CONTRIBUTION TO THE KNOWLEDGE OF THE NERVOUS SYSTEM IN THE TENTACLES OF SOME COELENTERATES

(Anemonia sulcata, Metridium senile, Cerianthus membranaceus, Tealia felina and *Hydra vulgaris)*

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SUMMARY

The nervous system in the tentacles of the sea anemones *Tealia felina, Anemonia sulcata, Metridium senile* and *Cerianthus membranaceus* was studied using light microscopic and electron microscopic techniques.

Because of the small dimensions of the nerve cells (6—7 μm) and of the neurites (diameter < 1 μm) satisfactory information could not be obtained using conventional histological techniques.

Electron microscopic investigation showed that the nervous system can be divided into three parts: the plexus round the mesogloea, a nervous system between the muscles (obviously connected with the plexus) and sensory cells in the outer layer of the tentacle connected to the plexus by nerve fibres. The latter nerve cells with their fibres are arranged radially in the tentacle ectodermis. These are the only sensory cells discovered in the tentacles of the sea anemones.

In these radial neurites and in a number of neurites in the plexus, dense core granules are found. In the nervous system between the muscles and in a number of neurites in the plexus, opaque granules are found. Neurites containing dense core as well as opaque granules were never observed. Only in the radial neurites and in the plexus a yellow F(ormol) I(nduced) F(luorescence) was observed. Analysis of the emission spectrum showed that the F.I.F. had developed from a catecholamine (most probably noradrenalin). Therefore the dense core granules contain a catecholamine.

On the basis of morphological similarity, the neurites containing opaque granules may be identified as purinergic as proposed by Burnstock (1972), since the innervation of the muscles in the sea anemones very much resembles the innervation of smooth muscles in vertebrates.

Synapses as described by a.o. Westfall (1973a) could not be demonstrated. However, desmosome-like structures were found between the epithelial cells and between the muscles, so that a non-neural conduction (c.q. myoepithelial conduction) is probable. This myoepithelial conduction may explain the presence of a "second nervous system" postulated by Bullock & Horridge (1965) which is supposed to be a slow-conductive system. A morphological indication for a "second nervous system" has never been found.

The two transmitter substances mentioned (no indication was found for the presence of GABA and acetylcholine), i.e. a catecholamine, most probably noradrenalin, and a purine derivate, both have an excitatory function. The possible role of glutamate as an inhibitor has been discussed. Glutamate acts as a possible inhibitor, since it is released from contracting muscles and inhibits the contraction via an unknown mechanism.

Hydra was investigated and the findings were discussed in relation to the existing literature. Only the existence of synapses was discrepant, since these structures could not be demonstrated.

Regarding the possible transmitters a catecholamine could be demonstrated with the F.I.F. method. A purinergic muscle innervation is possible in view of our experience with sea anemones.

CHAPTER I. INTRODUCTION

The structure of the nervous system of *Hydra* and various other coelenterates has been studied by a considerable number of authors. Light microscopically, the nervous systems have been investigated using silver impregnation methods and methylene blue staining. However, in coelenterates the application of these methods meets with great difficulties which is evident from the great variety of staining recipes and the variations in these recipes (literature survey: Bullock & Horridge, 1965). Moreover, the application of these methods may lead to erroneous results and interpretations. Nevertheless, by means of the above-mentioned methods insight has been obtained into the distribution of nerve cells in the animal, their sizes and their shapes, the length of the neurites and their distribution patterns and the number of neurites of one nerve cell.

The nervous system was found to consist of a network with local concentrations (e.g. in the mouth field).

Points at issue were:

(1) whether the network is present in all parts of the animal;
(2) which cells make contact with nerve cells;
(3) the continuity of the network.

Investigations were performed in particular on the stem, the septs and the mouth. However, the nervous system in the tentacles, which must be well developed considering the complicated pattern of behaviour, has hardly been investigated (literature survey: Bullock & Horridge, 1965).

It was only after 1960 that publications appeared on electron microscopic investigations of coelenterates (mainly *Hydra*) using an adequate fixation (e.g. Batham, 1960; Kawaguti, 1964; Jha & Mackie, 1967; Davis et al., 1968; Westfall, 1970 a & b; Westfall, 1973 a & b; Westfall et al., 1971; Peteya, 1973 a & b; Davis & Bursztajn, 1973; Hernandez-Nicaise, 1973 a, b & c).

Electron microscopic identification of nerve cells proved to be so difficult that it was considered doubtful whether nerve cells were present at all in coelenterates (Hess et al., 1957; Slautterbach & Fawcett, 1959). This difficulty can be explained when it is taken into account that Lentz (1965) showed that nerve cells develop from interstitial cells. These interstitial cells are non-differentiated cells, which are found in the

ectodermis and entodermis of coelenterates lying close to the mesogloea. From these interstitial cells, all types of cells present in the animals may originate. Each form of cell ranging from interstitial cell to fully developed nerve cell may be present in the animal, and therefore it is very difficult to make sure that a certain cell actually is a nerve cell.

In order to meet this difficulty, Jha & Mackie (1967) and also Davis et al. (1968) set up a number of morphological criteria that must be met if a cell was to be considered a nerve cell. These criteria are:

Jha & Mackie (1967):

(a) staining with silver impregnation methods;
(b) presence of neurosecretory material;
(c) presence of cilia (cilia were observed in all nerve cells in the species investigated. The authors do not comment on a sensory function of cells which cannot clearly be identified as receptor cells according to their place and shape. The structure and place of these non-receptor cells are not described);
(d) presence of synapses;
(e) if a, b, c and d do not apply: a general impression of nerve-like features.

Davis et al. (1968):

(a) specific staining with rongalit methylene blue (i.e. methylene blue in leucoform);
(b) specific staining with silver impregnation methods;
(c) positive staining for neurosecretion;
(d) presence of a cilium;
(e) a typical bi-, tri- or multipolar form;
(f) contact with other nerve cells or with myoepithelial cells.

None of these authors give an indication as to the number of criteria a cell must meet in order to be called a nerve cell.

A large number of cellular elements has been described as belonging to the nervous system. Thus the following general picture emerged: cells lying in the ectodermis with processes in contact with each other and with other elements of the ectodermis. Using the electron microscope, nerve cells have not been identified with certainty in the entodermis, except in *Hydra* (Davis, 1972).

Although some insight has been gained in the structure of the nervous system of coelenterates, a considerable number of points remains obscure.

The presence of synapses, as well as their structure (especially their polarity) is controversial. And so is the presence or absence of a thickened postsynaptic membrane. Some authors hardly mention synapses, while others stress their presence.

Whether the nerve cells make contact with every element of the ectodermis cannot be concluded from the various publications. Thus a coherent picture of the diverse elements of the ectodermis cannot be obtained, on the one hand on account of the high magnifications of the details given and on the other hand on account of the low magnifications used in survey pictures.

The innervation of the muscles and of the myoepithelial cells, if present, is not clear. There is no sharp distinction between muscle cells and myoepithelial cells. Possibly a gradual transition from one type to the other exists.

There is no certainty regarding the presence or absence of neurosecretion.

Only Burnett et al. (1964) give criteria for the identification of neurosecretion, viz.:

(a) light microscopy: positive staining for neurosecretion, i.e. staining with aldehyde thionine or with paraldehyde fuchsine;
(b) electron microscopy: granules bigger than 1000 Å, electron dense or with dense core.

In the same article the authors admit that the light microscopic feature, i.e. the specific staining, is difficult to observe. The second feature is arbitrary, since granules containing catecholamines or indolamines are often bigger than 1000 Å and are electron dense or have a dense core in a number of cases. However, these granules containing biogenic amines cannot be stained with the above-mentioned staining methods for neurosecretion.

Westfall (1970 a & b, 1973 a & b) and Westfall et al. (1971) examined various sea anemones and *Hydra*. They never use the term neurosecretion. In her most recent publication (1973b), Westfall identifies the dense core granules as the neurosecretion observed by others who examined *Hydra*.

Authors disagree about the number of types of nerve cells in *Hydra*. Light microscopically, bi-, tri- and multipolar neurons as well as receptor cells can be distinguished. The latter group is recognizable by a cilium which can be observed only with great difficulty. Neurosecretion can be

observed in both neurons and receptor cells.

Electron microscopy showed that in *Hydra* (sea anemones were investigated less thoroughly) either three, two or only one type of perikaryon was found, depending on the author.

Peteya (1973a) classified the neurites on the basis of their diameter. It is doubtful whether this classification corresponds to the classification of the perikarya since this was not investigated by the author.

The processes of the nerve cells and the network or plexus they form, are hardly mentioned. When the network is very dense and consists of a large number of densely packed neurites, it is called a plexus.

In *Hydra* the neurites are found everywhere in the tissue, often along the large interstitial spaces. This does by no means resemble the plexus in other coelenterates investigated, viz. *Oulastrea crispata* (Lamarck, 1816) (investigated by Kawaguti, 1964), *Nanomia cara* A. Agassiz, 1865, *Euphysa flammea* (Linko, 1905), *Sarsia tubulosa* (M. Sars, 1835), *Cordylophora lacustris* Allman, 1844 (investigated by Jha & Mackie, 1967) and *Ceriantheopsis* Carlgren, 1912 (investigated by Peteya, 1973a). In the two latter species almost all neurites are situated between muscles or in a plexus. This difference in structure has not been noticed.

Polarization within the plexus, either by the presence of synapses or by an orientation of the neurites has only been investigated in jellyfish, but not in *Hydra* or in sea anemones (Bullock & Horridge, 1965).

From physiological experiments and observations on behaviour, Bullock & Horridge (1965) concluded the presence of two different nervous systems, one for fast conduction and one for slow conduction. Other authors as well found evidence for the existence of different conduction systems (Josephson, 1961, 1966; Mc Farlane, 1969, 1973). However, morphological evidence either for the presence or for the absence of these two different systems has never been presented.

It is known that a number of transmitter substances play a part in the neuronal and neuromuscular transmission. Some of these substances have been identified in coelenterates: 5-hydroxytryptamine, adrenalin and noradrenalin were found in *Hydra, Sagartia, Metridium* and *Tealia* (Dahl et al., 1963; Lentz & Wood, 1964; Carlyle, 1969 a & b; Williams, 1972). Acetylcholinesterase and monoamine oxidase were identified in *Hydra* (Lentz & Barnett, 1961, 1963). These enzymes inactivate the transmitter substances, acetylcholine and catecholamines or indolamines, respectively. Consequently, the presence of these enzymes is possibly an indication for a transmitter role of acetylcholine and catecholamines or indolamines.

The above-mentioned results have often been published as short communications without illustrations. One of the authors (Lentz) does not refer to his earlier publications in his later articles regarding *Hydra*.

A great number of substances known to affect contraction, transmission or conduction in vertebrates, has been tested on sphincter and column preparations (Ross, 1960 a & b; Carlyle, 1969a, 1974). Most of the substances proved to have no effect at all. Those substances which were effective acted at such high concentrations that the effects can hardly be considered as physiological.

The present investigation deals with:

1. The structure of the nervous systems in the tentacles of *Hydra vulgaris* Pallas, 1766 and a number of sea anemones, viz. *Tealia felina* (Linnaeus, 1767), *Metridium senile* (Linnaeus, 1767), *Anemonia sulcata* (Pennant, 1777), and *Cerianthus membranaceus* (L. Spallanzani, 1784).
2. The identification and localization of possible transmitter substances.
3. A comparison between the nervous systems of *Hydra* on the one hand and the sea anemones on the other hand in order to determine similarities of and differences between the nervous systems in these species.

CHAPTER II. MATERIAL AND METHODS

Material

The sea anemones *Tealia felina, Metridium senile* and *Anemonia sulcata* were kept in aerated natural sea-water at 15°C, whereas *Cerianthus* was kept in sea-water at room temperature.

Hydra was kept at room temperature in fresh water from the place of collecting.

Methods

I. LIGHT MICROSCOPY

I.1. *Fixation and embedding*

The tentacles of the four sea anemones were fixed in Bouin's or Susa's solution (Romeis, 1968). (For the light microscopic study of *Hydra* 1 µm epon sections (see § II.2) were used). The tentacles were embedded either in paraplast or in diethylene glycol distearate (Taleporos, 1974) after slow dehydration.

Paraplast sections were cut at 4 µm. From the material embedded in diethylene glycol distearate 1 µm sections were cut on an L.K.B. Huxley microtome with glass knives.

I.2. *Staining*

I.2.1. General staining methods

The following general staining methods were used: hematoxylin/phloxine, toluidine blue (1% in water) and Azan (Romeis, 1968). Both material embedded in paraplast and in diethylene glycol distearate were stained after removal of embedding material through xylol and an alcohol series.

I.2.2. Neurological staining methods

The following methods for specific staining of nerve cells were used. Silver impregnation methods according to Bodian (Romeis, 1968), Batham et al. (1960), and Jha (1965). Methylene blue staining according to Batham et al. (1960) and Burnett & Diehl (1964).

I.2.3. Staining methods for neurosecretory material

The following methods were used for the demonstration of neurosecretion:

(1) paraldehyde fuchsin (Gabe, 1953); (2) aldehyde thionine (Paget, 1959); (3) pseudoisocyanin (Sterba, 1961); (4) phenanthrenequinone (Magun & Kelly, 1969); (5) astra blue (Bock & Schlüter, 1971); (6) acridine orange (Beattie, 1971).

The staining methods 1 to 5 were applied on material embedded in paraplast as well as in epon. Epon was removed according to Kuhlman & Greven (1973). Besides the normal staining procedure, staining was also performed after a previous oxidation (Stoeckel et al., 1972). Staining method no. 6 was performed on non-fixed cryostat sections.

I.3. *Maceration*

Schneider's maceration mixture (1 part 0.02% osmium tetroxide in distilled water and 4 parts 5% acetic acid) advised by Semal van Gansen (1952) was used. A maceration time of three to five days at 4°C was sufficient.

I.4. *Histochemistry*

I.4.1. Catecholamines and indolamines

Catecholamines and indolamines were demonstrated using the Formaldehyde Induced Fluorescence (F.I.F.) method. This reaction was performed on freeze dried material.

After freeze drying the tissue was treated with paraformaldehyde vapour, at 80°C for 1½ hour. The relative humidity of the paraformaldehyde used was 60—70% (Hamberger et al., 1965). After the paraformaldehyde treatment the tissue was embedded in paraplast under vacuum.

Instead of freeze dried material we also used cryostat sections cut at —30°C and dried over phosphorus pentoxide (Spriggs et al., 1966).

For a better localization of the fluorescence and in order to obtain material suited for electron microscopy, the freeze dried material, after paraformaldehyde treatment was embedded in epon under vacuum.

In order to test the specificity of the fluorescence developed we used the standard tests described in literature (Corrodi et al., 1964; Falck & Owman, 1965; Hökfelt, 1965; Eränkö, 1967; Corrodi & Jonsson, 1967).

Apart from the F.I.F. method, the chromaffine reaction of Hillarp and Hökfelt (in Pearse, 1972) was used to demonstrate the presence of catecholamines, and the diazonium method (Pearse, 1972) in order to demonstrate the presence of indolamines.

The behaviour of sea anemones is influenced by the nutritional state. A recently fed animal will react slowly to tactile stimuli. An animal which has not been fed for some time, however, reacts very quickly.

In order to study the effect of food on the nervous system i.e. the fluorescent part of the nervous system, the animals were starved during

a period of 1 to 8 weeks. Twice a week two tentacles were cut from a sea anemone (each time a different animal was used) to examine them for catecholamines and indolamines with the F.I.F. technique.

A possible effect of reserpine on the fluorescence pattern was tested by administering two different concentrations: 0.1 and 0.5 g/l sea-water (reserpine depletes the catecholamine and indolamine stores).

Monoamine oxidases, enzymes which degrade catecholamines and indolamines, were demonstrated according to Glenner et al. (1957).

I.4.2. Gamma-aminobutyric acid and gamma-aminobutyric acid metabolism

The method of Wolman (1971) was used for the demonstration of gamma-aminobutyric acid.

We tried to demonstrate the presence of a gamma-aminobutyric acid metabolizing system with the following two methods.

1. Demonstration of a gamma-aminobutyric acid transaminase/succinic semialdehyde dehydrogenase system (Van Gelder, 1965).
2. Demonstration of a succinic semialdehyde dehydrogenase (Sims et al., 1971). Succinic semialdehyde was synthesized according to Bruce et al. (1971).

Succinic acid dehydrogenase was always used as control (Pearse, 1972) both with succinic acid and with synthesized semisuccinic acid as a substrate. This was done to exclude false results, due to a reaction of succinic acid dehydrogenase with succinic acid which may be present in the preparation as an impurity.

I.4.3. Acetylcholinesterase and choline acetyltransferase

Acetylcholinesterase was demonstrated using the thiocholine method of Karnovsky & Roots (1964). An aspecific reaction caused by hydrolysis of acetylcholine by nonspecific cholinesterases was prevented by using iso-Ompa in the incubation medium in a concentration of 10^{-4} M.

Choline acetyltransferase was demonstrated using the method of Kasa et al. (1970) and Burt (1970). Instead of DFP (Kasa et al., 1970) or phospholine jodide (Burt, 1970) iso-Ompa was added to the medium (Ballard & Jones, 1972). The concentration of iso-Ompa in the medium was 10^{-3} M. This blocker is necessary to prevent splitting of acetyl coenzyme A by esterases other than choline acetyltransferase. Since choline acetyltransferase proved to be sensitive to lead ions, the lead concentration was reduced to ⅓ of the given value i.e. from 0.03 M to 0.01 M.

I.4.4. Adenosine triphosphatase

Adenosine triphosphatase (ATPase) was demonstrated according to Pearse (1968) and Geyer (1973). Several variants of the incubation medium were used in order to preclude aspecific reactions caused by other phosphatases and to determine the type of ATPase present.

As control a medium without substrate was used. Furthermore, incubation with adenosine diphosphate and adenosine monophosphate was performed to find out whether adenosine diphosphatase and adenosine monophosphatase were present (both enzymes are known to split adenosine triphosphate).

Acid and alkaline phosphatases are also known to split adenosine triphosphate. Their influence was assessed by using Na-β-glycerophosphate as a substrate and varying the pH of the incubation medium; pH tested: 5.8, 7.2 and 9.0.

In order to determine the type of adenosine triphosphate, Ouabaine (1 mM), DNP and cyanide (2 mM) were used as blockers. The magnesium dependence of the enzyme was decided by omitting magnesium sulphate from the incubation medium. Calcium dependence was determined by substituting calcium chloride for magnesium sulphate, so that the quantities of calcium ions and original magnesium ions were identical.

The effect of lead ions was studied by reducing the concentration of lead ions to one third of the original quantity.

II. ELECTRON MICROSCOPY

II.1. *Fixation and embedding*

II.1.1. Sea anemones

Tentacle pieces of the four sea anemones were fixed in 2.5% glutaraldehyde in 0.18 M phosphate buffer (pH 7.4) containing 5‰ NaCl at 4°C, and in 1% osmium tetroxide in barbital acetate buffer (pH 7.4 at 4°C). After dehydration the tissue pieces were embedded in epon.

Table I. Survey of methods used for the identification of catecholamines and indolamines.

