BIOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDIES OF THE EGG-LAYING HORMONE OF APLYSIA CALIFORNICA: PURIFICATION, PRIMARY STRUCTURE, NEUROSECRETION AND MORPHOLOGICAL DISTRIBUTION

Thesis by

Arlene Yuen-chin Chiu

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1981

(Submitted February 24, 1981)

to my parents

and my son

ACKNOWLEDGEMENTS

I am indebted to my advisor, Felix Strumwasser, for his tremendous patience, scientific acumen and unfailing ability to help me keep my goals in focus. His friendship and encouragement have sustained me through my graduate career. I am also very grateful to my thesis committee—Herschel Mitchell, Jeremy Brockes, Jim Hudspeth and Elias Lazarides—for sound scientific advice and for putting up with my constant use of their equipment. For support and assistance in ways too numerous to mention, I wish to thank all the members of the Strumwasser group. In particular, Duncan Stuart taught me the art of being positive, Len Kaczmarek offered constant good advice, Dan Viele showed us all how to be relaxed and productive at the same time and James Host assisted with great competence and humour. It has been a distinct pleasure collaborating with them.

My graduate education would not have been complete without the friendship of and many stimulating discussions with Dave Armstrong, Eileen Bagdonas, Dave Balzer, Steve Green, Mark Gurney, Mauri Krouse, Bruce Nicholson, Riz Sarmiento, Sandy Shotwell and Wilson Wu.

The financial support of the Lawrence A. Hanson Foundation, the Spencer Foundation, the Li Ming Scholarship Fund, the Jean Weigle Fund and the Biology Division of Caltech is gratefully acknowledged.

I am especially grateful to Richard Hammersclag who kindled my interest in neurobiology, and made me wonder about life beyond axonal transport.

Finally, my deepest appreciation goes to my husband Stanley for his seemingly endless patience, unwavering support and honest criticism. But for his contributions, the work leading to this thesis would not have been embarked upon.

ABSTRACT

Egg-laying behavior in Aplysia californica can be triggered by the introduction of a neuropeptide, the Egg-Laying Hormone (ELH) into the circulation. ELH is synthesized by the neurosecretory bag cells of the abdominal ganglion and released when these neurons are induced to fire repetitively. In this thesis, biochemical and immunohistochemical techniques have been employed to study the primary structure, release and distribution of ELH in the nervous system of Aplysia.

The purification of ELH to homogeneity from extracts of bag cell clusters, and the analysis of its primary structure are discussed in Chapter 1. A 100-fold enrichment of bioactive material was obtained by cation exchange chromatography (Sephadex SP C25) followed by gel filtration (BioRad P-6). This purified material was determined to be homogeneous by four lines of analysis: (i) SDS polyacrylamide gel electrophoresis, (ii) isoelectric focussing, (iii) microsequence analysis, and (iv) comparison of the amino acid compositions from acid hydrolysis and from microsequence data. ELH is a 36 amino acid, basic peptide with a calculated molecular weight of 4385 and an apparent isoelectric point of 9.0-9.2. Its amino acid sequence was determined as:

H-Ile-Ser-Ile-Asn-Gln-Asp-Leu-Lys-Ala-Ile-Thr-Asp-Met-Leu-Leu-Thr-Glu-Gln-Ile-Arg-Glu-Arg-Gln-Arg-Tyr-Leu-Ala-Asp-Leu-Arg-Gln-Arg-Leu-Leu-Glu-Lys-OH

Chapter 2 demonstrates the release of ELH, identified by molecular weight, pI and bioactivity, when bag cell clusters afterdischarge in vitro. During such synchronous and prolonged electrical activity, bag cell clusters, which have been pulsed in ^{35}S-Met, secrete at least four labeled presumed peptides of different molecular weights. One of these comigrates with ^{3}H-Leu labeled, purified ELH on gel filtration chromatography and causes egg laying when injected into a test animal. This material also comigrates with ^{3}H-ELH on isoelectrifocussing gels.

A second released peptide has a molecular weight of approximately 5-6 K and a pI of 4.8; its function, and the functions of the other released molecules, are unknown.

In order to study the distribution of ELH in the nervous system of Aplysia, antibodies were generated against the purified neuropeptide, coupled to a carrier molecule, thyroglobulin (Tg). Immune sera, enriched for anti-ELH antibodies by passage through an affinity column to remove antibodies which bound to Tg, was used for localizing ELH-like immunoreactivity in frozen sections of Aplysia ganglia. These results were discussed in Chapters 3 and 4.

When sections of the abdominal ganglion were stained by the PAP method for ELH, all neurons within the bag cell clusters were found to be immunoreactive. Except for occasionally displaced bag cells, all neurons within the ganglion remained unreactive, reflecting the specificity of the antiserum. Immunopositive processes from bag cells proliferate in the vascularized connective tissue capsule which serves as a neurohemal organ facilitating release of neurohormones. Some processes form a spiralling cuff around the nerve trunks of the pleuro-visceral connective and the vulvar nerves; others invade the ganglion in association with connective tissue septa which form partitions between groups of neurons. Immunoreactive fibers with varicosities are also found within the neuropile and the commissure between the two hemiganglia. This light microscopic visualization of the bag cell neuroendocrine system provides morphological support for the model of local hormone action of ELH upon other neurons in the abdominal ganglion. The immunoreactivity of all neurons within the clusters provides the strongest evidence to date of the homogeneity of the bag cell population.

Antibodies generated against ELH from A. californica selectively stained the bag cell systems of three other species of Aplysia - A. braziliana, A. vaccaria and A. dactylomela - which also share cross bioactivity. It is likely that receptor binding sites and antigenic determinants are conserved in their ELHs.

The fourth chapter describes the organization of cells and fiber tracts with ELH-like immunoreactivity, endogenous to the head ganglia. Each pleural ganglion has 1-5 immunopositive somata which are strikingly similar to bag cells in cell and nuclear sizes, process morphology and location. These similarities, coupled with the close developmental association of the pleural and abdominal ganglia, suggest a common heritage for both populations of ELH+ cells.

The ELH immunoreactive system in the cerebral ganglion consists of two laterally located clusters of small cells on the dorsal surface of the ganglion and extensive fiber tracts throughout the neuropile. The nature of immunoreactive molecules and the function of these systems within the cerebral and pleural ganglia are unknown. However, perfusion of ELH is known to induce long-term changes in the electrical activity of head ganglia neurons in vitro, and some of these changes may be linked to the suppression of feeding and locomotion during egg laying. The presence of these immunopositive systems in the pleural and cerebral ganglia raises the possibility that ELH target neurons in head ganglia may respond to local sources of ELH or ELH-like molecules.

TABLE OF CONTENTS

INTRODUCTION

With the exception of neurons which only make electrical contacts, all neurons secrete chemical signals to targets which receive the message by bearing the appropriate receptors. The functional distinction between neurosecretory cells and conventional neurons lies in the speed of delivery, the duration, and the spatial resolution and efficacy of the message. Conventional neurons release neurotransmitters which function over relatively small and limited areas for brief periods and mediate short-lived interactions between contacting neurons. The neurohormonal message, however, is generally slow in delivery, often long in duration and likely to be directed at a large number of widely dispersed, often nonneuronal targets. Often it is the actual 'command' for a behavioral act. These characteristics make neurosecretion a simple method for activating a complex program requiring the participation of many tissues, particularly if the response needs to persist for long periods of time. In simple systems, such as invertebrates, neurohormones control a wide range of functions such as growth (Schaller, 1973), development (Nijhout and Williams, 1974; Loeb, 1974; Truman and Schwartz, 1980), gonadal maturation (Clark, 1963; Baumann, 1974; Kanatani, 1979) and regeneration (Schaller, 1973; Marcel and Cardon, 1979) as well as slow regulatory processes such as osmoregulation (Golbard et al., 1970; Aston and White, 1974; Kupfermann and Weiss, 1976), heart rate (Traina et al., 1976; Greenburg and Price, 1980) and reproduction (Toevs and Brackenbury, 1969; Ram, 1977; Ram et al., 1977). With numerous sources of extraneuronal hormones in vertebrates, neurohormones frequently function as first order secretory products regulating the release of endocrine effector cells. Growth, development and ovulation are directly controlled by second or third order hormones.

The classical neurosecretory cell synthesizes, transports and releases a peptidergic neurohormone(s) into portal, systemic or hemocoelic circulation for distribution

to distant targets. However, recent experimental data now suggest a far broader role for many neurohormones. Immunohistochemistry has revealed the widespread presence of classical vertebrate neurohormones outside the hypothalamus (Swanson, 1977; Silverman and Zimmerman, 1978; Jackson and Reichlin, 1979; for review see Elde and Hökfelt, 1978); some have been specifically localized within conventional neurons and terminals in the central and peripheral nervous systems (Jan et al., 1980; Elde and Hökfelt, 1978). Electrophysiological studies demonstrate that neuropeptides can have profound and specific effects on the electrical activities of other neurons (Dyer and Dyball, 1974; Jan et al., 1979; for review see Renaud and Padjen, 1977). Moreover, posterior pituitary peptides have been implicated in memory p processes (de Wied and Verskeg, 1979) and LHRH in sexual behavior (Moss, 1979). It is now believed that neuropeptides, including neuroendocrine peptides, may be acting as modulators or as bona fide transmitters within the nervous system. Most recently, the intriguing finding that a neuroactive peptide and a conventional neurotransmitter can coexist within a single neuron has raised further speculations about the roles peptides may play in neurochemical communication (Hökfelt et al., 1980a,b).

Neurohormones are responsible for directly coordinating a much larger sector of activities in invertebrates (Berlind, 1977; Frontali and Gainer, 1977; Maddrell and Nordmann, 1979) than in vertebrates, and the questions raised about the roles of vertebrate neurohormones also apply to their invertebrate counterparts. However, because of the minute quantities of material present, information about the nature of most invertebrate neurohormones is limited. Identification of invertebrate neurosecretory cells has relied on patterns of staining with the conventional neurosecretory stains - paraldehyde-fuchsin and chromehematoxylin-phloxin - or has been characterized by an opalescent blue-white appearance under the microscope. Localization of bioactive material is determined by bioassaying extracts of various cells or ganglia.

However, details of specific intraganglionic distribution of a particular neurohormone are often difficult to ascertain. This thesis investigates the primary structure, the release and morphological distribution of an invertebrate neurohormone which acts on neuronal and nonneuronal targets to trigger egg-laying behavior in the sea hare, Aplysia californica.

The purification and amino acid sequence of this Egg-Laying Hormone (ELH) is described in Chapter 1. This basic (pI 9.0-9.2), 36 amino acid peptide is purified to homogeneity from extracts of the neurosecretory bag cell neurons of the abdominal ganglion. Its primary structure is unlike that of any known vertebrate hormone but shares some homology with the insect neuropeptide, Proctolin. Chapter 2 demonstrates the specific release of this pI 9, 4500 dalton molecule, along with several other presumed peptides, when bag cells are induced to a prolonged and synchronous discharge.

In order to visualize the distribution and organization of cells and processes bearing ELH or ELH-like molecules in the Aplysia nervous system, immunohistochemical procedures were employed. In Chapter 3, the topographical distribution of the bag cell system, including potential sites of release, is revealed when the abdominal ganglion is stained by the peroxidase-antiperoxidase method with antibodies generated against purified ELH. Bag cells send the majority of their hormone-laden processes to neurohemal areas in the vascularized connective tissue sheath overlying the whole ganglion. This greatly amplifies neurosecretory surfaces and facilitates release into the hemolymph. However other processes invade the interior of the ganglion and may be sites of local hormone action; ELH and other bag cell products are known to modulate the activities of other neurons within the abdominal ganglion (Mayeri, 1979).

By morphological, ultrastructural and electrophysiological criteria, bag cells appear very similar to each other (Frazier et al., 1967; Kupfermann and Kandel,

1970). We find all bag cells to be immunoreactive for ELH which strongly supports the argument that they are a homogeneous population of neurosecretory cells, synthesizing and releasing ELH. It is likely that bag cells also synthesize and release other peptides whose functions are as yet unknown.

Examination of other major ganglia in A. californica revealed two other systems with strong ELH-like immunoreactivity which are described in Chapter 4. The nature of the immunoreactive molecules in these ganglia, and the functions of these neurons are not presently known. Recently, an egg-releasing peptide, purified from the nonneuronal atrial gland of the Aplysia reproductive tract, is reported to share regions of homology with ELH (Schlesinger et al., 1981). There may be a family of ELH-like molecules with different functions and varying degrees of bioactivity. ELH is known to alter the electrical activity of certain neurons in the head ganglia (Stuart and Strumwasser, 1980), and these changes are likely to be involved in the inhibition of feeding and locomotion characteristic of egg-laying behavior. It is possible that these neurons respond to local sources of ELH or ELH-like peptides within the head ganglia.

Immunopositive cells and processes in the pleural ganglia bear a striking resemblance to those of the bag cell system and may also be neurosecretory in function. In contrast, the cerebral ganglion neurons with ELH-like immunoreactivity appear to send all their processes into the neuropile which bears massive tracts of stained fibers. These results raise the possibility that ELH, like many vertebrate neurohormones, may also function as a modulator or a transmitter in the Aplysia nervous system.

REFERENCES

Aston, R. J. and White, A. F. 1974. Isolation and purification of the diuretic hormone of Rhodinus prolixus. J. Insect Physiol. **20**:1673-1682.

Baumann, H. 1974. The isolation, partial characterization and biosynthesis of the paragonial substances PS1 and PS2 of Drosophila funebris. J. Insect Physiol. **20**:2181-2194.

Berlind, A. 1977. Cellular dynamics in invertebrate neurosecretory systems. Int. Rev. Cytol. **49**:171-251.

Clark, R. B. 1963. Endocrinology and reproductive biology of polychaetes. In: "Oceanography and Marine Biology, An Annual Review. 3.", H. Barnes, ed., London, Allen and Unwin.

de Wied, D. and Verskeg, D. H. G. 1979. Neurohypophyseal principles and memory. Fed. Proc. **38**: 2348-2354.

Dyer, R. G. and Dyball, R. E. J. 1974. Evidence for a direct effect of LRF and TRF on single unit activity in the rostral hypothalamus. Nature **252**: 486-488.

Elde, R. and Hökfelt, T. 1978. Distribution of hypothalamic hormones and other peptides in the brain. In: "Frontiers in Neuroendocrinology", L. Martini and W. F. Ganong, eds., Raven Press, New York, pp. 1-33.

Frazier, W. T., Kandel, E. R., Kupfermann, I., Waziri, R. and Coggeshall, R. E. 1967. Morphological and functional properties of identified neurons in the abdominal ganglion of Aplysia californica. J. Neurophysiol. **30**:1288-1351.

Frontali, N. and Gainer, H. 1977. Peptides in invertebrate nervous systems. In: "Peptides in Neurobiology", H. Gainer, ed., Plenum Press, New York, pp. 259-294.

Goldbard, G. A., Sauer, J. R. and Mills, R. R. 1970. Hormonal control of excretion in the American cockroach. II. Preliminary purification of a diuretic and antidiuretic hormone. Comp. Gen. Pharmacol. **1**:32-86.

Greenberg, M. J. and Price, D. A. 1980. Cardio-regulatory peptides in molluscs. In: "Peptides: Integrators of Cell and Tissue Function", F. E. Bloom, ed., Society of General Physiologists Series, Vol. 35, Raven Press, New York.

Hökfelt, T., Lundberg, J. M., Schultzberg, M., Johansson, O., Ljungdahl, Å, and Rehfeld, J. 1980a. Coexistence of peptides and putative transmitters in neurons. In: "Neural Peptides and Neuronal Communication", E. Costa and M. Trabuschi, eds., Advances in Biochemical Psychopharmacology, Vol. 22, Raven Press, New York, pp. 1-24.

Hökfelt, T., Johansson, O., Ljungdahl, Å, Lundberg, J. M. and Schultzberg, M. 1980b. Peptidergic Neurones. Nature **284**:515-521.

Jackson, I. M. D. and Reichlin, S. 1979. Distribution and biosynthesis of TRM in the nervous system. In: "Central Nervous System Effects of Hypothalamic Hormones and Other Peptides", Raven Press, New York, pp. 3-54.

Jan, Y. N., Jan, L. Y. and Kuffler, S. W. 1979. A peptide as a possible transmitter in sympathetic ganglia of the frog. Proc. Natl. Acad. Sci. USA **76**:1501-1505.

Jan, L. Y., Jan, Y. N. and Brownfield, M. S. 1980. Peptidergic transmitters in synaptic boutons of sympathetic ganglia. Nature **288**:380-382.

Kanatani, H. 1973. Maturation-inducing substance in starfishes. Int. Rev. Cytol. **35**:253-298.

Kupfermann, I. and Kandel, E. R. 1970. Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of Aplysia. J. Neurophysiol. **33**:865-876.

Kupfermann, I. and Weiss, K. R. 1976. Water regulation by a presumptive hormone contained in identified neurosecretory cell R15 of Aplysia. J. Gen. Physiol. **67**:113-123.

Loeb, M. J. 1974. Strobilation in the Chesapeake Bay sea nettle, Chrysaora quinquecirrha II: Partial characterization of the neck-inducing factor from strobilating polyps. Comp. Biochem. Physiol. **47A**:291-301.

Maddrell, S. H. P. and Nordmann, J. J. 1979. "Neurosecretion", Halsted Press, John Wiley and Sons, New York.

Marcel, R. and Cardon, C. 1979. Partial purification and characterization of the peptide which inhibits cephalic regeneration in Eisenia foetida Sav. (oligochaeta, Annelida). Comp. Biochem. Physiol. **63B**:233-237.

Mayeri, E. 1979. Local hormonal modulation of neural activity in Aplysia. Fed. Proc. **38**:2103-2108.

Moss, R. L. 1979. Actions of hypothalemic-hypophysiotropic hormones on the brain. A. Rev. Physiol. **41**:617-631.

Nijhout, H. F. and Williams, C. M. 1974. Control of moulting and metamorphosis in the tobacco hornworm, Manduca sexta (L.): growth of the last-instar larva and the decision to pupate. J. Exp. Biol. **61**:481-491.

Ram, J. 1977. Hormonal control of reproduction in Busycon: laying of egg capsules caused by nervous system extracts. Biol. Bull. **152**:221-232.

Ram, J., Salpeter, S. R. and Davis, W. J. 1977. Pleurobranchaea egg-laying hormone: localization and partial purification. J. Comp. Physiol. **119**:171-194.

Renaud, L. P. and Padjen, A. 1977. Electrophysiological analysis of peptide actions in neural tissue. In: "Centrally Acting Peptides", J. Hughes, ed., University Park Press, Baltimore, pp. 59-84.

Schaller, H. C. 1973. Isolation and characterization of a bow molecular weight substance activating head and bud formation in Hydra. J. Embryol. Exp. Morphol. **29**:27-38.

Schlesinger, D. H., Babirak, S. P. and Blankenship, J. E. 1981. Primary structure of an egg-releasing peptide from the atrial gland of Aplysia californica. In: "Symposium on Neurohypophyseal Peptide Hormones and Other Biologically Active Peptides", D. H. Schlesinger, ed., Elsevier, N. Holland Publ. Company.

Silverman, A. J. and Zimmerman, E. A. 1978. Pathways containing luteinizing hormone-releasing hormone (LHRH) in the mammalian brain. In: "Brain Endocrine Interaction III. Neural Hormones and Reproduction", 3rd International Symposium, Wurzburg, Kargon, Basel, pp. 83-96.

Stuart, D. K. and Strumwasser, F. 1980. Neuronal sites of action of a neurosecretory peptide, egg-laying hormone, in Aplysia californica. J. Neurophysiol. **43**:499-519.

Swanson, L. W. 1977. Immunohistochemical evidence for a neurophysin-containing autonomic pathway arising in the paraventricular nucleus of the hypothalamus. Brain Res. **128**:346-353.

Toevs, L. A. and Brackenbury, R. W. 1969. Bag cell-specific proteins and the humoral control of egg-laying in Aplysia californica. Comp. Biochem. Physiol. **29**:207-216.

Traina, M. E., Bellino, M., Serpietri, L., Massa, A. and Frontali, N. 1976. Heart accelerating peptides from cockroach corpora cardiaca. J. Insect Physiol. **22**:323-329.

Truman, J. W. and Schwartz, L. M. 1980. Peptide hormone regulation of programmed death of neurons and muscle in insects. In: "Peptides: Integration of Cell and Tissue Function", F. E. Bloom, ed., Society of General Physiologists Series Vol. 35, Raven Press, New York.

CHAPTER 1

Purification and primary structure of the neuropeptide egg-laying hormone of Aplysia californica

Reprinted from
Proc. Natl. Acad. Sci. USA
Vol. 76, No. 12, pp. 6656–6660, December 1979
Neurobiology

Purification and primary structure of the neuropeptide egg-laying hormone of *Aplysia californica*

(bag cells/amino acid sequence/neurosecretory peptide/molluskan neuropeptide/microsequence analysis)

A. Y. CHIU, M. W. HUNKAPILLER, E. HELLER, D. K. STUART*, L. E. HOOD, AND F. STRUMWASSER†

Division of Biology, California Institute of Technology, Pasadena, California 91125

Communicated by Theodore H. Bullock, August 15, 1979

ABSTRACT Egg-laying hormone (ELH), a neuropeptide synthesized by the bag cell neurons, induces egg laying and its correlated behavior in *Aplysia californica*. In the present study, ELH has been purified to homogeneity and its primary structure has been determined. We find this molecule to have 36 amino acid residues with a M_r of 4385 and a calculated isoelectric point of 9.7. Direct microsequence analysis revealed a single amino acid sequence that is in agreement with the amino acid composition determined after acid hydrolysis of ELH: H-Ile-Ser-Ile-Asn-Gln-Asp-Leu-Lys-Ala-Ile-Thr-Asp-Met-Leu-Leu-Thr-Glu-Gln-Ile-Arg-Glu-Arg-Gln-Arg-Tyr-Leu-Ala-Asp-Leu-Arg-Gln-Arg-Leu-Leu-Glu-Lys-OH. Enzyme data indicate that the COOH-terminal lysine may be modified but its exact nature remains to be determined. There is no similarity between the amino acid sequence of ELH and that of presently known vertebrate neuropeptides. The two-step purification procedure, starting with a homogenate of bag cell clusters, consisted of cation exchange chromatography on SP C25 (Sephadex) followed by gel filtration on Bio-Gel P-6. Our purification results in a 100-fold enrichment of ELH from bag cell homogenates and a 36% recovery of purified radiolabeled marker ELH. Analysis of purified ELH radiolabeled with [^{35}S]methionine or [^{3}H]leucine on isoelectric focusing gels and on 8 M urea/sodium dodecyl sulfate gels showed only a single peak containing 90% of the radiolabel. Radiolabeled ELH migrated with a pI of 9.0–9.2 and an apparent M_r of 3500–5700. ELH retained egg-laying bioactivity when eluted from this segment of the gel. We find that 2.5 nmol of pure ELH consistently induces egg laying at 20°C.

In molluscs, a clear example of neurohormone-induced behavior is the egg laying in *Aplysia californica* evoked by a neuropeptide egg-laying hormone (ELH) (1, 2). ELH is synthesized by two symmetrical populations of about 400 neurosecretory bag cells each, clustered around the base of the pleurovisceral connectives at the rostral edge of the abdominal ganglion (3). When a homogenate of bag cells, containing ELH, is injected into the hemocoel of a sexually mature animal, a pattern of egg-laying behavior is triggered which results in egg laying within 65 min at 14°C (1, 4). Egg-laying behavior typically consists of locomotion to a stationary position on a vertical surface, puckering of mouth musculature, inhibition of feeding, and increased frequency of head weaving movement, culminating in the extrusion and winding of the egg string into a compact knot attached to the vertical substrate (5, 6).

Bag cell neurons are normally electrically silent but can be triggered to produce a synchronous afterdischarge of action potentials lasting 20–60 min when electrically stimulated (7). Recent findings of gap junctions in bag cell clusters support the premise that this synchrony of activity is due to electrical coupling between bag cell neurons (8). *In vitro* experiments have demonstrated the release of radiolabeled and bioactive ELH only when bag cells afterdischarge (9). *In vivo*, such an afterdischarge, whether induced or spontaneous, is always followed by egg laying (10, 11).

Previous studies have characterized ELH as a basic peptide of M_r approximately 6000 with an isoelectric point of 9.0–9.3 (12, 13). We have purified ELH by a simple two-step procedure to yield a homogeneous product with biological activity. We find the molecule to be a polypeptide of 36 amino acids with a calculated M_r of 4385 and migrating with a pI of 9.0–9.2. We now report its amino acid sequence.

MATERIALS AND METHODS

Egg-Laying Bioassay. Material, at various stages of purification, was checked for its ability to induce egg laying by injection through the foot into the hemocoel of a sexually mature *A. californica* maintained at 20°C as described (9). A test was considered to be positive if eggs were sighted within 90 min of injection. Injection volumes ranged from 1 to 3 ml and usually consisted of an aliquot of test material in low-ionic strength buffer mixed with an equal volume of filtered seawater.

Bag Cell Clusters, Radiolabeling, and Homogenization. *A. californica* (>270 g) were dissected within 2 days after collection from the Palos Verdes Peninsula during October and November of 1977 and 1978. There is some correlation between body weight and reproductive tract weight, and the latter, in particular, is an indicator of sexual maturity (1). Our starting material of bag cell clusters was obtained from sexually mature animals. Bag cell clusters were cut free from the rest of the abdominal ganglia in ice-cold filtered seawater and stored at −20°C.

For radiolabeling ELH, Millipore-filtered (0.22 μm) natural seawater with a final concentration of 10 mM glucose was buffered to pH 8.0 at 25°C by 20 mM Na Hepes and supplemented with 150 μg each of K penicillin G and streptomycin sulfate per ml. L-[4,5-^{3}H]Leucine [250 μCi (1 Ci = 3.7×10^{10} becquerels); Amersham; specific activity, 100 Ci/mmol] or [^{35}S]methionine (350 μCi; New England Nuclear; specific activity, 450 Ci/mmol) was taken to dryness under forced air in a boiling water bath and redissolved in the above medium. Freshly dissected abdominal ganglia were incubated in radiolabeled medium for 16 hr at room temperature (20°C) and then subjected to a series of rinses in filtered seawater lasting 2 hr. The bag cell clusters were dissected out and kept frozen at −20°C until used. To obtain radiolabeled ELH, 6 pairs of labeled clusters were ground with 34 pairs of unlabeled tissue.

In a typical preparation, 40 pairs of frozen bag cell clusters

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: ELH, egg-laying hormone; >PhNCS, phenylthiohydantoin; IEF, isoelectric focusing; $NaDodSO_4$, sodium dodecyl sulfate.

* Present address: Department of Molecular Biology, University of California, Berkeley, CA 94720.
† To whom correspondence should be addressed at Division of Biology, California Institute of Technology, Pasadena, CA 91125.

were homogenized by hand in ice-cold 10 mM Na phosphate pH 6.0 (at 25°C; standard buffer) with phenylmethylsulfonyl fluoride. The homogenate was centrifuged in a Sorvall RC 2B at 12,000 × *g* for 20 min and the supernatant was dialyzed (Spectrapor 3 membrane, cutoff at M_r 3500) against two changes of 200 vol of standard buffer for 2 hr at 4°C. The retained material was then ready for purification.

Total protein in samples was measured by absorbance at 215 nm with a Beckman DB-G spectrophotometer. The sensitivity was 0.052 A_{215} units/10 μg of bovine serum albumin (used to generate a standard curve) per ml.

Amino Acid Composition. For analysis of amino acid content, ELH was hydrolyzed in two ways: in 6 M HCl under reduced pressure at 110°C for 24 hr, and in 3 M mercaptoethanesulfonic acid under reduced pressure at 110°C for 24 hr. Peptide fragments were subjected to the HCl hydrolysis only. All amino analyses were performed on a Durrum D500 amino acid analyzer.

Radiolabeled ELH was cleaved by 24-hr incubation in 6-fold (wt/wt) excess cyanogen bromide in 70% formic acid under nitrogen at 20°C. The reaction was stopped by dilution with distilled water, and the sample was frozen and lyophilized. In one experiment, the reaction products, a mixture of whole hormone and its cleavage products, were subjected directly to sequence determination in tandem. In a second experiment, the reaction products were redissolved in distilled water and separated on Bio-Gel P-6 in 10 mM Na phosphate, pH 7.5/50 mM NaCl. The radioactive peak migrating with a much higher K_{av} than ELH was again chromatographed on Bio-Gel P-2 and then on Bio-Gel P-4, both equilibrated in 1 M acetic acid. After these two additional steps of gel filtration, the major peak of radiolabel was subjected to microsequence analysis and to 6 M HCl hydrolysis and was found to be fragment 14–36 of ELH.

Sequence Determination. Automated Edman degradation was performed on the intact hormone and on the two peptides generated by cyanogen bromide cleavage by using a sequenator program similar to that described (14). The program included a 30-min coupling step using 0.3 M Quadrol buffer, a single 5-min cleavage step, and automated conversion of the anilinothiazolinones to phenylthiohydantoin (>PhNCS) amino acids by treatment with 25% aqueous trifluoroacetic acid for 45 min. The cup and conversion flask were maintained at 52°C.

The spinning cup sequenator used for the Edman degradation was designed and built at Caltech and will be described in detail elsewhere. It incorporates many of the design features described by Wittmann-Liebold (15) and Hunkapiller and Hood (14), with further refinements in the delivery head assembly and reagent/solvent reservoir system, and is equipped with a straight-edge cup from Beckman.

Polybrene (Aldrich, 6 mg) and glycylglycine (100 nmol) dissolved in 0.5 ml of distilled water were loaded into the sequenator cup and subjected to five complete degradation cycles. The peptide to be sequenced was then loaded and the sequencing program commenced with the coupling stage. >PhNCS amino acids were identified by high-performance liquid chromatography (HPLC) on a DuPont Zorbax CN column. Details on identification of >PhNCS amino acids and standard chromatograms are described elsewhere (16).

Isoelectric Focusing Gels (IEF). IEF gels were prepared and run as reported (9). On extrusion, gels were immediately sliced into 1.4-mm segments. To locate peaks of radioactivity, each slice was added to 5 ml of Wilson's cocktail (17) for assay in a Beckman LS scintillation counter.

Bioactivity was determined by incubation of gel segments in two changes of standard buffer and injecting the combined perfusates (total volume, 2 ml) into receptive test animals. To obtain the pH gradient, an equivalent gel was run concurrently and sliced into 2.8-mm segments, each of which was extracted in 1 ml of 100 mM KCl for several hours; pH was then measured directly with a Beckman Futura 39505 electrode and a Beckman Model 76 pH meter.

When 8 M urea IEF gels were used, normal IEF gels were made to 8 M urea and samples were brought to a final concentration of 3 M urea. Electrode buffers and other conditions were identical to those of normal IEF gels. Gradients of pH were similar in normal and urea IEF gels, and in both cases, cytochrome *c* was used in every gel as a reference marker.

