# Polypeptide Secretion from the Isolated Parietovisceral Ganglion of *Aplysia californica*

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ABSTRACT In vitro studies of the secretory behavior of the parietovisceral ganglion in *Aplysia californica* were performed. The aim of these studies was to investigate the release of polypeptides in response to depolarizing stimuli, and, in particular, to determine if a specific polypeptide known to induce egg laying in the intact animal is secreted into the bathing medium. During continuous perfusion of a ganglion preincubated in leucine-$^3$H the application of either high-potassium medium or a burst of electrical stimuli (via the pleurovisceral connective nerve) evoked a marked increase in the amount of trichloroacetic acid (TCA)–precipitable radioactivity recovered in the perfusate. Enhanced release could be detected within 80 sec of the initial exposure to high potassium; however, incubation of a ganglion in calcium-free media before the application of high-potassium medium abolished the increase of precipitable radioactivity. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of perfusate samples revealed a significant change in the polypeptide species washed from the ganglion during high-potassium depolarization. Bioassays confirmed that egg laying is induced when high-potassium medium used to bathe a ganglion is injected into a recipient animal. These and other results permit the conclusion that the bulk of the polypeptide material secreted from the ganglion in response to depolarization is a specific neurohormone produced by two identified cell clusters, the so-called bag cells.

## INTRODUCTION

The central ganglionic nervous system of the gastropod *Aplysia californica* has been the object of extensive electrophysiologic study for several years (Coggeshall et al., 1966; Strumwasser, 1967; general review, Tauc, 1967). However, only recently has attention been given to the biochemical properties of the identifiable neural elements in these tissues (Peterson and Kernell, 1970; Giller and Schwartz, 1968, 1971; McCaman and Dewhurst, 1970; Wilson, 1971). Among such studies is the discovery by Kupfermann (1967)

that a sea water extract of the bag cells of the parietovisceral ganglion (PVG) will induce egg laying when injected into a recipient animal. Since electron microscope studies (Coggeshall, 1967) had revealed that the bag cells are neurons packed with large, dense-cored vesicles, a tentative hypothesis that these cells have a neurosecretory function could be considered. Later work by Strumwasser et al. (1969) confirmed Kupfermann's principal observations and demonstrated the presence of the egg-laying stimulating substance in the remainder of the PVG. Toevs and Brackenbury (1969) reported that the egg-laying activity of bag cell extracts was sensitive to proteolytic enzymes and high temperature. Moreover, electrophoretic analyses by these authors revealed a protein band which was unique to the bag cells and the connective tissue sheath of the PVG. Additional work by Toevs (1969) led to the conclusion that the active substance was a polypeptide of approximately 6000 molecular weight. Although these findings are consistent with the presumed neuroendocrine role of the bag cells they do not permit a conclusion of neuroendocrine function.

Recently Kupfermann (1970) reported that egg laying was induced in animals after an injection of the bathing medium (sea water) of an isolated PVG which had been stimulated electrically via the right pleurovisceral connective nerve. With this result the hypothesis that the bag cells are neuroendocrine could be considered much closer to verification. However, it remained to be demonstrated that the release, from an isolated PVG, of a substance stimulatory to egg laying was, in fact, the neurosecretion of the previously identified polypeptide. It was the purpose of the studies reported here to provide this demonstration.

## MATERIALS AND METHODS

Adult *A. californica* weighing between 200 and 500 g each were obtained from Pacific Biomarine Supply Co. (Venice, Calif.). All animals were kept in large tanks containing filtered and aerated sea water at 13°–14°C. The light cycle was 12 hr light: 12 hr dark. All experimental manipulations were performed in a room held at 14°C. These studies were performed between July and December, 1970. *A. californica* is known to be responsive to bag cell extracts throughout this period (Strumwasser et al., 1969). Ganglia were taken from only those animals showing large and developed reproductive tracts.

