THE NEUROSECRETION OF THE POLYPEPTIDE EGG-LAYING HORMONE (ELH) FROM THE BAG CELLS, NEURONAL SITES OF ACTION OF ELH, AND CIRCADIAN RELEASE OF POLYPEPTIDES FROM THE EYE OF APLYSIA CALIFORNICA

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ABSTRACT

The thesis is about the neurosecretion of Aplysia peptides and demonstrates that one of them acts directly upon the nervous system. Such neuronal effects of neurosecretory substances may prove to be a general phenomenon; this is discussed in the introduction.

In chapter 1, radiolabeled peptides released from electrically active bag cell neurons in isolated bag cell clusters were compared with the polypeptide egg-laying hormone (ELH), 6,000 daltons, pI 9.0-9.3, as purified from homogenates of bag cell clusters. A labeled peptide which is selectively released from electrically active bag cell clusters comigrates with ELH from cluster homogenates on P-6 gel filtration columns and subsequent isoelectric focusing gels. When bag cells are activated, a released factor(s) induces egg-laying and comigrates with ELH from cluster homogenates on P-6 columns. At least three other presumed peptides of unknown function are also released. These experiments demonstrate that ELH (6,000 m.w., pI 9.0-9.3) as purified from bag cell cluster homogenates is the major, active form secreted from bag cells.

In chapter 2, the effects of ELH on neuronal activity of the attached head ganglia (buccal, cerebral, pleural, and pedal), on the isolated buccal ganglia, as well as on feeding in intact Aplysia were studied. Starved animals (n = 7) injected at 20°C with a crude extract containing ELH stopped eating algae at 17 $\pm$ 4 min and their eggs first appeared at 29 $\pm$ 4 min after injection. These data clearly indicate that a suppression of feeding activity occurs before the appearance of eggs. ELH applied to the paired buccal ganglia in vitro activates a pair of neurons into a tonic pacing mode ($\sim$1 spike/sec). The time for the full appearance of this activity in

vitro correlates well with the time for suppression of feeding in vivo. These neurons each have an ipsilateral axon in buccal nerve 3. ELH increases the rate of firing of a second pair of buccal neurons, each with an ipsilateral axon in the cerebrobuccal connective. ELH when applied to the attached head ganglia causes large bursts of neuronal activity in pedal nerves to the foot, and increased activity in the nerve to the penis. These in vitro effects were produced by ELH partially purified from bag cell cluster homogenates using ammonium sulfate precipitation, an anion exchange column, and a gel filtration column or by ELH released from activated bag cells in isolated abdominal ganglia and then frationated by gel filtration. The ELH effects upon the in vitro nervous system support the working hypothesis that ELH in vivo acts directly on the nervous system to suppress feeding activity. ELH may also activate neural circuits in the pedal and probably cerebral ganglia that produce characteristic movements of the head during egg-laying; the relevant neurons remain to be identified.

In chapter 3, a circadian rhythm (CR) of release of presumed peptides from the isolated eye of Aplysia is demonstrated. This isolated eye is known to have a CR of compound action potentials (CAPs) as recorded from its optic nerve. Substances labeled with radioactive amino acids and released into the perfusate were separated on gel filtration columns and SDS polyacrylamide gels. In the CR experiments, the perfusate of a single, labeled, dark-maintained eye was collected every 3 h for two days while simultaneously recording the CR of CAPs. Each 3-h perfusate was applied to a P-2 gel filtration column. Excluded substances (m.w. $\curvearrowleft$ 2000) and material fractionated in the region of m.w. $\curvearrowleft$ 1000 showed a CR which was in phase with the CR of CAPs. Much of these labeled substances can be precipitated with trichloroacetic acid. Their release is stimulated by a high

potassium solution and inhibited by a low calcium solution that also inhibits CAP activity. This and other previously published evidence suggests that the CAPs and the peptide release are directly produced by electrically coupled neurosecretory cells which may also contain the CR oscillator. One or more of these peptides may be a neurohormone and/or transmitter used for synchronizing, entraining and/or driving the rest of the animal's CRs.

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INTRODUCTION

The concept of neurosecretion was first advanced by Ernst Scharrer in 1928 (10) based upon a study of the fish midbrain. Berta Scharrer in 1935 (9) described neurosecretory neurons in invertebrates including several species of Aplysia. Descriptions of neurosecretory cells soon came to rely upon characteristic staining patterns using the Gomori chrome alum-hematoxylin or Gabe paraldehyde-fuchsin methods. Neurosecretory cells were generally treated as a distinct class of neurons that regulated peripheral endocrine targets (2). Their secreted substances were thought to be either peptides or monoamines (3).

Recently, it has become clear that neurosecretory cells are not physically distinct from other neurons in either their overall geometry, their ability to form synapses, or their capacity to produce action potentials. Current definitions of neurosecretory cells resort to requiring a peripheral, endocrine target for the secreted substance (4, 7). This function would usually require a site for secretion into the circulatory system, a neurohaemal organ, and an emphasis on the morphology needed to secrete large quantities of material, but even these requirements can be avoided by neurosecretory cells which make direct contact with their endocrine target cells (7). The requirement for a peripheral endocrine target is not only needed for monoamine neurons, but also for peptidergic neurons, since there are a growing number of small peptides, such as substance P, now thought to be acting as neurotransmitters in the central nervous system (6).

Earlier studies of primitive invertebrates, such as the sponge, hydra, and planaria, show that many of their neuronal or neuronal-like cells emphasize morphological characteristics of secretion (endoplasmic reticulum, Golgi apparatus, vesicles) in common with traditional neurosecretory cells. Therefore, Lentz (5) has postulated that neurosecretory cells might be evolutionarily close to primitive

neurons which he envisions to be cells releasing substances which act over a distance to modulate the responses, condition, or development of other cells. Release at local sites and at synapses would represent a more advanced state.

This perspective suggests that neurosecretory substances might also have evolved to act directly back on the nervous system as well as on peripheral sites. Experimental work with hypothalamic releasing factors (8), the silkmoth brain (11, 12), the crayfish sinus gland (1), and with the Aplysia bag cell neurons in this thesis support this concept: neurosecretory substances can act directly on the nervous system.

The first two chapters of this thesis are about a polypeptide neurohormone in Aplysia californica. This hormone, which is synthesized by a specific group of secretory neurons named bag cells, induces egg-laying and its associated behavior when injected into an animal. In the first chapter, it is demonstrated that the form of the hormone purified from homogenates of bag cells is the same form which is released from electrically activated bag cells in vitro. In the second chapter, it is shown that this hormone has specific effects on the Aplysia nervous system in vitro, suggesting that these effects may mediate some of the specific behavior, such as a suppression of feeding, associated with egg-laying.

Thus, this hormone has a property of a neurotransmitter in that it can directly change neuronal activity. This supports the argument that neurosecretory cells are not distinctly different from other neurons except to the degree that they need to secrete sufficient quantities of material to act over large distances.

In the final chapter, a circadian release of presumed peptides from the Aplysia eye is demonstrated in the absence of light or dark cues. This now means that there are two circadian rhythms produced by this eye in vitro, the well known

electrical activity cycle and the presumed secretory cycle described in this thesis. The eye contains morphologically defined neurosecretory cells postulated to be responsible for this peptide release. Work by others suggests that the eye may be secreting substances which can act elsewhere, such as the nervous system, to regulate the animal's circadian rhythms.

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Chapter 1

Neurosecretion of Egg-laying Hormone and other Peptides from Electrically Active Bag Cell Neurons of *Aplysia*[1]

[1]Work done in collaboration with Arlene Y. Chiu

SUMMARY AND CONCLUSIONS

1. Radiolabeled peptides released from electrically active bag cell neurons in isolated bag cell clusters were compared with the polypeptide egg-laying hormone (ELH), 6,000 daltons, pI 9.0-9.3, as purified from homogenates of bag cell clusters.

2. A labeled peptide which is released from electrically active bag cell clusters comigrates with ELH from cluster homogenates on P-6 gel filtration columns and subsequent isoelectric focusing gels. Other presumed peptides of unknown function are also released.

3. One of the released factors, when bag cells are activated, induces egg-laying and comigrates with ELH from cluster homogenates on P-6 columns.

4. These experiments demonstrate that ELH (6,000 m.w., pI 9.0-9.3) as purified from bag cell cluster homogenates is the major, active form secreted from bag cells. This purified ELH can, thus, 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, ophistobranch 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 (17, 27, 30) and in no other part of the nervous system (27). These bag cells fulfill the morphological requirements for neurosecretory cells since they contain 150-250 nM dense core granules and their endings are distributed throughout the surrounding connective tissue in neurohaemal spaces (8, 12). A brief train of electrical stimuli will set off an afterdischarge of bag cell action potentials lasting for tens of minutes (10, 11, 12, 16, 18, 19). In vivo this afterdischarge is followed by egg-laying (23).

ELH, as characterized from tissue homogenates, is a polypeptide with a molecular weight (m.w.) of about 6,000 (3, 29) and is basic with an isoelectric point (pI) of about 9.0-9.3 (3). It appears to be synthesized from a 29,000 m.w. precursor which could produce a number of bag cell peptides. These could include peptides of approximately 12,000, 6,000, and $\leq$ 3,000 m.w. (2, 5, 20, 21).

A stimulus associated with electrical activation of the bag cells (18, 19) or a high potassium solution (1) released a factor(s) that induced egg-laying into the perfusate surrounding the abdominal ganglion. However, there is no direct evidence relating this released factor to the pI 9.0-9.3, 6,000 m.w. peptide. In fact, the possibility has been suggested that a $\leq$ 3,000 m.w. peptide is the active form of the secreted ELH and the 6,000 m.w. form is a biologically active precursor transported and stored in the bag cells (21). This suggestion is based upon bag cell release stimulated by a high potassium solution. The substances released during an afterdischarge of bag cells have not been examined.

To study the physiological effects of the secreted hormone (9, 28), it is important to identify the form in which it is secreted. In experiments reported here, released ELH is compared with the 6,000 m.w. form from tissue homogenates by using gel filtration columns and isoelectric focusing gels. The results indicate that ELH is released from electrically active bag cells in the pI 9.0-9.3, 6,000 m.w. form. This form is responsible for at least the major proportion of the ability to induce egg-laying in such released material. 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. Any procedural variations in using bioassays to identify fractions with the ability to induce egg-laying will then be presented. 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 five days prior to their use. In order to monitor egg-laying activity, animals were isolated in individual baskets, and to assure the maturity of the animals, they were kept warm (6, 25) and well fed. The tank was maintained at 20 $\pm$ 0.5°C with a light-dark schedule of 12 h light and 12 h dark. Lights-on was at 0800 PDT and lights-off at 2000 PDT. 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 weeks before they were mature enough to lay eggs.

Animals weighed between 200 and 1500 grams and had reproductive tract weights (6, 27) of between 1.5 and 15 grams. 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 pleuro-abdominal connectives attached, was placed in a Millipore filtered (0.22 μ M) 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 units of K penicillin G and 100 μg of streptomycin (sulfate) per ml.

The bag cell bodies with their surrounding connective tissue were then dissected away from the rest of the abdominal ganglion. About 1/3 of the total length of the pleuro-abdominal connective, containing many bag cell processes (12), 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 cluster. 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 a medium of Millipore filtered (0.22 μM) natural seawater with 150 units of K penicillin G and 150 μg of streptomycin (sulfate) per ml, 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 the labeling medium.

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

Bag cell clusters were labeled in 1 ml of medium for 24 h in exp. Nos. 1, 2, 3, and 18 h in exp. No. 4. They were subsequently rinsed 6 h in 6 successive 10 ml rinses of the prelabeling solution.

STIMULATION. Following rinsing, the 2 to 4 largest and most cleanly dissected clusters were each placed, as a control, for 1 h in a separate 1 ml (0.5 ml for exp. No. 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 CRO, 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 msec duration repeated 6/sec for 30 sec. 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 1/2 ml of the control and stimulation perfusate was analyzed separately on a 50 x 1 cm Bio-Gel P-6, 400 mesh gel filtration column maintained

at 4°C. We used only the perfusates from the cluster which 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/cm^2/h. A Gilson micro fractionator 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-bungarotoxin (obtained from Professor Michael Raftery), pancreatic trypsin inhibitor, glucagon, bacitracin, leucine, and ^3H_2O. The P-6 column, nominally, should exclude globular polypeptides of m.w. > 6,000.

ISOELECTRIC FOCUSING. A 100-125 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 from 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 500-100 µl of a solution of 5% sucrose and .05% ampholines. The top buffer was 10 mM phosphoric acid, the bottom buffer 100 mM NaOH. The tops of all 8 gels were made positive, the reverse of the normal situation, with an applied voltage that was increased from 150 to 300 v, the current dropping from 9 to 1 mA, over the running period of

4-5 h at room temperature. The gels were then extruded, immediately sliced into 1.4 mm slices, and the radioactivity counted as reported previously (24).

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 the marker cytochrome c, pI = 9.4, present in each gel.

ELH PURIFICATION (work in progress in collaboration with Dr. Eri Heller). Six pairs of bag cell clusters were labeled as described above. The label was 150 μCi of L-Pro 2,3,4,5-^{3}H, 115 Ci/mM and 150 μCi of L-Leu 3,4,5-^{3}H(N), 110 Ci/mM (New England Nuclear).

After labeling the clusters were kept frozen (-20^{o}C) until homogenized with 34 pairs of nonlabeled clusters. The homogenate was centrifuged, the supernatant precipitated with ammonium sulfate, the pellet redissolved, and passed through an anion and then cation exchange column followed by a P-6 gel filtration column as described previously. The four 0.6 ml fractions from the ELH region, containing 84% of the recovered radioactivity in the P-6 fractions, were pooled, frozen, and vacuum evaporated to 0.2 ml. Equivalent amounts of this ^{3}H-ELH were added to the control and stimulation perfusate of exp. No. 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 hemocoele (27). Either released or homogenate forms of ELH were assayed after fractionation on P-6 columns.

Homogenate material was also assayed after elution from pieces 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 (6).

Animals used for bioassay, normally at 20°C, were proven egg-layers that had not laid eggs in the two days prior to the test. A given animal was injected no more than once a day. Eggs appear at 65 $\pm$ 16 min after injection at 14°C (4, 27). So, tests resulting in eggs within 90 min after injection were considered positive.

PREPARATION AND STIMULATION. 10 to 14 abdominal ganglia were dissected with most of their pleuro-abdominal connectives intact. Ganglia were transferred one at a time to 1 ml of sea water solution in a plastic beaker where the bag cells were directly stimulated with a suction electrode. When the bag cell after-discharge 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 x 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 to 1.0. One- or two-thirds of 1 to 4 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 Aplysia blood (15) by mixing with appropriate amounts of dried, filtered sea water.

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 1/2 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 one or two 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 h. At the beginning of the second h, 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 upon molecular weight, permits the direct comparison of labeled substances released before and after bag cell activation.

GEL FILTRATION. Bag cell clusters with a double label were used for release in three experiments while the fourth experiment used a single label. Fig. 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. 1B) perfusate show a 15- to 100-fold increase over the control (Fig. 1A) in the area where ELH consistently appears, K_{av} = 0.20-.25, fractions 36-37.

Labeled substances in four other regions also show large increases in the stimulation period (Fig. 1B). These regions include 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. No change, though, was observed in the number of counts recovered from either ^{14}C in the region for the label, leucine, K_{av} = 0.75, or from ^{3}H in the region for $^{3}H_2O$, K_{av} = 1.0 (Table 1). $^{3}H_2O$ occurs as a metabolite of the label.

Fig. 2 provides data on a pair of bag cell clusters labeled with ^{35}S-Met. In this experiment, ^{3}H-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. 2B) shows a 12-fold increase in ^{35}S counts in the ELH region, K_{av} = 0.2, compared to the control (Fig. 2A). The ^{35}S counts in this region correspond exactly with the ^{3}H counts of the ELH purified from cluster homogenates (Fig. 2B).

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 methionine, K_{av} = .75-.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 stimulated period contains more than

10-fold that of the control period. There is a similarly large increase in the pre-ELH region. For the labeled substances in the excluded region, a smaller increase of 3 to 5 times is apparent, while the radioactivity in the region of the label or ^3H_2O changes only by a factor of 1.0 to 1.5. In the remaining experiment, exp. No. 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. Since ELH is known to have a pI of 9.0-9.3, released material, first fractionated by P-6 gel filtration, was further separated on isoelectric focusing gels. To aid in establishing whether the pI 9.0-9.3, 6,000 m.w. ELH, itself, was released, the P-6 fractions used for focusing were from the experiment where ^{3}H-ELH had been first added to the ^{35}S-labeled, release substances (Fig. 2). A sample of a P-6 fraction in the ELH region, for both the control and stimulated period, is run on an isoelectric focusing gel (Fig. 3). Again, the stimulation perfusate shows 6-12 fold increase in a ^{35}S-labeled substance in the ELH region, pI 9.0-9.3 (Fig. 3B). The purified ^{3}H-ELH also comigrates to this position, demonstrating its probable identity with the ^{35}S-labeled, released substance.

Parts 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} = .10-.15, fraction 35, shows a similar increase in radioactivity for a substance with a pI of 4.5-5.0. Most of the radioactivity in a sample of fraction 60, K_{av} = .5-.7, did not enter the gel suggesting that the material is acidic with a pI <3.5

Bioassays

The bioassays for substances with the ability to induce egg-laying were

performed to answer two questions. First, which region(s) of radioactivity of the gel filtration fractions and isoelectric focusing gel corresponds to ELH? Secondly, what is the form(s) of biologically active ELH actually released from bag cells?

GEL FILTRATION. Five series of bioassays of P-6 fractions were carried out. Each of the first four series were of material released from the equivalent of 3-7 abdominal ganglia following bag cell activation. Normally, the entire range of fractions, K_{av} = 0-1, were bioassayed by injecting 3 or 4 consecutive fractions into each animal. Five animals, at least one per series, laid eggs, responding to fractions with a mean $K_{av} = .24 \pm .04$. Fig. 2 shows the corresponding active fractions for one of these series.

In the fifth series, material homogenized from the equivalent of 6 pair of bag cell clusters was bioassayed after P-6 fractionation, one fraction at a time. Six animals responded with eggs to fractions with a mean $K_{av} = .22 \pm .04$ (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 out of 15 slices were assayed. The eluate 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 eluate inducing egg-laying had a relative mobility of 0.84-0.92. Purified ELH has a relative mobility of .90 to .96.

