STUDIES ON THE NEUROSECRETORY SYSTEM OF IPHITA LIMBATA STAL. I. DISTRIBUTION AND STRUCTURE OF THE NEUROSECRETORY CELLS OF THE NERVE RING

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Neurosecretory cells have been discovered by several authors in different groups of insects (see reviews: Scharrer and Scharrer, 1954a, 1954b). In *Iphita limbata* (Pyrrhocoridae: Hemiptera) the presence of these cells in the brain and the metathoracic ganglion was reported by Nayar (1953) who also described certain changes in the activity of these cells correlated with reproduction in the female. The functions of the neurosecretory cells of the brain have been worked out in certain insects, especially by Scharrer (1952) in *Leucophaea maderae,* by Thomsen (1952) in *Calliphora erythrocephala,* and by Williams (1952) in *Platysamia cecropia.* The present series deals with a more detailed study of the structure, functions and development of the neurosecretory cells of *Iphita limbata* Stal.

Materials and Methods

Adult insects were used for the study. They were frequently collected fresh from the field and were kept in insectary boxes where they were fed on cotton seeds.

When the dorsal wall of the cranium is removed and the head is stretched forwards by pulling the rostrum and fixing it with plasticine, the brain becomes exposed. The dissection of the brain was done under a stereoscopic binocular microscope (magnification × 40). A longitudinal tracheal tube with a number of tracheoles traverses the middle of the cerebral ganglia. When that is removed, faintly whitish spots become visible underneath the firm and thin membrane investing the brain. When this membrane is teased with a fine needle, two groups of medial neurosecretory cells come into view as bluish-white masses on either side of the midline. Each contains about sixteen cells. The medial neurosecretory cells can be removed as a group from the pars intercerebralis of the brain with fine forceps (*cf.* Thomsen, 1952). When observed in insect Ringer, these remain without marked changes for about an hour.

For the study of topography and histological structure of the neurosecretory cells, the entire nerve ring was removed and fixed. The medial neurosecretory cells of the pars intercerebralis of the female were selected for the examination of finer cytological details; the corresponding male tissue shows no marked difference in cellular structure and distribution.

The following methods were used in this study:

1. For general histology: Bouin's, Helly's, Smith's, and Baker's formal-calcium were used as fixatives. Staining was done in Heidenhain's iron hematoxylin, Masson's trichrome (Foote, 1933), Gomori's chrome alum-hematoxylin-phloxin (Gomori, 1941), Gomori's aldehyde fuchsin (Pearse, 1953) and Heidenhain's Azan (Pantin, 1948).

2. For supravital observation: Phase-contrast and dark field microscopes. Light microscope for supravitally stained tissue (neutral red, methylene blue, and dahlia violet) in 0.001% stain for 10 to 15 minutes.
3. For the study of the granular system in the cytoplasm: Material fixed in Baker's ISO fixative (osmic acid in sucrose-iodate solution) stained in Altmann's acid fuchsin according to Metzner's method, and Helly-fixed material stained in Hirschler's hematoxylin (Baker, 1951).
4. For the study of the spheroidal system in the cytoplasm: Classical Golgi methods, such as fixation in Flemming-without-acetic and staining in iron hematoxylin; Weigl's Mann-Kopsch; Kolatchew's and Aoyama's methods (Baker, 1951); Thomas' (1948) method of study of gradual osmification in 2% osmium tetroxide; Baker's (1949) technique of sudan black staining; and Thomas' (1948) method of sudan black staining for paraffin sections.
5. For other structural details: Unna-Pappenheim's methyl green-pyronin method (Darlington and LaCour, 1947) after fixation in Heidenhain's saline-mercuric chloride, for nucleic acids; Baker's acid hematein test (1946) and pyridine extraction test for phospholipines; Nath's (1934) method of staining fats by Sudan III; Barnett and Bourne's (1942) method for ascorbic acid; treatment with Millon's reagent after Bouin-fixation, xanthoproteic reaction, Pollister's method after Bouin-fixation, Hartig-Zacharias' method after formal-calcium fixation, for proteins (Pearse, 1953); Best's carmine after Bouin-fixation for glycogen (Pearse, 1953); indole reaction, Vulpian reaction, Sevki's Giemsa-tannin method and Lison's chromaffin test (Pearse, 1953); and Schmorl's method for lipofuscins (Pearse, 1953).

