Molecular Weight Distribution of Proteins Synthesized in Single, Identified Neurons of *Aplysia*

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ABSTRACT   Parietovisceral ganglia from *Aplysia californica* were incubated in medium containing leucine-^{3}H. Single, identified nerve cell somas were isolated from the ganglia, and their proteins extracted and separated by electrophoresis on 5% SDS-polyacrylamide gels. The distribution of total or newly synthesized proteins from the single neurons was determined by staining or slicing and liquid scintillation counting of the gels. Experiments showed that: (*a*) a number of proteins were being synthesized in abundance in the nerve cells; (*b*) different, identified neurons showed reproducibly different labeling patterns in the gels; (*c*) cells R2 and R15, which showed different distributions of radioactivity in the gels, had similar staining patterns; and (*d*) there was significant incorporation into material of high (>75,000) molecular weight in most of the cells.

INTRODUCTION

The abdominal (parietovisceral) ganglion of *Aplysia californica* contains a number of large, identifiable nerve cells. The neurons have been characterized by their morphology and electrophysiology, but little research has centered on the biochemistry of *Aplysia* nerve cells (Giller and Schwartz, 1968; Berry, 1969; Toevs and Brackenbury, 1969; Peterson, 1970; Peterson and Kernell, 1970). The cells differ greatly from each other in terms of spontaneous electrical activity, neurosecretory function, and the effect of applied acetylcholine (Frazier et al., 1967; Tauc and Gershenfeld, 1962). One such cell, R15,[1] exhibits a circadian rhythm in spontaneous spike output, is whitish in color upon epiillumination (Strumwasser, 1965), and is presumed to be neurosecretory (Bernstein, 1967; Coggeshall, 1967). In contrast, the largest cell in the ganglion, R2, is brown-orange–pigmented, and shows little

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[1] The nomenclature of Frazier et al. (1967) is used in this paper. R2 is the giant cell of Tauc (1962), and R15 is Strumwasser's (1965) parabolic burster cell.

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spontaneous electrical activity (Frazier et al., 1967). Because these nerve cells are large (up to $\frac{1}{2}$ mm in diameter), identifiable, and have different properties, they are promising candidates for biochemical studies of single nerve cells.

In this paper, results are presented of studies of amino acid incorporation in single neurons. Isolated abdominal ganglia of *Aplysia* were incubated in a defined medium containing radioactive amino acid. Single nerve cell somas were removed and their proteins were extracted and analyzed by poly-acrylamide gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulfate (SDS).

The experiments presented here were designed to form a foundation for determining whether correlations between nervous function and protein synthesis exist. A knowledge of what proteins, if any, are synthesized in response to neuronal functioning will be required to understand the role of proteins in such complex processes as memory and learning.

MATERIALS AND METHODS

1. Animal

The gastropod mollusc, *Aplysia californica*, obtained from Pacific Biomarine Supply Co., Venice, Calif., was used throughout these experiments. Animals were maintained, unfed, in a circulating seawater system of 1,500 gal at 14°C under 12:12 LD light conditions. During the dark period red fluorescent lamps were on, but red light has been shown by Strumwasser (personal communication) not to influence the circadian locomotor rhythm. Each animal was weighed before use, and its reproductive tract weight (sexual maturity) was obtained after dissection.

2. Ganglion Labeling

Each abdominal ganglion was dissected from *Aplysia* with pleurovisceral connectives left long, rinsed in filtered seawater, and incubated for 4 hr or overnight (16–21 hr) at 14°C in 2.5 ml of the following medium (mM): 460 NaCl, 10.4 KCl, 11 $CaCl_2$, 55 $MgCl_2$, 0.20 sodium phosphate buffer (pH 7.8), 2.4 $NaHCO_3$, 1% glucose, 25 μCi L-leucine-4,5-^{3}H (New England Nuclear Corp., Boston, Mass., 30–50 Ci/mmole), vitamins, and amino acids. The vitamins and amino acids were obtained from Micro-biological Associates, Bethesda, Md., and are those used for Eagle's (1959) minimum essential medium (MEM). L-Glutamine was used at 29 mg/liter ($\frac{1}{10}$ MEM concentration), the other essential amino acids at $\frac{1}{50}$ MEM concentration (L-leucine at 1.05 mg/liter, giving 8.5×10^6 cpm/μg leucine), and nonessential amino acids at $\frac{1}{5}$ the concentration recommended by Microbiological Associates. The medium, minus label, was adjusted to pH 7.7 with 1 N NaOH, filtered (Millipore 0.22 μ filter), and stored frozen until use. Tests of electrophysiological activity indicate that neurons show normal action potentials and synaptic activity in the above medium.

3. Single Cell Dissection (After Strumwasser, Personal Communication)

The ganglion was transferred to a dissecting dish containing filtered seawater at 18–20°C, and the nerve trunks were pinned down. While viewing through a dissecting microscope, a small slit was made in the ganglion sheath, above the nerve cell of interest, using small iridectomy scissors and No. 5 Dumoxel tweezers (A. Dumont and Sons, Switzerland). The nerve cell was gently forced through the sheath. After removing any contaminating nerve cells, the nerve cell soma was plucked at its base with tweezers and transferred to a small test tube immersed in the seawater bath. The tube was taken from the bath, and upon removing the tweezers, the cell was released and trapped in the tube by surface tension. The cell was transferred to a glass grinding dish (depression slide) by micropipette. SDS-sample buffer (1 M urea, 10% glycerol, 0.0015% bromphenol blue, 0.1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 0.01 M sodium phosphate buffer, pH 7.2) was added, and the cell was ground with a glass rod. The sample (final volume of 20–25 μl) was frozen until use (1–10 days).

