Biochemical Isolation and Physiological Identification of the Egg-Laying Hormone in *Aplysia californica*

S. ARCH, P. EARLEY, and T. SMOCK

From the Biology Department, Reed College, Portland, Oregon 97202. Mr. Earley's present address is the Department of Medical Education, University of Cincinnati College of Medicine, Cincinnati, Ohio 45219.

ABSTRACT   It has been determined that the bag cells of *Aplysia californica* produce two polypeptide species that comigrate on electrophoretic gels containing sodium dodecyl sulfate. By this separation procedure both species can be assigned a molecular weight of approximately 6,000. One of these molecules has an R_f of 0.65 on alkaline discontinuous electrophoresis gels, an isoelectric point at pH 4.8, a gel filtration molecular weight of approximately 12,000, and has no known biological function. The other does not enter alkaline disk gels, has an isoelectric point at approximately pH 9.3, shows a gel filtration molecular weight consistent with that determined by SDS gel electrophoresis, and is the egg-laying hormone.

INTRODUCTION

The bag cell neuroendocrine organ associated with the parietovisceral ganglion of *Aplysia californica* has begun to receive attention as a possible model system for neuroendocrine effectors (review Arch, 1976). The morphology of the system, its proximity to the extensively studied parietovisceral ganglion, and the distinctive physiological and behavioral consequences of its hormonal output provide the prospect of a thoroughgoing characterization of the physiology and biochemistry of this effector system. Central to this characterization is information concerning the nature of the hormone (egg-laying hormone, ELH). While there is agreement that ELH is proteinaceous (Toevs and Brackenbury, 1969; Kupfermann, 1972; Loh et al., 1975), molecular weight estimates have varied widely (Toevs, 1969; Loh et al., 1975). A biosynthetic scheme has been proposed (Arch, 1972 b) on the assumption that the molecular weight is approximately 6,000 (Toevs, 1969). However, the possibilities of heterogeneity among low molecular weight species within the cell (Gainer and Wollberg, 1974) and of cleavage of 6,000-dalton material (Loh et al., 1975) have necessitated a reexamination of the nature of the biologically active material.

METHODS

Animals weighing between 200 and 400 g were obtained at 3-wk intervals from Pacific Bio-Marine Supply (Venice, Calif.). They were held in an aquarium containing recirculating

artificial seawater at 14°C. The light/dark cycle was 14/10. All experiments were performed between August and December, 1975.

In most experiments the bag cell organs (BCO) were removed from the parietovisceral ganglion (PVG), and, after preincubation in filtered artificial seawater (FSW), exposed to a medium (Arch, 1972b) containing an isotopically labeled amino acid. The incubation period varied in length depending on the experiment performed. After incubation, the BCO were transferred to a chase medium (Arch, 1972b) containing a large excess of the appropriate unlabeled amino acid. Amino acids used as precursors were L[4,5-^{3}H]leucine (44 Ci/mM), L[^{14}C]leucine (326 mCi/mM), and L[4,5^{3}H]lysine (40 Ci/mM) from Schwarz/ Mann (Orangeburg, N. Y.) and L[3-^{3}H]arginine (29 Ci/mM) from New England Nuclear (Boston, Mass.).

Gel Filtration Chromatography

Gel filtration chromatography was performed on low ionic strength extracts of the BCO. After 18-h incubation in [^{3}H]leucine and 10-h chase, the BCO were homogenized in 400 μl of ice-cold 0.01 M sodium phosphate buffer (pH 7.8). The crude homogenate was then centrifuged at 10,000 g_{max} for 10 min and the supernatant retained for application to the column. A Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) filtration bed 45 cm in height was prepared in a 1-cm diameter glass column. The elution was performed with 0.01 M sodium phosphate buffer (pH 7.8) at a rate of 2.5 ml/h/cm^2 regulated by a Sage (Orion Research, Cambridge, Mass.) tubing pump. Eluate fractions of 0.85 ml were collected automatically with a Gilson FC-80H (Gilson Medical Electronics, Middleton, Wis.) fraction collector. All chromatography was performed at 4°C.

Column calibration was achieved with protein standards, blue-dextran 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden), and K_2CrO_4. The protein standards and their nominal molecular weights were: bovin serum albumin, 67,000; horse heart myoglobin, 17,800; cytochrome C, 12,400; insulin, 6,000; and adrenocorticotropic hormone, 4,500. Assay of the elution position of the standards was performed by one or more of four methods. Visual identification was possible for the blue-dextran, K_2CrO_4, myoglobin, and cytochrome C. Ultraviolet absorbance at 280 nm was assayed in most cases. Protein concentration was determined by the method of Lowry et al. (1951), and bovine serum albumin and adrenocorticotropic hormone were made visible in ultraviolet light by dansylation (method after Gros and Labouesse, 1968; Earley, 1975).

