IDENTIFICATION AND CHARACTERIZATION OF THE EGG-LAYING HORMONE FROM THE NEUROSECRETORY BAG CELLS OF *APLYSIA*

Thesis by

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This thesis is dedicated to--

my husband, Jim, who through love and sacrifice, helped me grow into a complete and happy woman--wife, mother, and scientist;

my parents, Mr. and Mrs. L. B. Schloemer, who instilled in their seven children an undying desire to learn "why";

Mrs. Leonard Krause, my teacher for grades one through four, who never tried to bridle my curiosity, but only my mischievous ways;

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and, on too many occasions, has sacrificed a clean home and cheerful wife so that I could attain my goals.

ABSTRACT

This investigation has resulted in the chemical identification and isolation of the egg-laying hormone from Aplysia californica, Aplysia vaccaria, and Aplysia dactylomela. The hormone, which was originally identified as the Bag Cell-Specific protein (BCS protein) on polyacrylamide gels, is a polypeptide of molecular weight ≈ 6000, which is localized in the neurosecretory bag cells of the parieto-visceral ganglion and the surrounding connective tissue sheath which contains the bag cell axons. All three species produce a hormone of similar molecular weight, but varying electrophoretic mobility as determined on polyacrylamide gels. As tested, the hormone is completely cross-reactive among the three species.

Although the bag cells of sexually immature animals contain the active hormone, sexual maturation of the animal results in a 10-fold increase in the BCS protein content of these neurons.

A seasonal variation in the BCS protein content was also observed, with 150 times more hormone contained in the bag cells of Aplysia californica in August than in January. This correlates well with the variation in the animals' ability to lay eggs throughout the year (Strumwasser et al., 1969). There are some indications that the receptivity of the animal to the available hormone also fluctuates during the year, being lower in winter than in summer. The seasonal rhythm of the other species, Aplysia vaccaria and Aplysia dactylomela, has not been investigated.

A polyacrylamide gel electrophoresis analysis of water-soluble proteins in Aplysia californica revealed several other nerve-specific proteins. One of these is also located in the bag cell somas and stains turquoise with Amido Schwarz. The function of this protein has not been investigated.

TABLE OF CONTENTS

INTRODUCTION

A current problem of growing interest in the neurosciences is the determination of the role played by nerve-specific substances in neural functions. Few such nerve-specific substances have been chemically isolated or functionally identified. To investigate this and other related problems, the advantages of using simple systems for neurophysiological and behavioral studies have repeatedly been emphasized, recently in two symposia (Bullock, 1966; Wiersma, 1967). In these systems, it is possible to examine in detail parts of the nervous system which influence or control particular behavioral acts. The involved neurons are also amenable to detailed chemical analyses which may reveal substances unique to them. In view of the increasing knowledge of neuronal interactions and behavior in the gastropod mollusc Aplysia, an investigation was undertaken to try to detect nerve-specific proteins and relate them to neuronal functions. It later turned out that one of these proteins was involved in humoral control of behavior in the intact animal.

In Aplysia, ganglia are more or less grouped according to function and neurons are readily identifiable from one preparation to another (Hughes and Tauc, 1962; Chalazonitis and Arvanitaki, 1963; Coggeshall, 1967; Frazier et al., 1967; Strumwasser, 1967b). There is further evidence that even the neurons within a ganglion may be functionally grouped (Kupfermann and Kandel, 1968). Without question, the neurons are morphologically grouped into compartments in some ganglia by glial invaginations and vascular sinuses (Strumwasser and

Alvarez, unpublished observations; Frazier et al., 1967). This would facilitate microdissection of a ganglion into small groups of possibly related neurons.

In addition to the morphological advantages of its nervous system, the animal exhibits several stereotyped behavioral patterns: feeding behavior (Frings and Frings, 1965; Lickey and Berry, 1966; Lickey, 1968), locomotion (Strumwasser, 1967a), defensive responses (Kupfermann and Kandel, 1969), and reproductive behavior (Kupfermann, 1967; Strumwasser et al., 1968, 1969; Toevs and Brackenbury, 1969). An impressive study by Willows (1967) on a closely related nudibrach, Tritonia gilberti, has shown well-defined behavior patterns, such as turning and swimming, in response to stimulation of single neurons. These facts suggested the possibility of linking a homogeneous group of neurons to a specific behavioral response. Therefore, Aplysia is a useful system for study of the relationship between a nerve-specific molecule and behavior, if the molecule can be related to a particular cell or group of cells.

A qualitative examination of neuronal and non-neuronal tissues by discontinuous polyacrylamide gel electrophoresis was undertaken and revealed several water-soluble nerve-specific protein bands. In addition to being nerve specific, some of the proteins are unique to certain kind of ganglia and nerve trunks (Toevs and Brackenbury, 1968, 1969). Two such protein bands were localized in the bag cells, which are two clusters of small neurons located at the anterior pole of the

parieto-visceral ganglion (PVG) at its junction with the pleuro-visceral connective nerves (CN).

From all the nerve-specific proteins detected, only one of the bag cell-specific proteins was chosen for detailed chemical and physiological examination, since it appeared to offer the best opportunity to study the relationship between behavior and nerve-specific molecules. A preliminary study of this bag cell-specific protein (BCS protein) was already in progress when Kupfermann (1967) reported that application of a sea-water extract of isolated bag cells to the animal produced egg laying in Aplysia californica. Because of Kupfermann's result, this investigation then focused on characterizing both the bag cell-specific protein and the active egg-laying agent and determining whether a correlation existed between them. Strumwasser et al. (1968, 1969) have greatly elaborated the physiological aspects of the egg-laying agents, and found that both the isolated bag cells and the PVG proper can induce egg laying; that the induction of egg laying is seasonal in Aplysia californica; that the bag cell extracts of both sexually mature and immature animals induce egg laying; and that there is a stereotyped behavior pattern which accompanies egg laying.

Because of their ability to induce egg laying, Kupfermann (1967) suggested that these bag cell neurons may be of a neurosecretory nature. Coggeshall (1967) had previously made the same suggestion, after electron microscopic examination of the cells revealed that they were loaded with membrane-bound 2000 Å granules. However, to determine the neurosecretory nature of any neuron, it is necessary to establish

a set of criteria, since no single characteristic is by itself definitive. Six basic criteria are generally accepted to delineate the neurosecretory neuron (Bern, 1962; Gabe, 1966; Scharrer and Scharrer, 1963). A summary of these criteria with a focus on the evidence for the bag cells of Aplysia follows. A detailed discussion of the evidence for neurosecretion with emphasis on the gastropod mollusc Aplysia, is contained in Appendix I.

Morphology of the Neurosecretory Cell

A neurosecretory neuron generally contains a prominent Golgi complex, active endoplasmic reticulum, and is laden with electron-dense granules 1000 to 4000 Å in diameter (Bern and Knowles, 1966). The extensive electron microscopic studies by Rosenbluth (1963), Simpson et al. (1963) and Coggeshall (1967) on the PVG of Aplysia have revealed many cells in the ganglion which meet these requirements. Two prominent groupings are the bag cell clusters, which contain 250 to 400 small neurons each. Each neuron has a diameter between 30 and 50 μ, and is tightly packed with electron-dense 2000 Å granules (Frazier et al., 1967). Discrete clumps of electron-dense material can be seen in the marginal buds of the cisternae of the Golgi complex. The character of the granules changes as they are observed en route from the cell soma, where they appear round and electron-dense, to the axons, where they are ghostly, with broken, crenated membranes (Coggeshall, 1967). (See Figure 23.)

Neuronal Characteristics of Neurosecretory Neurons

Although transmission is not the primary business of neurosecretory fibers, conduction of impulses is essential to complete the neuroendocrine reflex arc. The bag cells exhibit action potentials similar to those exhibited by other Aplysia neurons, when induced by current passed through an intracellular microelectrode (Frazier et al., 1967). These cells generally do not fire spontaneously, but, by stimulating one of the pleuro-visceral connective nerves, all the bag cells of one cluster can be excited to spike in synchrony for up to 45 minutes. A prepotential has been observed which is similar and simultaneous in all the bag cells, and has led the investigators to believe either that all the cells of one cluster are electrically coupled, or that an interneuron with its axon in the ipsilateral connective nerve innervates all 400 bag cells in a cluster (Figure 1). There is little question that these cells act as typical neurons in their ability to produce and conduct an impulse.

Endocrine Function of the Neurosecretory Neuron

The neurosecretory cell, in addition to possessing typical neuronal function, shows a characteristic feature of its own: the ability to synthesize, store, and release hormonal substances, commonly polypeptides of low molecular weight. The ability to synthesize material is reflected in the presence of recognized cytological secretory apparatus. The storage and release of the material usually takes place in the axons which appear swollen with neurosecretory granules,

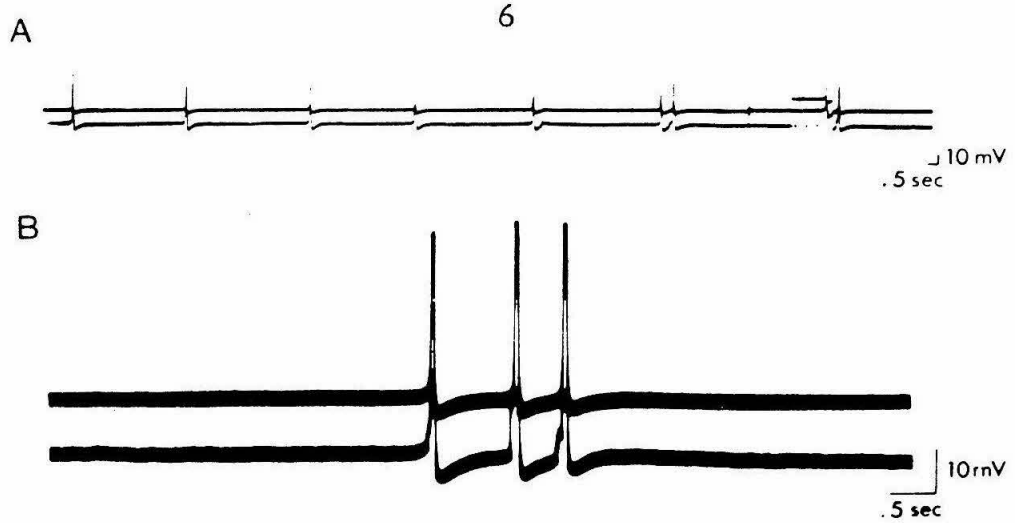


FIG. 5. *A*: simultaneous records from two ipsilateral bag cells cluster LA which have been set into activity by stimulating the left connective. *B*: at higher amplification to better illustrate the close synchrony of the two cells.

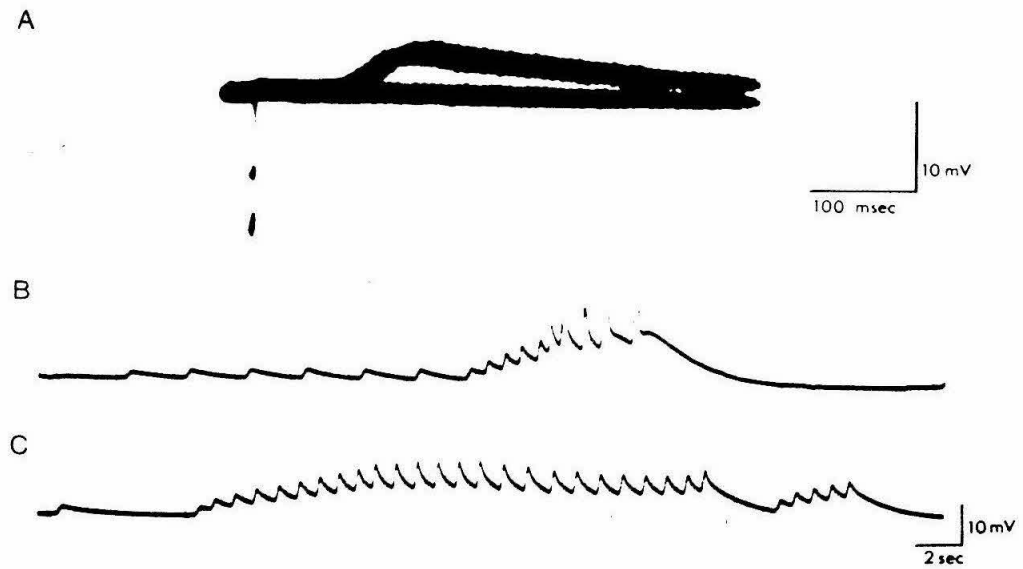


FIG. 6. Prepotentials in bag cells in response to stimulation of the ipsilateral connective. These records were made from a cell that had previously been set into activity by a stimulus train to the ipsilateral connective. At the time this record was made, spontaneous activity had ceased, and the cell could not be set into repetitive activity. *A*: a single shock produced an all-or-none prepotential. *B*: when the frequency of stimulation was increased, the prepotentials appeared to summate and trigger a spike. *C*: shows that the prepotential could disappear in an all-or-none fashion during constant-intensity repetitive stimulation of a connective. Although this prepotential resembles an EPSP, all attempts to alter the amplitude of the prepotential by changing the membrane potential of a bag cell have been unsuccessful; we therefore cannot distinguish between a remote EPSP or an electrotonically propagated axonal spike.

Figure 1: Internal microelectrode recordings from bag cells (from Figures 5 and 6 of Frazier et al., 1967).

and which terminate in close association with the vascular system. This association is referred to as a "neurohemal organ" (Carlisle and Knowles, 1953) and facilitates the distribution of the released hormone throughout the circulatory system of the animal. The naked axons of the bag cells terminate primarily in the connective tissue sheath of the ganglion, where they make intimate contact with venous sinuses or end blindly in the extracellular matrix of the connective tissue, which is liberally bathed in hemolymph (Coggeshall, 1967).

Although not applicable in all species, most neurosecretory neurons do not innervate effector organs, such as muscle, or other neurons (Knowles and Carlisle, 1956). There is no evidence that the bag cell axons leave the visceral ganglion to innervate any organ.

Chemical Nature and Hormonal Function of the Neurosecretory Material

The evidence from many species overwhelmingly indicates that neurosecretory hormones are of a polypeptide nature. Depending upon the age and species of the animal, the neurohormones exhibit a regulatory effect on almost every physiological function in the body, including water balance, pigment movements, regeneration, and reproduction (Bern and Hagadorn, 1965; Bern and Knowles, 1966; Hagadorn, 1967). No molluscan neurohormone had been isolated and characterized until this present study. This thesis will present evidence correlating the bag cell-specific protein and active egg-laying agent in Aplysia californica, Aplysia vaccaria, and Aplysia dactylomela.

Cyclical Variation of Neurosecretory Product

Neurosecretory cells exhibit a cyclical change in their granule content or hormone content, in relation to an altered state in the physiology of the target organ or the animal. This change can sometimes be artificially induced in the laboratory by the appropriate sensory stimulation, or it may reflect a diurnal, lunar, or seasonal aspect of the animal's behavior (Gabe, 1965; Brown, 1966). An annual variation in the BCS protein content of the bag cells has been observed, and correlated with the seasonal rhythms of the reproductive behavior of the animal.

In summary, there are six major criteria to delineate a neurosecretory neuron. The first four criteria--morphological characteristics, neurophysiological characteristics, and the two properties of axon termination--are met by the bag cells as previously reported in the literature. This thesis will deal with the last two criteria, concerning the isolation, chemical and physiological identification, and seasonal variation, of the active hormone of these cells.

MATERIALS AND METHODS

Maintenance of Species Used

Three species of the sea hare Aplysia were used in this study. Aplysia californica and Aplysia vaccaria were obtained from the intertidal zone around Corona del Mar, California, where the temperature of the sea water ranges from 12° to 18°C. Some Aplysia californica were obtained from Dr. Rimmon Fay, Pacific Bio-Marine Corporation, Venice, California. In the laboratory the animals were kept in constant light in 14°C circulating filtered sea water in a 1400 gallon system. Those animals which were to be the recipients in the egg laying experiments were separately isolated in a 15 gallon aquarium with circulating filtered sea water, for which the temperature was maintained at 16°C. The Aplysia dactylomela, located off the warm Florida coast, were obtained from Tropical Atlantic Marine Specimens, Big Pine Key, Florida. They were maintained in the laboratory in 15 gallon aquaria with aerated sea water kept at 25°C.

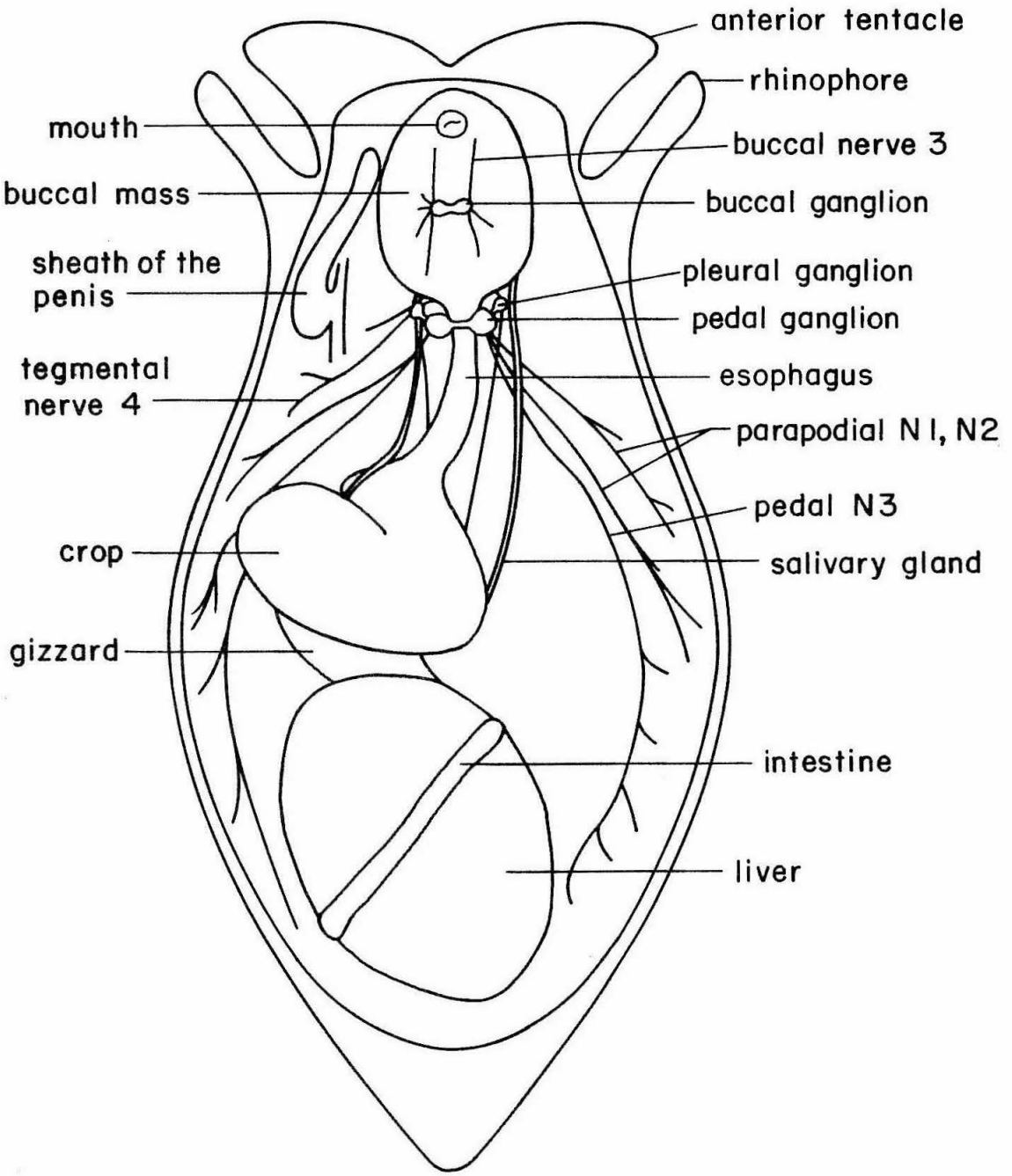
Unless otherwise stated, the experiments presented hereafter were conducted on Aplysia californica. However, the procedures for the extraction and analysis of the tissue are the same for all three species.

Dissection of Neural Tissue

The animal was pinned foot-up on a cork dissection board and opened along the midline to expose the viscera (Figure 2). Upon displacement of the esophagus, crop, and liver, the nervous system and

Figure 2: General dissection of Aplysia from the ventral side. The animal is pinned to a dissecting table through the foot, and a midline incision is made to expose the major viscera and nerves. The circulatory system is not illustrated. (After Eales, 1921.)

VENTRAL VIEW OF VISCERA OF APLYSIA



reproductive apparatus were clearly visible (Figure 3). The ovotestis is embedded in the posterior dorsal surface of the liver. The desired tissue was then removed, and, if it was to be extracted within two hours, it was stored in filtered 4°C sea water. However, if it was not to be used immediately, the tissue was blotted dry to remove excess surface sea water, quick frozen on dry ice, and stored at -20°C until extracted at a later time.

Often the intact parieto-visceral ganglion (PVG) was not used, but rather the bag cell clusters were removed and analyzed separately from the remainder of the ganglion. This was simply done by cutting between the small clustered white bag cells and the large white and orange neurons of the remaining PVG (Figure 4). The posterior pleuro-visceral connective nerves (first 5-10 mm) were then removed from the bag cell cluster and also analyzed separately. The remaining nerve trunks, branchial, anal, pericardial and all other smaller nerves, were trimmed off close to the PVG. A fine iridectomy scissors was used in this dissection.

By microdissection with fine stainless steel needles (20 μ tip), it was possible to remove the connective tissue sheath from the pleuro-visceral connective nerves and the PVG. To do this, each major nerve trunk of the PVG was tied with thread, and the intact PVG was secured to a dissecting microscope stage by applying sealing wax to each of the ties on the nerve ends. The connective tissue was removed from all sides of the first 10 mm of the posterior pleuro-visceral connective nerves by rotating them along their long axis. The bag

Figure 3: General dissection of the nervous system and reproductive apparatus as seen from the ventral side. The stomato-gastric system and the liver have been dissected away to expose the underlying reproductive tract. Only major nerves are diagrammed. (After Eales, 1921.)

VENTRAL VIEW OF REPRODUCTIVE SYSTEM AND NERVOUS SYSTEM OF APLYSIA

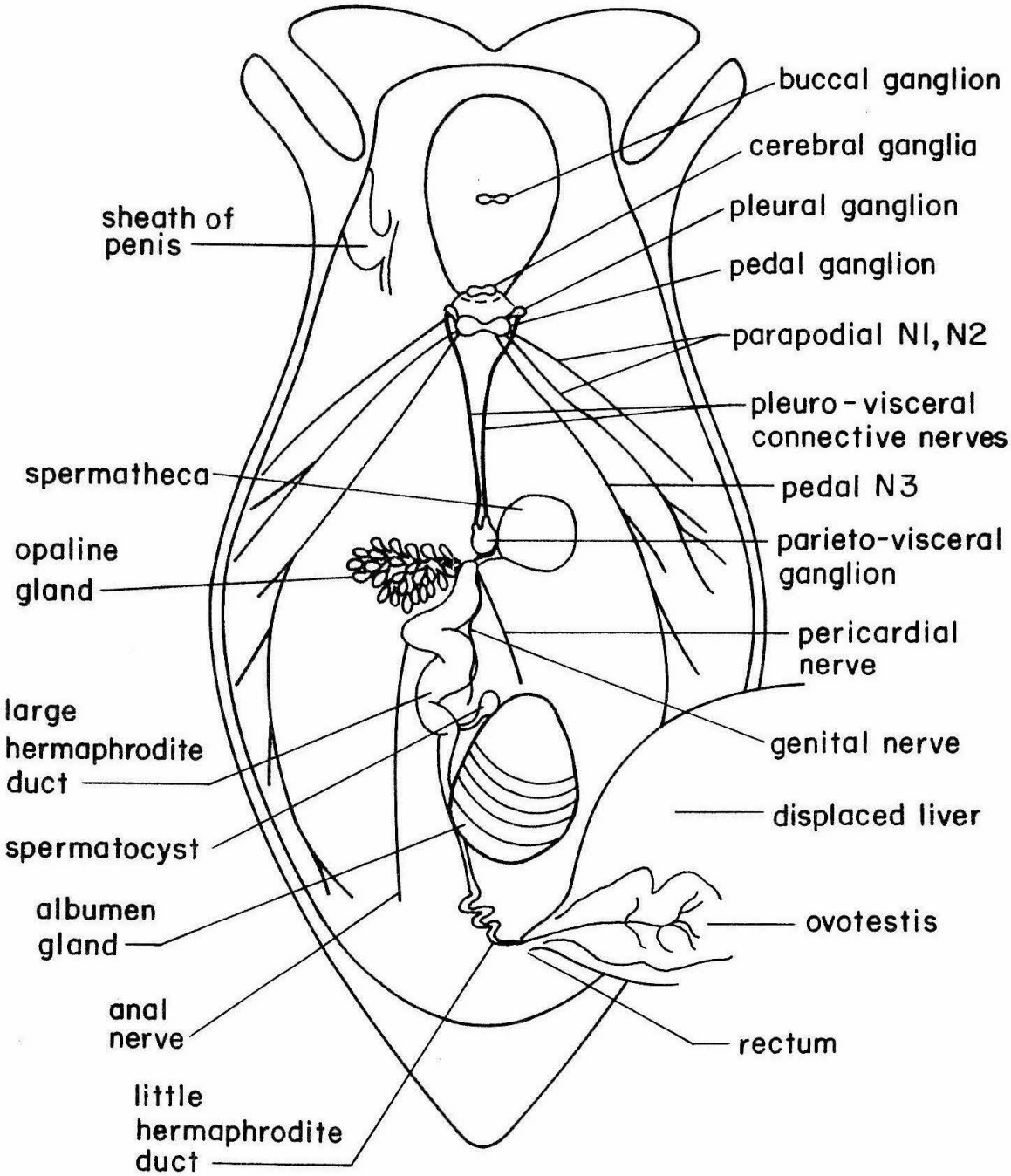
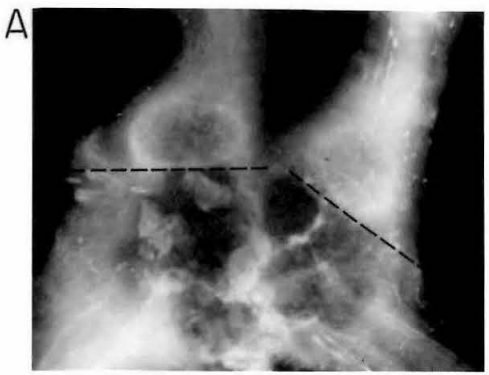
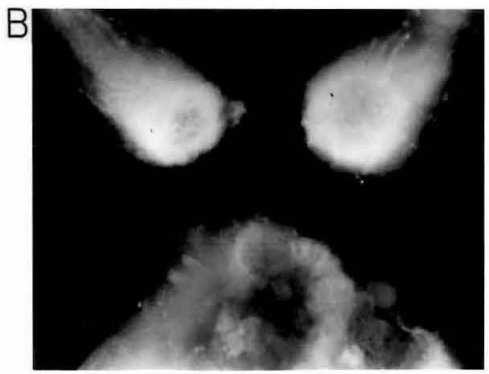


Figure 4A and B: Photograph of ventral surface of the PVG taken with epi-illumination. The intact PVG is shown in A; the dotted lines indicate where the incision between the two bag cell clusters and the rest of the ganglion were made. B shows the same ganglion after separation of the bag cell clusters. (From Figure 1A and B, Strumwasser et al., 1969.)

