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ELECTRON-MICROSCOPICAL OBSERVATIONS ON THE AURICLE OF SNAIL HEART (HELIX POMATIA L.) WITH SPECIAL REGARD TO THE STRUCTURE OF GRANULATED CELLS*

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It is known from earlier investigations that extracts from nerve, heart and other organs of *Helix pomatia* and other *Gastropoda* contain humorally transmissible biologically active substances (HABERLANDT, 1930; KERKUT and LAVERACK, 1960; JAEGER, 1966; S.-RÓZSA and ZS.-NAGY, 1967). For example, the presence of acetylcholine (Ach) and 5-hydroxytryptamine (5-HT) has been demonstrated by some authors (MENG, 1960; KERKUT and COTTRELL, 1963). Following stimulation of the heart nerves these substances are also released into the perfusion fluid (RIPPLINGER, 1957; JULLIEN, RIPPLINGER, JOLY and CARDOT, 1961; S.-RÓZSA and PERÉNYI, 1966). It has been generally accepted that the Ach acts as an inhibitory, and 5-HT, possibly together with other catecholamines, as an excitatory transmitter (WELSH, 1951, 1957, 1960; KERKUT and COTTRELL, 1963; GREENBERG, 1965; PHILLIS, 1966; S.-RÓZSA and ZS.-NAGY, 1967). The other effective components of the extracts and the sites of their origin, however, have not been exactly determined as yet. It was assumed by JAEGER (1966) that substances of a polypeptide nature may be involved. For this reason the recent investigations have concentrated not only on the examinations of the neurosecretum, but increasingly on cellular elements containing granulated substances, detected in the heart of several snail species (RIPPLINGER, 1957 *Helix pomatia* L.; JAEGER, 1966 *Strophocheilus oblongus;* S.-RÓZSA and ZS.-NAGY, 1967 *Lymnaea stagnalis*). These structural elements have not yet been studied in the edible snail and even the exact morphological determination seems to be rather questionable (nerve cells, neurohaemal organ, etc.).

It is generally felt that there is a need for a review of the structural elements of *Helix* heart. SCHLOTE (1962, 1968) has investigated the structure of *Helix* cardiac muscle and the ultrastructure of neuromuscular junctions, and other species have been studied by NORTH (1963) and KAWAGUTI (1963). The structure of n. visceralis, which innervates the heart, was studied by SCHLOTE (1957), SCHLOTE and HANNEROFTH (1963) and HANNEFORTH (1965), and the examination of the nervous system of some snail species was performed by GERSCHENFELD (1963) and ROSENBLUTH (1963).

The lack of the necessary morphological basis for the physiological investigations has led us to deal with the structural elements of Helix pomatia heart auricle by using different methods.

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Materials and Methods

For the microscopic and histochemical investigations, the isolated hearts of the edible snail (*Helix pomatia* L.) were fixed by the method of SUSA, BOUIN and CARNOY, and subsequently stanied with haematein-eosin, Van GIESON's method, HEIDENHAIN's iron haematoxylin, chrome haematoxylin-phloxin (CAH) and paraldehyde-fuchsin (PF). In addition, methyl green-pyronin, gallocyanin and Hale-PAS stains were also used.

For electron-microscopy, diastolized atria were fixed for 60 minutes in 1% OsO_4 buffered with the special Jullien-Ripplinger physiological Helix-solution (pH 7,4). 4% glutaraldehyde in physiological solution (pH 7,4) was also as a fixative in some samples, after 2 hours the material being postfixed in 1% OsO_4 for 30 minutes. Following fixation the specimens were washed in Ringer-solution, dehydrated in a graded series of ethanol and embedded in Araldite (DURCUPAN, Fluka). The sections were cut on a Tesla BS 478 utratome, and the electron-micrographs were taken on a Tesla BS 242 D table electron-microscope. The material was stained in block with 4% uranyl acetate in 70% ethanol, and the sections were contrasted with lead citrate by the Reynolds procedure.

The investigations were carried out on active animals freshly collected in the spring, summer and early autumn.

Results

Epicardial epithelium

The appearance and ultrastructure of the epicardial epithelium differ essentially from those of the epithelial cells of other organs SUMNER (1966), OVTRACHT (1967), and others. The membrane of the epithelial cells (EP) facing the pericardail cavity is only slightly segmented. The surfaces of the membranes in contact with the neighbouring cells are undulating; in some places these contacts are quite close, but desmosomal thickenings can not be observed. The cell surface facing the connective tissue layer is extremely segmented. The dilateted tubuli of the endoplasmic reticulum (ER) can be seen in the foldings entering the connective tissue, and the cell membranes make contact with each other over large areas (Fig. 1, a—b). The epithelial cells are poor in mitochondria (M), and most of their cytoplasm is filled by the nucleus (N). The ribosomes can be observed to be both free and bound to the endoplasmic reticulum. The cells in the examined physiological state exhibited a low degree of secretory activity. The secretion could be demonstrated in the vicinity of the nucleus as 0,2—2 μ electron-dense granules (DG), while a concentration of a secretion of a similar morphological character (Fig. 1, b) was also observed in the connective tissue (CT). The granules detected in our electron-micrographs are considered as material to be excreted, wich is transported across the epithelial cells towards the excreting organ.