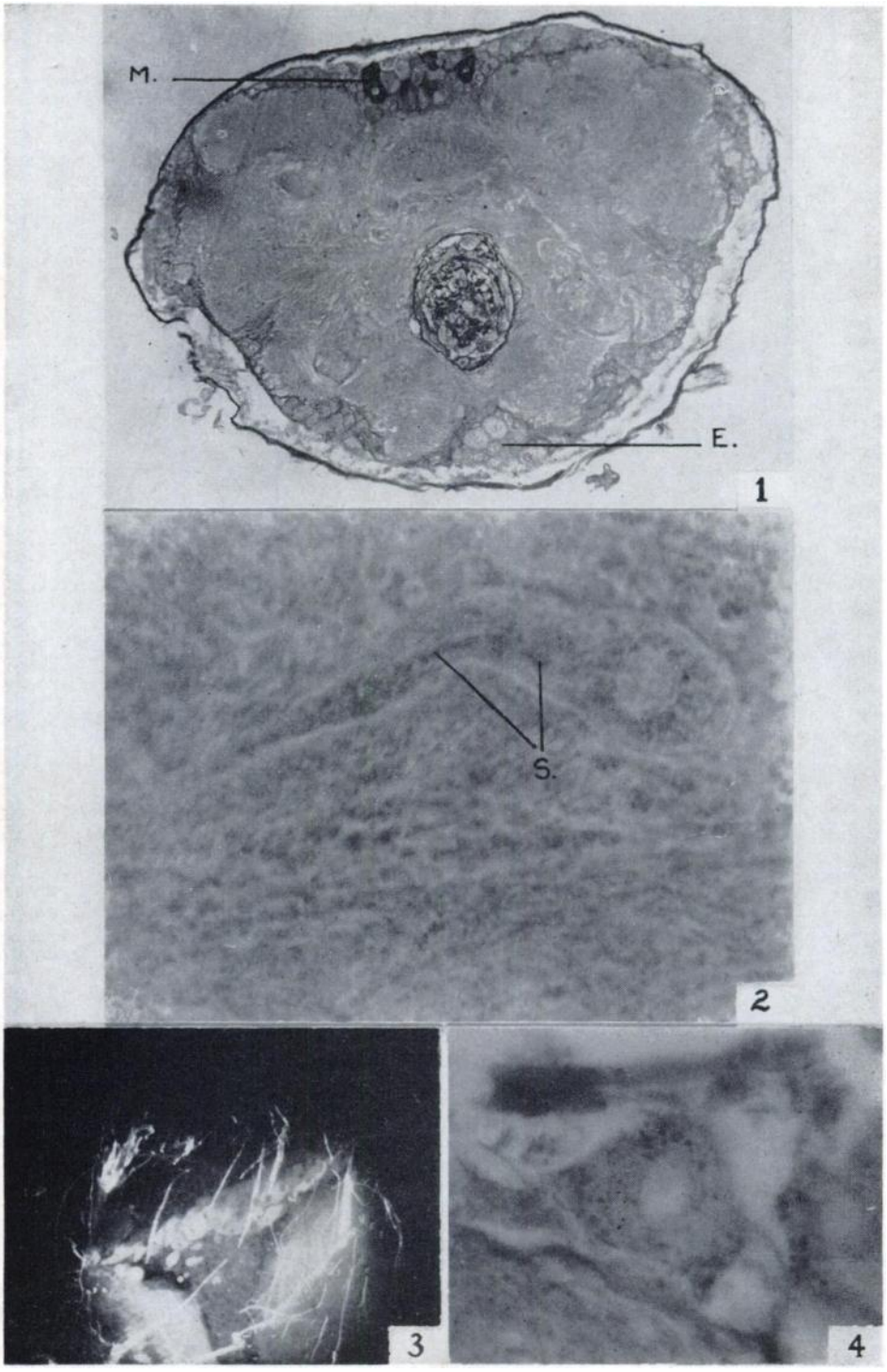
Fixed material was processed according to Peterfi's double embedding method with one-half to one per cent celloidin in methyl benzoate, and paraffin sections were cut at 5 μ for general staining. Some thick setions, 6 to 8 μ, were also cut for the study of the spheroidal constituents of the cells, while for mitochondria sections 2 to 3 μ were used. Frozen sections were cut at 10 and 15 μ after embedding in gelatine (25 per cent gelatine with trace of cresol).

