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Aspects of secretory phenomena within the sinus gland of *Carcinus maenas* (L.)

An ultrastructural study

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Summary. The sinus gland of *Carcinus maenas* contains small numbers of neurosecretory neurones ("intrinsic cells") as well as glial cells and nerve terminals. Intrinsic cells may be of the same cell type as some extrinsic cells, but are probably multipolar. Exocytosis is a common phenomenon, and may be either "basal" or "interterminal", and either "simple" or "compound" in character. Elementary neurosecretory granules may apparently fuse within the terminals to form "secondary" granules, and these also release their contents by exocytosis. A distinction is drawn between populations of vesicular inclusions which include elements of diverse size and form, and aggregations of synaptoid vesicles. The mixed populations may be involved in the retrieval of granule membranes. Typical synaptoid complexes are present in a minority of terminals and are characterized by a zonation of vesicular and granular inclusions, the aggregation of vesicles of fairly uniform size and shape adjacent to membrane thickenings, and an affinity of vesicle contents for the ZIO reagent. Granules within one type of fibre differ from typical peptidergic granules, but react negatively to cytochemical tests for amines.

Key words: Neurosecretion – Exocytosis – Synaptic vesicles – ZIO – Sinus gland

The sinus gland is a neurohaemal organ associated with the optic ganglia in the eyestalks of many Crustacea. Several studies of the morphology of the gland have included attempts to classify the different types of nerve terminals found within it on the basis of either staining affinity (e.g. Rehm 1959) or ultrastructure (e.g. Weatherby 1981). However, since exocytosis is, perhaps, more readily observed in this neurohaemal organ than within any other, the gland has also been used to investigate fundamental aspects of the biology of neurosecretion such as exocytosis (Bunt and Ashby 1967; Shivers 1976), membrane retrieval (Bunt 1969; Nordmann

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and Morris 1980), and the effects of electrical (Bunt and Ashby 1968), ionic (Andrew and Shivers 1976) and chemical stimulation (Strolenburg and Van Herp 1977).

According to Gabe (1966), "it appears certain that the cells of the sinus gland do not supply the elementary neurosecretory granules" in the nerve terminals. Ultrastructural studies have led to their identification as glial cells (Bunt and Ashby 1967; Smith 1974). In this study, we provide definitive evidence of the presence within the sinus gland of neuronal elements which, by analogy with insects (e.g., see Normann 1973) and annelids (review by Golding and Whittle 1977) we call "intrinsic cells". We also report on certain aspects of exocytosis and, in contrast to a recent study of the same species (Nordmann and Morris 1980), demonstrate that typical "synaptoid" complexes are a feature of this neurohaemal organ, as of most others. Furthermore, although the zinc iodide-osmium tetroxide (ZIO) reagent has been widely used to visualize synaptic (review by Akert and Sandri 1975) and synaptoid vesicles (e.g., see May and Golding 1982b), the present study represents, to our knowledge, the first application of this technique to the class Crustacea.

Materials and methods

Specimens of the shore crab *Carcinus maenas* were collected locally in the north-east of England. Some were adapted to 30% sea water prior to fixation. Sinus glands were dissected out and fixed at 0–4° C in the following solutions:

(1) 1% OsO_4 in veronal acetate buffer, pH 7.4.

(2) 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, containing 0.35 M sucrose, followed by 1% OsO_4, as recommended by Weatherby (1981) on the basis of an extensive study of fixation methods.

(3) 6.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, followed by exposure for 12–16 h to an ZIO mixture prepared according to the method of Sandri et al. (1978).

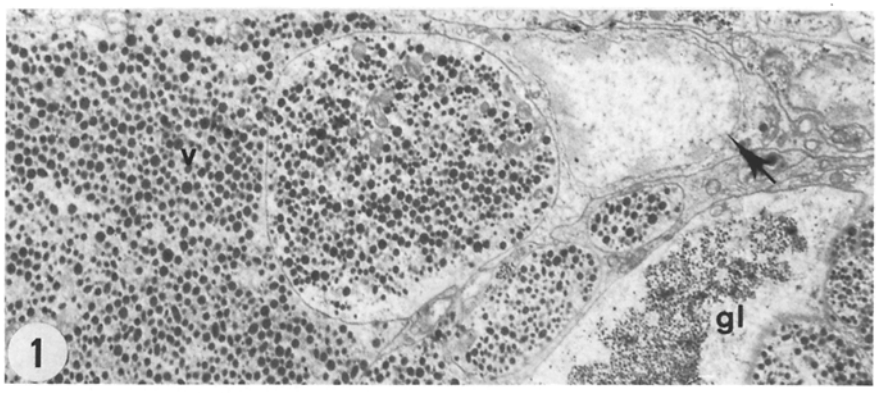
(4) Procedures for the cytochemical demonstration of amines according to the chromate method of Tranzer and Richards (1976) and the $KMnO_4$ method of Fillenz and Pollard (1976).

