

Biosynthesis of the Egg-Laying Hormone (ELH) in the Bag Cell Neurons of *Aplysia californica*

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ABSTRACT Biosynthesis of the egg-laying hormone in the bag cell neurons of *Aplysia californica* was studied. Bag cells were incubated with leucine-³H in vitro for 30 min and rinsed for variable periods of time in a chase medium. The distribution of incorporated label among proteins within the cells was assayed by electrophoresis of an homogenate on sodium dodecyl sulfate polyacrylamide gels. Results from rinse times shorter than 30 min revealed that the predominant synthetic product is a 25,000 dalton protein. With longer rinse times, this species was reduced and two species of lower molecular weight became prominent. This redistribution of radioactivity was quantitative and was not prevented by inhibition of protein synthesis during the rinse. A 10°C reduction in temperature (from 15°C) blocked the redistribution. These data are interpreted to indicate that the 25,000 dalton molecule is a precursor which is cleaved enzymatically to yield two lower molecular weight products. One product is a 12,000 dalton molecule which remains in the cell bodies. The other is a molecule of <10,000 daltons which is exported from the somata into the neurohemal regions of the connective tissue. Perfusion of these regions with high [K⁺] medium results in the release of this product into the medium. It is concluded that this product is the 6000 dalton egg-laying hormone (ELH).

INTRODUCTION

The bag cell neurons of the parietovisceral ganglion (PVG) in *Aplysia californica* provide a particularly favorable preparation for neurochemical study. Although relatively small cells (50 μm in diameter), they are grouped into two apparently homogeneous clusters of about 400 cells each (Coggeshall, 1967). These clusters are situated at the junctions between the two pleurovisceral connective nerves and the PVG proper. Thus both clusters can be dissected from the ganglion easily for biochemical study and for ensuring isolation from electrophysiological inputs from other cells in the ganglion.

A functional role for the bag cells was suggested by the finding that a crude

seawater extract of the clusters would induce egg laying when injected into another animal (Kupfermann, 1967). Subsequent study confirmed this observation and demonstrated that within the nervous system of *A. californica* only the bag cells and the connective tissue sheath surrounding the PVG contained egg-laying activity (Strumwasser et al., 1969). Electrophoretic studies of the water-soluble proteins in various nervous structures (Toevs and Brackenbury, 1969) revealed a unique protein with a distribution matching that of egg-laying activity. This protein, upon separation, was shown to have egg-laying activity and was deduced, from gel filtration studies, to have a molecular weight of approximately 6000 daltons (Toevs, 1970).

The final steps in establishing the neuroendocrine function of the bag cells were accomplished by Kupfermann (1970) and Arch (1972). Kupfermann (1970) showed that appropriate electrical stimulation of the connective nerves of a PVG *in vitro* induced the release of egg-laying activity into a bathing medium. A similar result was found when PVG's were perfused with a medium containing 11 times the normal potassium concentration (Arch, 1972). Moreover, electrophoresis of perfusate samples revealed the appearance of a single polypeptide peak of appropriate molecular weight whenever the PVG was depolarized by high-potassium medium. This depolarization-induced release was found to occur within the connective tissue sheath of the ganglion, to be a product of the bag cell clusters, and to be dependent on the presence of calcium in the bathing medium.

These investigations have indicated that the bag cells and surrounding connective tissue constitute a neuroendocrine organ responsible for the regulation of egg laying. Because of its morphology, the ease with which it can be isolated, and its hardiness *in vitro* the bag cell neuroendocrine organ (BCO) has many advantages as a model for study of the coupling between the electrophysiology and biochemistry of secretory neurons. In order for such study to proceed, however, information about hormone biosynthesis is necessary. The studies described in this paper were designed to provide this information.

METHODS

Adult *A. californica* weighing 200–500 g each were obtained from Pacific Biomarine Supply Co. (Venice, Calif). Animals were brought into the laboratory every 2–4 wk and kept, unfed, in large tanks containing filtered and aerated seawater at 14°C. The light cycle in the animal room was light:dark 12 hr:12 hr. The experiments reported in this paper were conducted over a 12 month period. No correlation between the phenomena reported and either time of year or reproductive state of the animals was noted.

Dissection and Preincubation

Preliminary dissection was performed as described by Strumwasser et al. (1969). The PVG was dissected carefully with 1 cm lengths of the major nerve trunks left intact.

In some cases the entire PVG was then rinsed in Millipore-filtered (0.22μ ; Millipore Corp., Bedford, Mass.) seawater (15°C) for 4 hr before the beginning of the experiment. In most cases, however, the bag cell organs (BCO) were cut away from the remainder of the PVG before the beginning of this preincubation. This further dissection was performed to ensure against artifacts arising from axonal processes originating at cell bodies in the PVG proper.

The preincubation in filtered seawater was found to be necessary for adequate incorporation of labeled amino acids. The amount of incorporation increased with the length of preincubation up to about 3 hr; after this time no improvement was detected. It seems likely that the improvement in incorporation with the duration of preincubation is the result of gradual depletion of endogenous precursor pools. Nevertheless, patterns of protein synthesis found upon polyacrylamide gel electrophoresis revealed no differences due to the length of preincubation (0–6 hr).