DISCUSSION

ELH

The results demonstrate that the pI 9.0-9.3, 6,000 m.w. ELH is released from isolated bag cell clusters following electrical activation and afterdischarge of the bag cells. Purified ^{3}H-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 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 which induce egg-laying correspond to 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.

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 (28).

It should be noted that ELH, like cytochrome c and α-bungarotoxin, is eluted late from the P-6 column (Figs. 1 and 2) which suggests a m.w. smaller than that identified by SDS gels (3). However, ELH, cytochrome c, and α-bungarotoxin all have a pI > 9, so their retardation on the column may relate to their charge.

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 released substances have been assayed for egg-laying activity in any form other than unfractionated perfusate (1, 18). Arch (1) first demonstrated that a high potassium solution applied to a ^{3}H-leucine labeled abdominal ganglion caused Ca^{+2} dependent release of trichloroacetic acid precipitable material that ran on SDS gels at a m.w. <10,000. The high potassium solution also released precipitable material from isolated, labeled bag cell clusters, but this was not analyzed on SDS gels. 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 (26). Perhaps the strongest evidence concurrent with our conclusion is from bioassay of homogenates of abdominal ganglia or clusters fractionated by gel filtration (3, 29), by isoelectric focusing gels (3), and by SDS gels (3). These bioassays show that there is only one major form of ELH in these homogenates, thus suggesting that it is also the released form.

Loh et al. (21) 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

m.w. polypeptides. This was determined by using SDS gels, designed to optimize low m.w. resolution, which were subsequently stained with Coomassie blue to reveal polypeptide bands. They found that within bag cell bodies where axonal transport has been blocked by colchicine a substantial amount of radioactive label is processed into the m.w. $\leq$3,000 substance(s). Therefore, they raised the possibility that such a substance is the major secreted form able to induce egg-laying, and that the 6,000 m.w. form is a biologically active precursor normally found in higher proportion in the bag cell bodies. The previous bioassays of fractionated homogenates (29) may have missed this smaller, active form due to starting with insufficient amounts of connective tissue containing the bag cell terminals. Our results rule out this possibility since the pI 9.0-9.3 6,000 m.w. ELH is released and is the major, if not exclusive, released form that induces egg-laying.

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 pI 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 one or more small acidic peptides, the sum of which show some increased release 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. (21). Their data include m.w. estimates only, so an exact correspondence cannot be made. More is known about peptides from cluster homogenates (3, 5, 13, 20, 21). A peptide with a pI of 4.8 (13) is reported by Arch et al. (5) to have a m.w. of 5,000 and is thought to be derived from a pI 4.6 peptide with a m.w. of 6,000. The pI 4.8 peptide may correspond to the peptide with a pI of 4.5-5.0 in the pre-ELH region of the P-6 fractions, K_{av} = .10-.15 (Figs. 1 and 2). Furthermore, the 1,000 m.w. peptide, hypothesized (5) to be cleaved from the pI 4.6 peptide in being processed to the pI 4.8 peptide, might correspond to one or more small, acidic peptides found in the K_{av} = .5-.7 region of the P-6 fractions (Figs. 1 and 2).

The histological and electron microscopic evidence to date (12, 26) 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, 20), playing a role in its transfer across a membrane, packaging and secretion (7, 22). These peptides may have additional hormonal effects when released, as may be the case with the joint secretion from the pituitary gland of two peptides, ACTH and β-endorphin, with a common precursor (14). These questions can best by 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, we examine the effects of ELH itself on neurons in the head ganglia.

ACKNOWLEDGEMENTS

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Table 1. Ratio of Released Counts in Stimulated versus Control Perfusates

Exp. 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 upon the appropriate isotope counts recovered in the P-6 fractions. For each profile the exclusion region is the sum of counts in 4 fractions, the pre-ELH region the sum in 3 fractions, the ELH or label region the sum in 5 fractions, and the ^3H_2O region the sum in 7 fractions. 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 stimulated period than the control period. Exp. No. 3 had the lowest percent recovery of an isotope from the stimulated period. In this experiment, the bag cells before labeling were also stimulated, producing

Table 1 (continued)

an afterdischarge. This was intended to increase the amount of labeled ELH, but also may have resulted in the release of less total ELH, leading to a relatively greater loss on the P-6 column and lower ratios in this table.

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 15-100-fold increase in released counts in B. The ^{14}C counts in the leucine region or the ^{3}H counts in the $^{3}H_2O$ region are not changed (Exp. No. 2, Table 1). The markers, not run concurrently, are ● (blue dextran 2,000, m.w. = 2,000,000; bovine pancreatic trypsin inhibitor (Kunitz), m.w. = 6,500), ▲ (cytochrome c, m.w. = 12,384), * (glucagon, m.w. = 3,485), ▼ (α-bungarotoxin, m.w. = 7,904), ■ (bacitracin, m.w. = 1,411), Δ (leucine), and o ($^{3}H_2O$). 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 stimulated period.

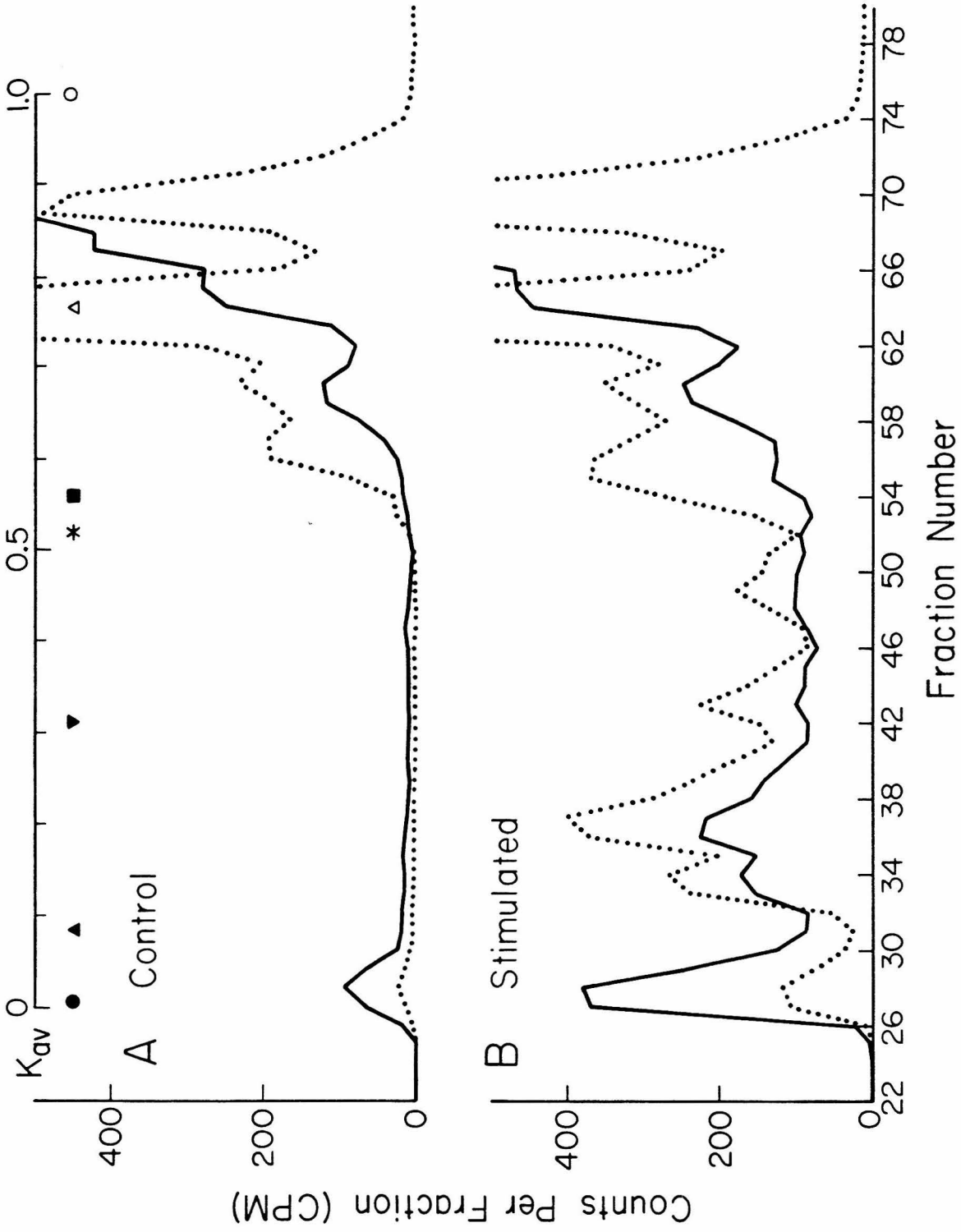

K_{av}
0
0.5
1.0
A Control
B Stimulated
Counts Per Fraction (CPM)
400
200
0
22
26
30
34
38
42
46
50
54
58
62
66
70
74
78
Fraction Number

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 in the methionine region are unchanged (Exp. No. 4, Table 1). The counts are from 25 µl samples of each 600 µl fraction. The 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 5 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.

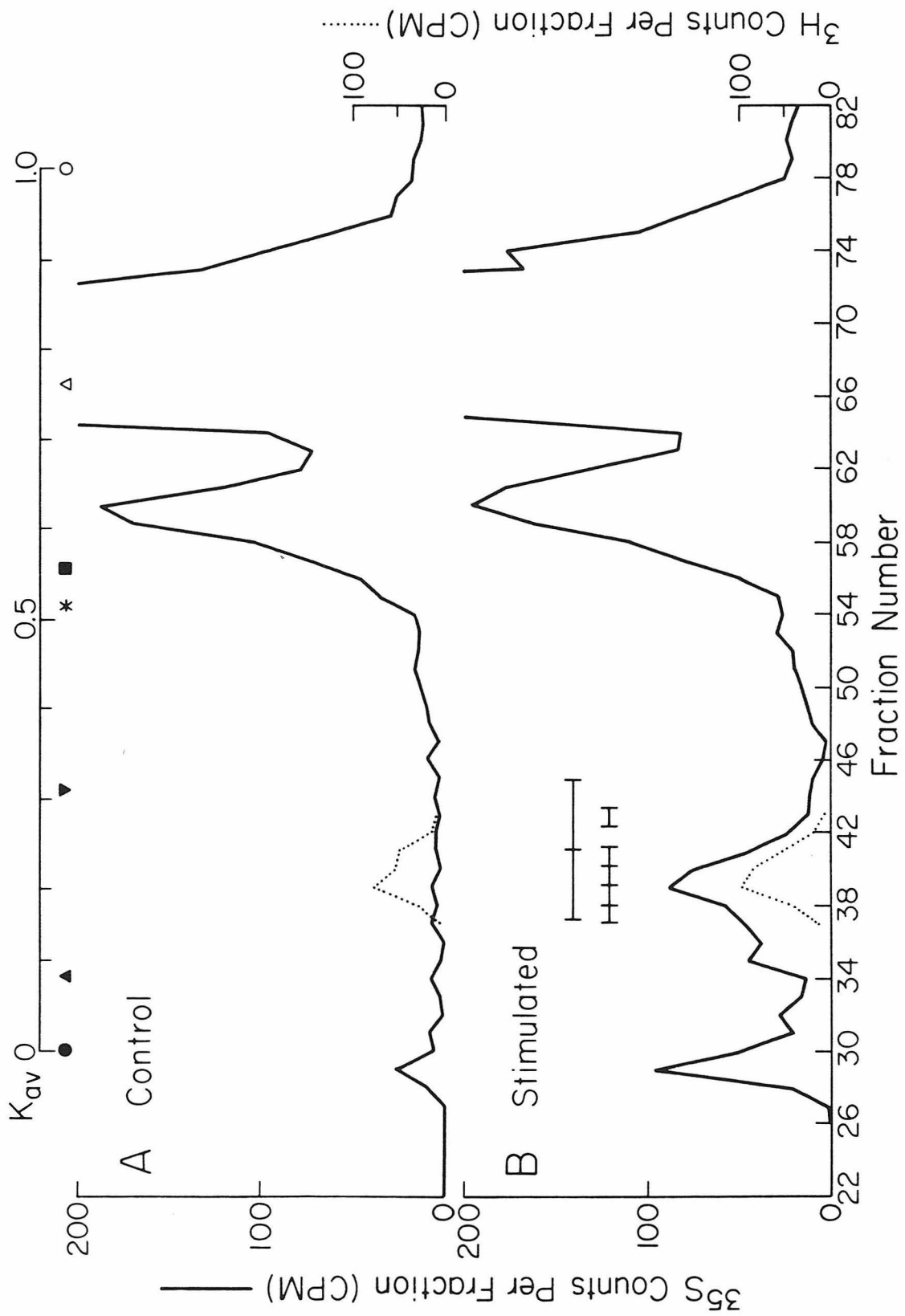
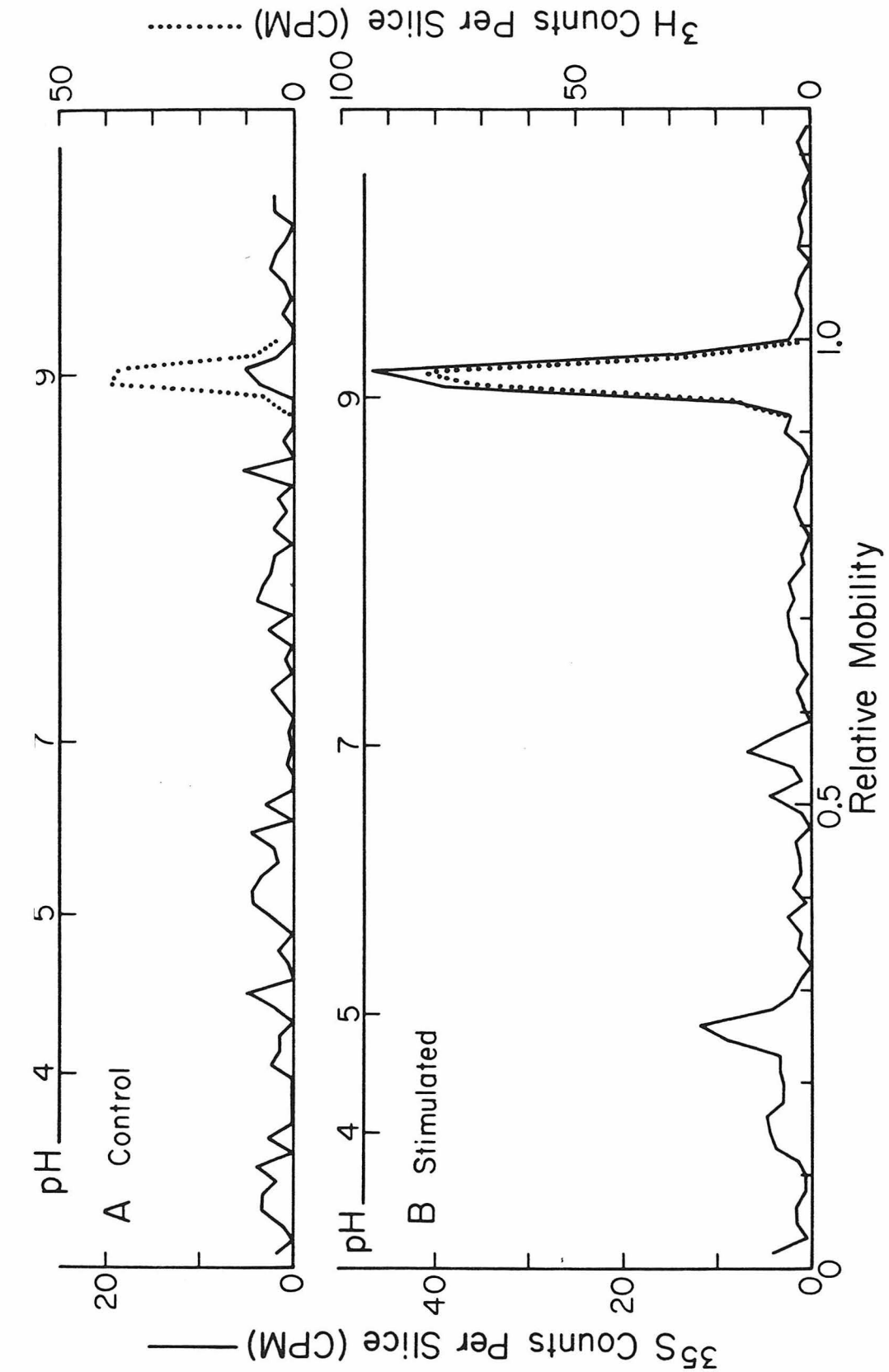

K_{av} 0
0.5
1.0
A Control
B Stimulated
^{35}S Counts Per Fraction (CPM)
^{3}H Counts Per Fraction (CPM)
Fraction Number
200
100
0
22
26
30
34
38
42
46
50
54
58
62
66
70
74
78
82

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. 2A and the stimulated profile in B is from the ELH peak in Fig. 2B. In both, the ^{35}S (——) profile represents released substances whereas the ^{3}H (····) profile represents purified ^{3}H-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-ELH purified from bag cell cluster homogenates. The pH of the gels is indicated along the top. Part of the respective fraction 40 (Fig. 2) was applied to each gel.


^{35}S Counts Per Slice (CPM) ——
^{3}H Counts Per Slice (CPM) .....
A Control
B Stimulated
pH
Relative Mobility

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Chapter 2

Neuronal Sites of Action of a Neurosecretory Peptide, Egg Laying Hormone, in *Aplysia californica*

SUMMARY AND CONCLUSIONS

1. Egg-laying hormone (ELH) is a polypeptide of about 6,000 m.w. synthesized in the bag cell neurons of the abdominal ganglion of Aplysia. We studied the effects of ELH on neuronal activity of the attached head ganglia (buccal, cerebral, pleural, and pedal) as well as the isolated buccal ganglia. We also studied the effects of ELH on feeding in intact Aplysia.

2. ELH applied to the paired buccal ganglia in vitro activates a pair of neurons into a tonic pacing mode ($\sim$ 1 spike/sec). These neurons each have an ipsilateral axon in buccal nerve 3.

3. ELH increases the rate of firing of a second pair of buccal neurons, each with an ipsilateral axon in the cerebrobuccal connective.

4. ELH when applied to the attached head ganglia causes large bursts of neuronal activity in pedal nerves to the foot, and increased activity in the nerve to the penis.

5. These effects were produced by ELH partially purified from bag cell cluster homogenates using ammonium sulphate precipitation, an anion exchange column, and a gel filtration column or by ELH released from activated bag cells in isolated abdominal ganglia and then fractionated by gel filtration.