Observations

For histological details fixation in Bouin's and Smith's fluids followed by Gomori's chrome alum-hematoxylin-phloxin gave the best results. The chrome hematoxylin selectively stains the neurosecretory cells a deep blue; sometimes in thick sections (6 or 8 μ) the cytoplasm appears blackish blue. Equally good results were obtained by using Azan stain where the cytoplasm is colored brilliant red by the azocarmine. The cytoplasm of these cells is fuchsinophilic in Masson's stain.

The neurosecretory cells are distributed in different parts of the nerve ring. In addition to the median neurosecretory cells of the brain (pars intercerebralis), there are the lateral groups of neurosecretory cells of the protocerebrum, numbering about three or four on each side. They are much smaller than the medial cells and rarely appear bluish in the fresh brain. The subesophageal ganglion contains scattered neurosecretory cells laterally and ventrally along the margin of the neuropile (Figs. 1, 5).

The neurosecretory cells of *Iphita* show two types of response to the staining procedures used. In one type, the cytoplasmic inclusions are stained deep blue in chrome hematoxylin-phloxin and dark red in Azan; these cells may be designated as "A cells." In the other type, the cytoplasmic contents stain red in chrome hematoxylin-phloxin and light blue in Azan; these may be designated as "B cells."



FIGURES 1–4.

In Heidenhain's iron hematoxylin, the "A cells" are colored bright blue, and the "B cells" light blue. No selective staining of any kind was obtained by Gomori's aldehyde fuchsin.

The distribution of "A cells" and "B cells" is characteristic. In all preparations, the majority of the medial neurosecretory cells of the protocerebrum belong to the "A type" while a few (varying in number from two to six) are similar to "B type" cells. The latter show in their cytoplasm scattered granules. It is possible that these cells may be "A cells" deprived of the bulk of secretory material. Under certain experimental conditions where the insects have been fed on salt water or where salt water has been injected into the hemocoele, all the cells of the medial clusters are colored blue. The lateral neuroscretory cells and most of the subesophageal cells belong to the "B type" with phloxinophilic cytoplasm. A few cells of the subesophageal mass show a resemblance to the "A type." A map showing the distribution of these cells in the ring is given in Figure 6.

Lying on the two sides of, and closely apposed to, the anterior end of the aorta are the tiny corpora cardiaca with a slender bridge-like mass of cells in between which represents the hypocerebral ganglion. The cells of the hypocerebral ganglion are phloxinophilic, resembling the "B type" cells of the nerve ring. Laterally are the compact corpora cardiaca which show in their cytoplasm, in the vicinity of their nerves, granules colored blue in chrome hematoxylin. The cytoplasm here is heterogeneous, unlike that of the cells of the hypocerebral ganglion. The few cytoplasmic granules in the corpora cardiaca resemble those in the "A type" neurosecretory cells.

The medial cerebral neurosecretory cells have been used for a more detailed study. They measure about 52 to 97 μ in length. The cell tapers towards the axon and the apical part is swollen and carries the eccentrically located nucleus. The broadest part measures 32 to 39 μ. The round nucleus is 13 μ in diameter.

