

Precursor and Product Processing in the Bag Cell Neurons of *Aplysia californica*

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ABSTRACT Posttranslational processing in the biosynthesis of the egg-laying hormone (ELH) by the bag cell neurons of *Aplysia californica* was studied. The precursor (pro-ELH) to ELH was found to be resistant to solubilization in denaturant-free media throughout its lifetime. Its principle products show a similar insolubility for 3 h, but two of these, ca. 6,000 daltons, subsequently become readily recoverable in the low-speed supernatant of a homogenate of the cells. The remaining product shows no change in solubility characteristics. From studies employing ultracentrifugation and examination of axoplasmic transport, the solubility shift for the lower molecular weight products is interpreted to represent the liberation of secretory vesicles into the cytoplasm from larger membranous associations. This event is accompanied by, but does not appear to be dependent upon, a 15% reduction in the molecular weight of one of the products. These findings are considered in the light of the extensively studied posttranslational processing regimen for the production of insulin in the pancreatic beta cell.

INTRODUCTION

The bag cell neurons (Frazier et al., 1967) of the parietovisceral ganglion of *Aplysia californica* have been shown to comprise, with their surrounding sheath of connective tissue, a neuroendocrine organ involved in the seasonal egg-laying activity of the animal (review, Arch, 1976). Because of its unusual tractability, this system permits a variety of detailed studies not so readily performed with neuroendocrine tissues in other organisms. Among these has been an examination of hormone biosynthesis within the bag cell somata (Arch, 1972*a, b*; Gainer and Wollberg, 1974; Loh et al., 1975; Arch et al., 1976). These studies revealed that a significant fraction of ongoing protein synthesis in the cells is devoted to the production of a 29,000-dalton protein. The use of a radioactive amino acid precursor in a pulse-chase protocol further disclosed that the 29,000-dalton species was broken down into at least three products during a 60-min chase after a 30-min pulse of labeling. One of the product species was found to be approximately 11,300 daltons and the others approximately 6,000 daltons. Since the egg-laying hormone (ELH) had been reported to be a polypeptide of 6,000 daltons (Toevs, 1969) it was concluded that the 29,000-dalton species represents a precursor (pro-ELH) that is subsequently cleaved to yield one 11,300-dalton product and two of 6,000 daltons.

The studies reported here were undertaken to examine this biosynthetic scheme in greater detail. Of particular interest were the subcellular dispositions of pro-ELH and its products preceding and during cleavage, and the relationship between cleavage and the production of secretory vesicles.

MATERIALS AND METHODS

Animals weighing 150–500 g were obtained at approximately 3-wk intervals from Pacific Bio-Marine Supply Co. (Venice, Calif.). Experiments were not performed with animals earlier than 24 h after their arrival in the laboratory, nor later than 3 wk. A 150-gallon aquarium was used to house the animals. Instant-Ocean (Aquarium Systems Inc., Eastlake, Ohio) maintained at 14°C, 34‰ salinity, and pH 7.8–8.1 was circulated through the aquarium continuously. The aquarium room was held at 14°C and had a light/dark cycle of 12/12. All procedures, unless otherwise noted, were performed at 14°C.

The parietovisceral ganglion (PVG) was removed from animals 1–2 h after daily light onset. It was immediately transferred to filtered artificial seawater (FSW; 0.22- μ m filter, Millipore Corp., Bedford, Mass.). Within an hour the bag cells with their surrounding sheath of connective tissue (BCO) were isolated from the remainder of the PVG and maintained for at least 3 h in FSW before further treatment. Incubation in a defined medium (Arch, 1972*b*) containing 25 μ Ci [³H]leucine (44 Ci/mM, Schwarz/Mann, Orangeburg, N. Y.) generally followed the FSW preincubation. In some cases, incubations were conducted with 2.5 μ Ci [¹⁴C]leucine (312 mCi/mM, Schwarz/Mann). Incubation periods for [³H]leucine were 60 min and for [¹⁴C]leucine, 120 min. After incubation, the BCO were transferred to a chase medium (Arch, 1972*b*) and maintained for various times until processed for analysis.

Tissue preparation was begun by dissection of the BCO and homogenization of either the bag cells alone, or the bag cells and the connective tissue sheath separately, in 10 μ l of 0.01 M phosphate buffer, pH 7.8. Each homogenate was either centrifuged immediately at 1,000 *g* for 20 min at 4°C in the JA-21 rotor of a Beckman J-21B centrifuge (Beckman Instruments, Fullerton, Calif.), or it was frozen and centrifuged subsequently. The supernatant solution was drawn off quickly and transferred to a separate tube. An aqueous sample buffer containing 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 1 mM ethylenediamine tetraacetic acid, 50% glycerol, and 0.01 M phosphate buffer (pH 7.0) was added to both supernatant solution and pellet before analysis by polyacrylamide gel electrophoresis.

