8-1970

Extraction and Partial Purification of Neurosecretory Substances from the Suprapharyngeal Ganglion of *Lumbricus terrestris*

William D. VandenBosch

Extraction and Partial Purification of Neurosecretory Substances from the Suprapharyngeal Ganglion of *Lumbricus terrestris*

by

William D. VandenBosch

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

August, 1970

ACKNOWLEDGEMENTS

The investigator wishes to express his sincere appreciation to his thesis committee - Dr. Jean McVay Lawrence, Dr. Stephen Friedman, and Dr. Jack Wood - and the many other persons who made the production of this paper possible.

The financial support of this thesis was provided in part by a Faculty Research Grant from Western Michigan University.

Special mention is due Dr. Edward Daniels and Dr. George B. Whitfield, Jr., for their instruction and aid during several phases of this project and to Miss Rita Sandstrom whose patience and secretarial skills contributed to the preparation of this paper.

William D. VandenBosch

DEDICATION

To my late wife, Sandra, whose love and devotion provided constant inspiration during this project.

TABLE OF CONTENTS

List of Tables

List of Figures

INTRODUCTION

Since Ernst and Berta Scharrer first described neurosecretory cells in the suprapharyngeal ganglion (brain) of the earthworm, *Lumbricus terrestris* (1937), there have been a number of studies carried out to determine the details of the morphology of these cells, the number of kinds of neurosecretory cells, and the role(s) they play in the functioning of the earthworm. However, nothing is known about the chemical nature of these secretions, nor have they been demonstrated to be true hormones. Extraction, isolation and purification of neurosecretory material has been carried out in insects, crustaceans and molluscs, but as of now there have been no known publications on the extraction, isolation and purification of neurosecretory substances or materials from the brain of *Lumbricus terrestris*.

The first purpose of this investigation was to develop procedures for the extraction of ninhydrin positive water-soluble substances from the brain of *L. terrestris* and the separation and initial purification of the fractions that were present in the water extract. Because some of the functions of this earthworm, which are controlled by substances present in the brain, are controlled in vertebrates by hormones which are polypeptides, only those factors in the water-soluble extract which were ninhydrin positive were studied. The decision as to what type of compound to look for was an arbitrary decision made by the investigator realizing other types of compounds which could be or are biologically active may also be present in the brain.

Following separation of fractions, the second objective was to determine if any function(s) in the earthworm could be demonstrated to be affected by any of the fractions in the water extract. If any specific regulatory activity or activities could be demonstrated for a fraction(s), the extraction and isolation procedures would then permit a partial chemical characterization of the factor(s). This would allow a comparison of substances, presumably neurosecretory, of *L. terrestris* with those hormones which play similar roles in other animals.

LITERATURE REVIEW

Because all neurones can be classified as neurosecretory due to their secretion of transmitter substances at their axonal endings, the term neurosecretory cell has been limited in this investigation to those nerve cells which contain characteristic granules and which produce products that, presumably, have physiological regulatory functions at a site which is distant from the brain (Bullock and Horridge 1965). Neurosecretory cells have been found in all animals studied except Porifera and Coelenterata (Novak 1966), although granules which have been described as neurosecretory granules have been isolated in hydra (Lentz 1965).

Scharrer and Scharrer (1963), Bullock and Horridge (1965), Gabe (1966) and Novak (1966) have reviewed the literature on invertebrate nervous systems, the neurosecretory cells found in these systems, and roles played by secretions of these cells. The present literature review will deal primarily with a consideration of neurosecretion in earthworms

with particular reference to *Lumbricus terrestris*. It is assumed that the neurosecretory cells found in *L. terrestris* produce factors, as correlation between regulatory function and neurosecretory activity have been made in growth, reproduction and osmotic regulation (Herlant-Meewis 1956, Gabe 1966, Goudie 1968).

Hubl (1955) stated that in four species of oligochetes, including *Lumbricus*, there are two types of neurosecretory cells and Herlant-Meewis (1956) reported two types of neurosecretory cells in the brain of *Eisena foetida*. Marapao (1959) described four types of cells (neurosecretory) in the brain of *L. terrestris* which he designated as A, B-1, B-2 and C cells. The A, B-1 and C cells are considered by Marapao to represent one type of neurosecretory cell in various stages of development or secretory activity. The B-2 cells were considered to be a single type of neurosecretory cell. Goudie (1968), as a result of histochemical study, concluded that there were two types of neurosecretory cells in the brain of *L. terrestris* on the basis of affinity of the granules for Gomori's aldehyde fuchsin stain. He designated these cells as types a and b. Goudie considered his a cells to be equivalent to the A, B-1 and C cells of Marapao and the b cells to be what Marapao designated as B-2 cells. As described by Gabe (1966), other investigators have reported up to six different types of neurosecretory cells in the brains of oligochetes. However, Gabe agrees with Herlant-Meewis (1956) that most differences seen were a function of age and secretory activity and that on the basis of location and appearance, there were only two types of neurosecretory cells in Lumbricidae. Teichman, *et al* (1966) in studies with *L. hurculeus*

and *Eisenia foetida* also reported only two types of neurosecretory cells in the brain of these earthworms which they designated as A-1 and A-2. The A-1 cells are apparently equivalent to the a cells of Goudie (1968) and the A-2 cells equivalent to the b cells of Goudie. Thus, on the basis of studies which have been done to date, it may be concluded that there are only two types of neurosecretory cells in the brain of *L. terrestris* on the basis of the appearance of and the staining affinities of the granules and the size of the cells.

The presence of physiologically active substances in the central nervous system of *L. terrestris* was first studied by McVay (1942). She showed that homogenates of *L. terrestris* brains or ventral nerve cords contained a substance or substances which gave positive chromatophorotropic results in the crayfish, *Cambarus* spp.