Cardiac muscle fiber system

Muscle fibers in the auricle show remarkable differences in size and form a characteristic architecture. The thinnest fibers are a few μ in diameter, but

Figure 1. *a)* The connection between the epicardial epithelium and its connective tissue.
b) The accumulation of excretum in the connective tissue layer adjoining the epicardial epithelium.
c) Longitudinal section of a muscle fiber showing a nuclear portion.
EP = epithelial cell; CT = connective tissue; N = nucleus; M = mitochondria; ER = endoplasmic reticulum; DG = dense granule; SR = sarcoplasmic reticulum; mf = myofilaments; Ax = axon; G = neuroglial cell with glial granules; ES = extracellular spaces; g = glycogen; S = sarcolemna; Go = Golgi apparate. Fix.: a., b. = glutaralde hyde-osmium, c. = osmium.

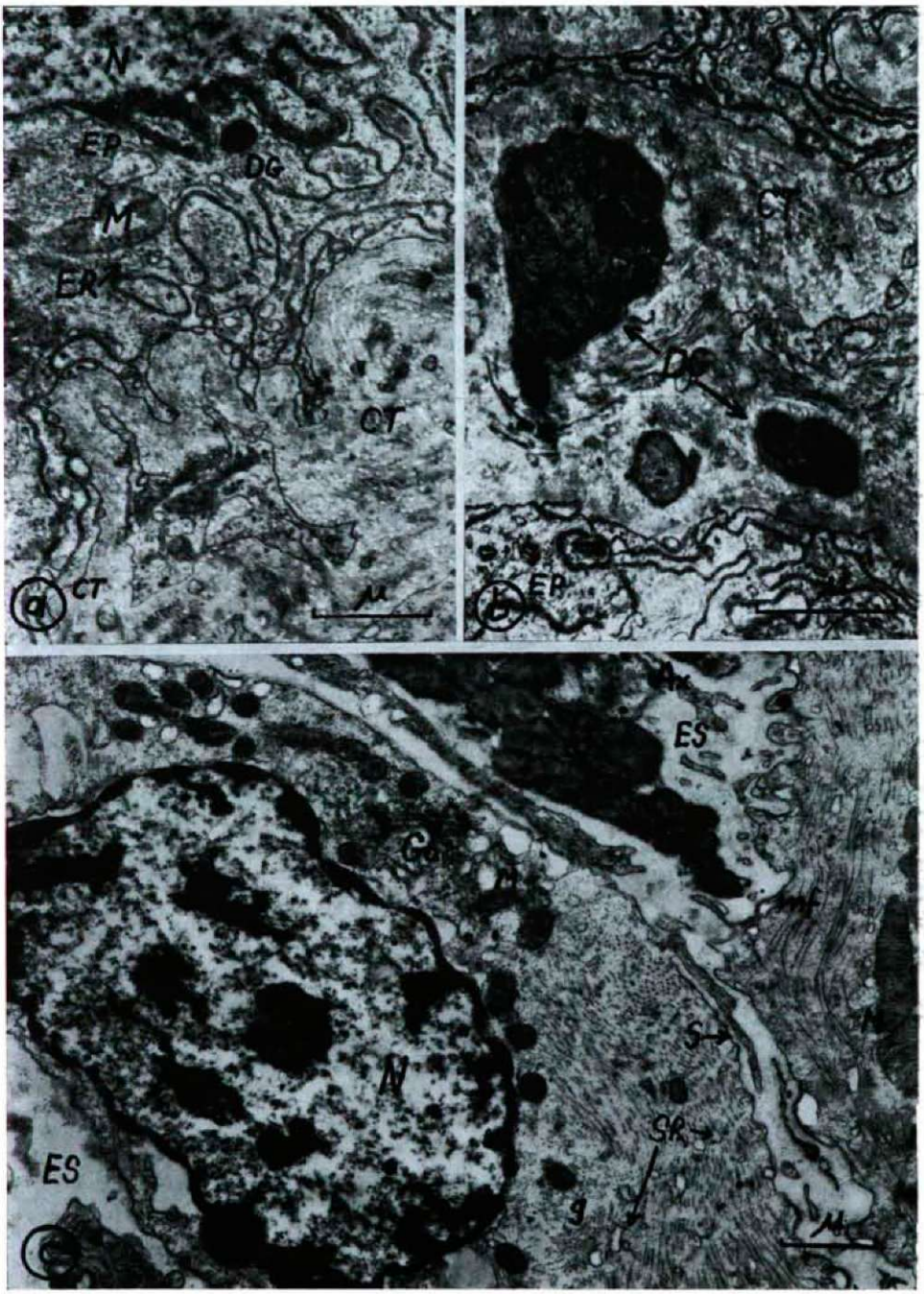

Figure 1

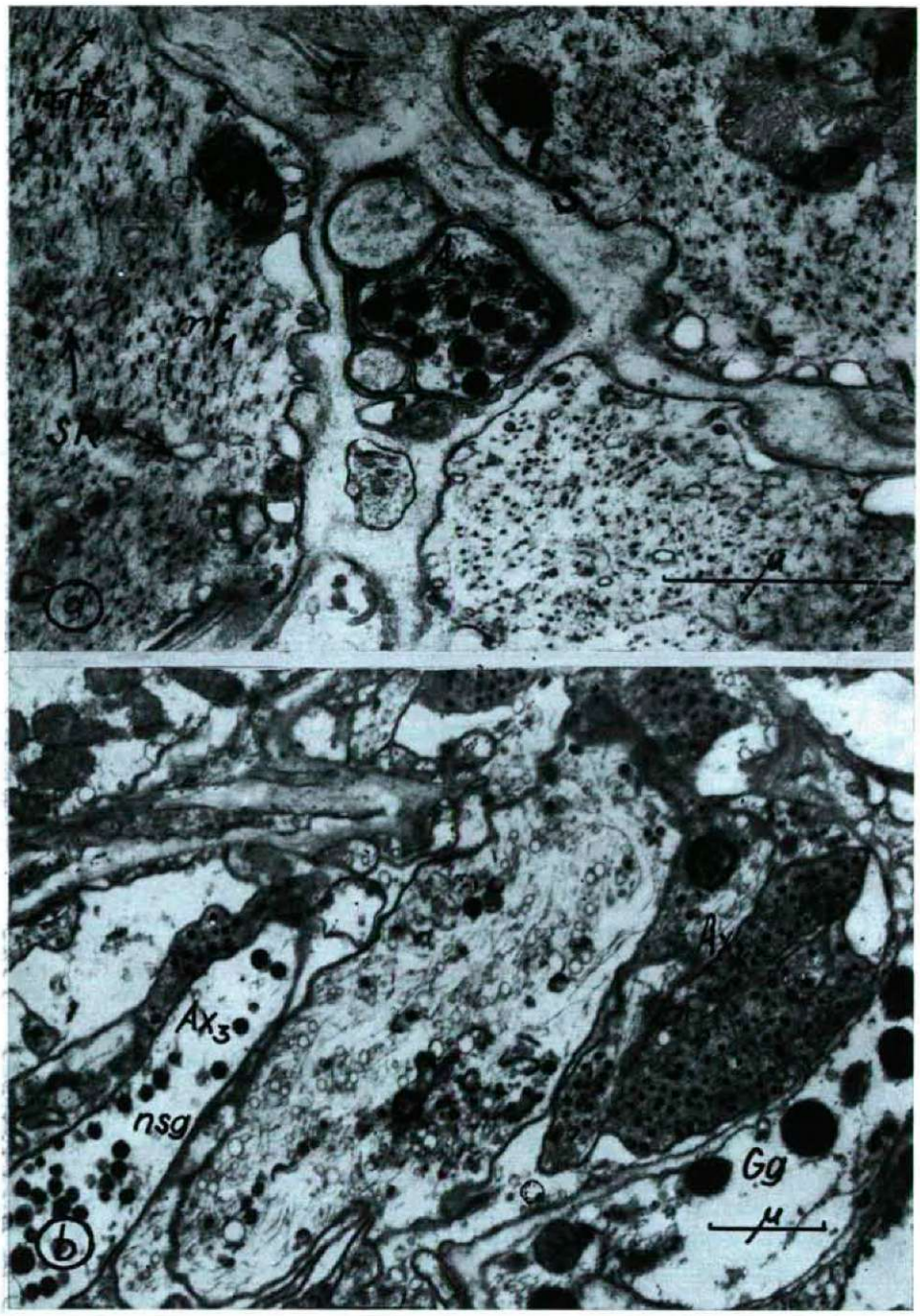

Figure 2

the thickest ones reach 10—12 μ. The length of the muscle fibers is 0,1—1 mm. The nuclei are situated in the centre or at the edge of the fibers; they are roundish and strikingly large, about 5 μ in diameter and 9 μ in length. Two parts can be distinguished electron-microscopically in the muscle fibers: a) the nucleus-containing part, which is richer in sarcoplasm; and b) the region containing the myofilaments.

In the immediate vicinity of the nuclei no myofilaments (mf) at all can be found (Fig. 1, c). The crista type mitochondria (M) are scattered evenly throughout the cytoplasm. A considerable number of granules reminiscent of glycogen (g), and also the Golgi apparatus (Go) are to be seen in the sarcoplasm. Dilateted tubuli of the sarcoplasmic reticulum (SR) can be observed near to the sarcolemma (S) and in the cytoplasm.

