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# FLUORESCENCE LOCALIZATION OF MONOAMINES IN CRAB NEUROSECRETORY STRUCTURES

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## INTRODUCTION

There is a growing body of evidence supporting the hypothesis that neurosecretory cells control the release of their own products by the same general mechanisms which operate in other neurones. They integrate inhibitory and excitatory synaptic input, propagate action potentials along their axons, and they release their products as a result of depolarization of their terminals by a calcium-dependent mechanism (review: Bern & Yagi, 1965; and, for example, Douglas & Paisner, 1964*a*, *b*; Haller *et al.* 1965; Dicker, 1966; Fridberg *et al.* 1966; Cooke, 1964, 1967; Kater, 1968*a*; Dyball & Koizumi, 1969). The uncertainty preventing acceptance of this hypothesis results from the presence in a number of neurohaemal structures of other nervous and non-nervous elements. These, on morphological grounds, may be considered for a role in the control of neurosecretory release (e.g. reviews: Bern, 1962; and Knowles, 1967; also, Enemar & Falck, 1965; Odake, 1967; Björklund, 1968; Kobayashi, Hirano & Oota, 1965; Knowles & Vollrath, 1966; Scharrer, 1963; Bern, Yagi & Nishioka, 1965; Normann, 1965). Here and in the following paper (Berlind, Cooke & Goldstone, 1970) we take up this problem in crab pericardial organs (Alexandrowicz, 1953). This paper describes monoamine-containing axons and terminals distributed in parallel with neurosecretory axons and terminals (Maynard, 1961*b*; Maynard & Maynard, 1963; Cooke, 1964). The following paper presents experiments designed to test the involvement of monoamines in release of heart-excitatory material from the pericardial organs. A third paper (Berlind & Cooke, 1970) adds new evidence that the heart-excitatory material released is peptide in nature and is not accompanied by detectable protein or enough monoamine to affect the heart. A brief summary of this work has appeared (Cooke, Berlind & Goldstone, 1970).

Alexandrowicz (1953), on morphological grounds, proposed a neurohaemal function for pericardial organs, and Alexandrowicz & Carlisle (1953) found that pericardial organ extracts had potent heart-excitatory effects. Cooke (1964) found that heart-excitatory material is released from isolated pericardial organs when stimulation results in a slowly conducted component of the compound action potential as recorded from the trunks of the plexus. There are cells in the ventral (thoracic) ganglion of crabs having the morphological features of neurosecretory cells (Matsumoto, 1958). These contribute axons and terminals to the pericardial organs (Maynard, 1961*a*, *b*) and are

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presumably responsible for the slowly conducted component of the action potential and for production of heart-excitatory material (Cooke, 1964; Berlind & Cooke, 1968). It seems probable that hormones released from the pericardial organs serve other as yet undetermined purposes (see, for example, Parrot, 1941).

All neurosecretory hormones that have been characterized are polypeptides (e.g. Du Vigneaud, 1956; Edman, Fänge & Östlund, 1958; Josefsson & Kleinholz, 1964; Davey, 1961; Brown, 1965; Kater, 1968*b*). This includes the material responsible for heart effects in crude extracts of pericardial organs (Maynard & Welsh, 1959) and in fluid which bathed pericardial organs during electrical stimulation (Berlind & Cooke, 1970; but cf. Carlisle, 1964). Maynard & Welsh (1959) also found significant amounts of 5-hydroxytryptamine (5-HT) (*c.* 4 $\mu$g/g wet weight in *Cancer* and *Libinia* pericardial organs). The heart responds similarly to 5–HT as to released or extracted pericardial organ material, but there is insufficient non-peptide material in either released or extracted material to affect the heart significantly (Maynard & Welsh, 1959; Cooke, 1966; Berlind & Cooke, 1970). The presence of heart-excitatory peptides and of 5-HT in extracts has been confirmed by Belamarich (1963), Carlisle (1964), Belamarich & Terwilliger (1966), Terwilliger (1967), and Clay (1968).

There are claims (Carlisle, 1956, 1964; Kerkut & Price, 1964) that other active amines are present in addition to 5-HT. None of these has been documented. Knowles (1967) has suggested that dense-cored vesicles (Type B, Knowles, 1960) in electron micrographs of a stomatopod pericardial organ may contain monoamine.

This study exploits the specificity (e.g. Falck *et al.* 1962; Jonsson, 1967; Jonsson & Sandler, 1969) and sensitivity (e.g. Norberg & Hamberger, 1964) of the formaldehyde induced fluorescence method for the microscopical demonstration of monoamines (Falck, 1962; Dahlström & Fuxe, 1964; Falck & Owman, 1965; Corrodi & Jonsson, 1967) to localize the 5-HT and explore for the presence of other monoamines in crab pericardial organs. By this method 5-HT is converted to a compound having yellow fluorescence which fades rapidly on exposure to ultraviolet (u.v.) light; catecholamines form compounds having stable, green fluoresence.

### *Review of anatomy*

Alexandrowicz (1953) recognized the neurohaemal function of the pericardial organs, though descriptions have been given by others (see, for example, Jolyet & Viallanes, 1893; Conant & Clark, 1896; Heath, 1941; Smith 1947). His terminology will be followed here (see Pl. 1; Text fig. 1). Alexandrowicz's description begins: 'The pericardial organs in crabs are composed of thick nervous trunks anastomosing with each other according to a characteristic pattern. They lie on the inside of the lateral pericardium wall in such a position that their stoutest parts span the three openings of the branchiocardiac veins. For facilitating the description these thickest parts will be called anterior and posterior bars, and those uniting the two bars will be called longitudinal trunks' (p. 564). He goes on to point out that nerves from the central ganglia reach the pericardial organs by means of three nerves entering the anterior bar and by others, whose number is difficult to determine, which enter at the posterior bar. 'Apart from those nerve elements which pass through the trunks to other destinations, the pericardial organs are composed of fibres which branch in the trunks themselves up to their last terminations. After entering the bars each of these

fibres gives off branches which pass into the prolongations of the bars and the different trunks and, running for a long distance, send subdivisions in various directions. A result of this distribution of branches is that each part of the pericardial organ has fibres belonging to various neurons, and it appears as if this intermingling of the elements of several neurones in the same area were of some particular importance for the functioning of the organ' (p. 568). Alexandrowicz describes the fine branches and terminals as forming a neuropile at the surface of the trunks and bars, and points out that such neuropiles are found only where the structures are exposed to blood. Maynard & Maynard (1962) describe the cross-section of trunks as consisting of a cortex formed by the branches and terminals surrounding a core consisting of axons, blood vessels, and connective tissue. The cortex is densely stained by procedures selective for neurosecretory material (Maynard & Maynard, 1962; Cooke, 1964).

Maynard (1961*a*, *b*) traced the nerves which enter the anterior bar of the pericardial organs from the ventral ganglion and identified them as segmental nerves 1, 2 and 3 (SN 1, SN 2, SN 3). He described another structure having the morphological characteristics of a neurohaemal organ formed by an anterior branch from SN 1. This he called the anterior ramification (AR) (Maynard, 1961*a*). In this study we have also examined this structure.

## METHODS

Pericardial organs from the following species of crabs were examined (number of individuals in parentheses): *Carcinus maenas* (51), *Libinia emarginata* (24), *Cancer irroratus* (22), *Cancer borealis* (8) obtained from Woods Hole, Mass., *Libinia dubia* (9) from Florida and *Cardisoma guanhumi* (10) from Puerto Rico. The first five species were kept at 10 °C in circulating artificial sea water; *Cardisoma* was maintained at room temperature in a dry tank with access to fresh water and sea water. The pericardial organs were dissected and pinned out on to a planchet coated with a thin layer of a silicone resin (Sylgard 184, Dow Corning Corp.). The tissue was frozen in isopentane cooled in liquid nitrogen, transferred to a modified (Rude, 1969) Pearse Tissue Freeze Dryer (Edward High Vaccum Ltd.) and maintained at $-45$ to $-55$ °C and 0·1 $\mu$ Hg pressure for 1–4 days. The dry tissue was exposed for 1–3 h at 80 °C to formaldehyde gas generated from paraformaldehyde which had been stored at 50–70% relative humidity (Hamberger, Malmfors & Sachs, 1965). Control tissues were treated similarly, including heating, but were not exposed to formaldehyde vapour. The whole pericardial organs were either mounted in non-fluorescent immersion oil or vacuum-embedded in degassed 'Maraglas' (Polysciences, Inc.). These were examined by darkfield fluorescence using a Leitz Ortholux microscope and an HBO 200 mercury vapour lamp. Schott UG 1, BG 12 or BG 3 excitation filters and a barrier filter with exclusion below 510 m$\mu$ or a Wratten 2E filter were used (Angelakos, 1964; Rude, 1969). Exposure times for black and white photography were minimized by using Kodak Tri-X or Plus-X pan film developed in Kodak D-11 or 'Diafine' (Acufine, Inc.).

