

BIOSYNTHESIS AND AXONAL TRANSPORT OF RAT NEUROHYPOPHYSIAL PROTEINS AND PEPTIDES

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ABSTRACT

³⁵S-cysteine injected adjacent to the supraoptic nucleus (SON) of the rat is rapidly incorporated into proteins. These ³⁵S-cysteine-labeled proteins in the SON (1–24 h after injection) were separated by polyacrylamide gel electrophoresis, and the distribution of radioactive proteins on the gels was analyzed. 1 h after injection, about 73% of the radioactivity appeared in two peaks (both about 20,000 mol wt). With time, these peaks (putative precursors of neurophysin) decreased, as a 12,000 mol wt peak (containing two distinct neurophysins) increased in radioactivity. Both the 20,000- and 12,000-mol wt proteins are transported into the axonal (median eminence) and nerve terminal (posterior pituitary) regions of the rat hypothalamo-neurohypophysial system. Conversion of the larger precursor protein to the smaller neurophysin appears to occur, in large part, intra-axonally during axonal transport. Six distinct ³⁵S-cysteine-labeled peptides (< 2500 mol wt), in addition to arginine vasopressin and oxytocin, are also synthesized in the SON and transported to the posterior pituitary where they are released together with labeled neurophysin by potassium depolarization in the presence of extracellular calcium. These data provide support for the hypothesis that the neurohypophysial peptides (vasopressin and oxytocin) and neurophysins are derived from the post-translational cleavage of protein precursors synthesized in the SON, and that the conversion process can occur in the neurosecretory granule during axonal transport.

The question whether neuronal peptides are synthesized by ribosomal mechanisms as protein precursors and then transformed to biologically active peptides by post-translational cleavage mechanisms, similar to those for insulin (47), or by direct enzymic (synthetase) mechanisms has received considerable attention in recent years (38). The answer to this question has several implications with regard to the cell biology of "peptidergic" neurons, i.e., neurons which synthesize and store specific peptides for release as intercellular mes-

sengers (3, 13, 30). Since ribosomes appear to be restricted to the neuron perikaryon (21), ribosomal mechanisms of peptide biosynthesis and their regulation would necessarily have to occur at this site in the neuron. In contrast, an enzymic mechanism of biosynthesis would allow these processes to occur in any part of the neuron (e.g., the axon terminal), analogous to the synthesis of conventional neurotransmitters (e.g., acetylcholine, gamma aminobutyric acid, catecholamines, etc.).

The peptidergic neurons of the mammalian hypothalamo-neurohypophysial system represent excellent models for such studies. The neurohypophysial peptides (oxytocin and vasopressin) and proteins (neurophysins) are synthesized by neurons in the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus and subsequently transported intra-axonally to the posterior pituitary (neurohypophysis) where they are stored in nerve terminals for release (2, 15, 22). As a result of their extensive investigations, Sachs and his colleagues (44) have hypothesized that vasopressin and neurophysin are derived from a common precursor protein which is synthesized by ribosomal mechanisms. The evidence in support of this hypothesis is as follows: (a) the synthesis of vasopressin occurs only at the site of the neuronal perikarya in the hypothalamus. The neurohypophysis, containing the axons and terminals of these same neurons, is incapable of vasopressin synthesis (40, 42). (b) In vivo and in vitro experiments on the incorporation of ^{35}S -cysteine into vasopressin showed that labeled vasopressin appeared only after a significant time lag (>1.5 h in the dog). Application of a protein synthesis inhibitor, puromycin, during the pulse-label period completely inhibits the incorporation of ^{35}S -cysteine into vasopressin, whereas if the puromycin is applied after the pulse (but before the appearance of labeled vasopressin) the incorporation of label into the peptide is not inhibited (42, 44, 50). Sachs and Takabatake (42) concluded from these findings that puromycin inhibited the synthesis of the precursor, but that once the precursor was synthesized the inhibitor did not affect the cleavage of the precursor to vasopressin. (c) Neurophysin and vasopressin appear to be synthesized with similar kinetics (43), and inhibition of neurophysin synthesis by incubation in the presence of analogues of amino acids found in neurophysin but not vasopressin also inhibited the synthesis of vasopressin (44). These data indicated a common precursor for both neurophysin and vasopressin. (d) Consistent with this hypothesis are the recent findings that rats with genetic defects in vasopressin synthesis, i.e., the Brattleboro strain, are also deficient in specifically the vasopressin-associated neurophysin (37, 52). (e) Various physical-chemical properties of neurophysin suggest that this protein is not in its originally synthesized form (8).