Herlant-Meewis (1956) suggested that neurosecretory cells are concerned in growth in Lumbricidae because of the morphological changes which occur in them during development of *E. foetida*. Hubl (1956) carried out a series of experiments on regeneration in the four species of Lumbricidae and showed the importance of the brain in the regeneration of lost posterior segments. Hubl's experiments indicated that the brain produced a substance(s) which was necessary for regeneration to occur. Herlant-Meewis (1961) in her studies on the regeneration of the nervous system in the earthworm, *E. foetida*, noticed that ablation of the ganglia caused a cessation of egg laying and a loss of secondary sex characteristics which reappeared only when the ganglia and nervous system connectives were completely regenerated.

Kamemoto (1964) and Kamemoto, *et al* (1962) investigated osmotic and ionic regulation in *L. terrestris*. These studies showed that the ability of the earthworm to regulate osmotically was controlled by substances present in the brain. In addition, they were able to demonstrate that not only were sodium levels of blood and coelomic fluid maintained at different concentrations, but that the earthworm was able to maintain this sodium concentration above that of the environment up to an environmental level of 0.1 molar sodium chloride. Thus, factors present in the brain were shown to be responsible for this ability of *Lumbricus* to regulate ionically. Goudie (1968) subjected these earthworms to hyper- and hypo-osmotic conditions and studied the effect of these conditions on the morphology of the a and b neurosecretory cells. The a cells were shown to lose granules and to develop vacuoles in the cytoplasm after an exposure of one hour. This depletion of granules and increase in vacuolization was sustained throughout the twenty-four hour period of exposure to hyper- or hypo-osmotic conditions. Goudie also observed that the b cells demonstrated what Marapao (1959) termed generalized response of b cells to a stressful situation which he caused by pricking the worms. The response was characterized by a more intense staining of the granules of the b cells.

Craig (1966) did a study which implicated the suprapharyngeal ganglion in the maintenance of blood glucose levels in *L. terrestris*. Her studies established an average blood glucose level of 6 mg percent in the earthworm. Decerebration caused the blood glucose levels to drop to zero within twenty-four hours after the operation. The levels

then increased for up to five days after decerebration, but never quite rose to the pre-operation blood glucose levels. When worms were subjected to sham operations the blood glucose levels tripled and were still above usual levels after twenty-four hours. Although Craig's studies did not show that the brain regulates blood glucose levels in the earthworm, they do implicate the brain as playing a role in the maintenance of blood glucose levels. It remains for further studies to establish that the brain does or does not produce factors, presumably neurosecretory, which play a role in the regulation of glucose levels in the earthworm. However, work by David Clough (personal communication) has demonstrated that injections of brain homogenate (2 brains/worm) elevate blood glucose levels above that elicited by injection of saline alone. Worms which were injected with albumin had blood glucose levels equivalent to worms given saline injections. This ability of substances in the brain to cause mobilization of carbohydrates so that blood glucose levels are elevated may well be evidence of a regulatory or hormonal role.

The presence of amine secreting cells in the central nervous system of the oligochete, *Octolasium complanatium*, was demonstrated by Bianchi (1967). The amine secreting cells are located in the lateral regions of the brain, subesophageal ganglion and the ventral nerve cord. Bianchi postulated that the amines were released from the perikaryon into capillaries which surround the cells and were carried through the blood stream exerting an effect on blood pressure and blood distribution. Rude (1969) demonstrated by fluorescent techniques that dopamine and noradrenaline were the major catechol-

amines present in the ventral nerve cord of *L. terrestris*. She also believed that adrenaline and DOPA were present but the techniques used were not sensitive enough to detect these compounds.

In the present investigation, procedures were developed to extract and separate polypeptide and polypeptide-like compounds from the brain of *L. terrestris* and to test the various fractions for their biological activity, because in vertebrates polypeptide hormones have been identified which regulate blood glucose levels (glucagon), osmoregulation (vasopressin or ADH), and growth (growth regulating hormone). It is these functions which have been demonstrated to be controlled in the earthworm by substances present in the brain. Although it is realized that in vertebrates and probably also in invertebrates, other hormones including non-polypeptides are also implicated in these functions, only procedures which were known to be effective in the extraction of polypeptides were used.

Extraction, isolation and purification of neurosecretory hormones have been carried out in insects, molluscs and crustaceans. Karlson and Hoffmeister (1963) extracted dried *Bombyx* pupae with methanol, dried the methanol extract and extracted this product with butanol. The butanol-soluble fraction contained ecdyson which was found to be a steroid. Ichikawa and Ishizaki (1961, 1963, 1967) homogenized 240 brains from the pupae of *Bombyx* in 1 ml of methanol and following centrifugation, the supernatant was dried *in vacuo*. They then did an ether:water separation and tested the fractions for their ability to cause metamorphosis in *Bombyx* pupae. The ether fraction was inactive but the water fraction was active. Ultraviolet absorption

analysis indicated the water fraction to contain a polypeptide material. The work of Karlson and Ichikawa show that the use of methanol as an initial extraction solvent can remove both polypeptide and steroidal compounds from tissue. Therefore, the procedures developed in this investigation and described in the methods section of this report were based on these methanol extraction procedures to insure that the final extract would most likely contain polypeptide compounds.

Mordue and Goldsworthy (1969) used methanol extraction and descending chromatography techniques to separate two biologically active fractions which were polypeptides from the corporum cardiaca of the locust, *Schistoceria gregaria* and *Locusta migratoria*. These two fractions were distinguished by their different effects in assay systems used which were tests for cardio-excitor activity.

Brown (1965) used both methanol and water techniques to isolate polypeptide hormones from the corpora allata of the cockroach, *Periplaneta americana*. Agarwal and Greenberg (1969) studied cardio-active agents in ganglia and hearts from four species of fresh water molluscs. They used water extraction techniques to isolate polypeptide compounds with hormonal activity. Kleinholz and Kimball (1965) used water extraction techniques to isolate polypeptide hormones from the eyestalk of the crustacean *Pandalus borealis* which are cardio-regulatory in action.