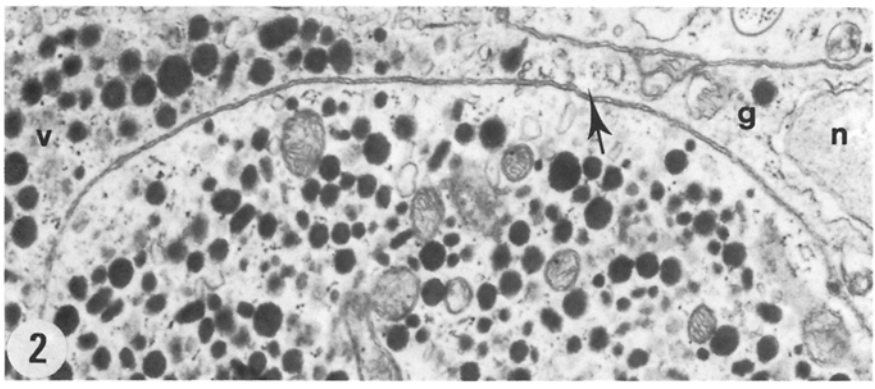
Specimens were dehydrated in graded alcohols, transferred to propylene oxide, and embedded in Araldite. The sinus gland was located by staining 1 μm thick sections in a solution of 1% toluidine blue in 1% borax. Thin sections were mounted on copper grids, stained with aqueous uranyl acetate and lead citrate (only the latter was used for specimens fixed in ZIO and for amine cytochemistry) and examined in an AEI 6B and a Kratos Cora electron microscope.

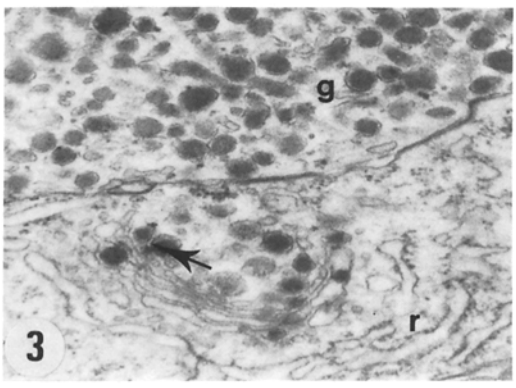
Results

Intrinsic cells

An extensive search was made for intrinsic cells within the gland. It is quite clear that the great majority of cells present are non-secretory glial cells. However, definitive secretory cells have been encountered, albeit very rarely. Figure 1 shows a cell body situated at the border of the gland, adjacent to the surrounding nervous tissue. The nucleus measures about 4 μm across in this profile and is surrounded by a thin layer of cytoplasm approximately 0.2 μm thick. The main feature of the perikaryon is the presence of ribosomes either associated with the outer leaflet of the nuclear envelope or free within the cytoplasm. The cisternae enclosed by the envelope are distended. A small number of mitochondria are present. Although the cell is, in some ways, an unlikely candidate for identification as a secretory cell, it is attached by a neck approximately 5 μm long to a large, typical, neurosecretory







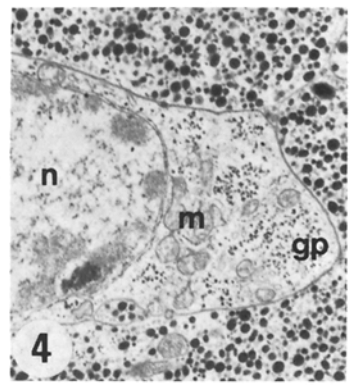


Fig. 1. Sinus gland of *Carcinus maenas*. Intrinsic cell body (*arrow*) connected to large neurosecretory varicosity (*v*); *gl* glia. OsO_4. ×7000

Fig. 2. Intrinsic cell body with nucleus (*n*) connected to varicosity (*v*) by short "neck" (*arrow*); *g* neurosecretory granule in perikaryon. OsO_4 ×22000

Fig. 3. Formation of secretory granules (*arrow*) by Golgi apparatus of intrinsic cell. *r* RER; *g* neurosecretory granules of adjacent varicosity. OsO_4 ×15000

Fig. 4. Glial cell with nucleus (*n*), mitochondria (*m*) and glycogen particles (*gp*). OsO_4 ×6000

varicosity packed with elementary granules (Fig. 2). In an adjacent section, a probable Golgi complex was observed just within the varicosity (not shown). Furthermore, five granules identical in appearance to those in the varicosity were observed in the perikaryon. Another process extending from the cell contained a similar granule. Thus the cell was multipolor, having at least three processes.