The most characteristic structural elements of the myofilament-rich part are the more or less oriented but rather loosely arranged myofilaments (mf). As reported by NORTH (1963), two types of filament can be demonstrated in *Helix pomatia*, too. The diameter of the thicker, more dense filaments (mf_1) is about 200 Å, whereas the diameters of the thinner, less osmiophylic ones (mf_2), which are situated among the former, are about 50 Å (Fig. 2, a). These filaments can be especially well observed in transverse sections, using a higher magnification. The presence of two types of myofilament is commonly characteristic of the molluscal muscles; the thick ones can be identified with myosin and paramyosin (Tropomyosin A), and the thin ones with actin and Tropomyosin B. (PROSSER, 1962; KAWAGUTI, 1963; MILLMAN, 1967; HEUMANN and ZEBE 1968). In the auricle the I-bands are frequently not sharply separeated. In some fibers the Z-bands are very faint, but in other cases they are striking as electron-dense J-granules. The transverse and longitudinal tubuli of the sarcotubular apparatus (SR) are well oriented and very obvious. They extend to the sarcolemma and come into close contact with both it and the Z-substance. The sarcolemma (S) is undulating and segmented by plasma outfoldings. The mitochondria (M) are found among the myofilaments (mf), either distributed or aggregated in groups beneath the sarcolemma. There are two types of granule in the muscle fibers, one of which can be considered as glycogen, in accordance with the findings of REVEL, NAPOLITANO and FAWCETT (1960). The other is an osmiophylic granule of mitochondrium size, and has not yet been identified. The Sjöstrand's disci intercalares characteristic of the vertebrate heart are totally missing, and we have failed to observe any structure indicating such a connection between the cells. It also proved impossible to demonstrate structures pointing to synaptic connections between the nerve and muscle elements, in spite of the fact that these structures can be seen to be in close contact (Fig. 5, c).