Belamarich and Terwilliger (1966) isolated two polypeptide compounds from the pericardial organs of *Cancer borealis* by acetic acid precipitation and extraction. The two compounds showed cardio-

excitor activity and seemed to complement each other. The authors used column chromatography (Silica Gel G-25) to separate and purify the two components. Identification was done through the use of paper chromatography in a butanol:acetic acid:water developing system. Ninhydrin spray reagent was used to locate the position of the polypeptides on the chromatography paper. Ultraviolet light absorption analysis showed the components to be polypeptide compounds.

The studies described in the preceding paragraphs, particularly those described in detail, show that methanol extraction followed by ether:water separation and/or the use of Sephadex chromatography is a realistic method to use for the isolation and the separation of polypeptide compounds. These investigations also showed that polypeptides which have been isolated from invertebrate neural tissue have biological activity in those particular organisms.

METHODS AND MATERIALS

Organisms and Preparation

The worms, species *Lumbricus terrestris*, purchased from a local bait store in mid-summer and late fall, were considered mature when a well-developed clitellum was present. No precautions were taken as to time of day or season when the brains were removed, although it was realized that seasonal and/or diurnal variations could be contributing factors as to the kinds and the concentrations of compounds which might be present.

The worms were anesthetized before decerebration by placing them in 10% ethyl alcohol for two minutes. The brain was exposed by

making a mid-dorsal incision from the second segment to the fifth segment and gently squeezing the worm in the region of the fourth segment. Unwanted tissue was gently teased away from the brain and the brain freed by cutting the connectives at their attachment to the brain. Isolated brains were placed in methyl alcohol (reagent grade) and stored at -20°C until sufficient brains were available for extraction. Over a six month period, 2,000 brains were collected.

Extraction and Fractionation

All extraction and separation procedures except for column chromatography were carried out at 4°C. Extraction was accomplished by homogenizing the brains in 4 ml of absolute methanol in a ground glass homogenizer for 15 minutes. The homogenate was then spun down at 12,100 x G for 20 minutes in an International refrigerated centrifuge at a temperature setting of 4°C. The supernatant was carefully poured off and the homogenization procedure repeated on the residue three more times, using 4 ml of methanol each time. All the supernatants were pooled.

The methanol was removed by vacuum evaporation using a Rinco evaporator and a Little Giant vacuum pump with a setting of 25 pounds per square inch. The wet material was then lyophilized. The yield of dried material from 2,000 brains was 62.4 mg.

Three extraction procedures were used to remove water-soluble lipids and monoamines. Accordingly, the lyophilized material was dissolved in 3 ml of chloroform:methanol:water (2 volumes $HCCl_3$MeOH [2:1] against 1 volume H_2O). The fractions were separated and the

water fraction re-extracted with an equal volume of chloroform:methanol (2:1). The chloroform:methanol and the water fractions were both lyophilized. The ether fraction was shown by chromatography to be devoid of polypeptide compounds and was discarded. The water-soluble fraction was then dissolved in 2 ml of ether:water (1:1 V/V) and the pH adjusted to 3.0 with concentrated acetic acid. The solution was shaken and the ether removed. The water fraction was then re-extracted with an additional 1 ml of ether. The pH of the water fraction was then adjusted to a pH of 8.0 with 5N sodium hydroxide and ether extractions carried out as with the acid pH. The pH adjustments were carefully done to avoid zones of hyper-acidity or -basicity in the extraction solution. These procedures were suggested by Dr. Edward G. Daniels of The Upjohn Company to remove the amines listed in Rude (1969). The water extract was lyophilized and stored at 4°C until used for fractionation. The period of storage ranged from 1 week to 2 months. The final yield was 2.7 mg of material from 2,000 brains which amounts to a yield of approximately 1.4 mcg of material per brain. Preliminary work had shown that from 4 to 8 ninhydrin positive fractions were present in water extract of brain; therefore, to determine if any ninhydrin positive substances could be demonstrated to be present, the water soluble extract was examined by thin layer chromatography (TLC) using a system of butanol:acetic acid:water (2:1:1 - V:V:V) and ninhydrin spray reagent to locate compounds. The plates were 8" x 8" silica gel (Gelman). Development took 45 minutes after which the plates were dried and then sprayed with ninhydrin reagent. The plates were then placed in a hot oven at 110°C for 5 minutes to

develop the ninhydrin positive spots. Chromatography showed there were at least four compounds present in the water soluble extract which were ninhydrin positive (Figure 1). The whole extract was also tested in a white chromatophore dispersing assay to confirm the presence of biological activity before fractionation procedures were begun. (See under Bioassay of Fractions)

Separation of the four fractions demonstrated to be present was accomplished by the use of Sephadex column chromatography. A 30 ml column was made using Sephadex G-25 which had been equilibrated for three hours in distilled water. The column size was 50 cm x 2.5 cm. Packing was accomplished in one step by pouring the Sephadex suspension through a funnel into the column and then allowing the column to pack by running distilled water through it for four hours. Care was taken to make sure the column was held in a vertical position while packing to prevent it from having any distortions which could result in poor fractionation.