4. Sample Treatment

After thawing, samples were heated to 65°C for 10–15 min, and centrifuged at 20,000 g for 10 min in 1 ml conical centrifuge tubes. The heating step minimizes aggregation (Burgess and Denhardt, 1969). Over 75% of nerve cell protein (Lowry et al., 1951) and TCA-precipitable counts was in the supernatant.

5. SDS-Polyacrylamide Gel Electrophoresis

The gel solutions and general procedure for polyacrylamide gel electrophoresis were those of Shapiro et al. (1967), Ornstein (1964), and Davis (1964) with the following modifications: gel tubes were acid-washed, 100 μl microsampling pipettes (Corning No. 7099S) cut to 9 cm length. A short length of tubing at the bottom of the gel tube allowed clamping during hardening of the gel solution. The gel solution was drawn into the tube to 6 cm height (about 100 μl volume of the 5% gel solution). Water was added to the top surface of the hardening gel with a 25 μl micropipette either by draining down the side of the gel tube or by layering water just above the gel surface and gently removing the trapped air bubble by drawing it off. Just before use, the water layer (and any unpolymerized gel solution) was removed by inserting an empty micropipette to just above the surface of the gel and "injecting" air, thereby forcing water out of the top of the tube where it was absorbed onto tissue paper. After sample and covering buffer (0.1 M sodium phosphate, pH 7.2, with 0.1% SDS) had been applied, the gels were subjected to electrophoresis (sample migration towards anode) at a constant voltage of 20 v for 4.5 hr or 30 v for 3 hr.

6. Staining

After electrophoresis, the gel was extruded from the tube with a tight-fitting plunger. The gels were stained in a filtered (Whatman No. 1), 0.25% solution of Coomassie Brilliant Blue in methanol:acetic acid:water (5:1:5) overnight. The gels were de-

stained in 7.5 % acetic acid, 5 % methanol for 24 hr, and were placed in a narrow trough for scanning with a Joyce, Loebl and Co. (Gateshead, England) microdensitometer.

7. Radioactive Gel Counting

More than 90 % of the TCA-precipitable counts in the sample entered the gel during electrophoresis. As discussed below, significant, unincorporated leucine-^{3}H is present only in the top three or four gel slices. Gels which were to be counted were not stained. Instead, the gel was extruded 1¼ mm at a time from its tube with a tight-fitting plunger, sliced, and placed in 3 or 5 ml of counting fluid. The counting fluid contained, per liter of toluene, 42 ml Liquifluor (New England Nuclear Corp.), 100 ml NCS (Nuclear Chicago solubilizer, Des Plaines, Ill.), and 20 ml 4 M NH_4OH

The apparatus used to extrude and slice the gels, and the counting fluid are described in detail elsewhere (Ward et al., 1970). Virtually all counts were released from the gel slices and counted at 38 % efficiency in a Beckman LS200 liquid scintillation counter.

RESULTS

1. Gel Staining

Single R2 and R15 nerve cell extracts were layered on SDS polyacrylamide gels and stained with Coomassie Brilliant Blue after electrophoresis. Fig 1 shows microdensitometer tracings of two of the gel patterns. R2, the largest cell in the ganglion, contains about 7 μg of protein (200-400 g animals; Giller and Schwartz, 1968). Despite the morphological and electrophysiological differences between R2 and R15, the staining patterns of proteins from the two cells are similar.

The migration of known (marker) proteins was used to calibrate the gels according to molecular weight (Shapiro et al., 1967; Weber and Osborn, 1969). As expected, protein migration relative to the bromphenol blue tracking dye was a function of the log of molecular weight (mol wt). Below about 10,000 mol wt, however, I found that the relationship no longer holds: insulin polypeptides (5,000 mol wt) and bacitracin (1,450 mol wt) both migrate at a rate corresponding to about 10,000 mol wt. There thus appears to be a piling-up of low molecular weight polypeptides in front of the tracking dye in the gels.

The marker proteins also give an indication of the resolving power in the gels (Fig. 1). Resolution can be increased by running the proteins further in the gels.