C. Schematic diagram of the PVG illustrating the dissection of it into three tissue samples which were used in many analyses.


A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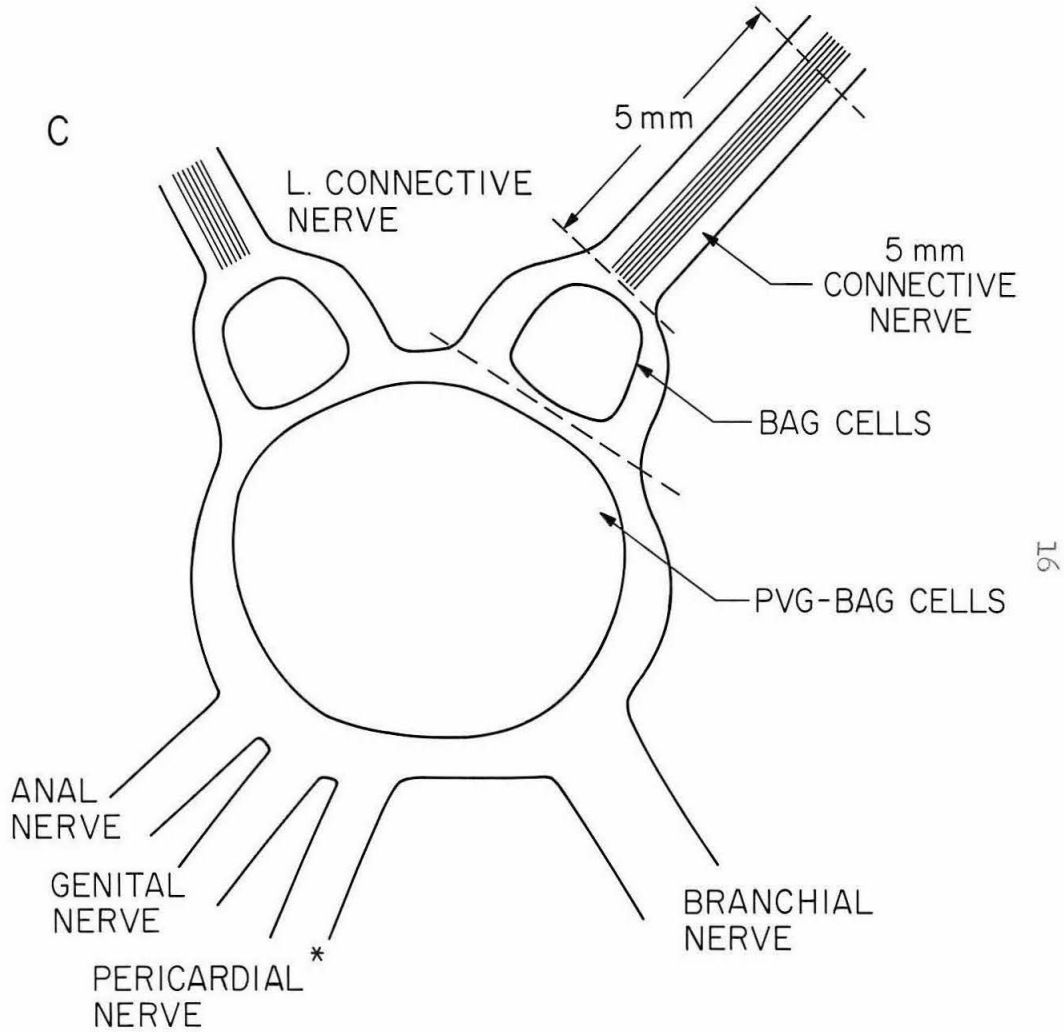

C
5 mm
L. CONNECTIVE NERVE
5 mm CONNECTIVE NERVE
BAG CELLS
PVG-BAG CELLS
ANAL NERVE
GENITAL NERVE
PERICARDIAL NERVE
*
BRANCHIAL NERVE

cells were then separated from the connective nerves and the rest of the PVG. The connective tissue sheath of the PVG was slit and peeled back and the PVG neurons and neuropile were gently sucked and washed out of the sheath. Care was taken not to squeeze the connective tissue sheath. It is technically difficult to remove the connective tissue sheath from the bag cells without rupturing the tiny neurons and thus contaminating the tissue samples, therefore the connective tissue sheath was not removed from the bag cells but was homogenized with it.

Examination of Reproductive Apparatus

The reproductive tract was also removed, weighed, and characterized. The sexual maturity of the individual was determined by qualitatively examining the size and color of the gonad, size of the albumen gland, and muscular development of the large hermaphrodite duct. The mature animal also contained his own sperm in the little hermaphrodite duct, and stored sperm from previous matings in the spermatocyst. If the animal had been previously mated, the spermatheca was enlarged and contained colored refuse, while, in the immature, unmated animal, the spermatheca was small and clear. The tract weights gave some indication of the sexual maturity of the animal but were not conclusive, since there was an overlap between the three categories due to great variation in animal size in the population tested. The average tract weight of 85 mature Aplysia was 0.94 ± 0.64 grams and of 50 immature Aplysia it was 0.079 ± 0.045 grams. Twenty-eight animals which fell

between these two categories were classified as "young." Their average tract weight was 0.24 ± 0.10 grams.

Extraction Procedures

Neuronal and non-neuronal tissues were dissected from Aplysia, blotted dry and usually weighed, quick frozen on dry ice, and then extracted with cold, 0.01 M potassium phosphate buffer (pH 7.0, μ = 0.05) in miniature (100 µl capacity) glass homogenizers. A higher ionic strength buffer (0.05 M sodium phosphate buffer, pH 7.0, μ = 0.11) was used when column chromatography was being employed as a method of protein purification. The homogenates were centrifuged at 12,000 g for 15 minutes at 10°C to remove chromatin, membrane fragments, mitochondria, and other cell debris. In an alternative method of preparation, also used to extract water-soluble proteins, the samples were homogenized in 0.05 M Tris (pH 8.0), 0.001 M $MgCl_2$, 0.25 M sucrose, and centrifuged at 105,000 g for 120 minutes at 10°C to remove chromatin, membranes, intact mitochondria and intact ribosomes. To extract water-insoluble proteins, tissues were homogenized in cold 10 M urea, and centrifuged at 12,000 g for 15 minutes at 10°C to remove insoluble cell debris. In a typical extraction for all three methods, 30 mg of wet tissue were homogenized in 150 µl of buffer.

Analysis of Proteins

When the total protein in the tissue was determined, the method of Lowry et al. (1951) was applied to a 1 M NaOH extract of the tissue.

The dry weight of the tissue was obtained by taking weighed, wet tissue, which had been blotted to remove excess surface water, and drying it in a 45-50°C oven for 10 to 15 hours or until no weight change occurred on further drying.

The extracts, containing the water-soluble materials, were analyzed by discontinuous polyacrylamide gel electrophoresis as outlined by Ornstein (1964) and Davis (1964). The solutions for acrylamide gel electrophoresis were prepared as described in Table I. Those extracts containing water-insoluble proteins were analyzed on acrylamide gels made up in 8 M urea rather than in distilled water. When it was necessary to know the total amount of protein layered on the gel, the method of Lowry et al. (1951) was used. Best separation was achieved when the proteins were run from cathode to anode at 2 milliamperes per gel on 3.5 mm by 120 mm gels of 7.5% or 15% gel concentration (pH 8.9). A marker dye, 0.001% bromphenol blue in water, was used to determine the salt front on the gel during electrophoresis.

Most gels were fixed and stained in 1% Amido Schwarz in 7.5% acetic acid, and destained by dialysis in 7.5% acetic acid, although some gels were stained with Coomassie Brilliant Blue (Colab Laboratories, Inc.). When this dye was used, gels were immediately placed in a 20-fold volume of 12.5% trichloroacetic acid (TCA) for 30 minutes, immersed for several hours in a 1:20 dilution of 1% aqueous Coomassie Brilliant Blue in 12.5% TCA, and then destained by dialysis in 10% TCA. The gels were not destained electrophoretically because this caused loss or distortion of some protein bands near the anode end of the gel.

TABLE I

Polyacrylamide Gel Solutions

	Proportion used
Separating gel stock solution	
1) for 7.5% gel acrylamide 30.0 gm N,N-methylenebisacrylamide 0.8 gm H_2O to make 100 ml	1
1) for 15% gel acrylamide 60.0 gm N,N-methylenebisacrylamide 0.8 gm H_2O to make 100 ml	1
2) 1 N HCl 24 ml Tris 18.15 gm N,N,N,N-tetramethylethylenediamine 0.23 ml H_2O to make 100 ml, adjust to pH 8.9	1
3) Ammonium persulfate 0.14 gm H_2O to make 100 ml	2
Stacking gel stock solutions	
4) acrylamide 5.0 gm N,N-methylenebisacrylamide 1.25 gm H_2O to make 100 ml	2
5) 1 M H_3PO_4 12.8 ml Tris 2.85 gm N,N,N,N-tetramethylethylenediamine 0.10 ml H_2O to make 100 ml, adjust to pH 6.9	1
6) riboflavin 2.0 mg H_2O to make 100 ml	1
Photopolymerize stacking gel	
Lower buffer	
1 N HCl 60.0 ml Tris to pH 8.1 H_2O to make 1000 ml	use full strength
Upper buffer	
Glycine 2.88 gm Tris 0.6 gm, adjust to pH 8.3 H_2O to make 1000 ml	use full strength

Although the Coomassie Brilliant Blue is 5-10 times more sensitive in detecting proteins than Amido Schwarz (Fazekas de St. Groth et al., 1963), it was not routinely used because the BCS protein band did not stain as well with it as with the less sensitive Amido Schwarz. Some gels were stained with periodic acid-Schiff reagent (Canalco procedure) to detect glycoprotein bands.

Quantitative Analysis of Proteins on the Gel

All the gels were scanned with a Joyce Loebl double-beam recording microdensitometer fitted with a one log-unit optical wedge, a one log-unit bias filter, and a 10X objective. To determine the relative amounts of the bag cell-specific protein in the various tissues, each gel was scanned at four to six equally spaced positions along its diameter. The area under the appropriate peak was measured by cutting out the peak and weighing the paper. In each case the weights for the 4 to 6 tracings were averaged. The absolute distance moved by a band depends on such parameters as current and time of electrophoresis, which vary because the gel conductivities vary. To directly compare protein band movements in each experiment, then, the distance moved by a band is expressed as a fraction of the distance moved by the bromphenol blue marker band.

Bioassay of Hormone

To determine the presence of the active egg-laying agent, aliquots of the phosphate buffer extract of tissues were diluted to

1 ml with cold, filtered sea water and injected through the foot into the anterior hemocoel of the previously isolated test Aplysia. In cases where the extract would not also be analyzed by acrylamide gel electrophoresis, the tissue was extracted directly with 1 ml of cold, filtered sea water. A typical control dose of an egg-laying agent extract contained 1-1 1/2 PVG. It has been shown (Strumwasser et al., 1969) that after induction of egg laying, an animal is in a "refractory" state and cannot lay eggs for at least two days. If an isolated animal does not lay eggs upon first injection of test material, it is assumed not to be refractory, and therefore no waiting time is necessary before the next injection. In our experiments, if an animal layed eggs, it was usually not injected again for seven days. In a given experiment, any animal which did not lay eggs was always injected with control extract to test its ability to lay eggs that day. This was an essential control for each experiment, since body size seems to be a poor indication of sexual maturity and the animals must be sexually mature to lay eggs. Also, it is necessary to control for any unknown factors that may cause the recipient to be in a refractory state that day. No conclusion, then, was drawn from an experiment in which the animal did not lay eggs after both test and control injections.

Enzymatic Degradation of BCS Protein and Active Agent

RNase and DNase digestion

The PVG extracts were subjected to enzymatic degradation to chemically characterize the bag cell-specific protein. Extracts treated

with RNase and DNase (Sigma Chemical Co.) were incubated at room temperature for 30 minutes at final concentrations of 20 μg/ml for both enzymes.

Pronase digestion

The multiple enzyme mixture Pronase (Sigma Chemical Co.) was also used for degradation studies but was pre-incubated for one hour at 37°C prior to use. The PVG extract was brought to the incubation temperature of 37°C before the appropriate quantity of pronase was added. Timing of the experiment began with the addition of the enzyme. The reaction was stopped by plunging the tube into a dry ice-acetone bath (-80°C). In order to test the pronase sensitivity of the BCS protein and the active egg-laying agent, the conditions of incubation were varied, with pronase concentrations ranging from 1 mg/ml to 0.0025 mg/ml and incubation times ranging from 6 hours to 2 minutes. Portions of the treated samples were run on acrylamide gels in order to determine the effect of the treatment on the BCS protein. The total time the extracts were thawed (from -80°C to 0°C) before electrophoresis began averaged two minutes. To test the activity of the egg-laying agent after pronase treatment, samples were thawed, quickly diluted with 1 ml of 0°C filtered sea water, and injected into mature test animals. An average of 1 minute elapsed between thawing of the sample and injection of the animal. Control animals were monitored for the effect of pronase itself on the reproductive behavior by injecting the animal with 5 μg pronase, diluted with 1 ml cold, filtered sea water, followed in

4 minutes by a second injection containing 0°C control PVG extract, which had also been diluted with 1 ml cold filtered sea water.

Trypsin digestion

Trypsin, which cleaves the lysyl- and arginyl-amino acid peptide bonds, was also used to degrade the BCS protein and active egg-laying agent. The chymotrypsin-free trypsin was supplied courtesy of the Prof. William Dreyer group. The stock solution (5 mg/ml) was stored at -20°C in 0.01 M HCl. It was diluted to 0.05 and 0.005 mg/ml with cold, pH 8.5, sodium phosphate buffer (0.05 M). The reaction mixture was incubated at 25°C at a pH of 8.3. The final concentrations of trypsin were 1%, 0.1%, and 0.01%, based on the total weight of protein in the solution. After some time at 25°C, the reaction was stopped by plunging the tube into a dry ice-acetone bath. The effect of the trypsin treatment on the BCS protein and active egg-laying agent was examined by the procedures described above for the pronase degradation. Again, the possibility of trypsin affecting the reproductive behavior was monitored by injecting the control animal with two separate injections, one of trypsin followed in 4 minutes by the 0°C control extract.

Thermal Stability

The thermal stability of the BCS protein and active egg-laying agent was examined at 37°C for 2, 4, and 6 hours and at 55°C for 5, 10, and 15 minutes. The heat denaturation was stopped by immersing the tubes in a dry ice-acetone bath. Both crude extract and the purified

BCS protein were so treated. The BCS protein was analyzed by its electrophoretic mobility on acrylamide gels and the activity of the egg-laying agent was examined in the animal by the procedure used for pronase degradation experiments.

Purification by Gel Filtration

A method was sought to isolate the BCS protein from other water-soluble proteins in the PVG. Due to the low quantities of material available from these small neurons, it was necessary to find a very simple method of purification. Gel filtration was chosen using G 50 fine Sephadex in a 1.5 x 60 cm column with 0.05 M sodium phosphate elution buffer (pH 7.0). Six to twenty-five intact PVG were homogenized in 450 to 1500 µl (60 to 70 µl per PVG) of sodium phosphate buffer (0.05 M, pH 7.0), and centrifuged at 12,000 g for 15 minutes. The resulting supernatant was layered on the column. Flow rate was adjusted to 2.5 ml/hour with a Mariotte flask, and 250 to 300 µl fractions were collected and frozen at -20°C until use. The column was maintained and run at 18° to 20°C. The elution and final volumes were monitored by a UV spectrophotometer (Beckman DB) at 280 mµ. The fractions were analyzed by polyacrylamide gel electrophoresis to find the BCS protein and by injection into mature test animals to localize the active egg-laying agent.

Molecular Weight Determination

In gel filtration, the elution volume of a given substance is dependent on its molecular weight and is easily reproducible on a given chromatography column. Therefore, the G 50 fine Sephadex column was calibrated with molecular weight markers, which were non-enzymatic proteins obtained from Mann Research Laboratories. Bovine serum albumin (67,000), chymotrypsin (25,000), cytochrome C (12,400) and bacitracin (1,400) were used. It was then possible to determine the molecular weight of an unknown substance from the relationship (Whitaker, 1963)

$$\frac{V_{elution}}{V_{void}} = k \cdot \log \text{molecular weight} \qquad (1)$$

For optimum fractionation and molecular weight determination of a protein, the gel must be carefully selected so that the protein to be isolated is recovered in the middle of the elution curve. Investigations of Andrew (1965) into the separation ranges of Sephadex gels, have shown that the middle of the elution curve obeys Equation 1, but that both ends deviated from Equation 1. G 50 fine Sephadex accurately follows Equation 1 over a separation range of 30,000 to 1,500 (Sephadex, 1967).

Seasonal Variation in Ganglion BCS Protein Content

January through October method of analysis

The seasonal variation of the content of the BCS protein in the bag cells, PVG-bag cells, and posterior 10 mm of the connective

nerve (CN) (Figure 4), was examined quantitatively from January to October on polyacrylamide gels. The tissue was blotted dry, weighed, and homogenized in 50 to 100 µl of sodium phosphate buffer pH 7.0 (0.05 M). The cell debris was pelleted, and Lowry determination for water-soluble protein was done on the supernatant. All or a fraction of the extract was then run on 7.5% gel at pH 8.9 as described above, stained in 1% Amido Schwarz in 7.5% acetic acid, and destained by dialysis in 7.5% acetic acid. The densitometer tracings were taken within 24 hours after destaining was completed.

October through December method of analysis

The amount of BCS protein contained in the intact PVG from October to December was calculated from the recovery of the BCS protein from the G 50 fine Sephadex column (to be discussed under Results). Fractions containing the BCS protein were run on polyacrylamide gels. Again, densitometer tracings were taken within 24 hours after destaining was completed.

Quantitative analysis of the BCS protein of the gel

To determine the amount of the bag cell-specific protein in the various tissues, each gel was scanned 4 to 6 times at equally spaced positions along the diameter of the gel. The gel was rotated along its length and rescanned. A Joyce Loebl double-beam recording microdensitometer fitted with a one log-unit optical wedge was used. The area under the appropriate peak was measured by cutting out the peak and weighing the paper. Keuffel and Esser tracing paper was

used throughout the analysis. In each case the weights from the multiple tracings were averaged.

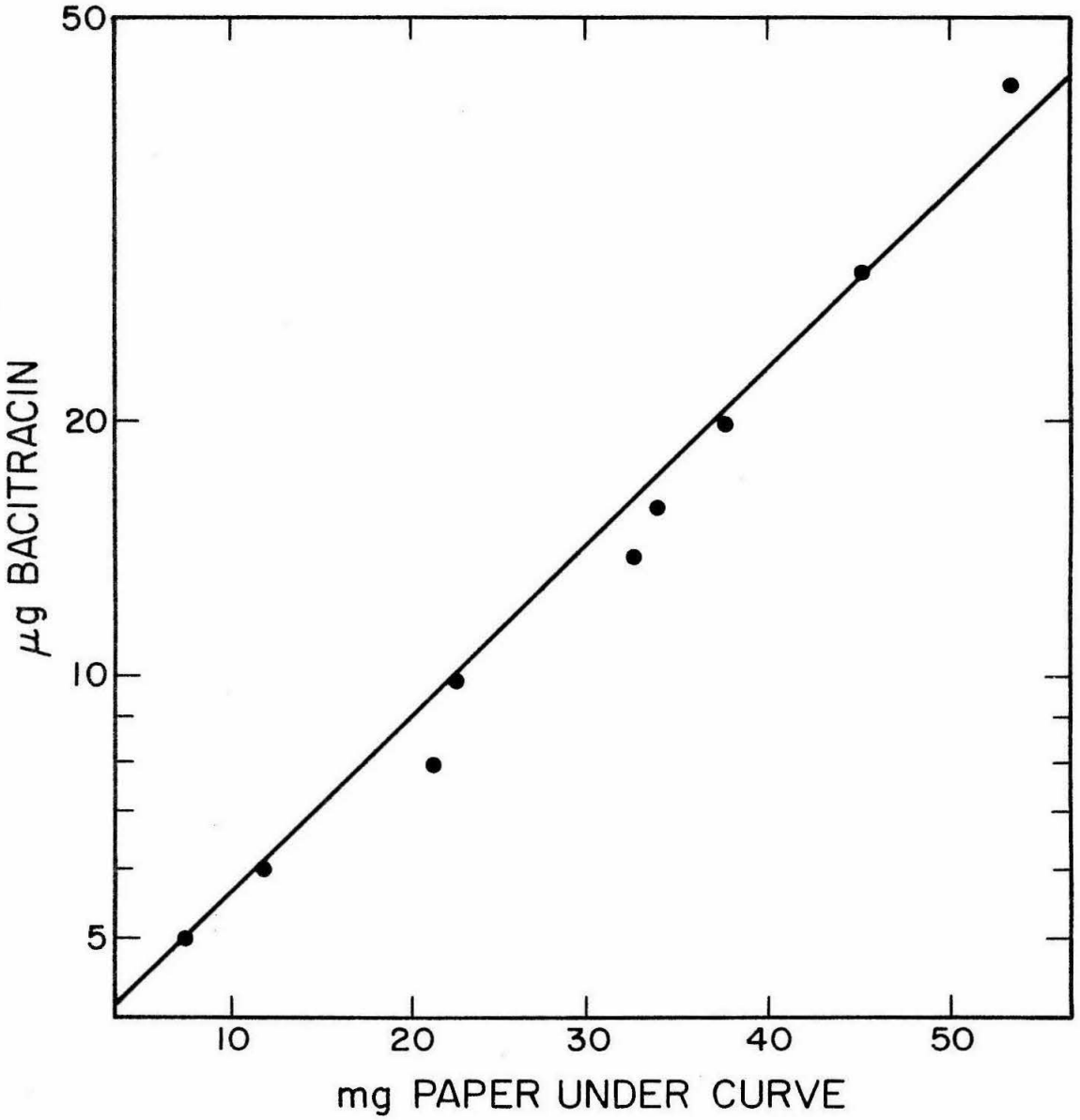
In order to relate this paper weight (area under the BCS peak) to a weight of protein, a standard curve of increasing amounts of bacitracin vs. area under the bacitracin peak was established (Figure 5). Bacitracin was chosen for the standard curve because it is acidic at pH 8.9, and the bacitracin-Amido Schwarz complex appears similar to the BCS protein-Amido Schwarz complex, e.g. a broad, grey-blue band on acrylamide gels. Most other protein-Amido Schwarz complexes appear as narrow, navy-blue bands. The area of the bacitracin peak was determined in the same manner as the area under the BCS protein peak, with both labeled in mg of paper. The total amount of the BCS protein in the tissue sample was computed by first converting the paper weight under the BCS protein peak on the gel to μg of BCS protein using the bacitracin standard curve. This number is then divided by the fraction of the total water-soluble protein in the extract pool layered on the gel.

$$\begin{bmatrix}\text{total BCS}\\ \text{protein in}\\ \text{tissue}\end{bmatrix} = \begin{bmatrix}\text{mg paper}\\ [\text{BCS on gel}]\end{bmatrix} \times \frac{[\mu\text{g of Bacitr.}]}{\begin{bmatrix}\text{mg of paper}\\ [\text{Bacitracin}\\ \text{on gel}]\end{bmatrix}} \times \frac{\begin{bmatrix}\text{total water-sol.}\\ \text{protein extracted}\end{bmatrix}}{\begin{bmatrix}\text{total protein}\\ \text{layered on gel}\end{bmatrix}}$$

Cross-reactivity of the BCS Protein

The test animals which had been previously isolated were injected with crude extract of the PVG from another species, and then closely watched for reproductive behavior and egg laying. Due to differences in the sizes of the animals of the three species, the amount

Figure 5: Standard curve of increasing amounts of bacitracin run on polyacrylamide gel electrophoresis as a function of area under the bacitracin peak on the gel. The gel was scanned by a Joyce Loebl recording microdensitometer and the area under the peak on the densitometer tracing was weighed and expressed as mg of paper.


