Proc. Nat. Acad. Sci. USA
Vol. 68, No. 11, pp. 2782–2786, November 1971

Supraoptic Neurosecretory Neurons of the Guinea Pig in Organ Culture. Biosynthesis of Vasopressin and Neurophysin

(hypothalamic median eminence/paraventricular nuclei/guinea pig/electron microscopy)

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Communicated by Sidney Udenfriend, August 18, 1971

ABSTRACT **Fragments of the anterior hypothalamus that contain supraoptic nuclei and short axonal segments from adult guinea pigs have been kept in organ culture for up to 15 days. Electron micrographs displayed intact nuclei, Nissl substance, Golgi bodies, and an ultrastructure characteristic of viable neurosecretory cells; by contrast, the surrounding neurophil showed extensive degeneration. The cultured hypothalamic tissues of the guinea pig that were pulsed with [^{3}H]uridine incorporated label into the RNA of neurosecretory neurons, as determined by radioautography and chemical analysis. Furthermore, and most important, these cells retained a complement of hormones and the ability to incorporate ^{3}H- and ^{35}S-labeled amino acids into vasopressin, neurophysin, and other polypeptides. This incorporation was inhibited by either puromycin or cycloheximide.**

The central nervous system exerts an influence on many diverse endocrine processes involved in homeostasis, adaptation, and normal growth and development (1). Hypothalamic neurosecretory neurons (2) constitute the means by which a vast array of neural information is channeled to endocrine glands and by which neural inputs are translated into endocrine function. These specialized neurons are found diffusely distributed throughout the medial basal region of the hypothalamus (hypophysiotropic area), as well as in specific aggregates in the anterior portion (supraoptic and paraventricular nuclei). The neuronal perikarya of the supraoptic and paraventricular nuclei send their axons to terminate in the distal segment of the neurohypophysis, where their respective secretory products, vasopressin and oxytocin, are stored and released.

Nerve cell bodies in the supraoptic nuclei are the essential sites for an apparent concerted biosynthesis (3, 4) of the octapeptide hormone, vasopressin, and its hormone-binding protein, neurophysin, with a subunit molecular weight of about 10,000 (5). Subsequently, the hormone–protein complex is packaged within neurosecretory granules of about 0.1-0.2 nm diameter that are then transported rapidly to the nerve terminal where the release of both vasopressin and neurophysin takes place in response to nerve excitation. Isotope studies performed *in vivo* and *in vitro* have, furthermore, indicated that the biosynthetic pathways for both vasopressin and neurophysin involve initially one or more precursor molecules produced by the synthesizing apparatus of the cellular protein (3, 4).

Abbreviation: SDS, sodium dodecyl sulfate.

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The intact animal and various freshly excised segments of the hypothalamo–neurohypophysial complex, incubated *in vitro*, have proved to be useful experimental systems for the study of many features of the biochemistry of "neurosecretion," neuroglial (pituicyte) RNA, and protein biosynthesis. These preparations, however, do not lend themselves readily to studies concerned with the interrelationships between neural inputs and those biochemical mechanisms controlling gene expression, the rate of production, packaging, transport, and secretion of neurohormones and binding proteins, and neuron-neuroglial interactions. In most instances, periods of hours or several days are involved and the complexity of responses in the *in vivo* situation may be formidable. Consequently, we have developed *in vitro* hypothalamic organ-culture preparations that contain supraoptic neurosecretory neurons that appear to be viable for periods up to 15 days, and are capable of synthesizing the hormone, the binding protein, and RNA.

MATERIALS AND METHODS

Culture of tissues

Male guinea pigs weighing between 150–250 g were decapitated and the brains were removed under sterile conditions. The bisected hypothalamic-median-eminence complex was lifted from the brain by undercutting to a depth slightly greater than 1 mm and placed on lens paper lying on stainless-steel grids in 60-mm Petri dishes containing 10 ml of incubation medium. The composition of the culture medium was Eagle's minimal essential (MEM), 85%; fetal-calf serum, 10%; 0.2 M glutamine, 1%; nonessential amino-acid mixture, 1%; HEPES, 2.8×10^{-2} M; Tricine, 10^{-2} M; glucose, 2.78×10^{-2} M; sodium penicillin G, 100 U/ml; the pH was adjusted to 7.2 with NaOH. The tissues were incubated in an atmosphere of 5% CO_2–95% air, at 35°C in a humidified incubator. No more than six sections were cultured in a single Petri dish; the medium was changed daily.