The small amount of stained material in the gels below about 25,000 mol wt could be due to a relative absence of low molecular weight material in the nerve cells, but could also result from a loss of such polypeptides during staining and destaining.
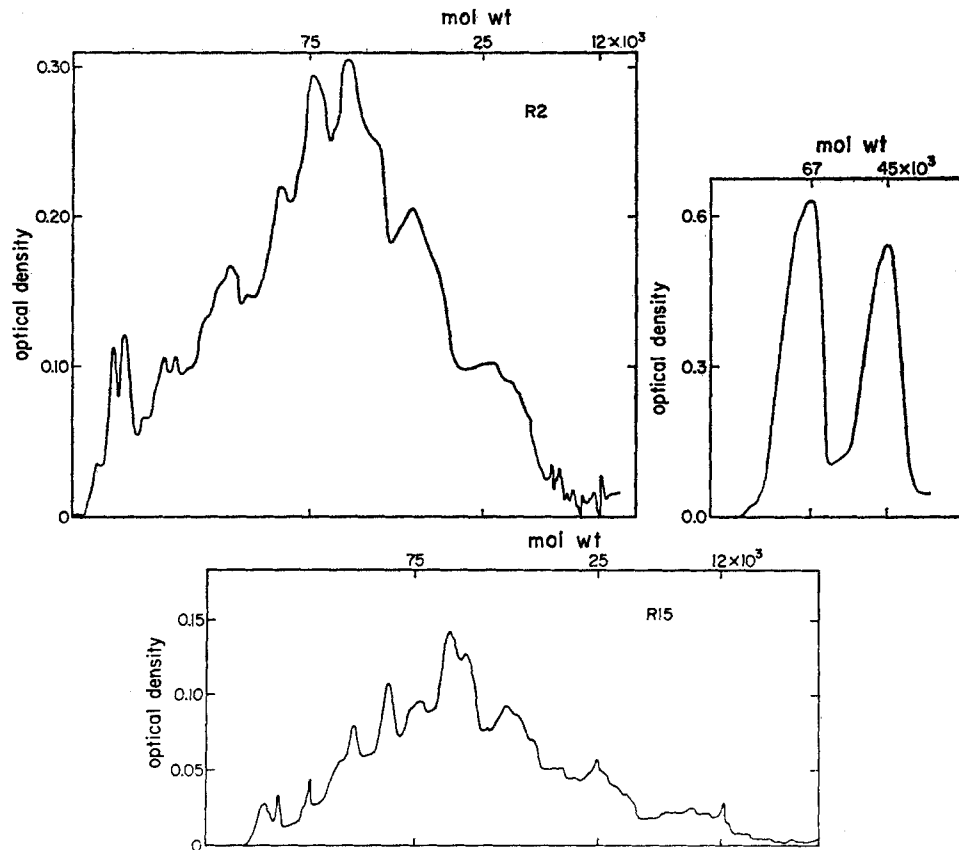
FIGURE 1. Coomassie Blue staining pattern of proteins from a single R2 and R15 nerve cell soma. Nerve cell extract preparation, SDS–polyacrylamide gel electrophoresis, and microdensitometer tracing as described in Materials and Methods. Protein molecular weight calibration of the gels is described in the text. Also shown, as an indication of the resolution of protein species in the gels, is the staining pattern of two marker proteins. 2.5 μg each of bovine albumin (67,000 mol wt) and ovalbumin (45,000 mol wt) in SDS-sample buffer were stained and traced after electrophoresis as described in Materials and Methods. The marker proteins were obtained from Mann Research Laboratories, New York.

2. Gel Labeling Patterns

Protein synthesis in the single neurons was studied by incubating the abdominal ganglion in media containing leucine-³H prior to single-cell dissection. The nerve cell extract was then layered on SDS-polyacrylamide gels, which were sliced and counted after electrophoresis. Typical patterns of label distribution for cell R2 after incubation of ganglia in leucine-³H are shown in Fig. 2 (a–d). The presence of radioactivity throughout the gel suggests, not surprisingly, that a number of proteins are being synthesized in quantity by

this cell. A few general observations can be made about the R2 patterns:
(*a*) there exists relatively little leucine-³H–containing protein with molecular
weight under 10,000; (*b*) there is considerable labeled material corresponding
to high molecular weight protein species in the gels; (*c*) incorporation patterns
at 4 and 19–20 hr are relatively similar.

There are control experiments which support points *b* and *c* above. Dis-
cussed below is an experiment with puromycin which supports point *b*. Next
there is a discussion of control experiments concerning unincorporated leu-
cine-³H, glial cell contamination, and the effects of ganglion isolation which
indicate that gel pattern similarity at 4 and 20 hr (point *c*) is not an experi-
mental artifact. Further support for point *c* comes from the different patterns
obtained for other ganglion neurons.

Concerning point *b*, the label in the gels corresponding to proteins with
molecular weight greater than 75,000 could be large polypeptide chains,
but could also be aggregated chains or polypeptides attached to other struc-
tures which are not broken apart by the extract treatment. There is some
evidence that they are, in fact, single, large polypeptides. Fig. 3 shows that
the R2 incorporation pattern is considerably altered if incorporation takes
place in the presence of puromycin. Puromycin is an inhibitor of protein
synthesis which causes premature release of growing polypeptides from ribo-
somes, resulting in the formation of peptidyl-puromycin (Nathans, 1964;
Allen and Zamecnik, 1962). Therefore, the decreased amount of label mi-
grating at higher molecular weight is expected, since the longer a polypep-
tide (normally) is, the greater the chance of premature termination. The
absence of significant label in gel slices corresponding to higher molecular
weights (other than the free leucine-³H counts) suggests that the high molecu-
lar weight material present in normal R2 extracts does not represent smaller
peptides which are part of incompletely dissociated complexes.

It is not known why leucine incorporation is only reduced to 23 % despite
the high levels of puromycin which were used. Low permeability, through
connective tissue and cells, could be a partial cause of the incomplete inhibi-
tion. Especially because of the high concentration of puromycin used, there
are reservations concerning the above interpretation of the experiment:
some secondary mode of action of puromycin might inhibit aggregation. In
addition, there is no independent evidence that high molecular weight poly-
peptides are more sensitive to puromycin in *Aplysia*.