Incubation and Rinse

The entire ganglion or the isolated BCO were incubated in 0.5 ml of a defined medium (A, Table I) for 30 min at 15°C . Incorporation in terms of total trichloroacetic acid (TCA)-precipitable radioactivity was linear over at least the last 20 min of this incubation period.

The incubation period was terminated by transferring the tissue to 2.5 ml of standard rinse medium (B, Table I). The duration of rinse depended upon the experiment. Unless otherwise noted the rinse medium was maintained at 15°C .

Sample Preparation

Tissues were removed from the rinse medium and immersed in a small volume of filtered seawater in a depression slide. The BCO, if not already free, were then cut away from the remainder of the PVG. The bag cell somata were obtained by slitting the connective tissue and squeezing it gently to force out the contents. Most often the cells came out as a clump; however, occasionally they were dispersed by the pressure applied. Thus it was not possible to obtain samples containing consistent numbers of cell somata and quantitative comparisons of incorporation could not be made.

The bag cell somata were next transferred to a small tissue grinder (1 ml volume) containing approximately $10 \mu\text{l}$ of sodium dodecyl sulfate (SDS) sample buffer (Arch, 1972). The cells were hand homogenized with a tight-fitting pestle. The homogenate was transferred to a 1 ml conical centrifuge tube and the grinder was rinsed with another $10 \mu\text{l}$ of sample buffer. After addition of the rinse volume, the homogenate was frozen and held at -20°C until analysis.

Gel Electrophoresis

Details of the preparation, fractionation, and scintillation counting of miniature SDS-polyacrylamide gels (5% acrylamide; 1.4×60 mm) are described by Ward et al. (1970) and Wilson (1971). Samples of bag cell homogenates were prepared for layering on SDS gels by heating to 60°C for 25 min and then centrifuging at 8000 rpm for 10 min (4°C). Over 80% of the TCA-precipitable radioactivity in the crude homoge-

nate could be recovered in the supernatant. 10–15 μ l of the supernatant was layered on the gels and the electrophoresis was performed at constant voltage (20 v) for 4 hr 35 min.

TABLE I
COMPOSITIONS OF MEDIA

Medium	Constituents, mM/liter*									Amino acids
	Na	K	Ca	Mg	Tris	Glucose	SO ₄	Cl	Leucine	
A Incubation	451	10	13	49	10	55	28	539	1.78 \times 10 ⁻³ †	+‡
B Rinse	451	10	13	49	10	11	28	539	1.78	—
C Low-Ca rinse	443	10	1	77	21	11	28	574	—	—
D High-K rinse	382	107	13	28	21	11	28	536	—	—
E High-K, low-Ca rinse	346	107	1	77	21	11	28	574	—	—

* All media were prepared by dissolving the crystalline constituents (except labeled leucine) in glass-distilled water. After preparation each medium was filtered through a 0.22 μ Millipore filter and then appropriate volumes were poured into clean vials which were frozen at -20°C until use. The pH of each medium was adjusted, if necessary, before filtration. The pH values ranged between 7.35 and 7.55.

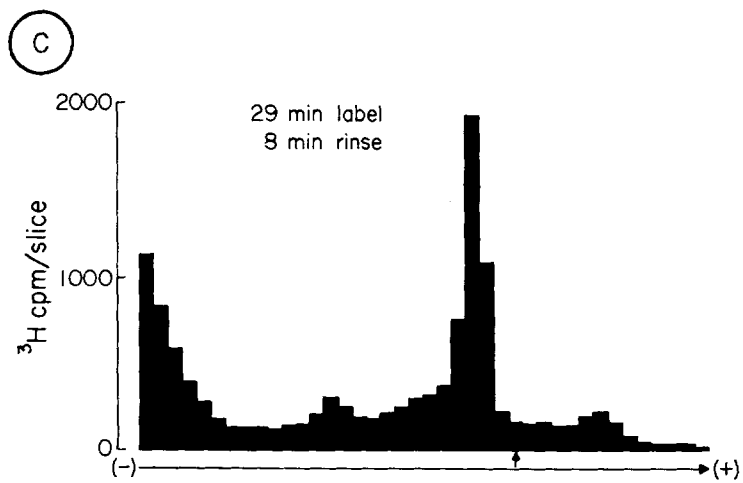
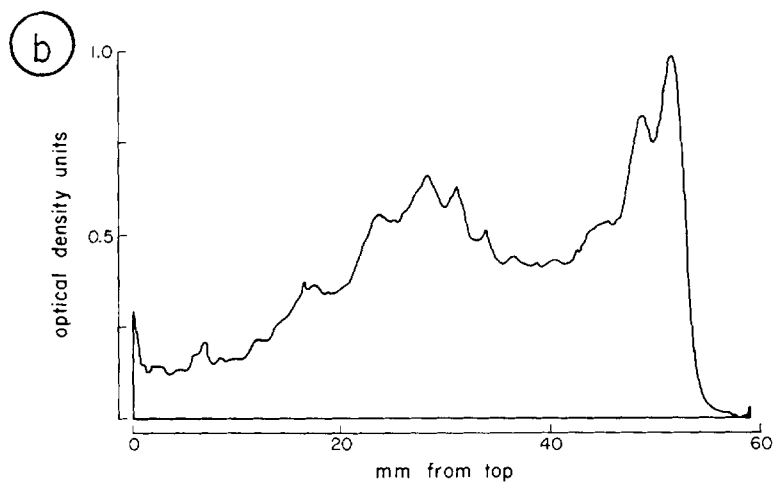
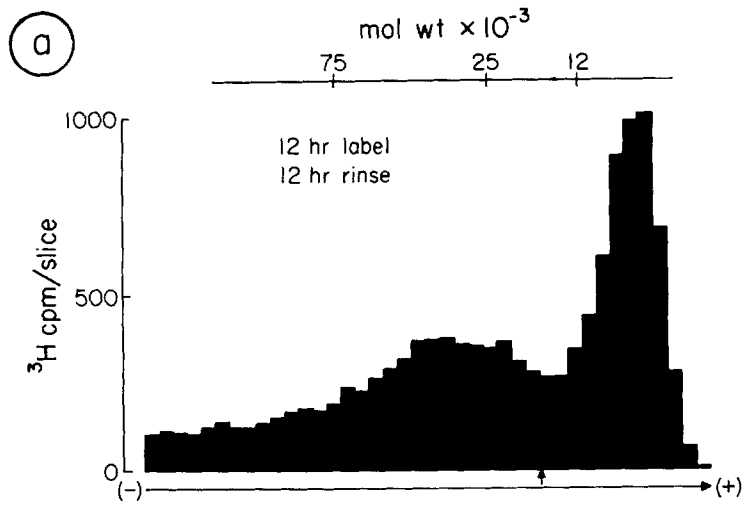
† Calculated leucine concentration of the 50 μ Ci of leucine-³H (L-leucine-4,5-³H, 56 Ci/mM, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y.). Since labeled leucine was in 0.01 N HCl solution, sufficient 0.05 N NaOH was added to neutralize the final solution.