Drugs were injected daily for 1–3 days in volumes not greater than 0·25 ml. The crabs were kept at 18–22 °C. Dissection of pericardial organs and freeze-drying were carried out 1 day after the final injection. Control injections were administered, and in no case did a solvent (*Carcinus* saline, Welsh, Smith & Kammer, 1968; distilled water; 0·8 M acetic acid) change the normal pattern of induced fluorescence. Pericardial

organs from uninjected crabs kept under the same conditions were also examined as additional controls. Following are listed the drugs used and their source: reserpine phosphate (CIBA), DL-α-methyl-*p*-tyrosine, methyl ester, hydrochloride (Regis Chemical Co.), DL-5-hydroxytryptophan (5-HTP) (Nutritional Biochemicals Corp.), nialamide (Chas. Pfizer and Co.), 6-hydroxytryptamine hydrochloride (6-HT) (custom synthesized by Regis Chemical Co.).

## OBSERVATIONS

### I. *Pericardial organs*

#### (*a*) *Fluorescent surface structures*

Pericardial organs which have been isolated, freeze-dried, and exposed for 1 h to formaldehyde vapour show rich amounts of green fluorescence and in some preparations a yellow fluoresence which is u.v.-labile. The majority of this is localized in a dense surface speckling of 'blebs' (Maynard & Maynard, 1962) (Pl. 2, figs. 1, 2; Pl. 7, fig. 2). These are sometimes distributed in bands or a honeycomb pattern (Pl. 5, figs. 1, 2). These can in some preparations be clearly seen as varicosities along very fine nerve branches (Pl. 3, figs. 2, 3). The structure of yellow surface fluorescence is frequently hard to resolve, perhaps chiefly because of rapid fading. When resolved, the yellow blebs are characteristically smaller (1–2 μ) than green ones (about 5 μ, Plate 5, fig. 4). In areas where blebs are not so frequent as to mask deeper structure, the fine surface branches can sometimes be followed to their point of emergence from deeper-lying, green or yellow axons. Only the neuropile-covered parts of the pericardial organ which are blue-white in fresh tissue have surface fluorescent blebs (Pl. 5, fig. 3). Thus proximal portions of SN 1, 2, and 3, before they emerge to be free in the blood spaces, and parts of the longitudinal trunks which run through pericardial tissue, do not have surface-fluorescent structures. At these points the more centrally situated fluorescent axons can be seen.

#### (*b*) *Fluorescent axons*

The following general picture results from drawing together the observations from many different preparations. Without exception, in each formaldehyde-exposed pericardial organ there is one extremely large and brilliantly green-fluorescing fibre whose point of entry, either SN 1 or SN 2, is constant for each species. This axon divides in the anterior bar to provide a major branch to each longitudinal trunk (Pl. 2, figs. 1, 2, 4). At the posterior bar these branches cross and perhaps anastomose before sending final branches to terminate in the processes anchoring the posterior bar to the pericardium (Pl. 4). Throughout its course this axon and its major branches give off small branches which contribute to the surface varicosities and blebs in all of the neuropile-covered parts of the pericardial organ.

Frequently, a small green-fluorescing axon has been found to enter via SN 1. This axon, before reaching the pericardial organs, branches to supply green-fluorescing blebs in the anterior ramification (see §II). Evidence suggesting that this and the major green-fluorescing fibre may represent branches of a single neurone is presented in §III.

Two to four small green-fluorescing axons in addition to those already mentioned have been seen in SN 1 or SN 2 but could not be clearly followed beyond the anterior

bar. Some of these may represent branches from the large axon which double back along the segmental nerves.

Yellow-fluorescing axons have been less easy to follow, except in *Cardisoma*, because they are less brilliant and fade rapidly during exposure to u.v. A pair of large axons are consistently observed to enter in SN 3. In addition, one or two other pairs of yellow-fluorescing axons are sometimes observed in SN 1 or SN 2. While in no case could the course of all of the less brilliant fibres be followed completely, it is clear that most of them distribute branches and contribute to the neuropile areas throughout the pericardial organ in a pattern similar to that seen for the large, green fibre (Pl. 3, figs. 1, 2; Pl. 4).

The fluorescent fibres conform very closely to the general description of methylene-blue-stained axons given by Alexandrowicz (1953; see quotation above). In a few studies in which we combined the methods we found that fibres which took up methylene blue were not fluorescent but ran parallel courses to the fluorescent fibres.

The green-fluorescing and yellow-fluorescing axons have distinguishing structural characteristics in addition to their differences in colour, fluorescent intensity and lability under u.v. illumination. The fluorescence of the yellow axons is distributed sparsely in a reticulated pattern along the course of the fibre while that of the green axons is more evenly distributed (Pl. 3, figs. 1, 2; Pl. 7, fig. 1). Whether this reflects a true cytological difference or is an artifact of fixation or of the concentration of the fluorescing material cannot be decided. Preparations re-examined months after exposure to paraformaldehyde sometimes show faded fibres with a reticulated distribution of green fluorescence. At points where green-fluorescing and yellow-fluorescing axons run parallel courses, the diameter of the yellow is usually greater than that of the green (Pl. 3, fig. 1; Pl. 4; but see Pl. 2, fig. 4).

Long lengths of the nerves which reach the posterior bar from the ventral direction, and which we therefore presume to be the posterior segmental nerves from the ventral ganglion, were dissected and examined for fluorescent fibres. None of these showed a significant continuation of the fluorescing fibres given off toward them from axons running in the posterior bar.

The somata of the neurones intrinsic to the pericardial organs (Maynard, 1961*b*) were not visible in u.v. illumination and hence can be assumed to make no contribution to the population of fluorescent fibres or terminals.

In summary, the green and yellow surface fluorescent structures represent an intermingling, in all areas of the pericardial organ, of fine branches, varicosities and terminals contributed by the fluorescing axons. There are few such axons; at most, six green-fluorescing and six yellow-fluorescing ones. They enter from the ventral ganglion via the anterior segmental nerves and divide to run in the core of all parts of the pericardial organ trunks and bars.

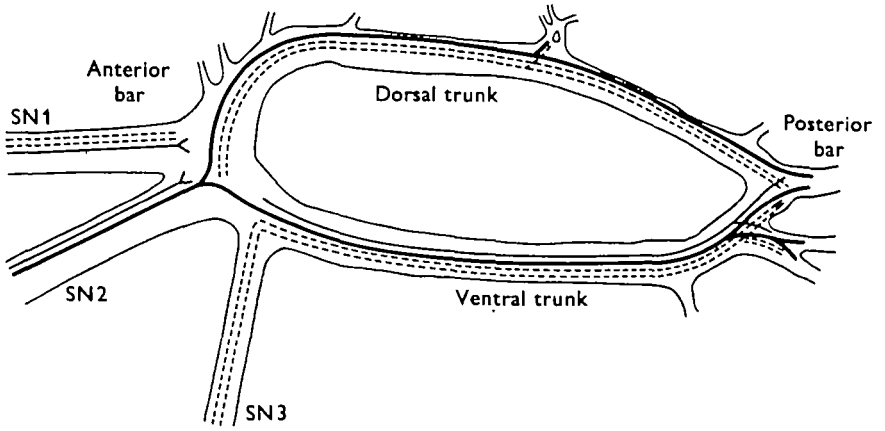
### (*c*) *Course of fluorescing axons, details by species*

Text-figs. 1–3 present diagrams showing the course of specifically fluorescing fibres consistently observed in the pericardial organs of *Carcinus*, *Libinia*, and *Cardisoma*.