Author	Formalin	Glut. ald.	Amm. Ag.sol.	Kbichr.	OsO_4	Organ tested	Adr.	Noradr.	Dopa	5-HT
Tramezzani et al., 1964		+	+			adrenal	—	+		
Wood & Barnett, 1964 Wood et al., 1971		+		+		adrenal	—	+		
Etcheverry &		+		+		adrenal,		+		—
Zieher, 1968	+	+		+		hypophyse		—		+
		+		+	+			+		—
	+	+		+	+			—		+
Cannata et al., 1968		+	+(1′)			various	—	+	+	—
		+	+(30′)			organs	—	+	+	+

Formalin: 8% formaldehyde solution, 24 hours at 4°C.
Glutaraldehyde: 6.25% in cacodylate or phosphate buffer, pH = 7.4, 24 hours at 4° C.
Ammoniacal silver carbonate solution: 300 ml 5% Na carbonate solution was added to 100 ml 10% silver nitrate solution. Dropwise ammonia was added till the precipitate formed, had disappeared. Incubation time was either 1 minute or 30 minutes.
Potassium bichromate: a 1.5% potassium bichromate and 1% natrium sulphate solution in 0.2 M acetate buffer at pH = 4, 14—16 hours.
OsO_4: solution mentioned in § II.1.1.
5-HT = 5-hydroxytryptamine

II.1.2. Hydra

Hydra tentacles were fixed in 2.5% glutaraldehyde in 0.01 M Hepes (pH 8.0, at 4° C). After osmication (1 hour in 1% osmium tetroxide in distilled water) the tentacles were dehydrated and embedded in epon.

The fixations mentioned by Westfall (1973b) were also used.

II.2. *Thin sections and 1 μm sections*

The 1 μm sections were stained on a hot plate with a 1% toluidine solution in 1% borax solution in water. Moreover, neurosecretory material was stained in 1 μm sections (see § I.2.3).

Thin sections with an inference colour of silver or gold were prepared on a Reichert ultratome for electron microscopy.

The sections were contrasted with uranylacetate and lead.

II.3. *Cytochemistry*

II.3.1. Identification of catecholamines and indolamines

For identification of catecholamines and/or indolamines in the dense core and opaque granules various methods according to the following publications were used: Tramezzani et al. (1964); Wood & Barnett (1964); Cannata et al. (1968); Etcheverry & Zieher (1968); Wood et al. (1971). These methods are summarized in table I.

Test tube experiments were performed in the way described by Cannata et al. (1968). The following substances were tested: 5-hydroxytryptamine, 5-hydroxytryptophane and tryptophane. The two latter substances were also tested in combination with creatinine sulphate. The creatinine sulphate was added in an equivalent quantity.

Creatinine sulphate is in fact always present in natural 5-hydroxytryptamine preparations and therefore may affect the reaction with the chemicals used in the above-mentioned acticles.

II.3.2. Ethanolic phosphotungstic acid method

The Ethanolic Phosphotungstic Acid (EPTA) method (Bloom & Aghajanian, 1968) was used for selective contrasting of postsynaptic membranes.

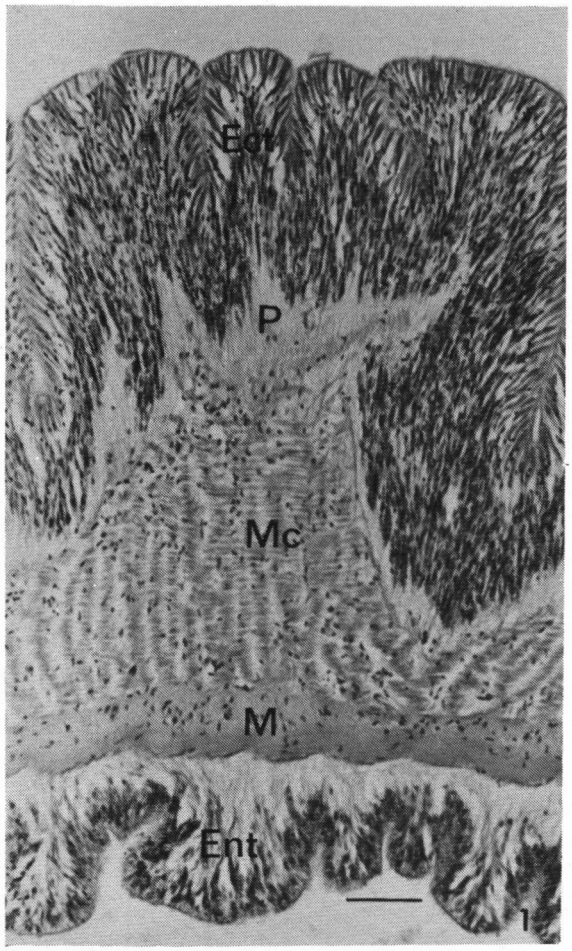
II.3.3. Adenosine triphosphatase

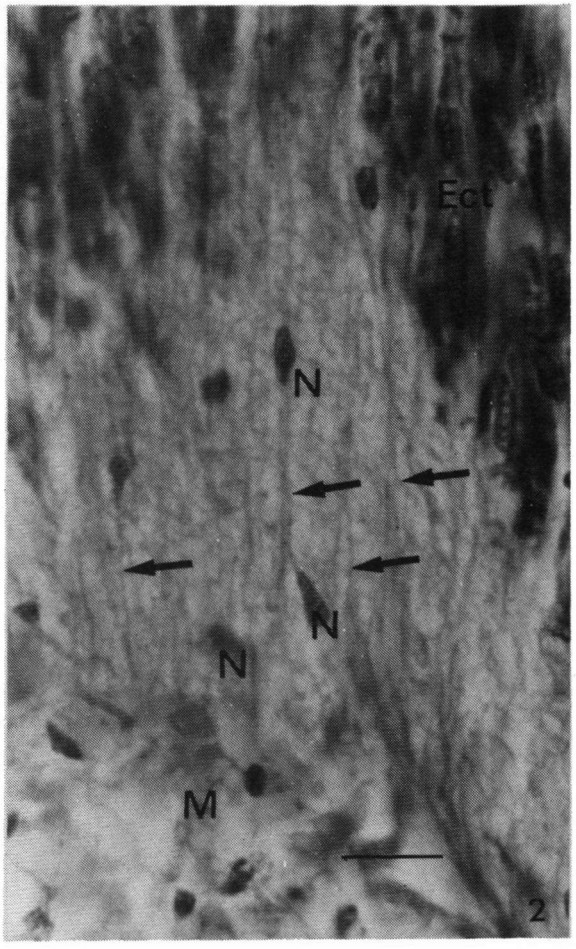
Adenosine triphosphatase (ATPase) was demonstrated as described by Geyer (1973). This reaction was mainly performed on *Anemonia sulcata*, for this animal was most convenient for cutting with the tissue chopper.

III. CULTURE AND AUTORADIOGRAPHY

III.1 *Culture*

Tealia felina was preferred for culture since in this animal muscles, nervous system between the





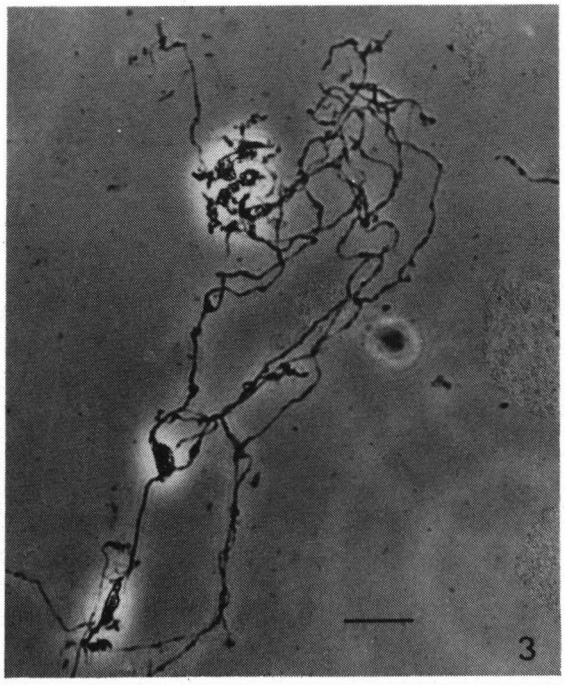


Fig. 1. *Tealia.* Transverse section of a tentacle. The tentacle consists of three layers: ectodermis (Ect), mesogloea (M) and entodermis (Ent). The layer indicated by Mc consists of mesogloea septs to which muscles are attached. These septs are longitudinally arranged in the tentacle. Bouin fixation, 6 µm paraffin section, HP staining. Scale: 50 µm.

Fig. 2. *Tealia.* The plexus (P) with nerve cells, only the nuclei of which (N) are visible. The vague net-like structure consists of neurites. The necks of the ectodermal cells go through the plexus (four of them are indicated by arrows). Bouin fixation, 6 µm paraffin section, HP staining. Scale: 10 µm.

Fig. 3. *Tealia.* Nerve cells in maceration preparation (phase contrast). Scale: 10 µm.

Ect = ectodermis; M = mesogloea; P = nervous plexus.

muscles and the plexus appeared to have a much wider spacial separation than in the other species.

Pieces of tentacles were incubated for 2, 4, 12, 24 and 48 hours in a culture medium. The medium was saturated with carbogen (95% O_2 and 5% CO_2). Temperature was 15°C, which is the same temperature at which the animals are kept in the aquaria. The size of the pieces was about 4 mm^3.

The medium used was developed in our laboratory by Veldsema & Veldsema-Currie.

The medium consisted of:
100 ml Eagles minimal essential medium + Earles salt solution,
10 ml foetal calf serum,
12 ml salt solution (= 2.32 g NaCl; 0.213 g KCl; 1.23 g $CaCl_2$; 1.5 g glucose per 50 ml),
29.2 mg L-glutamine,
0.75 ml 1.5 M Hepes-NaOH, pH = 7.2.
The pH must be 7.2. By adding NaCl the osmolarity of the medium was brought to 672 milli-osmol.

Culture took place in disposable culture vessels (Falcon Organ Culture Dish 3010). In each vessel four pieces of tissue were cultured in 1 ml of the above-mentioned medium.

At the start of the culture always one of the following labelled substances was added: ^{14}C tyramine, ^{14}C tyrosine, ^{14}C glutamate, ^{14}C tryptophane, ^{3}H gamma-aminobutyric acid, ^{3}H 5-hydroxytryptophane or ^{3}H 5-hydroxytryptamine, ^{3}H D,L, noradrenalin. Isotopes were added in such quantities that the activity of the medium was ca. 5 μCi per ml.

The maintenance of the cultured material was checked histologically by treating the cultured material as mentioned in § I.1 and § I.2.1.

Material cultured with ^{14}C tyramine, ^{14}C tyrosine, ^{14}C tryptophane and ^{14}C glutamate was tested biochemically. This was done according to the method described by Veldsema & Veldsema-Currie [1]).

III.2. *Autoradiography*

Material incubated for 1 hour or cultured for 24 hours was used for autoradiography.

The isotopes used for autoradiography were, respectively: ^{3}H 5-hydroxytryptophane, ^{3}H 5-hydroxytryptamine, ^{3}H D,L, noradrenalin and ^{3}H gamma-aminobutyric acid.

The tentacle fragments were fixed in glutaraldehyde/osmium (see § III.1). After dehydration the tissue was embedded in paraplast. Sections of 4 μm were used.

After removal of the paraplast the slides were coated with a layer of Kodak NTB-2 emulsion. This was done with the dipping technique described by Rogers (1973).

Processing and staining of the autoradiography was done according to Rogers (1973).

The autoradiographic methods are adopted from the following authors: Aghajanian & Bloom (1966, 1967); Hökfelt & Ljungdahl (1971); Evans (1974); Sotelo et al. (1972); Ericson et al. (1972).

CHAPTER III. RESULTS

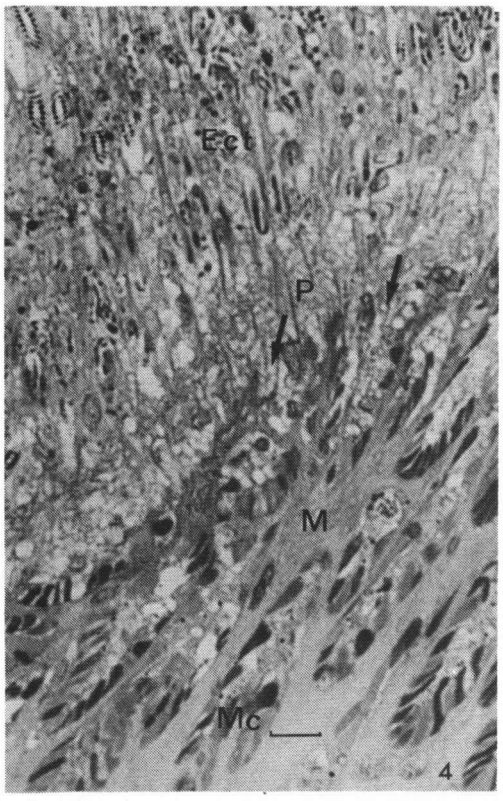
Since the structure, histology and cytology of *Anemonia, Metridium, Cerianthus* and *Tealia* are essentially similar, they are described together (see figs. 4—7). *Hydra,* which differs in structure from the four above-mentioned species has been dealt with separately.

[1]) Mr. and Mrs. Veldsema, Pharmacological Laboratory, University of Amsterdam, kindly performed these assays.

Figs. 4—7. Situation of the muscles and of the plexus with respect to each other in *Tealia, Anemonia, Cerianthus* and *Metridium,* respectively.

Fig. 4. *Tealia.* The muscles (Mc) lie against the walls of the longitudinally oriented spaces in the mesogloea. These spaces debouch all over the surface of the mesogloea, which is turned towards the ectodermis (outlets are indicated by arrows). Here the connection between the nervous system between the muscles and the plexus (P) is also situated. Toluidine blue staining, 1 μm section. Scale: 10 μm.

Figs. 5—7. Transverse sections of the tentacles of *Anemonia, Cerianthus* and *Metridium,* respectively. In figs. 5 and 7 mesogloeal ridges with muscles attached to them are indicated by arrows. The muscles in *Cerianthus* (fig. 6) as well as the mesogloeal ridges are considerably less developed than in the two other species. The ectodermal cells are attached to the tops of the mesogloeal ridges. Toluidine blue staining, 1 μm sections. Scale: 10 μm, 20 μm and 10 μm, respectively.
Ect = ectodermis; Ent = entodermis; M = mesogloea; Mc = muscle; P = plexus.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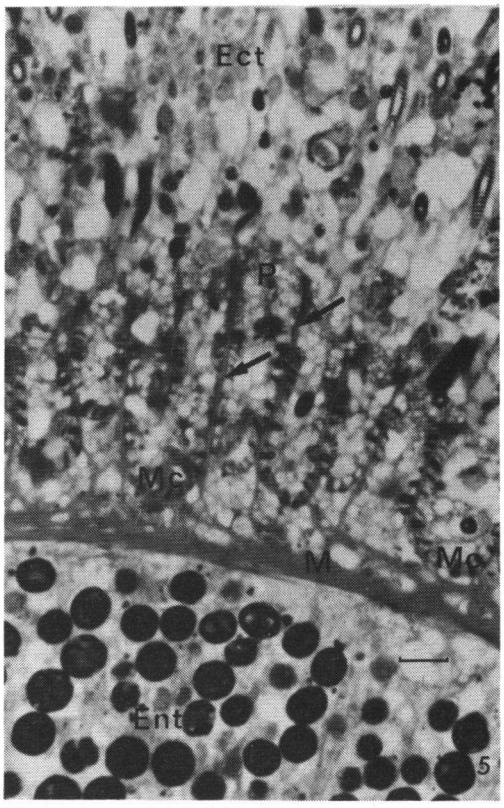
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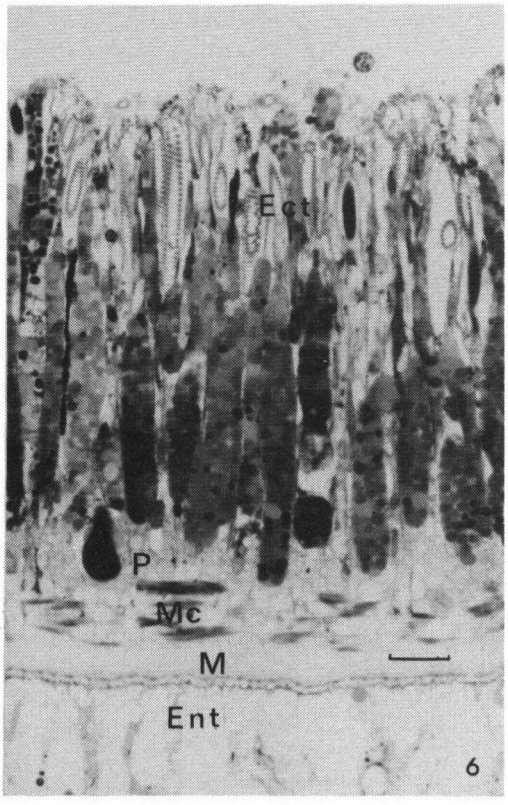
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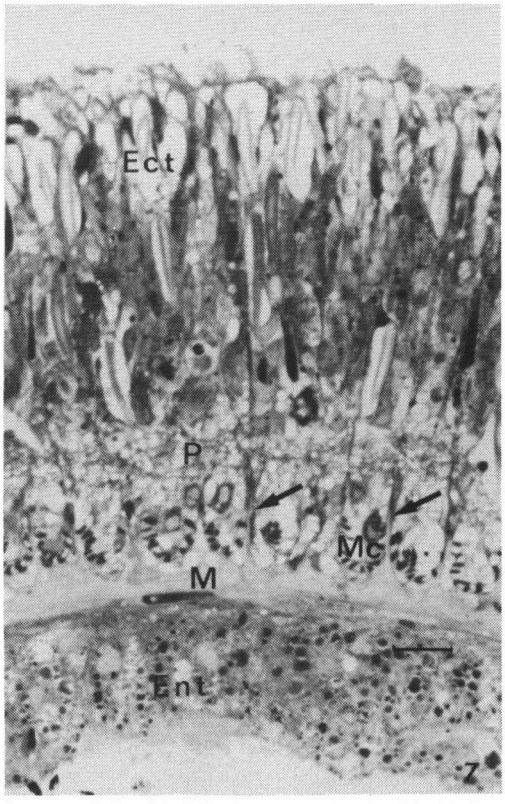
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Anemonia, Metridium, Cerianthus, Tealia

I. GENERAL STRUCTURE OF THE TENTACLES

A cross section shows that the tentacle consists of three concentric layers. From the outside to the inside these are ectodermis (Ect), mesogloea (M) and entodermis (Ent) (figs. 1 & 31). The muscles (fig. 1, Mc) are attached to mesogloea ridges which are oriented lengthwise in the tentacle. Mesogloea and entodermis are of no importance for the present study since they do not contain nerve elements.

In the ectodermis, epithelial cells, gland cells, nematocytes and spirocytes can be distinguished: all these elements are long cells which are implanted radially on the mesogloea perpendicular to the longitudinal axis of the tentacle (figs. 1 & 31).

The site of attachment to the mesogloea is connected with the cell body by a long thin neck (figs. 2, 8, 9 & 12, indicated by arrows). Apart from a nucleus, the cell body may contain secretory granules, a spirocyst or a nematocyst. The neck with which the cell body is connected to the mesogloea, is contractile.