The basic protocol for the experiments to be reported consisted of isolating the PVG from a mature animal by making an incision through the foot and then cutting the major nerve trunks connecting the PVG with the head ganglia and peripheral structures (Strumwasser et al., 1969). After a brief rinse in 0.22 $\mu$ Millipore-filtered (Millipore Corp., Bedford, Mass.) sea water the ganglion was placed in 2.5 ml of an artificial incubation medium (in millimoles per liter: NaCl 423, KCl 9.7, $CaCl_2$ 13.3, $MgCl_2$ 49, $Na_2SO_4$ 28.2, phosphate buffer 0.2, glucose 9; pH 7.35) supplemented with

a 40:1 dilution of Eagle's (1959) essential amino acids (Microbiological Associates, Inc., Bethesda, Md.) and containing 25 $\mu$Ci of leucine-$^3$H (L-leucine-4,5-$^3$H; 20–30 Ci/mM; New England Nuclear Corp., Boston, Mass.). Incubation was allowed to proceed for 12 hr and the ganglion was then transferred to a rinse medium identical to the incubation medium except that it contained a 1000-fold excess of unlabeled leucine. At the end of the 12 hr rinse the stump (approximately 2 cm) of the right pleurovisceral connective nerve was secured in a small cuff electrode with the ganglion rested on an integral wire loop.

The ganglion was perfused with drops of medium at a rate of 5 ml/hr. The standard perfusion medium was the same as the incubation medium except that it lacked amino acids. The high-potassium medium differed from the standard in that it contained 106.7 mM $K^+$ and 382.4 mM $Na^+$. According to Sato et al. (1968), H neurons (cells in the PVG which are hyperpolarized by applied acetylcholine) are depolarized by about 13 mv when $K_o^+$ is 120 mM. In contrast, Carpenter and Alving (1968) found in neuron R2 that a fivefold increase in $K_o^+$ (from 10 to 50 mM) decreased membrane potential by about 20 mv. By extrapolation from their Fig. 3 (p. 6) a 10-fold increase in $K_o^+$ to 100 mM would yield a depolarization of approximately 35 mv. Since systematic measurements of this kind are not available for most other neuron types, including the bag cells, in the PVG, estimation of the depolarizing effect of 106.7 mM $K_o^+$ is inferential. If one assumes that the resting potential of these neurons behaves as a simple potassium electrode the Nernst equation yields an estimate of 26 mv depolarization from a typical resting potential of $-45$ mv. For this calculation $K_i^+$ was taken to be 232 mM (Sato et al., 1968).

1-ml fractions were collected sequentially throughout the perfusion. To follow the release of labeled material from the ganglion, a 200 $\mu$l sample was withdrawn from each fraction and pipetted onto a glass fiber filter disc (Whatman GF/A, 2.4 cm). The discs were then dried in a 60°C oven for 1 hr and placed in a standard toluene–2,5-diphenyloxazole (PPO)–*p*-bis[2-(5-phenyloxazolyl)]benzene (POPOP) cocktail (10 ml). Scintillation counting was performed in a Beckman LS 230 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). The results of this count gave an estimate of the total radioactivity perfused from the ganglion. To estimate the proportion of this material which was macromolecular the discs were removed from the scintillation fluid, air dried, extracted in ice-cold trichloroacetic acid (TCA; three rinses lasting 15, 5, and 5 min, respectively), dried in a 60°C oven, and counted again in fresh scintillation fluid. Each sample was counted once for 20 min. The net counts per minute were determined by subtraction of the 18 cpm background.

For the electrophoretic analysis, sodium dodecyl sulfate (SDS) polyacrylamide gels (1 mm × 60 mm) were used. The gel preparation, fractionation, and scintillation counting are described in Ward et al. (1970) and Wilson (1971). Each gel slice was counted twice for 20 min each time and the 16 cpm background was then subtracted.

Because of the high salt content and low concentration of labeled macromolecules in the perfusate fractions, sample preparation for gel analysis posed some problems. Best results were obtained by precipitating the macromolecules (5 $\mu$g bovine serum albumin added to each milliliter of perfusate as carrier) with ice-cold 5% TCA and then collecting and redissolving the precipitate in 0.01 M sodium phosphate buffer

containing 0.2% SDS, 2% 2-mercaptoethanol, 10% glycerol, and 0.0015% bromphenol blue. The redissolved sample was then extracted 10 times with water-saturated ethyl ether to remove TCA.