Morphological Studies and Radioautography. Isolated hypothalamic tissues were immersed in phosphate-buffered formaldehyde fixative (6), followed by post fixation in osmium tetroxide, and routine embedding in Epon (7).

Radioautography was performed on 1-μm Epon-embedded sections, according to the standard dipping technique (8).

Radioactive compounds and analytical procedures

^{35}S-labeled cystine with a specific activity between 10^8–10^9 cpm/μmol was prepared from *Escherichia coli*, as described

(9); ^{3}H-labeled L-tyrosine and [^{3}H]proline with stated specific activities greater than 25 Ci/mmol were purchased from New England Nuclear Corp. The isotopic purity of the labeled amino acids was checked by thin-layer chromatography (TLC). [5-^{3}H]uridine >20 Ci/mmol) was from New England Nuclear Corp.

Counting was performed in a liquid scintillation spectrophotometer in an Aquasol scintillation mixture (New England Nuclear Corp.). Radioactivity in trichloroacetic acid-insoluble proteins was measured as described (9). Protein, RNA, and DNA were determined, respectively, according to Lowry *et al.* (10), Fleck and Munro (11), and Burton (12). Vasopressin was estimated by means of the pressor bioassay (13).

Incorporation studies

Isolation of Labeled Vasopressin. Hypothalamic-median eminence organ cultures were pulsed with either [^{35}S]cystine or a mixture of [^{3}H]tyrosine and [^{3}H]proline for periods of 8–20 hr in medium lacking the amino acid(s) that correspond to the added radioactive component(s). After the incubation period, the tissues (usually from 10 guinea pigs) were rinsed briefly in phosphate-buffered saline, and homogenized in 4–5 ml of an ice-cold mixture of 0.02 HCl–0.2 M acetic acid (HCl–HAc). The insoluble materials, removed by centrifugation, were washed twice with 4–5 ml of HCl–HAc and subsequently used for the determination of radioactivity in proteins. After concentration under reduced pressure, the extract was passed through a 0.9 × 20 cm column of Sephadex G-25 in HCl–HAc; vasopressin emerged after 2.5 void volumes. The pooled vasopressin fractions were purified by: (*a*) affinity chromatography with Sepharose-bound neurophysin, (*b*) CM-cellex chromatography (9, 14), (*c*) rechromatography on CM-cellex, and (*d*) 1- or 2-dimensional TLC.

