in amplitude and frequency of beat. This differs from the effect shown by 5-hydroxytryptamine which causes a rapid transitory increase in amplitude followed by a more prolonged increase in frequency, see Figure 41.

However vena cava extracts caused a depression of the beat of the isolated heart of Mya arenaria, (Figure 42). This was compared with extracts from another blood vessel of E. cirrosa, the dorsal aorta, which caused no effect on the amplitude or frequency of heart beat. Acetyl choline also depresses the beat but this effect is destroyed if the substance is boiled. Boiling of the vena cava extract did not alter its effect on the heart, see Figure 43.

COMMENT

Comparison of the effects of vena cava extract and 5-hydroxytryptamine on the isolated heart of H. aspersa showed that although

the pattern of the effect was similar for both substances there was a temporal difference. The effect caused by the vena cava extract was much more prolonged. This finding compares with the effects caused by these two substances on the isolated heart of E. cirrosa.

The vena cava extract caused a negative inotropic and chronotropic effect on the isolated heart of M. arenaria. This is similar to the effect caused by acetylcholine. However the effect of acetylcholine is abolished by boiling. Boiling does not appear to affect the activity of the vena cava extract since the extract is subjected to boiling during the extraction procedure. In 1966 Jaeger showed that extracts of the ventricle of Strophocheilus oblongus caused a negative inotropic and chronotropic effect on the isolated heart of the clam Mercenaria mercenaria. (This effect was reversed if the heart was treated with the ACh blocker Mytolon.)

Figure 41. The effect of extracts of the vena cava of Elëdone cirrosa on the isolated heart of Helix aspersa compared with the effect of the indolalkylamine, 5-hydroxytryptamine. These two substances have similar but very distinct effects.
V_a - 0.1 ml 1/100 standard VC extract; V_b - 0.1 ml 1/10 standard VC extract; V_c - 0.1 ml standard VC extract; H_a - 0.1 ml 5-hydroxytryptamine (at concentration of 10^{-9} g/l); H_b - 0.1 ml 5-hydroxytryptamine (at concentration of 10^{-6} g/l).

The blue arrow indicates the direction of contraction.

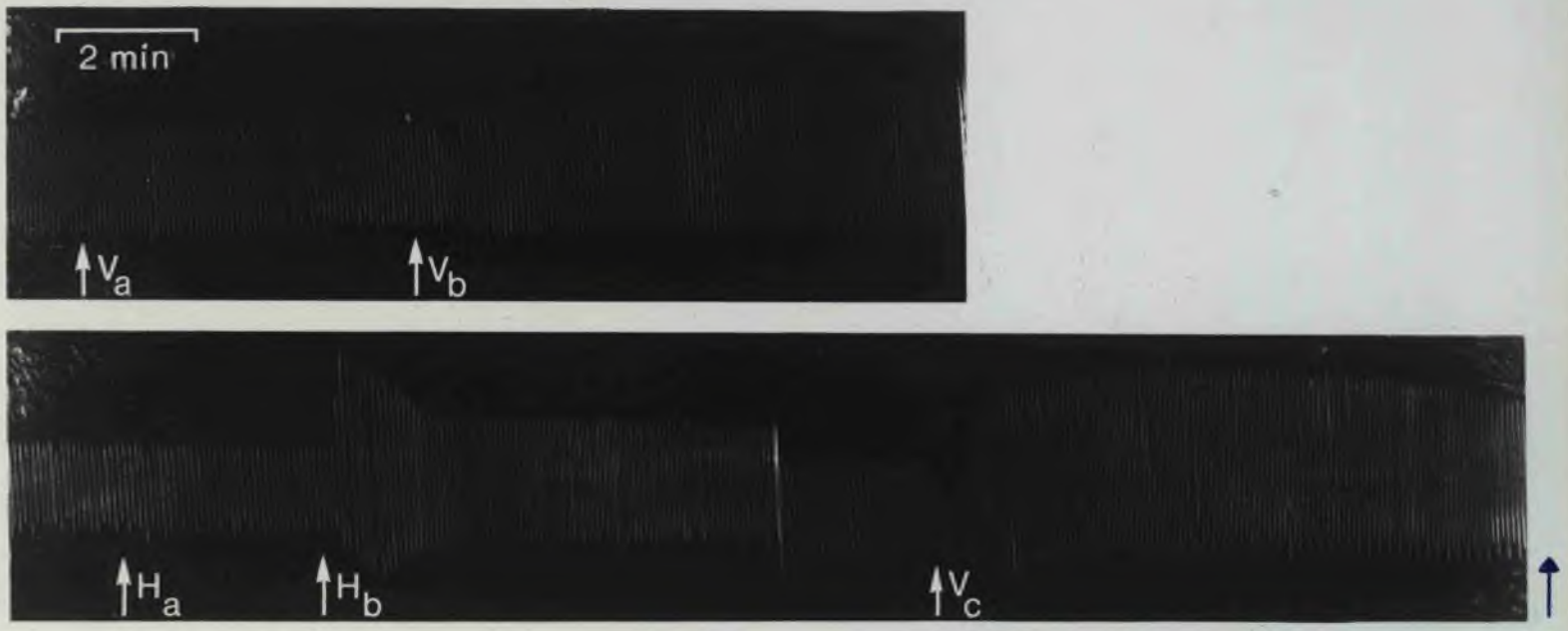

2 min
V_a
V_b
H_a
H_b
V_c

Figure 42. The effect of two different extracts of the vena cava (b) and (d) and an extract of the dorsal aorta (c) of Eledone cirrosa on the isolated heart of Mya arenaria. The suffix denotes an increase in concentration by a factor of ten.
a - injection of sea-water into the bathing medium as a control.

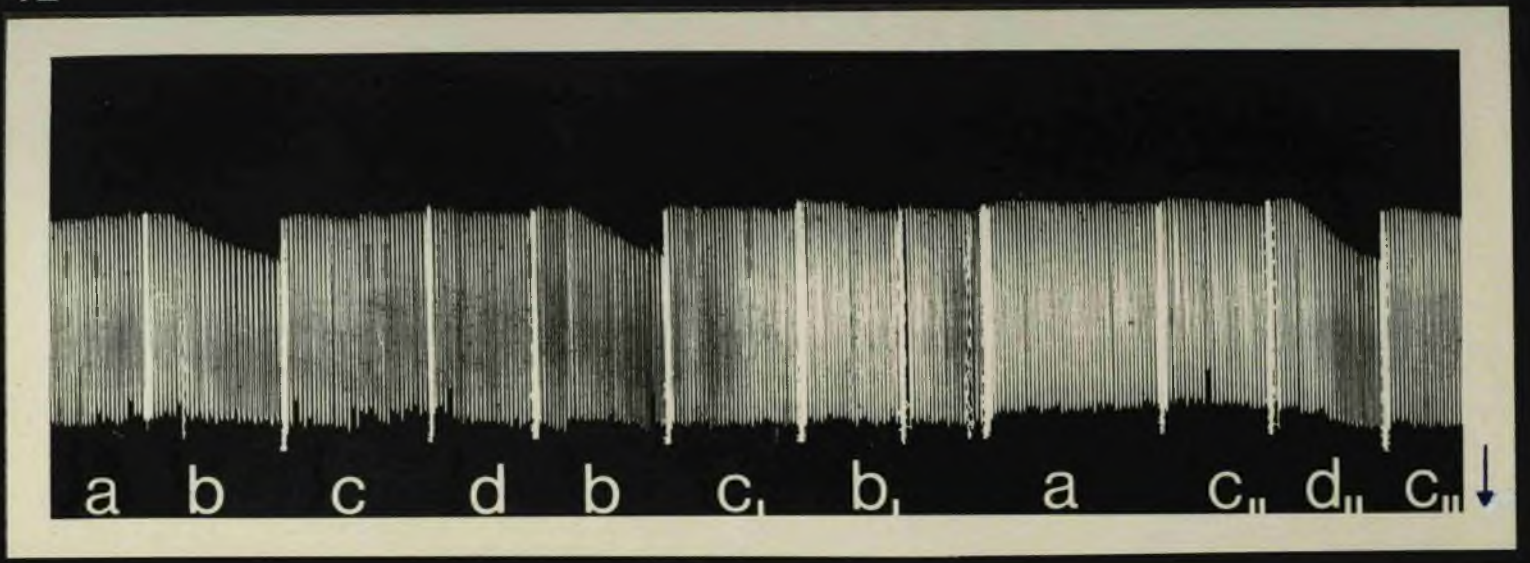
The blue arrow indicates the direction of contraction.

Figure 43. The effect of vena cava extract of Eledone cirrosa on the isolated heart of Mya arenaria compared with the effect of acetylcholine. Boiling destroys the effect of acetylcholine but does not alter the effect of the vena cava extract.
a - injection of 0.5 ml sea-water; b - 0.1 ml 1/100 standard VC extract; b_1 - 0.1 ml 1/10 standard VC extract; c - 0.1 ml acetylcholine (at concentration of 10^{-7} g/l); c_1 - 0.1 ml acetylcholine (at concentration of 10^{-7} g/l) after placement on a boiling water bath for 5 minutes; d - washing period.

The blue arrow indicates the direction of contraction.

The blue arrow indicates the direction of contraction.

42



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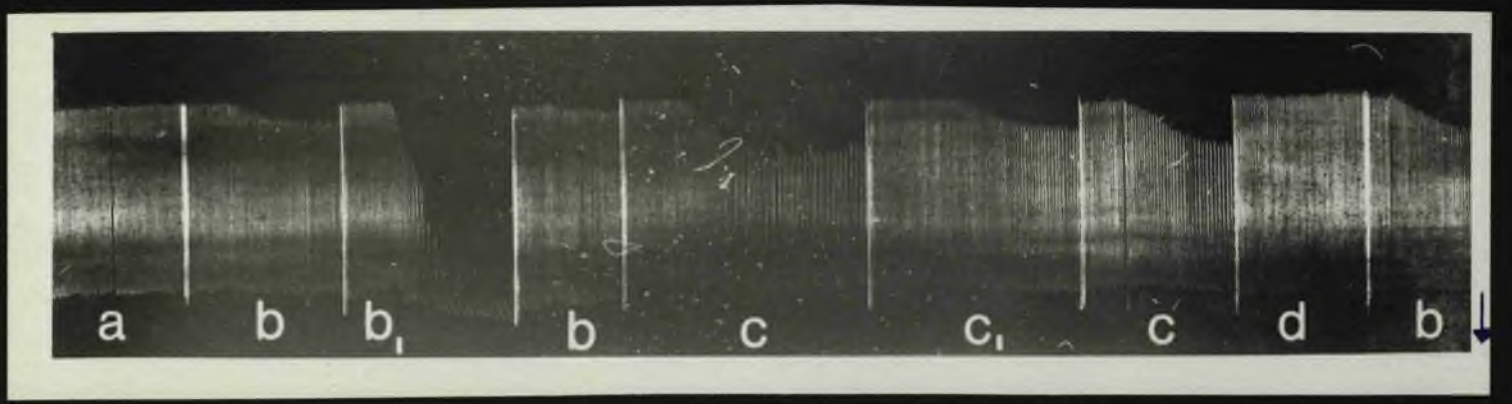


III. ASSAY OF VENA CAVA EXTRACTS ON SOME MAMMALIAN PREPARATIONS

To determine the effect of vena cava extract on the isolated rat uterus.

PERIMENTAL PROCEDURE

The uterus from a rat in oestrus was isolated and immediately aced in de Jalon's solution (the composition of de Jalon's solution noted in the Appendix, p. 175). The preparation was set up after e method reported in Perry (1968). A strip of tissue approximately mm wide was taken from one horn of the uterus. A threaded needle s passed through the distal end of the tissue and the thread was tached to a glass hook mounted in a 10 ml perfusing bath. A second read was tied onto the uterine strip immediately below the ovary and tached to a writing lever. Movements of the tissue were recorded ing a kymograph. The tissue was bathed in de Jalon's solution which s aerated with a 95% O_2/ 5% CO_2 gas mixture. The temperature of the th was maintained at 30°C. The tissue was allowed to equilibrate r 60 minutes. The following dose-cycle was adopted:

0 min. Started kymograph
1 min. Addition of drug
1 1/2 min. Stopped kymograph, drug washed out
4 min. Started kymograph and cycle repeated

e effect of vena cava extract was compared with a visceral nerve xtract from E. cirrosa and a standard acetylcholine solution. (The cetylcholine solution was obtained as described on p. 39 .) All extracts d drug solutions were added directly to the bathing fluid. In another

t of experiments the uterus from a rat in dioestrus was isolated. A trip of tissue was set up exactly as described above. In this case e effect of vena cava extract was compared with the effect of oxytocin.

ESULTS

Whereas acetylcholine had a marked effect on the rat uterus preparation, neither the vena cava extract nor the visceral nerve xtract showed any effect, see Figure 44.

Oxytocin also has a marked effect on the rat uterus. The vena ava extract again showed no effect, see Figure 45.

OMMENT

Although oxytocin, a neurosecretory substance from the mammalian ypothalamus, had a marked effect on the rat uterus the vena cava extract ppeared to have no effect on this preparation.

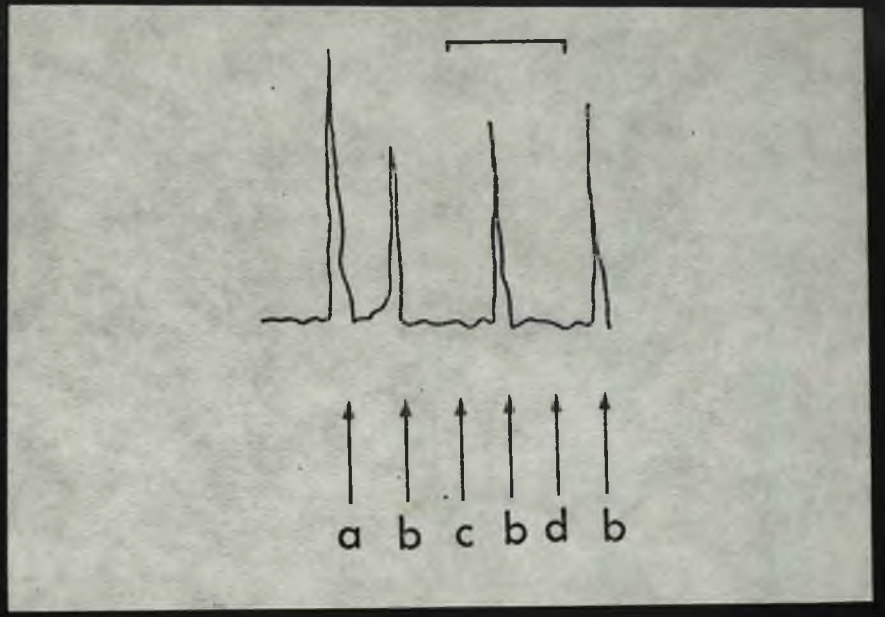
Another active substance from cephalopods, Eledoisin, has been hown to have only a weak action on the rat uterus (Erspamer and rspamer, 1962) and in 1970 Lederis showed that a potent smooth-muscle ontracting substance from teleost urophyses had no effect on the rat terus.

Figure 44. The effect of vena cava extract on an isolated rat uterus preparation compared with the effect of acetylcholine.
a - 100 µg acetylcholine; b - 10 µg acetylcholine; c - 0.1 ml standard vena cava extract; d - 0.1 ml visceral nerve extract. Time mark represents 4 minutes.

Figure 45. The effect of vena cava extract on an isolated rat uterus preparation compared with the effect of the neurohypophyseal octapeptide oxytocin.
a - 0.2 units oxytocin; b - 0.1 units oxytocin; c - control injection of De Jalon solution plus sucrose; d - 0.1 ml Fraction I, from sucrose gradient 11-6-70, unboiled; e - 0.1 ml Fraction I, from sucrose gradient 11-6-70, boiled. Time mark represents 4 minutes.

ml Fraction I, from sucrose gradient 11-6-70, boiled. Time mark represents 4 minutes.

44



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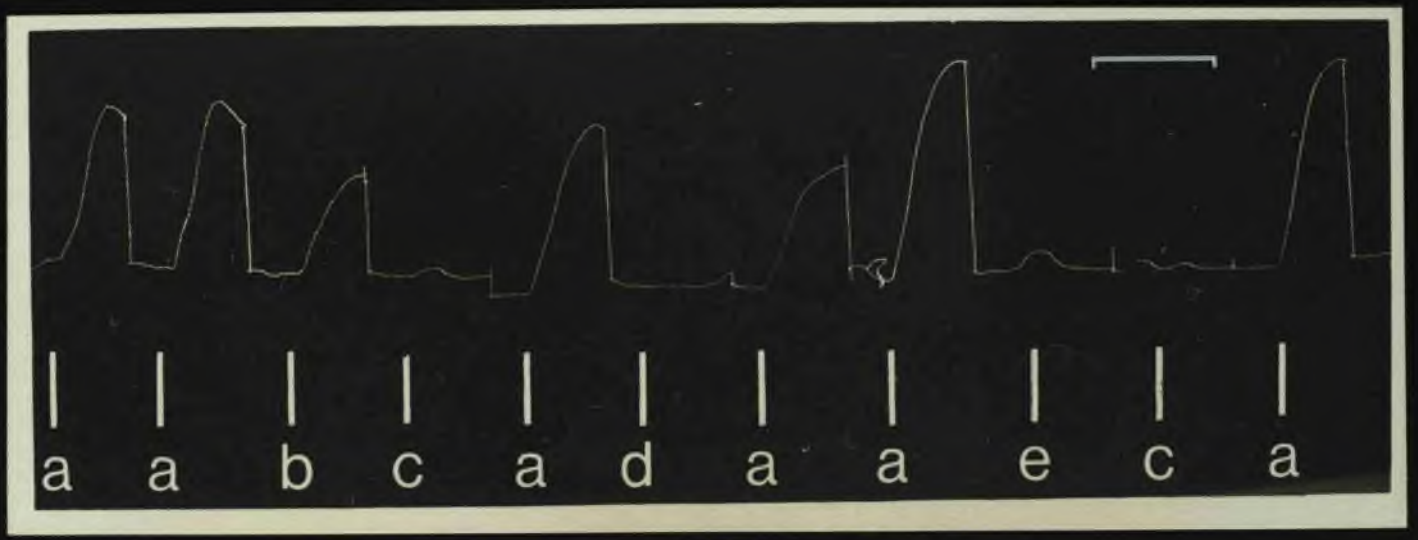


2. To determine the effect of vena cava extract on the isolated rabbit heart.

EXPERIMENTAL PROCEDURE

A rabbit was killed and the heart isolated as quickly as p into a dish of cold Locke's solution. (The composition of Locke's solution is noted in the Appendix, p. 175 .) Care was taken to ens that the blood was immediately emptied from the heart to prevent cl from forming inside the organ. The preparation was set up after th method reported by Perry (1968). The heart was perfused with oxyg Locke's solution at a constant head of pressure (40 mm Hg). The perfusing fluid was also maintained at a temperature of 37°C by a t jacket. A small spring clip carrying a thread was attached to the t of the ventricle, the other end of the thread being attached to a s lever. Recordings of the ventricular beat were made using a kymog Extracts were added to the perfusion fluid by injection through the rubber tubing above the heart.

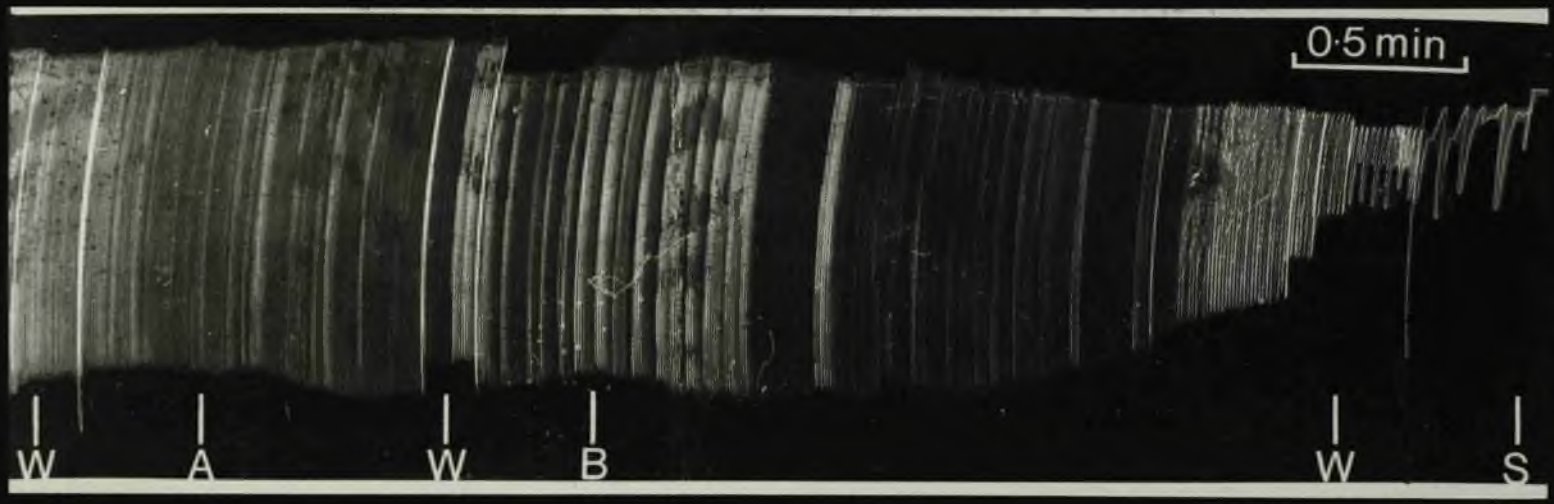
The preparation was allowed to equilibrate over a period o 30 minutes and then the effect of the vena cava extract was monito on the heart.

RESULTS

In all cases extracts of the vena cava of E. cirrosa caused a gradual cessation of beat of the isolated rabbit heart, see Figu

Figure 46. The effect of vena cava extract of Eledone cirrosa on the isolated perfused heart of a rabbit. (Since the vena cava extract was made up in sea-water, an injection of a SW-Locke's solution mixture was made as a control.)
A - injection of SW-Locke's solution into perfusing fluid;
B - injection of vena cava extract/Locke's solution into perfusing fluid, both additions in aliquots of 0.3 ml;
W - washing period of 3 minutes.

46



. To determine the effect of vena cava extract on the cardiovascular system of the anaesthetised cat.

XPERIMENTAL PROCEDURE

The preparation was set up as described by McLeod (1970).

A cat was weighed and then placed in an anaesthetisation box. ther was passed into the box until the animal was unconscious. The nimal was then placed on the operating table, the legs were tied own and one of the femoral veins was exposed and cannulated. A "main- enance" anaesthetic was then slowly administered via the femoral vein. n this case the anaesthetic was chloralose, 80 mg/kg, administered t a temperature of 37°C. This kept the cat anaesthetised for approxi- ately 5 hours. The trachea was then exposed, opened and cannulated. he cannula was attached to a pump which enabled ventilation of the ungs.

Next the carotid artery was exposed and cannulated. This annula contained heparinised saline to prevent formation of blood lots. Attached to this cannula was a strain gauge transducer. This ransducer recorded the blood pressure level within the animal which as displayed on a Devices recorder (Devices Type M4, Welwyn Garden ity, Herts, ENGLAND).

Drugs and extracts were administered to the animal via the moral vein. Care was taken to ensure that the pressure level eturned to a resting level before subsequent injections were made. the end of the experiment the cat was killed.

RESULTS

The results obtained in one blood pressure experiment are shown in Figure 47. 5 µg adrenaline caused an immediate drop in pressure followed by a very marked increase. Then there was a gradual return to resting pressure over a period of several minutes. Extracts of the visceral nerve and the vena cava of E.cirrosa both caused an immediate drop in pressure. This was not quite so marked in the case of the vena cava extract. 5 µg of adrenaline again caused a marked hypertension. In contrast, 5 µg of acetylcholine caused an immediate drop in pressure which was only slowly restored. The response was similar to that caused by the visceral nerve extract. A smaller dose of acetylcholine caused a response similar to that engendered by the vena cava extract. The addition of 35 µg eledoisin caused a very marked hypotensive effect which was maintained for several minutes. After the addition of atropine the previously observed effects of acetylcholine and the vena cava extract were abolished. Eledoisin and adrenaline however, retained their respective hypotensive and hypertensive effects.

COMMENT

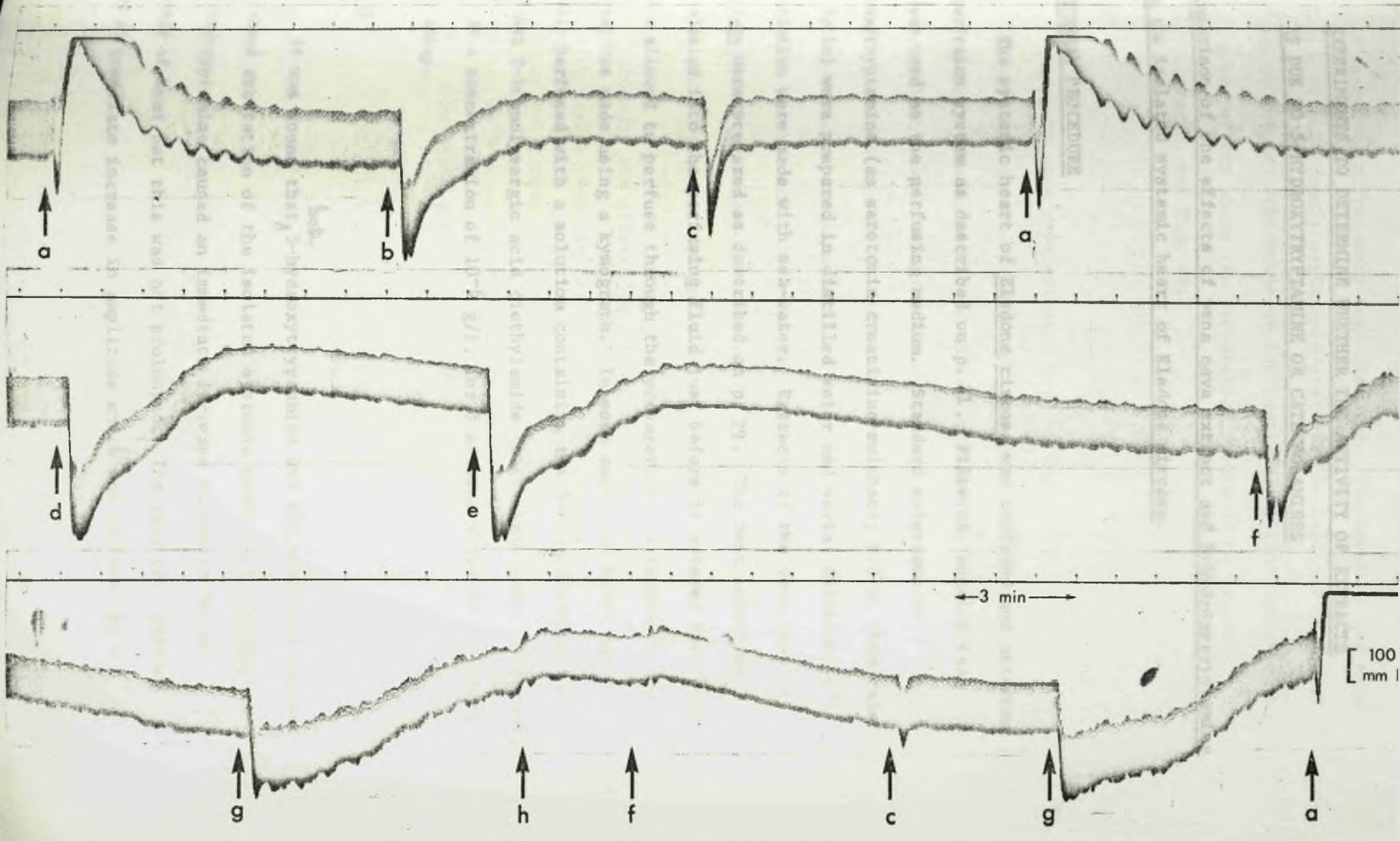
The initial injection of adrenaline indicated that the preparation was responding in a "normal" or expected manner. This was later confirmed by the pattern of responses to acetylcholine and eledoisin. Eledoisin is known to be a potent hypotensive agent in mammals. Although the visceral nerve and vena cava extracts also caused a hypotensive effect this was different in character from that caused by eledoisin and closely resembled the response caused by acetylcholine. After injection of atropine (an inhibitor of acetylcholine receptors) the response to acetylcholine was abolished and

the very transient response to vena cava extract indicated that the initial effects caused by this extract were probably due to the presence of acetylcholine.

The effects caused by eledoisin and adrenaline confirmed that the preparation still responded to hypotensive and hypertensive agents.

From these observations it is deduced that whereas the vena cava extract of E. cirrosa has a profound effect on the octopod heart it has very little effect on the mammalian vascular system. This is in contrast to another cephalopod compound, eledoisin.

Figure 47. Record of variations in pressure noted during the cat blood pressure experiment. Arrows denote time of introduction of substances into the femoral vein.
a - 5 µg adrenaline; b - visceral nerve extract;
c - vena cava extract; d - 5 µg acetylcholine;
e - 1 µg acetylcholine; f - 0.5 µg acetylcholine;
g - 35 µg Eledoisin; h - 3.5 mg atropine.


a
b
c
a
d
e
f
3 min
100
mm
g
h
f
c
g
a

IV. EXPERIMENTS TO DETERMINE WHETHER THE ACTIVITY OF EXTRACTS IS DUE TO 5-HYDROXYTRYPTAMINE OR CATECHOLAMINES

i. Comparison of the effects of vena cava extract and 5-hydroxytryptamine on the isolated systemic heart of Eledone cirrosa.

EXPERIMENTAL PROCEDURE

The systemic heart of Eledone cirrosa was isolated and attached to a perfusion system as described on p. 31. Filtered, aerated sea-water was used as the perfusing medium. Standard solutions of 5-hydroxytryptamine (as serotonin creatinine sulphate; B.D.H. Chemicals Ltd., Poole) were prepared in distilled water and serial dilutions of this solution were made with sea-water. Extracts of the vena cava of E. cirrosa were prepared as described on p. 29. The test solutions were injected into the perfusing fluid just before it entered the heart and were allowed to perfuse through the preparation. A record of the heartbeat was made using a kymograph. In some cases the heart was initially perfused with a solution containing the 5-hydroxytryptamine antagonist 2-bromolysergic acid diethylamide (Koch Light Labs, Colnbrook, Bucks) at a concentration of 10^{-6} g/l, for 5 minutes prior to the start of the assay.

RESULTS

It was found that both 5-hydroxytryptamine and the vena cava extract caused excitation of the isolated systemic heart of E. cirrosa. 5-hydroxytryptamine caused an immediate increase in amplitude and frequency of beat but this was not prolonged. The vena cava extract caused an immediate increase in amplitude which was followed by a

prolonged increase in frequency (see Fig. 48). Treatment of the assay preparation with 2-bromolysergic acid diethylamide caused a change in the effect of the two substances on the heart. 5-hydroxytryptamine no longer had any effect while the preparation continued to respond to vena cava extracts, see Fig. 49.

COMMENT

Assay of vena cava extracts and the indolalkylamine 5-hydroxytryptamine on the isolated systemic heart of E. cirrosa showed that both substances caused an increase in amplitude and frequency of heartbeat. However, the duration of action of the effects caused by these two substances differed. In the case of the vena cava extract there was a prolonged increase in frequency of beat, an effect which was not shown by 5-hydroxytryptamine. Similar effects to those caused by these substances on the isolated heart of E. cirrosa were elicited in the isolated heart of Helix aspersa (see p. 40).