If pressure is exerted on a fresh preparation, within a few minutes globule-like droplets, measuring up to 13 μ, often separate off from the abaxonal part of the cell.

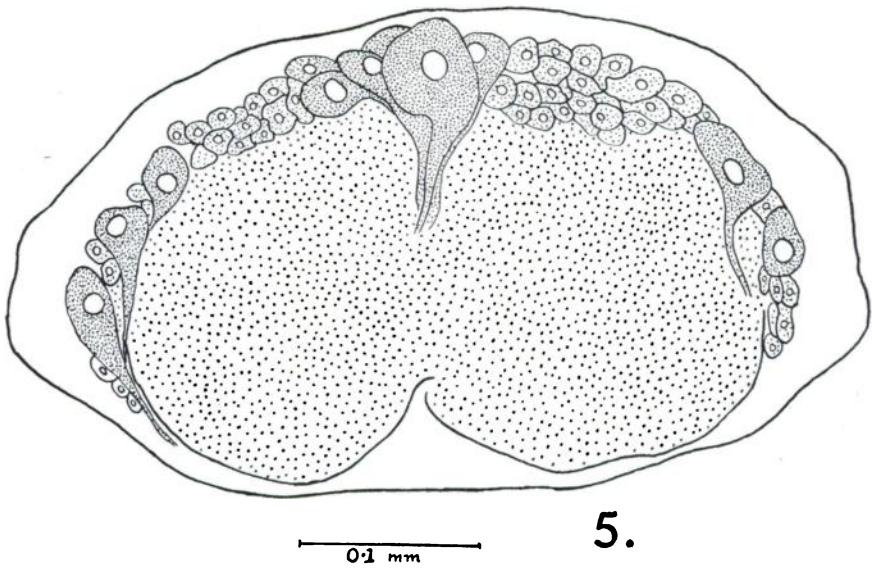
Under the phase contrast microscope, the cell is seen to contain a transparent and eccentric nucleus of low refractive index (Fig. 2). The chromocenters in the nucleus and the nucleolus appear dark, with a higher refractive index. The cytoplasm is filled with dark masses of granules which have generally a clumped appearance. Towards the broader edge of the cell are numerous tiny, dark granules which exhibit very active Brownian movement. The axons are traceable up to nearly three times the length of the cell. In the axons, as well as in the cytoplasm, are spheroids (see below) of variable size, with clear, dark rims and transparent

FIGURE 1. Transverse section of the nerve ring of *Iphita limbata* passing through the medial neurosecretory cells of the brain and the subesophageal ganglion. In the center is the esophagus. M = medial cells of the pars intercerebralis some of which are stained blue in Gomori's chrome-hematoxylin-phloxin. E = esophageal neurosecretory cells. Approx. × 95.

FIGURE 2. A medial neurosecretory cell of the pars intercerebralis under the phase contrast microscope. A few spheroids (S) are in focus. Approx. × 700.

FIGURE 3. Medial neurosecretory cells under the dark field microscope. They appear white; darker bodies in the cells are the nuclei. The white streaks are the tracheae. Approx. × 60.

FIGURE 4. The spheroids of the neurosecretory cell stained black in hematoxylin after fixation in Flemming without acetic acid. Approx. × 750.



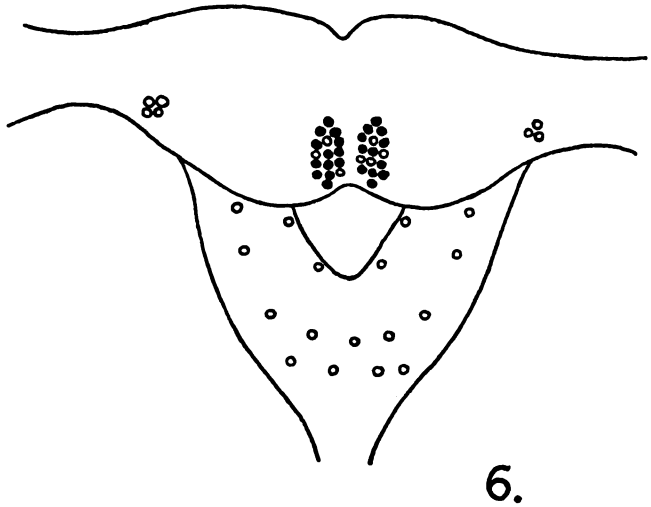


FIGURE 5. Camera lucida drawing (composite from a few adjacent sections) showing the marginal distribution of the subesophageal neurosecretory cells.