6. Starved animals ($n = 7$) injected at $20^{\circ}C$ with ELH stopped eating algae at 17 ± 4 min and their eggs first appeared at 29 ± 4 min after injection. This cessation of eating is significant when compared to the seven controls ($p < .01$). These data clearly indicate that a suppression of feeding activity occurs before the appearance of eggs.

7. The ELH effects upon the in vitro nervous system support the hypothesis that ELH in vivo acts directly on the nervous system to suppress feeding activity, controlled by the buccal and cerebral ganglia, and to produce characteristic movements of the head during egg-laying, that are presumably controlled by the pedal and probably cerebral ganglia.

INTRODUCTION

An extract of the neurosecretory bag cells induces egg-laying when injected into the hemocoel of an Aplysia (35), a marine, hermaphroditic, opistobranch gastropod. Specific behaviors are also associated with this egg-laying (2, 42, 52). The work reported here suggests that some of this behavior is mediated by characteristic effects of egg-laying hormone (ELH) upon specific neurons.

ELH is found in the bag cells of the abdominal ganglion (35, 52) and is a peptide (58) of molecular weight of about 6,000 (57) with an isoelectric point (pI) of about 9.0-9.3 (1, 53). The bag cells fulfill the morphological requirements of neurosecretory cells (7) with endings distributed through the sinus spaces of the connective tissue of the posterior pleuro-abdominal connectives and the abdominal ganglion (11, 19). When electrically stimulated in vitro, the normally inactive bag cells produce a series of synchronous action potentials lasting for tens of minutes (16, 30, 40) and release ELH into the dish (36, 53). A similar series of synchronous action potentials precedes spontaneous egg-laying in vivo (46).

A characteristic pattern of behavior is associated with egg-laying. Most readily identifiable are weaving and nodding movements of the head which appear to aid the lips (oral veil) in the attachment of the egg strand to the substratum (2, 42). During this process, an early puckering of the mouth and swelling of the genital groove occurs (2), the animal's locomotion is inhibited (2, 52), and feeding activity, at least in the opistobranch Pleurobranchaea, is suppressed (14).

The behavior associated with egg-laying might be directly controlled by ELH's or a secondary factor's effect on the nervous system and/or might be a reflex response to the egg's presence. The possibility of a direct neuronal effect of ELH was first considered by Strumwasser et al. (51). Recent evidence supporting this

concept is based on the application of crude bag cell extracts to the abdominal ganglion in vitro. Mayeri and Simon (44) have reported specific and reproducible effects of such extracts upon the neuronal activity of the abdominal ganglion; these effects also occur after activation of the bag cells in the same ganglion. Additionally, Treistman and Levitan (59) report that such extracts cause increases in the level of abdominal ganglion adenosine 3',5'-cyclic monophosphate (cAMP); this suggests that ELH might effect the nervous system via a secondary messenger, cAMP.

We decided to look initially for neuronal effects of ELH that might relate to suppression of feeding behavior or to the generation of the head weaving movements. Feeding behavior has been well characterized in the non egg-laying animal (4, 29, 37, 38, 39, 47, 54, 55, 56). The neuronal circuitry in the buccal and cerebral ganglia that is involved with this behavior has also been studied (13, 18, 22, 23, 24, 25, 31, 60). Furthermore, work related to feeding has been done with other gastropods such as Pleurobranchaea (15, 26, 27, 41, 49), Helisoma (32, 33, 34), Planorbis (8), and others (8, 61). The head weaving movements arise from movements of the anterior part of the foot. The control of this movement probably relates to the direct control of locomotion by the pedal ganglia. Work on the neuronal circuitry underlying locomotion has been started (28).

Effects of partially purified ELH on the neuronal activity of the head ganglia (buccal, cerebral, pleural and pedal) in vitro are reported here. Some of these effects may relate to the suppression of feeding, also reported here for Aplysia, and the head movements during egg-laying.

METHODS

Animals

Animals were maintained as previously described (53) and weighed between 300 and 1500 grams with reproductive tract weights (5, 52) of between 2 and 15 grams. Animals were normally used which had not laid eggs in the past two days, but which were observed to have laid eggs prior to that. The only exceptions were within the groups of animals used for preparation of ELH fractions.

Preparation

The dissection was performed at about 20°C. First, the abdominal ganglion with most of the pleuro-abdominal connectives was removed followed by dissection of the head ganglia (buccal, cerebral, pleural, and pedal; Fig. 1) with their connectives and commissures intact. Specific nerves, for recording purposes, were also left long. For some experiments, the head ganglia were separated into smaller groups.

These ganglia were subsequently maintained in a Millipore filtered solution (0.22 μM) containing 10 mM glucose, 5 mM Tris Cl, pH adjusted to 8.0 at 20°C, 100 units of K penicillin G and 100 μg of streptomycin (sulfate) per ml, and filtered natural seawater (GTPS-FSW). When control and experimental substances were in a non seawater solution, usually a NaCl solution with Tris Cl buffer, then the appropriate substances including concentrated filtered seawater were added to make the GTPS-FSW solution. Control and experimental solutions made up in this way might have caused a negligible increase of 1-2 mM NaCl in the chamber.

The recording chamber held about 7 or 8 ml of solution maintained at a constant temperature of 20 $\pm$ 0.5°C with a Lauda TK30D cooler. In the most

recent experiments, fresh solution was continuously pumped into the chamber at a rate of about 15 ml per h. Excess perfusate was continuously aspirated from the opposite end of the chamber. In early experiments, this perfusion was stopped when control and experimental substances were tested. This testing was performed no sooner than 18 h following dissection, that is, the first day following dissection. Additional experiments were sometimes done on the second day following dissection, but only if the neuronal activity still remained normal.

Recording

Usually 12 suction electrodes were attached to the nerves or connectives in the preparation. Each signal was then passed to a Tektronix 122 preamplifier before being displayed on a Tektronix 5000 series oscilloscope and/or recorded on one of 10 channels of two Grass 7B polygraphs at a speed of 300 mm per min.

J. Lillig built a 4-channel discriminator modeled after discriminators used by J. Olds (45). Each channel allowed both an amplitude and time window to be set to discriminate a particular waveform or class of waveforms in a signal from a nerve. The total number of waveforms meeting the criteria set for a channel were printed out every 15 min. The window settings could be monitored and changed, if necessary, to keep track of small changes in a unit's waveform.

The activity of a specific unit or waveform was followed either by the discriminator or by counting directly from the polygraph record. These two methods often agreed within a fraction of a percent. Small discrepancies were caused when simultaneous bursts of a number of units occurred at the same time. All ELH effects for discriminated single or multiple units were confirmed by direct inspection of the polygraph records.

Forms of ELH

ELH applied to the head ganglia was obtained as material released from electrically active bag cells in the abdominal ganglion or from homogenates of bag cells with their associated connective tissue. In some experiments, ELH from these two sources was also fractionated or purified before use. To minimize ELH losses due to adherance, Siliclad (Clay Adams) coated glass or plastic containers were always used.

RELEASED ELH. The bag cells were normally stimulated directly through a suction electrode resting against them. The normal stimulus was 30 v for 4 msec, repeated 6/sec for 30 sec. This supramaximal stimulus was used to assure its effectiveness in generating a long afterdischarge. The knowledge that this stimulus would be effective permitted an equivalent control stimulus to be given earlier to the siphon nerve. In both control and experimental periods, the bag cell activity or afterdischarge was monitored with the stimulating suction electrode and usually with a second electrode on the bag cells of the opposite side of the ganglion.

In some of these release experiments, the abdominal ganglion was positioned in the same chamber next to the head ganglia. The perfusion was stopped for several hours following a control stimulation of the siphon nerve, a nerve of the abdominal ganglion which does not excite the bag cells (36) and then again, after rinsing, for several hours following bag cell stimulation. In other release experiments, the abdominal ganglion was placed in a separate dish containing 1 ml of GTPS-FSW for a control period of 1 h. The solution was changed and the bag cells stimulated and allowed to sit for another h. This control and the experimental solution were then added or perfused into a second chamber containing the head ganglia.

FRACTIONATED, RELEASED ELH. ELH released from usually 12 bag cell stimulated abdominal ganglia was concentrated (in some experiments) with an Amicon UM-05 filter (retains substances of m.w. > 500 daltons), run on a 50 cm long Bio-Gel P-6, minus 400 mesh, gel filtration column, and the fractions frozen. Presumably, ELH was partially adsorbed to the gel filtration gel and so appeared later than it should for its reported m.w. of 6,000 (1). See Stuart et al. (53) for details. Part of each fraction was bioassayed for egg-laying activity by injection into an animal. Material from the active region, K_{av} = 0.2-0.3, was used in the experiments reported here. Pre-exclusion volume buffer or inactive fractions were used for controls. In a given experiment, the final material used was that recovered from starting with an equivalent of 2-4 abdominal ganglia. The column buffer (5 or 10 mM Tris Cl with or without 50 or 100 mM NaCl) was made equivalent to GTPS-FSW before the control or experimental material was added to or perfused into the recording chamber.

FRACTIONATED, HOMOGENIZED BAG CELL CLUSTERS. Right and left bag cell clusters, the bag cells with their surrounding connective tissue and about one-third of the pleuro-abdominal connective attached, were dissected away from 22 fresh abdominal ganglia (from which released ELH had just been collected for the above), and then frozen. Such clusters contain the cell bodies of the bag cells and glia (11, 19), the surrounding connective tissue, and axons from the abdominal and head ganglia. The connective tissue around the cell bodies and the connectives contain bag cell processes where ELH is released.

This material was homogenized in 10 mM Tris Cl, pH 8.0, at 4°C, centrifuged for 10 min at 12,000 g, 4°C and the 800 µl of supernatant applied to a

50 x 1 cm column of Bio-Gel P-6, minus 400 mesh, at 4°C. The column buffer was 100 mM NaCl and 5 mM Tris Cl, pH 8.0, at 4°C with a flow rate of about 4 $ml/cm^2/h$.

One quarter of each fraction coming off the column was saved for bioassay and the remainder frozen. The active region, K_{av} = .10 to .35, was thawed out, pooled and divided into nine aliquots which were then refrozen. Each aliquot, as used in an experiment, was the material obtained from 1.8 pair bag cell clusters. With losses due to the column, usually 20-30%, and freezing, the actual amount of ELH used was on the order of that in one pair of clusters. For controls either buffer from before or material from the exclusion volume (m.w. > 6,000 daltons) was used.

PARTIALLY PURE ELH. Partially pure ELH was prepared four times (in collaboration with Arlene Y. Chiu) for the experiments reported here. Bag cell clusters were incubated in ^{3}H-arginine, ^{14}C-leucine or ^{3}H-proline (53) to label ELH.

In the fourth preparation partially purified ^{3}H-arg, ^{14}C-leu labeled ELH was repurified a second time with nonradioactive material. A crude estimate of the percent recovery of ELH for this procedure can be made by following the radioactive label. The ELH obtained from this fourth preparation was used for half the experiments reported here and will be described in detail. The other three preparations employed the same steps with some variations in timing and buffers.

Thirty-two pairs of bag cell clusters from freshly dissected abdominal ganglia were frozen. All subsequent steps were at 4°C. The material plus a few crystals of phenyl methyl sulfonyl fluoride (PMSF) was homogenized with 0.7 ml of

10 mM Tris-glycine, pH 8.4, which contained the ^{3}H-arg, ^{14}C-leu labeled ELH and then spun at 12,000 g for 40 min. The supernatant was made up to 25% saturated $(NH_4)_2SO_4$, pH 8.4, stirred and spun again for 40 min, 12,000 g. The pellet was taken up in about 400 µl of 10 mM Tris-glycine, pH 8.4, and run on a Sephadex A-25, DEAE anion exchange column, 40 x 5 mm. The excluded material was applied to a gel filtration P-6 column as described above (50 mM NaCl replacing the 100 mM NaCl). The central ELH containing fractions (#37-40, 0.6 ml each, K_{av} = 0.2) were used and contained 8% of the labeled ELH, that is, the ELH in these fractions was that in about 2-3 pairs of bag cell clusters. Similarly, purified radioactive material was run on an isoelectric focusing gel. The ELH peak contained 50% of the gel's radioactivity suggesting that the ELH was at most 50% pure.

Feeding behavior during egg-laying

A procedure was designed to test for the presence or absence of feeding behavior in animals laying eggs. Bioassay animals (53) maintained at 20°C were starved for one day and then placed in separate buckets, each with several gallons of seawater at 20° ± 1°C. A double blind procedure was used whereby at time zero the animals were injected with either the homogenate of 2 or 3 pair bag cell clusters (experimental) or head ganglia homogenate (control). An equivalent number of experimental and control animals were tested on the same day.

A piece of algae, Plocamium coccinium (1 gm wet weight), was suspended from a piece of thread and placed a few cm from each animal's head at either 10 or 20 min after the injection. The animals were then observed continuously until they had eaten the algae and/or laid eggs. Algae not eaten or partly eaten were weighed again after the experiment.

RESULTS

A change in feeding behavior during egg-laying will be described first. Following this, three effects of egg-laying hormone (ELH) on neuronal activity recorded in vitro from nerves of the head ganglia (Fig. 1) will be described: 1) on a buccal nerve 3 unit (b.n. 3 unit), 2) on a cerebrobuccal connective unit (c.b.c. unit), and 3) on numerous pedal nerve units.

Feeding behavior during egg-laying

The work of Davis et al. (14) with Pleurobranchaea suggested that feeding behavior may also be altered in Aplysia during egg-laying. To test this hypothesis, experimental animals were injected with bag cell cluster homogenate, containing ELH, while control animals were simultaneously injected with head ganglia homogenates. Each animal was then presented with a small piece of algae, its behavior observed, and the time when feeding and egg-laying occurred noted.

For 5 pairs of animals that were given the algae 20 min after injection, none of the experimentals and all of the controls ate the algae. This difference is significant ($p < .01$, Fisher exact probability, 2 tailed). The egg strand, after exiting from the genital pore, moves in 2-3 min up to the animal's head via an external, ciliated groove. For the experimental animals, eggs started to appear at the head at 32 ± 3 min post injection whereas the control animals started to eat at 24 ± 3 min. This suggests that the change in feeding behavior occurs before the eggs have appeared.

To confirm this, algae was presented to another 7 pairs of animals only 10 min after injection. The experimentals started eating by 12 ± 1 min and stopped by 17 ± 4 min post injection, 12 ± 4 min before their eggs appeared at 29 ± 4 min.

When they stopped eating, they had consumed only 40 ± 20% of their algae. One control animal did not eat and the other 6 finished all their algae by 22 ± 8 min. The difference for the two groups with respect to eating all the algae is significant ($p < .01$). As suggested by these numbers, the experimental animals, before they actually stopped eating, appeared to be eating more slowly than the controls.

Buccal nerve 3 unit

If ELH is added to the in vitro head ganglia, a small continuously pacing unit (spike) reliably appears (Fig. 2) in buccal n. 3 (21). The cell body of the axon generating this unit spike is in the buccal ganglia. This effect is the best studied of the three.

In these experiments, there are two main sources for the ELH added to the solution surrounding the head ganglia: a) The ELH is released (along with other substances) from the disconnected abdominal ganglion into the perfusate following electrical stimulation of a bag cell afterdischarge. b) The ELH is obtained by homogenizing bag cell clusters. ELH from the first source either is directly added to the head ganglia or first fractionated on a P-6 gel filtration column. ELH from the second source is usually either fractionated on the P-6 column or partially purified employing ammonium sulfate precipitation, a DEAE anion exchange column, and a P-6 fractionation.

RELEASED ELH. In the ELH release type of experiment, the abdominal ganglion is placed a few millimeters away from the head ganglia. The chamber is slowly perfused with solution overnight. In the morning, after a few hours of recording neuronal activity, the perfusion is stopped and the siphon nerve of the abdominal ganglion is electrically stimulated (30 v, 4 msec, 5/sec for 30 sec). This control

stimulation does not activate the bag cells. The perfusion is resumed a few hours later so that the preparation is rinsed. For the experimental period, the perfusion is then again stopped and an identical stimulus is applied to the bag cells which now, in all but two of 20 cases, does produce an afterdischarge. The perfusion is resumed again a few hours later.

An early example of this type of experiment is seen in Fig. 2. Part of the polygraph record for an electrode attached to buccal n. 3 is shown for both the control (2B) and the experimental (2C) period. A continuously pacing unit spike is apparent in the record from the experimental period. The occurrence of this unit is quantified for the two days following the day of dissection in Fig. 2A. This unit appears following the bag cell afterdischarge which lasts for 30 min. The frequency of firing of this unit returns to baseline within 2-4 h after the perfusion is resumed. This occurred in all six experiments of this type where the siphon nerve was stimulated as a control. In the two experiments in which we failed to activate bag cell afterdischarge, this unit did not appear. On the next day, the perfusion is again stopped and this unit is reactivated following bag cell afterdischarge (2A).

A procedural variation consists of performing a control and experimental perfusion of the abdominal ganglion in a second dish separate from the head ganglia. These two perfusates can then be added separately at the appropriate time to the head ganglia chamber. The results of these two procedural variations are combined together in Table 1 under the category of released ELH. In 24 out of 29 such experiments, the b.n. 3 unit's appearance was identified in at least one of the two, left or right, nerves. In a majority of these 24 cases, the unit was also identified in the opposite nerve.

In other experiments, the ELH released from activated bag cells in abdominal ganglia is first fractionated on a P-6 gel filtration column. The ELH fractions, plus the equilibrating salts, etc. (see Methods), are then added directly to the chamber containing the head ganglia or to the perfusate entering the chamber.

Figs. 3B and C are recordings from a buccal n. 3 following addition to the chamber, respectively, of the pre-exclusion buffer and of the fractions containing the released ELH. Fig. 3A shows the frequency of occurrence of the ELH activated unit over the course of the experiment. Five out of six such experiments when compared to the control buffer showed a positive response to fractions containing ELH in at least the left or right nerve (Table 1).

ELH FROM BAG CELL CLUSTER HOMOGENATE. A second source of ELH is the homogenate of bag cell clusters. The supernatant of this homogenate or the ELH containing P-6 fractions of the supernatant usually activate this b.n. 3 unit (Table 1). In other experiments, ELH from this same source was partially purified using ammonium sulfate precipitation, an anion exchange column, and a P-6 column. Fig. 4 shows such an experiment. In this experiment, the perfusion is never stopped. The pre-exclusion volume P-6 buffer and the partially pure P-6 ELH fractions are present in the perfusate entering the dish during the times indicated. Table 1 shows that 5 out of 6 of these experiments were successful in activating this unit. In 3 of these successful experiments, the control was excluded material (protein) from the P-6 column.