Concerning point *c* above, the similarity of gel patterns at 4 and 20 hr is
surprising. Not only would differences in half-life of various protein species
result in an altered pattern, but also, since only the soma of the neuron is
being examined, protein species which are selectively transported down the
axon should produce changed patterns. (Transport out of the soma could
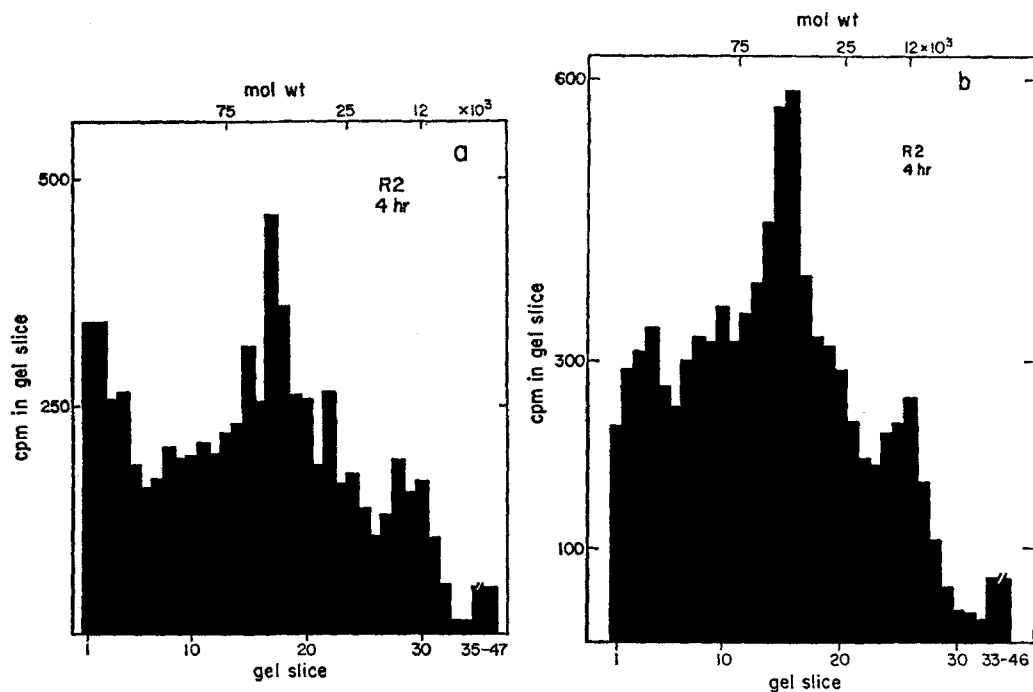eliminate some of the labeled bands.) It is possible that synthesis for shorter

FIGURE 2

or longer periods of time would be necessary to show such changes. Thus, incubation for shorter periods might increase the amount of label in some bands because the corresponding proteins had not yet been transported down the axon.

There is unincorporated leucine-^{3}H in the neuron extracts, but it cannot account for the similar patterns at 4 and 20 hr. Fig. 4 indicates that unincorporated leucine-^{3}H is found in significant quantity only in the top three or four gel slices. The amount of leucine in the top few slices indicates the maximum distortion of labeling patterns which leucine-^{3}H could cause. It is not surprising that leucine does not migrate far into the gel. The running pH of 7.2 is above the isoelectric point for leucine (pI = 5.98), but the basic ionization constant for leucine of 9.60 implies that only about 1 leucine molecule in 250 carries a net charge at pH 7.2 (calculated using the Henderson-Hasselbach equation).

Additional support for the notion that leucine and other small, labeled molecules do not alter the gel patterns significantly comes from the following experiment: an R2 extract was run on a gel which was then fixed and stained prior to slicing and counting. Small, unfixed molecules will have diffused out of the gel before counting. The resulting label pattern was not greatly changed. (Similarly, the label pattern for R15 does not change greatly after such a procedure.) Nevertheless, the procedure of staining and tracing
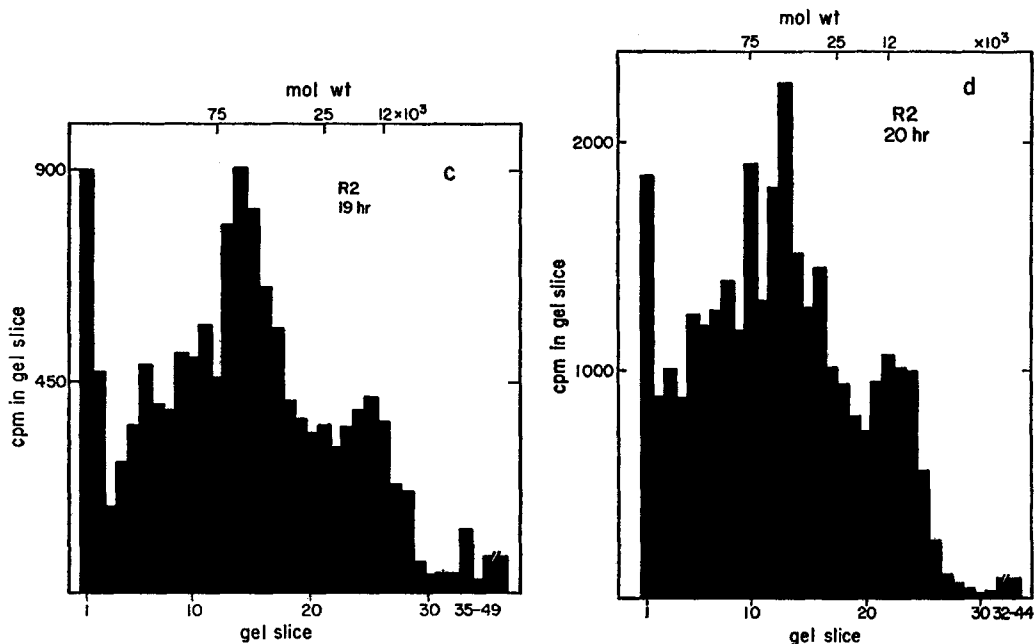
FIGURE 2 *continued*