The mesogloea has a number of ridges oriented lengthwise in the tentacle (figs. 5 & 7, arrows). These ridges branch and anastomose repeatedly. The impression is that the number of ridges over the whole length of the tentacle varies but little. Only in the most apical part of the tentacle their number decreases rapidly. These ridges are conspicuous in *Anemonia* and *Metridium*, which have a well developed muscular system (figs. 5 & 7, arrows). In *Cerianthus* whose muscular system is much less developed, the ridges are developed to a lesser extent. The sites of attachment of the ectodermal cells are situated on the tops of these ridges (fig. 32). The muscles are arranged lengthwise in the tentacle against the ridges. This is clearly visible in figs. 5 and 7.

The nervous system is visible forming a very fine-threaded network (fig. 31) with scattered cell bodies between the necks of the ectodermal cells (P in figs. 2 & 8) and between the muscles (fig. 17, round the cells marked with C).

Tealia is an exception to the above description in so far that the muscle cells do not lie against the ridges of the mesogloea, but against the walls of cavities in the mesogloea (fig. 4).

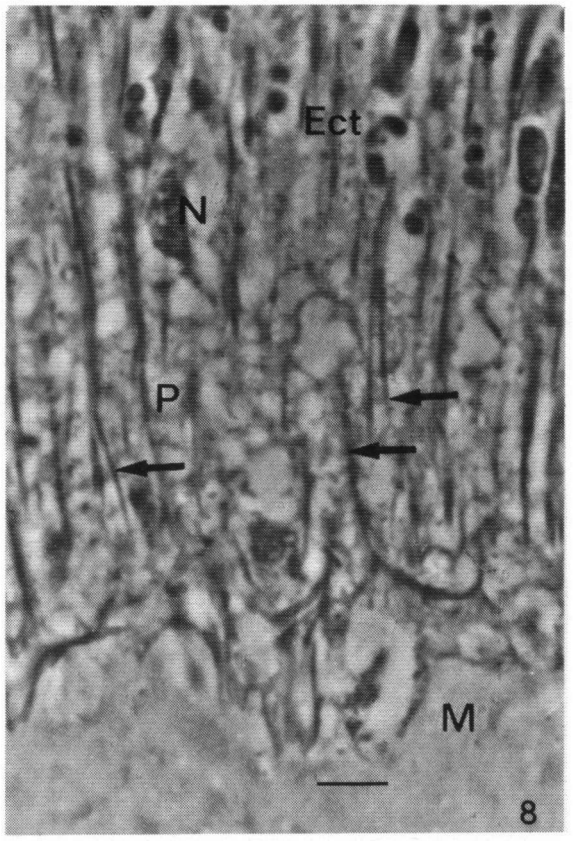


Fig. 8. *Tealia.* Plexus (P) with nerve cells, only the nuclei of which (N) are visible. The necks of the myoepithelial cells (three are indicated by arrows) go through the plexus. The neurites are better visible here than in fig. 2, owing to a different fixation and embedding technique. Glutaraldehyde/OsO_4 fixation, 1 µm epon section, stained with toluidine blue in borax. Scale: 10 µm.
Ect = ectodermis; M = mesogloea.

These cavities are oriented lengthwise in the tentacle just as the mesogloea ridges in *Metridium, Anemonia* and *Cerianthus*. They branch and anastomose often. Over the whole length of the tentacle these cavities end at the ectodermal side of the mesogloea (fig. 4, arrow). The muscles are oriented lengthwise in the tentacle.

II. STRUCTURE OF THE NERVOUS SYSTEM

II.1. *Perikarya*

II.1.1. Light microscopy

II.1.1.1. Nerve cells of the plexus and between the muscles

The nerve cells are visible as small cells (4—7

μm) with a relatively large nucleus (3—5 μm) (N in figs. 2 & 8). They often have an irregular, angular shape. Neither the exact shape of the perikarya nor the number of neurites emerging from them, could be established in the sections.

Maceration preparations showed that most of the nerve cells are tripolar (fig. 3). The transition from perikaryon to neurite is gradual. Bipolar or multipolar nerve cells were rarely observed in the maceration preparations. The shape of the nerve cells in the maceration sections was mostly irregular.

The above description can be applied to both nerve cells in the plexus and nerve cells between the muscles.

The number of nerve cells in the plexus is almost identical in the four species, viz. about 50 per 0.01 mm². This number was determined using 1 μm epon or diethylene glycol distearate sections. The number of nerve cells in a section is independent of the orientation of this section.

It should be noted that the number given (about 50 per 0.01 mm²) holds for fixed, i.e. contracted tentacles.

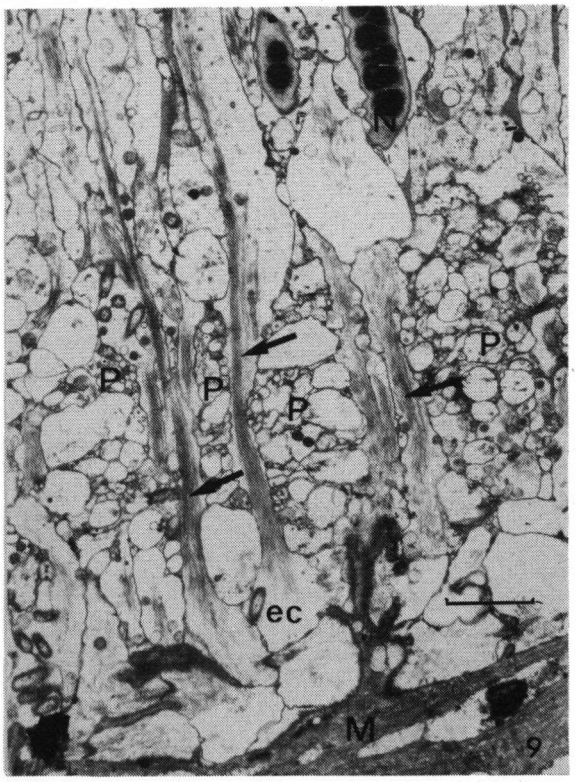


Fig. 9. *Tealia.* Electron microscopic survey of the plexus (P). The necks (arrows) of the ectodermal cells (ec) pass through the plexus. Transverse section. Scale: 2.5 μm.
M = mesogloea.

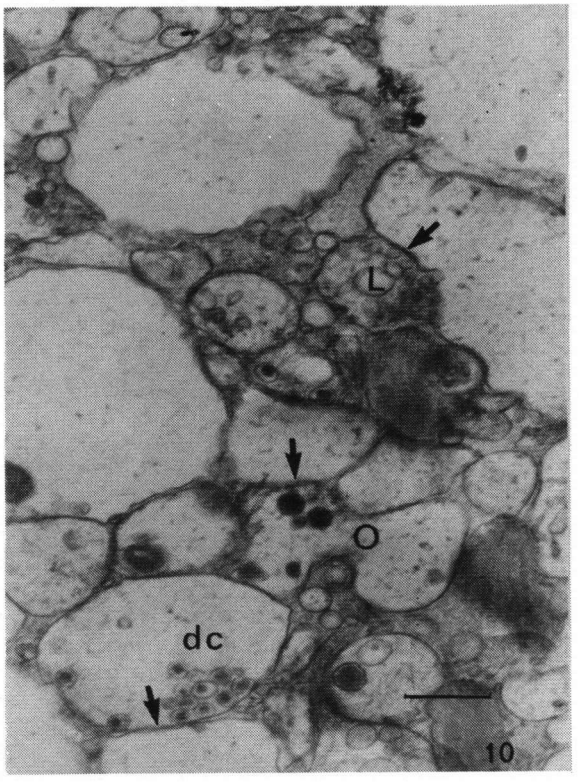


Fig. 10. *Metridium.* Detail of the plexus containing neurites with dense core (dc), opaque (O) granules and empty (L) vesicles. A few sites of contact of neurites with unidentifiable cross sections of cell fibres are indicated by arrows. Longitudinal section. Scale: 0.5 μm.

Fixation of the tentacle in an extended condition appeared to be impossible in all four species.

The number of nerve cells between the muscles could not be established with certainty on account of identification problems.

Small dark granules were seen infrequently in the perikarya. However, the perikarya did not stain with methods specific for neurosecretion. They remained unstainable even if the standard oxidation was preceded by an oxidation according to Stoeckel et al. (1972) (see Methods § I.2.3).

II.1.1.2. Receptor cells

The presence of receptor cells at the periphery is inferred from the results of the F.I.F. method. With this method thickenings are seen at the beginning of the fluorescent fibres running from the periphery to the plexus (fig. 21). Receptor cells were also found with the electron micro-

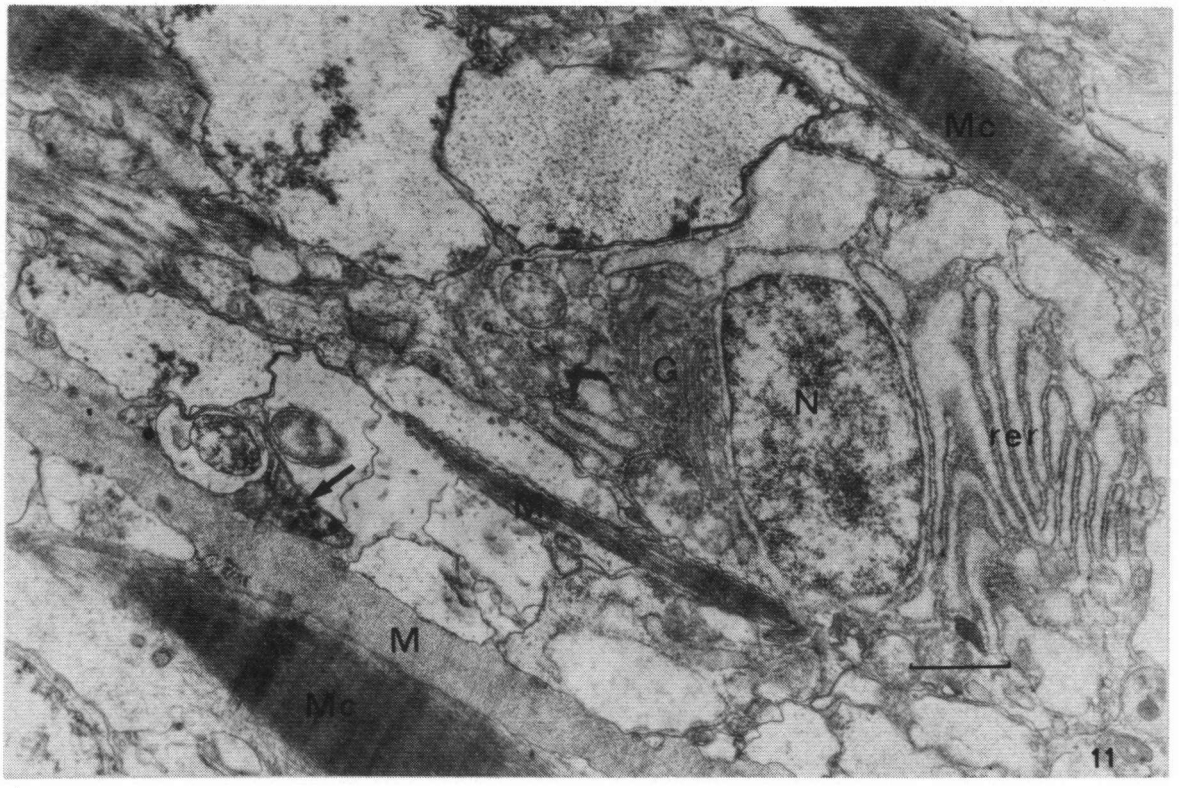


Fig. 11. *Anemonia.* Nerve cell between the muscles (Mc) showing the nucleus (N), the Golgi complex (G) and a strongly dilated, rough, endoplasmic reticulum (rer). A nerve fibre with opaque granules is indicated by an arrow. Scale: 1 μm.
M = mesogloea.

scope (see Results § II.1.2.1). However, these cells were never identified with the light microscope, whatever combination of fixative and embedding was used.

II.1.2. Electron microscopy

II.1.2.1. Nerve cells of the plexus and between the muscles

Electron microscopic investigation showed (figs. 11, 14) that the nerve cells contain little, but often strongly dilated, rough endoplasmic reticulum. The Golgi complex in the perikarya is well developed (G in figs. 11 & 14). The cytoplasm is densely packed with polysomes. Few mitochondria were observed.

The transition from perykaryon to neurite is gradual, the cytoplasm becoming less electron dense, while neurotubuli appear. An indication of this transition is shown in the nerve cell in fig. 11. It becomes really clear, however, when serial sections are studied.

Very few granules are found in the perikaryon, either of the dense core type or of the opaque type. Sometimes these granules lie close to the Golgi complex.

Cilia are absent in nerve cells lying either in or near the plexus or between the muscles. On the perikarya no sites of contact are seen (see Results § II.2.2).

Electron microscopically there is no difference between nerve cells of the plexus and nerve cells between the muscles.

II.1.2.2. Receptor cells

Only a few receptor cells (R in fig. 16) were observed electron microscopically, in spite of the great quantity of material investigated. They were situated at the edge of the tentacle of the sea anemones.

The cells were of a general type: a long cell lying between gland cells, having a small nucleus and a cilium protruding far outside the microvilli of the gland cells. The cilium is implanted in an invagination of the cell membrane. At the base of the cilium striated rootlets were found. In the direction of the plexus the cell is continued as a neurite which could not be traced further among

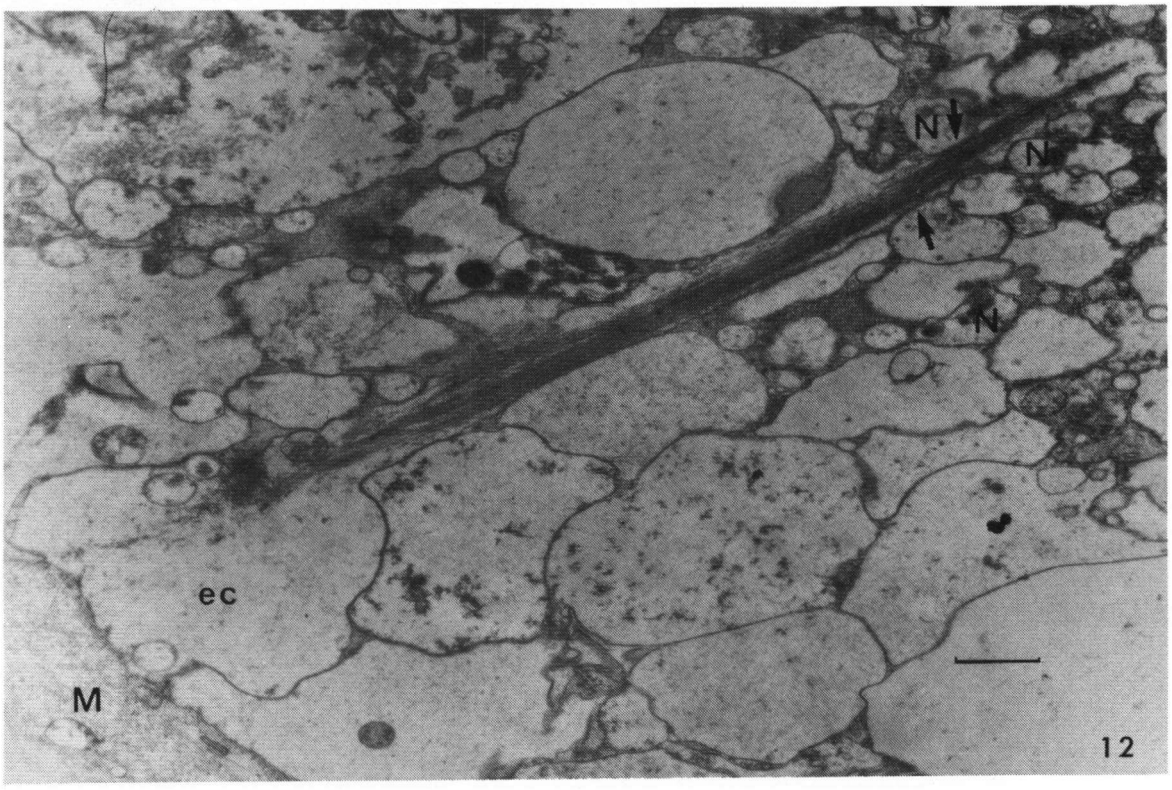


Fig. 12. *Tealia.* Ectodermal cell neck (ec) with neurites (N). The sites of contact between neurites and ectodermal cells are indicated by arrows. Longitudinal section. Scale: 1 μm.
M = mesogloea.

the gland cells. This neurite contained a few dense core granules. A number of big electron dense bulb-shaped objects were present in the receptor cells.

The receptor cells are connected with the surrounding ectodermal cells by means of desmosomes (fig. 16, arrows).

II.2 *Neurites*

II.2.1. Light microscopy

The neurites of the nerve cells are visible in the sections as thin threads. They lie between the necks of the ectodermal cells (arrows in figs. 2 & 8) where they form the plexus (P in figs. 2, 4—8). Besides they are visible between the muscles (fig. 17, round the cells marked C). Both groups of neurites are interconnected (see fig. 4, arrow). If the tentacles are fixed in Bouin or Susa and subsequently embedded in paraplast or diethylene glycol distearate, the result is rather disappointing (fig. 2). Fixation in glutaraldehyde/osmium (see Methods § II.1.1) and embedding in epon gave a far better result (fig. 8). However, even with this technique we could add nothing to the above description, since neurites are extremely small.

In maceration preparations the maximum length of a neurite is 200 μm. The real length, however, must be much more, for it is obvious that large parts of the neurites have broken off.

It was impossible to measure the diameter of the neurites. This was due partly to the small dimensions of the neurites (< 0.6 μm) and partly to the halo inherent to the use of phase contrast.

The perikarya gradually taper into neurites. These neurites rapidly divide into a number of considerably thinner fibres of varying length. The latter fibres in their turn give rise to a great number of fibres which are hardly distinguishable with the light microscope.