ISOLATED BUCCAL GANGLIA. In the prior examples, the rest of the head ganglia are present and attached to the buccal ganglia through the cerebrobuccal connectives (Fig. 1). But, the b.n. 3 unit appears when ELH is added to the buccal

ganglia only (Table 1). These responses are just as dramatic as when the rest of the head ganglia are attached. This means that the buccal ganglia must contain the responsible cell bodies.

OTHER CHARACTERISTICS. Some of the experiments provide useful information on the timing of the response. In these experiments, enough ELH to produce a substantial response (>1 spike/sec) is added directly to the chamber so that the zero point is clearly defined and there is no necessity for diffusion through the chamber to occur first. Diffusion through the connective tissue sheath, though, is still required. The unit appears intermittently during the first 5 min in 3 out of 4 such experiments. In all 4, the unit starts pacing continuously between 5 and 7 min after ELH was added and reaches its peak rate between 10 and 25 min post ELH. The average number of spikes, with the standard error, for these 4 experiments for each 5 min period is shown in Fig. 5. Also indicated, for comparison with the behavioral experiments, are the mean times when the experimental animals, fed food at 10 min, stopped eating and when they laid eggs.

In several preliminary experiments, it proved relatively easy to locate the vicinity of the cell body presumably responsible for the unit. This was accomplished in the presence of ELH by moving an extracellular electrode over the surface of the buccal ganglion while simultaneously recording from the ganglion's buccal nerve 3. The cell body lies on the rostral surface somewhere between buccal neuron 3 and 4 (21).

The size of the unit as recorded from the buccal n. 3 is typically about 10-15 μV with a range in different preparations of perhaps 5-40 μV. This relatively small size means that even with the extracellular electrode positioned near the cell body, it has not been possible to determine whether the unit participates in the

large, spontaneous rhythmic bursts of neuronal activity presumably involved in the control of feeding (60).

Much of the low background activity of this unit in the graphs before the addition of ELH may be due to artifacts in the method of counting spikes of the appropriate amplitude from the polygraph record. In some cases, though, the unit is identifiable before the addition of ELH and the discriminator can be set to follow it. In these cases, the unit appears a few sec after the large spontaneous rhythmic burst in buccal n. 3 has ceased. From 1 to about 15 relatively evenly spaced spikes (a few per sec) then occur. When, in the presence of ELH, spontaneous rhythmic bursts appears, this unit also reappears with the same phase, but with a higher frequency which does not terminate, unless another burst occurs.

It should be noted that in a majority of cases a corresponding unit appears in both the right and left buccal n. 3 with changes in their frequency of firing after activation by ELH generally occurring in parallel. There is never an exact one to one correspondence, though, of individual spikes, and their frequency of firing is often different by as much as 10-30%.

Cerebrobuccal connective

If ELH is added to the in vitro head ganglia, an identifiable unit whose cell body is in the buccal ganglia is present with increased frequency in the cerebrobuccal connective. This unit normally paces intermittently, but following the addition of ELH its frequency increases to the point where it is in some preparations continuously pacing.

RELEASED ELH. A summary of the effect of released, fractionated ELH is seen in Fig. 6A. During the control period, pre-exclusion volume buffer is present in the

perfusate entering the dish. During the experimental period released, fractionated ELH is in the perfusate. The unit responds by doubling its rate to about one spike every two sec. A significant increase in frequency for this unit occurred in 7 out of 7 experiments employing released, fractionated ELH (Table 1).

The frequency of occurrence of this unit (Fig. 6A) indicates a smaller response to the control, also. This, in fact, is due to a 15 min absence of perfusate at the end of the control period during which time small air bubbles are accidently pumped into the side of the chamber. Such a response to the air bubbles also occurs in pedal nerve activity. When a constant perfusion rate is maintained, none of these responses occur for the control buffer or exclusion volume fractions.

ELH FROM BAG CELL CLUSTER HOMOGENATE. The cerebrobuccal connective unit responds also to partially purified ELH by increasing its rate. In 8 out of 10 experiments a significant increase in rate occurs for the cerebrobuccal connective unit in response to fractionated homogenate or partially pure ELH (Table 1).

ISOLATED BUCCAL GANGLIA. This unit is more apparent in the detached buccal ganglia preparation (Fig. 6B, C, D) where it shows a large increase in rate. ELH is released from activated bag cells in a detached abdominal ganglion present in the same chamber. In two other experiments employing isolated buccal ganglia this unit's increase in rate in response to material containing ELH is also significant (Table 1).

OTHER CHARACTERISTICS. Information on the timing of the unit's initial response can be obtained from the experiments where ELH is directly added to the chamber. In 6 such experiments, there was always a response with a curve similar

to that of b.n. 3 unit (Fig. 5) except that the peak plateau rate was achieved in 30 min, that is, about 10 min later. This was the only response in the single experiment employing partially purified ELH. Material that contained ELH and also the 5,000-6,000 m.w. acidic peptide caused an additional early response. This response, of the same average magnitude, occurred at about 5 to 10 min after addition of these combined peptides in the 5 other experiments. Four of these experiments employed released ELH, one where the released material had been fractionated on a P-6 column, and the fifth employed BCC homogenate, fractionated on P-6. The P-6 fractions in both experiments included the pre-ELH region containing the pI 4.5-5.0, 6,000 dalton polypeptide (1, 20, 53). These results, then, are consistent with a hypothesis that the early response is due to some substance other than ELH, possibly the pI 4.5-5.0 polypeptide.

The vicinity of the cell body presumably responsible for this unit was located in two experiments. It lies on the caudal surface of each ganglion, slightly medial to buccal neuron 1 and 2 (21).

The size of this unit recorded from the cerebrobuccal connective is typically about 40-50 μV. In the isolated buccal ganglia there appears to be only one, infrequently occurring, spontaneous unit that is larger in amplitude. During a spontaneous buccal ganglia burst of impulses, the ELH sensitive unit normally starts firing preceding the main burst in the cerebrobuccal connective and buccal n. 3, slows or stops during the main burst and, most reliably, picks up in frequency at the end of the burst, continuing for a min or so thereafter. Periods of activity appear spontaneously, at other times, in the absence of such bursts.

This unit can usually be identified in both the left and right cerebrobuccal connectives, where there is a correlation in changes in frequency and times of

appearance. But, there is never an exact one to one correspondence of spikes, and the overall frequency can differ somewhat between sides by as much as 10-30%.

Pedal nerves

When ELH is added to the in vitro head ganglia, increases in neuronal activity appear in the pedal nerves, particularly the anterior nerve. Other stimuli elicit similar responses. Corresponding units in different preparations have not yet been repeatedly identified, so that it is not known if differences exist between the bursts of activity occurring in the presence of ELH and the bursts elicited by other stimuli.

The only systematic description of pedal ganglia nerves in Aplysia was made by Eales (17). The exact pattern of branches is variable, but usually the nerves and their major branches can be identified. Branches of the anterior, medial and posterior pedal nerves (Fig. 1) as described by Eales were usually recorded from as well as branches of the nerve to the penis (9). These 3 pedal nerves innervate the foot, leaving the pedal ganglia from their ventral sides and traveling in an anterior, medial posterior, and posterior direction, respectively.

In 7 preliminary experiments noncontinuous perfusion was used. In the control periods, a tendency existed for pedal nerve activity to build up slowly with time following cessation of perfusion. This control problem, which did not occur with continuous perfusion, somewhat obscured the ELH excitatory effects in the experimental period. All ELH effects that we now report were obtained with the continuous perfusion technique.

RELEASED ELH. Two experiments were performed while recording from a total of 11 pedal ganglia nerve branches in two attached head ganglia preparations. An

example of the effect of released, fractionated ELH is seen in Figs. 7B, C, D. Figs. 7C and D show a control and experimental recording from a branch of the anterior pedal nerve. The frequency of occurrence of the largest unit (7D) is plotted in Fig. 7B.

Positive responses occur in the one other continuous perfusion experiment with released ELH, in this case, nonfractionated. Perfusate from the abdominal ganglion prior to bag cell stimulation serves as a control. For the two experiments together activity in ten out of eleven pedal nerves or their branches show significant (student t test, $p < .05$, two tailed) increases in frequency of action potentials compared to control ($n = 4$ for nonfractionated and $n = 6$ for fractionated). These responses all belong to branches of either the right posterior or anterior pedal nerves. The one failure comes from a branch of the anterior pedal nerve in which many units diminished or disappeared between the control and the experimental period, probably due to a recording failure or axonal death.

ELH FROM BAG CELL CLUSTER HOMOGENATE. Two experiments were performed while recording from a total of 12 pedal ganglia nerve branches in two attached head ganglia preparations. An effect of partially purified ELH upon the activity from a branch of the anterior pedal nerve is shown in Fig. 7A where the frequency of occurrence of large, discriminated units is plotted. Significant increases in response to ELH occur for all six pedal ganglia nerve branches recorded from in this experiment.

Significant, positive responses in 3 out of 6 pedal ganglia nerve branches occur for a second continuous perfusion experiment that used P-6 fractionated BCC homogenate for the ELH source and the exclusion volume for the control. These two experiments together show significant excitatory responses that belong

to the branches of the right anterior, medial and posterior pedal nerves as well as the left posterior pedal nerve and the nerve to the penis.

Three other experiments were performed with partially purified ELH, but here the smaller amount of ELH reaching the dish was sufficient to only marginally activate the buccal nerve 3 unit (n = 2) or the cerebrobuccal connective unit (n = 3). Pedal ganglia nerve activity, in at least some branches, was significantly increased, but spontaneous changes in activity made the total data difficult to interpret.

OTHER CHARACTERISTICS. In the presence of ELH neuronal activity builds up almost simultaneously in all the pedal nerves followed. This increased activity lasts 10-20 min during which time neuronal bursts in different branches occur both in and out of phase with each other. This neuronal pattern of activity subsides and then may reoccur 15-45 min later (Fig. 7B). A similar, but not necessarily identical, pattern may occur spontaneously and can be elicited by other stimuli, for example air bubbles, jarring the table, or moving parts of the preparation.

Most recordings are from right pedal nerves, but the few examples from the left nerves show the same effect. The role of the other attached head ganglia in initiating, generating, or coordinating the increased activity in response to ELH is not yet known.

DISCUSSION

The results reported here demonstrate that in Aplysia the neurosecretory, polypeptide egg-laying hormone (ELH) has direct, specific effects in vitro on the animal's own nervous system. ELH activates a pair of neurons, into a tonic pacing mode, in the left and right isolated buccal ganglia, each with an axon in their respective buccal n. 3. ELH increases the rate of firing of another pair of neurons

in the isolated buccal ganglia, each with an axon in their respective cerebrobuccal connective. Finally, in the attached head ganglia, ELH activates simultaneous bursting activity in pedal nerves.

We think that ELH is responsible for these observed effects, because ELH as released or obtained from bag cell cluster homogenates always produces the same effect. First, the responsible substances must be released from the abdominal ganglion concurrently with bag cell activation, but not siphon nerve stimulation. Secondly, the responsible substance, as released, must run with ELH on a P-6 gel filtration column. Thirdly, the responsible substance must co-purify with ELH from homogenized tissue where ammonium sulfate precipitation, anion exchange chromatography and gel filtration chromatography are employed. Finally, control substances from the P-6 excluded volume do not produce these effects.

The behavior following injection of ELH into the animal might, in theory, be elicited by two very different stimuli. ELH may act on the gonad to cause release of eggs (12). The subsequent appearance of the eggs could then act as a stimulus for the egg-laying behavior. Alternatively, ELH or a secondary factor might act directly upon the nervous system to elicit the behavior. This latter hypothesis with ELH as the direct agent is supported by the results reported here. If a physiological concentration of ELH, as released from activated bag cells, surrounds the head ganglia in vitro, then specific neuronal activity is elicited (Table 1). These in vitro effects should be similar to the in vivo case where the bag cells preceding egg-laying produce a train of synchronous action potentials (46) which serves to release ELH (53). So, the heart should then pump blood containing ELH into and around the head ganglia resulting in effects similar to those described

here. These results do not exclude the possibility that the eggs or other secondarily released factors may also be stimuli for certain components of the behavior.

The hypothesis that ELH acts directly upon the nervous system is also supported by the well characterized effects occurring in the abdominal ganglion following bag cell activation (10). Extracts containing ELH will duplicate some of these effects (44). They are not necessarily "hormonally" mediated, though, in the manner proposed here. Since the bag cells are located in the ganglion where the effects occur, two other interesting possibilities exist: the effects could be mediated through synapses or by ELH diffusion through localized areas of the ganglion. The latter interpretation is favored by Mayeri and colleagues (43).

Some of the ELH effects described here may relate to specific behavior of the animal. The fact that at 20°C the timing of the initial appearance of the b.n. 3 unit corresponds to the timing of the initial behavioral increase in threshold for feeding (Fig. 5) suggests the hypothesis that this unit is concerned with this increased threshold for feeding. The unit may be tonically exciting a retractor muscle or inhibiting a protractor muscle.

The ELH induced increase in pedal nerve activity may relate to several aspects of behavior. First, it may be controlling the initial locomotor behavior observed in animals when injected with ELH (2). The head ganglia contains much of the program for locomotor movement and is capable of exhibiting it in vitro (28).

Alternatively or additionally, the ELH induced activity may be generating the weaving and nodding movements of the animal's head as it attaches its eggs to the substratum. Most of the pedal nerves studied here should be involved in such movements. The posterior pedal nerve changes were normally less substantial and of a more tonic and long lasting nature, and so, may not relate to these movements.

The rapid and coordinated buildup of bursting activity in these nerves seems to preclude the possibility that the ELH is directly modifying the characteristics of each affected neuron. It seems reasonable to assume that the neuronal network needed to produce the patterned output preexists, and that ELH helps to activate it and perhaps to modify and coordinate it via critical control points.

Part of the nerve to the penis innervates its retractor muscle (9) so that the ELH induced activity in this nerve may increase the threshold for penis eversion by activating this muscle's excitatory motor neurons (48). This is consistent with the observation that an animal laying eggs does not simultaneously evert its penis and mate with another animal, although it will act as a female and induce, perhaps by its eggs, mating in other animals (3, 6).

The neuronal ELH effects, here demonstrated, help explain the animal's selection of egg-laying behavior and simultaneous rejection of other behaviors such as feeding. This behavioral "choice" has been studied in terms of "behavioral hierarchies" by Davis et al. (14) who predicted that such a hormonal mechanism might explain the suppression of feeding occurring during egg-laying in Pleurobranchaea. Other behavior, such as inking, may be suppressed by a more "local" action of ELH in the abdominal ganglion (10).

Our results, then, provide a basis for further work in two directions. First, the relatively large and identifiable cells of Aplysia should permit work on identifying the exact neuronal sites of the ELH receptors in the head ganglia and the cellular mechanism(s) by which ELH effects its changes. Secondly, the growing knowledge of neural circuits underlying specific behaviors should stimulate investigation of the exact relationship between the ELH neural effects and the

behavioral changes associated with egg-laying.

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Table 1. Number of ELH responses for buccal ganglia units

	Unit			
	buccal nerve 3		cerebrobuccal connective	
ELH form	attached head g	detached buccal g	attached head g	detached buccal g
Released	24(5)	3	4	2
fractionated	5(1)		3	
Bag cell cluster supernatant	1	1		
fractionated	6(1)	3	3(1)	1
partially purified	5(1)		5(1)	
No. controls/No. preps.	20/30	2/6	11/11	2/3

The number of any failures is given in parentheses. For weak responses, most frequently when the amount of ELH present was marginal, the student t test was used. The 15 min, or sometimes 5 min, counts were compared with those of the earlier control period. The response was considered positive if the t value was significant ($p < .05$, two tailed) for the unit recorded from either the left or right ganglion.

FIG. 1. Ventral view of Aplysia head ganglia. The buccal, cerebral, pedal ganglia, and nerves are shown. The pleural ganglia lie underneath the pedal ganglia. The right anterior pedal nerve normally has 3 main branches. Each middle pedal nerve splits quite near the pedal ganglion into two branches. The posterior pedal nerve is the largest nerve from the pedal ganglion and normally has a reddish tint. All 3 nerves leave the pedal ganglion from its ventral side. Shown are the buccal musculature, the anterior esophagus, and the salivary glands, none of which were included in the head ganglia preparation used for experiments.

