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Thoracic Neurosecretory Structures in Brachyura
III. Microanatomy of Peripheral Structures

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The trunks of the brachyuran pericardial organs consist of an *inner core* of nerve fibers, connective tissue, and blood vessels and an *outer cortex* of secretory terminals. The terminals contain granules which can be visualized by darkfield, phase, or electron microscopy. A thin (less than 1 μ), amorphous, acellular epineurium separates the terminals from the surrounding hemolymph. Selective stains suggest that there may be three kinds of secretory terminal in the crab, *Carcinus*.

The cortex ranges from about 20% of total pericardial organ volume in some specimens of *Carcinus* down to a negligible percentage in *Libinia*. Secretory granules form less than 10% of the volume of the terminals in the cortex.

The electron dense secretory granules have an outer membrane. They range in diameter from about 0.05 to 0.5 μ. In *Carcinus* large (0.17 μ) and small (0.14 μ) granules occur in separate terminals. In *Cancer* only one population of granules has been found (0.15 μ). In *Libinia* terminals appear to contain either one (0.09 μ) or two populations (0.06 and 0.15 μ). In *Carcinus* and *Cancer* a third kind of terminal appears to contain vesicles about 0.03 to 0.05 μ in diameter.

INTRODUCTION

The first two papers of this series (Maynard, 1961a,b) have described the gross anatomy of brachyuran thoracic neurohaemal structures and the characteristics of the secretory neurons supplying them. This paper considers the histology of the pericardial organs (PO). Both conventional and electron microscopy indicate that the secretory terminations of the PO are analogous to those of the sinus gland in Crustacea (Hodge and Chapman, 1958; Welsh, 1959) and the system of dorsal trunks in Stomatopoda (Alexandrowicz, 1953b; Knowles, 1959). The pericardial organs are not identical in all forms, however, and interspecific differences appear to exist on both the microscopic and submicroscopic levels.

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MATERIALS AND METHODS

The species used and the methods of collection have been given in the preceding papers of this series (Maynard, 1961a,b). For light microscopy both fresh material and paraffin sections were used. Fixatives included Bouin with 1% $CaCl_2$, Susa, Helly, Zenker, Orth's, and 2% OsO_4 in buffered sea water. Stains included Masson's trichrome, Heidenhain's azan, aldehyde-fuchsin (Dawson, 1953), and chrome alum hematoxylin phloxine. Material to be used for electron microscopy was fixed with buffered 1–2% OsO_4, embedded in methacrylate, cut at about 0.05–0.1 μ, and mounted on a formvar film. These sections were examined with a Model EMU-3B, RCA electron microscope (EM) at initial magnifications of 1600 to 18,000. A diffraction grating replica was used for calibration of the EM.

Diameters of secretory granules were determined by measuring the granule area on enlarged electron micrographs with a planimeter and then making the appropriate corrections for diameter. With this method it is likely that the end slices of the granules (see Fig. 1) were neither sufficiently dense nor had sufficiently sharp boundaries to be counted. Such a systematic error,

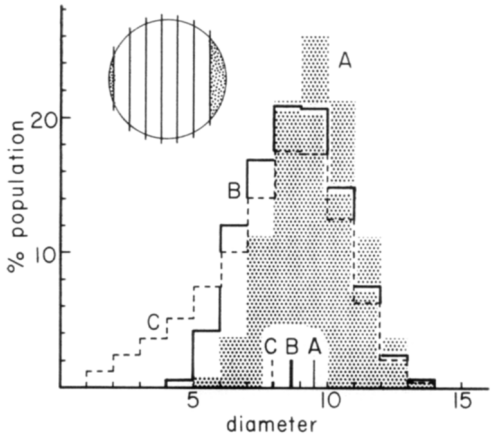


FIG. 1. Comparison between populations of diameters of spheres and of sections taken from such spheres. *Diameter* is given in units of section thickness, see inset. A. Original sphere population, normal distribution (stippled histogram); mean diameter, 9.5; standard deviation, about 1.5. B. Distribution of section population lacking all end slices (see stippled sections in inset); mean diameter, 8.7. C. Distribution of total section population; mean diameter, 8.0.

however would tend to shift the distribution of section diameters toward that of the original granule population. Consequently the difference between the average measured section diameter and the average granule diameter is probably less than 10%. More complete theoretical discussions of this "tomato salad" problem have been provided by Elias (1954), Lenz (1956), and Bach (1959). Additional errors resulting from variation of the initial magnification or from distortion during preparation of the specimen probably occurred. As a result of all these, the absolute diameter of the granule *in vivo* is probably rather larger than that reported in this paper, possibly as much as 10 to 15%.