Electrophoresis was performed on miniature polyacrylamide gel columns (1 \times 60 mm) containing SDS (gel preparation adapted from Murray et al., 1972; Arch et al., 1976). Before layering, each sample was heated to 65°C for 30 min, and then centrifuged at 10,000 *g* for 10 min. The resultant supernatant was withdrawn and a 5- μ l portion was layered on the prepared gel column. Procedures for preparation and electrophoresis of isoelectric focusing gels are detailed in Arch et al. (1976). After electrophoresis, gels were extruded, sliced, and prepared for scintillation counting. Scintillation counting was performed at 48% efficiency for ³H and 96% efficiency for ¹⁴C in a Beckman LS-230 liquid scintillation counter. Typical background values were 21 cpm in the wide ³H channel and 8 cpm in the narrow ¹⁴C channel. Subsequent data reduction, statistical treatment, and graphical analysis were performed on an IBM digital computer (IBM World Trade Corp., Armonk, N.Y.).

RESULTS

Since cleavage of the prohormone requires more than 60 min to be completed (Arch, 1972*b*), we assumed that a rate-limiting step other than enzymatic activity

is interposed between pro-ELH synthesis and the production of the hormone. The most likely candidate for this time-requiring process is translocation of the pro-ELH from the sites of synthesis to some different site(s) within the cell where the cleavage enzymes are located. If translocation is by simple diffusion through the cytoplasm, pro-ELH should be recovered from the supernatant of an aqueous homogenate prepared during the period between its synthesis and cleavage.

The solubility of pro-ELH was examined by homogenizing pulse-labeled bag cells in a variety of different media. In our initial experiments we used iso-osmotic salines with potassium and calcium concentrations adjusted to approximate intracellular conditions. Over 80% of the labeled pro-ELH in such homogenates was retained in the pellet after centrifugation for 20 min at 1,000 g. This observation led us to investigate the possibility that the precursor was trapped in large membranous vesicles that were not disrupted by the iso-osmotic homogenization. However, homogenization in either distilled water (pH 6.2) or 0.01 M phosphate buffer (pH 7.8) produced substantially similar results (Fig. 1). The only media that effectively solubilized pro-ELH contained denaturants. Sodium dodecyl sulfate was most effective followed by 8 M urea and Triton X-100.

In the course of these experiments we were surprised to note that the products of pro-ELH cleavage displayed solubility characteristics similar to those of the precursor. While we had no a priori expectations regarding the 11,300-dalton product, there was considerable reason to expect the ca. 6,000-dalton material to be soluble in aqueous media. We have routinely used hypo-osmotic buffers for the preparation of extracts with which to induce egg laying in receptive animals (Arch and Smock, 1976).¹ If ELH is not soluble in such media immediately after cleavage it must undergo some further processing which makes it susceptible to solubilization.

To determine when substantial 6,000-dalton solubility develops, we performed a series of pulse-chase experiments employing 0.01 M phosphate buffer for homogenization. The BCO's were incubated for 60 min in [³H]leucine and then chased for variable lengths of time. The isolated bag cells were homogenized and, in most cases, the crude homogenate was frozen and maintained at -25°C before centrifugation and gel analysis. To examine the effect of freezing the crude homogenate before centrifugation, several preparations were centrifuged and separated before freezing. Since no consistent differences were detected between the two procedures, the data have not been analyzed separately. Centrifugation was performed for 20 min at 1,000 g in all cases, and yielded a clear supernatant and visible white pellet.

Examples of electrophoretic profiles prepared from samples homogenized at two different times after the beginning of pulse labeling are shown in Fig. 2. It is apparent that a significant change in the solubility of the 6,000-dalton material has occurred during the period intervening between the sampling times. For the sample taken at 210 min, the soluble radioactive material represents 15% of the total at that molecular weight in the cells, while in the sample taken at 405 min,

¹ Arch, S., and T. Smock. Egg-laying behavior in *Aplysia californica*. *Behav. Biol.* In press.

63% is present in the supernatant. The full time course of this change is shown in Fig. 3 and summarized in Table I.

Since the bag cells had been homogenized in a low ionic strength medium, these data did not permit us to determine whether ELH was becoming free in

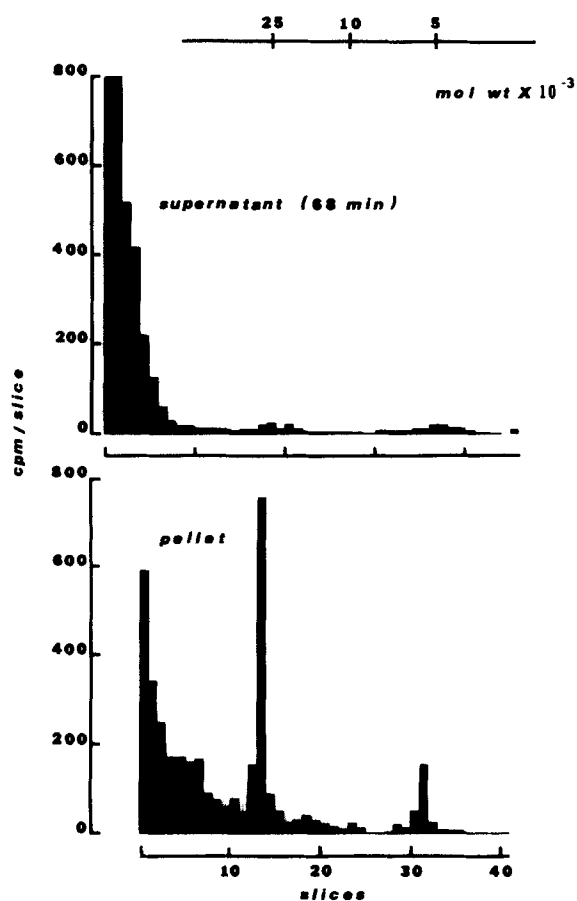


FIGURE 1. Electrophoretic profiles of radioactively labeled proteins produced by the bag cells. The cells were incubated for 60 min in [^3H]leucine and, after 8 min in chase medium, homogenized in 0.01 M phosphate buffer. The homogenate was then centrifuged at 1,000 g for 20 min and the supernatant and pellet separately analyzed on SDS-containing polyacrylamide gels. The prominent peak of radioactivity in the pellet sample is pro-ELH, the smaller peak represents product species. The first few slices in the gels are contaminated by free leucine. Approximate molecular weights are indicated on the abscissa across the top.