50
20
10
5
μg BACITRACIN
10
20
30
40
50
mg PAPER UNDER CURVE

of extract differed for each species. Table II indicates the number of ganglia injected into the recipient in each type of experiment. Super-threshold doses were used in all cases.

TABLE II

Dosage Used for Cross-Reactivity of the BCS Protein Experiments

donor / recipient	A.c.	A.v.	A.d.
A.c.	1	1/2 to 1	not done
A.v.	3	1	not done
A.d.	1	1	not done

Species code: A.c. = Aplysia californica

A.v. = Aplysia vaccaria

A.d. = Aplysia dactylomela

RESULTS

Protein and Water Content of Tissue

As a foundation for a detailed chemical analysis of the protein content of Aplysia neurons, some basic parameters of the ganglia, nerve trunks, and selected non-neural tissues were necessary (Table III). For convenience in calculations and in order to directly compare tissues, dry weights of the tissues were determined. The ganglia consisted of about 17 to 25% dry tissue while the nerve trunks had a higher water content and contained only 12 to 20% dry tissue. When expressed as mg protein per mg dry tissue times 100%, the total NaOH-extracted protein of all ganglia examined was 42-45%. The nerve trunks differ significantly; the third pedal nerves contain (45±3)% protein and the pleuro-visceral connective nerves contain (57±2)%. Four different solvents were used to extract the tissues. About 5 to 10% of the dry weight of the tissues consisted of protein solubilized by low ionic strength phosphate buffer, sucrose-Tris-$MgCl_2$ buffer, or sea water. Three times that amount was extracted by 8 M urea.

Neural Specific Proteins

A general survey of polyacrylamide gel (pH 8.9) discontinuous electrophoresis patterns of liver, buccal muscle, gonad, blood vessel, albumen gland, pedal, pleural, parieto-visceral ganglia, connective nerves and third pedal nerve, revealed numerous neural-specific protein bands (Toevs and Brackenbury, 1968). The typical banding patterns on

TABLE III

Dry Weight of Tissue and Amount of Protein Extracted by Various Solvents

Tissue	(N) % dry tissue	(N) % protein* soluble in 1 N NaOH	(N) % protein* soluble in phosphate buffer
PVG	(6) 24.8 ± 1.0	(5) 42.3 ± 1.7	(9) 11.0 ± 0.9
Pedal Ganglion	(11) 17.0 ± 1.2	(5) 45.6 ± 1.7	(6) 12.4 ± 1.4
Pleural Ganglion	(4) 19.3 ± 1.2	(2) 43.2 ± 3.0	(1) 11.9
Pedal Nerve**	(8) 19.9 ± 1.2	(4) 44.9 ± 3.0	(5) 12.2 ± 1.9
Connective Nerve**	(8) 11.9 ± 1.2	(4) 57.4 ± 2.2	(10) 6.6 ± 0.9
Buccal Muscle	(10) 16.5 ± 1.2	(6) 78.8 ± 2.8	(8) 12.7 ± 1.7
Liver	(5) 37.4 ± 1.4	(8) 36.4 ± 2.9	(5) 17.6 ± 2.4

(N) Number of separate determinations. Each determination was carried out on a tissue pool consisting of at least two ganglia or nerve trunks and may be on as many as ten ganglia or nerve trunks.

* mg protein/mg dry tissue x 100% extracted by various methods ± 1 standard deviation. Although the number of determinations is insufficient to be certain that the distribution is Gaussian, standard deviations are shown to indicate the spread of the data.

** Entire length of nerve used.

TABLE III (continued)

Tissue	(N) % protein* soluble in sucrose, Tris $MgCl_2$	(N) % protein* soluble in sea water	(N) % protein* soluble in 8 M urea
PVG	(3) 8.8	not done	not done
Pedal Ganglion	(3) 5.7	(6) 10.9 ± 1.2	(4) 18.3 ± 0.8
Pleural Ganglion	not done	(3) 5.0 ± 1.0	(5) 13.1 ± 2.6
Pedal Nerve**	(3) 7.8	(4) 13.9 ± 1.8	(3) 29.8 ± 2.9
Connective Nerve**	(3) 4.0	(4) 10.1 ± 0.5	(4) 22.2 ± 3.6
Buccal Muscle	(3) 8.9	not done	not done
Liver	(3) 9.3	not done	not done

(N) Number of separate determinations. Each determination was carried out on a tissue pool consisting of at least two ganglia or nerve trunks and may be on as many as ten ganglia or nerve trunks.

* mg protein/mg dry tissue x 100% extracted by various methods ± 1 standard deviation. Although the number of determinations is insufficient to be certain that the distribution is Gaussian, standard deviations are shown to indicate the spread of the data.

** Entire length of nerve used.

7.5% gel (pH 8.9) of several tissues are illustrated in Figure 6. In addition to being nerve specific, some of these proteins are unique to certain kind of ganglia and nerve trunks. The pedal ganglia, which are primarily motor in function, and the third pedal nerve which innervates the muscles of the parapodia and foot, contain at least two such protein bands. One protein band is found only in the ganglia tested and not in the nerve trunks.

Two other protein bands are found uniquely in the bag cells of the parieto-visceral ganglion (PVG). One of these proteins is present only in bag cells and is not detectable in the surrounding connective tissue suggesting that it is present in the bag cell somas but not in their axons. This protein is present in low concentration and thus is only detected when the gels are heavily loaded with extract. It exhibits a characteristic staining appearance with Amido Schwarz and will be referred to as the turquoise band (Figure 7). The other bag cell-specific protein band also travels rapidly toward the anode in polyacrylamide gels, but it appears as a broad, grey-blue band when stained with 1% Amido Schwarz (Figure 8).

Active Egg-Laying Agent

In addition to the two bag cell-specific proteins, this same extract contains an active agent which, when injected into a mature Aplysia, induces a typical reproductive behavior response. It was observed that the animal usually climbs the vertical wall of the tank within several minutes after the injection, remains quietly in this

Figure 6: Polyacrylamide gel profiles of various neural and non-neural tissue extracts on 7.5% gel at pH 8.9. Note the bag cell-specific protein band at arrow on gel 7. Tissues from left to right are: 1, liver; 2, buccal muscle; 3, gonad; 4, blood vessels removed from the PVG; 5, pedal ganglion; 6, pedal nerve; 7, PVG.
(From Figure 1 of Toevs and Brackenbury, 1969.)

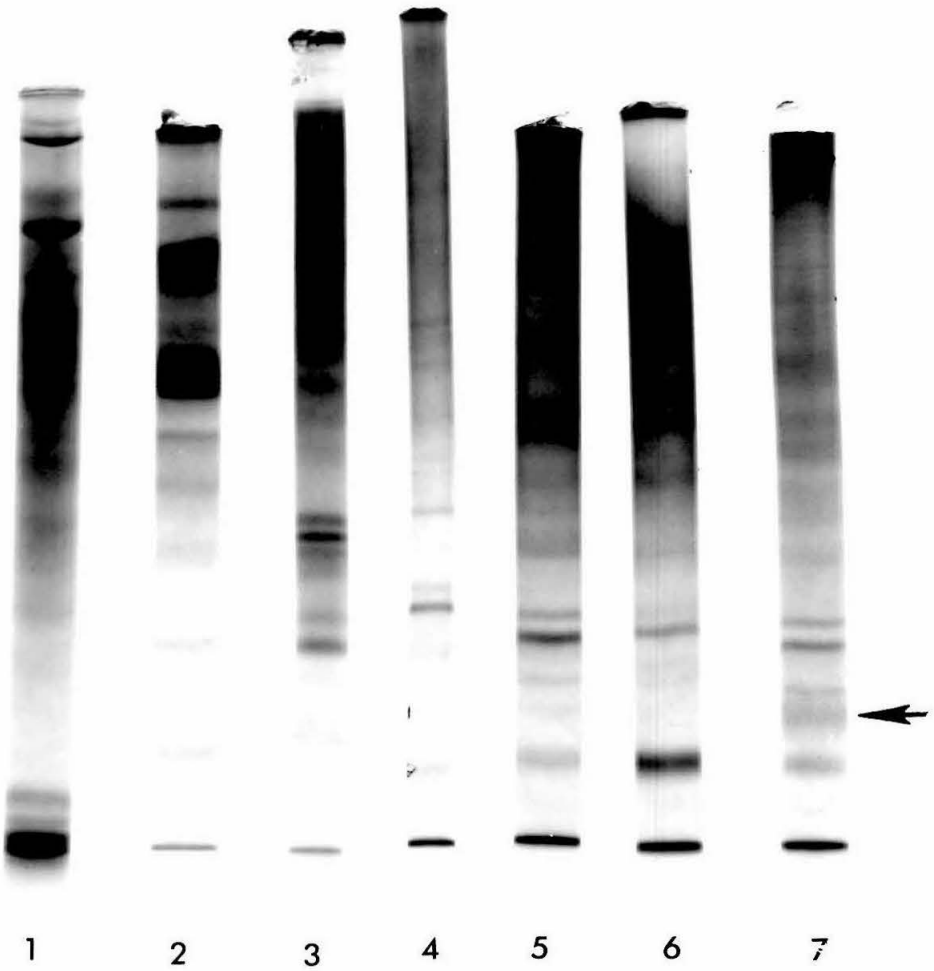
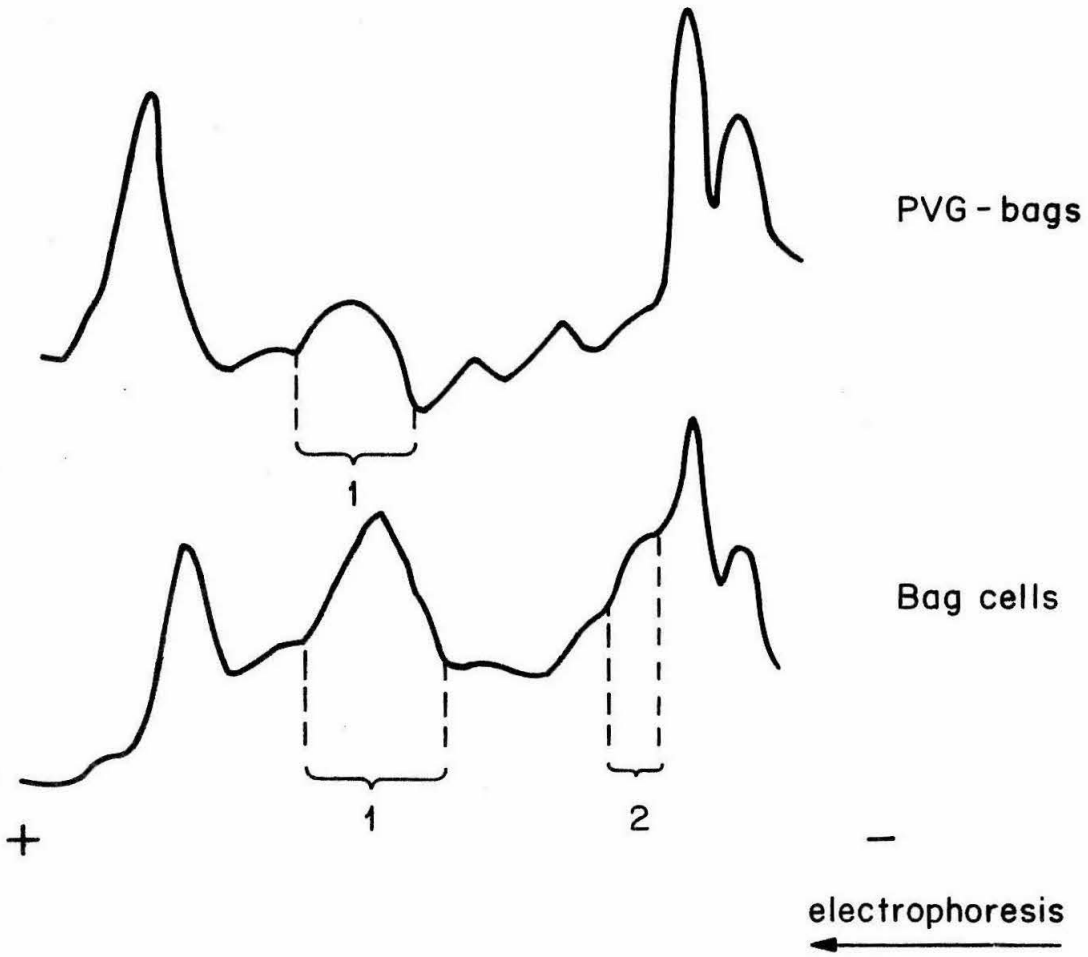

1
2
3
4
5
6
7

Figure 7: Microdensitometer tracings of polyacrylamide gels showing electrophoretic patterns of proteins in the isolated (A) PVG-bag cells and (B) isolated bag cells. One of the bag cell-specific proteins (the BCS protein) is noted at region #1. The bag cell extract also contains a protein which stains turquoise with 1% Amido Schwarz, in contrast to the other proteins which stain dark blue-black. The tracing of this turquoise band is indicated by region #2. This protein is absent in the PVG-bag cell extract suggesting that it is probably localized in the bag cell somas.

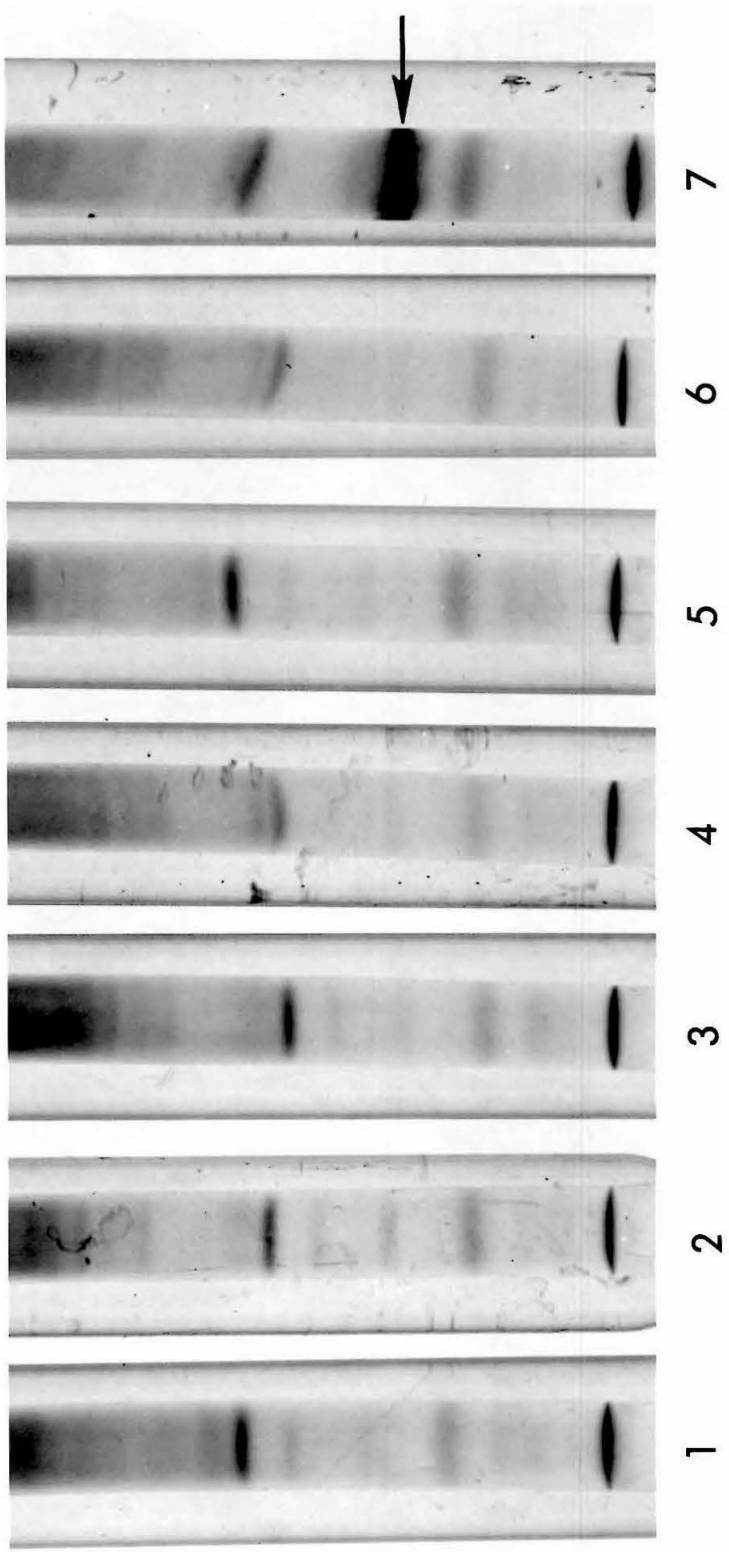
TURQUOISE PROTEIN BAND IN THE BAG CELLS



1 BCS - protein band

2 Turquoise band

Figure 8: Polyacrylamide gel profiles of the PVG and its major nerve trunks. The BCS protein band is clearly seen at the arrow in gel 7, while it is only faintly visible in the PVG-bag cells (gel 1), and the connective nerve (gel 2). It is not detectable in any of the other major nerves of the PVG, suggesting that the bag cell axons do not exit through any of these nerve trunks, but rather terminate in the PVG. Tissues from left to right are: 1, PVG-bag cells; 2, Connective nerve; 3, Branchial nerve; 4, Genital nerve; 5, Anal nerve; 6, Pericardial nerve; and 7, bag cells.


7
6
5
4
3
2
1

position for 45 to 60 minutes and then commences to move its head in a weaving fashion and lay the eggs in a tightly woven mound (Figure 9). The fertilized eggs, which are spontaneously layed or which are induced to be layed by a PVG extract, develop at the same rate and in the same manner (see Appendix II for stages of normal Aplysia development up to free-swimming larval stage).

The remainder of the results section describes morphological localization, maturational effect, seasonal variation and chemical characterization of this grey-blue BCS protein band and the active egg-laying agent.

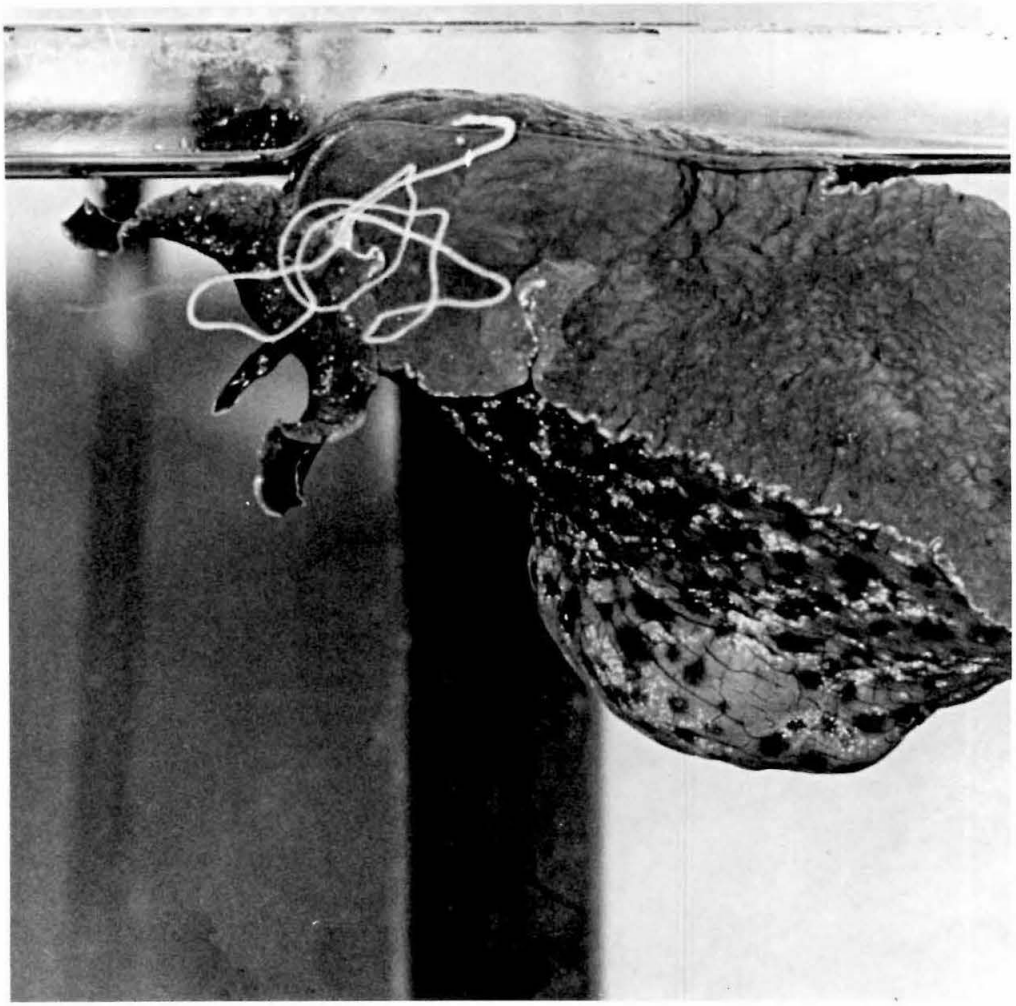
Morphological Localization of the BCS Protein and Egg-Laying Agent

A characteristic rapidly moving protein band was detected in the PVG extract. This is absent from the following non-neural tissue extracts: liver, buccal muscle, albumen gland, gonad and connective tissue from the circumesophageal ganglia. It was also absent from the following neural tissue extracts: buccal, pleural, pedal, cerebral and genital ganglia; pericardial, genital, branchial and anal (siphon) nerves, third pedal nerve and the anterior two-thirds of the pleuro-visceral connective nerves (Toevs and Brackenbury, 1968). The total protein placed on each of the gels was approximately 200 μg (Figures 6, 8).

By microdissection, it was possible to remove the connective tissue sheath from the connective nerves and PVG. It is technically difficult to remove the connective tissue sheath from the bag cells without rupturing the neurons and thus contaminating the tissue samples.

Figure 9: Egg laying induced by an extract of two PVGs. The sea hare is on the vertical glass wall of an aquarium and was photographed 60 minutes after the injection. Egg laying had started 10 minutes prior to the photograph.
(From Figure 1C of Strumwasser *et al.*, 1969.)

C



The bulk of the bag cell-specific protein is present in the bag cell somas. It is present in successively lower amounts in the connective tissue sheath of the PVG, the connective tissue sheath of the posterior 5 mm of the connective nerves and the desheathed 5 mm of the connective nerves. The cells and neuropile of the PVG do not contain any detectable quantity of this protein (Figure 10). The bag cell-specific protein (hereafter referred to as BCS protein) could be detected on gels layered with as little as 15 μg of total protein. To ensure that the variance in amount was not a result of inability to extract significant quantities of bound BCS protein, the tissue was homogenized in urea, which liberates membrane-bound and water-insoluble proteins. The apparent distribution of BCS protein was not altered by this procedure.

Egg-laying activity was localized in the isolated bag cells and in the connective tissue sheath of the PVG. Egg-laying activity was, in general, not found in the neurons and neuropile of the PVG. The posterior 10 mm of the connective nerves also contain the active agent but an extract of the anterior portion of these nerves did not induce egg laying (Table IV).

Effect of Animal Maturation on the BCS Protein Content

The effect of maturation on the presence and total amount of the BCS protein was studied by examining sexually immature, young and mature animals. Sexual maturity was based on the following identifying features: muscularized genital tract, large differentiated albumen

Figure 10: A diagram of the PVG showing the five tissue areas which were isolated by microdissection and assayed for both the BCS protein and the active egg-laying agent. The densitometer tracings, taken from the lower third of typical gels, show the total amount of the BCS protein in the different tissues. This protein is marked by an arrow under the appropriate peak on the tracings. On the basis of these tracings, it is suggested that the BCS protein is synthesized in the bag cell somas and transported down their axons (see text).

*It has been suggested (Strumwasser et al., 1969) that the "Pericardial Nerve" is a misnomer since this nerve does not innervate the heart or surrounding tissue but rather terminates in a secretory organ situated at the base of the kidney. (From Figure 2 of Toevs and Brackenbury, 1969.)