‡ All essential amino acids, except leucine, were present in 1/40, the concentration suggested by Eagle (1959).

RESULTS

Long-Term Labeling

For the initial studies of protein synthesis in the bag cells, ganglia were labeled for 12 hr in 2.5 ml of incubation medium containing 25 μ Ci of leucine-³H. After a 60 min rinse these cells were treated as described above and yielded synthesis profiles on electrophoretic gels like that shown in Fig. 1 *a*. Perhaps the most interesting aspect of this profile is the very large amount of labeled polypeptide material which migrates in the low molecular weight region. From the work of Toevs (1970) it is known that the bag cells contain large amounts of the egg-laying hormone (ELH), and that this hormone has a molecular weight of about 6000 daltons. Thus, although it was anticipated that the bag cells would show an accumulation of polypeptide with a low molecular weight, it was surprising to find that synthesis of this material proceeds at such a high rate (30–50% of the newly labeled polypeptides are found in the <10,000 dalton region of the gels after a 12 hr incubation). A microdensitometric tracing of a gel stained with Coomassie Brilliant Blue is shown in Fig. 1 *b* to illustrate the rather close correspondence between the resident protein constituents of the bag cells and their synthesis profile.



Short-Term Labeling

Short-term incubations (30 min) were undertaken as a means of pulse labeling the bag cells in order to examine both short-lived polypeptide species (if any) and the rate of export of ELH from the somata. The results of these studies were quite unexpected. As can be seen in Fig. 1 *c* there is little material in the low molecular weight region but a significant accumulation of label at about 25,000 daltons. Since the staining patterns on such gels are identical to that shown in Fig. 1 *b* it seemed likely that the 25,000 dalton peak represented either a polypeptide which was rapidly exported or a short-lived intermediate.

To examine these possibilities, the bag cells were exposed to a chase-type rinse medium (B, Table I) for variable lengths of time after incubation. Examples of the results of these rinse periods are shown in Fig. 2. If the 25,000 dalton material were a rapidly exported product, it would be expected to disappear from the gels with the longer rinse times. If, on the other hand, it were an intermediate, the pattern of radioactivity would be expected to exhibit a redistribution with time in rinse. The latter possibility is clearly favored by the evidence in Fig. 2.

A more detailed examination of the apparent conversion of 25,000 dalton material into low molecular weight material is presented in Fig. 3. For the first 20–30 min after the end of incubation there is an obvious preponderance of 25,000 dalton material in the cells. This period is followed by a period of about 30 min during which the low molecular weight material begins to predominate. After 60 min of rinse the distribution of radioactivity remains stable with the low molecular weight species accounting for approximately 30% of the radioactivity and the 25,000 dalton species for about 10%.

This time-dependent shift in the distribution of radioactivity suggests the conversion of the 25,000 dalton species into the low molecular weight species.

FIGURE 1. (*a*) Incorporation profile derived from an homogenate of the bag cells after electrophoresis on an SDS-polyacrylamide gel. The bag cells had been allowed to incorporate leucine-³H for 12 hr after which they were rinsed for 12 hr in medium B (Table I). (*b*) Staining profile derived from a bag cell homogenate after SDS-gel electrophoresis. At the end of electrophoresis the gel was extruded into 12.5% trichloroacetic acid (TCA) (4°C) and left overnight. It was then stained in a solution of 0.25% Coomassie Brilliant Blue in methanol:water:acetic acid (10:10:2) for 4 hr. Destaining was by diffusion in 7.5% acetic acid. (*c*) Incorporation profile from bag cells incubated for 29 min and rinsed for 8 min. Ordinates in (*a*) and (*c*) are in counts per minute corrected for a background of 12 cpm. The efficiency of tritium counting was approximately 40%. The ordinate in (*b*) is calibrated in arbitrary optical density units. The abscissae in all three profiles represent the length of the gels. In (*a*) and (*c*) sequential slices were made at 1.3 mm intervals and each slice was counted separately. Molecular weight calibrations are derived by calculation from the position of the bromphenol blue marker dye (vertical arrows).