Sodium Dodecyl Sulfate ($NaDodSO_4$) Gel Electrophoresis. $NaDodSO_4$/8 M urea slab gels were made according to the method of Swank and Munkres (18) with a final concentration of 12.5% acrylamide and a bisacrylamide/acrylamide ratio of 1:10. Standard mixtures of myoglobin (17,200), cytochrome *c* (12,300), α-bungarotoxin (7800), bovine pancreatic trypsin inhibitor (6160), insulin (5700), and glucagon (3500) were applied to wells on either side of the sample and run simultaneously. Gels were run at 15°C, to minimize diffusion, at a constant voltage of 65 V for 12–18 hr. The length of the gel containing the sample was then cut out and sliced, crosswise, into 1.4-mm segments for assay of radioactivity as described for IEF gels. The flanking slabs were stained and destained to reveal standards.

RESULTS

Hormone Purification. All operations were carried out at 4°C.

Cation exchange chromatography (SP C25 Sephadex). A dialyzed homogenate of bag cell clusters was diluted with standard buffer to a final volume of 30 ml and applied to a 6.0 × 1.5 cm column of SP C25, equilibrated to the standard buffer. After a rinse with 50 ml of standard buffer, a linear gradient made with 50 ml of standard buffer and 50 ml of 20 mM Na phosphate, pH 6.5/250 mM NaCl was applied. Two major peaks of protein were eluted at approximately 65 and 144 mM NaCl (Fig. 1). Biological activity was observed only in the first peak. Excluded material and rinse fractions also did not show any biological activity. A flow rate of 30 ml/hr was generated by gravity, and a Gilson microfractionator collected 80 fractions (1.1 ml).

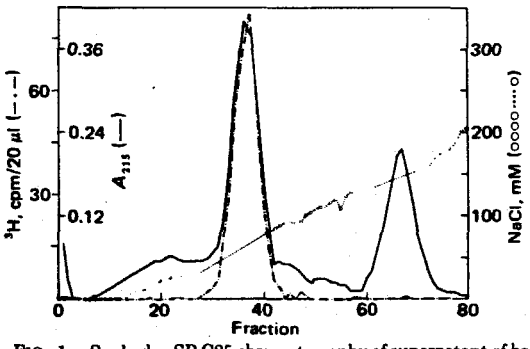


FIG. 1. Sephadex SP C25 chromatography of supernatant of bag cell extract after dialysis in standard buffer. Purified [^{3}H]leucine-labeled ELH was added as a marker to the supernatant. The column (6.0 × 1.5 cm) was equilibrated with standard buffer, and a gradient of 50 ml each of standard buffer and 20 mM Na phosphate, pH 6.5/250 mM NaCl was applied. The fraction volume was 1.1 ml and flow rate was 30 ml/hr. A background of 20 cpm was subtracted. Bioactivity coincided with the labeled marker ELH peak. Salt concentration was measured by conductivity on a conductivity meter (type CDM 2d, Radiometer, Copenhagen).

Gel filtration (Bio-Gel P-6). After cation exchange chromatography, fractions of the biologically active peak (Fig. 1, fractions 32–42) were pooled, lyophilized to a final volume of 0.8 ml, and applied onto a 50 × 1.0 cm Bio-Gel P-6 column equilibrated with 10 mM Na phosphate, pH 7.5/100 mM NaCl. Three protein peaks were seen in the A_{215} profile (Fig. 2). A small peak of material was excluded followed by the major peak of protein with a K_{av} of 0.2 (fractions 36–43) and another peak eluting with a K_{av} of 0.9 (fractions 70–75). Biological activity was only found in the major, middle peak, and this peak contained only ELH.

When radiolabeled pure marker ELH was copurified with an unlabeled bag cell cluster homogenate, the radioactive peak of ELH comigrated only with biologically active fractions in both steps of purification (Figs. 1 and 2).

In early experiments, a four-step procedure was used to purify ELH, which included ammonium sulfate precipitation and anion exchange chromatography followed by the cation exchange chromatography and gel filtration steps described above. ELH purified by this lengthy procedure was subjected to partial sequence analysis and was found to be homogeneous as judged by the presence of only one NH_2-terminal sequence. Later, the simpler two-step procedure was found to yield ELH which, when sequenced, was identical in purity and content to ELH obtained by the four-step method. Radiolabeled material purified by either procedure produced only one radioactive peak on IEF gels and on rechromatography on Bio-Gel P-6.

Amino Acid Sequence and Composition of ELH. The amino acid sequence of ELH and high-performance liquid chromatography data from a sequenator run with 4 nmol of intact hormone are shown in Fig. 3. Similar results were obtained from runs with 2 nmol of the cyanogen bromide-cleaved fragments. In Table 1, amino acid composition of ELH after acid hydrolysis is compared with that obtained from sequence data of the intact hormone. Because there is a decrease in yield of subsequent amino acids as one progresses toward the COOH terminus during sequence determination, ELH_{14-36} was first isolated and then examined to confirm the COOH-terminal half of the molecule. Fragment 14–36 was obtained by cyanogen bromide cleavage and was separated from undigested hormone and from fragment 1–13 by gel filtration. This COOH-terminal fragment was subjected to sequence determination and separately hydrolyzed for amino acid composition (Table 1) For

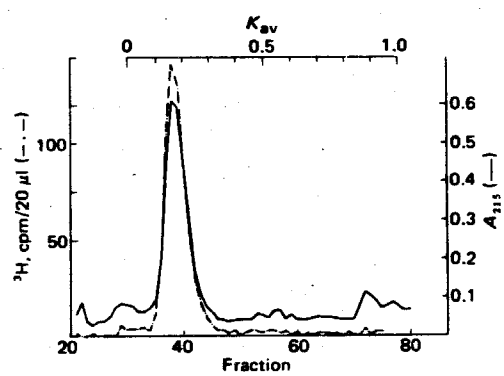


FIG. 2. Bio-Gel P-6 fractionation of biologically active material after cation exchange chromatography (Fig. 1, fractions 32–42). This material was pooled, concentrated to 0.6–0.8 ml, and applied to a 50 × 1 cm P-6 column equilibrated with 10 mM Na phosphate, pH 7.5/100 mM NaCl. Each fraction was 0.6 ml and flow rate was 4 ml/hr. Radioactivity was from [^{3}H]leucine-labeled marker ELH.

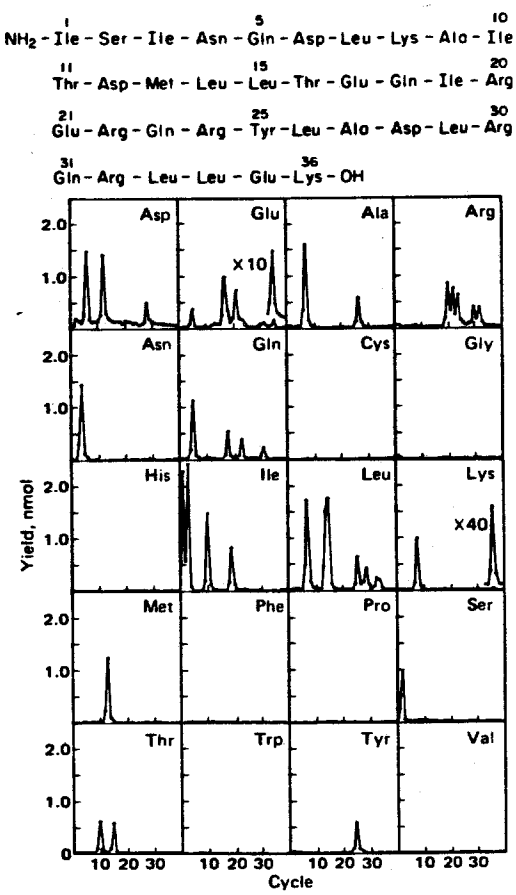


FIG. 3. Amino acid sequence of ELH and yields of >PhNCS amino acids from an NH_2-terminal sequenator analysis of 4 nmol of the neuropeptide. Aliquots of each cycle were analyzed by high-performance liquid chromatography; peak heights were converted to nmol for each derivative by using values for standard mixtures of >PhNCS amino acids and the yields were normalized to a 100% injection.

both whole ELH and ELH_{14-36}, sequence content and amino acid composition results match well. The COOH-terminal sequence was also confirmed by isolation of a tetrapeptide (Lys, Glu, Leu_2), from a tryptic digest of ELH succinylated in 5 M guanidinium hydrochloride, that corresponds to the terminal Leu-Leu-Glu-Lys sequence.

Isoelectric Focusing and Sodium Dodecyl Sulfate ($NaDodSO_4$) Gel Analyses. When [^{3}H]leucine-labeled purified ELH was analyzed on normal IEF gels, 90% of total radioactivity was recovered as a single peak in gel segments corresponding to a pI of 9.0–9.2 (Fig. 4). Bioassayable activity was eluted only from these regions of an equivalent gel. These results are identical to those of Arch and colleagues (13) in their characterization of ELH. The profiles of [^{3}H]ELH, biological activity and marker cytochrome *c* were similar on both 8 M urea and normal IEF gels.

For M_r analysis of polypeptides, radiolabeled ELH was run on a 12.5% $NaDodSO_4$/8 M Urea gel; 90% of total radioactivity comigrated with insulin (M_r, 5700) and slightly ahead of a marker of lower molecular weight, glucagon (M_r 3500) (Fig. 5).

ELH Recovery and Content. Table 2 summarizes the re-

Table 1. Comparison of amino acid contents of ELH and ELH_{14-36} determined from microsequence analysis and from acid hydrolysis

	ELH			ELH_{14-36}*	
	a	*b*	*c*	*a*	*b*
Asx	4	3.6	4.0	1	1.3
Thr	2	1.7	1.9	1	0.7
Ser	1	0.7	0.7	—	—
Glx	7	7.2	7.4	6	6.4
Ala	2	2.1	2.4	1	1.1
Met	1	1.0	1.3	—	—
Ile	4	4.2	3.0	1	1.2
Leu	7	7.7	7.1	6	6.4
Tyr	1	0.9	1.1	1	0.5
Lys	2	1.9	2.2	1	1.1
Arg	5	4.5	4.8	5	5.0

Column *a* lists amino acid composition derived from microsequence analysis. Column *b* lists molar ratios of amino acids obtained after 6 M HCl hydrolysis of the peptide. Column *c* lists molar ratios of amino acids obtained after hydrolysis by mercaptoethanesulfonic acid.

* ELH_{14-36} was obtained from cyanogen bromide cleavage of intact ELH followed by gel filtration.

covery of ELH during the two-step purification. When 117.5 μg of purified [^{3}H]leucine-labeled ELH was added to a dialyzed homogenate of 35 pairs of unlabeled bag cell clusters, the specific activity of this mixture was 628 cpm/mg of total protein. Upon elution with a salt gradient, 45% of counts applied to the SP C25 column was recovered in the protein peak with biological activity (Fig. 1) which had a specific activity of 46.8 cpm/μg; 24% of the total radiolabel input was excluded by the cation exchanger. Because the excluded material never exhibited any biological activity, these excluded counts may represent inactive fragments of ELH after proteolytic degradation as a result of extraction. An alternate possibility is that ELH has a high affinity for some other cell product and the complex formed by their interaction neutralized the basic nature of free ELH and prevented the retention of ELH by the cation exchanger. If this were the case, the complexed ELH also would have no biological activity.

After fractionation on Bio-Gel P-6, 80% of the applied radioactivity was recovered in fractions with a K_{av} of 0.2 (Fig. 2). The specific activity of this purified ELH was 62.7 cpm/μg, a 100-fold increase from the initial specific activity of 0.628 cpm/μg of the homogenate. A total recovery of 36% was derived from the product of the percentage recoveries of radiolabel in the ELH peaks after cation exchange and gel filtration.

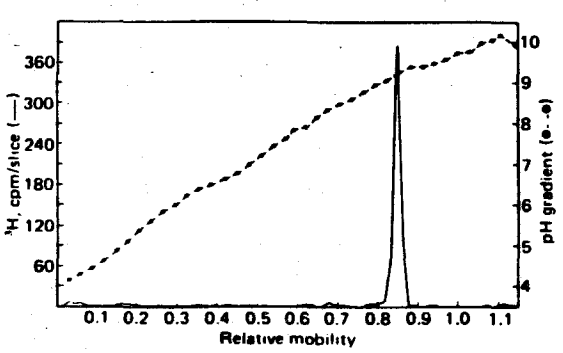


FIG. 4. Isoelectric focusing of purified [^{3}H]leucine-labeled ELH from fractions 36–43 of the Bio-Gel P-6 gel filtration (Fig. 2). The pH gradient was obtained from an equivalent gel run concurrently. Mobility is relative to the internal marker cytochrome *c*, pI 9.4–9.6.

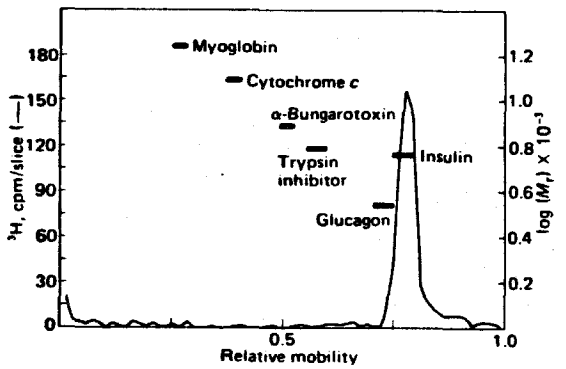


FIG. 5. $NaDodSO_4$/8 M urea/12.5% polyacrylamide gel electrophoresis of purified [^{3}H]leucine-labeled ELH from fractions 36–43 (Fig. 2). Slab gels were used and the six standards were applied to wells flanking the sample well. Mobility of Coomassie blue-stained standards and of radioactivity in gel slices were calculated relative to that of bromphenol blue.

DISCUSSION

We have established the purity and homogeneity of ELH prepared by our procedures by four types of analysis: amino acid sequence, amino acid composition, IEF, and $NaDodSO_4$/polyacrylamide gel electrophoresis. Microsequence analysis of purified ELH consistently revealed only one polypeptide chain. Moreover, it shows a good match with the amino acid composition after conventional acid hydrolysis of biologically active ELH. There is therefore no evidence of the presence of a contaminant with a blocked NH_2 terminus that may have copurified with the molecule analyzed. Gel analyses of radiolabeled ELH revealed one band of radioactivity that migrated with a pI of 9.0–9.2 on IEF gels and an apparent mass of 5700–3500 daltons on 8 M urea/$NaDodSO_4$ gels. In each case, the single peak contained 90% of the total radioactivity recovered on the gel. Biologically active material was eluted only from these same regions of equivalent gels. A conclusive demonstration of the identity of ELH will require that the peptide be synthesized and shown to be bioactive.

Based on the amino acid sequence, ELH has a M_r of 4385. Previous studies have characterized ELH as a 6000-dalton peptide on the basis of gel filtration on Sephadex G-50 (12). However, on Bio-Gel P-6 (exclusion size, M_r 6000) ELH is in the included volume with a K_{av} of 0.2 which is compatible with a M_r of 4000–5000. On 8 M urea/$NaDodSO_4$ gels, ELH comigrates with insulin (M_r 5700) and glucagon (M_r 3500). However, the rate of migration on such gels may be greatly affected by other considerations such as charge and shape, particularly in the case of oligopeptides (18). Clearly, $NaDodSO_4$ gels and gel filtration studies at best provide approximations of the M_r for such small peptides, and previous and present data from such analyses are not incompatible with the M_r of 4385 calculated from amino acid content. It is conceivable that this mass could be increased if polysaccharides were associated with ELH, although sequence analysis shows no polysaccharides.

The amino acid sequence revealed a greater number of basic residues than acidic ones, which explains the basic nature of ELH that persists even after denaturing by 8 M urea. If the COOH-terminal residue is an unblocked lysine, calculated by the method of Edsall and Wyman (19), the pI of ELH should be 9.7, which agrees with the apparent pI of 9.0–9.2 on normal and 8 M urea IEF gels. However, the inability of carboxypeptidases A and B to cleave ELH suggests a blocked COOH ter-

Table 2. Recovery of purified, [^{3}H]leucine-labeled ELH during copurification with unlabeled bag cell extract

	Specific radioactivity, cpm/mg total protein	Total radioactivity, cpm	% recovery of initial radioactivity
Bag cell extract supernatant*	628	50,000	100
After cation exchange chromatography, fractions 32–42 (Fig. 1)	46,800	22,390	44.8
After gel filtration, fractions 35–45 (Fig. 2)	62,700	17,920	35.8

* Bag cell clusters (35 pairs) were homogenized and, after dialysis of the supernatant, 117.5 μg of [^{3}H]leucine-labeled marker ELH was added prior to purification.

minus, possibly by amidation. The majority of biologically active neuropeptides, including substance P, LHRH, TRH, oxytocin, and vasopressin, have amidated COOH termini. In many cases, biological activity of the molecules requires the presence of the terminal amide. Alternatively, cleavage of the terminal lysine residue by carboxypeptidase B may be inhibited by the presence of the penultimate glutamic acid residue or by a peptide tertiary structure that shields the lysine from the enzyme. At present, we do not have an answer to the nature of the block at the COOH terminus of ELH, which may affect the calculated pI of the molecule.

Besides the basic character of ELH, the composition of this molecule is also unusual in that it has no valine, proline, tryptophan, histidine, glycine, phenylalanine, and cysteine. The absence of cysteine means that disulfide linkages do not play a role in intramolecular or intermolecular interactions with ELH.

Our two-step procedure resulted in a 100-fold purification of ELH based on specific activities in the recovery experiment in Table 2. This suggests that ELH may make up as much as 1% of the soluble protein in bag cell clusters. On the average, 7–14 μg of hormone is recovered from a pair of bag cell clusters. Because there is a 36% recovery of marker radiolabeled ELH, the calculated initial level of the hormone is 19–39 μg per cluster pair. The actual value may be higher because we do not know how efficient our method of extraction is, and ELH contained in some of the bag cell terminals extending into the abdominal ganglion and connective tissue have not been included. Most of the clusters used were dissected from sexually mature animals during October and November, at the height of the reproductive season. This high ELH content may not be maintained all year round.

The neuropeptide, ELH, whose primary structure has been determined, is a molluskan neurohormone that is synthesized by identifiable neuron clusters. A cardioexcitatory factor, the tetrapeptide amide Phe-Met-Arg-Phe-NH_2, has been extracted from the ganglia of clams, but the precise neuronal source and its normal physiological role have still to be defined (20). The atrial gland in the large hermaphroditic duct of *Aplysia* has been shown to contain peptide factors capable of inducing egg laying in *Aplysia* (21). Two of these peptide factors have recently been purified in this laboratory and their sequence has been determined (22). The two atrial peptides are rather similar but they bear no resemblance to ELH, and their mode of action in egg laying appears to be to produce bag cell afterdischarge (22). ELH therefore remains as the only known neuropeptide directly implicated in egg laying in *Aplysia*.

We thank Dr. L. K. Kaczmarek for comments on the manuscript and Mr. Vince Farnsworth, Mr. Dan Viele, and Ms. Annette Yuen for technical assistance. The work was funded by National Institutes of Health Grants NS-07071 and NS-15183 to F.S., Grant GM-06965 to L.E.H., and Biomedical Research Support Grant RR 07003 and by the B.W. Foundation. Predoctoral support came from the Lawrence A. Hanson Foundation Fellowship, Spencer Foundation Fellowship (A.Y.C.), National Institutes of Health Training Grant GM-0086, and the Arthur McCallum Fund (D.K.S.).

1. Strumwasser, F., Jacklet, J. W. & Alvarez, R. B. (1969) *Comp. Biochem. Physiol.* **29**, 197–206.
2. Arch, S. (1976) *Am. Zool.* **16**, 167–175.
3. Arch, S. (1972) *J. Gen. Physiol.* **60**, 102–119.
4. Kupfermann, I. (1967) *Nature (London)* **216**, 814–815.
5. Arch, S. & Smock, T. (1977) *Behav. Biol.* **19**, 45–54.
6. Stuart, D. K. & Strumwasser, F. (1979) *J. Neurophysiol.*, in press.
7. Kupfermann, I. & Kandel, E. R. (1970) *J. Neurophysiol.* **33**, 865–876.
8. Kaczmarek, L. K., Finbow, M., Revel, J. P. & Strumwasser, F. (1979) *J. Neurobiol.*, in press.
9. Stuart, D. K., Chiu, A. Y. & Strumwasser, F. (1979) *J. Neurophysiol.*, in press.
10. Pinsker, H. M. & Dudek, F. E. (1977) *Science* **197**, 490–493.
11. Dudek, F. E., Cobbs, J. S. & Pinsker, H. M. (1979) *J. Neurophysiol.* **42**, 804–817.
12. Toevs, L. A. S. (1970) Dissertation (California Institute of Technology, Pasadena, CA).
13. Arch, S., Early, P. & Smock, T. (1976) *J. Gen. Physiol.* **68**, 197–210.
14. Hunkapiller, M. & Hood, L. E. (1978) *Biochemistry* **17**, 2124–2133.
15. Wittmann-Liebold, B. (1973) *Hoppe-Seyler's J. Physiol. Chem.* **354**, 1415–1431.
16. Nelson, N. J., Hunkapiller, M. & Hood, L. E. (1979) *Anal. Biochem.*, in press.
17. Ward, S., Wilson, D. L. & Gilliam, J. J. (1970) *Anal. Biochem.* **38**, 90–97.
18. Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39**, 462–477.
19. Edsall, J. T. & Wyman, J. (1958) in *Biophysical Chemistry* (Academic, New York), pp. 507–510.
20. Price, D. A. & Greenberg, M. J. (1977) *Science* **197**, 670–671.
21. Arch, S., Smock, T., Gurvis, R. & McCarthy, C. (1978) *J. Comp. Physiol.* **128**, 67–70.
22. Heller, E., Kaczmarek, L. K., Hunkapiller, M. & Strumwasser, F. (1979) *Soc. Neurosci. Abstr.* **5**, 808.

CHAPTER 2

Neurosecretion of egg-laying hormone and other peptides from electrically active bag cell neurons of Aplysia

JOURNAL OF NEUROPHYSIOLOGY
Vol. 43, No. 2, February 1980. *Printed in U.S.A.*

Neurosecretion of Egg-Laying Hormone and Other Peptides From Electrically Active Bag Cell Neurons of *Aplysia*

DUNCAN K. STUART, ARLENE Y. CHIU, AND FELIX STRUMWASSER

Division of Biology, California Institute of Technology, Pasadena, California 91125

SUMMARY AND CONCLUSIONS

1. Radiolabeled peptides released from an isolated cluster of bag cell neurons, during an afterdischarge, were compared with the polypeptide egg-laying hormone (ELH), 4,500 daltons, p*I* 9.0–9.3, as purified from homogenates of bag cell clusters. A peptide, labeled with methionine, leucine, and arginine, which is selectively released from afterdischarging bag cell clusters comigrates with marker ELH, purified from cluster homogenates, on P-6 gel filtration columns and subsequent isoelectric focusing gels. At least three other presumed peptides of unknown function are also released, including one of 5,000–6,000 mol wt, p*I* 4.5–5.0.

2. When bag cells afterdischarge in vitro, bioactive material is released that will induce egg laying when injected into an *Aplysia*. This released bioactive material also comigrates with bioactive material from cluster homogenates on P-6 columns.

3. These experiments demonstrate that ELH (4,500 mol wt, p*I* 9.0–9.3), as purified from bag cell cluster homogenates, is also the major form secreted from bag cells that induces egg laying. This purified ELH can now be used to study the physiological effects of a secreted neurohormone and their relationship to behavior.

INTRODUCTION

The injection of the egg-laying neurohormone (ELH) into the marine, opisthobranch mollusk *Aplysia* causes it to lay eggs. ELH, as determined by the bioassay for egg laying, is located in the two groups of bag cells and surrounding connective tissue of the abdominal ganglion (22, 34, 37) and in no other part of the nervous system (34). These bag cells fulfill the morphological requirements for neurosecretory cells since they contain 150- to 250-nm dense-core granules and their endings are distributed throughout the surrounding connective tissue in neurohemal spaces (11, 16). A brief train of electrical stimuli will set off an afterdischarge of bag cell action potentials lasting for tens of minutes (14–16, 21, 24). In vivo this afterdischarge is followed by egg laying (29).

ELH, as characterized from tissue homogenates, is a basic polypeptide with an isoelectric point (p*I*) of about 9.0–9.3 (3). In earlier studies it was assigned a molecular weight of 6,000 (3, 36), but based on its amino acid sequence, it now appears to be a ~4,500 dalton molecule (10). It appears to be one of the posttranslational product peptides of a 29,000 mol wt precursor, which could produce a number of bag cell peptides. Other sibling-product peptides may be 12,000, 6,000, and ≤3,000 mol wt (2, 5, 25, 26).

When bag cells are stimulated electrically (23, 24) or by a high-potassium solution (1) they release a factor(s) that induces egg laying into the perfusate surrounding the abdominal ganglion. However, there is no direct evidence relating this released material to the p*I* 9.0–9.3, 4,500 mol wt peptide recovered from tissue homogenates. Previous studies (1, 2, 26) have analyzed peptides released, during high-potassium stimulation, on SDS gels. However, substances released during the normal afterdischarge of bag cells were not examined until this study.

To study the physiological effects of the

secreted hormone (9, 12, 35), it is important to identify the form in which it is secreted. In experiments reported here, released ELH is compared with the 4,500 mol wt form from tissue homogenates by using gel filtration columns and isoelectric focusing gels. The results indicate that ELH is released from afterdischarging bag cells as the p*I* 9.0–9.3, 4,500 mol wt form. This form is certainly responsible for the ability of released material to induce egg laying. We also show that peptides other than ELH are released during bag cell afterdischarge.

METHODS

The procedure for radioactively labeling bag cells and then examining their released substances with gel filtration and isoelectric focusing will be described first. We will then present procedural variations used to identify, by bioassay, fractions with the ability to induce egg laying. To minimize ELH losses due to adsorption, plastic containers or Siliclad-(Clay Adams) coated glass was always used.

Labeled release

ANIMALS. *Aplysia californica* were collected locally off the Palos Verdes peninsula, California, and maintained in a 100-gallon tank of aerated, recirculating natural seawater for at least 5 days prior to their use. In order to monitor egg-laying activity, animals were isolated in individual baskets and, to assure their maturity, they were kept warm (7, 32) and well fed. The tank was maintained at 20 ± 0.5°C with a light-dark schedule of 12 h light and 12 h dark. Lights-on was at 0800 and lights-off at 2000 Pacific daylight time. The animals were fed with locally collected *Plocamium coccinium* and *Laurentium*, or dried green algae (Ko Kyu Wakame, Nishimoto Trading Co., Japan). From January through April, it was usually necessary to keep animals in the 20°C tank for 3–4 wk before they were mature enough to lay eggs.

Animals weighed between 200 and 1,500 g and had reproductive-tract weights (7, 34) of between 1.5 and 15 g. Animals used for release and bioassay experiments had laid eggs previously.

PREPARATION. Dissection, labeling, and stimulation were performed at ~20°C. The abdominal ganglion, with most of the pleuroabdominal connectives attached, was placed in a Millipore-filtered (0.22 μM) natural seawater solution containing 10 mM glucose, 5 or 10 mM Tris Cl, or 10 mM Na HEPES, pH adjusted to 8.0 at 20°C, 100 U of K penicillin G and 100 μg of streptomycin (sulfate) per milliliter. The bag cell bodies with their surrounding connective tissue were then dissected away from the rest of the abdominal ganglion. About one-third of the total length of the pleuroabdominal connective, containing many cell processes (16), was left attached. Such a preparation will be referred to as a single bag cell cluster. In one of four experiments, a small bridge of connective tissue was left joining the right and left bag cell clusters. Between 1 and 12 pairs of bag cell clusters were labeled together for each of the four experiments.

LABELING. Bag cell clusters were labeled in 1 ml of Millipore-filtered (0.22 μM) natural seawater with 150 U of K penicillin G and 150 μg of streptomycin (sulfate) per milliliter, and a concentration of 10 mM glucose, 10 mM Tris Cl or 20 mM Na HEPES (pH 8.0 at 20°C), and 5 μM of all amino acids except glutamine and the amino acids that were to be labeled. Labeled amino acids were usually first taken to dryness under forced air in a boiling-water bath and then redissolved in this labeling medium.

The labeled amino acids were, for expt. *1*, 50 μCi of L-[4,5-^{3}H]Leu, 48 Ci/mM and 5 μCi of L-[^{14}C]Leu, 312 mCi/mM, from Schwarz-Mann; for expt. *2* and *3*, 125 μCi of L-[N-3-^{3}H]Arg, 23 Ci/mM and 25 μCi L-[U-^{14}C]Leu, 320 mCi/mM from New England Nuclear; and for expt. *4*, 350 μCi L-[^{35}S]Met, 440 Ci/mM from New England Nuclear. In expt. *4*, the medium contained no cold amino acids.

The durations of labeling were 24 h in expt. *1*, *2*, *3*, and 18 h in expt. *4*. The bag cell clusters were subsequently rinsed for 6 h in six successive 10-ml rinses of the prelabeling solution.

STIMULATION. Following rinsing, the two to four largest and most cleanly dissected clusters were each placed, as a control, for 1 h in a separate 1 ml (0.5 ml for expt. *4*) of fresh solution. For each cluster the electrical activity was followed with an extracellular suction electrode coupled to a Tektronix 122 preamplifier, monitored on a Tektronix 5000 series cathode-ray oscilloscope, and usually recorded on a Grass model 7 polygraph. The control solution was then replaced with an equivalent volume of solution, and the bag cells were stimulated through the electrode with a 30-V stimulus of 4 ms duration repeated 6/s for 30 s. Resulting compound action potentials were recorded and the bag cell cluster removed from the stimulation solution at the end of the second hour. Both solutions were held on ice until applied to gel filtration columns.

GEL FILTRATION. A 0.5-ml quantity of the control and stimulation perfusate was analyzed separately on a 50 × 1 cm Bio-Gel P-6, 400-mesh

gel filtration column maintained at 4°C. We used only the perfusates from the cluster(s) that had produced the longest series of compound action potentials. The column buffer was 10 mM Tris Cl and 50 or 100 mM NaCl, pH 8.0. The buffer reservoir was maintained 30 cm above the column top producing a flow rate of 4 $ml \cdot cm^{-2} \cdot h^{-1}$. A Gilson microfractionator collected 80 fractions of 0.6 ml each.

Part or all of each resulting fraction was then added to 5 ml of Aquasol (New England Nuclear) and counted for at least 20 min on a Beckman L230 scintillation counter. All data have been corrected for background and crossover. The recovery of each isotope in the perfusate applied to the column varied between 24 and 80%.

