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A Histochemical Study of the Neurosecretory Substance(s) Controlling Osmoregulation of Lumbricus Terrestris

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A HISTOCHEMICAL STUDY
OF THE NEUROSECRETORY SUBSTANCE(S)
CONTROLLING OSMOREGULATION OF LUMBRICUS TERRESTRIS

by
William
John Wm. Goudie

A Thesis
Submitted to the
Faculty of the School of Graduate
Studies in partial fulfillment
of the
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
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John Wm. Goudie

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INTRODUCTION

The presence of neurosecretory cells in the supraesophageal ganglia of Lumbricus terrestris has been confirmed by numerous investigators since they were first described by B. and E. Scharrer in 1937. In common with neurosecretory cells of all animals, these specialized neurons of earthworms have been shown to synthesize, elaborate, and release neurosecretory substances, which have been presumed to function as hormones (Marapao, 1959; B. Scharrer, 1959).

The control and regulation of maturation of gametes, reproduction, and regeneration have been definitely assigned to secretions of specific neurosecretory cells in brains of earthworms. More recently it has been suggested that osmotic and ionic regulation are controlled by neurosecretory substances also. Earthworms have been shown to regulate osmotically (Bahl, 1947; Ramsey, 1949) as well as ionically (Kamemoto, Spalding, and Keister, 1962) and Kamemoto (1964) demonstrated that some substances or substance present in the brains of these organisms controlled their osmotic and ionic regulation. Although it is presumed that these regulatory substances are products of neurosecretory cells, there is no evidence to substantiate this conclusion.

The purpose of this investigation was to study the characteristics of neurosecretory cells in the cerebral ganglia of L. terrestris using histochemical procedures and to attempt to demonstrate that neurosecretory substances played a role in ionic and osmotic regulation. The second part of this investigation was accomplished by

forcing earthworms to regulate ionically and osmotically by subjecting them to hypotonic and hypertonic environments and observing the effects of these osmotic and ionic stresses on the histological appearance of the neurosecretory cells.

Literature Review

The existence of modified nerve cells capable of glandular secretions, as evidenced by the possession of secretory granules, has been established in nearly all groups of animals possessing a centralized nervous system (Bullock and Horridge, 1965). These specialized neurons, which possess many if not all of the functions associated with ordinary neurons, have come to be known as neurosecretory cells in invertebrates as well as vertebrates.

Neurosecretory cells in the earthworm, L. terrestris were described by B. and E. Scharrer (1937) in a review of the literature of this field. Although neurosecretory cells in polychaetes and leeches (Bullock and Horridge, 1965), have also been described, this literature survey will be confined to that of the neurosecretory cells in the oligochaetes.

Numerous neurosecretory cells stained by various histochemical procedures have been described in the supraesophageal ganglia of L. terrestris. Schmid (1947) confirmed the existence of these cells and Marapao (1959) recognized five morphologically different cell types in the brain of L. terrestris after chrome alum haematoxylin phloxin staining. The first cell type designated as an a cell was located in the lateral and dorsal regions of the brain, according to

Marapao, and can occasionally be found scattered among other cell types. This a cell type was characterized by an acidophilic staining reaction. The second cell type designated by Marapao as b-1 was the largest of the cell types and was found in the dorsal, lateral and ventral regions of the brain. These cells, according to Marapao, have an affinity for haematoxylin of the chrome alum haematoxylin stain. The third designated cell type was the c cell which was similar to the b-1 cell type, the most prominent difference being the presence of vacuoles in the cytoplasm. The fourth cell type, b-2 cells, according to Marapao stain intensely blue with the haematoxylin and are smaller than the previously mentioned b-1 cells. He has found b-1 cells to be rare in what he considers control or normal earthworm brains. The final cell type designated by Marapao are neuroglia cells. These cells are small, oval and their chromatin material stains deep blue with haematoxylin. Marapao calculated that the a, b-1, and c cells of the control worms, together made up 68.4% of the grand total of secretory cell types.

After describing the neurosecretory cells of L. terrestris, Marapao attempted to demonstrate that their secretory products were hormonal in function. This involved the non-specific stress of needle pricking as well as brain extract injections to determine effects of excess secretory products on the neurosecretory activity of the brain cells of L. terrestris. He concluded from these experiments that there actually were only two types of secretory cells in the cerebral ganglia. The first secretory cell type included three stages corresponding to his previously described a, b-1, and c cells.

The second secretory types include his previously described b-2 type neurosecretory cells.

Some investigators have studied and classified the neurosecretory cells in the brains of other oligochaetes. Herlant-Meewis (1956) described two types of cells in the cerebral ganglia of Eisenia foetida, which she labeled a cells and b cells. The a cells, situated peripherally on the posterior side of the cerebroid ganglia, were generally ovoid in shape when they contained neurosecretory granules. The type b cells localized much deeper in the brain were fusiform in shape and their secretory product had a granular appearance.

Hubl (1956) described a neurosecretory cell in L. terrestris which he designated as a c cell and whose activity he correlated with regeneration of the posterior end of these worms. Neurosecretory cells in L. terrestris, and E. foetida have also been found in the subpharyngeal ganglia and ventral ganglia (Herlant-Meewis, 1955, 1956; Hubl, 1953).

Bianchi (1967) described amine secreting neurons in the ganglia of the earthworm, Octolasion complanatum. From his cytological findings, he believes that the secretions from these cells are discharged into the blood stream and act as true hormones.

The neurosecretory cells of oligochaetes described by each investigator may or may not be completely equivalent, since neurosecretory cell types that have been given the same name by different investigators sometimes seem not only to be related to different physiological conditions (Bullock and Herridge, 1965), but also are different morphologically. Marapao (1959) concluded from his

experiments as described above that some of the cell types which he classified originally as 3 separate types are actually the same cell type in different phases of the secretory cycle.