the cellular cytoplasm or entering an osmotically labile subcellular compartment. To distinguish between these possibilities, we pulse-labeled and chased bag cells as before, but homogenized them in an iso-osmotic sucrose solution (1.1 M made in 0.01 M phosphate, pH 7.8). The 20- μl homogenate was centrifuged as before at 1,000 g for 20 min. The resultant pellet was suspended in SDS sample buffer

and frozen until electrophoretic analysis. The supernatant was added to 0.7 ml of the homogenizing buffer and placed in a 0.8-ml cellulose nitrate centrifuge tube. This sample was then centrifuged at 105,000 g for 90 min in the SW-50L rotor on a Beckman model-L ultracentrifuge. The supernatant was discarded and the tube blotted of adhering sucrose solution. No pellet could be seen, but SDS sample buffer was added and the sample frozen pending electrophoresis.

Gel analysis of the samples derived from these experiments revealed low-

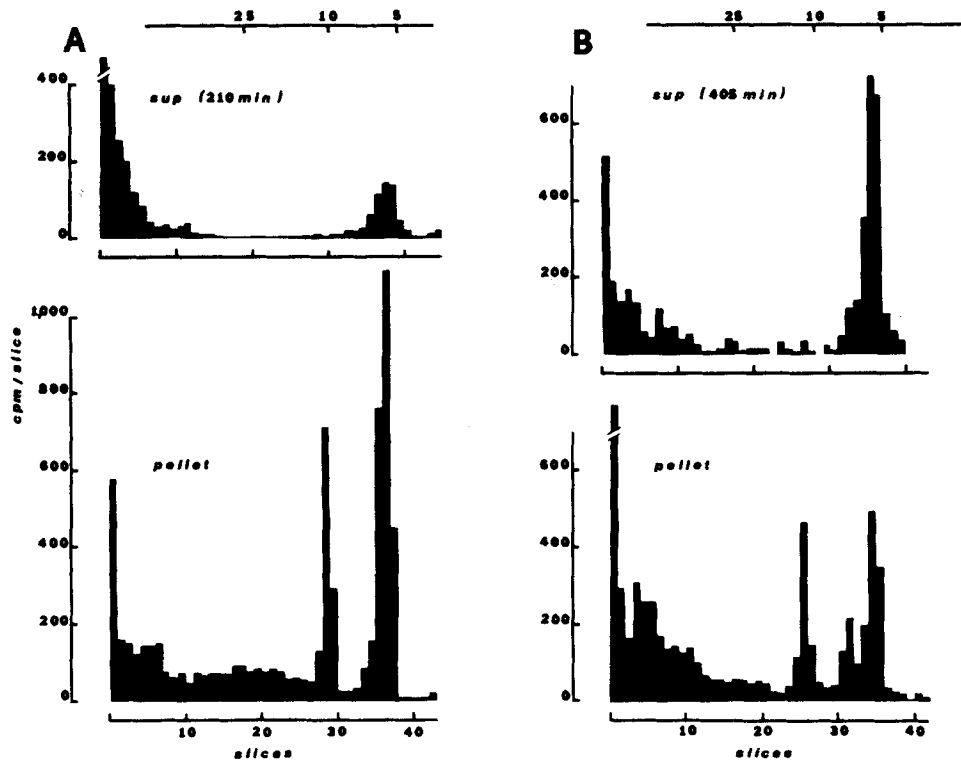


FIGURE 2. (A) Electrophoretic profiles of supernatant and pellet derived from bag cells incubated for 60 min in [^3H]leucine and chased for 150 min. (B) Electrophoretic profiles of supernatant and pellet from bag cells incubated under identical conditions but chased for 345 min.

speed pellets essentially similar to those seen after 0.01 M phosphate homogenization. The high-speed pellets from the low-speed supernatants showed that the 6,000-dalton material that becomes soluble is associated with a sedimentable fraction (Fig. 4). Since the pellets were so small, microscopic analysis was not attempted.