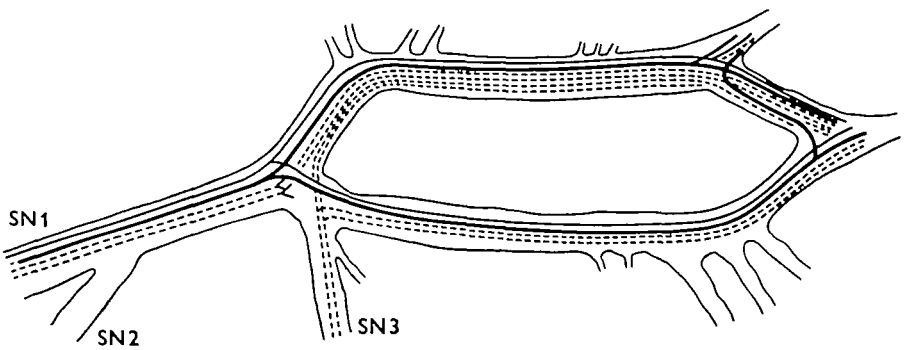
In *Carcinus* (Text-fig. 1) the largest (13 $\mu$) green-fluorescing axon enters in SN 2. Small (5 $\mu$), green-fluorescing axons appear in SN 1 and SN 2. A pair of yellow-fluorescing axons (8 $\mu$) is present in SN 3 and occasionally in SN 1 or SN 2.

In *Libinia* (Text-fig. 2) the major (8–13 $\mu$ diameter) and a small (5 $\mu$ diameter) green-fluorescing axon enter via SN 1. A pair of yellow-fluorescing axons enters in SN 3, and sometimes a pair is also seen in SN 1. In two out of 33 preparations examined, small green-fluorescing axons were see in SN 2.

*Cardisoma quanhumi.* The morphology of the pericardial organs of *Cardisoma*, the large, blue land-crab of southern Florida and the Caribbean, has apparently not been previously described. A striking feature of the gross morphology is a large and elaborate



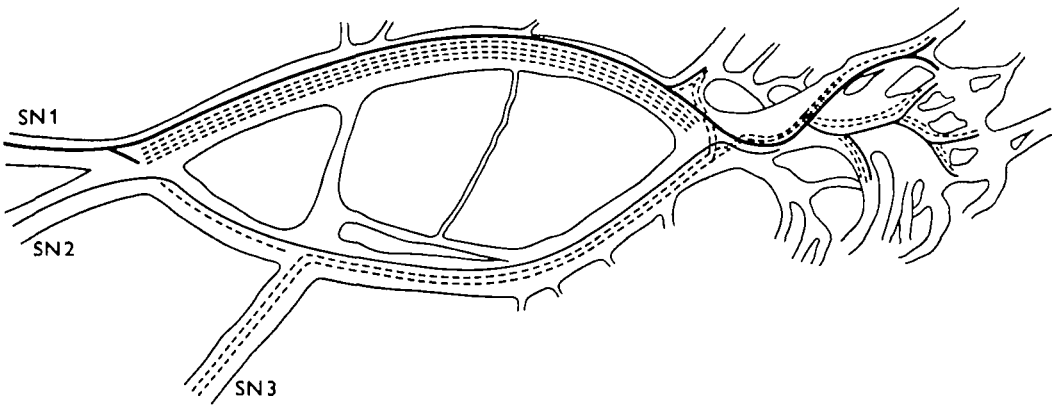

Text-fig. 1. Diagram of the course of specifically fluorescent axons in *Carcinus* pericardial organs. Composite of all observations (exceptions excluded). Each axon gives off fine branches (not shown) and supplies terminals throughout its course. Axons which are shown ending in the anterior bar could not be followed further, but probably continue to dorsal and ventral trunks. Heavy continuous line, the large green-fluorescing axon; thin continuous lines, small green-fluorescing axons; interrupted lines, yellow-fluorescing axons; width of nerves, bars and trunks exaggerated.




Text-fig. 2. Diagram of fluorescing axons in *Libinia* pericardial organ. Conventions as in Text-fig. 1.

arbor of processes posterior to the meeting point of the dorsal and ventral longitudinal trunks (Pl. 1). In fresh tissue, this arbor has the same brilliant iridescent white appearance that characterizes the neuropile-covered portions of pericardial organs of all species. This iridescence is attributed to refraction of light from dense accumulations of intracellular granules (Maynard, 1961*b*). The arbor vastly expands the surface of the neurohaemal organ exposed to the haemolymph in this species. SN 1 and 2 reach the pericardial organ together anteriorly; slightly more posteriorly, SN 3 reaches the

ventral trunk. Some non-fluorescent fibres of SN 3 continue upward to the dorsal trunk and then leave by a pair of nerves given off dorsally near this same point (*n. mot.* and *n. dors.*, Pl. 1). Near the posterior bar the dorsal trunk gives off a thin sheet of tissue about 200 $\mu$ wide which joins it to the pericardium (*s.*, Pl. 1). In methylene-blue preparations this displays a regular array of fine, parallel processes. Four or five nerves enter the ventral trunk from the ventral side anterior to its union with the dorsal trunk at the posterior bar. Since there are no other nerves entering from the ventral direction these may represent the more posterior segmental nerves. Two to four large cell bodies could be distinguished in freshly dissected pericardial organs. One or more lay at the junction between SN 1 and 2, and one at the point at which SN 3 enters the ventral trunk.




Text-fig. 3. Diagram of fluorescing axons in *Cardisoma* pericardial organ. Conventions as in Text-fig. 1.

Fluorescent blebs and surface varicosities are less densely spaced in this species than in the others (Pl. 5, figs. 1, 2). Thus the course of axons from which they arise could be consistently followed (Pl. 2, fig. 2; Pl. 4; Text-fig. 3). The major green-fluorescing axon (13–18 $\mu$) enters via SN 1, divides in the anterior bar and sends a large branch to the dorsal trunk. A small branch may run to the ventral trunk, but could never be completely linked to its apparent continuation in the ventral trunk. The green axon regularly found in the ventral trunk could also be the continuation of the small green fibre which enters via SN 2 but which has not been seen to divide. Both green axons can be seen to divide many times in the posterior arbor, each branch ending in blebs (Pl. 4).

The pattern of yellow-fluorescent fibres has been very consistent in the *Cardisoma* preparations (Text-fig. 3). The yellow fluorescence is more intense and fades less rapidly than in any other species examined. Two large yellow fibres (22–30 $\mu$ diameter) enter via SN 3 and proceed posteriorly in the ventral longitudinal trunk (Pl. 2, fig. 3). They each branch at the posterior bar and send a thin axon a short distance anteriorly in the dorsal trunk. Their main axons branch to each process in the posterior arbor; each branch ends in yellow blebs. Four small (9 $\mu$) yellow fibres are seen in the dorsal trunk; their point of entry is unknown. A yellow fibre is seen in the ventral trunk between the point of entry of SN 1 and 2 and of SN 3, but cannot be followed anteriorly and posteriorly of these points.

(*d*) *Dorsal nerves*

In about half of our preparations a small number of axons can be distinguished which pass from SN 1 and SN 3 through the anterior bar to the nerves which leave the pericardial plexus dorsally. These fibres have a dull grey-green fluorescence which, together with their large diameter, smooth, unvarying profile, lack of branches, and direct course distinguishes them from the fluorescent axons which supply terminals in the pericardial organ (Pl. 5, fig. 3). We are uncertain whether their fluorescence is specific since they have also been visible in some of the preparations not exposed to formaldehyde. We have seen at most four of these fibres in SN 1 and four in SN 3. Those in SN 1 pass to the second most anterior of the dorsal nerves; those in SN 3 take their course to the most anterior dorsal nerve. In none of our preparations could we distinguish any fluorescence in axons of the cardioregulator nerve.

## II. *Anterior ramifications*

The anterior ramifications (AR's) stand out from other structures by reason of their iridescent white appearance in fresh tissue. After treatment by the monoamine histochemical procedure the AR's present the same general appearance as neuropile-covered parts of the pericardial organs (Pl. 2, fig. 3). They show a dense surface-speckling of green-fluorescent blebs. These can sometimes be seen to be connected by fine branches to a single branch of a green axon traversing SN 1 and continuing into the pericardial organ. Yellow blebs have been discerned in the AR's; as in pericardial organs they fade very rapidly. A yellow fibre has been seen in SN 1 where it passes the point of origin of the AR, though connexions between it and terminals in the AR have not been discernible. The soma of the neurone intrinsic to the AR (Maynard, 1961*b*) can usually be discerned in dark-field tungsten light but not in u.v. Hence it is reasonably certain that this cell does not contribute fluorescing terminals to the AR.