Although the effects caused by the vena cava extract and 5-hydroxytryptamine were similar they were shown to be caused by two different substances. Whereas the effect of 5-hydroxytryptamine was inhibited by 2-bromolysergic acid diethylamide, the activity of the vena cava extracts was not affected.

Figure 48. A comparison of the activities of extracts of the anterior vena cava (C) and abdominal aorta (A) and the effect of 5-hydroxytryptamine at a concentration of 10^{-6} g/l (B) assayed on the isolated systemic heart of Eledone cirrosa. Only the vena cava extract caused a prolonged cardio-acceleratory effect.

Figure 49. Evidence to indicate that 5-hydroxytryptamine is not the excitatory factor present in vena cava extracts. The isolated heart was previously perfused with the 5-HT antagonist, 2-Bromolysergic acid diethylamide for 5 min. Subsequent addition of 5-hydroxytryptamine (concentration of 10^{-6} g/l) caused no effect (A) whereas the activity of the blood vessel extracts was not inhibited.
(B) Efferent branchial vessel; (C) abdominal aorta;
(D) anterior vena cava.

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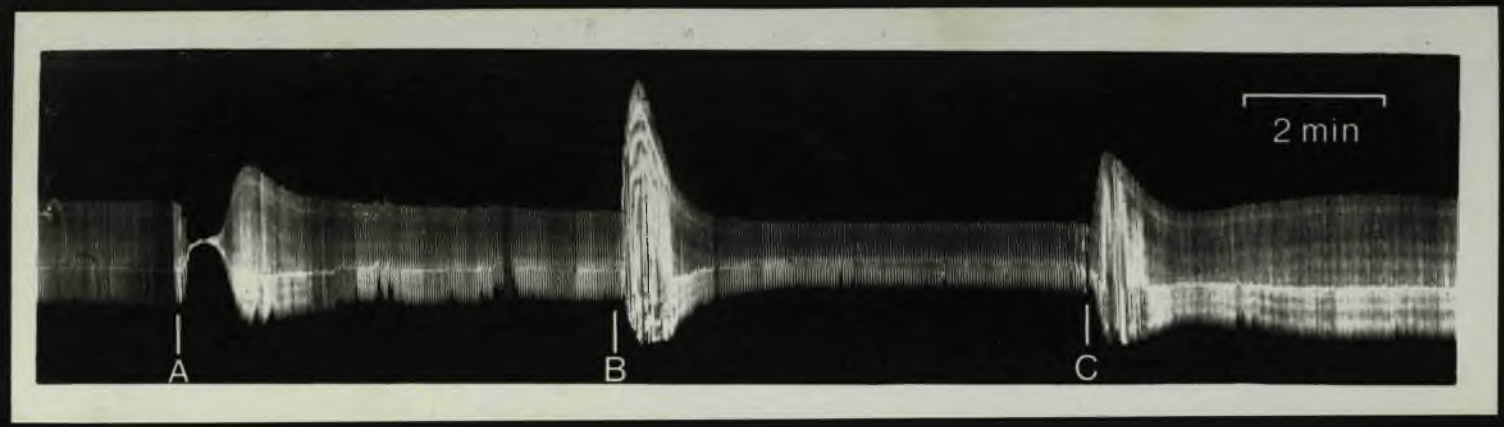

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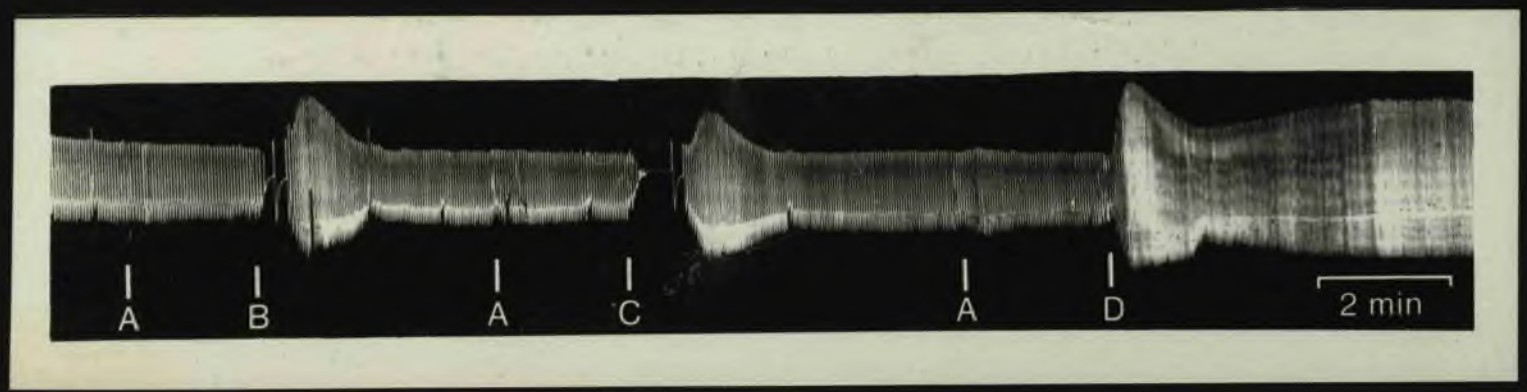


. Comparison of the emission wavelengths for vena cava extracts and 5-hydroxytryptamine using a spectrophotofluorimetric technique.

The extraction procedure used was adopted from Quay (1963).

Standard solutions of 5-hydroxytryptamine were prepared from erotonin creatinine sulphate (B.D.H. Chemicals Ltd., Poole) in 0.1 N Cl 0.5% ascorbic acid solution.

Scrapings of the inner wall of the vena cava of Eledone rrosa, 250 - 300 mg wet weight, were also placed in 0.5 ml 0.1 N Cl 0.5% ascorbic acid solution and the mixture was macerated with glass rod until the tissue was finely suspended. Care was taken ensure that the pH of the suspension was acid, i.e. between pH2 d pH4, and that during the procedure there would be no interference om the extraction reagents.

Two aliquots containing suboesophageal ganglia from the marine stropod, Neptunea antiqua, were also prepared in a similar manner. e aliquot contained 5 ganglia weighing 150 - 180 mg wet weight and e second aliquot contained ten ganglia weighing 300 - 350 mg wet weight.

All extraction aliquots, i.e. a reagent blank, 5-hydroxytryp- ine solutions, the vena cava suspension and the Neptunea ganglia spensions, were of the same volume, viz. 0.5 ml, and were placed in ppered centrifuge tubes.

1. Into each tube were placed in the following order:
   0.1 ml 3% ascorbic acid in 1% EDTA,
   300 mg NaCl,
   3 ml diethyl ether.
2. All tubes were shaken 1 min, centrifuged 1 min and then the ether phase was drawn off and discarded.

3. 3 ml diethyl ether was added and step 2 was repeated.
4. 3 ml diethyl ether and 0.5 ml borate buffer, pH 10, were added and step 2 was repeated.
5. 3 ml n-butanol was added, the tubes were shaken 1 min, centrifuged 1 min and the butanol phase was transferred to a set of similar tubes containing 8 ml heptane and 0.5 ml 0.1 N HCl, 0.5% ascorbic acid solution.
6. All tubes were shaken 2 min, centrifuged 2 min and the heptane-butanol phase was removed.
7. Sufficient conc. HCl was then added to each tube to give a final solution concentration of 3N-HCl.

Each solution was then placed, in turn, in an Aminco-Bowman Spectrophotofluorimeter and readings were taken at an activation wavelength of 295 mμ and an emission wavelength of 540 mμ. This final step was completed rapidly to ensure that readings were not inaccurate due to breakdown of 5-hydroxytryptamine in the 3NHCl solution.

A series of standard solutions of 5-hydroxytryptamine that had not been subjected to the extraction procedure were submitted to step 7 above and a calibration reading for the amount of 5-hydroxytryptamine present in each solution was determined using the spectrophotofluorimeter. This series of readings was run as a control so that the percentage of 5-hydroxytryptamine lost during the extraction procedure could be determined.

RESULTS

All solutions of 5-hydroxytryptamine and the Neptunea ganglia gave an emission peak at 540 mμ whereas no peak was recorded for the vena cava tissue. The photometer readings are given in Table 2.

TABLE 2. PHARMACOLOGICAL STUDIES. Results obtained when using a photofluorimetric technique to determine the amount of 5-hydroxytryptamine present in various tissues.

Solution	Photometer Readings
Reagent blank	0.0005
1 µg 5-HT	0.0280
4 µg 5-HT	0.0420
8 µg 5-HT	0.0710
10 µg 5-HT	0.0840
267 mg Eledone tissue	0.0010
175 mg Neptunea tissue	0.0850
304 mg Neptunea tissue	0.1250

COMMENT

This method is extremely sensitive and under optimum conditions may be used to detect amounts of 5-hydroxytryptamine (5-HT) of the order of 10 nanograms (Quay, 1963).

Using the above procedure the suboesophageal ganglia of Neptunea antiqua were found to contain approximately 5 µg 5-HT/100 mg wet weight of tissue. However, the vena cava tissue of Eledone cirrosa did not give a reading for 5-hydroxytryptamine.

Thus this spectrophotofluorimetric analysis provided evidence that 5-hydroxytryptamine was not present in the neuropile region of the vena cava wall of E. cirrosa.

Examination of the vena cava tissue using fluorescence histochemistry.

The vena cava of Eledone cirrosa was prepared for examination for the presence of fluorescence using the method of Falck and Hillarp (see Falck and Owman, 1965; Corrodi and Jonsson, 1967) as interpreted by Osborne (1970).

Whole venae cavae and strips of vena cava wall were carefully removed from freshly killed specimens of Eledone cirrosa and were rapidly frozen in liquid propane (Calor gas). The frozen specimens were freeze-dried in a Pearse freeze-dryer at -40°C and 10^{-3} torr for a period of 3 days.

Subsequently the tissue was exposed to paraformaldehyde gas (paraformaldehyde stored at 70% R.H.) for 1 or 3 hr. at 80°C (This difference in exposure time can be used to separate fluorescence caused by monoamines and that caused by noradrenaline, Corrodi and Hillarp, 1963). The tissue was then infiltrated with paraffin wax under reduced pressure. Ten micron transverse and longitudinal serial sections were cut and mounted in a non-fluorescent medium, liquid paraffin. Slides were viewed in a suitably adapted Leitz fluorescence microscope. (For catecholamines and 5-hydroxytryptamine the maximum excitation wavelength of the fluorophore is 410 mμ while the emission wavelengths are 480 mμ and 525 mμ respectively. There is also a difference in colour of light emitted although this is sometimes difficult to ascertain. Fluorophores emitting light at 480 mμ appear green while those emitting light at 525 mμ appear yellow.)

Fluorescent structures were subjected to the sodium borohydride test recommended by Falck, Haggendal and Owman (1963) as a check for

the specificity of the fluorescence. In this test sodium borohydride in alcoholic solution reduces the fluorescence present due to catecholamines but this fluorescence can be regenerated in the presence of paraformaldehyde. Non-specific fluorescence however is usually unaffected by sodium borohydride and if it is reduced it cannot be regenerated.

At present there is no specific test for autofluorescence and protein fluorescence, i.e. non-specific fluorescence. Control slides, not subjected to sodium borohydride treatment, were examined alongside experimental slides. This allowed direct comparison of experimental and control slides and excluded the possibility that fluorescent compounds were extracted during the treatment with sodium borohydride.

RESULTS

Small areas of yellow fluorescence appeared to be dotted throughout the tissue. There were no large fluorescent areas. Treatment with sodium borohydride caused no observable effect on the fluorescence.

COMMENT

The fluorescence present in sections of the vena cava tissue was not altered by the sodium borohydride test and was thus shown to be non-specific. It is therefore concluded that the fluorescence was due to autofluorescence and protein fluorescence and not to the presence of 5-hydroxytryptamine or catecholamines.

The method employed here had been used with success in the examination of tissues from other marine animals. It was used to demonstrate the presence of catecholamines in the radial nerve of the

starfish, Asterias rubens (Pentreath, 1970; Cottrell and Pentreath, 1970) and the presence of monoamines in the stomatogastric ganglion of the lobster, Homarus vulgaris (Osborne and Dando, 1970). The method is extremely sensitive and can be used to detect 1 - 5 µg of primary catecholamine or 5-hydroxytryptamine per gm of protein in protein layers that are 10 µ thick (Corrodi and Jonsson, 1967).

Therefore it is unlikely that the lack of specific fluorescence in this case is due to methodology.

It would appear from these results that neither indolalkylamines nor catecholamines are present in the neuropile region of the vena cava in Eledone cirrosa.

EXPERIMENTS TO DETERMINE SOME PROPERTIES OF THE VENA CAVA EXTRACT.

Assay preparation

The assay preparation used for all these experiments was the isolated systemic heart of Eledone cirrosa. The preparation was set up as described on p. 31 . All experiments were conducted at room temperature.

Perfusing medium

Filtered, aerated sea-water was used to perfuse the preparation.

1. Experiments to determine the effect of acid and alkaline hydrolysis on the vena cava extract.

EXPERIMENTAL PROCEDURE

An extract of the inner layer of the vena cava of Eledone cirrosa was prepared as described on p. 29. Three aliquots of 0.5 ml vena cava extract were treated as follows:

(a) addition 0.5 ml 0.1N HCl → final pH 2.0.

(b) addition 0.5 ml 0.1N NaOH → final pH 10.5.

(c) addition 0.5 ml sea-water → final pH 7. (control)

All three aliquots were placed on a boiling water bath for 60 minutes. They were then allowed to cool to room temperature. 0.1 ml of each aliquot was added to separate 0.4 ml portions of sea-water. The resulting solutions were then adjusted to pH 7 using NaOH or HCl.

0.1 ml of each solution was injected into the sea-water perfusing the isolated systemic heart. Samples for assay were taken at

30 minutes and 60 minutes after the start of the experiment. The experiment was repeated on several preparations.

RESULTS

Activity was retained by all samples after 60 minutes on a boiling water bath, see Figure 50. However the activity of the acid and alkali treated samples was decreased compared to that of the control. There was a 50% reduction in the maximum amplitude achieved and a 25% reduction in frequency of heartbeat, see Table 3.

TABLE 3. PHARMACOLOGICAL STUDIES. Effects of acid and alkaline hydrolysis on the vena cava extract. Results from one experiment where samples were placed on a boiling water bath for 60 minutes.

Aliquot	Original Amplitude	New Amplitude	% change
Extract, pH 10.5	3	14	+ 360%
Extract, pH 2.0	3	16	+ 430%
Extract, pH 7.0	3	24	+ 700%

Aliquot	Original Frequency	New Frequency	% change
Extract, pH 10.5	6	8	+ 33%
Extract, pH 2.0	6	8	+ 33%
Extract, pH 7.0	7	10	+ 43%

There was also a change in the shape of the response curves. The experimental extracts did not cause the pronounced rapid increase

in amplitude shown by the control extract.

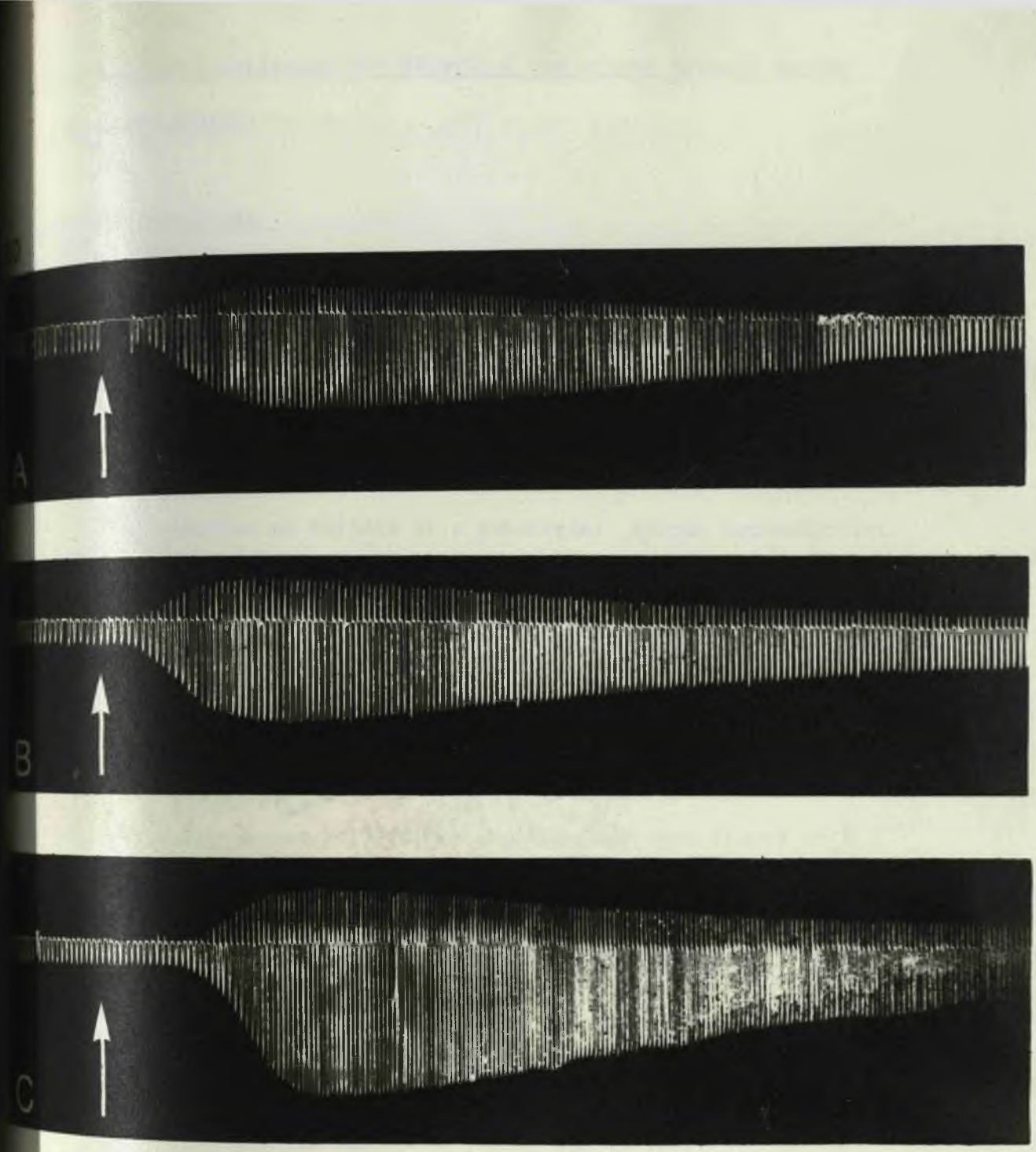
COMMENT

Similar experiments have been carried out on other cardio-excitatory substances. In 1966 Jaeger showed that there was no decrease in the activity of brain extracts of the gastropod Strophocheilus oblongus subjected to heating for 30 minutes at pH 4.8 or pH 8.8. Maynard and Welsh (1959) made similar findings with extracts of pericardial organs from brachyuran crustacea that had been subjected to heating for 20 minutes at an acid pH. However activity was not retained after heating at pH 11.8. Recently Lederis (1970) reported that the activity of urophyseal extracts of the rainbow trout, Salmo gairdnerii, was decreased after heating for three minutes at pH 10.0.

In the present experiments, the differences shown in the shape of response supports the hypothesis that the vena cava extract contains two substances, one responsible for the initial rapid increase in amplitude and the other responsible for a gradual increase in amplitude accompanied by a prolonged increase in frequency. From the results above acid and alkaline hydrolysis appeared to have a pronounced effect on the rapid increase in amplitude.

Figure 50. Acid and alkaline hydrolysis. The effect of successive doses of A - vena cava extract subjected to acid hydrolysis, B - vena cava extract subjected to alkaline hydrolysis and C - vena cava extract alone on the isolated systemic heart preparation of Eledone cirrosa. All extracts were placed on a boiling water-bath for 60 minutes prior to assay.

The blue arrow indicates the direction of contraction.


A
B
C

2. Experiments to determine the effect of the enzyme trypsin on the vena cava extract.

EXPERIMENTAL PROCEDURE

An extract of the inner layer of the vena cava of Eledone cirrosa was prepared as described on p. 29. Equal aliquots of the vena cava extract (at a concentration of 100 mg/ml) were treated as follows:

(a) addition of trypsin at a substrate: enzyme concentration of 50:1 in Gomori's Tris buffer (0.04M) containing 0.01 M $CaCl_2$, at pH 8.1.

(b) addition of Tris buffer alone at pH 8.1.

(c) addition of sea-water.

This method was adapted from that used by Lederis (1970). All samples were incubated at 37°C for one hour and then placed on a boiling water bath for 3 minutes to destroy the enzyme. After cooling to room temperature, 0.1 ml aliquots were taken for assay on the isolated systemic heart of E. cirrosa.

RESULTS

The buffer did not appear to affect the activity of the vena cava extract whereas incubation with trypsin caused a marked decrease in activity. There was a reduction both in the amplitude and frequency of beat, see Fig. 51 and Table 4.

TABLE 4. PHARMACOLOGICAL STUDIES. Effect of incubation with the enzyme trypsin on the vena cava extract.

Aliquot	Original Frequency (beats/min)	New Frequency (beats/min)	% change
Extract and buffer (A)	18	26	+44.5%
Extract and buffer and trypsin (B)	20	22	+10%
Extract alone (C)	20	30	+50%

Aliquot	Original Amplitude	New Amplitude	% change
Extract and buffer (A)	4	11	+170%
Extract and buffer and trypsin (B)	6	12	+100%
Extract alone (C)	6	15	+150%

COMMENT

These results indicated that the activity of vena cava extracts was decreased after incubation with trypsin. The shape of the response after trypsin treatment did not show the usual marked increase in amplitude. If the vena cava extract contained two substances and the normal rapid increase in amplitude was caused by a different substance to that causing the prolonged increase in frequency of beat it is possible that trypsin is having a more marked effect on the former substance. The loss of activity indicated that a peptide was possibly present in the extract.

Other cardio-excitatory substances have been subjected to enzyme digestion experiments. In 1966 Jaeger found that the activity

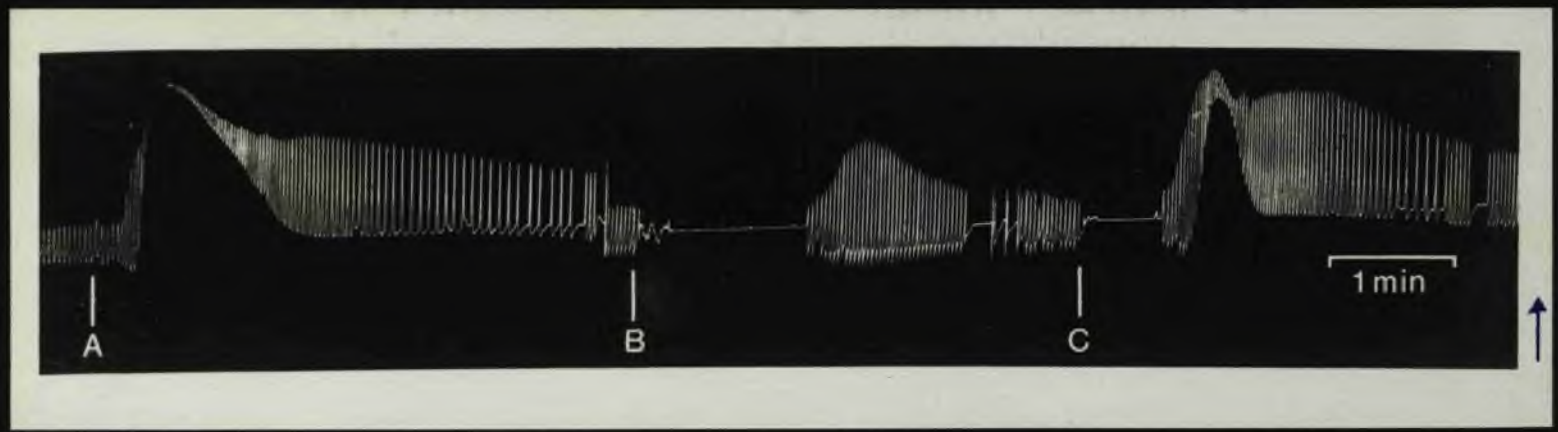
of brain tissue extracts from the gastropod Strophocheilus oblongus were not affected by incubation with pepsin and trypsin whereas incubation with pronase caused a decrease in activity. In contrast, activity of the pericardial organ extracts from the decapod crustacean Cancer borealis was destroyed after incubation with trypsin (Maynard and Welsh, 1959; Belamarich, 1963). A possible explanation of these differences is that the peptides present in S. oblongus extracts are not broken down by pepsin and trypsin, or that the extract is not in a sufficiently pure form for breakdown by enzymes which demonstrate specificity. (Pronase splits a high proportion of peptide bonds whereas trypsin and pepsin are more specific). Hampe, Tesch and Jaeger (1969) later found that brain tissue extracts of Strophocheilus oblongus subjected to Sephadex gel filtration contained two cardio-excitatory fractions. The activity of one of these fractions was destroyed after incubation with the proteolytic enzymes pepsin, trypsin or α-chymotrypsin while the activity of the second was not. Frontali, Williams and Welsh (1967) found that trypsin and chymotrypsin abolished activity in three of four cardio-excitatory fractions from Mercenaria mercenaria ganglia and trypsin and pronase destroyed one of two cardio-excitatory fractions from ganglia of Busycon canaliculatum. Lederis (1970) also found that trypsin and chymotrypsin decreased the activity of urophyseal extracts of the rainbow trout, Salmo gairdnerii. Recently Blanchi, Noviello and Libonati (1973) have reported that activity in crude vena cava extracts of Octopus vulgaris was reduced after incubation with pronase but not after incubation with trypsin.

Experiments designed to assay the effect of incubation of vena cava extracts with trypsin over a longer period (up to 18 hours) and with other enzymes i.e. chymotrypsin and pronase, had to be postponed due to lack of vena cava material.

Figure 51. Enzyme digestion. The effect of successive doses of A - vena cava extract with buffer, B - vena cava extract with buffer and trypsin and C - vena cava extract alone on the isolated systemic heart preparation of Eledone cirrosa. All extracts were placed on a water-bath at 37°C for 60 minutes prior to assay. (Highly concentrated doses of extract appeared to affect the isolated heart preparation adversely, sometimes leading to a cessation of heart beat for short periods. The response shown at A is typical when a high concentration of extract has been administered. In this experiment temporary cessation of beat occurs when further doses of extracts are added to the preparation.)

The blue arrow indicates the direction of contraction.

51



Experiments to compare the effect of vena cava extract with three other pharmacologically active substances: Bradykinin, Eledoisin and Vasopressin.

An extract of the inner layer of the vena cava of Eledone osa was prepared as described on p. 29. The remaining assay tances were obtain as follows:

ykinin - Synthetic Bradykinin, Sandoz, BRS 640
SANDOZ, Basel, Schweiz.
Used at a concentration of 0.1 µg/ml

isin - Eledoison, Batch no. 011.22
SANDOZ, London
Used at a concentration of 5 mg/ml

ressin - Vasopressin injection, B.P. 20 units/ml
Batch no. LHL 198
PARKE, DAVIS & Co., London
Used at a concentration of 20 units/ml

The assay preparation was allowed to equilibrate over a period minutes. 0.1 ml aliquots of the above solutions were then ed into the sea-water perfusing the isolated heart preparation. ing period of 30 minutes was maintained between the addition of liquot. The experiment was repeated on several preparations.

S

Bradykinin, eledoisin and vasopressin all caused inhibition isolated heart preparation while the vena cava extract was the bstance to have an excitatory effect, see Fig. 52.

The vena cava extract caused a very marked increase in ampli- d frequency. This was then followed by a period where beating

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The vena cava extract caused a very marked increase in ampli- d frequency. This was then followed by a period where beating

was intermittent. However, a normal beating pattern was restored after washing for 30 minutes. 0.01 µg bradykinin caused a slowing of the heart beat followed by cessation of the beat and 50 µg of vasopressin and 0.5 µg eledoisin caused immediate cessation of the beat. In all cases a normal beating pattern was restored after the washing period. The preparation still showed a positive response to vena cava extract.

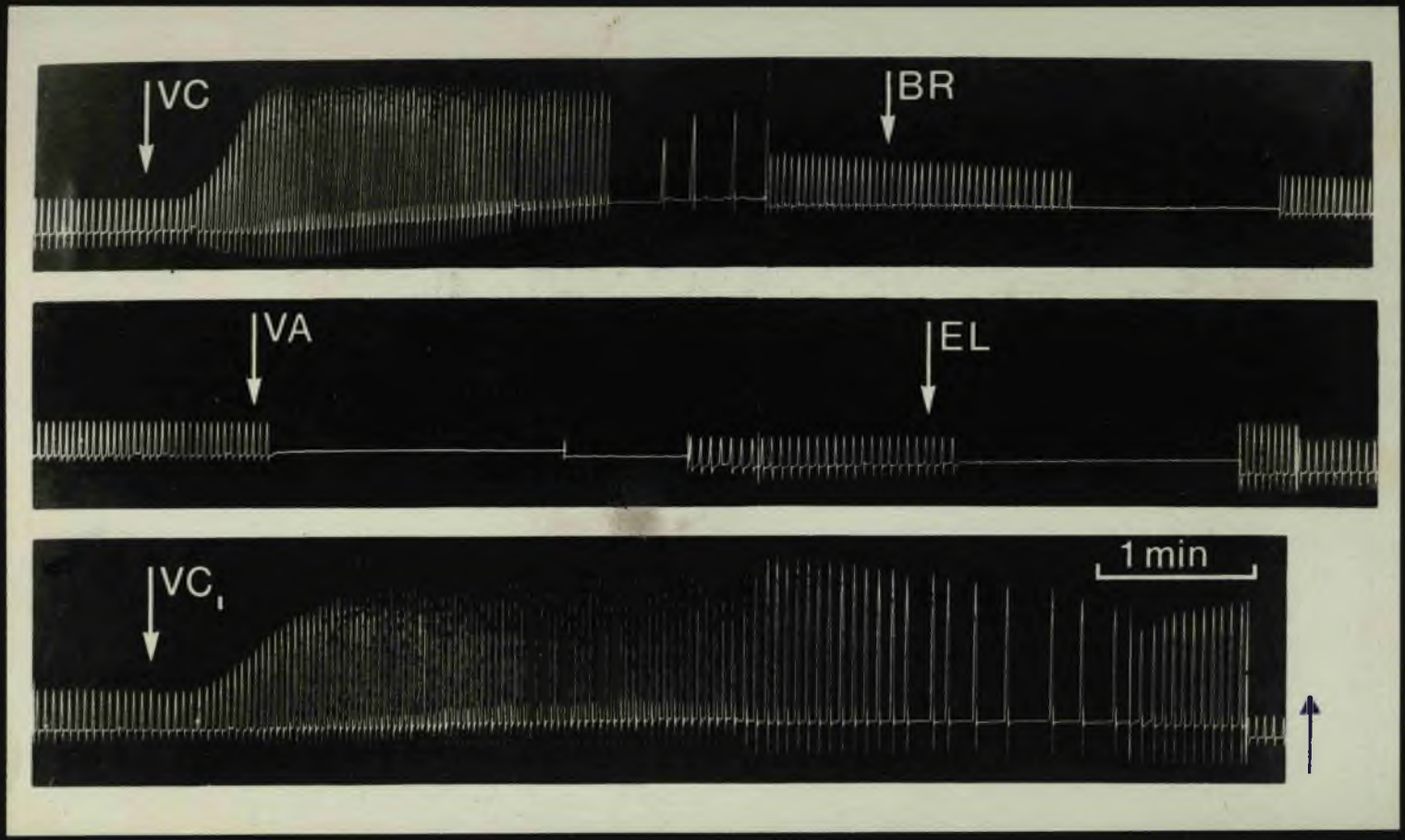
COMMENT

The results of these experiments indicated that the vena cava extract of Eledone cirrosa does not contain substances similar in composition or effect to the pharmacologically active compounds vasopressin, bradykinin or eledoisin.