FIGURE 2. Gel pattern from labeled R2 somas. Gel-labeling patterns from extracts of single R2 cell bodies obtained after ganglion incubation in leucine-^{3}H-containing medium for 4 hr (a, b), 19 hr (c), and 20 hr (d). 8.5 × 10^6 cpm = 1 μg L-leucine. A gel slice is 1¼ mm in length. A portion of the gels, in front of the tracking dye, was extruded and counted as a single piece; its length is indicated by the corresponding number of gel slices. Protein molecular weight calibration, as determined by migration relative to the bromphenol blue tracking dye, is indicated. 45 cpm background was subtracted.

the gels before slicing and counting was not followed routinely because of the possibility of selective loss of some polypeptides during the fixing and staining process.

There is a layer of glial cells around the cell soma of R2. Similar patterns at 4 and 20 hr could result if most protein synthesis were found to occur in this layer. Such a finding would be surprising because of the relatively small mass of the glial cells. In addition, radioautographic evidence of Strumwasser (1967) shows that this is not the case for some abdominal ganglion neurons.

In order to control for the effects of ganglion removal from the animal (cut nerves, incubation medium effects, etc.), leucine-^{3}H injections into whole animals were performed. Subsequently the abdominal ganglion was removed, and single cells were dissected and extracts prepared for electrophoresis. Fig. 5 shows the resulting pattern for an R2 cell. Incorporated counts were low. One difficulty was the rapid excretion of label from the
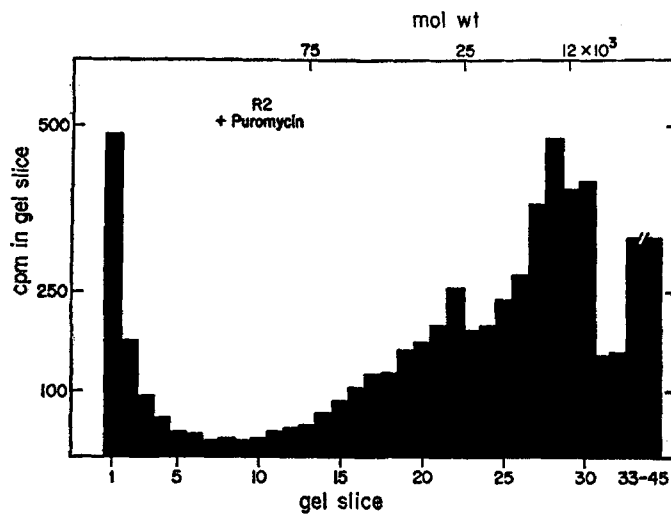
FIGURE 3. Effect of puromycin on R2 gel-labeling pattern. The *Aplysia* ganglion was preincubated for 2 hr in filtered seawater containing 1 mg/ml puromycin, and incubated in medium containing 1 mg/ml puromycin for $19\frac{1}{2}$ hr. Otherwise, details as in caption to Fig. 2 and in Materials and Methods.
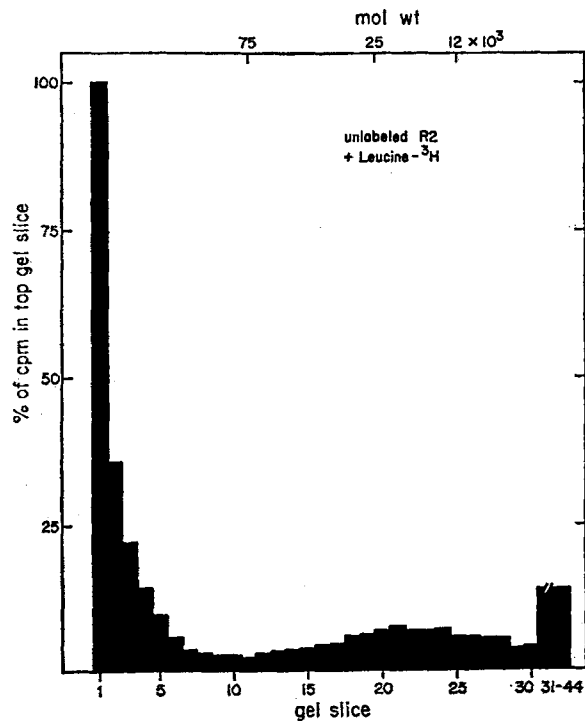