Although considerable evidence for the existence of a precursor of neurophysin and vasopressin has been available, efforts to isolate and char-

acterize this postulated precursor in biosynthetic experiments have been unsuccessful. In this paper, we present evidence for the existence of a protein precursor to neurophysin in the rat hypothalamo-neurohypophysial system, its tentative identification as a protein of 20,000 mol wt, and evidence for its post-translational modification during axonal transport. With the use of the biosynthesis-axonal transport paradigm described in this paper, we have detected several cysteine-containing peptides, other than vasopressin and oxytocin, which appear to be transported to the posterior pituitary for release. A preliminary account of this work has been published (14).

MATERIALS AND METHODS

Animals and Operative Procedures

Rats of the Osborne-Mendel strain were used in these experiments. The animals were anaesthetized with ether, and their heads were fixed in a stereotaxic instrument (5° , nose down). A flap of skull bone was removed, exposing the brain, and two 31-gauge, stainless steel needles were positioned 7 mm rostral to the interauricular line, 2.5 mm on each side of the midline. The needles were lowered 8.6 mm beneath the dural surface, and 1 μl of a solution containing 10 μCi of ^{35}S -1-cystine (50 Ci/mmol, New England Nuclear, Boston, Mass.) in 0.9% NaCl and 10 mM dithiothreitol was injected through each needle over a period of 10 min. After the injection, the needles were left in position for 10 min, and were then removed from the brain. Histological examination of the brains showed that the tips of the needles had been just above and lateral to the supraoptic nuclei (SON). The scalp was closed with wound clips, and the animals awoke 10 min postoperatively. At various times post-injection, the animals were sacrificed by decapitation, and their brains and pituitaries were quickly removed and frozen on dry ice. Serial frontal sections (300 μm in thickness) of the brain were cut in a cryostat at -9°C . The SON where oxytocin and vasopressin containing neuronal perikarya are found (49), and the median eminence (ME) through which axons from the SON neurons pass, were dissected by the Palkovitz punch technique (35). The posterior pituitary, where the axons of the hypothalamo-neurohypophysial system terminate, was also studied. These tissue samples were homogenized in 0.1 N HCl in order to destroy degradative enzymes (11), and the homogenates were stored at -70°C .

Polyacrylamide Gel Electrophoresis of Labeled Proteins

Four types of polyacrylamide gel electrophoresis (PAGE) were used to separate ^{35}S -labeled proteins from the isolated tissues: acid-urea gels, basic gels (with a running pH of 9.5), electrophoresis in the presence of

sodium dodecyl sulfate (SDS), and isoelectric focusing (IEF) gels. Two methods were used to prepare the tissue samples for electrophoresis. (a) The tissues were directly homogenized in a sample buffer (see below) appropriate for the gel system to be used, and the homogenate was centrifuged at 12,000 g_{\max} . The supernate was then applied directly to the gel for electrophoresis. (b) The tissues which had been homogenized in 0.1 M HCl were treated with 10% TCA, and the precipitated proteins were separated from the TCA-soluble proteins by centrifugation. Both the TCA-precipitable and-soluble fractions were washed extensively with ether to remove the TCA and stored at -70°C until use. The TCA-precipitable fraction was solubilized by the specific sample buffer and subjected to electrophoresis. The TCA-soluble peptides were analyzed as will be described later.