If the development of solubility of the 6,000-dalton species is the consequence of the liberation of secretory vesicles from larger membranous associations in the cell somata, all the pulse-labeled material should eventually become recoverable in the low ionic strength buffer. To assess this prediction we pulse-labeled BCO's with either [^3H]leucine or [^{14}C]leucine. After a 4-h chase, the tissues were

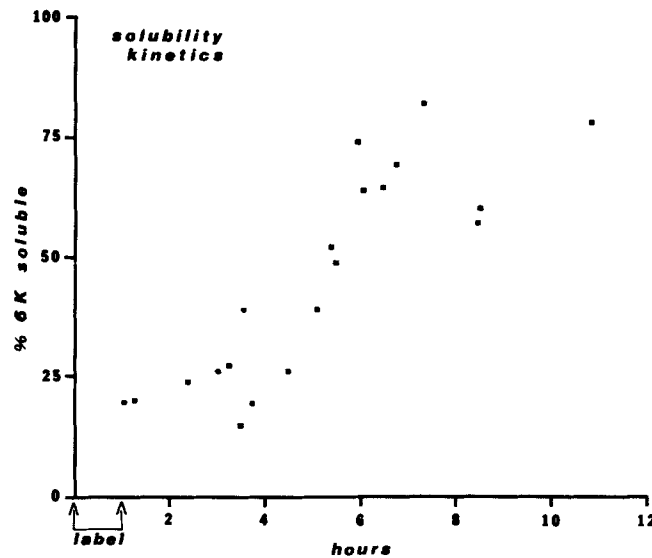


FIGURE 3. Kinetics of the solubility change undergone by 6,000-dalton products. The solubility is expressed as the ratio between radioactive material present in a low-speed supernatant of a 0.01 M phosphate homogenate, and the total labeled material present at the same molecular weight in the homogenate. Time is along the abscissa and includes the initial 60-min period of incubation in radioactive precursor.

TABLE I
DISPOSITION OF Pro-ELH CLEAVAGE PRODUCTS

| | (N) | Solubility* Mean \pm SD |
|-------------|-----|------------------------------|
| Pro-ELH | (4) | 0.18 \pm 0.10 |
| Products | | |
| 11,300 | (7) | 0.14 \pm 0.11 |
| 6,000-early | (7) | 0.24 \pm 0.08 |
| 6,000-late | (7) | 0.67 \pm 0.09 |

* Solubility calculated by summing the radioactivity in the three gel slices at the designated molecular weight in the supernatant sample and dividing by the total radioactivity at this position in both supernatant and pellet samples. Pro-ELH values taken at the four earliest sampling times after labeling (Fig. 3). The 11,300-dalton value was determined for the samples taken 6 h or later after incubation. 6,000-early and 6,000-late refer to the earliest and latest groups of samples shown in Fig. 3.

maintained in 2.5 ml of incubation medium (without labeled leucine) for 48 h. Both chase and long-term rinse media were made 1 mM for colchicine to prevent axoplasmic transport (Arch, 1972*b*; Loh et al., 1975) and thereby confine the pulse-labeled material to the somata. Each tissue was then incubated in the alternate isotopically labeled precursor to determine that metabolic activity

remained normal. A second 2-4-h chase followed the second labeling period. The BCO's were then transferred to a medium containing 1 mM Ca^{++} and 77 mM Mg^{++} (Arch, 1972*b*) on ice. This medium was employed to minimize vesicle discharge during dissection. Two preparations were made from each BCO. The

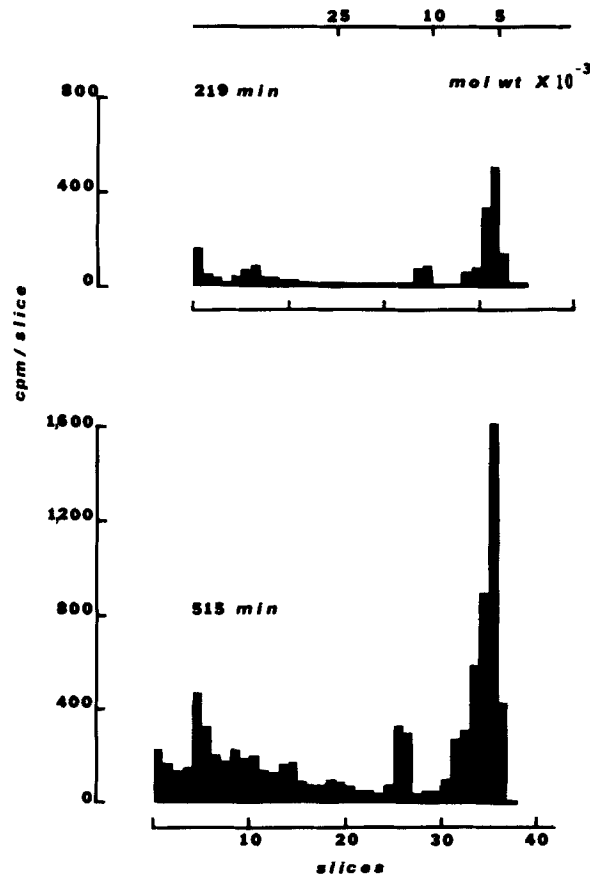


FIGURE 4. Electrophoretic profiles derived from pellets obtained by centrifugation of low-speed supernatants at 105,000 *g* for 90 min. Times of tissue homogenization are indicated on the profiles and include the 60-min period of incubation in radioactive precursor. That essentially all the 6,000-dalton material in the low-speed supernatant is recovered in the high-speed pellet can be seen by comparing the ratio between this material in the high-speed pellet to the total in the sample with the values in Fig. 3. This ratio for the 219-min sample is 0.19, and for the 515-min sample is 0.60. The examples shown in this figure are taken from a series of 10 preparations.

bag cell somata were removed and homogenized as before. The remaining connective tissue sheath was then homogenized separately in the same manner. Thus, after centrifugation, four samples were obtained: low-speed supernatants and pellets for both the bag cells and the connective tissue.