Figure 2. *a)* Cross-section of muscle fiber.
b) Nerve fibers with different vesicle contents.
M = mitochondria; S = sarcolemma; CT = connective tissue; SR = sarcoplasmic reticulum; Ax = axon; mf_1 = thick filaments; mf_2 = thin filaments; nsg = neurosecretory granules; Gg = glial granules; Ax_2 = axon of type II; Ax_3 = axon of type III; Fix.: glutaraldehyde-osmium.

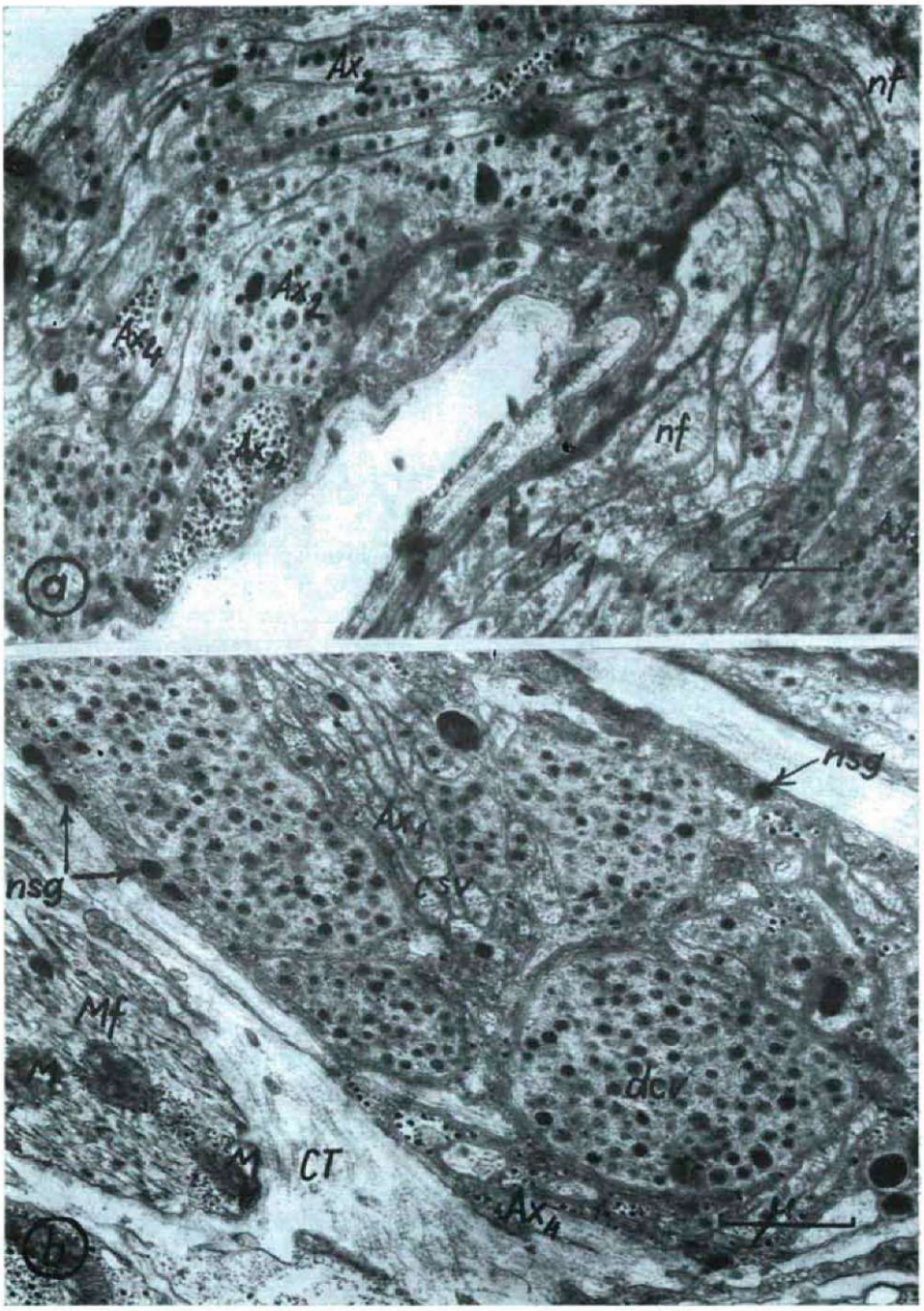

Figure 3

Figure 3. *a)* Bundle of nerve fibers in longitudinal section.
b) Bundle of nerve fibers in cross-section.
nf = neurofilaments; Ax_1 = axon with clear synaptic vesicles; Ax_2 = axon with dense-core vesicles; Ax_4 = axon with dense vesicles; Mf = muscle fiber; M = mitochondria; CT = connective tissue; csv = clear synaptic vesicles; dcv = dense-core vesicles; nsg = neurosecretory granules. Fix.: osmium.