FIGURE 6. Diagram showing the distribution of the neurosecretory cells in the nerve ring of *Iphita limbata*. "A type cells" shown as black dots; "B type cells" shown as circles.

interior, appearing as extremely tiny droplets. Dark patches of a blotchy nature are seen on the nuclear membrane. The spheroids could be made out only with difficulty, but they could easily be distinguished when the distribution had been made out previously by vital staining methods.

In the dark field microscope the cytoplasmic content of the cell looks shiny and bluish-white, in the form of granules (Fig. 3). Besides granules there are larger bodies which are probably spheroids. The granules seem to flow along the axons.

Supravital staining in 0.001% neutral red gave good and uniform pictures of the cytoplasmic content. The cytoplasm shows red spheroids of variable size (Fig. 7). The neutral red spheroids measure from 0.71 μ to 2.86 μ in diameter. Somewhat similar results have been obtained by staining in 0.001% methylene blue. The spheroids here appear blue but the general staining effect is not quite as good as in neutral red.

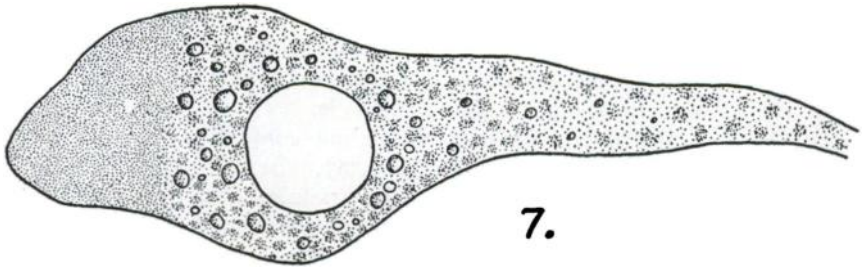
Supravital staining in 0.001% dahlia violet also gives a satisfactory picture of the cytoplasmic structures. The entire cell shows a very faint violet tint. The granulated mass stands out as dark greyish-violet bodies performing active movements. These granules show a blotchy appearance due to clumping. The spheroids do not always show up well; sometimes they appear as rounded bodies with dark violet rims and clear interior. Dahlia violet staining is not quite as good as neutral red; but in good preparations the granules and spheroids are traceable as conspicuously colored materials into the axons also.

The abaxonal broad part of the cell contains a vacuole-like structure which enlarges in due course into a conspicuous watery vacuole. Dense masses of granules fill this vacuole which show continual Brownian movement (Fig. 7). In the course of time these vacuoles part from the cells and appear as transparent drops filled with colored granules. These droplets sometimes take a faint reddish tint in neutral red. Such vacuoles have a low refractive index. The granules stain in the same way as mitochondria with Metzner's and Hirschler's methods. It is possible that these granules are derived from mitochondria, but this cannot be demonstrated conclusively with the methods used in this investigation.

Thus the study of living cells under phase-contrast and after supravital staining reveals that the cytoplasmic content of the medial neurosecretory cells of the brain is a complex of two substances: (1) a granular mass of small bodies, stainable dark greyish-violet by dahlia violet supravitally, contained in a fluid-filled vacuole; (2) a spheroidal system of tiny vacuole-like bodies, variable in size, stainable by neutral red, dahlia violet, and somewhat poorly by methylene blue.