Columns were calibrated with blue dextran, cytochrome *c*, ^{125}I-labeled bungarotoxin (obtained from Professor Michael Raftery), pancreatic trypsin inhibitor, glucagon, bacitracin, leucine, and $^{3}H_2O$. The P-6 column, nominally, should exclude globular polypeptides of molecular weight > 6,000.

ISOELECTRIC FOCUSING. A 100- to 125-μl sample from a P-6 column fraction was made up to 10% sucrose and 0.1% LKB ampholines, pH 3-10, with cytochrome *c* as a marker for a total volume of 150–200 μl. Gels, 105 x 4 mm, were made with a final concentration of 6.7% acrylamide monomer, 0.4% methylene bisacrylamide, 4.7% ampholines, pH 3–10, 0.047% ammonium persulfate, and 0.4% tetramethylethylenediamine (TEMED). The sample was applied to the top of the gel and overlaid with 100–500 μl of a solution of 5% sucrose and 0.05% ampholines. The top buffer was 10 mM phosphoric acid, the bottom buffer 100 mM NaOH. The tops of all eight gels were made positive during the running period of 4–5 h at room temperature with an applied voltage that was increased from 150 to 300 V, the current dropping from 9 to 1 mA. On extrusion, the gels were immediately sliced into 1.4-mm slices, and the radioactivity counted as reported previously (30).

To determine the pH gradient an equivalent gel was concurrently run and sliced into 2.8-mm pieces. Each piece was then added to 1 ml of 100 mM KCl, and after 12 h the pH was measured with a Beckman model 76 meter. Gels were compared by computing the mobility of substances in each piece relative to that of the marker cytochrome *c*, p*I* = 9.4, present in each gel.

ELH PURIFICATION. Six pairs of bag cell clusters were labeled, as described above, with 150 μCi of L-[2,3,4,5-^{3}H]Pro, 115 Ci/mM and 150 μCi of L-[N-3,4,5-^{3}H]Leu, 110 Ci/mM (New England Nuclear).

After labeling the clusters were kept frozen (−20°C) until homogenized with 34 pairs of unlabeled clusters. The homogenate was centrifuged, the supernatant precipitated with ammonium sulfate, the pellet redissolved, and separated on an anion and then cation exchange column, followed by P-6 gel filtration as just described. The four 0.6-ml fractions from the ELH region, containing 84% of the radioactivity recovered in the P-6 fractions, were pooled, frozen, and vacuum evaporated to 0.2 ml. Equivalent amounts of this marker ^{3}H-labeled ELH were added to the control and stimulation perfusate of expt. *4* before fractionation on the P-6 column.

Bioassays

Fractions with the ability to induce egg laying were assayed by injecting them through the foot of an *Aplysia* into its hemocoel (34). Either released or homogenate forms of ELH were assayed after fractionation on P-6 columns. Homogenate material was also assayed after elution from slices of isoelectric focusing gels.

ANIMALS. A number of abdominal ganglia were needed to provide the released substances for P-6 fractionation and bioassay. Because of space limitation in the 20°C tank, the dissection and stimulation of bag cells were performed on the same day the animals were collected. Experiments were performed in late summer and early fall when the ocean temperature was around 20°C and all the animals mature (7).

Animals used for bioassay, kept normally at 20°C, were proved egg layers that had not laid eggs in the 2 days prior to the test. A given animal was injected no more than once a day. Eggs appear at 65 ± 16 min after injection at 14°C (4, 34). Tests resulting in eggs within 90 min after injection were considered positive.

PREPARATION AND STIMULATION. Ten to 14 abdominal ganglia were dissected with most of their pleuroabdominal connectives intact. Ganglia were tranferred one at a time to 1 ml of seawater solution in a plastic beaker, where the bag cells were directly stimulated with a suction electrode. When the bag cell afterdischarge was observed, each abdominal ganglion was transferred to a second beaker containing 2 ml of solution. All the ganglia were stimulated within about 30 min, the contents of both beakers combined, and the bag cells allowed to discharge collectively for 1 h.

GEL FILTRATION. The 3-ml solution containing the released substances was then applied to a 50 × 2.5 cm P-6 column, and 3.9-ml fractions were collected. For comparison, at 4°C 19 pairs

of bag cell clusters were homogenized in 10 mM Tris Cl, centrifuged at 12,000 *g* for 10 min, and the 0.5 ml supernatant run on a 50 x 1 cm P-6 column.

A series of bioassays usually included all the P-6 fractions from K_{av} = 0–1.0. One-third or two-thirds of one to four consecutive fractions was injected into each animal for bioassay. Before injection, the low osmotic pressure of the column buffer was first corrected to that of *Apylsia* blood (19) by mixing with appropriate amounts of salts from filtered seawater taken to dryness.

ISOELECTRIC FOCUSING. Two series of bioassays of material extracted from isoelectric gels were performed. Supernatant from abdominal ganglia homogenate was focused on one gel while partially pure ELH was focused on the second. The first gel was sliced into 5-mm and the second into 3-mm slices. Each slice was extracted for 48 h in two successive 0.5-ml solutions of 10 mM potassium phosphate, pH 6.5. Each pair of successive solutions was then combined and injected into an animal for bioassay.

RESULTS

Labeled release

In each of four experiments a bag cell cluster, previously labeled with radioactive amino acids, is placed in a perfusate solution for a control period of 1 h and then transferred to an equivalent volume of perfusate for a second hour. At the beginning of the second hour, the bag cells are given a brief electrical stimulus, setting off an afterdischarge of compound action potentials. Following this, the control perfusate and the stimulation perfusate are applied separately to a P-6 gel filtration column and the radioactivity in the resulting fractions counted. This separation, based mainly on molecular weight, permits the direct comparison of labeled substances released with and without bag cell activation.

GEL FILTRATION. Bag cell clusters were incubated with a double label for release in three experiments, while the fourth experiment used a single label. Figure 1 provides data on one cluster labeled with [^{3}H]-Arg and [^{14}C]Leu. The P-6 ^{3}H and ^{14}C profiles for the stimulation (Fig. 1*B*) perfusate show a 15- to 100-fold increase over the control (Fig. 1*A*) in the area where ELH consistently appears, K_{av} = 0.20–0.25, fractions 36–37.

Labeled substances in four other regions also show large increases in the stimulation period (Fig. 1*B*). These regions are the excluded region, K_{av} = 0; a pre-ELH region appearing between the excluded and ELH region, K_{av} = 0.10–0.15; and two later eluting regions, K_{av} = 0.3–0.5. The region K_{av} = 0.5–0.7 shows a more moderate increase in labeled substances. No change, though, is observed in the amount of radioactivity recovered either from ^{14}C in the region for [^{14}C]Leu, K_{av} = 0.75, or from ^{3}H in the region for ^3H_2O, K_{av} = 1.0 (Table 1). ^3H_2O occurs as a metabolite of the label.

Figure 2 provides data from a pair of bag cell clusters labeled with [^{35}S]Met. In this experiment, ^{3}H-labeled ELH, purified from bag cell homogenates, was added to both the control and stimulation perfusates after the stimulation period. The P-6 profile for the stimulation perfusate (Fig. 2*B*) shows a 12-fold increase in ^{35}S counts in the ELH region, K_{av} = 0.2, compared to the control (Fig. 2*A*). The ^{35}S peak in this region coincides exactly with the marker peak, ^{3}H-labeled ELH, purified from cluster homogenates (Fig. 2*B*).

Other regions, which again show increases, are the excluded region, K_{av} = 0 and the pre-ELH region, K_{av} = 0.10–0.15, while the region for the label, [^{35}S]Met, K_{av} = 0.75–0.80, shows no change (Table 1).

Table 1 summarizes the results of the four experiments. In three of them, the radioactivity in the ELH region during the stimulation period is more than 10-fold that of the corresponding control region. There is a similarly large increase in the pre-ELH region. For the labeled substances in the excluded region, a smaller increase of 3–5 times is apparent, while the radioactivity in the region of the label or ^3H_2O changes only by a factor of 1.0–1.5. In the remaining experiment, expt. *3*, the radioactivity in the ELH region for the stimulation period increases by only 2–3 times, while there is no increase for that of the label and ^3H_2O regions.

ISOELECTRIC FOCUSING. To aid in establishing whether the p*I* 9.0–9.3, 4,500 mol

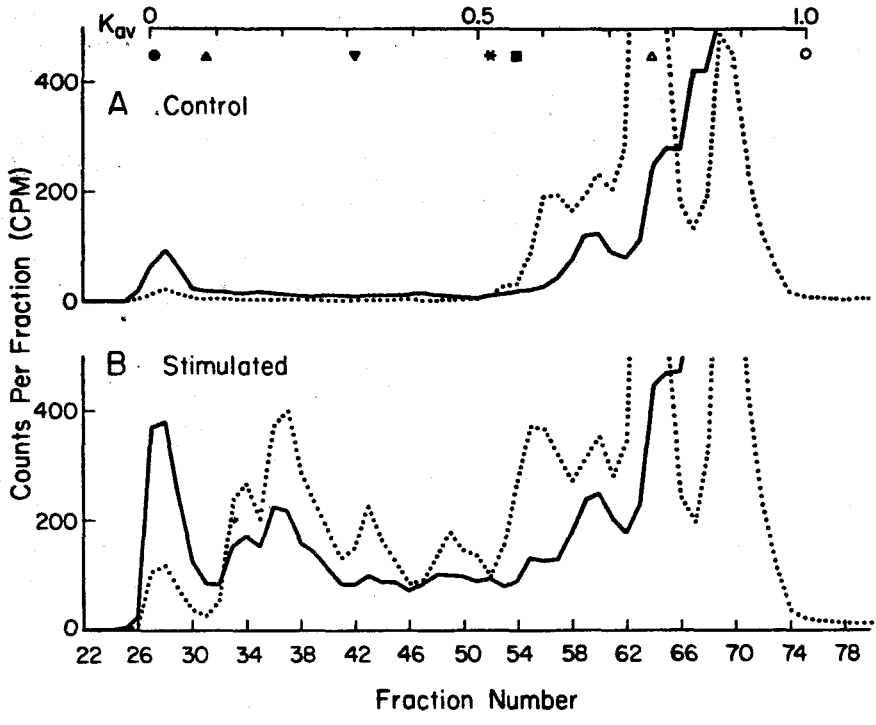


FIG. 1. P-6 fractionation of consecutive 1-h perfusates from a single bag cell cluster. The profile in *A* is from the control hour and in *B*, from the second hour where the cluster was stimulated briefly and produced an afterdischarge for more than 25 min. The cluster was labeled with [^{3}H]Arg and [^{14}C]Leu. The profile for both ^{3}H (——) and ^{14}C (· · · · ·) are shown. The ELH region, K_{av} = 0.2, shows a 15- to 100-fold increase in released counts in *B*. The ^{14}C counts in the leucine region or the ^{3}H counts in the ^3H_2O region are not changed (expt. *2*, Table 1). Markers, not run concurrently, are ● (blue dextran 2,000, mol wt = 2,000,000; bovine pancreatic trypsin inhibitor [Kunitz], mol wt = 6,500), ▲ (cytochrome *c*, mol wt = 12,384), * (glucagon, mol wt = 3,485), ▼ (α-bungarotoxin, mol wt = 7,904), ■ (bacitracin, mol wt = 1,411), △ (leucine), and ○ (^3H_2O). It will be noted that two of the markers (▲, ▼) do not elute ideally according to molecular weight. For ^{3}H and ^{14}C, each, 59% of the applied radioactivity was recovered in the collected fractions of the control period, and 42 and 46%, respectively, of the stimulation period.

wt ELH itself was released, aliquots of P-6 fractions containing the marker ^{3}H-labeled ELH (Fig. 2), for control and stimulation periods, were further separated on isoelectric focusing gels (Fig. 3). A ^{35}S-labeled peak again comigrates with ^{3}H-labeled ELH to a position with a p*I* of 9.0–9.3. Furthermore, stimulated released samples show a 6- to 12-fold increase in the ^{35}S-labeled material, which comigrates with marker ELH on both P-6 and isoelectric focusing gels.

Aliquots of other P-6 fractions (Fig. 2) were also focused on gels. It is of interest that for the stimulation period the pre-ELH region, K_{av} = 0.10–0.15, fraction 35, shows a similar increase in radioactivity for a substance with a p*I* of 4.5–5.0 (Fig. 4). Most of the radioactivity in a sample of fraction 60, K_{av} = 0.5–0.7, did not enter the gel, suggesting that this low (~1,000) mol wt, ^{35}S-labeled material is acidic with a p*I* < 3.5.

Bioassays

Bioassays were performed to locate the gel filtration fractions and IEF gel slices with egg-laying activity.

GEL FILTRATION. Five series of bioassays of P-6 fractions were carried out. Each of the first four series were of material released from three to seven abdominal ganglia following bag cell activation. Normally, the entire range of fractions, K_{av} = 0–1, were bioassayed by injecting three or four consecutive fractions into each animal. Five animals, at least one per series, laid eggs, responding to fractions with a mean K_{av} = 0.24 ± 0.04. Figure 2 shows the cor-

TABLE 1. *Ratio of released counts in stimulation versus control perfusates*

Expt No.	Label	Gel Filtration Region				
		Exclusion	Pre-ELH	ELH	Label	^3H_2O
1	[^{3}H]Leu	4.6	>10	>10	1.5	1.2
	[^{14}C]Leu	3.4	7.0	>10	1.4	
2	[^{3}H]Arg	4.5	10	>10		1.0
	[^{14}C]Leu	5.5	>10	>10	1.0	
3	[^{3}H]Arg	2.6	3.0	3.2		1.0
	[^{14}C]Leu	3.5	1.5	2.4	1.0	
4	[^{35}S]Met	4.0	>10	>10	1.0	

Ratios are based on the appropriate isotope counts recovered in the P-6 fractions. For each profile the exclusion region, the pre-ELH region, the ELH region, the label region, and the ^3H_2O region are the sum of counts in 4, 3, 5, 5, and 7 fractions, respectively. The [^{3}H]Arg peak was not readily distinguishable from the ^3H_2O peak, so no ratio is given for this label. The percent recovery for each isotope in the P-6 fractions was always lower for the stimulation period than the control period. Experiment *3* had the lowest percent recovery of an isotope from the stimulation period. In this experiment, the bag cells before labeling were also stimulated, producing an afterdischarge. This was intended to increase the amount of labeled ELH, but also may have resulted in the release of less total ELH following the experimental stimulation, leading to a relatively greater loss on the P-6 column and lower ratios in this table.

responding active fractions for one of these series.

In the fifth series, material homogenized from six pairs of bag cell clusters was bioassayed after P-6 fractionation, one fraction at a time. Six animals responded with eggs

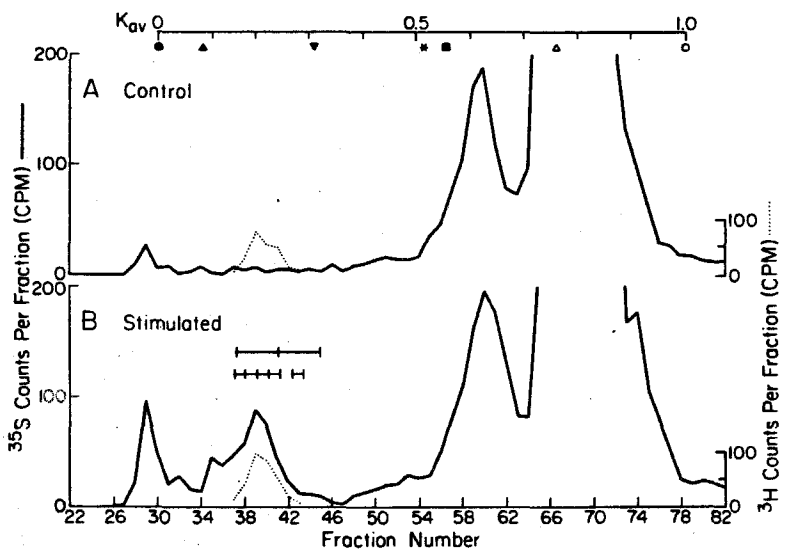


FIG. 2. P-6 fractionation of consecutive 1-h perfusates from a pair of bag cell clusters. The profile in *A* is from the control hour and in *B*, from the second hour where the clusters were stimulated briefly and produced an afterdischarge for 23 min. The pair of clusters was labeled with [^{35}S]Met. Purified ^{3}H-ELH was mixed with each of the two perfusates before P-6 fractionation. The profile for both ^{35}S (——) and ^{3}H (· · · · ·) are shown. The ELH region, K_{av} = 0.2, shows a 12-fold increase in released counts in *B* that correspond to the ^{3}H counts of the purified ELH. The ^{35}S counts are from 25-μl samples of each 600-μl fraction. Markers are the same as in Fig. 1. The two long bars above the ELH region in *B* represent the combined fractions of released ELH that produced egg laying in one series of bioassays. The five short bars represent single fractions of bag cell cluster homogenate that produced egg laying in a second series. In both series, the entire range, K_{av} = 0–1, was assayed.

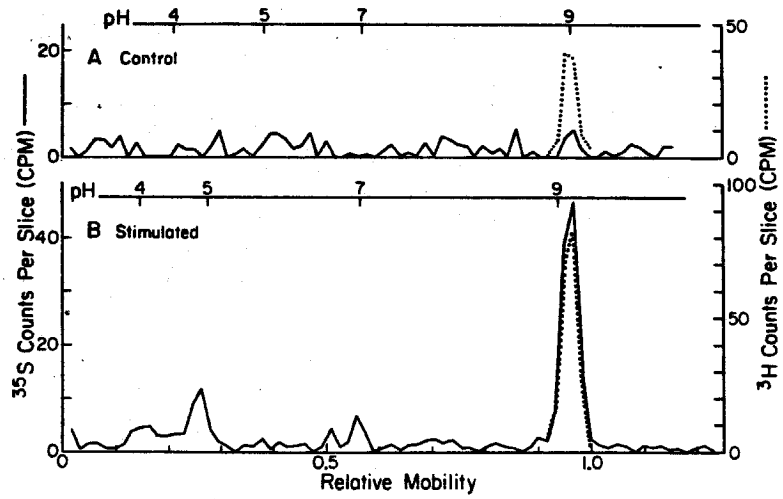


FIG. 3. Isoelectric focusing of released ELH first fractionated on P-6 column (Fig. 2). The control profile in *A* is from the ELH peak in Fig. 2*A* (fraction 40) and the stimulation profile in *B* is from the ELH peak in Fig. 2*B* (fraction 40). In both, the ^{35}S (——) profile represents released substances, whereas the ^{3}H (· · · · ·) profile represents purified ^{3}H-labeled ELH mixed with each of the two perfusates before P-6 fractionation. In *B* the ^{35}S-labeled, released ELH appears identical to the ^{3}H-labeled ELH purified from bag cell cluster homogenates. The radioactivity from each slice is plotted versus its mobility relative to the internal marker cytochrome *c*, p*I* 9.4–9.6. The pH of the gels is indicated along the top.

to fractions with a mean $K_{av} = 0.22 \pm 0.04$ (Fig. 2). This active region is clearly the same as that for the released ELH.

ISOELECTRIC FOCUSING. In each of the two series of bioassays, the eluate of only one specific slice produced egg laying. In the first series, 8 of 15 slices were assayed. The slice producing egg laying had a mobility relative to cytochrome *c* of 0.83–0.96. In the second series, all 31 slices were assayed. The slice inducing egg laying had a relative mobility of 0.84–0.92. Purified radiolabeled ELH has a relative mobility of 0.90–0.96. The existence of this one region, p*I* 9.0–9.3, with egg-laying activity confirms the findings of Arch et al. (3).

DISCUSSION

ELH

The results demonstrate that the p*I* 9.0–9.3, 5,000 mol wt ELH is released from isolated bag cell clusters following electrical activation and afterdischarge of the bag cells. Purified ^{3}H-labeled ELH comigrates with ^{35}S-labeled material, released from electrically activated bag cells, on a P-6 gel filtration column (Fig. 2) followed by isoelectric focusing gels (Fig. 3). The former

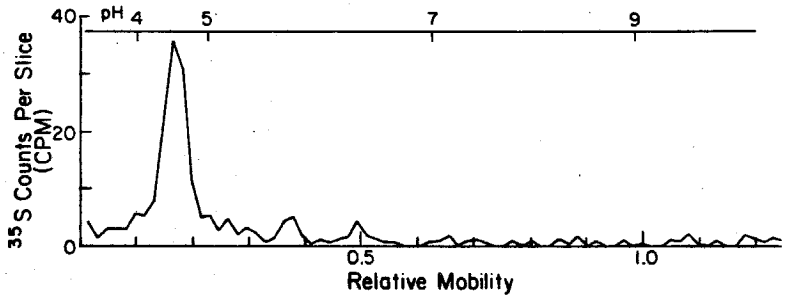


FIG. 4. Isoelectric focusing of acidic, 5,000–6,000 mol wt released peptide from the P-6 pre-ELH peak, fraction 35 (Fig. 2*B*). This ^{35}S profile is from the stimulation period; there was no radioactivity in this region during the control period (Fig. 2*A*). The radioactivity from each slice is plotted versus its mobility relative to the internal marker cytochrome *c*, p*I* 9.4–9.6. The pH of the gel is indicated along the top.

separates molecules predominantly by size while the latter does so by charge. This is reasonably convincing evidence that the two substances are identical. A comparison of composition and amino acid sequence would be most convincing.

The bioassays of released substances after P-6 gel filtration indicate that fractions that induce egg laying coincide with those fractions in which ELH purified from cluster homogenates is found (Fig. 2). The results do not exclude the possibility that there are other released substances capable of inducing egg laying, but they must then be either less active and/or present in lower concentrations than ELH and, so, are not detected by our bioassay. We think that this possibility is unlikely.

This conclusion, that much if not all of the released activity is due to the purified form of ELH rather than other released peptides (see below), is important for physiological research concerned with the effects of the hormone elsewhere in the animal. The finding that purified ELH is the same form as that released from bag cell clusters into medium and probably into the circulatory system during spontaneous egg laying, permits meaningful interpretation of the effects of applying the purified hormone to potential target sites in the animal, such as to the in vitro nervous system described in the accompanying paper (35).

These results also demonstrate that the released form of ELH is similar, if not identical, to the form in which it is stored, providing that purification from cluster homogenates does not alter the stored form of ELH. Although released neuropeptides have been detected by radioimmunoassay in vertebrate systems, for example substance P (27), and more routinely by bioassay (6, 38) in invertebrate systems, our characterization of released ELH has involved bioassay, apparent molecular weight, isoelectric point, and comparison of these characteristics with the purified molecule.

Previous work

Our conclusion that ELH is released in a form equivalent to that purified from bag cell cluster homogenates is consistent with other published work. It should be emphasized, though, that this is the first report where bag cell released substances have been first fractionated and then assayed for egg-laying activity. Previously, released substances were bioassayed only in unfractionated perfusate (1, 23). Arch (2) first demonstrated that a high-potassium solution applied to a [^{3}H]leucine-labeled bag cell cluster caused Ca^{2+}-dependent release of acetone-precipitable material that ran on SDS gels at a mol wt $< 10{,}000$. A high-potassium solution applied to a cluster induced the release of Formalin-precipitable material, which binds to eriochrome cyanine, a stain specific for basic proteins. The high-potassium solution also decreased the amount of stainable material in histological sections of the bag cells (33).

Loh et al. (26) demonstrated that bag cell clusters, exposed to a high-potassium solution, showed a Ca^{2+}-dependent release of 12,000, 6,000, and $\leq 3{,}000$ mol wt polypeptides. This was determined by SDS gels, designed to optimize low molecular weight resolution, that were subsequently stained with Coomassie blue. They found that within bag cell bodies where axonal transport has been blocked by colchicine a substantial amount of radioactive label is processed into $\leq 3{,}000$ mol wt substance(s). Therefore, they raised the possibility that such a substance is the major secreted form able to induce egg laying, and that a 6,000 mol wt form is a biologically active precursor normally found in higher proportion in the bag cell bodies. No bioassays were carried out in their study to identify the form(s) with egg-laying activity. Our results rule out this possibility since the p*I* 9.0–9.3, 4,500 mol wt ELH is released and is the major, if not exclusive, released form that induces egg laying.

Although the Ca^{2+} dependence of bioassayable ELH release has not been demonstrated, Ca^{2+}-dependent release of peptides in the above-mentioned studies of Arch (1, 2) and Loh et al. (26) support the expectation that ELH is also secreted by the conventional Ca^{2+}-dependent process (13).

Other peptides

Our results show that other presumed peptides besides ELH are released from electrically active bag cell clusters. Peptides falling into the P-6 exclusion region and the

pre-ELH region (K_{av} = 0.10–0.15) seem also to be released preferentially following bag cell stimulation and can be labeled by either leucine, arginine, or methionine (Figs. 1 and 2). The major, released peptide in this pre-ELH region is acidic with a p*I* of 4.5–5.0 according to our isoelectric focusing gels. Leucine label also reveals the presence of several preferentially released peptides in the region K_{av} = 0.3–0.5 (Fig. 1). Finally, in the K_{av} = 0.5–0.7 region there appear to be several small peptides, at least one of which is acidic. The radioactivity in this region shows some moderate increased release, by a factor of 1.2–5, following bag cell activation (Figs. 1 and 2).

The presumed peptides, that we have shown to be released from electrically activated bag cells, should correspond to the polypeptides released on high-potassium stimulation, as demonstrated by Loh et al. (26). Their data include molecular weight estimates only, so an exact correspondence cannot be made. More is known about peptides from cluster homogenates (3, 5, 18, 25, 26). A peptide with a p*I* of 4.8 (18) is reported by Arch et al.(5) to have a molecular weight of 5,000 and is thought to be derived from a p*I* 4.6 peptide with a molecular weight of 6,000. The p*I* 4.8 peptide may correspond to the peptide with a p*I* of 4.5–5.0 in the pre-ELH region of the P-6 fractions, K_{av} = 0.10–0.15 (Figs. 1, 2, and 4). Furthermore, the 1,000 mol wt peptide, hypothesized (5) to be cleaved from the p*I* 4.6 peptide in being processed to the p*I* 4.8 peptide, might correspond to one of the small peptides found in the K_{av} = 0.5–0.7 region of the P-6 fractions (Figs. 1 and 2).

The histological and electron microscopic evidence to date (14, 31) is consistent with the hypothesis that only one type of neuron is contained in the bag cell cluster. However, glial and connective tissue cells are also present. Thus, not every released peptide described here has to come from the bag cells, although the probability that this is the case is certainly higher for peptides preferentially released during bag cell afterdischarge. This preferential release should reflect substances released by bag cells in vivo more closely than previous studies utilizing high-potassium stimulation.

It is interesting to ask whether some of the released peptides described here are part of the original ELH precursor (5, 25), perhaps playing a role in the transfer of ELH across endoplasmic reticulum and its packaging and secretion (8, 28). The 5,000–6,000 mol wt, p*I* 4.5–5.0 peptide might be analogous to the neurophysins in vertebrates (17, 31) since it appears to be synthesized from the ELH precursor (5), is acidic, and is released with ELH (Figs. 1, 2, and 4). These non-ELH bag cell peptides may have additional hormonal effects when released, as might be the case with the joint secretion from the pituitary gland of two peptides, ACTH and β-endorphin, with a common precursor (19). These questions can best be answered by determining the amino acid sequences of the ELH precursor and the released peptides as well as by searching for physiological effects of the latter. In the following paper (35) we examine the effects of ELH on neurons in the head ganglia.

ACKNOWLEDGMENTS

We thank Dr. Eri Heller for his participation in the ELH purification and Robin Paltis for technical assistance.

The research reported was funded by the National Institues of Health Research Grants NS-07071 and NS-13896.

Predoctoral support came from the National Institutes of Health Training grant GM-00086 and the Arthur McCallum Fund to D. K. Stuart, and the Spencer Foundation Fellowship and Lawrence A. Hanson Foundation Fellowship to A. Y. Chiu.

Please address reprint requests to F. Strumwasser.

Present address of D. K. Stuart: Dept. of Molecular Biology, University of California, Berkeley 94720.

Received 13 July 1978; accepted in final form 30 July 1979.

REFERENCES

1. ARCH, S. Polypeptide secretion from the isolated parietovisceral ganglion of *Aplysia californica*. *J. Gen. Physiol.* 59: 47–59, 1972.

2. ARCH, S. Biosynthesis of the egg-laying hormone (ELH) in the bag cell neurons of *Aplysia californica*. *J. Gen. Physiol.* 60: 102–119, 1972.