## III. *Central origin of green-fluorescing axons*

Whole mounts and sections of the central nervous system of *Carcinus* have been examined for the purpose of locating the cell bodies of the fluorescing axons having their terminals in the pericardial organs and anterior ramifications (Goldstone and Cooke, in preparation). Yellow-fluorescing axons could not be followed, but green-fluorescing axons have been traced to the ganglion of the circumoesophageal connectives.

Each half of the connective ganglion has a very large (about 90 $\mu$ diameter) green-fluorescing cell at the anterior-lateral margin (Pl. 6, figs. 1, 2, 4). The border of the cell is irregular and indented, in marked contrast to other fluorescing neurones in the central nervous system of the crab. Fluorescence in the peripheral cytoplasm has a reticulated distribution. Centrally, it is more evenly spread but shows scattered bright points. The cell has a single major axon (about 15 $\mu$ in diameter) which gives off several collaterals within the ganglion and then travels toward the brain at the lateral edge of the connective. Two or three small (about 30 $\mu$ diameter) green-fluorescing cells send their major process toward the axon of the large cell (Pl. 6, fig. 3). It has not been possible to resolve whether the small cell axons fuse with that of the large cell or whether they run parallel courses. Thus there is a possibility that what appears to be a

single green-fluorescing axon emerging from the connective ganglion of each side, in fact represents a tract.

At a point a few hundred microns posterior to the emergence of the connectives from the brain, the green-fluorescing axon from the connective ganglion doubles back on itself and travels posteriorly at the lateral edge in the same connective to the ventral ganglion (Pl. 6, fig. 1). At its turning-point lies a large (75 $\mu$ diameter) green-fluorescing cell. In some preparations, sections give the appearance of a very close association between this cell body and the axon.

In the ventral ganglion the large green-fluorescing axon travels dorsolaterally directly to SN 2 of the ipsilateral side. In passing it gives off a small axon to SN 1 which continues peripherally to supply terminals in the anterior ramifications and pericardial organs. The major axon continues in SN 2 to its final terminations in the pericardial organ.

By reason of its size and of its course from the circumoesophageal connective to SN 2 it seems likely that the large, green-fluorescing axon is the same as Maynard's 'a' fibre (Maynard, 1961*b*), though we have not examined the same species.

The large, green-fluorescing cell of the connective ganglion, by reason of its position, relative size, distribution of collaterals and course of its axon may be homologous with a cell described by Orlov (1929) from methylene-blue studies of the crayfish (see cell 42, fig. 16.52 in Bullock & Horridge, 1965). Orlov mentions that this neurone rarely took up methylene blue, another characteristic observed for the proposed homologous crab neurone.

It is difficult to assign with certainty the number of green-fluorescing axons which innervate the pericardial organ in addition to the large one of SN 2 and small one of SN 1, because different numbers are seen from preparation to preparation, and because some which appear to be separate axons near the anterior bar of the pericardial organ may represent branches of the major green-fluorescing axon which double back. The observations are consistent with the suggestion that the green-fluorescing cells of the connective ganglion give rise to the entire green-fluorescing system of axons and terminals in the pericardial organs and anterior ramifications, though they do not exclude the possibility of a contribution by other cells.

## IV. *Specificity of the fluorescence*

### (*a*) *Chemical test for specificity of the fluorescence*

Pericardial organs and AR's were carried through the freeze-drying procedures, including heating at 80 °C for 1 h, but were not exposed to formaldehyde vapour. Such preparations had a dim green background fluorescence and a few small, round, orange-yellow-fluorescent cells. These cells were present in greater numbers in adherent tissues of the pericardium occasionally included in the dissection. It was sometimes possible to discern the course of one or more axons. This can also sometimes be done in dark-field tungsten illumination.

Treatment of tissues with sodium borohydride reversibly quenches the fluorescent product of monoamines condensed with formaldehyde in the presence of protein (Corrodi, Hillarp & Jonsson, 1964). This provides a critical test for distinguishing non-specific fluorescence from that resulting from monoamines. Pericardial organs

treated with formaldehyde vapour were covered for 2 min with 0·1% $NaBH_4$ in 90% isopropanol. This treatment removed all fluorescence located in fibres and terminals. After re-exposure to formaldehyde vapour, green fluorescence was again localized in axons and terminals, though background fluorescence remained higher than before treatment with $NaBH_4$.

(*b*) *Effects of pharmacological agents on fluorescence*

Experiments were performed to test the effect of pharmacological agents found, in other preparations, to have specific effects on neurones containing catecholamine or 5-HT. The immediate objective of the experiments was to obtain further evidence for the specificity of the green and yellow fluorescence observed in pericardial organs. The experiments were carried out on *Carcinus maenas* and on *Libinia emarginata* and *L. dubia*. The results were similar for the three species.

*Reserpine*. Studies in several animal phyla including arthropods (e.g. Eloffson *et al.* 1966; Frontali, 1968) have been consistent in showing that treatment with reserpine results in depletion of tissue stores of catecholamines and 5-HT (e.g. Carlsson, 1966; Stjärne, 1964; Malmfors, 1965; Hökfelt, 1968). Animals were held at room temperature (18–20 °C) and injected on two successive days with a total of 3 mg reserpine phosphate (20–115 mg/kg). The pericardial organs and AR's were dissected on the third day. Behavioural changes in the crabs could be observed 12–24 h after the final injection of reserpine. Injected crabs became slow-moving and would not respond with defence postures when provoked. In seven out of eight preparations all fluorescent surface varicosities and blebs were absent (Pl. 7, figs. 1, 2). In the eighth, they were markedly less numerous and less bright than in controls. As a result of this 'unmasking', the deeper-lying major branches of yellow and/or green axons could be followed clearly in several preparations. In others the fluorescence of the fibres was also depleted to varying extents. The degree of depletion was not correlated with dosage within the narrow range used.

In two preparations from *Carcinus* treated with reserpine a number of yellow-fluorescing neurone-like cell bodies were visible. These have never been seen in control or uninjected animals. Their numbers and positions suggest that they correspond to the intrinsic cells (Maynard, 1961*b*). We can offer no explanation for their visualization in these two reserpinized preparations.

In summary, reserpine injected into whole crabs results in disappearance of material in pericardial organs and AR's producing fluorescence after treatment of the dried tissue with formaldehyde vapour. The terminals and fine processes are more rapidly or more completely depleted than the larger axons. This is evidence that the materials responsible for fluorescence are monoamines.

*α-Methyl*-p-*tyrosine methyl ester hydrochloride* (*H* 44/68). In the mammalian central nervous system H 44/68 causes rapid and complete depletion of fluorescence attributable to nor-epinephrine (NE), less-pronounced effects on fluorescence of neurones thought to contain dopamine (DA) and no effect on fluorescent products of 5-HT (Andén *et al.* 1966). It does not appear to have been previously tested on invertebrate nervous tissue.

Crabs were injected on three successive days and the pericardial organs were dissected for histological preparation on the fourth day. Preparations from animals

which received a total of up to 40 mg/kg were indistinguishable from controls. Doses totalling 185 mg/kg or more produced marked changes in formaldehyde-induced fluorescence. However, about half of the animals injected died during or immediately after the series of injections. All four of the preparations dissected from survivors showed marked reduction in green-fluorescing surface structures and the number of fluorescing axons was reduced or they were absent. A yellow-fluorescing 'haze' of surface blebs was visible in some specimens, though yellow-fluorescing axons were not prominent.

The experiments with H44/68 have not been extensive enough to allow definite conclusions. They suggest that injection of this drug results in a selective depletion of material responsible for green fluorescence in pericardial organs. If the effects of the drug are the same as in the mammalian central nervous system, then these observations support the conclusion that the green-fluorescing material represents catecholamine.

*Other pharmacological procedures.* A limited number of experiments were performed with combinations of drug injections designed to increase specific fluorescence. The injection of reserpine (45 mg/kg) or nialamide (200–300 mg/kg) followed by 5-HTP (70–230 mg/kg) (Dahlström & Fuxe, 1964) or 6-HT (3 mg/kg); or the incubation (Jonsson *et al.* 1969) of isolated pericardial organs in nialamide (saturated solution in saline, 15 min) followed by 6-HT ($10^{-7}$–$10^{-5}$ M, 3 h, with 2 mg/ml glucose and 0·1 mg/ml ascorbic acid), all resulted in preparations with yellow-fluorescing fibres which were more pronounced than in controls. In the preparations treated with 6-HT, green-fluorescing fibres and blebs appeared to be brighter than in normal preparations (Pl. 2, fig. 4). These procedures all produced preparations which showed increased background fluorescence.