The partially purified lyophilized water-soluble extract was dissolved in 5 ml of water and 1/2 ml aliquots were run through the column, one at a time, for 10 runs. The column was allowed to run until the water head had reached the top of the Sephadex and then the 1/2 ml aliquots of extract solution were placed on the column using a 1 ml pipette. Care was taken to be sure the top of the Sephadex was not disturbed when the sample was added to the column. The sample was allowed to run into the Sephadex and then water was added to form a head of 3 cm above the Sephadex and maintained throughout the run. The solvent used throughout fractionation

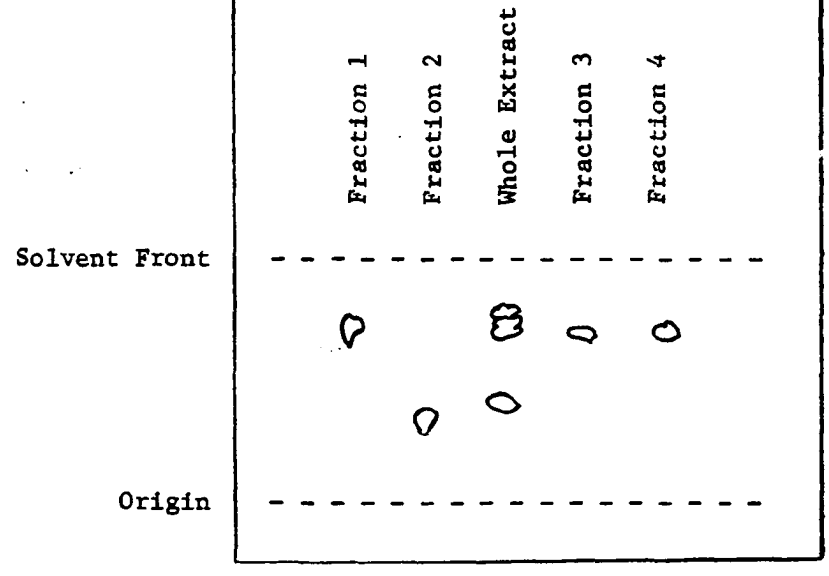
Figure 1

Chromatographic Patterns of Water Extract of Earthworm Brains

Solvent System
BuOH:Acetic Acid:Water
2:1:1

Run was for 45 minutes

Fraction	rF on TLC	rF Column
1	.769	.422
2	.382	.466
3	.727	.533
4	.745	.844



was distilled water at a flow rate of 3 ml an hour. Flow was controlled by keeping the water in a 1,000 ml separation funnel and adjusting the flow rate by means of a stopcock. The funnel was connected to the column by a rubber tubing. One half milliliter samples were collected for a period of 24 hours. These samples were then examined for the presence of ninhydrin positive compounds. Twenty lambda of solution from each sample was spotted on a silica gel thin layer plate (Gelman Corporation). The plates were then sprayed with ninhydrin reagent and placed in a hot oven at 110°C for 5 minutes. Samples which contained an amino acid, polypeptide or protein were indicated by the development of a purple color.

Those samples which contained a ninhydrin positive substance were run in thin layer chromatography (TLC) using 8" x 8" silica gel chromatography plates in order to determine which samples contained identical fractions. The developing system was butanol:acetic acid: water (2:1:1). Ten to 20 lambda of the samples were spotted on a thin layer plate. The amount spotted was a subjective decision based on the color intensity of the ninhydrin spots. While the plates were being spotted, a chromatography tank was set up by pouring 200 ml of the developing system into the tank and covering to allow the atmosphere of the tank to become saturated with the developing system. After spotting and making sure all spots were dry, the plates were placed into the tank. Ascending chromatography was carried out for 45 minutes. The plates were removed and the solvent front marked. Drying of the plates was by forced warm air. After drying, the plates were sprayed with ninhydrin and developed in

a hot oven at 100°C for 5 minutes. Those samples with similar rF values were combined as fractions and used in tests for biological activity.

Bioassay of Fractions

The four fractions which were isolated from the water extract were tested for biological activity by four bioassays.

Chromatophore dispersion assay

The chromatophore dispersing assay was used by McVay (1942) to demonstrate that homogenates of earthworm brains contained a substance(s) which had biological activity. In this investigation this assay was used initially to demonstrate chromatophorotropic activity of brain homogenates and the unfractionated water-soluble portion of the initial methanol extract. The fractions were then tested by means of this assay to determine if one or more of them could be responsible for the chromatophore dispersing activity of brain homogenates. The white chromatophores in the carapace from median sections of each half of the thorax of *Cambarus* spp. were the test objects for the bioassay. Carapace sections were placed in physiologic saline until used. The chromatophores adhere to the exoskeleton when it is removed and are easily observed with the use of a dissecting microscope. Although both red and white chromatophores were present, it has been demonstrated that central nervous system homogenates of *L. terrestris* caused contracted white chromatophores to disperse while the red chromatophores were unaffected. To insure full contraction of the

white chromatophores, the crayfish were kept in an aquarium with a dark bottom. The chromatophores found in the dorsal region are very sensitive and readily disperse in physiologic saline and those at the ventral edge are relatively unreactive; therefore, to insure reproducible results, the dorsal and ventral regions (approximately one centimeter) were removed and discarded.

Readings of chromatophorotropic activation were made on a scale of 0 to 5; five meaning full dispersion of the chromatophores. All tests were run in physiologic saline. The control solution was saline and the test samples were whole brain homogenate (20 brains/ml) in saline, brain residue from methanol extraction resuspended in 1 ml saline, unfractionated whole extract and individual extract fractions. The piece of carapace which was to be used for testing was immersed in 0.5 ml of physiologic saline. To this was added 0.1 ml of the solution to be tested. All pieces of carapace that were used for one test came from the same crayfish.

The dispersion of white chromatophores is a continuous process and the time intervals for observation were arbitrarily chosen by the author. Readings were made at zero time (no test solution added) and then every 15 minutes for 1 hour after the test solutions had been added. The samples were not randomized and because of the subjective nature of the readings, it is admitted by the author that it was possible that a positive bias was involved in the results of the assay. To calculate dispersion results the zero time readings were subtracted from the test readings. Epinephrine was also tested as to its ability to cause white chromatophore dispersion. Dilutions of $1{:}10^3$ to

$1:10^9$ were tested. Results were calculated as with the other solutions.