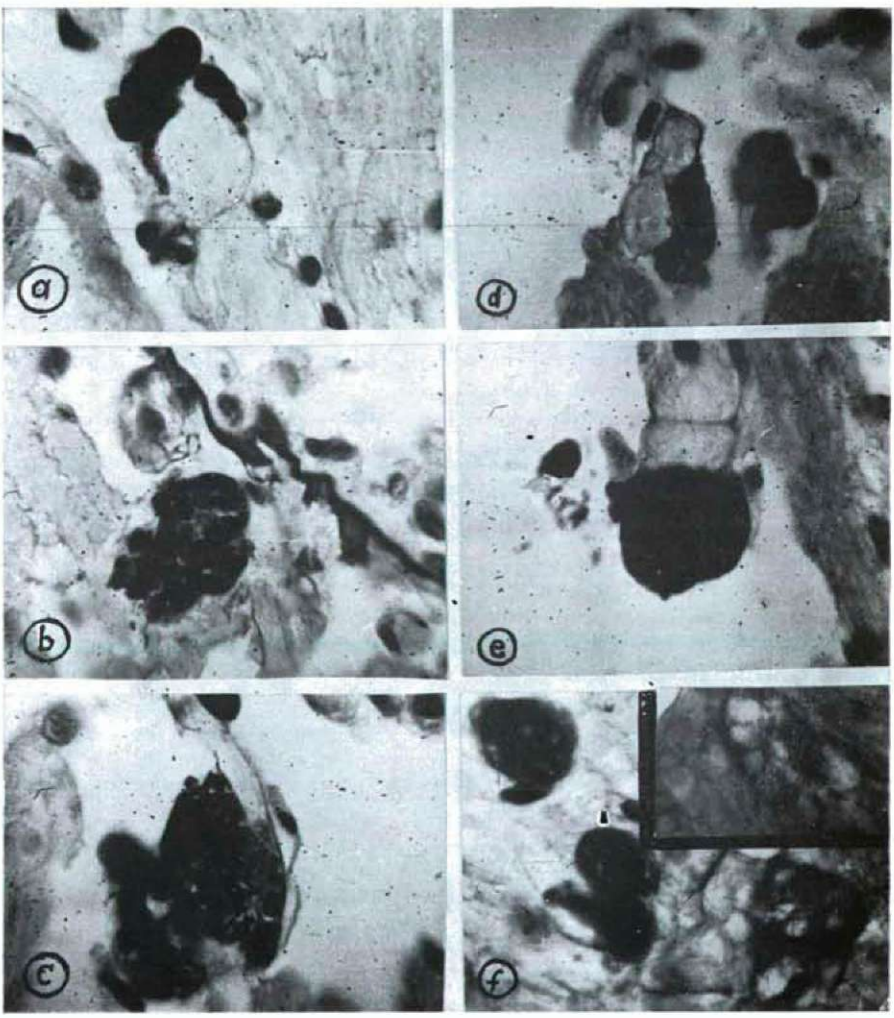

Figure 4

Figure 4. Micrographs of granulated cells. 750 x.
a., b. d. = β-cells,
c., e. = α-cells. Chrome haematoxylin-phloxin,
f. = α- and β-cells. Paraldehyde-fuchsin.

Nerve fiber system

The nerve elements of the auricular branch of the n. visceralis occur as nerve bundles and single nerve fibers among the muscle fibers (Fig. 3, a—b). The larger nerve bundles are linked with the musculature by interstitial connective tissue rich in collagen fibers (CT). In support of BAECKER (1932), BEY and RIPPLINGER (1966) and others, we failed to find neurons in the wall of the auricle.

The nerve fibers have very different diameters; 5—8 μ for the thick ones, and only 0,1—0,5 μ for the thin ones. In agreement with the findings of SCHLOTE (1957, 1963), SCHLOTE and HANNEFORTH (1963), GERSCHENFELD (1963), ROSENBLUTH (1963) and HANNEFORTH (1965), the axons (Ax) can be classified upon the basis of their fine structures and granule contents as follows (Figs. 2, and 3):

1. Gerschenfeld type I:

These axons (Ax_1) contain purely clear synaptic vesicles (csv) of different sizes (average diameter 700 Å) (Fig. 5, c).

2. Gerschenfeld type II:

This type of axon (Ax_2) can be observed mainly in the auricle of the *Helix pomatia* heart. They have very different diameters and contain only dense-core vesicles (dcv, average diameter 900 Å) or a mixed vesicle population (csv, mvb) predominantly of the dense-core type. These axons exhibit Falck positive fluorescence as a result of their catecholamine content.

3. Gerschenfeld type III:

These axons (Ax_3) contain neurosecretory vesicles (nsg) (average diameter 1300 Å) alone or mixed with other vesicles. Their physiological significance can not easily be determined, but their appearance in the invertebrate nervous system seems to be fairly general (BERN and HAGADORN, 1965).

We have succeded in separating a fourth type of axon (Ax_4); these contain merely electron-dense material (Fig. 3). This corpuscule is smaller then the dcv, and differs both in size and appearence from the granulation mentioned by GERSCHENFELD (1963) in csv-containing axons treated with uranyl acetate.

The appearence of the axon (Ax) membranes, the connections of the different types of axon and the structures of the glial cells, including the glial granules (Gg), exhibit a general agreement in the case of the molluscs investigated, irrespective of the different species (Fig. 2, b).

Granulated cells

These cells occur abundantly in the auricle of the *Helix pomatia*, but only a few can be observed in the wall of the aorta. The literature data indicate that the granulated cells are present in the heart of other snail species, too, and this suggests their general occurrence (JAEGER, 1966; S.-RÓZSA and ZS.-NAGY, 1967). The secretory activity of the cells is not at all obvious in certain periods of time and certain functional states (general emptying phase in the resting period?); at other times, however, these cells occur in large numbers and filled with granules (active period). Referring to RIBEIRO JAEGER (1966) mentions that the emptying of the cells can also be observed in *Strophocheilus* following

nerve stimulation. In other snail species these cells are considered by some authors to be neurons, but in the case of the *Helix pomatia* this view can not be accepted. The innervation of the internal organs of *Helix pomatia* is well known morphologically in the case of the intestinal tract and stomach (ÁBRAHÁM, 1940). If the granulated cells of the auricle are compared with the neurons in the stomach and in the ganglia, significant differences can be observed both in the size of the cell and in the appearance of the nucleus, and this casts doubt on the neuronal character of the auricular cells. The results of our microscopic (Fig. 4) and electron-microscopic investigations may serve as further evidence of this, by showing significant differences from the well-known structures of neurons (ROSENBLUTH, 1963; LANE, 1964; SCHMEKEL and WECHSLER, 1968).