The formation of secretion granules has been studied through use of the electron microscope. Scharrer and Brown (1961), upon the basis of ultra-structural evidence, concluded that the granules of neurosecretory cells in the supraesophageal ganglia of the earthworm, L. terrestris, were formed in association with the Golgi apparatus. Other investigators have found that elementary neurosecretory granules occurred in association with systems of membranes and the tubules of the Golgi organelle (Bern, Nishioka and Hagadorn, 1961). In all cases, however, most investigators agreed that the neurosecretory material was synthesized in the soma of the cell.

The site of release and the mode of transport of neurosecretory material within the cell is a highly speculative subject for annelid worms. Many authors have postulated that the release of material in organisms possessing a neurosecretory system, including annelids, is through the axonal bulb with a preceding axonal migration (Bern, 1962; E. Scharrer and B. Scharrer, 1963; Herlant-Meewis, 1956). A recent study of the literature shows that there is no concensus regarding the release of neurosecretory materials through axonal bulbs of neurosecretory cells, not only in annelids but also in all invertebrates. However, Dogra (1967) concluded that perhaps ultrastructural studies of isolated neurons and their axon bulbs in oligochaete worms would show that this was the means of release.

A number of histochemical techniques have been adapted to demonstrate neurosecretory cells of the neuroendocrine systems of invertebrates including annelids. The two staining techniques which have been used mainly are the chrome alum haematoxylin method and the aldehyde-fuchsin method of Gomori (1950). A third technique now used to demonstrate this material is the performic acid/alcian blue method introduced by Adams and Sloper (1956). Adams and Sloper concluded that neurosecretory material was rich in cystine and cysteine. Their technique depended on the oxidation of cystine and cysteine to cysteic acid and the resulting uptake of the basic dye, Alcian blue. Various modifications of Adams and Sloper's technique usually also involved oxidation of cystine to cysteic acid followed by demonstration of the acid with basic stains as Victoria blue (Dogra and Tandan, 1964) and Gomori's aldehyde-fuchsin. These authors' use of a Performic acid/Victorian blue method has been successful in staining oxidized sections of ganglia in insects and annelids.

Dogra (1967) investigated neurosecretory phenomena in the earthworm, Pheretima posthuma, using three staining techniques. These were the paraldehyde-fuchsin (PF), performic acid/Victorian blue (PAVB), and the paraldehyde thionin (PATH) methods. All three techniques are Adams and Sloper modifications. He found that in P. posthuma the neurosecretory system is composed of two main types of cells located in the supraesophageal ganglia. The a cells stain purple with PA, greenish-blue with PAVB, and blue with PATH. The b cells stain light purple or brick red with PA, light blue with

PATH and show a negative response to the PAVB.

In this same study, Dogra traced the axons of groups of neurosecretory cells into the subesophageal ganglia and showed that the axons did converge in the vicinity of the blood capillaries where, he concluded, they probably released their secretory products.

Dogra, (1967) in agreement with Clark (1963), came to the conclusion that the presence of stainable material in annelid neurosecretory cells indicated that the rate of synthesis of the material outpaced its rate of release and that the accumulation of it in the cell may be caused by a change in either rate.

A number of functions have been attributed to the secretions from the neurosecretory cells of earthworms. However, extreme caution must be used when neurosecretory substances are correlated to particular functions in any animal. Simpson, Bern, and Nishioka (1966) have proposed a number of criteria which can be applied in determining neurosecretory functions of a particular neurosecretory cell. Although their criteria were developed in connection with their study of neurosecretory phenomena among gastropods, they can be applied to other organisms, including annelids. These authors emphasized the unreliability of staining images, stating that there was no specific stain for neurosecretion and that various structures and inclusions have similar affinities for these stains. They concluded, also, that the presence of stainable neurosecretory granules does not necessarily establish a neuroendocrine status of function. Secondly, caution must be used in the correlation of changes in neurosecretory neurons and changes in body function and/or structure.

For instance, subjection of animals to stress alone may, according to Simpson, et. al., (1966), cause changes in supposed neurosecretory cells and may also cause changes in other neurons and in tissue cells in general. However, specificity of response of neurosecretory cells was considered by these authors to be an important basis upon which to construct tentative physiological hypotheses.

The role of neurosecretion in regeneration in oligochaete worms has been known for some time (Birrell, 1957). The details of this regulation has been worked out recently. Experimental evidence has shown that in these animals the supraesophageal ganglia produced hormones essential for regeneration (Herlant-Meewis, 1961; Clark, et. al., 1961).

Hubl (1956) in studying the effects of the cerebral ganglia upon regeneration in L. terrestris has shown that type b cells located in the cerebral ganglia at the junction with the circumoesophageal commissures, become vacuolated following loss of posterior segments. From these observations, he concluded that type b neurosecretory cells are the source of the hormones involved in regeneration.

Neurosecretory hormones have also been associated in annelids with control over reproduction. Durchon (1962) demonstrated that reproduction in oligochaete worms was under a hormonal control of cerebral ganglionic origin by carrying out several operations upon Eisenia foetida during their egg laying period and when they showed well developed sexual characteristics. Elective ablation of the cerebral ganglia, the subesophageal ganglia or both these structures

always resulted in a reduction in body size, the progressive disappearance of somatic sex characteristics, and an arrest in the egg laying process. All these modifications were temporary. Within a period of 4-7 weeks, the operated individuals were found to return to their primary condition, when the resected ganglia regenerated. From these results Durchon concluded that the secondary sex characteristics are probably controlled by a hormonal factor.

From histological studies of the supraesophageal ganglia of E. foetida and L. terrestris, Herlant-Meewis (1955), discovered that the secondary sex characteristics, after regeneration of resected nerve centers, always coincided with the recovery of neurosecretory cells which she designated as type a. It has been pointed out that in these organisms a seasonal cycle of secretion occurred which resulted in the draining off of secretions at the beginning of spring when somatic sex characteristics were developing. In periods of inactivity these organisms stored colloid in their parakaryon (Bullock and Horridge, 1965). From these observations Herlant-Meewis concluded that type a neurosecretory cells in the cerebral ganglia of E. foetida and L. terrestris was the origin for the production of factors indispensable for the appearance and maintenance of somatic sex characteristics.