The acid-urea polyacrylamide gels were prepared as described elsewhere (24), except that the separating gel also contained 0.1% Triton X-100. Electrophoresis in the system is toward the cathode, and the running pH of the gel is 2.7. The sample buffer contained 8 M urea, 1% Triton X-100, and 0.9 M acetic acid. SDS gel electrophoresis was done with the buffer system described by Neville (29). The running pH of this system is 9.5, and the sample buffer contained 1% SDS, and 2% β -mercaptoethanol made up in the upper gel buffer (29). This same gel system, in the absence of SDS and β -mercaptoethanol, was used to separate proteins on the basis of their charge and size, and is referred to as the "pH 9.5 gel" in the text. IEF in polyacrylamide gels was done according to the method described by O'Farrell (34). The sample buffer used with this gel system contained 8 M urea, 1% Triton X-100, and 2% ampholytes (pH 3-10), and the sample was loaded at the anode end of the gel. Conventional disc gels were used when the radioactive patterns on the gels were determined by slicing techniques, and slab gels were used for radioautographic analysis. After electrophoresis, the gels were stained with Coomassie Blue and destained by conventional techniques. Several marker proteins were used to standardize each gel run. The marker proteins were either added to the sample buffer containing the labeled proteins (providing an internal standard for the gel) or co-run during the same gel electrophoresis run on a separate gel. The marker proteins used for acid-urea gels were bovine serum albumin, cytochrome *c*, and bovine neurophysin I and II (obtained as a gift from Dr. E. DiLiberto). The same marker proteins, except for cytochrome *c*, were used for the pH 9.5 gel, and bovine serum albumin (68,000 mol wt), ovalbumin (43,000 mol wt), carbonic anhydrase (29,000 mol wt), cytochrome *c* (12,000 mol wt), and insulin α and β subunits (2,500 and 3,500 mol wt) were used in SDS gel electrophoresis.

In some experiments, the labeled proteins were electrophoresed on acid-urea gels, and after electrophoresis, the gels were sliced longitudinally into two halves. One half was sliced and counted for its radioactive profile, and the other half was frozen at -70°C . When the

migration proteins on the gels of the labeled proteins were determined, the slices containing the labeled proteins of interest were sliced out of the frozen gel, fragmented, and eluted in either SDS or IEF sample buffer. The sample buffer and gel fragments were then applied to either SDS or IEF gels for electrophoresis.

Analysis of TCA-Soluble Peptides

The TCA-soluble fractions were chromatographed on Sephadex G-25 columns (0.9×30 cm, Pharmacia-Uppsala, Sweden) with 5% acetic acid as the eluant. The TCA-soluble fractions were lyophilized, dissolved in 0.2 ml of 5% acetic acid and applied to the column without disturbing the gel, and eluted. Flow rates were about 40 ml/h, and blue dextran, arginine vasopressin, and riboflavin were used as markers on the G-25 column. Aliquots from the resulting fractions were counted for their radioactivity, and the radioactive fractions preceding the riboflavin fraction were pooled and lyophilized. The lyophilized peptides were dissolved in a small volume of 5% acetic acid, and spotted on cellulose (500 μm thick) thin-layer chromatography (TLC) plates (Analtech, Inc., Newark, Del.), for two-dimensional TLC. The solvent used for the first dimension (solvent 1) contained butanol: H_2O :pyridine in the ratio 1:1:1, while solvent 2 contained butanol: H_2O :acetic acid:pyridine in the ratios of 3:3:1:3. The antioxidant thioglycol (0.1% vol/vol) was also added to both solvents. Arginine vasopressin and oxytocin were added to each sample and detected on each plate at the end of the chromatographic separation by fluorescamine treatment of the plate (17). The addition of these peptide markers served as internal standards for each run, and also provided information as to the position of these peptides on the plate. The plates were radioautographed as described below.