Fig. 4 shows granulated cells in various functional states. The cells are closely attached to the surface of the muscle fibers and are in free contact with the cavity of the auricle. In Figure 4, b, c, e the cells are filled with secretory substance, but are in different stages of the emptying. The round or ovoid cells are 20—40 μ in size, and their nuclei (1—4 μ in diameter, 10 μ in length) can be seen at the edges of the cells. The largest secretory granules can reach a size of 5 μ. Finely (α-cells) and much more coarsely granulated (β-cells) secretory substance can be distinguished in the cell, even with a microscope. The former can be stained very intensely, and the latter less electively with chrome haematoxylin-phloxin. Using paraldehyde-fuchsin, however, only the α-cells are enhanced as a result of the affinity of their secretory substance for the dye (Fig. 4, f).

The close connection between the granulated cells and the muscle fibers is also striking in the electron-microscopic pictures. The cell membrane is smooth, but in some places it is a little segmented. In the cells a considerable number of elementary RNA granules can be observed. At the same time the RNA content is quite obvious in preparations stained with methyl green-pyronin and gallocyanin. The Golgi apparatus (Go) is localized near the nucleus (N) and has a regular appearance. The cytoplasm is poor in mitochondria (M), but the dilatated endoplasmic reticulum is very striking everywhere. The granulated form of the endoplasmic reticulum often occurs, too. These morphological characteristics of the granulated cells produce the impression of secretory activity. The secretory substance appears in several forms, and electron-microscope observations indicate that it is distinguish between α- and β-cells. In the α-cells the characteristic outward form of the secretum (DSG) is an aggregate of a substance with high electron density, which in general does not reach a size of 1— 3μ. In the formation procedure, at first only the substance can be observed in the ergastoplasm, but later a vacuole (V) is formed gradually around it in such a way that the aggregate is surrounded by a separating membrane. These vacuoles can join together, in which case they occupy most of the cytoplasm. Figure 5, a and c show the early stages of the secretory granule formation, while Figure 5, b and d depict granulated cells which have already excreted their content, the remaining vacuoles being very obvious. Figure 6, a shows a granulated α-cell.

The granules of the β-cells are much larger (1—5 μ) and have lower electron density. During their formation they fill the whole vacuole and are bordered by membranes arranged next to each other. The large secretory granules can fill the whole cell, as seen in Figure 6, d, and are very similar to the substances

of the protein-secreting cells, although they exhibit PAS negativity (HOLLMAN, 1965; OVTRACHT, 1967) (Figure 6, b).

In Figure 6, c the cell has already excreted its granule content; it appears as if the vacuole of the secretory substance (V) has remaind in the cytoplasm. The filamentous process (cf) of the cytoplasm extends over the majority of the vacuole, and here the close contact of the membranes can be seen. Nevertheless the cell is situated very near to both the muscle (Mf) and nerve elements (Ax), and this suggests the connection of the different structural elements.

Discussion

Our histological, histochemical and electron-microscopic investigations lead us to believe that only some of the substances found in different structural elements are biologically active. Such materials are the well-known mediator substances (Ach, 5-HT) and the substances of neurosecretory granules (nsg) and granulated cells. On the other hand, the granules in the epithelial cells (DG), which are in all probability transported toward the excretory organs, the glia-granas localized in glia cells, and the accumulated material in some muscle cells, can not be considered as biologically active substances.

It is rather difficult to outline the functional connections of the different structures, which are known partly from our morphological results, partly from the physiological data. It is thought, however, that the three or four types of axon can control the *Helix* heart by several different coordinating systems. This possibility is supported by the physiological fact that five sorts of nerve fiber exist in n. intestinalis with different conduction velocities, thresholds to stimulation and action potentials. One of these is expressed in acetylcholine (Ach)-mediated inhibitory effects (csv-containing axons), and another in excitatory effects, mediated by 5-HT and different catecholamines (dcv-containing axons). At the same time, however, one has to consider the regulative effect of the neurosecretory-neurohaemal hormone-releasing (neuro-endocrine) system, which is well known from other groups of invertebrates. This assumption is supported by seasonal changes in the functional state of granulated secretory cells, and by functional changes related with the resting and active periods. Further evidence is provided by the findings of JAEGER (1966) and VOLKMER-RIBEIRO (1970), who described the emptying of the granulated cells following nerve stimulation, as well as the terminating of the neurosecretory (nsg-containing axons) tract in

Figure 5. Cells in different states of producing and emptying granules.
a. and *c.* = Initial state of the granule-forming process.
b. and *d.* = Cells emptying their granules partly or completely.
N = nucleus; DSG = dense secretory granules; M = mitochondria; ER = endoplasmic reticulum; Mf = muscle fiber; csv = clear synaptic vesicles; V = vacuole. Fix.: osmium.