SDS Gel Electrophoresis

Samples were prepared for analysis on miniature (0.8 × 60 mm) gel columns by mixing equal volumes of sample in 0.01 M sodium phosphate buffer with sample buffer containing 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 1 mM EDTA, 50% glycerol, and 0.01 M sodium phosphate buffer (pH 7.0). The gels were prepared containing 10% acrylamide monomer, 1% methylene bisacrylamide, 0.2% SDS, and 0.1 M sodium phosphate buffer (pH 7.0) essentially as described by Murray et al. (1972). Before beginning electrophoresis, the sample was heated at 65°C for 30 min after which it was centrifuged for 10 min at 10,000 g_{max}. From the supernatant, 5 μl were layered on the gel column, and after casting of the cap gel, subjected to continuous electrophoresis at 60 V until the bromphenol blue tracking dye reached a mark 1 cm from the anodal end of the gel. The electrode buffers were 0.01 M sodium phosphate (pH 7.0) containing 0.2% SDS.

SDS gels were calibrated with standard proteins run alone or in combinations. The proteins used and their nominal molecular weights were: bovine serum albumin, 67,000; amylase, 45,000; chymotrypsinogen, 25,000; cytochrome C, 12,400; insulin, 6,000; and glucagon, 3,500. In all cases, the position of the protein was determined relative to that of

the tracking dye by staining with Coomassie Brilliant Blue (after Fairbanks et al., 1971). The relationship between log molecular weight and relative migration was essentially linear over the range between 50,000 and 3,500 daltons. The best-fit line relating the molecular weight and distance moved is described by log mol wt $= -1.376 R + 5.069$, where R is the relative mobility of the species of interest.

Discontinuous pH Gel Electrophoresis

Cell homogenates or eluates derived from other separation procedures were dissolved in a sample buffer containing 8 M urea, Triton X-100 at its critical micellar concentration (CMC; Kushner and Hubbard, 1954), and 0.05% bromphenol blue in Tris-glycine buffer (pH 8.9). All other procedures were essentially as described in Brewer and Ashworth (1966) except that the buffer concentrations for stacking and running gels were doubled.

Isoelectric Focusing Gel Electrophoresis

Samples were generally prepared in either distilled water or 0.01 M sodium phosphate buffer. Gels were made under red light at 4°C. They contained, in addition to 10-100 μl of sample, 7.5% acrylamide monomer, 0.2% methylene bisacrylamide, 2% ampholines (Bio-Lyte 3/10, batch no. 13979, Bio-Rad Laboratories, Richmond, Calif.), mixed-bed ion exchange resin purified Triton X-100 at its CMC, and 0.1% riboflavin. After the gels were poured (4.5 × 90 mm) they were photopolymerized under fluorescent light at 4°C. Isoelectric focusing was performed at 200 V for 20 h during which time the amperage fell from 2 mA per tube to 0.01 mA. The electrolyte solutions were 0.06 N H_2SO_4 and 0.01 N $Ca(OH)_2$ in 0.05 N NaOH. The latter solution was stirred continuously throughout the run.

Calibration of the pH gradient was obtained by slicing control gels into 20-25 segments and eluting each into several milliliters of distilled water. The distilled water had been boiled immediately before elution to purge CO_2. After 3 h of elution the pH of the eluate was determined at room temperature. The pH gradient thus determined was resolved into two linear segments. For isoelectric points (pI) between pH 3.2 and 6.8, pI $= 5.21 D + 3.21$, and for the range pH 6.8 to 9.8, pI $= 9.69 D + 0.119$. In both equations D is the distance moved from the acidic end of the gel divided by the total gel length. In all cases myoglobin was included in isoelectric focusing gels as a monitor of the progress of the separation. The pI calculated for myoglobin in seven randomly selected runs was at pH 7.1 ± 0.13 with a secondary band appearing at pH 7.5 ± 0.19 and was in excellent agreement with the value reported by Salaman and Williamson (1971).

Analysis of Radioactivity in Gels

To assess the distribution of radioisotopically labeled species after electrophoresis, gels were extruded and sliced into sequential segments. If contamination from free amino acids was not significant the gel slices were immediately placed in Wilson's cocktail (Ward et al., 1970). After overnight elution at room temperature the eluted radioactivity was determined by scintillation counting in a Beckman LS230 (Beckman Instruments, Fullerton, Calif.). When it was necessary to remove nonmacromolecular radioactivity, the individual gel slices were incubated for 20 h in 10% trichloroacetic acid at 4°C. This was then decanted, the gel slices dried at 125°C for 60 min, and elution with Wilson's cocktail begun. In instances where elution of gel separated material was performed for bioassay, the isotope distribution was monitored by scintillation counting of 10 μl eluates dissolved in Aquasol (New England Nuclear, Boston, Mass.).

In all cases, data obtained from scintillation counting were stored on perforated paper tape. Data reduction and graphical presentation was performed on an IBM 1130 computer (IBM World Trade Corp., Armonk, N. Y.).