An important structure in the cytoplasm of the neurosecretory cell is the spheroidal system. It consists of vacuole-like tiny spheres of variable size, supravitally stainable by neutral red, methylene blue, and dahlia violet.

The spheroids are demonstrable by the classical "Golgi" methods. When the cells are fixed in Flemming-without-acetic and stained in Heidenhain's iron hematoxylin, the spheroids appear as black bodies (Fig. 4). The spheroids are osmiophilic, and many of them appear as definite rings, while others look like large granules in Mann-Kopsch preparations after impregnation with osmic acid for two and a half days. These granules of different sizes could be seen on the nuclear membrane also. Similar bodies are discernible when cells are treated for about three days according to Kolatchew's method. The method of Aoyama is excellent to demonstrate the "Golgi system." The cytoplasm contains a system



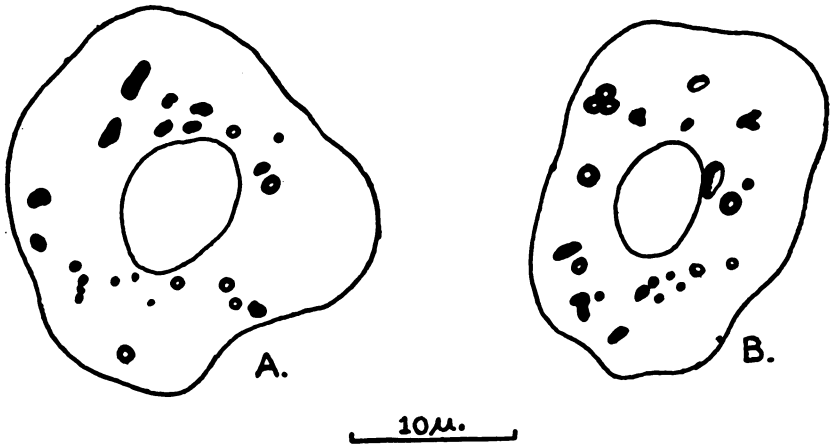


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FIGURE 7. Camera lucida drawing of a medial neurosecretory cell of the brain, stained in 0.001% neutral red. The neutral red vacuoles forming the spheroids are shown as circles. At the abaxonal part of the cell is a vacuole filled with mitochondria (fine stipples). The mitochondria of the rest of the cell appear in groups.

FIGURE 8. Camera lucida drawings of: A. A medial neurosecretory cell after treatment according to Aoyama's method, showing the "Golgi apparatus." The small spheroids which have coalesced appear as irregular masses, while the larger ones are ring-shaped. B. A neurosecretory cell after treatment according to Baker's sudan black method. Note similarity of the spheroids in both preparations.

of spheroids which show a deposit of silver around the periphery of the large spheroids, while the smaller vacuoles are more or less completely blackened. The picture in Aoyama preparations closely resembles the cells stained by neutral red; the neutral red spheroidal structures corresponding to the black ones in silver preparations (Fig. 8A).

Thomas' technique is very useful for the study of the development of the osmiophilic structures in the spheroids of the live cells. Freshly dissected neurosecretory cells were placed on a slide in a drop of 2% osmic acid. The coverslip was then sealed off. Within about five minutes the cells appear brownish. This becomes well marked in about thirty minutes; after about fifty minutes a few fine black granules and crescent-shaped black rims make their appearance. These are the developing osmiophilic elements. The subsequent deposition of osmium is comparatively slow. By about sixteen hours, the spheroids look like rings with the periphery almost completely blackened. In the deeper parts of the cell, such complete ring-like formation occurs in about a day. At room temperature (28–29° C.) the spheroids of all sizes become completely blackened after the fifth day. The cytoplasm as a whole then gets tinged with dark grey.

This method showed the gradual blackening of the margin of the spheroids which ultimately produced the configuration seen in the classical "Golgi preparations."

Baker's sudan black method shows that these spheroids are lipoidal in constitution. This is further supported by the acid hematein test which gives a positive result. Sudan black staining in both frozen and paraffin sections has similar results, and the preparations strikingly resemble those described above (Fig. 8B).