Release Experiments with the Posterior Pituitary

Posterior pituitaries were removed from rats which had been injected in the SON with ^{35}S -cysteine 24 h before and suspended at 37°C in normal saline solution (composition in mM/liter: 130 NaCl, 5.4 KCl, 1.3 CaCl_2 , 0.8 MgSO_4 , 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid [HEPES], and 10 mM glucose) equilibrated with 95% O_2 , 5% CO_2 . All incubating solutions were adjusted to pH 7.3-7.4 and 335-340 mosmol/liter with NaCl and contained bovine serum albumin (1 mg/ml). The pituitaries were then incubated in the above-described medium at 37°C for a total of 30 min (each pituitary was incubated in a separate Falcon microtest culture dish well in 100 μl of medium for 10 min, and transferred to a new well containing fresh medium). The medium from the final 10-min incubation was made 0.1 M with HCl and saved for analysis. The TCA-precipitable and-soluble cpm in this sample is referred to as the "basal release" in the text. The pituitaries were then transferred consecutively to wells contain-

ing normal K^+ - Ca^{++} -free medium, high K^+ - Ca^{++} -free medium, and high K^+ - Ca^{++} -containing medium (incubation periods were 10 min each, for a total of 20 min in each medium). The composition of the normal K^+ - Ca^{++} -free medium (in mM/liter) was 130 NaCl, 5.4 KCl, 0.8 $MgSO_4$, 4.2 $MgCl_2$, 1 Na-ethylene glycol-bis(β -aminoethyl ether) N,N,N',N' -tetraacetate (EGTA), 10 mM HEPES, and 10 mM glucose; the high K^+ - Ca^{++} -free medium was the same except that NaCl was lowered to 51 mM and the KCl was raised to 80 mM; the high K^+ - Ca^{++} -containing medium was the same as the normal saline solution (see above) except that the NaCl was lowered to 51 mM and the KCl raised to 80 mM. Each medium was acidified with HCl (to a concentration of 0.1 M HCl), and the pituitary (after the experiment) was homogenized in 0.1 M HCl as described above. TCA-precipitable and-soluble cpm was determined from each of the medium samples, and was expressed as a fraction of the basal release into the medium (per 10 min of incubation). The labeled proteins and peptides in the release medium, i.e., the high K^+ - Ca^{++} -containing medium, were analyzed by acid-urea gel electrophoresis and thin-layer chromatography, respectively, as described above.

Analysis of Radioactivity

Polyacrylamide gels in tubes were sliced by conventional methods and processed for their radioactivity as described elsewhere (24). Slab gels were dried in a Bio-Rad slab gel drier (Bio-Rad Laboratories, Richmond, Calif.) onto 3 MM Whatman filter paper, covered with X-ray film (Kodak Royal X-Omat film, CGR Medical Associates, Rockville, Md) and exposed at $-70^\circ C$ (4). The radioautograms were scanned with an E-M densitometer (EM Laboratories, Inc., Elmsford, N. Y.) for a qualitative representation of the radioactive pattern on the gels, and quantitative data were obtained by slicing out the radioactive bands of interest, solubilizing them in an appropriate cocktail (54), and counting their radioactivity in a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). Radioactivity in each band was expressed as a percentage of total cpm on the gel.

The TCA-precipitable and-soluble cpm of the tissue and media were obtained by conventional techniques using Whatman 3 MM filter paper pads for TCA precipitation. The counting efficiency for ^{35}S was 68% and the background was 18 cpm.

RESULTS

Incorporation of ^{35}S -Cysteine into Neurohypophysial Proteins and Their Axonal Transport to the Posterior Pituitary

The time-course of appearance of ^{35}S -labeled protein, i.e., TCA-precipitable cpm, in the various

regions of the hypothalamo-neurohypophysial system is shown in Fig. 1. The data in Fig. 1 A (filled circles, solid line) show that, at 30 min after injection of ^{35}S -cysteine into the SON, about 30% of the radioactivity in the SON region was TCA precipitable, and that this value did not change significantly until about 6 h postinjection. Thus,