We were unable to determine whether the thin layer of cytoplasm which apparently comprised the perikaryon was truly typical of the cell described above. However, it is clearly not a feature of all intrinsic cells. That shown in Figure 3 contains much RER and a Golgi complex from which inclusions, similar in appearance to those in the adjacent neurosecretory fibre, are apparently being produced. Similarly, an expansive perikaryon containing typical neurosecretory granules was seen in this species by Smith (1974). In our material, glial cells differ in appearance from intrinsic cells in that their cytoplasm contains much glycogen but no secretory granules (Fig. 4).

Exocytosis

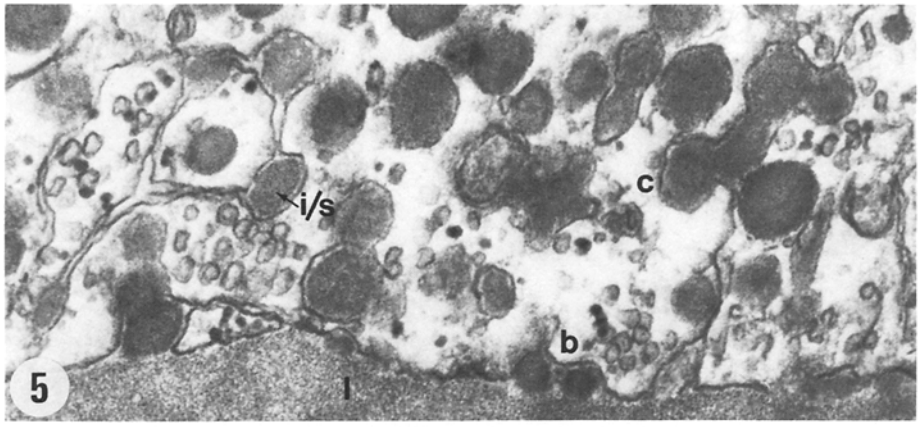
We have provisionally identified five types of neurosecretory terminals on the basis of the size and appearance of the elementary granules they contain, but we present no detailed analysis in this regard. Signs of exocytosis were very common in our specimens (Fig. 5). Omega profiles may be *basal*, i.e., situated adjacent to the external lamina of the blood sinus; or *interterminal* and involve the extrusion of granule contents adjacent to neighbouring endings. *Simple* profiles each apparently involve only a single granule. In contrast, *compound* figures have an elongate, beaded shape, and may contain several granule cores which remain partially discrete. They are presumably formed by the fusion of one or more granules with a pre-existing simple profile.

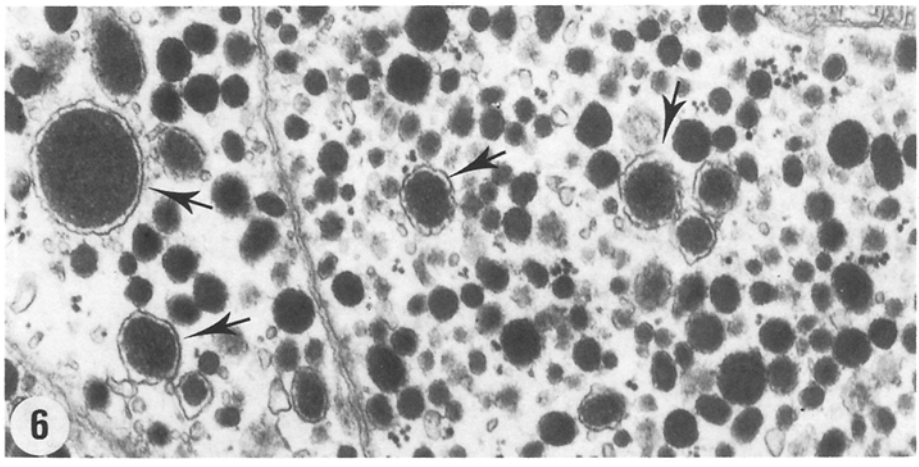
Intermingled with typical *elementary* granules in both terminal and preterminal regions are inclusions which we call *secondary* granules (Fig. 6). They are 200–400 μm in diameter and are probably formed by the fusion of two or more elementary granules. Inclusions with a dumb-bell shape have been observed and may be the products of fusion which occurred immediately prior to, or during, fixation. Secondary granules with circular profiles have crenelated bounding membranes separated from the dense core by a wide halo. Exocytotic omega profiles apparently involving inclusions of the secondary granule type have been observed in a basal position (Fig. 7).