The spheroidal system of the neurosecretory cells could be reasonably described as lipochondria of variable size, characteristically osmiophilic, argentophilic, and sudanophilic.

In addition to the secretory granules and the spheroid system, other constituents of the neurosecretory cells were examined. In sections fixed in Heidenhain's mercuric-saline, and stained with methyl green-pyronin according to a modification of Unna-Pappenheim, the cytoplasm of the neurosecretory cells showed red or dark pink coloration indicating a concentration of ribose nucleic acid. The large nuclei of these cells are colored light pink, having in some cases a faint greenish tinge also, which indicates a comparatively low concentration of chromosome nucleic acid.

Baker's acid hematein test showed that the medial neurosecretory cells of the brain react strongly positively. The cytoplasmic products are colored a brilliant blue in both A and B types of cells. The mitochondrial and lipochondrial materials of the cytoplasm react like this: Pyridine extraction followed by acid hematein test shows no coloration at all. This is positive indication of the presence of phospholipines in the cytoplasm.

Though sudan black selectively stains the lipochondria found in the spheroids, simple staining by Sudan III according to Nath's method was unsuccessful. There was no indication of any coloring in these cases.

Barnett and Bourne's method for vitamin C revealed the presence of scattered black granules in the cytoplasm of the neurosecretory cells. They are more numerous in a perinuclear zone and close to the nuclear membrane.

Best's carmine test for glycogen on Bouin-fixed material was negative in the cytoplasm of the neurosecretory cells.

An indication of the presence of protein material in the cytoplasm was observed. A pink or light brick-red color developed after treatment with Millon's reagent for one hour at 60° C. after Bouin-fixation. There was no definite result with the xanthoproteic reaction. Sections of brain fixed in formal-calcium and treated with potassium ferrocyanide and ferric chloride showed an especially bright blue color in the cytoplasm of the neurosecretory cells, indicating the presence of protein. Bouin-fixed material treated according to Pollister's method also revealed the presence of a high protein content in these cells (development of a brick-red color in contrast to the light color of other parts of the brain).

Repeated tests were made to determine whether chromaffin granules are present in the cytoplasm of the neurosecretory cells. The chromaffin test of Lison, indole reaction, Vulpain reaction and Sevki's Giemsa staining method all gave negative results.

In cytochemical studies, Gomori's chrome-hematoxylin-phloxin method has been described to be selective for lipofuscins. The deep blue neurosecretory material of *Iphita limbata* may be considered as lipofuscins. But Schmorl's method for melanin and lipofuscin is not very useful for the characterization of neurosecretory cells.

Discussion

The distribution of the neurosecretory cells of the brain resembles that reported in other groups of higher insects. Those of the hemipteran brain have been described by Hanström (1938). The presence of neurosecretory cells has also been reported in the subesophageal ganglion, which forms an important neurosecretory center in orthopteroid insects (Scharrer, 1941). In an earlier note (Nayar, 1953) neurosecretory cells in the brain of *Iphita limbata* were described which stain blue with Gomori's chrome-hematoxylin-phloxin. In the present study, two types of neurosecretory cells are described in this species, A and B cells, which can be distinguished by their staining properties.

From a study of histological sections and from experimental investigations, various authors (Scharrer and Scharrer, 1944; Scharrer, 1952; E. Thomsen, 1952, 1954; Arvy, Bounhiol and Gabe, 1953; M. Thomsen, 1954) concluded that the neurosecretory products are transported along the axons and, in the case of the protocerebral neurosecretory cells, reach the corpus cardiacum, where they are stored. The observations in *Iphita limbata* support this view, the neurosecretory material being traceable along the nervi corporis cardiaci.