Bahl (1947) and Ramsey (1948) have demonstrated that earthworms regulate osmotically. In addition, Bahl concluded that when an earthworm was in its natural environment its nephridia functioned adequately as volume and osmoregulatory organs. However, when kept in water and made to live like freshwater animals, large quantities of water were

absorbed through the skin which cannot be eliminated by the nephridia alone. At this point the gut took over a share in osmo- and volume regulating by eliminating water through the mouth and anus.

Ionic regulation has been demonstrated in earthworms by Kamemoto, Spalding, and Keister (1962). L. terrestris, E. foetida and Helodrilus caliginosus were shown to regulate sodium, potassium and calcium because concentration of these electrolytes was higher in the blood than in the coelomic fluid. These authors presented further evidence for ionic regulation by showing that the earthworms were capable of maintaining the sodium levels of their blood higher than environmental sodium levels up to a sodium concentration of 0.1M in the medium.

The influence of the brain upon osmotic and ionic regulation in E. foetida and L. terrestris has been explored by Kamemoto (1964). Removal of the brains of these animals resulted in increased body weight due to the uptake of water, as compared to the normal animals, when placed in tap water. This increased uptake of water by debrained worms was accompanied by a decreased sodium concentration in the coelomic fluid and the blood. It was demonstrated that this was not due to dilution alone. Changes in sodium concentrations in the body fluids were prevented in brainless animals when the brain was reimplanted or with the injection of brain homogenates. These results indicated to the authors that there was a factor or factors present in the brain of these organisms which exerted influence upon ionic and osmoregulation. Kamemoto suggested that neurosecretory cells might be the source of the regulatory factors.

A histochemical study was carried out by Wall and Ralph (1964) in which the production by neurosecretory cells of an "antidiuretic hormone" was suggested in the cockroach, Periplaneta americana. This suggestion was based on the histological changes which occur in the neurosecretory cells in response to dehydration and salt-loading, and on studies on the uptake of dyes by the isolated Malpighian tubules. Dehydration or salt-loading of the animal caused a decrease in the rate of uptake of the dye by the tubules which was indicative of decreased rate of water uptake. The active factor has been demonstrated in extracts of the brain and corpora allata of normal animals but not in dehydrated or salt-loaded animals. Wall and Ralph suggested that the "antidiuretic hormone" was secreted in the brain and was transported to the corpora allata for storage, and then released from the corpora allata when there was a need for conservation of water.

METHODS AND MATERIALS

These investigations were carried out on the cerebral ganglia of the earthworm, Lumbricus terrestris. The worms were purchased from a local bait shop and were collected by them during the late fall. Only those displaying a mature clitellum were used. Animals were maintained in the laboratory at 2°C in moist bedding material. To avoid the possibility of diurnal variations, the ganglia were always extirpated between seven and seven-thirty P.M.

The specimens were anesthetized by placing them in 10% ethyl alcohol. The brains were exposed by a longitudinal mid-dorsal incision and fixed insitu in Bouin's fluid. After 30 minutes the ganglia were removed and placed in fresh fixative for 24 hours, embedded in paraffin (M.P. 56.5°C), and sectioned on a longitudinal frontal plane. Standard histological techniques were employed to prepare the paraffin section for staining. The stains used in this investigation were Gomori's aldehyde-fuchsin with Halmi's mixture as a counterstain and Harris' haematoxylin with eosin Y as a counterstain. In all cases duplicate brains were processed, each being stained with one of the two staining procedures used in this investigation. This was done so that each brain which was stained specifically for neurosecretory granules, would have a "reference" brain stained with standard haematoxylin and eosin.

In the procedure involving staining with Gomori's aldehyde-fuchsin (Humason, 1962) the mounted sections, following removal of the paraffin, were subjected to oxidation by means of a strong oxidant, so that the

cystine and cysteine which Adams and Sloper (1956) considered to be characteristic of neurosecretory granules would be oxidized to cysteic acid. This sulphonic acid contained highly ionized acid radicals which would take up basic dyes readily. The oxidant used in this investigation was a sulfuric acid-potassium permanganate oxidant (0.3 g KMnO_4 in 100 ml of water containing 0.30 ml concentrated H_2SO_4). The sections were then bleached with a 4% sodium bisulphate solution. The basic dye used to stain these oxidized neurosecretory granules was, as indicated above, Gomori's aldehyde-fuchsin. This particular technique of oxidation was developed by Dogra and Tandan (1964) to demonstrate neurosecretory material and thus neurosecretory cells in whole mounts of the central nervous system of insects and annelids. Their technique was a modification of one originally developed by Adams and Sloper (1956).

Since Gomori's aldehyde-fuchsin stained only neurosecretory material, the present investigation used Halmi's mixture (Humason, 1962) as a counterstain so that the other cell structures could be distinguished.

Worms maintained in the laboratory in moist bedding material at 20°C were designated as standard controls. A number of these ganglia were processed according to the 2 procedures described above.

The effects of osmotic stress on the neurosecretory cells in the supra-pharyngeal ganglia were studied in the following manner. Earthworms in groups of 10 were placed in isotonic (0.14M or 0.8%), hypotonic (distilled water), and hypertonic (0.28M or 1.6%) solutions for 1, 6, and 24 hours. The exact composition of the isotonic

saline solution is presented in Table 1 and the hypertonic solution is presented in Table 2.