The protocol for bioassay experiments consisted of isolating PVG's from animals with mature reproductive tracts (Strumwasser et al., 1969) and rinsing the ganglia in filtered sea water for 60 min. Next the ganglia were transferred singly into small beakers containing 1 ml of high-potassium medium. After 60 min in the high-potassium medium the ganglia were discarded and the medium was injected into recipient animals. The injections were made through the anterior portion of the foot directly into the hemocoel (Kupfermann, 1967).

Each figure presented in this paper was taken from a single experiment and is representative of the findings from replicate studies. The number ($N$) of replicates is indicated in the text. No statistical analysis of the data has been undertaken since no negative or ambiguous results were obtained.

## RESULTS

My initial experiments were designed to determine whether depolarizing stimuli could alter the amount of TCA-precipitable, $^3$H-labeled material released from a prelabeled ganglion. The control perfusion profile (Fig. 1; $N = 5$) illustrates the result of continuous perfusion with the standard medium. The first fraction is not shown in this or any other profile presented in this paper because it routinely contained several thousand counts per minute, varied in content of label with the duration of rinse, and likely represented the washout of extracellular material accumulated during the rinse. As can be seen in the control profile the second fraction often was highly labeled as well. By the third and subsequent fractions, however, a relatively consistent trend of slowly decreasing radioactivity was established. In contrast, if the potassium concentration of the perfusing medium was elevated abruptly from 9.7 to 106.7 mM, the resultant profile (Fig. 1; $N = 10$) showed a large increase in the amount of TCA-precipitable radioactivity coincident with this treatment. In the 10 experiments the first three fractions taken during high-potassium perfusion contained a mean of 230% more precipitable radioactivity than was found in the previous three control fractions. After reaching a peak, the enhanced level of release fell off sharply until it returned to the control level. A similar profile was produced when a 30 sec electrical stimulation was applied to the right pleurovisceral connective nerve (Fig. 1; $N = 4$). In the four experiments the mean increase of precipitable radioactivity after electrical stimulation was 175% of prior levels. Control studies of the effect of electrical stimulation were performed. Stimulation of the siphon (= anal) nerve at 10 v elicited no release from ganglia which when subsequently stimulated at 10 v via the right connective nerve showed substantial release ($N = 4$). It was only at 40 v on the siphon nerve that any release could be evoked. In one of three experiments

there was a 20% increase in precipitable radioactivity in the perfusate. Again stimulation at 10 v via the right connective produced a full release with precipitable radioactivity levels at least 140% above the unstimulated levels.

Additional high-potassium perfusion experiments were carried out using isolated bag cell clusters instead of intact PVG's. In these studies ($N$ = 3)