The response of the heart preparation to vena cava extract did not follow the usual pattern. There was a pronounced excitation followed by intermittent beating. This was a typical response when a highly concentrated dose of extract had been administered. In some cases highly concentrated doses lead to cessation of beat. However, in all cases cessation was always preceded by excitation. After prolonged washing the preparation returned to a normal beating frequency. A similar reaction was also noted by Blanchi, Noviello and Libonati (1973) when assaying vena cava extracts on the isolated systemic heart of O. vulgaris.

Figure 52. Comparison of the effect of successive doses of vena cava extract (VC), bradykinin (BR), vasopressin (VA) and eledoisin (EL) on the same isolated systemic heart preparation of Eledone cirrosa. Dosing: VC - 0.1 ml standard vena cava extract; BR - 0.01 µg bradykinin; VA - 2 units vasopressin; EL - 0.5 µg eledoisin; VC_1 - 0.1 ml 50% standard vena cava extract.

The blue arrow indicates the direction of contraction.


VC
BR
VA
EL
VC1
1 min

CUSSION

The pharmacological experiments indicated that there was a stance associated with the wall of the vena cava of Eledone cirrosa ch stimulated both cardiac and smooth muscle. The site of the active stance was very specific, activity only being found where nerve minations were present in the blood vessel wall. Greatest activity always associated with the inner layer of the vessel wall. There no such activity associated with other blood vessels i.e. the erent branchial vessel or the abdominal aorta.

The activity of vena cava extracts was assayed on three ferent Eledone preparations: the isolated systemic heart, the lated branchial heart and the isolated vena cava, and of these the temic heart preparation was undoubtedly the most sensitive. On s preparation the vena cava extract caused an immediate inotropic ponse followed by a chronotropic response which was maintained for eral minutes. The shape of the response suggested that there might ore than one "active" substance present in the extract. This othesis was supported by results obtained from the hydrolysis and me experiments which indicated that the inotropic response was ected more by these treatments than was the chronotropic response. effect caused by the vena cava extract was unusual in that it was remely prolonged. This is particularly interesting as the extract perfused through the heart and the contact time of the extract very small compared with the time-length of response. There are eral possible explanations for this. The active substance might oined to a carrier molecule and be released relatively slowly or substance might have an immediate effect but only be broken down

gradually and therefore the response would be prolonged.

Blanchi (1969a)* has conducted a series of experiments investigating the effect of extracts of the vena cava of another cephalopod, Octopus vulgaris, on the isolated systemic heart of the same animal. Results were identical to those obtained with E. cirrosa. Blanchi (1969b)* and Blanchi, Noviello and Libonati (1973) have also shown that the effect of these extracts is interspecific since extracts from Octopus macropus and Eledone moschata were effective on the isolated heart of O. vulgaris. Extracts from the venae cavae of the decapod cephalopods, Loligo vulgaris and Sepia officinalis demonstrated activity on isolated decapod hearts but were not so effective on octopod hearts, although the pattern of response was the same. Extracts had to be concentrated ten times to produce a marked effect on an octopod heart. This might be expected as the innervation or neuropile of nerve terminations in the vena cava wall of decapods is not nearly as abundant as that found in octopods, (Alexandrowicz, 1965). However it was also found that decapod hearts were much more sensitive to octopod extracts than extracts from decapods -- octopod extracts had to be diluted 10 - 20 times to give an effect comparable to a decapod extract. The functional significance of such differences is not clear.

There is a marked similarity between the system described in E. cirrosa and the pericardial organ system of decapod crustacea (Alexandrowicz, 1953). In both cases there is a site of release from nerves into the blood stream at a point close to the main vascular pump. Extracts of the nerve terminations of the pericardial organs of

* This 1969 edition of Boll. Sper. was not published until February 1971.

Cancer borealis cause an acceleration of the rate of beat of the isolated heart of C. borealis (Alexandrowicz and Carlisle, 1953). One of the substances responsible for the activity of pericardial organ extracts was thought to be an indolalkylamine, possibly 5-hydroxytryptamine (5-HT) or a related compound (Carlisle, 1956). However, it was later shown that although 5-hydroxytryptamine was present in extracts, the major activity was due to a substance with similar physiological action but differing from 5-hydroxytryptamine (Maynard and Welsh, 1959).

Since the initial increase in amplitude caused by the vena cava extract on the isolated heart of E. cirrosa was similar to the effect caused by 5-hydroxytryptamine on the same preparation it was possible that some 5-HT was present in vena cava extracts. However the experiments using 2-bromolysergic acid diethylamide, an inhibitor of 5-hydroxytryptamine, indicated that 5-hydroxytryptamine was not present in vena cava extracts and this finding was confirmed by fluorescence histochemistry and spectrophotofluorimetry. The results obtained using fluorescence histochemistry also showed that there were no monoamines present in the vena cava tissue.

Simpson (1969) examining a possible neuroendocrine tissue, the intercerebral commissure of the gastropod Helisoma tenue, found vesicles with an electron-dense core similar in appearance to those found in E. cirrosa. Fluorescence histochemistry on this tissue indicated that 5-hydroxytryptamine was not present. In the sub-cellular localisation of 5-hydroxytryptamine both Zs-Nagy and associates (1965) and Cottrell and Maser (1967) concluded that 5-hydroxytryptamine is not bound to vesicles of the type with an electron-dense core and

being 100 - 200 nm diameter. This is interesting in the light of the evidence presented in this thesis that 5-hydroxytryptamine is not associated with the nerve terminals but that marked pharmacological activity is associated with vesicles 80 - 150 nm diameter with an electron-dense core. Recently, Blanchi, Noviello and Libonati (1973) using thin-layer chromatography have shown that the active substance in vena cava extracts from O. vulgaris cannot be identified with 5-hydroxytryptamine.

Johansen and Huston (1962) reported that adrenaline, noradrenaline and tyramine cause retardation of the rate of beat of the systemic heart in the intact, non-anaesthetised cephalopod, Octopus dofleini. This is in contrast with the findings of Fange and Østlund (1954) and Berry (1968) working with isolated cephalopod hearts. Johansen and Huston also showed that 5-hydroxytryptamine caused stimulation of the systemic and branchial hearts in the intact animal.

The vena cava substance was shown to withstand heating on a boiling water bath, freezing and thawing, and to be relatively stable to acid and alkaline hydrolysis. In these respects it resembles brain and heart tissue extracts from the gastropod, Strophocheilus oblongus (Jaeger, 1966) and pericardial organ extracts of the crustacean, Cancer borealis (Belamarich, 1963). Such properties are similar to those of some peptides and in 1966 Belamarich and Terwilliger showed that in the pericardial organ extracts of Cancer borealis, two peptides were responsible for the cardio-excitatory activity. It may be that similar compounds are present in the vena cava extracts of Eledone cirrosa. However, see General Discussion, p.141. Experiments designed to measure the effect of various enzymes on the vena cava extract were not completed due to difficulties with the supply of animals. There

was an indication that the activity of extracts was reduced after incubation with trypsin. This provides another property in common with pericardial organ extracts (Maynard and Welsh, 1959). Enzyme digestion experiments of this type would probably be more meaningful if carried out on purified extracts.

The activity shown by vena cava extracts on other pharmacological preparations did not establish a specific similarity with any other known pharmacologically active substance nor did it compare with another potent cephalopod preparation, eledoisin.

The present work has established that there is a substance or group of substances with unusual pharmacological effects associated with the vena cava in E. cirrosa. Future experiments should be directed towards the standardisation of the assay procedure of this active substance. Although at present the isolated systemic heart of E. cirrosa is the most sensitive assay preparation found it is sometimes variable in its sensitivity and is extremely sensitive to pressure change (Berry, personal observation). A more reliable assay preparation would allow standardisation of the extract and the formulation of a standard extraction procedure.

A more sensitive assay preparation would also allow assay of the nerve trunks leading to the neurosecretory neuropile in the blood vessel wall. This would provide a comparison of the activity of these two regions and possibly indicate whether the active substance is modified on its passage to the nerve terminals.

Cardio-excitatory activity has been shown to be present in extracts of various molluscan tissues (for reviews see Hill and Welsh,

1966; Agarwal, Ligon and Greenberg, 1972). Early work (Kerkut and Laverack, 1960; Kerkut and Cottrell, 1963; Cottrell, 1966; Jaeger, 1966) indicated that extracts of gastropod and lamellibranch ganglia contained a potent cardio-excitatory factor, different from 5-hydroxytryptamine, which was called "substance X" (Hill and Welsh, 1966). Later Frontali, Williams and Welsh (1967) showed that substance X was a mixture of compounds. There is now considerable evidence that a number of cardio-excitatory substances are present in extracts of molluscan nervous tissue, see Table 5.

Experiments to identify these substances and determine their distribution within various molluscan classes have been carried out by Agarwal and Greenberg (1969) and Agarwal, Ligon and Greenberg (1972). Agarwal et al.(1972) state that there are seven cardio-active agents, designated by them as A, B, B°, C, D, acetylcholine and 5-hydroxytryptamine, which are probably characteristic of all molluscan ganglia. However, at present these substances are defined by the extraction and assay procedures used. Employment of different methods may give different results.

As indicated in Table 5 there is increasing evidence that there is at least one cardio-excitatory substance present which is not a catecholamine or 5-hydroxytryptamine but has a small molecule.

In 1969 Agarwal, Ligon and Greenberg proposed that the universal presence of a substance designated peak C in the extracts of all molluscs examined by them and its known effect on isolated hearts might indicate that the substance acts as a cardio-regulator.

More recently they (Agarwal, Ligon and Greenberg, 1972) have expressed the opinion that, since peaks C and B° are universally distributed and concentrated in the molluscan ganglia they have studied and these peaks contain highly active pharmacological agents, many of the effects of molluscan extracts, including the vena cava extract of cephalopods, are probably attributable to these substances. It would be interesting to see which active substances were obtained if vena cava extracts from E. cirrosa were subjected to the procedure used by Agarwal and his associates.

TABLE 5. PHARMACOLOGICAL STUDIES. Distribution of cardio-excitatory substances in molluscs.

Species	Class	Tissue	Assay preparation	Number of cardio-excitatory substances present	Proposed identity	Proposed function	Reference
Cryptochiton stelleri	Amphineura	"Brain"	Isolated ventricle of C. stelleri	1	--	--	Greenberg, 1962
Cryptochiton stelleri	Amphineura	Buccal ganglia	Isolated heart of Mercenaria mercenaria & Amblema neisleri	6	1. A - 2. B - 3. B° possibly a catecholamine but not DA, A or NA. 4. C unknown small molecule probably not a polypeptide 5. D - 6. 5-HT	-- -- -- -- -- --	Agarwal, Lig and Greenbe 1972
		Heart	"	2	1. B° as above 2. C as above	-- --	"

Species	Class	Tissue	Assay preparation	Number of cardio-excitatory substances present	Proposed identity	Proposed function	Reference
Helix aspersa	Gastropoda	Brain, heart, mantle, foot	Isolated heart of H. aspersa	1	Aromatic amine -(not 5-HT).	control of respiration?	Kerkut and Laverack, 1960
Helix aspersa	Gastropoda	"Brain"	Isolated heart of H. aspersa	3	1. 5-HT 2. unknown 3. unknown	-- -- --	Kerkut and Cottrell, 1963
Strophocheilus oblongus	Gastropoda	"Brain" and heart	Isolated heart of S. oblongus & M. mercenaria	2	1. 5-HT 2. unknown - some protein properties but heat stable at different pH.	-- --	Jaeger, 196
Strophocheilus oblongus	Gastropoda	Cerebral ganglia	Isolated heart of S. oblongus	2	1. F_1 unknown - not peptide 2. F_{11} unknown - probably small peptide	-- --	Hampe, Tesch and Jaeger, 1969

Species	*Class*	Tissue	Assay preparation	Number of cardio-excitatory substances present	Proposed identity	Proposed function	Reference
Lymnaea stagnalis	Gastropoda	Heart	Isolated heart of L. stagnalis	3	1. 5-HT 2. unknown 3. unknown hypothesise a peptide	-- -- --	S.-Rózsa an Zs - Nagy, 1967
Busycon canaliculatum	Gastropoda	Cerebral ganglia	Isolated heart of M. mercenaria	2	1. Peptide 2. unknown - (probably not a peptide)	-- --	Frontali, Williams, and Welsh, 1967
Pomacea palludosa	Gastropoda	Pooled cerebral pedal, pleural and buccal ganglia	Isolated heart of M. mercenaria & A. neisleri	5	1. A - 2. B° as for 3. C C.stelleri 4. D - 5. 5-HT	-- -- -- -- --	Agarwal, Lig and Greenber 1972
Mercenaria mercenaria	Bivalvia	Pooled cerebral visceral and pedal ganglia	Isolated heart of M. mercenaria	4	1. A small mol. heat stable - protein or large peptide	--	Frontali, Williams, and Welsh, 1967

Species	Class	Tissue	Assay preparation	Number of cardio-excitatory substances present	Proposed identity	Proposed function	Reference
					2. B Peptide 3. C Peptide 4. D unknown (probably not a peptide) none of these identified with 5-HT	-- -- --	
Mercenaria mercenaria	Bivalvia	Heart	Isolated heart of M. mercenaria	3	1. A } cf. to 2. B } ganglia 3. C } above	-- -- --	Frontali, Williams, and Welsh, 1967
Elliptoides sloatianus	Bivalvia	Ganglia	Isolated heart of Amblema neisleri & M. mercenaria	3	1. 5-HT 2. A - mol.wt. above 1500 3. C - cf. C of Frontali et al, 1967	-- -- C - helps in maintaining rhythm of heart.	Agarwal and Greenberg, 1969
		Heart	"	2	1. A } as for 2. C } ganglia		

Species	Class	Tissue	Assay preparation	Number of cardio-excitatory substances present	Proposed identity	Proposed Function	Reference
Amblema neisleri	Bivalvia	Ganglia	Isolated heart of Amblema neisleri & M. mercenaria	2	1. A - unknown 2. C - as above	C - as above	Agarwal and Greenberg, 1969
		Heart	"	2	1. A - unknown 2. C - as above		
Macrocallista nimbosa	Bivalvia	Pooled cerebral, visceral & pedal ganglia	Isolated heart of A. neisleri & M. mercenaria	6	As indicated for C. stelleri	--	Agarwal, Lig and Greenberg 1972
Eledone cirrosa	Cephalopoda	Nerve-endings in vena cava wall	Isolated systemic heart of E. cirrosa	1 or 2	1. unknown 2. unknown - not 5-HT or catecholamine, possibly peptidergic	May act in response to stress.	Berry and Cottrell, 1970
Octopus vulgaris	Cephalopoda	Nerve-endings in vena cava wall	Isolated systemic heart of O. vulgaris	1	--	--	Blanchi, 1969

Species	Class	Tissue	Assay preparation	Number of cardio-excitatory substances present	Proposed identity	Proposed function	Reference
Octopus vulgaris	Cephalopoda	Nerve-endings in vena cava wall	Isolated systemic heart of O. vulgaris O. macropus E. moschata L. vulgaris S. officinalis	1	1. umknown - resists heating, destroyed by Pronase, some carbohydrate present - not 5-HT	Hormone regulating activity of heart, may also influence a number of physiological activities	Blanchi, 1969b; Blanchi, Noviello and Libonati, 1973
Octopus macropus	Cephalopoda	Nerve-endings in vena cava wall	Isolated systemic heart of O. macropus O. vulgaris E. moschata L. vulgaris S. officinalis	1	--	Hormone regulating activity of heart	Blanchi, 1969b; Blanchi, Noviello and Libonati, 1973
Eledone moschata	Cephalopoda	As for O. macropus	As for O. macropus	1	--	As for O. macropus	Blanchi, 1969b; Blanchi, Noviello and Libonati, 1973

Species	*Class*	Tissue	Assay preparation	Number of cardio-excitatory substances present	Proposed identity	Proposed function	Reference
Loligo vulgaris	Cephalopoda	As for O. macropus	As for O. macropus	1	--	As for O. macropus	Blanchi, 1969b; Blanchi, Noviello and Libonati, 1973
Sepia officinalis	Cephalopoda	As for O. macropus	As for O. macropus	1	--	As for O. macropus	Blanchi, 1969b; Blanchi, Noviello and Libonati, 1973
Octopus bimaculatus	Cephalopoda	Brain, excluding optic lobes	Isolated heart of M. mercenaria & A. neisleri	4	1. A - 2. B - 3. B° } as for 4. C } C.stelleri	-- -- -- --	Agarwal, Ligon and Greenberg 1972

Species -	Class	Tissue	Assay preparation	Number of cardio-excitatory substances present	Proposed identity	Proposed function	Reference
Octopus bimaculatus	Cephalopoda	Systemic heart	Isolated heart of M. mercenaria & A. neisleri	3	1. A - 2. B - 3. C - as for C.stelleri	-- -- --	Agarwal, Ligon and Greenberg, 1972

SUB-CELLULAR LOCALISATION

It was important to determine whether there was a direct correlation between the kinds of structures in the nerve endings and the activity shown in the vena cava extracts. Separation of the neurosecretory structures within the nerve terminals would allow the activity of each component to be compared with the activity of other components of the nerve terminals.

Previous work had indicated that separation of subcellular components can be achieved by differential centrifugation. This method was used by Evans (1962) in the separation of neurosecretory granules from the corpora cardiaca of the cockroach, Periplaneta americana, by Keller and Lederis (1962) in the separation of neurosecretory granules from neural lobes of the rabbit and by Knight (1964) in the separation of neurosecretory granules from the neural lobes of the rabbit and the sheep.

Subsequently it was found that a better separation could be achieved by the application of differential and density gradient centrifugation, a technique which has also been employed in the separation of subcellular components (Whittaker 1959; Morrison [illegible] 1964). This technique was used to separate neurosecretory granules from neurosecretory axons and to characterise neurosecretory granules from various invertebrates (Bertoll, Hay and Pfeiffer, 1961; Hay and Lederis, 1963; Bird, Lederis and Hay and Delamarter, 1970). [illegible] to the isolation of pure fractions of neurosecretory granules and to the characterisation of the neurosecretory products contained within these granules from the neurohypophysis of the rat (Bird and Lederis, 1967). The method has also been used to characterise

SECTION 4

SUB-CELLULAR LOCALISATION

It was important to determine whether there was a direct correlation between the kinds of structures in the nerve endings and the activity shown in the vena cava extracts. Separation of the different structures within the nerve terminals would allow the activity of one component to be compared with the activity of other components of the nerve terminals.

Previous work had indicated that separation of subcellular particles can be achieved by differential centrifugation. This method had been used by Evans (1962) in the separation of neurosecretory granules from the corpora cardiaca of the cockroach, Periplaneta americana; by Heller and Lederis (1962) in the separation of neurosecretory granules from neural lobes of the rabbit and by Lederis (1962) in the separation of neurosecretory granules from the cod, Gadus morrhua.

Subsequently it was found that a better separation was achieved with a combination of differential and density-gradient centrifugation, a procedure which has also been employed in the characterisation of transmitter substances (Whittaker 1959; Whittaker, Michaelson and Kirkland, 1964). This technique was used successfully to isolate secretory granules from neurosecretory systems both in vertebrates (Barer, Heller and Lederis, 1963; Bindler, LaBella and Sanwal, 1967) and in the invertebrates (Bartell, May and Fingerman, 1968; Terwilliger, Terwilliger, Clay and Belamarich, 1970). Critical application of the technique led to the isolation of pure fractions of neurosecretory granules and characterisation of the neurosecretory hormones and their carrier proteins from the neurohypophysis of the bovine pituitary (Dean and Hope, 1966; 1967). The method had also been used for examination

of sub-cellular fractions of cephalopod material, in particular the brain tissue of Octopus vulgaris (Jones, 1967; 1970).

Therefore differential and density-gradient centrifugation were employed to obtain a pure fraction of the most numerous structure within the nerve terminals of E. cirrosa i.e. the electron-dense vesicles. This fraction might then be compared with other fractions of the gradient. Fractions were examined in two ways: 1) by examination with the electron microscope, and 2) by bioassay.

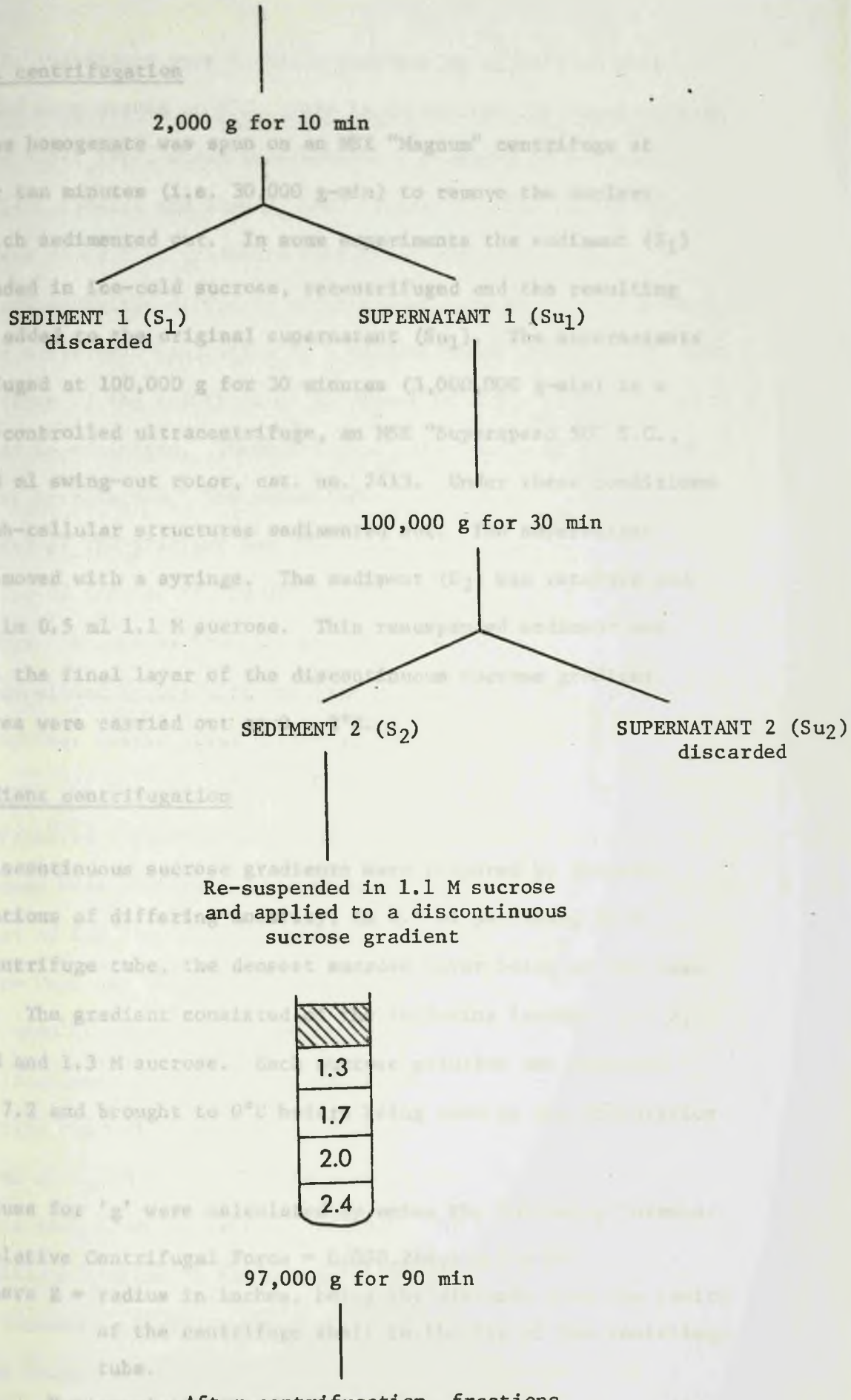
EXPERIMENTAL PROCEDURE

The fractionation procedure used was adapted from that employed by Cottrell, 1966. An outline of the procedure is shown in Fig. 53.

Homogenisation

Animals were killed by "decapitation" and the vena cava was isolated in an ice-cold wax dish. The vessel was slit longitudinally and pinned out to expose the inner surface. (Only the inner ridged layer of the vessel was used for extraction. This region had been shown to correspond with the area containing the nerve terminals and thus provided very suitable material from which to isolate the electron-dense vesicles.) The inner layer was scraped off with a cooled metal spatula and a known amount of tissue was placed in a glass homogeniser containing 1.1 M sucrose at pH 7.0. A ground-glass pestle was used with an homogeniser clearance of 0.05 mm. The tissue was homogenised (at 1000 rpm) for two one minute periods with two upward and downward thrusts in each period. This procedure was repeated exactly for each experiment.

Figure 53. Outline of the centrifugation procedure used to separate sub-cellular structures from the nerve terminations of the vena cava wall.


2,000 g for 10 min
SEDIMENT 1 (S_1)
discarded
SUPERNATANT 1 (Su_1)
100,000 g for 30 min
SEDIMENT 2 (S_2)
SUPERNATANT 2 (Su_2)
discarded
Re-suspended in 1.1 M sucrose
and applied to a discontinuous
sucrose gradient
1.3
1.7
2.0
2.4
97,000 g for 90 min
After centrifugation, fractions
collected from gradient
using a syringe

Differential centrifugation

The homogenate was spun on an MSE "Magnum" centrifuge at 3,000 g* for ten minutes (i.e. 30,000 g-min) to remove the nuclear material which sedimented out. In some experiments the sediment (S_1) was resuspended in ice-cold sucrose, recentrifuged and the resulting supernatant added to the original supernatant (Su_1). The supernatants were centrifuged at 100,000 g for 30 minutes (3,000,000 g-min) in a temperature-controlled ultracentrifuge, an MSE "Superspeed 50" T.C., with a 3 x 3 ml swing-out rotor, cat. no. 2413. Under these conditions all small sub-cellular structures sedimented out. The supernatant (Su_2) was removed with a syringe. The sediment (S_2) was retained and resuspended in 0.5 ml 1.1 M sucrose. This resuspended sediment was then used as the final layer of the discontinuous sucrose gradient. All procedures were carried out at 0 - 2°C.

Density-gradient centrifugation

Discontinuous sucrose gradients were prepared by layering sucrose solutions of differing molarity, in 0.5 ml portions, in a polythene centrifuge tube, the densest sucrose layer being at the base of the tube. The gradient consisted of the following layers: 2.4 M, 2.0 M, 1.7 M and 1.3 M sucrose. Each sucrose solution was adjusted to pH 6.8 - 7.2 and brought to 0°C before being used in the preparation

*All the values for 'g' were calculated by using the following formula:

Relative Centrifugal Force = $0.000,284,4 \times R \times N^2$.

where R = radius in inches, being the distance from the centre of the centrifuge shaft to the tip of the centrifuge tube.

N = revolutions per minute.

of a gradient. Gradients were normally prepared up to half an hour before use and were stored at 0°C. This is in contrast to other workers (see Dean and Hope, 1967, where gradients were prepared 18 hr. before use and Bindler, LaBella and Sanwal, 1967, where the time period was 2 hr.). It was found that gradients containing marker dyes (e.g. methylene blue) exhibited diffusion effects when left at 0°C for several hours. Since structures separating in a discontinuous gradient separate at the interfaces of the solutions of different molarity any diffusion effects should be minimised. Therefore gradients were used as soon as possible after preparation. The resuspended sediment (S_2) was added as the final layer of the gradient and the gradient was then centrifuged at 97,000 g for 90 minutes (8,730,000 g-min) in the MSE "Superspeed 50" ultracentrifuge. Fractions from the gradient (as indicated in Fig. 54a) were collected either by piercing the base of the centrifuge tube with a "denecked syringe needle" (see Cottrell, 1966) or by pipetting off using a syringe. Of the two collecting methods tried, the pipetting method was preferred since this gave the most consistent fractions. Fraction volumes were difficult to standardise with the piercing method since the size of the drop differed with the molarity of the sucrose (also found by Dean and Hope, 1967). A series of trial gradients with markers were used to calculate the volume required in each fraction to ensure that fractions corresponded to interface regions (see Fig. 54a). After collection the volume and molarity of each fraction was adjusted to give 1.0 ml of 1.1 M sucrose. This ensured that all structures present within each fraction were subjected to the same osmotic conditions, that is conditions similar to the normal cell environment, during the remaining processing. Fractions were then processed for electron microscopy or frozen and stored at -20°C until required for bioassay or protein estimation.

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The molarity of the sucrose used in this procedure is much higher than that used by workers in the separation of vertebrate material, where normally a 0.32 M sucrose solution is used. In the case of E. cirrosa the higher molarity is similar to the osmotic conditions normally found in this marine animal. Jones (1967) has pointed out that use of 0.32 M sucrose with material from Octopus vulgaris results in severe damage to membranes due to hypo-osmotic effects. Cottrell (1966) used glucose in preference to sucrose since glucose has a lower viscosity. However in the case of E. cirrosa glucose did not appear to offer any advantages over sucrose. A similar finding was made by Jones (1967). Therefore sucrose solutions were used throughout.

Analysis of gradients

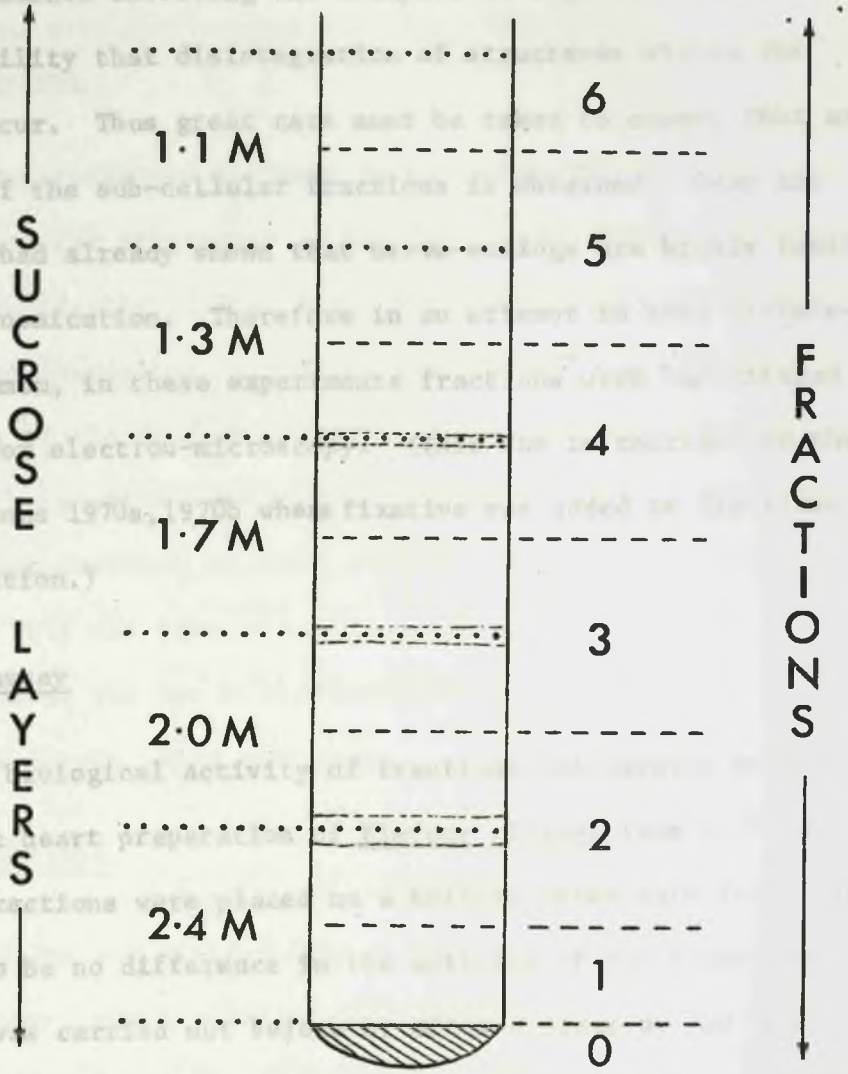
1. Electron Microscopy

Fractions taken from the gradient were centrifuged at 97,000 g for 60 minutes (5,820,000 g-min) to sediment any structures present. The supernatants were discarded and the sediments prepared for examination with the electron microscope. The sediments were retained within the centrifuge tubes to prevent disintegration and disorientation. The pellets were fixed according to the scheme on p. 13 . The material was dehydrated through an acetone series, embedded in Araldite and heated at 60°C for 36 hr. The tubes were then discarded and the Araldite pieces were replaced in the oven for a further 36 hr. to ensure complete hardening of the Araldite. Pellets were mounted for cutting so that sections were cut as shown in Fig. 54b. This ensured that if there was layering of material within the pellet this would be seen on microscopic examination. Each pellet was sampled from at least four levels.