Table 1 -- Composition of Isotonic Saline Solution (0.14M or 0.8%)
(Welsh and Smith, 1960)

0.54M	NaCl	-----	250 ml
0.54M	KCl		5 ml
0.36M	CaCl ₂		5 ml
0.36M	MgCl ₂		1 ml
0.44M	Na ₂ SO ₄		1 ml

The solution was brought to 1 liter with distilled water.

Table 2 -- Composition of Hypertonic Saline Solution (0.28M or 1.6%)
(Welsh and Smith, 1960)

0.54M	NaCl	-----	500 ml
0.54M	KCl		10 ml
0.36M	CaCl ₂		10 ml
0.36M	MgCl ₂		2 ml
0.44M	Na ₂ SO ₄		2 ml

The solution was brought to 1 liter with distilled water.

Immediately upon removal from the experimental solutions, the cerebral ganglia were processed according to the previously described techniques.

The length of time that the worms were maintained in the isotonic, hypotonic, and hypertonic media was purely arbitrary. It was considered that if any neurosecretory material was involved in osmotic and/or ionic regulation that these particular time intervals would result in evidence of an immediate response by the cells as well as response to a stimulation continuing for a more extended period.

The sections of the cerebral ganglia of standard controls and experimental controls, maintained in an isotonic media for the 3 time intervals, and those worms subjected to hypotonic and hypertonic conditions for the same stated time intervals, were studied. In all cases the brain sections stained specifically for neurosecretory granules were compared with those brains stained with haematoxylin and eosin. The neurosecretory cells in the brains of standard control worms were identified on the basis of their staining characteristics and were examined for morphological characteristics, location in the ganglia, staining reaction, and amount of stainable material. The brains of all experimental worms were similarly examined and observations were made of any changes in any of the neurosecretory cells which could be correlated with osmotic and ionic regulatory activity by the worms.

Photomicrographs were taken to aid in comparison of neurosecretory cells in all groups as well as to illustrate the characteristics of the types of neurosecretory cells and changes correlated with osmotic regulation by these worms.

RESULTS AND DISCUSSION

The supra-pharyngeal ganglion, or the brain, of L. terrestris is a simple, bilobed structure lying on the dorsal surface of the pharynx between the third and fourth segments. All ganglia were sectioned in a frontal, longitudinal plane. In viewing these sections, the viewer is looking down on the dorsal aspects of the brain. On all plates the photographs are oriented so that the anterior end of the ganglion is to the top of the page.

Standard Control Brains Stained with
Haematoxylin and Eosin

All brains stained with haematoxylin and eosin were studied to establish the histological details when standard staining procedures were used. A standard control brain from a worm maintained in moist bedding material at 2^oC is shown in Figure a, Plate 1. The tough connective tissue sheath is still in place. A number of blood vessels can be observed in this covering as well as striated muscle tissue from the pharynx.

The cellular organization of these brains is typical of all invertebrate ganglia. The cell bodies of the neurons are located around the periphery, and the neuropile occupies the medullary region. The neurons, whose cell bodies are spherical in shape and stain typically (blue to purple with the haematoxylin) indicating the position of chromatin material and other chromatophilic structures. In the photographs these structures are darker in color. The eosin

stains the cytoplasm of the neurons a pink color which shows as a lighter color in the photographs. Cells which contain secretory granules can also be observed in the photographs and are indicated by arrows. However, it is difficult to characterize them. One type, which is identified as type b cells, in sections stained with Gomori's aldehyde-fuchsin stain, may be observed in the anterior regions. The larger neurosecretory cells located posteriorly in Figure a, Plate 1 are classified later as type a cells with the above stain.

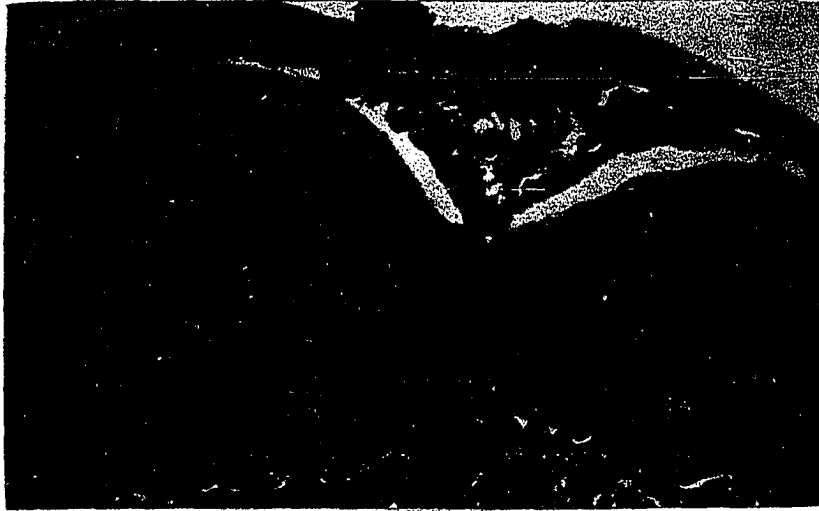
It is not possible to classify type a and type b cells as neurosecretory cells on the basis of their appearance with the haematoxylin and eosin stain. The appearance of the brain in Figure a, Plate 1 is typical of all the brain sections stained in this investigation with haematoxylin and eosin. All brains were stained specifically with Gomori's aldehyde-fuchsin and compared with brains stained with haematoxylin and eosin. No further analysis of haematoxylin and eosin sections will be given in this paper since this stain is not specific for neurosecretory granules.

Standard Control Brains Stained with Gomori's Aldehyde-Fuchsin

In brains stained with Gomori's aldehyde-fuchsin following oxidation, two main types of neurosecretory cells were demonstrated in the cerebral ganglia of L. terrestris in this investigation. All results of staining both control and experimental brains with Gomori's aldehyde-fuchsin are summarized in Table 3, page 19. The cells have been named type a and type b by this investigator (Plate 2, Figures a, b, and c).