RESULTS

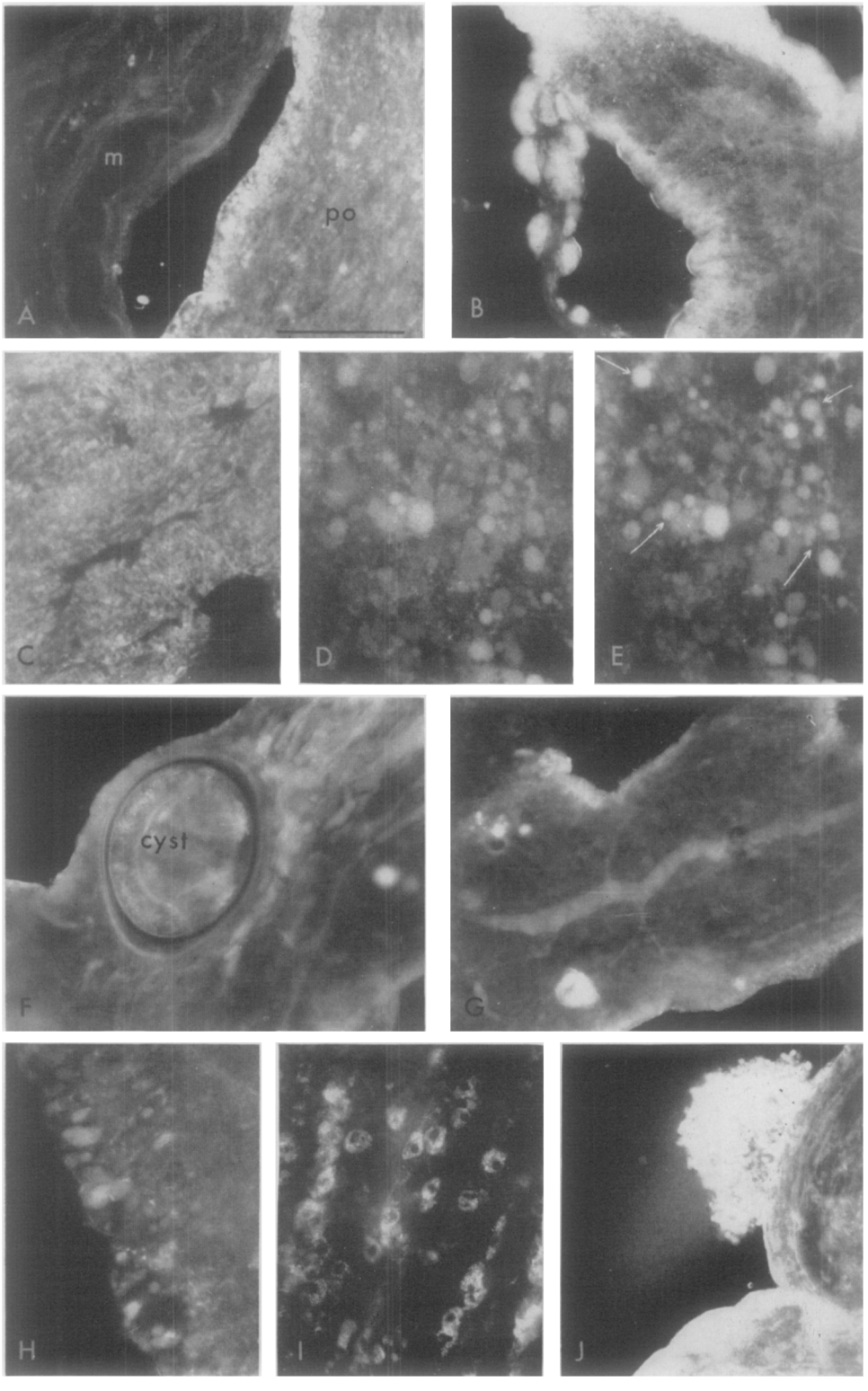
OBSERVATIONS ON LIVING, UNSTAINED ORGANS

The PO of many species (*Carcinus maenas, Goniopsis cruentata, Gecarcinus lateralis, Grapsus grapsus, Plagusia depressa, Pachygrapsus crassipes, Callinectes ornatus*) usually appear opaque and white or bluish-white with incident illumination (Fig. 2). Organs in different individuals vary with respect to relative content of refractile material and in extreme cases, the posterior bars may be covered with opaque, blister-like protuberances (Figs. 2B, 4C, 5B). In *Libinia emarginata* and *Cancer borealis* the PO is translucent, not opaque. Under intense illumination it has a characteristic smokey-blue hue which distinguishes it from the usual nerve.

The opacity and whiteness of the PO under darkfield or incident illumination result mainly from an outer cortex of granular cytoplasmic blebs (Figs. 2A, 2H). Granular hemocytes (Fig. 2I) and nerve fibers containing refractile material contribute to a lesser extent. The peripheral blebs are presumably terminations of secretory nerve fibers. Sometimes connections can be observed between bleb and fiber (Fig. 3). We could not be certain however, that all blebs normally possessed such connections. Certainly in preparations which have been isolated for some time or excessively compressed, blebs are present which have no connection with nerve fibers and which yet maintain their structural integrity and can be moved within the PO by judicious pressure on the coverglass. Although these isolated blebs certainly originate as terminals and many are obviously produced by adverse treatment of the PO, the possibility of such "pinching off" within the normal course of events in the crab remains. Both blebs and nerve fibers stain selectively with methylene blue and so correspond to the structures described by Alexandrowicz (1953a).

The surface texture of the PO cortex varies among individuals and species. In many instances the mass of terminations spreads like the bark of a tree over the more transparent core of fibers and connective tissue (Figs. 2B, 2C). In others, spherical or oval terminal blebs are scattered over the PO just beneath the epineurium which forms the outermost surface of the organ (Figs. 2D, 2E, 2H) (Knowles, 1959). The epineurium can be distorted without breaking, and therefore has some mechanical strength.

The terminals vary in hue and brilliancy under darkfield illumination. Many are a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bright, opaque white; others are blue- or green-white; and still other blebs are a much dimmer, iridescent blue. The color of the light scattered by the terminals can be demonstrated with red and blue filters (Figs. 2D, 2E). In some cases, white, blue-white, and green-white masses occur in the same nerve fiber (Fig. 3). The opaque blebs have a granular texture under high magnification, and when crushed release granular, refractile material into the surrounding solution (Fig. 2J). They are apparently filled with secretory granules similar to those observed in the PO cells and in the fibers from the C-cells (Maynard, 1961b). Such blebs may also contain clear vacuoles one or more microns in diameter. When the granular masses occur in nerve fibers rather than terminal blebs, they are often concentrated along the sides of the axon leaving a central channel of clear axoplasm. Such concentrations may cause extensive localized swelling of the axon and occur in fixed as well as fresh preparations (Figs. 4D, 4E, 4G, 4H, 8A).

Although direct attempts to observe normal granule movement in the excised PO fibers were unsuccessful, there is indirect evidence for such movement. In *Callinectes* infested with encysted flatworms, cysts occasionally occur in the PO (Fig. 2F). Their presence occludes secretory fibers and leads to accumulation of granular material in fibers on one side of the cyst. The approximate position of the cyst and the resulting accumulations of material suggest that the fibers involved must originate in the C-cells or the B-cells of the first three segmental nerves (Maynard, 1961b).

Anterior Ramifications (AR)

The structure of the anterior portion of the secretory complex, the AR, is analogous to the posterior portion, the PO. Both branching nerve fibers containing granules (Figs. 2G, 4G, 4H) and terminal blebs are present.

Observations on Fixed and Stained Organs (*Carcinus, Cancer, Libinia*)

The pericardial organs can be divided into a cortex and a central core (Fig. 4A). The cortex contains the nerve terminals, some connective tissue or glial cells, and often the posterior pericardial neuron somata. The core contains secretory and, at least in some places, motor nerve fibers, connective tissue cells, cuboidal cells forming walls of small arteries, hemocytes contained within these vessels, and usually, the anterior PO neuron somata. The cortex may be absent in some regions of the ventral and longitudinal trunks and is most prominent in the anterior and posterior bar regions.

The epineurium of the PO has no closely associated nuclei and usually stains like the connective tissue fibers of the core. The cortex has little or no fibrous connective tissue and nuclei are comparatively rare in *Carcinus* and *Cancer*, but fairly common

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Fig. 2. Pericardial organs and anterior ramifications. Fresh, unstained preparations, darkfield illumination with tungsten filament, slightly compressed with cover slip. A. Normal nerve trunk, left, compared with portion of PO, right. m, a large nerve fiber (*Plagusia depressa*). B. Posterior bars of PO. Note cortical accumulation of refractile material and blister-like protuberances (*Goniopsis cruentata*). C. Surface texture of PO trunk (*Plagusia depressa*). D. Surface texture of PO trunk, photographed with "red" light (Corning glass filter No. 3480) (*Grapsus grapsus*). E. Same preparation as 2D, but photographed with "blue" light (Corning glass filter No. 5031). Note terminals selectively scattering blue light (arrows). F. PO trunk containing trematode cyst. Note accumulation of refractile material in occluded fibers (*Callinectes* sp.) G. Portion of AR. Note branching secretory fiber and peripheral accumulation of refractile material (*Callinectes ornatus*). H. Edge of PO. Note terminal blebs just beneath epineurium (*Gecarcinus lateralis*). I. Granular hemocytes in PO (*Gecarcinus lateralis*). J. Crushed trunk of PO, excessive pressure on cover glass. Note (1) localized nature of epineurium rupture; (2) evagination of terminal blebs; (3) cloud of secretory granules released into surrounding sea water (*Plagusia depressa*). Calibration line in A represents 167 μ for A, B, F, G, J; 83 μ for C; 76 μ for I; 58 μ for D, E; and 55 μ for H.