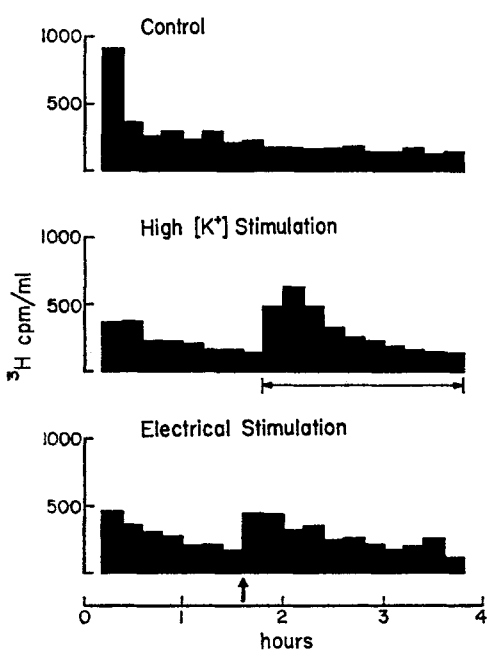


FIGURE 1

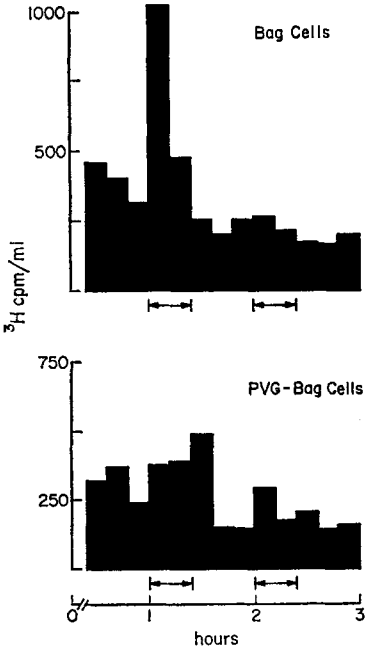


FIGURE 2

FIGURE 1. These profiles represent the amounts of TCA-precipitable, $^3$H-labeled material released from ganglia over a 4 hr period of perfusion. The *control* profile was derived from continuous perfusion with the standard medium. *High* [$K^+$] *stimulation* was performed by changing from the standard medium (9.7 mM $K^+$) to a medium containing 11 times as much potassium (106.7 mM $K^+$). The duration of high-potassium perfusion is indicated by the horizontal line beneath the abscissa. *Electrical stimulation* was performed by applying 10 v square-wave pulses (4 msec/pulse, 6 pulses/sec) to the right pleurovisceral connective nerve for 30 sec during continuous perfusion with the standard medium. The time of stimulation is indicated by the arrow. The values on the ordinate represent the TCA-precipitable counts per minute per sample adjusted to represent the total number of counts per minute for each 1 ml fraction. Time is along the abscissa.

FIGURE 2. *Top*, perfusion profile from an experiment during which two pulses of high-potassium medium (pulse = 2 ml = 24 min) were administered to the isolated bag cell clusters. The clusters had been cut away from the remainder of the PVG before incubation and rinse under the standard conditions. *Bottom*, perfusion profile derived from the PVG remnant after removal of the bag cell clusters. Other conditions were as described for the bag cell clusters. High-potassium pulses are indicated by the horizontal lines beneath the abscissa.

the bag cell clusters were cut away from the PVG before incubation in labeled medium. The top profile in Fig. 2 illustrates a perfusion run on the isolated bag cell clusters and the bottom profile shows a perfusion run on the PVG remnant after bag cell cluster removal ("PVG-Bag Cells"). It is apparent that a substantial release of precipitable radioactivity from the isolated bag cells was produced by the first high-potassium pulse (mean = 205%). Perfusion of the PVG remnant with high-potassium medium also produced some release. However, the level of release is small compared to that of the isolated bag cells. This observation suggests that although there are other cells in the PVG which will release labeled macromolecules in response to high-potassium perfusion, the contribution of these other cells to the total release from an intact ganglion is minimal in comparison to the release from the bag cells.

The connective tissue sheath surrounding the bag cells and proximal regions of the ganglion is riddled with hemal sinuses. Recent electron microscope studies (Strumwasser and Alvarez, unpublished) have revealed the presence of neuronal processes, filled with dense-cored vesicles, closely apposed to these hemal spaces. Hence if the material perfused from the ganglion during stimulation is released from neuroendocrine cells, a likely site of release would be in these blood sinuses. If release is occurring into these blood spaces one would expect the appearance of labeled secretory substances almost immediately after the onset of high-potassium perfusion. Alternatively if release of polypeptides is occurring at some intraganglionic site, a considerable delay in the appearance of label in the perfusate might be expected. Sato et al. (1968) found that membrane depolarization began in cell somata about 15 sec after the onset of high-potassium (221 mM) perfusion. Since they studied desheathed ganglia this time-course must be considered minimal. However, if it is assumed to be representative of intact ganglia, an estimation of the time required for a polypeptide released within the ganglion to appear in the perfusate can be made. Since diffusion rates for polypeptides are at least one order of magnitude less than those for small ions this estimate would be about 165 sec.