Fig. 5 provides examples from these experiments. The prediction of complete

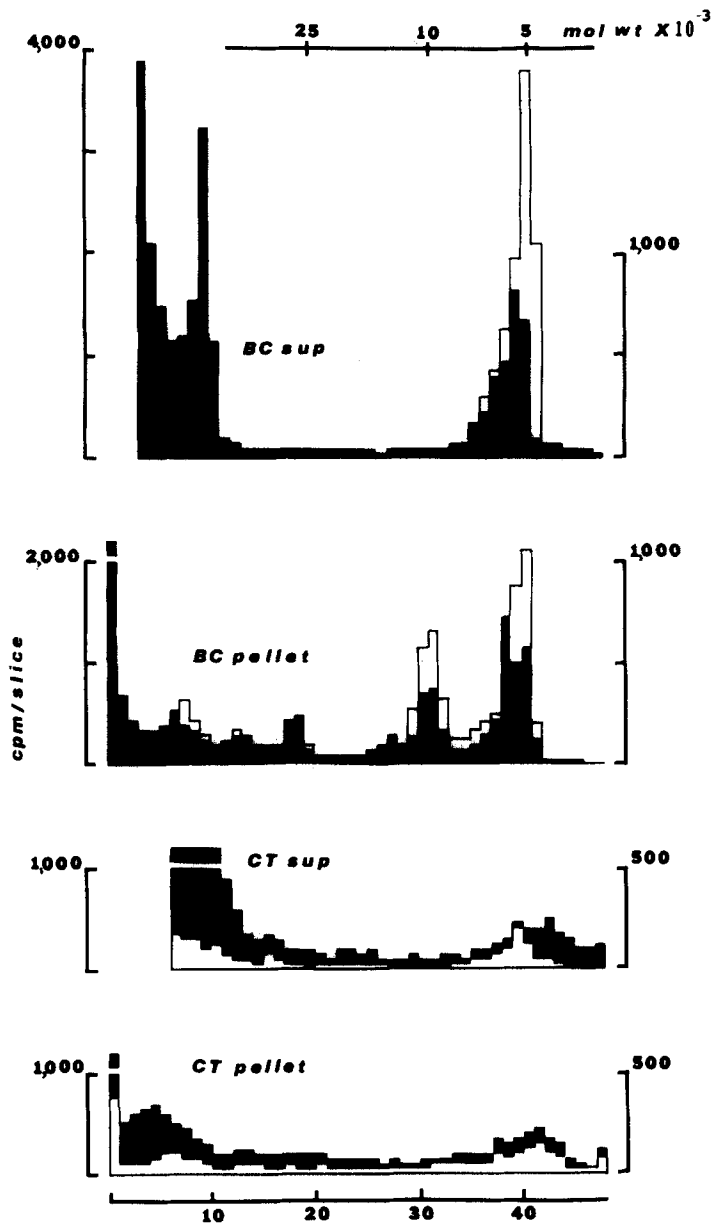


FIGURE 5. Electrophoretic profiles derived from experiments employing dual labeling of the bag cell organs. Supernatants and pellets were prepared from 0.01 M phosphate homogenates of the bag cell somata (BC) and the connective tissue sheath (CT) of a bag cell organ incubated first in [¹⁴C]leucine and then 48 h later in [³H]leucine. During the period intervening between labeling times and for 4 h after the second labeling, the tissues were bathed in media containing 1 mM colchicine. The left ordinate registers ¹⁴C radioactivity, the right ordinate refers to tritium. The filled profiles represent tritium radioactivity. This is an example taken from a series of four experiments.

solubility was not borne out in these studies. While it is evident that material labeled during the earlier incubation is substantially more soluble than that labeled later, the bag cell pellet still contains approximately 39% of the 6,000-dalton material labeled earlier. Since the profile labeled later demonstrates that the cells were still functional with respect to protein synthesis and pro-ELH cleavage, the possibility that colchicine had blocked the completion of the solubility shift was examined. In these experiments the same procedure was followed; however, colchicine was not added to any of the media. Thus, the solubility of transported, and presumably vesicular material, could be assessed. In Fig. 6, it can be seen that 6,000-dalton material labeled during the first incubation is predominantly located in the connective tissue preparation, and, within this, over 80% is in the supernatant. While the connective tissue supernatant does not contain all the material at this molecular weight, it is evident from Fig. 5 that cells in the connective tissue synthesize polypeptides of approximately the same weight as well. It is likely, therefore, that all, or nearly all, the transported material is soluble in phosphate buffer. It is interesting to note that the 6,000-dalton material labeled at the same time but remaining in the cell somata is soluble only to about the same extent as that labeled 48 h later. Thus, colchicine had not interfered with the solubility shift in our previous experiments. It appears, instead, that the 6,000-dalton species exist in two pools in the cell bodies. One of these is particulate and cytoplasmic, while the other is confined to larger membranous material sedimented at low speed from 0.01 M phosphate homogenates.