In summary, all of the changes observed after pharmacological treatment are consistent with the conclusion that fluorescence induced in pericardial organs by formaldehyde vapour represents monoamines contained in axons and their terminals.

## DISCUSSION

Examination of whole-mount preparations of crab pericardial organs and anterior ramifications (AR's) by the specific histochemical procedures for monoamines reveals a green-fluorescing and a yellow-fluorescing system of axons and terminals. The axons enter these neurohaemal organs in nerves from the ventral ganglion. Each axon branches to supply varicosities and terminal blebs to the surface neuropile in all parts of the structures which are directly exposed to the haemolymph. There are probably, at most, six green-fluorescing and six yellow-fluorescing axons entering the pericardial organ. Their numbers and points of entry are relatively constant in each species. A single green-fluorescing and a single yellow-fluorescing axon supply the AR.

The major green-fluorescing axon supplying the pericardial organ and the small axon supplying green-fluorescing terminals in the AR have been traced to a single large neurone and a few small neurones of the circumoesophageal connective ganglion. The possibility remains open that these neurones are the source of all the green-fluorescing terminals of the pericardial organ and AR.

A small number of axons which sometimes show dim, but probably not specific,

fluorescence have been observed to pass through the anterior bar of the pericardial organs into dorsal nerves (but never to the cardio-regulator).

The courses of the fluorescing fibres seen in this study are remarkably similar to those of methylene-blue-staining axons pictured and described by Alexandrowicz (1953). It is clear, however, that they represent a population distinct from that of the peptide neurosecretory cells. We have seen methylene-blue-stained fibres running parallel courses with non-stained, green-fluorescing fibres. Specific fluorescence (Goldstone & Cooke, in preparation) has not been detected in the cell groups of the ventral ganglion which have been classified as neurosecretory by morphological and histological criteria (Matsumoto, 1958) and which send their axons to the pericardial organs (Maynard, 1961*b*). These include the group of about 200 C-cells on each side whose axons reach both the anterior ramification and the pericardial organ. These axons are smaller than any of the fluorescing axons visualized. In addition, about 25 $B_{SN}$ cells on each side terminate in the pericardial organ. Thus there is a numerous population of non-fluorescent terminals in these neurohaemal organs.

Electron micrographs (Cooke, Goldstone & Coggeshall, unpublished; Maynard & Maynard, 1962; also Knowles, 1960, 1962, of a stomatopod) show nerve terminals in the pericardial organ distinguishable by morphologically different kinds of included granules. In *Libinia* two sorts of 'elementary neurosecretory granules,' 'dense-cored' granules, and at least one other class of inclusion are seen, each in separate nerve processes. Neurosecretory granules have been isolated together with peptide neurohormones from several neurosecretory systems (e.g. Perez-Gonzales, 1957; Evans, 1962; Barer, Heller & Lederis, 1963) including crab pericardial organs (Terwilliger, 1967). There is evidence that associates dense-cored granules with monoamines (e.g. Wolfe *et al.* 1962; Wood & Barrnett, 1964; Wood, 1965; Rude, Coggeshall & van Orden, 1969). Thus, the morphology at the ultrastructural level adds additional evidence that the monoamine-containing axons and terminals form a separate population from those of the peptide neurosecretory cells.

Work to be published separately (Cooke, Goldstone, van Orden & King, in preparation) presents microspectrofluorometric analyses of the excitation and emission maxima for green and yellow fluorescence of whole-mount preparations of pericardial organs and chromatographic analyses of pericardial organ extracts. These data establish that the green fluorescence represents the intracellular localization of dopamine, and the yellow fluorescence that of 5-HT. The presence of 5-HT in pericardial organs has been reported previously by Maynard & Welsh (1959).

Thus we conclude that, besides the classical neurosecretory cells, presumably responsible for synthesis, transport and release of the peptide hormone(s) from pericardial organs and AR's, there are two further groups of neurones which supply axons and terminals to the pericardial organs and AR's. One of these contains dopamine, and another 5-HT.

The intermingling of monoamine-containing and peptide neurosecretory axons and terminals in these crustacean neurohaemal organs has a remarkable parallel in the mammalian hypophysial system where green-fluorescing axons end alongside neurosecretory terminals in the median eminence (Enemar & Falck, 1965; Odake, 1967; Björklund, 1968; Björklund, Enemar & Falck, 1968). Whether there is any functional significance in the intermingling and proximity of neurosecretory and

monoamine-containing axons and terminals is unknown. Certainly there are peptide-secreting neurohaemal organs which do not have monoamines. For example, crab (*Carcinus*, *Cardisoma*) sinus glands show no specific monoamine fluorescence (Cooke & Goldstone, unpublished observations). Physiological experiments designed to test whether the monoamines play a role in the release of peptide neurosecretory material from crab pericardial organs are described in the following paper (Berlind *et al.* 1970). They lead to the conclusion that the systems of axons and terminals containing dopamine and 5-HT serve roles which are independent of peptide neurosecretion.

## SUMMARY

1. The pericardial organs and anterior ramifications (both neurohaemal structures) of six species of crabs have been examined as whole mounts by the histochemical method for monoamines based on formaldehyde-induced fluorescence.

2. A small number of specifically fluorescing axons (not more than six green and six yellow) innervate the pericardial organ; one of the green-fluorescent and one yellow-fluorescent axon branches and also innervates the anterior ramification.

3. All of the fluorescing axons enter via segmental nerves 1, 2 and 3 from the ventral ganglion.

4. One large, brilliant green-fluorescing axon, and the small green-fluorescing axon which branches to the AR, have been traced in *Carcinus* to cell bodies in the circumoesophageal connective ganglion. These cells may give rise to the entire population of green-fluorescing axons and terminals in the neurohaemal organs.

5. Each axon, throughout its course in the pericardial organ, supplies a dense array of varicosities (blebs) at surfaces which are directly exposed to the haemolymph. The anterior ramifications are also supplied with blebs.

6. Lack of fluorescence in controls not exposed to paraformaldehyde, reversible quenching of fluorescence by treatment with sodium borohydride, and depletion of the fluorescence by reserpinization of the crabs, all confirm that the fluorescence is specific and represents the intracellular localization of monoamines.

7. With the aid of data available elsewhere we conclude that there are distributed, in parallel with peptide-secreting axons and terminals in the pericardial organs and anterior ramifications, a group of dopamine-containing and a group of 5-hydroxytryptamine-containing axons and terminals.

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## EXPLANATION OF PLATES

### PLATE 1

Fresh *Cardisoma* pericardial organ pinned for freeze-drying. The majority of axons enter from the thoracic ganglion via the anterior segmental nerves, SN 1, 2 and 3. The complex array of processes at the right, termed the posterior arbor, has been seen only in *Cardisoma*. *n. mot.*, Nerve which continues to dorsal musculature; *n. dors.*, presumed cardioregular nerve; *s.*, thin, flat sheet of tissue described in text. Scale, 1 mm.

### PLATE 2

Fig. 1. Fluorescence induced by formaldehyde vapour in a pericardial organ. A dense surface distribution of green-fluorescent blebs gives the white granular appearance. Yellow-fluorescing blebs are also observed, but fade during u.v. exposure for photography. A deeper-lying, green-fluorescing axon enters anteriorly via SN 2 and divides (arrow) sending a branch to the dorsal (upper) and ventral trunks. All figures are from whole mounts. Unless noted, preparations are embedded in oil, and are not drug-treated. *Cancer irroratus*; scale, 100 $\mu$.

Fig. 2. Anterior part of a *Cardisoma* percardial organ. Blebs are sparser in this species revealing deeper-lying structures. A green-fluorescing axon enters via SN 1 (right) and passes to the dorsal trunk. A

number of fine branches given off by it can be discerned. Two yellow-fluorescing axons enter via SN 3 (from bottom) and continue into the ventral trunk. Scale, 100 $\mu$.

Fig. 3. Part of an anterior ramification. Fluorescent blebs cover the AR. The nerve entering from the bottom is SN 1 from the thoracic ganglion. It continues (upper left) toward the pericardial organ. *Carcinus*; scale, 100 $\mu$.

Fig. 4. Branching of the large green-fluorescing axons in the anterior bar. The axon enters via SN 2 and sends a branch to the dorsal trunk (upper right). Another branch leaves and immediately divides to supply terminals in the region. The major process continues into the ventral trunk (lower right). A pair of yellow-fluorescing axons enters via SN 3. This preparation is exceptional in showing these branching rather than passing directly to the ventral trunk. Embedding in 'Maraglas' has quenched surface fluorescence. *Carcinus*; nialamide and 6-HT incubation. Scale, 100 $\mu$.