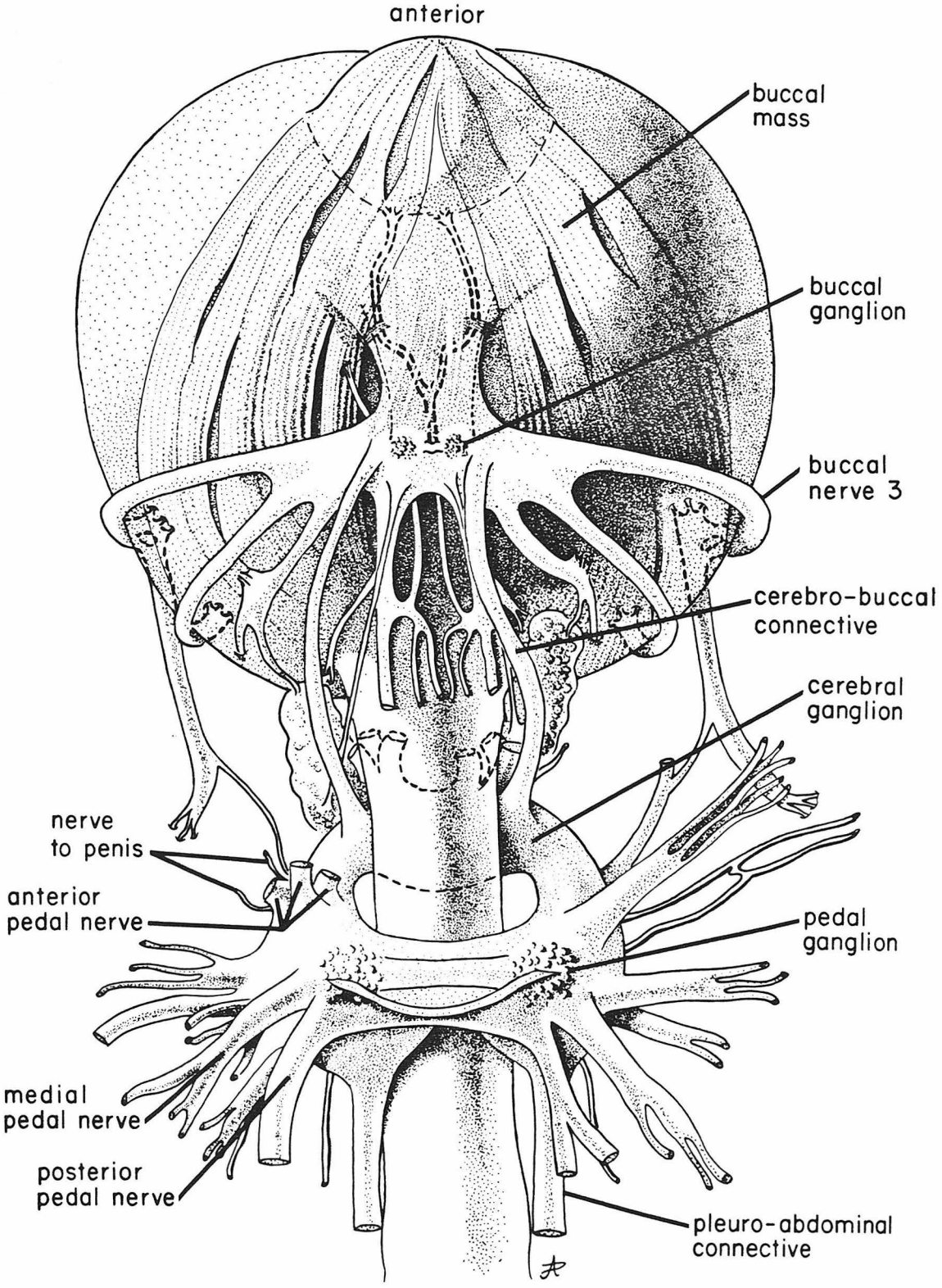

anterior
buccal mass
buccal ganglion
buccal nerve 3
cerebro-buccal connective
cerebral ganglion
nerve to penis
anterior pedal nerve
pedal ganglion
medial pedal nerve
posterior pedal nerve
pleuro-abdominal connective

FIG. 2. Activation by released ELH of the buccal n. 3 unit (attached head ganglia). A) The frequency of firing over the first two days post dissection of the continuously pacing unit spike evident in "C". For the control at the first arrow the perfusion is stopped, and the siphon nerve of the detached abdominal ganglion present in the same chamber is stimulated. The two bars labeled bag cells show the periods when the activated bag cells of the abdominal ganglion are releasing ELH into the chamber. At the beginning of each bar the perfusion is stopped and the bag cells stimulated. The 3 arrows marked "rinse" are the points when perfusion is resumed. For the second day, two fresh abdominal ganglia have been added to the chamber and the bag cells of all 3 are stimulated. B) A control recording from the right buccal n. 3 following stimulation of the siphon nerve. C) Recording from this nerve following stimulation of the bag cells in the abdominal ganglion.

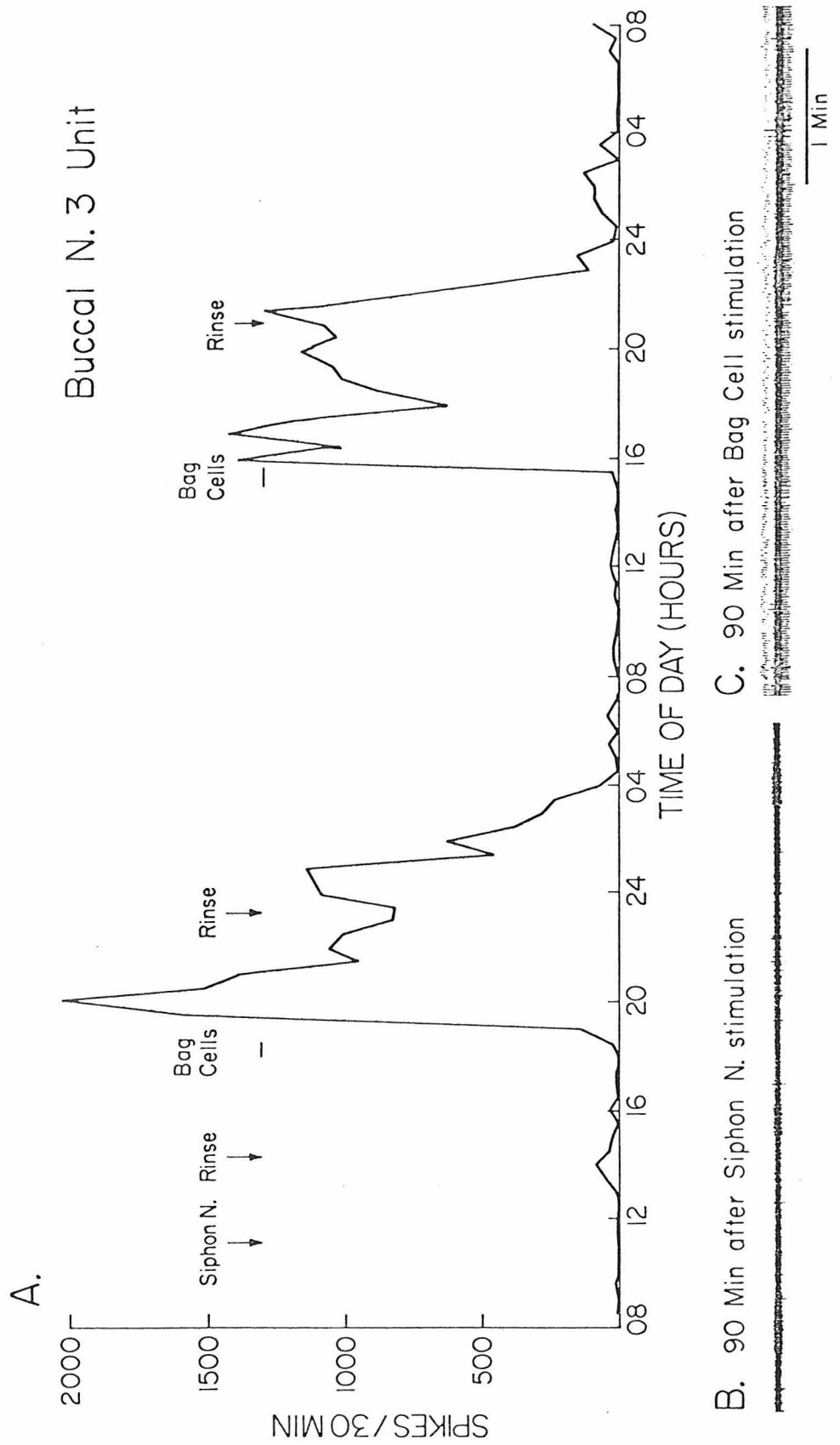

A.
Buccal N. 3 Unit
SPIKES / 30 MIN
2000
1500
1000
500
Siphon N.
Rinse
Bag Cells
Rinse
Bag Cells
Rinse
08
12
16
20
24
04
08
TIME OF DAY (HOURS)
B. 90 Min after Siphon N. stimulation
C. 90 Min after Bag Cell stimulation
1 Min

FIG. 3. Activation by fractionated, released ELH of the buccal n. 3 unit (attached head ganglia). A) Frequency of firing of unit evident in "C". The perfusion through the chamber stops when the buffer control or released ELH is added (arrows) and resumes at the "rinse" arrows. B) Control recording from the left nerve following addition of pre-exclusion volume buffer to the chamber. C) Recording from the same nerve following addition of P-6 fractions of released ELH.

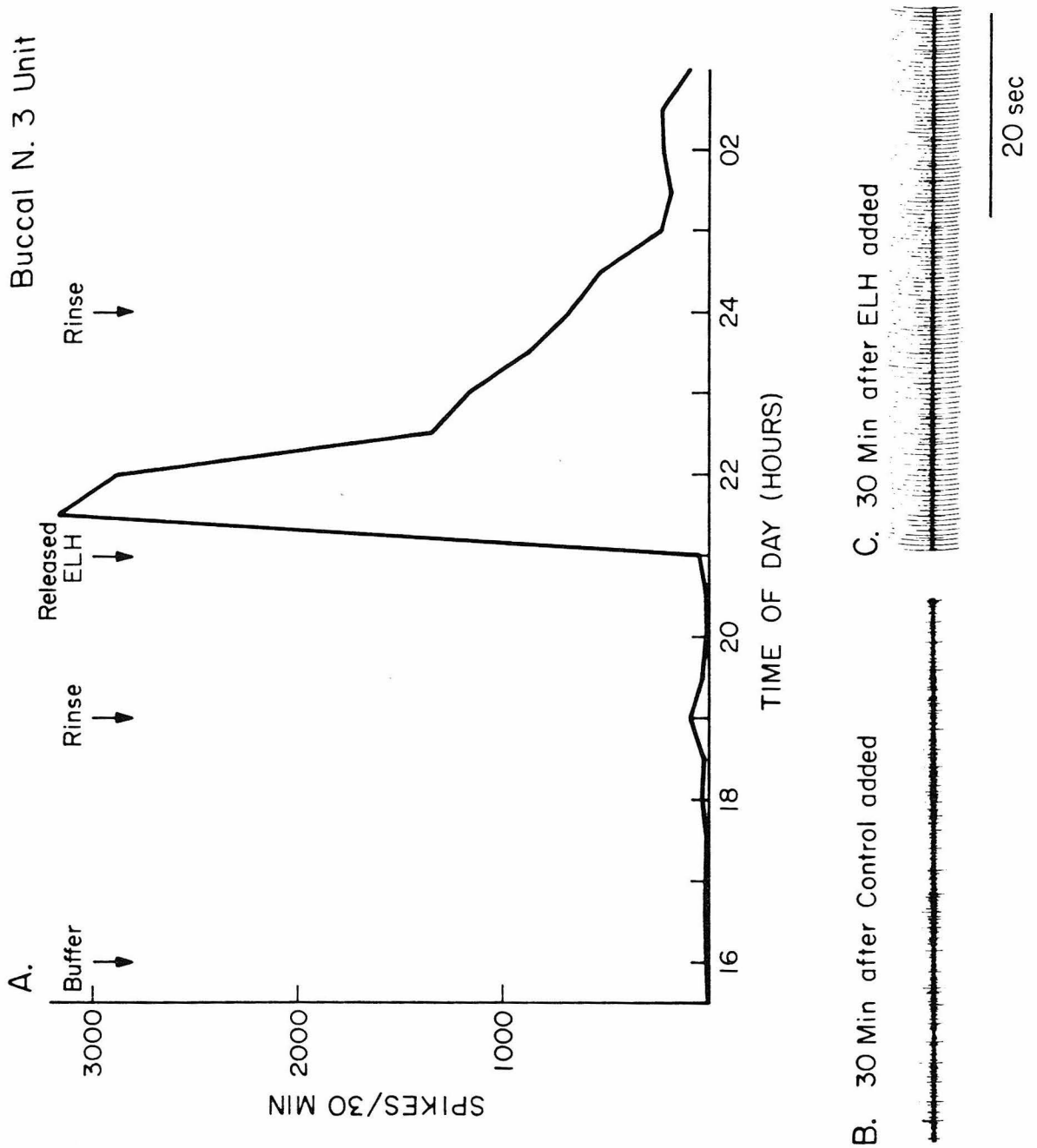

A.
Buccal N. 3 Unit
Buffer
Rinse
Released ELH
Rinse
3000
2000
1000
SPIKES/30 MIN
16
18
20
22
24
02
TIME OF DAY (HOURS)
B. 30 Min after Control added
C. 30 Min after ELH added
20 sec

FIG. 4. Activation by partially pure ELH of the buccal n. 3 unit (attached head ganglia). A) Frequency of firing of unit evident in "C". The perfusion through the chamber does not stop and the buffer control or partially pure ELH are present in the perfusate entering the chamber during the times indicates. B) Control recording from the right nerve following addition of pre-exclusion volume buffer to the perfusate entering the chamber. C) Recording from the same nerve following addition to the perfusate of P-6 fractions of partially purified ELH equivalent to the ELH of 1 to 1-1/2 pair of bag cell clusters.

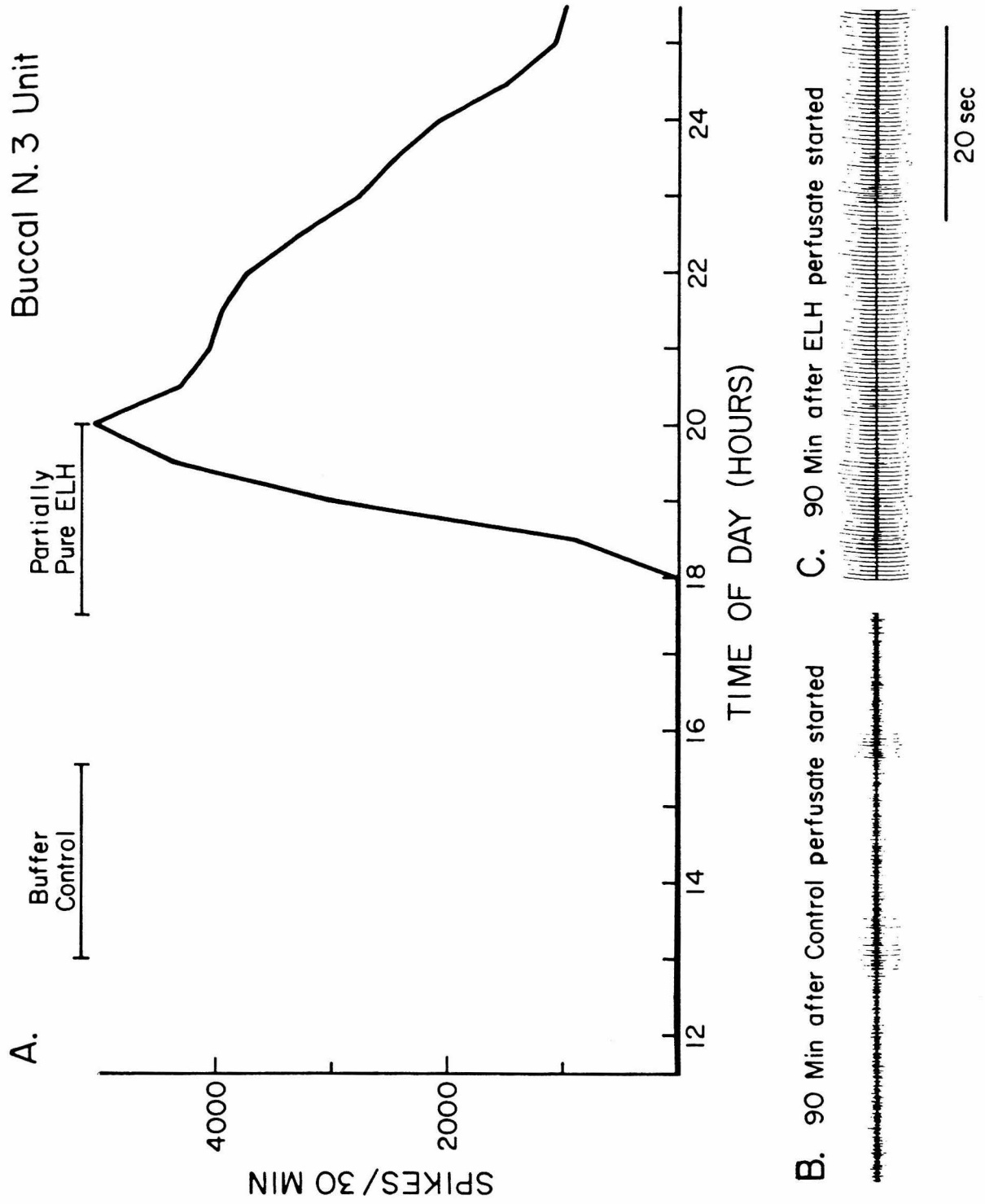

A.
Buccal N. 3 Unit
Buffer Control
Partially Pure ELH
SPIKES/ 30 MIN
4000
2000
12
14
16
18
20
22
24
TIME OF DAY (HOURS)
B. 90 Min after Control perfusate started
C. 90 Min after ELH perfusate started
20 sec

FIG. 5. ELH is mixed into the chamber at zero time. The number of spikes for the buccal n. 3 unit during each 5 min period is shown at the middle of that period. The first arrow indicates the mean time when the animals from the behavioral experiment stopped eating, and the second arrow indicates the mean time when they started laying eggs. Each 5 min point is a mean (n = 5) and the standard error is shown. Four of the units were responding to released ELH and the fifth to P-6 fractionated, released ELH.