An alternative hypothesis is suggested by our recent finding that the 6,000-dalton material observed on SDS gels is not homogeneous (Arch et al., 1976). It is composed of two species that can be resolved by isoelectric focusing gel electrophoresis (IEF). One of these has an acidic isoelectric point (pI 4.8) and labels strongly with leucine. The other has an alkaline isoelectric point (pI 9.3), labels less strongly with leucine, and is the egg-laying hormone. Thus, the incomplete solubility of the 6,000-dalton material could reflect differential solubility of these two species. We therefore performed IEF analyses of phosphate buffer extracts of the bag cells at selected times during the solubility series. The results of these analyses (Fig. 7) show that both the pI 4.8 and 9.3 species appear in increasing amounts in the low-speed supernatants with increasing time after incubation. Since IEF cannot be used effectively to assess the content of the low-speed pellet, we cannot determine that the two species are becoming soluble to the same extent; however, it is apparent that both are readily recovered in the soluble fraction at longer chase times.

The profiles shown in Fig. 7 reveal an additional phenomenon observed in these studies. During the period of 6 h spanning the solubility shift, IEF gels prepared on the earlier samples show two closely spaced peaks of radioactivity in the acidic region. Early during the chase, the more prominent is at pI 4.6 (Fig. 7a). With increasing time, this peak diminishes and the pI 4.8 material becomes predominant (Fig. 7b). Interestingly, SDS gels prepared from the same samples also show dual peaks in the 6,000-dalton region early during this period (Fig. 7c). Subsequently, only a single major band appears at ca. 6,000-daltons (Fig.

7d). Since the dual peaks on SDS gels are found in both pellet and supernatant samples, the molecular weight shift (Table II) appears to occur independently of the solubility change.

The disposition of the 11,300-dalton product throughout these studies was

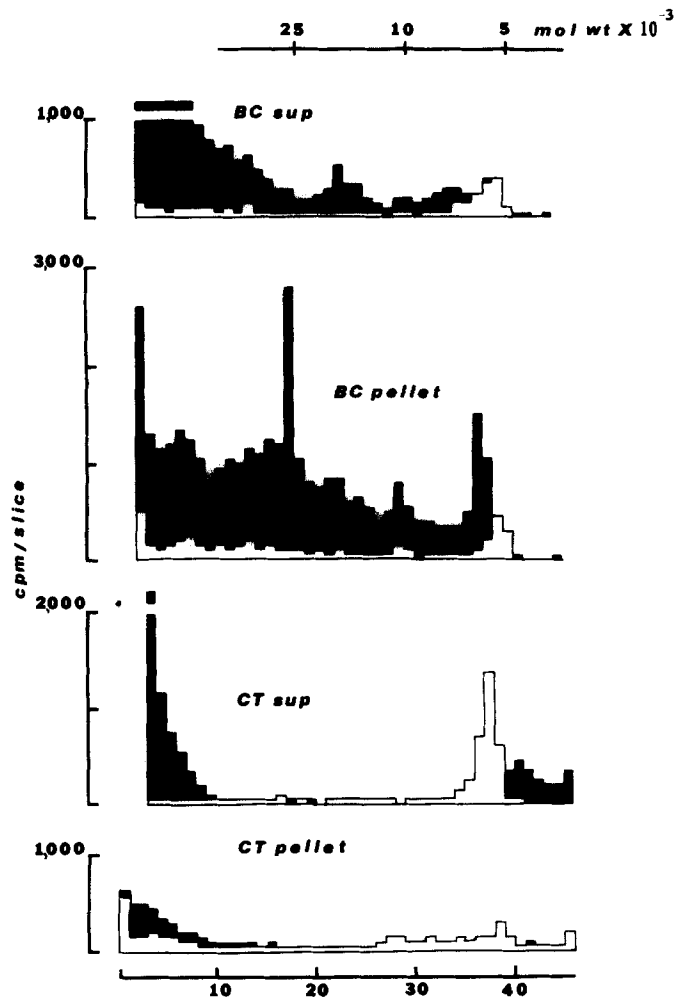


FIGURE 6. Electrophoretic profiles derived from a tissue handled in the same fashion as that shown in Fig. 5, with the exception that colchicine was omitted from all media and the rinse after the second labeling was only 2 h in length. The ordinate applies to both isotopes. The filled profile represents tritium radioactivity. This example is taken from a series of 10 experiments.

also examined. At the completion of precursor cleavage, this species contains approximately 30% of the radioactivity found at 6,000 daltons. As summarized in Table III, entirely different proportionalities are found in the supernatant and pellet of low ionic strength homogenates. The 11,300-dalton material is far less