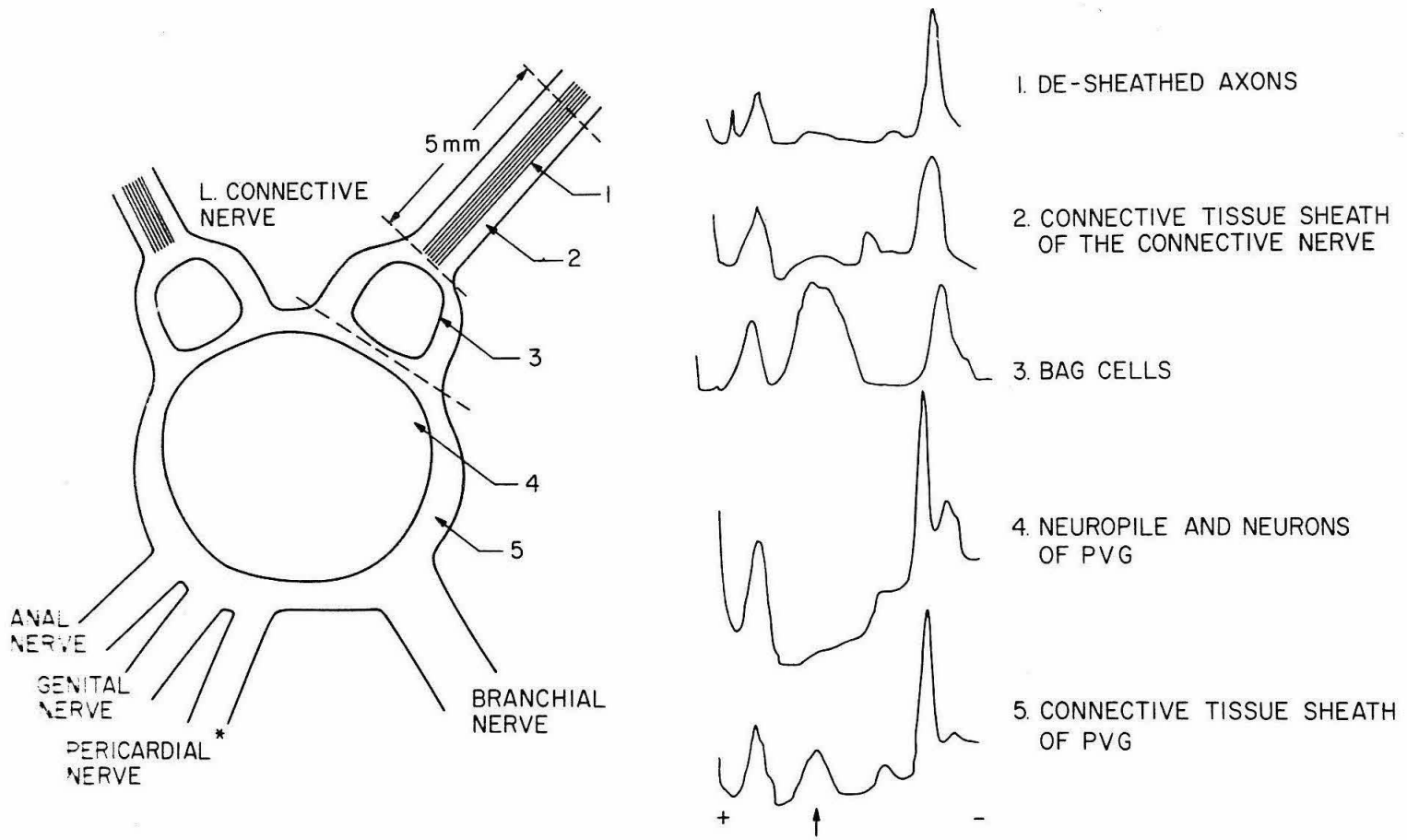

5 mm
L. CONNECTIVE NERVE
1
2
3
4
5
ANAL NERVE
GENITAL NERVE
PERICARDIAL NERVE
*
BRANCHIAL NERVE
1. DE-SHEATHED AXONS
2. CONNECTIVE TISSUE SHEATH OF THE CONNECTIVE NERVE
3. BAG CELLS
4. NEUROPILE AND NEURONS OF PVG
5. CONNECTIVE TISSUE SHEATH OF PVG
+
-

TABLE IV

Localization of the Active Egg-Laying Hormone

Recipient	PVG neurons and neuropile	PVG connective tissue sheath	Bag cells	Lower connective nerves	Upper connective nerves
1	+*	+	x	x	x
2	0	+	x	x	x
3	0	+	+	+	0
4	0	+	+	+	0
5	+	0‡	+	+	0
6	0	+	+	+	0

*In this experiment, the connective tissue sheath around the PVG was slit open and the cells were squeezed out. We now feel that this method of separation liberates active egg-laying substance, and thus contaminates the PVG cell extract. In all experiments following this one, the cells were gently washed out of the sheath in an attempt to minimize such contamination.

‡Some animals which had been in captivity for a month or longer and had previously layed eggs four or five times could no longer be induced to lay eggs.

+ = animal layed eggs within 2 hours after injection; 0 = no eggs layed; x = experiment not performed.

From Table III (Toevs and Brackenbury, 1969).

gland, large-sized and dark-colored gonad, sperm in the little hermaphrodite duct and spermatocyst, and large brown spermatheca [according to Eales (1921) the spermatheca contains the refuse from previous matings]. The average tract weight of eighty-five mature Aplysia was 0.941 g, S.D. = 0.643 g. Animals classified as sexually immature possessed a small, light-colored gonad, undeveloped genital tract and albumen gland, a hermaphrodite duct containing no sperm, and a small clear spermatheca, indicating that the animal had not been previously mated. Of the fifty immature Aplysia analyzed, the genital tract weight average was 0.079 $\pm$ 0.045 g. Twenty-eight animals which fell between these two categories were classified "young." These animals represented a continuum in sexual development between the two extremes of immaturity and maturity. Although the gonads of these animals were small and light-colored, the little hermaphrodite duct was filled with sperm, indicating that they were capable of producing gametes. In these animals the spermatheca was brown, and the spermatocyst, which stores sperm introduced during copulation, was opaque white, indicating that they had been mated. The average tract weight was 0.243 $\pm$ 0.105 g. There was some overlap in genital tract weights among the three categories because there was great variation in animal size.

The BCS protein is present in increasing amounts in the bag cell somas of sexually immature, young and mature Aplysia. The number of bag cells increases approximately threefold (Coggeshall, 1967) during maturation, while the total amount of the BCS protein increases

ninefold (Table V). The amount of this protein present in the connective tissue sheath of the PVG and the posterior 5 mm of the connective nerve also increases with age (Figure 11). The material first appears in the connective tissue sheath of the PVG and then in the posterior portion of the connective nerve.

Chemical Characterization of BCS Protein and Active Agent

Preliminary examination of the BCS protein

The bag cell-specific band binds two well-known protein-specific stains, Amido Schwarz and Coomassie Brilliant Blue (Fazekas de St. Groth et al., 1963; Chrambach et al., 1967). However the protein band does not stain with periodic acid-Schiff reagent, indicating that it is not a glycoprotein. In extracts treated by procedures known to remove intact ribosomes and mitochondria, the band was still present with unaltered electrophoretic mobility. Mobility and staining appearance were also unchanged after treatment with RNase and DNase. Three possible invertebrate neurotransmitter agents, acetyl-choline, 5-hydroxytryptamine, and γ-amino butyric acid, were examined by acrylamide gel electrophoresis. None is similar in electrophoretic properties to the bag cell-specific substance. All the transmitter agents are presumably at the salt front with the bromphenol blue marker dye when run on polyacrylamide gel electrophoresis.

TABLE V

Effect of Sexual Maturation on the Total Amount of the BCS Protein

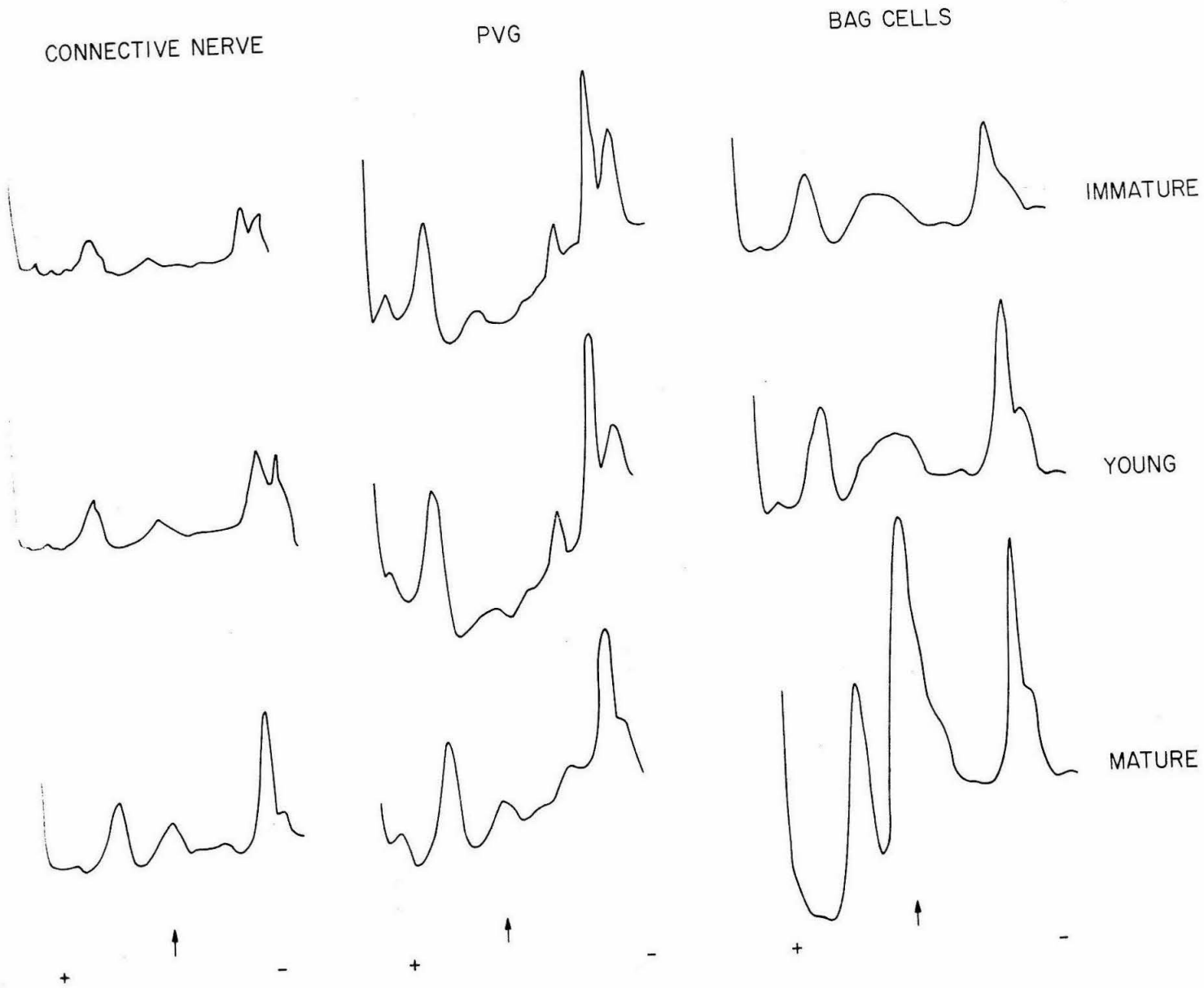
	No. of animals	Tract wt. range (g)	Total amount of BCS protein (in arbitrary units)		
			Posterior portion of connective nerves	PVG minus bag cells	Bag cells
Immature	5	0.056-0.099	5	9	38
Young	5	0.142-0.302	10	10	55
Mature	4	0.533-1.330	15*	13*	314*

*These values have been normalized to five animals for direct comparison.

For each of the tissues, extracts from the animals were pooled and run on a single acrylamide gel. The tract weights listed are for the animals used in this experiment only.

From Table II (Toevs and Brackenbury, 1969).

Figure 11: Densitometer tracings of the lower third of typical polyacrylamide gels. The total amount of bag cell-specific protein (labeled with an arrow) in each of the three tissues increases with the sexual maturation of the animal. The quantitative data are presented in Table V. (From Figure 3 of Toevs and Brackenbury, 1969.)


CONNECTIVE NERVE
PVG
BAG CELLS
IMMATURE
YOUNG
MATURE
+
-
+
-
+
-

Molecular homogeneity of the BCS protein band

The molecular homogeneity of the BCS protein band from crude extract was investigated. A 3-4 mm section of the gel containing the BCS protein band was removed from the 7.5% gel before staining, cut into small pieces, layered on top of a 15% gel and re-run at the same pH and current. After staining, this gel revealed four bands: one dark, broad band and three faint narrow ones. When the same procedure was followed with a control connective nerve (anterior 20 mm), five bands were revealed, three of which matched the faint bands, but none of which matched the dark, dense band of the bag cell extract. This homogeneous dark band is the material called the BCS protein.

Enzymatic degradation of the BCS protein and active agent

The crude extract of the intact PVG was subjected to enzymatic digestion with RNase, DNase, pronase, and chymotrypsin-free trypsin to determine whether the active egg-laying agent and the bag cell-specific (BCS) substance are both proteins or nucleoproteins. Mobility and staining appearance were unchanged after treatment with RNase or DNase.

Pronase digestion. Many different incubation times and pronase concentrations were used to determine the sensitivity of the substances to pronase (Table VI). In all cases, both the BCS protein band and the egg-laying activity were destroyed. None of the reproductive behavior was induced; the animal did not climb the vertical wall, nor did it sit still for any length of time anywhere in the tank. (The animal was checked every ten minutes.) It should be noted

TABLE VI

Pronase Degradation of BCS Protein and Active Egg-Laying Agent

Dates of experiments	Pronase concentration	Time of incubation at 37°C	BCS-protein band	Egg-laying	Ability to lay eggs	(N)
7-26-68 9-26-68	0.9 mg/ml	15 min	-	-	+	2
		30 min	-	-	+	1
		60 min	-	-	+	1
11-2-68	0.05 mg/ml	15 min	-	-	+	1
		30 min	-	-	+	1
		60 min	-	-	+	1
11-14-68 11-21-68	0.025 mg/ml	2 min	-	-	+	1
		5 min	-	-	+	2
		8 min	-	-	+	1
		15 min	-	-	+	1
11-21-68	0.005 mg/ml	5 min	-	-	+	2
11-21-68	0.0025 mg/ml	5 min	-	-	+	2
all dates	no pronase, control	0	+	+	+	6
7-26-68 9-26-68 11-2-68	5 µg pronase, control *single injection	0		-	+	3
11-8-68 11-14-68 11-21-68	5 µg pronase, control **dual injection	0		+	+	3

TABLE VI (continued)

N = number of determinations.

*Control 0°C extract and pronase combined in 1 ml filtered sea water immediately prior to injection. 5 µg pronase injected was equal to 0.005 mg/ml pronase concentration.

**5 µg of pronase in 1 ml filtered sea water injection followed in 4 minutes by 0°C control extract injection. 5 µg pronase that was injected is equal to 0.005 mg/ml pronase concentration.

that the lowest concentration of pronase used, 2.5×10^{-3} mg/ml, actually corresponded to 0.17 μg pronase in a reaction mixture containing approximately 200 μg of total protein. The BCS-protein constituted about 3-5% of the total protein at the time of the year when this experiment was performed.

In these experiments, it was essential to monitor the effect of the pronase itself on the reproductive behavior of the test animal. It was necessary to first inject the animal with 5 μg of pronase followed in 4 minutes by a second injection containing the 0°C control extract. If the control extract and the pronase were combined immediately prior to the injection, the animal never layed eggs. It was concluded that active egg-laying agent was very rapidly destroyed when exposed to pronase even when diluted with 1 ml sea water at 0°C.

Trypsin. The extract was also treated with chymotrypsin-free trypsin which is more specific than pronase in its mode of protein degradation. Again various concentrations of trypsin and times of incubation at 25°C were used to ascertain the trypsin-sensitivity of the BCS-protein and the active agent in the extract (Table VII). It is seen that at lower trypsin concentrations the activity of the egg-laying agent is lost but the BCS-band is still present on the gels. At 1% trypsin, both the BCS-band and all egg-laying behavior is destroyed. However, at 0.1% and 0.01% trypsin, although the animal does not lay eggs, it does climb immediately onto a vertical wall and remain silent in this position for about one hour (N = 5).

TABLE VII

Trypsin Degradation of BCS Protein and Active Egg-Laying Agent

Dates of experiments	Trypsin concentration*	Time of incubation at 25°C	BCS-protein band	Egg-laying	Ability to lay eggs	N**
12-5-68, 12-18-68, 7-25-69	1%	5 min	-	-	+	3
		15 min	-	-	+	2
		60 min	-	-	+	1
7-25-69	0.1%	5 min	+	-	+	1
12-18-68, 7-25-69	0.01%	5 min	+	-	+	2
		15 min	+	-	+	1
		30 min	+	-	+	1
12-5-68, 12-18-68, 7-25-69	control‡ dual injection	0		+	+	3

*Trypsin concentration expressed as per cent of total weight of protein in reaction mixture.

**N = number of determinations.

‡Animal injected first with 2 μg trypsin in 1 ml sea water, followed in 4 minutes by a second injection containing 0°C control extract in 1 ml sea water.

It is concluded from the pronase and trypsin degradation studies, that both the BCS-substance and the active egg-laying agent are proteins sensitive to enzymatic degradation.

Purification

The BCS-protein was isolated, from all other water-soluble proteins extracted from the PVG, by the method of gel filtration which separates substances solely by their size and not by charge. This one step purification procedure (Figure 12) yielded a 1 to 1.2 ml fraction which contained only one protein and this was shown to be the BCS protein on 7.5% and 15% polyacrylamide gels (Figure 13). It was also determined that this fraction and only this fraction contained the active egg-laying agent which had been previously shown to be a protein.

Molecular weight determination

This same column was calibrated with four molecular weight markers so that the molecular weight of unknown substances could be determined by the relationship

$$\frac{V_{elution}}{V_{void}} = k \cdot \log MW \qquad \text{(Whitaker, 1963)}$$

Although the elution volumes for the proteins varied somewhat from experiment to experiment, the ratio Ve/Vo was very constant (Table VIII) and gave a good linear plot (Figure 14).

Figure 12: Chromatographic separation of the BCS protein and active egg-laying agent by gel filtration. A 1 ml sample of crude PVG extract was applied to a 1.5 x 60 cm column containing G 50 fine-Sephadex equilibrated with 0.05 M sodium phosphate buffer pH 7.0. The protein was eluted at 18°C, at a flow rate of 2.5 ml per hour, and collected automatically in 0.25 ml fraction. Aliquots of each fraction were assayed for the BCS protein and the active egg-laying agent. Both were localized in the same fractions which had a total volume of 1.2 ml. The molecular weight of this fraction centered around 6000, as determined by calibrating the same column with four molecular weight markers.

PURIFICATION AND MOLECULAR WEIGHT DETERMINATION

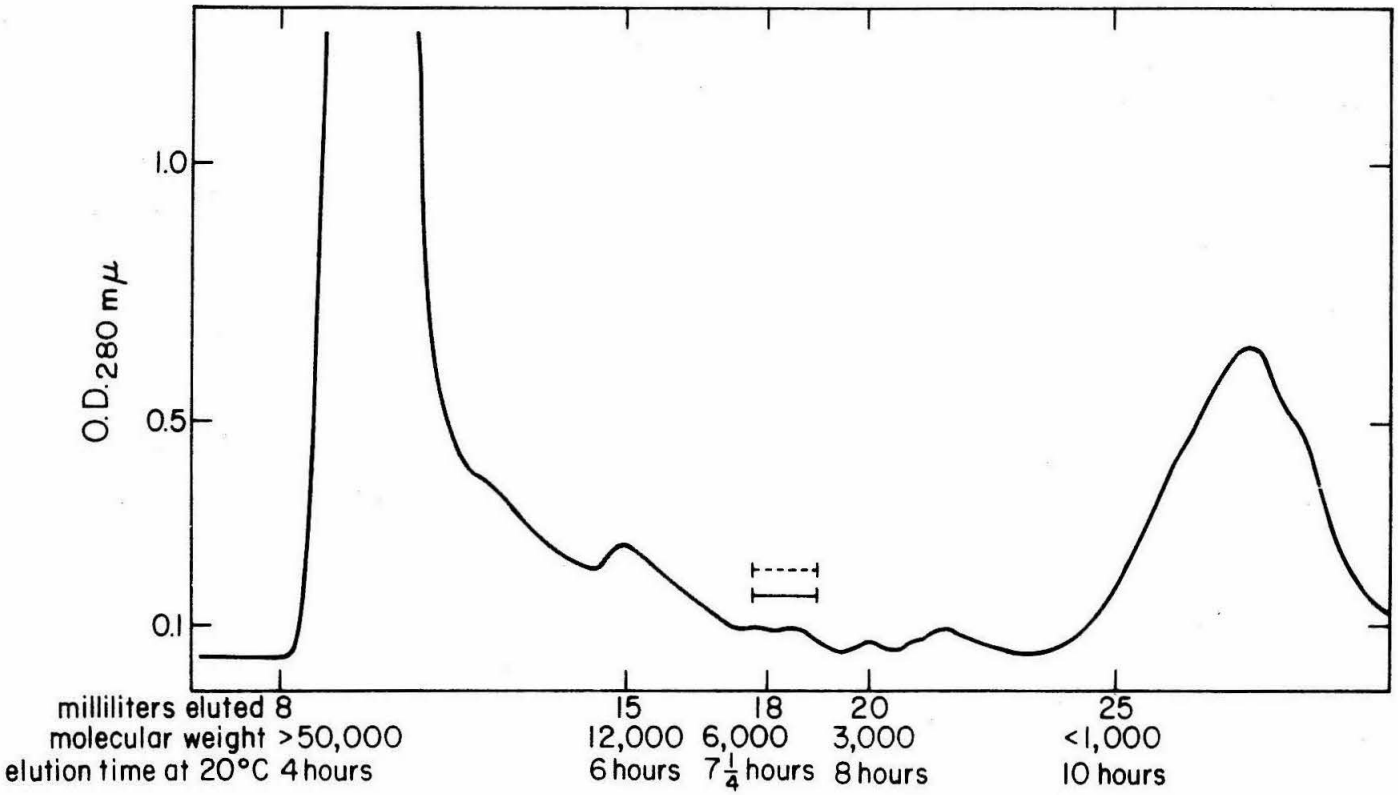


Figure 13: A. Photograph of 7.5% polyacrylamide gel containing purified BCS protein from a G 50 fine-Sephadex column. This fraction and only this fraction contained the active egg-laying hormone which was also shown to be a protein.

B. Microdensitometer tracing of this gel, showing that only one protein band is present on it. This type of experiment was used to show that the BCS protein and the active egg-laying hormone are identical and have a molecular weight of 6000.

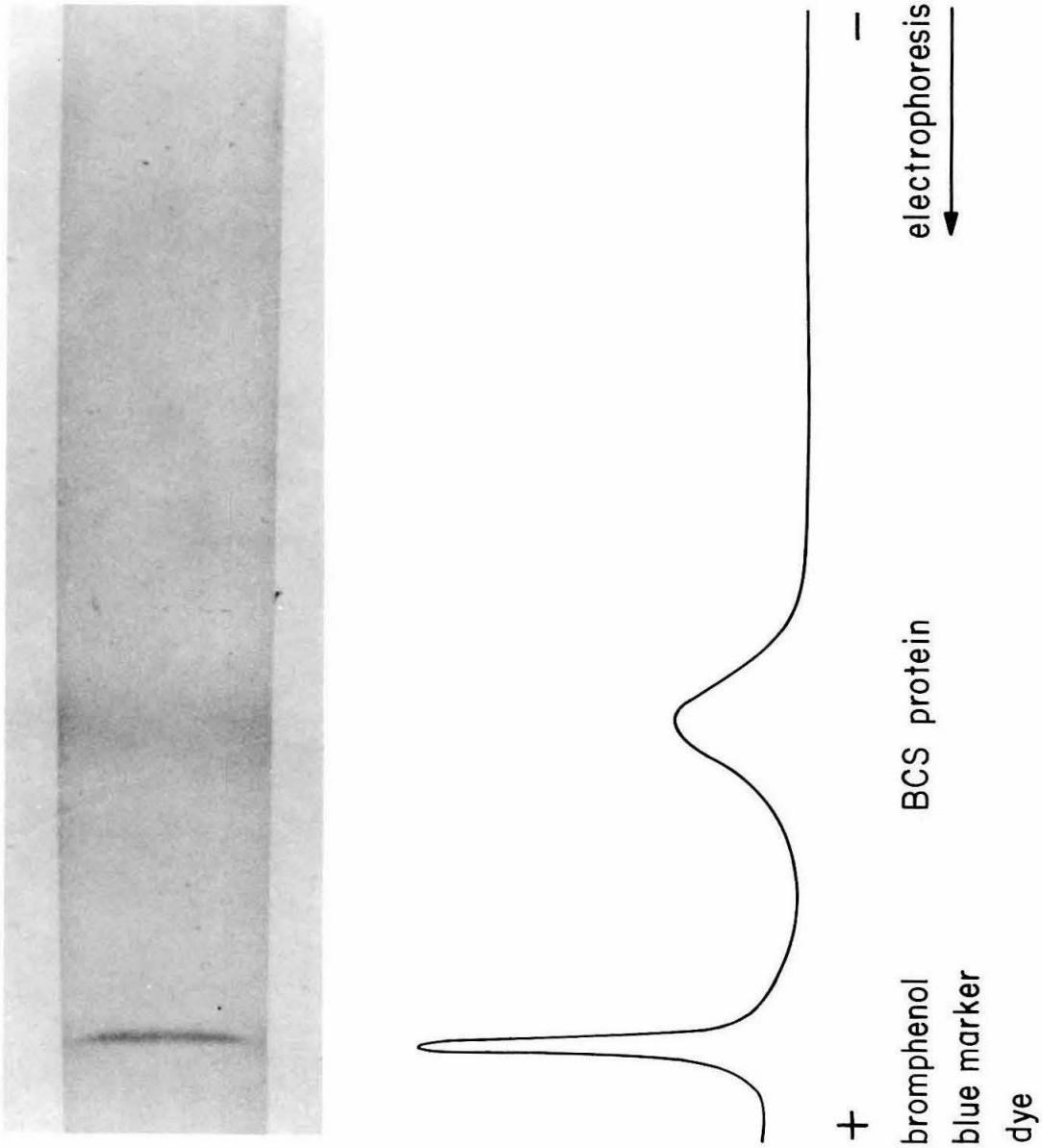

+
−
bromphenol blue marker dye
BCS protein
electrophoresis

TABLE VIII

Relationship of Elution Volume to Molecular Weight of Substance

µl applied to column	Ve (ml)				Ve/Vo			
	67 K	25 K	12.4 K	1.4 K	67 K	25 K	12.4 K	1.4 K
800	8.5	11.6	14.3	22.5	1	1.37	1.68	2.65
1000	8.4	11.45	14.0	22.2	1	1.36	1.67	2.65
200	9.6	13.2	16.2	25.2	1	1.37	1.69	2.62
300	11.2	15.2	19.0	29.5	1	1.36	1.69	2.63
600	8.8	12.1	15.2	24.4	1	1.37	1.72	2.77
500	9.3	12.6	15.8	23.5	1	1.36	1.70	2.53

67,000 = Bovine serum albumin

25,000 = Chymotrypsin

12,400 = Cytochrome C

1,400 = Bacitracin

Figure 14: Molecular weight calibration curve for G 50 fine-Sephadex column. Four pure non-enzymatic proteins were used to establish this curve: bovine serum albumin (67,000), chymotrypsin (25,000), cytochrome C (12,400), and bacitracin (1400). The elution volume of each protein divided by the column void volume is a characteristic of the column. Therefore, approximate molecular weights, of unknown substances separated on this column, can be determined from this plot simply by knowing the elution volume of the unknown material. Four separate column fractionations are illustrated here to indicate the spread of the data from different elutions. See Table VIII for original data.