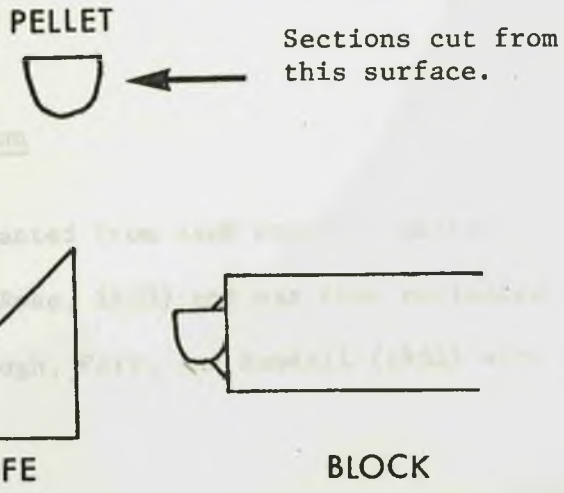
Figure 54. a) Diagram illustrating the appearance of the discontinuous sucrose gradient after centrifugation. Note that banding appeared at the interfaces of the different sucrose solutions. The most noticeable band was at the 2.0 M - 2.4 M sucrose interface.

b) Diagram illustrating the orientation of the fraction pellet when cutting sections for electron microscopy.

54a


SUCROSE LAYERS
1·1 M
1·3 M
1·7 M
2·0 M
2·4 M
FRACTIONS
6
5
4
3
2
1
0

54b


PELLET
Sections cut from this surface.
KNIFE
BLOCK

In experiments involving the analysis of a gradient there is a distinct possibility that disintegration of structures within the gradient might occur. Thus great care must be taken to ensure that an accurate record of the sub-cellular fractions is obtained. Gray and Whittaker (1962) had already shown that nerve-endings are highly labile structures after osmication. Therefore in an attempt to keep disintegration to a minimum, in these experiments fractions were centrifuged before fixation for electron-microscopy. (This was in contrast to the method used by Jones 1970a, 1970b where fixative was added to fractions before centrifugation.)

2. Bioassay

The biological activity of fractions was assayed on the isolated systemic heart preparation of Eledone cirrosa (see p. 31). Prior to assay fractions were placed on a boiling water bath for 3 minutes. There appeared to be no difference in the activity of the fractions whether boiling was carried out before or after storage at low temperature. Sucrose at the concentration used in the fractionation procedure causes excitation of the isolated heart preparation of E. cirrosa. Therefore each fraction was diluted 1:5 in sea-water prior to assay so that the final concentration of sucrose would have no detectable effect on the isolated heart. 0.1 ml aliquots of the diluted fractions were taken for assay.

3. Protein estimation

Protein was extracted from each fraction using 1.0 M and 0.2 M perchloric acid (after Rose, 1970) and was then estimated using the method of Lowry, Roseborough, Farr, and Randall (1951) with bovine serum albumin as a standard.

RESULTS

Appearance of gradient

When the resuspended sediment S_2 was submitted to density-gradient separation the appearance of the tube after centrifugation was as shown schematically in Fig. 54a. Structures within the homogenate distributed themselves throughout the gradient, particularly at the interfaces of the different sucrose solutions, and were apparent as bands within the gradient and as a small pellet at the bottom of the tube. The most distinct band was that between the 2.4 M and 2.0 M sucrose layers. This was fawn in colour. Banding at higher interfaces in the gradient was not so distinct, the band between the 1.3 M and 1.1 M sucrose layers being very faint.

Morphology of fractions

The gradient was divided into six fractions as indicated in Fig. 54a. Fraction 0 was the pellet that sedimented at the bottom of the tube and fraction 1 was the quantity of 2.4 M sucrose between the pellet and the first band. Fractions 2 - 5 corresponded to the interface regions between 2.4 M and 2.0 M sucrose, 2.0 M and 1.7 M sucrose, 1.7 M and 1.3 M sucrose and 1.3 M and 1.1 M sucrose respectively. Fraction 6 consisted of the remaining sucrose and homogenate.

The colours of the precipitates of individual fractions after centrifugation are given in Table 6.

TABLE 6. SUB-CELLULAR LOCALISATION. Appearance of precipitates obtained from the various fractions after centrifugation.

Fraction Number	Fraction 0	Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6
Colour of Precipitate	Fawn/ White	White	Fawn	Fawn	Yellow	no ppt.	very small ppt.

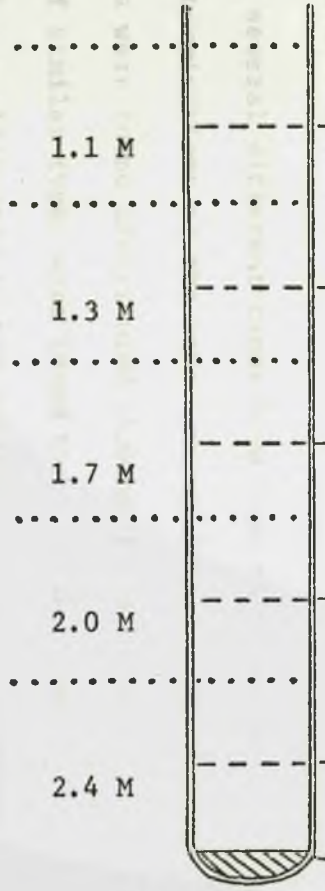
Each fraction was examined using the electron microscope and the findings are outlined in Table 7.

Within fraction 0 there were vesicles of assorted types. The most predominant of these were electron-dense vesicles, 80 - 150 nm diameter, which were isolated throughout the fraction. Amorphous material, considered to be cell debris, was also seen (Fig. 55a).

Fraction 1 was composed of electron-dense vesicles, 80 - 150 nm diameter; some electron-lucent vesicles of a similar size; and some larger vesicular structures, 50 - 500 nm diameter, which were probably derived from nerve-ending membranes (Fig. 55b).

The most homogeneous layer of the gradient was seen in fraction 2 (Fig. 56). This consisted almost entirely of isolated electron-dense vesicles, 80 - 150 nm diameter. Occasionally electron-lucent vesicles were seen. When examined at high magnification the electron-dense vesicles were shown to possess a limiting membrane (Fig. 57). A small amount of cell debris was also present.

TABLE 7. SUB-CELLULAR LOCALISATION. Identification of components within individual fractions.

Sucrose Layers	Gradient	Fraction	Morphological Identification
		6	Nerve-ending bodies. Assorted vesicle types.
1.1 M		5	No precipitate.
1.3 M		4	Nerve-ending bodies. A few vesicles.
1.7 M		3	Assorted vesicle types. Nerve-ending "ghosts" and a few nerve-ending bodies.
2.0 M		2	Electron-dense vesicles. A small amount of cell debris with a few electron-lucent vesicles.
2.4 M		1	Electron-dense vesicles. Electron-lucent vesicles. Cell debris.
		0	Assorted vesicle types. Cell debris.

In fraction 3 assorted vesicle types were seen, viz. electron-dense vesicles, electron-lucent vesicles and "microvesicles". Large vesicular structures, ranging from 200 - 600 nm diameter, were also detected (Fig. 58). These are thought to be derived from nerve-ending membranes and were designated "nerve-ending ghosts". Vesicles were isolated throughout the fraction or were contained within these larger vesicular structures. Often, vesicles of different types were seen contained within the same "nerve-ending ghost".

Larger membrane structures ranging from 250 nm - 1 μm diameter, containing high concentrations of vesicles, particularly of the electron dense type, were found in fractions 4 and 6. These have been named "nerve-ending bodies". They were not found in the lower fractions. "Nerve-ending ghosts" and isolated vesicles of assorted types were also present in both of these fractions, see Fig. 59 and Fig. 60. The "nerve-ending bodies" usually contained vesicles of one specific type while the smaller "nerve-ending ghosts" appeared to contain an assortment of vesicle types.

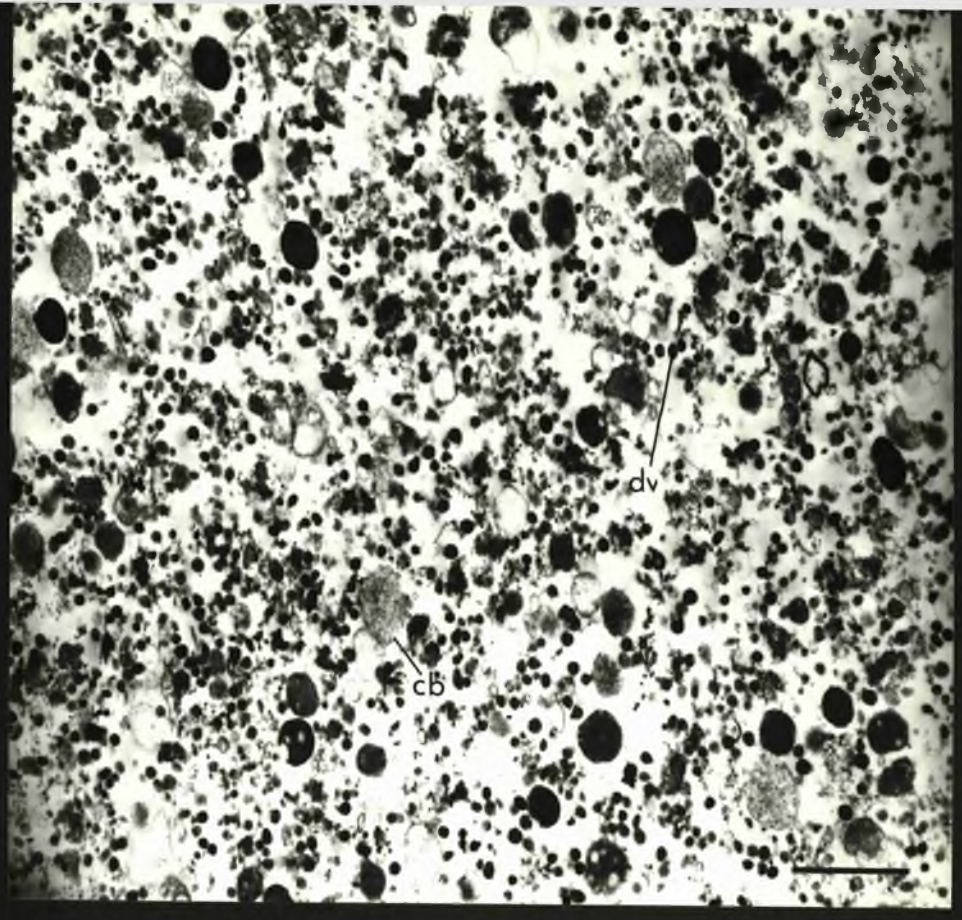
No sediment was obtained after centrifugation of fraction 5.

Characteristics of isolated vesicles

Vesicles of several different types have been shown to be present in the nerve terminations of the vena cava wall (see p. 15). The same vesicle types were found distributed through the sucrose gradient. Vesicles of similar types were found to be of the same dimensions wherever they equilibrated in the gradient and the dimensions were comparable to those of the vesicles found in normal tissue. The general appearance of the vesicles in these different situations was

Figure 55a. Electron micrograph of Fraction 0. Electron-dense vesicles (dv) and cell debris (cb). Bar represents 1 μm.

Figure 55b. Electron micrograph of Fraction 1. Electron-dense vesicles (dv); electron-lucent vesicles (lv) and large vesicular structures (lav). Bar represents 1 μm.



55b

Figure 56. Electron micrograph of Fraction 2. This fraction displayed the most homogeneous layer of the gradi and consisted almost exclusively of isolated elec dense vesicles. This shows a longitudinal sectio through the entire pellet, the top of the pellet being towards the top of the page.
Bar represents 1 μm.

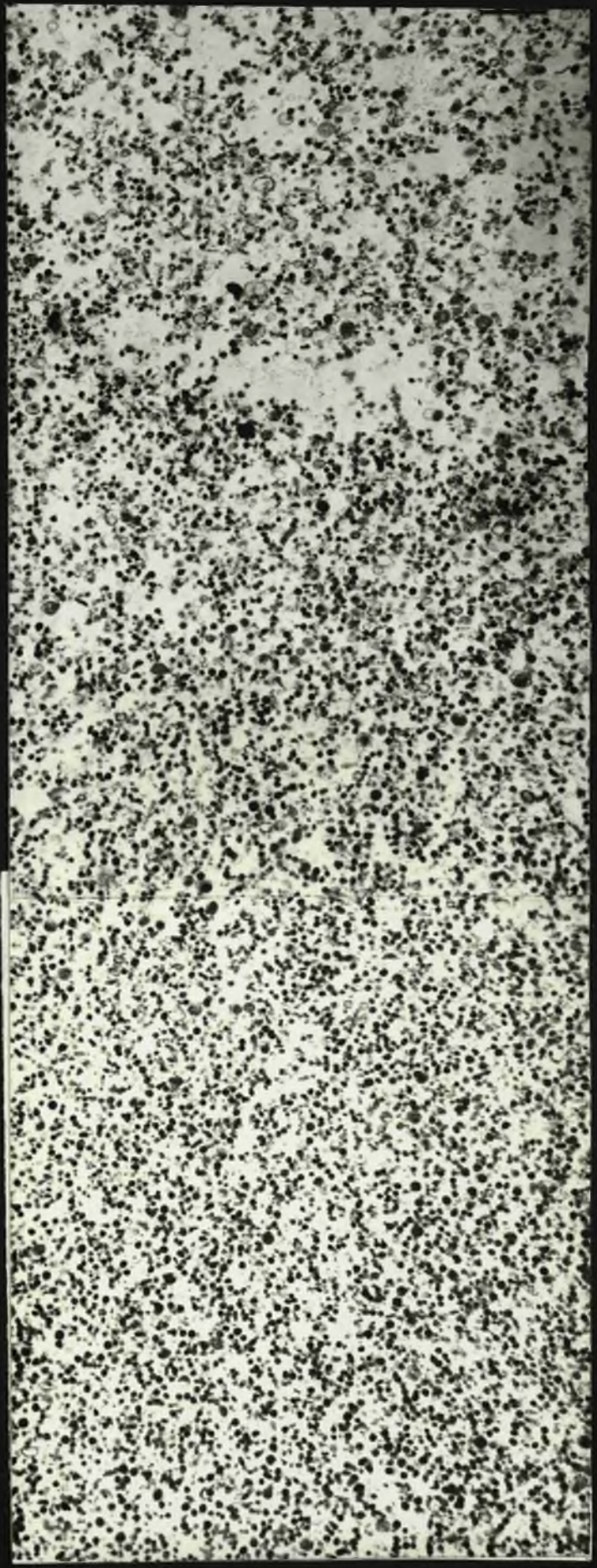
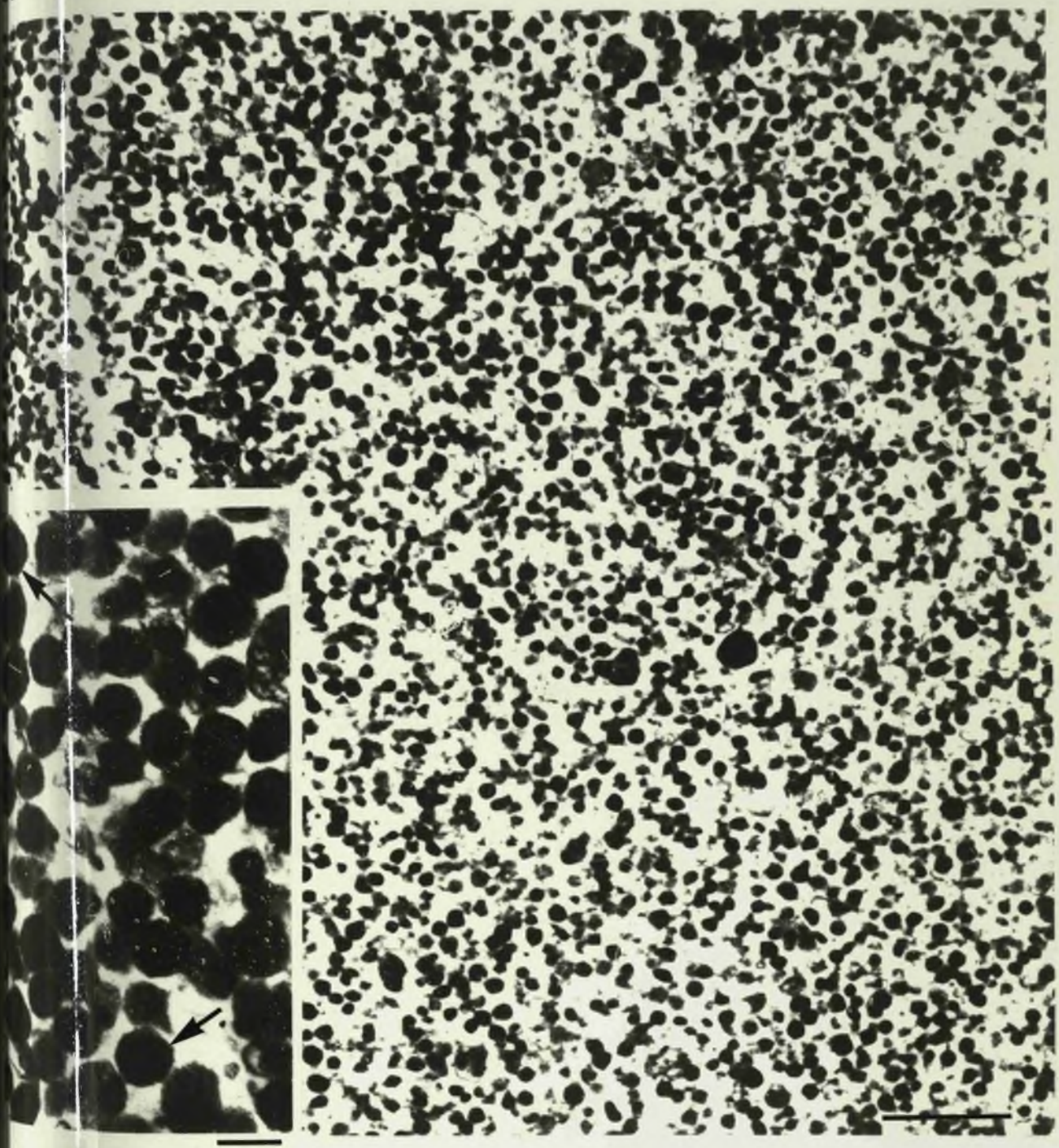


Figure 57. Electron micrograph of part of Fraction 2 at high magnification. Note the membrane which envelopes each electron-dense vesicle. In some cases this appears crenelated (indicated by arrows).
Bar represents 1 µm.
Inset bar represents 100 nm.



2 at high
envelopes
ses this
.

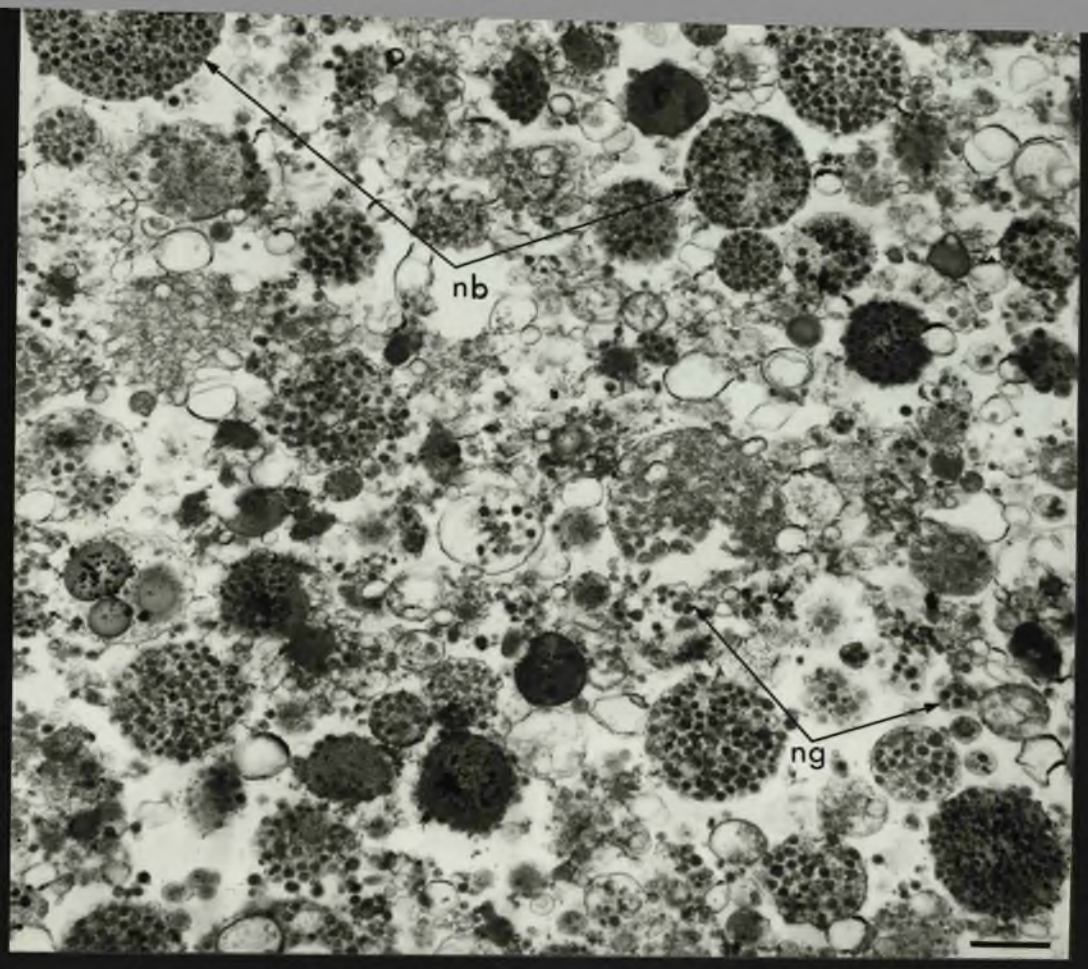
Figure 58. Electron micrograph of Fraction 3. Electron-dens
vesicles (dv); electron-lucent vesicles (lv);
microvesicles (mv) and nerve-ending ghosts (ng).
Arrows indicate nerve-ending ghosts that contain
an assortment of vesicles.
Bar represents 1 μm.

tron-dens
(lv);
ts (ng).
contain

Figure 59. Electron micrograph of Fraction 4. Nerve-ending bodies (nb); nerve-ending ghosts (ng).
Bar represents 1 μm.

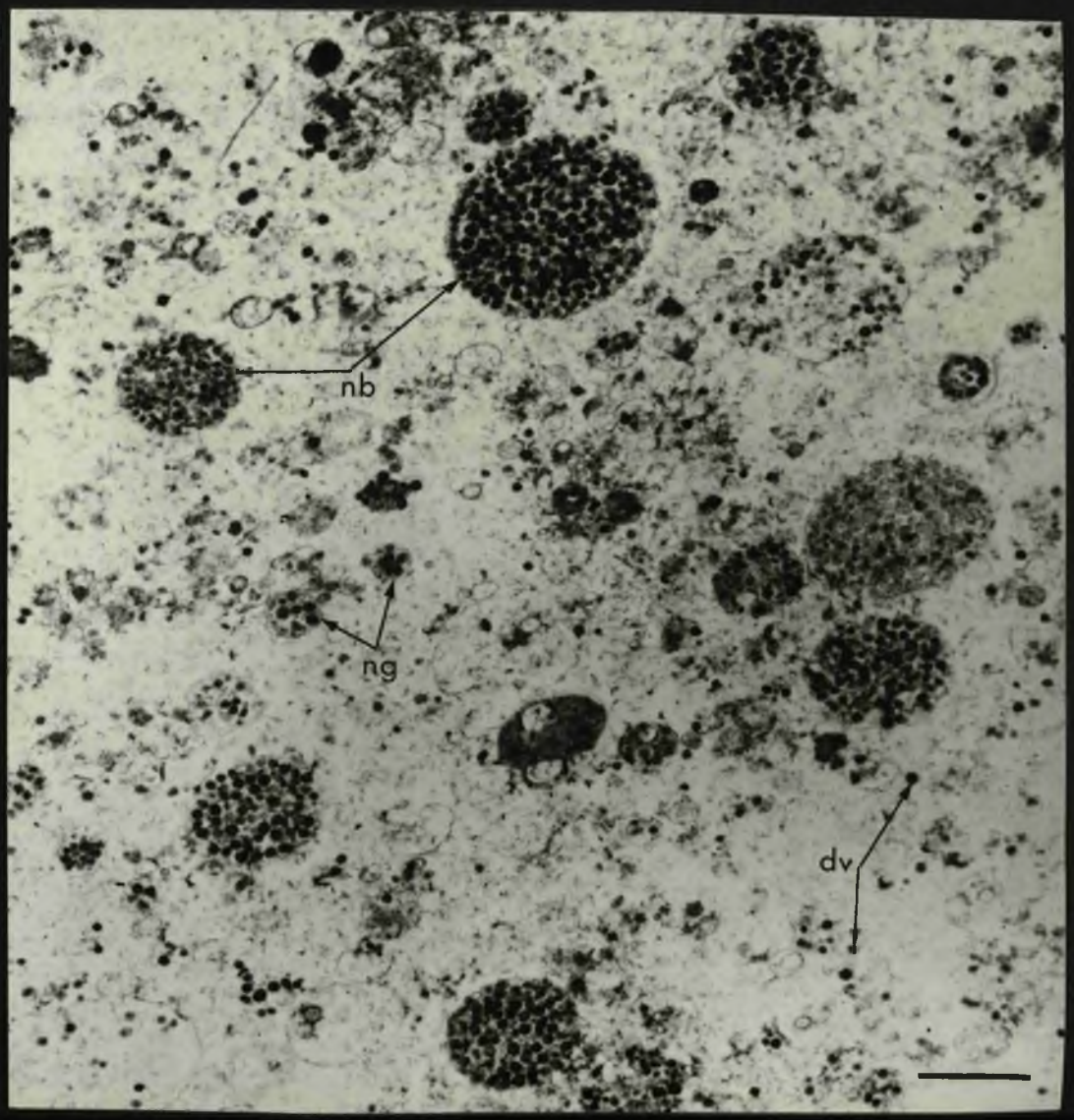
Figure 60. Electron micrograph of Fraction 6. Nerve-ending bodies (nb); nerve-ending ghosts (ng); electron-dense vesicles (dv).
Bar represents 1 μm.

e-ending



60

ve-ending
electron-



also similar. However at high magnification (see Fig. 57) the outer membrane of the electron-dense vesicles was seen to be crenelated.

An estimate of the number of isolated electron-dense vesicles present within each fraction was made and expressed as a percentage of the total number of vesicles counted. This indicated that 50% of the isolated electron-dense vesicles within the gradient equilibrated at the 2.4 M - 2.0 M interface, see Table 8.

TABLE 8. SUB-CELLULAR LOCALISATION. Assessment of the number of isolated electron-dense vesicles present in each fraction of the gradient. The figures represent the number of electron-dense vesicles present in each fraction expressed as a percentage of N, the total number of isolated vesicles counted in each fraction (N = 1532).

Fraction Number	1	2	3	4	5	6
Dense Vesicle Content	19.5	49.5	22.7	5.37	--	3.7

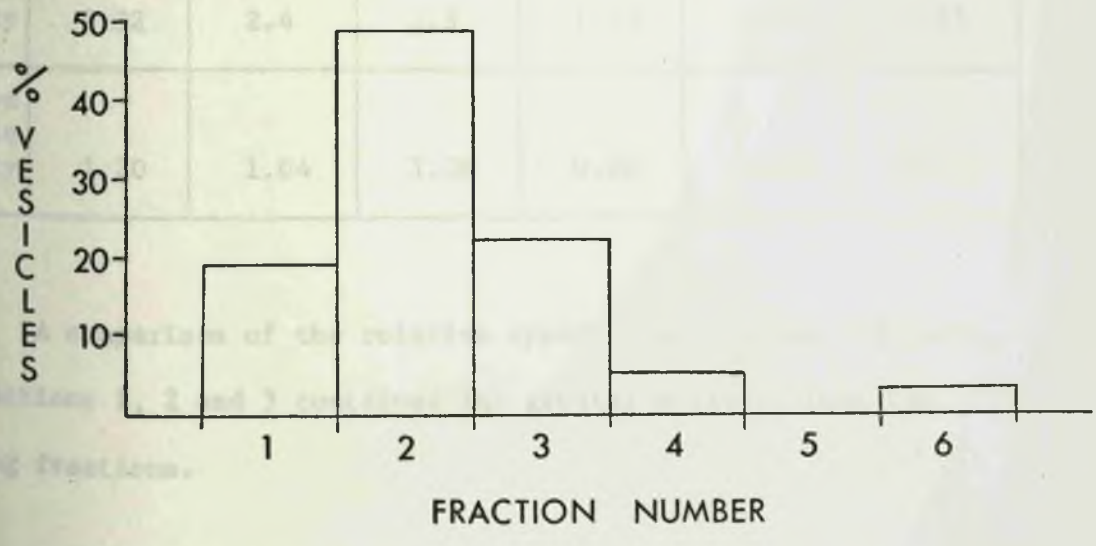
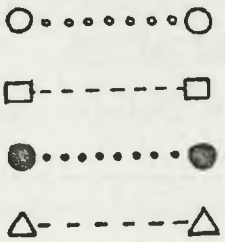


Figure 61. Graph indicating the biological activity of individual fractions from a discontinuous sucrose gradi The results were obtained from four separate experiments.



Gradient 9-7-70

Gradient 10-7-70

Gradient 5-9-70

Gradient 5-10-70

Activity was measured as the increase in amplitud relative to the basal heart amplitude.