3. ARCH, S., EARLY, P., AND SMOCK, T. Biochemical isolation and physiological identification of the egg-laying hormone in *Aplysia californica*. *J. Gen. Physiol.* 68: 197–210, 1976.
4. ARCH, S. AND SMOCK, T. Egg-laying behavior in *Aplysia californica*. *Behav. Biol.* 19: 45–54, 1977.
5. ARCH, S., SMOCK, T., AND EARLY, P. Precursor and product processing in the bag cell neurons of *Aplysia californica*. *J. Gen. Physiol.* 68: 211–225, 1976.
6. ARECHIGA, H., HUBERMAN, A., AND MARTINEZ-PALOMO, A. Release of a neurodepressing hormone from the crustacean sinus gland. *Brain Res.* 128: 93–108, 1977.
7. AUDESIRK, T. E. *The Role of Seasonal Periodicity, Chemical Communication and Chemoreceptive Organs in Reproduction in Aplysia californica Cooper*. (Ph.D. Dissertation). Los Angeles: University of Southern California, 1976.
8. BLOBEL, G. AND DOBBERSTEIN, B. Transfer of proteins across membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* 67: 835–851, 1975.
9. BROWNELL, P. AND MAYERI, E. Hours-long inhibition produced by peptide-secreting bag cells of *Aplysia*, including inhibition of cells controlling inking behavior. *Neurosci. Abstr.* 3: 173, 1977.
10. CHIU, A. Y., HUNKAPILLER, M., HELLER, E., STUART, D. K., HOOD, L. E., AND STRUMWASSER, F. Purification and primary structure of the neuropeptide egg-laying hormone of *Aplysia californica*. *Proc. Natl. Acad. Sci. USA* 76: 6656–6601, 1979.
11. COGGESHALL, R. E. A light and electron microscope study of the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* 30: 1263–1287, 1967.
12. COGGESHALL, R. E. A cytologic analysis of the bag cell control of egg-laying in *Aplysia*. *J. Morphol.* 132: 461–468, 1970.
13. DOUGLAS, W. W. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. *Br. J. Pharmacol.* 34: 451–474, 1968.
14. DUDEK, F. E. AND BLANKENSHIP, J. E. Neuroendocrine cells of *Aplysia brasiliana*. I. Bag cell action potentials and afterdischarge. *J. Neurophysiol.* 40: 1301–1311, 1977.
15. DUDEK, F. E. AND BLANKENSHIP, J. E. Neuroendocrine cells of *Aplysia brasiliana*. II. Bag cell prepotentials and potentiation. *J. Neurophysiol.* 40: 1312–1324, 1977.
16. FRAZIER, W. T., KANDEL, E. R., KUPFERMANN, I., WAZIRI, R., AND COGGESHALL, R. E. Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* 30: 1289–1351, 1967.
17. GAINER, H., SARNE, Y., AND BROWNSTEIN, M. J. Biosynthesis and axonal transport of rat neurohypophysical proteins and peptides. *J. Cell Biol.* 73: 366–381, 1977.
18. GAINER, H. AND WOLLBERG, Z. Specific protein metabolism in identifiable neurons of *Aplysia californica*. *J. Neurobiol.* 5: 243–261, 1974.
19. GUILLEMIN, R., VARGO, T., ROSSIER, J., MINICK, S., LING, N., RIVIER, C., VALE, W., AND BLOOM, F. β-Endorphin and adrenocorticotropin are secreted concomitantly by the pituitary gland. *Science* 197: 1367–1369, 1977.
20. HAYES, F. R. AND PELLUET, D. The inorganic constitution of molluscan blood and muscle. *J. Mar. Biol. Assoc. UK* 26: 580–589, 1947.
21. KACZMAREK, L., JENNINGS, K., AND STRUMWASSER, F. Neurotransmitter modulation, phosphodiesterase inhibitor effects and cAMP correlates of afterdischarge in peptidergic neurites. *Proc. Natl. Acad. Sci. USA* 75: 5200–5204, 1978.
22. KUPFERMANN, I. Stimulation of egg-laying: possible neuroendocrine function of bag cells of abdominal ganglion of *Aplysia californica*. *Nature London* 216: 814–815, 1967.
23. KUPFERMANN, I. Stimulation of egg-laying by extracts of neuroendocrine cells (bag cells) of abdominal ganglion of *Aplysia*. *J. Neurophysiol.* 33: 877–881, 1970.
24. KUPFERMANN, I. AND KANDEL, E. R. Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of *Aplysia*. *J. Neurophysiol.* 33: 865–876, 1970.
25. LOH, Y. P., SARNE, Y., DANIELS, M. P., AND GAINER, H. Subcellular fractionation studies related to the processing of neurosecretory proteins in *Aplysia* neurons. *J. Neurochem.* 29: 135–139, 1977.
26. LOH, Y. P., SARNE, Y., AND GAINER, H. Heterogeneity of proteins synthesized, stored, and released by the bag cells of *Aplysia californica*. *J. Comp. Physiol.* 100: 283–295, 1975.
27. OTSUKA, M. AND KONISHI, S. Release of substance P-like immunoreactivity from isolated spinal cord of newborn rat. *Nature London* 264: 83–84, 1976.
28. PALADE, G. Intracellular aspects of the process of protein synthesis. *Science* 189: 347–358, 1975.
29. PINSKER, H. M. AND DUDEK, F. E. Bag cell control of egg-laying in freely behaving *Aplysia*. *Science* 197: 490–493, 1977.
30. ROTHMAN, B. S. AND STRUMWASSER, F. Phase shifting the circadian rhythm of neuronal activity in the isolated *Aplysia* eye with puromycin and cycloheximide. *J. Gen. Physiol.* 68: 359–384, 1976.
31. SACHS, H., FAWCETT, P., TAKABATAKE, Y., AND PORTANOVA, R. Biosynthesis and release of vasopressin and neurophysin. *Recent Prog. Horm. Res.* 25: 447–491, 1969.
32. SMITH, S. T. AND CAREFOOT, T. H. Induced maturation of gonads in *Aplysia punctata* Curier. *Nature London* 215: 652–653, 1967.
33. SMOCK, T. AND ARCH, S. A cytochemical study of the bag cell organs of *Aplysia californica*. *J. Histochem. Cytochem.* 25: 1345–1350, 1977.
34. STRUMWASSER, F., JACKLET, J., AND ALVAREZ, R. B. A seasonal rhythm in the neural extract induction of behavioral egg-laying in *Aplysia californica*. *Comp. Biochem. Physiol.* 29: 197–206, 1969.
35. STUART, D. K. AND STRUMWASSER, F. Neuronal sites of action of a neurosecretory peptide, egg-

laying hormone, in *Aplysia californica*. *J. Neurophysiol.* 43: 499–519, 1980.

36. TOEVS, L. *Identification and Characterization of the Egg-Laying Hormone From the Neurosecretory Bag Cells of Aplysia* (Ph.D. Dissertation). Pasadena: California Institute of Technology, 1970.
37. TOEVS, L. AND BRACKENBURY, R. W. Bag cell-specific proteins and the humoral control of egg-laying in *Aplysia californica*. *Comp. Biochem. Physiol.* 29: 207–216, 1969.
38. TRUMAN, J. W. Physiology of insect ecdysis. I. The eclosion behavior of saturnid moths and its hormonal release. *J. Exp. Biol.* 54: 805–814, 1971.

CHAPTER 3

An immunohistochemical study of the neuropeptidergic bag cells of Aplysia

ABSTRACT

The Bag Cell neurosecretory system of Aplysia californica synthesizes and releases a neuropeptide, the Egg Laying Hormone (ELH), which can induce egg laying behavior. Using antibodies generated against pure ELH, we have studied the distribution of the neurohormone within the abdominal ganglion of A. californica as well as in primary cultures of A. californica bag cells. We also find ELH-crossreactivity in the bag cell systems of three other species of Aplysia.

In sections of abdominal ganglia stained for ELH by the peroxidase-antiperoxidase method, bag cell clusters were found to be homogeneous populations of ELH-immunoreactive neurons extending processes in a complex array within the connective tissue sheath overlying the ganglion and the proximal regions of neighboring nerves. Stained processes also invade the interior of the ganglion in association with septal sheets of connective tissue and as scattered fibers in the neuropil. A few ELH-positive ectopic cells were seen outside the confines of the bag cell clusters as far displaced as the base of the branchial nerve. Specificity of the antiserum was confirmed by the total lack of staining of all other neurons within the abdominal ganglion.

As in the intact ganglion, 1-4 day old cultured bag cells appear to maintain a high ELH content in somata and all neurites, including newly extended ones, when stained by the indirect immunofluorescent method. Similar cultures of buccal ganglion and eye neurons showed no difference in staining between preimmune and immune sera.

Although the ELHs of A. vaccaria, A. braziliana and A. dactylomela have not been biochemically characterized, it is known that abdominal ganglion extracts from A. californica can induce egg laying in all three species. We find that antiserum against A. californica-ELH stains the bag cell systems of A. vaccaria, A. braziliana and A. dactylomela and not other parts of their abdominal ganglia. It is likely that this staining represents cross-immunoreactivity of the egg-laying neurohormones from these species which also share cross-bioactivity.

INTRODUCTION

Immunohistochemistry has proven a powerful tool for tracing neuronal fiber tracts and nuclei which contain putative peptide transmitters and marker enzymes in the vertebrate nervous system. (For review, see Hökfelt et al., 1980). In invertebrate systems, the distribution of peptidergic neurons has traditionally been based on histochemical and ultrastructural observations. Recently, antibodies were generated against two intrinsic invertebrate neuropeptides and employed to identify cells of origin in Arthropods. Thus neurosecretory cells in the X-organ of Carcinus have been found to contain the Hyperglycemic Hormone (Jaros and Keller, 1979). Using antibodies specific for Proctolin, a pentapeptide which causes contraction of the cockroach hindgut, revealed 10-15 immunoreactive cell bodies in abdominal ganglia of the cockroach, Periplaneta americana (O'Shea, personal communication).

Invertebrate neurons have also been immunohistochemically stained with antisera generated against vertebrate neuropeptides. Cells with enkephalin-like immunoreactivity have been visualized in both earthworm cerebral ganglia (Alumets et al., 1979) and leech segmental ganglia (Zipser, 1980). The neural complex of the sea squirt, Ciona intestinalis, bears somatostatin- and Substance P-immunoreactive cells (Fritsch et al., 1979). Antisera, specific for nineteen vertebrate peptides including ACTH, vasopressin, oxytocin, Met-enkephalin and TRH, have lately been reported to stain an array of neurons in the pond snail Lymnaea stagnalis (Boer et al., 1979; Schot et al., 1981). In each of these cases, the structure of the immunoreactive molecule in the invertebrate has not been characterized because of difficulties in obtaining sufficient quantities of pure material.

In this study, we have sought to determine the distribution of an endogenous molluscan neuropeptide, the Egg Laying Hormone (ELH), in sections of Aplysia abdominal ganglion and in cell culture by conventional immunohistochemical procedures. ELH of A. californica is produced by the bag cell neurons which form two clusters at the junctions of the pleurovisceral connectives (PVCs) and the abdominal ganglion.

These neurons are characterized by unique electrophysiological properties, such as synchronization and afterdischarge, and biochemical products which relate to their function as neurosecretory cells. During an afterdischarge they release a heterogeneous mixture of peptides (Stuart el al., 1980; for peptide release induced by high K^+, see Arch, 1972a; Loh et al., 1975).

One of these neurosecretory peptides, ELH, has recently been purified and its amino acid sequence determined (Chiu et al., 1979a). It is a 36 amino acid, basic molecule which, when injected into a test animal, induces egg laying and associated behaviors (review see Arch, 1976). Although it is clear that bag cells synthesize (Arch, 1972b; Arch ef al., 1976; Stuart et al., 1980) and maintain (Chiu et al., 1979a) high levels of the neuropeptide, it is not clear if ELH is unique to bag cells. The possibility exists that small amounts of ELH, undetectable by bioassay procedures, or biologically inactive forms of the molecule could be present in other neurons in Aplysia. In this study, we endeavor to locate ELH+ cells in the abdominal ganglion within and beyond the confines of the bag cell clusters.

Histological and cytological studies of bag cell clusters suggest that they contain only one type of neuron based on the size and appearance of secretory granules ($\sim$170 nm) and on basic protein specific stains (Coggeshall, 1967; Smock and Arch, 1977). The electrophysiological properties of bag cells within a cluster also appear similar (Kupfermann and Kandel, 1970; Blankenship and Haskins, 1979; Kaczmarek et al., 1979). However, a functional and biochemical criterion for a homogeneous population of neurosecretory cells requires all bag cells to synthesize and release ELH. Our results demonstrate the presence of ELH immunoreactivity in all somata within bag cell clusters of four species of Aplysia while other abdominal ganglion neurons remain unreactive.

From neurophysiological data, it has been postulated that bag cells exert a neuromodulatory influence on other neurons in the abdominal ganglion via a "local

hormonal action" (Mayeri, 1979). It is therefore important to localize possible sites of release where such local effects can occur. Earlier studies have described the general distribution of bag cell processes, identified by a high content of neurosecretory granules, similar to those found in bag cell somata (Frazier et al., 1967). Using a specific bag cell product, ELH, as marker, our studies are the first to show the topography and distribution of hormone-bearing processes in the neurohaemal areas of the connective tissue sheath enveloping the ganglion as well as within the abdominal ganglion. An abstract of some of this work has appeared (Chiu et al., 1979b).

METHODS

1. Generation and partial purification of antibodies

ELH, labeled with ^{3}H-leucine and purified to homogeneity as described elsewhere (Chiu et al., 1979a), was desalted by dialysis (Spectrapor 3 membrane, cut-off 3500 mol wt) or by passage through a 20.0 x 1.0 cm column of P-2 (Bio-Rad, minus 400 mesh) equilibrated in 1 M acetic acid. Approximately 0.5 mg of ELH was lyophilized and redissolved in 50 µl of distilled water. An equal volume of Tg (Sigma, 10 mg/ml in distilled water) was added with 2.5 mg of 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl (Sigma) as the coupling agent. Incubation at room temperature for 1-3 h was followed by dialysis against distilled water to remove unreacted carbodiimides. Material retained after dialysis was mixed with an equal volume of Freund's Complete Adjuvant (Difco Labs) for primary immunizations.

Multiple site, intradermal injections were given to 2 female New Zealand rabbits according to the method of Vaitukaitis et al. (1971). About 0.6 mg of the ELH-Tg complex, containing coupled as well as unreacted hapten and carrier, in a total volume of 2-3 ml was injected at 15-20 sites on the backs of animals. 4-6 weeks later, the animals received intravenous booster injections of 50-150 µg ELH-Tg complex in phosphate buffered saline (PBS, 0.1 M sodium phosphate, 0.9% NaCl, pH 7.5), and

harvesting of antisera was started a week later. Protein content in solutions of ELH or ELH-Tg complex was estimated by absorbance at 215 nm.

Partial purification of gamma globulins (IgG) from immune and preimmune sera included precipitation by 50% saturated ammonium sulfate followed by an ion exchange chromatography on a Whatman DEAE cellulose DE 52 column and a second ammonium sulfate precipitation (Garvey et al., 1977). After dialysis against PBS to remove excess salt, the total protein in these IgG fractions was estimated from absorbance at 280 nm. All antisera were kept frozen in aliquots until used.

Antibodies specific for Tg were removed by passing whole sera through an affinity column of cyanogen bromide-activated Sepharose 4B coupled to Tg according to the method of March et al. (1974). The column contained a maximum of 7 mg of Tg coupled to 2.5 ml packed resin and equilibrated in 0.3 M borate buffer, pH 8.7. 2 ml of immune sera was applied and allowed to incubate at 15°C for 10 min before outflow from the column was resumed. Fractions containing protein which did not bind to the Tg-Sepharose column were pooled; the column was washed with borate buffer until absorbance at 280 nm was undetectable. Tg-specific antibody was then eluted with a 0.2 M glycine-HCl buffer, pH 2.4. With the column reequilibrated in borate buffer, the cycle was repeated by reapplying the pooled unbound protein fractions until no more protein was detected on elution with glycine-HCl buffer. The recycled unbound protein fractions, now containing immune IgG minus anti-Tg (antisera C), were then pooled and purified for IgG as described above. Preimmune sera is designated antisera A, whole immune sera is antisera B.

2. Enzyme linked immunosorbent assay (ELISA)

ELISA was carried out according to Engvall and Perlmann (1972), with slight modifications, for quantitation of antibodies against ELH and Tg. All reactions were performed in microtiter plates (Scientific Products) and kept in a moist chamber

during incubations. Antigens were diluted (ELH at 5 µg/ml; Tg at 20 µg/ml) in 100 mM sodium carbonate buffer, pH 9.6; 35 µl of either antigen solution was added to each microwell for overnight incubation at 4°C. The coated wells were washed with 4 changes of 200 µl of PBS-TWEEN (50 mM Na phosphate buffer, 0.9% NaCl, pH 7.4 with 0.05% TWEEN-20) followed by a 2-4 h incubation at room temperature with 50 µl of antisera, diluted with PBS-TWEEN, which prevents nonspecific adhesion of antisera but does not interfere with antibody-antigen binding. After microwells were again well rinsed, 50 µl of goat-anti-rabbit alkaline phosphatase conjugate (Miles) at a dilution of 1:50 in PBS-TWEEN was added and plates were incubated overnight at 37°C. Enzyme activity was revealed by reacting bound enzyme with 40 µl of 1 mg/ml p-nitrophenyl-phosphate (Sigma) in 50 mM Na carbonate, pH 9.8 with 1 mM $MgCl_2$ for 15 min at 37°C. Reaction was stopped on addition of 50 µl of 4N NaOH and the antisera titer expressed as absorbance at 405 nm.

3. Peroxidase-anti-peroxidase (PAP) staining of ganglion sections

Freshly dissected abdominal ganglia were fixed in 3.7% formaldehyde solution of Leibovitz L-15 (Gibco) for at least 24 h at 4°C. After several hours of rinsing in L-15 to remove excess formaldehyde, the tissue was cryoprotected in 30% sucrose for 24 h at 4°C. Whole ganglia were then rapidly frozen in OCT Compound (Miles) over dry ice and 40 µm frozen sections were cut on a cryostatic microtome. Sections were rapidly thawed and well rinsed in PBS, and treated with sodium nitro-ferrocyanide according to the method of Straus (1971) to inactivate endogenous peroxidase activity before incubation with immunohistochemical reagents.

All incubations with antisera were carried out for 30 min to 1 h at room temperature with mild agitation. Tissue was rinsed for 2 h in several changes of PBS following the use of each serological reagent. All serological agents were absorbed against acetone pellets of extracts of Aplysia head ganglia to reduce nonspecific background

staining; dilutions were made in PBS. Following a short preincubation in whole goat serum (Gibco, 1:20 dilution), experimental sections were incubated with a 0.2 mg/ml solution of antisera C IgG while control sections were incubated in a similar concentration of antisera A IgG (preimmune IgG). All sections were then incubated in goat-anti-rabbit (Miles, diluted 1:20) followed by incubation in rabbit peroxidase-anti-peroxidase (Bionetics, 1:20 dilution). Horseradish peroxidase reaction product was visualized by reaction in 3,3'-diaminobenzidine-4HCl (Sigma, 0.6 mg/ml in PBS) and 0.006% H_2O_2 for 15 min. After a final rinse in PBS, the sections were floated on 5% gelatin in 50% ethanol and picked up on microscope slides precoated with 0.5% gelatin with 0.05% chromium potassium sulfate. The sections on slides were then dehydrated in ethanol, cleared in xylene and mounted with Permount for viewing.

4. Immunofluorescent staining of tissue cultured cells

Tissue cultures of bag cells from A. californica were generated on microcoverslips as described elsewhere (Strumwasser et al., 1978) and maintained at 15°C in L-15 media supplemented with 0.5% of Neomycin and Polymyxcin B for 3-4 days. The medium in culture dishes was gradually replaced by 3.7% formaldehyde in L-15 at 15°C and fixation of cells allowed for 15 min. The cells were always kept immersed as they ruptured if exposed to air at this stage. The fixative was removed by several rinses of filtered sea water followed by increasing concentrations of ethanol. When cells were finally in 100% ethanol, they were incubated in an ice cold solution of 5% acetic acid in ethanol and kept for 15 min at -20°C, followed by a gradual rehydration at room temperature in PBS. Binding with different sera was carried out by applying 150-200 µl of reagent onto cells on coverslips balanced on rubber stoppers inside a moist chamber at room temperature for 30 min to 1 h. A preincubation with whole goat serum was followed by the first layer of primary rabbit IgG (antisera A or C) and a second layer of fluorescein isothiocyanate conjugated goat anti-rabbit antibodies

(Keppel Labs) at a dilution of 1:30. In between layers, the cells were returned to separate culture dishes and extensively rinsed in PBS for 1 h. After staining and final rinses in PBS and distilled water to remove excess antibody, the coverslips were mounted on slides with UV inert mountant (Hopkin and Williams) and sealed with nail varnish. Slides were viewed with a Leitz Diavert microscope illuminated with light from a 200 W mercury lamp filtered through a BG 12 filter.

5. Animals

Most of the Aplysia californica were collected at the Palos Verdes Peninsula from September 1979 to March 1980, and were mature (body weight $\geq$ 150 gm, reproductive tract weights $\geq$ 0.9 gm); two exceptionally large animals (body weights > 3 kg, reproductive tract weights 20 gm) were obtained from the Monterey Peninsula. A. vaccarria were also collected off the Palos Verdes Peninsula between October 1979 to February 1980; the A. dactylomela was obtained from Hawaii. These animals were maintained in aerated sea water at 20°C, until sacrificed. A. braziliana, collected at Padres Island, Texas, were immediately dissected and their ganglia preserved in fixative as described above.

RESULTS

1. Presence of antibodies specific for ELH

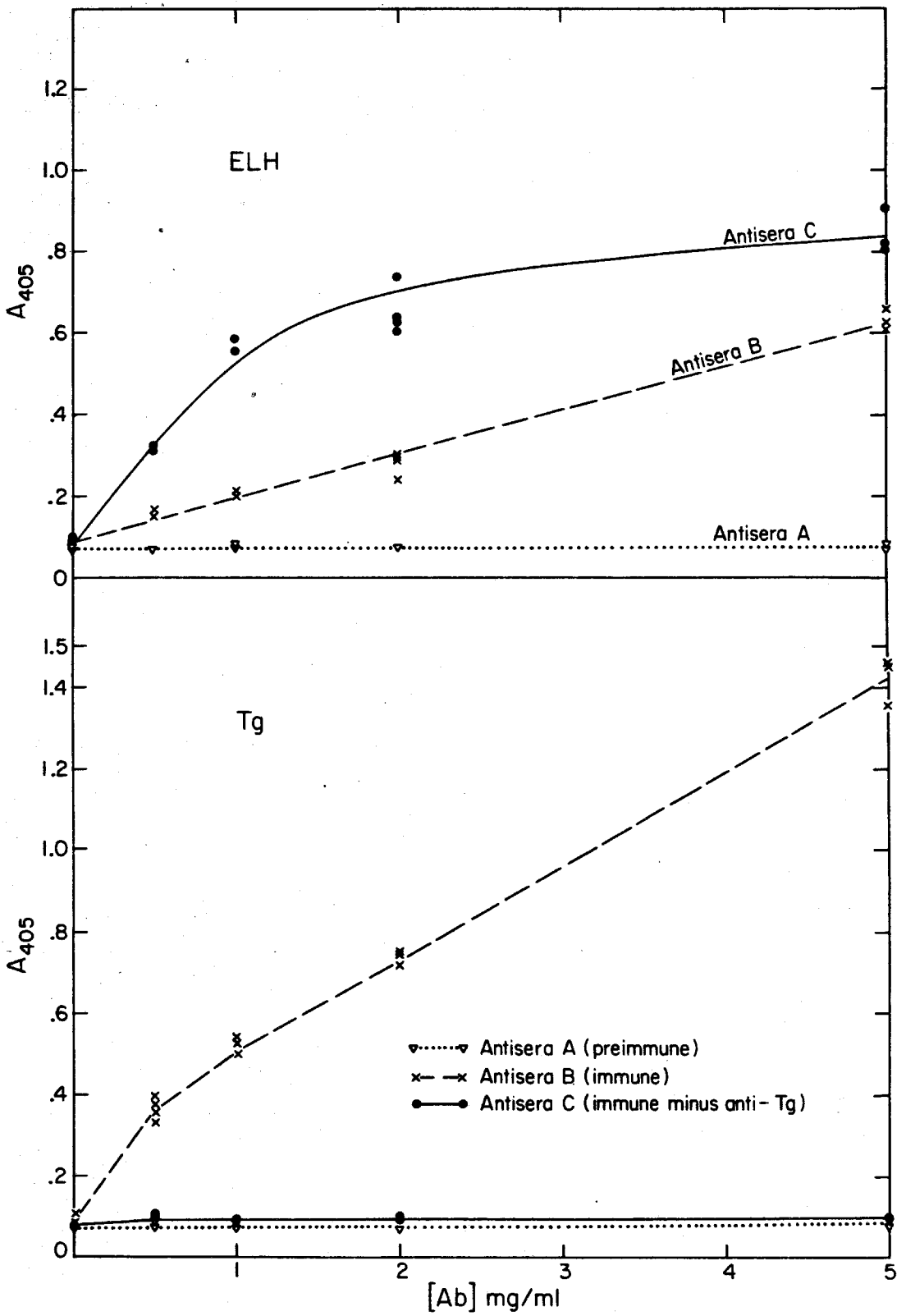
Purified ELH and Tg were separately reacted against IgG fractions of antisera A, B and C by ELISA to determine whether antibodies specific for the hapten had been generated. Figure 1 shows that immune sera (B) had an apparent higher titer of anti-Tg antibodies than antibodies which bind to ELH. However, while specific removal of anti-Tg antibodies by affinity chromatography greatly reduced the response of antisera C to Tg (Fig. 1, lower graph), there is an increase in response of antisera C to ELH (Fig. 1, upper graph). Therefore at the same total antibody protein concentrations, the specific activity of antibodies binding to ELH is higher in sera where

FIG. 1. ELISA quantitation of anti-ELH and anti-Tg titer. Rabbit preimmune IgG (antisera A), immune IgG (antisera B) and immune IgG after removal of anti-Tg antibodies by affinity chromatography (antisera C) were each tested against hapten (ELH) or carrier (Tg) coated wells. Bound rabbit antibodies, after rinsing, were treated with goat-anti-rabbit alkaline phosphatase conjugate, and enzyme activity was measured by reaction with p-nitrophenyl-phosphate. Antisera titer is expressed as absorbance at 405 nm.

Upper: When tested in wells coated with 5 μg/ml of pure ELH, both immune antisera B and C show increasing binding with increasing antibody concentrations; however, antisera C has a higher titer than antisera B, which still contains antibodies against Tg.

Lower: Strong binding to Tg (wells coated with 20 μg/ml) is only seen with antisera B. Antisera C, which has passed through a Tg-conjugated Sepharose column, shows a reduced titer of anti-Tg, not anti-ELH antibodies.

No binding is seen when antisera A is reacted against either antigen.


ELH
Antisera C
Antisera B
Antisera A
A_{405}
1.2
1.0
.8
.6
.4
.2
0
Tg
1.5
1.4
1.2
1.0
.8
.6
.4
.2
0
Antisera A (preimmune)
Antisera B (immune)
Antisera C (immune minus anti-Tg)
1
2
3
4
5
[Ab] mg/ml

the predominant Tg antibodies have been removed. Antisera C IgG fractions are thus enriched for anti-ELH antibodies which do not recognize determinants on the carrier molecule. As expected, preimmune sera (A) shows no response to either antigen.

Approximately 7 mg of total protein was recovered in the IgG fractions from 1 ml of antisera A, 34 mg/ml of antisera B and 14 mg/ml of antisera C.

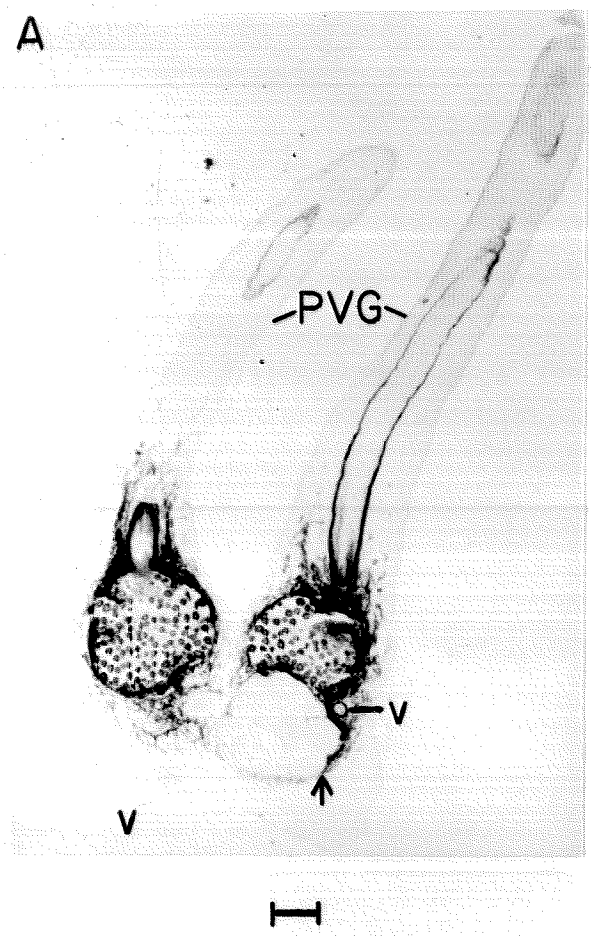
2. Peroxidase-anti-peroxidase staining of abdominal ganglion of Aplysia californica

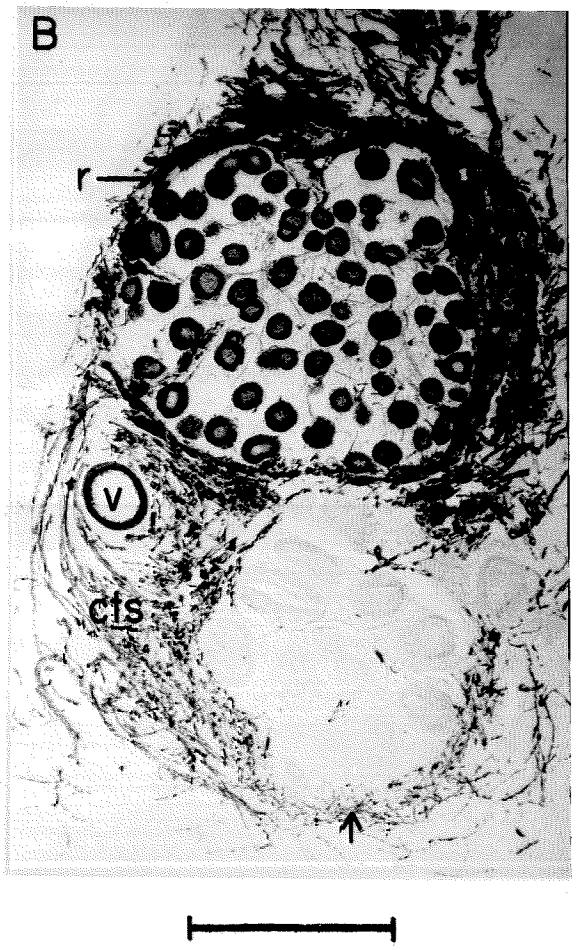
A pocket of connective tissue encloses 200-400 bag cell somata at the junction of the abdominal ganglion and each pleurovisceral connective nerve. In immunostained sections, all the somata within a bag cell cluster show strong, positive immunoreactivity for ELH in contrast to the great majority of neurons in the rest of the ganglion (Fig. 2A and B). In the 12 ganglia examined, the large identifiable neurons of the ganglion, such as other neurosecretory cells including R15 and the white cells (R3-R14) and neurons which are nonsecretory in function, such as L10 and R2, always remain unreactive to anti-ELH antisera. Within the abdominal ganglion, none of the other neurons stained for ELH, except for a few ectopic bag cells described later.