E. and M. Thomsen (1954) described the appearance of living neurosecretory cells in the darkfield microscope. The fresh cells of Iphita studied in ordinary, darkfield, and phase contrast microscopes show signs of a pronounced glandular activity. The large nucleus resembles that of other glandular cells with conspicuous nucleolus and chromonemata with large chromocenters. The cytoplasm is densely filled with secretory material which is seen to flow along the axons. This product resembles that observed in the corpora cardiaca of *Locusta migratoria* (Nayar, 1954).

The spheroids are osmiophilic and argentophilic and so give rise to the classical

"Golgi" pictures. A similar spheroidal system has been described in *Locusta migratoria* (in the metathoracic motor neurons by Shafiq, 1953, and in the corpus cardiacum by Nayar, 1954). Baker (1950) has pointed out that spherical or spheroidal bodies in live cells are of a lipoidal nature; the smaller ones are lipoidal throughout and the others contain a spherical vacuole of non-lipoid material within, so that the lipoid is in the form of an enveloping sheath or externum. This description also applies to the spheroids seen in the neurosecretory cells of *Iphita limbata.*

The entire content of the cytoplasm of the median neurosecretory cells of the brain is rich in phospholipines. This is evidenced by the positive bright blue coloration with acid hematein and by the lack of coloration with acid hematein after extraction with pyridine. Baker (1946) has pointed out that mitochondria in many cases react positively to the test, and Cain (1947) has shown that the lipochondria contain phospholipines. The phospholipines of the neurosecretory cells may be the combined lipines of the spheroidal and mitochondrial substances.

Part of the cytoplasmic content is ascorbic acid appearing as black granules when the cells are subjected to treatment according to the method of Barnett and Bourne (1942). These authors have described the presence of vitamin C granules in the neurons of the chick. The distribution in the neurosecretory cells of *Iphita limbata* is somewhat similar with granules scattered in the cytoplasm and apposed to the nuclear membrane.

The different tests for proteins, precipitin, etc. have indicated the presence of some type of protein in the cytoplasm. Tests for chromaffin inclusions and glycogen gave negative results. Cameron (1953) has suggested that the chromaffin content of the corpus cardiacum in the locust is secreted by the gland itself and is not elaborated by the neurosecretory cells. This may also apply to *Iphita limbata,* because chromaffin material is not seen in the cytoplasmic content of these cells.

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Summary

1. In *Iphita limbata* (Pyrrhocoridae: Hemiptera) the brain contains paired medial and lateral groups of neurosecretory cells, and the subesophageal ganglion scattered marginal neurosecretory cells. The medial cells (pars intercerebralis) number about 16 on each side, the lateral three or four.

2. On the basis of their staining properties two types of neurosecretory cells can be distinguished in the nerve ring of Iphita. "A cells" staining a deep blue with Gomori's chrome-hematoxylin-phloxin and a bright red with Azan make up most of the neurosecretory cells of the brain. The subesophageal ganglion contains "B cells," staining red with Gomori's method and light blue with Azan.

3. The corpora cardiaca and their nerves (nervi corporis cardiaci) contain a material similar in its staining properties to that of the "A cells."

4. The medial neurosecretory cells of the protocerebrum contain large nuclei with a low, and nucleoli with a higher, refractive index. The nucleoli are phlox-

inophilic and azocarminophilic. The cytoplasm contains granules and spheroids. The granules appear black in the phase contrast microscope, violet with dahlia and, like mitochondria, red with acid fuchsin; they exhibit continual Brownian movement in the living cells. The granules are associated with a fluid material. The spheroidal system which is osmiophilic, argentophilic, and sudanophilic, represents the lipochondria. Both the granular and spheroidal systems are revealed by supravital staining methods.

5. The cytoplasm of the neurosecretory cells contains a high concentration of ribose nucleic acid. The nuclear membrane and the cytoplasm show granular concretions of vitamin C. The secretory material contains proteins as indicated by various tests. The granular and spheroidal constituents are rich in phospholipines.

6. Tests for chromaffin substances and glycogen gave negative results. Staining methods for lipofuscins show that the product in the "A cells" probably contains these very complex substances.

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