FIGURE 4. Unincorporated leucine-^{3}H migration in the gels. An unlabeled R2 neuron extract was mixed with 0.4 μCi leucine-^{3}H before electrophoresis. Less than 30% of the leucine-^{3}H entered the gel, and half of that was in the top three slices. 70% of the leucine-^{3}H was recovered from the buffer in the gel tube above the gel. See caption to Fig. 2 and Materials and Methods for details.
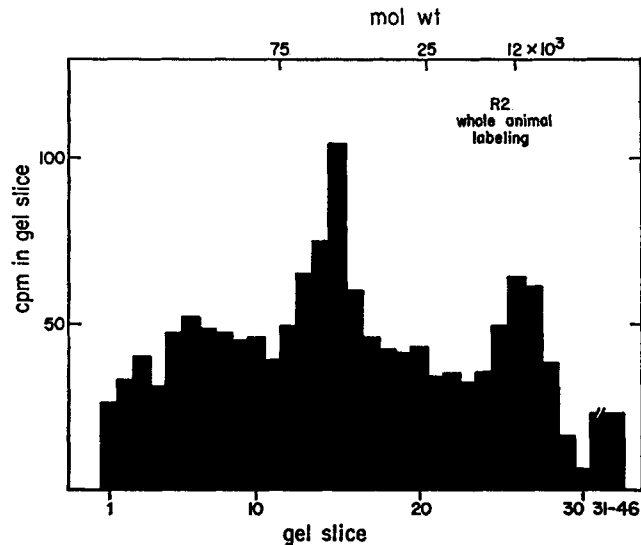
FIGURE 5. R2–gel-labeling pattern obtained after whole animal labeling. SDS–poly-acrylamide gel–labeling pattern for the extract of an R2 neuron obtained from an *Aplysia* which had been injected with 200 μCi of L-leucine-^{3}H-4,5 (58 Ci/mmole) at 11 and 23 hr before sacrifice. The 0.20 ml of label in 0.01 M HCl was neutralized with 0.020 ml of 0.1 M NaOH before injection into the haemocoel through the anterior third of the foot. See caption to Fig. 2 and Materials and Methods for details.

animals: 3 hr after injection of leucine-^{3}H (or uridine-^{3}H) into the blood cavity of *Aplysia*, 80–90 % of the label was in the aquarium seawater. There are, at least, no radical differences between the R2 pattern in Fig. 5 and that obtained after incubation of the isolated ganglion.

After 20 hr incubation of left pedal and pleural ganglia, the left pleural giant cell (LGC; Hughes and Tauc, 1963) has a gel-labeling pattern indistinguishable from that for R2. The LGC and R2 are considered to be symmetrical cells: they are similar in size, coloration, electrical activity, and axon distribution.

At least two large, left-side neurons (precise identification has not been made, but the cells are in the area of L6 and L7) have gel-labeling patterns similar to that for R2.

The gel-labeling pattern obtained for R15 is significantly different from that for R2 (Fig. 6 [a–d]). As with R2, however, incorporation patterns at 4 and 17–21 hr are similar.

Fig. 7 (a, b) shows gel-labeling patterns for cells R14 and L11. R14 is a slow pacer (about 2 spikes/min) and is thought to be neurosecretory, while L11 is a fast pacer (2/sec; Frazier et al., 1967). The pattern shown for R14 is not always observed: other R14 cells show a pattern more similar to that
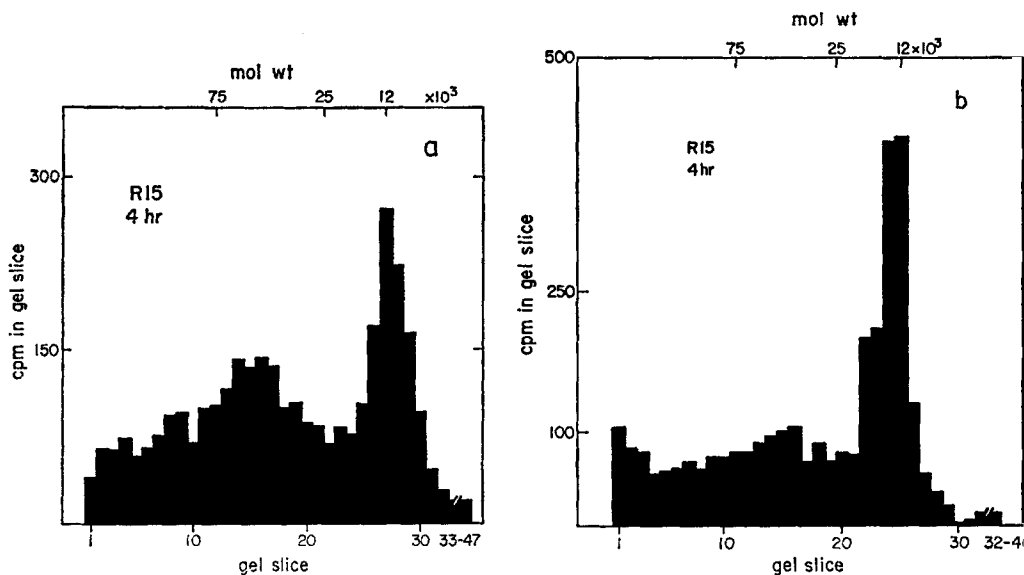
FIGURE 6

of R15. Since R14 is fragile and difficult to isolate intact, variation in the pattern of R14 may be caused by damage occurring during cell isolation.