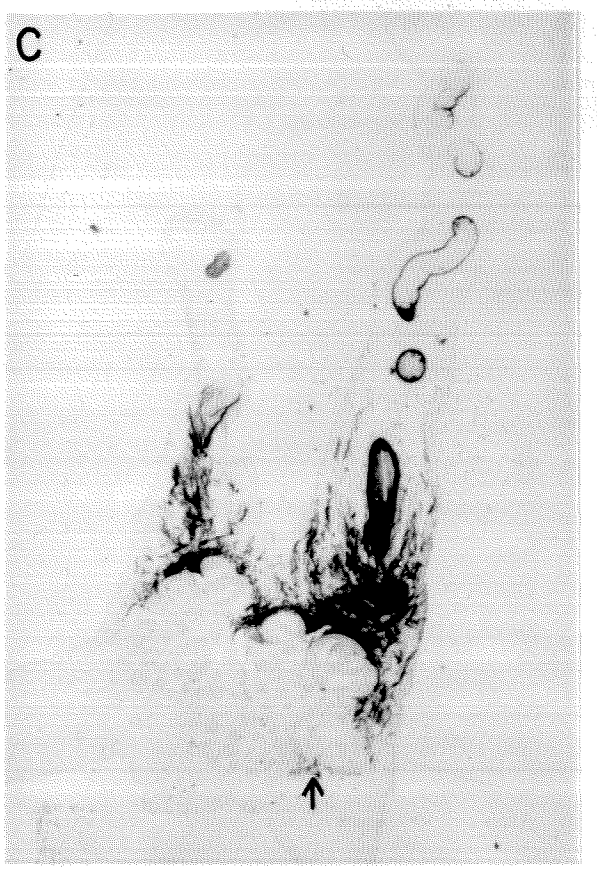
Within bag cells, perinuclear staining is intense while the nuclei remain free of stain. Bag cell somata appear ovoid with a mean major somal diameter of 87 microns and a minor somal diameter of 62 microns when averaged over six animals. Mean nuclear major and minor diameters measure 47 microns and 29 microns respectively (Table I). Inside the cluster stained processes are relatively sparse suggesting that each bag cell may only send out a small number of neurites to the periphery, or that the detectability of ELH was limiting in finer processes. Extensive branching of these ELH-bearing processes occurs within the connective tissue sheath at the rim of the cluster and radiate out over the whole abdominal ganglion (Fig. 2B).

FIG. 2. Cryostat sections of the abdominal ganglion of A. californica immunostained with antisera C by the PAP method. (A) Whole transverse section shows immunoreactive bag cell somata confined within two clusters with processes ringing the periphery and extending into the ganglionic sheath as well as anteriorly into both PVCs. A cuff of ELH+ processes outlines each PVC nerve trunk; v points to another cuff, appearing as a ring in cross section around the vulvar nerve. (B) Higher magnification reveals darkly stained perikarya within all bag cell somata. Though ELH+ processes are sparse within the cluster, they ramify at the rim (r), sending branches rostrally, caudally and medially into the connective tissue sheath (cts). A few stained branches are seen, associated with septa, among the large, unstained neurons of the ganglion. (C) A section dorsal to the bag cell clusters shows stained processes densest in the rostral part of the ganglionic sheath; some still extend to the caudal margins (arrows) of the ganglion. Stained cross-sections of the PVC cuff diminishes in intensity with distance from the ganglion. All abdominal ganglion neurons in this and other sections are unreactive. (D) Several cross sections of the spiralling cuff of processes show, in detail, the reduction of staining in more anterior regions of the PVC.

(A) and (C) share the small scale bar, (B) and (D) share the large bar. Both bars represent 500 μm.


A
PVG
v
v


B
r
v
cis


C

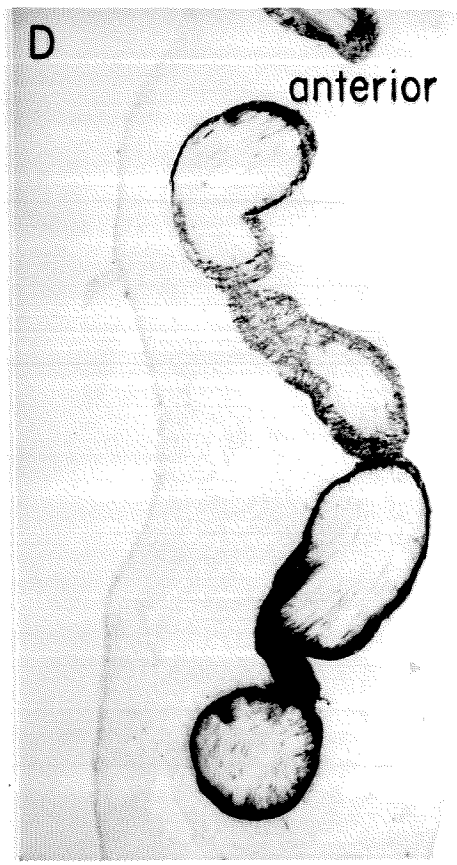

D
anterior

TABLE 1. Somal and nuclear dimensions of bag cells and ectopic cells

	n	Major somal diameter, µm		Minor somal diameter, µm		n	Major nuclear diameter, µm		Minor nuclear diameter, µm	
		mean	(S.E.M.)	mean	(S.E.M.)		mean	(S.E.M.)	mean	(S.E.M.)
Animal I										
Bag cells	51	99.91	(2.95)	66.43	(2.07)	42	52.80	(2.15)	29.74	(2.09)
Ectopic cells	8	103.40	(6.35)	69.34	(2.79)	2	48.40		26.40	
Animal II										
Bag cells	381	100.68	(1.10)	71.35	(0.90)	295	54.53	(0.82)	34.82	(0.66)
Ectopic cells	42	107.91	(4.90)	73.96	(3.27)	36	58.42	(2.85)	37.89	(2.04)
Animal III										
Bag cells	171	103.59	(2.87)	70.45	(1.94)	118	57.35	(1.65)	32.02	(1.16)
Ectopic cells	6	139.33	(23.01)	70.40	(11.94)	3	64.63	(19.01)	44.00	(12.45)
Animal IV										
Bag cells	285	67.65	(0.86)	55.12	(0.61)	257	36.57	(0.59)	26.78	(0.40)
Ectopic cells	4	79.20	(16.07)	46.20	(6.39)	4	37.40	(7.62)	26.40	(4.15)

Table 1 (continued)

Animal V										
Bag cells	136	79.72	(1.15)	66.26	(1.21)	115	43.09	(1.08)	30.54	(0.87)
Ectopic cells	8	80.30	(5.16)	63.80	(6.83)	5	47.52	(6.68)	31.68	(6.68)
Animal VI										
Bag cells	193	67.94	(0.98)	43.60	(0.62)	169	35.88	(0.68)	22.08	(0.44)
Ectopic cells	11	72.80	(4.67)	43.20	(2.31)	9	37.15	(3.02)	16.62	(2.29)
Mean of Animals I-VI[a]										
Bag cells		86.58		62.20			46.70		29.33	
Ectopic cells		97.16		61.15			48.90		30.50	

[a]Mean of the six animals weighted equally.

These neurosecretory processes are densest over the bag cell clusters and can be traced medially to the region of connective tissue between clusters at the rostral margins of the ganglion (Fig. 3, arrow). The density of bag cell processes diminishes with distance from the clusters but stained processes can often be seen to extend to the caudal edge of the abdominal ganglion (Fig. 2A, B and C, arrows).

Immunoreactive neurites invade the pleurovisceral connectives (PVCs) in two ways. Within the thick connective tissue sheath of the proximal PVC, processes radiate toward the outermost surface. These neurosecretory processes seldom extend anteriorly beyond 1 cm from the clusters. Immunoreactive processes also spiral around the PVC nerve to form a cuff between the longitudinally oriented axons travelling to the pleural ganglion and the external cylinder of connective tissue sheath (Fig. 2C and D). This circularly oriented cuff of processes extends farther into the PVC than the radiating neurites described above, and though they also diminish in intensity as they travel away from the clusters, faint staining can be seen as far as 2 cm anteriorly into the PVC. In the majority of A. californica examined (body weight $\geq$150 gm; reproductive tract weight $\geq$0.9 gm), there is no discernible staining as one proceeds anteriorly in the PVC until within 1 cm of the pleural ganglia. Immunoreactive processes at this other extreme of the PVC appear to emanate from cells in the head ganglia and are under further investigation.

In most animals, there appears to be a region of discontinuity in staining, in the middle of the PVC, of up to 2 cm. However, in two very large A. californica (body weight >3 kg; reproductive tract weight $\leq$20 gm), a few longitudinal immunopositive fibers are seen as far as 5.5 cm from the bag cell clusters, running within the PVC nerve trunk, which had a total length of 7 cm. It is possible that a small number of longitudinal fibers, or fibers of small caliber in this middle region of the PVC, were not detectably stained in the smaller animals.

FIG. 3. Medially directed immunoreactive processes (arrow) in the connective tissue sheath between bag cell clusters. These presumably serve to couple the electrical activities of the two clusters of neurosecretory cells. ELH+ processes also invade the ganglion with septa (s) adjacent to the unstained large neurons caudal to the right cluster. Three immunopositive ectopic cells are seen outside and below the left cluster. Bar represents 200 μm.


S

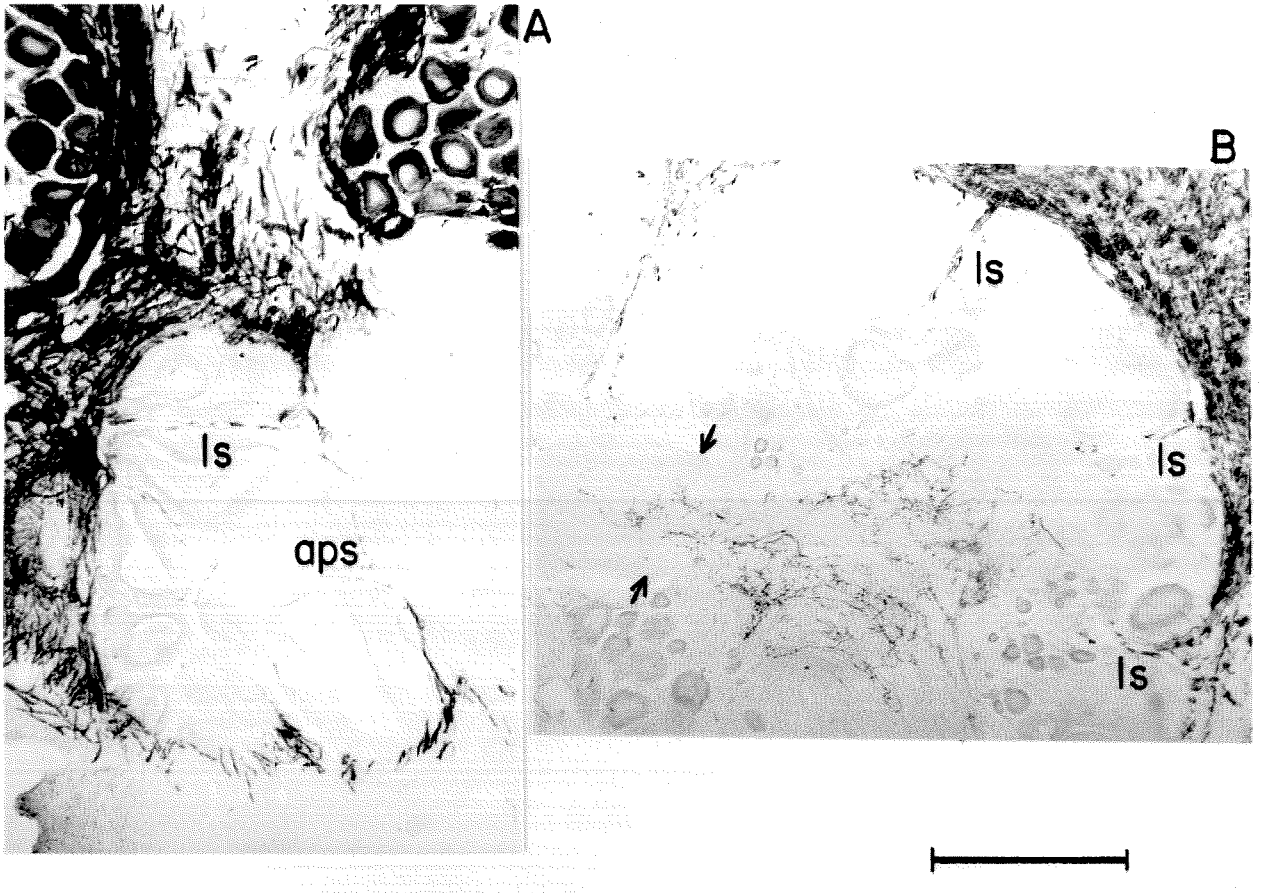
The tendency of bag cell processes to spiral around neighboring nerves is also expressed as a ring of stained neurites encircling the vulvar nerve (Fig. 2A and B, "v"). Other peripheral nerves are free of such a cuff, probably because they are more distant from the clusters. The example of spiraling neurites from an ectopic bag cell in the branchial nerve, described below, suggests that bag cell processes do not discriminate against cuff formation around the more caudally located peripheral nerves.

Bag cell processes often invade the interior of the abdominal ganglion by being associated with septal sheets of connective tissue (Fig. 3 and 4). Where such septa partition off groups of abdominal ganglion neurons, ELH-bearing processes may trace the septal divisions and surround some neuronal clusters within the abdominal ganglion. When a septum bisects the ganglion, bag cell processes run along in an anteroposterior corridor between the two hemiganglia (Fig. 4A, C and D, "aps").

The majority of stained processes appear to be neurosecretory in function because of their close association with the vascularized connective tissue sheath. However, some immunoreactive fibers are seen arising from bag cells which ascend within the PVC nerve trunk or descend into the neuropil of the abdominal ganglion where they course across the commissure which links left and right halves of the ganglionic neuropil (Fig. 4B and 5).

Although all neurons within bag cell clusters stain for ELH, and none of the large ganglionic neurons are immunoreactive, a few scattered immunoreactive cells can be seen in different parts of the ganglion outside the confines of the clusters in 11 of the 12 animals examined (Fig. 4C and D). Some of these ectopic, ELH-immunoreactive cells have also been located within the PVC nerve (Fig. 6A), others adjacent to a septum and one as far displaced as in the caudally located branchial nerve (Fig. 6B). Ectopic or displaced immunoreactive cells have dimensions similar to those of bag cells (Table 1). Although they are not uncommon, ectopic cells are

FIG. 4. Immunopositive processes, associated with connective tissue septa, travel into the abdominal ganglion. In (A), (C) and (D), bundles of processes course along an antero-posterior (aps) corridor within the ganglion. More laterally oriented septa in (A), (B) and (C) also bear processes (ls). (B) shows shallow invasions of neurites from the connective tissue sheath, dense with bag cell processes, in the upper right corner. ELH+ fibers in the neuropile travel into the commissure (arrows). Ectopic bag cells, one with a process, are seen among unreactive neurons in the ganglion in (C) and (D). Bar represents 500 μm.


A
ls
aps
B
ls
ls
ls

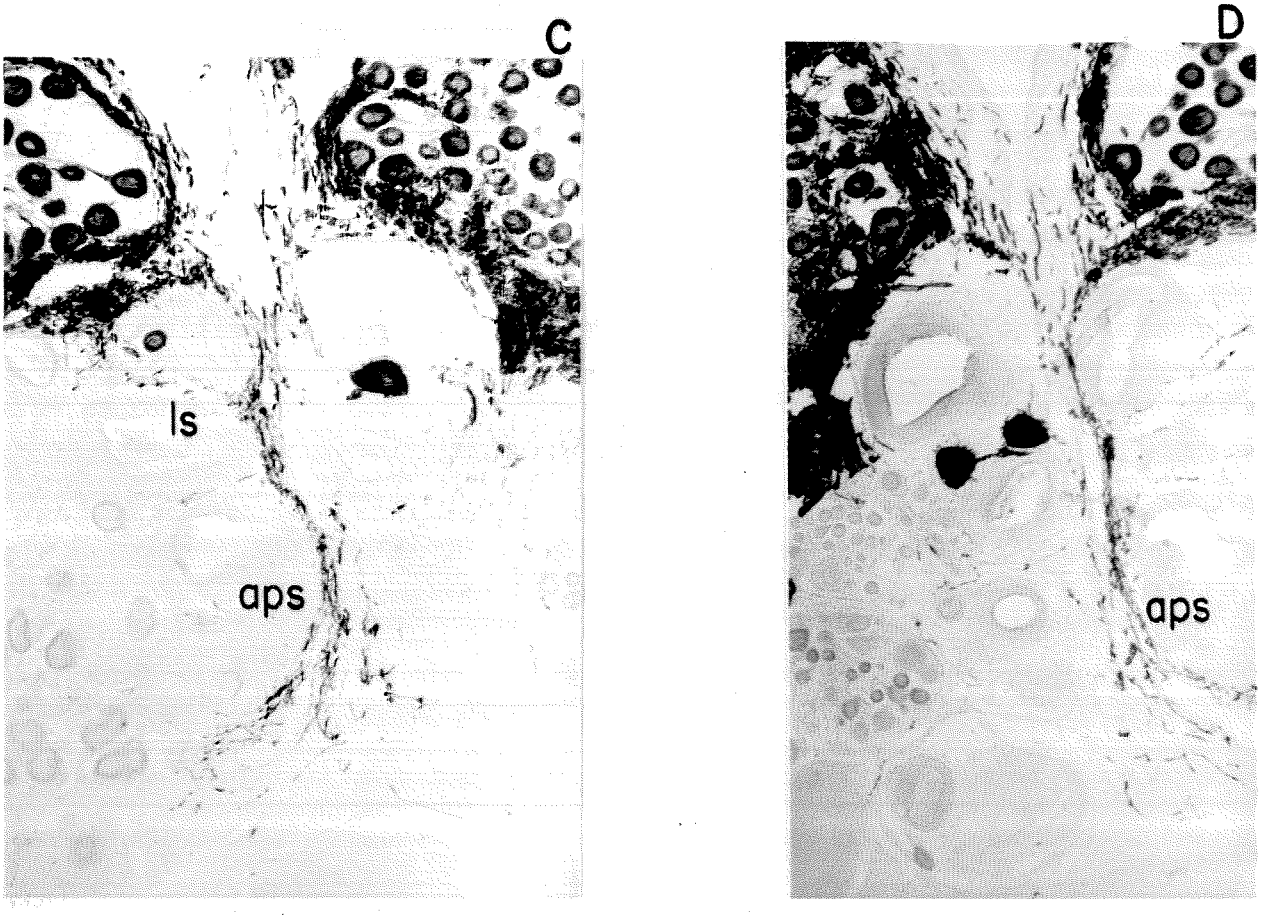

C
ls
aps
D
aps

FIG. 5. Immunoreactive fibers within the neuropile and commissure of the abdominal ganglion. (A) A network of fibers is present within the neuropile on both sides of the ganglionic commissure (box over right side only). (B) These fibers descend from the bag cell clusters along the PVC nerve into the neuropile, some travel across the commissure (arrows) to the neuropile of the contralateral hemiganglion. A longitudinal section of the medial side of the PVC nerve cuff appears at the upper right. (A') and (B') show beaded fibers, within the boxed areas of (A) and (B) respectively, at higher magnification. Bars represent 200 μm.

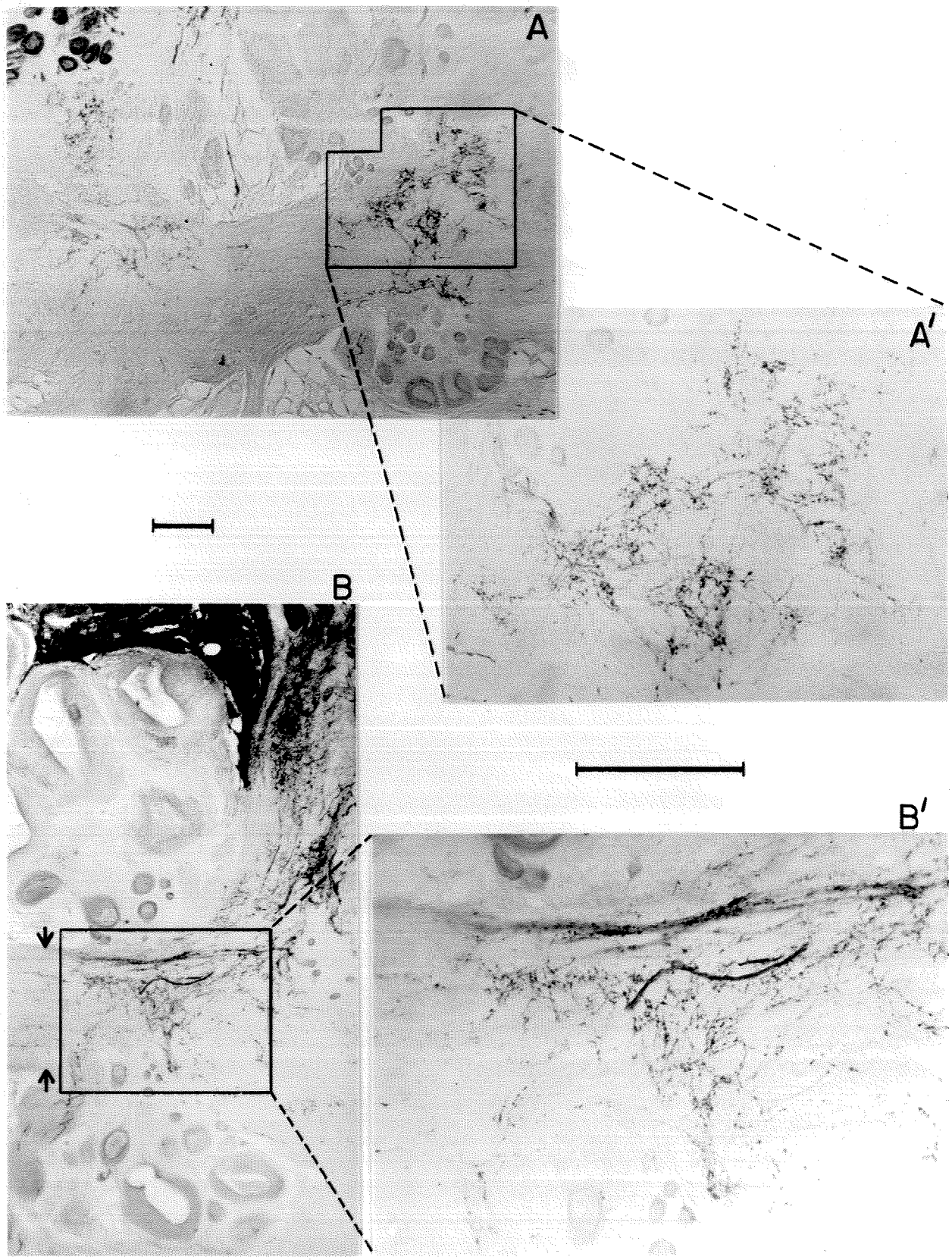
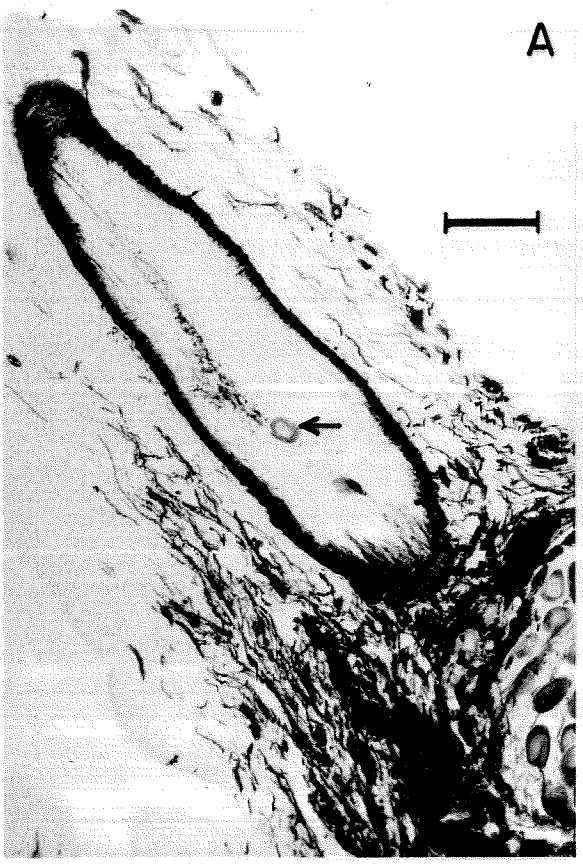
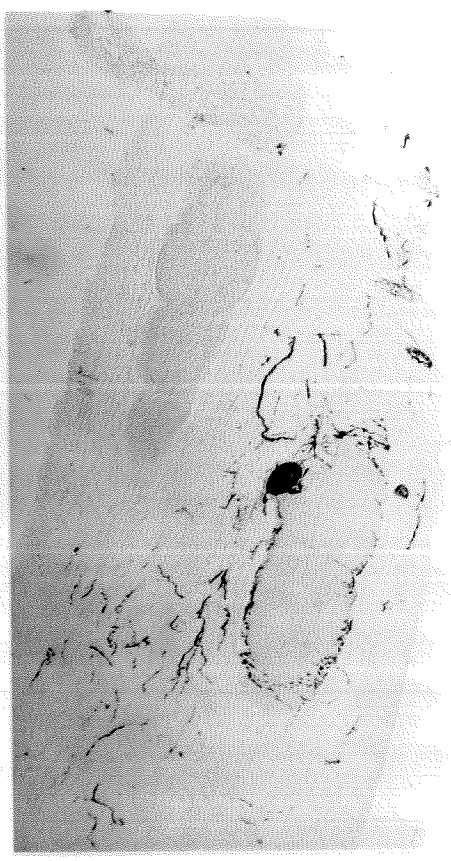

A
A′
B
B′

FIG. 6. Ectopic bag cells within nerve trunks leading from the abdominal ganglion. (A) Cross section of a PVC nerve trunk, encircled by a deeply stained cuff of processes, reveals soma (arrow) with ELH+ perikaryon and unstained nucleus similar to those within the bag cell cluster at lower right. (B) A single displaced immunoreactive cell in the branchial nerve gives rise to processes wrapped around the branchial nerve trunk, as well as neurites ramifying in the sheath of the nerve. Bar represents 250 μm on (A) and (B).


A


B

never consistently seen in any one location; their distribution varies greatly from ganglion to ganglion. In general, they tend to be more common in the rostral half of the ganglion, but as mentioned above, may be found as far away from the clusters as in the branchial nerve. Because of its untypical location, isolated from other immunoreactive cells and processes, this branchial nerve ectopic cell illustrates the variety and complexity of axonal projections that a single "bag" cell is capable of producing. Examined in multiple sections, this single stained cell extended neurosecretory processes into the connective tissue sheath of the nerve as well as a cuff of spiraling processes around the branchial nerve (Fig. 6B).

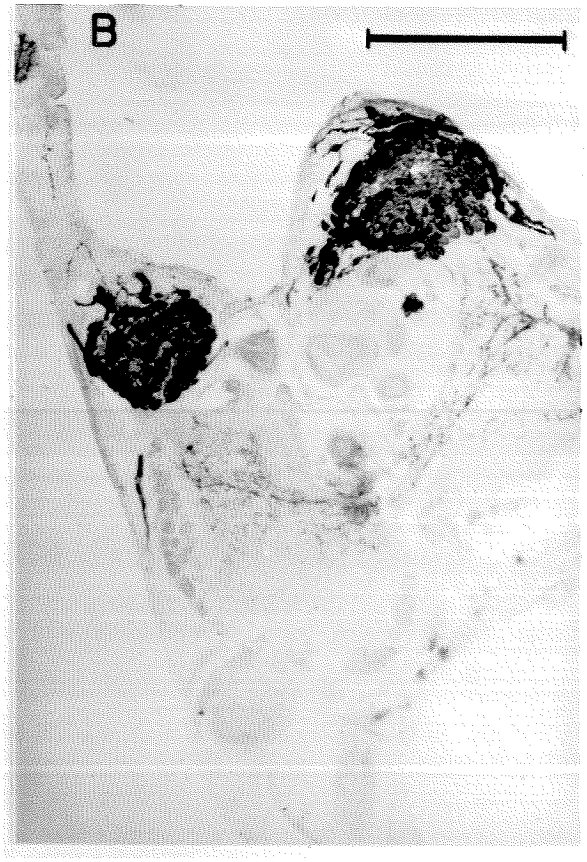
3. Aplysia braziliana, Aplysia dactylomela and Aplysia vaccaria

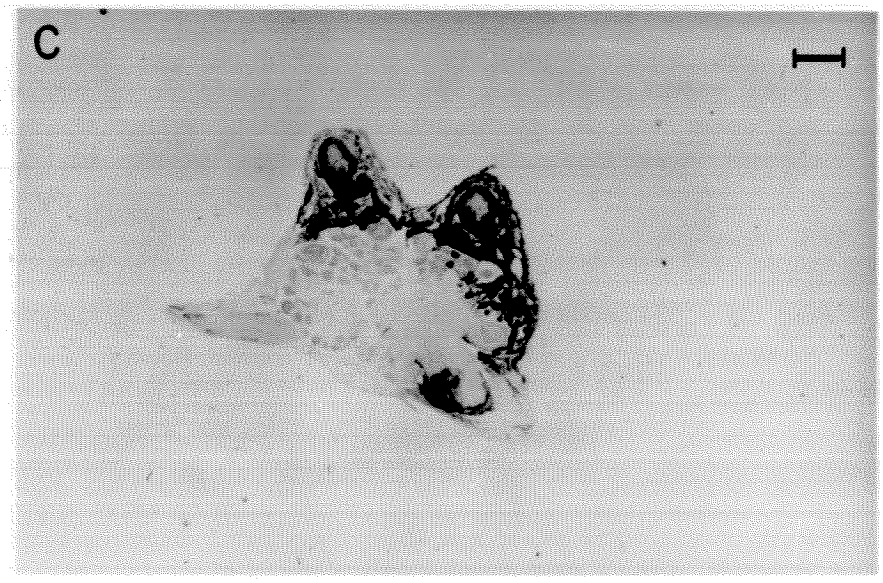
In these three species of Aplysia, the bag cell clusters are consistently strongly stained by immune antisera C to A. californica ELH (Fig. 7). A. braziliana (n=3) shares the extensive and complex branching pattern of processes seen in A. californica (Fig. 7C). Because the A. braziliana abdominal ganglion has a shorter anteroposterior axis, bag cell processes of the connective tissue sheath appear to envelope the whole ganglion more completely than in A. californica with dense immunoreactive processes frequently seen at the caudal margins. Groups of ectopic cells also appear more frequently and stained processes have been seen to invade the connective tissue sheath of the caudally placed peripheral nerves. In A. californica ganglia this was only seen in the one instance when an ectopic cell and its processes were located in the branchial nerve. The bag cells of A. braziliana also send a cuff of processes around the proximal end of the PV nerve as well as medial processes between the clusters.

The immunopositive bag cell system of a small A. dactylomela (n=1) does not appear to differ greatly from those of A. californica and A. braziliana except for a general reduction in size commensurate with the smaller dimensions of its abdominal

FIG. 7. ELH-like immunoreactivity in the bag cell systems of other Aplysia species. (A) A. vaccaria: While bag cells and processes at the edge of the cluster and cuffing the PVC nerves are strongly immunoreactive, no other stained processes can be seen within the sheath of the ganglion. Conspicuously absent are neurites between the two clusters. (B) A. dactylomela: Two clusters of tightly packed ELH+ cells send some stained processes into the sheath. Fibers can clearly be seen within both sides of the neuropile, crossing the commissure in the middle. A small group of ectopic cells are present beneath the right cluster. (C) A. braziliana: This section cuts through both PVC nerves displaying the dense cuff. Processes within the sheath continue to the caudal aspect of the ganglion; ectopic cells can be seen as far as at the junction of the branchial nerve and the abdominal ganglion. Bars represent 500 μm.