MOLECULAR WEIGHT
50,000
20,000
10,000
5,000
2,000
1.1
1.5
2.0
2.5
Ve/Vo

Using this method, the molecular weight of the BCS-protein from Aplysia californica was estimated to be well under 12,000 centering about 6000. This value is based on five separate isolations of the BCS protein from G 50 fine-Sephadex. If the elution profile of all five PVG fractionations are superimposed by aligning two optical density peaks, one at about 12,000 molecular weight and the other at 1000 molecular weight, the fractions containing the BCS protein coincide in all cases to within ± 0.2 ml out of a 1.2 ml total volume of the fraction. The PVG extract from Aplysia vaccaria was also fractionated in this manner, and the BCS protein was isolated from all but two other proteins as determined on 15% polyacrylamide gels. The fraction containing the BCS protein also contained the egg-laying activity and was approximately 6000 molecular weight. The investigations of Andrews (1965) into the separation ranges of Sephadex gels has shown that linearity of the relation $Ve/Vo = k \cdot \log MW$ exists only in the middle of the elution curve and that both ends deviate from linearity. Since pure proteins in the molecular weight range of 6000 were either not obtainable or not suitable for calibration purposes, the column was calibrated with cytochrome C, 12,400, and bacitracin, 1,400. Therefore, the molecular weight of the BCS protein cannot be more accurately assigned.

From these data, it was concluded that the BCS protein and the active egg-laying agent are indeed identical and have a molecular weight of around 6000.

Effect of freeze-thawing on purified hormone

An effect from repeated freeze-thawing of the purified BCS protein was observed. The BCS protein-hormone, from Aplysia californica, had been recovered from the G 50 fine-Sephadex column in pH 7.0 phosphate buffer, and the fraction shown to be a single band on 7.5% and 15% polyacrylamide gel and to contain egg-laying activity. The same procedure was carried out with BCS protein from Aplysia vaccaria, however, the purified BCS protein was as usual contaminated with two other proteins. The fractions were lyophilized, resuspended in a small volume of buffer and thawed and refrozen 7 or more times during the course of several experiments. The BCS protein from Aplysia californica then exhibited two bands on 15% polyacrylamide gel. The purified Aplysia vaccaria BCS protein also now exhibited two new bands on 15% gel. The purified protein from both species lost its biological activity after this extensive freeze-thawing (Table IX). It is suggested that the proteins may be aggregating during this procedure.

Thermal stability

The thermal stability of the BCS protein and the active egg-laying agent was examined at 37°C and 55°C. These data are summarized in Table X. Although the BCS protein loses its egg-laying activity within 5 to 10 minutes of 55°C incubation, it is not sufficiently denatured in 15 minutes at this temperature to alter its electrophoretic

TABLE IX

Effect of Repeated Freeze-Thawing on Purified Hormone

	Aplysia californica		Aplysia vaccaria	
	Distance BCS band moved*	Biological activity	Distance BCS band moved*	Biological activity
Initial conditions	62-65 mm	+	91-94 mm	+
After repeated freeze-thawing	42-46 mm 62-65 mm	-	31-33 mm 75-76 mm	-

*Distance traveled by bromphenol blue marker dye equals 100 mm.

The gels, 3.5 mm by 120 mm, were run at a constant current of 2 mA/tube. The distance moved by the BCS protein band is expressed as a range which represents the width of the stained protein band on the gel.

TABLE X

Thermal Stability of BCS Protein and Egg-Laying Agent

Incubation		BCS protein on gel	Eggs layed	Ability to lay eggs	N
Temp.	Time				
37°C	1 hr	+	+	+	1
37°C	2 hr	+	+	+	1
37°C	4 hr	-	-	+	1
55°C	5 min	+	+	+	3
55°C	10 min	+	-	+	3
55°C	15 min	+	-	+	3

N = number of determinations.

mobility. Both BCS protein from crude PVG extract and purified BCS protein were examined at 55°C and gave identical results.

Electrophoretic mobility

The electrophoretic mobilities of the BCS protein from the three species of Aplysia were compared on 7.5% and 15% polyacrylamide gels (Table XI). The 15% gel has smaller pores and thus retards the movement of the molecules giving better separation. The gels are run from cathode to anode at pH 8.9. At this pH, the Aplysia vaccaria BCS protein is most acidic and the Aplysia californica BCS protein is least acidic.

Seasonal Variation in Extractable BCS Protein

A seasonal variation in the amount of extractable BCS protein was observed when the PVG extracts were analyzed on polyacrylamide gels (Figure 15). From January to October the PVG was divided into three tissue samples (Figure 4) which were extracted with buffer and the supernatant analyzed by polyacrylamide gel electrophoresis (Table XII). Values from October to December were obtained by determining, on polyacrylamide gels, the amount of purified BCS protein contained in fractions recovered from a G 50 fine-Sephadex column (Table XIII). The calculation of the quantity of BCS protein on the polyacrylamide gels was the same for both the January to October and the October to December analysis. This determination was based on a comparison of optical density tracings of the test BCS-protein gels and the standard bacitracin gels.

TABLE XI

Electrophoretic Mobility of BCS Protein from Three Species of Aplysia

Species	Distance BCS band moved toward anode**	
	on 7.5% gel	on 15% gel
Aplysia californica*	78 mm	62 mm
Aplysia dactylomela	88 mm	not done
Aplysia vaccaria*	100 mm	93 mm

*Purified BCS protein.

**Distance traveled by bromphenol blue marker dye equals 100 mm.

The gels, 3.5 mm by 120 mm, were run at a constant current of 2 mA/tube. At this current a typical electrophoresis run was completed in 50 to 60 minutes.

Figure 15: The seasonal variation of the BCS protein content of the bag cells, PVG-bag cells, and lower connective nerve. The BCS protein values from January to October were obtained by directly analyzing tissue extracts on polyacrylamide gels and quantitatively determining the amount of BCS protein on the gel. The October through December values were obtained from the BCS protein recovery from the fractionation of crude extract of the intact PVGs. These points include a correction factor of 5 to take into account differences in amount of total protein extracted from the tissue in the two different sized tissue grinders, and the loss of protein on the column. The per cent of material recovered from the column in each case is unknown and probably over-estimated, and, therefore, the October through December figures may be too low. The quantitative analysis of the BCS protein on the gel includes several errors (see text). There is a 16 to 24% error on all values plotted.

SEASONAL VARIATION IN THE BCS-PROTEIN CONTENT

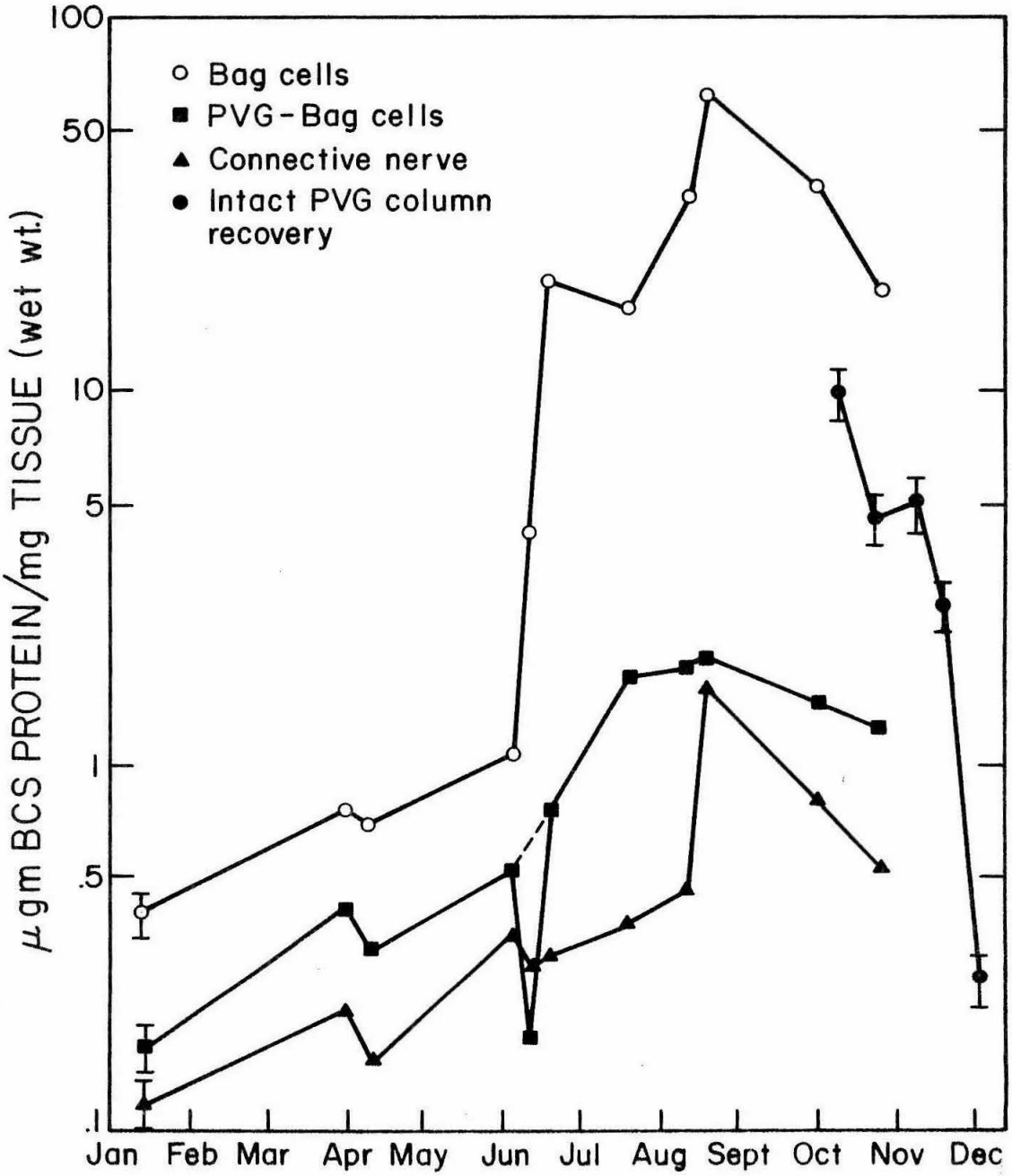


TABLE XII

Seasonal Variation in the BCS Protein Content

	BAG CELLS					
Date	1-12	3-29	4-10	5-14	6-5	6-11
Number and maturity of animals	3M	5M	4M 1Y	5M 1Y	3M	4M
Tissue wt. (mg) Calculated (c) Weighed (w)	12 (c)	17 (w)	17.7 (w)	24 (c)	14.3 (w)	16 (c)
*Total BCS protein in tissue (µg)	4.8	13.1	12.2	32.8	15.2	69.0
µg BCS protein per mg wet tissue	0.4	0.77	0.69	1.36	1.06	4.3
µg BCS protein per ganglion	1.6	2.6	2.4	5.5	5.1	17.2
% BCS protein of total protein	5%	9.6%	7.8%	17.1%	3.8%	17.3%

Animal maturity code: M = mature, Y = young adolescent, I = immature (according to criteria in Toevs and Brackenbury, 1969).

*Bacitracin equivalent µgms of protein. The determination of this value is discussed in text under Error analysis in BCS protein content.

TABLE XII (continued)

	BAG CELLS					
Date	6-17	7-18	8-12	8-19	10-1	10-26
Number and maturity of animals	2M	8M	2M	3M	2M	2M
Tissue wt. (mg) Calculated (c) Weighed (w)	11.6 (w)	65.4 (w)	21.0 (w)	24.0 (w)	15.4 (w)	12.8 (w)
*Total BCS protein in tissue (µg)	>225	1100	690	1360	535	236
µg BCS protein per mg wet tissue	>20	16.8	33.0	56.5	34.8	18.4
µg BCS protein per ganglion	>100-125	138	345	440	267	118
% BCS protein of total protein	23%	55%	not done	not done	not done	not done

Animal maturity code: M = mature, Y = young adolescent, I = immature (according to criteria in Toevs and Brackenbury, 1969).

*Bacitracin equivalent µgms of protein. The determination of this value is discussed in text under Error analysis in BCS protein content.

TABLE XII (continued)

	PVG-BAG CELLS					
Date	1-12	3-29	4-10	6-5	6-11	6-17
Number and maturity of animals	3M	5M	4M 1Y	3M	4M	2M
Tissue wt. (mg) Calculated (c) Weighed (w)	24 (c)	39.8 (w)	37.4 (w)	23.3 (w)	32 (c)	26.4 (w)
*Total BCS protein in tissue (μg)	4.0	16.8	12.9	12.4	5.8	20.3
μg BCS protein per mg wet tissue	0.17	0.42	0.34	0.53	0.18	0.77
μg BCS protein per ganglion	1.3	3.4	2.6	4.1	1.45	10.1
% BCS protein in total protein	2.1%	7.1%	4.3%	2.0%	2.3%	2.0%

Animal maturity code: M = mature, Y = young adolescent, I = immature (according to criteria in Toevs and Brackenbury, 1969).

*Bacitracin equivalent μgms of protein. The determination of this value is discussed in text under Error analysis in BCS protein content.

TABLE XII (continued)

	PVG-BAG CELLS				
Date	7-18	8-12	8-19	10-1	10-26
Number and maturity of animals	8M	2M	3M	2M	2M
Tissue wt. (mg) Calculated (c) Weighed (w)	90.7 (w)	24.8 (w)	26.9 (w)	19.6 (w)	13.6 (w)
*Total BCS protein in tissue (μg)	157	45	52	28	17.1
μg BCS protein per mg wet tissue	1.73	1.81	1.93	1.43	1.26
μg BCS protein per ganglion	19.6	22.5	17.3	14.0	8.5
% BCS protein in total protein	7.5%	8.6%	not done	not done	not done

Animal maturity code: M = mature, Y = young adolescent, I = immature (according to criteria in Toevs and Brackenbury, 1969).

*Bacitracin equivalent μgms of protein. The determination of this value is discussed in text under Error analysis in BCS protein content.

TABLE XII (continued)

	Connective Nerve (posterior 10 mm)					
Date	1-12	3-29	4-10	6-5	6-11	6-17
Number and maturity of animals	3M	5M	4M 1Y	3M	4M	2M
Tissue wt. (mg) Calculated (c) Weighed (w)	30 (c)	17.6 (w)	25.0 (w)	16.6 (w)	20 (c)	13.2 (w)
*Total BCS protein in tissue (µg)	3.7	3.8	4.1	6.0	5.6	4.1
µg BCS protein per mg wet tissue	0.12	0.22	0.16	0.36	0.28	0.31
µg BCS protein per ganglion	1.2	0.76	0.8	2.0	1.4	2.0
% BCS protein in total protein	4.1%	8.2%	5%	2.3%	9.3%	2.4%

Animal maturity code: M = mature, Y = young adolescent, I = immature (according to criteria in Toevs and Brackenbury, 1969).

*Bacitracin equivalent µgms of protein. The determination of this value is discussed in text under Error analysis in BCS protein content.

TABLE XII (continued)

	Connective Nerve (posterior 10 mm)				
Date	7-18	8-12	8-19	10-1	10-26
Number and maturity of animals	8M	2M	3M	2M	2M
Tissue wt. (mg) Calculated (c) Weighed (w)	69.1 (w)	23.0 (w)	30.0 (w)	9.0 (w)	13.1 (w)
*Total BCS protein in tissue (µg)	26.5	10.7	50.0	7.4	7.1
µg BCS protein per mg wet tissue	0.38	0.47	1.66	0.82	0.54
µg BCS protein per ganglion	3.3	5.35	16.6	3.7	3.5
% BCS protein in total protein	6.6%	7.4%	16%	4.1%	3.9%

Animal maturity code: M = mature, Y = young adolescent, I = immature (according to criteria in Toevs and Brackenbury, 1969).

*Bacitracin equivalent µgms of protein. The determination of this value is discussed in text under Error analysis in BCS protein content.

TABLE XIII

Recovery of BCS Protein from G 50 Sephadex Column

Date	10-9	10-22	11-8	11-18	12-3
Number and maturity of animals	12M	12M	13M 2I	7Y 7I	8M 5Y 12I
*Tissue wt. (mg) calculated	120	120	140	94.5	182.5
**Total BCS protein recovered (μg)	240	110	145	51	10
μg BCS protein per mg wet tissue	2.0	0.92	1.03	0.54	0.05
μg BCS protein per ganglion	20	9.2	9.7	6.7	0.4
% BCS protein in total protein	7.3%	3.3%	3.8%	1.9%	0.2%

Animal maturity code: M = mature, Y = young adolescent, I = immature (according to criteria in Toevs and Brackenbury, 1969).

*"Mature" PVG = 10 mg, "Young" PVG = 8.5 mg, "Immature" PVG = 5 mg.

**Bacitracin equivalent μgms of protein. The determination of this value is discussed in text under Error analysis in BCS protein content.

Error analysis of BCS protein content

The total BCS protein content of the tissue was calculated from the following equation:

$$\mu g \text{ BCS protein} = (A \pm \sigma_A) \cdot \frac{(B \pm \sigma_B)}{(C \pm \sigma_C)} \cdot \frac{(D \pm \sigma_D)}{(E \pm \sigma_E)}$$

where A = area under the BCS protein curve on gel.

errors in A: gel tracing 5%

curve tracing 10%

B = μg bacitracin layered on gel

error in B: Lowry determination 5%

C = mg paper under bacitracin peak on gel

error in C:	3-7 μg	10-30 μg	>30 μg
gel tracing	10%	2%	4%
curve tracing	15%	2%	5%

D = total protein in tissue

error in D: Lowry error 5%

loss of extract during homogenization of tissue and transfer of extract 5%

E = protein layered on gel

error in E: Lowry error 5%

loss of extract applied to gel 5%

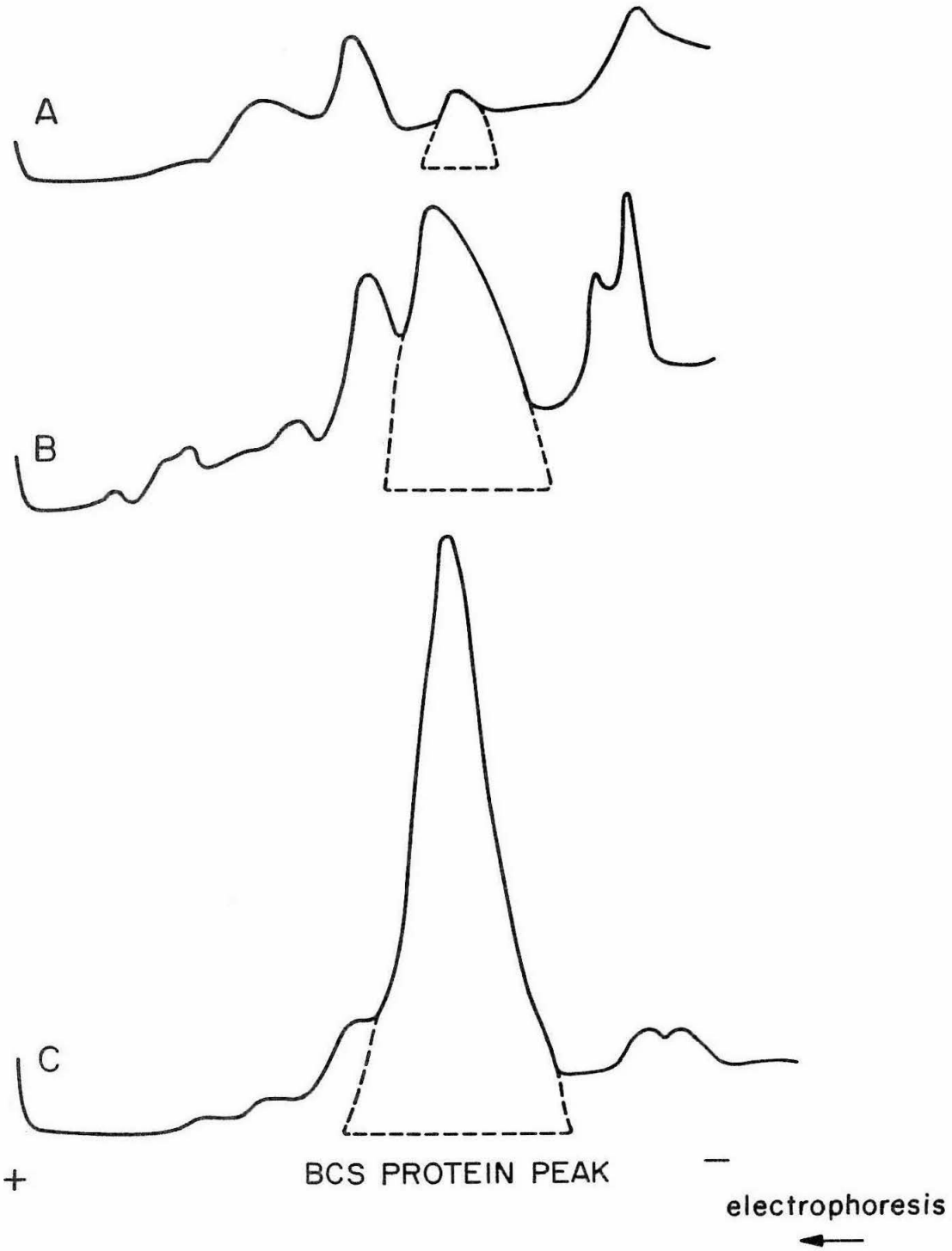
Relative errors were calculated from the formula

$$\frac{\sigma}{\bar{X}} = \frac{\left(\frac{\Sigma(\Delta x_i)^2}{n(n-1)}\right)^{1/2}}{\bar{X}}$$

Perhaps the largest error is in taking the area under the peak of either the bacitracin or BCS protein. At very low values (3-7 μg protein), it is difficult to distinguish the peak and the background in tracing the curve; in addition, there is much scatter in the individual tracings. In the range of 10-30 μg of bacitracin or BCS protein, the error in measuring the area under the curve is about 16%. At values above 30 μg of protein, the protein-dye complex saturates and one can then only put a lower limit on the amount of protein present in the band.

When determining the area under the BCS protein peak, other difficulties are encountered, the background color of the gel changes along its length. The cathode end of the gel appears blue and becomes continuously lighter until the gel is clear near the marker dye at the anode end of the gel. In all cases, the baseline of the BCS protein peak was chosen to be the background level of the anode end of the gel. Another error is incomplete resolution of the BCS protein band with one other protein band. One must therefore sketch by hand the boundaries and baseline of the BCS protein. This was done in a consistent manner in each of the tracings for each gel (Figure 16). The total error in determining the total BCS protein in the tissue varies from 16% to 24%

Figure 16: Densitometer tracings from A. lower connective nerve, B. PVG-bag cells, and C. bag cells to show the manner in which the boundaries and baseline of the BCS protein peak were chosen for quantitative analysis of the area under the peak. The area under the peak was traced onto K & E tracing paper, cut out, and weighed. See text for a discussion of the difficulties and error analysis of these measurements.