The nerve fibres connecting the peripheral receptor cells to the plexus, can be made visible with the F.I.F. method (see Methods § I.4.3). These radial fibres (fig. 21) were never observed when other methods were used, not even in material in which their localization had become

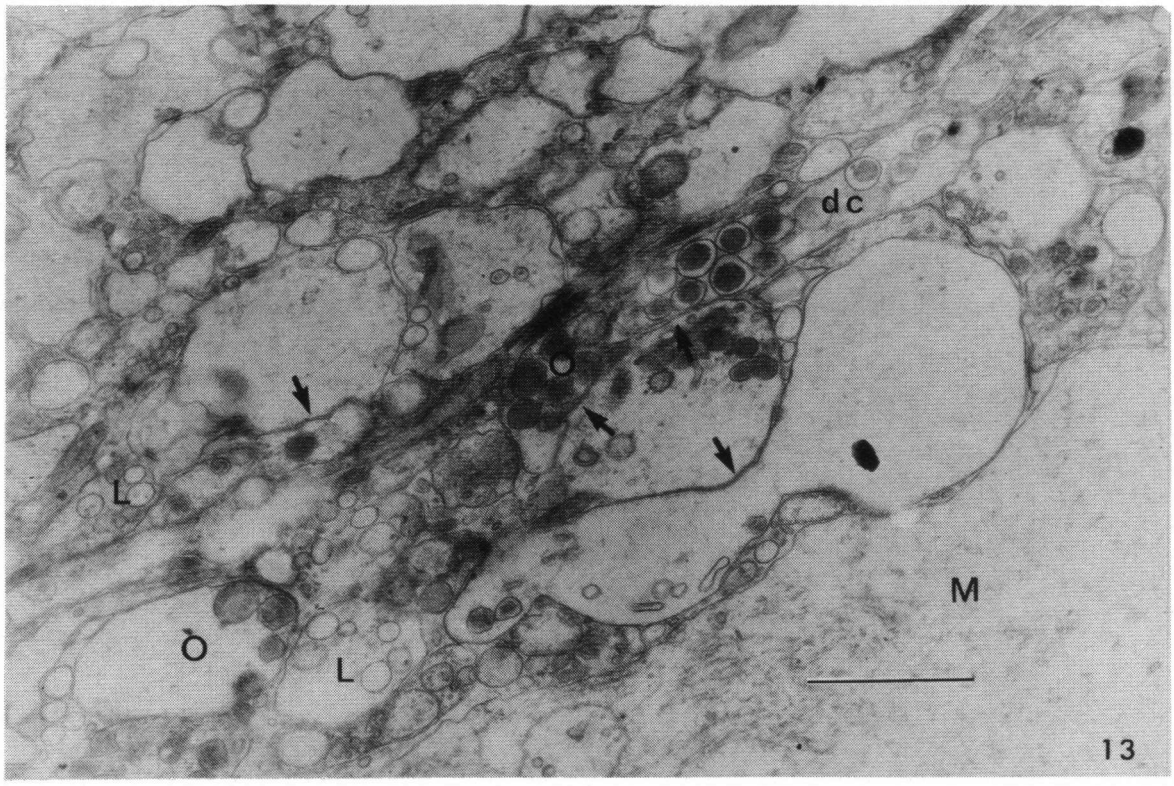


Fig. 13. *Tealia.* Neurites in the plexus with dense core (dc), opaque (O) granules and empty vesicles (L). Arrows indicate sites of contact formed by the neurites (whether or not with fibres identifiable as neurites). Transverse section. Scale: 1 μm.
M = mesogloea.

known from the pictures obtained with the F.I.F. method.

Staining the plexus on neurosecretory material gave negative results as was also the case with the perikarya (Methods § I.2.3).

Attempts to stain the nervous system with (leuco-)methylene blue or silver impregnation in order to obtain reproducibly specific results, failed. Consequently, these methods were no longer applied.

II.2.2. Electron microscopy

The osmotic value of the fixative appeared to be of vital importance for an acceptable fixation. However, in spite of all efforts to improve (application of other buffers) and standardize the procedure, variations in fixation of the membranes and granules remained. These variations do not show as much in the investigation of the perikarya as they do in the investigation of the neurites.

It was therefore impossible to investigate the spaces between the membranes at the contact sites and to apply the controlled aldehyde fixation methods of Bodian (1970) for granules.

II.2.2.1. Neurites of the plexus

The neurites of the nerve cells outside the muscle area form the plexus (P in fig. 9). This plexus lies round the mesogloea. The necks of the ectodermal cells go right through the plexus (fig. 9, arrow).

In *Tealia* and *Metridium* the cross sections of the neurites are mostly circular. In *Cerianthus* and *Anemonia* there is a greater number of irregular cross sections. Possibly this is correlated with the fact that the contraction of the tentacles of the first group of species is stronger than the contraction of the tentacles of the second group. The shape of the cross sections of the neurites is independent of the orientation of the section (figs. 10, 12, 13 & 15).

More than 90% of the cross sections of the neurites have a diameter of 1 μm or less. The mean diameter is about 0.6 μm.

Neurites with a diameter up to 4 μm did occur, but they are rare. From serial sections it became

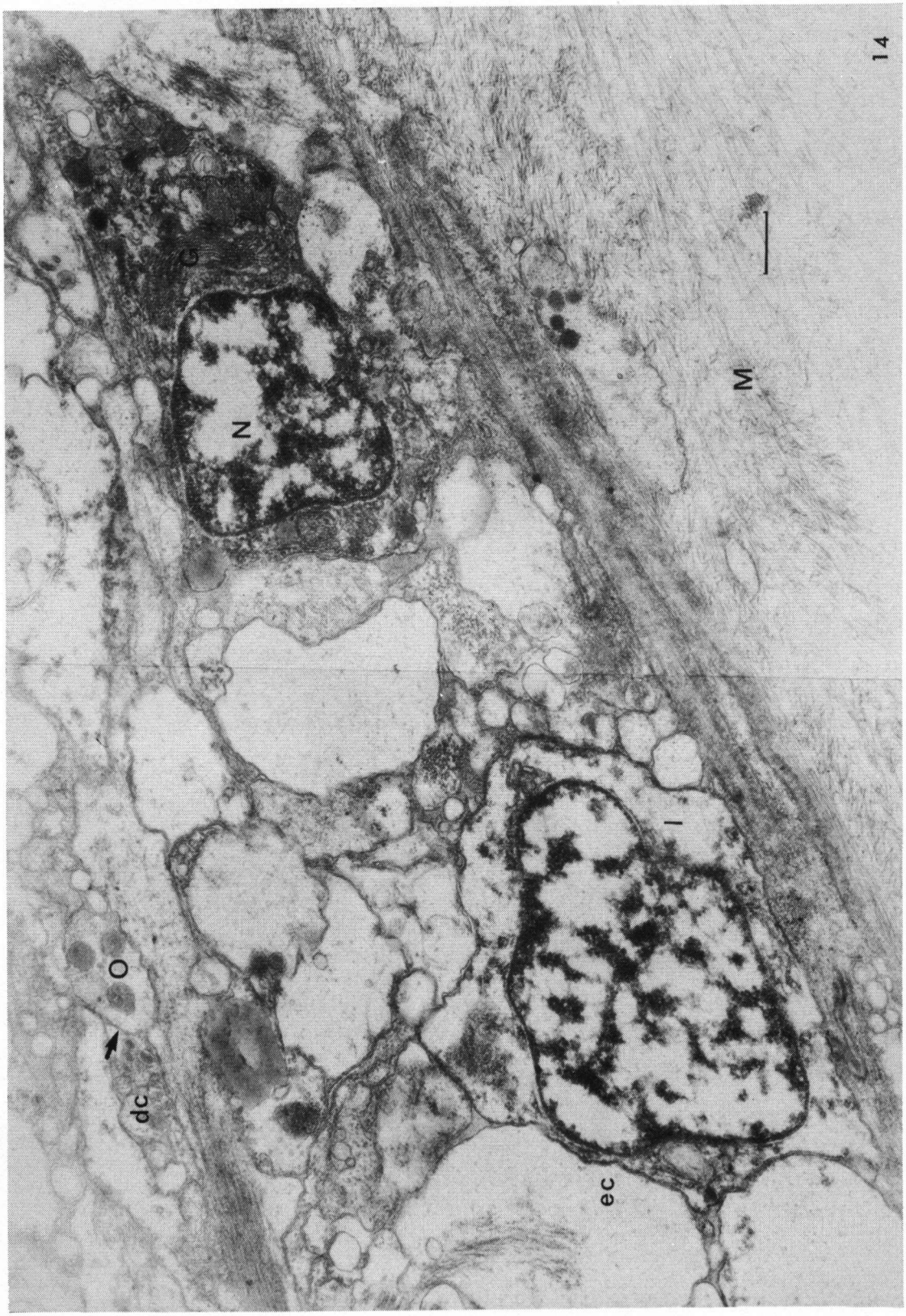


Fig. 14. *Tealia.* Nerve cell (N) belonging to the plexus. The site of contact between a neurite with dense core granules (dc) and a neurite with opaque granules (O) is indicated by an arrow. Scale: 0.5 µm.
ec = ectodermal cell; G = Golgi complex; I = interstitial cell; M = mesogloea.

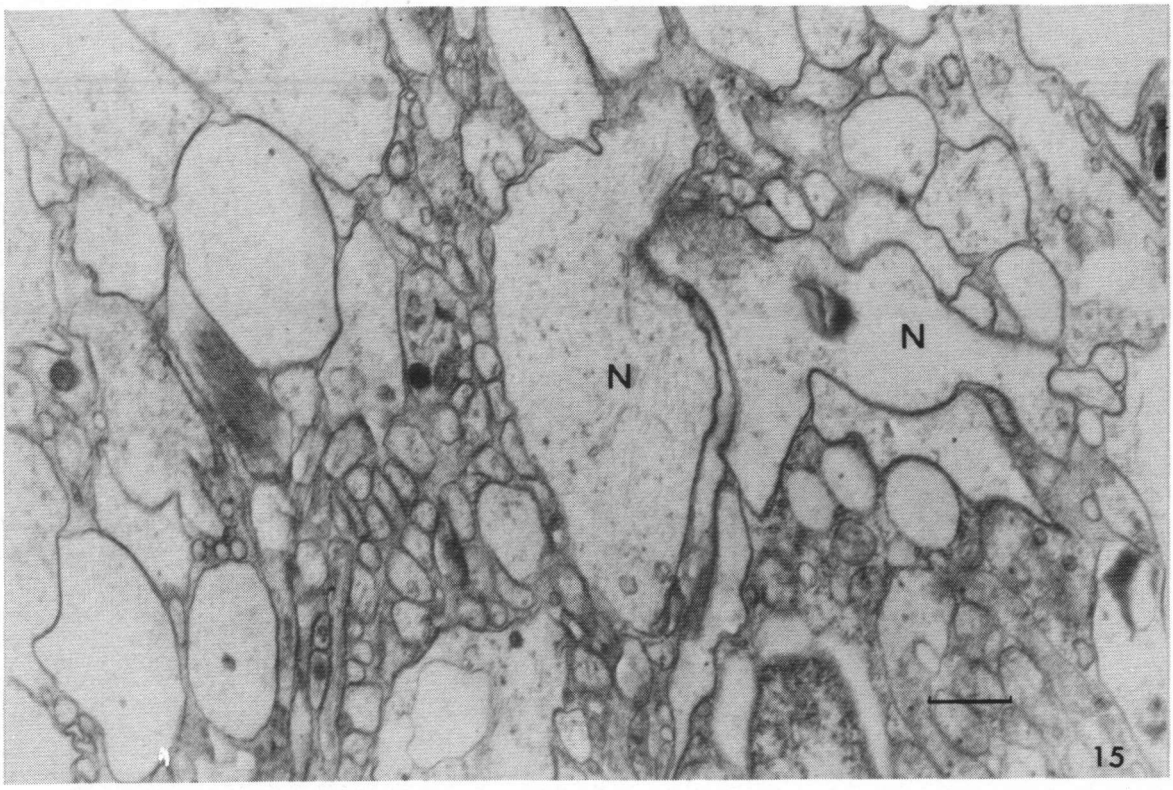


Fig. 15. *Cerianthus.* Plexus with irregular cross sections of neurites. Since granules are absent in this instance, a definite identification of the cross sections is impossible. The large cross sections indicated by N appeared to be neurites after serial sections were cut. They gradually pass into nerve cells. Transverse section. Scale: 0.5 μm.

apparent that these large neurites always lie close to the perikaryon from which they originate. These neurites branch rapidly and give rise to many fibres with diameters smaller than 1 μm.

Three types of granules are observed in the neurites of the plexus: dense core, opaque granules and empty vesicles (dc, O and L, respectively, in figs. 10, 13 & 14). These three types can be distinguished as follows:

Dense core granules have a light zone between the core and the membrane of the granule. The opaque granules are either completely electron dense or show a less electron dense zone between core and granular membrane. However, this zone is always more electron dense than it is in dense core granules. The empty vesicles have no electron dense contents.

The diameters of the dense core, the opaque granules and the empty vesicles are approximately 1300 Å, 1100 Å and 1500 Å, respectively.

In the different preparations the number of cross sections of neurites containing granules varies considerably. Especially the dense core granules are subject to variations: on the whole, the neurites in the plexus in which granules are observed are divided as follows:
50% with dense core granules;
20% with opaque granules;
10% with empty vesicles.

The remaining 20% contain empty vesicles in combination with either dense core or opaque granules. The combination of dense core and opaque granules in one neurite was never observed.

The neurites in the plexus make contact with each other and with the ectodermal cells.
(a) Neurite-neurite contacts (arrows in figs. 10, 13 & 14).

In this type of contact the cell membranes run parallel over a certain distance. The distance between the membranes at the site of contact is 150—200 Å (the normal distance between two neurites is about 300 Å). At these sites usually but not always, granules are observed in one of the two neurites. Granules are rarely observed in both neurites at the site of contact.

From serial sections it appeared that two

neurites often make contact at several sites. The granules at these various sites may occur in both neurites.

At the contact sites the structure of the cell membranes of both neurites is identical. It was never observed that one of the cell membranes is clearly thickened. Nothing can be said about the material between the two cell membranes owing to the precariousness of the fixation.

(b) Neurite-ectodermal cell contacts.

Between neurites and ectodermal cells, sites of contact are frequently observed. Structurally they resemble the neurite-neurite contact (fig. 12, arrow). Again there is no thickening of the membrane. The granules in these neurites are always of the dense core type.

II.2.2.2. Neurites between the ectodermal cells

The receptor cells in the outer layer of the ectodermis are connected with the plexus by fibres which can be made visible with the F.I.F. method (fig. 21) (see Results § III.1).

With the electron microscope nerve fibres between the ectodermal cells could not be identified.

II.2.2.3. Neurites between the muscles

The neurites between the muscles do not differ from the neurites of the plexus as regards diameter and shape (O in figs. 18 & 19). They only contain opaque granules and empty vesicles. Dense core granules were never observed in the nerve fibres between the muscles.

This difference from the neurites in the plexus is most obvious in *Tealia,* because here the sharpest spacial separation between plexus and nervous tissue between the muscles (cf. fig. 4 and figs. 5, 6 & 7) is present.

Generally the neurites between the muscles end at a distance of more than 150—200 Å from the muscle cells. In those rare cases in which there is contact between nerve and muscle i.e. when the distance between nerve membrane and muscle membrane is 150—200 Å and the two membranes run parallel over a certain distance, the muscle membrane at this site of contact is neither thickened nor in any other way differen-

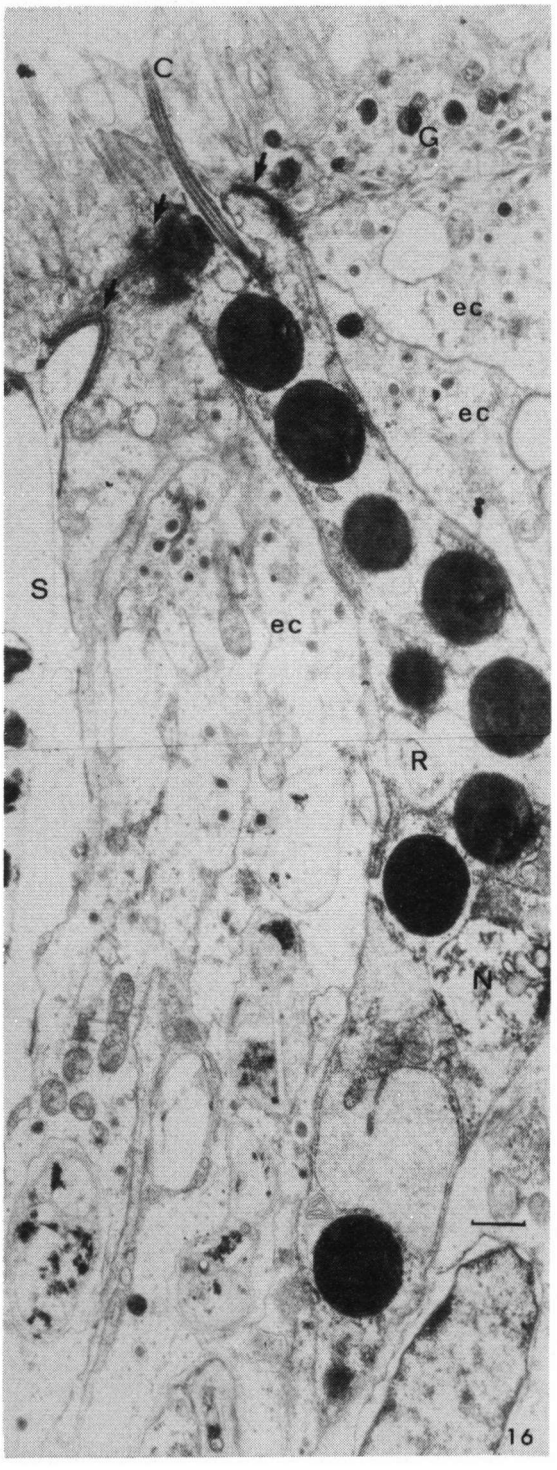
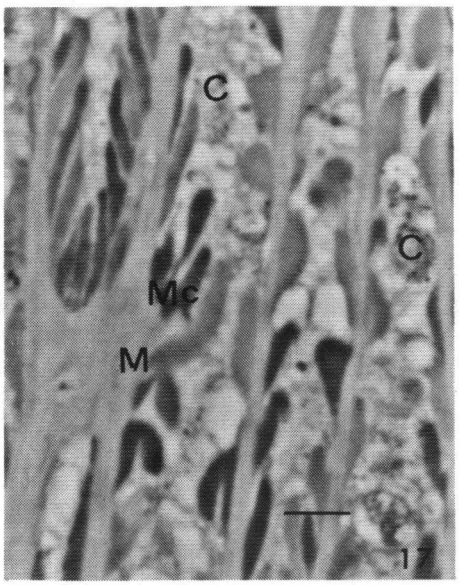


Fig. 16. *Tealia.* Receptor cell (R) in the outer layer of the tentacle between the cells of the ectodermis (ec). Specialized membrane segments (arrows) are observed between epithelial cells and receptor cells as well as between epithelial cells and spirocysts (S) (cf. fig. 20). The granules in these epithelial cells must not be confused with dense core granules in the neurites having the same dimensions. The receptor cell has a cilium (C) standing in an invagination of the cell. Scale: 0.5 µm.
G = gland cell; N = nucleus of the receptor cell.



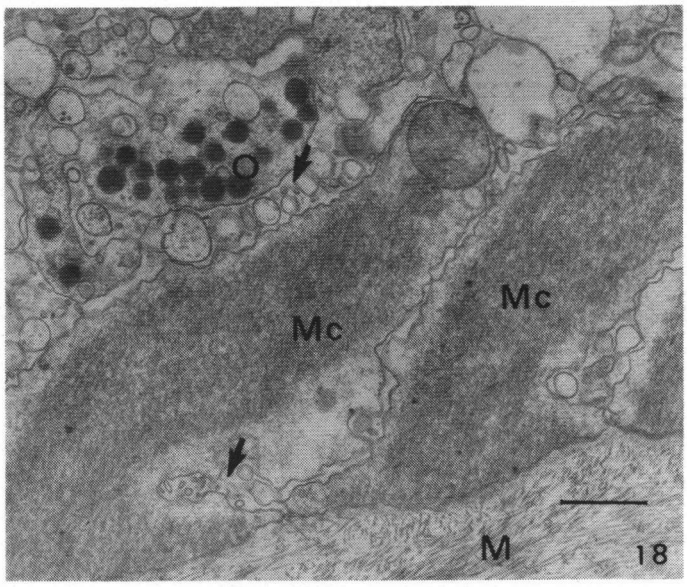


Fig. 17. *Tealia.* Muscles (Mc) against the walls of spaces in the mesogloea (M). Cells (C) in these spaces are not identifiable with the light microscope. The vague structures round the cells (C) consist largely of neurites as was shown electron microscopically. Transversally cut 1 μm epon section. Scale: 5 μm.