A


B


C

ganglion (Fig. 7B). While a prominent cuff of neurites extends up each PVC, neurosecretory processes within the connective tissue sheath are less dense than the networks of A. californica and A. braziliana and do not appear to extend beyond the rostral half of the ganglion. We cannot exclude the possibility that the range of processes is a function of sexual maturity.

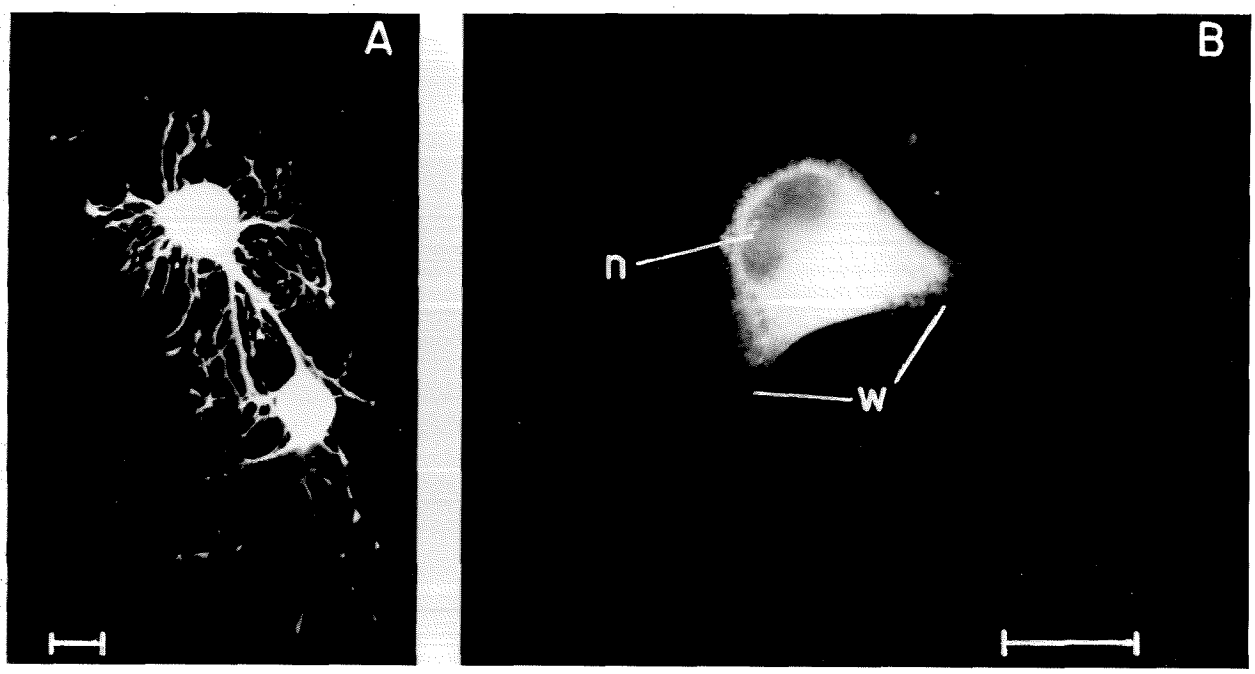
In marked contrast to their counterparts in the other three species of Aplysia, the bag cells of A. vaccaria (n=6; reproductive tract weights from 0.2 g to 9.3 g) show a very limited array of extended processes (Fig. 7A). No network of stained neurites can be seen in the connective tissue sheath of their abdominal ganglia and there is a conspicuous lack of processes running between clusters. Within the prominent clusters, intercellular spaces are densely packed with stained material which may represent a high investment of coiled bag cell processes. Except for the presence of a reduced cuff spiraling around the proximal PVC nerves, no other stained process can be seen beyond the confines of the cluster in all A. vaccaria examined (over 150 sections). However, as these animals were collected during a four month period, between October 1979 and February 1980, we cannot rule out seasonal variability in the distribution of bag cell processes in A. vaccaria.

4. Immunofluorescent studies on primary cultures of Aplysia neurons

There are advantages in applying immunofluorescent staining to bag cells in primary culture since a single bag cell, with all of its processes, could be viewed in its entirety as opposed to sectioned material. Intensely fluorescent immunoreactivity to ELH was displayed by all bag cell neurons in cultures of from 1-4 days (Fig. 8) in sharp contrast to controls of bag cells treated with preimmune sera and of other cultured Aplysia neurons from two other sources after reaction with either antisera A or antisera C.

In many preparations, it was possible to note heterogeneity of staining within the bag cell soma: the nucleus can be clearly distinguished as a dark, asymmetrically

FIG. 8. Immunofluorescent staining of primary cultures of bag cells. These 4-day-old cultured bag cells were fixed in formaldehyde and treated with cold acid-alcohol prior to reaction with antisera C, followed by fluorescein isothiocynate conjugated goat-anti-rabbit IgG. (A) Cultured bag cells often become electrically recoupled (Kaczmarek et al., 1979). The proximity of this pair of intensely stained somata, and the presence of two, thick, immunoreactive processes between them makes it likely that these cells are coupled. (B) At this level of focus, the unstained ovoid nucleus (n) of this bag cell is contrasted with strongly immunoreactive material within the perikaryon. A thin "web" (w) or sheet of cytoplasm, spreading from the lower left and right corners of the soma, leads to numerous processes. Bars represent 50 μm.


A
B
n
w

located ovoid encircled by a richly fluorescent perinuclear ring which fills up most of the rest of the cell body (Fig. 8B). Around the edge of the cell body, there are occasional unstained vacuoles which may represent lysosomes and are not expected to contain immunoreactive ELH. Often the edge of the soma extends in a sheet or web over the substrate before processes project away from it. Such extensions and all neurites of bag cells are also immunoreactive though to a lesser degree than the perinuclear ring. Putative glial cells, present in bag cell cultures and identified by their small size, showed faint background staining similar to bag cells and glial cells stained with preimmune serum.

Primary cultures of A. californica eye and buccal ganglion neurons, 1-2 days after seeding, were also reacted with preimmune or immune sera. These are heterogeneous populations of neuronal cell types but in all cases, no specific immunoreactivity was seen associated with any cell type.

DISCUSSION

These immunohistochemical studies are the first to show the topographic distribution of ELH+ cells and processes in cryostat sections of the abdominal ganglion and its neighboring nerves. Our observation that all neurons within bag cell clusters are immunopositive in all 12 A. californica examined establishes bag cell clusters as homogeneous populations of ELH-producing, neurosecretory cells antigenically distinct from the other neurons of the abdominal ganglion. The extensive array of immunoreactive processes provides important anatomical support for the model of local hormonal action of bag cells on the activities of other neurons within the ganglion (Mayeri, 1979). This distribution of neurites is also consistent with electrophysiological studies on the coupling of afterdischarges between clusters (Kupfermann and Kandel, 1970; Haskins and Blankenship, 1979). The observed "cuff", organized in close apposition to the proximal PVC nerve trunk, may contain contacts between

putative driver neurons in the head ganglia and the bag cell system. Finally, in studies carried out on three other species of Aplysia, we report the presence of a molecule(s) in their bag cell systems which shares antigenic determinants with A. californica-ELH.

Are bag cells homogeneous?

It is known that besides ELH, at least three other peptides are released by bag cell clusters during a single afterdischarge in vitro (Stuart et al., 1980). The functional significance of these peptides is not presently known and raises the question whether the bag cells are heterogeneous in their production of these peptides. Our observation that all neuronal somata within bag cell clusters show strong immunoreactivity for ELH supports the hypothesis that all bag cells produce ELH. Our results cannot rule out the possibility that bag cells may still be heterogeneous in their ability to synthesize and release some of the other, less well characterized neurosecretory products which are also released during an afterdischarge. While it is clear from the cell culture results that the glial cells are not immunoreactive for ELH, it is not known whether any of these other secretory products are glial in origin.

In studies where intracellular injections of Lucifer Yellow allowed visualization of the neuritic arborization of single cells, the direction of bag cell process extension was variable (Kaczmarek et al., 1979). We have described several types of immunoreactive processes—those associated with the sheath, cuff processes and fibers within the neuropile. The possibility then arises that there may be several subgroups of ELH-bearing bag cells each producing a separate class of process which would argue against morphological homogeneity. While we cannot completely exclude the possibility that a small but distinct population of bag cells only send their processes to the neuropile, the example of an ectopic immunoreactive cell in the branchial nerve demonstrates the ability of one cell to send out the other two types of processes.

Not all immunoreactive cells are found within the confines of the BC clusters; a few stained cells have been found in other parts of the abdominal ganglion or neighboring nerve tracts in every animal examined. One possibility is that these cells contain some product which shares immunoreactive determinants with ELH but bear no other kinship to BCs. A second and more attractive explanation is that these are ELH-bearing ectopic cells of BC origin which have migrated to inappropriate locations during development. Several points are consistent with the latter explanation: (i) location of these cells vary greatly from ganglion to ganglion suggesting that they are not members of another defined group of abdominal ganglion neurons; (ii) their cell size does not differ greatly from that of BCs (Table 1); (iii) when neurites are seen emanating from these cells, they resemble those of BCs, again attesting to a common origin (Fig.6B).

In developing vertebrate nervous systems, many cells, including displaced ones, are often eliminated when they fail to make appropriate synaptic contacts with post-synaptic targets (Lund, 1978). However, since BCs are neuroendocrine cells which presumably do not make synaptic contacts with their targets, these ectopic cells may not have been programmed for elimination.

Immunofluorescent staining of all dissociated bag cells in culture demonstrates that, as in the intact cluster, these cells bear a high ELH content in somal and neuritic cytoplasm. The lack of immunoreactivity in cultures of buccal and eye neurons rules out the remote possibility that ELH immunofluorescense is an artifact of, or triggered by, culture conditions. Since bag cells extend new processes while in culture (Strumwasser, et al., 1980), the staining of all processes indicate ongoing transport of ELH in culture as in the intact system.

Primary cultures of bag cells appear to retain their normal neurophysiological characteristics of afterdischarge (Kaczmarek et al., 1978a) and electrical coupling (Kaczmarek et al., 1979). It is therefore not surprising to find that they also maintain

their neurosecretory properties such as synthesis and transport of ELH. Whether ELH persists in primary cultures of bag cells over weeks remains to be determined.

Morphological correlates of local hormone action

Within the abdominal ganglion we find ELH+ processes associated with septa as well as stained fibers within the neuropil and traversing the commissure which links left and right hemiganglia. Septa are extensions of the connective tissue sheath which invade and partition the ganglion as the animal grows. A very simple septal pattern can already be seen in small Aplysia with a body weight of 5 gm. In larger animals, the increasingly complex septal partitions appear to subdivide the ganglion into segments (Kandel, 1979). Since the majority of bag cell neurosecretory processes are intimately associated with the superficial envelope of connective tissue overlying the ganglion, this association appears to continue with the development of septa as the ganglion matures.

Mayeri and colleagues have proposed that bag cells modulate the activity of other neurons within the abdominal ganglion by local release of neurohormones during an afterdischarge (Branton et al., 1978, Mayeri 1979). The responses of the target neurons are characteristically long-lasting with slow and smoothly graded onsets and differ from responses at conventional synapses. They have further shown that some of the excitatory effects can be mimicked by direct application of ELH onto a target neuron, R15 (Branton et al., 1978), while inhibitory responses may be mediated by a different product of the bag cell cluster (Rothman et al., 1979). Our results provide morphological support for this model; we note three routes through which such local hormonal action can be brought about. Firstly, the cortex of neuronal somata in the abdominal ganglion is enveloped by an overlying sheath densely infiltrated by ELH+ processes. Secondly, in the interior of the ganglion, septa bearing bag cell neurites frequently lie in close proximity to cell bodies. Finally, immunoreactive fibers course through the neuropil amidst processes of other neurons. It is likely,

in these three instances, that bag cell neurohormones can readily reach somal or dendritic receptors on nearby neurons when released during an afterdischarge.

Our studies suggest that A. vaccaria provides an ideal preparation in which to test the local hormone action model. We notice that except for a PVC cuff and processes within the cluster, its abdominal ganglion lacks all other types of immunoreactive bag cell processes. Target neurons within the ganglion would only have access to bag cell neurohormones through the hemolymph. It would therefore be of great interest to see if prospective target neurons respond to bag cell products and whether these responses still occur concomitant with a bag cell afterdischarge as they do in A. californica (Mayeri, 1979).

Possible roles of the BC organized cuff

One of the unique morphological features of the bag cell system is the highly organized cylindrical cuff of ELH+ processes arranged just outside the core of the nerve trunks (the pair of PVC and the vulvar nerve). While the presence of a cuff of neurosecretory processes, presumably from bag cells, has been described by ultrastructural criteria by Frazier and colleagues (1967), the PAP immunocytochemical technique has allowed a clear demonstration of this structure at the light microscope level.

The function for this cuff of bag cell processes in direct contact with the PVC nerve is not known. Release of neurohormones at these sites may modulate communications between abdominal ganglion neurons and those in the head ganglia at axonal levels, another form of local hormone action. A second possibility is that this region represents an area of direct synaptic inputs from the head ganglia onto bag cell processes. Stimulation of the peripheral nerves of the abdominal ganglion will not activate bag cells (Kupfermann and Kandel, 1970; Kaczmarek, personal communication), while direct stimulation of the pleural end of the PVC or of certain cell clusters in the cerebral ganglion will induce bag cells to afterdischarge (Kaczmarek and

Chiu, unpublished results). This suggests the presence of an orthodromic pathway which descends from the head ganglia to the bag cells. Consistent with this hypothesis are studies which show that bag cell action potentials typically originate from the distal neurites of the PVC nerve and propagate inward toward the somata in the isolated abdominal ganglion (Dudek and Blankenship 1977; Kaczmarek et al., 1978b).

Alternatively, if bag cell processes extended all the way to the head ganglia, stimulation of the distal PVC would trigger bag cells antidromically. In all but two of the animals examined, immunoreactive processes from the bag cells are contained within the proximal 2 cm of the PVC. There appears to be no continuity between these processes and stained neurites at the pleural end of the connective, usually 2-3 cm away, which most likely originate from several immunoreactive cells within the pleural ganglia (Chiu and Strumwasser, unpublished results). However, a very few ELH+ fibers were found in the midsection of the PVCs and as far anteriorly as 5.5 cm from the BC cluster in two exceptionally large A. californica whose PVCs were 7 cm long. The presence of a small number of similar fibers in the PVC nerve of the smaller animals may have escaped detection by this technique. Or such long processes may appear late in development and are therefore only seen in these exceptionally large animals. It is unclear whether such fibers are bona fide bag cell processes or neurites from immunoreactive neurons in the head ganglia. The role, if any, they play in triggering bag cell afterdischarge is equally ambiguous. It is clear that afterdischarge, however, can be initiated from the pleural end of the PVC in animals that clearly have no immunocytochemically evident bag cell processes in the middle portion of the PVC.

Intercluster coupling

The initiation of an afterdischarge in one cluster will result in the activation of the contralateral cluster to afterdischarge within seconds. When the sheath capsule

between two clusters was cut, such coupling also ceased (Kupfermann and Kandel, 1970). It is more than likely that such intercluster coupling is effected by the profusion of medially directed immunoreactive processes we see emanating from each cluster. In A. vaccaria where such processes are conspicuously missing from stained sections, there is a concomitant lack of coupling between the pair of bag cell clusters (Kaczmarek and Chiu, unpublished results).

In the neuropil immunoreactive fibers are present but sparsely represented compared with the profusion of processes in the sheath. Since a shallow cut between clusters is sufficient to uncouple the activity of the two clusters, any intercluster communication via these deeper fibers probably does not play an important role in the transfer of afterdischarge. As discussed above, they may contribute to local hormonal effects as release sites within the neuropil. Earlier ultrastructural studies have revealed no chemical synapses between bag cell processes and other neurons within the sheath. It is however not known if bag cells make synaptic contacts with other neurons within the neuropil.

Neurohaemal areas

Rapid introduction of neurohormones into the general circulation, a requisite of any effective neurosecretory system, is dependent upon the extent and efficacy of the neurohaemal areas where neurosecretory surfaces are brought into close contact with the vascular system. In the abdominal ganglion, Frazier and colleagues (1967) described the general distribution of processes in the vascularized connective tissue sheath which contain 1700 Å, moderately dense core granules resembling those found in bag cell somata. They argued that such processes must be neurosecretory in function based on their content of granules, their proximity to sinus spaces within the sheath and their conspicuous lack of functional contact with other axons and cells within the sheath. We extend their description in our visualization of the full

scope of this neuritic network which is densest around the clusters but stretches to the caudal edges of the ganglion, especially on the ventral surface where the bag cell clusters are most prominent. The Bag Cell Organ thus increases its neurosecretory surfaces immensely by utilizing the major part of the ganglionic surface as its neurohaemal organ. More importantly, this study establishes the presence of the neurosecretory product, ELH, in these hitherto presumed neurosecretory processes. It is highly likely that the 1700 Å granules actually contain ELH and other secreted bag cell peptides; however, this awaits verification by immunohistochemical studies at the EM level.

Comparative studies in other species

Antiserum, generated against ELH purified from A. californica, subgenus Neoaplysia, specifically crossreacts with the bag cell systems of three other species of Aplysia from two other subgenera: A. braziliana and A. dactylomela, subgenus Varria, and A. vaccaria of subgenus Aplysia. As in A. californica, the bag cell clusters of these three species are also homogeneous populations of immunoreactive neurons. It is reassuring to note that in the three species where intercluster transfer of afterdischarge has been reported (Haskins and Blankenship, 1979), we also find the medially extending processes in the sheath between clusters which couple such activity.

Other investigators have found that extracts of A. californica abdominal ganglia will induce egg laying activity in these other three Aplysiads which we have studied. Extracts containing the ELH of A. vaccaria or A. braziliana will in turn cause oviposition in A. californica (A. braziliana, J. Blankenship, personal communication; A. vaccaria and A. dactylomela, see Toevs, 1969). Our demonstration of cross-immunoreactivity and the evidence of cross bioactivity between these species make it likely that if the ELHs of these four species of Aplysia are not identical, at least the receptor binding sites and antigenic determinants of these molecules must be highly conserved.

REFERENCES

1. Alumets, J., Håkanson, R., Sundler, F. and Thorell, J. (1979) Neuronal localization of immunoreactive enkephalin and β-endorphin in the earthworm. Nature, 279: 805-806.

2. Arch, S. (1972a) Polypeptide secretion from the isolated PVG of Aplysia californica. J. Gen. Physiol., 59: 47-59.

3. Arch, S. (1972b) Biosynthesis of the ELH in the bag cell neurons of Aplysia californica. J. Gen. Physiol. 60: 102-119.

4. Arch, S. (1976) Neuroendocrine regulation of egg laying in Aplysia californica. Amer. Zool., 16: 167-175.

5. Arch, S., Smock, T. and Earley, P. (1976) Precursor and product processing in the bag cell neurons of Aplysia californica. J. Gen. Physiol., 68: 211-225.

6. Blankenship, J. E. and Haskins, J. T. (1979) Electronic coupling among neuroendocrine cells in Aplysia. J. Neurophysiol., 42: 347-355.

7. Boer, H. H., Schot, L. P. C., Roubos, E. W., Ter Maat, A., Lodder, J. C. Reichelt, D. and Swaab, D. F. (1979) ACTH-like immunoreactivity in two electrotonically coupled giant neurons in the pond snail Lymnaea stagnalis. Cell Tissue Res., 202: 231-240.

8. Branton, W. D., Arch, S., Smock, T. and Mayer, E. (1978) Evidence for mediation of a neuronal interaction by a behaviorally active peptide. Proc. Natl. Acad. Sci. USA 75: 5732-5736.

9. Chiu, A. Y., Hunkapiller, M., Heller, E., Stuart, D. K., Hood, L. E. and Strumwasser, F. (1979a) Purification and primary structure of the neuropeptide egg-laying hormone of Aplysia californica. Proc. Natl. Acad. Sci. USA 76: 6656-6601.

10. Chiu, A. Y., Hunkapiller, M., and Strumwasser, F. (1979b) The neuropeptide, ELH of Aplysia: purification, amino acid sequence and antibodies. Society for Neurosciences Abstracts 5:, No. 789.

11. Coggeshall, R. E. (1967) A light and electron microscope study of the abdominal ganglion of Aplysia californica. J. Neurophysiol., 30: 1263-1287.

12. Dudek, F. E. and Blankenship, J. E. (1977) Neuroendocrine cells of Aplysia braziliana. I. Bag cell action potentials and afterdischarge. J. Neurophysiol., 40: 1301-1311.

13. Engvall, E. and Perlmann, P. (1972) Enzyme-linked immunosorbent assay, ELISA III Quantitation of specific antibodies by enzyme-labeled antiimmunoglobin in antigen-coated tubes. J. Immunol., 109: 129-135.

14. Frazier, W. T., Kandel, E. R., Kupfermann, I., Waziri, R. and Coggeshall, R. E. (1967) Morphological and functional properties of identified neurons in the abdominal ganglion of Aplysia californica. J. Neurophysiol., 30: 1288-1351.

15. Fritsch, H. A. R., Van Noorden, S. and Pearse, A. G. E. (1979) Localization of Somatostatin-, Substance P- and Calcitonin-like immunoreactivity in the neural ganglion of Ciona intestinalis L. (Ascidiaceae). Cell Tissue Res., 202: 263-274.

16. Garvey, J. S., Cremer, N. E. and Sussdorf, D. H. (1977)"Methods in Immunology," 3rd Edition, W. A. Benjamin Inc., Reading, Mass.

17. Haskins, J. T. and Blankenship, J. E. (1979) Interactions between bilateral clusters of neuroendocrine cells in Aplysia. J. Neurophysiol., 42: 356-367.

18. Hökfelt, T., Johansson, O., Ljungdahl, Å, Lundberg, J. M. and Schultzberg, M. (1980) Peptidergic Neurones. Nature, 284: 515-521.

19. Jaros, P. and Keller, R. (1979) Immunocytochemical identification of Hyperglycemic Hormone-producing cells in the eyestalk of Carcinus maenas. Cell Tissue Res., 204: 379-385.

20. Kaczmarek, L. K., Jennings, K. R. and Strumwasser, F. (1978a) Cyclic AMP analog generates afterdischarge in Aplysia bag cell neurons. Soc. for Neurosci. Abstr. 4:, No. 814.

21. Kaczmarek, L. K., Jennings, K. and Strumwasser, F. (1978b) Neurotransmitter modulation, phosphodiesterase inhibitor effects and cyclic AMP correlates of afterdischarge in peptidergic neurites. Proc. Natl. Acad. Sci. USA 75: 5200-5204.

22. Kaczmarek, L. K., Finbow, M., Revel, J. P. and Strumwasser, F. (1979) The morphology and coupling of Aplysia bag cells within the abdominal ganglion and in cell culture. J. Neurobiol., 10: 535-550.

23. Kandel, E. R. (1979) "Behavioral Biology of Aplysia," Freeman Press, San Francisco, Pg. 150-151.

24. Kupfermann, I. and Kandel, E. R. (1970) Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of Aplysia. J. Neurophysiol., 33: 865-876.

25. Loh, Y. P., Sarne, Y. and Gainer, H. (1975) Heterogeneity of proteins synthesized, stored, and released by the bag cells of Aplysia californica. J. Comp. Physiol., 100: 283-295.

26. Lund, R. D. (1978) "Development and Plasticity of the Brain," Oxford University Press, New York.

27. March, S. C., Parikh, I. and Cuatrecasas, P. (1974) A simplified method for cyanogen bromide activation of agarose for affinity chromatography. Anal. Biochem. 60: 149-152.

28. Mayeri, E. (1979) Local hormonal modulation of neural activity in Aplysia. Fed. Proc., 38: 2103-2108.

29. Rothman, B. S., Brownell, P. and Mayeri, E. (1979) Purified bag cell peptide mimicks some but not all responses of central neurons to bag cell stimulation in Aplysia. Soc. for Neurosci. Abstr. 5:, No. 857.

30. Schot, L. P. C., Boer, H. H., Roubos, E. W. and Swaab, D. F. (1981) Peptidergic neurons in the pond snail Lymnaea stagnalis (L.) In: Neurosecretion - Molecules, Cells, and Systems." D. S. Farner and K. Lederis, eds., Plenum Press, New York.

31. Smock, T. and Arch, S. (1977) A cytochemical study of the bag cell organs of Aplysia californica. J. Histochem. and Cytochem., 25: 1345-1350.

32. Straus, W. (1971) Inhibition of peroxidase by methanol and by methanol-nitro-ferricyanide for use in immunoperoxidase procedures. J. Histochem. and Cytochem., 19: 682-688.

33. Strumwasser, F., Kaczmarek, L. K. and Viele, D. (1978) The peptidergic bag cell neurons of Aplysia: morphological and electrophysiological studies of dissociated cells in tissue culture. Soc. for Neurosci. Abstr. 4: No. 645.

34. Strumwasser, F., Kaczmarek, L. K., Chiu, A. Y., Heller, E., Jennings, K. R. and Viele, D. P. (1980) Peptides controlling behavior in Aplysia. In: Peptides: Integrators of Cell and Tissue Function. F. E. Bloom, ed., Society of General Physiologists Series Vol. 35, Raven Press, New York.

35. Stuart, D. K. Chiu, A. Y. and Strumwasser, F. (1980) Neurosecretion of egg-laying hormone and other peptides from electrically active bag cell neurons of Aplysia. J. Neurophysiol. 43: 488-497.

36. Toevs, L. (1970) Identification and characterization of the egg-laying hormone from the neurosecretory bag cells of Aplysia. Ph.D. Dissertation, Pasadena, California Institute of Technology.

37. Vaitukaitis, J., Robbins, J. B., Nieschlag, E. and Ross, G. T. (1971) A method for producing specific antisera with small doses of immunogen. J. Clin. Endocr. 33: 988-991.

38. Zipser, B. (1980) Identification of specific leech neurons immunoreactive to enkephalin. Nature, 283: 857-858.

CHAPTER 4

ELH-like immunoreactive systems in the head ganglia of *Aplysia californica*

ABSTRACT

Egg Laying Hormone (ELH), a polypeptide released by the neurosecretory bag cell system in the abdominal ganglion of Aplysia californica, produces marked changes in the electrical activity of neurons in the head ganglia. We have used immunohistochemical procedures to reveal organized groups of cells and fibre tracts within the pleural and cerebral ganglia of the head which are immunopositive when reacted with antibodies generated against ELH. Moreover, these ELH+ systems persisted in two animals 43 days and 67 days after surgical removal of their abdominal ganglia. It is therefore likely that these immunoreactive neurons and their processes are independent of the bag cell system.

The pleural ganglia neurons, with ELH-like immunoreactivity, bear a striking resemblance to bag cells in somal and nuclear dimensions, process morphology and location, which suggests that both populations of cells may have descended from a common precursor pool.

In the cerebral ganglion, a pair of bilateral clusters of 6-10 small immunopositive neurons are located on the dorsal surface in the vicinity of the C clusters, and send their processes into the neuropile. Intensely stained tracts of ELH+ fibres are prominent at all levels of section in the cerebral neuropile; stained fibres can also be traced down most of the nerves emanating from the cerebral ganglion.

The functions of these systems, as well as the specific nature of the immunoreactive molecule(s) they contain, remain unknown. However, this demonstration of the presence and distribution of ELH-like molecules endogenous to the head ganglia raises the possibility that ELH target neurons in the head ganglia may be activated by local sources of ELH-like neuroactive peptides.

INTRODUCTION

Neuropeptides have been known since the early 1950s to function as hormones upon release into the circulation (Bargmann and Scharrer, 1951). The effects of biologically active hypothalamic peptides, such as oxytocin and vasopressin, and the more recently discovered hypothalamic regulatory factors, TRH, LHRH and Somatostatin, were first found to be directed at nonneuronal targets. In recent years, the sensitivity of immunohistochemical techniques and radioimmunoassays have allowed in situ localization and quantitation of minute amounts of neuropeptides which had escaped detection by less refined methods of earlier investigators. These major advances in methodology revealed hitherto unsuspected, widespread distribution of most of the vertebrate neuroendocrine peptides within the central nervous system (Swanson, 1977; Jackson and Reichlin, 1979; Silverman and Zimmerman, 1978; for reviews: Elde and Hökfelt, 1978; Hökfelt et al., 1980), prompting a re-evaluation of the scope and functions of these chemical signals (Moss et al., 1978; Renaud, 1978). A growing body of evidence now suggests that some of these peptides, such as TRH, LHRH and Somatostatin, may be interneuronal messengers, acting as bona fide neurotransmitters or modulating the activity of other, more conventional transmitter compounds (Jan et al., 1979, 1980; Renaud et al., 1975; Dyer and Dyball, 1974; for review: Renaud et al., 1979).

We have studied a molluscan neurosecretory peptide, the Egg-Laying Hormone (ELH) which triggers a program of behavior involving feeding, locomotion and oviposition in Aplysia (Strumwasser et al., 1980). Using immunohistochemical techniques in a previous investigation, we have described the distribution of the neuroendocrine bag cell system in the abdominal ganglion of Aplysia (Chiu and Strumwasser, 1981), which is known to release ELH upon adequate stimulation (Stuart et al., 1980). Although some of the targets of ELH are believed to be outside the nervous system (Coggeshall,

1972; Dudek and Tobe, 1978; Rothman et al., 1979; for review: Blankenship, 1980), ELH is capable of having pronounced and long-term effects on the electrical activities of other neurons. Some of these responsive neurons are found within the abdominal ganglion (Branton et al., 1978); a model of local hormonal modulation discusses the implementation of these effects (Mayeri, 1979). Other putative target neurons have been located in distant ganglia, such as the buccal ganglion (Stuart and Strumwasser, 1980); ELH, released by activated bag cells, may be circulated to these sites via the hemolymph. However we also wondered if bag cell processes extended as far as the head ganglia and whether some of these target neurons are influenced by ELH from neuronal sources other than the bag cells. It therefore became important to know whether this peptide, or ELH-like molecules, are located in other parts of the Aplysia nervous system.

In this study, we report the organization of ELH-like immunoreactive cells and fibre tracts within the head ganglia of A. californica. We also discuss the consequences of long-term surgical removal of the abdominal ganglion on these ELH+ systems.