We cannot rule out altogether the possibility that all secondary granules merely represent compound exocytotic profiles in which the point of contact between granule and plasma membranes is not present in the section concerned. However, our observations, which include examination of serial sections, suggest otherwise. Secondary granules seemed more common in crabs adapted to 30% sea water – a natural regime designed to induce a measure of stress (cf. Zanders 1980). However, we have yet to substantiate this objectively.

Vesicular elements

Lucent vesicles 20–30 μm in diameter are often closely associated with secondary granules and their origin as protrusions of the granule membrane is suggested by





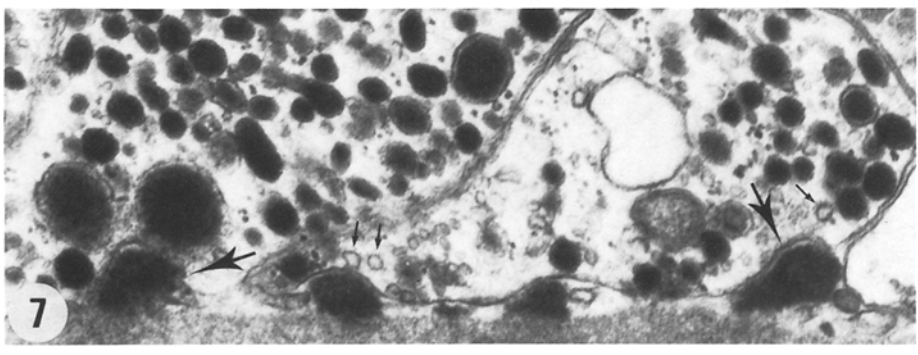


Fig. 5. Patterns of exocytosis – basal (*b*), interterminal (*i*), simple (*s*) and compound (*c*); *l* basal lamella. Glutaraldehyde/OsO_4 × 60 000

Fig. 6. Giant "secondary" granules (*arrows*) intermingled with typical elementary granules. OsO_4 × 40 000

Fig. 7. Omega profiles (*arrows*) involving probable secondary granules, and associated coated vesicles (*small arrows*). OsO_4 × 40 000