Bioassay Procedure

Samples obtained from gel filtration or electrophoretic separations were bioassayed to determine their activity in the induction of egg-laying. As shown elsewhere (Arch and Smock, 1976)[1] egg-laying hormone is soluble and stable in low-molarity phosphate buffer at neutral pH; thus, elutions were performed in this medium. Injection of eluate was made into mature recipient animals through the anterior third of the foot and directly into the hemocoel. If an animal produced more than 10 cm of egg string within 2 h after the injection, the assay was considered positive. Since some eluates were contaminated with SDS or ampholines, control injections of these substances alone and mixed with active extracts were made. The results of these injections were dependent solely on the presence or absence of the active material.

RESULTS

Electrophoresis of a low molarity phosphate buffer extract of the BCO on an SDS gel is shown in Fig. 1. The major proportion of radioactivity can be seen to be located in a single band at a position corresponding to approximately 6,000 daltons. The narrow width of this band indicates essential identity of molecular weights for the isotopically labeled, low molecular weight species. However, additional evidence of identity would be desirable. Unfortunately, the difficulty of reliably stripping SDS from a polypeptide makes further analysis of SDS gel-separated material problematic. We therefore attempted to isolate the presumed hormonal species by other procedures.

Toevs and Brackenbury (1969) reported that a species of polypeptide unique to the bag cells could be located at approximately R_f 0.65 on disk electrophoretic gel. We attempted to locate this material by disk electrophoresis of extracts prepared from BCO subjected to radioisotope labeling. Fig. 2a illustrates the radioactive profile of such cells and shows a prominent concentration of label at the R_f reported by Toevs and Brackenbury. From a duplicate gel, the region from R_f 0.60 to 0.70 was excised and eluted into 0.01 M phosphate buffer. This eluate was then used to prepare samples for analysis on isoelectric focusing (IEF) and SDS gels. The IEF profile (Fig. 2b) showed a single peak of radioactivity at pH 4.8 containing 92% of the labeled material in the gel. An equally sharp peak (95% of the retained radioactivity) was seen on the SDS gel (Fig. 2c).

To determine if this material was ELH we prepared IEF gels from phosphate extracts of BCO after 6-h labeling (Fig. 3). The pH 4.5–5.0 region was then eluted into two changes of 0.5 ml of phosphate buffer at 4°C for 24 h. On the basis of radioactivity retained in the slice after elution, the procedure was determined to recover at least 90% of the labeled material. The results of injection of the eluate into mature animals were uniformly negative.

To determine whether this result was caused by aggregation and thus inactivation of smaller biologically active monomers, we treated eluates with half-concentrated filtered seawater, 2-mercaptoethanol (1%), 4 M urea, and 1% SDS. None of these treatments led to biological activity.

These findings raised two possibilities. Either extraction, electrophoresis, and elution had inactivated the hormone, or hormonal activity resided with another

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[1] Arch, S., and T. Smock. Analysis of egg-laying behavior in *Aplysia californica*. *Behav. Biol.* In press.

species. To examine these alternatives, IEF gels were prepared as before with long-term labeled extracts and sliced into 20–25 equal length segments. These were then eluted in the same manner and the eluates were bioassayed. Biological activity was located consistently at the alkaline end of the gel at approximately pH 9.3 (Fig. 3). On the possibility that more specific labeling of this alkaline material could be obtained, we used both [^{3}H]lysine and [^{3}H]arginine as precur-
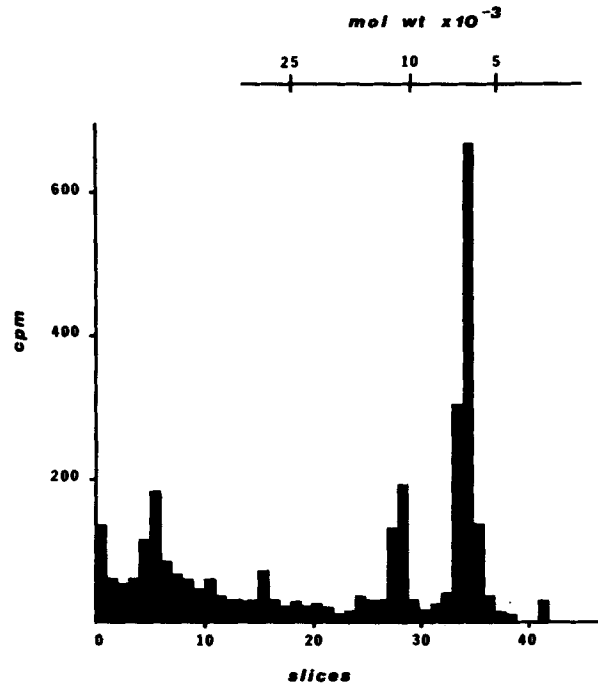

Figure 1.   The histogram represents a typical profile produced when an extract of the bag cells is subjected to electrophoresis on a miniature SDS-containing polyacrylamide gel. In this case the cells had been incubated in a medium containing [^{3}H]leucine for 60 min. They were then rinsed in a chase-type medium for 120 min before extraction in a sample buffer containing SDS. After electrophoresis, the gel was extruded and sliced into equivalent sections, each approximately 1.3 mm in length. These were then eluted and the eluate assayed for radioactivity. The ordinate represents counts per minute with a background of 25 cpm subtracted. The upper abscissa indicates the approximate molecular weights.

sors in 18-h labeling. The IEF profile derived from lysine labeling is similar to that obtained with leucine. Arginine, on the other hand, provided a more specific labeling of the hormonal material; however, after adjustment for specific activity of the radioactive amino acids, more leucine is incorporated into this material than arginine.