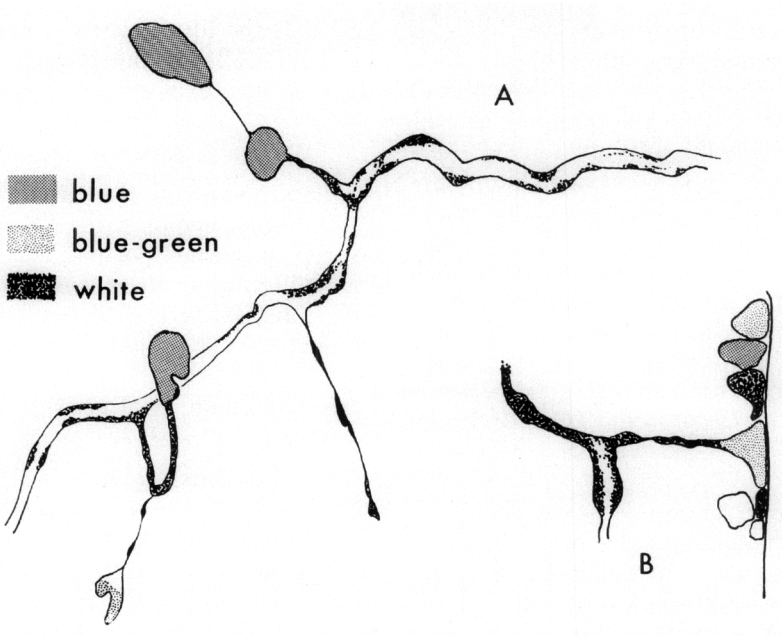


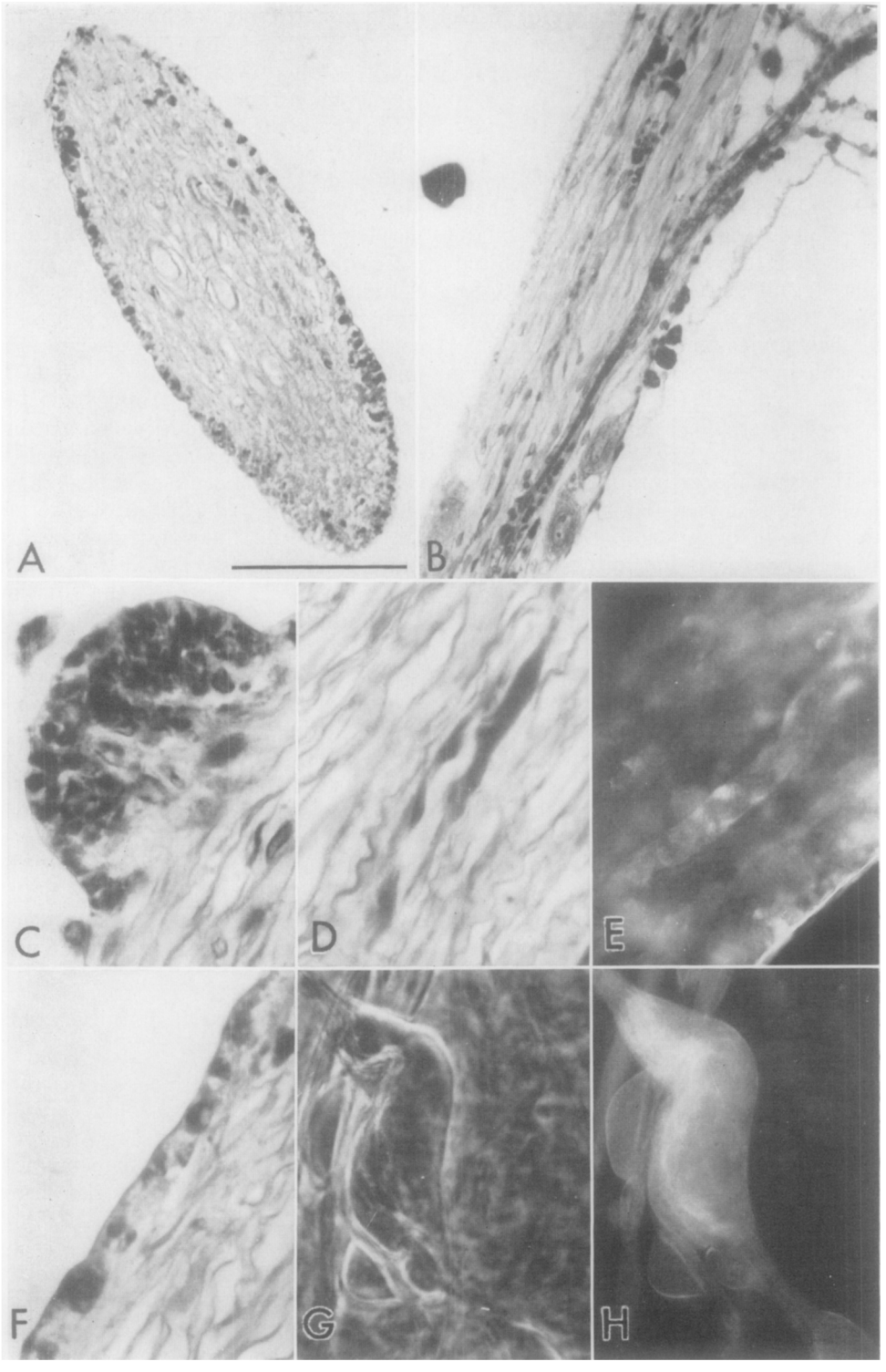
FIG. 3. Free-hand sketches of living terminal blebs in PO under dark-field illumination to show connections with nerve fibers. A. *Plagusia depressa.* B. *Callinectes ornatus,* epineurium to the right. Neighboring terminals without obvious fiber connections are also indicated.

in *Libinia.* Within the core connective tissue cells and extracellular fibrous material are plentiful. Many of the larger nerve fibers have a definite sheath. In *Carcinus* and *Cancer* connective tissue nuclei are particularly common at the junction between core and cortex.

Small arteries enter the PO at several points where there is contact with the pericardial lining (Fig. 4B). These arteries have a well developed, acellular intima which is surrounded by a layer of cuboidal cells. Their terminations are not obvious, and they seem to open into irregular cavities within the PO which contain large numbers of hemocytes. We could not be sure whether these cavities represent sinuses bounded by a thin epithelium or true interstitial spaces. Hemocytes were less common in the cortex than in the core of the PO.

The secretory terminals of the cortex

FIG. 4. A. Diagonal section of PO trunk. Note nerve fibers in core and layer of azocarmine-staining terminals in cortex (*Carcinus maenas,* OsO_4 fixation, Heidenhain's azan, 3 μ section). B. Section of posterior bar region of PO. Note small artery entering PO trunk from upper right corner. Cortex is very thin, but there are two posterior PO cells (*Carcinus maenas,* Bouin's, Heidenhain's azan, 10 μ section). C. Section of blister-like protuberance, see Fig. 2B (*Carcinus maenas,* Bouin's, Heidenhain's azan, 10 μ section). D. Longitudinal section of nerve fiber in PO trunk. Note peripheral accumulations of azocarmine-stained material, see Fig. 8A (*Carcinus maenas,* OsO_4 fixation, Heidenhain's azan, 3 μ section). E. Nerve fiber in PO trunk containing peripheral accumulations of refractile material (*Plagusia depressa,* fresh, unstained preparation, slightly compressed, darkfield illumination). F. Edge of PO trunk. Note cortical layer of terminals, some staining with azocarmine, that lies just beneath epineurium (*Carcinus maenas,* OsO_4 fixation, Heidenhain's azan, 3 μ section). G. Swollen nerve fibers in trunk of AR (*Callinectes* sp., fresh, unstained preparation, slightly compressed, Leitz phase optics). H. Same preparation as 4G, but with darkfield illumination. Note course of fibrous material in nerve fiber and refractile material, probably secretory granules, filling swellings. Calibration line in A represents 110 μ for B; 100 μ for A; 68 μ for E; 38 μ for G, H; 36 μ for C; and 23 μ for D, F.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are best considered separately for each species, and for each stain. In *Carcinus*, at least, there is tinctorial evidence for three types of secretory termination.