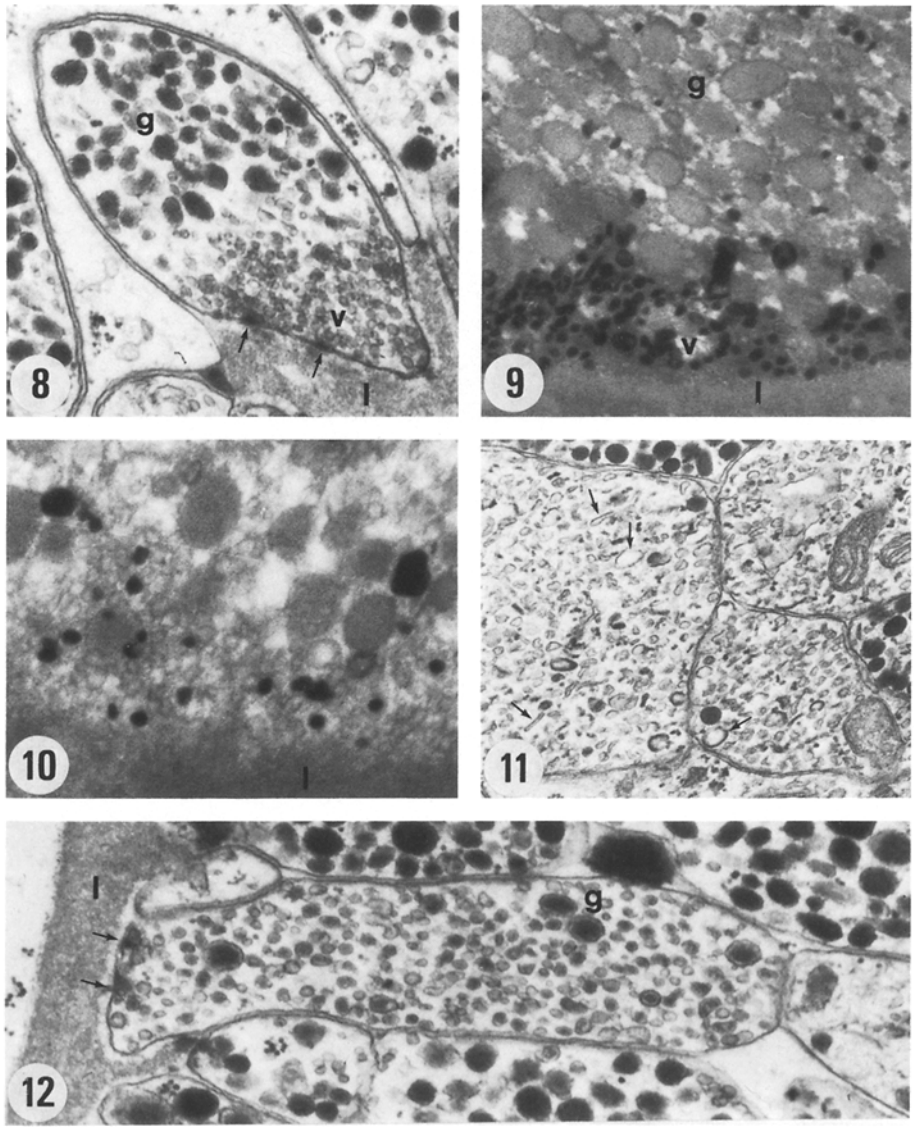


Fig. 8. Synaptoid terminal with neurosecretory granules (*g*), lucent vesicles (*v*), and membrane thickenings (*arrows*); *l* basal lamella. OsO_4 ×40 000

Fig. 9. Densely stained synaptoid vesicles (*v*) clustered adjacent to basal lamella (*l*); *g* neurosecretory granules. Glutaraldehyde/ZIO ×40 000

Fig. 10. Intermingling of stained and unstained vesicles; *l* basal lamella. Glutaraldehyde/ZIO ×60 000

Fig. 11. Neurosecretory terminals containing mixed populations of vesicular inclusions including vacuoles and flattened sacs (*arrows*). Glutaraldehyde/OsO_4 ×24 000

Fig. 12. Synaptoid terminal containing granulated vesicles and some larger granules (*g*). *Arrows*, membrane thickenings and associated electron lucent vesicles; *l* basal lamella. OsO_4 ×40 000

some micrographs (Fig. 6). Coated vesicles about 30 μm in diameter sometimes bear a similar relation to the exocytotic profiles of these granules (Fig. 7).

Prominent aggregations of vesicular and vacuolar elements are present in some terminals. We tentatively distinguish two categories of vesicle clusters. A minority of terminals contain synaptoid complexes (Fig. 8) characterized by vesicles which are comparatively uniform in shape and size (20–40 μm in diameter) and which are clustered adjacent to membrane thickenings and the external lamina of the blood sinus. Neurosecretory granules "stack back" from the presumed site of secretory release and dominate the body of the terminal and the preterminal axon.

Synaptoid complexes within typical neurosecretory endings have been identified in specimens impregnated with the ZIO reagent. Vesicles are typically densely "stained" (Fig. 9), although in some cases inclusions with positively reacting cores intermingle with those whose contents remain lucent (Fig. 10). Lysosomes and mitochondria are often deeply stained, but the cores of neurosecretory granules typically give a negative reaction.

In contrast to synaptoid complexes, some terminals contain aggregations of vesicles and vacuoles whose members vary widely in size (20–200 μm) and form (Fig. 11). Neurosecretory granules may intermingle with these lucent inclusions. Such mixed populations have not been identified in specimens impregnated with ZIO, possibly due to the comparatively poor state in ultrastructural preservation of the latter.

A small minority of fibres within the gland do not resemble typical neurosecretory elements, being characterized by their content of inclusions of the "granulated vesicle" type approximately 50 μm in diameter (Fig. 12). Granules more than 100 μm in diameter have been encountered in terminal regions, but since they are apparently absent from preterminal axons, it is possible that they are "secondary" granules. The terminals form synaptoids with small numbers of lucent vesicles clustered around membrane thickenings (Fig. 12).

Amine cytochemistry

Our study indicates that granules within the sinus gland, including the small inclusions described above, give a negative reaction to the cytochemical methods employed (in contrast to certain elements in annelid nervous systems; Golding, unpublished).