Since the biologically active material is different from the ca. 6,000-dalton acidic species, two approaches were taken to estimate its molecular weight. First, labeled BCO were extracted with SDS sample buffer and prepared for SDS gel
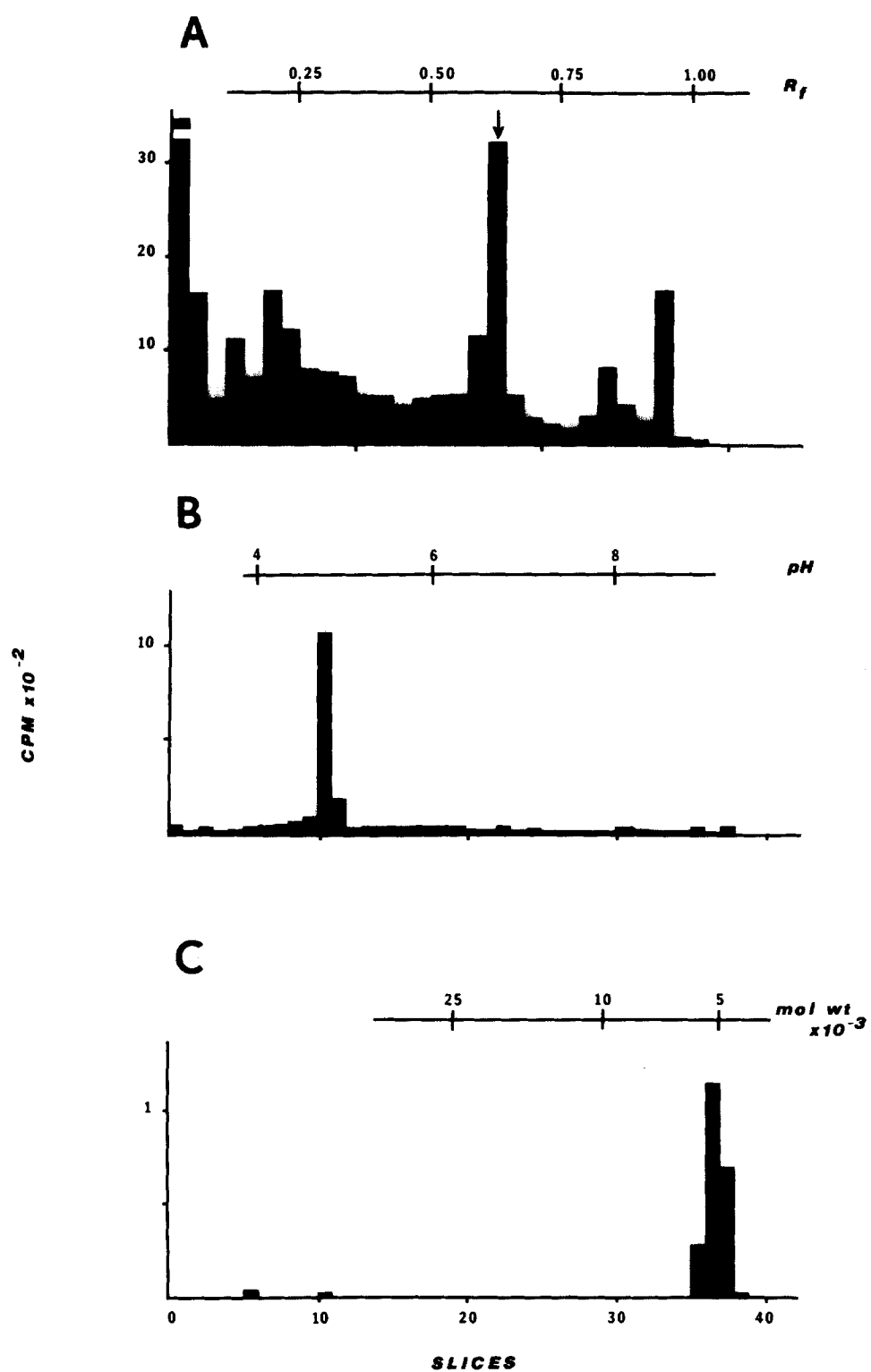
electrophoresis. The heating step was omitted before electrophoresis in view of the heat lability of the egg-laying hormone reported by Toevs and Brackenbury (1969). After electrophoresis the SDS-containing gels were sliced into 20–25 slices and eluted into 0.01 M phosphate buffer. Only the region corresponding to 5,000–7,000 daltons contained egg-laying activity when bioassayed (Fig. 4).