Carcinus maenas. The cortex is typically 4 to 8 μ thick. This is about 10% of the PO trunk radius, or treating the PO as a cylinder, about 20% of PO volume. There is variation in cortex thickness from place to place along the PO trunks. In localized regions, terminals may accumulate to a depth of 10 μ or more, or even protrude in blister-like projections (Figs. 4C, 5B).

Heidenhain's azan. *Type I* terminals generally stain brilliant red with azocarmine, even after OsO_4 fixation (Fig. 4F). These endings may be scattered among Type II terminals, or may occur together producing large areas of almost pure red cortex. Masses of material with similar affinities for azocarmine often occur in nerve fibers in the core and occasionally in the PO neurons (Maynard, 1961b). Such masses resemble the clumped granules in living axons (Figs. 4D, 4E) and there is little doubt that Type I terminals represent terminal blebs of neurons filled with secretory granules as observed in fresh preparations.

Type II endings usually stain with orange G, but may appear pink or purple according to vagaries of staining. After OsO_4 fixation they are brown or grey. Clumped orange material does not occur in axons, but orange terminals do occur in masses and occasionally form the major component of PO blisters (Fig. 4C). Some terminals give staining reactions between Type I and Type II. Small vacuoles occur in both red and orange endings.

Type III endings are the least common and occur widely scattered among the other terminals. They characteristically stain blue or blue-grey with aniline blue. The granules of the hemocytes stain brilliant red.

Aldehyde-fuchsin (Rosa-Halmi according to Dawson, 1953). Terminations in the PO cortex stain deep purple, orange, or light blue. Although it is likely that these correspond to Types I, II, and III respectively, the absence of consistent localization of terminal types within the PO makes such correlations uncertain, even when adjacent sections of the same organ are used. Hemocyte granules stain purple or orange.

Chrome-alum-hematoxylin-phloxine. Terminals appear grey or grey-violet and brilliant red. Many of the axons are red or pinkish. Hemocyte granules stain red and purple.

Cancer irroratus (and *C. borealis*). Staining is generally not as satisfactory with these species as with *C. maenas*. The general histology of the PO is very similar to that of *Carcinus*, but the cortical layer may be less thick. With Heidenhain's azan, pure red terminals are less common than in *Carcinus*, the majority are purple, or after OsO_4, brown. Hemocyte granules are red or blue. After aldehyde-fuchsin, the terminals are purple, and there are no pure orange endings. Hemocyte granules are also purple. In *Cancer*, therefore, the division of PO terminals into three tinctorial types does not seem to be justified.

Libinia emarginata. The PO cortical layer is almost absent. Terminals are small (2–4 μ in diameter), widely scattered, and are generally one color blue-grey, when stained with Heidenhain's azan. Occasional red spheres 1 to 2 μ in diameter located beneath the epineurium may represent a second kind of termination. The tissue surrounding the terminals stains pink and appears to be the cytoplasm of connective tissue cells. Other stains were not used in *Libinia*.

Observations with the Electron Microscope

Pericardial organs from *Cancer irroratus*, *Carcinus maenas*, and *Libinia emarginata* were examined. One PO from *Carcinus* was fixed in 10% formalin to demonstrate the intrinsic electron density of the secretory granules and epineurium (Fig. 5E).

Epineurium. The acellular sheath or epineurium separating the PO secretory terminals from the surrounding hemolymph ranges between 0.1 and 0.7 μ in thickness in *Carcinus maenas*. In our pictures it is generally a moderately dense, amorphous material which is slightly darker at the

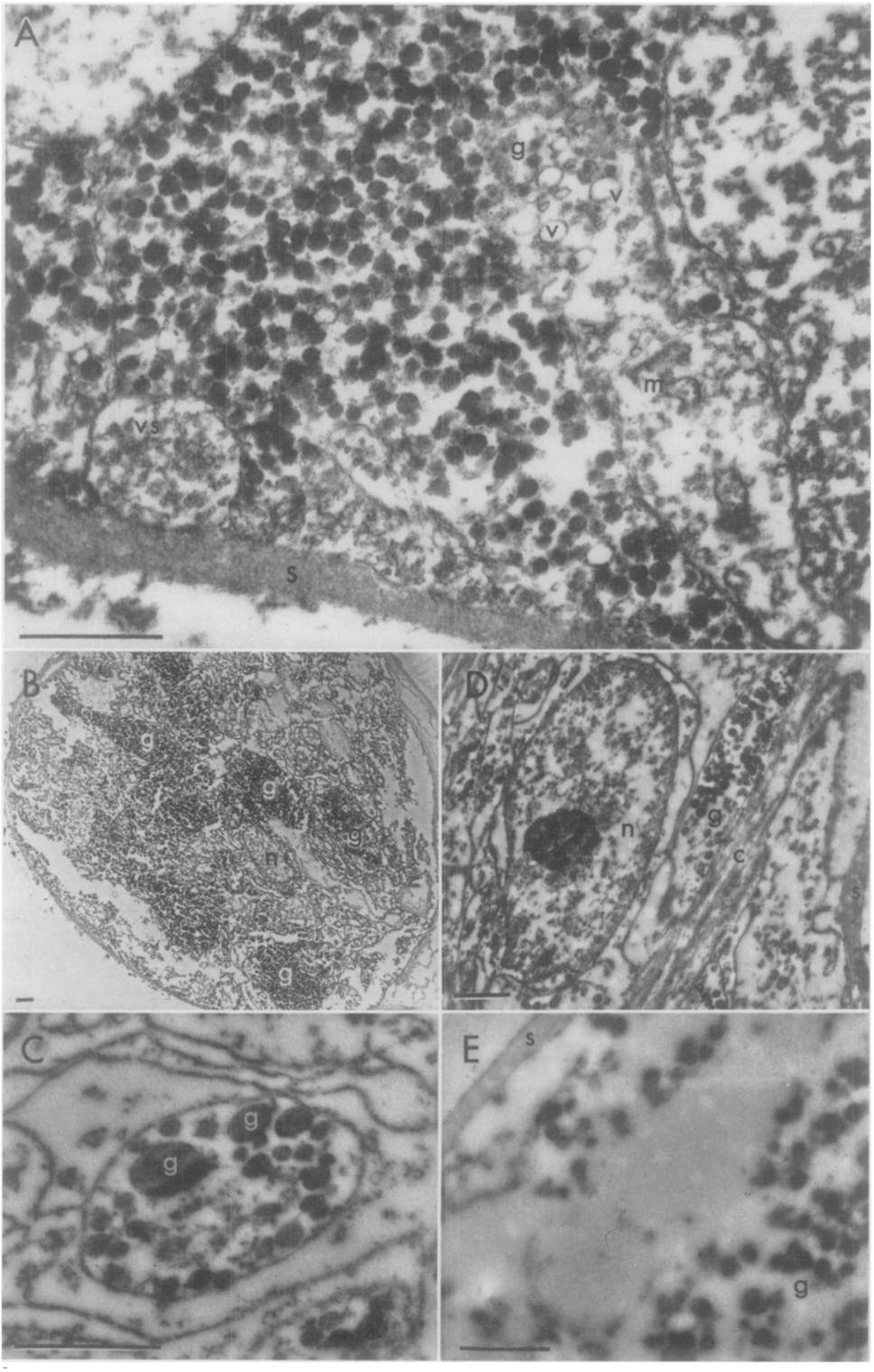


FIG. 5. A. Edge of PO (*Carcinus maenas*). B. Section through presumed blister-like protrusion (*Carcinus maenas*). C. Small, preterminal process (*Libinia emarginata*). D. Edge of PO (*Libinia emarginata*). E. Edge of PO, formalin fixation (*Carcinus maenas*). c, fibrous connective tissue; g, secretory granules; m, mitochondrion; n, nucleus of connective tissue cell; s, epineurium; v, vacuoles; vs, vesicles (secretory?). Electronmicrographs, all calibration lines represent 1 μ.

outer edge and which occasionally seems to be torn or riddled with open spaces along its inner margin (Fig. 6B). The epineurium is not bounded by a well-defined membrane, and neuron terminals come into direct contact with its inner edge (Figs. 5–8). In *Libinia,* and occasionally in *Carcinus,* fingers of extracellular material reach from the inner edge of the epineurium into the core of the PO. In *Libinia* these projections and the neighboring inner border of the sheath may show a laminar structure. Specific cells associated with sheath production were not identified.