Fig. 18. *Tealia.* Muscles (Mc) against the mesogloea (M). The nerve-ending contains opaque granules (O). There is no contact between nerve-ending and muscle. The small structures indicated by arrows are folds in the cell membrane of the muscle cells. Scale: 0.5 μm.

tiated (fig. 19, thin arrow, upper left).

Owing to irregularities in fixation nothing can be said about the space between the membranes.

Specialized membrane areas on the muscle cells are often found but only between muscle cells (fig. 19, thick arrow, lower right).

The many microvilli-like protrudings of the muscle cell membrane are indicated by arrows (fig. 18).

III. HISTOCHEMISTRY AND CYTOCHEMISTRY

III.1. *Catecholamines and indolamines*

Application of the F.I.F. method on the four sea anemones resulted in a yellow to yellowish green fluorescence. The plexus appeared to be fluorescent (P in fig. 21). Radially fluorescent fibres ran from the plexus to the periphery of the tentacle. They often ended in a thickening (fig. 21).

The number of radial fibres in a transversal section of 10 μm was 4 to 5, distributed at random, sometimes in groups, sometimes further apart. The number of these fibres per section is the same over the whole length of the tentacle.

No fluorescent material was found in the entodermis and in the mesogloea. Neither was F.I.F. observed between the muscles.

The fluorescence appeared to be sensitive to UV (365 nm) radiation. If exposed to UV radiation the fluorescence disappeared almost completely after a few minutes.

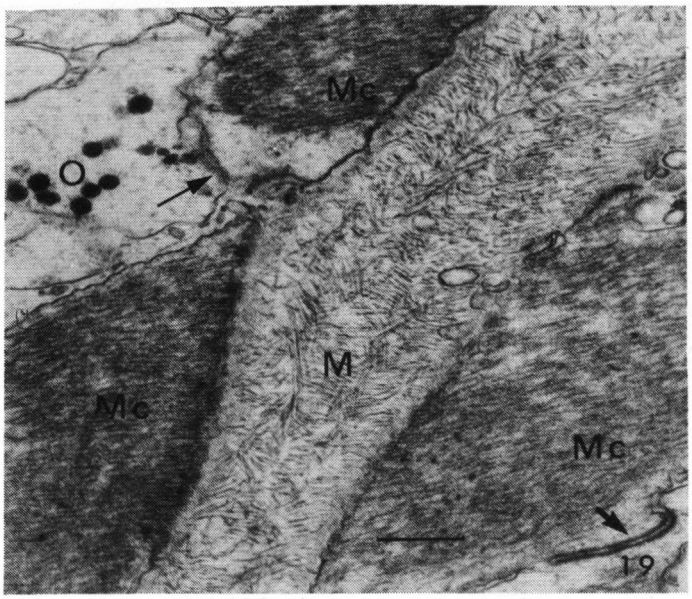
Treatment with Na-borohydride caused the fluorescence to disappear. On renewed treatment with paraformaldehyde the fluorescence appeared again.

The excitation and emission spectra of the fluorophore were analysed with a Leitz microspectrofluorimeter [2]). It appeared that the excitation maximum and the emission maximum were 390 nm and 485 nm, respectively, indicating that the fluorescence is due to the presence of catecholamines. The complete excitation and emission spectra are given in fig. 33.

The characteristics of these spectra (excitation maximum 390 nm, emission maximum 485 nm) are the same everywhere, irrespective as to whether it is the fluorescence of the plexus or the fluorescence of the radial fibres.

In order to get a better localization and to obtain material suitable for electron microscopy, the material was embedded in epon under

[2]) Ing. F. H. van Dijk (Laboratory of Histochemistry and Cytochemistry, Leyden University) kindly performed these analyses for me.



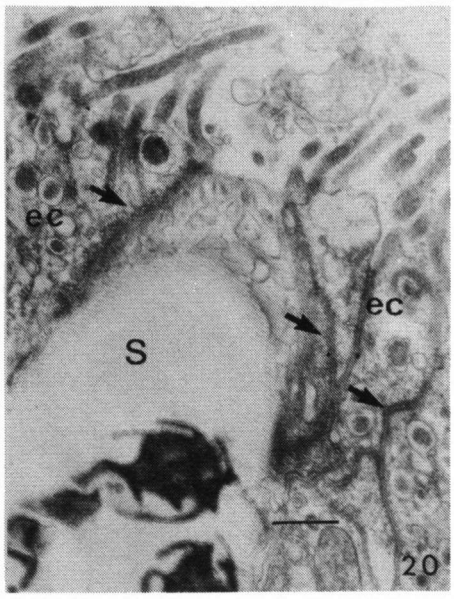


Fig. 19. *Tealia.* Muscle cells (Mc) attached to the mesogloea (M). Opaque granules (O) in the nerve endings. At the site of contact between nerve and muscle (thin arrow) no specialized membranes are present. Specialized membrane segments (thick arrow) are observed, however, between two muscle cells. Scale: 0.5 μm.

Fig. 20. *Tealia.* Outer layer of tentacle with spirocysts (S) and epithelial cells (ec). Thickened membrane segments are observed between the cells (arrows). Scale: 0.5 μm.

vacuum after freeze drying and paraformaldehyde treatment.

The F.I.F. could easily be observed in the 1 μm sections, however, unfortunately the material could not be used for electron microscopy. In spite of all precautions, freezing disturbed the ultrastructure of the cells in such a way that the result was not worth risking contamination of the oil of the diffusion pump with epon vapours.

Reserpine, which depletes catecholamine and indolamine stores, was added in various concentrations with a maximum of 5 mg/ml sea-water. The animals remained in the reserpinated seawater up to three weeks. The F.I.F. remained the same during this period, both as regards the intensity of the fluorescence and the number of radial fibres.

The effect of the nutritional condition on this part of the nervous system was ascertained by starving the animals during various periods of time (the maximum time of starving was 8 weeks).

The fluorescence was in no way affected by starving.

The silver and chromate methods used for identification of catecholamines and indolamines gave negative results both with light microscopy and electron microscopy.

Sometimes electron microscopy showed a precipitation on the granules in those circumstances (see Table I) which are specific for 5-hydroxytryptamine. But this was neither clear nor reproducible.

It was shown in test tube experiments that 5-hydroxytryptamine with glutaraldehyde gives a precipitation, as was already known from literature (see Methods § II.3.1). Moreover, tryptophane and 5-hydroxytryptophane (see Culture results) did not give a precipitation, not even if creatinine was added.

The diazonium method according to Pearse (1972) for the demonstration of indolamine also gave a negative result.

Monoamine oxidases, the only catecholamine and indolamine degrading enzymes which can be demonstrated histologically, do not occur in the four sea anemones *Tealia, Metridium, Cerianthus* and *Anemonia.*

III.2. *Gamma-aminobutyric acid and gamma-aminobutyric acid metabolism*

From the culture results (§ IV.2) it appeared that if culture took place with labelled glutamate, 2% of the total amount of radioactivity present in the tissue was recovered in the fraction identifiable as gamma-aminobutyric acid (GABA).

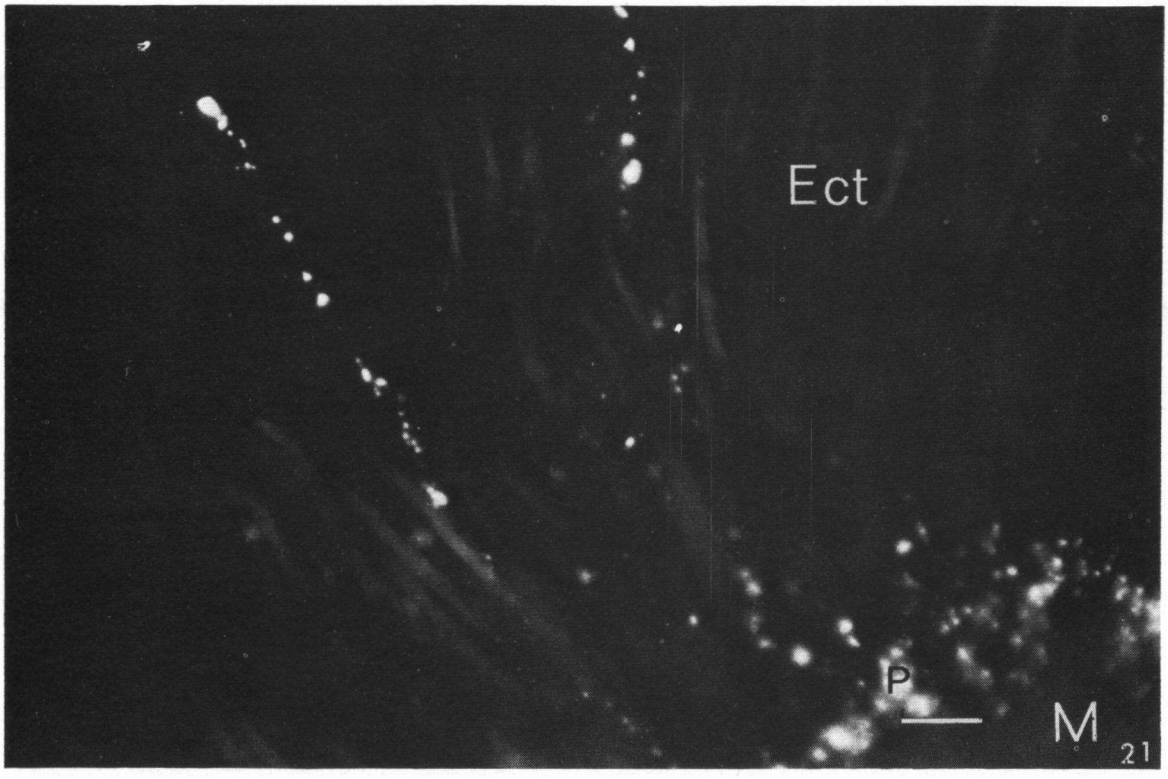


Fig. 21. *Tealia.* F.I.F. of the plexus (P) and of radial fibres from the periphery to the plexus. The background fluorescence consists mainly of autofluorescence of the spirocytes. Scale: 10 μm.
Ect = ectodermis; M = mesogloea.

Histochemically gamma-aminobutyric acid could not be demonstrated according to the method described by Wolman (1971). The enzymes connected with gamma-aminobutyric acid metabolism were not demonstrable either.

Glutamate → GABA → Semisuccinic acid → Succinic acid
under influence of
Glutamate decarboxylase
GABA-ketoglutaric acid transaminase
Semisuccinic acid dehydrogenase

Gamma-aminobutyric acid transaminase — semisuccinic acid dehydrogenase complex, and semisuccinic acid dehydrogenase could not be demonstrated, no matter how the incubation medium and pretreatment of the material were varied. These reactions were performed both on freeze dried or freshly prepared cryostat sections and on sections from the tissue chopper, with or without fixation.

As control of the purity of the synthesized semisuccinic acid, it was used as a substrate for an assay of succinic acid dehydrogenase, with negative results.

III.3. *Choline acetyltransferase and acetylcholinesterase*

Both choline acetyltransferase and acetylcholinesterase did not occur in the four sea anemones. Titrimetric and spectrophotometric analysis in a tentacle homogenate did not show the presence of acetylcholinesterase.

III.4. *Adenosine triphosphatase*

The nerve cells showed a positive adenosine triphosphatase (ATPase) reaction (fig. 22, arrows). If natrium-beta-glycerophosphate, adenosine monophosphate or adenosine diphosphate were used instead of adenosine triphosphate, no reaction in the nerve cells was found.

If incubation with one of the four above-mentioned substrates took place at pH 5.8 or pH 9.2 instead of pH 7.2, not any reaction in the nerve cells was observed either.

A 50% reduction of the lead ion concentration did not decrease the reaction of the nerve cells. It was impossible to increase the lead concentration, for in this case a precipitation was the

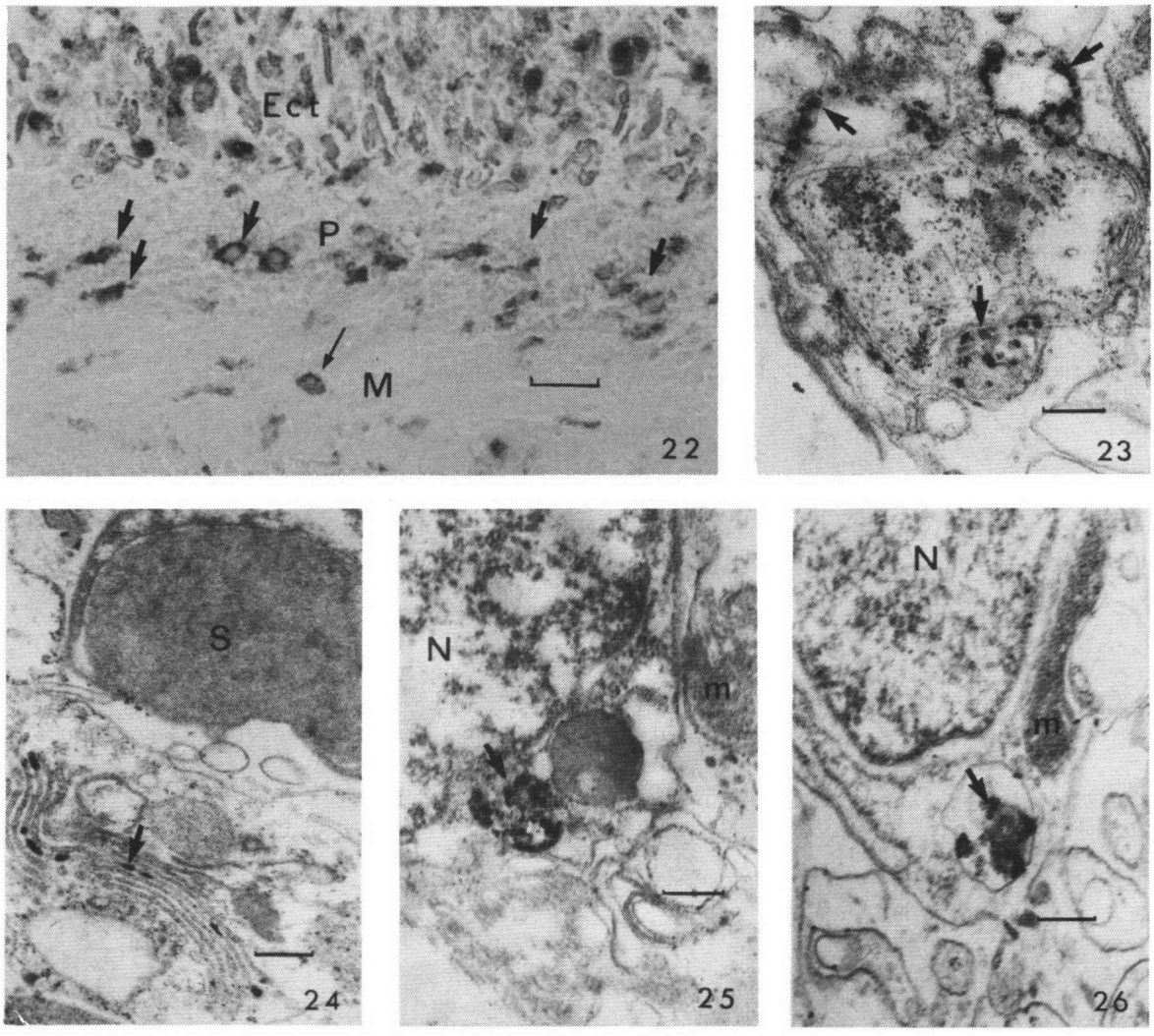


Fig. 22. *Anemonia.* Light microscopic demonstration of ATPase positive nerve cells (indicated by thick arrows) in a 10 µm frozen section. An ATPase positive nerve cell between the muscles is indicated by a thin arrow. Scale: 20 µm.
Figs. 23—26. *Anemonia.* Electron microscopic localization of the ATPase in the Golgi complex (fig. 24, arrow) and different vesicles (fig. 25 and 26, arrow). The ATPase is also observed in the intercellular spaces (fig. 23, arrows). This enzyme is not observed in mitochondria (fig. 25 and 26, m). In a number of cases an aspecific precipitation was unavoidable (figs. 23, 24). Scale: 0.25 µm.
Ect = ectodermis; M = mesogloea; N = nucleus; P = plexus; S = developing spirocyte.

immediate result.

The ATPase appeared to be sensitive to magnesium ions. Omission of magnesium ions gave a negative reaction for ATPase in the nerve cells. If magnesium ions were replaced by calcium ions, the ATPase reaction remained positive. The ATPase was insensitive to KCN and Ouabaine.

Identification problems kept us from ascertaining whether all nerve cells in the plexus and between the muscles have a positive reaction. It is certain, however, that the percentage of nerve cells with a positive reaction is high.

Electron microscopy showed that the ATPase was localized in the Golgi complex of the nerve cells (fig. 24, arrow), and in vesicles lying in the perikarya (figs. 25 & 26, arrows) and in the neurites. The ATPase is also present in the intercellular space between the neurites (fig. 23, arrows). The enzyme is not present in the mitochondria (M in figs. 25 & 26). Unfortunately, an aspecific precipitation was inevitable in a number of cases.

The great mechanical resistance of the mesogloea made it very difficult to prepare 75 µm

sections with a tissue chopper. Freezing of sea anemone tissue caused very severe damage to the ultrastructure of the cells, so that frozen sections were unsuitable for electron microscopy (see Results § III.1). These circumstances made it very difficult to get satisfactory results from the ATPase reaction on electron microscopic level.

The various incubation mediums for the ATPase reaction are summarized in table II.

III.5. *Ethanolic phosphotungstic acid method*

Contacts between neurites, between neurites and ectodermal cells and between neurites and muscles (even though the latter two groups are scarcely present), have been described above.

The ethanolic phosphotungstic acid method (EPTA) was developed for selective contrasting of postsynaptic membranes. It proved to be impossible to contrast either one or both membranes at the site of contact with this method, even if the water content of the EPTA solution was varied as advised by the authors.

Table II. Effects of different substrates, inhibitors and metal ions on the ATPase reaction of nerve cells.

Substrates Inhibitors Metal ions	ATPase reaction		
	pH=5.8	pH=7.2	pH=9
Natrium-beta-glycerophosphate	—	—	—
Adenosine triphosphate	—	+	—
Adenosine diphosphate	—	—	—
Adenosine monophosphate	—	—	—
Ouabaine		+	
DNP		+	
KCN		+	
Mg^{2+} or Ca^{3+} present		+	
Mg^{2+} absent		—	
$[Pb^{2+}]$ high		± ?	
$[Pb^{2+}]$ low		+	

IV. CULTURE

IV.1. *Histology*

When pieces of tentacles of *Tealia* were cultured for a period of 24 or 48 hours at 15°C, light microscopically no degeneration was visible. In comparison with the starting material the number of spirocysts in the ectodermis had significantly decreased. The discharged threads of the spirocysts were visible in the sections as a thick mass lying close to the ectodermis.