The second approach was with gel filtration chromatography. We prepared extracts of the BCO from four to seven animals in phosphate buffer for application to the column. Egg-laying activity was recovered from these column eluates only at K_{av} 0.54 $\pm$ 0.09 (SD). The molecular weight corresponding to this K_{av} value is 7,000 $\pm$ 1,000 (Fig. 5).

The results from SDS gel analysis suggest that both the pI 4.8 and the alkaline species should appear in IEF gels prepared from the column eluates inducing egg laying. This is not the case. The alkaline species is the only material present in abundance in the biologically active eluates (Fig. 6b). The acidic species was located at K_{av} 0.23–0.27 (Fig. 6a). While this datum suggests a molecular weight assignment of approximately 12,000, it may be reconciled with the SDS gel findings by positing a dimerization of ca. 6,000-dalton units. Samples of eluate from K_{av} 0.25 region when prepared for SDS-gel analysis either with or without 2-mercaptoethanol show a single, preponderant peak of radioactivity at the 6,000-dalton position (Fig. 7).

DISCUSSION

The results we have reported indicate the presence of more than one polypeptide of approximately 6,000 molecular weight in the bag cells. The substance Toevs and Brackenbury (1969) termed the bag cell-specific protein is homogeneous by both isoelectric focusing and SDS-gel electrophoresis. It has an isoelectric point at pH 4.8 and a molecular weight in the SDS system of approximately 6,000. When isolated from IEF gels or gel filtration columns it does not possess biological activity. Attempts to produce biological activity by affecting changes in H bonding, disulfide linkages, and hydrophobic interactions in this substance were unsuccessful. The reason for this lack of success became clear with the

FIGURE 2. (A) This histogram represents the results of seven experiments in which an extract of the bag cells was subjected to disk electrophoresis. The bag cells had been incubated in [³H]leucine for 6 h and rinsed for 1 h. Since the cells contained an appreciable amount of unincorporated amino acid, the gel slices prepared after electrophoresis were incubated overnight in 10% TCA. Subsequently they were processed for scintillation counting. The arrow indicates the material described by Toevs and Brackenbury (1969). This area was eluted from duplicate gels not subjected to TCA incubation. These eluates served as starting samples for the analyses illustrated in B and C. The ordinate indicates net counts per minute. The lower abscissa marks the sequential slices. The upper abscissa shows migration relative to the position of the tracking dye. (B) This histogram represents the result of isoelectric focusing electrophoresis on an eluate sample. The major peak is located at pH 4.8. This gel was incubated in TCA before scintillation counting. The upper abscissa indicates approximate pH. (C) This histogram represents the result of SDS-gel electrophoresis on an eluate sample. The upper abscissa shows approximate molecular weight.

discovery that the biological activity resided with a different substance. This material was located by bioassay at the alkaline end of IEF gels and at positions corresponding to molecular weight ranges of 6,000-8,000 on column chromatography (cf. Toevs, 1969) and 5,000-6,500 on SDS-gel electrophoresis. In the
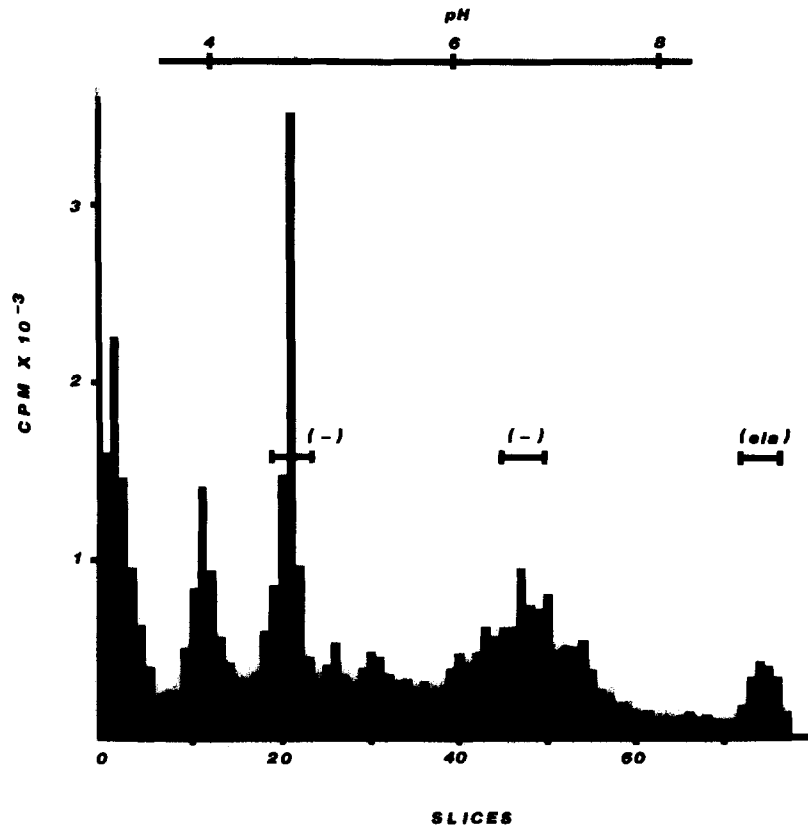