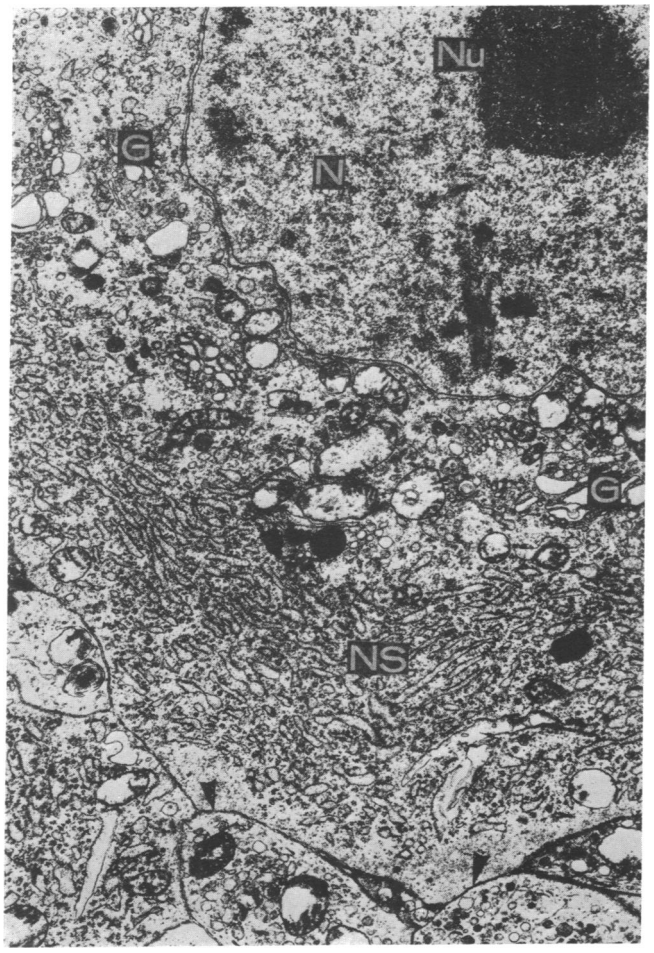

Fig. 1. Electron micrograph of supraoptic neuron from the hypothalamic median eminence maintained in culture for 15 days. Fine structure of cell resembles day-0 preparations, in that the cytoplasm contains well developed elements of the Nissl substances (*NS*), Golgi apparatus (*G*), nucleus (*N*), nucleolus (*Nu*), and is bounded by an intact plasmalemma (*arrows*). × 13,320.

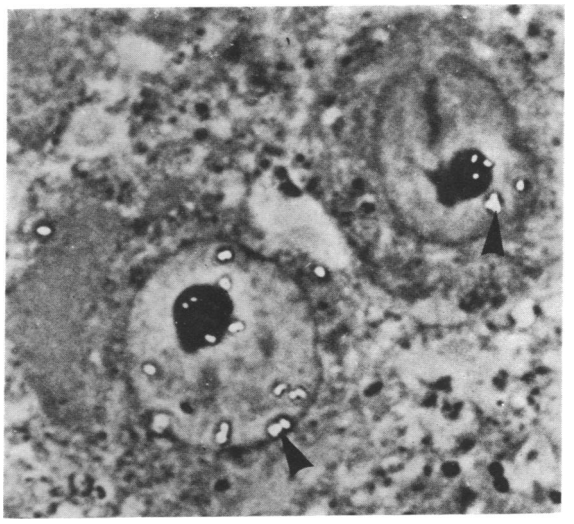

Fig. 2. Autoradiograph of the supraoptic nucleus maintained in culture 15 days, and incubated in medium containing [^{3}H]-uridine for 45 min. In this light micrograph, by the use of phase optics, silver grains may be seen as bright areas (*arrows*) overlying the nuclei of two supraoptic neurons, ×3,500.

Isolation of Labeled Neurophysin. The void-volume fractions from Sephadex G-25 columns were pooled, concentrated to a volume of 1 ml by rotary evaporation, and then passed through a 0.9 × 30 cm column of Sephadex G-75, equilibrated with HCl–HAc. The fractions comprising the radioactive peak that emerges at an elution volume 2.5 times the void volume (neurophysin region) were pooled, lyophilized, and used for gel electrophoresis, electrofocusing, and reaction with rabbit antisera to porcine neurophysin.

Gel electrophoresis and electrofocusing

Gel electrophoresis was performed (15) on 7.5 and 15% gels at a running pH of 9.5; gels were stained with Amido Schwartz and destained with 7% acetic acid. Radioactivity was detected on 7.5% gels by freezing the extruded gels on dry ice and then sectioning the frozen gels into 1-mm slices by the use of a Joyce-Loebl gel slicer. Gel slices were swollen in 0.5 ml of NCS solubilizer–0.1 ml of 4 M NH_4OH in scintillation vials, heated at 40°C overnight, and then counted in 5 ml of Liquifluor.