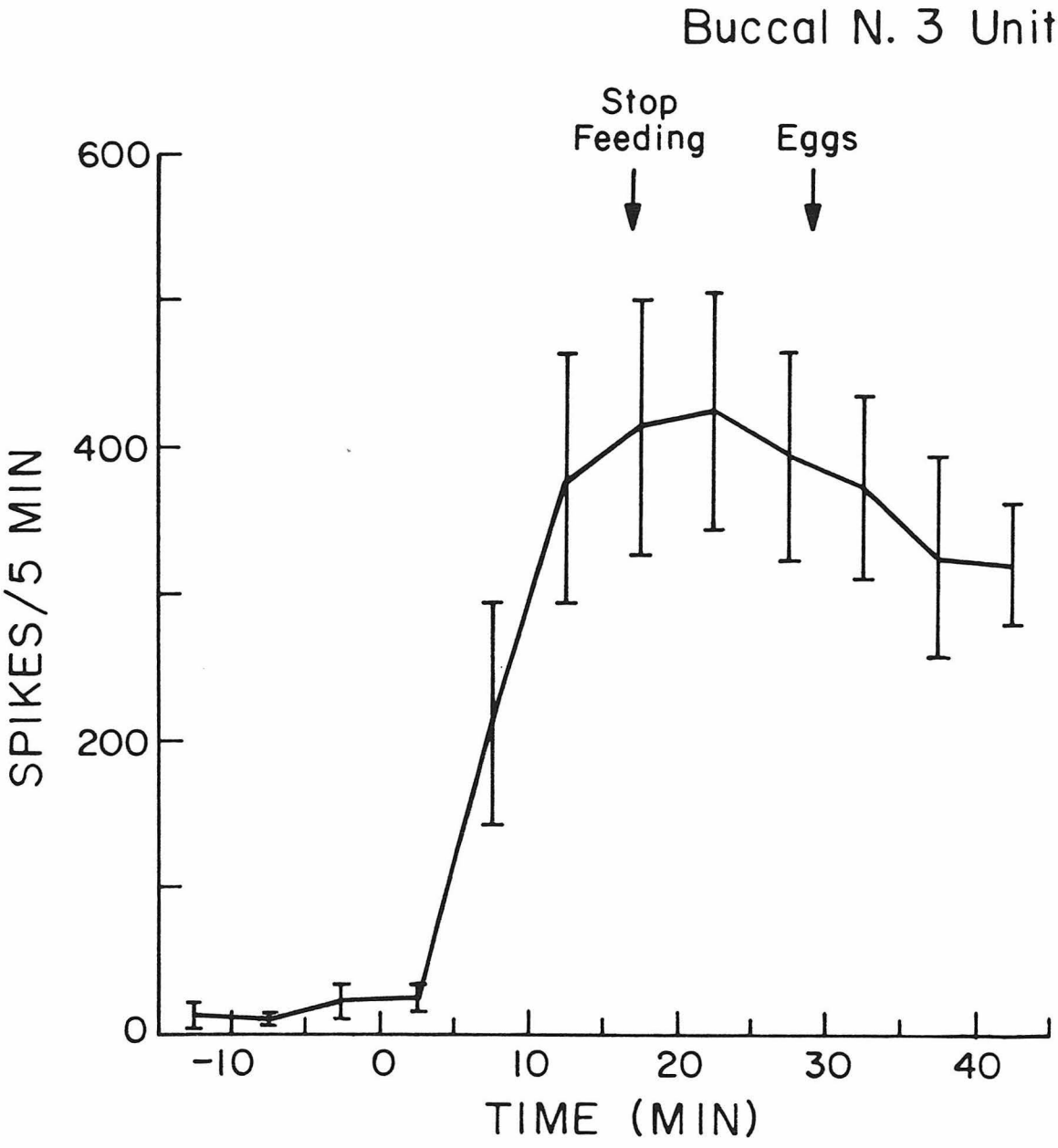

Buccal N. 3 Unit
Stop
Feeding
Eggs
600
400
200
0
SPIKES/5 MIN
-10
0
10
20
30
40
TIME (MIN)

FIG. 6. Activation by ELH of the c.b.c. unit. A) Frequency of firing of this unit (attached head ganglia) in the right connective. The pre-exclusion volume buffer control or P-6 fractions of released ELH are present in the perfusate entering the chamber during the times indicated. The perfusion through the chamber stops only momentarily at the end of the control. B) Frequency of firing of the unit in the right connective (detached buccal ganglia) evident in "D". The bar indicates the duration of the period when the activated bag cells of the detached abdominal ganglion are releasing ELH into the chamber. The perfusion stops at the first arrow and resumes at the second. At the beginning of the bar, the bag cells are stimulated briefly. C) Control recording for "B" from the right cerebrobuccal connective before bag cell stimulation. D) Recording from the same nerve after bag cell activation. The large unit is now continuously pacing.

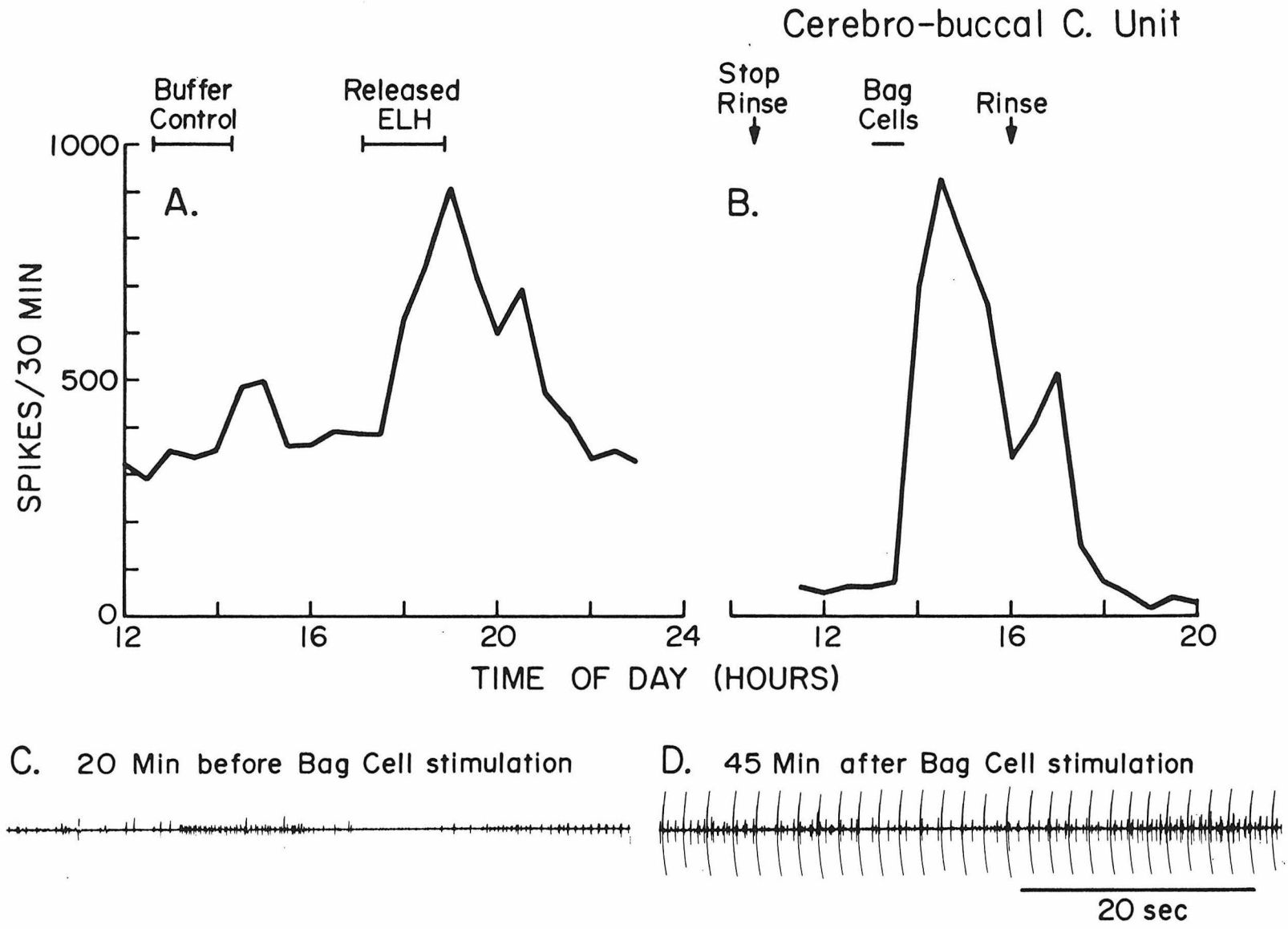
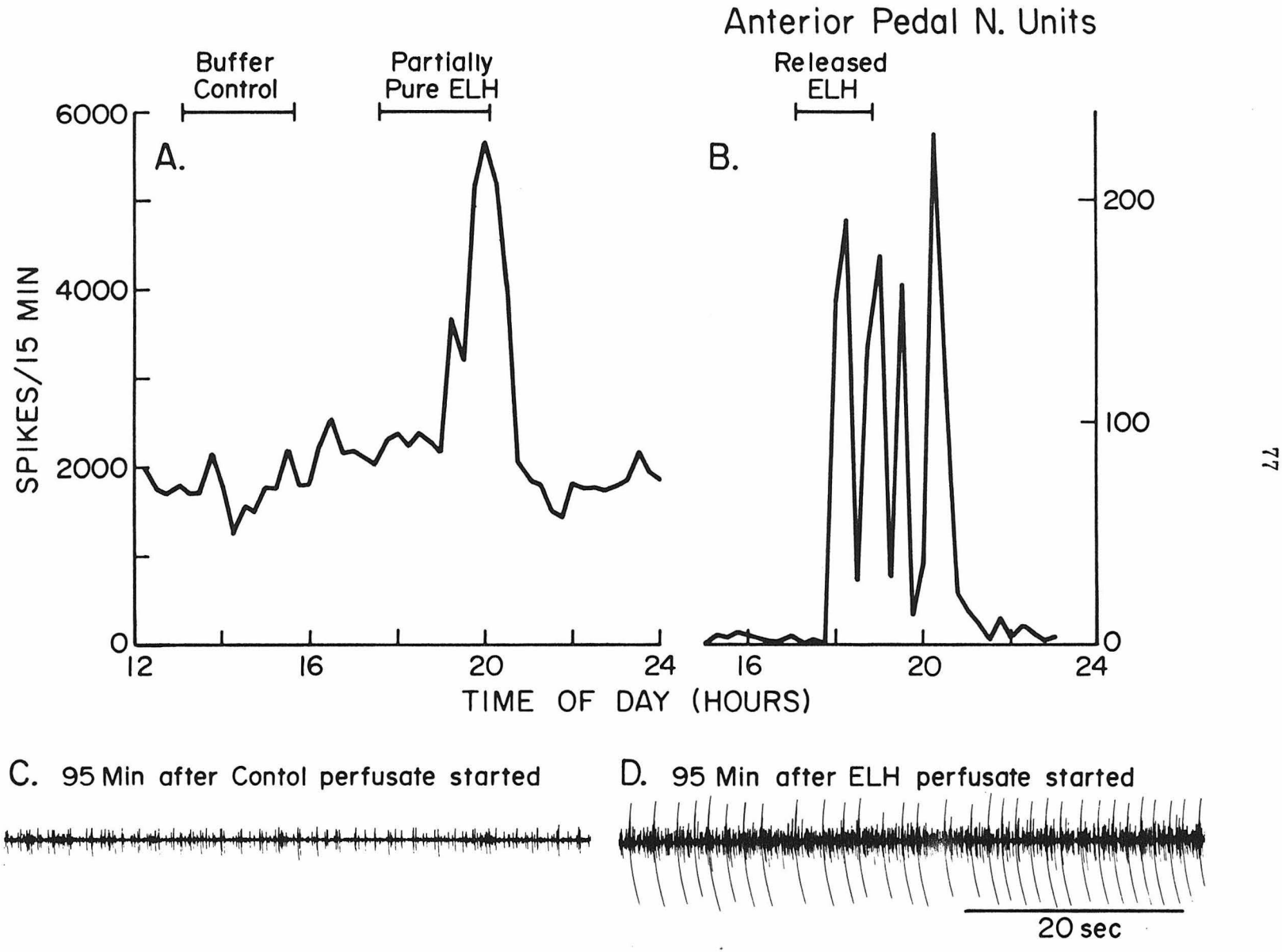

Cerebro-buccal C. Unit
Buffer Control
Released ELH
Stop Rinse
Bag Cells
Rinse
A.
B.
1000
500
0
SPIKES/30 MIN
12
16
20
24
TIME OF DAY (HOURS)
C. 20 Min before Bag Cell stimulation
D. 45 Min after Bag Cell stimulation
20 sec

FIG. 7. Activation by ELH of anterior pedal nerve activity (attached head ganglia). A) Frequency of firing of larger, discriminated units from the main branch of the nerve. During the control pre-exclusion volume buffer is added to the perfusate continuously entering the chamber. Partially pure ELH, equivalent to the ELH of 1 to 1-1/2 pair of bag cell clusters, is then added. B) Frequency of firing of the largest unit evident in "D". The perfusion through the chamber does not stop and the P-6 fractions of released ELH are present in the perfusate entering the chamber during the time indicated. C) Control recording from a lateral branch of the nerve following addition of pre-exclusion volume buffer to the perfusate. D) Recording from the same branch following addition to the perfusate of P-6 fractions of released ELH.


Anterior Pedal N. Units
Buffer Control
Partially Pure ELH
Released ELH
A.
B.
6000
4000
2000
0
SPIKES/15 MIN
12
16
20
24
200
100
0
TIME OF DAY (HOURS)
C. 95 Min after Contol perfusate started
D. 95 Min after ELH perfusate started
20 sec

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Chapter 3

Circadian Release of Presumed Peptides from the Isolated Eye of *Aplysia californica*[1]

[1]Work done in collaboration with Gerald Audesirk

SUMMARY

A circadian rhythm (CR) of release of presumed peptides from the isolated eye of Aplysia californica is demonstrated. Substances labeled with radioactive amino acids and released into the perfusate were separated on gel filtration columns and SDS polyacrylamide gels. In the CR experiments, the perfusate of a single, labeled, dark-maintained eye was collected every 3 h for two days while simultaneously recording the CR of compound action potentials (CAPs) from the optic nerve. Each 3-h perfusate was applied to a P-2 gel filtration column. Excluded substances (m.w. > 2000) and material fractionated in the region of m.w. ∿ 1000 showed a CR which was in phase with the CR of CAPs. Over half of these substances can be precipitated with trichloroacetic acid. Their release is stimulated by a high potassium solution and inhibited by a low calcium solution that also inhibits CAP activity. This and other previously published evidence suggests that the CAPs and the peptide release are directly produced by electrically coupled neurosecretory cells which may also contain the CR oscillator. One or more of these peptides may be a neurohormone and/or transmitter used for synchronizing, entraining and/or driving the rest of the animal's CRs.

INTRODUCTION

Only a few in vitro systems for the study of circadian rhythms (CRs) have been described. Such systems include the adrenal gland[1], the pineal gland (M. Menaker, personal communication), the crayfish eyestalk[36], and neurons of the Aplysia abdominal ganglion[38,39]. The eyes of opisthobranch gastropods Navanax[12] and Aplysia are more suitable than any other presently described system for in vitro CR study; these isolated eyes have a reproducible, long lasting, and large amplitude CR of neuronal activity.

A series of investigations initiated in this laboratory has described the CR of the Aplysia eye. Jacklet[15] first showed that the isolated eye, in vitro, produces a CR of compound action potentials (CAPs) recorded from the optic nerve under constant dark conditions. This CR could be phase shifted in the isolated eye by application of an appropriate light stimulus[9,16,19] or by a pulse of 100 mM potassium[10]. Thus, the isolated eye contains the neuronal circuitry of a CR oscillator (clock) that can be entrained to external stimuli such as the normal 24-hour light cycle.

At 14°C eye CAPs occur in bursts of one to five. In filtered sea water, the period of the CR is 23.4 h, and the overall CAP frequency has a maximum near projected dawn and a minimum during projected night[35]. In culture medium, the CR can last for up to two weeks[20].

In other work[13], the eye released radiolabeled material, which was trichloroacetic-acid-precipitable, when challenged with a high potassium solution. This leucine or lysine labeled release was calcium dependent and contained a substance with a molecular weight (m.w.) of approximately 7,000.

Histological evidence employing electron microscopy[14,22,32,33,40,41] has provided evidence for neurosecretory cells in the outermost retina, clustered in groups closely associated with sinus blood spaces. A first type contains numerous dense core granules of about 100 nM diameter in its cell body, axon and synaptic endings. These granules are thought to contain a catecholamine, probably dopamine[32,33]. Autoradiographic evidence indicates that these neurosecretory cells, as compared to other eye cells, avidly incorporate leucine into substances that are preserved by formalin[40]. This implies that these neurosecretory cells actively synthesize proteins, perhaps for export. Axons and synaptic endings in the eye containing granules of about 150 nM diameter have also been described[30,33,40], but the cell bodies of this second type have not been located and, so, may not be in the retina.

One function of the eye is to control the animal's circadian locomotion. Experiments on Aplysia with eyes removed indicate that they play a primary role in generating the freerunning CR of locomotion exhibited in constant dim light or dark conditions[29,42]. This function of the eye could be mediated by either a CR of neurosecretion and/or the CR of CAPs in the optic nerve synaptically controlling other neurons in the head ganglia.

A second function of the eye may be to control the activity of the neuron R15[7]. Evidence suggests that this function is mediated by a CR of eye secretion. Eyes placed into a chamber with the Aplysia abdominal ganglion influence for several days the activity of R15. Significantly, the phase of the eye's CR of CAPs determines the timing of this influence[6]. A similar humoral dependence between the eyes and R15 appears to also exist in vivo[6].

The following experiments were undertaken as a result of this evidence in the eye for neurosecretory cells and for a CR of release of a physiologically active substance. We wished to follow the CR of secretion of one or more substances and relate this CR to the CR of CAPs.

MATERIAL AND METHODS

Aplysia californica were collected locally or obtained from Pacific Biomarine Supply Co. They were maintained under constant temperature and light-dark conditions as previously described[7]. Lights-on was at 0800 Pacific Daylight Time (by definition Circadian Time 0000) and lights-off at 2000 PDT (CT 1200). Dissections were carried out at 14°C and consisted of removing the eye and its attached optic nerve. The optic nerve was cut where it connects to the cerebral ganglion.

Maintenance Solutions

Except for the labeling medium, the eyes were subsequently maintained in a Millipore filtered (0.22 M) solution containing 10 mM glucose, 5 mM Tris Cl, pH adjusted to 8.0 at 14°C, 100 units of penicillin and 100 µg of streptomycin per ml, and filtered natural seawater (GTPS-FSW). The solution for the control eyes in Table I lacked glucose and Tris Cl (PS-FSW). In some experiments, 0.1 mM dithiothreitol (DTT) was also added (GTPS-FSW-D) to minimize aggregation of secreted polypeptides. In control experiments, the 0.1 mM DTT made no apparent difference in the eye's pattern of CAPs.

For the high potassium or low calcium experiments, the compositions of the experimental solutions and normal, control solution were the same as the perfusion solutions of Arch[2] except that they lacked glucose, the buffer was 10 mM

Na HEPES (pH 7.8) and the low calcium solution contained 0.1 mM Ca^+ instead of 1.0 mM. These experiments were conducted during the projected day. The perfusion solution was changed every 1 or 2 h with two control perfusions followed by two experimental and then two more control perfusions. This permitted comparison of the experimental period with the average of the pre and post controls.

Eye Recording

Recordings were made with the optic nerve doubled over and taken up into a suction electrode with a piece of Intramedic polyethelene (PE 20) tubing at the end. The electrical signal was amplified by a Tektronix 122 preamplifier and continuously recorded on a Grass Model 7 polygraph. The eye, hanging free from the electrode end, was positioned in 0.5-1.0 ml of the appropriate solution in a plastic beaker (1.5 ml capacity). For a given experiment, the temperature of the solution was maintained within 0.25°C by a Haake-Brinkman cooler with a range of 14.0-15.5°C over all experiments.