The spirocytes which are always present in the ectodermis, were more developed and present in greater quantities than in non-cultured material.

The F.I.F. pattern did not change under the culture circumstances used.

IV.2. *Biochemistry*

If ^{14}C tryptophane was added to the culture medium, 90% of the activity present in the tissue was recovered in the tryptophane fraction. Of the activity present in the tissue, 2% was present in the 5-hydroxytryptophane fraction.

Furthermore, some activity was present in a fraction identifiable as 5-hydroxy indol acetic acid; 5-hydroxytryptamine was not found.

With the use of ^{14}C tyramine all the activity present in the tissue was recovered in the tyramine fraction. None of the following substances was formed: noradrenalin, dopa, dopamine, octopamine and tyrosine.

If culture took place with ^{14}C tyrosine, a number of metabolites was found. These substances and their percentages of activity incorporated in the tissue pieces are presented in table III.

Table III. Labelled substances in tissue pieces of sea anemones after a culture period of 24 hours in a culture medium with ^{14}C tyrosine and their respective percentages of the total incorporated activity.

Labelled substance	%
Noradrenalin	13
Dopa	10
Dopamine	7.5
Octopamine	3.5
Tyramine	5
Tyrosine	12

However, it was shown that the tyrosine added to the medium was contaminated with tyramine, dopamine and octopamine in amounts up to 1% of the added amount of activity.

If glutamate was added to the medium, 2% of the activity present in the tissue pieces was recovered as gamma-aminobutyric acid.

In contrast to the tyrosine used, the tyramine, tryptophane and glutamate were not contaminated.

IV.3. *Autoradiography*

After an incubation period of 1 hour or a culture period of 24 hours with one of the following

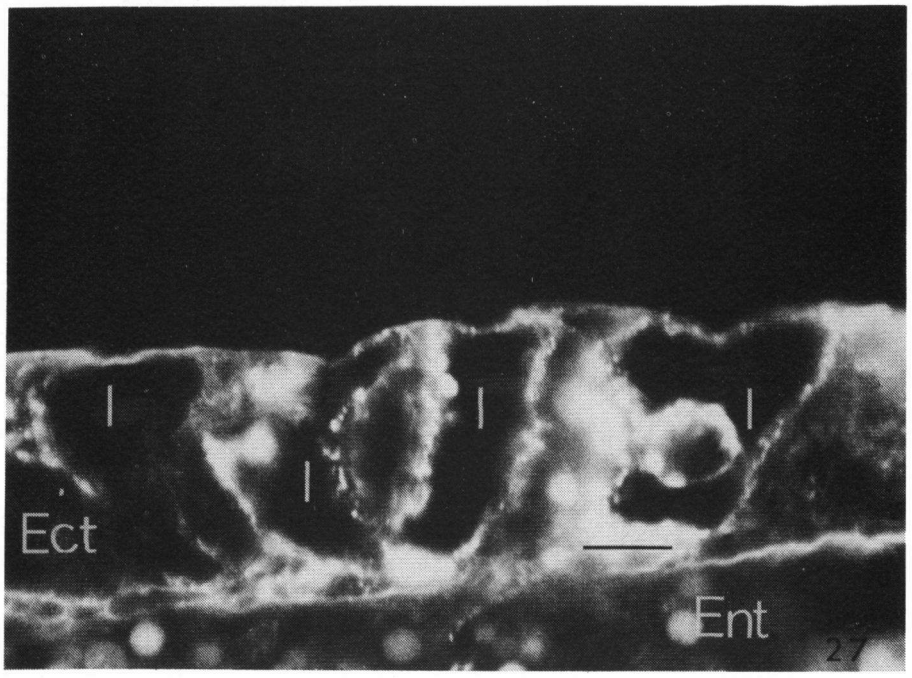


Fig. 27. *Hydra.* F.I.F. in a tentacle. In contrast to the F.I.F. pattern in the sea anemones investigated (fig. 21), this fluorescence is spread through the whole ectodermis. Scale: 10 µm.
Ect = ectodermis; Ent = entodermis; I = interstitial space.

isotopes, no specific accumulation of radioactivity was observed anywhere in the autoradiographs. These isotopes were: ^{3}H 5-OHtryptamine, ^{3}H 5-OHtryptophane and ^{3}H gamma-aminobutyric acid.

If incubation or culture took place with ^{3}H D,L, noradrenalin, a specific accumulation of radioactivity became apparent in some perikarya in the plexus (fig. 31). Specific labelling of the plexus itself i.e. the neurites, did not occur. The perikarya between the muscle cells were not labelled.

Mention should be made of the sometimes heavy labelling of interstitial cells lying in the mesogloea.

Hydra

Since the perikarya have been described extensively in the literature and since our own observations were completely in accordance with what has been found in the literature, a description of the perikarya of *Hydra* is omitted here (for literature survey, see Westfall, 1973b).

It should be mentioned here that, in general, the perikarya in *Hydra* resemble the perikarya in the sea anemones. They deviate from the sea anemone perikarya in the presence of a cilium. However, not in all *Hydra* perikarya a cilium occurs.

A description of the electron microscopic structure of the neurite is given, for this has not been done before.

V. ELECTRON MICROSCOPY OF THE NEURITES

The neurites are scattered through the ectodermis (see also fig. 27) and do not form a plexus as in the four sea anemones. A number of neurites is oriented lengthwise in the tentacle. Besides they often run parallel with the interstitial spaces as the F.I.F. sections show (fig. 27). The neurites contain tubuli, mitochondria and granules (figs. 28—30).

The granules are often irregular in shape. They contain a small, electron dense core (about 500 Å) which is often situated excentrically. There is probably only one morphologically recognizable type of granules.

The contacts of the neurites with other neurites, with ectodermal cells and with muscle cells are similar to those in the sea anemones (figs. 28 & 29, arrow; fig. 30, thin arrows on the left). The distance between the membranes at the sites of

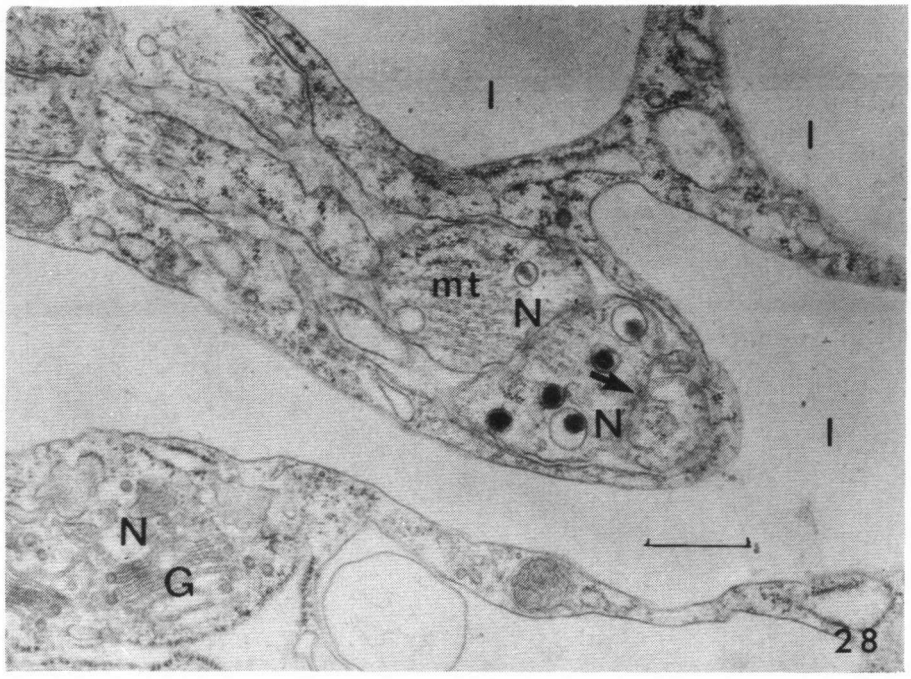


Fig. 28. *Hydra.* Neurites (N) one of which containing granules. Microtubuli (mt) and Golgi complexes (G) were observed in the neurites. Scale: 0.5 μm.
I = interstitial space.

contact is 150—200 Å. Thickened membranes were never observed in *Hydra,* nor could they be made visible with the EPTA method.

Contacts of neurites and muscles seldom occur. The nerves between the muscles mostly end at a distance larger than 300 Å. *Hydra* shows many specialized membrane areas between the muscles (fig. 30, thick arrows on the right) identical to those found in the sea anemones.

Notable is the great number of mitochondria in the neurites, for they are seldom found in sea anemones.

VI. CATECHOLAMINES AND INDOLAMINES

The colour of the F.I.F. is yellow to yellowish green. The fluorescence is sensitive to UV radiation and disappears in water. After Na-borohydride treatment the fluorescence disappears. It returns after renewed paraformaldehyde treatment.

Occasionally the fluorescent fibres run parallel to the mesogloea over some distance. The rest of the fibres is scattered through the whole of the ectodermis. They mostly follow the large intercellular spaces. Specific fluorescent material was never observed in the mesogloea or in the entodermis.

VII. HISTOCHEMISTRY

The presence of acetylcholinesterase and choline acetyltransferase and monoamine oxidase could not be demonstrated in *Hydra.* Gamma-aminobutyric acid and the semisuccinic acid dehydrogenase and gamma-aminobutyric transaminase/semisuccinic acid dehydrogenase complex could not be demonstrated either.

ATPase did occur. Identification problems in the cryostat sections caused uncertainty whether nerve cells react positively, but the impression was gained that they do react. Electron microscopically this could be confirmed.

VIII. STAINING OF NEUROSECRETORY MATERIAL

Neurosecretion could not be demonstrated with light microscopy, neither in paraffin sections nor in 1 μm sections, independent of the staining method used or previous oxidation.

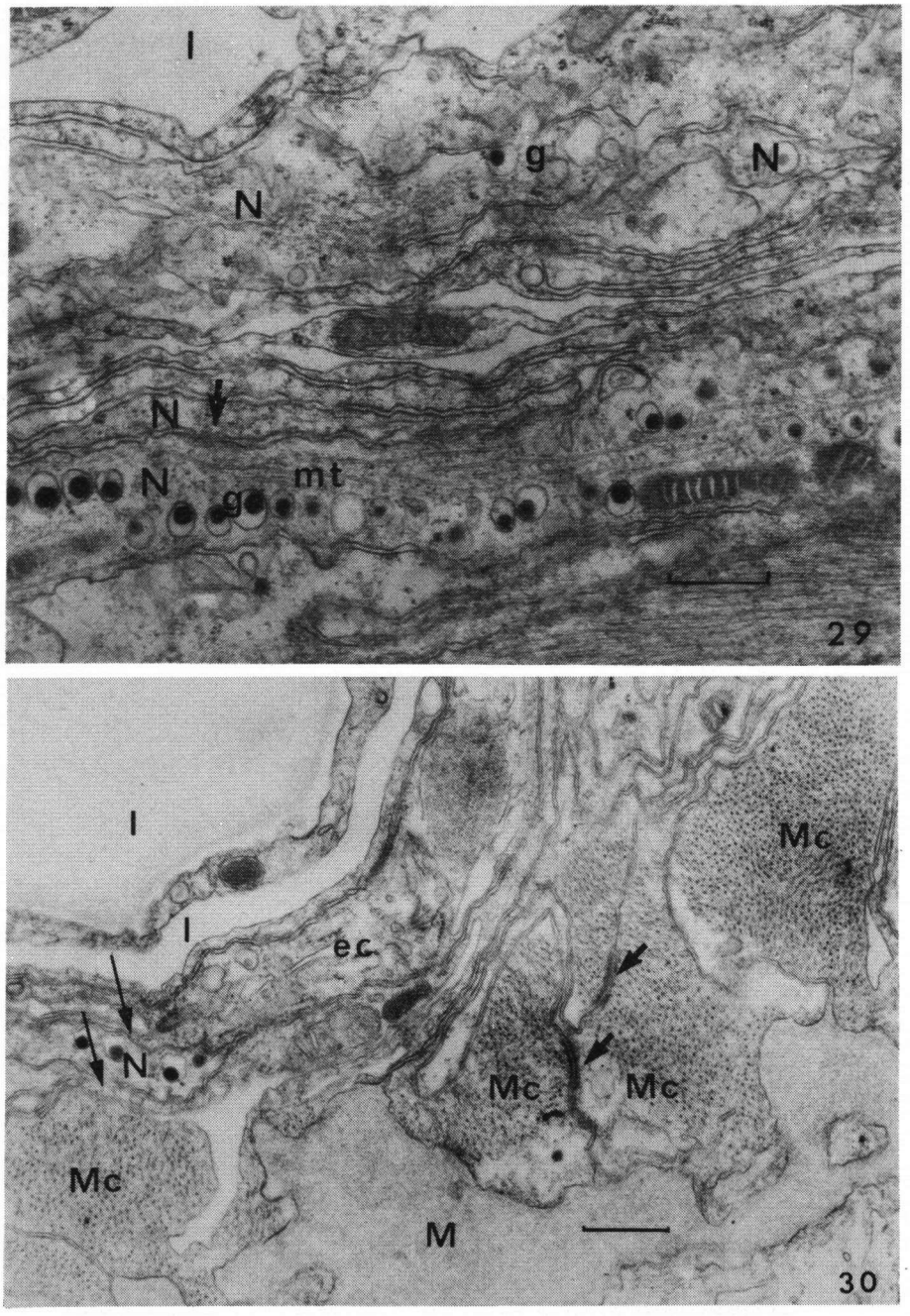


Fig. 29. *Hydra.* Some layers of neurites with granules (g) and microtubuli (mt) and mitochondria (the latter are almost completely absent in the neurites of the four species of sea anemones). A site of contact between two neurites is indicated by an arrow. Scale: 0.5 μm.

Fig. 30. *Hydra.* Mesogloea (M) with muscles (Mc). Between the muscles thickened membrane segments (big arrows on the right) (cf. fig. 19). Neurite (N) has contact with a myoepithelial cell (ec) and with the muscle (thin arrows on the left). However, the sites of contact do not consist of specialized membranes. Scale: 0.5 μm.

I = interstitial space; N = neurite.

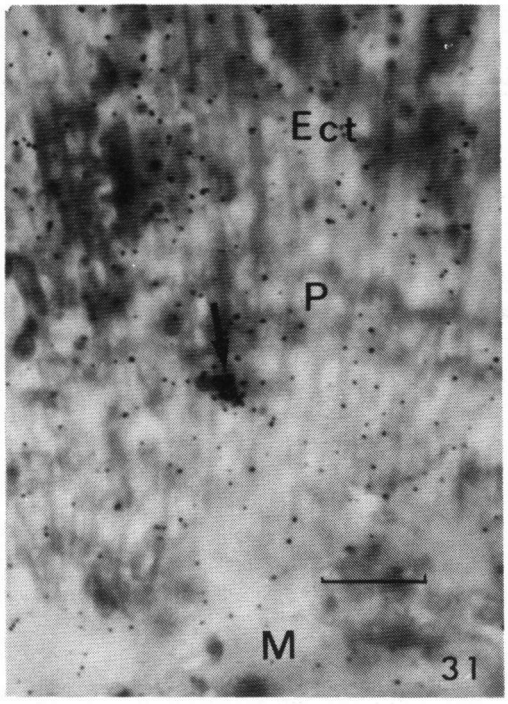


Fig. 31. *Tealia.* A ^3H noradrenalin labelled neuron in the plexus (P) is indicated by an arrow. Other, non-labelled neurons are present in the plexus as well. Transverse section, glutaraldehyde/OsO_4 fixation, 4 μm paraplast section. Scale: 10 μm.
Ect = ectodermis; M = mesogloea.

CHAPTER IV. DISCUSSION

I. IDENTIFICATION OF NERVE CELLS

The major problem in the investigation of a nervous system is, how to recognize the cells which form the nervous system. In other words, what is the definition of nerve cells, and how can this definition be used.

As already mentioned in the Introduction, Jha & Mackie (1967) and Davis et al. (1968) established criteria which a cell must meet in order to be called nerve cell. However, these authors do not indicate the exact number of criteria decisive for calling a certain cell a nerve cell, or which criteria are most important.

If these criteria (see Introduction) are used to recognize nerve cells, their inadequacy soon becomes apparent, especially in the sea anemones.

1. In our investigation we did not succeed in reproducibly staining the nervous system (neither perikarya nor neurites) specifically with (leuco-) methylene blue or silver impregnation methods.
2. Staining for neurosecretory material always gave a negative result, whatever method was used. However, these staining methods are difficult to apply. Since the staining solutions are tenable for a short time only, they must be prepared freshly repeatedly. The staining properties of these freshly prepared solutions may vary considerably as was shown with control experiments. Rat hypophyses, rat endocrine pancreas and the central nervous system of *Locusta* and *Periplaneta* were used as control material.

   The small dimensions of the cells and the granules within the cells make it difficult to see whether they react positively on the staining. Davis et al. (1968) acknowledged this. For electron microscopy, neurosecretory material has been defined as electron dense granules or dense core granules bigger than 1000 Å (Davis et al., 1968). According to this criterion a great number of cross sections of fibres in sea anemones and in *Hydra* may belong to the nervous system. However, epithelial and some gland cells in the tentacles of the sea anemones also contain granules which meet the above-mentioned criterion. Moreover, the perikarya would not belong to the nervous system according to this criterion, since they contain either very few granules or no granules at all.

   In nerves known to contain catecholamines or indolamines the dense core and opaque granules are often larger than 1000 Å. Therefore they meet the electron microscopic definition of neurosecretion. However, these granules cannot be demonstrated with light microscopic neurosecretory staining methods.
3. Both groups of authors often describe very short sensory cilia in all perikarya of the nervous system. Whether all these cells have a sensory function as well is not mentioned. Neither do they mention what contacts these cilia make. In the sea anemones studied by us, cilia were only observed in receptor cells at the periphery between the epithelial cells. These cilia protrude further than the microvilli of the epithelial cells, since they are longer than the cilia of the perikarya as described in the literature.

Cilia were never found in the perikarya of nerve cells lying in the plexus or between the muscles.

So this criterion could not be used either.

4. Since descriptions are not given, only the photographs in both articles can show what the authors mean by contacts and synapses. The criterion that nerve cells can be recognized from the contacts they make with other cells can be used only if the following definition, concluded from their photographs, is used. Two cross sections of cells lie next to each other. Their membranes at the site of contact run parallel, and in one or both cross sections granules are present.

   Both Jha & Mackie (1967) and Davis et al. (1968) make use of the words (site of) contact and synapse indiscriminately. However, a thickened membrane, which is characteristic of a synapse, is not visible in any of their photographs.

5. What remains is the "criterion": a general impression of nervelike features (Jha & Mackie, 1967) which covers a typical bi-, tri- or multipolar form.

   In our investigation the total impression of shape, size and position of the cells and their fibres appeared to be the only workable criterion.

Apparently it is not possible to define, in- or excludingly, on the basis of morphological criteria, the nerve cells or the nervous system in our sea anemones.