A
B
C
+
BCS PROTEIN PEAK
−
electrophoresis

using the following equation:

$$\frac{\sigma_x}{\bar{X}} = \sqrt{\Sigma\left(\frac{\sigma_i}{x_i}\right)^2}.$$

When analyzing the fractions from the Sephadex column, the BCS protein was the only protein found on the gels. Therefore, there was no difficulty determining the baseline or resolving it from other protein peaks. However, since there were always small amounts of BCS protein on each gel, there were large errors in reproducibility of the area under the curve for the several traces done on each gel. The error analysis is the same as for the January to October data and therefore the total error in determining the μgm BCS protein/mg wet weight is 22%.

Comparison of values from the two methods of analysis

It is difficult to quantitatively compare the results of the two methods--column recovery of the BCS protein vs. direct gel analysis of the three tissue samples of the PVG. Approximately three times more water-soluble protein was extracted from the tissue when the PVG was cut into three tissue samples and homogenized in miniature grinders (January through October data) than when a large number of intact PVG were ground in large homogenizers (October through December data). The per cent recovered from the column is unknown. Therefore, taking into account the extraction efficiency and the column efficiency, the values of BCS protein recovered from the column were arbitrarily increased by

a factor of five. This was done so that the value of μgm BCS protein/mg wet tissue fit the curve of January to October data. The difference in the BCS protein content between October (10 μgm BCS protein/mg wet tissue) and December (0.25 μgm BCS protein/mg wet tissue) is significant as can be seen from the error bars on Figure 15.

Effect of prolonged freezing on tissue

Two sets of intact ganglia from mature Aplysia californica were isolated in mid-February (1969) and early March (1969) and frozen at -20°C until mid-May (1969) at which time they were thawed and dissected into the three tissue samples and extracted with cold buffer. The extracts were analyzed for the amount of BCS protein on polyacrylamide gels as usual. Considerably more BCS protein was extracted from the three month frozen tissue of February and early March than was expected as compared to an interpolation of the BCS protein levels of January and late March when the tissues were extracted immediately after dissection (Table XIV). It is suggested that this discrepancy was caused by the long three month storage of the tissue at -20°C.

Cross-Reactivity of the BCS Protein

Three species of Aplysia were used to test the interspecies cross-reactivity of the hormone action of the BCS protein. Aplysia californica and Aplysia vaccaria, from the cool California coastal waters, were used both as donors and as recipients in the egg-laying experiments. Aplysia dactylomela, from the warm Florida waters, was used only as a recipient in these experiments. As tested, there was

TABLE XIV

Effect of Prolonged Freezing of Tissue on BCS Protein Content

Tissue	μg BCS protein extracted/mg tissue									
	Jan. 12*		Feb. 20**			March 6**			March 29*	
	(N)	(measured)	(N)	(measured)	(expected)	(N)	(measured)	(expected)	(N)	(measured)
C. N.	(3)	0.12	(3)	0.35	0.16	(2)	0.69	0.18	(5)	0.22
PVG-Bags	(3)	0.17	(3)	0.34	0.26	(2)	0.38	0.31	(5)	0.42
Bag cells	(3)	0.40	(3)	8.0	0.55	(2)	1.8	0.62	(5)	0.77

*Tissue extracted immediately after dissection.

**Tissue extracted after being frozen for three months.

N = one determination of N ganglia. All ganglia are from sexually mature animals.

complete reciprocity of action of the injected BCS-protein among the three species (Table XV). All three species exhibited the same reproductive behavior within the same time periods. The test animal usually climbed a vertical wall of the tank within 3 to 10 minutes after injection, then remained quietly in this position for 45 to 60 minutes after which time she commenced egg laying. Egg laying itself could continue for several hours.

Dose-Response Relationship

An estimate of the effective dose of BCS hormone which would cause egg laying was established. Intact PVG from mature Aplysia californica were extracted with buffer in mid-August (1969). The BCS protein content of the bag cells is at its maximum during this period. Test Aplysia were isolated for one week prior to injection with the extract. Each animal would receive a series of injections starting with the equivalent of 1/10 PVG. The animal was observed and if after 4 hours it did not lay eggs, a larger dose, 1/4 or 1/2 PVG, was administered to test this animals ability to lay eggs (Table XVI). Since the sampling number was not large enough to average out random behavior observed, the details of the reproductive behavior such as time to climb vertical wall, time sitting still prior to egg laying, total time of egg laying, etc. are not tabulated. However, each animal that did respond to the dose administered did so in 45 to 60 minutes and layed from 3 to 15 grams of eggs. For example, the animal which responded to 1/10 PVG dose started laying eggs 45 minutes after the

TABLE XV

Cross-Reactivity of the BCS Protein

donor / recipient	A.c.	A.v.	A.d.
A.c.	+	+	not done
A.v.	+	+	not done
A.d.	+	+	not done

+ indicates that the recipient showed the typical reproductive behavior and layed eggs within 60 minutes after the injection of the donor PVG.

Species code: A.c. = Aplysia californica

A.v. = Aplysia vaccaria

A.d. = Aplysia dactylomela

TABLE XVI

Dose-Response Relationship

Date injected	Animal number	Dose*		
		1/10 PVG	1/4 PVG	1/2 PVG
8-15-69	1	+	+	not done
8-15-69	2	-	+	+
10-1-69	3	-	-	+
10-1-69	4	-	+	not done
10-12-69	4	-	-	+

*All doses were from a common pool of 6 intact PVG which were dissected and extracted in mid-August 1969. Extract was stored at -20°C in a single container.

1/10 PVG = 7.5 μg unpurified BCS protein (bacitracin equivalents)

1/4 PVG = 19 μg unpurified BCS protein (bacitracin equivalents)

1/2 PVG = 37.5 μg unpurified BCS protein (bacitracin equivalents)

injection and continued to lay eggs for 8 hours. The total weight of eggs layed was 14.5 grams. In other words, the low doses did not elicit partial responses but gave typically positive results. However, it was observed in a number of cases that subthreshold doses would elicit some reproductive behavior without the egg laying. The animal climbed the vertical wall, and sat still for about 60 minutes after which it proceeded to freely move around the tank. This was not seen in all cases of subthreshold doses and the number of animals sampled makes it difficult to establish any predictable pattern.

The quantity of BCS protein injected into each animal was estimated from polyacrylamide gels which had been layered with the volume equivalent to 1/10 and 1/4 PVG from the mid-August (1969) extract pool. The method of calculating the amount of protein in the BCS protein band was the same as employed in the seasonal variation calculations. In this extract, 1/10 PVG contained 7.5 μgm (bacitracin equivalent) of unpurified BCS protein; 1/4 PVG contained 19 μgm. These values represent an over-estimation of the actual amount of pure BCS protein present, since three other faint protein bands run at the same banding position on the gel.

DISCUSSION

Identity of Egg-Laying Hormone of Aplysia

The isolation and chemical characterization of a molluscan neurohormone from the identified bag cells of the abdominal ganglion (PVG) of Aplysia are described in this thesis. This hormone, which was originally identified as the Bag Cell-Specific protein (BCS protein) by polyacrylamide gel electrophoresis (Toevs and Brackenbury, 1968, 1969), has the biological activity to induce egg laying in sexually mature Aplysia. The function of Bag Cell-Specific protein #2, the "turquoise protein band," has not been investigated. The cells from which these proteins are extracted have been shown to be of a neurosecretory nature by meeting morphological (Coggeshall, 1967), and physiological criteria (Kupfermann, 1967; Strumwasser et al., 1968, 1969; Toevs and Brackenbury, 1969).

Bag Cell Axon Path and Terminations

Morphological investigations by Coggeshall (1967) have indicated that the bag cells send granule-filled processes into the highly vascularized connective tissue sheath of the rostral PVG and that they wrap around the axon trunk of the pleuro-visceral connective nerve and terminate in the connective tissue sheath of the posterior half of the connective nerves (Frazier et al., 1967). In the present study, it has been determined both by assaying for the BCS protein on acrylamide gels and by bioassaying for the egg-laying activity, that a series of

decreasing quantities of the protein-hormone in the tissue exists: bag cell somas and connective tissue sheath of the bag cells > connective tissue sheath of PVG > connective tissue sheath of connective nerve > desheathed connective nerve, and is absent in the neurons and neuropile of the PVG. It is therefore proposed that the BCS protein-hormone is synthesized in the bag cell somas and transported into their axonal processes, which are found primarily in the connective tissue sheaths of the bag cells and PVG and secondarily in the posterior portion of the connective tissue sheath and nerve fibers of the connective nerve.

The light microscope studies of Alvarez and Strumwasser (unpublished) have revealed several other modes of bag cell axon termination; some on vascular invaginations into the bag cell cluster itself (Figure 17), some on vascular components in the connective tissue sheath, and some on a highly vascularized "brush-border" (Figure 18). This prominent anuclear structure can be seen, with the Frazer-Rowell silver stain, in the outer region of the connective tissue sheath of the bag cell cluster and extends anteriorally into the connective tissue sheath of the connective nerve as far as bag cell somas are located. The function of this vascularized border is unknown at present but it is presumed to be related to the secretory nature of the bag cells. There is also the possibility that this highly structured "brush border" and its accompanying bag cell processes may be serving a chemosensory function to detect levels of circulating hormones from other neurons or reproductive organs for feedback control.

Figure 17: Termination of a bag cell axon on a vascular sinus invagination of the bag cell cluster. This constitutes part of the bag cell neurohemal organ which facilitates the distribution of the hormone, presumably released by the bag cell axons, throughout one circulatory system to the target organ(s). Frazer-Rowell silver stain. Magnification 1000X.

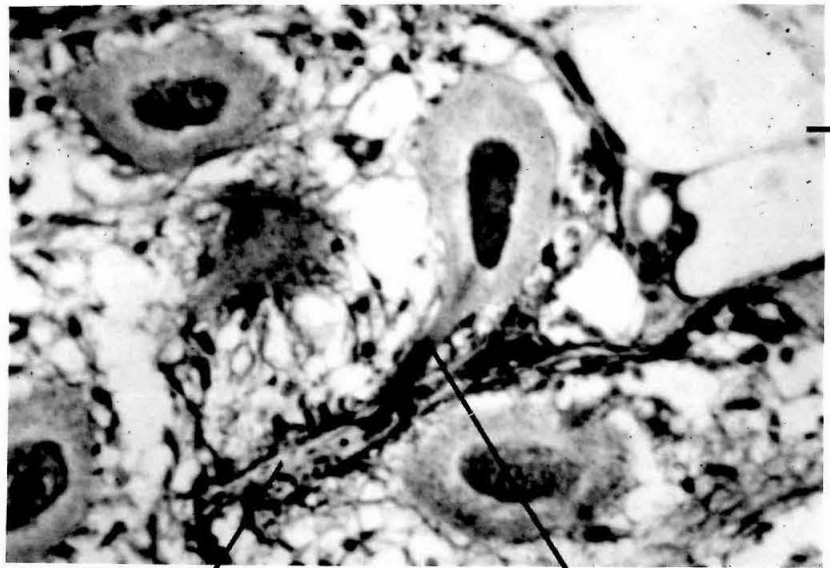
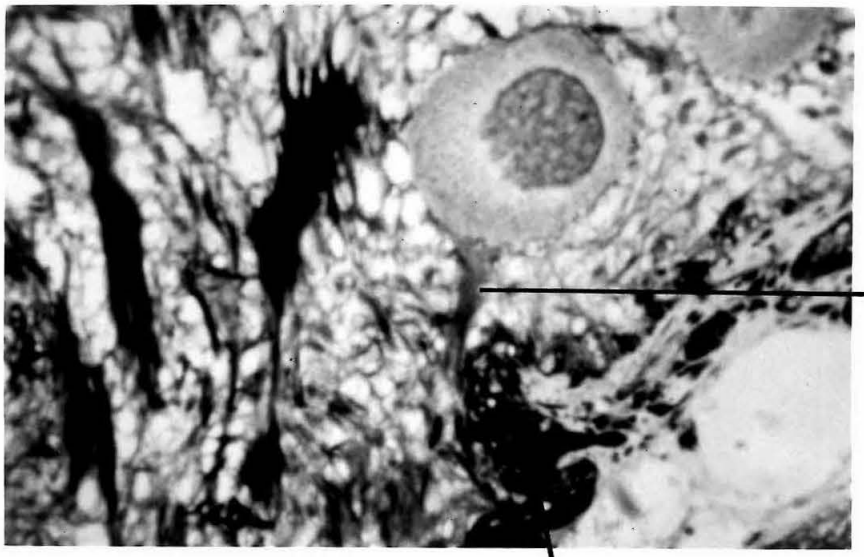

Vascular sinus
Vascular sinus invagination
Bag cell axon

Figure 18: A. Direct termination of a bag cell axon on the highly vascularized brush border located at the edge of the connective tissue sheath surrounding the bag cell clusters. B. Detail of this complex, anuclear border showing similarity of the structure to microvilli. The function of the structure is unknown but believed to be related to the secretory function of the bag cells.

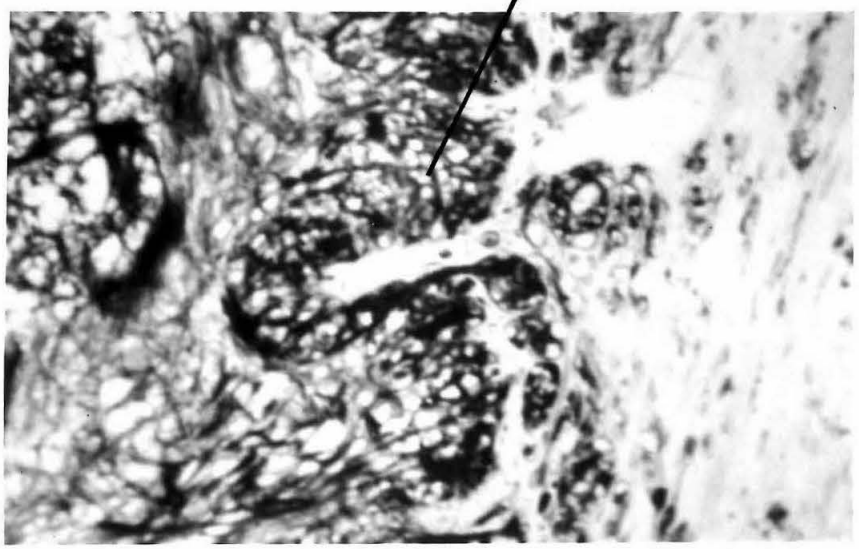
Frazer-Rowell silver stain. Magnification 1000X.

A



"brush border"

B



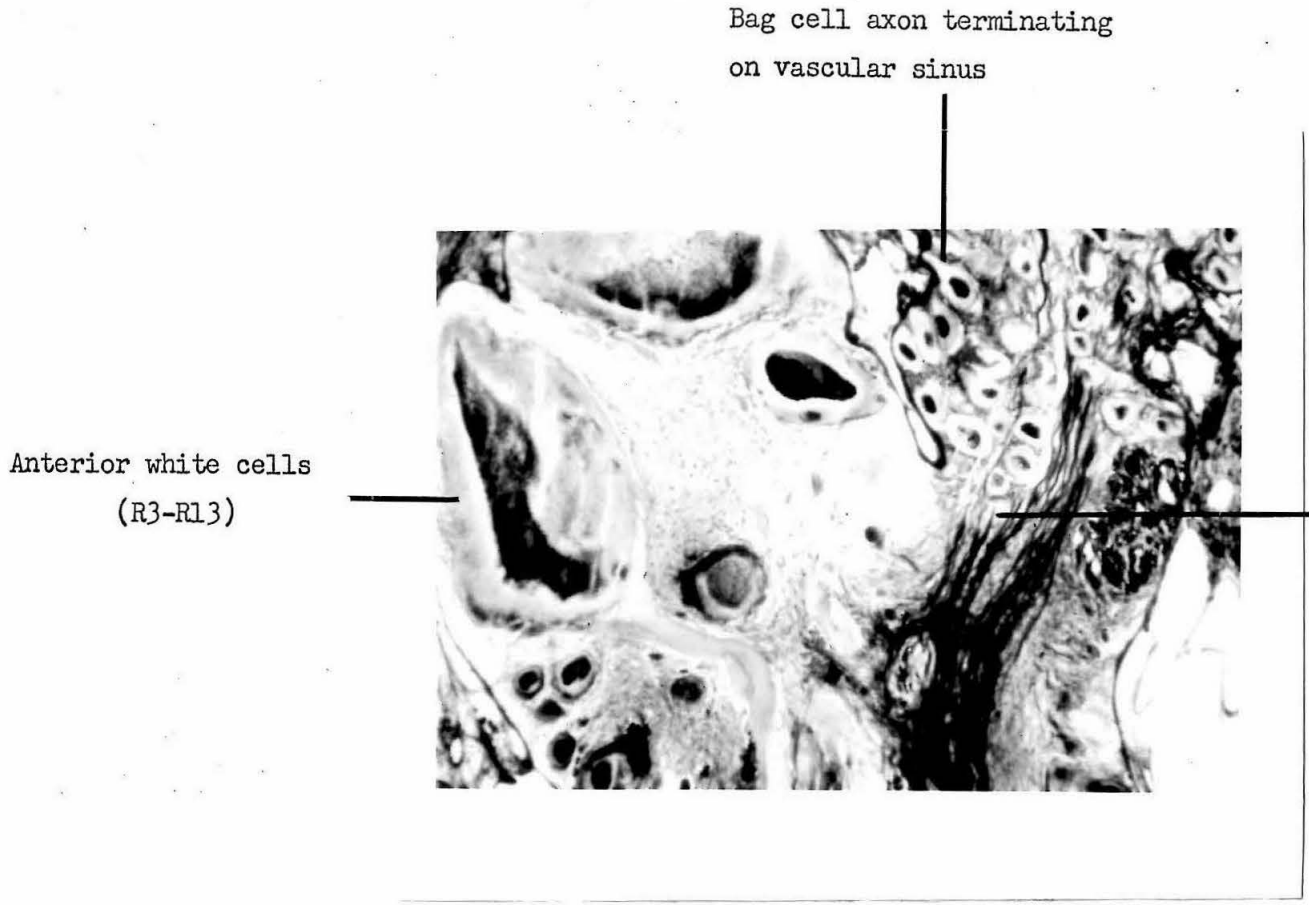
The entire variety of axon paths and terminations of the bag cells probably has not been revealed by the investigations of Coggeshall (1967), but it is clear that the main termination of these axons is on some component of the circulatory system of the ganglion presumably for the rapid distribution of the released egg-laying hormone to the target organ(s).

Possible Bag Cell "Dendrites"

There are interesting aspects of the bag cell morphology which have not been clarified yet. Coggeshall (1967) reported that the granule-filled axons of the bag cells terminate blindly in the connective tissue sheath of the connective nerve, bag cell cluster and anterior pole of the PVG. By chemical localization of the BCS protein and the activity of the hormone, I would agree with this conclusion. I have found no evidence for the presence of the hormone in the PVG neurons or neuropile. However, it is estimated that 10-30% of the branches of the bag cell axons, as seen by light microscopy (Alvarez and Strumwasser, personal communication) dive back into the neuropile of the PVG (Figure 19). Since no BCS protein has been detected in the neurons or neuropile of the desheathed PVG, it is possible that these axon branches do not transport neurosecretory material but rather make synaptic connections in the PVG neuropile. From simultaneous intracellular microelectrode recording from two or more bag cells, Frazier et al. (1967) have observed that electrical stimulation to the ipsilateral connective nerve caused all the bag cells of one cluster to

Figure 19: Bag cell processes diving into the neuropile of the PVG. A probable secondary termination of branches of bag cell processes may be in the PVG neuropile rather than in the vascularized connective tissue sheath of the ganglion. The possible "dendritic" function of these processes is discussed in the text.

The large neurosecretory, anterior white cells (R3-R13) can be seen on the left of the photograph. In sharp contrast to the highly stained axons of the bag cells, the axons of R3-R13 neurons do not exhibit an affinity for the silver stain. Frazer-Rowell silver stain. Magnification 175X.


Bag cell axon terminating
on vascular sinus
Anterior white cells
(R3-R13)
Bag cell axons
entering
neuropile

fire in synchrony. They concluded that this was due to a prepotential which was similar and simultaneous in all bag cells on one side. It was, however, not possible to ascertain if this was due to electrical coupling of all the bag cells or to the innervation of the 400 bag cells by an interneuron. These bag cell processes observed by Alvarez could be entering the neuropile, acting as "dendrites," to make a typical molluscan axo-axonal synapse with just such an interneuron.

The possibility that certain neuronal processes of a neurosecretory cell are not neurosecretory themselves has also been raised by Maynard (1961) in the crab pericardial organ where he has noted that the dendritic processes of the monopolar "C-cells," in contrast to the axons, do not contain secretory granules. The initial process of the C-cell extends a few cell diameters from the soma at which point it divides into an axonic branch with masses of characteristic neurosecretory granules, and a dendritic branch which is devoid of granules. The dendritic branches pass through the neuropile of the first and second segment where they presumably terminate. The axons proceed to the pericardial organ neurohemal organ.

Both the crab "C-cells" and the Aplysia "bag cells" are monopolar and have a single process which branches into a granule-laden axon that progresses to a neurohemal area, and a dendrite probably devoid of granules which proceeds into the ganglionic neuropile presumably to receive synaptic connections. For conservation of neurosecretory material, this morphological arrangement appears sensible, but must require some type of "valve system," so that the large neurosecretory

granules are shunted only into the axons and not into the dendritic branches.

Vascular System of the PVG

The question of the vascularization of the ganglion and neuropile has not yet been conclusively answered. Coggeshall (1967), using india ink injections and electron microscopy, concluded that only the connective tissue sheath is vascularized, while Eales (1921) described a vascular sinus between the sheath and the ganglion (also seen by Strumwasser and Malhotra using an injection of ferritin into the dorsal aorta, unpublished observation). Chalazonitis (1961), after injecting a solution of Janus green and examining the ganglion in a dissecting microscope, described capillaries that passed over the surface of the neurons. Alvarez and Strumwasser (unpublished observations) have observed many invaginations of connective tissue, containing vascular sinuses, into the ganglion thus forming compartments of neurons within the PVG. Regardless of the exact location of the capillaries and sinuses within the ganglion, it appears safe to assume that the cells and axons are liberally bathed in hemolymph which freely diffuses from the capillaries and vascular lacunae.

Effect of Maturation on BCS Protein Content

The effects of maturation on the BCS hormone content and localization have been studied. The BCS protein is present in sexually immature animals although its amount increases in the bag cells by a

factor of 10 with the sexual maturation of the animal. This increase in BCS protein correlates well with the maturational increase in number and development of bag cells as reported by Coggeshall (1967). In the very youngest animals examined, the BCS protein could not be detected in the lower portion of the connective nerves, whereas it was present in the bag cells and to a lesser degree in the connective tissue sheath of the PVG. We take this to indicate that the first axon pathway is into the connective tissue sheaths of the bag cell cluster and PVG and only in larger animals do the axons also grow into the posterior connective nerve region. The small amount of hormone which is produced by sexually immature animals is fully active and can induce egg laying when injected into sexually mature recipient Aplysia (Strumwasser et al., 1969).

Differences Between Bag Cell Neurons and the Anterior Neurosecretory Neurons (R3-R13)

A second group of presumably neurosecretory cells exist in close proximity to the bag cells. They are located in the right anterior quarter ganglion, being labeled R3-13 by Frazier et al. (1967), and appear white under epi-illumination. These cells also send their axons into the connective tissue sheath of the PVG. The axons of the bag cells can be differentiated from the axons of these white cells by the striking difference in the morphology of their neurosecretory granules (Coggeshall, 1967) (see Figure 23). Besides the morphological difference, the absence of the BCS protein and egg-laying activity in the neurons

and neuropile of the PVG indicates that the neurosecretory product of the bag cells is different from the neurosecretory product of the granule-filled white cells. It is not surprising though that neurons with different morphological and electrophysiological characteristics would produce different neurosecretory products and most likely have different functions. The functions of these white cells remains unknown but Jahan-Parwar et al. (1969) conclude from physiological studies, that osphradial stimulation (osmo-, chemo-, and mechano-stimulation) activates the electrically silent pacemaker neurons or modifies the discharge rate of the active ones; and, therefore, the cells may regulate autonomic reactivity to the internal environment, such as regulation of water balance.

Chemical Nature of Neurohormones

The vast literature on the chemistry of hormones does not permit a detailed discussion on the various active principles, but does indeed show that all purified neurosecretory hormones are of a polypeptide nature. The thoroughly studied vertebrate neurohypophysial octapeptides, vasopressin and oxytocin, have a molecular weight around 1000 (du Vigneaud, 1956). The seven releasing factors produced in the hypothalamus and transported to the adenohypophysis via the portal circulatory system, are all polypeptides with molecular weights between 1000 and 1400 (McCann and Dhariwal, 1967). In invertebrates, several polypeptide neurosecretory hormones have been isolated, primarily from crustacea--several eyestalk hormones (Josefsson and

Kleinholz, 1964; Kleinholz, 1966), cardioexcitor hormone (Belamarich and Terwilliger, 1966); and from insect cerebral neurosecretory system--cuticle tanning hormone (Fraenkel and Hsiao, 1963), hormone for protein digestion (Thomsen and Møller, 1959), and brain hormone (Gersch, 1959; Ichikawa, 1962; Ishizaki and Ichikawa, 1967; Yamazaki and M. Kobayashi, 1969). No molluscan neurosecretory hormone had been isolated or identified prior to the "bag cell-specific" or "egg-laying" hormone.