FIGURE 3. This histogram represents the distribution of radioactivity in an IEF gel prepared from BCO incubated for 18 h in [^{3}H]leucine. This gel was incubated in TCA before preparation for scintillation counting. The brackets indicate regions eluted from a duplicate gel, not exposed to TCA, and bioassayed. The (−) indicates the absence of biological activity; (ela) indicates the position of the egg-laying activity. Similar elution and bioassay experiments were performed on five separate BCO and all regions of the gels were assayed. Only the area indicated by (ela) was effective.

alkaline buffered disk gel systems used by Toevs and Brackenbury (1969) and in our studies, the biologically active polypeptide would be either uncharged or carry a net positive charge, and thus remain in the cathodal buffer compartment.

These findings differ in important respects from previous reports of heterogeneity among low molecular weight peptides in the bag cells. Gainer and Wollberg (1974) reported that the region corresponding to molecular weights
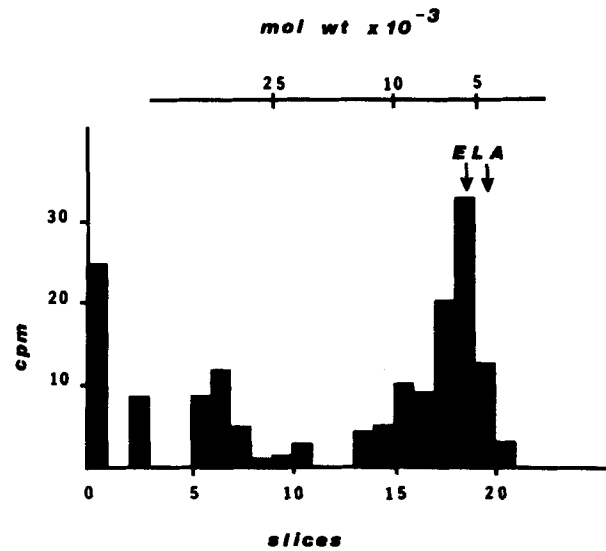
$$mol \ wt \ x \ 10^{-3}$$

FIGURE 4.   This histogram was prepared by scintillation counting of 10-μl samples of the eluates from sequential slices of an SDS gel prepared from BCO incubated for 6 h in [^{3}H]leucine. The remainder of the eluates were bioassayed directly by injection into mature animals. Only the samples indicated by arrows contained egg-laying activity.
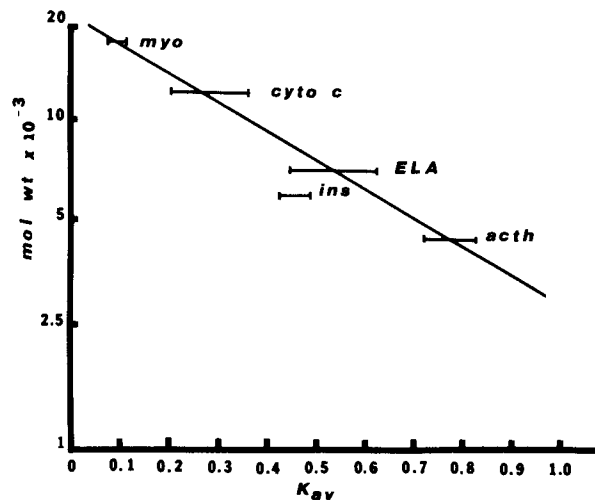