Eye recordings were carried out with the eye maintained in a light-tight box. Solution changes were made with Intramedic polyethylene (PE 160) tubing which led from the beaker to the outside of the light-tight box where solutions were added or removed via plastic syringes. Such changes normally took about 30 sec.

Eye Labeling

Eyes were labeled in a medium of Millipore filtered (0.22 µM) natural seawater with 150 units of penicillin and 150 µg streptomycin per ml and a concentration of 10 mM glucose, 10 mM Tris Cl (pH 8.0 at 14°C), and 5 µM of all amino acids except glutamine and the one or two that were to be labeled. This

medium was stored frozen at twice its final concentration until just before its use when the label, 0.01 N NaOH to neutralize the 0.01 N HCl with the label, and glass distilled water was added. In the later experiments, the HCl was first removed by taking the label solution to dryness under forced air in a boiling water bath.

The labeled amino acids obtained from New England Nuclear were L-Pro [2,3-^{3}H(N)], 22 Ci/mM; L-Leu [3,4,5-^{3}H(N)], 83 Ci/mM; L-Leu [^{14}C(U)], 283 mCi/mM; and L-Met [^{35}S], ~200 Ci/mM. One pair of eyes was normally labeled for 10-12 h beginning between CT 0200 and 0800 in 0.5-1.0 ml of labeling medium which contained 40-50 µCi/ml of the ^{3}H label and, if a second amino acid label was included, 5-10 µCi/ml of ^{14}C-Leu or 100 µCi/ml of ^{35}S-Met. Preliminary experiments indicated that ^{3}H-Pro labeled a number of presumed peptides released from the eye.

Gel Filtration

The perfusate (0.5 ml of GTPS-FSW or GTPS-FSW-D) which had contained one or two eyes for a known period was stored at 0°C for no longer than 72 h before application to a 50 cm x 1 cm diameter Bio-Gel P-2 (or P-10), 400 mesh gel filtration column (Bio-Rad, Richmond, Calif.) maintained at 4°C. The column buffers used were 2 mM Tris Cl with 0.1 mM DTT; 5 mM Tris Cl, 100 mM NaCl, with 0.1 mM DTT; and most recently, 5 mM Tris Cl, 50 mM NaCl, with 1.0 mM DTT. All were adjusted to pH 8 at 4°C. The buffer reservoir was maintained 30 cm above the column top producing a flow rate of 4-5 ml/cm^{2}/h. Two micro fractionators (Gilson Medical Electronics, Middleton, Wisconsin) were used. Each collected 80 fractions of 0.6 ml each. The exact volume differed by 4% for the two collectors.

Part or all of each resulting fraction was then added to 5 ml of Aquasol (New England Nuclear, Boston, Massachusetts) and counted for 20 min on the liquid scintillation counter. All data have been corrected for background and crossover.

The gel filtration columns were calibrated with substances which were detected in the fractions: 1) by eye for blue dextran 2000 (Pharmacia), cytochrome c, or phenol red (Matheson, Coleman & Bell); 2) by radioactivity for $^{3}H_2O$, ^{35}S-Met (both New England Nuclear), ^{3}H-Val (Schwartz-Mann), or ^{125}I-bungarotoxin (obtained from Professor Michael Raftery); 3) by UV light absorption measured with a Beckman DB spectrophotometer for myoglobin, bacitracin (Calbiochem), oxidized gluthathione, reduced glutathione, dopamine, tyrosine, or serotonin; 4) by the ninhydrin reaction for Arg, Asp, Glu, Leu, Lys, Phe, Pro, or Trp. Substances not otherwise indicated were obtained from Sigma.

Circadian Rhythm Experiments

The general procedure for the CR experiments reported here was as follows. Two pairs of eyes were dissected between CT 0000 and CT 0400 and placed in darkness between CT 0100 and CT 0430. Electrical recording was then begun immediately from the optic nerve of each eye. Each pair was then labeled, while recording was in process, for 11 or 12 h starting at CT 0400 or CT 0500. After labeling, the light-tight box was opened under dim red light, and each eye was placed in a separate beaker. The box was again closed and the eyes were rinsed with 1.0 ml of perfusate solution 4 or 5 times over a period of 2-4 h. At CT 1900, the first 0.5 ml of perfusate for analysis was added to each eye and subsequently changed every 3 h. Perfusate collections were continued only for the eye with the most normal CAP pattern of activity (Table I) during the prelabel, label and, most importantly, postlabel period.

The perfusate collections from a given eye were run on P-2 gel filtration columns for CR analysis. A given gel filtration column was only used twice. The gel beads were then recycled after being washed in distilled water. This helped maintain a constant percent recovery of applied radioactivity of about 80% for ^{3}H.

The position of materials coming off these columns was quite reproducible. For either fraction collector, each peak corresponding to the void and to $^{3}H_2O$ always fell into one of two fractions. To prevent the small difference in the collectors from obscuring a CR, one collector was used for all the 3 h perfusates of a given 24 h period.

Polyacrylamide Gel Electrophoresis

Either the 0.5 ml of perfusate (GTPS-FSW) which had contained the eye for a 3 h period or parts of the gel filtration fractions of such were centrifuged at 12,000 g (4°C) for 10 min, and the supernatant was made up to 10% trichloroacetic acid (TCA) and 2 μM bovine serum albumin (BSA) resulting in a 50% increase in volume. This was precipitated at 0° for 4 h and then centrifuged. The pellet was washed by resuspension in 1 ml of 1:1 ether:ethanol and centrifuged again to remove the TCA. This was repeated three times followed by three 1 ml washes of ether. The final pellet was dried and redissolved in a 70 μl solution of 0.015 M sodium phosphate buffer, pH 7.2, containing 10% glycerol, 0.2% sodium dodecyl sulfate (SDS), 0.0015% bromophenol blue, and 2% 2-mercaptoethanol. This was heated for 30 min at 60°C and 20-40 μl was applied to the top of the gel.

The 5% acrylamide gel and the procedure for running, slicing, and counting it were essentially identical to that of Rothman and Strumwasser[35]. The major difference was that the gel was fixed in 12-1/2% TCA for 30 min instead of 2 days to allow visualization of the BSA marker as well as the bromophenol blue

marker. Their calibration, based on the mobility of four protein standards relative to the tracking dye, bromophenol blue, was used. Between a m.w. of 70 k and 10 k, this system exhibits a linear relationship between the log m.w. of the standard proteins and their mobility relative to the bromophenol blue tracking dye[35].

RESULTS

Circadian rhythm experiments were carried out by analyzing on P-2 gel filtration columns, the radioactive substances which were spontaneously released from eyes maintained in constant darkness. These substances were also examined on P-10 columns and SDS gels.

Circadian Rhythm

In the CR experiments, the eye perfusate was changed every 3 h. It was then fractionated on a P-2 column, producing a profile of radioactive counts. These profiles have been examined in two ways. First, the total counts recovered in any given P-2 region have been followed over time. Secondly, the counts for a given P-2 region have been expressed as a percentage of the total counts recovered from the column and this percentage followed over time. This latter method not only compensates for the decline in released radioactivity with time, but also compensates for small variations in the volume of perfusate recovered and applied to each column.

The most successful CR experiment as judged by the close to normal CR pattern of CAPs for the first and to a lesser extent the second day (post label) of 3 h collections (exp. No. 1 in Table I) is shown in Figs. 1 and 2. The eye was labeled with ^{3}H-Pro and each subsequent 3 h perfusate collection was run on a P-2 gel filtration column. Examples of the total recovered radioactivity from the P-2

columns are seen in Fig. 1 which superimposes patterns from three consecutive periods, each 12 h apart.

When the recovered counts from four different regions of these P-2 columns, regions I (m.w. $\geq$ 2000), II (m.w. $\curvearrowleft$ 1000), the proline region (region III), and the $^{3}H_{2}O$ region (region IV), are examined over time, a CR of release is apparent for substances in the first 3 regions (I, II, III). In general, more counts are released during the projected day than during the projected night in all fractions appearing with or earlier than the label (region III). The counts in the $^{3}H_{2}O$ region (region IV) may move in antiphase to the former regions (Fig. 1). Metabolism of the label presumably produces $^{3}H_{2}O$ which then is free to diffuse into the perfusate.

When these data are converted to percentages of the total recovered counts (Fig. 2), labeled substances in regions I and II exhibit a CR in phase with each other and just about in phase with the CAP CR for both cycles. For both days, the peak percentages of radioactivity occurs for the 3 h period starting at CT 0400, while the peak CAP frequency occurs for the 1 h period starting at CT 0300. Also, the percentage of radioactivity starts increasing for both days during the 3 h period starting at CT 1900 while the CAP frequency starts increasing for the hour starting at CT 2000.

The Pearson coefficient of correlation, r, is another way of expressing this relationship between the percentage of counts in region I or II and the corresponding number of CAPs. In the first cycle for region I, $r = 0.83$ ($p < 0.02$) and for region II, $r = 0.93$ ($p < 0.001$). In the second cycle for region I, $r = 0.66$ ($p < 0.10$) and for region II, $r = 0.78$ ($p < 0.05$).

These results suggest a possible correlation between the release and the number of spontaneous action potentials in the optic nerve. But, the amplitude of

the eye CAPs is not constant and, in fact, varies with a CR[17,19]. So, a better approximation to the relative number of action potentials is achieved by weighting the number of CAPs by their average amplitude. CAPs of larger amplitude, presumably due to more action potentials, are given proportionally more weight. The above correlations are higher when the number of CAPs is replaced by this amplitude-weighted number of CAPs. In the first cycle for region I, $r = 0.85$ ($p < 0.01$) and for region II, $r = 0.91$ ($p < 0.01$). In the second cycle for region I, $r = 0.79$ ($p < 0.05$) and for region II, $r = 0.83$ ($p < 0.02$).

A second CR experiment gave results supporting the first. A single eye was labeled using a similar protocol but with a combination of ^{14}C-leucine and ^{3}H-proline. The number of CAPs produced by this eye was 25% of the control value (exp. No. 2, Table I). Nevertheless, the percentage of the total recovered ^{3}H counts in regions I and II of the P-2 fractions showed a weak CR with the peaks 20-50% above the troughs. During the first cycle, the peak percentage of 3 h radioactive release for region I and II followed the peak number of CAPs by 6 h. This lag might have been due to slow label uptake and processing, but other possibilities are not ruled out. During the second cycle, the CR of the labeled, released substances appeared in phase with the CR of CAPs (unweighted). For region I, $r = 0.95$ ($p < .01$) and for region II, $r = 0.83$ ($p < .05$). The absolute number of recovered ^{14}C counts in region II, which was small, also showed a weak CR. This appeared in phase with the CAP CR during a 24 h period overlapping the first and second cycle, $r = 0.81$ ($p < 0.10$).

Condition of Eye

Eyes that were labeled did not show normal CAP activity patterns in most experiments. Prior to the two experiments reported, more than 20 CR release

experiments were attempted. The nonexistent or poor CAP and radioactive release CRs that occurred in these experiments appeared to be due to nonradioactive contaminants or incorrect levels of HCl in the radioactive amino acids obtained from Schwartz-Mann. These problems were less severe with New England Nuclear labels.

It was necessary to evaluate the physiological condition of individual eyes by quantifying the CAP cycle as well as the release of labeled products. A set of measures was developed for evaluating CAP records. Measurements were made on each of the first and second cycle post label: total number of CAPs, time of onset, time of peak, and duration of active part of the cycle (Table I). See Rothman and Strumwasser[35] for other control data on eyes in PS-FSW and Jacklet[15,19] for control records of eyes in culture medium.

Incorporation of label and the release of products was evaluated from the ^{3}H counts released from individual eyes labeled with ^{3}H-Pro. These include the percentage of total recovered counts for substances in regions I and II. Eyes with more normal patterns of CAP activity, as judged by phase, amplitude and duration, had a higher percentage of recovered counts for substances in these two regions as well as a generally larger value for the ratio of region II to I counts. For the eyes in Table I, these two criteria were best met by the eye in exp. No. 1 (Figs. 1 and 2), followed by exp. No. 2, and then exp. No. 3 (Fig. 3).

Normal Release

Fig. 3 shows the Bio-gel P-2 column fractionation of a 3 h perfusate from a single eye starting at CT 2200. During this period, the ^{3}H-Pro and ^{14}C-Leu labeled eye achieved its maximum CAP frequency (exp. No. 3, Table I). It will be noted that the labeled substances in the exclusion volume, region I (m.w. $\geq$ 2000),

incorporate more ^{3}H counts than ^{14}C counts while the reverse is true for region II (m.w. $\curvearrowleft$ 1000). It is also apparent that region II contains more than one substance.

When another pair of eyes is labeled with both ^{3}H-Pro and ^{35}S-Met, it is again apparent that region II contains more than one substance. In this case, the ^{35}S-Met strongly labels a substance that appears 3 or 4 fractions later than the peak ^{14}C-Leu labeled substance (Fig. 1). Part of the same perfusate run on a P-10 column shows that about 10% of the ^{3}H counts excluded on the P-2 column is also excluded on the P-10 (m.w. $\geq$ 20,000). The other 90% is distributed in at least two major peaks suggesting a m.w. for them of between 20,000 and 6,000.

High Potassium or Low Calcium Release

In a high potassium experiment on a ^{3}H-Leu labeled eye, the counts in region I were increased by 1.6, in region II by 2.4, in the leucine region (III) by 4.2, and in the $^{3}H_2O$ region (IV), they were unchanged. In a low calcium, high magnesium experiment on a ^{3}H-Pro, ^{14}C-Leu labeled eye, the counts in most regions were reduced to 0.1-0.3 of the control periods. The major exception was the ^{3}H (not ^{14}C) counts in region I which were only reduced by 1/2. Since the CAP frequency and amplitude during the low calcium period were about 1/4 and 1/3, respectively, of the preceding and subsequent control period, this experiment does not prove that the release is Ca^{+2} dependent, but again suggests a relationship between CAP activity and release.

SDS Gels of Released Substances

Released substances were precipitated with 10% TCA and then run on SDS gels. With leucine labeled substances, over 90% of the counts in region I was precipitable and 30-50% in region II. For leucine or proline labeled eyes, the most prominant peak of gel radioactivity usually occurred at a position suggesting a m.w.

of 40-50 k. Other regions frequently containing counts corresponded to m.w. of 100-120 k, 17-20 k, and 10-12 k.

In some cases, released substances were first separated on P-2 columns before being TCA precipitated and applied to SDS gels. Region II included substances in the 6-7 k region as well as smaller substances. Since the gel system is not entirely linear below 10 k, this 6-7 k figure is tentative.

DISCUSSION

The analysis of perfusate from radioactively labeled Aplysia eyes reported here demonstrates that a number of presumed peptides are cyclically released during the continuous darkness of the experiments. Moreover, the presumed peptides are released with a circadian rhythm (CR) which is in phase with the CAP CR as measured electrically from the optic nerve (Fig. 2).

Peptides

Several points argue that the released material in region I and II of the P-2 effluent includes peptides. First, the material is labeled with at least three different radioactive amino acids, proline, leucine and methionine. Second, the material comes off the P-2 column before any of the 10 amino acid markers tested (Figs. 1 and 3) and hence must be larger than a single amino acid. Finally, much of this material is TCA precipitable (this paper and [13]).

SDS gels of radiolabeled eyes[24,30,35] suggest that larger proteins may be broken down in the eye into smaller peptides, possibly with a CR. Some of these may correspond to the released peptides described here.

Several points favor the argument that this release also occurs in vivo. First, the eye for the CR experiment No. 1 had a normal pattern of CAP activity

(Table I) with a CR of CAPs that continued for 2 days beyond the 3 reported here. This maintenance of a complex and sensitive system suggests that the eye was relatively normal. Secondly, eyes with more normal CAP activity patterns released more labeled substances in region I and, particularly II; again suggesting this is a property of normal eyes.

Neurosecretory Cells

We presume that at least some of these released substances are being secreted from granules in the processes of intact cells in a normal calcium dependent manner[8]. The calcium dependence is not demonstrated here since the low calcium, high magnesium solution also inhibited the CAPs. So, some of this release may be simply dependent on the neuronal activity of the CAP producing cells. Such a dependence has been demonstrated for glutamate, glutamine, and asparate release occurring from isolated bull frog spinal roots[45]. This release was increased several fold by electrical stimulation of the root, but not by a high potassium solution. The stimulated release was also not blocked by a low calcium solution. For the Aplysia eye, an experiment employing a high potassium solution versus a high potassium, low calcium, high magnesium solution might help distinguish this process from the normal calcium dependent release process.

The cell type(s) in the eye releasing these presumed peptides is presently unknown. But, evidence suggests that neurosecretory cells, or at least neurons in the eye, are responsible for this peptide release. First, using morphological criteria[23,34], there may be at least two types of peptidergic neurosecretory cells in the eye, as previously discussed. The first type contains dense core vesicles of 100 nM diameter and the second type contains 150 nM granules, although the cell bodies of the latter are probably not in the retina[40]. Secondly, the cell bodies of

the first type of neurosecretory cell incorporate radioactive leucine into substances preserved by formalin much more rapidly than do any other kind of cell in the eye[40]. Finally, a high potassium solution causes increased release of substances in region I and II of the gel filtration column (this study) as well as the 7,000 m.w. peptide[13].

A simple hypothesis is that neurosecretory cells are responsible for not only the peptide release, but also the CAPs, that is, neurosecretory cells release at least some of the presumed peptides described here by generating CAPs which can be recorded from the branches of their axons in the optic nerve. The following data support this position: first, electrophysiological, pharmacological and optic nerve backfilling experiments[5,11,14,19,21] indicate that a CAP is produced by the synchronous firing of a population of non-receptor "secondary" cells which are electrically coupled to one another and send axons down the optic nerve. The synchronous nature and bursting pattern of CAPs is similar to that of known peptide neurosecretory cells in Aplysia, the bag cells[26]. A bursting pattern also occurs in mammalian neural endocrine systems[27]. Secondly, the first type of neurosecretory cells have gap junctions between them[33,40,41] and send axons down the optic nerve[32,33]. These gap junctions could mediate the synchronous firing of these secondary cells. The second type of neurosecretory cell also has axons in the optic nerve[33,40]. Finally, this paper demonstrates a spontaneous CR of release which is in phase with the CAP CR (Fig. 2). This is consistent with neurosecretory cells directly producing the CAPs while simultaneously releasing the presumed peptides.