Hyperglycemic assay

The fractions were assayed for their hyperglycemic effect. This was done by injecting the fractions into earthworms and comparing and observing their effects on blood glucose levels with those effects of saline injections alone. Blood glucose levels were determined by the glucostat method as modified by Craig (1966) for microsamples. This method involved the preparation of a 1:10 dilution of the blood sample followed by precipitation of proteins with zinc sulfate and barium hydroxide. The protein-free filtrate was used for glucose determinations (by the Glucostat method - Worthington Biochemical Corporation). The glucostat method is specific for β-D-glucose and does not determine pentoses, other hexoses, or other reducing substances.

Blood samples tested for glucose content were from worms that had been injected with one of the following: saline, brain homogenate in saline (20 brains/ml), and each of the 4 fractions of brain extract. One tenth milliliter of test solution was injected into test worms which had been debrained 24 hours prior to test. The worms were sacrificed one hour after injections were made and blood glucose levels were determined. Blood samples obtained from each worm were about 10 lambda in volume. To insure enough blood per sample, 10 worms were used for each test and their blood samples pooled to make one sample. In making glucose analyses, duplicate samples were run from each pooled sample and the results averaged.

Glycogenolysis assay

The next bioassay was a test of the effect of Fraction 1 on glycogenolysis. In earthworms, glycogen is stored in chlorogonen cells located in the wall of the intestine. This was an *in vitro* test and involved quantitative analysis of glycogen in samples of intestine before and after exposure to the brain extract fractions. The intestinal wall glycogen assay was developed by Radke (1969) and was a modification of the anthrone reagent procedures of Roe and Daily (1966) in which tissue was homogenized in NaOH and protein precipitated. The glycogen was precipitated from protein-free solution by ethanol and was then assayed colorometrically. For each assay three samples of washed intestinal tissue were weighed and placed into test tubes labeled control (a), 10 minutes hold (only saline added) (b), and 10 minutes hold plus extract (c). Homogenization of tissue in tube a was started immediately by addition of 3 ml of 1N NaOH. At the end of 10 minutes, 3 ml of 1N NaOH was added to tubes b and c. Treatment of all solutions was then according to the method of Roe and Daily (1966).

Hypo-osmotic assay

The assay for hypo-osmotic activity by any of the water extract fractions was conducted by determining the ability of decerebrated worms to regulate in a hypo-osmotic medium following injections of the fractions. The procedures used for the assay were developed with the aid of Dr. Jean Lawrence. Worms were decerebrated three days before use and stored at 4°C in wet toweling. Control groups of worms, which

were not decerebrated, were isolated at the same time and maintained at 4°C in wet toweling. On the day of the test the worms were grouped into 10 classifications with from 2 to 5 worms in a group. The treatment to which each group was subjected is tabulated in Table 1.

All injections which contained material derived from brain had an actual or calculated value of 2 brains for each worm. The injections were given in two portions, 0.05 ml injected anterior to the clitellum and 0.05 ml injected approximately half-way between the clitellum and the posterior end. All worms which were injected were first anesthetized in 10% ethanol. The worms were maintained in aerated distilled water which had been aerated for 15 minutes prior to immersion of worms. All worm groups were weighed at the start of the assay after blotting in paper toweling. All weights were rounded off to the nearest 0.1 gram. After immersion in distilled water, worm groups were weighed every fifteen minutes for the first two hours and then hourly for seven more hours (9 hours total time). Results were calculated as percent weight gain for each time interval.

RESULTS AND DISCUSSION

At the onset of this investigation the material obtained from the methanol extraction procedure was shown to contain at least four ninhydrin positive (Figure 1) fractions by means of ascending chromatography. The fractions were considered to be polypeptide in nature due to their reaction to ninhydrin. The material was then partially purified by performing extraction procedures to remove lipids which are water soluble. It is admitted by the author that it is possible

Table 1

Classification of Worm Group for Osmoregulation Assay

Designation	
Ia	Decerebration
Ib	Decerebration + anesthesia*
Ic	Decerebration + anesthesia + saline injection**
Id_1	Decerebration + anesthesia + Fraction 1 injection
Id_2	Decerebration + anesthesia + Fraction 2 injection
Id_3	Decerebration + anesthesia + Fraction 3 injection
Id_4	Decerebration + anesthesia + Fraction 4 injection
Ie	Decerebration + anesthesia + Brain homogenate injection (20 brains/ml)
IIa	Normal (no treatment)
II b	Normal + anesthesia

* Anesthesia = worms placed in 10% EtOH for 2 minutes

** Injection = 0.1 ml of sample injected

not all of the water-soluble lipids were removed either because the extraction procedures were not effective or the possibility of bound lipids. Thus, any water-soluble steroids should have been removed which might have been included with the four fractions. Since catechol and indol amines are present in nervous tissue of *L. terrestris* (Rude 1969 and Bianchi 1967) and in our laboratory, epinephrine has been demonstrated to have a positive chromatophoric effect, to elevate blood glucose levels and to increase water uptake, it was necessary to remove them as their presence would not permit definite determination that any biological activity was due to the ninhydrin positive fractions, although it is admitted that the possibility exists that a minor contaminent of epinephrine could still be present in the fraction and could have been responsible for the activity seen (Table 2).