Chemical Similarity of Egg-Laying Hormone from Three Species of Aplysia

This investigation used three species of Aplysia, two from cool California waters and one from the warm Florida coast. The egg-laying hormone, which is located in the bag cells in all three species, was completely cross-reactive as tested. Besides their similar activity, the hormone from Aplysia vaccaria and Aplysia californica were eluted from a G 50 fine Sephadex column in the same fraction, indicating they were of similar molecular weight. However, the isoelectric point of the hormone was different for each species. These data suggest that there is a region of the molecule, essential for activity, which is the same in all three species. However, due to differences in isoelectric point, there may be amino acid substitutions in a nonessential length of the polypeptide. The amino acid composition or sequence of the hormones from the three species would settle this question. However, to be feasible, the hormone from Aplysia californica would have to be obtained during the summer months since the quantity

of the BCS protein is so low during the winter. The seasonal variation of the other two species Aplysia vaccaria and Aplysia dactylomela has not been studied.

Intensive chemical investigations have shown that the neurohypophysial octapeptides, oxytocin, arginine-vasopressin, lysine-vasopressin and vasotocin, differ from each other by only one or two amino acid substitutions. Depending upon the amino acid substitution, this basic octapeptide can have eight different functions.

Correlation of Seasonal Rhythms in BCS Protein Content, Reproductive Behavior and Amino Acid Uptake

A seasonal rhythm in the extractable BCS protein content of the bag cells was observed. The bag cells contain 150 times more BCS protein/mg wet weight in August than in January. It is interesting to compare this curve with those determined by Strumwasser et al. for the inducibility of egg-laying throughout the year (1969) and for the H^3-leucine uptake/mg wet wt by the intact PVG throughout the year (Strumwasser and Alvarez, unpublished) (Figure 20). Figure 21 clearly shows that ability of the animal to lay eggs parallels the egg-laying hormone content in the PVG. The increase in H^3-leucine uptake, presumably for increased protein synthesis, also follows the same curve, although it is higher during December, January and February than the other two curves.

Figure 20: Correlation of seasonal rhythm in the A. egg-laying ability of the animal, and B. H^3-leucine uptake by the intact PVG and the bag cells. The egg-laying ability of the animals was assayed by injecting each test animal with an extract of 2 PVG or 2 pairs of bag cell clusters. The curve represents a compilation of 2 years data (two complete cycles) with a minimum of 12 experiments per month. The amino acid uptake curve represents a minimum of 2-4 experiments per month, each experiment consisting of the examination of H^3-leucine uptake in 3 ganglia or pairs of bag cell clusters.
(Figure compliments of F. Strumwasser. A. similar to Figure 4 of Strumwasser et al., 1969. B. unpublished data.)

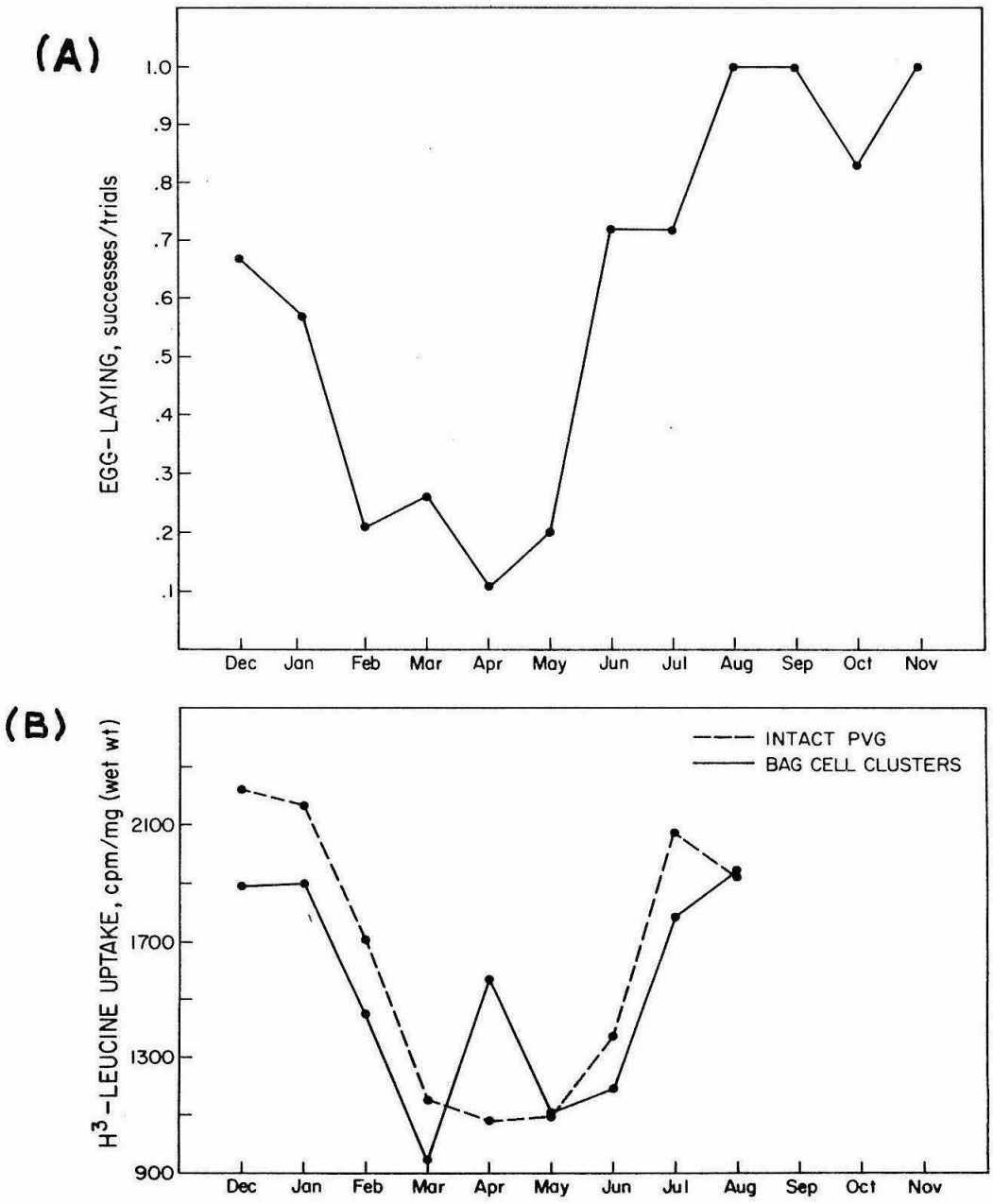
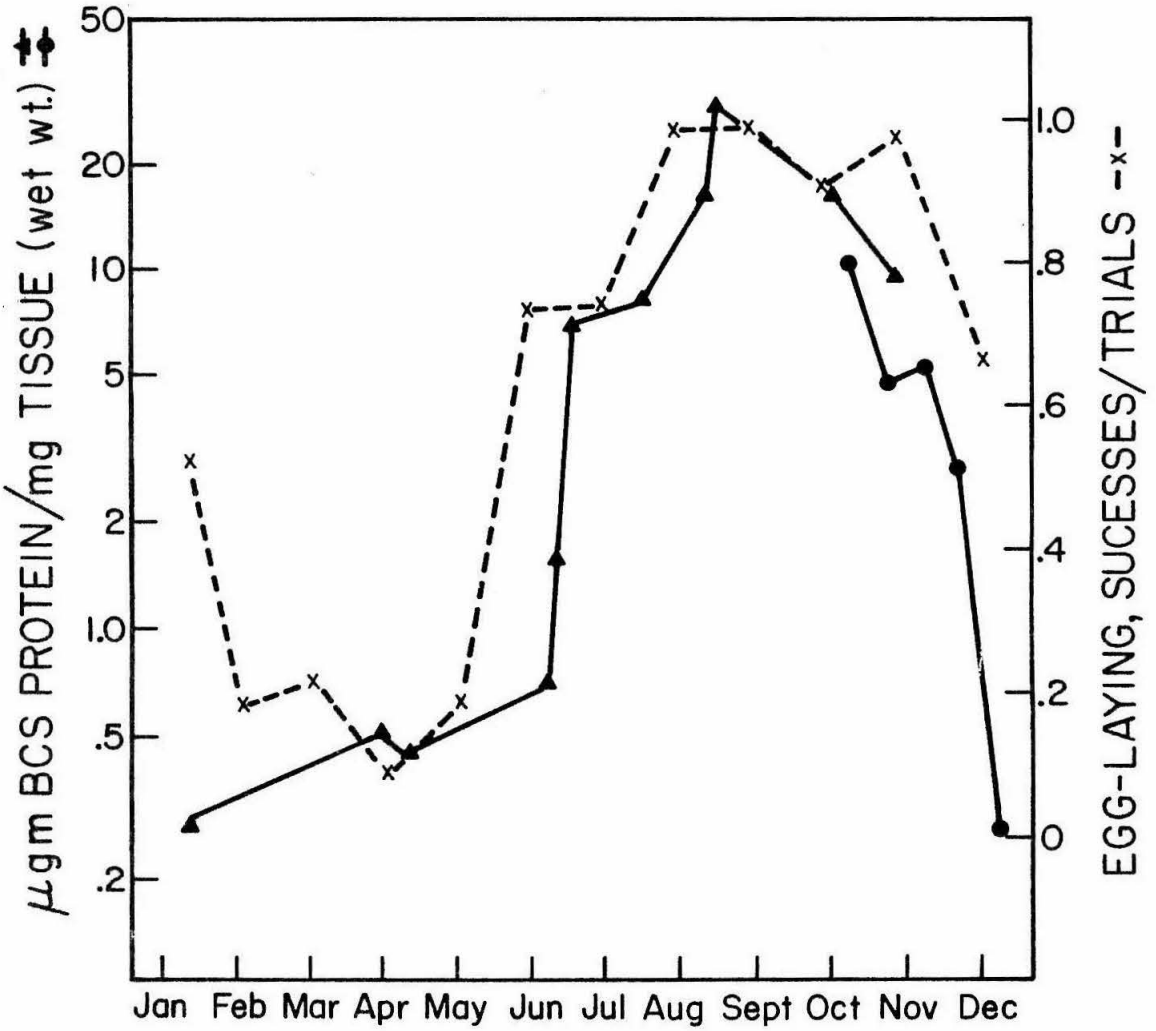

(A)
EGG-LAYING, successes/trials
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov
(B)
H³-LEUCINE UPTAKE, cpm/mg (wet wt)
INTACT PVG
BAG CELL CLUSTERS
2100
1700
1300
900
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
Nov

Figure 21: Correlation of seasonal rhythm in the BCS protein content of the PVG and egg-laying ability of the animal. The BCS protein content per mg wet weight of tissue, for the intact PVG, was calculated by adding the BCS protein in the bag cells and in the PVG-bag cells and dividing this value by the sum of the two tissue weights. The egg-laying ability was assayed as described in Figure 20. By comparing Figures 20 and 21 it can be seen that all three curves sharply rise in May and June. The BCS protein content decreases abruptly in early winter, slightly sooner than the other two curves; but, all three remain at their lowest levels during late winter and spring. For interpretation of the correlation see text.


μgm BCS PROTEIN/mg TISSUE (wet wt.)
50
20
10
5
2
1.0
.5
.2
EGG-LAYING, SUCESSES/TRIALS -x-
1.0
.8
.6
.4
.2
0
Jan Feb Mar Apr May Jun Jul Aug Sept Oct Nov Dec

There are two interpretations of the seasonal variation in the BCS protein content. The cells may be synthesizing the hormone at a constant rate throughout the year, releasing it from December through May and storing it from June through November. Alternatively, the synthesis of the BCS protein may be minimal during the winter and abruptly turn on about the second week of June. The protein is synthesized at a high rate during June through November and stored during this time for use to induce egg laying whenever the appropriate set of stimuli are present. The second proposal appears to be more sound since both amino acid uptake and the ability to lay eggs increase considerably from June through the summer months.

Receptivity of Animal to Egg-Laying Hormone

The ability of the animal to lay eggs may not be solely controlled by the amount of egg-laying hormone present in the ganglion. It appears that another factor, at present unknown, controls the receptivity of the animal to the egg-laying hormone. This is suggested by two experiments. First, if the animal is given a large dose of extract (containing 4 or 5 PVG) in February it still cannot be induced to lay eggs. Second, the dose-response relation (Table XVI in Results section), using the same pool of extract in all experiments, showed a change during the fall months, so that 1/10 PVG (containing 7.5 µg unpurified BCS protein) was sufficient to cause egg laying in an animal in mid-August, but the animals tested from the population in late October require 1/2 PVG (containing 37 µg unpurified BCS protein).

Morphological Studies on Neurosecretory Cycles and Their Relation with Rhythmic Reproductive Processes

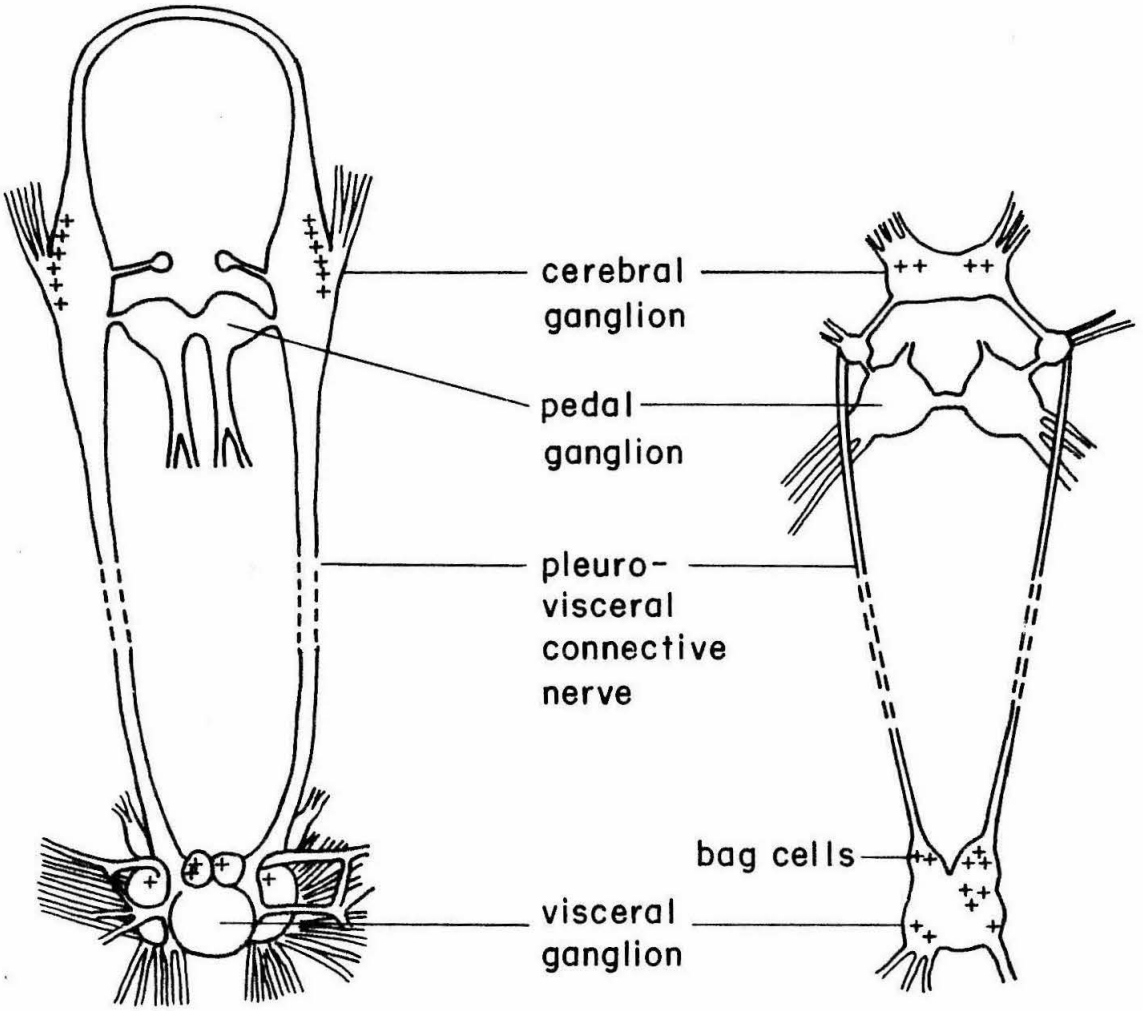
The granule content of the cerebral ganglia and the visceral ganglion neurosecretory cells and the reproductive cycle in two lamellibranches, Chlamys varia and Mytilus edulis, were correlated by the investigations of Lubet (1955, 1956). He showed that there is a definite relation between the granule content and the release of gametes. By examining the normal reproductive cycle of a population of bivalves, he concluded that the acidophilic neurosecretory product accumulated in the neurosecretory perikarya during the period of gamete maturity. He has also shown that certain mechanical stimuli can cause oviposition but that the period of maximum efficacy of the stimulus corresponds to the period of maximum granule accumulation in the neurosecretory cells. At certain times during the year the animal is completely refractory to mechanical stimuli to induce ejaculation or egg laying even though the gametes are completely mature (as demonstrated by artificial fertilization and normal development of the embryos).

Evacuation of the gametes and oviposition were preceded, by about one day, by the definite emptying of the neurosecretory perikarya. The return of the neurosecretory activity in the cells appears to precede, by a short time, the return of gametogenesis. Although surgical removal of neurosecretory cells in molluscs means the removal of a major portion of a ganglion, leading to definite trauma in the animal, Lubet (1956) has extirpated the cerebral or visceral neurosecretory

cells. Ablation of the cerebral ganglia, during the period of mature gametes, has the same effect as mechanical stimulation; it has no effect on maturation of gametes but accelerates their discharge. On the other hand, removal of the visceral ganglion greatly retards oviposition. Lubet concludes that the visceral ganglion contains a product favorable to egg laying and that the disappearance of the neurosecretory product of the cerebral ganglia seems necessary for the animal to become receptive to external stimuli causing emission of gametes. He has also shown that these two factors fluctuate with the seasons.

This study seems particularly relevant to the situation found in Aplysia. Even the morphology of the nervous system suggests some homologous system (Figure 22). The location of the neurosecretory cells of the visceral ganglion of Chlamys closely resembles the bag cells of Aplysia. Lubet's suggestion of a visceral ganglion factor facilitating egg laying, seems to have the physiological characteristics of the Aplysia egg-laying hormone. Also, his proposal that the cerebral ganglion contains a neurosecretory factor which affects the receptivity of the animal to egg-laying stimuli, corresponds to the hypothesis presented here that some unknown factor in Aplysia effects the receptivity of the animal to the egg-laying hormone. It would be possible to inject an Aplysia in the summer with a winter extract of the cerebral ganglion, followed by a summer extract of the bag cells, to determine if, in the winter, the cerebral ganglion does indeed produce a factor which inhibits the egg-laying ability of the animal. It

Figure 22: Schematic representation of the nervous system of (A) the lamellibranch gastropod Chlamys and (B) the opisthobranch gastropod Aplysia. The similarity of the location of the neurosecretory neurons in the visceral ganglion is striking, and is possibly significant since this ganglion, in both species, contains a hormone which facilitates egg laying. Lubet (1955, 1956) has demonstrated the seasonal variation in the granule content of the neurosecretory cells of the visceral ganglion and states that it is closely correlated with the seasonal rhythm of gamete maturity, and the seasonal rhythm of receptivity of a mature animal to oviposition stimulation. The present study correlates the BCS hormone content of the Aplysia visceral ganglion with both the animal's seasonal egg-laying cycle and receptivity to hormonal induction of oviposition. (A. from Figure 1 of Lubet, 1955.)


cerebral ganglion
pedal ganglion
pleuro-visceral connective nerve
bag cells
visceral ganglion
(A)
(B)

is possible that this same factor may play a role in possible inactivation of the BCS protein synthesis during the winter months.

A relationship between neurosecretory cells and the reproductive process in molluscs was first suggested by Gabe (1951) in connection with his histological investigations on neurosecretory perikarya in Heteropoda, free-swimming, pelagic gastropods. Comparison of the neurosecretory cells of the cerebral ganglia of sexually immature and sexually mature animals and comparison between cells from the animal "fixed during the period of reproduction" and cells from an animal "fixed during sexual repose" effectively showed an appearance of "emptiness" of acidophilic products in sexually mature animals that were reproductively active and also in sexually immature animals. Sexual rest presented a picture of a "storage stage" with heavily stained granules in the secretory neurons.

Nagabhushanam (1963) has demonstrated that the period of "gonad exhaustion" (October to December) in the bivalve Crassostrea virginica, coincides chronologically with the rarity or absence of the secretory product of neurosecretory perikarya in the cerebral and visceral ganglia.

An accessory endocrine gland, located on the posterior dorsal surface of the cerebral ganglia in Aplysia punctata, also exhibits a seasonal variation in the size of its cells which correlates well with the reproductive cycle. The volume of the cells increases tremendously in the sexually differentiating animal but decrease in size

in the over-wintering animal (Simpson et al., 1966). The function of the organ is unknown.

The functional significance of neurosecretory cycles with relation to reproductive processes has been reviewed by Gabe (1965) for many molluscs. The neuroendocrine and endocrine relationships controlling gamete maturation, maturation of accessory sexual apparatus, oviposition and sperm release, appear to be quite complex and involve several different ganglia and accessory glands in gastropod molluscs. Some features appear to be similar in the various species; the neurosecretory cells of the visceral ganglion produce some factor which facilitates oviposition (i.e. the bag cell-specific, egg-laying hormone of Aplysia), and the cerebral ganglion or an associated endocrine gland may control gamete development or inhibit oviposition by lowering the animals receptivity to reproductive stimuli or internal hormonal agents.

SUMMARY

In summary, this investigation has resulted in the chemical identification and isolation of the egg-laying hormone from Aplysia californica, Aplysia vaccaria, and Aplysia dactylomela. The hormone, which was originally identified as the BCS protein on polyacrylamide gels, is a polypeptide of molecular weight 6000, which is localized in the neurosecretory bag cells of the parieto-visceral ganglion. All three species produce a hormone of similar molecular weight, but varying electrophoretic mobility as shown on polyacrylamide gels. As tested, the hormone is completely cross-reactive among the three species.

Although the bag cells of sexually immature animals contain the active hormone, sexual maturation of the animal results in a ten-fold increase in the BCS protein content of these neurons.

A seasonal variation in the BCS protein content was also observed, with 150 times more hormone contained in the bag cells of Aplysia californica in August than in January. This correlates well with the variation in the animals ability to lay eggs throughout the year (Strumwasser et al., 1969). There are some indications that the receptivity of the animal to the available hormone also fluctuates during the year, being lower in winter than in summer. The seasonal rhythm of the other species, Aplysia vaccaria and Aplysia dactylomela, has not been investigated.

The study of this neurosecretory system has just begun and there are many important questions yet unanswered. The author feels

the following are of top priority: 1) Is the hormone released into the circulatory system upon electrical stimulation of the bag cells? A preliminary finding (Kupfermann, private communication, August, 1968) indicates that this may be so. 2) Is the hormone localized in the 2000 Å neurosecretory granules? Is any other protein, such as a carrier or synthetic enzyme, also in the isolated granules? 3) What is the target organ for the neurohormone--another neuron or part of the reproductive system such as the gonad? What is the nature of the hormone action on this target organ? 4) Is there another factor, possibly neurosecretory, which varies with the reproductive season and controls the receptivity of the target organ to the available egg-laying hormone?

APPENDIX I

Concept and Evidence for Neurosecretion

Morphology of Neurosecretory Cell

The earliest reports of the phenomenon of neurosecretion (Nansen, 1886; Speidel, 1919; E. Scharrer, 1928; B. Scharrer, 1935, 1937; Weyer, 1935) were based on staining affinities of neurons using various combinations of stains. Neurons appeared "secretory" with the light microscope, containing granules, globules, and highly stained "colloid," and were labeled "Gomori positive," "Gomori negative," or "Gomori unreactive" based primarily on the chrome hematoxylin-phloxine conditions of Gomori (1941) or the highly selective paraldehyde fuchsin stain (Gomori, 1950).

It should be noted here that there are many structures in the nervous centers of gastropods which are in no way related to neurosecretion and yet stain very selectively with chrome hematoxylin in the Gomori method and with paraldehyde fuchsin. The nerve cells of molluscs in general and gastropods in particular are very rich in lipid oxidation products. Most lipofuscin structures such as lysosomes, pigment granules, broken down mitochondria, and many other particles appear light yellow to brown in the unstained state with the light microscope; permanganate oxidation, a routine step in the staining procedure, gives them sufficient basophilia to ensure their selective demonstration by the Gomori method (Gabe, 1966). The sheer abundance of neurosecretory cells seen after staining should warn the investigator

to do proper controls. However, Lemche (1955, 1956) has reported that the majority of the cells in the tectibranch mollusc Cylichna are neurosecretory and that these supposed neurosecretory cells innervated muscles and made synapses with other effectors or neurons. While the affinity of the secretory products for the Gomori stains may vary from intense to very weak, depending on the species and stage of the secretory cycle of the cell, other inclusions which have no relationship to neurosecretion always stain with chrome hematoxylin and paraldehyde fuchsin (Gabe, 1966). Pearse (1954) even recommends the use of these stains to detect the "wear and tear" pigment granules of the neurons. For these reasons, tinctoral methods are not reliable in molluscs to define a neurosecretory cell, although one should not forget that the discovery of neurosecretion was due to the application of histological techniques and that most recent advances in this field have been made possible by the development of selective staining methods for certain secretions elaborated in nerve cells.