FIGURE 5.   This is a summary of calibration and bioassay gel filtration experiments. The K_{av} values calculated for standard proteins are plotted against their molecular weights on a logarithmic scale. The line was fitted by eye to the mean K_{av} values. The brackets represent 1 SD of the mean. These values were calculated by including all fractions containing a detectable amount of a given standard. The position of biologically active material calculated in the same manner is indicated by ELA. These results are based on seven separate filtration runs. Protein standards: myo, myoglobin; cyto, cytochrome C; ins, insulin; acth, adrenocorticotropic hormone.
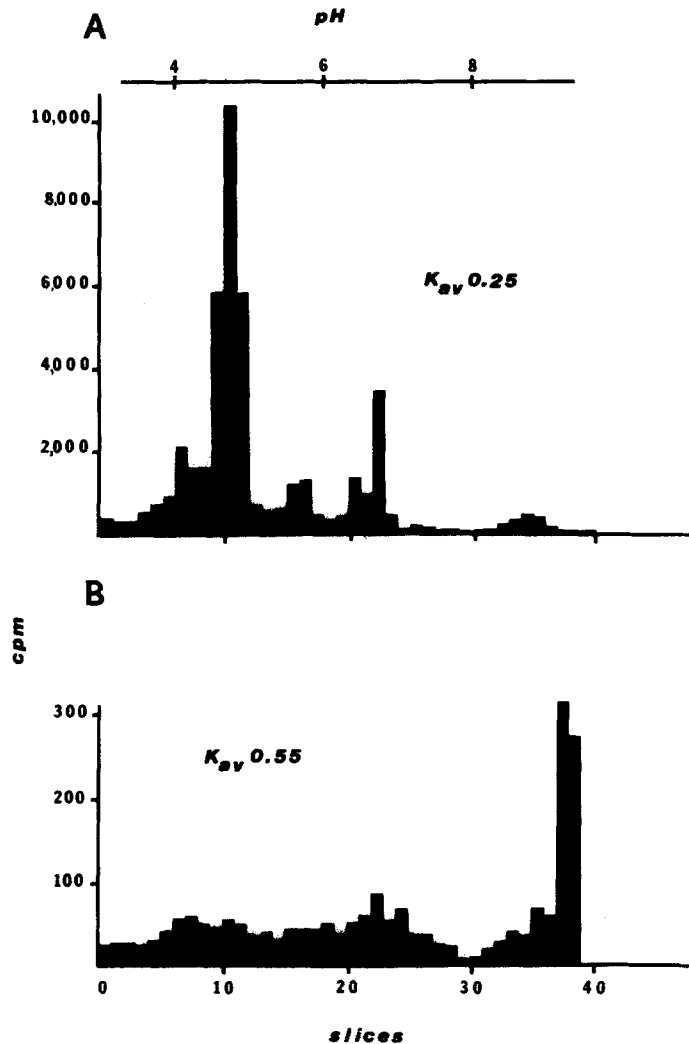
FIGURE 6. (A) A column fraction at K_{av} 0.25 was subjected to isoelectric focusing electrophoresis. The predominant peak of radioactivity is located at pH 4.8. The positions of subsidiary peaks and their amplitudes varied depending on the K_{av} value sampled between 0.20 and 0.30. In all cases, the pH 4.8 peak was dominant. (B) A portion of a column fraction of K_{av} 0.55 was subjected to IEF analysis. The distribution of radioactivity illustrated was characteristic of those obtained from fractions with K_{av} values corresponding to those determined for the biologically active material.

lower than 12,500 on SDS gels contained three or more species distinguishable by isoelectric point. One of these species had a pI of approximately 4.8, but neither of the other two was the strongly alkaline material we have identified as the biologically active substance. It is possible that this material was lost during their

precipitation and subsequent long-term dialysis of the SDS eluate preparatory to isoelectric focusing. Loh et al. (1975) have also described heterogeneity among the low molecular weight peptides in the bag cells. From their observations, they argue for the production of a <3,000-dalton species from an approximately
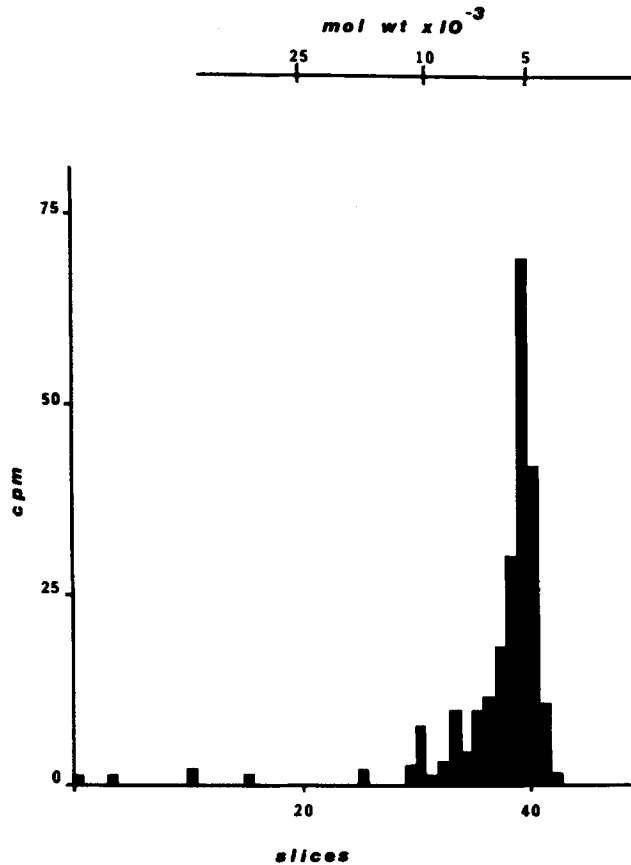

FIGURE 7.   A 10-μl sample of the eluate fraction at K_{av} 0.27 from gel filtration was exposed to SDS gel electrophoresis in the presence of 2-mercaptoethanol. The resultant profile reveals that over 75% of the radioactivity is recovered in a single band at 5,000–7,000 daltons. Essentially identical profiles were obtained when 2-mercaptoethanol was deleted from the sample buffer mixed with the eluate sample.