After removal of water-soluble lipids and amines, the material was run through a Sephadex G-25 column which separated the four fractions into individual components. It can be see from Figure 1 that Fraction 2 did not quite match the lowest spot in the chromatograph of the whole brain extract. This might have been due to an effect on the movement of the fraction when it was mixed with the other three fractions. No further chemical procedures were carried out and because of this, nothing can be said about the homogeneity of each component. It is possible that each component could consist of more than one compound. Because of the extraction and isolation techniques used and the know characteristics of Sephadex G-25, it is considered by the author that the components isolated are polypeptide compounds having a molecular weight in the range of 1500 to 3400. Definitive proof of

Table 2

Chromatophore Dispersing Activity of Brain Homogenate, Water Extract of Brains, and Extract Fractions

Values obtained are the average of four experiments. In each experiment the ability of treatments to cause dispersal of white chromatophores was expressed on a scale of from 0 to 5. Zero indicated no activity and 5, maximum white chromatophore dispersing activity. All test solutions had a calculated concentration of 20 brains/ml. Results of the tests were read after one hour.

Treatment*	Chromatophore Dispersing Value
Epinephrine (1:10^3 dilution)	3.75
Whole Extract (Methanol)	2.5
Fraction 1	1.75
Fraction 2	0.75
Fraction 3	0.75
Fraction 4	0.25
Brain Homogenate (20 brains/ml)	3.75
Control (saline)	0.25

n = 4

* 0.1 ml of the indicated solution in saline was added to the bathing medium for each test. Results were read after one hour.

the kind and size of molecule can be obtained by a complete chemical analysis of each of the components.

The fractions obtained by column chromatographic separation were initially tested for the presence of biologically active factors in the chromatophorotropic assay of McVay (1942). This assay tests the dispersing effect of compounds on white chromatophores of the crayfish. The dispersion and contraction of these chromatophores is hormonally controlled in the crayfish (Bullock and Horridge 1965). Although any activity shown by the fraction in this assay would not necessarily implicate that the fractions contained factors which are involved in any physiological process in earthworms, it would account for the chromatophorotropic activity seen with whole brain homogenates.

Table 2 shows the results of the chromatophorotropic assay. As can be seen, whole brain homogenate had a chromatophorotropic value of 3.75. As it had been shown by McVay, 1942, that epinephrine also has such an effect, epinephrine was included in the series of substances tested in the assay. Results obtained from a series of dilutions of epinephrine are presented in Table 3. An epinephrine solution with a dilution of $1:10^{-3}$ was used in testing along with extract solutions as it had the greatest effect in the assay. As seen in Table 2, whole brain homogenate and the epinephrine solution had the greatest effect on dispersion of white chromatophores. The chromatophorotropic value for the water extract was 2.5 compared to 3.75 for the whole brain homogenate. Since the monoamines had been removed from the water extract, the fact that considerable chromatophorotropic activity was still present would indicate the presence of biologically active

Table 3

Chromatophore Dispersing Activity of Epinephrine

Values obtained are the average of four experiments. In each experiment the ability of treatments to cause dispersal of white chromatophore was expressed on a scale of from 0 to 5. Zero indicated no activity and 5, maximum white chromatophore dispersing activity.

Treatment*	Chromatophore Dispersing Value
$1:10^3$	2.25
$1:10^4$	1.0
$1:10^5$	1.25
$1:10^6$	0.75
$1:10^7$	0.5
$1:10^8$	0.75
$1:10^9$	Trace
Control (saline)	0.25

n = 4

* 0.1 ml of the indicated epinephrine dilutions in saline was added to the bathing medium for each test. Results were read after one hour.

factors other than epinephrine with this activity. The results from tests with the four fractions indicates that Fraction 1 has the most activity, Fractions 2 and 3 much less activity and Fraction 4 no activity. This does not necessarily mean there are no factors in Fraction 4 which could show chromatophorotropic activity as there may be factors present which would show activity if they were present in a higher concentration. It is also possible that the extraction and isolation procedures or environmental factors could have caused changes in molecular structure so as to alter or destroy biological activity. The results of the assay indicate that it is possible that at least part of the chromatophorotropic activity seen with whole brain homogenates is the result of factors present in the fractions obtained from whole brain extraction.

The first assay to test for biological activity of the fractions in earthworms was a hyperglycemic assay. Craig (1966) had shown that decerebration resulted in a decrease in blood glucose to zero over the succeeding 24 hour period, which was followed by a gradual increase of blood glucose levels to near normal. Although Craig did not prove the brain has a regulatory function in the control of blood glucose levels, the implication was still there. Clough (personal communication) did demonstrate that injections of brain homogenates (2 brains/worm) elevated blood glucose levels above that caused by injection of saline alone. Solutions of the fractions were made up to contain 2 brain equivalents per 0.1 ml. Table 4 shows that Fractions 1 and 2 contain a factor or factors which elicited a definite hyperglycemic response when injected into earthworms. Fractions 3 and 4 gave a response that

Table 4

Blood Glucose Levels of Earthworms Following Injection of Saline and the Four Fractions of Brain Extract

Treatment	Result (mgs %)
Saline	14.0
Fraction 1	23.6
Fraction 2	28.4
Fraction 3	13.7
Fraction 4	16.25

n = 3

All injections were 0.1 ml. Worms were allowed to stand for 1 hour and then blood samples were obtained.

was equivalent to saline injection alone. As none of the fractions caused a decrease in blood glucose levels, it is only possible to say that the active factor or factors in Fractions 1 and 2 may possibly play a role in the elevation of blood glucose in the earthworm. Statistical analyses were not done because the dramatic rise in glucose levels caused by Fractions 1 and 2 were obviously different from the saline controls.

Table 5 shows the results of testing Fraction 1 in a glycogenolysis assay. Only Fraction 1 was tested in this assay due to the small amount of material available for the other three fractions. The assay as used was in the process of design and has since been refined to give a better assay. As can be seen by examining results presented in Table 5, the worms treated with Fraction 1 had slightly more loss of glycogen from the intestinal wall than the sample left without treatment with Fraction 1 (Sample B). If the results of this assay are combined with the results of the hyperglycemic assay, it shows a good possibility that at least Fraction 1, or a factor or factors present in Fraction 1, may play a role in the mobilization of glycogen from the chlorgonen cells in the intestinal wall and as a result raise blood glucose levels.