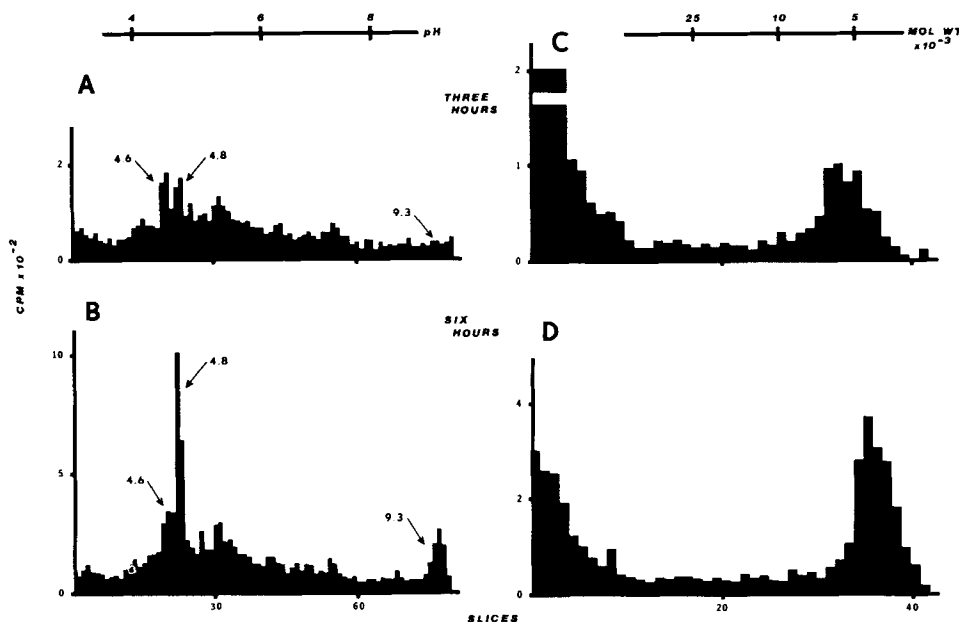


FIGURE 7. Low ionic strength supernatants derived from low-speed centrifugation of bag cell organs were prepared at the times shown. Each was separated into two portions that were subjected to IEF and SDS electrophoresis, respectively. Profiles A and C were derived from the same preparation, and B and D from another. The arrows indicate the positions and isoelectric points of species of interest. The differences in overall radioactivity at the two times reflect the difference in solubility. The differences in overall radioactivity in IEF and SDS gels prepared on the same supernatant is a function of the proportions of the sample used for each procedure. These examples were taken from a series of eight such preparations.

TABLE II
ACIDIC PRODUCT MOLECULAR WEIGHT CHANGE*
(Mean \pm SD)

| | Heavy | (N) | Light | (N) |
|-------------|-----------------|------|-----------------|------|
| | daltons | | daltons | |
| Supernatant | 5,725 \pm 615 | (15) | 4,800 \pm 400 | (16) |
| Pellet | 5,845 \pm 590 | (11) | 4,970 \pm 565 | (13) |

* Molecular weight estimates are based on a calibration curve (Arch et al., 1976) prepared with protein standards for the SDS-polyacrylamide gel used in these studies. Assignment of molecular weight for each case was performed by computer on the basis of the calibration data. Both dual-labeled and single-labeled samples were used. For dual-labeled samples, material synthesized within 4 h of the sampling time was considered heavy while that synthesized previously (usually 48 h) was considered light. Only those single-labeled samples that displayed a double peak in the ca. 6,000-dalton region were used in this compilation. Tests of significance (*t* test) for the molecular weight differences detected in both supernatants and pellets gave $0.01 > P > 0.001$.

soluble than the 6,000-dalton species at long chase times. The presence of some 11,300-dalton product in high-speed pellets like those in Fig. 4 may, however, suggest some association with the particulate fraction occupied by the lower molecular weight products.

DISCUSSION

The products of cleavage of radioactive pro-ELH become detectable at about 10 min after the end of a 30-min pulse labeling (Arch, 1972*b*). Whether this latency represents an interval during which no cleavage is occurring or is an artifact of the limitations of sensitivity of the techniques employed is not yet known. Once under way, the rate of disappearance of the 29,000-dalton precursor accelerates between 25 and 45 min after incubation, then slows again as labeled pro-ELH becomes exhausted. Assuming the kinetics of peptide hydrolysis to be considerably more rapid than the time course of pro-ELH breakdown, it is probable that the proteolytic enzymes responsible are not located in close proximity to the sites of synthesis. An adequate account of the kinetics for pro-ELH cleavage can be constructed by supposing the specific enzymes to be confined to a small number

TABLE III
PROPORTION OF 11,300- TO 6,000-DALTON PRODUCTS

| Ratios* | (N) | Mean ± SD |
|-------------|------|-----------|
| Overall | (20) | 0.29±0.10 |
| Supernatant | (7) | 0.08±0.07 |
| Pellet | (7) | 0.61±0.18 |

* The ratios were calculated by dividing the radioactivity contained in three sequential gel slices at 11,300 daltons by the radioactivity in three slices at the 6,000-dalton region.

of discrete sites within the cell. Cleavage would begin slowly as only those pro-ELH molecules synthesized near the cleavage sites move into the proximity of the enzymes. With increasing time, the number of labeled precursor molecules liable to enzymatic attack would increase, and the cleavage rate would appear to accelerate. After this, an apparent deceleration would occur as labeled substrate was consumed. The rate-limiting process in this model would be the translocation of pro-ELH from synthesis to cleavage sites.

It seems clear from our studies that translocation is not accomplished by free cytoplasmic diffusion. More than 80% of the pro-ELH labeled in our experiments was confined to the low-speed pellet when homogenization was performed in denaturant-free media. Even strongly hypo-osmotic media were ineffective in liberating pro-ELH into a soluble fraction (Fig. 1). Thus, it appears that the precursor retains close membrane associations throughout its processing.