PO Cortex. Nerve terminals and connective tissue cell processes lie immediately beneath the epineurium. Dense secretory granules characterize many of the neuron terminations, but when these granules or connective tissue cell nuclei are lacking, distinction between neuron and nonneuron cytoplasm is not always certain (Fig. 5D). It is clear, however, that many of the secretory terminals are free from enveloping glial cells, for granule-containing structures may lie side by side with no intervening cytoplasm, and as indicated above, the cell membrane of neuron terminals usually borders directly on the PO sheath (Figs. 6, 8E). Granule-containing terminals are much more common in the *Cancer* and *Carcinus* specimens examined than in *Libinia,* confirming the impression obtained from light microscopy.

Extracellular fibrous material and connective tissue cell nuclei are usually absent from the most peripheral cortical layer in *Cancer* and *Carcinus.* They begin to appear a few microns in from the epineurium, however, often in conjunction with small, preterminal nerve fibers containing both neurofilaments and secretory granules or vesicles (Fig. 5D). In favorable sections the extracellular material appears in the form of long fibrils embedded in a "spongy" matrix. The cytoplasm of connective tissue cells contains scattered vesicles and short tubules similar to those described below in the neurosecretory terminals. Dense secretory granules, however, were never found in cytoplasm containing nuclei.

PO Core. Nerve fibers of reasonable size (5 μ) with thick connective tissue sheaths contain varying numbers of secretory granules and vesicles. As suggested by light microscopy, the granules often aggregate in dense masses in the peripheral parts of the nerve fibers, while a filamentous axoplasm winds around the clumped material (Fig. 8A). Extensive observations were not made on the core of the PO, and information on the PO neuron somata and PO blood vessels is lacking.

PO Terminals. The typical secretory terminal is filled with dense, spherical or elliptical granules (Figs. 5A, 6, 7). These granules often show no internal structure and seem to be enclosed within a double membrane (Figs. 7, 8B, 8D, 8E). Their diameter may range from 0.05 to 0.5 μ, but the size distribution within any one terminal is usually limited. Consequently, neurosecretory terminations may be characterized by their granule populations (Figs. 6, 7, 9).

In *Carcinus maenas* there are at least two kinds of terminal with dense granules. One contains granule populations with average measured section diameters of about 0.14 μ; the other, populations with average measured section diameters of about 0.17 μ (Figs. 7, 9). Terminals with large and small granules may lie side by side, and although the population means vary slightly among different specimens, intermediate populations do not seem to occur.

Although most granules seem to be dense masses enveloped by a smooth membrane as described above, variations may occur (see Knowles, 1960). In *Carcinus,* the membrane often seems to separate from the smooth surface of dense material. It may appear wrinkled or occasionally, seem to break up into vesicles surrounding the denser core of the granule (Figs. 7, 8D, 8E). In one specimen the usual homogeneity of the dense material of the granule was replaced in all terminals by a complex of wrinkled membranes and vesicles embedded in a less dense matrix (Fig. 8C). Granules with such internal

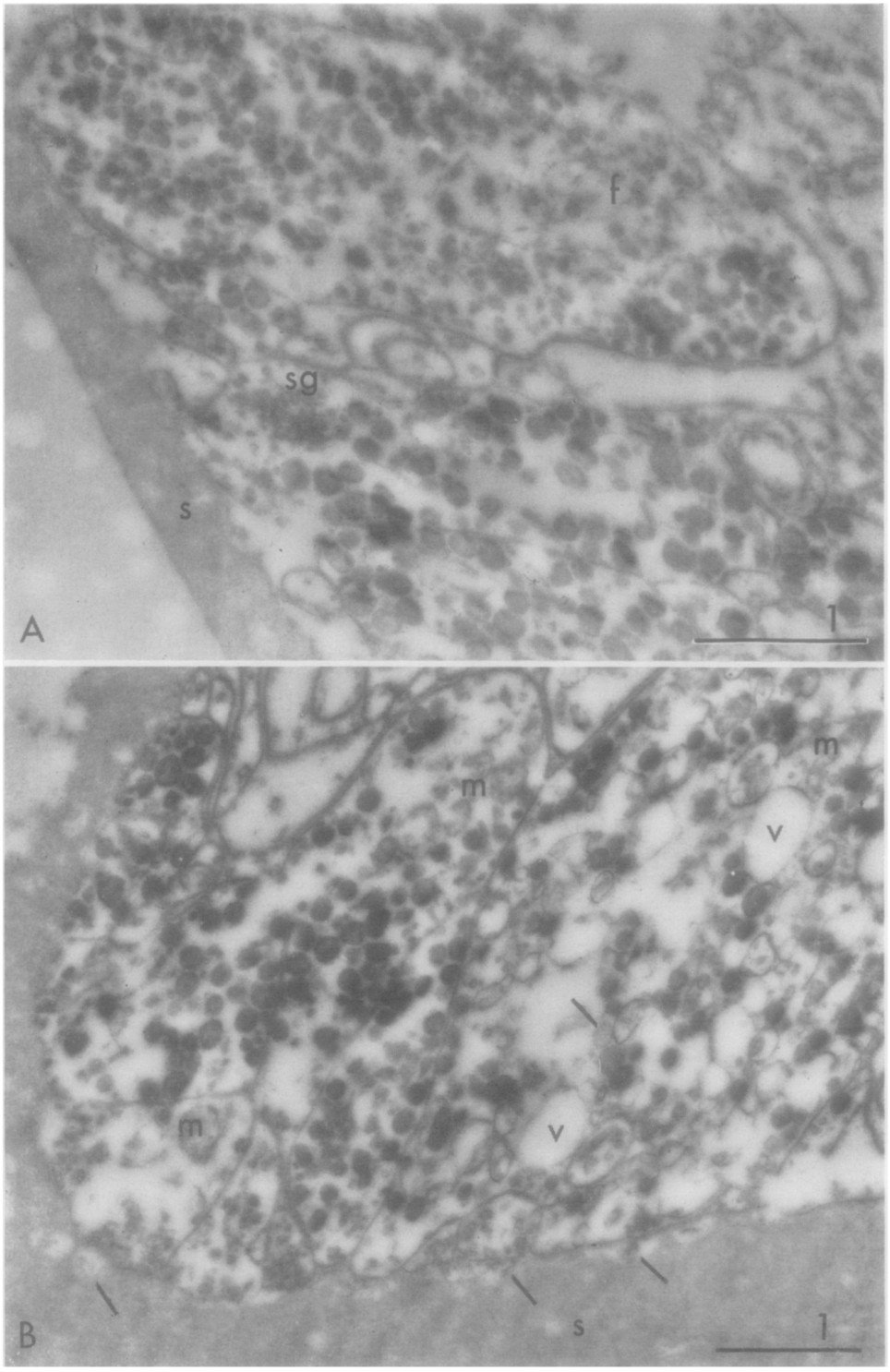


FIG. 6. Edge of PO showing accumulations of terminals (*Libinia emarginata*). In B: Arrow within terminal points to granule-sized object with internal structures. Arrows in epineurium point to material apparently located just outside the terminal membrane. f, neurofilaments at inner pole of terminal; m, mitochondrion; s, epineurium; sg, accumulation of small granules; v, vacuole. Electronmicrographs, calibration lines represent 1 μ.