The studies of Kamemoto, *et al* (1962) and Kamemoto (1964) have shown that the brain contains a substance or substances which are responsible for the control of osmotic and ionic regulation. Table 6 shows the results of testing the isolated fractions in an osmoregulation assay. When comparing results, it is interesting to note the effect caused by ethanol on the ability of the earthworms to regulate

Table 5

Effect of Extract Fraction 1 on Glycogen Content of Intestinal Wall

Results are expressed in terms of milligrams glycogen per gram of tissue. A:no treatment, B:10 minute hold and C:10 minute hold plus extract Fraction 1.

Trial	A	B	C
1	1.80	0.00	----
	----	0.00	----
2	2.05	----	1.85
	1.76	1.17	1.11
3	0.28	1.39	0.38
	1.38	1.54	0.89
4	1.53	0.92	1.16
	1.53	0.92	0.97
5	5.45	3.23	1.85
	4.95	3.37	2.03
$\Sigma X(n)$	22.52(9)	12.54(9)	10.98(8)
$\bar{X} = \frac{X/2}{n}$	1.13	0.63	0.55

Table 6

Bioassay of Extract Fractions for Osmoregulation Activity

(percent weight gain for each sample at each time period)

Time	IIa	IIb	Ia	Ib	Ic	Id_1	Id_2	Id_3	Id_4	Ie
15 min	0	0	0	0	0	0	0	0	0	0
30 min	4.0	3.9	2.7	8.4	4.6	6.8	4.7	4.3	1.1	5.5
45 min	8.0	7.8	5.0	14.3	8.6	10.4	9.9	11.9	5.7	10.6
60 min	8.8	9.8	5.4	17.1	11.1	13.3	14.8	15.4	8.3	17.1
75 min	9.7	11.8	6.2	18.7	14.1	15.3	16.1	17.0	9.7	18.8
90 min	11.4	13.1	7.8	21.5	15.8	17.4	17.4	19.9	11.2	21.1
105 min	11.1	14.4	7.8	21.9	17.5	17.4	18.1	21.1	11.5	20.8
120 min	12.0	15.6	7.5	23.0	19.2	18.1	17.4	31.3	11.5	20.5
3 hrs	13.7	16.7	4.6	27.2	23.4	20.5	20.2	26.4	14.4	24.2
4 hrs	14.3	18.3	8.1	30.3	23.7	21.4	21.2	27.4	16.8	25.5
5 hrs	14.6	20.0	9.2	32.8	25.4	22.9	23.9	26.7	17.6	22.4
6 hrs	12.6	22.4	9.1	34.1	26.2	23.1	23.6	25.2	15.8	23.5
7 hrs	12.6	20.9	8.2	35.5	27.0	23.0	24.1	25.2	17.3	23.6
8 hrs	8.0	18.3	7.2	34.0	24.1	19.5	20.3	23.4	11.8	20.6
9 hrs	7.5	17.9	7.2	34.0	24.1	18.3	18.6	21.7	8.9	21.0
	Intact	Intact + Ethanol	Debrain	Debrain + Ethanol	Debrain + Ethanol + Saline injection	#1 + Ethanol	#2 + Ethanol	#3 + Ethanol	#4 + Ethanol	Homogenate + Ethanol

osmotically. The treatment of ethanol anesthetization caused intact worms to gain about twice as much weight as intact worms which had had not been anesthetized. When the worms were subjected to decerebration in addition to ethanol anesthetization, they gained about three times as much weight as the non-anesthetized worms. Because the worms that were used to test the extract fractions were subjected to both an injection of test solution and to ethanol anesthetization, the important control group is Group 1b. Anesthetization was carried out with 10% ethanol for a period of 2 minutes.

The results indicate that each of the fractions contain a factor or factors which are active in this assay. All worms in the test groups gained less weight than the worms in the control group (1b). The assay design limits the test only to an evaluation of the ability of the worms to regulate the uptake of water (expressed in the assay as weight gain), using the criteria that less weight gain means more ability to regulate osmotically. The results show that Fraction 4 had the most activity (least weight gain) and Fractions 1, 2 and 3 were about equal in activity. As can be seen, all test groups showed an ability to regulate water uptake after 7 hours in the hypo-osmotic solution. Fractions 1, 2 and 3 had an effect about equal to that of brain homogenate, which was a slower rate of water uptake than shown by debrained, anesthetized worms and showed a leveling off after three or four hours in the hypo-osmotic solution. Fraction 4 showed a greatly increased osmoregulatory effect resulting in very little weight gain by that group of worms. The mechanism of action can not be deduced by the assay system used, but the pattern of inital rapid weight gain

followed by a leveling off which is then followed by weight loss would seem to indicate that the action is by way of stimulating diuresis. The marked prevention of weight gain by Fraction 4 could be by an immediate stimulation of marked diuresis, by a prevention of water uptake through some mechanism or a combination of the two.

SUMMARY

Extraction of the suprapharyngeal ganglion was carried out using methanol. The methanol extract supernatant was dried, then re-suspended in water and the water-insoluble material discarded. The water-soluble extract was further purified by ether:water and the ether fractions discarded and the water-soluble fraction saved for biological testing. The water extract was separated into four ninhydrin positive fractions by sephadex column chromatography. The fractions were tested for biological activity in a chromatophorotropic assay using the white chromatophores of the crayfish *Cambarus* spp. and in three assays in the earthworm *Lumbricus terrestris*; the first a hyperglycemic blood assay, the second a glycogenolysis assay, and the third an osmoregulation assay. Results of the assays have shown that Fraction 1 was active in all assays but showed the highest activity in the chromatophorotropic assay, the hyperglycemic assay, and the glycogenolysis assay. Fraction 2 was active in all assays in which it was tested showing the greatest activity in the hyperglycemic assay. Fraction 3 was active in the chromatophorotropic and the osmoregulation assays and inactive in the hyperglycemic assay. Fraction 4 was the most active in the osmoregulation assay and inactive in the chromatophorotropic and the

hyperglycemic assays. Fractions 2, 3 and 4 were not tested in the glycogenolysis assay due to a lack of material.