METHODS

A. californica were collected off the Palos Verdes Peninsula, California, and maintained in 14°C filtered sea water until used. The intact circumesophageal ring of ganglia was dissected with long lengths of peripheral nerves and connectives attached. The preparation was pinned onto a Silgard dish with nerves stretched to align the ganglia and to facilitate identification of specific nerves in section. After trimming off excess connective tissue, the ganglia were fixed, while pinned out, in 3.7% formaldehyde solution of L-15 (Gibco) for at least 48 hours at 4°C. After rinsing in several changes of L-15, to remove excess fixative, the ring of ganglia was unpinned and cryoprotected in 30% sucrose. The cerebro-pleural, cerebro-pedal and pleuro-pedal connectives were carefully severed to free the individual ganglia

which were then separately frozen in O.C.T. Compound (Miles) and sectioned in a cryostatic microtome. Details of treatment of sections and peroxidase-anti-peroxidase staining are described elsewhere (Chiu and Strumwasser, 1981).

For a serial reconstruction of the cerebral ganglion, groups of five consecutive sections were kept separate through the phases of the staining procedure. After the sections were mounted on microscope slides, camera lucida tracings were made, and these were subsequently retraced onto transparent sheets of plastic. Each set of five was internally aligned and ordered; finally the whole sequence was established by collating the sets.

Deganglionation surgery

All A. californica were maintained in 12:12 LD cycle for at least five days, and starved for 24 hours prior to surgery. To anaesthetize the animal, it was immersed for approximately 4 hours in a three liter bath of 230 mM $MgCl_2$ in 33% filtered sea water ($MgCl_2$-FSW). Animals were considered anaesthetized when they did not respond to pinching of the rhinophores and when posture could not be maintained. With the animal placed dorsal side up, immersed in $MgCl_2$-FSW, the skin around incision sites was pinned up away from the body to avoid excessive loss of hemolymph. Through a 10-12 mm incision, made along the dorsal midline at the posterior edge of the rhinophores, the pleuro-visceral connectives were located and severed as close to the pleural ganglia as possible. A second incision was made, starting 2 mm medial to the seminal grove and ending 2 mm anterior to the osphradium. The free ends of the pleuro-visceral connectives and then the attached abdominal ganglion were pulled out; branchial, siphon, pericardial and genital nerves are tied off in two groups with surgical silk (5-0) in order to clamp off the posterior blood vessel. After cutting these nerves, the abdominal ganglion and pleuro-visceral connectives were removed and the incisions stitched closed with surgical silk.

Post operative animals were weighed and antibiotics (4 µl/gm body weight of 25,000 units Penicilin, 25,000 µg Streptomycin per ml) injected into the muscle of the foot. Animals were then maintained in separate baskets in 14°C circulating sea water and weighed weekly until sacrificed.

Bioassays

Maintenance of test animals and the procedure for the egg-laying bioassay have been described elsewhere (Stuart et al., 1980). For ganglionic extracts, cerebral, pedal and pleural ganglia were dissected at 14°C and kept frozen till used. Pooled cerebral ganglia from 20 donors were hand homogenized, in a ground glass homogenizer, in 2 ml of 10 mM Na phosphate, pH 6.5, over ice. After centrifugation for 10 min at 12,000 x g the supernatant was adjusted to a final volume of 3 ml with FSW. Three competent test animals were each injected with 1 ml of this material and observed for egg-laying activity within 2 hours of injection. Pleural and pedal ganglia from 20 donor animals were extracted in the same way, and similar dosages tested for egg-laying.

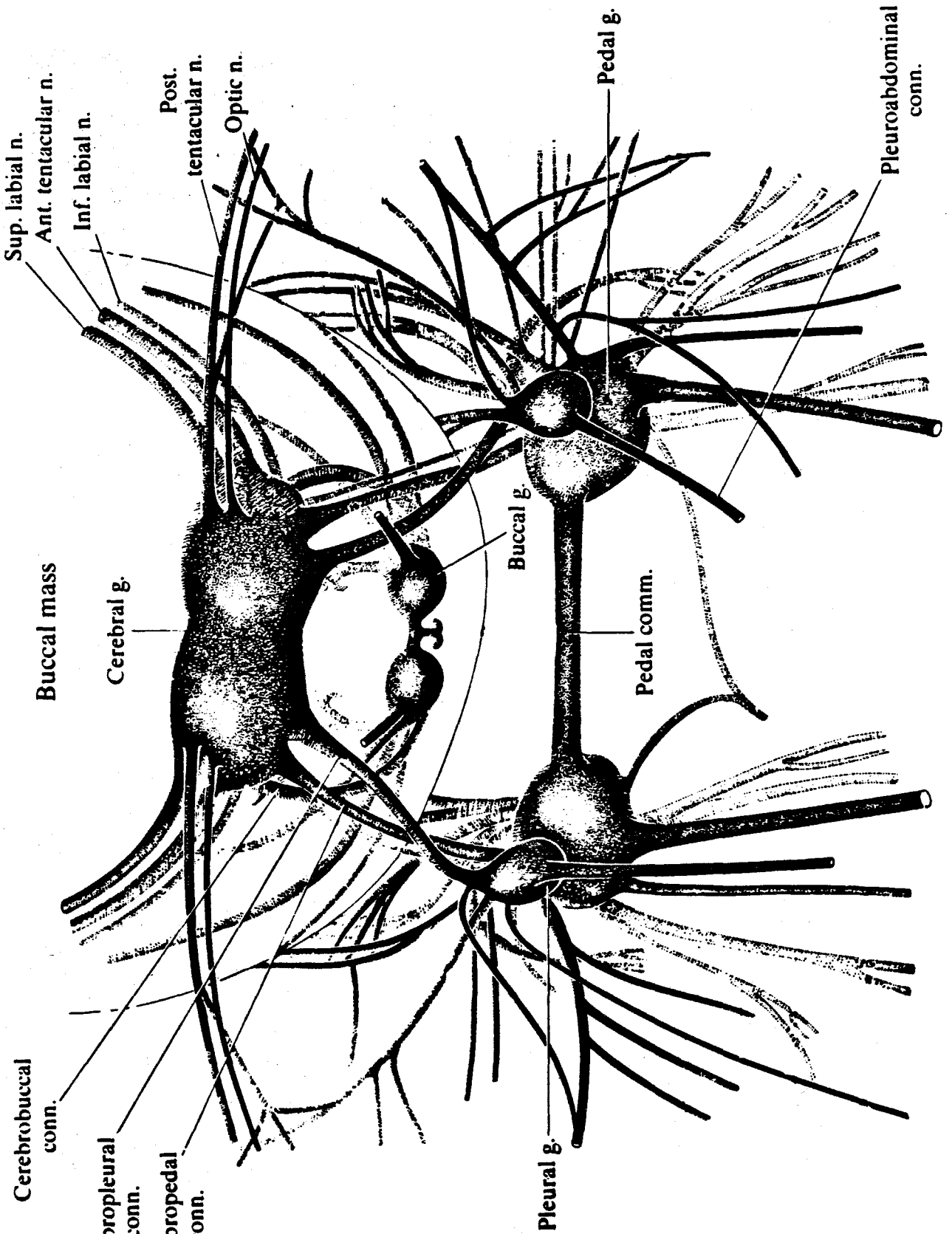
RESULTS

The central nervous system of A. californica consists of a rostrally located group of head ganglia linked to an abdominal ganglion, in the midsection of the animal, by long pleuro-visceral connectives, also known as pleuroabdominal connectives as indicated in Figure 1. The head ganglia are organized into a circumoesophageal ring made up of paired pleural, pedal and buccal ganglia all connected to a single, fused cerebral ganglion (Fig. 1).

Pleural ganglia

Each pleuro-visceral connective arises from a pleural ganglion and descends into an abdominal hemiganglion at its posterior end. A cluster of 200-400 bag cells is always found at the junction of a pleuro-visceral connective and the abdominal

Figure 1. The head ganglia of A. californica. The head ganglia consist of paired pleural, pedal and buccal ganglia all linked by connectives to the single cerebral ganglion to form a circumesophageal ring (unpublished figure of Jahan-Pawar in Kandel, 1979).


Sup. labial n.
Ant. tentacular n.
Inf. labial n.
Post. tentacular n.
Optic n.
Pedal g.
Pleuroabdominal conn.
Buccal mass
Cerebral g.
Buccal g.
Pedal comm.
Cerebrobuccal conn.
Cerebropleural conn.
Cerebropedal conn.
Pleural g.

ganglion in a sexually mature animal; at the anterior terminal where this connective joins the pleural ganglion, up to five ELH+ neurons can be seen in stained sections (Fig. 2C). As in bag cells, the immunoreactivity within these pleural neurons is confined to perikaryon and neuritic cytoplasm; the nuclei remain unstained (Fig. 2D). Somal and nuclear dimensions are also comparable to those of bag cells (Table 1), and the somata are always found in the vicinity of the pleural-visceral connective junction (Fig. 2). Occasionally, a cell may even be seen within the pleuro-visceral connective nerve trunk, caudal to the pleural ganglion (Fig. 2C and D). Similarly, at the other end of the pleuro-visceral connective, ectopic bag cells have been located within the pleuro-visceral connective nerve trunk, rostral to a bag cell cluster. Unlike bag cells, however, these neurons do not always appear to aggregate tightly (Fig. 2C). This lack of obvious clustering may be due to the very small number of immunoreactive cells in the pleural ganglion compared with those in the abdominal ganglion.

When multiple horizontal sections of one left pleural ganglion, cut parallel to a plane through the three connectives (pleuro-visceral, pleuro-pedal and cerebro-pleural) were examined, immunoreactive somata are absent in sections where the Left Giant Cell can clearly be identified on the basis of size (Fig. 3A). Since this easily recognizable neuron lies on the ventral surface of the ganglion, the ELH+ cells appear to be located on the dorsal side of the left pleural ganglion where an immunoreactive cuff round the pleuro-visceral connective nerve as well as fibres in the neuropile are most dense (Fig. 3C). Whether immunoreactive cells of the right pleural ganglion are also found on the dorsal surface could not readily be determined from sectioned material since a convenient marker such as the Left Giant Cell is not available.

Immunopositive processes invade the connective tissue sheath and the neuropile of the pleural ganglia. Some processes can clearly be seen emanating from stained

Figure 2. Cells and processes within the pleural ganglia bearing ELH-like immunoreactivity. In cryostat sections of pleural ganglia, stained by the PAP method with antibodies generated against ELH, up to 5 immunopositive somata were found at the junction of the ganglion and the pleuro-visceral connective. Some stained processes formed a cuff around the pleuro-visceral nerve trunk while other neurites branched into the connective tissue sheath. The multipolar immunoreactive cell in (A), situated at the edge of the ganglion, is seen at higher magnification in (B) to contribute a process to the spiraling cuff. Other processes emanating from this cell appear to extend into the connective tissue sheath. All other neurons in this section are unreactive. Three immunopositive cells are clearly seen in (C). Two of these (D) are intimately associated with the pleuro-visceral connective: one cell, bearing an unstained nucleus, is found within the nerve trunk which is encircled by an immunoreactive cuff. The second cell is extending processes into the cuff. Note the numerous processes in the surrounding connective tissue sheath. (A) and (C) share the small scale bar, (B) and (D) the large bar; both bars represent 200 μm.

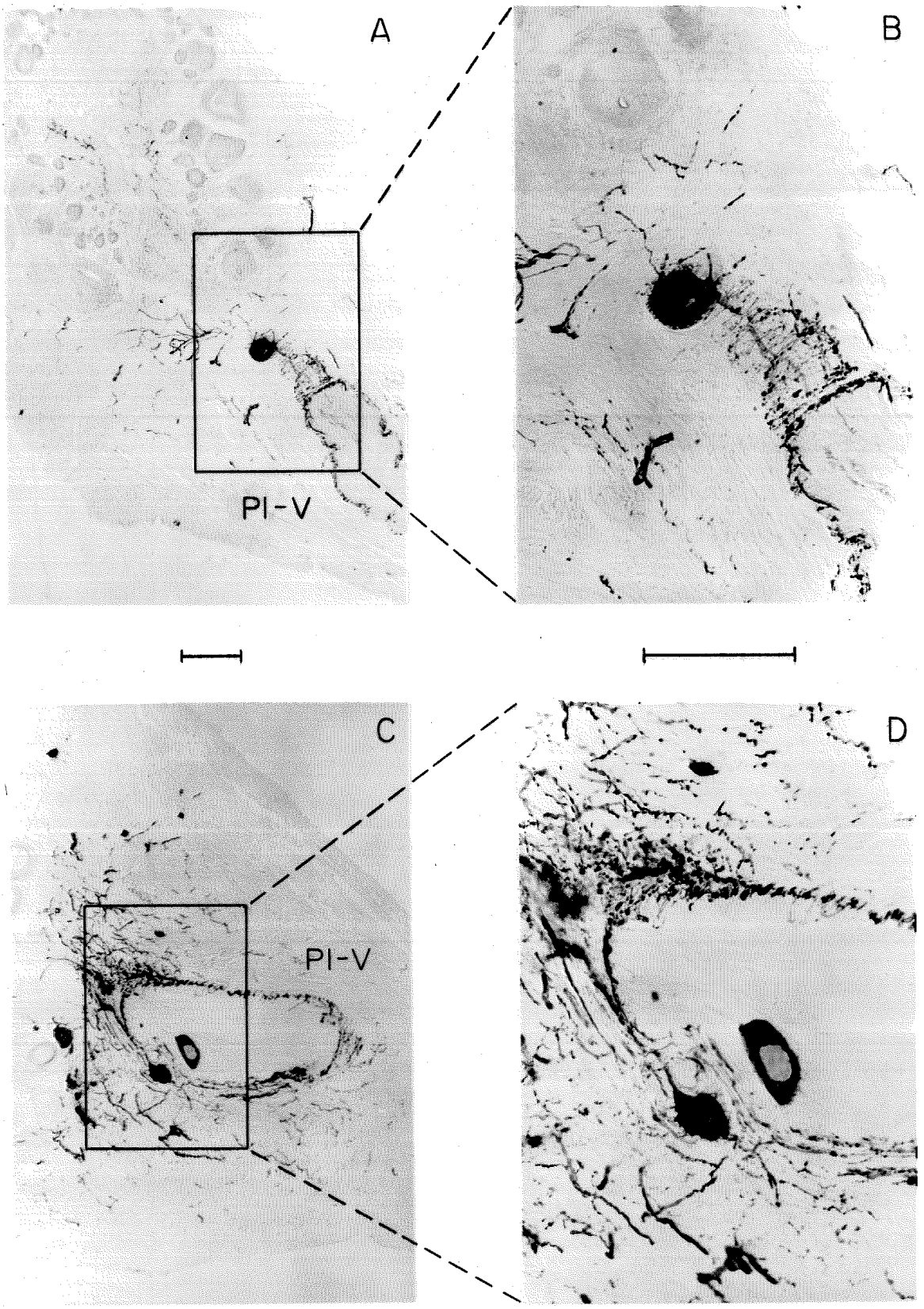

A
B
PI-V
C
D
PI-V

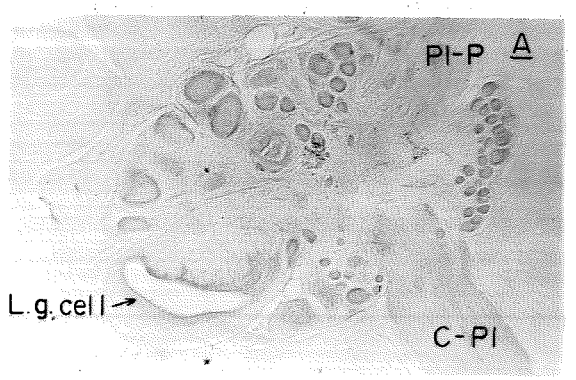
TABLE 1

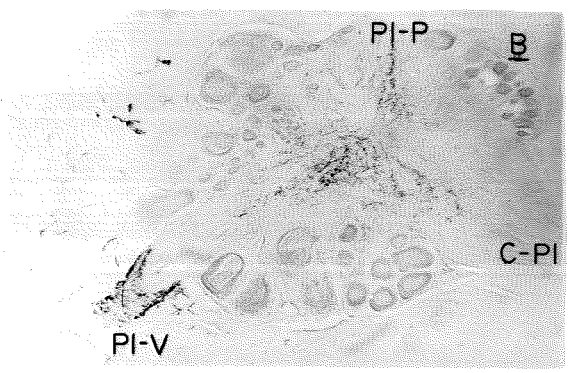
Dimensions of Bag Cells and ELH-Immunoreactive Pleural and Cerebral Neurons

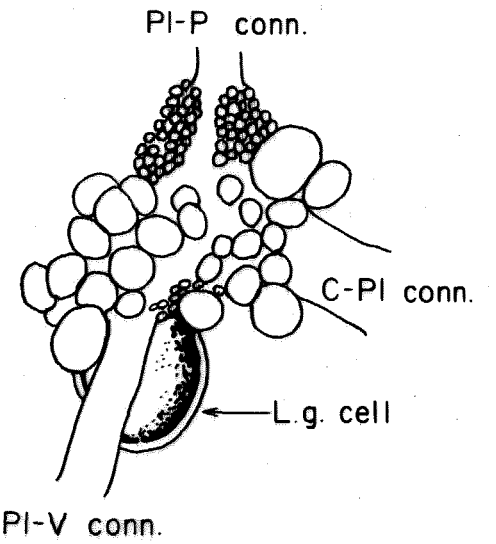
	n	Major somal diameter, µm	Minor somal diameter, µm	Major nuclear diameter, µm	Minor nuclear diameter, µm
Bag cells mean of 10 animals	1453	84.84	61.03	45.77	28.81
Pleural cells mean of 8 animals	36	82.78	58.16	41.20	29.52
Cerebral cells mean of 5 animals	44	21.57	15.97	-	-

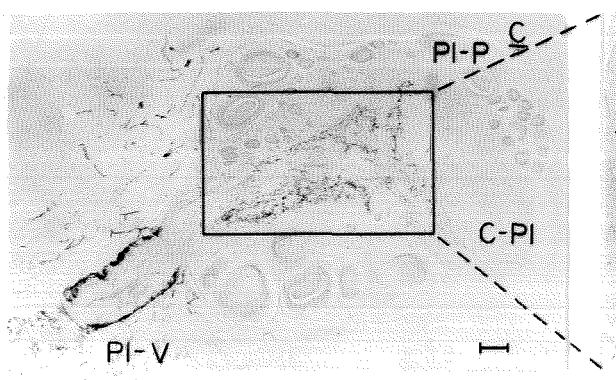
Figure 3. Transverse sections of the left pleural ganglion, immunostained for ELH. Three sections (A, B and C), taken from a single left pleural ganglion, show increasing numbers of immunoreactive processes toward the dorsal aspect of the ganglion. It is therefore likely that the immunopositive pleural somata are positioned on the dorsal surface. The line drawing is a dorsal view of the left pleural ganglion and shows the Left giant cell (L. g. cell) on the ventral surface (Shimahara and Tauc, 1976).

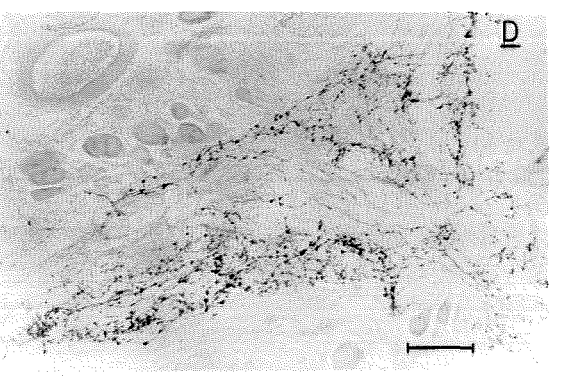
(A) A ventral section, with the Left giant cell, bears few immunostained fibers. (B) Toward the center of the ganglion, stained fibres are clearly present in the neuropile. A small cuff encircles the pleuro-visceral connective nerve (P1-V) but is absent from the pleuro-pedal (P1-P) and the cerebro-pleural (C-P1) connectives. (C) A dorsal section of the ganglion has numerous ELH+ processes in the neuropile, connective tissue sheath and the pleuro-visceral connective cuff. (D) Fibres, dotted with varicosities, are shown at high magnification within the pleural ganglion neuropile. Scale bars represent 200 μm; (A), (B) and (C) share the small bar.


Pl-P
A
L.g.cell
C-Pl


Pl-P
B
C-Pl
Pl-V


Pl-P conn.
C-Pl conn.
L.g. cell
Pl-V conn.


Pl-P
C
C-Pl
Pl-V


D

somata (Fig. 2B and D). Within the sheath, such processes proliferate near the pleuro-visceral connective, and bear a strong resemblance to the neurosecretory bag cell processes in the neurohemal areas of the abdominal ganglion sheath. A second type of ELH+ process spirals around the rostral end of the pleuro-visceral connective nerve trunk, forming a cuff between axons of the trunk and the sheath, and appears identical to the cuff of bag cell processes at the caudal end of the pleuro-visceral connective (Fig. 2). This immunoreactive cuff is confined to the proximal 1 cm of the pleuro-visceral connective nerve, diminishing in staining with distance from the ganglion. In the majority of animals, we found no stained fibres between the immunopositive cuffs at both ends of the connectives. However, in two exceptionally large A. californica, a few faint longitudinal fibres were detected in the middle segment of the pleuro-visceral connective nerves (Chiu and Strumwasser, 1981). It is not known whether these occasional processes originate in the abdominal or pleural ganglion.

ELH+ fibres, intermittently dotted with varicosities, are frequently present within the neuropile of the pleural ganglion (Fig. 3B, C and D). Sometimes a few long processes can also be seen within the nerve trunks of the cerebro-pleural and the pleuro-pedal connectives. The origin of such fibres, located in the neuropile or within the nerve trunks, is unclear at present. The results of deganglionation experiments suggest that they may not arise exclusively from the pleural immunoreactive somata (see below).

Cerebral ganglion

The cerebral ganglion receives sensory input from the tentacles, mouth and eyes through five pairs of peripheral nerves - the upper and lower labial, the anterior and posterior tentacular and the optic nerves - and is linked to all the other head ganglia through paired cerebro-pleural, -pedal and -buccal connectives. In A. californica, the cerebral ganglion is the fused product of two bilaterally symmetrical hemiganglia

which communicate across a short commissure. Investigators have grouped neurons of the cerebral ganglion on the basis of size and location. On the dorsal surface, Fredman and Jahan-Pawar (1975) have described seven pairs of clusters (Clusters A to G) and an eighth, asymmetric rostral group (H cluster). Four additional cluster pairs (Clusters J to M) have been localized on the ventral surface (Ono and McCaman, 1980).

We observe both prominent immunoreactive fibre tracts and cell bodies within the cerebral ganglion. A pair of bilaterally symmetric ELH+ clusters, each consisting of six to ten neurons, is located, amid unstained cells, on the dorsal surface in the vicinity of the C clusters (Fig. 4). Each of these small cells (mean major diameter 23 µm, mean minor diameter 17 µm; see Table 1) projects a single stout process toward the center of the ganglion.

In serial sections of the cerebral ganglion, we find immunoreactive fibres in the neuropile beginning to appear at the level of the ELH+ cell bodies. These fibres increase in extent and intensity of staining in a dorsal to ventral gradient (Fig. 5) and travel across the commissure in the center of the neuropile (Fig. 5E, F, G and Fig. 6). In sections of the ventral portion of the ganglion, these large immunoreactive fibre tracts bear the appearance of bilaterally symmetric "fans" within both halves of the ganglionic neuropile (Fig. 5H).

ELH+ fibres also travel along many of the connective and peripheral nerve trunks associated with the cerebral ganglion. There is frequently a plexus of stained neurites at the junction of a nerve trunk and the neuropile (Fig. 6); longitudinal fibres appear to travel from neuropile into nerves, diminishing in staining intensity and number with distance from the ganglion. The optic, posterior tentacular and superior labial nerves are the only nerve trunks which consistently show no immunoreactivity. Strongest immunostaining is always associated with the anterior tentacular nerves; however,ELH+ fibres can also be seen in the inferior labial nerves and in all three sets of connectives - cerebro-pleural, -pedal and -buccal.

Figure 4. Cell clusters with ELH-like immunoreactivity in the cerebral ganglion. In each cerebral ganglion, two clusters of immunopositive cells are located on the dorsolateral surface. Each cluster typically consists of 6-10 small, apparently monopolar cells which direct their processes toward the central neuropile. (A) and (B) show clusters from two different ganglia. Notice the scarcity of stained fibers within the neuropile in these dorsal sections. The arrow points at the commissure in (A). All scale bars represent 100 μm. (A) and (B) share the shortest bar, (C) and (D) the intermediate bar and (E) and (F) the longest bar.

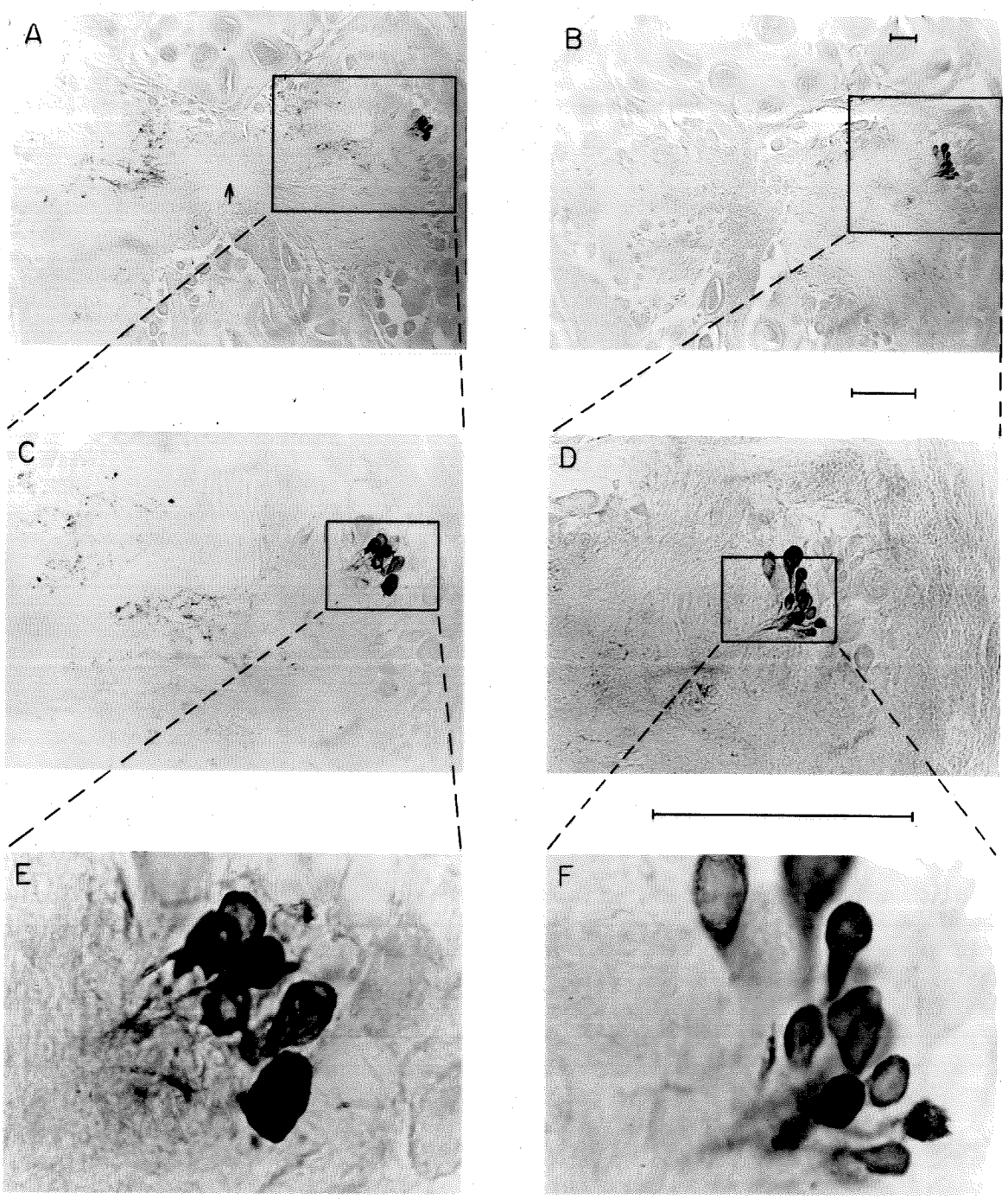
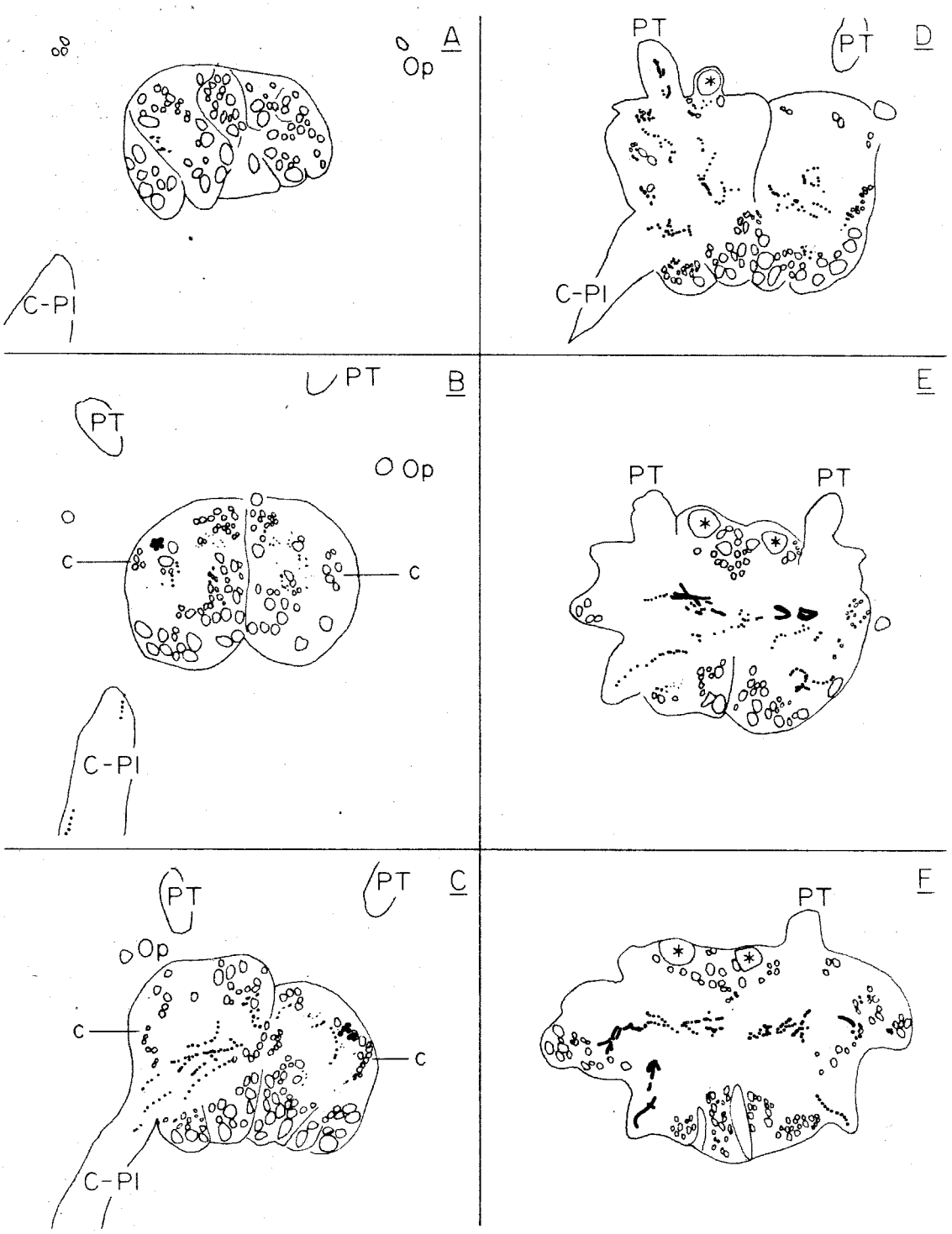

A
B
C
D
E
F

Figure 5. Camera lucida tracings of ELH+ cells and fiber tracts in serial sections of the cerebral ganglion. (A) is the dorsal-most section of this series and (J) the most ventral. These representative sections are spaced approximately 80 µm from neighbours with the following exceptions: the distance between (F) and (G) is 120 µm, and that between (I) and (J) is 40 µm. The bilaterally symmetrical ELH+ clusters of cells, seen in (B) and (C), are located in the vicinity of the dorsolaterally placed "C clusters". While immunoreactive fibers are found in all levels of section within the neuropile, they appear increasingly intense and widespread in the ventral part of the ganglion (G, H and I). Metacerebral giant neurons (*) are present in sections (E) and (F).