Many neurons in the parieto-visceral ganglion of Aplysia appear opaque white with epi-illumination, and are suspected to be neurosecretory cells. Two prominent groupings are the large anterior white cells (Coggeshall et al., 1966; R3-13 according to the terminology of Frazier et al., 1967) and the two clusters of bag cells. The supposed neurosecretory cells of the PVG show different staining affinities; whereas paraldehyde fuchsin intensely stains the large anterior white cells, the small bag cells show no affinity for this common neurosecretory stain (unpublished observation, R. B. Alvarez).

The application of electron microscopic methods further refined the cytological criteria that could be employed in defining the neurosecretory neuron. Discriptions by Palay (1957), Bargmann et al. (1958) and many others (see Bern and Hagadorn, 1965) of membrane-bound, electron-dense granules measuring 1000 to 4000 Å in the neurohypophysis of vertebrates, the urophysis of caudal neurosecretory system of fishes and in the neurohemal organs of many invertebrate species, gave additional means of delineating a neurosecretory neuron. But other non-neurosecretory neurons occasionally contain granules in this size range, such as the neurons in the leech (Hagadorn, Bern and Nishioka, 1963) or rotifer brain (Eakin and Westfall, 1965), so one must be cautious in labeling a neuron as neurosecretory solely on the evidence of 1000 to 4000 Å granules.

Although a well-developed endoplasmic reticulum and prominent Golgi complex are characteristic of all neurons, the perikaryon of the neurosecretory neuron can generally be distinguished from that of the ordinary neuron by the presence of typical elementary granules and the association of electron-dense material with the Golgi membranes (Bern et al., 1961). However, there are other electron-dense bodies, including the pigmented droplets of molluscs, that also may take their supramolecular origin as electron-dense material in the Golgi regions (Bern and Knowles, 1966).

Extensive electron microscopic studies by Rosenbluth (1963), Simpson et al. (1963), and Coggeshall (1967) on the parieto-visceral ganglion of Aplysia have revealed discrete clumps of electron-dense

material in the cisternae of the Golgi apparatus in supposed neurosecretory cells (Bern et al., 1962). Some clumps of material can be seen as marginal buds of the cisternae possibly in the process of becoming elementary neurosecretory granules. Coggeshall (1967) describes in detail the granule appearances from two different groups of neurosecretory cells in the PVG of Aplysia californica. The anterior white cells (R3-R13) and their axons contain very electron-dense, round granules of 1000 to 4000 Å in diameter. The small bag cell somas contain round 2000 Å granules which are less electron-dense than the granules in the white cells. However, the granules in the axons of these bag cells appear ghostly with broken, crenated membranes and are clearly distinguishable from the granules in neighboring white cell axons (Figure 23). But it is not unusual to find neurosecretory granules of varying morphology in the same ganglion. For example, Hagadorn (1962) has identified four types of granules (classified on the basis of average diameter and morphology) in perikarya of the brain of the leech Theromyzon.

Neuronal Characteristics of Neurosecretory Neurons

The primary function of an ordinary neuron is to conduct and transmit impulses. Although transmission is not the business of neurosecretory fibers, conduction may be an essential activity. A neuroendocrine reflex arc consists of sensory input, central integration of the stimulus with other information of the internal and external environment, and a final common path of activation of a neurosecretory

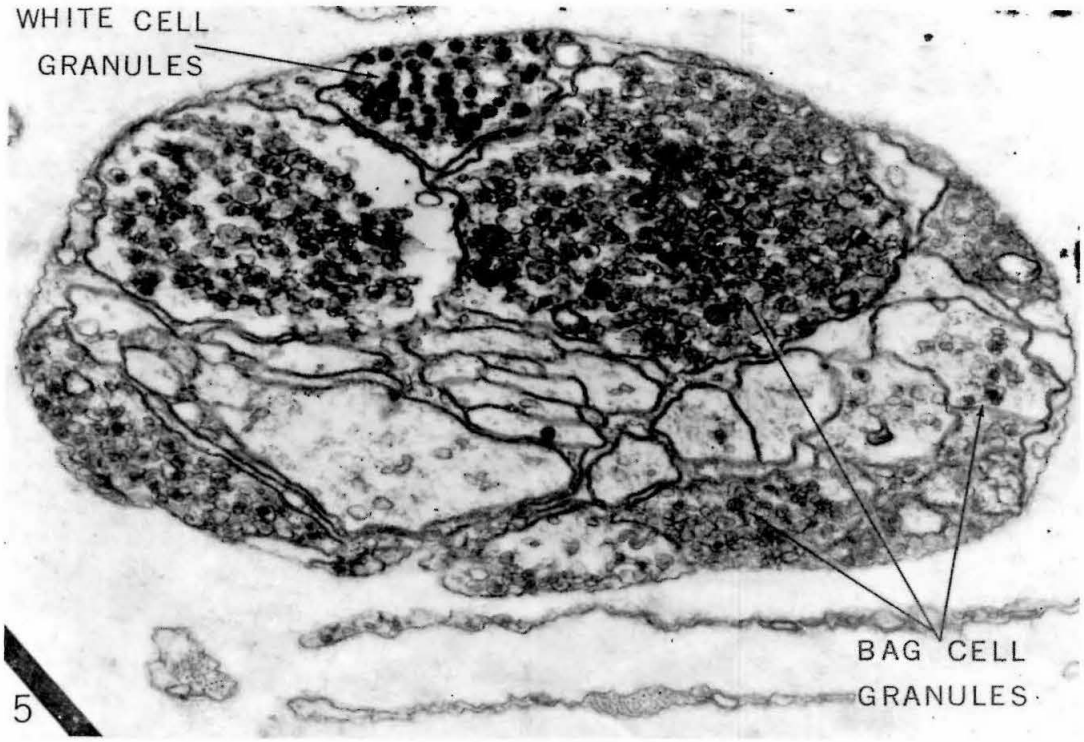


FIG. 5. A cross section of a small nerve in the sheath. One of the axons in the nerve contains round granules with very electron-dense cores. These granules arise in the perikarya of the white cells R3-R14. Most of the other axons contain granules with relatively irregular crenated membranes and less electron-dense cores. Such axons have been traced to the bag cells which are found in clusters at each ganglion-connective junction. (A more complete description of the white cells and bag cells is given in the following paper (14).) Note that many of the granule-filled axons in this nerve are directly exposed to the extracellular fluid in the sheath. ×27,000.

Figure 23: Comparison of neurosecretory granules in axons of anterior white cells and bag cells of Aplysia californica. (From figure 5 of Coggeshall, 1967).

cell to release its hormonogenic product. Conduction of information from presynaptic sources to the point of release of a neurohormone is vital to the completion of neuroendocrine reflexes. For many years attempts have been made to obtain intracellular recordings from identified neurosecretory cells. This has now been accomplished for several invertebrate and vertebrate species (Bern and Knowles, 1966), and reveals that action potentials, both induced and spontaneous, are similar in form to those recorded from motorneurons. A consistent finding, however, is that these action potentials are of a longer duration (2 to 10 times longer) than those of adjacent ordinary neurons (Bern and Yagi, 1965). Some neurosecretory neurons show not only a long duration action potential, but also, a very slow conduction velocity (Bennett and Fox, 1962). It has been suggested that these features are related to the need for sustained release of neurohormone from these fibers once they are activated.

In Aplysia, it is possible to impale one or more neurons with an intracellular microelectrode for the purpose of stimulation and recording. All neurons thus impaled, both ordinary and neurosecretory neurons, show similar action potentials and synaptic potentials (Frazier et al., 1967). Upon synaptic activation, all the bag cells of one cluster fire synchronously for up to 45 minutes. It is believed that the cells of one cluster are all innervated by a single interneuron or that they are electrically coupled.

Endocrine Function of Neurosecretory Neurons

The neurosecretory cell, in addition to possessing typical neuronal functions, shows a characteristic feature of its own: the ability to synthesize, store, and release hormonal substances, commonly polypeptides of low molecular weight. The ability to synthesize material is reflected by the presence of recognized cytological secretory apparatus, such as elaborate endoplasmic reticulum, prominent Golgi complex and numerous membrane-bound, electron-dense granules. The storage and release of the neurosecretory material usually takes place in the axons which appear swollen with tightly packed elementary neurosecretory granules. These axons terminate in close relation to the vascular or coelomic systems, and generally do not directly innervate a target structure. The non-innervation of effector organs by a neurosecretory axon is considered by some to be one of the major criteria for neurosecretion (Knowles and Carlisle, 1956). However, this may not be universal since some neurosecretory processes do not terminate in a neurohemal organ but accompany ordinary axons to the periphery and make intimate contact with other neural and non-neural structures (Bern and Hagadorn, 1965). The secretomotor axons fall in this category.

The close association of the axons with vascular system is referred to as a neurohemal organ (Carlisle and Knowles, 1953) and facilitates the distribution of the released hormone through the circulatory system of the animal. The neurohypophysis of vertebrate

tetrapods, the corpus cardiacum of insects, and the sinus gland of crustaceans are the best known examples of the highly developed neurohemal organ, where the normal blood-brain barrier apparently does not function, and where the hormones are released directly into the circulatory system.

At their simplest, the neurohemal organs in lower phyla are little more than a loose association of axons ending on the walls of capillaries or other vascular species. In Aplysia, which has primarily an open circulatory system, two main blood vessels branch off the dorsal aorta and enter the visceral ganglion at the septal area of the posterior PVG and at the base of the left bag cell cluster. Arterial blood is pumped by the two chamber heart (Hill and Welsh, 1966) through the two vessels as they branch extensively in the connective tissue sheath and the septal region of the PVG. The fine structure of these vascular channels has been examined by Coggeshall (1967), who reports that there are many intercellular gaps, of as much as 2 μ, along the walls of the vessels which either empty into large venus lacunae or end blindly in the connective tissue sheath. Therefore he concludes that the connective tissue sheath, which contains many granule-filled, unsheathed axons embedded in an extracellular matrix, is liberally bathed in arterial and venous hemolymph.

Chemical Nature and Hormonal Function of Neurosecretory Material

The morphological evidence of the organellar apparatus of the neurosecretory cell suggests, to most workers, the proteinaceous nature

of the neurosecretory material. The neurosecretory granule, which is produced by the cell's protein synthetic apparatus, is definitely visualized as a protein, however it has an unresolved relationship with the actual hormonal product. Numerous relationships have been proposed: 1) The neurosecretory material is a carrier molecule which sequesters the hormone from the cytoplasm, as illustrated by neurophysin which is the hormonally inert carrier of the active neurohypophysial octapeptides (Acher and Fromageot, 1957; Chauvet et al., 1960; Acher, 1968). 2) Neurosecretory material may be protein of high molecular weight which is a parent molecule that is involved in the continual synthesis of the hormone as the granule is transported along the axon. This is implied by the "growth" of elementary neurosecretory granules en route from the perikaryon to the axon terminal (Bodian, 1951; Green and Maxwell, 1959; Knowles, 1959). 3) Neurosecretory material and the active agent are identical. Isolation and characterization of elementary neurosecretory granules should clarify the relationship between the granule material and the hormone itself.

Few neurosecretory hormones have been isolated and chemically identified. However, the vertebrate neural lobe hormones have been fully characterized and are a group of cyclic, cystein-containing octapeptides (du Vigneaud, 1956). The polypeptide nature of several other neurohormones from both vertebrates and invertebrates has also been confirmed. The activity of these low molecular weight hormones exhibits some regulatory effect, depending on the age and specie of the animal, on almost every physiological function in the body, including growth,

maturation, regeneration, somatic and retinal pigment movements, water balance, cardioregulation, morphogenesis, and reproduction (ranging from maturation of gametes and secondary sexual apparatus to oviposition). The references are too numerous to mention individual investigators; see reviews by Bern and Knowles (1966), Bern and Hagadorn (1965), and Hagadorn (1967).

Cyclical Variation of Neurosecretory Product

The sixth major criterium for neurosecretion is that the neurosecretory cells exhibit a cyclical variation related to an altered state in the physiology of the target organ or the animal. Many investigators have reported morphological changes in the granule content of neurosecretory cells after an appropriate sensory stimulation has been presented to the animal, such as hypertonic sea water, change in photoperiod, or a blood or high protein meal. A word of caution must be presented before examining the morphological evidence of cyclical variation in the neurosecretory granule content. Gabe (1966) has presented three categories of relationships between the morphologically detected neurosecretory material and the physiologically detected active agent. 1) The neurosecretory granules contain the active principle, in which case microscopic examination of the granules would furnish direct information of the functional stage of the cell. 2) The granule contains compounds which are more or less related to the metabolism of the active principle, such as a pro-hormone or the synthetic enzyme for the hormone. 3) The neurosecretory cell produces neurosecretory

granules and an active hormone, however the elaboration of the two may not run in parallel. Solely from the morphological and histochemical characteristics of these secretory granules, one could not determine the stage of the secretory cycle of the neuron. According to Gabe, unfortunately this type of situation is found most often.

It is difficult to assess the significance of the morphological evidence until the hormone produced by the particular neurosecretory cell has been isolated. It would also be necessary to examine the isolated granules for this hormone and any other molecules associated with it.

The present study describes evidence for an annual variation in the BCS protein content of the bag cells. This is correlated with other seasonal rhythms in the reproductive behavior of the animal. The annual variation in the neurosecretory granule content of these bag cells has not been investigated.

To summarize, there are six major criteria to delineate a neurosecretory neuron. All six criteria may not be met by all neurosecretory neurons from all phyla. However, since each criterium has its exceptions, it is important for the investigator to consider this set before labeling his system as neurosecretion.

1. Morphologically the cell should contain elementary neurosecretory granules of 1000 to 4000 Å diameter, and also contain the cytological apparatus for active protein synthesis.

2. The neurosecretory neurons are capable of generating and conducting an action potential.

3. The neurosecretory axons generally do not make synaptic contact with other neurons or effector organs.

4. The neurosecretory axon terminates in intimate contact with the circulatory system, often in a neurohemal organ.

5. The cell synthesizes, stores, and releases a unique substance, often a polypeptide, which has a hormonogenic function.

6. The cell exhibits a cyclical change in granule or hormone content related to a change in the physiology of the animal.

APPENDIX II

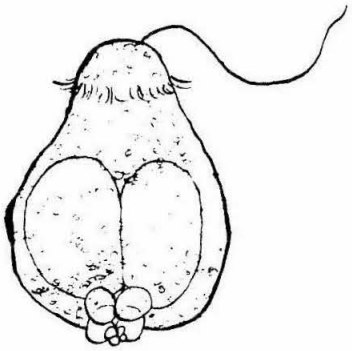
Development of Aplysia californica

An attempt was made to raise Aplysia californica to adulthood under laboratory conditions so that the development of its nervous system could be studied and manipulated. Although it was not possible to bring the veligers through metamorphosis, data were collected on the animals' development (Figures 24, 25) and behavior until metamorphosis begins.

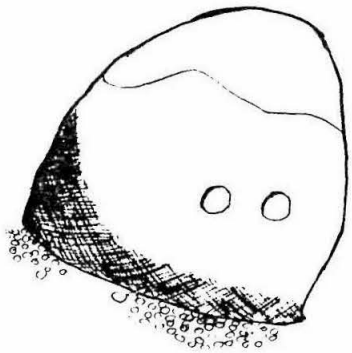
The hermaphroditic adult lays its eggs in linear strands which it forms into a tight mound upon sea weed or rocks. The ribbon, which contains over 100,000 eggs, is composed of compartments each holding 15-20 eggs. In general, all eggs within a compartment develop at the same rate; however, development in different compartments is not synchronized. The eggs closest to the cut end of the ribbon start to develop five to ten days sooner than those in the interior portions.

Spiral cleavage of the 100 μ fertilized egg begins 15-20 hours after laying with the first division complete within 45 minutes at 13°C. Within four days the embryo is an amorphous group of cells with the two large anal cells (Carazzi, 1905) protruding from the posterior pole. Epibolic gastrulation (Saunders and Poole, 1910) occurs on day six. The single-coiled pigmented shell is formed after the animal has developed a vellum and begins to rotate in the compartment. This occurs at about day 13-14.

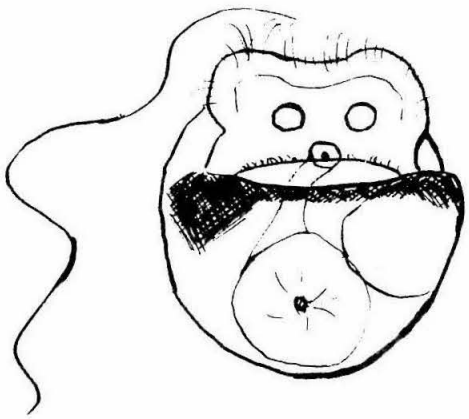
Figure 24: Drawings of various stages of development in live Aplysia californica. By 5 days after the first cleavage, the embryo is pear shaped with a row of cilia and a single flagellum at its anterior end, and 4 large protruding anal cells at the posterior pole. A nine day old embryo has secreted a hemispherical, pigmented shell, and has 2 hollow otocysts. Two major organs, the esophagus-stomach and the liver, are visible in the 19 day old rotating embryo, which also possesses a highly ciliated vellum, or oral hood. The single-coil shell is now completed. The 25 day old embryo, which has hatched from the egg case, is now a free-swimming veliger with a ciliated esophagus, gut, and intestine; a large pigmented, donut shaped liver and a smaller right liver; and a primordial kidney. The otolyths are now present in the otocysts. (Labeling of organs after Saunders and Poole, 1910.)



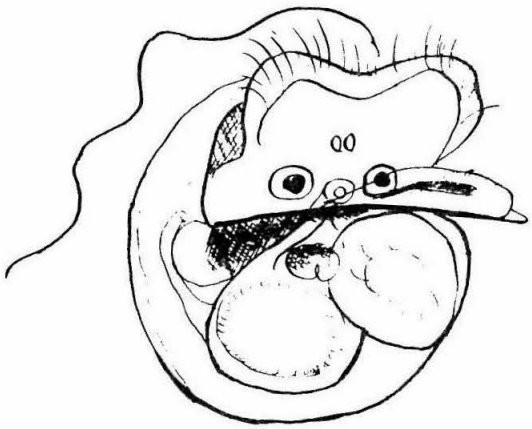
5 days
← 50μ →



9 days
← 50μ →

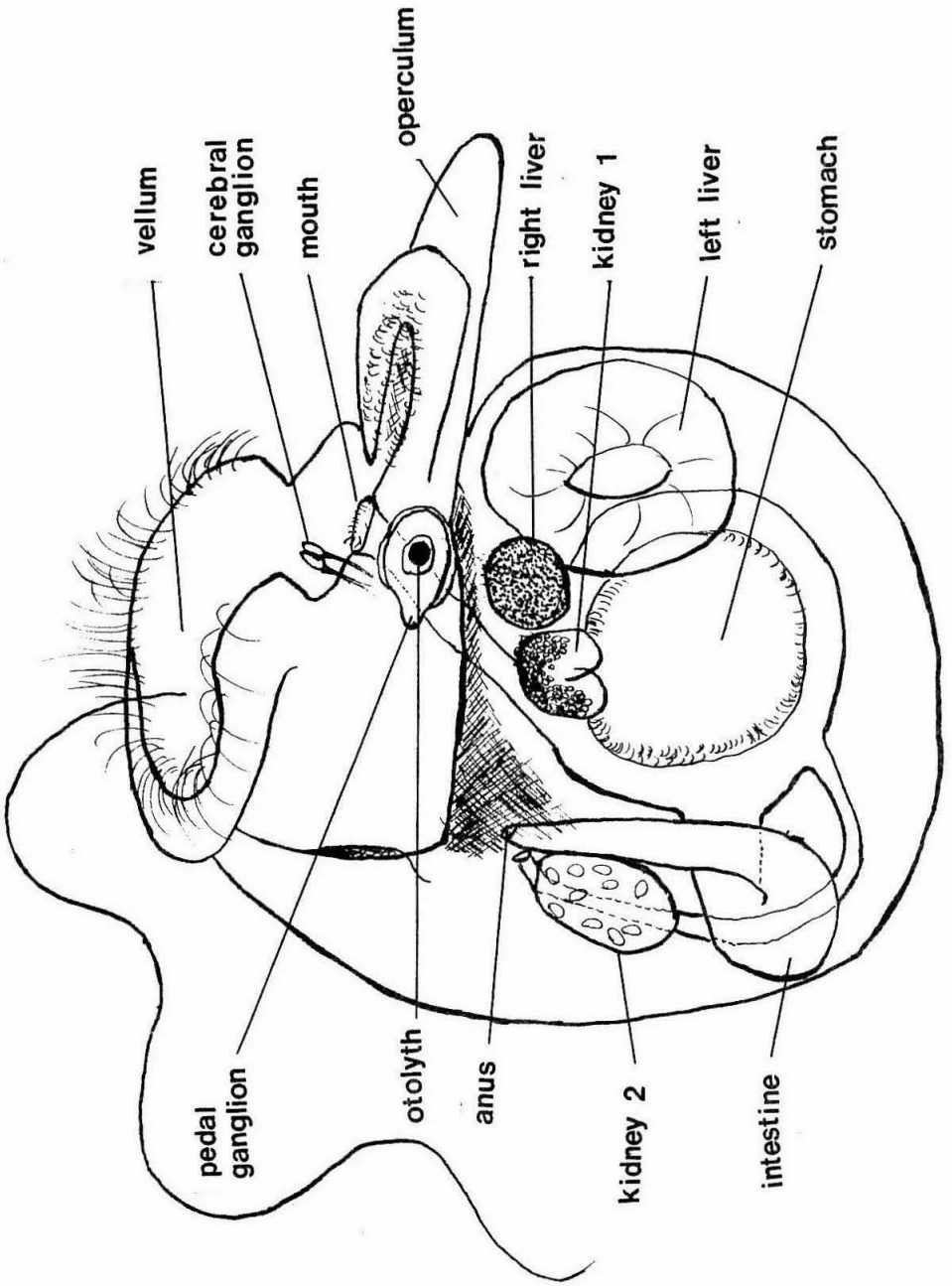


19 days
← 50μ →



25 days
← 50μ →

Figure 25: Drawing of a live 40 day free-swimming veliger from color photographs. The stomato-gastric system now has a second tube extending from the ciliated stomach to the mantle, and is surrounded by a structure having large vacuoles or cells. This structure is presumed to be one of the kidneys. Parts of the nervous system can now be seen in the living embryo. Two small, egg-shaped bodies are located directly above the mouth. These are presumed to be the cerebral ganglia, each having a nerve trunk traveling along beside either side of the esophagus. The presumed pedal ganglia can be seen budding from the otolyth containing otocysts. (Labeling of organs after Saunders and Poole, 1910.)


vellum
cerebral ganglion
mouth
operculum
right liver
kidney 1
left liver
stomach
pedal ganglion
otolyth
anus
kidney 2
intestine
40 days
←25μ→

The unhatched embryos exhibit an interesting sensitivity to ultraviolet light. With white, green, or blue light the animal spins in a counter-clockwise fashion when viewed through a compound microscope with epi- or trans-illumination; however under UV illumination within five to seven seconds the embryo stops rotating and retracts completely into its shell. A 0.01% acridine orange solution sensitizes the embryo to all light so that it now stops within five seconds upon illumination with white light and within one second upon illumination with ultraviolet light. At a stage of development four days later, the autofluorescence of the operculum is decreased and the responses to UV is gone unless the animal is first sensitized with acridine orange. Coincidental with the decrease in UV sensitivity is the development of the otolyths within the otocysts.

Hatching occurs at day 15-20 when the compartments break open and release the rotating and forward swimming veligers. The embryos, which were then fed daily from a sterile diatom (Nitchia) culture, showed a stereotyped feeding behavior. If a food-source was not present in the culture dish, the animal could be found on the bottom of the dish, motionless and completely retracted into its shell. Within two to five seconds, after a drop of culture medium containing diatoms was introduced into the dish, the embryo emerged and started rotating and swimming very actively up and down. They rotate counter-clockwise and catch bits of food with their cilia. When food is caught, the oral hood or vellum contracts and the food is swallowed; it can be seen entering the highly ciliated esophagus and entering the stomach.

Then the vellum opens and the animal again emerges and starts rotating in search of more food.

The metamorphic period, of an undetermined length, begins in week six when the 100 μ shell is lost and the 60-90 μ long veliger is free to swim in all directions or fasten itself securely to the culture dish with its foot. The operculum is retained with the shell-less animal. Shell-less embryos often die within one week, after transferring them to a sterile dish, even though diatoms are presented to them. This could be due to handling, starvation, or, perhaps, just a lack of the proper stimulus to trigger metamorphosis.

The developmental rate is doubled at 20°C, compared to the rate at 13°C. At the higher temperature, hatching occurs on day nine and metamorphosis begins at day 20 to 25. At 4°C, the rate of development is greatly reduced. In its final morphogenetic stage, the embryo raised in the cold still has surface anal cells, a vellum and only a fragment of the shell which never fully developed. This embryo never progresses beyond the stage of rotating movements to the free-swimming stage.

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