A summary of incorporation data obtained for the various neurons is shown in Table I. A comparison of incorporation at 4 and at 16–21 hr suggests that incorporation is roughly linear for R2 and R15. Also shown, as one measure of differences in patterns of radioactivity between the neurons, is the ratio of counts in the gel corresponding to about 60,000 mol wt (three slices) to the counts at about the 12,000 mol wt position (three slices). R2 and R15 are significantly different by this criterion. As shown, ganglion incubation in the presence of cycloheximide, a protein synthesis inhibitor in mammalian cells (Ennis and Lubin, 1964), could reduce radioactivity in the gels by 80 to 90 %. For unknown reasons, cycloheximide was not always so effective in blocking leucine incorporation. Even at the high level of 200 $\mu g/ml$, some experiments showed synthesis rates as high as 50 % of normal.

DISCUSSION

The experiments presented in this paper were designed to form the groundwork for the eventual determination of what proteins are required in the greatest abundance by nerve cells, and whether there are specific proteins whose synthesis is correlated with electrical activity in neurons. The following results were obtained: (a) The leucine-^{3}H incorporation patterns for R2 and R15 were quite dissimilar. Thus, different neurons in the same ganglion can be synthesizing proteins at different rates, if not different proteins. (b) For both R2 and R15 there was no significant difference between 4 and
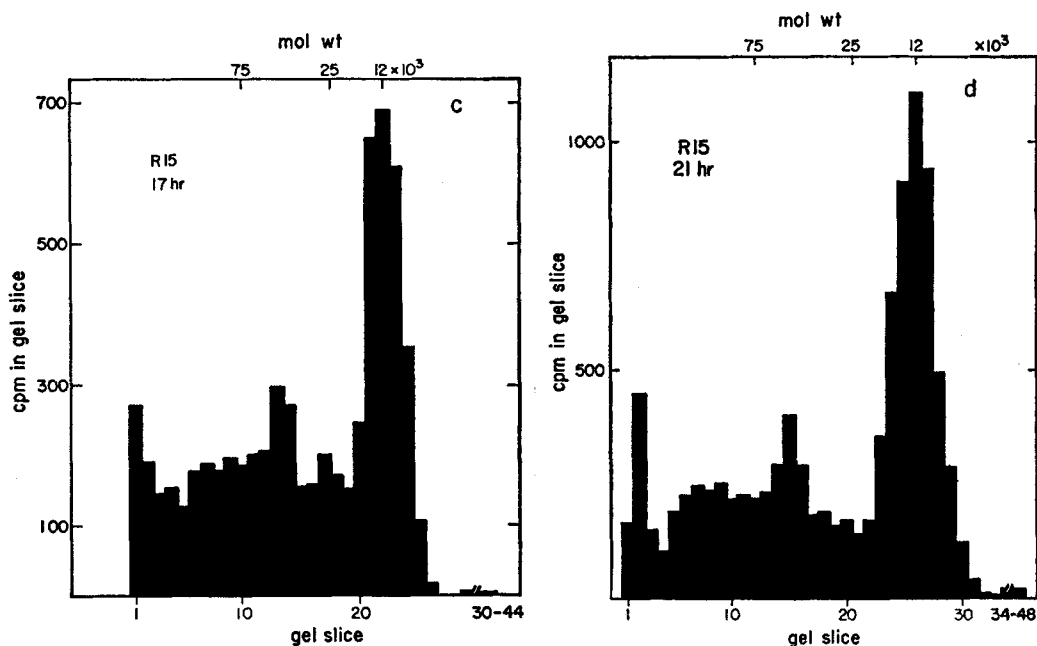
FIGURE 6 *continued*

FIGURE 6. Gel pattern from labeled R15 somas. SDS–polyacrylamide gel–labeling patterns from extracts of single R15 neuron cell bodies obtained after ganglion incubation in leucine-^{3}H–containing medium for 4 hr (a, b), 17 hr (c), and 21 hr (d). Details are described in Materials and Methods and caption to Fig. 2.

16–21 hr labeling patterns. Total incorporation of leucine was greater after 16–21 hr, but the ratio of counts at various gel positions was not significantly different from the 4 hr values. However, the variance among cells labeled for the same period was surprisingly large (Table I), and could have masked some differences. Such variance could have been due to experimental technique (cell damage during isolation or contamination with smaller neurons). Alternatively, there are numerous differences between animals which could contribute to the state of the neurons. Variables which do not singly account for differences in total incorporation or 60 K:12 K label ratios between different R2 cells (or R15 cells) include animal size (animals weighed from 150 to 400 g), number of days in the laboratory, degree of starvation, and sexual maturity (reproductive tract weights ranged from 0.08 to 2.0 g. Strumwasser et al. (1969) discuss the relation between tract weight and sexual maturity). (*c*) There was significant incorporation into high (>75,000) molecular weight material. (*d*) A number of proteins are synthesized in abundance in most of the single nerve cells.