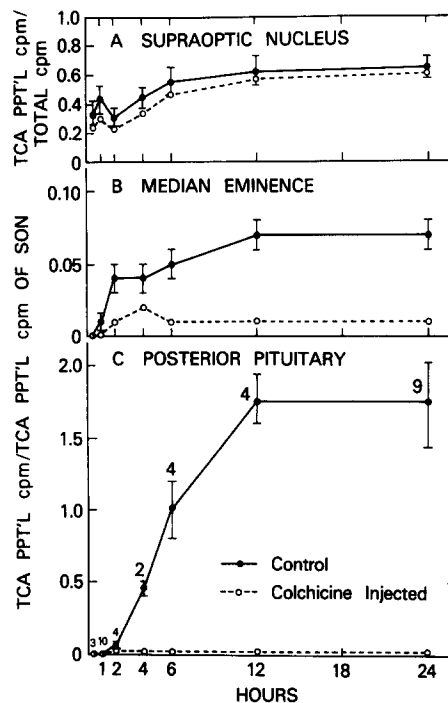


FIGURE 1 Time-course of appearance of ^{35}S -labeled proteins in the supraoptic nucleus (A), median eminence (B), and posterior pituitary (C) after a bilateral microinjection of ^{35}S -cysteine into the SON of female rats. Filled circles show data from control rats; open circles represent data from rats which were intraventricularly injected with colchicine 12 h before injection of the ^{35}S -cysteine. Note that although there is considerable labeled protein in the SON after 30 min, labeled protein first appears in the median eminence only after 1 h, and in the posterior pituitary after 2 h. In addition, the appearance of axonally transported labeled protein is inhibited by colchicine (B) and (C), whereas the percent of incorporation in the SON was unaffected (A) by colchicine. The incorporation into the SON (A) is expressed as the fraction of total cpm in the punched tissue that was TCA precipitable (PPTL). In (B) and (C) the TCA-precipitable cpm in the tissue was expressed as a fraction of the TCA-precipitable cpm in the punched SON sample of the same rat. Data (filled circles) are expressed as means \pm standard deviations. The number of experiments for each time-point is shown in (C).

the incorporation of ^{35}S -cysteine into protein by the SON was very rapid, and approximated a pulse incubation.

Labeled proteins did not appear at the level of the ME (Fig. 1 B, solid line) until 1 h post-injection, and rose rapidly between 1 and 2 h, reaching a maximum at about 12 h. In the posterior pituitary (Fig. 1 C, solid line), labeled proteins were first detected at 2 h and rose rapidly to a maximum value at 12 h. These kinetic data are consistent with the notion that the proteins are first synthesized in the SON region and then transported intra-axonally to the posterior pituitary via axons traversing the median eminence. The time lag of 60–120 min between injection in the SON and arrival of labeled proteins in the posterior pituitary is comparable to values found by others in similar experiments with rats (32, 36) and consistent with the expected axonal transport rates of neurohypophysial proteins (see Table 4 in reference 52). Further evidence for the role of axonal transport in the arrival of labeled proteins in the median eminence and posterior pituitary comes from the colchicine studies shown in Fig. 1. Colchicine, an inhibitor of axonal transport (10, 19, 20, 26), was injected into the lateral ventricles (100 μg dissolved in 10 μl of 0.9% NaCl) 12 h before the bilateral injection of ^{35}S -cysteine in the rat SON. Although this colchicine treatment did not appreciably affect ^{35}S -cysteine incorporation into proteins in the SON (Fig. 1 A, broken line), the appearance of labeled proteins in the ME (Fig. 1 B, broken line) and posterior pituitary (Fig. 1 C, broken line) was dramatically reduced. Similar effects of colchicine on axonal transport of neurohypophysial principles were demonstrated by Norstrom (31) and Sachs et al. (45).

Analysis of Proteins Transported to the Posterior Pituitary

In an attempt to evaluate the diversity of proteins transported to the posterior pituitary after ^{35}S -cysteine injection into the SON, we separated the labeled proteins from the posterior pituitary (24 h post-injection) by using four different PAGE systems (see Materials and Methods).