PLATE 3

Fig. 1. Green-fluorescing and yellow-fluorescing axons. Detail from the *Cardisoma* preparation of Plate 4. The large axon between the major green-fluorescing (left) and small green-fluorescing (right) fibres shows the characteristic reticulated distribution of yellow fluorescence. Scale. 100 $\mu$.

Fig. 2. Origin of blebs. Two green-fluorescing axons each bifurcate in the posterior bar and form a beaded thread of blebs. Two yellow-fluorescing axons travel parallel through the field from anterior to posterior (top and bottom). *Cardisoma*; scale, 100 $\mu$.

Fig. 3. Origin of blebs. The major green axon gives off branches to processes of the posterior arbor. Two of the branches can be seen in this field breaking into blebs (extreme right and at centre). *Cardisoma*; scale, 100 $\mu$.

Fig. 4. Typical distribution of blebs. Green-fluorescing terminals at the surface are aligned in rows indicating their origin as varicosities along fine axon branches. The thick bright line is the major green axon lying deeper and out of the plane of focus. *Cardisoma;* scale, 100 $\mu$.

PLATE 4

Posterior part of a *Cardisoma* pericardial organ. This montage shows the branching of several fluorescent axons to reach numerous processes of the posterior arbor. The major green-fluorescing axon can be followed from the dorsal trunk where it is seen alone at the upper right edge of the field. The two other axons in the dorsal trunk more posteriorly are branches from the large yellow-fluorescing axons of the ventral trunk. Four small yellow-fluorescing axons in the dorsal trunk are not resolved in this photograph. The small green axon of the ventral trunk is visible between the large axons. Scale, 1 mm.

PLATE 5

Figs. 1, 2. Distribution of fluorescent terminals, *Cardisoma*. Green-fluorescing and yellow-fluorescing surface varicosities are distributed together in bands (Fig. 1) or an irregular honeycomb (Fig. 2). The bright points represent green fluorescence and the paler areas within the bands represent yellow fluorescence. In Fig. 2, deeper-lying axons are out of the plane of focus. Scales, 100 $\mu$.

Fig. 3. Dorsal nerves. Four dully fluorescent axons are visible in *n.mot.* (upper right). Compare the brightness of the green-fluorescing blebs on *n.dors.* where it crosses over *n.mot.* The abrupt end of terminals on *n.dors.* corresponds to its exit from the blood sinus. Non-fluorescent axons are discernible in *n.dors.* The crossing of these nerves is a preparation artifact. *Cardisoma*; scale, 100 $\mu$.

Fig. 4. Green-fluorescing and yellow-fluorescing blebs. The left arrow is aligned with a row of green-fluorescing terminals, the right arrow indicates a row of yellow-fluorescing ones. Yellow-fluorescing blebs fade rapidly in u.v. light. They are always smaller than green-fluorescing terminals. *Cardisoma*, negative image. Scale, 10$\mu$.

PLATE 6

Fig. 1. Origin and course in the central nervous system of the large green-fluorescing axon of the pericardial organ. At lower left and right the large green-fluorescing cell body is visible at the lateral margin of each half of the circumoesophageal connective ganglion. The axon gives off several collaterals within the ganglion and then travels toward the brain (upward). It doubles back abruptly at the location of another green-fluorescing cell (arrow, upper left, but not in focus) and travels posteriorly (downward) in the connective toward the ventral ganglion (not shown). In this preparation both portions of the axon lie almost one above the other and are thus not seen separately. The axon as here described may represent a tract including axons of two or three small green-fluorescing cells of the connective ganglion. A green-fluorescing tract not related to the neurohaemal structures is visible running more centrally in the connectives. Montage of a whole mount, *Carcinus;* scale, 100 $\mu$.

Fig. 2. Section through the cell body of the large, green-fluorescing neurone of the connective ganglion. The cell border is indented and irregular. Fluorescence in the cytoplasm has a reticulated distribution at the periphery and shows bright points more centrally. *Carcinus*; scale, 25 $\mu$.

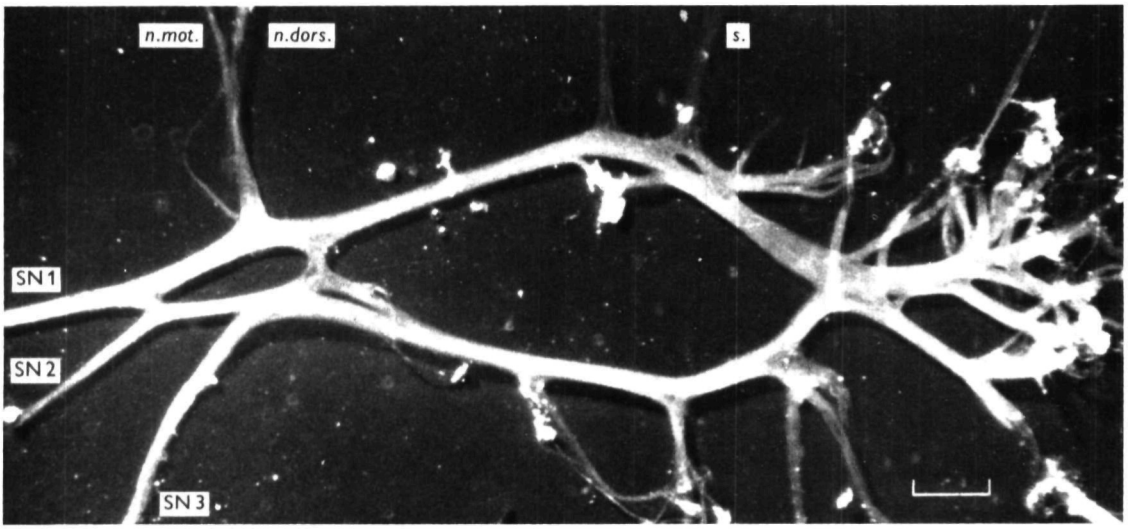
Fig. 3. Section of a connective ganglion showing a small, green-fluorescing cell in close association with axon of the large, green-fluorescing cell. The sections have not resolved whether the axons of the small cells fuse with that of the large cell or form a tract with it. *Carcinus*; scale, 25 $\mu$.

Fig. 4. Whole mount of the right half of a circumoesophageal connective ganglion showing the large, green-fluorescing cell body and the system of collaterals given off by its major axon. Two bright areas between the cell body and the first collaterals represent small, green-fluorescing neurones out of the plane of focus. *Carcinus*; scale, 100 $\mu$.

PLATE 7

Fig. 1. Example of partial depletion of fluorescence by reserpine. No surface structures exhibit fluorescence (compare Pl. 7, fig. 2). Two yellow-fluorescing axons enter via SN 1 and divide in the anterior bar. *Libinia dubia*; photographic exposure $\times 2$ that in Pl. 7, fig. 2. Scale, 100 $\mu$.