Fig. 3 ($N$ = 5) illustrates a profile obtained by determining the radioactivity in each drop of perfusate falling from the ganglion during the first 10 min of high-potassium perfusion. The temporal resolution of this procedure was approximately 80 sec. Thus, since the first high-potassium drop contained about 65% of the increased radioactivity seen in the subsequent (peak) drop, it is probable that much less than 80 sec was required for the onset of a significant release of labeled material. It is likely, therefore, that the initial release in response to high potassium is taking place near the surface of the ganglion and not at some intraganglionic site.

The sharp fall-off of radioactivity, after the peak, during continued high-

potassium perfusion, as seen in Fig. 1, could be taken as evidence for the depletion from the ganglion of a limited store of labeled secretory material. However, the possibility that the ganglion was deteriorating during the perfusion or that membrane repolarization was occurring could not be ex-

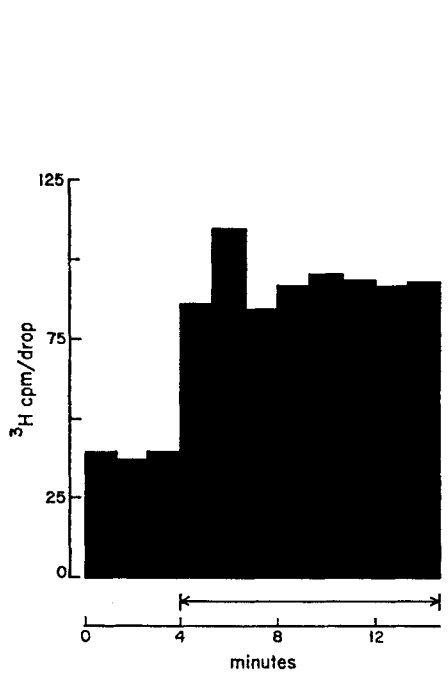


FIGURE 3

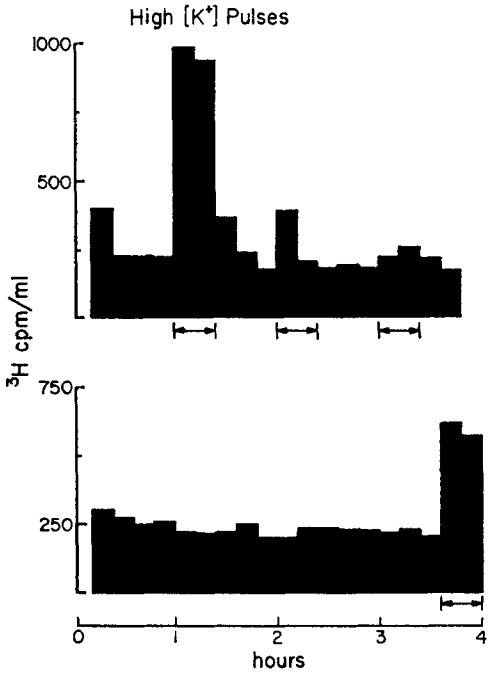


FIGURE 4

FIGURE 3. Detail of the initial fraction taken during high-potassium perfusion. Just before the onset of high-potassium stimulation and for the succeeding 10 min each drop of perfusate (approx. 100 $\mu$l) falling from the ganglion was collected separately. The time between successive drops was approximately 80 sec. Onset and continuation of high-potassium perfusion is indicated by the horizontal line beneath the abscissa.