PLATE 1

Anterior



Posterior

Figure a. Frontal longitudinal section of supra-pharyngeal ganglion of L. terrestris stained with haematoxylin and eosin. 150X Cells presumed to be neurosecretory granules are indicated by arrows 1 and 2. Typical neurons are indicated by arrow 3. The darker colored structures stained with haematoxylin; these were dark blue to purple in color. The lighter colored structures stained with eosin are light pink.

Table 3 -- Summary of Results

Cell type	Characteristics of neuro-secretory cells of standard controls stained with Gomori's aldehyde-fuchsin	Experimental controls stained with Gomori's aldehyde-fuchsin	Hypo-osmotic condition	Hyper-osmotic condition
a	<p>Large coarse granules loosely packed in cytoplasm</p> <p>Stain light purple</p> <p>Located in posterior regions of ganglion</p> <p>Cell size - 16.8-48.2 by 16.8-31.0 microns</p>	<p>Same as standard controls with Gomori's aldehyde-fuchsin</p>	<p>Loss of granulation in cytoplasm</p> <p>Numerous vacuoles</p>	<p>Greater loss of granulation from cytoplasm than hypo-osmotic stress</p> <p>Numerous vacuoles</p>
b	<p>Fine granulation tightly packed in cytoplasm</p> <p>Stain dark purple to brick red</p> <p>Located in dorsal and anterior of ganglion</p> <p>Cell size - 12.0-26.4 by 9.6-24.0 microns</p>	<p>Greater intensity of stain causing cells to be darker than standard controls</p>	<p>Same as in experimental control</p>	<p>Same as in hypo-osmotic except intensity of stain more pronounced</p>

Standard Controls: worms maintained at 2°C in moist bedding material

Experimental Controls: worms subjected to a 0.14M isotonic saline solution for 1, 6 and 24 hours

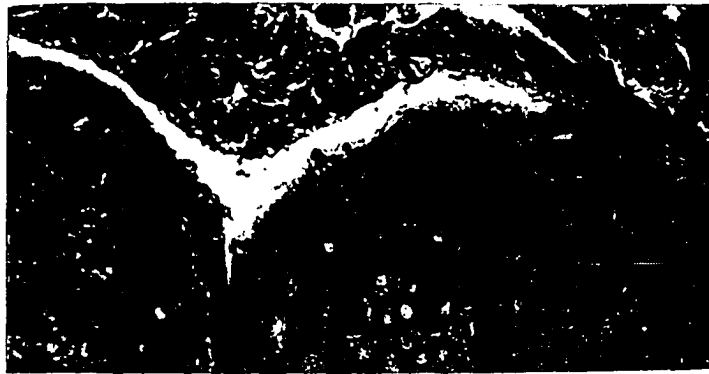
The type a cells (Plate 2, Figure a and b) are located in the posterior regions of the brain. These cells are oblong to spherical in shape and range in size from 16.8-48.2 by 16.8-31.0 microns. Type a neurosecretory cells when stained with Gomori's aldehyde-fuchsin showed neurosecretory material in the form of large coarse granules which were not packed tightly together in the cytoplasm of the cell. This can be seen in Plate 2, Figure b. This photograph shows type a cells at 1000X. The granules in the cytoplasm of this cell type stained light purple.

The second cell type recognized in this investigation were designated as type b. This cell type can be found in the dorsal and anterior parts of the brain. The secretory granules were very fine and tightly packed together. They stained purple to brick red with the Gomori stain (Plate 2, Figure a and c). As a result, these cells were very dark in the photographs. As in the case of type a cells, the nuclei stained more lightly and were thus easily distinguished. Type b neurosecretory cells were found to be oval to spherical in shape. They are, in general, smaller than type a cells, with cell dimensions of 12.0-26.4 by 9.6-24.0 microns.

The cell type designated as type a in this study corresponded to those cells named a, b-1, and c by Marapao (1959) and which he considered to be three different secretory stages of the same cell type. Type b cells were considered to be the cells which he named b-2. Other investigators (Herlant-Meewis, 1955, 1956; Hubl, 1953) have described neurosecretory cells in the brains of other earthworms which they have named a and b cells. However, it was not possible to

PLATE 2

Anterior



Posterior

Figure a. Frontal longitudinal section of supra-pharyngeal ganglion of *L. terrestris*. 150X. Stained with Gomori's aldehyde-fuchsin. Arrow 1 indicates type b cells found in anterior region of ganglion. These cells contain fine granules which are tightly packed together and stain dark purple to brick red. Arrow 2 indicates type a cells which are larger than type b cells. Type a cells contain coarse granules which are not tightly packed and which stain a light purple. These cells are located in the posterior region of the ganglion.



Figure b. Higher magnification, 1000X, of type a cells as indicated in Figure a. Coarse granules can be seen in cytoplasm. These granules stain light purple.



Figure c. Higher magnification, 1000X, of type b cells indicated in Figure a. Fine granulation in cytoplasm is tightly packed. These granules stain dark purple to brick red.

determine whether the type a and type b of this investigation were equivalent to these.

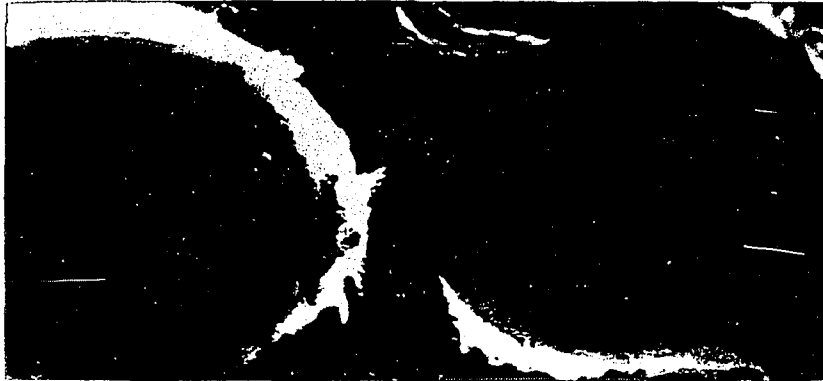
Experimental Control Brains Stained with
Gomori's Aldehyde-Fuchsin