FRACTION / EXPT.	% ACTIVITY					
	1	2	3	4	5	6
1 ○	50	100	75	43	43	28
2 ●	75	100	25	—	—	—
3 △	50	170	100	50	33	—
4 ■	83	100	66	16	33	—

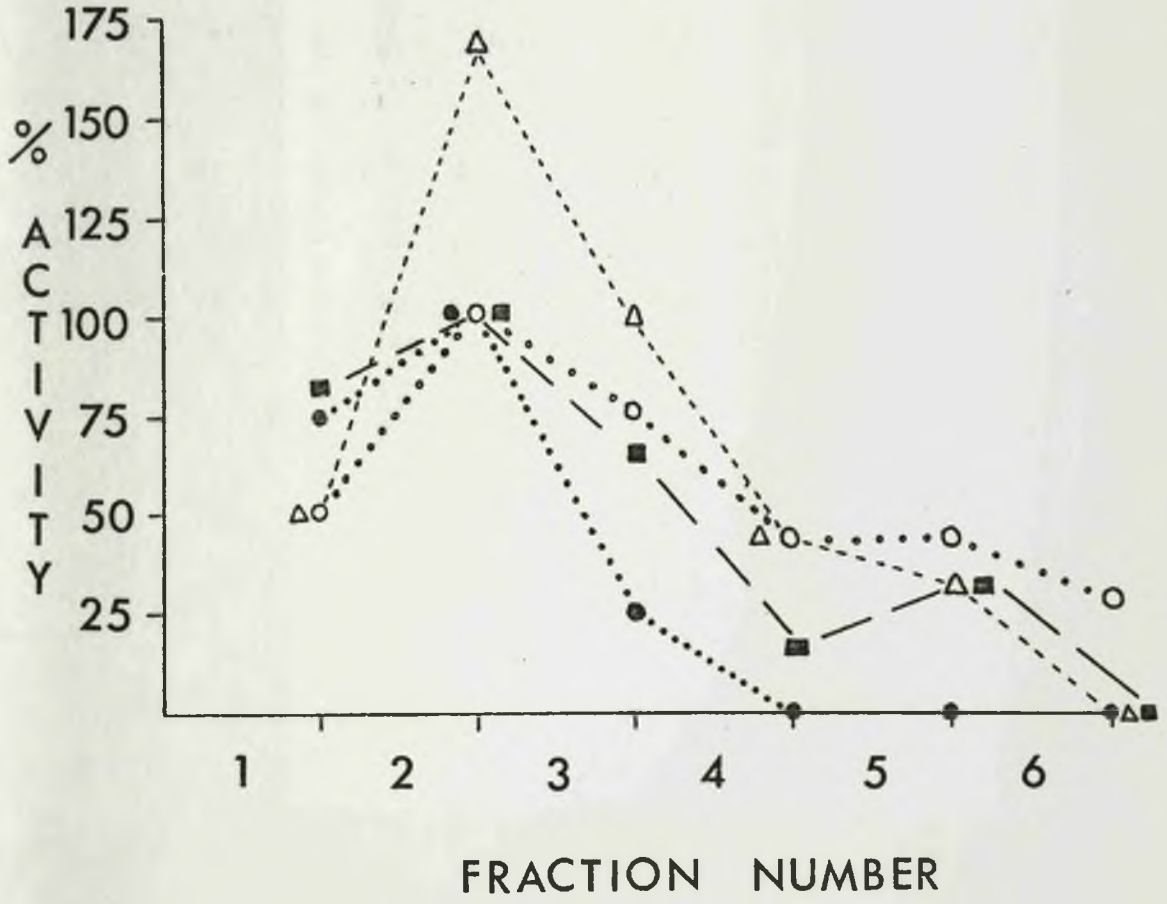
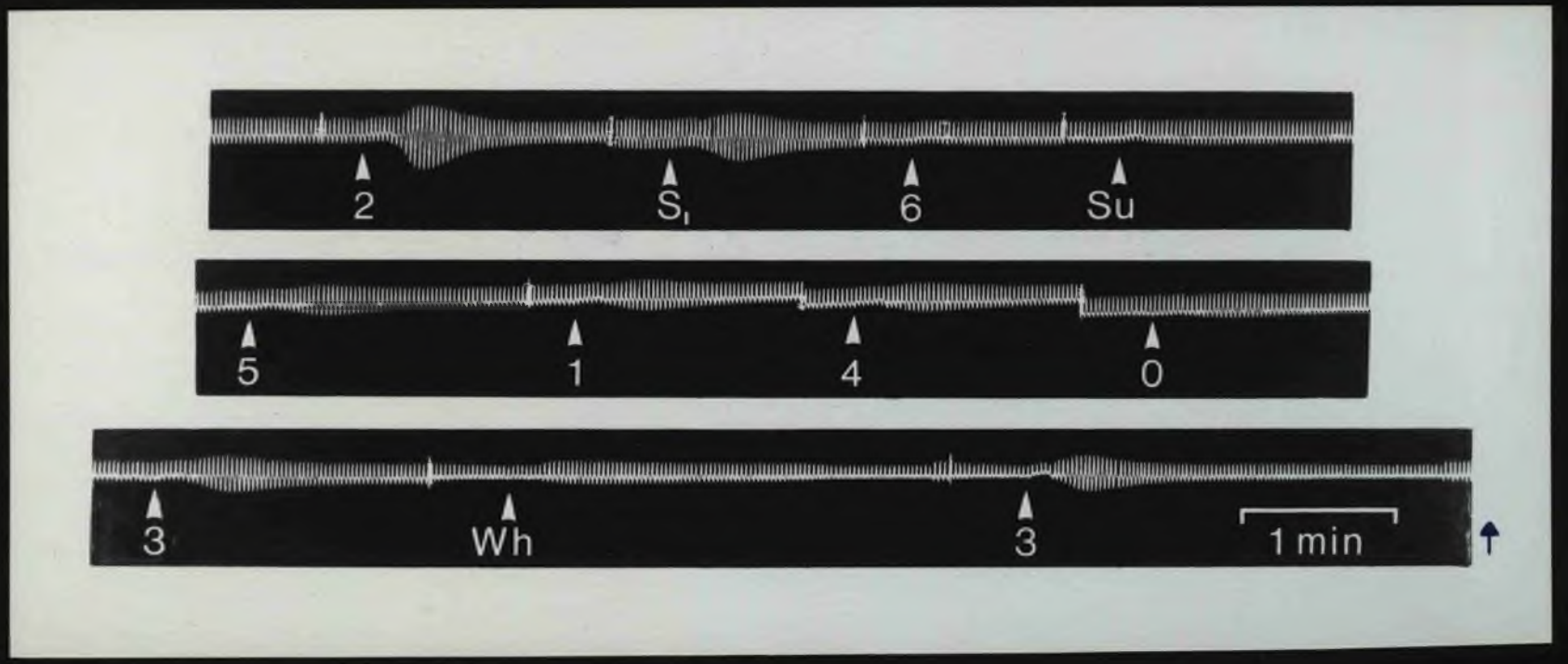


Figure 62. Bioassay of fractions from the discontinuous sucrose gradient experiment of 6-10-70. The assay was carried out on the isolated systemic heart preparation of E. cirrosa. Figures correspond to fraction numbers; Wh - whole homogenate; S_1 - sediment 1; Su - supernatant 2.

The blue arrow indicates the direction of contraction.

62



DISCUSSION

The results indicated that by using differential and density gradient centrifugation it is possible to separate electron-dense vesicles from other structures contained in the nerve endings associated with the vena cava in Eledone cirrosa. In addition, fractions containing partially ruptured nerve endings and assorted vesicles were obtained.

Although Jones (1970) has separated synaptic vesicles from the brain tissue of Octopus vulgaris, previous attempts to separate electron-dense vesicles from molluscan tissue have resulted in fractions containing heterogeneous material. In 1966 Nisbet and Plummer attempted the isolation of vesicles of this type from the heart tissue of the pulmonate Archachatina marginata. However, they were unable to make a complete isolation of the vesicles. The vesicles were contained in structures comparable to the "nerve-ending bodies" described on p. 88 . In experiments to determine the sub-cellular localisation of 5-hydroxytryptamine in the ganglia of the clam Mercenaria mercenaria Cottrell and Maser (1967) obtained heterogeneous vesicle fractions. The results obtained with E. cirrosa show that an almost homogeneous layer of electron-dense vesicles was found at the 2.4 - 2.0 M sucrose interface. The preparation of a homogeneous fraction thus allowed a direct comparison of the biological activity of this fraction i.e. the electron-dense vesicles, with other fractions which contained a heterogeneous mixture of nerve-ending material.

It was found that cardio-excitatory activity was present in all fractions but the greatest activity was always associated with the homogeneous fraction of the gradient. From this it would appear that the substance or substances responsible for this pharmacological effect

are represented by the electron-dense vesicles.

Although a precipitate was never obtained from centrifugation of fraction 5, activity was also associated with this fraction. This finding supports the hypothesis that some activity is lost from the granules during the extraction procedure. Loss of this type may also be the explanation for the finding that the relative specific activity of fraction 1 is higher than that of fraction 2. Nevertheless readings of relative specific activity confirmed that greatest activity was associated with the fractions containing the majority of the isolated electron-dense vesicles.

The finding that half of the total number of electron-dense vesicles present within the whole gradient form an almost pure fraction of electron-dense vesicles suggests that these structures have attained their equilibrium position within the gradient. The electron-dense vesicles present in the higher fractions of the gradient may be in this different position because of the late rupture of nerve-ending bodies. In the isolation of synaptic vesicles from the brain of O. vulgaris Jones (1970) found that vesicles were present in 3 of the 5 fractions resulting from density gradient centrifugation. As an explanation of this Jones argued that as the vesicles in each fraction were morphologically similar the different sedimentation properties might reflect differences in molecular constitution. It may be however that in this case the equilibrium position for the vesicles had not been attained. In the case of the E. cirrosa gradients the electron-dense vesicles in each fraction appeared morphologically the same and the pharmacological activity associated with each fraction was identical. From these observations it is unlikely that there are differences in molecular

constitution between electron-dense granules from different parts of the gradient.

Since the solutions of the gradient provided conditions that were hyper-osmotic to those prevailing within the normal tissue one might have expected shrinkage of the vesicles with an attendant crenelation of the outer membrane. However, the diameter of the vesicles remained remarkably constant. It was only at high magnifications that evidence of slight shrinkage could be seen.

There were two types of structure in the gradient that had not been seen in previous electron microscopic examination of the vena cava tissue viz. the "nerve-ending ghosts" and the "nerve-ending bodies". The "nerve-ending ghosts" were smaller in size than the "nerve-ending bodies" or the normal nerve terminations. They were probably fragments of membrane which had rounded off in a manner similar to that proposed for the formation of "synaptosome ghosts" (Whittaker, Michaelson and Kirkland, 1964; Whittaker and Sheridan, 1965). The "ghost" structures were always found in fractions lower in the gradient than the "synaptosome bodies".

Comparison of the "nerve-ending bodies" with the size of the normal nerve terminations suggested that the former are pinched off nerve terminals analogous to "synaptosomes" (see Gray and Whittaker, 1962). The structures isolated in the E. cirrosa gradient between 1.7 M - 1.3 M sucrose and at 1.1 M sucrose are homologous to structures described by Bindler, LaBella and Sanwal (1967) in the fractionation of bovine pituitary extracts. They called the structures "neurosecretosomes". Neurosecretosomes and nerve-ending bodies probably form in a manner similar to synaptosomes. Bindler et al stated that the sedimentation

of the neurosecretosomes is probably largely determined by the type of neurosecretory granules they contain. In the case of E. cirrosa it is unlikely that this is the sole factor involved since nerve-ending bodies containing different types of vesicles equilibrated at the same position in the gradient.

Terwilliger, Terwilliger, Clay and Belamarich (1970), using similar techniques, have recently shown that the cardio-excitatory peptide present in the pericardial organs of the crab, Cancer borealis, is associated with electron-dense granules. There is a very striking similarity in the structure and properties of this pericardial organ system and the neurosecretory system of the vena cava in E. cirrosa. In both cases cardio-excitatory activity has been shown to be associated with electron-dense vesicles 100 - 150 nm diameter. Terwilliger and associates also provided evidence that the active substance, thought to be a peptide, is enclosed within a membrane-bound particle.

There is now increasing evidence, from both vertebrate and invertebrate systems, to indicate that the neurohormones elaborated by neurosecretory systems are associated with the "elementary neurosecretory granule" seen with the electron microscope. In 1963 Barer, Heller and Lederis showed that this was the case with the neurohormones from the posterior pituitary of the rabbit. Evans (1962) demonstrated a similar relationship in the corpora cardiaca of insects and Terwilliger, Terwilliger, Clay and Belamarich (1970) did so for the pericardial organs of a crustacean, Cancer borealis. The results obtained with E. cirrosa provide further proof that this is so.

SECTION 5

BIOCHEMICAL CHARACTERISATION

The fractionation experiments showed that the pharmacological activity present in the vena cava extracts was associated with specific sub-cellular structures viz. the electron-dense vesicles. However, there was still no evidence to indicate whether the activity was due to the presence of one or more than one substance and there was limited data on the chemical properties of the active substance. Further progress required the isolation and purification of the substance or substances present. If purified preparations were obtained these could then be tested for the specificity of their physiological effects.

Gel-filtration is a very sensitive method of separating substances from crude extracts. This technique consists of the passage of a solute through columns of porous gel particles. Provided there is no adsorption of the substances within the solute onto the gel particles, elution rates from the column are correlated with molecular size. Thus substances of a molecular size larger than the gel particles pass straight through the column while substances of a similar size or smaller are retarded in the column. This method has been used in the purification of several different types of biologically active extracts of invertebrates e.g. extracts from the corpora cardiaca of insects (Natalizi and Frontali, 1966; Ishizaki and Ichikawa, 1967); in the separation of neurosecretory hormones of the crustacean eyestalk (Kleinholz and Kimball, 1965) and the separation of heart-excitatory substances from molluscan ganglia (Frontali, Williams and Welsh, 1967; Lempe, Tesch and Jaeger, 1969 and Agarwal, Ligon and Greenberg, 1972).

Filtration through a gel column was used to fractionate vena cava extracts of E. cirrosa and thus separate the different substances

present. This procedure should also give some indication of the molecular weight of the substance or substances present (see Carnegie, 1965).

XPERIMENTAL PROCEDURE

aterials

Sephadex G-10 and G-25 (coarse grain) were obtained from harmacia, Uppsala. Dextran Blue, mol. wt. 500,000, was also obtained rom Pharmacia.

el-filtration

Dry Sephadex G-10 and G-25 were suspended in sea-water and llowed to swell at 0°C for several days. After swelling, the Sephadex lurry was poured into a vertical glass column (volume 46.5 ml., diameter 5 mm) which was fitted with a porous disc at its base. (The column as supplied by Pharmacia). A gel column of 400 mm was then allowed o settle under a flow of solvent for at least 2 hours before application f a sample. Sea-water was used as the solvent throughout. Samples ere applied in a total sample volume of 0.5 ml. The sample was allowed o settle in the gel, a solvent layer was added above the gel column, a reservoir was attached and elution was allowed to proceed at a rate of 0.9 ml/min. Fractions of 2.0 ml were collected using an automatic fraction collector and were stored at -20°C until assayed for protein nd/or biological activity. All experiments were conducted at room mperature, i.e. 20°C.

Dextran Blue, mol. wt. 500,000, was used as a marker for the alibration of the column. The behaviour of the marker as it was eluted rom the column indicated whether the column was properly packed and ould therefore give a true separation of extracts.

Preparation of extracts

Extracts of vena cava tissue were prepared as previously described on p. 29 . The amount of material taken for each extract was 50 mg. In some experiments tissue had to be collected from several animals over a period of one to two weeks in order to obtain the required weight of material. In such cases scrapings from the inner wall of the vena cava were collected and kept frozen at -20°C until required for extraction.

Bioassay

The biological activity of fractions was assayed on the isolated systemic heart preparation of E. cirrosa (see p. 31).

Protein estimation

Protein was extracted from each fraction and estimated by the methods indicated on p. 84.

RESULTS

Using Dextran Blue as a marker the void volume of the column was found to be 16.0 ml. For Sephadex G-10, substances with a molecular weight greater than 700 would be excluded at the void volume i.e. between 15 - 20 ml, while substances with a molecular weight smaller than 700 would be retarded in the column and eluted later. For Sephadex G-25 one would expect any substances with a molecular weight greater than 5,000 to be excluded between 15 - 20 ml and substances with a molecular weight less than 5,000 to be retarded in the column and eluted later.

Using Sephadex G-10:

Activity, as monitored on the E.cirrosa heart preparation (Fig. 63), was associated with two distinct parts of the elution curve, see Fig. 64 and Table 10.

The first activity peak, associated with an eluant volume of 17-21 ml, was characterised by a gradual increase in amplitude and frequency of heartbeat which was then maintained for several minutes.

The second activity peak was associated with an eluant volume of 23-29 ml, the most pronounced activity being at a volume of 23-27 ml. This peak was characterised by a transient increase in amplitude of heartbeat with no attendant increase in frequency.

Using Sephadex G-25:

Activity, as monitored on the E.cirrosa heart preparation (Fig. 65), was found to be present at elution volumes of 14-21 ml and 30-42 ml (see Fig. 66 and Table 11). The first activity peak coincided with the void volume, indicating that this activity was due to a substance that is completely excluded from the gel. However, the second activity peak, eluted much later than the first, appeared to be due to a substance that is retarded by the gel. The activities present were of two types:

Type I, excluded at the void volume, was characterised by a slow increase in frequency and amplitude of heartbeat.

Type II, retarded by the gel, demonstrated a faster, transient increase in amplitude of heartbeat with no attendant increase in frequency.

These responses also demonstrated different time courses for the assay period between injection of the fraction and the attainment of a peak response. For Type I this was found to be 3.12 min while for Type II it was 1.5 min.

Initial experiments were conducted with a Sephadex G-10 column.

This did not give a good separation of activity. Extracts were then applied to a Sephadex G-25 column at a concentration four times greater than the concentration of extracts applied to the G-10 column. There was a far better separation achieved with the G-25 column (compare Fig. 64 and Fig. 66). It should be noted that the material for the extracts used for these two types of column was initially treated in different ways. For the G-10 column the material was collected from several animals and stored over a period of several weeks at -20°C before being extracted while for the G-25 column extracts were prepared from fresh material.

Activity in fractions eluted from columns and subsequently stored at -20°C for several weeks was found to be highly stable (compare with the findings of Berlind and Cooke, 1970).

An attempt was made to estimate the amount of protein present in each fraction. However the readings obtained on every occasion were abnormally low and did not fit onto the standard curve. Therefore it was not possible to compare the activity recovered from the column with the protein content of the active peak and determine a specific activity (i.e. activity per mg protein). Comparison of the specific activities of the initial extract and the active fraction obtained from the column would have indicated the degree of purification achieved with this procedure.

TABLE 10. *BIOCHEMICAL CHARACTERISATION.* Data for Sephadex column, G-10, run 15 - 2 - 71.

Assay of the original vena cava extract applied to the column showed no response.

The assay preparation was no longer sensitive.

Fraction number	% increase in amplitude	% increase in frequency	Eluant volume	Fraction number	% increase in amplitude	% increase in frequency	Eluant volume
1	--	--	2.9 ml	8	16.5%	--	17.0 ml
2	--	--	4.8 ml	9	25%	20%	19.1 ml
3	--	--	6.9 ml	10	39%	20%	21.1 ml
4	--	--	9.0 ml	11	24%	20%	23.0 ml
5	10%	--	10.9 ml	12	35%	20%	25.1 ml
6	15%	--	12.9 ml	13	39%	--	27.1 ml
7	10%	--	14.9 ml	14	31.5%	--	29.2 ml

TABLE 11. *BIOCHEMICAL CHARACTERISATION.* Data for Sephadex column, G-25, run 22 - 2 - 71. Fractions beyond no. 20 were not retained. Assay of the original vena cava extract applied to the column gave a percentage increase in amplitude of 40% and a percentage increase in frequency of 50%.

Fraction number	% increase in amplitude	% increase in frequency	Eluant volume	Fraction number	% increase in amplitude	% increase in frequency	Eluant volume
1	--	--	0.75 ml	12	16.5%	16.5%	24.55 ml
2	--	--	2.85 ml	13	--	--	26.65 ml
3	--	--	4.95 ml	14	--	--	28.65 ml
4	--	--	7.15 ml	15	7%	--	30.75 ml
5	--	--	9.45 ml	16	27%	--	32.85 ml
6	--	--	11.65 ml	17	21%	--	34.95 ml
7	--	--	13.75 ml	18	18%	--	37.15 ml
8	91%	71.5%	15.75 ml	19	41.5%	16.5%	39.15 ml
9	43%	21%	18.05 ml	20	82%	16.5%	41.35 ml
10	38%	7%	20.25 ml				
11	--	16.5%	22.45 ml	Original VC extract	40%	50%	

Figure 63. Bioassay of fractions eluted from Sephadex G-10 column of 15-2-71. Figures correspond to fraction numbers.

The blue arrow indicates the direction of contraction.

63

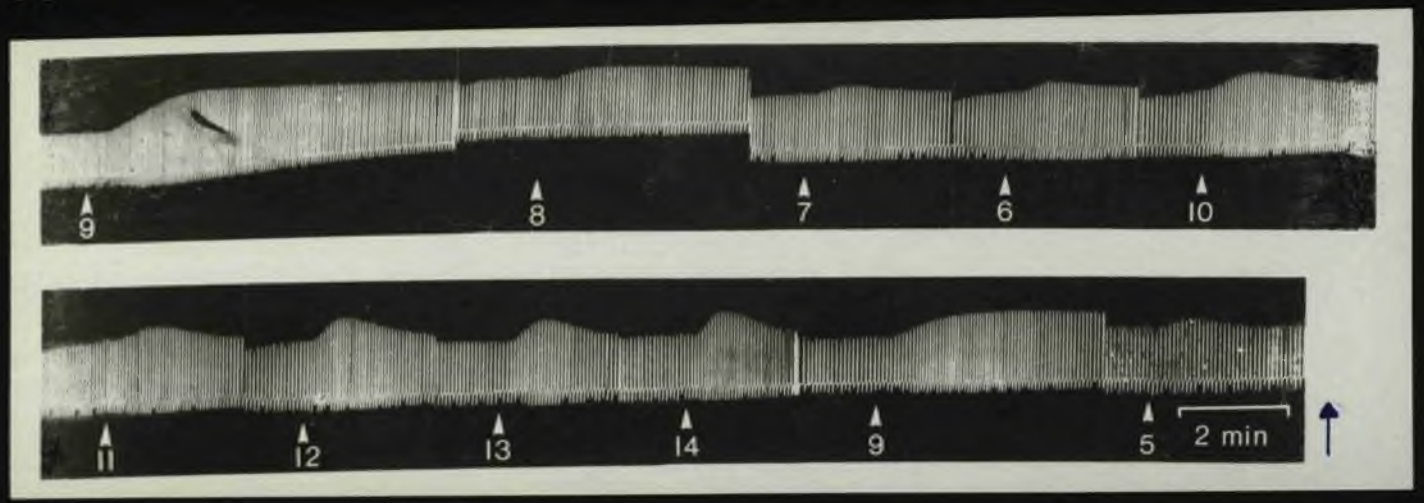


Figure 64. Graph indicating the biological activity of fractions eluted from a Sephadex G-10 column of 15-2-71.

increase in amplitude of heartbeat.

increase in frequency of heartbeat.

The figures from which this graph is plotted are given in Table 10.

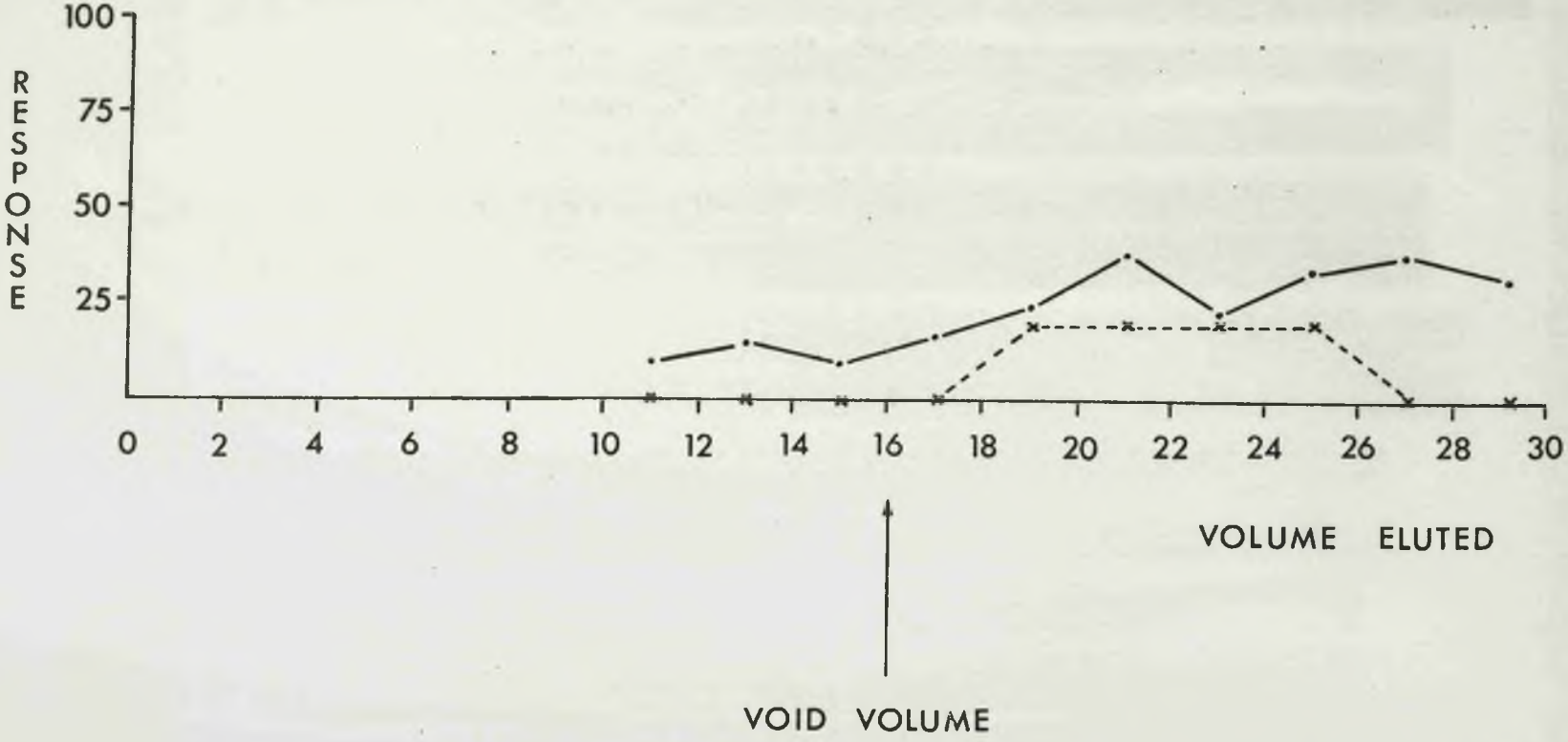

100
75
50
25
RESPONSE
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
VOLUME ELUTED
VOID VOLUME

Figure 65. Bioassay of fractions eluted from Sephadex G-25 column of 24-2-71. Figures correspond to fraction numbers. Note that the response to fraction 8 is a typical response to a highly concentrated extract. 8_I - fraction 8 diluted 1:3; 8_{II} - fraction 8 diluted 1:2, VC - original vena cava extract that was applied to the gradient diluted 1:3. The original vena cava extract was prepared at a concentration of 100 mg/ml, i.e. 50 mg wet tissue in 0.5 ml.

The blue arrow indicates the direction of contracti

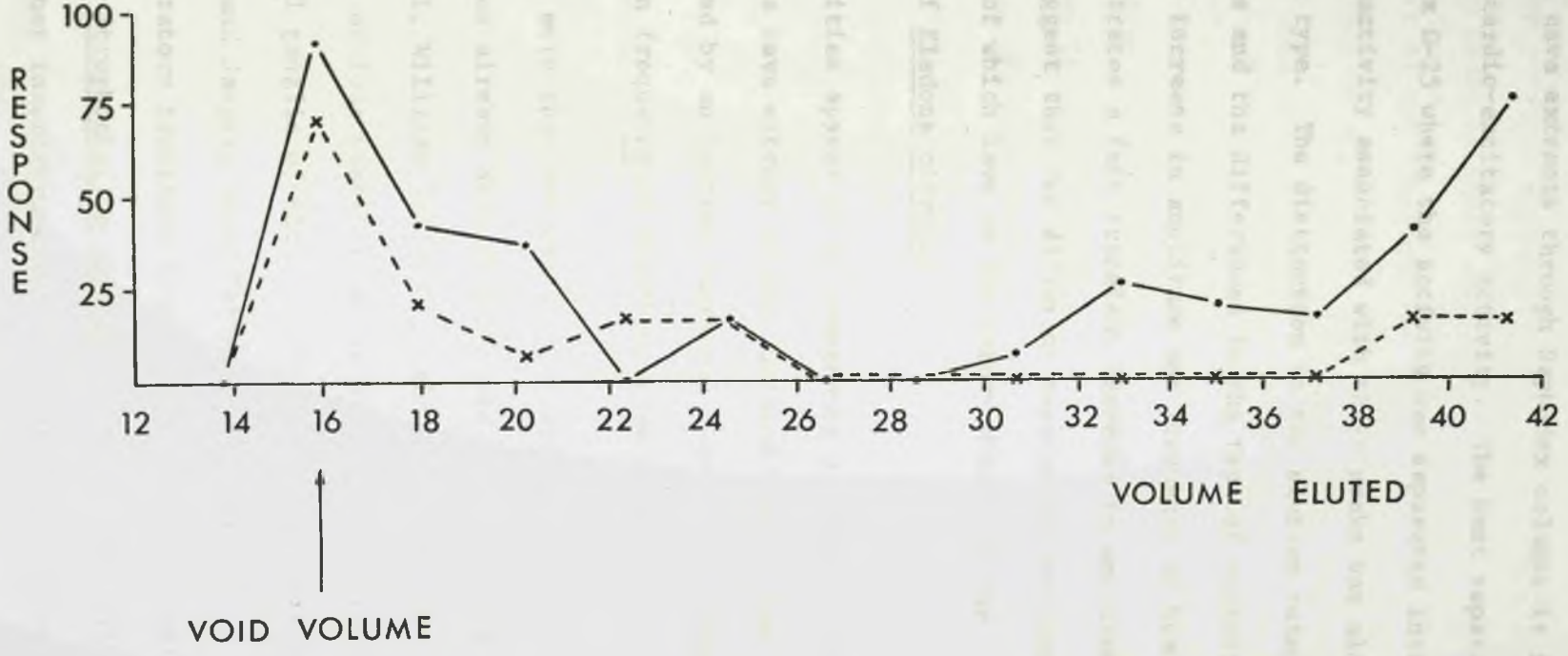
65

Figure 66. Graph indicating the biological activity of fractions eluted from a Sephadex G-25 column of 24-2-71.

increase in amplitude of heartbeat.

increase in frequency of heartbeat.

The figures from which this graph is plotted are given in Table 11.