FIGURE 4. *Top*, perfusion profile for an experiment during which three successive pulses of high-potassium medium (pulse = 2 ml = 24 min) were administered to the ganglion at the times indicated by the horizontal lines. *Bottom*, profile from an experiment in which perfusion with the standard medium was continued for 3 hr 36 min and then high-potassium medium was applied.

cluded in that experiment. Therefore the experiment illustrated in Fig. 4 was performed. Instead of continuous perfusion with high potassium, three pulses of high potassium were delivered. The top profile ($N$ = 11) shows that it is unlikely that the apparent depletion noted in Fig. 1 was the result of membrane repolarization. The mean levels of increased release for the three successive pulses in the 11 experiments were 280%, 180%, and 25%, respectively. It is clear that the successive pulses evoked less release even though

the tissue was bathed in the standard ionic medium for an hour between each pulse. That tissue degeneration over the duration of the perfusion was not occurring can be seen in the bottom profile in Fig. 4 ($N$ = 4). The sharp increase in released material (mean = 250%) after nearly 4 hr of standard perfusion indicated that the tissue was still fully capable of release.

The release of labeled material under high-potassium stimulation was investigated further using calcium-free media. Initially ganglia were isolated, incubated, and rinsed as previously described, but the perfusion and high-potassium stimulation were carried on in calcium-free preparations of the standard media. The results of these studies revealed that some release occurred, but less than 20% of that seen under standard ionic conditions. This low-level release could be abolished altogether by transferring the ganglion out of the standard rinse medium and into a calcium-free rinse for the last 2 hr of the 12 hr rinse period. The perfusion profile in Fig. 5 ($N$ = 5) demonstrates both the abolition of high-potassium stimulated release in calcium-free conditions and the reversibility of this phenomenon.

In order to characterize the labeled materials released from the ganglion, perfusate fractions were taken from four different regions of standard high-potassium perfusions (see Fig. 1), TCA precipitated, and analyzed electrophoretically on SDS-polyacrylamide gels. The four regions consisted of one at the beginning and one at the end of the initial period of control perfusion, and one at the beginning and one at the end of the subsequent high-potassium perfusion. The electrophoresis resulted in the separation, by molecular weight, of most polypeptide species present in the samples (polypeptides with molecular weights below 10,000 migrate as a single band). Representative results of this investigation are illustrated in Fig. 6 ($N$ = 4). Perfusion fractions 1–3 (f 1–3, Fig. 6) contained predominantly 25,000 molecular weight material with a smaller peak of material located somewhat below 75,000 molecular weight. In fractions 8–10, the three fractions immediately preceding the onset of high-potassium perfusion, a similar distribution was evident. With the onset of high-potassium perfusion (f 11–13), a radical change in the distribution of polypeptides was apparent. The 25,000 molecular weight peak disappeared while a very prominent peak at less than 10,000 molecular weight appeared. The distribution of labeled polypeptides in fractions 18–20, taken at the end of high-potassium perfusion, showed no clear prominence of material at any molecular weight.

The bottommost distribution in Fig. 6 ($N$ = 8) was derived from an aqueous supernatant of an homogenate of labeled bag cells themselves. The major concentration of material (over 40% of the total counts per minute) found in the region below 10,000 molecular weight was consistent with the findings of Toevs and Brackenbury (1969) and Toevs (1969) that the bag cells store large amounts of egg-laying substance and that the molecular weight of this material is less than 10,000.