In order to make a statement after all, one is obliged to compare assumed nerve elements with a schematized ideal picture of a nerve cell. This picture is derived from vertebrate histology. Firstly, this ideal nerve cell is a cell with one long fibre and a number of short fibres, secondly, but far less well-known, this ideal nerve cell is a cell with a number of equally long fibres. This latter type is, to the best of our knowledge, present in sea anemones and this very type of nerve cell is unfamiliar. This makes a recognition as nerve cells, certainly of ill-defined, multipolar cells in sections, difficult. Since a general impression of hardly defined features such as form, size and situation had to be the basis of our judgment, it is possible that a number of elements which play a part in the conduction and integration of stimuli (having another form and therefore being not in agreement with the ideal picture of a nerve cell) were not recognized. Identification of these elements cannot be attained with the techniques used and their inherent definitions. To obtain this identification the use of other disciplines is necessary.

In our opinion only those cells or fibres containing dense core or opaque granules (see Results § II.2.2.1) can be identified with certainty as belonging to nervous tissue and can thus be distinguished from processes of epithelial cells. It is assumed that an identification of these granules on morphological grounds with granules such as occur in nervous systems of other animal species and are typical of these nervous systems, is justifiable.

If no granules are present in the cells or their fibres, which is often the case, the identification of a certain structure as nervous tissue remains difficult, if not impossible.

In *Hydra* the identification problems are essentially similar, with a few exceptions.

1. The silver and (leuco-)methylene blue methods were more workable for the demonstration of the nervous system in *Hydra* than in sea anemones.
2. In some, though not all, perikarya of *Hydra* a cilium was observed.

II. STRUCTURE OF THE NERVOUS SYSTEM

The nervous system in the four sea anemones consists of a plexus round the mesogloea connected by fibres with the periphery and with nerve cells between the muscles (fig. 32).

In *Hydra* there is no such concentration of nervous tissue forming a plexus. The neurites can be found everywhere between the ectodermal cells. This is visible especially by comparing fig. 27 with fig. 21 in which the fibres of the nerve cells have been made visible with the F.I.F. method.

II.1. *Plexus*

In the four sea anemones the plexus is about 10 neurites thick. This confirms the findings of Kawaguti (1964), Jha & Mackie (1967) and Peteya (1973a). Most of the perikarya belonging to the plexus are found between the plexus and the mesogloea.

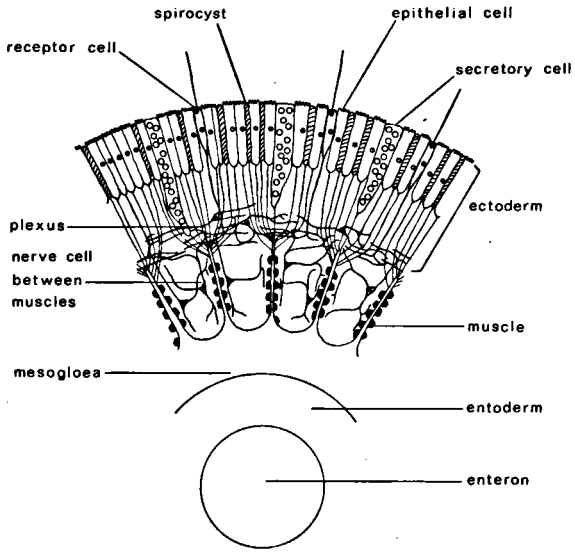


Fig. 32. Schematic representation of a transverse section of a tentacle of a sea anemone (i.e. *Anemonia, Cerianthus, Metridium*). In *Tealia* the situation is more complicated. In this animal the muscles are not attached to ridges, but to walls of spaces in the mesogloea.

Perikarya containing a cilium, which therefore may have a sensory function, were not observed in the plexuses of the four sea anemones investigated, whereas in *Hydra* cilia were found in a number of perikarya (cf. Westfall, 1973b; Davis & Bursztajn, 1973; Davis et al., 1968; Davis, 1969). In *Ceriantheopsis* (Peteya, 1973a) and in some Hydrozoa (Jha & Mackie, 1967) the presence of cilia in the perikarya has also been described. Whether and how these cells function as receptors is not mentioned by these authors.

Cross sections of the neurites are quite similar in size, 90% of the cross sections have a diameter smaller than 1 μm. In *Tealia* (figs. 9 & 12) and *Metridum* (fig. 10) they are mostly round to slightly oval; in *Cerianthus* (fig. 15) and *Anemonia* there are more cross sections with a pronounced elongated shape. This uniformity is caused by the very strong contraction due to fixation. Anesthesia with an isotonic magnesium chloride solution (according to Batham et al., 1960) gives no improvement. This is explicable since extension of the tentacles is caused by the pressure of the column muscles on the water skeleton. This pressure can only be maintained when neither the column nor the sphincter muscles are paralysed. If the pressure disappears, the tentacles contract by the mechanical properties of the mesogloea (see Chapman, 1953).

The number of fibres containing granules, differs from preparation to preparation. Since only cross sections containing granules can be identified as nervous tissue, the number of identifiable neurites varies. The photographs show (figs. 9 & 12) that it is impossible to identify neurites on the basis of localization and shape, since neurites without granules cannot be distinguished from processes of epithelial cells. If the epithelial cells are cut in such a way that no contractile material (fig. 9, arrow) appears in the cross section, the cross section is empty and has the same dimensions as a neurite.

Classification of the neurites based on size, as done by Peteya (1973a) with *Ceriantheopsis*, was not possible. The dimensions of the neurites identified with certainty varied too little (90% have a diameter of less than 1 μm). The neurites with a larger diameter (up to 4 or 5 μm) rapidly taper into a perikaryon (this is also the case with the two large neurites in fig. 15). This was checked as much as possible, using serial sections, or using 1 μm sections (the latter are easier to prepare, but more difficult to study).

The identification problems described and the erratic course of the neurites (caused by contractions of the tentacles due to fixation) rendered it quite impossible to ascertain what types of granules occur in one neurite, even if serial sections were used.

The impression was gained that one neurite may contain either dense core or opaque granules, either alone or together with empty vesicles. This seems to be confirmed by photographs in Stokes' publication (1974b). If the above is true then two types of neurites are present in the plexus, viz. neurites with dense core granules and neurites with opaque granules.

The dense core granules are found in the plexus and in the radial fibres exclusively. They were never observed in the entodermis, in the mesogloea or in nervous tissue between the muscles. The plexus and the radial fibres are the only parts showing a F.I.F. having the characteristics of a catecholamine (most probably noradrenalin). Just like dense core granules, the F.I.F. does not occur in the entodermis, in the mesogloea and between the muscles (see Discussion § II.3). Because of these two facts, we assume that the dense core granules contain a catecholamine and therefore the neurites in which these granules are observed, are catecholaminergic. This catecholaminergic system serves

the transport of the stimuli in the plexus and the transport of the stimuli of the receptor cells at the periphery of the tentacles to the plexus. For reasons explained in § II.3 of the present chapter, we assume that the neurites with opaque granules are purinergic. The function of this purinergic nervous system is discussed in the same section.

It could not be established whether the granules in all neurites belonging to one perikaryon, are of the same type or that some neurites contain dense core and other neurites opaque granules. In our opinion the former is the most probable.

No indications were found that different classes of perikarya could be distinguished. Light microscopically as well as electron microscopically their shape varies but little (the perikarya are always triangular, figs. 11, 14). They all have the same ultrastructure, i.e. little but strongly dilated rough endoplasmic reticulum, a Golgi complex and few mitochondria. The nucleus is relatively large (the cell is 5—7 μm, the nucleus 4—5 μm). Granules observed in the cells are either dense core or opaque. Their number is too small, however, to justify a classification on this basis.

A histochemical division was not possible either. Nerve cells did not react to most of the reactions performed. Only the reaction for adenosine triphosphatase was positive, a great number of nerve cells showing a positive reaction (fig. 22, arrows). The ratio, number of non-reacting cells to number of positively reacting cells, could not be established, especially since interstitial cells show a negative reaction and are difficult to distinguish from nerve cells.

Contacts of the neurites of the plexus with each other and with other cells.

Several authors (Westfall, 1970 a & b, 1973 a & b; Westfall et al., 1971; Hernandez-Nicaise, 1973c; Peteya, 1973a; Davis, 1974; Stokes, 1974b) described the presence of synapses in the nervous system of coelenterates.

Synapses are structures defined by the aforementioned authors as follows:

1. The membranes of the nerve cell and the cell on which the stimulus is transferred run parallel over a certain distance, the distance between both membranes being 150—200 Å.
2. The membrane of the cell on which the stimulus is transferred is thickened.
3. The nerve cell contains granules or vesicles.

The sites of contact of the nerve cells with other nerve cells in the plexus and with ectodermal cells observed in the four sea anemones and in *Hydra* answer to the criteria mentioned under 1 and 3. One neurite can make contact with various other cells in one section. Thickened membranes, however, were observed neither in the four sea anemones (figs. 10, 12 & 13, arrows), nor in *Hydra* (figs. 28—30, arrows). This discrepancy is difficult to explain. At first it was ascribed to a difference in fixation and the buffer solutions used. Especially the buffer solution influences the reaction of the membranes with osmium tetroxide and uranyl acetate. When using the same fixations as described in the literature it appeared, however, that the fixation prescribed by Westfall (1970a) crystallized at 4°C and produced useless material. Even when a more recent fixation described by Westfall (1973b) was used, again thickened membranes could not be observed. Thickened membranes could not be observed either when other types of fixations were used (glutaraldehyde in Hepes buffer, osmium tetroxide in veronal acetate buffer).

The fixations of Westfall (1970a & 1973b) were chosen because the thickened membranes were most obvious in these publications.

Not even the E.P.T.A. method produced thickened membranes in spite of all variations in incubation time and in water content (according to the authors the latter is very critical).

As already stated, the neurites form contacts with each other and with the ectodermal cells in the plexus but no sites of contact were ever observed on the perikarya.

Nerve-nerve contacts.

In the nerve-nerve contacts (figs. 10 & 13, arrows) granules are present in only one of the two neurites but a few sections further the other neurite may be the one that contains the granules. The presence of granules in both neurites was rarely observed in one section.

The contacts described above may be present between two neurites both containing either dense core or opaque granules, or between a neurite with dense core and a neurite with opaque granules. The sites of contact are polarized because the granules lie in only one of the two

neurites. The overall exchange between two neurites is not polarized.

The above-mentioned data together with the fact that the neurites are not oriented (the form of the neurite is independent of the orientation of the section) make it probable that the plexus is not polarized. If a polarization does indeed exist then it is physiological and not morphological (according to Pantin, 1935).

Nerve-ectodermal cell contacts (fig. 12, arrows). Only in the plexus contacts are present between identifiable or probable neurons and the necks of ectodermal cells (i.e., with gland cells, nematocytes and spirocytes). Granules were rarely observed. If present they are of the dense core type. The small number of granules may be a preparation or fixation artefact.

Innervation of the ectodermal cells by the fluorescent fibres as Dahl et al. (1963) assumed, is unlikely. According to our findings the fluorescent fibres always end in a thickening at the periphery of the tentacle. They never end within the ectodermis. Besides, their number is too small (3 to 4 fibres in a transversal 10 µm section) to be important for innervation. Furthermore, they are distributed at random whereas, if they would innervate the gland cells, spirocytes or nematocytes, a regular pattern of distribution is to be expected. Picken & Skaer (1966) found indications of a nervous regulation of nettle cell-discharge. In a swimming sea anemone *(Stomphia)* the excitation level of the nettle cells is higher than when the animal is attached. Pantin (1942), who worked with *Anemonia sulcata*, found that stimulation of the nervous system did not give a higher percentage of discharged nettle cells.

If an innervation of the gland and nettle cells is present, then it is inhibiting and takes place in the plexus, although it is doubtful whether such a general conclusion may be drawn from these two data regarding animals with such different behaviour patterns.

Hydra differs from sea anemones in that the nervous system is not concentrated to form a plexus. The perikarya are generally situated on the mesogloea. The neurites often run parallel with the large interstitial spaces, as is also demonstrated in the F.I.F. preparations. The granules are large and irregular in shape. The core is small and often has such an excentric position that one granule in successive sections may present itself as an opaque, dense core granule or empty vesicle. Perhaps there is only one type of granule in *Hydra* (according to Westfall, 1973b), though it seems unlikely that only one substance is involved in the conduction, considering the results obtained with the sea anemones.

Just as in the four sea anemones, synapses were not observed in *Hydra*. The contacts have the same structure as the contacts in the sea anemones.

This is again in contradiction to the observations of Davis et al. (1968), Davis & Bursztajn (1973), Westfall (1970 a & b, 1973a) and Westfall et al. (1971).

II.2. *Receptor cells and the radial fibres running from the periphery of the tentacle to the plexus*

The radial fibres are clearly visible in F.I.F. preparations (fig. 21). Their small number (4 to 5 in a transversal 10 µm section, this is ⅓ of the number given by Dahl et al., 1963) and the identification problems already described make it understandable that these fibres could not be identified between the ectodermal cells. The thickenings at the end of the fibres most probably indicate the presence of receptor cells. The latter were found a few times with the electron microscope (fig. 16). They are of a common mechanoreceptor type (Stokes, 1974b). The cilium which stands in an invagination of the cell protrudes far outside the microvilli of the gland cells. In a few receptor cells and in the neurites belonging to them, dense core granules were found. The receptor cell is connected with the surrounding ectodermal cells by means of very well developed desmosomes. Evidence for other receptor cells has not been found.

The periphery of the tentacle is the only place where receptor cells were found in the four sea anemones.

In *Hydra* the receptor cells are of the same structure, but the cell body is more variable in shape, often triangular to almost circular, in contrast to the long drawn shape in the sea anemones. Moreover, the receptors can be observed in the outer layer of the tentacle as well as everywhere in the ectodermis.

Consequently, in *Hydra* there are no radial fibres present, but in the ectodermis there are only fibres with an irregular course, as can also be observed with the F.I.F. method (fig. 27) (cf. Discussion § II.1).

II.3. *The nervous system between the muscles*

The muscles of *Tealia, Anemonia, Cerianthus, Metridium* and *Hydra* are smooth. Striated muscles, as described by Schmid (1974) in the Anthomedusa *Podocoryne carnea* M. Sars, 1846, were not present. The variations in staining of the muscle cells as seen in the 1 µm epon sections were caused by a different state of contraction, as could be shown in the electron microscopic investigation.

Perikarya and neurites lie between the muscles. They do not differ in structure from the perikarya and neurites which were found in the plexus. In the four sea anemones studied, no receptor cells are present between the muscles (in contrast to the observation of Peteya, 1973b, in *Ceriantheopsis*), whereas in *Hydra* they are present (cf. Westfall, 1973b; Davis et al., 1968).

In contrast to those in the plexus, the neurites between the muscles contain opaque granules exclusively, sometimes together with empty vesicles. Dense core granules were not present in the neurites between the muscles. Since the same identification problems apply here as in the plexus, it was not possible to ascertain the correct amount of nervous tissue.

The neurites between the muscle cells rarely form contacts with each other or with the muscle cells. The distance between the neurites and the muscle cells is seldom smaller than 250 Å, even at the sites at which the membranes of both cells run parallel. Therefore, there is not a genuine contact as is the case between the neurites in the plexus. The muscle cells never show thickened membrane parts at the sites of contact, comparable to postsynaptic membranes. The finding that there are few contacts and no thickened postsynaptic membranes corresponds with findings by Peteya (1973a) who studied *Ceriantheopsis* and Stokes (1974b) who studied *Hydractinea* Van Beneden, 1841. It is not in accordance with the observations of Westfall (1970 a & b, 1973a) and Westfall et al. (1971) in *Hydra* and *Metridium* and in various Ctenophora studied by Hernandez-Nicaise (1973c).

The number of contacts on one muscle cell could not be established. Neither could be determined whether one single neurite or a number of neurites make contact with one muscle cell. This was caused by the following factors:

1. Even after fixation and embedding in epon the mesogloea differs strongly in mechanical characteristics from the epithelia of the ectodermis and entodermis. Therefore long series of thin sections could not be made.
2. The muscle fibres are extremely long (5—7 mm, i.e. ⅓ to ½ of the tentacle length).
3. The erratic course of the neurites due to the strong contractions of the material during fixation causes identification problems.

As in the four sea anemones, synapses between nerves and muscles could not be observed in *Hydra*. Contacts between neurites and muscle cells are rare. This is in contradiction to Westfall (1970 a & b, 1973 a & b) and Westfall et al. (1971). It was impossible to determine the type of granules in the neurites which innervate the muscles (figs. 29, 30). In fact, there is no spacial separation between the neurites belonging to the muscles and the neurites in the ectodermis. Furthermore, the granules, owing to their irregular shape and often small excentric core, are not always easily identified.

In *Anemonia, Metridium, Cerianthus* and *Tealia* only opaque granules were observed in the neurites between the muscles (figs. 18 & 19). Granules of the same type are also present in neurites localized in the plexus. It is therefore possible that the nerve cells containing these granules and the substance in these granules are involved in transport of the stimulus from the plexus to, and for innervation of, the muscles.

Innervation of the muscles in the sea anemones and in *Hydra* strongly resembles the situation in smooth muscles in vertebrates such as described by Thaemert (1966), Merrillees (1968), Burnstock (1970, 1972) and Burnstock & Iwayama (1971). On the basis of physiological experiments, Burnstock assumes that beside the well-known cholinergic and aminergic nervous systems also a third, a purinergic nervous system exists which innervates the smooth muscles. In this purinergic system the transmitter is a purine derivative (ATP?). The neurites belonging to this purinergic system are supposed to be characterized morphologically and histochemically by opaque granules (1400 Å diameter) and a strong positive

ATPase reaction of perikarya and neurites. In the four sea anemones a number of perikarya and neurites in the plexus and all the perikarya and neurites between the muscles show these characteristics, so that the presence of a purinergic system, conducting the stimulus from the plexus to the muscles and innervating them, seems an acceptable hypothesis.

ATP is one of the few substances causing contraction in sea anemones (Ross, 1960 a & b). Hence a purinergic innervation of the muscles is possible. However, in this case it is supposed to have an excitatory function and not an inhibitory function as in vertebrates.

It could not be ascertained whether the innervation of the muscles always goes via a nerve cell between the muscles, or whether there is also a direct innervation from the plexus.

The fact that ATPase was also localized in the intercellular space between the neurites (fig. 23) is not necessarily an argument supporting the existence of a purinergic transmitter system. It is possible that an ATPase is responsible for the transmitter release, or its regulation (Gilbert et al., 1975).

The enzyme demonstrated by us has the same characteristics as the Na-ATPase demonstrated by Gilbert et al. (1975) in rat synaptosomes (i.e. Ouabaine insensitive, Mg^{2+} dependent) (see Discussion § III.4).

It is noteworthy that the latter ATPase is acetylcholine insensitive (the other ATPase described by the author, the Na-K-ATPase is Ouabaine sensitive), and that acetylcholine was not present in the sea anemones (Discussion § III.2).

Whether a purinergic system is also present in *Hydra* could not be determined. The type of granule could not be established and because of the identification problems arising from the use of frozen sections it was impossible to ascertain whether the nerve cells react positively to the ATPase reaction, although they probably do react positively.

III. HISTOCHEMICAL AND CYTOCHEMICAL RESULTS, CULTURE RESULTS

III.1. *Biogenic amines*

With the application of the F.I.F. method a yellow to yellowish green fluorescence develops.