Fig. 2. Control. Dense distribution of fluorescing blebs in this pericardial organ from an animal injected with solvent is indistinguishable from that seen in uninjected animals. *Libinia dubia*; scale 100 $\mu$.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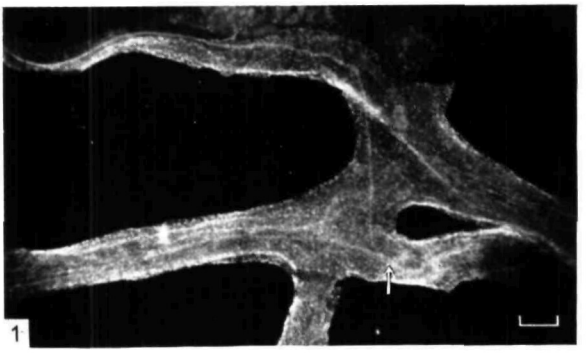
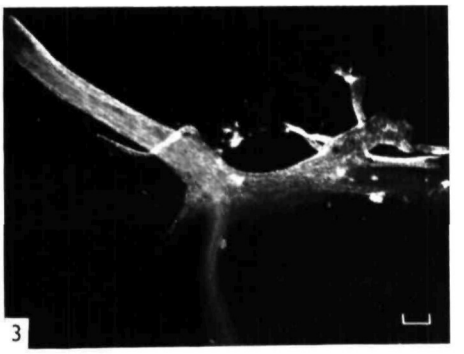
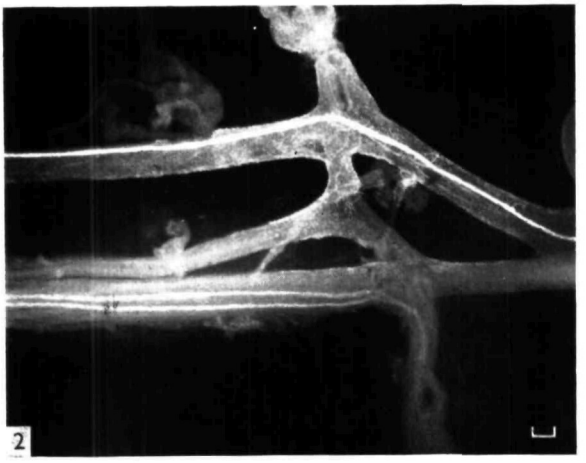
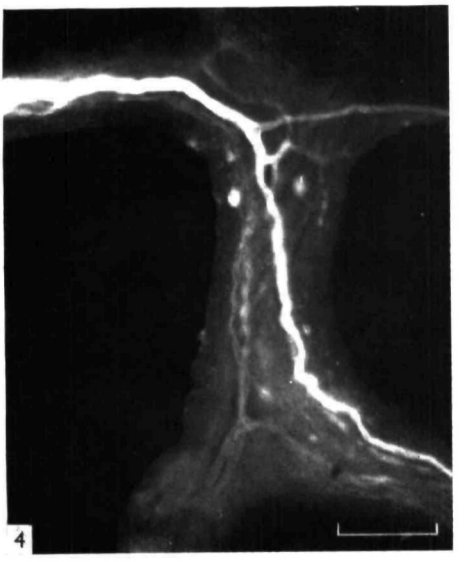
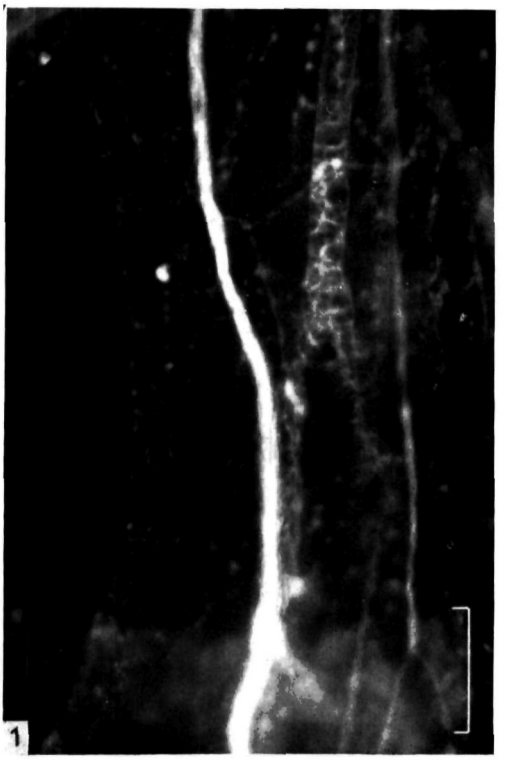
n.dors.
s.
SN 1
SN 2
SN 3







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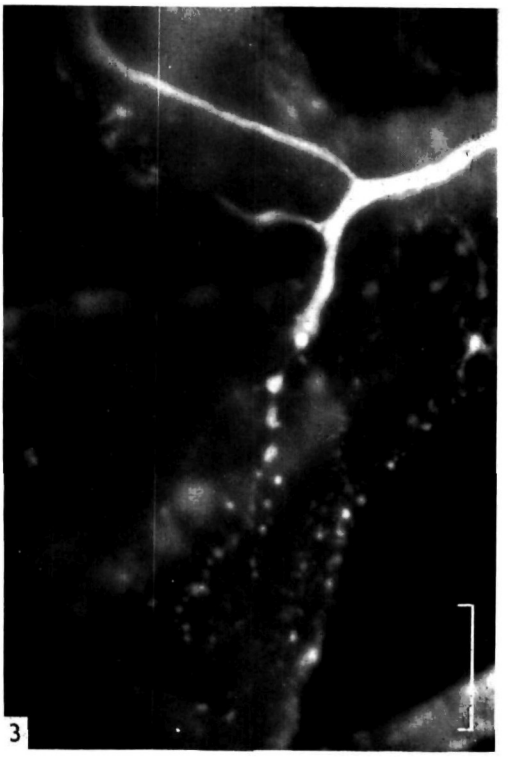
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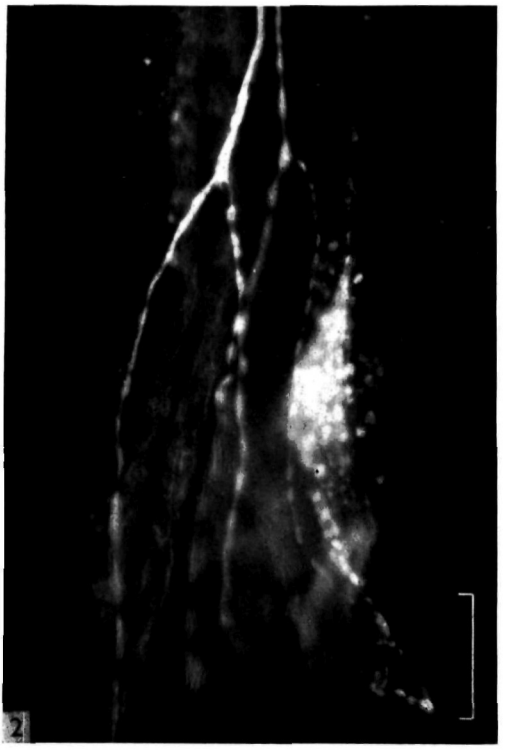
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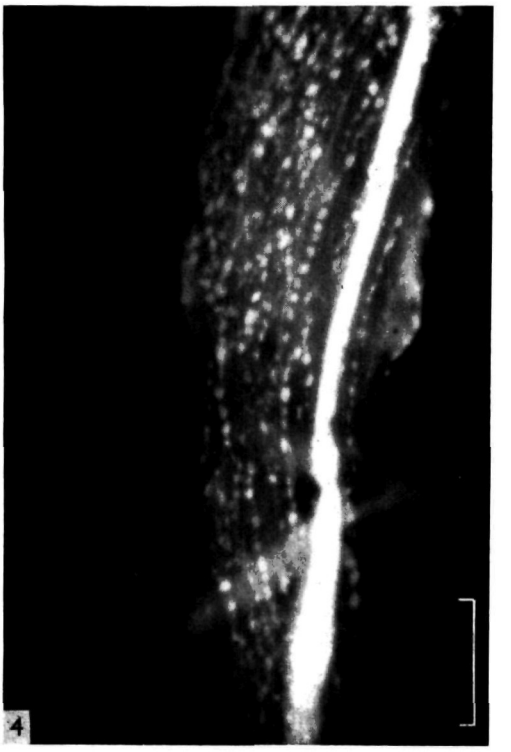
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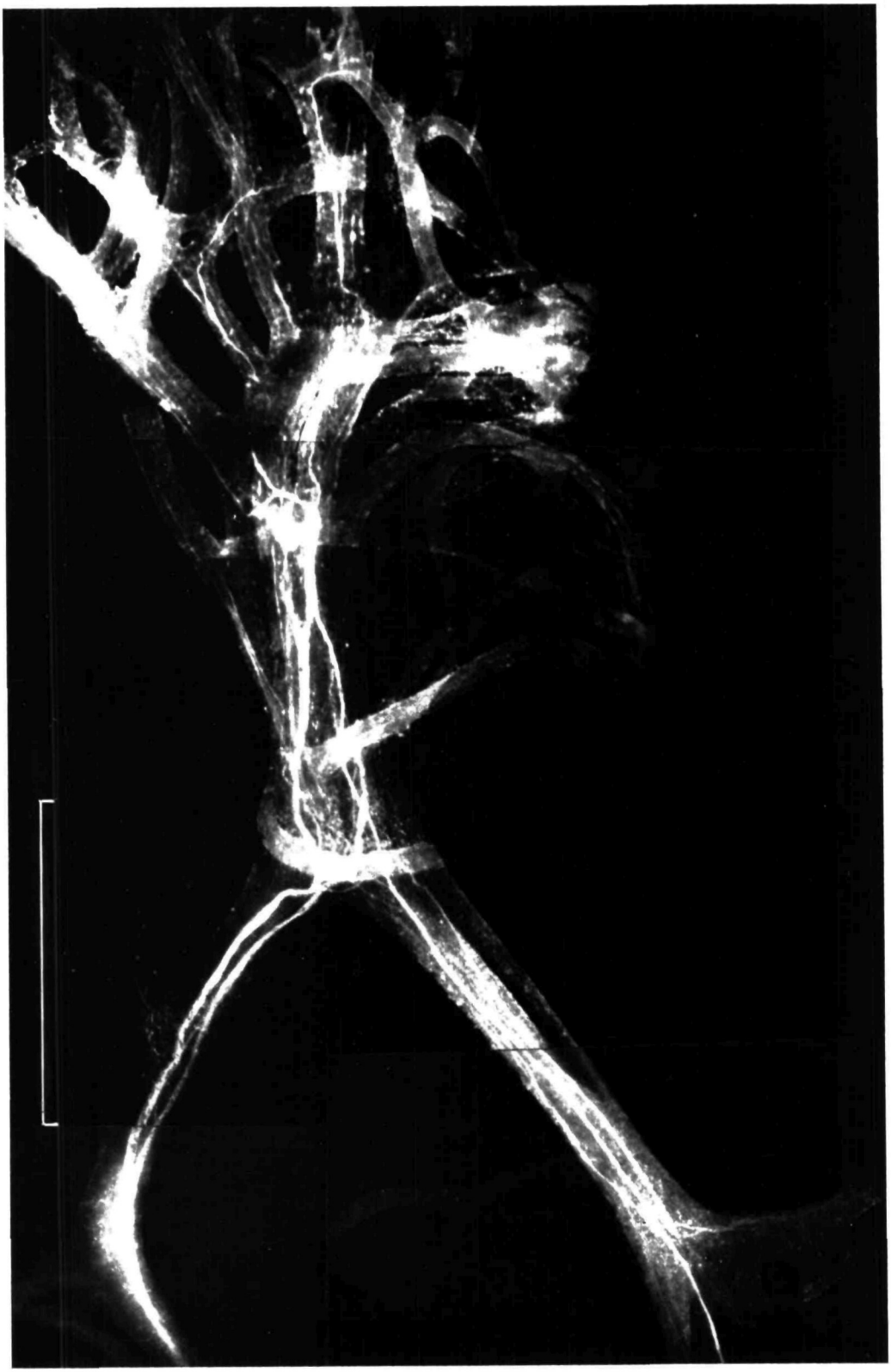