The results of these separations are illustrated in Fig. 2. Fig. 2 A shows that the pattern of labeled proteins transported to the posterior pituitary was relatively simple with electrophoresis on pH 9.5 gels. Only one major peak of radioactivity was detected. Separation of the labeled neurohypophysial proteins on acid-urea gels (pH 2.7 gel) also

revealed only one major radioactive peak which migrated on the gel between bovine neurophysins I and II (Fig. 2 B). We believe that these labeled peaks (Fig. 2 A and B) represent transported neurophysin(s) for several reasons. (a) Neurophysin is known to be the major cysteine-rich, acidic protein transported to the posterior pituitary (16). A similar labeling pattern of transported neurophysins has been reported by Norstrom et al. (33) using a gel system comparable to our pH 9.5 gel system. (b) These radioactive protein peaks were highly soluble in alcohol, just as has been reported for neurophysin (1, 25, 55). (c) The labeled peak found on acid-urea gels (Fig. 2 B) corresponds in migration position on the gel to a major Coomassie Blue-stained band, which is specifically decreased in staining intensity when the rats are osmotically stressed by 2% NaCl in their drinking water (Fig. 3). (d) These labeled proteins are released from the pituitary by potassium depolarization in the presence of calcium (see later) as is expected for neurophysin (12).

With regard to the salt treatment of the rats mentioned above, it is of interest to note that the labeling pattern shown in Fig. 2 B was the same independent of whether the experiments were done on normal or salt-treated, i.e., after 5–10 days of exposure to 2% NaCl in the drinking water, rats. The only difference was that the absolute cpm in the peak from salt-treated rats was about fourfold greater than that from normal rats. This was probably due to the fact that salt-treated rats incorporated four times more ^{35}S -cysteine into SON proteins and transported four times more labeled protein to the pituitary in 24 h than normal rats (Table I). In the Coomassie Blue-staining patterns shown in Fig. 3, at least seven major stained bands were detected by acid-urea PAGE of proteins from normal rat pituitary. However, only the most rapidly migrating band contained ^{35}S -labeled proteins. Consistent with this was the observation that this stained band selectively disappeared with salt treatment, although the same radioactive peak was still present. Since both vasopressin and oxytocin are depleted in the posterior pituitary of rats after prolonged osmotic stress (18), it is possible that both the vasopressin- and oxytocin-associated rat neurophysins, which appear to be distinct proteins (5, 37, 48), comigrated in this gel system. In this regard, it is important to note that we have detected both ^{35}S -labeled arginine vasopressin and oxytocin in the posterior pituitary 24 h after injection of ^{35}S -cysteine in the