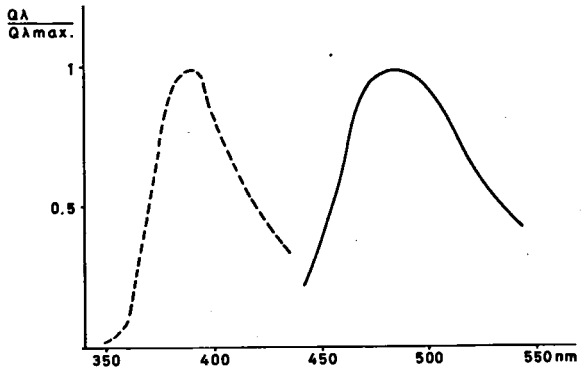


Fig. 33. Excitation (- - -) and emission (—) spectra of the F.I.F. in the tentacle of *Tealia felina*.

A blue colour was never observed. From the tests (Results § III.1, Corrodi et al., 1964; Häkanson et al., 1971) it appeared that the developed fluorescence was due to the presence of a biogenic amine. Using the narrow band excitation (Ploem, 1971), the yellowish fluorescence would indicate the presence of an indolamine or a peptide with an NH_2-terminal indolamine.

However, analysis of the emission spectrum showed a maximum at 485 nm, which shows that the F.I.F. developed from a catecholamine. The possibility of a peptide with an NH_2-terminal catecholamine is unlikely. Staining methods for neurosecretion always gave negative results and these methods are most probably specific to the protein to which the real transmitter is bound (Stoeckel et al., 1972). So it should be stressed again here that only a colour identification of F.I.F. can give erroneous results.

From culture experiments it appeared that no significant synthesis of adrenalin, noradrenalin, octopamine, dopamine, and dopa takes place in a culture with tyramine as precursor. However, if tyrosine is used as a precursor, noradrenalin, dopa, dopamine and octopamine are present in the tissue in significant amounts.

The presence of tyramine and octopamine (5.1% and 3.6% of the total uptake, respectively) is ascribed to an aspecific uptake from the medium, which is known to be contaminated with these substances (both in a concentration of about 1% of the amount of radioactivity added to the medium).

After incubation for 1 hour or after a culture period of 24 hours in a medium containing ^3H D,L, noradrenalin, a specific uptake in some perikarya of the plexus, but not in all, could be

demonstrated (fig. 31). No uptake was present in the perikarya between the muscles.

From the results discussed above it can be assumed that the transmitter in the plexus, which develops into a fluorophore after application of the F.I.F. method, is a catecholamine and most probably noradrenalin (for this is present in the highest concentration after culture with ^{3}H D,L, noradrenalin, see Results § IV.2).

The metabolic pathway runs via dopa and dopamine, and not via tyramine and/or octopamine, for culture with tyramine gives no metabolites.

Noradrenalin being the transmitter is in accordance with the assumption of Dahl et al. (1963). The small quantities of dopa, dopamine and noradrenalin, as claimed by Carlyle (1969b) to be present in *Actinia equina* Linnaeus, 1767, are confirmed by our results. Mention should be made of the fact that adrenalin has a distinct effect on sphincter and column preparations (Ross, 1960 a & b). However, in sea anemones adrenalin has never been demonstrated. Adrenalin could not be demonstrated in our culture experiments either. Moreover, adrenalin develops into a fluorophore after a prolonged period under quite rigorous conditions and a high relative humidity (2—3 hours, 80°C, 70%), (cf. Dahl et al., 1963). The fluorescence in our preparations develops already after ½ hour, at 60°C and a relative humidity of 40—50%. According to Ross (1960 a & b) noradrenalin has hardly any effect on the contraction of the muscles of sphincter or column preparations. This fits in with the assumed function of noradrenalin in the scheme of the nervous system as is discussed in § V of the present chapter, i.e. noradrenalin functions only in the plexus as a transmitter, whereas the innervation of the muscles is supposed to be purinergic.

After culture of pieces of tissue for 24 hours in a medium containing labelled tryptophane, a small but just significant enough amount of 5-OHtryptophane was demonstrable. Neither tryptamine, nor 5-OHtryptamine could be demonstrated.

In the spectra of the F.I.F., in spite of their yellow to yellowish green colour, no maximum at 520 nm (specific to indolamines) was present. Incubation or culture with ^{3}H 5-OHtryptophane or ^{3}H 5-OHtryptamine did not lead to a specific accumulation anywhere in the nervous system as was demonstrated with autoradiography.

Therefore we do not assume an indolamine to be present in the sea anemones. The synthesis of 5-OHtryptophane may be due to aspecific hydroxylation. The absence of an indolamine in sea anemones is confirmed by Carlyle (1969a).

Apart from the F.I.F. method no catecholamine or indolamine could be demonstrated light microscopically as well as electron microscopically. The diazonium method (Pearse, 1972) and the chromaffine reactions (Hillarp & Hökfelt in Pearse, 1972) for a light microscopic demonstration of indolamines and catecholamines, respectively, always gave negative results.

Electron microscopic identification for the demonstration of these substances also failed.

To exclude the possibility of 5-OHtryptophane being present after all in the sea anemones, test tube experiments were performed according to Etcheverry & Zieher (1968) and Cannata et al. (1968) in order to determine whether it is possible to demonstrate this substance with an argentaffine reaction. Unfortunately, this appeared to be impossible: 5-hydroxytryptophane never gave a precipitation, independent of the presence of creatinine, whereas under the same circumstances (see Methods § II.3.1, table I) 5-hydroxytryptamine did give a precipitation. Since 5-hydroxytryptamine is always accompanied by creatinine and the latter compound may be responsible for the precipitation found, creatinine alone was also tested, with negative result.

Of the many enzymes inactivating or degrading biogenic amines, monoamine oxidases are the only enzymes that can be demonstrated histochemically (Kopin, 1972). They could neither be demonstrated in the sea anemones nor in *Hydra* (in contradiction to Lentz & Barnett, 1961).

Therefore, either other enzymes play a part in the inactivation, or diffusion is the main factor causing the elimination of the transmitter from the receptor sites. The latter is a possibility in view of the high concentration of a.o. adrenalin necessary to obtain an effect in the sphincter or column preparations (Ross, 1960 a & b).

Reserpine which is known to inhibit the storage of catecholamines (Goodman & Gilman, 1975) does not affect the fluorescence. Neither the intensity nor the number of fluorescent fibres is changed. The behaviour of the animals is not affected either. Perhaps this indicates that an uptake mechanism as present in vertebrates is

absent in sea anemones.

Feeding does not affect the fluorescence, whereas the shape of the tentacle and the speed of reaction did change, which may indicate that the nervous system is affected. Possibly it is the purinergic nervous system which is affected by the nutritional state of the animal.

III.2. *Acetylcholinesterase and choline acetyltransferase*

An indication of acetylcholine being a transmitter in coelenterates, was found by Lentz & Barnett (1961). They demonstrated acetylcholinesterase in nerve cells of *Hydra.* In order to investigate this possibility closer, attempts were made to demonstrate acetylcholinesterase in the four sea anemones and in *Hydra,* but in our experiments the enzyme could not be demonstrated. The reaction for the enzyme necessary for the synthesis of acetylcholine, the choline acetyltransferase, was negative in *Hydra* as well as in the sea anemones.

These results together with the fact that acetylcholine cannot be demonstrated in sea anemones (Carlyle, 1969a) and the fact that acetylcholine has no effect on sphincter or column preparations (Ross, 1960 a & b) make it highly improbable that acetylcholine plays a role as a transmitter in coelenterates.

III.3. *Glutamate, gamma-aminobutyric acid and gamma-aminobutyric acid metabolism*

Carlyle (1974) showed that after stimulation and successive contraction of her preparations, a great quantity of glutamate was released. She demonstrated that glutamate has an inhibiting effect on either the muscles or the neurons, but she could not determine which of the two. However, we consider the existence of an inhibiting neuron which might be excited by released glutamate, unlikely. Between the muscles there is only one type of neuron which is probably purinergic, and this neuron is supposed to have an excitatory function (cf. Discussion § II.3).

If glutamate has an inhibiting effect on neurons, the most likely supposition is that it acts on the neurons between the muscle cells. The nerve cells of the plexus are too far removed from the muscles, and the environment between the muscles, especially in *Tealia,* is too well isolated.

The action of glutamate on the neurons between the muscle cells can work two ways: a direct inhibition of transmitter (a purine derivate) release (the most obvious); an inhibition of the plexus via the nerve cells between the muscles. In the latter case the glutamate must induce a release of purine derivate from the nerve cells between the muscles in the plexus. This would implicate that the purine derivate acts excitatory on the muscles and inhibitory on the plexus.

Since there are no receptor cells between the muscles in sea anemones, a neural feed-back is absent. Possibly the release of glutamate acts as an inhibitory feed-back on the muscles in the sea anemones (and *Hydra?*).

Even more difficult to interpret is the 3% gamma-aminobutyric acid synthesis from glutamate. According to Ross (1960 a & b) gamma-aminobutyric acid does not affect sphincter and column preparations. Nor is gamma-aminobutyric acid formed after stimulation of sphincter preparations (Carlyle, 1974).

Gamma-aminobutyric acid synthesis takes place in glia cells (Csillik et al., 1971), but glia cells were observed neither in sea anemones nor in *Hydra.* If pieces of tentacle were incubated in a solution containing tritiated gamma-aminobutyric acid, selective uptake of gamma-aminobutyric acid was not found in the autoradiograms. This is in contrast to the observation of Sotelo et al. (1972) and Evans (1974) that gamma-aminobutyric acid neurons selectively take up gamma-aminobutyric acid. Histochemically, neither gamma-aminobutyric acid nor the mechanism by which it is degraded, was demonstrable; neither the gamma-aminobutyric acid transaminase — semisuccinic acid dehydrogenase complex nor semisuccinic acid dehydrogenase were demonstrable with histochemical methods.

Conclusively, we are of the opinion that gamma-aminobutyric acid does not play a role as transmitter in the coelenterates investigated.

III.4. *Adenosine triphosphatase (figs. 22—26)*

The possible implications of a positive adenosine triphosphatase reaction are already discussed in § II.3 of the present chapter.

The adenosine triphosphatase appeared to be localized in the Golgi complex. Furthermore it was present in vesicles in both the perikarya and the neurites, of the plexus as well as the nervous

system between the muscles. The technical problems accompanying the preparation of the tissue chopper sections and the differences in characteristics of the mesogloea make it very difficult to preserve the ultrastructure. Therefore, a false localization caused by cell damage artefacts cannot be excluded.

The enzyme is insensitive to Ouabaine, DNP and KCN, but it is sensitive to magnesium ions and probably also to lead ions.

Together with the localization described above, the latter indicates a cytoplasmic adenosine triphosphatase (Pearse, 1968; Geyer, 1973). There is no difference in properties between the enzymes of sea anemones and of *Hydra*.

Light microscopic identification problems were so great in *Hydra* that it could not even be stated with certainty that the nerve cells react positively. Electron microscopically this could be established.

IV. NON-NEURAL CONDUCTION

Well developed specialized membrane regions were found at sites of contact between muscle cells in the sea anemones and in *Hydra*. At these sites the cell membranes run parallel over some distance and are thickened on both sides. The distance between the two membranes is about 180 Å. For convenience' sake we call them desmosomes. We did not succeed in obtaining an optimal fixation and therefore it was impossible to investigate the ultrastructure of these contact sites (especially the intercellular space) any further. Corresponding structures were observed between the smooth muscles in vertebrates, where they are supposed to play an essential role in myoid conduction (Burnstock, 1970; Burnstock & Iwayama, 1971).

Therefore it is plausible to assume a myoid conduction in the coelenterates investigated.

On the basis of physiological experiments, Mackie & Passano (1968), Spencer (1971) and Stokes (1974a) concluded that a myoid conduction is present in the species they studied. Incorrect in the work of Mackie & Passano (1968) is that they assume that nervous tissue is absent, an assumption which is based on an article by Jha & Mackie (1967). In the latter article the authors in fact emphasize the difficulty of identification of nervous tissue, which rendered the argument of Mackie & Passano (1968) in favour of myoid conduction (i.e. the absence of neurons) less valid.

Similar desmosomes as found between the muscles were also found between the gland cells, nematocytes, spirocytes and receptor cells in the outer layer of the tentacle. It is possible that these desmosomes may have a function in conduction, apart from a barring function.

If it is assumed that a well developed conduction system through muscle cells and epithelial cells (according to Mackie, 1970, a separation between both is impossible in view of the myo-epithelial character of the cells) does exist in coelenterates, then this conductive system could be an explanation for the second "nervous system" with slow conduction, as described by Bullock & Horridge (1965), Josephson (1961, 1966), McFarlane (1969, 1973) and McFarlane & Lawn (1972) (see Introduction). Morphologically no indication was found in the present study that a second nervous system exists. According to the literature, evidence for the existence of a second nervous system has never been found, except by Leghissa (1965). In *Actinia equina* Leghissa (1965) described an outer and an inner plexus above the mesogloea ridges with connections to the muscles in the ectodermis and in the mesogloea, respectively. We could not find any evidence for a second nervous system, not even in *Actinia equina* (investigated summarily in order to verify Leghissa's results). According to our experience with silver impregnations it often happened that a confusing precipitation developed both on the mesogloea and on the attachment of the epithelial cells and muscles in the mesogloea (cf. Perkins et al., 1971).

CHAPTER V. CONCLUSION

We may conclude that the plexus in the sea anemones investigated is composed of two types of neurons. One type is catecholaminergic, noradrenalin most probably being the transmitter, possibly stored in dense core granules. The other type is purinergic, containing ATP (?), perhaps stored in opaque granules.

The nervous tissue between the muscles only contains the latter type, i.e. purinergic neurons.

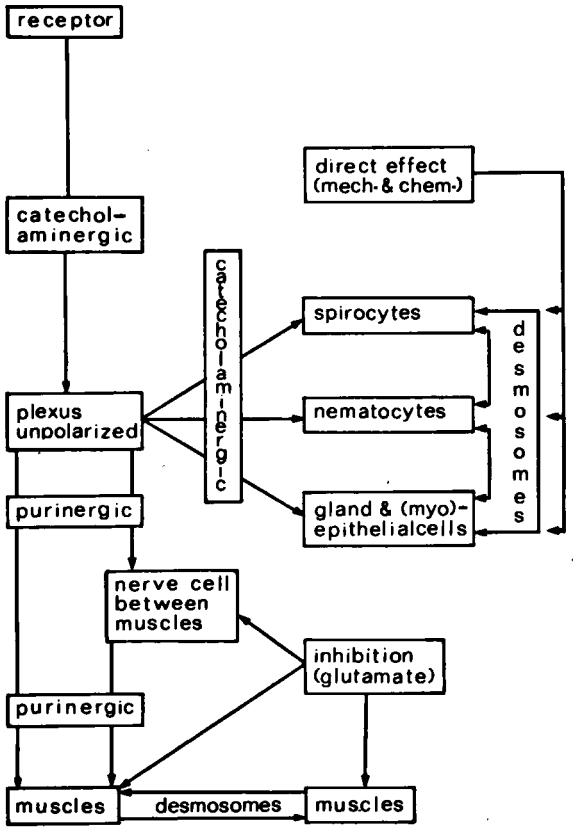


Fig. 34. Schematic representation of the supposed interrelations between nervous and other conduction systems in the tentacles of a coelenterate.

The experiments described in the present study together with results obtained by other investigators, may lead to the following hypothetical outline of the function of the nervous system in sea anemones (see diagram, fig. 34).

A catecholaminergic system provides the conduction of the stimuli from the receptor cells or other peripheral cells to the plexus. The stimulus is spread in all directions in the plexus by the catecholaminergic system. Possibly an inhibition of gland cells, nematocytes and spirocytes takes place via the plexus.

Stimuli from the plexus are transmitted to the muscles via a purinergic system. Glutamate may have a direct inhibitory effect, either on the muscle cells, or on the neurons between the muscle cells. This inhibition is discussed in the previous chapter, § III.3.

Apart from this neural conductive system we assume the existence of a myoid-epithelial conduction. This myoid-epithelial conduction provides a further conduction of a stimulus leading to contraction of the muscles. Moreover, a comparable system enables the ectodermal cells to react to influences from the surroundings, independent of the nervous system.

However, it should be borne in mind that besides the neural and myoid-epithelial conductive systems other conductive and integrative systems may exist, but they could not be recognized on account of the limitations inherent to the techniques used.

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SAMENVATTING

Lichtmicroscopisch zowel als electronenmicroscopisch werd het zenuwstelsel van de zeeanemonen *Tealia felina, Anemonia sulcata, Metridium senile* en *Cerianthus membranaceus* onderzocht.

Wegens de geringe afmetingen van de zenuwcellen (6—7 μm) en van de neurieten (diameter < 1 μm) kon nauwelijks informatie verkregen worden met de gebruikelijke histologische technieken.

Electronenmicroscopisch onderzoek toonde aan dat het zenuwstelsel in drie delen verdeeld kan worden: de plexus rond de mesogloea, een zenuwstelsel tussen de spieren (duidelijk verbonden met de plexus) en sensorische cellen in de tentakelrand, die door een uitloper verbonden zijn met de plexus. De uitlopers van deze cellen liggen radiair gerangschikt in de tentakel ectodermis. Deze cellen zijn de enige sensorische cellen die in de tentakels van de zeeanemonen aangetoond kunnen worden.

In deze radiaire neurieten en in een aantal neurieten in de plexus waren dense core granula aanwezig. In het zenuwstelsel tussen de spieren en in een aantal neurieten in de plexus liggen opake granula. Neurieten waarin zowel dense core als opake granula voorkomen zijn nooit waargenomen. Alleen in de radiaire neurieten en in de plexus ontstaat een gele F.I.F.. Analyse van het emissiespectrum toonde aan dat de fluorophoor ontstaan was uit een catecholamine (waarschijnlijk noradrenaline). De dense core granula bevatten dus een catecholamine.

Op grond van een morfologische overeenkomst is het aannemelijk dat de neurieten met opake granula purinerg zijn (Burnstock, 1972). De innervatie van de spieren van de zeeanemonen lijkt n.l. sterk op de innervatie van glad spierweefsel bij vertebraten.

Synapsen, zoals beschreven door o.a. Westfall (1973a) konden niet worden aangetoond. Desmosoomachtige structuren waren wel aanwezig tussen epitheel en spiercellen, zodat een niet-neurogene (i.e. myoepitheliale geleiding) aannemelijk is.

Deze myoepitheliale geleiding is mogelijk een verklaring voor het „tweede zenuwstelsel" gepostuleerd door Bullock & Horridge (1965), waarvan wordt aangenomen dat dit een langzaam geleidend systeem is. Een morfologische indicatie voor dit systeem is nooit gevonden.

De twee bovengenoemde stoffen (de aanwezigheid van GABA en actylcholine kon niet aangetoond worden), een catecholamine en een purine zijn beide excitatorisch. De rol van glutamaat, mogelijk een inhibitor, werd besproken. Glutamaat is misschien een inhibitor, daar het wordt vrijgemaakt door de contraherende spiercellen en het de contractie via een onbekend mechanisme remt.

Hydra werd eveneens onderzocht en de resultaten werden vergeleken met de bestaande literatuur. Alleen het voorkomen van synapsen was afwijkend. Deze structuren konden niet worden aangetoond. Met behulp van de F.I.F. methode kon een catecholamine worden aangetoond. Een purinerge spierinnervatie is gezien onze ervaring met de zeeanemonen mogelijk.