100
75
50
25
RESPONSE
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
VOLUME ELUTED
VOID VOLUME

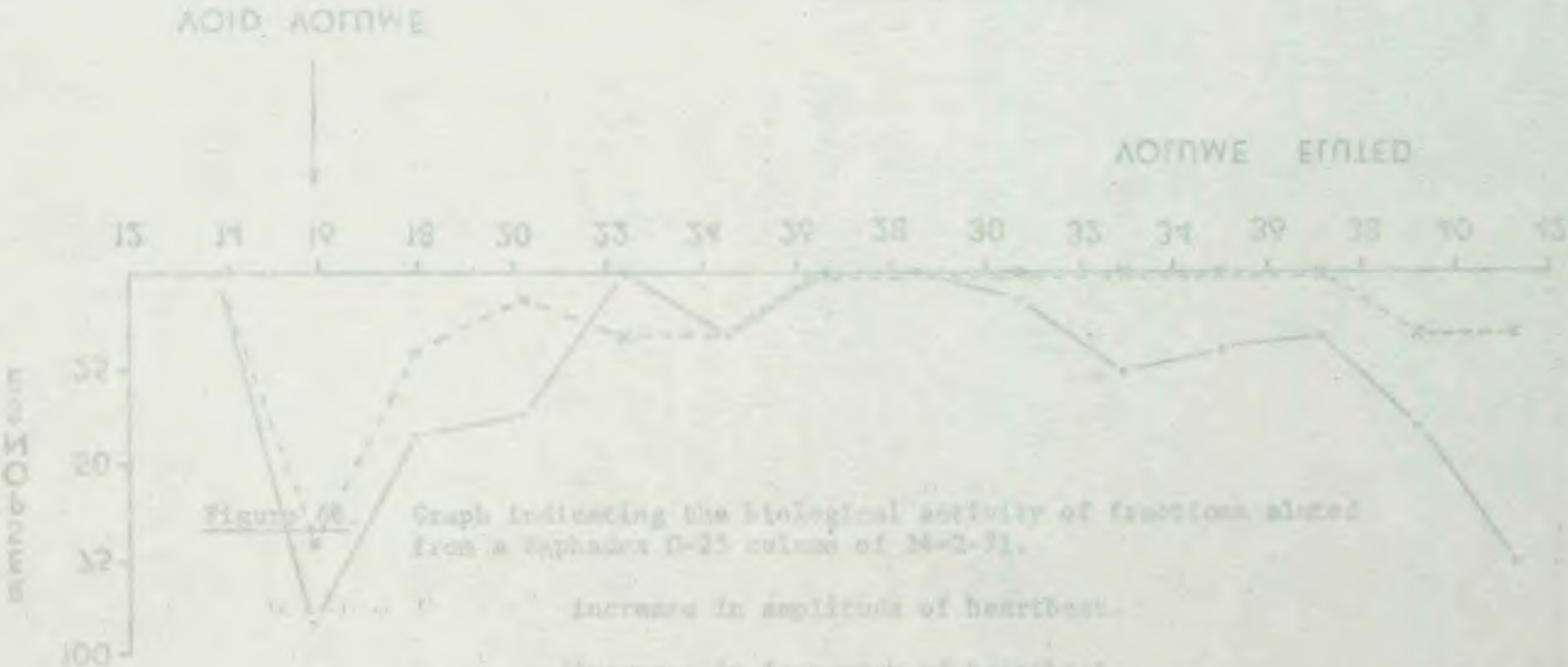


Figure 18. Graph indicating the biological activity of fractions eluted from a Sephadex G-25 column of 24-2-71.

Increase in amplitude of heartbeat.

Increase in frequency of heartbeat.

The figures from which this graph is plotted are given in Table II.

DISCUSSION

By passing vena cava extracts through Sephadex columns it is possible to separate the cardio-excitatory activity. The best separation was obtained with Sephadex G-25 where the activity was separated into two distinct peaks. The activity associated with these peaks was also shown to be of a specific type. The distinction in the elution rates of these active components and the differences in the type of activity (i.e. Type I demonstrates increase in amplitude and frequency of heartbeat while Type II demonstrates a fast transient increase in amplitude of heartbeat) strongly suggest that two different substances are present within the extract, both of which have an excitatory effect on the isolated systemic heart of Eledone cirrosa.

These two activities appear to be components of the activity normally shown by the vena cava extract on the isolated heart since the latter is characterised by an initial increase in amplitude followed by a prolonged increase in frequency of heartbeat (see p. 34).

The presence of more than one cardio-excitatory factor in nervous tissue extracts has already been demonstrated in a molluscan species. In 1967 Frontali, Williams and Welsh, using Sephadex G-15, demonstrated the presence of four peaks of cardio-excitatory activity in extracts of the central ganglia of the clam, Mercenaria mercenaria and in 1969 Hampe, Tesch and Jaeger, using Sephadex G-25 and G-15, separated two cardio-excitatory fractions from extracts of the central ganglia of the gastropod, Strophocheilus oblongus. Similar activity has also been shown in other invertebrate species using paper chromatography i.e. in corpora cardiaca extracts of Periplaneta americana (Brown 1965) and in pericardial organ extracts of crustacea (Belamarich, 1963; Belamarich and Terwilliger, 1966).

Frontali, Williams and Welsh have stated that the physiological significance of the presence of several cardio-excitatory factors was not clear. One possible explanation for this is that the cardio-excitatory activity shown by these substances may not be the normal physiological effect of the substance but just an attendant effect. (For a consideration of the cardio-excitatory activity of the substances present in the vena cava extract of E. cirrosa see the General Discussion, p.145).

In an investigation of the activity of pericardial organ extracts of the spider crab Libinia emarginata and L. dubia Berlind and Cooke (1970) found that when extracts were chromatographed on Sephadex G-25 columns, no cardio-excitatory activity was found in fractions comparable to the elution fractions for 5-hydroxytryptamine or dopamine although cardio-excitatory activity was associated with other fractions. A similar finding was made by Natalizi and Frontali (1966) when investigating insect heart-accelerating hormones from the corpora cardiâca of the cockroach, Periplaneta americana. Earlier experiments with vena cava material from E. cirrosa had indicated that neither 5-hydroxytryptamine nor catecholamines were present (see p. 48). In this respect, heart-accelerating substances obtained from widely different tissues are similar. It would be interesting to know if any chemical similarity exists between them.

Some time ago Belamarich (1963) suggested that all neurosecretory substances might be peptides. There is now increasing evidence to support this statement as reviewed by Ishizaki and Ichikawa (1967) and Berlind and Cooke (1970).

It is possible that the substances present in the E. cirrosa

extract are peptidic in character. The properties of the extract i.e. heat stability, activity unaffected by evaporation to dryness, extractability in acetone, resemble those of a heart-accelerating substance extractable from the corpus cardiacum of Periplaneta americana (Davey, 1961) which appears to be a peptide. Also, the E. cirrosa type II activity was found in fractions which corresponded to the volume of elution for small peptides. Confirmation of this hypothesis would be obtained by subjecting the eluant corresponding to the activity peaks of the column to enzymatic digestion and to the ninhydrin test outlined by Berlind and Cooke (1970). At present there is insufficient evidence to define their chemical nature.

Andrews (1964, 1965) has demonstrated that for globular proteins there is a good correlation between their elution behaviour from Sephadex columns and their molecular weight. This property has been applied by several workers in the estimation of molecular weights of impure proteins separated by gel-filtration (Carnegie, 1965; Siegel and Monty, 1966). However, application of this relationship must be made with caution since it has only been shown to hold for globular proteins. It does not hold for glycoproteins or peptides that contain aromatic amino-acids. The latter are retarded in the column due to adsorption and are eluted in a volume that would indicate an anomalously low molecular weight. If the active substances in the E.cirrosa extracts are globular proteins then type I activity would be due to a substance with a molecular weight greater than 5,000 and type II activity would be due to a substance with a molecular weight less than 5,000.

Due to lack of material only preliminary experiments on separation of the extract were possible. Further experiments using Sephadex G-75 and G-100 would help to characterise the activity within the extracts more exactly. It should also be possible to carry out enzyme digestion experiments, acid and alkaline hydrolysis and thin-layer chromatography on the active fractions from the column and thus determine the chemical composition of the active substances. Such experiments would be more valid than those carried out on whole vessel extracts since the substances eluted from the column are in a much purer form.

Recently Blanchi, Noviello and Libonati (1973) have used Sephadex gel and thin layer silica gel chromatography to purify vena cava extracts from the octopod, Octopus vulgaris. Using Sephadex G-75 one cardio-active substance was separated which was estimated to have a molecular weight of 1300. The activity of the substance was increased ninety five times by these procedures. The activity, as assayed on the isolated systemic heart of O. vulgaris, was described as a positive inotropic and chronotropic effect which was long lasting. It was not seen as two distinct effects as found with extracts from E. cirrosa.

It is difficult to compare the results obtained from these two species since there were differences in the procedures used. Vena cava tissue of O. vulgaris was extracted with distilled water and extracts were initially separated on Sephadex G-75. Vena cava tissue of E. cirrosa was extracted with 50% acetone and extracts were separated on Sephadex G-25. It would be interesting to subject extracts from both species to identical procedures and then to compare the results.

SECTION 6

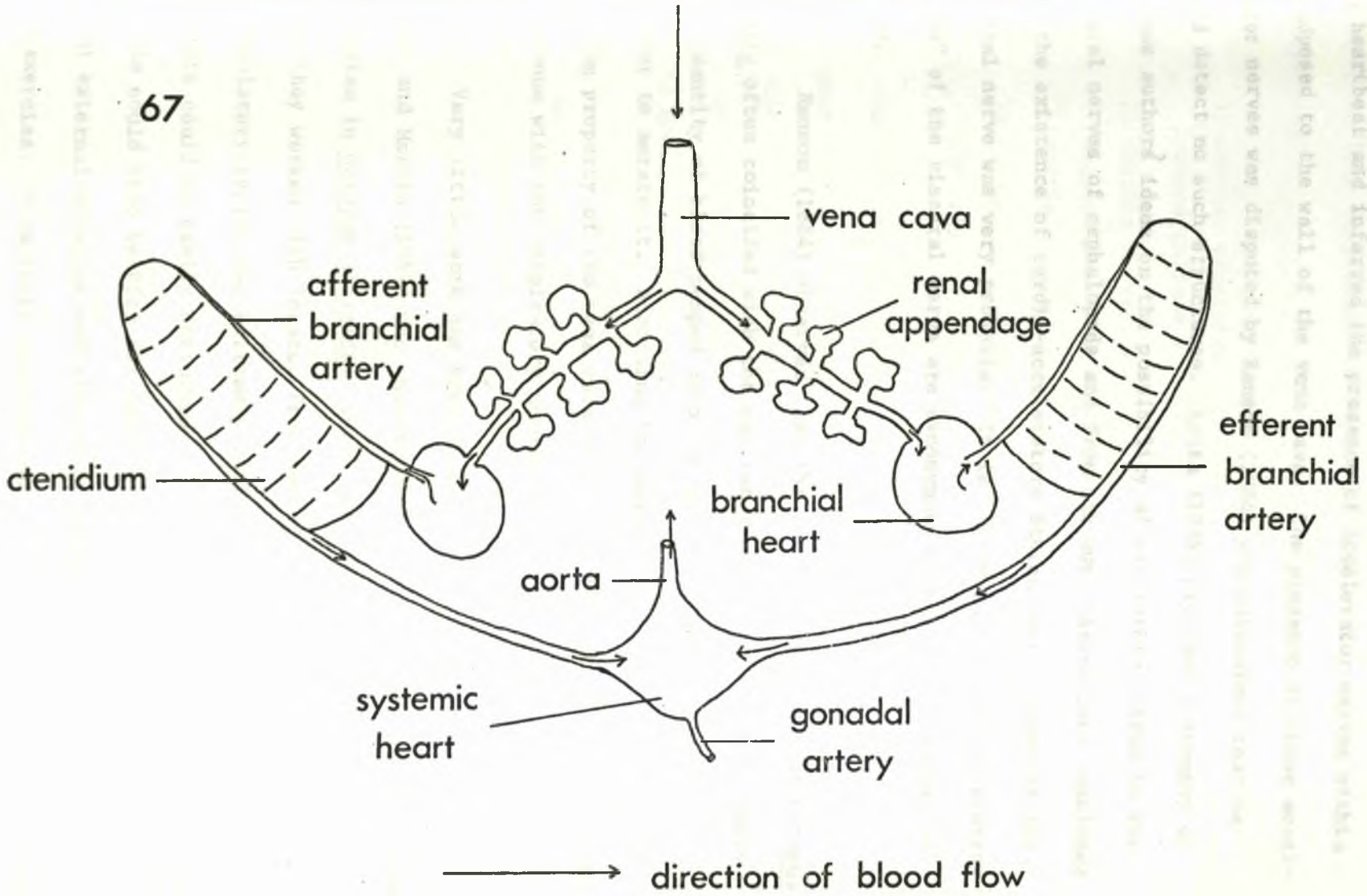
PHYSIOLOGICAL STUDIES

The previous experiments indicated that an active substance was synthesised in the cell bodies of the "neurosecretory neurons", was passed along the axons and stored in a neurohaemal organ adjacent to the lumen of the vena cava. This substance was also shown to have a potent excitatory effect on the isolated systemic heart of Eledone cirrosa. It was thus of interest to demonstrate release of the active substance, determine factors which cause release of the substance and determine the effect of the substance in the intact animal.

The distribution of structures forming the central vascular system of E. cirrosa is shown in diagrammatic form in Fig. 67. Blood passes from the cephalic region along the anterior vena cava to the afferent branchial vessels. It then passes through the branchial hearts to the ctenidia where it is oxygenated. The oxygenated blood passes through the efferent branchial vessels to the systemic heart from whence it is pumped to the remainder of the body. Thus an active substance released at the site of the neurosecretory terminations would pass directly into the central vascular system and might affect any organ within this complex. For a general discussion of the central vascular system in cephalopods see Martin and Harrison (1966).

Probably the first consideration of the physiology of the cephalopod heart was made by Fredericq in 1878. He showed that in Octopus vulgaris the rhythm of beat of the systemic heart was accelerated under differing conditions e.g. mechanical excitation by touching the animal, and especially by electrical stimulation. He also asserted that stimulation of the vena cava by an interrupted current accelerated

Figure 67. A simplified diagram of the central vascular system of Eledone cirrosa.


vena cava
afferent branchial artery
renal appendage
efferent branchial artery
ctenidium
branchial heart
aorta
systemic heart
gonadal artery
direction of blood flow

the heartbeat and inferred the presence of accelerator nerves within or adposed to the wall of the vena cava. The presence of these accelerator nerves was disputed by Ransom (1884) who maintained that he could detect no such structures. Kruta (1936) provided a summary of various authors' ideas on the possibility of excitatory fibres in the visceral nerves of cephalopods and from his own observations concluded that the existence of cardio-acceleratory fibres in the trunk of the visceral nerve was very probable. It is likely that the "acceleratory fibres" of the visceral nerve are synonymous with the neurosecretory nerves.

Ransom (1884) observed that the respiratory rhythm of Octopus vulgaris often coincided with the cardiac rhythm, ensuring that every fresh quantity of blood pumped into the gills had a fresh supply of sea-water to aerate it. According to Ransom, Fredericq noticed the pulsating property of the vena cava and stated that they appeared to be synchronous with the respiratory movements.

Very little work has been done recently apart from that of Johansen and Martin (1962) who investigated the dynamics of the circulatory system in Octopus dofleini. Their work was of particular interest because they worked with intact, free-moving animals. They found that the circulatory system was extremely labile and the function of its components could be easily disturbed. They also showed that the systemic ventricle could stop beating for considerable periods without any apparent external stimulus and also often showed great acceleration during exercise. From their observations they concluded that the nervous system played a crucial role in the co-ordination of the activity of the octopus circulatory system.

In this investigation experiments were conducted to demonstrate the following:

1) whether electrical stimulation of the neurosecretory neurons caused release of an active substance.

2) whether electrical stimuli, chemical stimuli or physiological stress were factors effecting release of an active substance.

3) to determine the function of the active substance within the intact animal.

One of the standard procedures used in the establishment of an endocrine organ is to perform an ablation experiment. This was used successfully in establishing the function of the optic gland in Octopus vulgaris (Wells and Wells, 1959). However, in the system under investigation the position of the neurosecretory trunks with their close association with the visceral nerves and the nerve endings forming a neuropile in the vena cava wall, made an experiment of this type technically extremely difficult. Therefore less direct methods i.e. ligation of the neurosecretory nerves and injection of extracts into living specimens, had to be used.

1. TO DETERMINE WHETHER ELECTRICAL STIMULATION OF THE NEUROSECRETORY NERVE TRUNKS ADJACENT TO THE VENA CAVA WOULD CAUSE RELEASE OF A PHARMACOLOGICALLY ACTIVE SUBSTANCE.

EXPERIMENTAL PROCEDURE

Eledone preparation

Specimens of Eledone cirrosa were allowed to rest quietly in a tank for several days prior to an experiment. This precaution was taken in case the substance within the neurosecretory trunks was released in response to stress.

The animal was removed from the tank and dissected without the use of an anaesthetic. The brain, visceral nerves and medial neurosecretory nerve trunks, and the anterior vena cava were isolated from the animal and this preparation was placed in a bath containing continuously aerated sea-water. Both ends of the isolated vena cava were cannulated and the vessel was perfused with aerated sea-water. This perfusion system could be closed so that a specific amount of sea-water could be retained within the vessel.

Stimulation

Stimuli were passed through platinum suction electrodes which were applied to the medial neurosecretory nerve trunks at a position 5 mm from where the trunks entered the wall of the vena cava. The parameters of stimulation were as follows:

Series of stimuli were delivered at a rate varying between 1 - 100 per minute; each series was of 1 - 30 msec duration

at a frequency of 3 - 10 Hz. Each stimulus was a 0.5 msec rectangular pulse ranging in strength from 1 - 20 volts.

Stimulation was continued for time periods of 15, 30 or 60 minutes.

Method

The preparation was set up as indicated in Figure 68. Before stimulation commenced the cannulation system of the vena cava was closed for fifteen minutes. At the end of this period the sea-water contained within the vessel (2.0 ml) was collected and retained for subsequent assay.

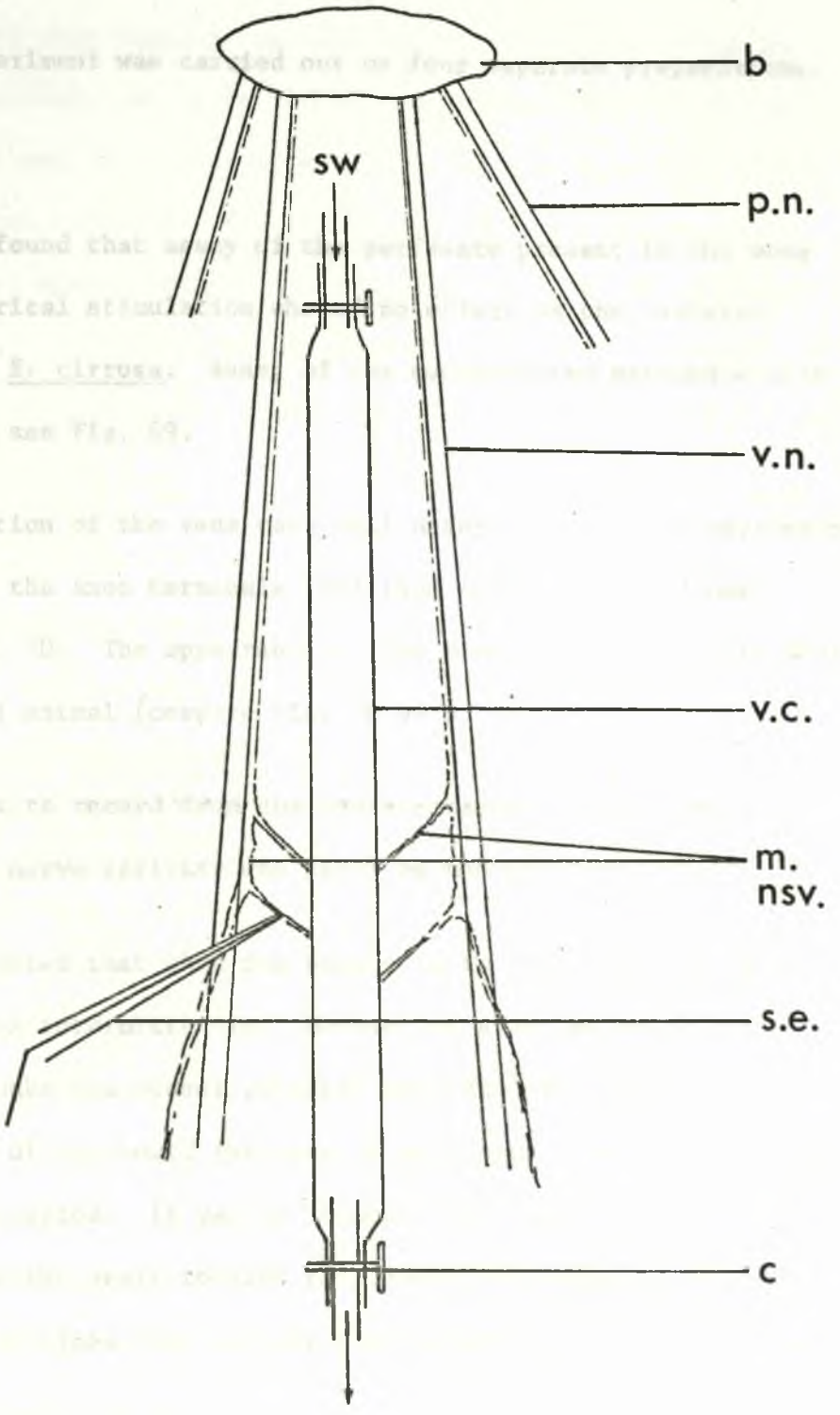
The vena cava was filled with a similar amount of fresh sea-water, the cannulation system was closed and the same volume of sea-water was contained within the blood vessel throughout a fifteen minute period of stimulation. At the end of this period the sea-water was again collected and retained for assay on the isolated systemic heart preparation of E. cirrosa, see p. 31 .

In some cases the aliquots of sea-water collected during the experiment were concentrated by dehydration under reduced pressure. The products were then taken up in 0.1 ml sea-water and applied to the assay preparation.

During the period of stimulation an attempt was made to record from the neurosecretory nerve trunks. This was achieved through a further set of platinum suction electrodes which were connected through a conventional differential amplifier to a Tektronix oscilloscope.

At the completion of the experiment the vena cava tissue at the point of stimulation was processed for examination with the electron

Figure 68. Diagram of the preparation set-up used in the electrical stimulation experiments. b - brain; c - clamp; m. nsv. - medial neurosecretory nerve; p.n. - posterior infundibular nerve; s.e. - suction electrode; sw. - sea-water; v.c. - vena cava; v.n. visceral nerve.


b
sw
p.n.
v.n.
v.c.
m.
nsv.
s.e.
c

microscope using the methods described on p. 13 .

The experiment was carried out on four separate preparations.

RESULTS

It was found that assay of the perfusate present in the vena cava during electrical stimulation showed no effect on the isolated systemic heart of E. cirrosa. Assay of the concentrated perfusate also showed no effect, see Fig. 69.

Examination of the vena cava wall using the electron microscope demonstrated that the axon terminals contained many electron-dense vesicles, see Fig. 70. The appearance of the tissue was similar to that seen in the normal animal (compare Fig. 70 with Fig. 22.)

Attempts to record from the neurosecretory trunks were unsuccessful. No nerve activity was shown on the oscilloscope.

It was noted that when the vena cava is filled with fluid the vessel pulsates intermittently. However on stimulation of the neurosecretory trunks the vessel pulsated continuously. The pulsations ceased at the end of one hour, the rate of pulsation slowly decreasing through this time period. It was of interest that each pulse moved in the direction from the heart towards the brain i.e. opposite to the normal direction of blood flow and the flow of perfusion fluid through the vessel.

COMMENT

The results above indicated that there had been no release of an active substance from the nerve endings in the vena cava wall.

The fact that no nerve activity was recorded from the neurosecretory trunks may indicate that these nerves are too small for recordings to be made extracellularly. At first it was thought that the pulsations of the vessel were due to release of an active substance, since it is known that vena cava extracts cause contraction of the isolated vessel (Berry, unpublished observations). However, it now seems more likely that these were due to stimulation of vasomotor fibres that are present within the neurosecretory nerve trunks, see Alexandrowicz, 1964.

Recently Blanchi, Noviello and Libonati (1973) have reported that electrical stimulation of the medial neurosecretory nerves of Octopus vulgaris resulted in release of a cardioexcitatory substance. Their preparation (similar to that described on p. 109) was bathed in 5 ml oxygenated sea-water. However, the vena cava was not perfused. Instead 50 µl aliquots from the bathing medium were diluted in 1 ml sea-water and assayed on the isolated systemic heart of O. vulgaris.

There are some differences in the procedure outlined by Blanchi et al. and that described in this thesis. The volume of bathing fluid taken for assay was very small compared with the total bathing volume. Thus for any activity to be shown there must have been a massive release of cardioexcitatory substance. The rate of stimulation used was one train of impulses every 10 seconds. The frequency of stimulation with E. cirrosa was varied between 1 stimulus every 60 seconds and 16 stimuli per second. Previously Cooke (1967), when stimulating neurosecretory nerves passing to the pericardial organ of the crab, Libinia emarginata, had provided evidence that the amount of neurosecretory material released depended on the frequency of stimulation. With the crab preparation he found that the rate of release

during stimulation was less at a frequency of 20 stimuli per second than at a frequency of 10 stimuli per second. Bunt and Ashby (1968) when stimulating the sinus gland of the crayfish Procambarus clarkii, found that greatest release occurred with a stimulation rate of 0.5 - 2 stimuli per second. The results obtained by Blanchi et al. indicate that a slow rate of stimulation is most effective.

Blanchi and her associates also found that with preparations which had been stimulated for thirty minutes samples from both the control and the stimulated preparation caused an increase in amplitude and frequency of heartbeat. However, a stronger effect was noted in the case of the stimulated preparation. They suggested that the small excitatory effect of the control sample indicated that neurohormone is released by the tissue when it is not being stimulated. Similar evidence of activity in control solutions has not been reported for other invertebrate neurosecretory systems.

Figure 69. Assay of the perfusate present in the vena cava during electrical stimulation of the medial neurosecretory nerve trunks. a - 0.1 ml perfusate; a_1 - 0.2 ml perfusate; b - 0.1 ml standard vena cava extract; a_{11} - 0.1 ml concentrated perfusate; c - 0.1 ml sea-water, control. Arrow indicates kick due to pressure change. Time mark represents 1 minute.

The blue arrow indicates the direction of contraction.

69

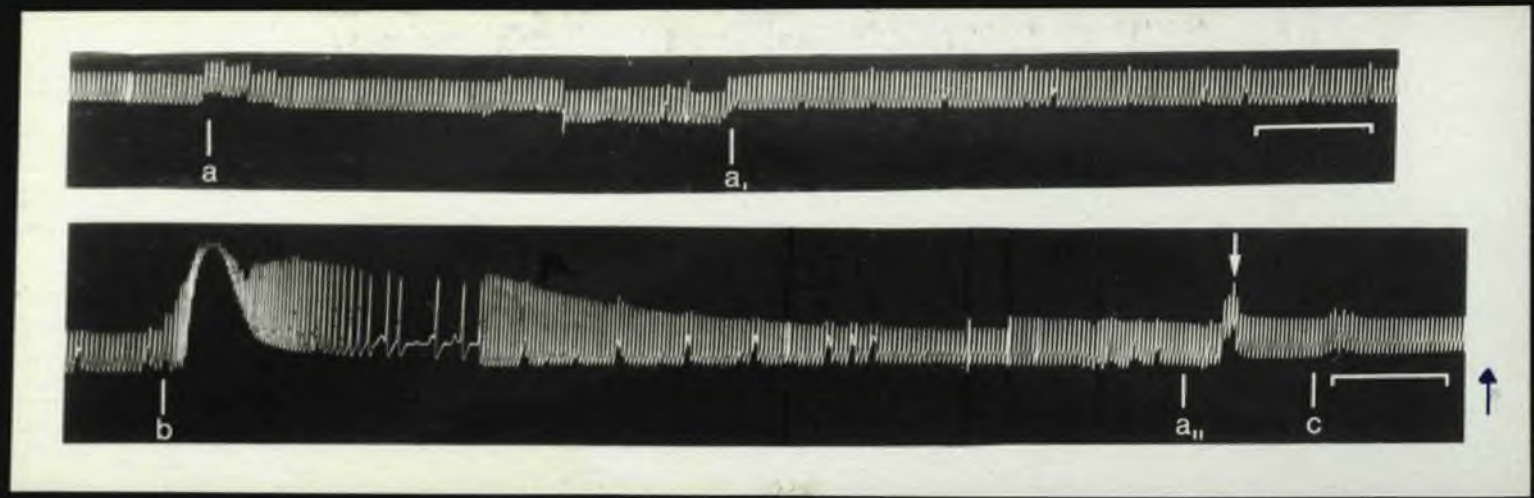
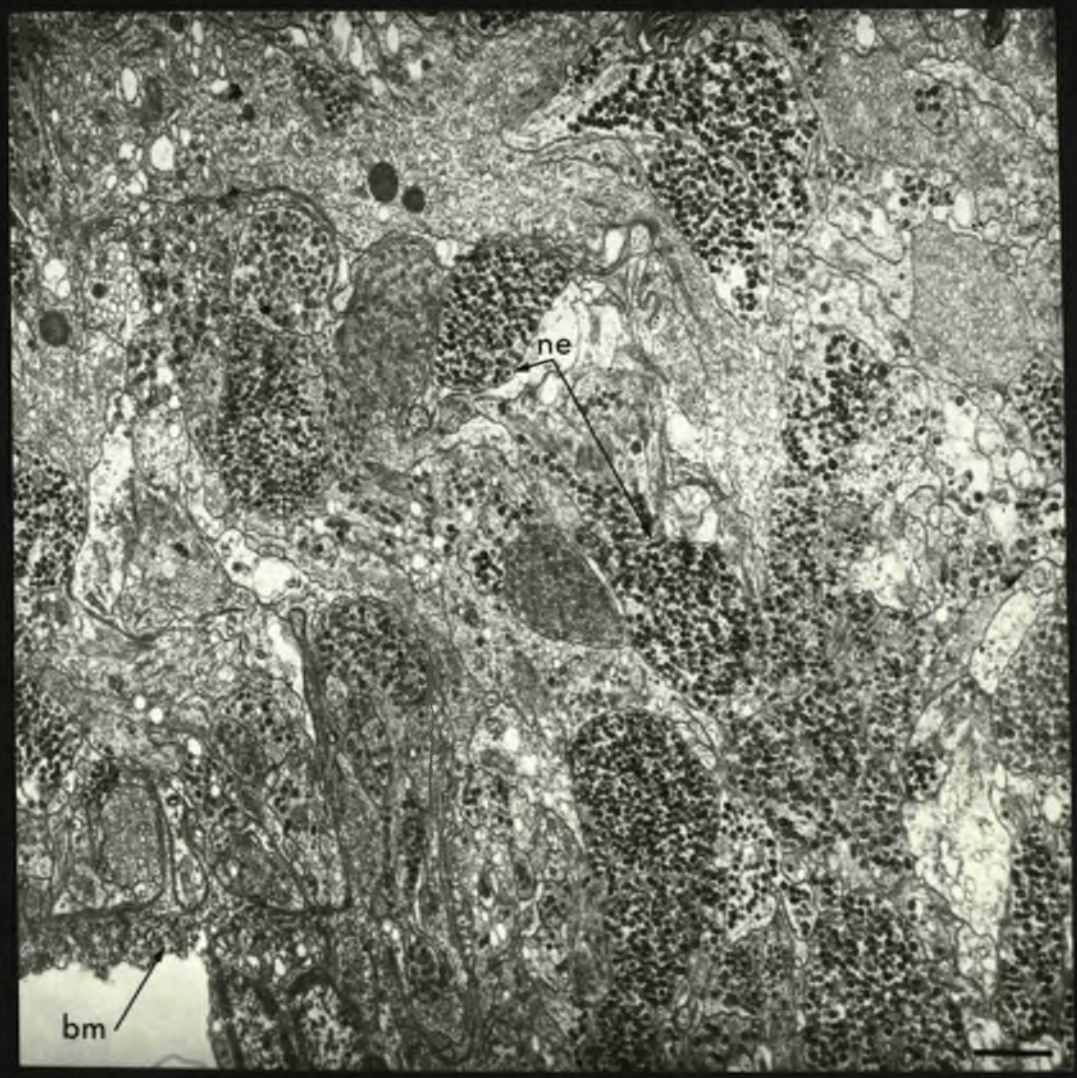


Figure 70. Electron micrograph showing the appearance of the nerve-endings in the vena cava wall after electri stimulation of the medial neurosecretory nerve tr bm - basement membrane; ne - nerve-endings. Bar represents 1 µm.