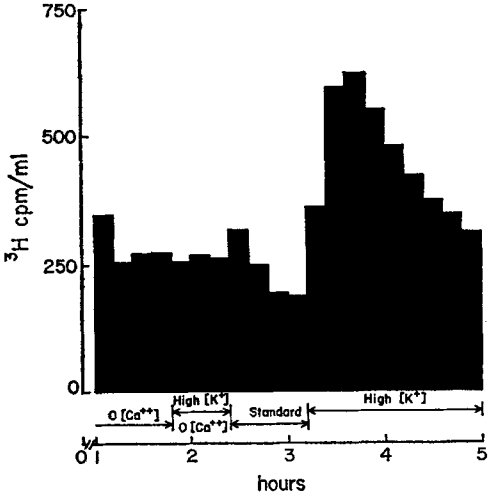


FIGURE 5

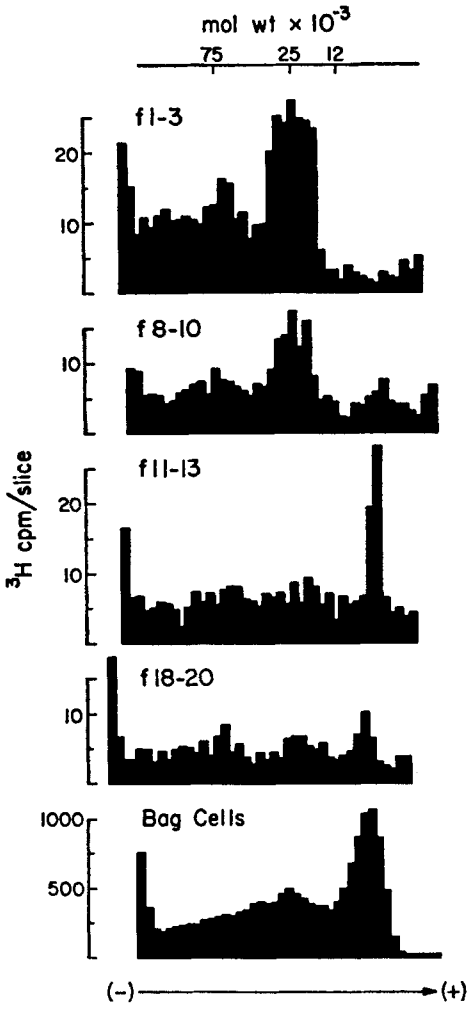


FIGURE 6

FIGURE 5. Perfusion profile from an experiment during which a ganglion preincubated in calcium-free medium was sequentially exposed to calcium-free medium, calcium-free high-potassium medium, standard perfusion medium, and standard high-potassium medium.

FIGURE 6. The distributions of $^3$H-labeled polypeptides after electrophoresis on SDS-polyacrylamide gels are illustrated. The uppermost distribution was derived from perfusate fractions 1–3 collected at the outset of control perfusion in a high-potassium stimulation experiment like that shown in Fig. 1. The second distribution was derived from fractions 8–10 which immediately preceded high-potassium stimulation. Fractions 11–13 were the first three collected after the onset of high-potassium perfusion, and fractions 18–20 were collected at the end of high-potassium perfusion. The bottommost distribution was derived from an aqueous supernatant of an homogenate of the bag cells. Sample preparation from this supernatant was the same as that described for perfusate samples. Due to variation in the length of each gel, the distance of tracking dye migration, and the fractionation procedure comparison of molecular weights from gel to gel is approximate. In this figure the distributions have been aligned according to the location of the 12,000 mol wt marks.

The likelihood of egg-laying hormone release under high-potassium stimulation of the PVG, although supported fully by the foregoing data, was investigated further by means of bioassay. Ganglia were incubated for 60 min in high-potassium medium and then the medium was injected into test animals. Medium bathing PVG's from animals with mature reproductive tracts induced egg laying in five of seven animals tested. Neither high-potassium medium alone ($N = 8$) nor filtered sea water used as the incubation medium ($N = 4$) evoked egg laying in recipients.

## DISCUSSION

During my investigation of the secretory behavior of the PVG in vitro I have posed several questions which the experiments reported in this paper were designed to answer. The first question concerned the feasibility of studying this phenomenon in vitro. By perfusing ganglia previously allowed to incorporate radioactive leucine it was possible to demonstrate that the application of a depolarizing stimulus resulted in a significant and repeatable change in the amount of labeled macromolecules washed out of the tissue (Fig. 1). But is this change in TCA-precipitable material the result of a secretory process? Two lines of evidence suggest that it is. The first of these is the apparent exhaustion of releasable material following a period of stimulation. After an initial abrupt increase of labeled material during high-potassium perfusion (estimated to evoke a depolarization of from 13 to 35 mv), there is a sharp decay of release until the level of precipitable radioactivity returns to background. That this pattern is not the result of membrane or tissue viability changes during the perfusion is indicated by the similar decrement of release when successive pulses of high potassium are administered (Fig. 4). An exhaustion of releasable material would be expected if the high-potassium perfusion is triggering release from a cell population with a limited store of labeled product.