However, such a change in the relative peak heights might come about by differential rates of synthesis such that early during rinse there is an excess of 25,000 dalton synthesis while late in the rinse series synthesis of the low molecular weight material is favored. The fact that the rinse medium contains a

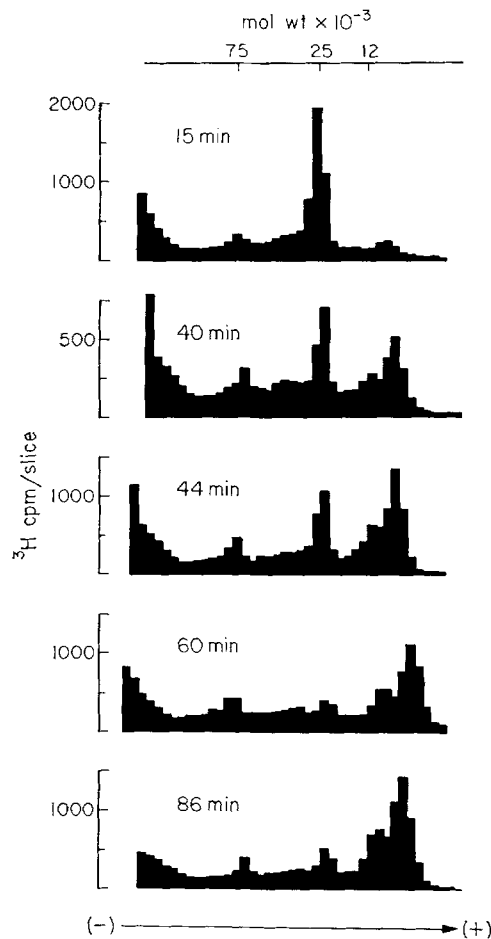


FIGURE 2. Examples of incorporation profiles derived from bag cells incubated for 30 min and rinsed for the times indicated.

large excess of unlabeled leucine militates against the possibility of significant continued incorporation of labeled amino acid into any polypeptide species. Nonetheless, experiments were performed in which the rinse medium contained anisomycin (50 μ g/ml; Pfizer Inc., New York). This antibiotic is a particularly effective inhibitor of protein synthesis in the neurons of *A. californica* (Schwartz et al., 1971) and in these experiments was found to inhibit over 90% of the protein synthesis in the bag cells. As can be seen in Fig. 4 in-

hibition of protein synthesis to this extent had no apparent effect on the conversion process.

Temperature Blockade

Several attempts were made to influence the conversion process. After standard 30-min incubations in label, BCO were placed in rinse media containing various compounds at 1 mM concentrations. Neither Na azide nor KCN had any effect on the conversion. Similarly, both vinblastine and colchicine failed to prevent conversion. However, conversion could be stopped completely for

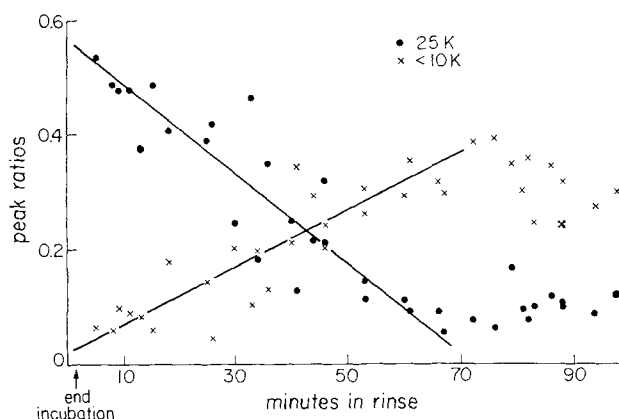


FIGURE 3. Time-course of change in the relative amounts of 25,000 dalton and <10,000 dalton material. Peak ratios were calculated by dividing the total radioactivity contained in three slices centered on the indicated molecular weight region by the total radioactivity contained in the entire gel at molecular weights below 75,000 daltons. Least squares regression lines are plotted for points between 0 and 70 min of rinse. The slopes for 25,000 dalton and <10,000 dalton lines are -0.00784 and 0.00484 , respectively.

up to 100 min by holding the rinse medium at 5°C (Fig. 4). This temperature blockade could be reversed by returning the tissue to 15°C medium.

Quantitation

As is apparent from the slopes in Fig. 3, the total radioactivity contained in the 25,000 dalton region at the outset of the rinse period is not found in the low molecular weight region after conversion. A suggestion of the fate of the remaining labeled material can be seen in the later profiles in Fig. 2. At about the midpoint of conversion a relatively small accumulation of labeled polypeptides begins to appear at 12,000 daltons. That this material, in conjunction with the low molecular weight substance, can account for the original 25,000 dalton material is illustrated quantitatively in Fig. 5. Summation of the peak ratios from the 25K, 12K, and low molecular weight (6K) regions yields an

almost horizontal least-squares regression line, while summed 25K and 6K peaks alone give a clear negatively sloping line. As can be seen in Fig. 4 the accumulation of radioactivity at the 12,000 dalton peak represents about 40% of that at the low molecular weight peak.