Discussion

Secretory "intrinsic" cells are a constituent of the sinus gland of *Carcinus*, although such elements are encountered very rarely. Thus the organ is a gland in what for Gabe (1966) was "the true sense of the term" in that "an on-site process of secretion" takes place within it.

The association of intrinsic cells with a neurohaemal complex is a feature of several invertebrate systems (e.g., the pericardial organs of crabs, Cooke 1964; the lateral nerve plexus of isopods, Juchault and Kouigan 1975; the corpus cardiacum of insects, Normann 1973; Krogh 1973; the infracerebral gland of annelids, Golding and Whittle 1977). In each case it is probable that the cells are

neurosecretory neurones. Smith (1974) characterized a cell containing secretory granules, which he encountered in the sinus gland, as a "glial" cell. Our observations indicate that such cells are continuous with typical neurosecretory varicosities and should therefore be regarded as secretory nerve cells.

The secretory granules in the cells we have examined in *Carcinus* are apparently identical to those within one type of neurosecretory terminal, which is present in abundance. Consequently, it is probable that one category of neurone has some representatives within the gland, whereas the majority are extrinsic to the latter and are located in the ganglionic X-organs (review by Gabe 1966). Similarly, although the perilamellar neurohaemal complex of the polychaete *Nephtys* is typically devoid of secretory cells (Zahid and Golding 1975), a single intrinsic cell containing granules apparently identical to those within the neurosecretory terminals has now been encountered (Al-Yousuf, personal communication).

The formation of giant granules, apparently by fusion of two or more elementary neurosecretory granules, has been observed previously only in the corpora cardiaca of locusts treated with lindane (Normann and Samaranayaka-Ramasamy 1977). Our study indicates not only that such "secondary" granules can be formed in unpoisoned material, but that these inclusions discharge their contents by exocytosis. It is noteworthy that elevated concentrations of calcium induce fusion of secretory vesicles isolated from pancreatic islets (Dahl and Gratzl 1976). The phenomenon suggests that neurosecretory granules are more labile than is often thought.

Nordmann and Morris (1980) distinguished between, on one hand, microvesicular inclusions and, on the other, vacuolar elements thought to be involved in membrane retrieval following exocytosis, but apparently failed to find typical synaptoid complexes. Our study of the same species establishes that such complexes are present in a minority of terminals, and similar findings relate to the sinus gland of, for example, the crayfish *Procambarus clarkii* (Bunt 1969 – see Fig. 1). As in other groups, synaptoids in *Carcinus* are characterized, first, by the zonation of vesicular and granular inclusions within the terminal (May and Golding 1982a); second, by the aggregation of vesicles around membrane thickenings (Binnington 1980); and last, by the affinity of the vesicle contents for the ZIO reagent (Rufener and Dreifuss 1970; Scharrer and Wurzelmann 1974), although the significance of this reaction remains unknown (see May and Golding 1982b, for brief discussion). The role of synaptoid vesicles remains controversial (review by Morris et al. 1978).

The mixed populations of vesicular elements present in some terminals include larger vacuoles and flattened cisterns of the type thought to mediate membrane retrieval (Nordmann and Morris 1980). However, small vesicles are present also, in abundance. Furthermore, coated microvesicles have been observed in close association with exocytotic profiles (as at central sites in annelids, Golding and May 1982). This suggests that elements of diverse sizes may be involved in membrane retrieval, particularly if compound exocytosis has occurred. The existence of two distinct populations of lucent inclusions, comparable to synaptoid vesicles and vesicles involved in membrane retrieval, respectively, is more readily recognized within terminals in certain invertebrate neuropiles (Golding and May 1982).

Membrane fragmentation may also follow the formation of secondary granules, since smaller areas of membrane are required to envelop such inclusions. It is noteworthy that possible signs of such vesiculation feature not coated, but smooth surfaced profiles.

Fibres of one type present in the sinus gland (see Fig. 12) contain secretory granules more reminiscent of aminergic than of typical peptidergic neurones (Knowles 1965). However, our cytochemical investigation, like previous histochemical tests (Goldstone and Cooke 1971; Strolenberg et al. 1977), has yielded negative results. The fibres concerned probably correspond to the small diameter, "non-neurosecretory" and possibly "sensory" axons which, in the crayfish, have their origin in the brain rather than within the eyestalk, and which form a discrete component of the neurosecretory tract (Andrew et al. 1978). Our observations vindicate their endocrine status.

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