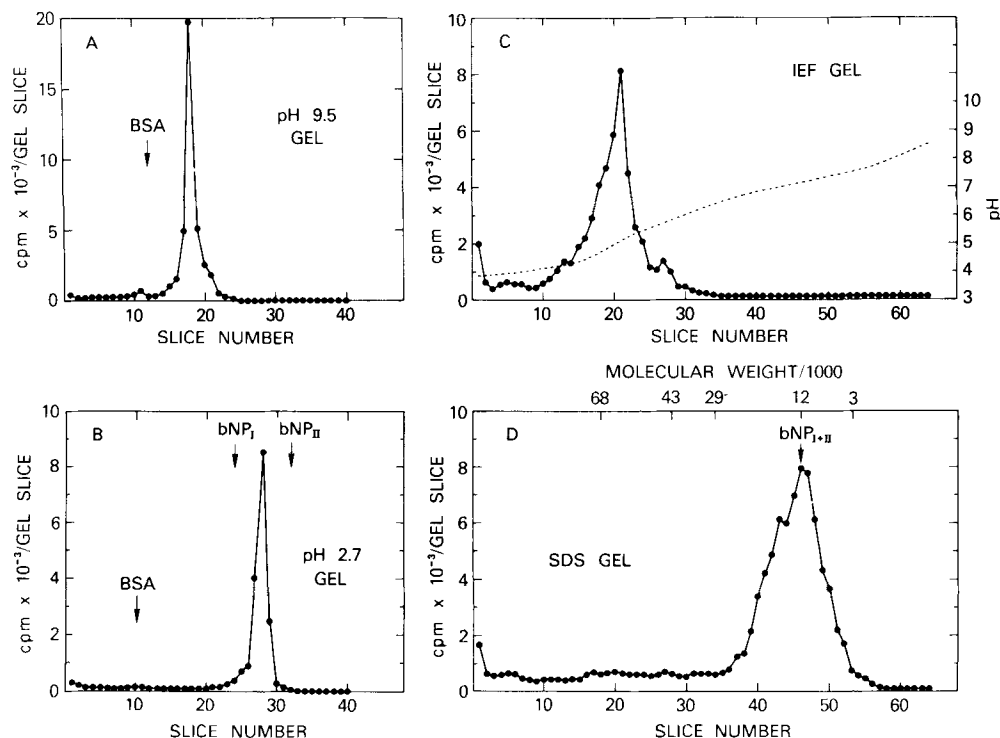


FIGURE 2 Polyacrylamide gel electrophoresis (PAGE) of ³⁵S-labeled proteins extracted from rat pituitaries 24 h after injection of ³⁵S-cysteine into the SON. (A) Disc PAGE (7.5% acrylamide) in a pH 9.5 (running pH) system. Internal marker protein was bovine serum albumin (BSA). (B) Disc PAGE (7.5% acrylamide) in a pH 2.7 system (acid-urea gel). Internal markers: BSA; bovine neurophysins I and II, bNP_I and bNP_{II}. (C) Isoelectric focusing (IEF) (7.5% acrylamide) with pH 3-10 ampholytes. The broken line shows the actual pH gradient measured using a co-run gel. (D) Disc PAGE in sodium dodecyl sulfate (SDS). Upper abscissa shows slice positions of internal marker proteins of known molecular weight, and arrow shows position of bNP_I and bNP_{II} in a co-run gel. Polyacrylamide concentration of gel was 11%.

SON (see Fig. 8). The ratio of labeled vasopressin to that of oxytocin in the neurohypophysis was about 1.8 in our experiment, which was comparable to the value of 1.49 obtained by Burford et al. (6) for the ratio of radioactivity in vasopressin-neurophysin to that in oxytocin-neurophysin after intracisternal injections of ³⁵S-cysteine.

In view of our inability to resolve more than one major radioactive pituitary peak with the above-described gel systems, which separate proteins on the basis of both electrical charge and molecular size properties, we turned to IEF separation procedures. Fig. 2 C shows the results of such experiments. The labeled neurohypophysial proteins electrofocused principally in the acidic region of the gel ranging in isoelectric points from pH 4.5 to 5.2 with the peak value at about pH 4.8. In subsequent IEF experiments using expanded pH gradients on the gel, i.e., from pH 3.5 to 7, we

found that the labeled neurohypophysial proteins are represented by two distinct closely migrating peaks with pI values equal to 4.6 and 4.8. These may correspond to the two rat neurophysins reported by Burford and Pickering (5) who used a different separation procedure.

Separation of the labeled neurohypophysial proteins by PAGE in SDS showed that the transported proteins were of relatively low molecular weight (Fig. 2 D). Similar findings that the transported, labeled neurohypophysial proteins showed a peak on SDS gels at around 12,000 mol wt (ranging between 10,000 and 14,500 mol wt) have been reported by others (31, 33). It should be pointed out that, although the peak in Fig. 2 D comigrated with the 12,000-mol wt marker-protein in the gel, bovine neurophysins I and II (which are about 10,000 mol wt) also comigrated with this marker-protein (see arrow in Fig. 2 D).