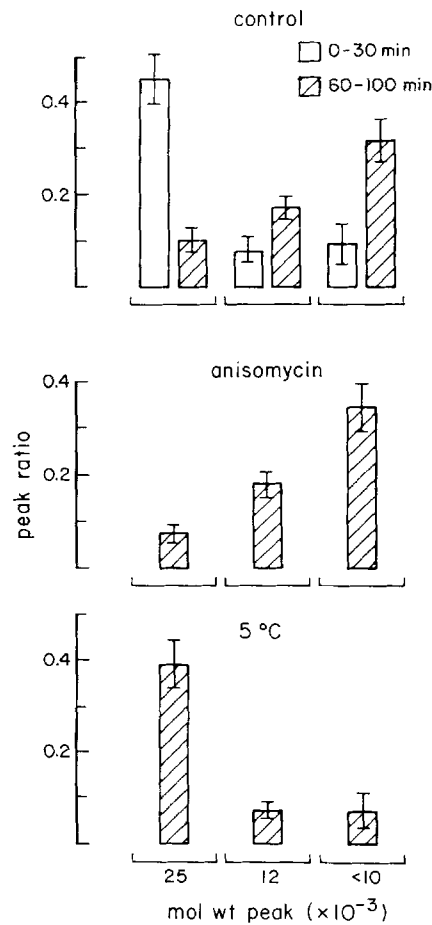


FIGURE 4. *Top*, mean (\pm SD) peak ratios for the indicated molecular weights of the control gels. Open histograms indicate rinse times of less than 30 min (number of samples $N = 10$). Hatched histograms represent times in rinse greater than 60 min ($N = 15$). *Middle*, mean (\pm SD) peak ratios for cells exposed to 50 μ g/ml anisomycin in rinse for greater than 60 min ($N = 5$). *Bottom*, mean (\pm SD) peak ratios for cells rinsed for more than 60 min at 5°C ($N = 6$).

Identity of the Low Molecular Weight Material

Because of the morphology of the bag cell organ it is possible by careful dissection to isolate a segment of the connective tissue containing numerous bag cell axonal processes but lacking bag cell somata. The bag cell somata are tightly packed in a roughly spherical cluster. This cluster is easily distinguish-

able from the surrounding connective tissue sheath by the slight orange cast of the bag cells. Immediately rostral, along the pleurovisceral connective nerve, to each cluster is a region of connective tissue devoid of bag cell bodies but containing many bag cell processes. These processes appear to be coursing rostrally from the cell cluster but many end on hemal spaces in this region of the sheath as well (Strumwasser and Alvarez, unpublished). Thus, by cutting just rostral to the cluster and again about 0.5 mm ahead of the first cut, one can isolate a disc of connective tissue, surrounding a short segment of the connective nerve, which is "enriched" for bag cell processes and terminals but which contains no bag cell bodies. If the low molecular weight product of the breakdown of the 25,000 dalton precursor is ELH, examination of the connective tissue at

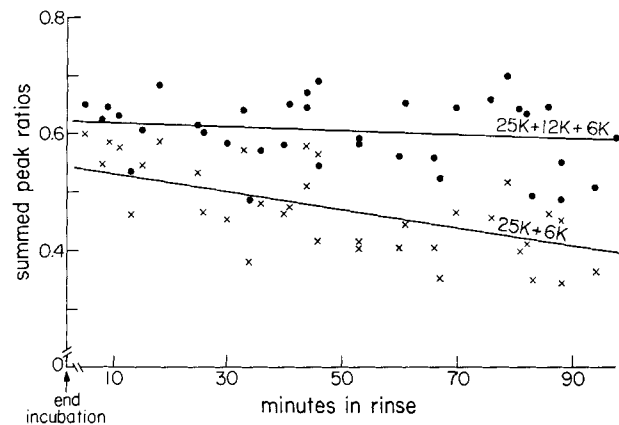


FIGURE 5. Summed peak ratios over time in rinse. Lines are least squares regression fits for the data with or without the inclusion of the relative amount of material contained in the 12,000 dalton region. 6K in this figure refers to the material designated <10,000 daltons elsewhere in the paper.

various times after pulse labeling should reveal a progressive increase in low molecular weight substance as ELH is transported to the axonal terminals where release will occur (Arch, 1972).