Aliquots of the concentrated G-75 neurophysin fraction were dialyzed against distilled water and subjected to electrofocusing in 7% acrylamide gels containing 2% (w/v) Ampholine, pH 4–6, (LKB) electrofocusing ampholyte according to Pliska, McKelvy, and Sachs (in preparation). After electro-

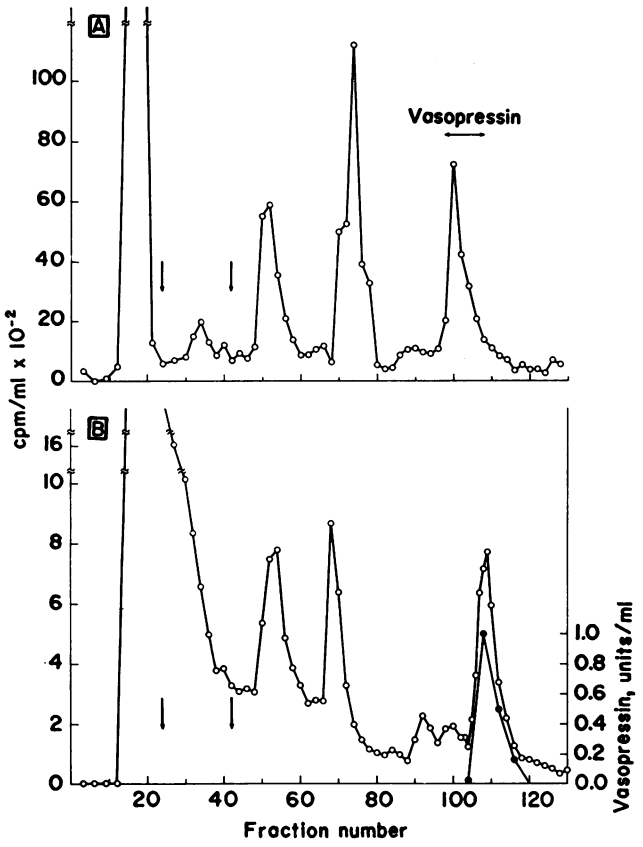

Fig. 3. Ion-exchange chromatography of labeled peptides bound to neurophysin–Sepharose: CM-cellex, 0.9 × 29 cm column, equilibrated in 2mM ammonium acetate (pH 5.0); elution was performed with the same buffer up to the position of the *first arrow;* thereafter, with a gradient consisting of 0.05 M ammonium acetate (pH 6.9) in a 50-ml mixing chamber of constant volume that received 0.1 M and then 0.2 M buffer (pH 6.9) (at positions of *first and second arrows,* respectively). The flow rate was 15 ml/hr and 1.75-ml fractions were collected until the 0.2 M buffer change; thereafter, the fraction volume was adjusted to 0.8 ml; in each experiment, tissues of hypothalamic median eminence from 10 animals, were pulsed with [^{3}H]tyrosine and [^{3}H]proline for 20 hr; (*A*) After 5 days in organ culture, (*B*) at day 0. O——O, cpm; ●——●, vasopressin (units).

focusing, gels were visualized for protein either by immersion in 10% Cl_3CCOOH or staining with Coomassie blue. Radioactivity was determined on gel slices as described above. The ampholyte had no effect on the scintillation process.

Reaction with rabbit anti-porcine neurophysin

The antiserum was a generous gift of Dr. Henry Friesen; its ability to crossreact with neurophysin of guinea pig neural-lobe extracts was established by double diffusion in agar gel. Aliquots of the concentrated G-75 neurophysin region were incubated with 0.1 ml of a 1:50 dilution of antibody in a final volume of 0.7 ml of phosphate-buffered saline at 4°C for 48 hr. As a control, normal rabbit serum, diluted 1:50, replaced the antiserum. Goat antiserum to rabbit globulin (0.2 ml) was then added, and after 24 hr at 4°C, the incubation mixtures were centrifuged at 15,000 × *g* for 10 min and washed twice with saline. The washed precipitates were taken up in 100 μl of 0.01 M sodium phosphate buffer (pH 7.8) containing 2% sodium dodecyl sulfate (SDS) and 2% 2-mercaptoethanol, and heated at 56°C for 1.5 hr. Aliquots were then taken for radioactivity measurement and for electrophoresis on 5% SDS gels. Radioactivity in the gels was determined as described above.

Incorporation of [^{3}H]uridine into RNA

The incubation mixture consisted of two guinea pig hypothalamic sections (4 halves) in 1 ml of culture medium containing 20 μCi of [^{3}H]uridine. After the desired labeling period, tissues were removed for radioautography and determination of radioactivity in RNA (11). The $HClO_4$-insoluble precipitates were used to estimate DNA.