The methods used in the chemical extraction and the chromatographic procedures indicated that the fractions contained compounds which were probably polypeptide in nature. The results of the assays used for testing the biological activity indicated that the extraction and the isolation procedures used resulted in the isolation of fractions which contained a factor(s) that may play a role in the regulation of the physiological processes of carbohydrate metabolism and osmoregulation in the earthworm *Lumbricus terrestris*.

LITERATURE CITED

Agarwal, A. and M. J. Greenberg. 1969. A comparative study of cardioactive agents from bivalve tissue. Comp. Biochem. Physiol. 31(6): 835-850.

Belamarich, F. A. and R. C. Terwilliger. 1966. Neurosecretion of invertebrates other than insects. Part I. The nature and localization of neurosecretory substances. Isolation and identification of cardio-excitor hormone from the pericardial organs or *Cancer borealis*. Am. Zool. 6:101-106.

Bianchi, S. 1967. The amine secreting neurones in the central nervous system of the earthworm (*Octolasium complanatum*) and their possible neurosecretory role. Gen. Comp. Endocrinol. 9:343-348.

Brown, B. C. 1965. Pharmacologically active constituents of the cockroach corpus cardiacum: resolution and some characteristics. Gen. Comp. Endocrinol. 5:387-401.

Bullock, T. H. and G. A. Horridge. 1965. Structure and Function in the Nervous System of Invertebrates. Freeman Company. San Francisco. 354-377.

Craig, J. 1966. The effect of neurosecretion from the cerebral ganglia on blood glucose levels in *Lumbricus terrestris*. Thesis (M.A., Biology) Western Michigan University, Kalamazoo, Michigan.

Gabe, M. 1966. Neurosecretion. Pergamon Press. New York. 38-82.

Goudie, J. W. 1968. A histochemical study of the neurosecretory substance(s) controlling osmoregulation of *Lumbricus terrestris*. Thesis (M.A., Biology) Western Michigan University, Kalamazoo, Michigan.

Herlant-Meewis, H. 1956. Croissance et neurosecretion chez *Eisenia foetida* (Sav). Ann. Sci. Nat. Zool. et Biol. Animale 11th Ser. 18:185-198.

Herlant-Meewis, H. 1961. Regeneration du systeme nerveux chez *Eisenia foetida*. Bull. Biol. France Belgique 95:695-730.

Hubl, H. 1953. Die inkretorischen zelldemente im gehirn der *Lumbriciden*. Arch. Entw. Mech. Org. 146:421-432.

Ichikawa, M. and H. Ishizaki. 1961. Brain hormone of the silkworm *Bombyx mori*. Nature 191:933-934.

Ichikawa, M. and H. Ishizaki. 1963. Protein nature of brain hormone of insects. Nature 198:308-309.

Ishizaki, H. and M. Ichikawa. 1967. Purification of the brain hormone of the silkworm *Bombyx mori*. Biol. Bull. 133:355-368.

Kamemoto, F. I. 1964. The influence of the brain on osmotic and ionic regulation in earthworms. Gen. Comp. Endocrinol. 4:420-426.

Kamemoto, F. I., A. E. Spalding and S. M. Keister. 1962. Ionic balance in blood and coelomic fluid of earthworms. Biol. Bull. 122:228-231.

Karlson, P. and H. Hoffmeister. 1963. Zur biogenese des Ecdysons. I. Umwandlung von cholesterin in ecdyson. Hoppe-Seyl, Z. 331: 298-300. In Insect Hormones by U. J. A. Novak, 1966, p. 77.

Kleinholz, L. H. and F. Kimball. 1965. Separation of neurosecretory pigmentary-effector hormones of the crustacean eyestalk. Gen. Comp. Endocrinol. 5:336-341.

Lentz, T. L. 1965. Hydra induction of supernumery heads by isolated neurosecretory granules. Science 150:633-635.

Marapao, B. P. 1959. The effect of nervous tissue extracts on neurosecretion in the earthworm, *Lumbricus terrestris*. Cath. U. Amer. Biol. Stud. 55:1-33.

McVay, J. A. 1942. Physiological experiments upon neurosecretion, with special reference to *Lumbricus* and *Cambarus*. Dissertation, Northwestern University, Evanston, Illinois.

Mordue, W. and G. J. Goldsworthy. 1969. The physiological effects of corpus cardiacum extracts in locusts. Gen. Comp. Endocrin. 12: 360-369.

Novak, V. J. A. 1966. Insect Hormones. Methnen and Company Ltd. London. p. 77.

Radke, C. D. 1969. Glycogen levels and hormone effects in the intestine of *Lumbricus terrestris*. Special Biology Project. Western Michigan University, Kalamazoo, Michigan.

Roe, J. H. and R. E. Dailey. 1966. Determination of glycogen with the anthrone reagent. Anal. Biochem. 15:245-250.

Rude, S. 1969. Catecholamines in the ventral nerve cord of *Lumbricus terrestris*. Comp. Biochem. Physiol. 28:747-752.

Scharrer, E. and B. Scharrer. 1937. Uber drusen-nervelzellen und neurosekretorische organe bei wirbellosen und wirbellieren. Biol. Rev. 12:185-216.

Scharrer, E. and B. Scharrer. 1963. Neuroendocrinology. Columbia University Press. New York and London.

Teichman, I., B. Aros and B. Vigh. 1966. Histochemical studies on Gomori-positive substances. III. Examination of the earthworms neurosecretory system. Acta Biol. Hung. 17:329-357.