Experiments designed to test this hypothesis followed a somewhat different protocol from those previously described. After the usual preincubation period, BCO were incubated for 90–120 min. This increased period of labeling was employed to ensure that any materials being transported down the bag cell axons would have a high specific activity and be easily detectable against the background of connective tissue synthesis. Following incubation the tissues were rinsed under the same conditions as in the earlier studies except that now rinse times were in hours rather than minutes. For the last 2 hr of all rinse periods the tissues were bathed in a low-calcium medium (C, Table I) in order to inhibit release of ELH during dissection. The results of these experi-

ments are illustrated in Figs. 6 and 7. With longer rinse times progressively more labeled low molecular weight polypeptide can be found in the connective tissue. From inspection of the profiles (Fig. 6) this appears to be the only

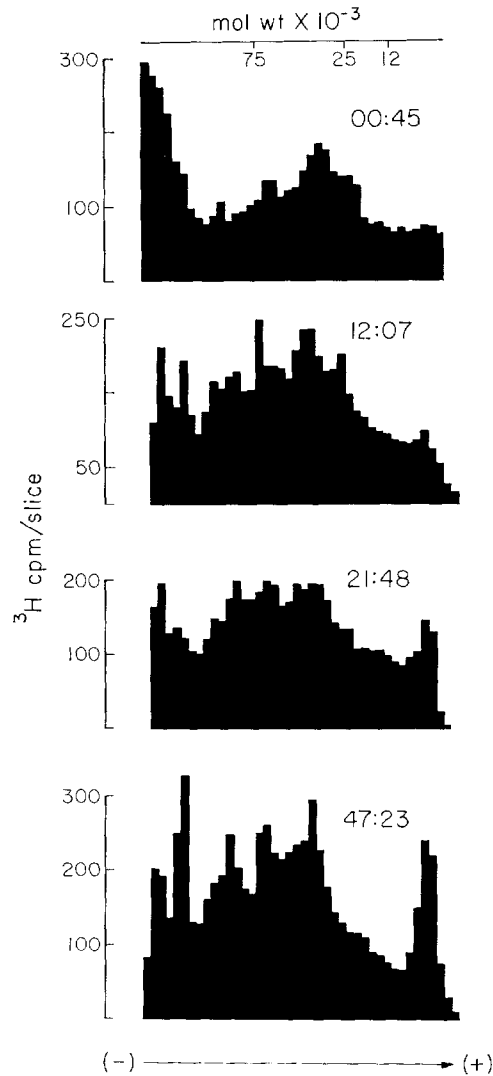


FIGURE 6. Examples of incorporation profiles derived from samples of the connective tissue sheath taken at the indicated times after a 2 hr incubation.

major time-dependent change in the connective tissue. Between 36 and 48 hr the proportion of low molecular weight material reaches 210% of the initial value (Fig. 7). Note that no similar accumulation of labeled material occurs in the 12,000 dalton region.

Neurosecretion

If this material appearing in the connective tissue sheath with time after labeling is ELH, it should be released by perfusing the ganglion with high-potassium medium (Arch, 1972). To examine this hypothesis BCO were treated as above except that they received an additional treatment at the end of rinse. After the 2 hr in low-calcium medium BCO were placed for an additional 2 hr in either high-potassium medium (D, Table I) or in high-potassium, low-calcium medium (E, Table I). It was expected that medium D would evoke

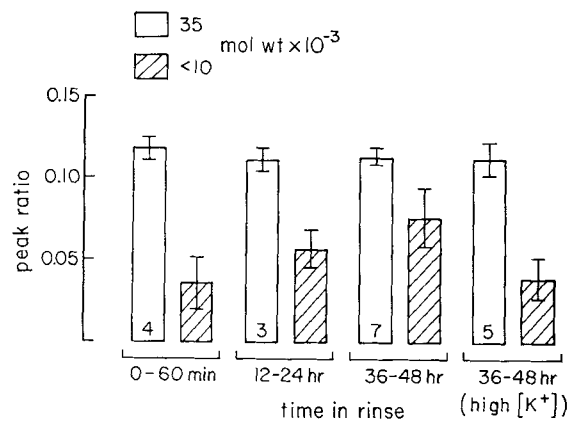


FIGURE 7. Comparison of peak ratios from two different regions of gels derived from connective tissue samples after three different durations of rinse. The 35,000 dalton peak is located near the middle of a typical gel (Fig. 6). It was chosen as a convenient control region against which the change in the proportion of <10,000 dalton material could be compared. The effect on the proportion of <10,000 dalton material remaining in the tissue after a terminal rinse in high [K⁺] medium (D, Table I) is shown on the far right. Peak ratios were calculated as before. The histograms represent mean peak ratio \pm SD. The number in each group is shown at the base of the 35,000 dalton histograms.

the release of ELH from terminals while medium E, although having a similar depolarizing effect, would not permit release because of the low calcium and high magnesium concentrations. Either one or two BCO were rinsed in 100 μ l of either medium D or medium E. Connective tissue samples were taken as usual from these tissues.

After removal of the BCO the rinse media were made 90% with ice-cold acetone. The precipitate resulting from this treatment was collected by centrifugation (8000 rpm for 20 min). The pellet was washed once with acetone and then dissolved in SDS sample buffer (supplemented with 0.35 mM ethylenediaminetetraacetic acid [EDTA]) and treated as other samples in prepara-

tion for gel electrophoresis. Fig. 8 illustrates examples of both connective tissue samples and acetone precipitates from the media. These results demonstrate that medium D caused a reduction in the amount of low molecular weight material in the connective tissue and, concurrently, the large accumulation of this material in the medium. By comparison, tissues treated with medium E show a normal profile and the medium lacks any prominent accumulation of polypeptide. The data from several such experiments indicate that the effect of medium D is to reduce the proportional content of low molecular weight material in this region of the connective tissue by an average of 49% at 36–48