Figure 6. *a)* α-cell in the state of increased granule formation.
b) β-cell with its large granule content.
c) Granulated cel in empty state.
d) β-cell filled with granule.
N = nucleus, Mf = muscle fiber; M = mitochondria; ER = endoplasmic reticulum; Go = Golgi apparatus; DSG = dense secretory granules; LSG = large secretory granules; V = vacuole; cf = cell fimbria; dcv = dense-core vesicles.
Fix.: osmium.

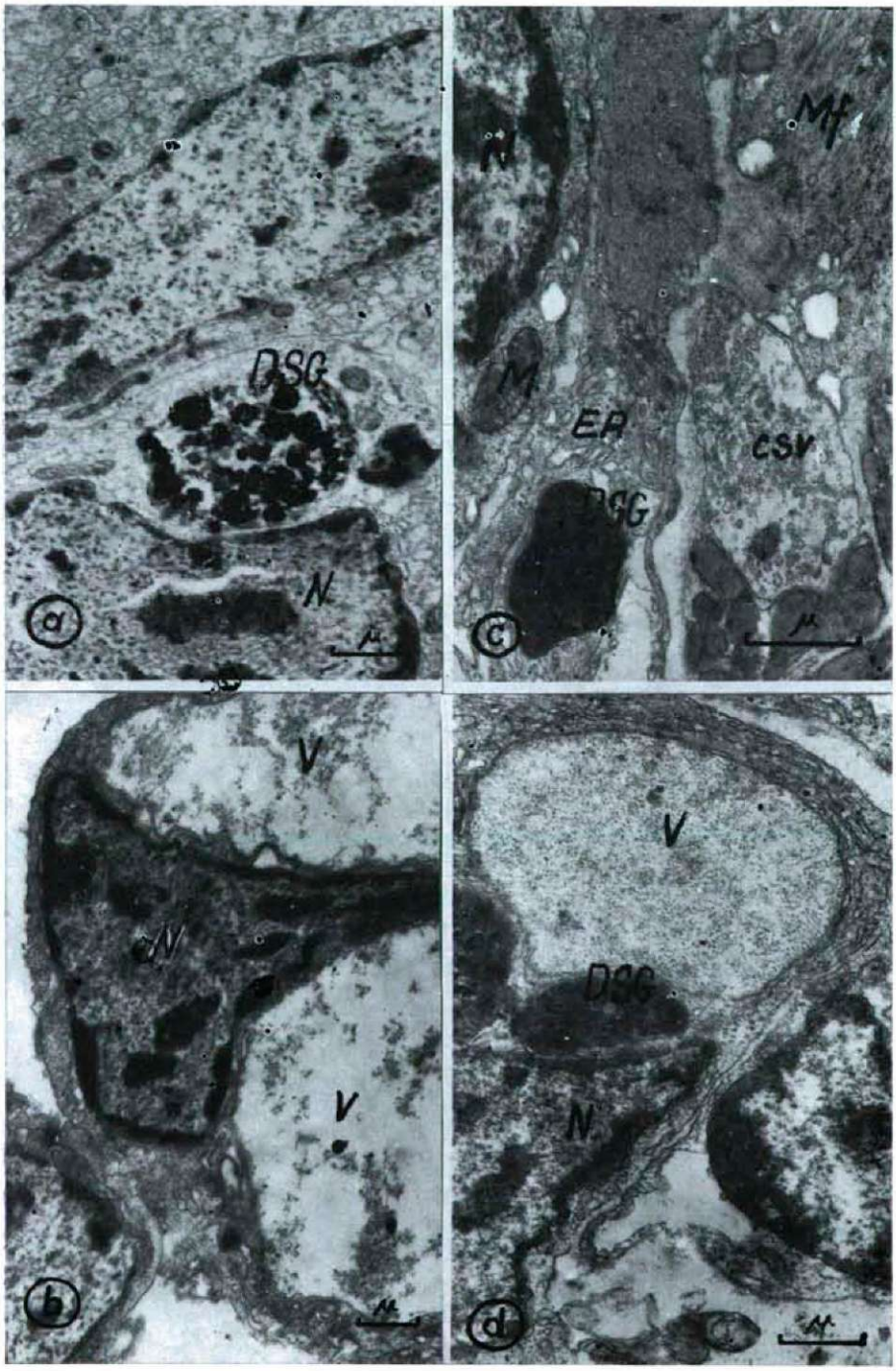

Figure 5

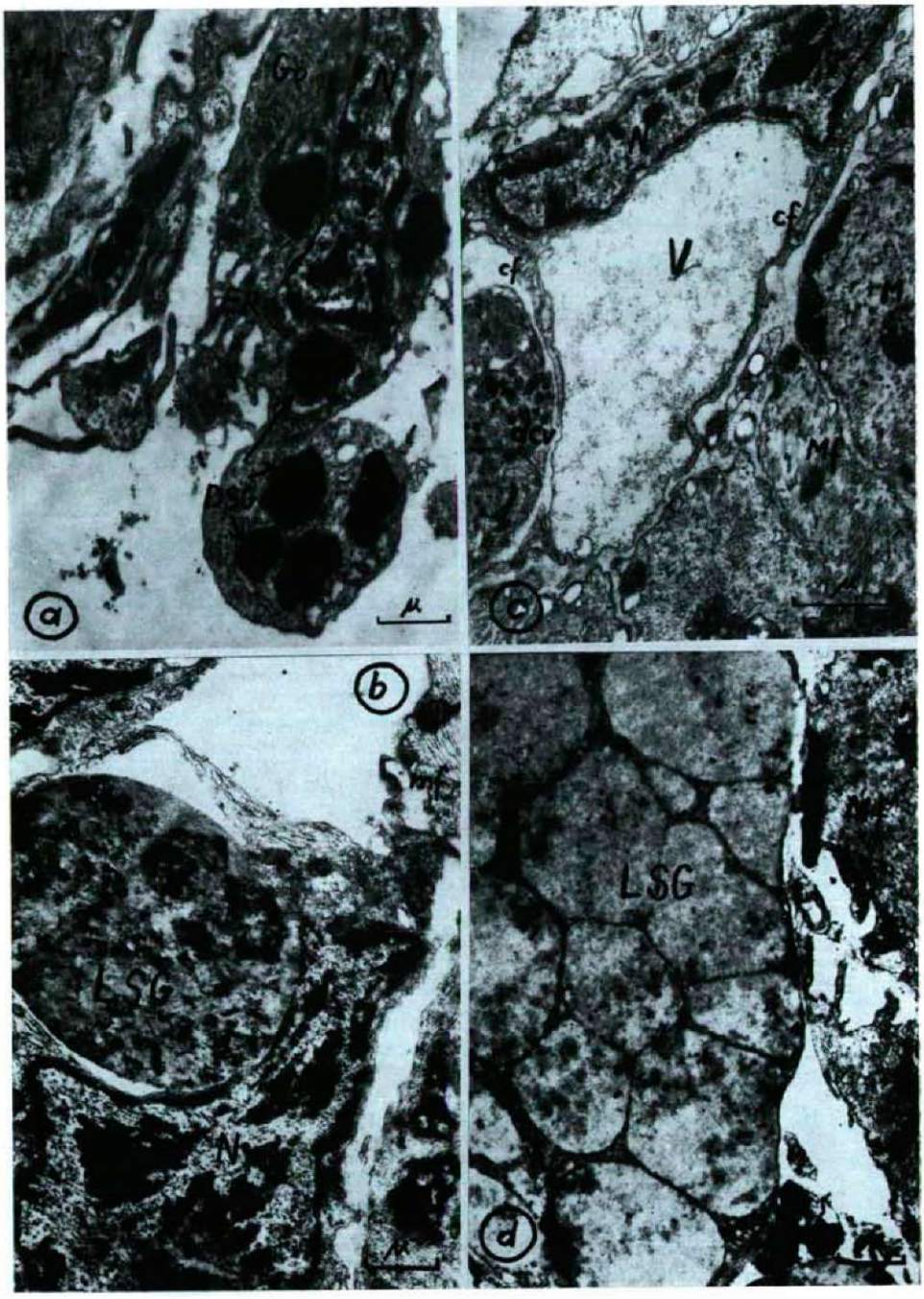

Figure 6

heart, which was also demonstrated by us. The granulated cells (Sc) are in many respects very similar to the cells in the medio-dorsal body (MDB) are formed in groups, while in the auricle they are diffusely arranged (BOER, SLOT, VAN ANDEL, 1968).

The question finally arises of how the regulation of muscle activity by these above-described systems can be imagined. In this regard the neuro-endocrine system should play a role in establishing long-lasting changes connected with periodic factors and the functional state of the animal; and perhaps it could be identified with the effect of cor-hormone or X-substance. At the same time, the inhibitory and excitatory fibers should transmit direct effects of a nervous character from the integration centers by means of their mediator substances. It is interesting that the stimulation of the heart nerve during certain periods of time produces only inhibitory effects, and at other times mainly excitatory effects. This clearly indicates a functional connection between the two coordinating system, and in so far as regards effectivness they may somehow influence or perhaps regulate each other. Interesting data were reported by NICAISE, DE CECATTY and BALEYDIER (1968) on the presence of mediatory elements in the muscle innervation of *Glossodoris*. We have failed to find these connections in the heart innervation of *Helix pomatia*, but the possibility can not be excluded that the granulated cells have a similar mediatory role in the neuroendocrine system. In a recent paper, COTTRELL and OSBORNE (1969) suggest that the neurosecretory tract, which terminates in the heart, plays a direct role in liberating the general stimulating substance. A similar functional role is presumed in the case of the granulated cells in *Lymnaea stagnalis* ZS.