The product species from pro-ELH breakdown are also confined to the pellet fraction during the first 3 h after labeling (Fig. 2). Subsequently, the ca. 6,000-dalton products begin to appear in increasing proportion in the supernatant fractions (Fig. 3). While the 11,300-dalton product is also found in supernatants

as postcleavage time increases, the largest proportion of this species remains in the pellet (Table III). By 6 h after the beginning of synthesis, over 65% of the 6,000-dalton material is present in the soluble fraction while less than 15% of the 11,300-dalton species is soluble. Studies employing IEF revealed that both species of ca. 6,000-dalton polypeptide take part in the solubility change.

Two kinds of experiments were performed to assess the significance of the shift in solubility of the smaller product species. Ultracentrifugation of low-speed supernatants prepared in iso-osmotic sucrose showed that they are in a particulate association at the time they become soluble in low ionic strength buffer. This observation is complemented by the results of the dual-label studies. Material of the same approximate molecular weight and extensive solubility as that in the particulate fraction in the soma is transported to neural-hemal regions of the connective tissue by a colchicine-sensitive process. Thus, it seems plausible that the solubility shift represents the liberation of mature secretory vesicles into the cytoplasm from larger membranous associations.

During the investigation of the change in solubility of the low molecular weight products, it became evident that another process was occurring as well. Profiles of radioactive proteins on SDS gels prepared from samples taken during the first 5 h of chase indicated that a reduction in molecular weight was taking place in the low molecular weight products. This molecular weight decrease had a mean value of approximately 1,000 (Table II). When representative samples were analyzed by isoelectric focusing (Fig. 7), the prominent acidic species in the cells appeared to shift its isoelectric point from pI 4.6 to 4.8. Since this species is known to migrate at a position corresponding to approximately 6,000 mol wt on SDS gels (Gainer and Wollberg, 1974; Arch et al., 1976) it appears likely that the observed molecular weight and isoelectric point shift describe the same event. The significance of the event is not known; however, Loh et al. (1975) have reported evidence of a <3,000-dalton oligopeptide released from the bag cells when they are challenged with high-potassium superfusion. It is possible that this released material is the segment removed from the acidic product. If so, it would appear that the prohormone gives rise to three species of peptides that are included in the secretory vesicles. At present, only one of these is known to have biological activity (Toevs, 1969; Arch et al., 1976).

Similarities between the production of insulin by beta cells of the pancreatic islets and the events described for ELH production have been mentioned previously (Arch, 1972*b*; Loh et al., 1975). The data reported in this paper can be interpreted as a further extension of the analogy between the two systems. After translation on the rough endoplasmic reticulum, proinsulin is inserted into the cisternal spaces of the reticulum (Sharp et al., 1975). Once in this compartment, it is translocated to the Golgi region of the beta cell by an energy-dependent process (Steiner et al., 1970). In our experiments pro-ELH was found to be confined to a noncytoplasmic fraction of the cells throughout its lifetime. Although we do not know the membranous fraction with which pro-ELH is associated, the persistence of its association suggests either that it is membrane bound or enclosed in a subcellular compartment resistant to osmotic disruption. As noted previously (Arch, 1972*b*) and confirmed in conjunction with the

present studies, interruption of energy production by metabolic poisons and anaerobiosis after pulse labeling does not inhibit prohormone cleavage. While this suggests that the early posttranslational events are not energy dependent, it is possible that the energy demands are not sufficient to render ambient ATP levels limiting. Since processing time is decreased when the chase is performed at 25°C instead of the usual 15°C (unpublished), the possibility of an energy requirement must remain open.

After cleavage, the ca. 6,000- and 11,300-dalton fractions remain, for a period, largely inaccessible to 0.01 M phosphate buffer. The extent of solubility of 6,000-dalton material during the initial 3 h after incubation is, however, slightly greater than that observed for the precursor (Table I). This may suggest that 6,000-dalton species are occupying a more labile compartment within the cell. The 11,300-dalton product appears to retain the less soluble characteristics of the precursor (Table III). Thus, at the time of precursor cleavage, the product species may become separated, with the 11,300-dalton product maintaining the same membrane association as the precursor, while the smaller products enter the early stages of secretory vesicle production. The 3-h period after incubation before they become substantially soluble would then represent the time required for vesicle assembly. This time course differs markedly from that determined for the production of cytoplasmic vesicles in the beta cells. Passage through the Golgi apparatus in that system requires only about 20 min (Steiner et al., 1974). However, the large discrepancy in the time requirements for the production of cytoplasmic secretory vesicles in these two cell types may reflect the large difference in the temperatures at which they are studied rather than significantly different processes.

The coincidence of the solubility change for both the acidic and the hormonal product suggests that both are included in secretory vesicles. In addition, the molecular weight reduction in the acidic product suggests the presence of a peptide as well in the vesicles. While it would be incautious to speculate about the likelihood of heterogeneity or homogeneity of individual vesicle contents it should be noted that there is precedence for heterogeneity of vesicle contents in that the beta cell vesicles contain the C fragment and related peptides as well as insulin.

While the similarities between production of ELH by the bag cells and insulin by the beta cells have been emphasized, comparable mechanisms may operate in a variety of other polypeptide-secreting cells (Palade, 1975; Steiner et al., 1974). The emerging evidence that these systems may share common processes for the production of their secretory proteins, presents the intriguing prospect that, despite widely divergent evolutionary histories, these tissues have adopted closely similar biochemical strategies in response to common contingencies presented by the cellular environment.

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