Neurosecretory cells of worms subjected to the isotonic saline solution, and termed experimental controls, showed results comparable to the standard controls. Plate 3, Figure a, shows a section of a ganglion stained with the Gomori stain from a worm maintained in isotonic saline for 1 hour. Type b neurosecretory cells can be seen in the anterior region of the brain. In Figure b, Plate 3, these cells are shown at a magnification of 1000X. As in the standard controls, these cells stained very darkly because of the compact arrangement of fine granules found in their cytoplasm. When these cells were compared to type b cells of the standard controls (Plate 2, Figure a) it could be observed that they were more prominent in the experimental controls because they are, apparently, darker in appearance. This may be due to a generalized stress reaction which is discussed in the next section. The stressful condition encountered by the worms in the isotonic solution may have resulted from the additional handling involved and the buffeting of the worms by air being bubbled through the solution.

In Figure a, Plate 3, type a cells located in the posterior regions of the brain are also shown. When these cells from worms maintained in isotonic solution for 1 hour were compared to type a cells of the standard controls, no differences in their appearances could be detected. The granules are coarse and not tightly packed

PLATE 3

Anterior



Posterior

Figure a. Experimental controls. Frontal longitudinal section of supra-pharyngeal ganglion of a worm subjected to isotonic saline solution for 1 hour. 150X. Stained with Gomori's aldehyde-fuchsin. Arrow 1 indicates type b cells which possess fine, tightly-packed granules staining dark purple to brick red. These cells are located in the anterior regions of the ganglion. Type a cells, indicated by arrow 2, are located in the posterior region of the ganglion. These possess granules which stain light purple and are not packed tightly.

Figure b. Higher magnification, 1000X, of type b cells as indicated by an arrow in Figure a. The fine granules stain dark purple to brick red and are tightly packed in the cytoplasm.



together. In addition, these type a cells in worms kept in the isotonic medium for 6 and 24 hours (Figures b and c, Plate 4) were similar in all respects to those of the standard control worms and experimental control worms in saline for 1 hour. Therefore, being subjected to an isotonic medium for up to 24 hours caused no visible change in the type a neurosecretory cells.

Effects of Osmotic Stress on Type b Cells

When type b cells of worms subjected to hypotonic conditions for periods of 1, 6, and 24 hours were compared to the same cells of control worms no decrease of neurosecretory material was observed. However, these cells appeared to show what may be a generalized response to stress in worms subjected to these conditions of osmotic stress. After the worms were exposed to hypo-osmotic stimulation, these type b cells appeared to have a greater intensity of stain. This can be seen when a standard control brain (Plate 2, Figure a) is compared to an experimental brain (Plate 5, Figure a). This change may result from an increase in the amount of the very fine granules or it may result from some change in the granules which caused them to take up more of the stain. In addition, the cells appeared to be greater in number. This apparent increase in number was not investigated by means of actual cell counts. Marapao (1959) however, was able to demonstrate that these cells actually increased in number when specimens of L. terrestris were stressed with the non-specific stimulus of needle-pricking. They also stained with greater intensity and it was his conclusion that this was a general response to a

PLATE 4

Figure a. Experimental controls. Type a cells at 1000X of worms subjected to isotonic saline solution for 1 hour. The coarse granules in these cells stain light purple with Gomori's aldehyde-fuchsin stain. These cells are found in the posterior region of the ganglion.

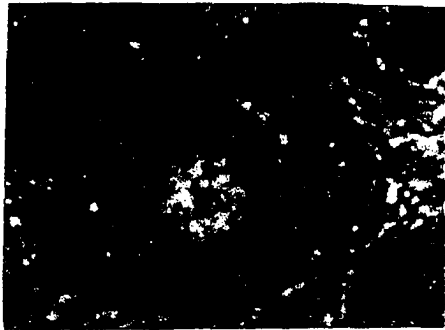


Figure b. Type a cells at 1000X of a worm subjected to isotonic saline for 6 hours. Granulation in cytoplasm stains light purple with Gomori's aldehyde-fuchsin stain.



Figure c. Type a cells at 1000X of worms subjected to isotonic saline for 24 hours. Granules are coarse and stain light purple with Gomori's aldehyde-fuchsin stain.



PLATE 5

Anterior



Posterior

Figure a. Frontal longitudinal section of supra-pharyngeal ganglion of worm subjected to 1 hour hypotonic stress. 150X. Stained with Gomori's aldehyde-fuchsin. Arrow 1 indicates type b cells which possess fine, tightly-packed granules staining dark purple to brick red. These cells are located in the anterior region of the ganglion. Arrow 2 indicates type a cells in the posterior region of the ganglion. These cells have lost stainable granules from their cytoplasm, as indicated by lighter coloration in the cytoplasm, as compared to type a cells of the standard controls and experimental controls.

stressful condition.

The same results were observed at all the time intervals when worms were subjected to hypertonic conditions (Plate 7, Figure a). Plate 7, Figure a, shows the b cells in the brain of a worm subjected to hypertonic stress for 1 hour. There was no lessening in the amount of granulation. The cells were again prominent in appearance because of an increased intensity of staining and there were apparently greater numbers of them.

Effect of Osmotic Stress on Type a Cells

Definite changes were observed in type a secretory cells when worms were subjected to osmotic and ionic stress. The cytoplasm of type a cells in the standard controls (Plate 2, Figure a) and in the experimental controls (Plate 4, Figures a, b, and c) contained a large amount of neurosecretory material in the form of coarse granules which stained light purple with Gomori's aldehyde-fuchsin.