-NAGY and S.-RÓZSA (1970) and *Helix aspersa* and *Storphocheilus oblongus* VOLKMER-RIBEIRO (1970). Considering these results, an exact knowledge of the assumed relations can only be expected from further investigations.

Summary

The results of our morphological investigations on the auricle of *Helix pomatia* L. heart are as follows:

A close morphological and functional connection was observed between the epicardial epithelium and the connective tissue layer. This is revealed in the formation of large membrane surfaces in contact with each other and in the removal of their material concent towards the excretory organ.

Two types of myofilament (50 and 200 Å) and a well-developed sarcotubular system are characteristic of the muscle fibers in the auricle. An essential difference was observed between the muscle fibers in the appearance of the cross-striations and the Z-band. A direct neuromuscular connection is not obvious, and it is possible that in additon the nerve regulation, the heart is affected by some hormonal mediator.

The axons innervating the heart contain csv (type I), dcv (type II), nsg (type III), and as we have succeeded in showing, there is a network of nerve fibers of a fourth type, too, containing purely electron-dense substance of csv size.

Two types of granulated cell can be separated (α- and β-cells). The α-cells produce electron-dense granules of a maximum size of 1—3 μ which are accumulated in vacuoles. The less electron-dense granules of the β-cells reach 1—5

μ in size and their morphological appearance is very similar to the secretum with complex protein content, but they have PAS negativity.

It is presumable that the granulated cells play a role in the neuro-hormonal process in connection with the neuronal regulation of the heart function.

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