6,000-dalton precursor. Moreover, on the basis of studies of the materials released from the bag cells in response to depolarization they hypothesize that the <3,000-dalton species may be the actual hormone. Our results permit an evaluation of this hypothesis. Gel filtration chromatography of BCO extracts showed egg-laying activity to be restricted to a single volume of eluate appearing after the elution of cytochrome C and before that of ACTH. Hence, this separation procedure indicates that the molecular weight of the active substance must be greater than 4,500. The possibility that this estimate is in error because

of aggregation of smaller molecules in the low ionic strength medium is countered by the positive results of bioassay from the 5,000–6,000-dalton region of SDS gels. Since the gels contain both SDS and mercaptoethanol, it is unlikely that quaternary structure would be retained.

A relationship between the two substances we have identified is suggested by earlier studies on protein biosynthesis in the bag cells (Arch, 1972b, 1976). Bag cells allowed to incorporate [³H]leucine for 30 min in vitro and then immediately prepared for SDS-gel electrophoresis show as much as 60% of the incorporated label in a single band corresponding to 25,000–30,000 mol wt. If an increasing length of time is permitted to elapse between the termination of the 30-min incubation and preparation of the cells for analysis, this major band can be seen to diminish in radioactivity while 12,000- and 6,000-dalton bands show accumulation of radioactivity. This shift in the distribution of radioactivity is quantitative and will occur under conditions inhibitory to protein synthesis. The uniformity of the 6,000-dalton band on SDS gels could be taken to indicate that the cleavage of the precursor molecule results in one product of approximately 12,000 daltons and two identical 6,000-dalton products. Our present data challenge this model. While the molecular weights on SDS gels of the acidic and alkaline substances are not differentiable it is evident that they have quite different compositions. Thus, either the precursor yields the two different 6,000-dalton products, or one of these molecules arises independently of the precursor. The latter possibility, although it cannot be excluded, is not likely in view of the essential absence of labeled material at 6,000 daltons immediately after a 30-min incubation of the bag cells and the conservation of radioactivity in the 12,000-and 6,000-dalton species as precursor cleavage progresses (Arch, 1972b).

Because our bioassay studies were directed at locating the hormonal activity and not at quantification of the amount of material present, we cannot assert that all the activity present in a crude homogenate is recovered at pH 9.3 on the IEF gels, in the 5,000–7,000-dalton region on SDS gels, or at K_{av} 0.54 on gel filtration. However, estimates of the minimum potency of the material recovered can be derived from the bioassay studies. IEF gels prepared from two BCO yielded at least enough active material at pH 9.3 to evoke egg laying from two animals. SDS gels prepared from one BCO contained enough hormonal substance at 5,000–7,000 daltons to cause egg laying in at least one animal. Gel filtration eluates from 8 to 14 BCO contained at least enough material at K_{av} 0.54 for positive bioassay results from four to six animals. The last case is the most informative since one can assume that the concentration of active material falls off exponentially on either side of the one, or at most two, fractions containing the highest concentrations of active substance. Thus, in the absence of ambiguous data from any of the separation procedures, we have no reason to doubt that we have located in each case the region of major concentration of the hormone molecules.

Our results raise two important questions. The first of these concerns the molecular nature of the egg-laying hormone. Use of radioactive leucine, lysine, and arginine as precursors, and preliminary amino acid analyses (Earley and Arch, unpublished), have shown that it contains all three amino acids. It is

quantitatively richer in leucine than in either of the other two amino acids. Of particular interest is its insensitivity to denaturation by SDS and mercaptoethanol. In view of its leucine content, it seems likely that SDS can interact with the hormone. Thus, neither tertiary structure nor disulfide bonding appears to be paramount for its biological activity. Further molecular characterization will be necessary before the relationship between the amino acid composition of ELH and its biological activity can be explained.

The second question arising from our studies concerns the physiological role of the acidic polypeptide. Previous (Arch, 1972*a*, *b*; Loh et al., 1975) and unpublished studies have shown the colchicine-sensitive transport and depolarization-induced release of leucine-labeled material with a molecular weight of approximately 6,000. It is certain that this material represents ELH, but is the acidic polypeptide present as well? Since 6,000-dalton material is greatly reduced in the somata of bag cells and appears in the connective tissue when samples are prepared 12–24 h after a short period of incubation, it is likely that the acidic polypeptide as well as ELH is transported. If it is also released it may have a hormonal function independent of egg laying. Alternatively, it is possible that the acidic species acts as a counterion or, in some other role, is essential for the stability or retention of ELH in secretory vesicles.

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