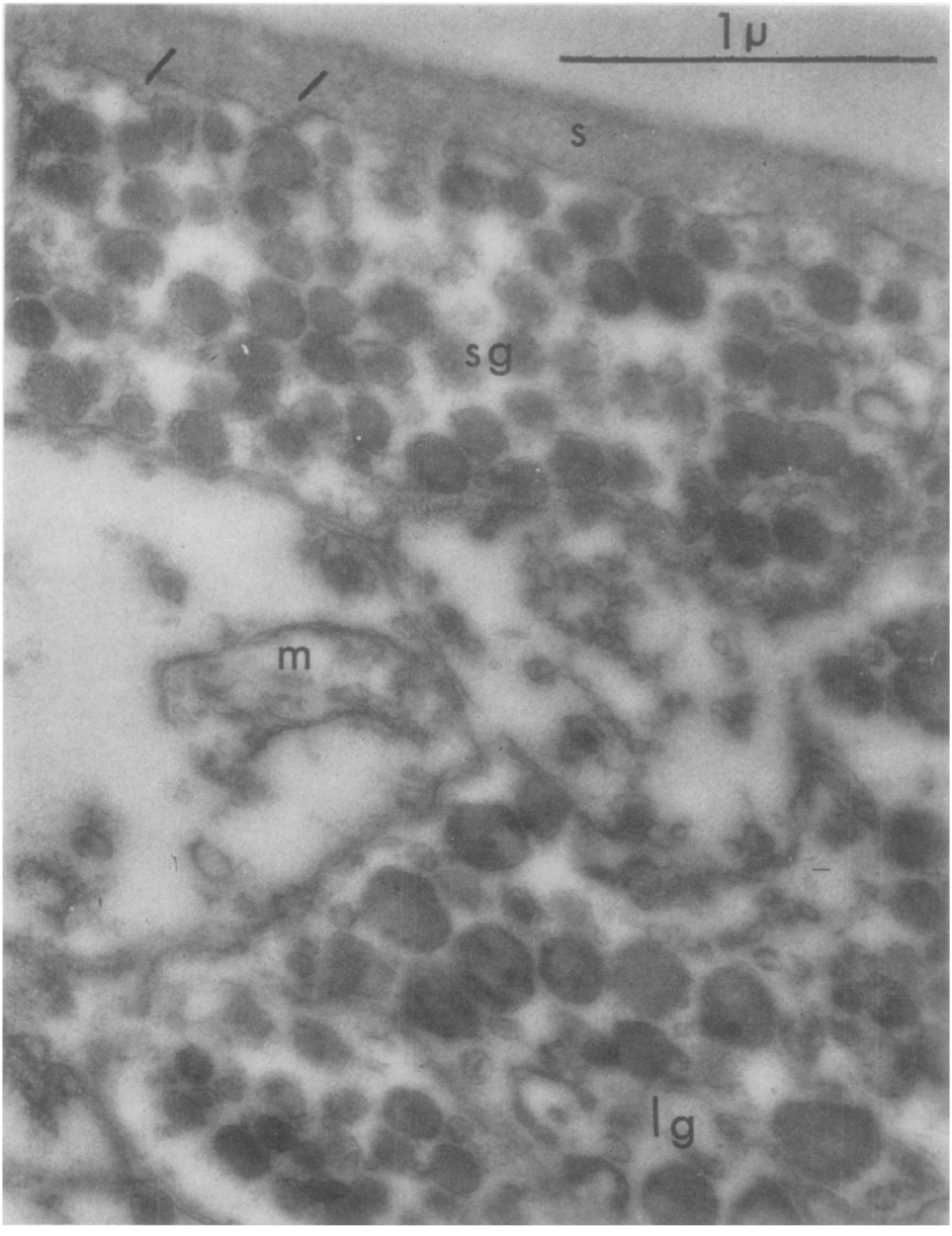


FIG. 7. Edge of PO (*Carcinus maenas*). Arrows point to regions of close association of granules with epineurium. lg, large granules (see Fig. 9); m, presumed mitochondrion; s, epineurium; sg, small granules. Note that small and large granules occur in different terminals. Electronmicrograph, calibration line represents 1 μ.

structure also occur among the more usual dense granules (Figs. 6B, 7, 8).

In sections of two typical *Carcinus* terminals containing average numbers of small granules, the measured granular material occupies 28% and 32.5% of the total terminal area. This is equivalent to slightly less than 10% of the volume.

In the single specimen of *Cancer* examined, only one kind of granule-contain-

ing terminal is present. This corresponds with the picture from stained material described above. The average measured granule section diameter is about 0.15 μ (Figs. 5D, 9).

In both *Cancer* and *Carcinus* certain processes were filled with vesicles about 0.03 to 0.05 μ in diameter (Figs. 5A, 8B). These probably represent an additional type of neuron terminal in which secretory material is associated with vesicles rather than granules. If so then one may distinguish three kinds of terminals in *Carcinus* by electron micrography and two in *Cancer*.

The neuron terminals in *Libinia* contain the widest size range of granules, and unlike *Cancer* and *Carcinus*, mixed populations occur within single terminals. There seem to be three granule populations: (1) mean section diameter is 0.06 μ; (2) mean section diameter, 0.09 μ; (3) mean section diameter, 0.15 μ, but including occasional giant granules up to 0.5 μ or more in diameter (Figs. 5C, 6, 9). There is great variability in the granule distribution within terminals, so characterization of the terminals by granule population is less apt in this species than in *Carcinus*. Nevertheless, there do seem to be two kinds of terminal. One contains either large granules only or large granules plus appreciable numbers of the smallest granules. The latter often clump near the outer pole of the terminal (Fig. 6A). The second kind of terminal with large numbers of medium-sized granules (0.08–0.11 μ) usually does not contain either giant granules or clumps of the very small granules (Figs. 6A, 9).

The relationship between granules and terminal membranes was not clearly established. Granules and vesicles certainly come into close contact with the membrane, but whether such contact is associated with changes in granular or membrane structure is not clear (Figs. 7, 8E).