70



II. TO DETERMINE WHETHER CHEMICAL STIMULI ARE FACTORS EFFECTING RELEASE FROM THE NEUROSECRETORY NERVES.

EXPERIMENTAL PROCEDURE

Eledone preparation

The specimens used in these experiments had not been anaesthetised but their brain had been split in half. The mantle was cut longitudinally and deflected back to expose the vena cava. The specimen was placed in a dish containing continuously aerated sea-water. The vena cava was cannulated close to the brain and at the region where the vessel splits to pass to the renal appendages. The vessel was perfused with filtered, aerated sea-water. Care was taken to ensure that no blood remained in the vessel.

Chemicals

Standard solutions of acetyl choline, as acetyl choline chloride (B.D.H.), were made up in filtered sea-water.

Procedure

The cannulation tubes to the vena cava were clamped and a known amount of sea-water was retained within the vessel for 10 minutes before being collected. A further amount of sea-water was then passed into the preparation.

Acetyl choline solution, at a concentration of 10^{-5} g/l was applied to the external surface of the blood vessel. A total of 6 ml of this solution was dripped onto the surface of the vessel from a hyphypodermic syringe. In subsequent experiments acetyl choline, at

concentrations of 10^{-6} g/l and 10^{-4} g/l, was used.

Aliquots from the vena cava were collected in separate containers at 10 minute intervals and these were then assayed on the isolated systemic heart (see p. 31) of the same experimental animal. The experiment was continued for 40 minutes.

At the completion of the experiment the region of the vena cava subjected to acetyl choline treatment was processed for examination with the electron microscope using the methods described on p. 13.

The above procedure was carried out on three separate preparations.

RESULTS

Assay of aliquots on the isolated systemic heart of E. cirrosa showed no activity (see Fig. 71) and the appearance of tissue examined with the electron microscope might be compared with that shown by normal tissue, with mass accumulation of electron-dense vesicles adjacent to the basement membrane, (see Fig. 72).

COMMENT

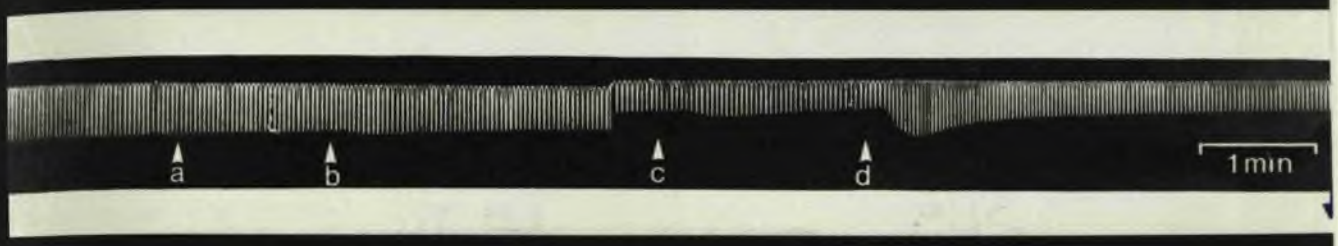
Application of acetyl choline appeared to cause no effect on the vena cava tissue. Therefore it would seem that chemical depolarisation of membranes effected by acetyl choline does not lead to the release of an active substance from the neurosecretory nerves.

Figure 71. Assay of the perfusate present in the vena cava during treatment with acetylcholine solution.
a - 0.1 ml sea-water, control injection;
b - 0.1 ml control perfusate; c - 0.1 ml experimental perfusate; d - 0.1 ml standard vena cava extract.

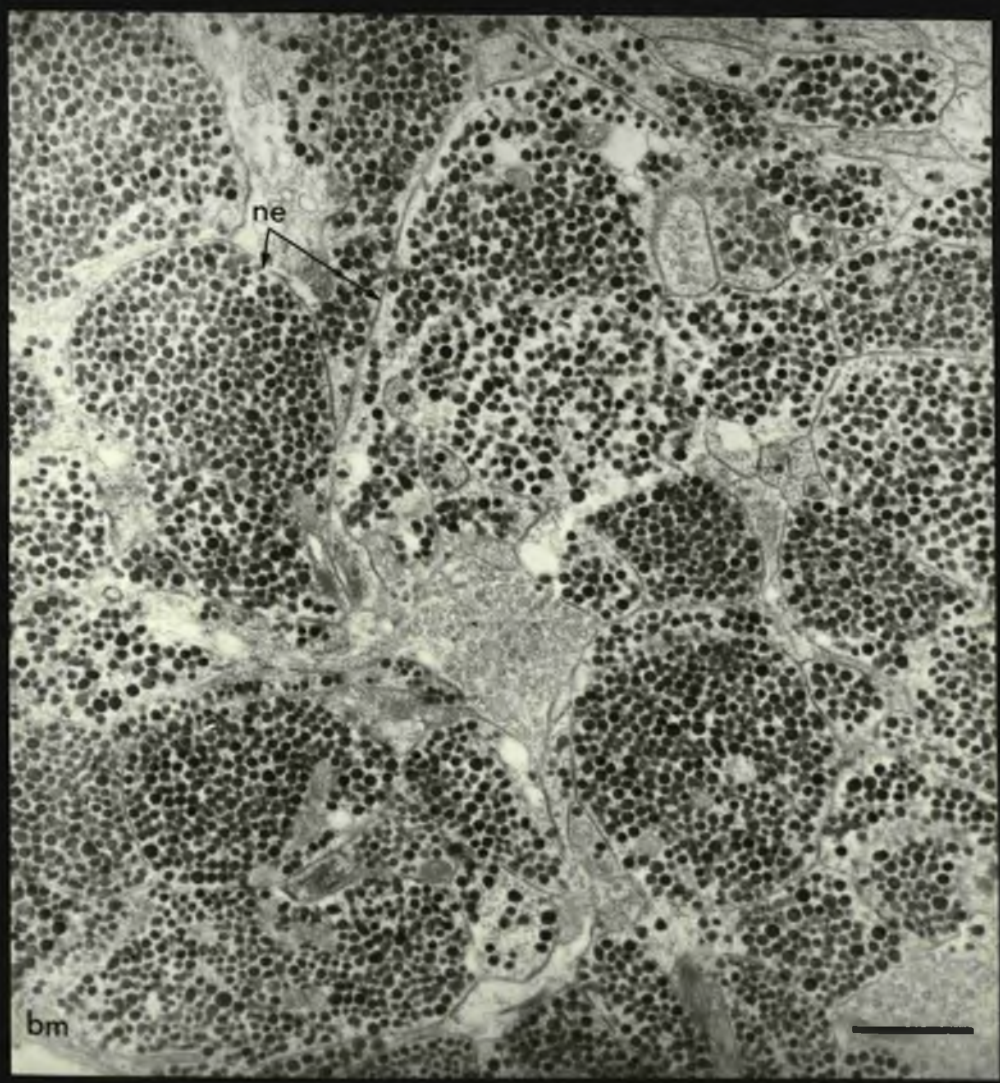
The blue arrow indicates the direction of contracti

Figure 72. Electron micrograph showing the appearance of the nerve-endings in the vena cava wall after treatment with acetylcholine.
bm - basement membrane; ne - nerve-endings.
Bar represents 1 µm.

71



72



III. TO DETERMINE WHETHER PHYSIOLOGICAL STRESS IS A FACTOR EFFECTING RELEASE FROM THE NEUROSECRETORY NERVES.

EXPERIMENTAL PROCEDURE

An experimental animal was kept out of sea-water on a cold damp concrete slab for 5 hours. The specimen was then allowed to recover in a tank of aerated sea-water for one hour. At the end of this period the animal was still alive but respiratory movements were weak. A control animal was placed in a tank containing aerated sea-water for the duration of the experiment.

At the completion of the experiment both animals were decapitated, the venae cavae were isolated and processed for examination with the electron microscope as described on p. 13 .

RESULTS

When tissue from the experimental and control animals was compared no difference was seen in the appearance of inclusions found within the vena cava wall, see Fig. 73.

COMMENT

The above results indicated that severe physiological stress did not cause any apparent change in the appearance of the neurosecretory "neuropile" of the vena cava wall.

This experiment was undertaken to corroborate findings by Martin (1968). He had taken a specimen of Octopus vulgaris that had been out of a tank for several hours and allowed it to recover. When the vena cava tissue was examined with the electron microscope Martin

found that the majority of nerve endings close to the lumen contained electron-lucent vesicles. He concluded that the transparent appearance of the vesicles might indicate that a substance had been released. From his paper it would appear that only one specimen of this type was examined. No further work on this topic has been published.

The experiment with E. cirrosa was repeated twice but on neither occasion did the nerve-endings contain transparent vesicles. A possible explanation of the discrepancy in results found between O. vulgaris and E. cirrosa is that the neurosecretory system may carry out differing functions in these two animals i.e. in Octopus it might respond to physiological stress whereas in Eledone it might not. However, the great similarity between the two systems, both in position and structure, would tend to preclude this.

It is interesting to note that in the axon profiles presented by Martin there are very few microvesicles or "synaptic vesicles" present, i.e. structures which are normally associated with sites of release from neurosecretory terminals (Gerschenfeld, Tramezzani and De Robertis, 1960; Normann, 1969; Bunt and Ashby, 1968). Douglas, Nagasawa and Schulz (1971) have shown that large electron-lucent vesicles can be produced by various treatments including exposure of the tissue to cold or the use of osmic acid as the primary fixative. (Such results are not obtained with glutaraldehyde fixation.) Martin stated that both glutaraldehyde and osmic acid were used as primary fixatives for the Octopus material and indicated in the figure legends that chrome-osmium fixation was used for the "stressed" tissue while glutaraldehyde fixation was used for the control or "normal" tissue. The temperature at which the material was fixed was not stated. There is now evidence that, in tissue depleted of its neurohormone content, the number of electron-dense

vesicles present is markedly decreased. However all remaining vesicles retain a normal appearance and aspects of electron-transparent or "empty" vesicles are not seen (Boudier, Boudier et Picard 1970). It would appear that in Martin's experiment the electron-lucent vesicles do not represent release of a substance in response to stress but rather a reaction to the fixative used.

Much more information is required before one could ascertain whether there is any relationship between physiological stress and the neurosecretory system of the vena cava in cephalopods.

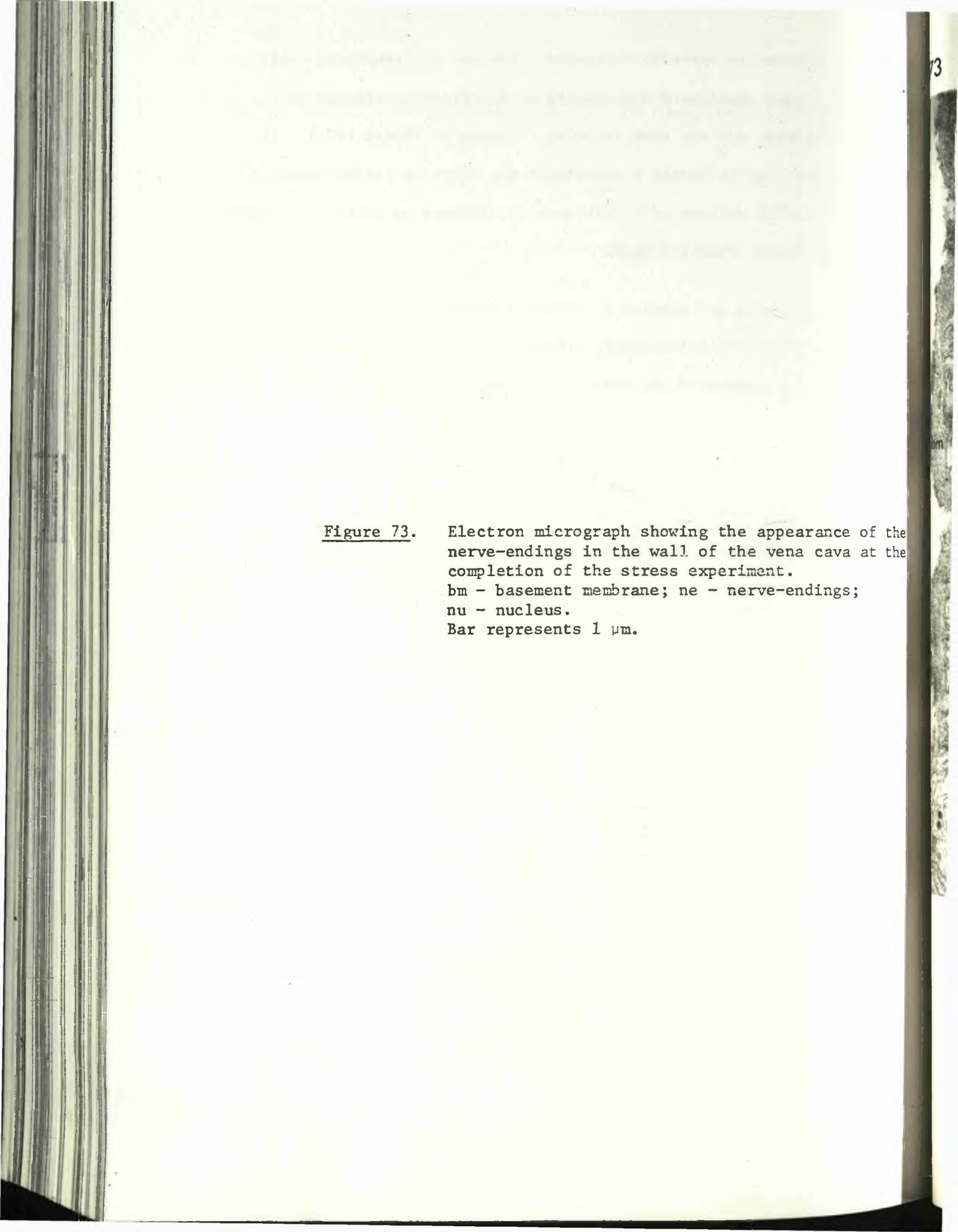
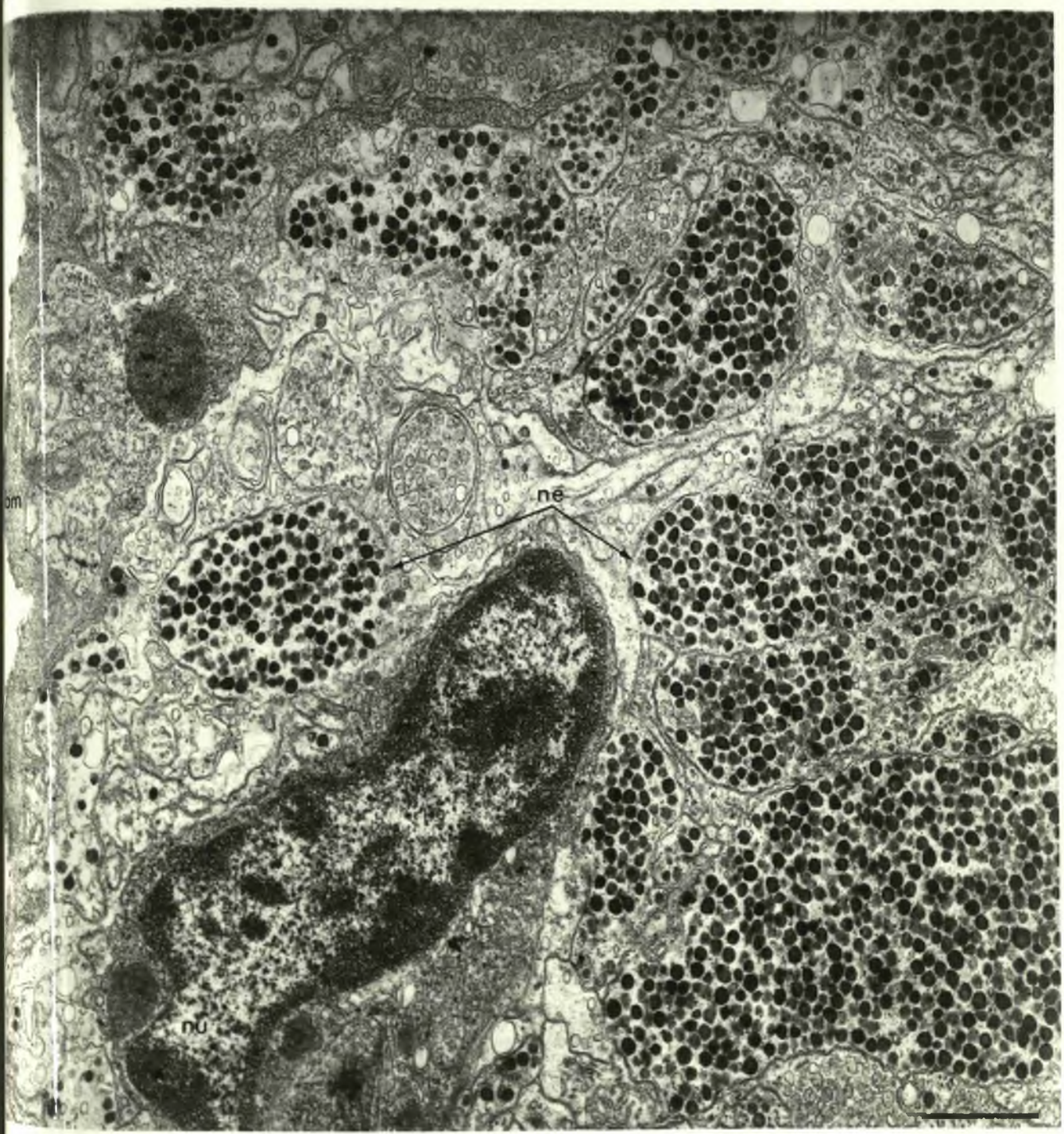


Figure 73. Electron micrograph showing the appearance of the nerve-endings in the wall of the vena cava at the completion of the stress experiment.
bm - basement membrane; ne - nerve-endings;
nu - nucleus.
Bar represents 1 μm.


ne
nu

IV. TO DETERMINE THE EFFECT OF LIGATION OF THE NEUROSECRETORY NERVES WITHIN THE INTACT ANIMAL.

EXPERIMENTAL PROCEDURE

Specimens of *E. cirrosa* used for these experiments weighed between 500 and 800 g wet weight.

Specimens were anaesthetised in a solution of 3% urethane (ethyl carbamate, B.D.H.) in sea-water. After approximately 5 minutes in the solution, all movement of the animal had ceased and the specimen was completely blanched. The animal was removed to a dissecting board and from the dorsal surface an incision was made through the skin, muscle, and connective tissue surrounding the coelomic sac. The digestive gland was then deflected to expose the posterior infundibular and visceral nerves with their associated neurosecretory trunks. The nerves and associated trunks on the left hand side of the animal were ligatured with cotton thread. The digestive gland was replaced and the inner layers of tissue stitched together with thread. The skin was not stitched - this procedure was adopted because the animal usually "plays" with the stitches and loosens them. The operative procedure normally took ten minutes. The mantle cavity was perfused with aerated sea-water until the animal attained normal respiratory movements. The animal was then placed in a tank which was supplied continuously with fresh, aerated sea-water. The colour of the animal was normal apart from an area on the left hand side of the body which was permanently blanched. A control specimen was treated in exactly the same way except that the nerves were left intact. The control animals did not exhibit any change in colour. All experimental and control animals were isolated in separate tanks.

In later experiments the posterior infundibular nerve or the visceral nerve alone were ligatured.

In one case, 76 hours after ligation of the visceral nerve and the neurosecretory nerve trunks on the left hand side of the animal, the vena cava and left and right visceral nerves were prepared for examination with the electron microscope, for method see p. 13.

RESULTS

The results obtained are shown in Table 12.

TABLE 12. PHYSIOLOGICAL STUDIES. Details of the ligation experiments.

Specimen	Nerves ligatured	Blanching effect	Survival time
1	Left posterior infundibular and left visceral nerves	Blanching on left hand side of animal	48 hours
2	Left posterior infundibular nerve	Blanching on left hand side of animal	48 hours
3	Left and right visceral nerves	- - - - -	- - - - -
4	Left visceral nerve	No blanching	76 hours

Due to the intimate relationship of the neurosecretory trunks with their associated nerves it was found to be impossible to ligature the neurosecretory trunks alone. Thus the attendant nerve trunks were always included in the ligature.

If the posterior infundibular nerve or visceral nerve of one side were ligatured the animals recovered from the operation. However when both visceral nerves were ligatured the animal failed to recover.

(In a subsequent experiment it was found that when both visceral nerves were ligatured there were no respiratory movements. If these ligatures were removed, respiratory movements were restored and the animal recovered.)

When the posterior infundibular nerves were ligatured there was a blanching effect which was entirely confined to the left hand side of the animal. This blanching was usually over the body sac but sometimes extended across the head and tentacles.

Although animals appeared to recover from the anaesthetic and operation very well their survival time was extremely limited and no animal survived longer than 76 hours. Examination of the wound and nerves at death showed no abnormal appearance of the tissue.

The tissue of the animal surviving to 76 hours was examined under the electron miscroscope but the appearance of the vena cava, visceral nerve, and neurosecretory trunks paralleled that of normal tissue. Previous specimens were not examined in this way since they had all been dead for several hours before being found.

The sham-operated animals did not show any increase in survival time.

COMMENT

Experiments of this type could provide valuable information on the effect of the substance contained in the neurosecretory nerves on the living animal.

However, the neurosecretory system of E. cirrosa is not an easy system to manipulate. The intimate association of the neurosecretory nerve trunks with other important nerves and the blood system

makes the system extremely difficult to isolate. Also, E. cirrosa appears to be much more sensitive to surgical manipulation than O. vulgaris (personal observation).

Further information on the best methods of manipulating E. cirrosa and an abundant supply of animals are required before proceeding with experiments of this type.

V. TO DETERMINE THE EFFECT OF INJECTING VENA CAVA EXTRACTS INTO A LIVING SPECIMEN OF ELEDONE CIRROSA.

EXPERIMENTAL PROCEDURE

Specimens used for these experiments were approximately 500 g wet weight. The specimens were anaesthetised according to the procedure outlined on p.119 and were removed to a dissecting board. The mantle was slit longitudinally on the ventral side, and the tissue was deflected exposing the visceral mass. The specimen was removed to a dish supplied with a constant circulation of aerated sea-water and the mantle and tentacles were pinned down to prevent movement of the animal. The tentacles were not removed in order to ensure that the blood system remained intact. Structures within the central vascular system e.g. branchial hearts, ctenidia, were freed from their association with the mantle so that movements of the mantle did not affect the record of beat of the systemic heart. An entomological pin attached to a thread was placed in the tip of the systemic ventricle. The thread was attached to a lever and the beat of the systemic ventricle was recorded using a kymograph apparatus. A record of the beat of the renal appendages was obtained in a similar manner. Extracts of the vena cava were prepared as described on p. 29 . When the animal had fully recovered from the anaesthetic, aliquots of 0.5 ml vena cava extract were injected into the afferent branchial vessel. (Injections were not made directly into the vena cava as this might have caused damage to the vena cava wall and lead to the release of other substances which would affect the central vascular system.) Aliquots of sea-water were used for control injections. At the end of the experiment a marker dye was injected into the afferent branchial vessel so that the passage of the injected

substances could be traced.

RESULTS

The effect of injection of vena cava extract into the afferent branchial vessel was noted on two structures in the central vascular system, the renal appendages and the systemic heart. The vena cava extract was shown to regularise the beat of the renal appendages and caused a marked increase in the beating frequency of the systemic heart. Control injections of sea-water did not demonstrate this effect. The marker dye injections showed that an injected substance circulated through the branchial hearts, ctenidia, and eventually passed to the systemic heart. The presence of the extract had no apparent effect on other organs of the central vascular system although specific monitoring is required before this possibility can be completely ruled out.

It was noted that the normal rate of beat of the systemic heart in the intact animal was extremely labile and was dependent on the activity of the animal. Thus movement of the mantle caused an increase in the frequency of beat and flexion of the whole animal caused a further increase in the frequency of beat (Fig. 74).

COMMENT

This experiment was undertaken to determine whether the pharmacological activity shown by vena cava extracts on isolated preparations was in fact likely to occur within the intact animal. In the intact animal a substance released into the vena cava does not pass directly to the systemic heart. Therefore it is possible that such a substance could be broken down before it reached that particular structure.

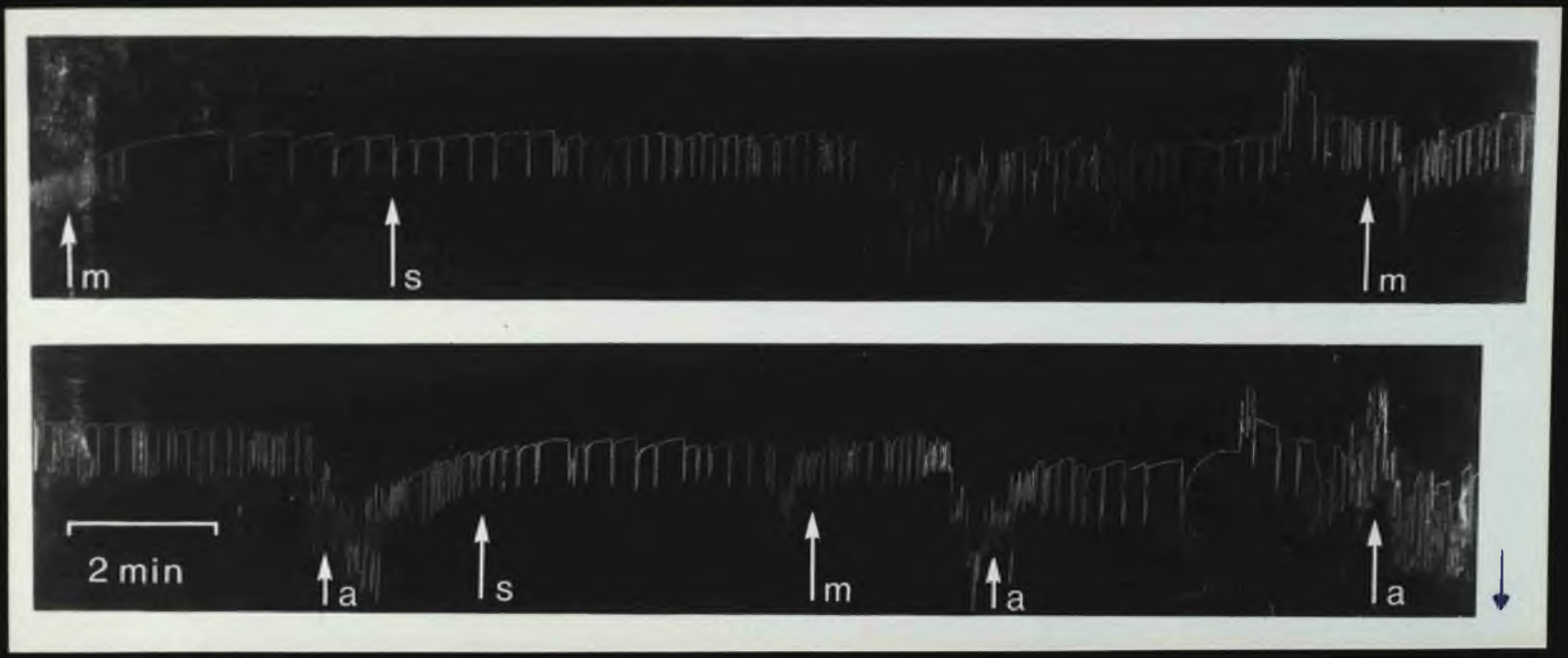
However these experiments indicated that the activity shown by extracts on the isolated systemic heart is reproducible within the intact animal.

The results also indicated that the central vascular system within Eledone cirrosa is extremely labile.

Figure 74. Monitoring, on the systemic heart, of the effect of vena cava extract injected in a living specimen of Eledone cirrosa. m - animal moving; s - animal still; a - injection of 0.5 ml vena cava extract.

The blue arrow indicates the direction of contraction.

74



DISCUSSION

A few workers have been able to show that electrical stimulation induces neurohormone release. Probably the first demonstration of this in invertebrates was made by Cooke (1964) when he showed that electrical stimulation of the nerve trunks passing to the pericardial organs of the spider crab, Libinia emarginata lead to the release of a cardio-acceleratory factor. Later, Cooke (1967) amplified this work and Kater (1967, 1968) showed that stimulation of nerves leading to the corpora cardiaca in Periplaneta americana also lead to the release of a cardio-acceleratory substance.

No similar type of relationship was established when the nerve trunks leading to the vena cava in Eledone cirrosa were stimulated. Previous attempts by Martin (1969, personal communication) to elicit release from the neurosecretory nerve-endings in Octopus vulgaris by electrical stimulation of the nerve trunks had also produced negative results. Recently Blanchi, Noviello and Libonati (1973) have shown that electrical stimulation of the medial neurosecretory nerves in O. vulgaris does cause release of a cardio-excitatory substance.

It is also interesting to note that vena cava tissue examined with the electron microscope after being subjected to electrical stimulation, change of ionic environment or extreme physiological stress also showed no apparent difference from vena cava tissue of a normal animal. In 1968 Bunt and Ashby conducted an ultrastructural examination of the sinus glands of the crayfish, Procambarus clarkii after electrical stimulation in vivo and in vitro. They found significantly fewer neurosecretory granules with an attendant increase in the number of large and small electron-lucent vesicles present. The depletion of secretory contents from some terminals in preference to others was not

shown. When Scharrer and Kater (1969) examined neurosecretory tissue from the corpora cardiaca of Periplaneta americana which had been subjected to electrical stimulation they found that the effect on the ultrastructural appearance of the tissue was less pronounced than anticipated. Again no preferential depletion of terminals was demonstrated.

At present, the criterion used for demonstration of release at the ultrastructural level is not depletion of the neurosecretory contents of the vesicles as shown by a loss of electron-density but is an increase in the number of microvesicles within the nerve terminations. This is in line with the hypothesis that release of the neurosecretory product is by a process of reverse pinocytosis or exocytosis (Douglas, 1967; Normann, 1969; Douglas Nagasawa and Schultz, 1971). An increase in the numbers of microvesicles within the nerve terminations of the vena cava wall was not seen.

Thus all the results obtained with E. cirrosa were consistent in indicating that no release had occurred. There are several possibilities that may be put forward as an explanation of these negative results:

1. the substance is never released from the nerve terminals. This seems highly unlikely.
2. the mechanism of release may be very specific. Perhaps the methods employed so far have not provided the necessary conditions for release. It has already been shown that relatively harsh treatment of the tissue is required for the release of the active cardio-excitatory factor in situ (Berry and Cottrell, 1970).

3. there may have been release but the methods employed for detecting this phenomenon i.e. assay and electron microscope examination, may not have been sensitive enough to detect the amount of material released. It has previously been shown that release is difficult to show at the electron microscope level (Scharrer and Kater, 1969).
4. the cardio-excitatory factor may be associated with a carrier molecule and this substance may be masking the effects noted in the in situ experiments.

Only further experimentation will show whether any of these possibilities applies.

Certain features of the central vascular system noted in E. cirrosa concur with results obtained by Johansen and Martin (1962) who were working with Octopus dofleini. They showed that in the unanaesthetised unrestrained animal the heart sometimes showed great acceleration during exercise.

They also noted the pulsation of the anterior vena cava and suggested that the pressure changes were passively mediated from pressure changes created by the respiratory movement of the animal. However, some other explanation is required for the pulsations noted in the isolated preparation during electrical stimulation.

The experiments of Johansen and Martin also indicated the importance of the nervous system for the coordination of activity in the octopod vascular system.

In E. cirrosa, injection of vena cava extracts into the central vascular system showed that the active substance would pass

from the efferent branchial vessel to the systemic heart without being broken down and would have an effect on the systemic heart in vivo which was similar to the effects already shown in in situ experiments. No apparent effects on other organs were noted in the living animal.