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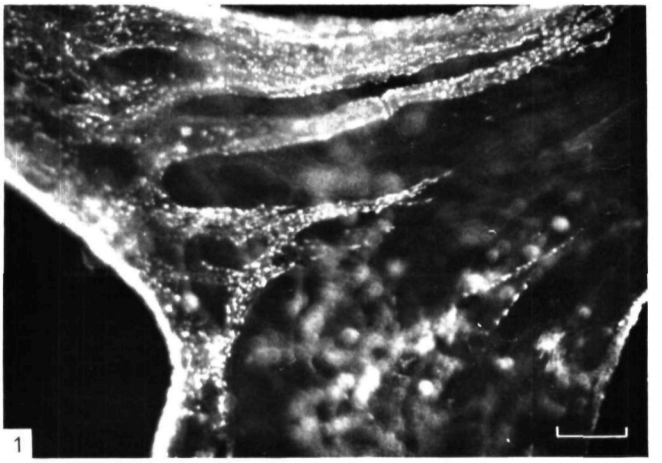

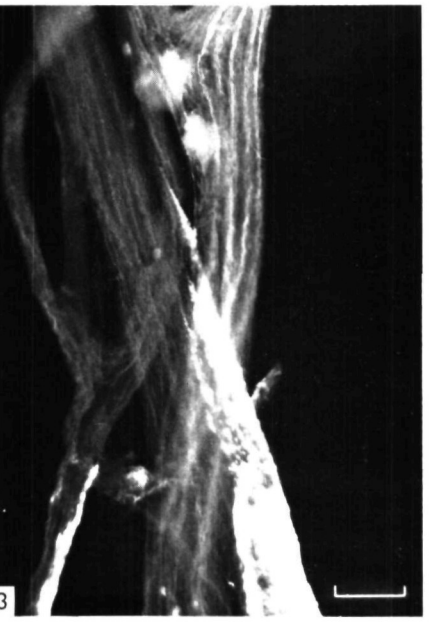

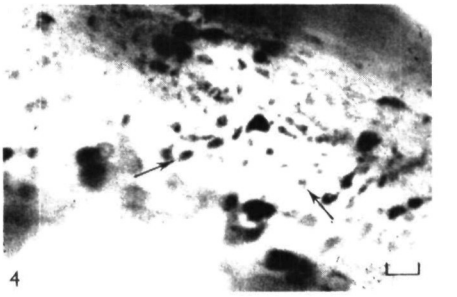

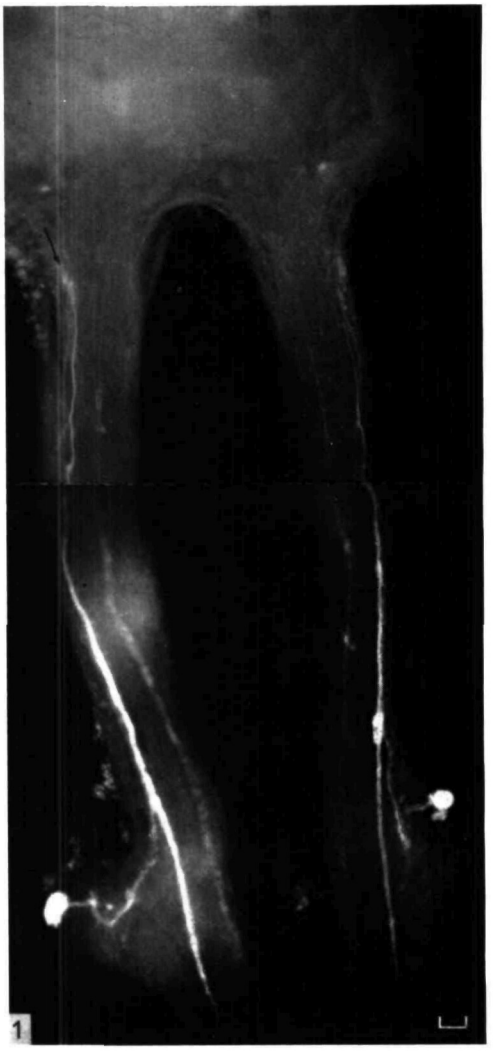
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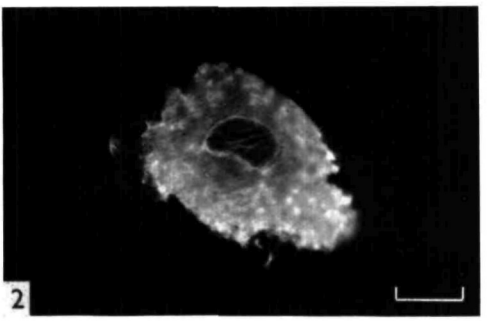
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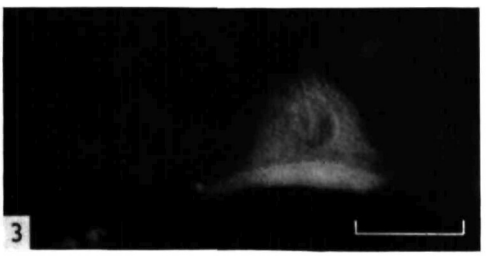
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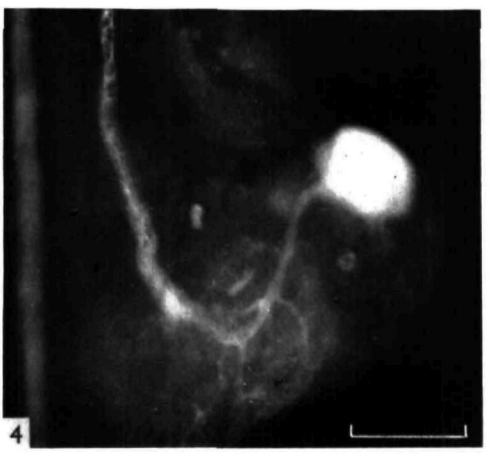
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