Abbreviations for cerebral nerves and connectives are: AT - anterior tentacular, PT - posterior tentacular, SL - superior labial, IL - inferior labial, Op - optic, C-P - cerebro-pedal, C-P1 - cerebro-pleural, C-B - cerebro-buccal.


A
Op
C-PI
D
PT
PT
C-PI
B
PT
PT
Op
c
c
C-PI
E
PT
PT
C
PT
PT
Op
c
c
C-PI
F
PT

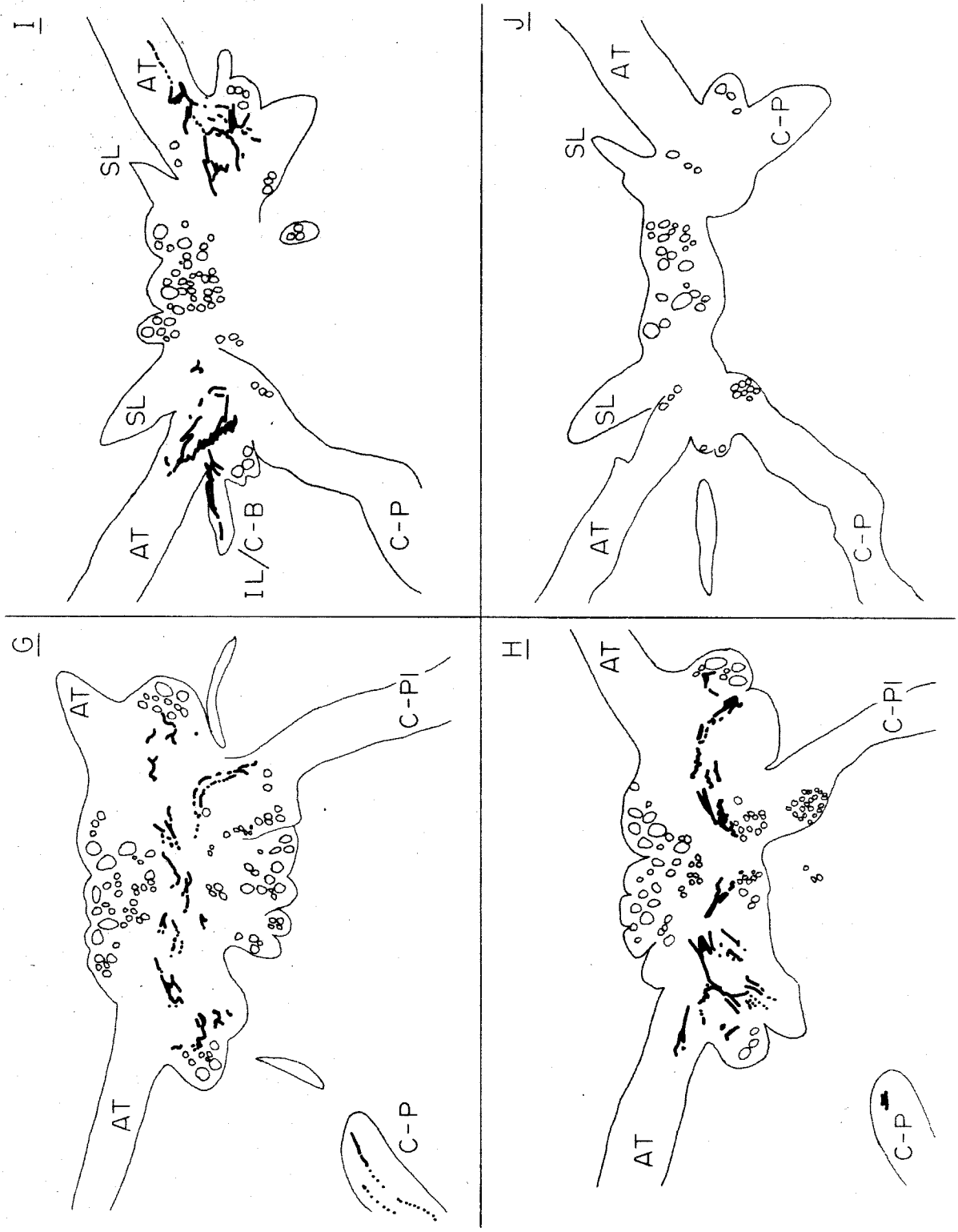
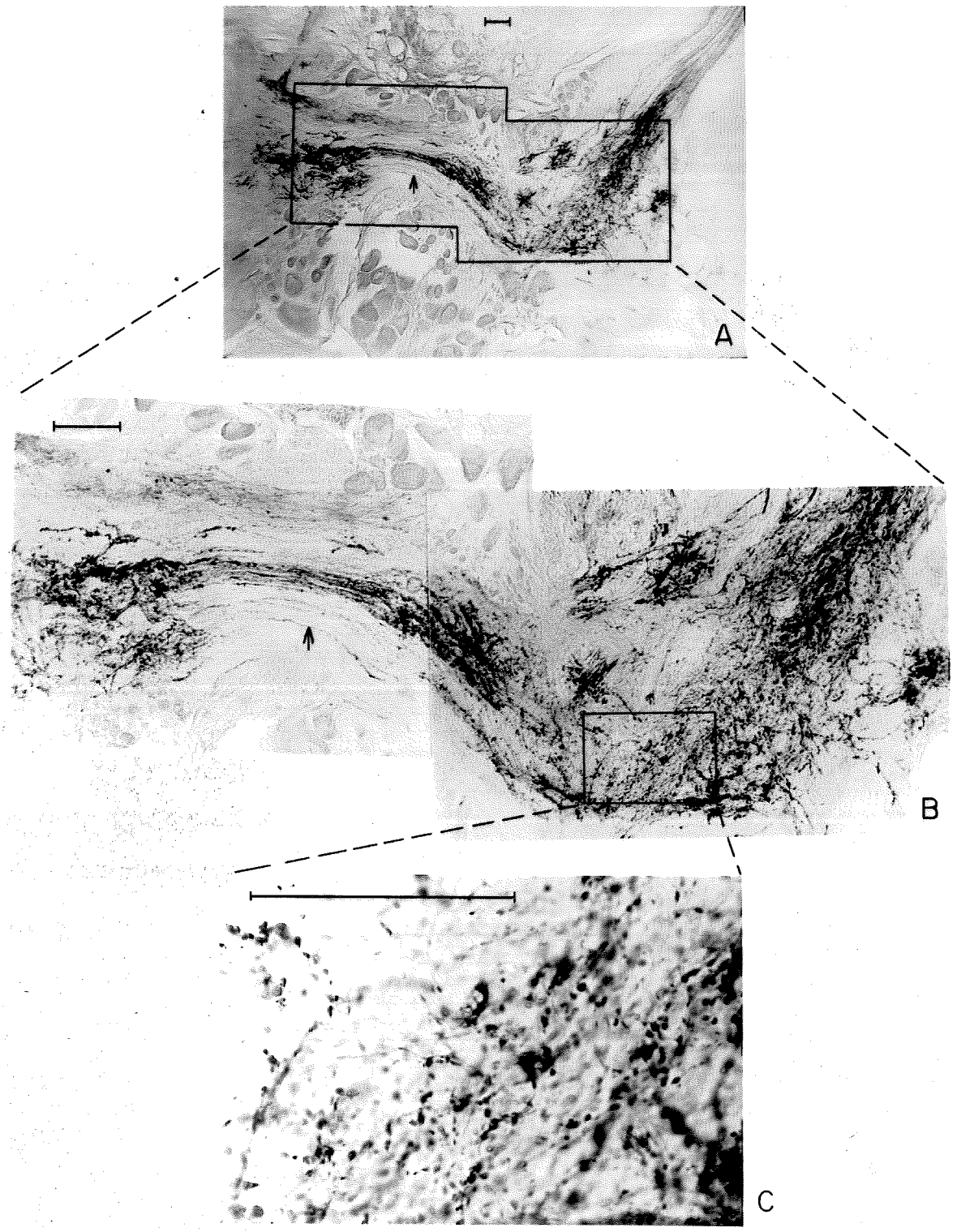

I
AT
SL
SL
AT
IL/C-B
C-P
J
SL
AT
C-P
SL
AT
C-P
G
AT
C-PI
AT
C-P
H
AT
C-PI
AT
C-P

Figure 6. Immunoreactive fibres within the neuropile and commissure of the cerebral ganglion. In this section of the ventral cerebral ganglion, shown at three magnifications, densely stained networks on both sides of the neuropile are joined by longitudinal fibres traversing the commissure (arrow). In (A) and (B), processes also can be seen to emerge from a dense plexus to travel into the anterior tentacular nerve. In (C), fibres in the right neuropile have a beaded appearance at high magnification. All scale bars represent 100 μm.


A
B
C

Pedal and buccal ganglia

Buccal ganglia are devoid of any ELH+ cells or fibres. The pedal ganglia, which "make up the largest neural aggregation of the CNS" (Kandel, 1979) bear no immunoreactive somata. Some stained fibres however can often be found within its neuropile. Since a few of the incoming nerve trunks - the cerebro-pedal and the pleuro-pedal connective nerves - contain immunoreactive fibres, it is likely that the source of immunoreactivity within the pedal neuropile is not in the pedal ganglion.

Deganglionate animals

It is well known that the bag cell neurons of the abdominal ganglion synthesize and release ELH. Bag cells project extensive arrays of ELH-bearing processes some of which ascend into the pleuro-visceral connectives (Chiu and Strumwasser, 1981). In order to study whether the immunoreactive systems within the head ganglia arise from the bag cells, we removed the abdominal ganglia and pleuro-visceral connectives of several mature A. californica and examined the head ganglia of animals which survived well months after surgery.

Two A. californica were sacrificed for immunohistochemistry 43 days and 67 days after the surgical removal of their abdominal ganglia and almost total removal of both pleuro-visceral connectives. In the cerebral ganglia of these animals, strongly immunoreactive neuropile fans, nerve trunk fibres and cell clusters are all present. Similarly, in their pleural ganglia, immunoreactive processes within the sheath and the cuff of neurites also persist and are not obviously different from those in unoperated control animals. Stained fibres within the pleural ganglion neuropile, although present, appear somewhat diminished in both animals. ELH+ fibres within the pedal neuropile are also greatly reduced.

These results demonstrate that much of the ELH-like immunoreactivity we report is not a consequence of the bag cell system in the abdominal ganglion. Bag cell processes usually extend no more than 2 cm beyond the caudal end of the pleuro-

visceral connectives, and in most animals, no stained fibres can be detected in the middle of the connectives (Chiu and Strumwasser, 1981). It is likely that the rare bag cell process, which may have extended all the way to the pleural ganglion, would have degenerated after such long periods of isolation from the somata. In Aplysia, as in vertebrate systems, degeneration profiles can be seen after deafferentation (Strumwasser, unpublished results). Furthermore, ELH, released by bag cells and retained by receptors, would not be expected to persist for up to 67 days. The dimunition of neuropile fibres in both pleural and pedal ganglia may be a consequence of abdominal deganglionation. However the intensity of staining of these fine neuropile processes shows great variability even in control animals, and it would be premature to conclude from these results that bag cells send processes all the way to pleural and pedal ganglia. It is clear that, with the possible exceptions of fibres in pleural and pedal neuropiles, all other ELH+ cells and processes of the cerebral and pleural ganglia are endogenous to the head ganglia.

Bioassays

Egg-laying bioassays were performed to locate egg-laying activity in extracts of the different head ganglia. For each test, the supernatant of a homogenate containing the equivalent of ganglia from 6-7 donor animals was injected into a competent test animal. In two out of three trials, extracts of pleural ganglia induced egg laying. When similarly extracted pedal (n = 3) or cerebral (n = 3) ganglia material were tested, no egg laying was observed.

DISCUSSION

Within the Aplysia nervous system, the evidence had, prior to this report, suggested that ELH may be a unique neurosecretory product of the bag cells within the abdominal ganglion (Kupfermann, 1967; Strumwasser et al., 1969; Toevs, 1970). Its effects on neurons in other ganglia were therefore believed to be mediated by circulation

in the hemolymph. Our results demonstrate the presence of neurons and organized fibre tracts with ELH-like immunoreactivity in the head ganglia, which persist within the animal months after removal of the whole abdominal ganglion, and thus raise the question whether these local sources of ELH, or ELH-like material, also act on neurons in the head ganglia of Aplysia. Since the immunoreactive molecules of the head ganglia have not been characterized, it is possible that, other than sharing antigenic determinants with ELH, they may share no other property. However, the striking resemblance between the pleural ELH+ neurons and bag cells suggests a common heritage and hence a similar product.

ELH-like molecules in Aplysia

In an earlier survey of the Aplysia nervous system, egg-laying activity was consistently found only in extracts of the abdominal ganglion, although pleural ganglia homogenates did induce oviposition on one occasion out of six trials (Strumwasser et al., 1969). However these results are dependent on a number of unresolved factors including (i) the sensitivity of the test animal, (ii) ease of extraction of bioactive material from resident structures such as somata or fibres, and (iii) the presence of degradative agents or of factors which can inhibit target response. While the bag cell system clearly has a very high ELH content (Chiu et al., 1979), small amounts of ELH present in cerebral and pleural ganglia may easily have escaped detection by an all-or-none egg-laying assay.

Using a high dosage of ganglionic extract, we have repeated these bioassays of material from cerebral, pleural or pedal ganglia, and have found egg-laying activity only in homogenates of pleural ganglia. This supports the immunohistochemical evidence indicating the presence, albeit slight, of ELH-like material within the pleural ganglia. The nature of immunoreactive material within the cerebral ganglion is less clear. It may be much more difficult to extract bioactive material from fibres deep in the neuropile, as in the case of the cerebral ganglion, than from processes

in the connective tissue sheath capsule or from the large somata of the pleural ganglia.

At present nothing more is known about molecules within the head ganglia which show binding to antibodies generated against ELH. It is entirely possible that immunoreactive molecules in the cerebral ganglion are different from those in the pleural ganglia, and neither are identical to the ELH purified and sequenced from bag cells. Recently, a bioactive peptide purified from the atrial gland of the reproductive tract of A. californica, has been found to be identical to ELH in residues 1-3 and 8-19 of the amino-terminus (Schlesinger et al., 1981). The second half of its amino acid sequence curiously resembles those of two other atrial peptides which can induce egg laying by causing bag cells to afterdischarge and consequently to release ELH (Heller et al., 1980). The roles played by these atrial peptides in spontaneous egg laying is not clear. However, the presence of this "hybrid" atrial peptide strengthens the possibility that ELH-like molecules may also be present in the nervous system of Aplysia. Perhaps, like the diverse endophins in the vertebrate nervous system, there is a family of ELH-like molecules, sharing regions of homology, but possessing different degrees of bioactivity.

ELH circuits mediating egg-laying behavior

The injection of pure ELH into an Aplysia is sufficient to elicit a typical program of egg-laying behavior which includes lip puckering, lateral weaving motions of the head, reduction in locomotion and feeding and, finally, the extrusion of an egg strand (Blankenship, 1980; Strumwasser et al., 1980). There is evidence that this neuropeptide acts on neuronal and nonneuronal targets whose collective response is egg-laying behavior (for review, see Blankenship, 1980). Purified ELH causes egg release by fragments of ovotestis (Rothman et al., 1979) while bath perfusion of the neurohormone results in long term excitatory responses in identified neurons in Aplysia. Some of these putative targets are within the abdominal ganglion where local diffusion of released peptide can modulate the activity of neurons such as R15 (Branton et

al., 1978; Mayeri, 1979). Other responsive neurons have been located in the buccal and pedal ganglia (Stuart and Strumwasser, 1980), and may be involved in changes in locomotion and feeding seen during egg-laying. While it is probable that many of these distant targets are triggered by ELH, released into the hemolymph when bag cells afterdischarge, our results raise the possibility that some neuronal circuits in the head ganglia respond to intraganglionic sources of ELH, or ELH-like molecules. Such peptides may function as neuromodulators through local circulation from sites in the connective tissue sheath of the pleural ganglia, or as neurotransmitters within the neuropile of the cerebral ganglion.

Furthermore, it is tempting to speculate that these systems of ELH-like immunoreactivity endogenous to the head ganglia are also directly involved with the egg-laying program and are activated by the same conditions which stimulate bag cells to afterdischarge. This idea is totally consistent with the "one neuroactive agent-one-behavioral function" hypothesis (Bloom, 1979) which attempts to account for the increasing numbers of transmitters and modulatory compounds found to act on neurons. Studies of antidromic responses in cells, upon stimulation of cerebral nerves, show that C cluster neurons most frequently send processes down the ipsilateral cerebro-buccal connective, presumably to the buccal ganglion (Jahan-Pawar and Fredman, 1976). Some of the C neurons also act as command neurons for pedal locomotion (Jahan-Pawar, personal communication). The immunostained cerebral cells, which lie in the vicinity of the C cluster, may be among those neurons projecting to the buccal ganglion, or may be involved in the control of locomotion. Stuart and Strumwasser have identified, by intracellular recording, a pair of buccal neurons which is activated to pace at rates of up to 90 spikes per minute when ELH is applied in vitro (Stuart and Strumwasser, 1980). Other lines of evidence suggest that the activation of these neurons may be connected with the termination of the feeding motor program (Strumwasser et al., 1980). Bath application of ELH to a preparation

of the circumoesophageal ring of head ganglia will also produce bursts of neuronal activity in the pedal nerves to the foot (Stuart and Strumwasser, 1980). Having established the size and locations of ELH+ neurons in the cerebral and pleural ganglia, it now remains to be seen if direct stimulation at these sites will produce the same responses in target neurons of the buccal and pedal ganglion as superfusion of ELH.

Are ELH+ systems related

The immunoreactive system within the cerebral ganglion, with its small somata and massive neuropile fibre tracts, has few morphological features in common with bag cells which send the great majority of their processes into the connective tissue sheath of the abdominal ganglion. However, ELH+ pleural cells and processes bear such a striking resemblance to the bag cell system in location, morphology and a special association with the pleuro-visceral connective nerve as to raise the likelihood of a common lineage. Both bag cell clusters and the immunoreactive pleural cells are found where the pleuro-visceral connective meets the ganglion. These pleural cells clearly match bag cells in somal and nuclear dimensions (Table 1) and process morphology. They both wrap a cuff of processes around the base of the pleuro-visceral connective nerve; other neurites meander through presumed neurohemal areas in the overlying sheath, and beaded fibres can be discerned within the neuropiles of both abdominal and pleural ganglia.

This hypothesis of a shared heritage is consistent with a number of events which occur during Aplysia development. All central ganglia in Aplysia start out as ectodermal thickenings; both pleural and abdominal ganglia primordia are present at hatching (Stage 1) linked by short pleuro-visceral connectives in the young veliger (Kandel et al., 1980). However, immature bag cells identified only by location and distinguished from glia by prominent cytoplasmic structures, are not seen until 35 days after metamorphosis at Stages 12-13. These small cells aggregate on the surface of each PVC nerve above the ganglion and are the last components of the

abdominal ganglion to multiply, develop and become morphologically recognizable (Frazier et al., 1967). It is possible that bag cell precursors, as yet unidentified by current techniques, are located within the short, immature pleuro-visceral connective nerves. During development, the great majority of them stay at one end, eventually to give rise to the bag cell clusters. Some are separated out when the connectives increase in length (Stage 8-12), ending up in the pleural ganglia end of the nerve; a rare few are left to mature within the pleuro-visceral connective nerve trunk (Fig. 2C; also see Chiu and Strumwasser, 1981).

Other morphological and physiological data also suggest a close developmental relationship between the pleural and abdominal ganglia. Hughes and Tauc (1961, 1963) demonstrated a remarkable similarity in the axonal pathways taken by two giant neurons, R2 and the Left Giant Cell (LGC) in A. depilans, even though these cells were located in different ganglia. R2, which is found in the right abdominal hemiganglion, and LBC, in the left pleural ganglion, also share a similar appearance as well as a common transmitter - acetylcholine. If these two neurons were originally a bilaterally symmetrical pair of cells early in development, their adult locations support the existence of a close relationship between the pleural and abdominal ganglia during development. ELH+ cells in the abdominal and pleural ganglia may similarly have arisen from a single population of neurons and have migrated to these two destinations in the course of development.

If bag cells and immunoreactive pleural neurons are indeed descended from the same precursor pool, and, upon maturation, maintain common features such as morphology, location and ELH immunoreactivity, one might then expect them to also share physiological properties such as electrical coupling and afterdischarge (Kupfermann and Kandel, 1970). Furthermore, these pleural homologues of bag cells should release ELH or ELH-like peptides when appropriately triggered, and the input(s) may be common to both cell populations. It remains to be seen if purified

atrial peptides, which can induce bag cell afterdischarge (Heller et al., 1980; Schlesinger et al., 1981), can also drive pleural neurons to repetitively fire and secrete ELH-like material. The development of a quantifiable assay, sensitive to low concentrations of ELH, could verify simultaneous release of the neuropeptide by these two, physically separated groups of neurons.

REFERENCES

Bargmann, W. and Scharrer, E. 1951. The site of origin of the hormones of the posterior pituitary. Amer. Sci. **39**:255-259.

Blankenship, J. E. 1980. Physiological properties of peptide-secreting neuroendocrine cells in the marine mollusc Aplysia. In: "The Role of Peptides in Neuronal Function", J. L. Barker and T. G. Smith, Jr., eds., Marcel Dekker Inc., New York.

Bloom, F. E. 1979. Contrasting principles of synaptic physiology: peptidergic and non-peptidergic neurons. In: "Central Regulation of the Endocrine System", K. Fuxe, T. Hökfelt, and R. Luft, eds., Plenum Press, New York.

Branton, W. D., Arch, S., Smock, T. and Mayeri, E. 1978. Evidence for mediation of a neuronal interaction by a behaviorally active peptide. Proc. Natl. Acad. Sci. USA **75**:5732-5736.

Chiu, A. Y., Hunkapiller, M., Heller, E., Stuart, D. K., Hood, L. E. and Strumwasser, F. 1979. Purification and primary structure of the neuropeptide egg-laying hormone of Aplysia californica. Proc. Natl. Acad. Sci. USA **76**:6656-6701.

Chiu, A. Y. and Strumwasser, F. 1981. An immunohistochemical study of the neuropeptidergic bag cells of Aplysia. Submitted for publication.

Coggeshall, R. E. 1972. The muscle cells of the follide of the ovotestis in Aplysia as the probable target organ for bag cell extract. Am. Zoologist **12**:521-523.

Dudek, F. E. and Tobe, S. S. 1978. Bag cell peptide acts directly on ovotestis of Aplysia californica: Basis for an in vitro bioassay. Gen. Comp. Endocrinol. **36**:618-627.

Dyer, R. G. and Dyball, R. E. J. 1974. Evidence for a direct effect of LRF and TRF on single unit activity in the rostral hypothalamus. Nature **252**:486-488.

Elde, R. and Hökfelt, T. 1978. Distribution of hypothalamic hormones and other peptides in the brain. In: "Frontiers in Neuroendocrinology", L. Martini and W. F. Ganong, eds., Raven Press, New York, pp. 1-33.

Frazier, W. T., Kandel, E. R., Kupfermann, I., Waziri, R. and Coggeshall, R. E. 1967. Morphological and functional properties of identified neurons in the abdominal ganglion of Aplysia californica. J. Neurophysiol. **30**:1288-1351.

Fredman, S. M. and Jahan-Parwar, B. 1975. Synaptic connections in the cerebral ganglion of Aplysia. Brain Res. **100**:209-214.

Heller, E., Kaçzmarek, L. K., Hunkapiller, M. W., Hood, L. E. and Strumwasser, F. 1980. Purification and primary structure of two neuroactive peptides that cause bag cell afterdischarge and egg-laying in Aplysia. Proc. Natl. Acad. Sci. USA **77**:2328-2332.

Hökfelt, T., Johansson, O., Ljungdahl, Å., Windberg, J. M. and Schultzberg, M. 1980. Peptidergic neurones. Nature **284**:515-521.

Hughes, B. M. and Tauc, L. 1961. The path of the giant cell axons in Aplysia depilans. Nature **191**:404-405.

Hughes, G. M. and Tauc, L. 1963. An electrophysiological study of the anatomical relations of two giant nerve cells in Aplysia depilans. J. Exp. Biol. **40**:469-486.

Jackson, I. M. D. and Reichlin, S. 1979. Distribution and biosynthesis of TRM in the nervous system. In: "Central Nervous System Effects of Hypothalamic Hormones and Other Peptides", Raven Press, New York, pp. 3-54.

Jahan-Parwar, B. and Fredman, S. M. 1976. Cerebral ganglion of Aplysia: cellular organization and origin of nerves. Comp. Biochem. Physiol. **54A**:347-357.

Jan, Y. N., Jan, L. Y. and Kuffler, S. W. 1979. A peptide as a possible transmitter in sympathetic ganglia of the frog. Proc. Natl. Acad. Sci. USA **76**:1501-1505.

Jan, L. Y., Jan, Y. N. and Brownfield, M. S. 1980. Peptidergic transmitters in synaptic boutons of sympathetic ganglia. Nature **288**:380-382.

Kandel, E. R., Kriegstein, A. and Schacher, S. 1980. Development of the central nervous system of Aplysia in terms of the differentiation of its specific identifiable cells. Neuroscience **5**:2033-2063.

Kupfermann, I. 1967. Stimulation of egg-laying possible neuroendocrine function of bag cells of abdominal ganglion of Aplysia californica. Nature **216**:814-815.

Kupfermann, I. and Kandel, E. R. 1970. Electrophysiological properties and functional interconnections of two symmetrical neurosecretory clusters (bag cells) in abdominal ganglion of Aplysia. J. Neurophysiol. **33**:865-876.

Mayeri, E. 1979. Local hormonal modulation of neural activity in Aplysia. Fed. Proc. **38**:2103-2108.

Moss, R. L., Dudley, C. A. and Kelly, M. 1978. Hypothalamic polypeptide releasing hormone: modifiers of neuronal activity. Neuropharmacology **17**:87-93.

Ono, J. K. and McCaman, R. E. 1980. Identification of additional histaminergic neurons in Aplysia: Improvement of single cell isolation techniques for in tandem physiological and chemical studies. Neuroscience **5**:835-840.

Renaud, L. P. 1978. Peptides as neurotransmitters or neuromodulators. In: "Psychopharmacology: A Generation of Progress", M. A. Lipton, A. DiMascio and K. F. Killam, eds., Raven Press, New York, pp. 423-430.

Renaud, L. P., Martin, J. B. and Brazeau, P. 1975. Depressant action of TRH, LMRH and somatostatin on activity of central neurons. Nature **255**:233-235.

Renaud, L. P., Pittman, Q. J., Blume, H. W., Lamour, Y. and Arnauld, E. 1979. Effects of peptides on central neuronal excitability. In: "Central Nervous System Effects of Hypothalamic Hormones and Other Peptides", Raven Press, New York, pp. 147-161.

Rothman, B. S., Brownell, P. and Mayeri, E. 1979. Purified bag cell peptide mimicks some but not all responses of central neurons to bag cell stimulation in Aplysia. Soc. Neurosci. Abstracts **5**, No. 857.

Schlesinger, D. H., Babirak, S. P. and Blankenship, J. E. 1981. Primary structure of an egg-releasing peptide from the atrial gland of Aplysia californica. In: "Symposium on Neurohypophyseal Peptide Hormones and Other Biologically Active Peptides", D. H. Schlesinger, ed., Elsevier, North-Holland Publishing Company.

Shimahara, T. and Tauc, L. 1976. Identification of a neuron inducing heterosynaptic facilitation on a specific synapse in Aplysia. Brit. Res. **118**:142-146.

Silverman, A. J. and Zimmerman, E. A. 1978. Pathways containing luteinizing hormone-releasing hormone (LHRH) in the mammalian brain. In: Brain endocrine interaction III. "Neural Hormones and Reproduction", 3rd International Symposium, Wurzburg Kargen, Basel, pp. 83-96.

Strumwasser, F., Jacklet, J. W. and Alvarez, R. B. 1969. A seasonal rhythm in the neural extract induction of behavioral egg-laying in Aplysia. Comp. Biochem. Physiol. **29**:197-206.

Strumwasser, F., Kaczmarek, L. K., Chiu, A. Y., Heller, E., Jennings, K. R. and Viele, D. P. 1980. Peptides controlling behavior in Aplysia. In: "Peptides: Integrators of Cell and Tissue Function", F. E. Bloom, ed., Society of General Physiologists Series Vol. 35, Raven Press, New York.

Stuart, D. K. and Strumwasser, F. 1980. Neuronal sites of action of a neurosecretory peptide, egg-laying hormone, in Aplysia californica. J. Neurophysiol. **43**:499-519.

Stuart, D. K., Chiu, A. Y. and Strumwasser, F. 1980. Neurosecretion of egg-laying hormone and other peptides from electrically active bag cell neurons of Aplysia. J. Neurophysiol. **43**:488-497.

Swanson, L. W. 1977. Immunohistochemical evidence for a neurophysin-containing autonomic pathway arising in the paraventricular nucleus of the hypothalamus. Brain Res. **128**:346-353.

Toevs, L. 1970. Identification and characterization of the egg-laying hormone from the neurosecretory bag cells of Aplysia. Ph.D. Dissertation, Pasadena, California Institute of Technology.