It is relevant to note that Luborsky-Moore and Jacklet[33] have histochemically shown that the dense cores of vesicles in some cell bodies of the eye bind

chromium; these cells were interpreted to be the first type of neurosecretory cells with the chromium binding to dopamine. A potential conflict with this conclusion is the finding of Corrent, McAdoo, and Eskin (unpublished) that there is 10 times more serotonin in the eye than dopamine. If these neurosecretory vesicles contain dopamine, it does not exclude the possibility that they also contain biologically active peptides.

Histological evidence does present an alternative to CAP production by neurosecretory cells. Another secondary cell type, the lower retinal neuron, sends axons into the beginning of the optic nerve and has gap junctions with both cells of its own type and with photoreceptors[40,41]. Thus, it could play a role in producing the CAP, perhaps in response to light. These cells, though, do not have the histological properties of neurosecretory cells, so attributing CAP production to them does not as readily explain the correlated CR of presumed peptide release or the burst pattern of the synchronous CAP activity.

It has been hypothesized that "secondary cells" produce the CAPs and contain the CR oscillator[18,23]. Our work has shown that the eye releases substances with a CR. If a CR of release is to be considered the major function of the CR oscillator, then the simplest, most direct place for it to reside is in the secondary cells producing this release, that is, as suggested here, the neurosecretory cells. Available evidence is consistent with this hypothesis, but does not rule out other possibilities[11].

Function

The work reported here supports the hypothesis that the CR of CAP activity functions to release neurosecretory substances, that is, peptides and/or amines, which normally synchronize, entrain and/or drive the CRs of different

parts of the animal. Since information about light intensity is required, such a central CR oscillator at least in its primitive form, might be expected to be closely associated with photoreceptors.

One function of the eyes' CR of neurosecretion may relate to the eyes' ability in vitro to drive or entrain the CR of R15 in a detached abdominal ganglion, as has already been mentioned. A second function may involve the control of the CR of locomotion which is largely absent in blinded animals[29,42]. The secretion of the eye neurosecretory cells postulated to control these behavioral CRs may occur from release sites in the eye and/or in the cerebral ganglion[31,32,33,40]. The released products may then act via the general blood circulation and/or more locally, including synaptically. These questions are approachable by the cutting of nerves or the removal of ganglia from animals. The effects of eye extracts or gel filtration fractions on locomotor activity can also be evaluated in blinded animals.

Work on other systems suggests that a CR of neurosecreted substances from structures evolutionarily associated with photoreceptors may be a general mechanism by which an animal's diverse CRs are kept synchronized, entrained, or driven[3,4,43,44,46]. The widespread use of such regulation would be consistent with evidence that neurosecretory cells are evolutionarily close to the primitive nervous system[28,37].

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TABLE I. Quantification of CAP cycle and released radioactivity

Exp. No.[a]	Σ CAPs[b] Cycle I Cycle II	$\frac{\text{Cycle II}}{\text{Cycle I}} \times 100$ (%)	Onset[c] Cycle I Cycle II (CT)	Peak[d] Cycle I Cycle II (CT)	Duration[e] Cycle I Cycle II (h)
Controls	2038 ± 314		18.5 ± .5	00.6 ± .7	10.8 ± .5
n = 10	1460 ± 386	68 ± 8	17.5 ± 1.0	23.3 ± .9	10.8 ± 1.0
1	1989		17	03	8
	1294	65	17	03	12.7
2	426		17	00	9.7
	180	42	18	08	9.3
3	1592		-	00	-
	1064	67	02	11	12

[a]The first paired row gives the mean values and standard deviations for 10 unlabeled control eyes from seven animals maintained continuously in FSW. The next two paired rows are for the single eyes for which a CR of released substances is reported. The last paired row is for the eye upon which Fig. 3 is based.

[b]The total number of CAPs are given for the first and second cycle post label followed by the percentage the second cycle is of the first.

[c]Circadian time (CT) when the CAP frequency started to increase for the following hour.

[d]CT when the CAP frequency was highest for the following hour.

[e]Length of period from when the CAP frequency had accomplished 50% of its increase to when the CAP frequency had accomplished 50% of its decrease.

Fig. 1. Sequential gel filtration profiles of collections taken 12 h apart in a CR experiment of released radioactivity. The single eye was labeled in the dark with ^{3}H-Pro, and 3-h perfusate collections (GTPS-FSW-D) were started at CT 1900. The CAP activity of the eye was normal (Table I; Fig. 2). The 0.5 ml of perfusate from each 3-h collection was subsequently applied to a Bio-Gel P-2 gel filtration column.

A. The 3-h period starting at CT 0400 of Day I of collections.

B. The 3-h period starting at CT 1600 at the end of Day I.

C. The 3-h period starting at CT 0400 of Day II of collections.

For Fig. 2, the region I (void) was considered to be five fractions (#29-33 in A,B; #30-34 in C). Region II was considered to be five fractions (#41-45 in A,B; #40-44 in C; K_{av} = 0.21-0.33; minimum m.w. 800-500). Region III (Pro) was six fractions (#50-55 in A,C: #51-56 in B; K_{av} = 0.43-0.57). And region IV ($^{3}H_2O$) ten fractions (#66-75 in A,B,C). The markers (not run concurrently) are blue dextran for the void (●), bacitracin (m.w. ∿ 1400; ■), oxidized glutathione (m.w. ∿ 610; ▼), reduced glutathione (m.w. ∿ 310; ▲), Pro or Leu (O), and $^{3}H_2O$ (□).

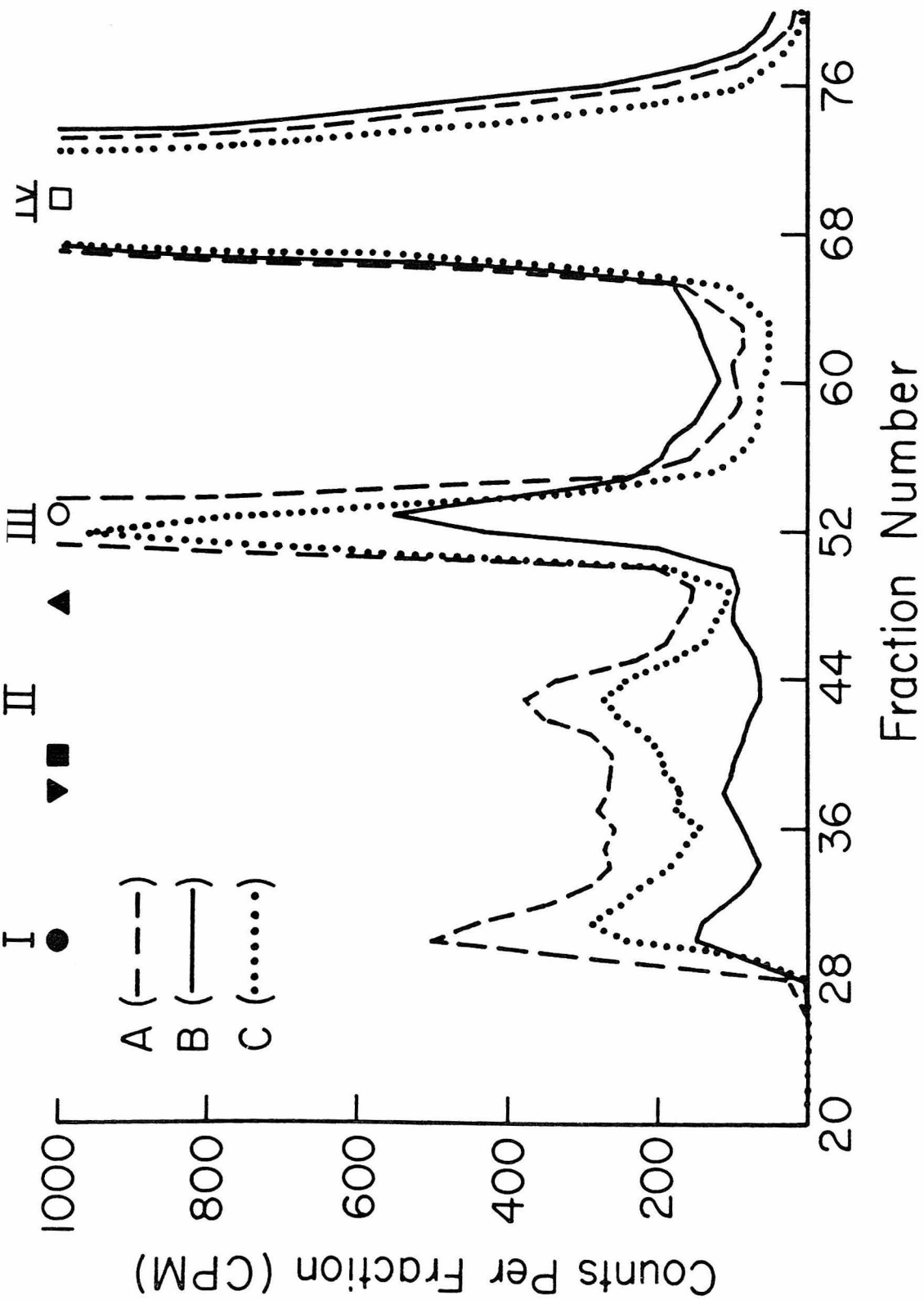

Counts Per Fraction (CPM)
1000
800
600
400
200
20
28
36
44
52
60
68
76
Fraction Number
I
II
III
IV
A (— — —)
B (——)
C (·····)

Fig. 2. Percent of the total recovered counts in region I (- - -) and II (——) of the gel filtration columns (Fig. 1) over a 2-day period. Each consecutive 3-h perfusate from a single eye in constant darkness was applied to a column. The percents are graphed at the mean circadian time of each 3-h perfusate collection. The number of CAPs for each hour are plotted (o——o) at their mean circadian time. The projected dark periods are indicated by the horizontal dark bars.

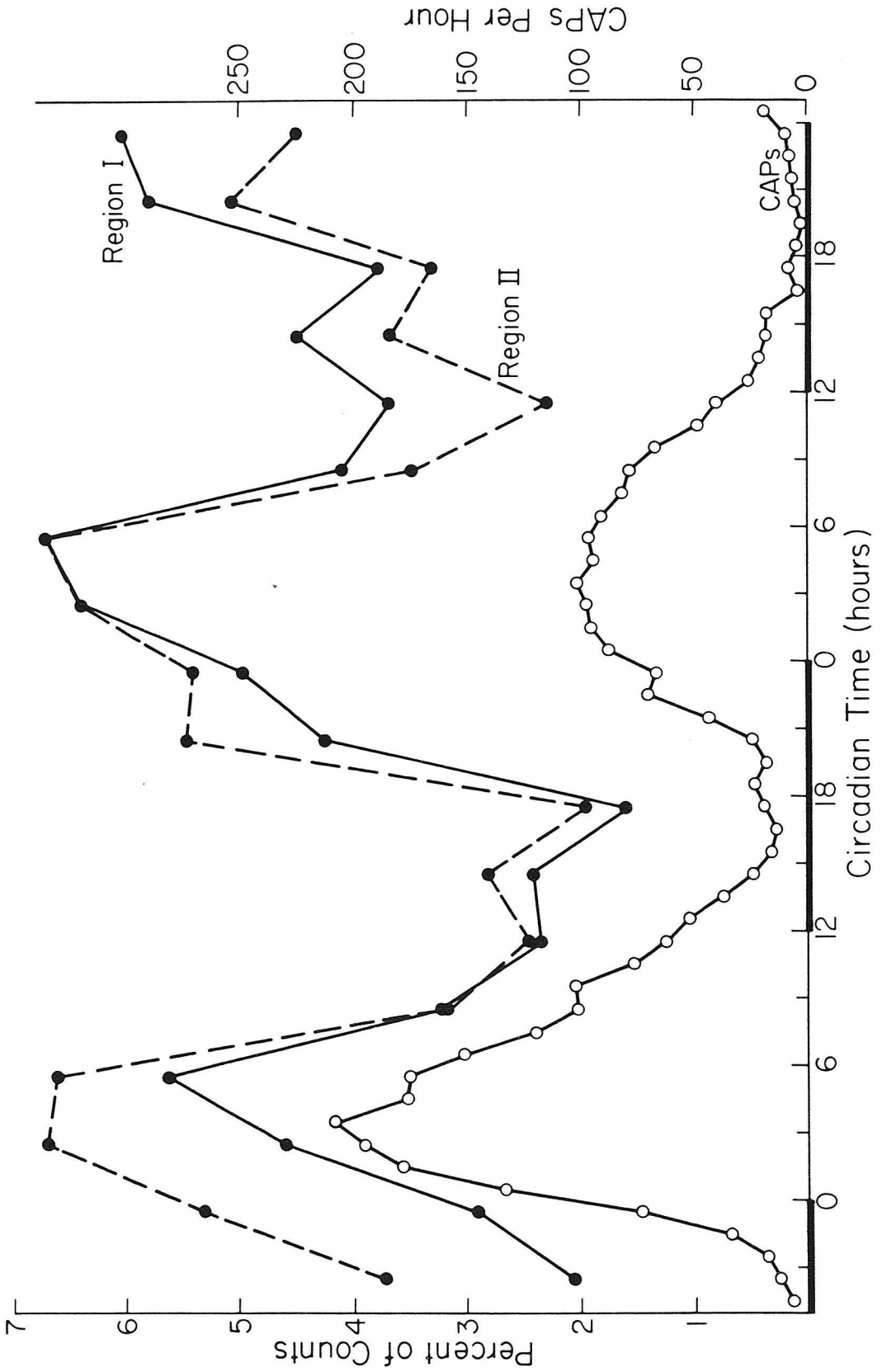
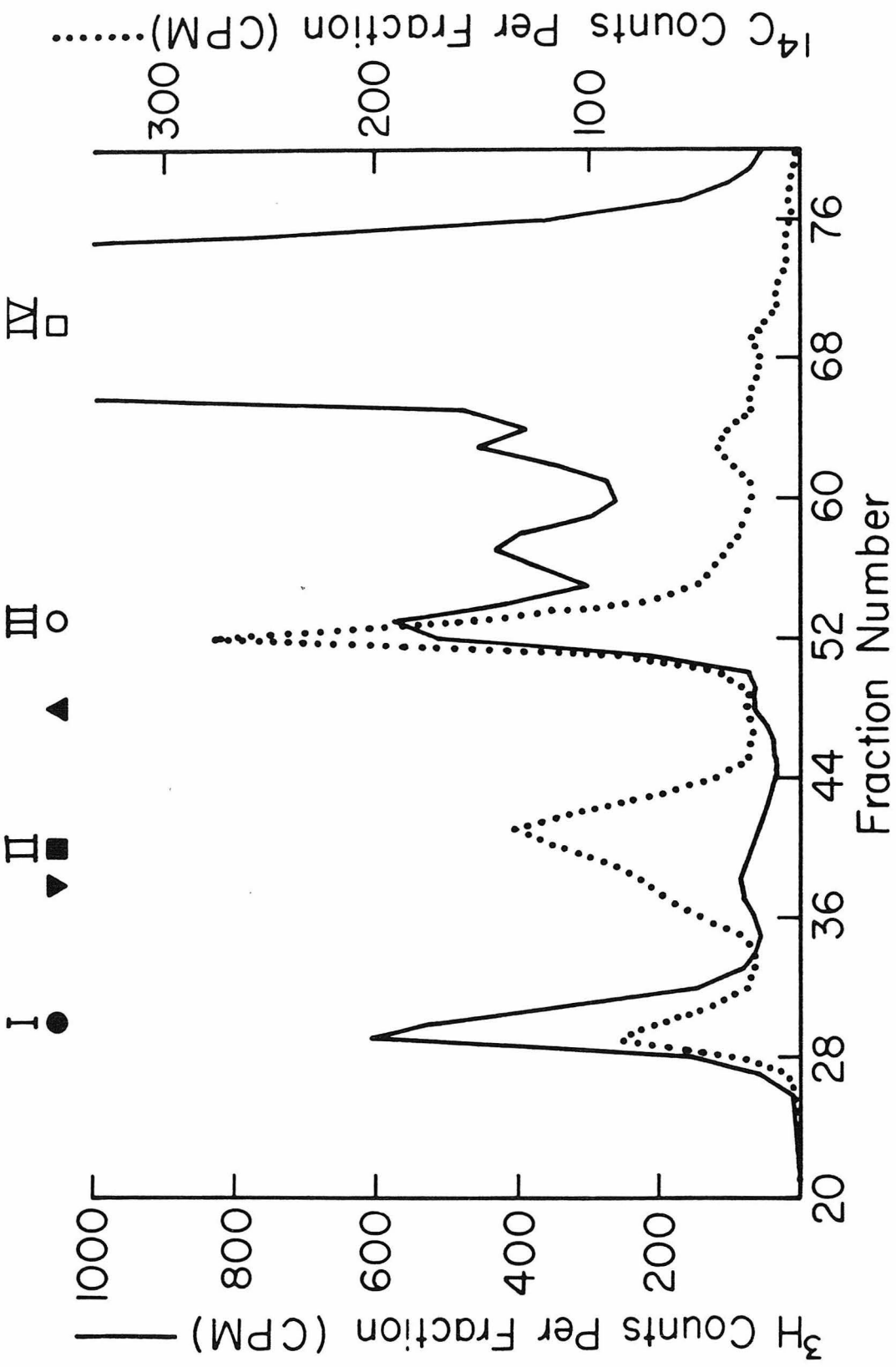

Region I
Region II
CAPs
CAPs Per Hour
250
200
150
100
50
0
Percent of Counts
7
6
5
4
3
2
1
0
6
12
18
0
6
12
18
Circadian Time (hours)

Fig. 3. A P-2 gel filtration profile of radioactivity released into the perfusate of a single eye labeled with ^{3}H-Pro and ^{14}C-Leu. The voided region I, fractions #28-32, should contain substances with a m.w. larger than 2000. The region II, fractions #34-45, K_{av} = 0.13-0.34, minimum m.w. 1300-400, appears to contain at least two peaks, region III, fractions #51-55, corresponds to the radioactive label, and region IV, fractions #66-75, corresponds to $^{3}H_{2}O$. Recovery for ^{3}H counts applied to the column was 80% and for ^{14}C counts, 34%. The 3-h perfusate (GTPS-FSW) was started at CT 1900 of the first day post label. The eye CAPs peaked during this period, which is normal, although the peak was phase shifted the next day (Table I).


^{3}H Counts Per Fraction (CPM) ——
^{14}C Counts Per Fraction (CPM).....
1000
800
600
400
200
300
200
100
20
28
36
44
52
60
68
76
Fraction Number
I
II
III
IV

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