The second line of evidence for secretion is the sensitivity of the potassium-induced release to the calcium content of the medium. Although little work on the calcium sensitivity of invertebrate secretory systems has been performed, in those cases that have been studied (neurotransmitters: Holye, 1955; Miledi and Slater, 1966; Katz and Miledi, 1967; neuroendocrine secretion: Berlind and Cooke, 1968) the results are consistent with the more numerous cases examined in vertebrates (Douglas and Poisner, 1964; Rubin, 1970; Rasmussen, 1970). In virtually every secretory system so far examined the presence of extracellular calcium is a requirement if release is to occur. The abolishment of the potassium-induced release from calcium-deprived PVG's thus constitutes strong evidence that the released material is secreted (Fig. 5).

With this evidence for the secretory cell origin of the released material the next problem was to determine from which cell population the release is

occuring. The rapid onset of secretion after the application of high-potassium medium (Fig. 3) suggests that the initial secretory events are taking place in the rind of connective tissue covering the ganglion. Hence cells having terminal processes in the connective tissue are the most likely to be the secretory elements. From morphological studies only two groups of neurosecretory-type cells are known to send processes into the sheath: the bag cells (two clusters of cells each consisting of about 400 small cells) and the "white cells" of the right rostral quadrant (Frazier et al., 1967).

Several lines of evidence permit a tentative conclusion that the $^3$H-labeled material released from the ganglion in the studies I have reported is primarily secreted from bag cell processes. Frazier et al. (1967) determined that bag cell processes are 10 times more numerous in the connective tissue than are those from the white cells. Thus unless the white cells secrete disproportionately large amounts of material the bulk of the labeled macromolecules in the perfusate originate from the bag cells. This contention is supported by the observation that bag cell clusters isolated from the PVG before incubation and rinse will release only a small amount less precipitable radioactivity in response to high-potassium stimulation (Fig. 2) than will an intact PVG. A bag cell origin of the released material is also suggested by the correspondence between the perfusion studies reported here and previously reported electrophysiologic studies. Frazier et al. (1967) found that electrical stimulation of the pleurovisceral connective nerves had little or no effect on the spontaneous firing rate of the white cells, while similar stimulation triggered repetitive firing of the normally silent bag cells. A later study by Kupfermann and Kandel (1970) showed that brief stimulation of either connective nerve could initiate repetitive discharges in all the cells of both bag cell clusters. These discharges generally continued after the termination of stimulation and occasionally lasted for more than 30 min. In light of these findings the release of labeled material after brief electrical stimulation of the right connective nerve (Fig. 1) almost certainly represents prolonged secretion from repetitively firing bag cells.

Considered alone the gel analysis presented in this paper does not permit a definite conclusion that egg-laying hormone is being secreted from the bag cells during high-potassium perfusion. What can be said is that (*a*) the egg-laying hormone is a polypeptide with molecular weight of approximately 6000 (Toevs and Brackenbury, 1969; Toevs, 1969), (*b*) the gel analysis of the bag cells reveals a prominent concentration of labeled polypeptide in the molecular weight region below 10,000, and (*c*) the perfusate fractions collected before high-potassium perfusion do not contain an obvious accumulation of material in this region while those collected during high-potassium perfusion show a sharp peak. However, when these observations are coupled with the bioassay results they provide a strong argument for the secretion of the egg-laying hormone in response to high-potassium perfusion.

In sum, the evidence presented and discussed above permits the conclusion

that the bag cells and their surrounding connective tissue constitute a neuroendocrine organ involved in the regulation of egg laying. Because of the morphology of the bag cell clusters and their contiguity to other cells of known electrophysiologic function, it is anticipated that fruitful studies of the electrical and biochemical regulation of this neuroendocrine system in vitro will be possible. For example, since *A. californica* shows a seasonal cycle of egg laying (Strumwasser et al., 1969), it would be of considerable interest to know whether the synthesis and/or release of the egg-laying hormone is seasonally modulated, and, if there is seasonal modulation, how the transduction from environmental input to biochemical regulation is achieved.

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