RESULTS

Characteristics of organ cultures: morphologic and quantitative aspects

Light microscopy was used to identify supraoptic neurosecretory cells by their characteristic morphology (i.e., large perikaryon, conspicuous nucleolus, and vesicular nucleus), and by their position immediately lateral to the optic tract. Neurosecretory cells were observed in 4-, 7-, 15-, and 28-day-old cultures despite the widespread cellular degeneration that occurred in the surrounding hypothalamus and the optic tract. Surviving neurosecretory cells have intact nuclei, nuclear membranes, and well-defined cell boundaries. In electron micrographs, highly organized elements of the rough endoplasmic reticulum of Nissl substance were well preserved in many of these cells (Fig. 1). Smooth-surfaced elements of the Golgi apparatus were as prominent in the cultures as in the day-0 preparations. Mitochondria sometimes appeared swollen and clear, although this is considered to be a fixation artifact since it was also observed in the day-0 culture.

Initially, each hypothalamic median eminence (2 halves) contained 2.69 ± 0.21 mg of protein and had protein/DNA and RNA/DNA rations of about 123 and 2.1, respectively. The DNA content declined by about 20% after 11 days in culture, whereas RNA/DNA and protein/DNA ratios fell dramatically, to about half their initial value, by the third day

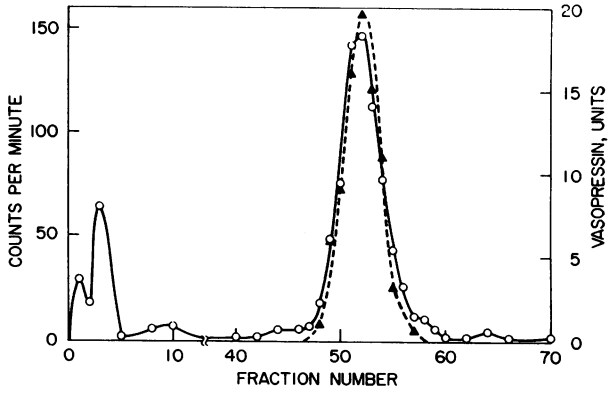

FIG. 4. Rechromatography of [^{35}S]vasopressin on CM-cellex, 0.9 × 5.0 cm column, equilibrated in 2mM ammonium acetate (pH 5.0) and eluted with the same buffer to fraction number 5; thereafter, by gradient elution from 0.05 to 0.2 M buffer in a 50-ml mixing chamber of constant volume. Hypothalamic median eminence was cultured for 5 days and pulsed with [^{35}S]cysteine for 16 hr. ▲——▲, vasopressin (units); O——O, cpm of ^{35}S.

and then remained relatively constant for the next 9 days, except for a small rise in the RNA/DNA ratio at the eighth and ninth days. Vasopressin concentration was 20–30 mU per hypothalamic median eminence and did not show any significant change over the same period (0–12 days).

Biosynthetic studies

RNA. The tissues were pulsed with [^{3}H]uridine for 90 min on each consecutive day and the specific activities of the total RNA were determined in each of six halved hypothalamic median-eminence segments. After 1 or 2 days in culture, the RNA specific activity was about twice that of the RNA of the freshly excised tissue. This could probably be attributed to the decline in RNA content that occurred over this time interval. Thereafter, the RNA specific activity continued to rise to a peak at the seventh and eighth day, to a value about 25 times the initial value, and subsequently showed a continuous decline. The respective uptake of label into the $HClO_4$-soluble tissue protein (cpm/mg) did not show any marked differences. Radioautography confirmed that in addition to many other cells present in the cultured hypothalamic median eminence, the supraoptic neurons were heavily labeled, and that even after 15 days in culture, these neurosecretory cells still demonstrated a vigorous incorporation of [^{3}H]uridine (Fig. 2).