Do these results indicate that the function of the substance stored in the nerve terminations of the vena cava is to have a simple and direct effect on the systemic heart? The experiments undertaken so far have only shown an effect on the systemic heart. However if the main function of this substance is to provide some control for the central vascular system, it is difficult to see why a similar effect is not seen on the branchial heart. Also the system of nerve terminations is extremely complex. It would appear to be wasteful for the animal to have such an elaborate system present for control of the systemic heart alone. A system such as that described at the auriculoventricular junction in Helix pomatia (Cottrell and Osborne, 1969) would seem much more appropriate.

Perhaps the effect of the substance is a very general one in which case it will not be easy to detect.

SECTION 7

GENERAL DISCUSSION

The previous sections of this thesis have outlined the data obtained in an attempt to establish that nerves passing to the vena cava in Eledone cirrosa have a neurosecretory function. In this final section this information is considered with reference to the general concept of neurosecretion and the criteria used to define neurosecretory systems. The possible function of the vena cava system in E. cirrosa is discussed and a comparison made between this system and the neurosecretory systems described in some other invertebrates.

The concept of neurosecretion

The idea that certain neurons might function in a manner similar to gland cells was first put forward by Speidel (1919) when he noted that certain neurons in the posterior region of the spinal cord in elasmobranchs appeared to contain secretory droplets. Later, notably due to the work of Ernst and Berta Scharrer, similar cells were found in other vertebrates and in invertebrates. Ernst Scharrer hypothesised that these neurosecretory neurons were specialised for the manufacture of substances which resembled hormones. These neurohormones were released into the blood stream and were circulated to affect a secreting organ some distance away. Cells of this type thus provided a link between the nervous and endocrine systems (E. Scharrer and B. Scharrer, 1963).

It was found that some neurosecretory cells showed an affinity for particular stains e.g. paraldehyde-fuchsin and alcian blue. The introduction of the electron microscope refined the cytological criteria for the identification of a neurosecretory neuron. Electron microscopy

revealed that neurosecretory cells contain membrane bound, electron-dense vesicles, 100 - 300 nm diameter. These elementary neurosecretory granules (see p. 19) are thought to represent the neurosecretory product plus a carrier molecule. However these techniques have not proved to be completely specific and can only be used to indicate the possibility of neurosecretory activity (Bern, 1966).

Observations from light microscopy indicated the perikaryon of the neurosecretory cell as the site of synthesis of the secretory product. This view was extended when electron microscopy showed that the Golgi apparatus within the cell was involved in the formation of the elementary neurosecretory granules. It is thought that synthesis of neurosecretory material follows a pattern similar to that seen in epithelial exocrine or endocrine glands i.e. the site of synthesis is the endoplasmic reticulum and the neurosecretory material is then passed to the Golgi apparatus for packaging (Scharrer and Brown, 1962; Beams and Kessel, 1968). There have been proposals that synthesis of neurosecretory material occurs at sites other than the perikaryon. Lederis (1962b) suggested that there might be synthesis throughout the length of the axon and Knowles (1964) hypothesised that there was synthesis of the secretory product within the axon terminals. However other evidence for local synthesis has not been forthcoming. Use of isotope labelling techniques has indicated that the cell perikarya are the site of synthesis for mammalian neurohypophyseal hormones (Sachs, 1969).

Information obtained with the electron microscope indicates that the neurosecretory product is present in all parts of the neuron and is found in rows along the axon (see Fig. 16). This finding supports the idea that the neurosecretory product is transported along

the axon from the perikaryon to the nerve terminal by axoplasmic flow. The neurosecretory product is stored in the nerve terminals. These are often enlarged and show great accumulations of electron-dense vesicles. Terminals of this type which are intimately associated with the blood system have been termed neurohaemal organs (Knowles and Carlisle, 1956).

Initially it was thought that one of the definitive characteristics of neurosecretory cells was that these neurons did not form synapses with other neurons or effector organs but terminated in close association with the bloodstream. This is the case for many neurosecretory systems e.g. neurohypophysis in vertebrates; corpus cardiacum in insects; sinus gland in crustaceans. However, as more neurosecretory systems are examined it is apparent that neurosecretory neurons also terminate at other sites. Bern (1966) broadened the "classical" view by citing a number of ways in which neurosecretory neurons contribute to endocrine regulation and introduced the concept of "neurosecretomotor" junctions where neurosecretory fibres terminate directly onto endocrine cells. Bern proposed that neurosecretory neurons may provide control in the following manner:

a) by releasing into the circulation neurosecretory substances which directly affect a "target organ", e.g. pericardial organ in crustaceans.

b) by releasing neurosecretory substances which diffuse across a basement membrane to affect an adjacent endocrine tissue e.g. neurohypophysis/pars intermedia relation in teleosts.

c) by releasing neurosecretory substances directly onto the effector (neurosecretomotor fibres) e.g. corpus allatum of insects.

Recently B. Scharrer (1973) has stated that one can hypothesise that neurosecretory fibres that pass to other neurons will also be found.

The method of release of neurosecretory products has been a subject of much discussion. There are four main theories that have been put forward: 1) acetylcholine theory 2) complex dissociation 3) intracellular fragmentation 4) exocytosis. These are discussed in turn:

1) acetylcholine theory -- the presence of small, electron-lucent "synaptic-type" vesicles in the terminations of neurosecretory neurons led to the hypothesis that these vesicles contained acetylcholine which facilitated the release of the neurosecretory product from its containing vesicle (Gerschenfeld, Tramezzani and De Robertis, 1960; Koelle and Geesey, 1961; De Robertis, 1962; 1964). However several workers failed to demonstrate the presence of acetylcholine at sites where these small vesicles occur (Douglas and Poisner, 1964a; 1964b; Daniel and Lederis, 1966b; Lederis and Livingston, 1968; 1969). It is now thought that this method of release is unlikely. (In 1967 Lederis suggested that small vesicles that did contain acetylcholine might facilitate transfer of hormones into the blood stream by causing vasodilatation but not by acting directly on the neurosecretory vesicles themselves.)

2) complex dissociation -- this hypothesis was put forward by Thorn (1966; 1970). In this mechanism of release calcium ions are thought to detach the hormone from its carrier protein and thus make it available in a pool of easily releasable hormone within the nerve-endings. Free hormone then passes through the cell

barriers to the bloodstream.

3) intracellular fragmentation -- in this theory (Herlant, 1967) the neurosecretory vesicles are thought to fragment within the nerve-ending with an attendant release of neurosecretory material and the formation of small electron-lucent vesicles. Support for this theory is given by Johnson (1966), Scharrer (1968) and Weitzman (1969).

4) exocytosis -- the hypothesis that has received most attention is that neurosecretory products are released by the process of exocytosis (Douglas, 1967; Normann, 1969; Douglas, Nagasawa and Schulz, 1971). In this case the neurosecretory vesicle is thought to fuse with the terminal membrane. The contents of the vesicle i.e. the neurohormone plus the carrier molecule, are extruded from the neurosecretory cell. Meanwhile the vesicle membrane is resorbed, by a process resembling reverse pinocytosis, and several small "microvesicles" are formed. Thus according to this hypothesis the small vesicles found in neurosecretory neurons represent the form in which excess membrane is removed from the terminal surface. There is now considerable ultrastructural evidence to support this theory, both from vertebrates e.g. in mammals (Douglas, Nagasawa and Schulz, 1971) and from invertebrates e.g. in insects (Normann, 1965; Smith and Smith , 1966) and in crustaceans (Bunt and Ashby, 1967; 1968; Weitzman, 1969). The theory of exocytosis puts forward an explanation for the observation that in some neurosecretory systems the number of microvesicles present appeared to increase after a period of stimulation (Nolte, 1968). Douglas et al. (1971) have also argued convincingly that exocytosis is a very efficient

method for the resorption of the vesicle membrane while creating space for new neurosecretory vesicles to accumulate at the nerve terminal.

As more data is collected it should be possible to determine the exact method of release. At present the evidence appears to favour the theory of exocytosis. However there is no reason why more than one mode of release cannot occur, a view also held by Bern (1966). Weitzman (1969) has suggested that both exocytosis and intracellular fragmentation occur in the sinus gland of the crab.

There is still relatively little information to indicate which processes will initiate release from neurosecretory neurons. Cooke (1964) was probably the first to show that electrical stimulation of neurosecretory nerves led to the release of material from a neurohaemal organ in invertebrates. This has been followed by other examples including the work of Kater (1967; 1968) and Cooke (1967) with invertebrates and that of Kandel (1964); Ishikawa, Koizumi and Brooks (1966) and Cross (1971) with vertebrates. Berlind and Cooke (1968) showed that release in response to electrical stimulation requires the presence of calcium ions in the external medium. Later Berlind, Cooke and Goldstone (1970) showed that it is unlikely that monoamine-containing neurons control release from neurosecretory cells. Neither 5-hydroxytryptamine nor dopamine caused release when applied directly to the isolated pericardial organs of the crustaceans Libinia emarginata and Libinia dubia. Thus our present state of knowledge indicates that neurohormone release results from depolarisation of the terminal by action potentials conducted along the axon of the neurosecretory cell.

Only in a few cases is the identity of a neurohormone known e.g. the octapeptide neurohypophyseal hormones of vertebrates. As early as 1963 Belamarich suggested that all neurosecretory substances might be peptides. Evidence for the peptidergic nature of these substances has come from various invertebrate systems e.g. in insects (Ishizaki and Ichikawa, 1967; Natalizi and Frontali, 1967) and in crustaceans (Belamarich and Terwilliger, 1966; Berlind and Cooke, 1970). However some neurosecretory substances demonstrate heat stability and resistance to certain peptidases. Williams (1967) called attention to this and suggested caution in describing such substances as proteinaceous. He further suggested that the brain hormone of insects might be a mucopolysaccharide rather than a protein. Another group of substances which may be associated with neurosecretory systems are biogenic amines. This possibility has been discussed by Scharrer and Weitzman (1970). Much more information is required to establish these substances as neurohormones. However it seems unlikely that all neurohormones belong to a single class of substances.

The large amount of work that has been carried out on neurosecretory systems, particularly in the last decade, has emphasised the importance of neurosecretory substances as integrators between the nervous and endocrine systems. Neurosecretory substances have been shown to influence such diverse functions as growth, maturation, regeneration, reproduction, metabolism and homeostasis and facilitate adaptation to external factors. In most cases the effect of the neurosecretory substance is only known in general terms. The specific site of action is unknown. It is interesting to note that in the lower invertebrates e.g. coelenterates, flatworms and annelids, neurosecretory substances are the only source of hormones since epithelial endocrine

glands have not been developed. This indicates that the neurosecretory function is a very primitive one. For reviews of the function of neurosecretory substances in invertebrates see Hagadorn, 1967; Scharrer & Weitzman, 1970.

There has been considerable discussion about the degree of specialisation exhibited by neurosecretory cells. In 1963 Yagi, Bern and Hagadorn recorded action potentials from neurosecretory neurons in the leech, Theromyzon rude, providing evidence that neurosecretory cells could conduct impulses and substantiating the view that the neurosecretory cell is a neuron with glandular properties. More recently Adiyodi and Bern (1968) showed that the gross structure of the neurosecretory neuron is similar to that of conventional neurons ("neurons banaux"). It has also been pointed out that nearly all neurons synthesise and release active substances (Welsh, 1955; 1961). However there does appear to be some differences between neurohormones and neurotransmitter substances. Whereas neurohormones normally cause a prolonged effect on a tissue at some distance from the site of release, neurotransmitter substances act immediately at the site of release and are rapidly broken down. It has also been demonstrated that after release, specific neurotransmitter substances can be taken up by sympathetic nerves, stored and be released again (Iversen, 1967; 1971). This recycling of material has not been shown to occur with neurosecretory substances.

As more information on neurosecretion is acquired it is obvious that there is not a sharp division between conventional and neurosecretory neurons. Knowles and Bern (1966) have stated that it is the functional aspects of these cells which set them apart from other neurons. They gave the following definition for a neurosecretory cell -- "a neuron

which is engaged directly or indirectly in endocrine control and which may form all or part of what may be regarded as a traditional endocrine organ". However, as indicated by Sachs (1969b) and Scharrer (1973) one should probably consider the types of neurons present in nervous systems as members of a continuum which passes from conventional neurons to the classical neurosecretory neuron.

For reviews on the concept of neurosecretion see Scharrer and Scharrer, 1963; Bargmann, 1966; Bern and Knowles, 1966; Knowles and Bern, 1966; Picard and Stahl, 1966; Hagadorn, 1967; Hofer, 1968; Sachs, 1969a; 1969b; Scharrer, 1970; Scharrer and Weitzman, 1970.

Criteria employed to determine neurosecretion

Many groups of cells have been described as being neurosecretory purely on the basis of histological appearance and this has led to many mistakes in the identification of neurosecretory systems. Recently there has been an attempt to correlate morphological findings with physiological and biochemical data. Tombes (1970) has put forward the view that the criteria employed to determine neurosecretion should be similar to those used in the identification of normal endocrine organs viz. 1) identification of the neuron or group of neurons from which a neurohormone is being secreted.

2) demonstration that a correlation exists between the variation in appearance of the secretory cells with a demand made for the hormone in the organism.

3) removal or immobilisation of the tissue with subsequent appearance in the body of clearly defined signs of hormone deprivation.

4) removal of deficiency by replacement therapy.

5) demonstration of the presence of the hormone in the circulation.

6) purification, characterisation and synthesis of the hormone.

However, rigorous application of these criteria, particularly to invertebrate neurosecretory systems is not always possible. Variation in the appearance of the system is often not seen. This may be due to the amount of synthesis and storage of material that is taking place as put forward by Highnam (see p. 24). In many cases removal of the tissue is not practicable due to the intimate relationship of the neurosecretory neurons with other organs and tissues.

Therefore a modified scheme has to be adopted. I suggest that the following criteria are used:

1) identification of a neuron or group of neurons which display secretory activity
2) identification of storage and release sites associated with the neurohormone
3) demonstration of the presence of an active substance in tissue homogenates
4) demonstration that the active substance is associated with the secretory product seen in morphological studies
5) purification, identification and synthesis of the active substance
6) demonstration of the function of the active substance, where possible by ablation/replacement therapy

Methods presently employed in the identification of neurosecretory systems have been outlined by Scharrer and Weitzman (1970).

These include bulk staining techniques, histochemistry, electron microscopy, density-gradient centrifugation, gel-filtration, enzymatic digestion, electrical stimulation and radioactive incorporation studies.

Consideration of the results obtained with Eledone cirrosa

Electron microscope studies of the nerves associated with the vena cava indicated the presence of numerous electron-dense vesicles, 80 - 150 nm diameter. These were found to be elaborated by the Golgi systems in the perikarya of the nerve cells. Thus the vena cava nerves were found to demonstrate secretory activity in a manner similar to that described by Scharrer and Brown (1962).

Electron microscope studies also indicated that the terminals of the vena cava nerves were enlarged and contained large numbers of the electron-dense vesicles. The terminals formed a neuropile adjacent to the basement membrane of the blood vessel. The appearance of the tissue is similar to that seen in the sinus gland of crustacea (Bunt and Ashby, 1968). This arrangement represents a typical neurohaemal organ. The presence of such a structure establishes that the nerves passing to the vena cava are neurosecretory.

There was a pronounced cardio-excitatory activity associated with extracts of the neurohaemal organ. This activity was shown to be a prolonged effect, lasting up to twenty minutes, a property which is normally attributable to neurohormones rather than neurotransmitter substances. It was found that the cardio-excitatory activity was associated with the most numerous inclusion within the nerve terminals, the electron-dense vesicles.

This is the first time that a neurohormone has been demonstrated to be associated with a specific sub-cellular particle in molluscs.

Preliminary pharmacological and biochemical procedures indicated that there may be two active substances contained within the vena cava nerves. These substances are not 5-hydroxytryptamine or catecholamines. Some of the properties possessed by these active substances e.g. reduction in activity after incubation with trypsin, are indicative of peptides. Extracts retained activity after heating on a boiling water bath also suggesting that peptides are present. If there are two active substances it may be that one substance is a peptide while the other is not. This would compare with findings made with other cardio-excitatory substances (see p. 61). Blanchi, Noviello and Libonati (1973) have obtained evidence suggesting that the active substance present in vena cava extracts from O. vulgaris may be a peptide with some carbohydrate also present. It is obvious that further analysis is required before the chemical nature of these substances can be determined.

As indicated in the discussions at the end of each section, similar findings have been made on neurosecretory systems from both vertebrates and invertebrates. There is a very great similarity between the vena cava system described for E. cirrosa and the pericardial organ system of crustacea. This is indicated in the following table:

TABLE 13. GENERAL DISCUSSION. Comparison of the vena cava system in Eledone cirrosa with the pericardial organ system of decapod crustacea.

Characteristic	Vena cava system (from this thesis)	Pericardial organs (reference)
Presence of elementary neurosecretory granules	Section 2	Maynard and Maynard, 1962
Presence of a neurohaemal organ	Section 2	Maynard and Maynard, 1962
Presence of cardio-excitatory activity	Section 3	Maynard and Maynard, 1959
Association of cardio-activity with elementary neurosecretory granules	Section 4	Terwilliger, 1967 as quoted in Berlind and Cooke, 1970

There is also evidence that in the crab pericardial organs the cardio-excitatory substance is a peptide (Belamarich and Terwilliger, 1966; Berlind and Cooke, 1970). Thus although no evidence of release of a neurohormone was obtained and the function of the system was not diagnosed the evidence obtained indicates that the nerves passing to the vena cava in E. cirrosa are neurosecretory.

At present there are three major problems to be solved:

1) demonstration of release of the neurosecretory material.
2) characterisation of the neurosecretory substance and the determination of whether the substance is associated with a carrier molecule.
3) determination of the function of the neurosecretory substance.

The following methods might be used to obtain this information:

1) Further electrical stimulation experiments should demonstrate release of the neurohormone. This method has been used on a variety of preparations and recently Blanchi, Noviello and Libonati (1973) have shown that electrical stimulation of the nerves from the same system in O. vulgaris will elicit release of a cardio-excitatory substance. A more critical assessment of synthesis and release from the system might be obtained using the technique of auto-radiography.
2) It should be possible to determine the nature of the neurosecretory substance using extracts purified and concentrated by chromatographic techniques. The density-gradient

centrifugation procedure, outlined on p. 81 , allows the isolation of a very pure fraction of electron-dense vesicles which might also permit studies on other problems e.g. determination of whether the neurosecretory substance is associated with a carrier molecule, determination of which factors effect release of the neurosecretory substance. At present the existence of specific binding proteins analogous to the vertebrate neurophysins has not been shown in invertebrate neurosecretory systems. In a recent study of the cardio-excitatory factor present in crab pericardial organs Berlind and Cooke (1970) found that when using procedures modified from studies of the mammalian neurohypophysis, there was no indication that the cardio-excitatory factor was bound to a large non-structural protein molecule intracellularly. It is therefore clear that if a specific binding protein is present its properties are not analogous with those of the vertebrate neurophysins.

3) The determination of the function of the neurosecretory substance is a difficult problem. As stated earlier neurosecretory substances in invertebrates have been shown to influence a large number of processes and often this influence is of a very general nature. At present there is difficulty in determining which criteria denote that release of a neurosecretory substance is occurring or that a neurohaemal organ has been depleted. More information in these areas is required before changes in neurosecretory systems can be followed with any certainty. In many cases, especially in invertebrate systems, experiments

tracing changes in the system would be easier if the chemical composition of the neurohormone was known.

While normal ablation experiments in E. cirrosa would be difficult to carry out, knowledge of the composition of the neurohormone might allow chemical ablation of the system.

Speculation on the function of the vena cava system

At present there is no definite evidence to indicate the function of the neurohormone. However some general remarks may be made. The vast number of neurons involved in the system and the observation that the wall of the vena cava is ridged only in the neuro-haemal region, thus increasing the area available for release, suggests that the system is of some importance to the animal. The pulsating property of the vena cava may also facilitate release of the neurohormone.

As indicated previously (p. 106) the system is situated so that any neurohormone released from the nerve-endings is passed into the main blood vessel returning blood to the central vascular system. Thus the substance immediately comes into contact with some of the major organs of the body, i.e. the renal appendages; the ctenidia, and the hearts.

The normal effect of the neurohormone may be to act as a cardio-regulator, allowing rapid increase in the amplitude and frequency of the systemic heart. This has been shown to be possible by some of the pharmacological and physiological experiments. If this is the case, the neurohormone may be released in response to "stress" and facilitate adaptation of the animal to a higher heart rate. This has been suggested

by Carlisle (1964) as the function of the secretory product of the pericardial organs in decapod crustacea. It was also envisaged by Martin (1966) as the function of some secretory cells in the brain of a deep sea cephalopod, Illex coindetti, and of the neurosecretory nerves of the vena cava in Octopus vulgaris (Martin, 1968). However there was no indication of release of the neurohormone in stressed specimens of E. cirrosa. Therefore this explanation seems unlikely.

There is evidence that a given cardio-excitatory substance may also have an additional hormone function. In 1969 Mordue and Goldsworthy showed that extracts of the corpora cardiaca of the locust, Schistocerca gregaria, accelerated the heart beat, increased amaranth excretion and increased active phosphorylase levels in the fat body. They stressed the fact that one must not assume that the active factors would necessarily have a physiological role in vivo in regulating heart beat. The major role in this case is probably in the regulation of other homeostatic mechanisms. It is possible that a similar situation exists for the vena cava system of E. cirrosa and that the cardio-excitatory activity is secondary to some other effect.

It has also been shown that there are a large number of substances with cardio-acceleratory activity in molluscs (see Table 5, p. 71). It seems unlikely that the primary function of all these substances is the control of cardiac rhythm. However the substance in E. cirrosa was shown to initiate activity in hypodynamic hearts. Perhaps one of the functions of the substance is cardiac regulation while the major function is to influence a number of physiological activities in a manner analogous to that of adrenaline in vertebrates. A similar suggestion has been put forward by Blanchi,

Noviello and Libonati (1973) for the function of the vena cava system in Octopus vulgaris.

Another reason why the primary function of the substance isolated from the vena cava tissue may not be associated with the control of cardiac function is the finding that some of the neurosecretory axons, containing electron-dense vesicles i.e. neurohormone, pass directly between the muscles of the septum (see p. 15). There is also some evidence that there is a similar type of tissue found in a different region of the same animal. This juxta-ganglionic tissue (see p. 6) contains nerve cells and ends in close association with the wall of the venous sinus that surrounds the buccal mass. In O. vulgaris the fine structure of this tissue (Barber, 1967) is similar to that of the vena cava system in O. vulgaris (Martin, 1968) and E. cirrosa (Berry and Cottrell, 1970). No pharmacological or biochemical studies have been carried out on the juxta-ganglionic tissue to indicate whether other similarities occur. These findings may be significant. It may be inferred that if the same substance is produced and released from these regions the effect of the substance is probably not specific to the site where it occurs.

J. Z. Young has also put forward various proposals for the function of the juxta-ganglionic and the vena cava tissue. In 1970 he proposed that these tissues be called "neurovenous tissue" and suggested that its function might be the regulation of the volume, composition and disposition of body fluids. He also drew attention to the fact that the juxta-ganglionic tissue (a sub-buccal tissue) is very highly developed in the pelagic Argonautidae whereas there is very little sub-buccal tissue present in Sepia officinalis or Loligo vulgaris. (This is interesting since the neurosecretory system of

the vena cava in these species is also reduced compared to that of octopods; Alexandrowicz, 1965). Later Young (1971) suggested that this finding might indicate that the juxta-ganglionic tissue is concerned with the composition of body fluids in relation to flotation and/or changes in depth.

In 1970 Young proposed that since the vena cava system in octopods consists of such an enormous number of very small nerves it was possible that a surface phenomenon was involved and the system might be concerned with the transport of ions from the nerves into the blood stream or vice versa. From the evidence presented in this thesis it would appear that even if this did occur, it would not be the primary function of these nerves.

Another possibility was that the nerves formed a chemoreceptor analogous to the carotid body found in vertebrates. However a comparison of the ultrastructure of these two tissues indicated some major differences. Biscoe and Stehbens (1966) have shown that the carotid body is composed of two types of cells. Only one of these contains electron-dense vesicles 35 - 190 nm diameter while both cell types were shown to contain cilia. Therefore it would appear that the vena cava nerves are not acting as a chemoreceptor.

The function of the nerves passing between the septal muscles must also be considered. This is difficult to assess as the endings of these nerves were not observed. Bern (1966) has suggested that the function of neurosecretory nerves which project onto non-endocrine effectors may be associated with an aspect of general nervous activity which is not yet understood. It may be an example of the concept of "chemical addressing" which was developed by Horridge (1961).

The evidence presented here suggests that the function of the neurohormone associated with the vena cava in E. cirrosa may be of a general nature associated with preserving homeostasis. A more thorough understanding of the physiology of cephalopods may be required before the function of the system can be determined.

Neurosecretion in cephalopods

Until recently there has been very little evidence for the occurrence of neurosecretion in cephalopods (see Bern and Hagadorn, 1965; Durchon, 1967; Tombes, 1970). However several structures in cephalopods have been proposed as being neurosecretory and these are outlined in Table 14.

Two of these structures, the subpedunculate tissue and the pulsating ganglion have only been examined with the light microscope. Therefore there is insufficient evidence to verify their neurosecretory status. However Young (1970) has already stated that nerve fibres are probably absent from the subpedunculate tissue and thus the likelihood of the tissue being neurosecretory seems remote. The epistellar body and the juxta-ganglionic tissue have both been examined with the electron microscope. This work has indicated that the epistellar body is a photoreceptor, see p. 6, and the juxta-ganglionic tissue as possibly having a neurosecretory function, see p. 6. Thus at this time the only established neurosecretory system in cephalopods is the system of nerves that is associated with the vena cava.

Neurosecretion in other molluscs

The evidence for the occurrence of neurosecretion in molluscs has been reviewed by Bern and Hagadorn, 1965; Gabe, 1966; Simpson,

TABLE 14. GENERAL DISCUSSION. Analysis of proposed neurosecretory structures in octopods.

Tissue	Reference proposing n/s activity	Level of analysis	Remarks
Brain cells	Hagadorn unpublished in Bern & Hagadorn 1965	Light microscopy	Insufficient information
Brain cells	Martin, 1966	Light microscopy	Insufficient information
Epistellar body	Cazal & Bogoraze, 1949	Light microscopy	Insufficient information
	Nishioka et al, 1966a 1966b	Electron microscopy	Photoreceptor
Juxtaganglionic tissue	Barber, 1967	Electron microscopy	May be neurosecretory
Nerves passing to the vena cava	Alexandrowicz, 1964, 1965	Light microscopy	May be neurosecretory
	Martin, 1968 Berry & Cottrell, 1970	Electron microscopy	Probably neurosecretory
	Blanchi et al, 1973 Berry, 1974	Electrical stimulation Pharmacological & Biochemical Studies	Definitely neurosecretory
Pulsating ganglion	Alexandrowicz, 1963	Light microscopy	Insufficient information
Subpeduncular tissue	Cazal & Bogoraze, 1949	Light microscopy	Unlikely to be neurosecretory

Bern and Nishioka, 1966b; Durchon, 1967 and Tombes, 1970. It is apparent that while many molluscs possess a highly evolved nervous system knowledge concerning neurosecretion in this group is very poor. The first demonstration of neurosecretory cells in molluscs was made by B. Scharrer (1935) on opisthobranch gastropods. Since then the majority of investigations have been carried out on members of the gastropoda and the lamellibranchiata. There is also evidence that neurosecretory cells are present in the ganglia of some members of the scaphopoda (Gabe, 1966). However in most cases the work presented has only been on the morphology of these cells. There have been few attempts to determine their physiological role. Bern and Hagadorn (1965) report that Lever and Joosse (1961), working with Lymnaea stagnalis, showed that neurosecretory cells in the pleural ganglia may secrete a hormone concerned with the regulation of water balance. Lubet (1955, 1956), working with the lamellibranchs Mytilus edulis and Chlamys varia, showed that neurosecretory cells had a definite influence on the discharge of gametes.

More recently some interesting work has been carried out on a group of neurosecretory cells, sometimes referred to as bag cells, found in the parieto-visceral ganglion of the opisthobranch, Aplysia californica. The morphology of these cells has been shown to be similar to that of other neurosecretory cells (Coggeshall, 1967) and sea-water extracts were found to induce egg-laying (Kupfermann, 1967). The effect of these extracts was further investigated by Strumwasser, Jacklet and Alvarez, 1969. They found that extracts can induce egg-laying, that egg-laying is seasonal and is accompanied by a stereotyped behaviour pattern. Toevs and Brackenbury (1969) have conducted preliminary experiments to characterise the active agent. They showed that there was a specific protein, associated with the bag cell soma and surrounding

connective tissue sheath, which was destroyed by pronase and heating. The distribution of this specific protein was the same as the active egg-laying agent.

Jahan-Parwar and his associates (1969) have indicated that other neurosecretory cells in Aplysia californica may regulate water balance. They recorded changes in the electrical activity of specific neurosecretory cells after osmotic stimulation of the osphraduim.

Wendelaar Bonga (1972) has reinvestigated the involvement of neurosecretory cells in osmoregulation in Lymnaea stagnalis. He showed that two types of neurosecretory cells demonstrated release activity when animals were exposed to deionised water.

In a morphological study Cottrell and Osborne (1969) described a neurosecretory system in the heart of Helix pomatia. This consists of a network of nerve fibres which terminate at the auriculo-ventricular junction and contain electron-dense vesicles similar to those seen in the vena cava nerves of E. cirrosa. They suggested that this system might be responsible for a "general stimulating substance". It would be interesting to compare the pharmacological and biochemical properties of this system with those associated with the vena cava system in E. cirrosa.

As indicated in Table 5 (p. 71) cardio-excitatory activity has been shown to be present in nervous tissue extracts of various molluscs. It is likely that some of these substances are neurohormones. However there have been very few studies designed to determine the exact location of these substances. In 1967, Cottrell and Maser attempted to correlate cardio-excitatory activity in ganglion extracts from Mercenaria mercenaria with specific sub-cellular structures.

However, the fractions obtained from density-gradient centrifugation were found to be heterogeneous. The results of similar experiments with vena cava extracts from E. cirrosa as reported in this thesis (p. 79) provide the first demonstration in molluscs of a direct correlation between cardio-excitatory activity and elementary neurosecretory granules.

Further investigation of neurosecretory systems in molluscs is required. Integrated examination of these systems, combining morphological, pharmacological, biochemical and physiological studies, should provide information on the importance of neuroendocrine integration in this phylum.

Conclusion

The findings reported in this thesis establish that the nerves passing to the vena cava in the cephalopod, Eledone cirrosa, form a neurosecretory system.

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APPENDIX I

PHYSIOLOGICAL SALINE SOLUTIONS

1. MENG'S RINGER

Salt	Amount (g/l)
Sodium chloride (NaCl)	3.450
Potassium chloride (KCl)	0.430
Calcium chloride ($CaCl_2$)	0.117
Sodium bicarbonate ($NaHCO_3$)	0.110
Magnesium chloride (MgCl)	0.155

2. LOCKE'S RINGER

Salt	Amount (g/l)
Sodium chloride (NaCl)	9.20
Potassium chloride (KCl)	0.40
Calcium chloride ($CaCl_2$)	0.24
Sodium bicarbonate ($NaHCO_3$)	0.15
Glucose	1.00

3. DE JALON'S SOLUTION

Salt	Amount (g/l)
Sodium chloride (NaCl)	9.0
Potassium chloride (KCl)	10% 4.2 ml
Sodium bicarbonate ($NaHCO_3$)	5.0
Calcium chloride ($CaCl_2$)	1 M 0.27 ml
Aerating gas	95% O_2 + 5% CO_2

APPENDIX II

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