There are, however, a number of complicating factors: (*a*) Only the somas of the nerve cells are obtained. Any (*b*) synthesized proteins which are trans-
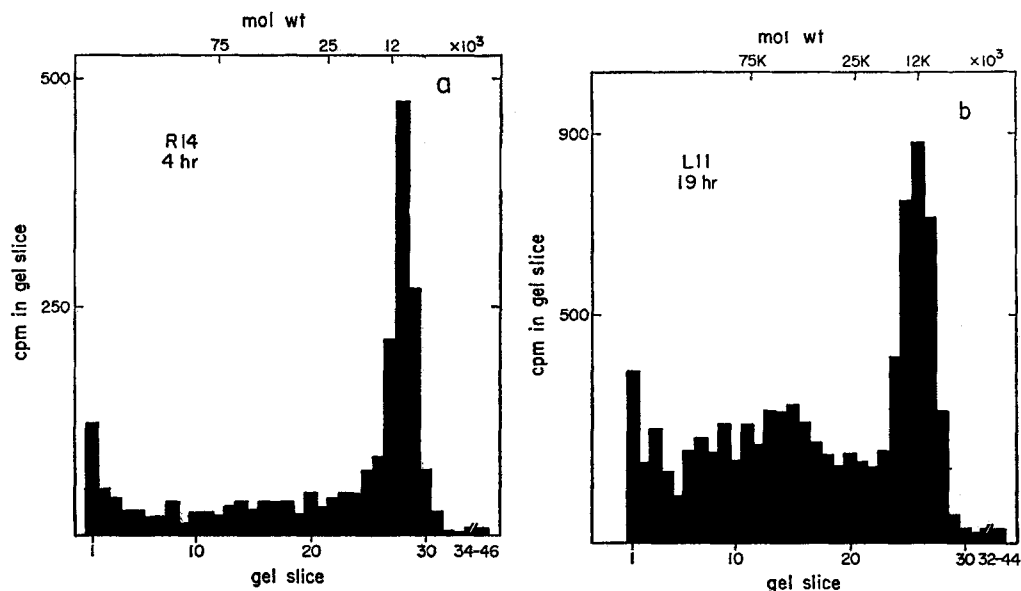
FIGURE 7. R14 and L11 gel-labeling patterns. SDS–polyacrylamide gel-labeling patterns from extracts of single R14 (a) and L11 (b) cell somas obtained after ganglion incubation in medium containing leucine-³H for 4 and 19 hr, respectively. Details are described in Materials and Methods and caption to Fig. 2.

TABLE I

SUMMARY OF DATA FOR R2 AND R15 NEURONS

Cell	Incorporation time	Leucine-³H in gel*	60 K:12 K ratio‡	No. of cells
	hr	*mμg*		
R2	4	0.47±0.29	2.3 ±0.8	6
	16–21	2.5 ±1.3	2.0 ±0.6	7
	19	0.27§		
R15	4	0.25±0.13	0.40±0.16	4
	16–21	1.0 ±0.2	0.45±0.15	4
	18	0.09§		

* Amount in gel ± sample standard deviation. The counts in the top three gel slices were not included.

‡ Ratio of counts in three gel slices at about 60,000 mol wt to counts in three gel slices at about 12,000 mol wt ± sample standard deviation.

§ Ganglia were preincubated for 2 hr in filtered seawater containing 200 μg/ml cycloheximide, and the incorporation medium also contained 200 μg/ml cycloheximide.

ported down the axons are lost. (*b*) A contaminating layer of glial cells deeply invaginates the surface of the neurons. There is, however, radioautographic evidence indicating that glial cells are not the site of most leucine incorporation at 4 hr (Strumwasser, 1967). (*c*) Because of pool effects and permeability

differences, leucine-³H incorporation cannot be equated with total leucine incorporation. (*d*) There may be effects caused by removal of the ganglion from *Aplysia* prior to labeling (cut nerve trunks, etc.), but whole animal labeling experiments indicate that such effects are not large. (*e*) Significant, unincorporated leucine-³H is present in the top three or four slices. (*f*) Leucine-³H incorporation patterns in the gels do not correspond to total protein because of different relative amounts of leucine in different proteins. Experiments with other labeled amino acids show similar incorporation patterns, but with relative peak heights changed. (*g*) Unlike R2, the gel-labeling patterns for R15 appear quite different from the staining pattern shown in Fig. 1. There is little stain corresponding to the peak of labeled material at about 12,000 mol wt. It is tempting to conclude that such material either has a short half-life or is selectively transported out of the soma. Alternatively, the relative staining of the 12K peak polypeptide(s) may be weak, or the polypeptides may contain a large number of leucine residues. (That the latter is not a sufficient explanation is suggested by experiments involving other labeled amino acids.)

Why do two neurons in the same ganglion, R2 and R15, have such different incorporation patterns? A major difference between the neurons is that, as mentioned in the Introduction, R15 is neurosecretory, while R2 is not. However, there are two reasons why the 12K peak material may not be involved in neurosecretion. (*a*) L11, which is not classed as neurosecretory (Frazier et al., 1967), has a gel-labeling pattern much more similar to R15 than to R2. (*b*) It is very unlikely that the 12K peak material in R15 is the neurosecretory product itself because it is aqueous insoluble. This is also true of the 12K peak material in R14 and L11 (unpublished observations). An understanding of why R2 and R15 have such different incorporation patterns will require further experiments.

The experiments described above indicate the kind of information obtainable from *Aplysia* neurons using SDS–polyacrylamide gel electrophoresis. With this technique, one can begin to ask questions about the coupling of the synthesis of particular proteins to neuronal functions. Is there a connection between protein metabolism and electrical activity? Do rhythms (circadian, seasonal, etc.) of synthesis exist for certain proteins? What are the proteins which nerve cells synthesize in abundance?

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