Vasopressin and Polypeptides. Organ cultures of the hypothalamic median eminence were capable of incorporating the ^{3}H- and ^{35}S-labeled amino acids into many proteins and polypeptides, especially into vasopressin and neurophysin. Because of the widespread labeling, it was necessary to rigorously establish the isotopic purity of the newly synthesized vasopressin and neurophysin. This was done by a modification of previously published procedures (3) by the use of gel filtration, affinity, ion-exchange, and thin-layer chromatography, gel electrophoresis and electrofocusing, and antibody interactions. Although the affinity resin (neurophysin bound to Sepharose) removed about 99% of the contaminating radioactivity from the low molecular weight fraction (from Sephadex G-25), it also bound a number of labeled peptides other than vasopressin, as seen in Fig. 3, which shows the labeled-peptide profile in two representative experiments obtained after pulsing segments of the hypothalamic median eminence with [^{3}H]tyrosine and [^{3}H]proline (Fig. 3*B*, day 0, *A*, after 5 days in culture). Although qualitatively the labeling patterns were similar, the extent of incorporation was considerably greater after 5 days in culture. At this stage of isolation of vasopressin, radioactive contaminants were still present. Purification was readily accomplished, however, by TLC and by rechromatography on CM-cellex, as shown in Fig. 4, which illustrates the incorporation of [^{35}S]cystine into vasopressin by 5-day-old organ cultures. This incorporation into

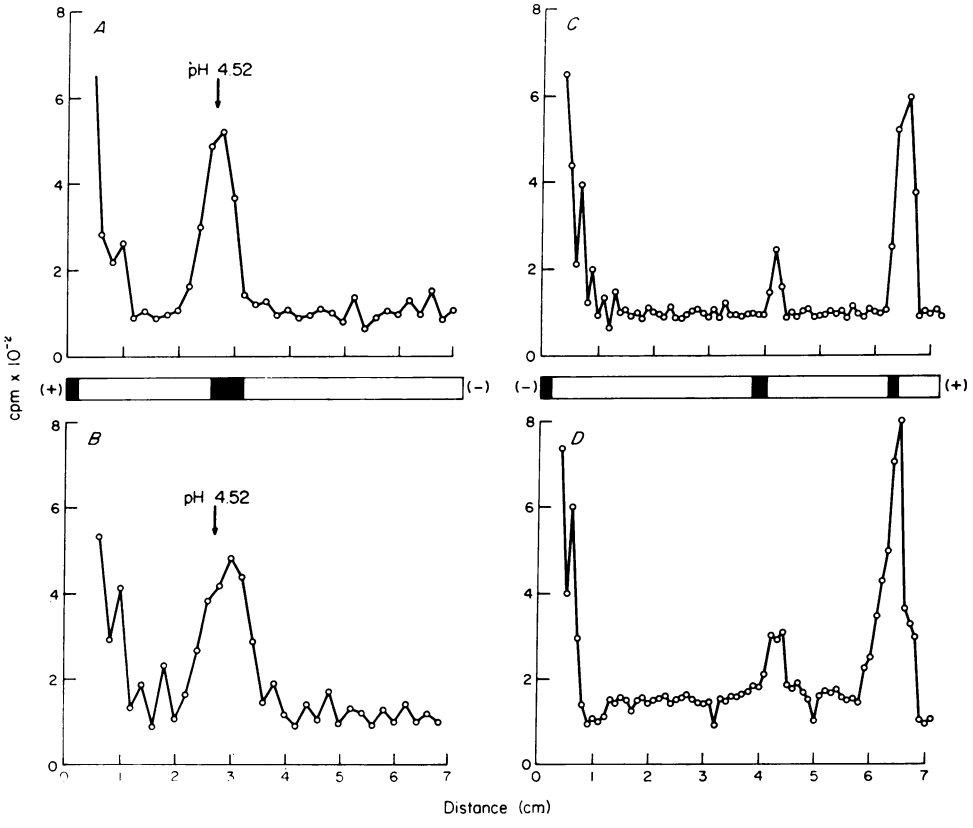

FIG. 5. Acrylamide gel electrofocusing (*A,B*) and electrophoresis (*C,D*) of [^{3}H]neurophysin. Tissue segments of the hypothalamic median eminence were incubated with [^{3}H]tyrosine and [^{3}H]proline (each at 100 μCi/ml) for 20 hr, either immediately after dissection (0 time, *A,C*) or after 5 days in organ culture (*B, D*). Aliquots of the "neurophysin region" emerging from Sephadex G-75 were run as described in *Methods;* radioactivity (*ordinate*), pH (*abscissa*). Center diagram represents the stained gel after electrofocusing of an extract of guinea pig posterior pituitary glands.