When type a cells in brains of worms subjected to hypo-osmotic stress were examined, it was found that the loss of stainable secretory granules was readily apparent as illustrated in Plate 5, Figure a, at the lower magnification of 150X. This effect on these cells was relatively rapid as it was observed after subjecting the worms for 1 hour to a hypo-osmotic environment. The decrease of coarse granulation is also seen at 1000X in Plate 6, Figure a. This same lack of secretory granules was also observed after 6 and 24 hour subjection to hypo-osmotic stress (Plate 6, Figures b and c). Since the depletion of the secretory granules continued through the 24 hour period, it

would appear that the neurosecretory material was being used continuously to maintain a regulatory mechanism (s). Since it is known that earthworms can regulate indefinitely in tap water, a hypo-osmotic medium, it would be interesting to observe these neurosecretory cells in brains of worms subjected to hypo-osmotic stress for longer periods of time.

The photographs at all time intervals (Plate 6, Figures a, b, and c) at the higher magnification showed that considerable vacuolization has occurred in the cytoplasm. A possible significance of this was pointed out by Marapao (1959). He stated that when aggregates or granules of neurosecretory material were discharged from the cell, that this was accompanied by the formation of conspicuous vacuoles in the cytoplasm once filled by the discharged material.

Clark (1963) points out that it is difficult to interpret neurosecretory phenomena in annelids on the basis of histological appearance of the cells. According to him the presence of stainable material in annelid neurosecretory cells indicates only that the rate of synthesis of the material has outpaced its rate of release and that accumulation of it in the cells may be caused by a change in either rate. However, in the opinion of this investigator, the obvious lack of neurosecretory substances from the cytoplasm of type a cells in the cerebral ganglia of worms subjected to hypo-osmotic stress resulted because this material was discharged from the cells in response to this osmotic stimulus. This neurosecretory material then would function to initiate and maintain the appropriate regulatory activities. This might be further investigated by isolation of these particular neurosecretory

PLATE 6

Figure a. Type a cells at 1000X of worm subjected to hypo-osmotic stress for 1 hour. Stained with Gomori's aldehyde-fuchsin stain. This cell type showed loss of coarse light purple granulation from cytoplasm. Extreme vacuolation in cytoplasm is evident. Type a cells are located in posterior region of ganglion.



Figure b. Type a cells at 1000X of worms subjected to hypo-osmotic stress for 6 hours. Loss of granules and extreme vacuolization apparent as in Figure a.



Figure c. Type a cells at 1000X of worms subjected to hypo-osmotic stress for 24 hours. Loss of granules and extreme vacuolization apparent as in Figures a and b.



granules by differential centrifugation of cell free homogenates of brains followed by injection of the particular fractions in debrained worms to see the effects of those granules upon osmotic and ionic regulation.

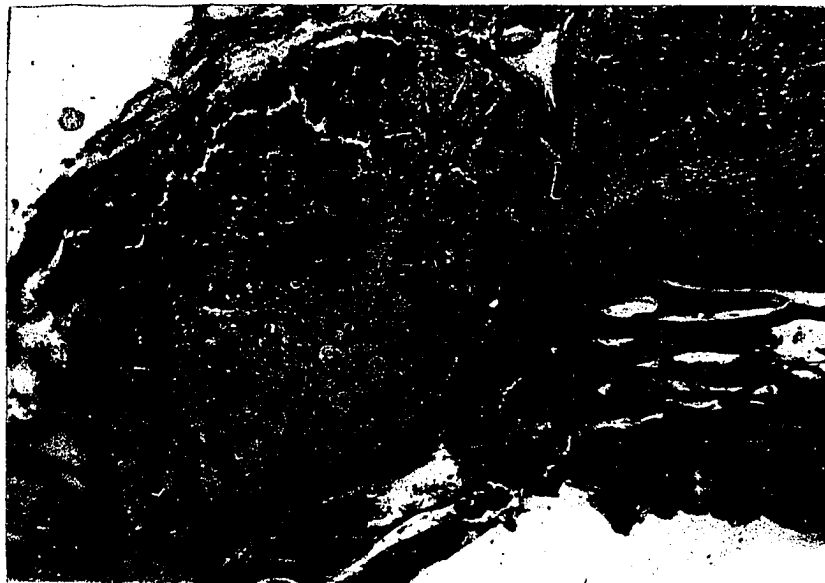
When type a cells in brains of worms subjected to hyper-osmotic stress were examined, it was found that the depletion of secretory granules was distinctly greater (Plate 7, Figure a, and Plate 8, Figures a, b, and c) than that found under hypo-osmotic stress. This can be seen in the lower magnification (150X) in Plate 7, Figure a.

The effect of osmotic stress on these neurosecretory cells was rapid, since it was observed after subjecting the worms to a hyper-osmotic stress after 1 hour (Plate 8, Figure a). The decrease of coarse granulation by those cells after 6 and 24 hours (Plate 8, Figures b and c) of subjection of worms to hyper-osmotic stress was the same as that after only 1 hour exposure. This would be expected as the worms have been shown to regulate in hyper-osmotic conditions for more than 24 hours. In addition, the photographs at all time intervals (Plate 8, Figures a, b, and c) at the higher magnifications showed that considerable vacuolation has occurred in the cytoplasm under these conditions also. A further time study should be done at shorter time intervals than 1 hour in order to determine how quickly the loss of granulation can be observed.

In the opinion of this investigator, the loss of neurosecretory substances from the cytoplasm of type a cells in the cerebral ganglia of these worms was due to stimulation resulting from hyperosmotic stress. As a result, the neurosecretory material was secreted from the

PLATE 7

Anterior



Posterior

Figure a. Frontal longitudinal section of supra-pharyngeal ganglion at 150X of a worm subjected to hypertonic stress for 1 hour and stained with Gomori's aldehyde-fuchsin stain. Type b cells, indicated by arrow 1, possess fine, tightly-packed granules staining dark purple to brick red. These cells are located in the anterior region of the ganglion. Arrow 2 indicates type a cells located in the posterior region of the ganglion. These cells have lost stainable granules from their cytoplasm as indicated by lighter coloration in cytoplasm when compared to type a cells of standard and experimental controls.