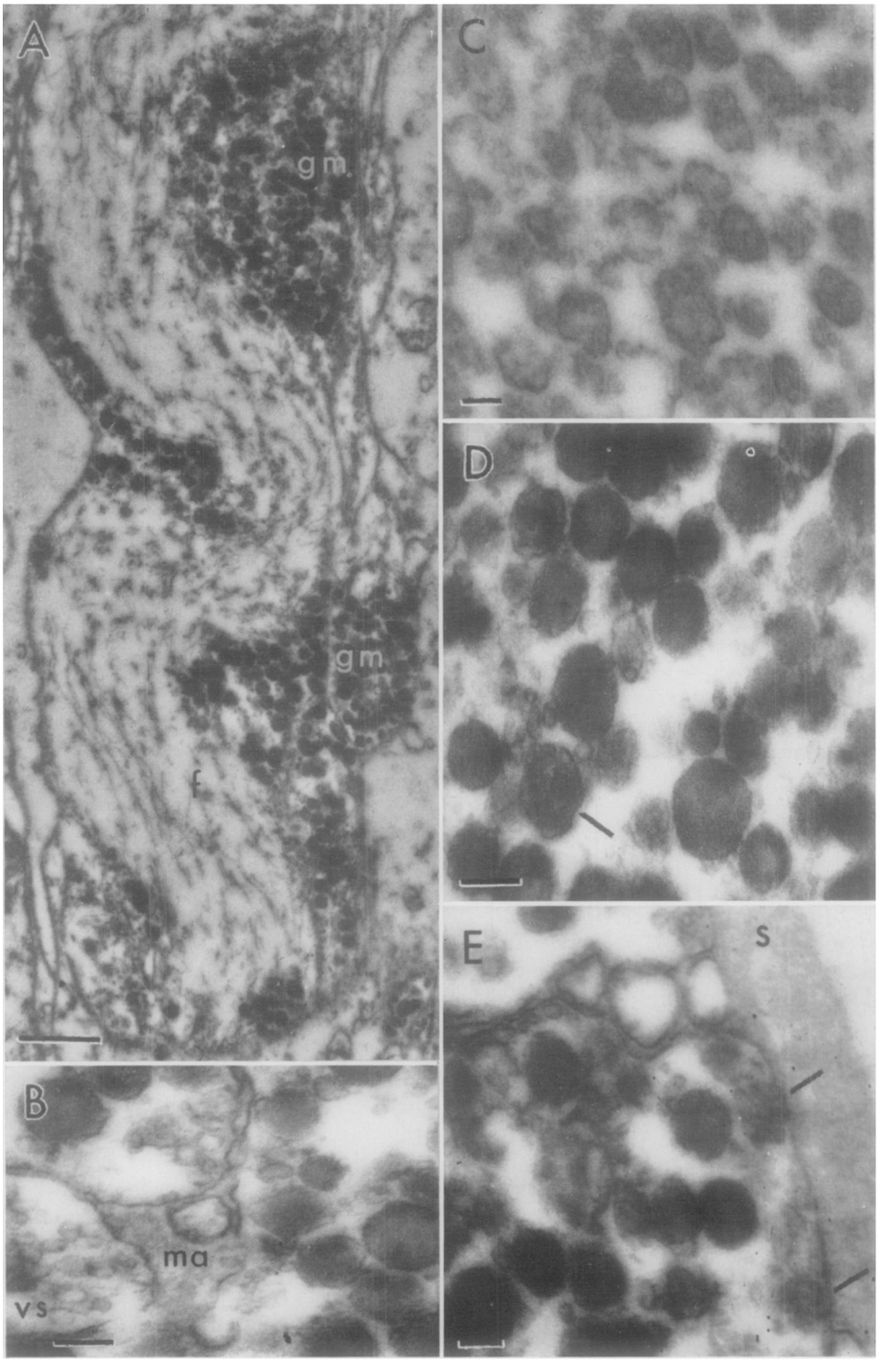
In addition to granules and vesicles, neuron terminals often contain small mitochondria, occasional neurofilaments at the inner pole of the terminal (Fig. 6A), small vesicles or short tubules 0.02–0.05 μ in diameter, and clear vesicles or vacuoles one or more microns in diameter. The vacuoles are particularly prominent in terminals with few or deformed granules (Figs. 5A, 6B).

DISCUSSION

The results reported here emphasize the similarity between the pericardial organ-anterior ramifications complex and the typical neurohaemal structure (Carlisle and Knowles, 1959). Axons of the three kinds of secretory neuron contributing to the PO (Maynard, 1961b) terminate in arborizations and blebs which are separated from the circulating hemolymph of the pericardium by the acellular epineurium alone. These terminals color with the usual neurosecretory stains and are filled with masses of electron dense granules characteristic of neurosecretory structures elsewhere (Hartmann, 1958; Hodge and Chapman, 1958; Knowles, 1959; Palay, 1957; Sano and Knoop, 1959; Scharrer and Brown, 1961). Darkfield or incident illumination also reveals opaque blebs characteristic of granule-filled terminals *in vivo*.

In grosser morphology, however, the PO-AR complex shows a typical form which differs from that of the other major crustacean neurohaemal organ, the sinus gland. Rather than the compact mass of terminals surrounding a central sinus found in the latter organ, the comparable region of the PO forms a thin layer over branches of a nerve plexus, thus greatly increasing its surface-volume ratio. In this it is like the post-commissure organ (Knowles, 1953). The PO also stretches across the lumens of hemolymph channels, and does not simply line them. It will be interesting to discover whether the potentially more rapid release of secretory material permitted by the location and greater surface of the PO has physiological significance.

In at least some crabs, three kinds of secretory neurons contribute to the PO (Maynard, 1961b), and as shown here, three kinds of neuron terminal (on the basis of staining affinities) and three kinds of secretory granule or vesicle occur. Correlations between neuron, terminal, and granule are desirable, but are difficult to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establish. There is no doubt that at least some of the dense granules seen in electron micrographs and visualized *in vivo* with darkfield illumination are associated with substances staining with azocarmine. Exactly which granules, and from which cells, however, is not clear. All cell types, with the possible exception of the posterior PO neuron, appear capable of producing granular material. They also contain material staining with azocarmine (Parameswaran, 1956; Maynard, 1961b). At present, therefore, it is possible though unlikely that all three neuron types contribute to Type I terminals, and that the tinctorial variations represent differences in physiological state, rather than different morphological entities. More systematic studies will be necessary to show whether this is so, and if not, to link granule with terminal with neuron soma.

The interspecific variability in PO histology is appreciable. It is perhaps significant that those forms with the relatively larger PO also seem to have larger amounts of secretory material in their cortex, thus accentuating differences in storage volume (Maynard, 1961a). The relative distribution of terminals with specific staining properties also varies. If these tinctorial differences reflect qualitative differences in secretory material, then one may anticipate corresponding variability in PO–AR function between species (see Alexandrowicz and Carlisle, 1953). Although the dense secretory granules in all three species examined are similar to those found in other neurohaemal organs, there are minor differences in size and homogeneity. Some species have terminals characterized by rather homogeneous granule populations while others show great variability in granule size and indeed, often appear to contain two granule populations within a single terminal. The functional significance of these differences is not known.

The problem of the mechanism and control of neurohormone release remains one of the more enticing aspects of neurosecretion. Most current speculations seem to postulate either breakdown of the secretory granule within the terminal followed by general diffusion of the active material out of the terminal cytoplasm, or they propose some specific evagination of the granule or its contents at the cell membrane (see Hartmann, 1958; Palay, 1957; the observations of Farquhar, 1961 are also relevant). In either case, specific destruction of the granule membrane seems desirable for most efficient hormone release (Pérez-González, 1957). Some of the findings accumulated in the present investigations suggest that a third hypothesis is worthy of brief discussion. This feeling originates with the observation that terminal blebs in the isolated PO "pinch off" with extreme ease. Speidel (1933) has observed terminal swelling and pinching-off in the normal course of events in growing tips of amphibian neurons which encounter an impassable barrier. Possibly neurosecretion can be an analogous process, an *apocrine secretion* involving destruction of the secretory terminal. The terminal bleb would presumably pinch off and subsequently disintegrate to release its contents of secretory material into the interstitial spaces of the PO. The very fine connections often found between terminal bleb and the main secretory fiber (Fig. 3) and the "blister-like" accumulations of secretory terminals could be understood if an apocrine process normally occurs. There are, however, certain complicating aspects of such apocrine secretion. First, the amount

FIG. 8. A. Longitudinal section of nerve fiber in PO-containing accumulations of secretory granules (*Cancer*). Compare with Figs. 4D and 4E. B. Section of terminals in PO (*Carcinus maenas*). C. Granules in PO terminal (*Carcinus maenas*). Note wrinkled outer membrane and internal structure of granules. D. Granules in PO terminal (*Carcinus maenas*), another preparation. Compare with Fig. 8C. Arrow points to granule showing "internal structure," in this case possibly a tangential section revealing vesiculation of granule membrane. E. Edge of PO (*Carcinus maenas*). Arrows point to presumed granules in contact with terminal membrane. f, neurofilaments; gm, secretory granule masses; ma, matrix; s, epineurium; vs, vesicles. Electronmicrographs, calibration line in A represents 1 μ; in B through E, 0.1 μ.