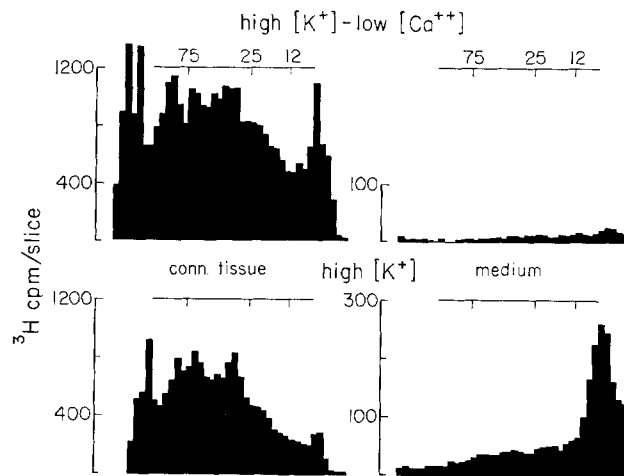


FIGURE 8. On the left are incorporation profiles from connective tissue samples rinsed for 2 hr (46–48 hr of total rinse) in either high $[K^+]$ -low $[Ca^{++}]$ medium or high $[K^+]$ with normal $[Ca^{++}]$. On the right are profiles of the acetone-precipitable material obtained from the respective media. Differences in the connective tissue patterns at the high molecular weights are characteristic of intrinsic variability in these samples and not a consequence of the medium employed during the terminal rinse. Molecular weights ($\times 10^{-3}$) are indicated across the top of each figure.

hr after labeling (Fig. 7). If the proportion of low molecular weight material at 0–60 min after incubation (Fig. 7) is considered to represent background polypeptide synthesis within the sheath, then the consequence of high $[K^+]$ treatment was a reduction of the transported low molecular weight species by more than 90%.

DISCUSSION

The studies reported in this paper represent the first examination of the biosynthesis of a polypeptide hormone in an invertebrate. The results have revealed an unusual synthetic scheme.

Although long-term incubation (12 hr) of the BCO in radioactive leucine

yielded electrophoretic profiles which were consistent with the expectation that the bag cells synthesize the 6000 dalton egg-laying hormone, short-term incubations (30 min) revealed a sharply contrasting synthesis profile. Instead of the expected peak at less than 10,000 daltons, a prominent peak was seen at 25,000 daltons, and little or no labeled material had accumulated at the lower molecular weight. The significance of this 25,000 dalton species became evident when bag cell somata were sampled after various periods of rinse following incubation. Increasingly longer rinse periods resulted in progressively less 25,000 dalton material and increasing amounts of lower molecular weight species.

This molecular weight redistribution of labeled products is almost certainly the result of the breakdown of the 25,000 dalton material and not the result of *de novo* protein synthesis during the rinse. The rinse medium contained unlabeled leucine at 1000 times the concentration of the labeled amino acid used during the incubation period. Hence it can be assumed that the intracellular pools of labeled leucine were quickly diluted by the unlabeled leucine, and that label incorporation was greatly reduced as a consequence. An even more direct argument against *de novo* synthesis of the low molecular weight species can be made from the results of inhibiting protein synthesis with the antibiotic anisomycin. Used at a concentration known to inhibit protein synthesis by over 90%, anisomycin failed to prevent the redistribution of label in the cells.

A third argument for the specific breakdown of the 25,000 dalton species is made by the conservation of radioactivity throughout the time-course examined. Summation of the peak ratios in the 25,000, 12,000, and <10,000 dalton regions of the gels reveals essentially no change from the first few minutes after the end of incubation to more than 1.5 hr later. These data indicate that the radioactive leucine contained in the 25,000 dalton molecule immediately after incubation is found in both a 12,000 dalton species and species of lower molecular weight after longer periods of rinse.

The 25,000 dalton precursor molecule is not simply an oligomer of the lower molecular weight products. Since the sample preparation for electrophoresis involves exposure of the labeled proteins to both SDS and mercaptoethanol as well as high-temperature treatment (60°C for 25 min), the resulting polypeptides are likely to be denatured and monomeric. Thus, the 25,000 dalton precursor is almost certainly covalently bonded. This fact makes it likely that the conversion to lower molecular weight products is an enzymatic step. The nature of the enzyme cannot be inferred from present knowledge of the conversion process. However, it is interesting to note that lowering the temperature by 10°C during the rinse completely blocks conversion. It would appear that the enzyme responsible for cleaving the precursor has a high Q_{10} and very low activity at 5°C.

There are two products of the breakdown of the 25,000 dalton precursor, one with a molecular weight of 12,000 daltons and the other with a molecular weight of less than 10,000 daltons. Because there is an abrupt increase in the slope of the line relating molecular weight to relative migration distance on these gels at the 10,000 dalton level, it is not possible to derive the precise molecular weight of the smaller breakdown product. The simplest case would be that the remaining half of the precursor is cleaved in half to yield two molecules of between 6000 and 7000 daltons each. Such a model is consistent with the estimate of molecular weight for ELH (6000 daltons) made by Toevs (1970).