PLATE 8



Figure a. Type a cells at 1000X of worms subjected to 1 hour hyper-osmotic stress and stained with Gomori's aldehyde-fuchsin. Note extreme vacuolization in cytoplasm. Coarse light purple granules lacking. These cells are located in posterior regions of ganglion.



Figure b. Type a cells at 1000X of worms subjected to hyper-osmotic stress for 6 hours and stained with Gomori's aldehyde-fuchsin. Extreme vacuolization in cytoplasm. Coarse granules lacking in cytoplasm of these cells.



Figure c. Type a cells at 1000X of worms subjected to hyper-osmotic stress for 24 hours and stained with Gomori's aldehyde-fuchsin. Extreme vacuolization observed in cytoplasm along with loss of stainable granules. Granules are coarse and stain light purple in standard and experimental controls.

cells and functioned to control the appropriate regulatory activities.

As discussed above in the works of Bern (1962) and Simpson, et. al. (1966), three levels of evidence for neurosecretion have been suggested. These categories are: (1) morphological description of neurosecretion, (2) correlation of cyclic changes or altered physiological activity with changes in secretory activity, and (3) determination of physiological effects of an agent definable as a hormone originating from a specialized neuron.

The first category of evidence, namely morphological description of neurosecretion, has been fulfilled by the histological studies reported not only in this investigation, but also by Marapao (1959), Herlant-Meewis (1956), and Hubl (1953). The neurosecretory cells of L. terrestris could be classified into two groups of cells which have been designated as type a and type b by this investigator.

In order to demonstrate a specific function for any type of neurosecretory cell, the second and third levels of evidence described above must be fulfilled. In this investigation, a definite function for type a cells was demonstrated on the basis of the loss of secretory granules when the worms were forced to regulate osmotically and ionically. Kamemoto (1964) had previously demonstrated that some factor or factors present in the brains of these worms controlled ionic and osmotic regulation. Thus, the second level of evidence has been fulfilled and it can be concluded that type a cells produce a hormone or hormones involved in osmotic and ionic regulation.

It should be noted again, that Clark (1963) warned against mis-interpretation of histological evidence in attributing response to neurosecretory cells. However, in this study, the definite loss of granulation was considered to be a specific neuroendocrine response and an indication that the release of this neurosecretory material resulted in ionic and osmotic regulatory activity.

The depletion of neurosecretory material from type a cells by both hypo- and hyperosmotic stress is an interesting aspect of this investigation. The regulatory mechanisms which must function to maintain the internal environment of the earthworm at a constant level when it is placed in a hypo-osmotic medium are quite different from those which would function in the opposite, or hyperosmotic, situation. Thus, it would be presumed that different neurohormones would be needed to control these regulatory activities. The histological evidence would indicate that perhaps a single hormone is produced by these type a cells. However, the secretory granules which are visible histologically may be only carriers of the hormones and thus would be no indicator of the numbers or kinds of hormones secreted by the cell.

SUMMARY

- 1) Histochemical procedures were used to demonstrate neurosecretory cells in the brain of L. terrestris. This was done by specifically staining neurosecretory granules with Gomori's aldehyde-fuchsin stain following oxidation of cystine and cysteine, characteristic components of the granules, to cysteic acid.
- 2) Haematoxylin and eosin stained sections of earthworm brains were studied and compared with brains stained with Gomori's aldehyde-fuchsin. Neurosecretory cells could not be definitely identified by means of the haematoxylin and eosin procedure.
- 3) As a result of staining procedures using Gomori's aldehyde-fuchsin, two main types of neurosecretory cells were identified. They were named type b and type a cells.
- 4) Type b cells, located in the dorsal anterior regions of the brain possess five, tightly-packed secretory granules in their cytoplasm. These granules stain dark purple to brick red with Gomori's aldehyde-fuchsin.
- 5) Type a cells, located in the posterior area of the brain possess large, coarse granules which are not tightly packed together in the cytoplasm. These granules stain light purple with Gomori's aldehyde-fuchsin.
- 6) The earthworms were subjected to osmotic stress by placing them

in distilled water as a hypotonic medium, and a 0.28 M hypertonic saline solution. Specimens maintained at 2°C in a refrigerator were used as standard controls. Other worms in .16 M isotonic saline solution were considered to be the experimental controls.

- 7) No differences in type a cells were observed between standard and experimental controls.
- 8) A greater intensity of the staining of the b cells of experimental controls with Gomori's aldehyde-fuchsin may indicate a generalized stress reaction to ambient conditions.
- 9) Upon subjection of worms to hypo-osmotic stress for periods of 1, 6, and 24 hours, a loss of stainable secretory granules was seen in the cytoplasm of type a cells by 1 hour and this was sustained for the 24 hours. The loss of granules was accompanied by extreme vacuolization of cytoplasm at all time intervals.
- 10) Upon subjection of worms to hyper-osmotic stress for periods of 1, 6, and 24 hours, there was a greater loss of stainable secretory granules seen in the cytoplasm of type a cells, than in the type a cells observed after hypo-osmotic stress. The loss of granules after hypertonic stress was also accompanied by extreme vacuolization of cytoplasm at all time intervals.
- 11) Since it had been previously demonstrated that some factor or factors in the brain of L. terrestris controlled osmotic and ionic regulation in these worms, it is concluded that those

factors are products of type a neurosecretory cells because of the depletion of granules as a result of osmotic and ionic stress.

- 12) There was no loss of stainable material from type b cells as a result of osmotic stress. However, a generalized response to this stress on the part of the cells was indicated because of greater intensity of staining of the granules and an apparent increase in numbers of all types present.

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