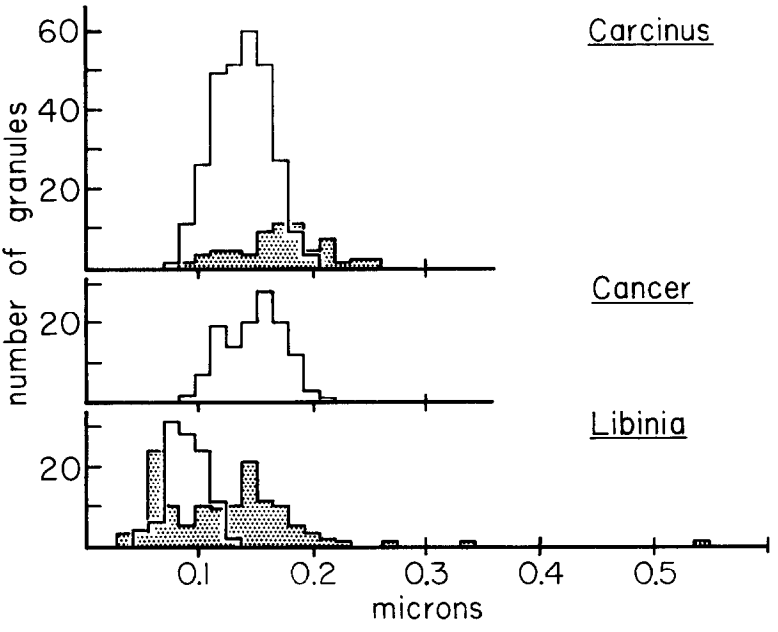

FIG. 9. Distribution of diameters of sections of granules in PO terminals from three crab genera. Open histograms, granules found in one kind of terminal; stippled histograms, granules found in a second kind of terminal. Number of terminals whose granules were counted: Open; *Carcinus*, 4; *Cancer*, 2; *Libinia*, 1. Stippled; *Carcinus*, 2; *Libinia*, 4. See text for details.

of secretory material released would depend upon the size of the terminal and its contents rather than the intensity of the releasing stimulus. Second, assuming continual growth of the secretory terminals, spontaneous "pinch-offs" should occur. Third, assuming that there is active neural control of hormone release (Carlisle and Knowles, 1959), some lag-time between the releasing stimulus and pinch-off with subsequent disintegration of granules and terminal bleb seems inevitable. Observations indicating relative lack of degeneration in sinus gland fibers 15 hours after nerve fiber section (D. D. Potter, personal communication) imply that some active disintegrative mechanism would be required to keep the lag-time within reasonable physiological limits. Further discussion is unwarranted at this time, but it is clear that experiments demonstrating either spontaneous secretion or the time course of events in evoked PO secretion would be profitable.

Our electron micrographs give very little information on release mechanisms. Terminals containing wrinkled, apparently empty granules might be considered isolated degenerating blebs: but in other pictures, granules appear to approach and fuse with the terminal membrane, suggesting quantal release as at the vertebrate motor end plate (del Castillo and Katz, 1954). The possibility of several mechanisms of release is not excluded, of course.

With respect to passage of hormones into the hemolymph in contradistinction to release from the terminal, it seems significant that openings in the epineurium sufficiently large to pass dense granules were not found, and that in isolated PO, the epineurium has to be pierced or broken before granules pass to the surrounding medium (Fig. 2J). Evidently the secretory material is in solution before it reaches the hemolymph of the pericardium.

In concluding this series of papers on the brachyuran PO-AR complex, a brief review seems in order.

Secretory material is stored in and presumably released from two peripheral, paired sites (Maynard, 1961a). The larger is the pericardial organ located in the lateral pericardium and branchio-pericardial veins; the smaller site is the anterior

ramifications found in an anterior, ventral sinus. In both, the structures are extended nerve plexuses floating in or bordering a venous hemolymph channel. The secretory terminals form an outer cortex over the branches of the plexus. Consequently, though the volume of the storage area is probably about equal to that of the sinus gland, the surface through which secretion may reach the hemolymph is considerably greater. Some secretory neurons seem to send terminale to both PO and AR.

At least three kinds of secretory neurons send fibers to the PO-AR complex (Maynard, 1961b). One group, the C-cells, occurs in the anterior portion of the ventral ganglion mass as paired clumps of monopolar neurons. There are about 200 C-cells per clump. A second group, the B-cells, also occurs in the ventral ganglia, but the cells are segmentally arranged and there are 3 to 4 per segment. Axons carrying secretory granules from B- and C-cells pass to the PO-AR complex in the appropriate segmental nerves. The third group of neurons is located in the PO and AR themselves, but presumably retains some central connection via processes extending into the segmental nerves. The homologous neurons in the stomatopod, *Squilla*, form somewhat separate peripheral structures (Alexandrowicz, 1952, 1953b), suggesting that the common site of termination of the three neuron types may represent a secondary coalescence in the Brachyura.

The three kinds of tinctorial endings found in some crabs agree in number with the kinds of secretory neurons, and with the number of size classes of secretory granules or vesicles observed in some PO with the electron microscope. Although correspondence between specific terminal, cell body, and secretory granule has not been established, there seems little doubt that at least three kinds of secretory material are released from the PO, at least in some species. Thus far cardio-acceleration is the only known effect of PO extracts (Alexandrowicz and Carlisle, 1953; Maynard and Welsh, 1959). The size and secretory content of the PO, however, tends to be larger in active or euryhaline species than in lethargic or stenohaline species (Maynard, 1961a), so one may expect to find other general regulatory effects of PO extracts.

Observations on living secretory C- and PO-cells show that the secretory granules are formed by the time they leave the cell body and move relatively freely in the processes. They accumulate on the perikaryal side of partial occlusions of the axon and seemingly do not pass into the dendritic branch at the splitting of the initial segment of the monopolar C-cells. In heteropolar PO-cells, however, granules seem to enter all processes. Iridescent blue and green patches in C-cells are considered indicative of some stage in granule synthesis (Maynard, 1961b). They could be caused by a regular, crystal-like arrangement of the secretory granules themselves, (see Smith and Williams, 1958; Klug *et al.*, 1959) or more likely, by alternating layers of high and low optical density about 0.1 μ apart. Structures seeming to meet the latter requirements have been found in earthworm neurons in the Golgi region (Scharrer and Brown, 1961).

Finally, we would like to point out that the superficial, two-dimensional morphology of the PO, combined with its ease of exposure, make it one of the more appropriate preparations for the study of mechanisms of hormone release in neurosecretory structures. One hopes that future studies will reveal not only the function of the PO-AR complex, but also the mechanisms of neurohormone release and its control.

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