Inspection of Fig. 4 reveals that different proportions of the radioactivity originally contained in the precursor species appear in the product species. There is nearly 2.5 times the radioactivity in the region below 10,000 daltons as appears in the 12,000 dalton region. Among several hypotheses that could account for this distribution of the product radioactivity, two commend themselves on the basis of parsimony. Perhaps there are two species of 25,000 dalton precursor, one being 2.5 times more abundant than the other. The less abundant precursor would give rise to the 12,000 dalton product while the more abundant would yield the species of less than 10,000 daltons. An alternative hypothesis could account for the distribution of radioactivity in the two products by positing an unequal distribution of leucine residues in a single precursor species. If one half of the 25,000 dalton molecule contains 2.5 times the leucine contained in the other half, the observed distribution would be explained. The only argument that would tend to support one of these models over the other is that the two-precursor hypothesis requires a coincidence in the time-course of cleavage of two different molecules by the same or two different temperature-sensitive enzymes. The necessary coincidences for the two-precursor hypothesis make it somewhat less straight-forward than the single-precursor model.

Although little can be said about the 12,000 dalton product, the smaller product can be identified with some certainty. Toevs (1970) demonstrated large accumulations of ELH in the bag cells. During some months of the year this 6000 dalton polypeptide was present at levels near 50% of the total protein. The densitometric tracing presented in Fig. 1 of this paper is consistent with Toevs's findings in that a large band of stainable material is located in the region below 10,000 daltons. That this material is the principal synthetic product of these cells is shown by the large incorporation of leucine-³H into polypeptides that migrate to the same position on the gels. The identity between this labeled material and ELH can be shown (*a*) by the finding that it is transported away from the cell somata and into axon-packed regions of the connective tissue during long periods of rinse, and (*b*) by the result of bathing BCO's in high-potassium medium with the consequent recovery of significant

levels of labeled material in the medium. When coupled with the bioassay studies reported in a previous publication (Arch, 1972) there can be little doubt that the more abundant product of precursor cleavage is the 6000 dalton ELH molecule.

The existence of a precursor molecule in polypeptide hormone biosynthesis is not without precedent. It is clear in the case of insulin synthesis that a larger, covalently bonded molecule is specifically cleaved to yield the active hormone and a residual species without hormone activity (Steiner and Oyer, 1967; Steiner et al., 1971). There is, as well, a suggestion that the neurophysins and the octapeptide hormones of the vertebrate posterior pituitary may be related through the occurrence of a larger precursor species (Fawcett et al., 1968). However, the significance of such a similarity in three functionally and phylogenetically distinct hormonal systems could be only a matter for speculation at present.

Two additional observations of biochemical and functional interest were made during these studies. At the earliest time points assayed after a typical 30 min incubation period there is no evidence for accumulations of either the 12,000 or the 6000 dalton product; only the precursor is present at levels clearly above background. This observation seems to imply that there is a latency between the time of precursor synthesis and its enzymatic cleavage. Such a latency, in turn, suggests that cleavage is not taking place close to the synthesis site but instead at some more distant location in the cell. Since it is likely that ELH is contained in the abundant dense-cored vesicles seen in the bag cells, an explanation for the latency might be the time required for the precursor to move into the region of the Golgi apparatus where cleavage and vesicle filling would take place. Although the data are incomplete, there is some evidence that the movement of the precursor does not require the synthesis of high-energy phosphates, nor does it involve somatic microtubules. The failure of either 1 mM KCN or 1 mM Na azide in the rinse medium to block cleavage argues against the involvement of the usual energy metabolism system in this process. Similarly, the failure of 1 mM colchicine in the rinse to block cleavage, although it does prevent the appearance of ELH in the connective tissue (Arch, unpublished), makes it unlikely that microtubular transport of the precursor occurs within the soma. Hence, at present, the latency should probably be considered a result of the time required for the precursor to diffuse into the site of cleavage.

In terms of the function of the BCO it has been most surprising to find that they appear to be engaged in significant levels of ELH synthesis during both the reproductively refractory and active seasons. Although, because of the dissection technique, it is not possible to assert that the absolute level of hormone synthesis does not change with the season, it is clear that the proportion of the over-all synthesis devoted to ELH production (30% +) remains nearly con-

stant. This observation prompts the speculation that the hormone serves some additional function beyond its role in the release of eggs from the ovotestis (Coggeshall, 1970). A possible role for ELH that would require a relatively constant rate of synthesis might be one involving tonic release throughout the year. Perhaps ELH has a dual role in reproduction analogous to that played by the vertebrate gonadotropins. Its tonic and low-level release might be important to the maintenance of the reproductive system while its acute and relatively massive release leads to ovulation.

The results of this examination of hormone biosynthesis have raised several interesting questions with regard to this secretory system. For example: What is the significance of the latency between precursor synthesis and breakdown? Is the breakdown associated with vesicle filling? What is the function of the 12,000 dalton product? Why is the proportion of ELH synthesis so large throughout the year? In a broader perspective, these studies have provided a useful foundation of information for the study of regulatory coupling between membrane and cytoplasmic events in these neurons. It will now be possible to examine the effects on hormone biosynthesis of ionic and electrophysiologic modifications of membrane potential. It is hoped that the results of such studies will suggest models for regulatory processes that may be applicable to neurons involved in synaptic transmission and the more complicated integrative functions of nervous systems.

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