Gel electrophoresis, 7% acrylamide gels, run at pH 9.5, and then sliced into 1-mm segments for radioactivity measurements (*ordinate*); center diagram between *C* and *D* represents the two major stainable bands after electrophoresis of an extract of guinea pig posterior pituitary glands.

vasopressin was blocked by inhibitors of protein synthesis such as puromycin or cycloheximide (see ref. 3).

After 12 days in culture, the hypothalamic median-eminence segments could still incorporate radioactive amino acids into vasopressin, although at 28 days their capacity to do so was negligible. Light and electron microscopy of 28-day-old cultures showed neurosecretory neurons that were healthy in appearance, but few in number, indicating that extensive degeneration had occurred.

Neurophysin. After extraction of the labeled hypothalamic median-eminence sections, soluble proteins were subjected consecutively to Sephadex G-25 and G-75 gel filtration. Gel electrophoresis of the proteins from the neurophysin region (G-75, 2.5 void volumes) gave a major radioactive species with the greatest mobility at pH 9.5 (Fig. 5 *C,D*), that corresponds to the fast moving component in guinea pig neural-lobe extracts (Fig. 5) and is characteristic of the neurophysin family of proteins. Gel electrofocusing substantiated the identification of a neurophysin-like molecule, showing a single radioactive zone with a pI of about 4.5 (Fig. 5 *A,B*); bovine Neurophysin I under the same conditions gave a pI of 4.43 (Pliska, McKelvy, and Sachs, unpublished). In all gel experiments, considerable amounts of radioactivity and stainable protein did not enter the gels.

Further evidence for the presence of labeled neurophysin was obtained by reaction of aliquots of the G-75 neurophysin fractions with rabbit anti-porcine neurophysin, followed by precipitation with goat anti-rabbit serum. When aliquots of the dissociated immunoprecipitates were subjected to SDS–gel electrophoresis, a single major radioactive peak was observed for both 0- and 5-day cultures that corresponds to the neurophysin peak observed when guinea pig neural-lobe extracts were treated in the same way.

DISCUSSION

The technology of culture of highly differentiated eukaryotic cells and tissues is receiving widespread application for studies concerned with the control of gene expression and cell metabolism. In most instances, it has been possible to examine various cellular biochemical processes, which could be clearly related to cell or organ function. By contrast, the culture of neurons has proved to be more difficult; equally formidable has been the task of relating cellular biochemistry to the functions of nerve tissue. The specialized neurosecretory neurons of the hypothalamus, however, offer some unique opportunities in this regard, i.e., their function is to produce and secrete polypeptide hormones in association with discrete macromolecular structures; this function can be readily translated into biochemical language. Furthermore, the expression of function appears to hinge on cellular and molecular structures, as well as on intra- and intercellular interactions common to that of conventional neurons. The supraoptic neurosecretory neuron receives both cholinergic and adrenergic synaptic inputs, which modulate transmembrane ionic movements and the generation of action potentials. As with conventional neuronal systems, these phenomena take place over milliseconds, and furthermore, there is a demonstrable secretion of neurohormone and binding protein within seconds after a stimulatory input. Only after prolonged stimulation of days or even weeks, however, are such inputs measurably translated into an enhanced biosynthesis of polypeptide hormone and RNA of the neurosecretory neuron, and probably their satellite or neuroglial cells (4). Thus, in order to probe the neuronal cellular control mechanisms involved in these phenomena, it was imperative to develop a system whereby differentiated neurosecretory neurons of the supraoptic nuclei could be maintained *in vitro* for extended time periods, with retention of all or part of their functional activities.

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