Labeling Patterns of Proteins Synthesized in the Hypothalamo-Neurohypophysial System

In the preceding section, it was shown that the principal ³⁵S-cysteine-labeled proteins (neurophysins) transported to the posterior pituitary were represented by a single rapidly migrating peak on acid-urea gels. Since the biosynthetic process is in the neuronal perikarya (15, 16, 22, 44) located in

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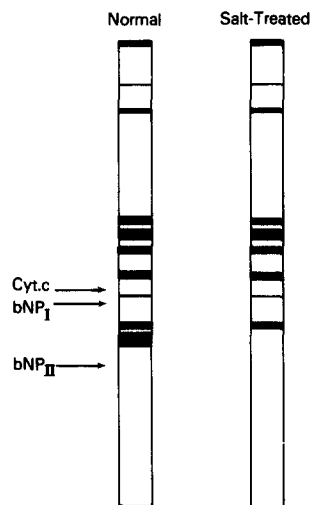


FIGURE 3 Diagram of Coomassie Blue-stained bands of proteins extracted from posterior pituitaries of normal rats and rats that had been drinking 2% NaCl for 10 days (salt treated), which were separated by electrophoresis on 15% acid-urea gels. Note disappearance of the most rapidly migrating band after salt treatment. Arrows denote migration positions of cytochrome c, bovine neurophysins I and II in co-run gels.

the SON and PVN of the hypothalamus, we analyzed the labeling profiles of proteins, separated on acid-urea gels, which were extracted from the SON at various times after injection of ³⁵S-cysteine. In these experiments the labeled proteins were electrophoresed on acid-urea slab gels (15% acrylamide) and radioautographed. Fig. 4 shows autoradiograms of such gels containing labeled proteins from the SON and posterior pituitaries of salt-treated rats. These autoradiograms were overexposed in order to emphasize that only one highly labeled, rapidly migrating peak is found in the pituitary after 24 h, in comparison to the complex Coomassie Blue-staining pattern of pituitary proteins shown in the same figure (Fig. 4, COOM. BLUE). In contrast to the posterior pituitary data, the SON labeling pattern, 1 h postinjection, shows a heavy zone of radioactivity (composed of two different peaks) with a lower migration rate on the gel than the neurophysin peak. At 24 h postinjection, this zone of radioactivity virtually disappeared from the SON (Fig. 4).

The data in Fig. 5 show the results of a time study of the labeling patterns found in the SON, ME, and posterior pituitary after injection of ³⁵S-cysteine in the SON of normal rats. In these experiments, the autoradiograms of the gels were exposed for shorter times in order to improve the resolution of the individual bands of radioactivity. The autoradiograms were then densitometrically scanned to produce the traces shown in Fig. 5. Several peaks with distinct R_f values can be discerned in Fig. 5, and have been designated a-g. Specific peaks of interest were cut out of the gel and counted for their radioactivity by liquid scintillation counting techniques.

1 h postinjection, the SON labeling profile was dominated by two closely migrating peaks a and b

TABLE I
Effects of Salt Treatment on the Incorporation of ³⁵S-Cysteine into SON and Posterior Pituitary Proteins*

Tissue	Time	Control	Salt-treated	Ratio‡	p§
	<i>h</i>	<i>cpm × 10⁻³</i>			
SON	1	129 ± 34 (9)	565 ± 73 (9)	4.4	<0.001
Posterior pituitary	24	147 ± 39 (6)	585 ± 97 (6)	4.0	<0.001

* Female rats were given 2% NaCl to drink for 5-7 days (salt-treated), in comparison to normally treated (control) female rats. The rats were injected with ³⁵S-cysteine in the SON (bilaterally), and SON (1 h after injection) and posterior pituitaries (24 h after injection) were taken for analysis of their TCA-precipitable cpm. Data are expressed as means ± SEM. Number of experiments is shown in parentheses.

‡ Ratio of salt-treated CPM/control CPM.

§ p Value determined by Fisher *t* test for small numbers.

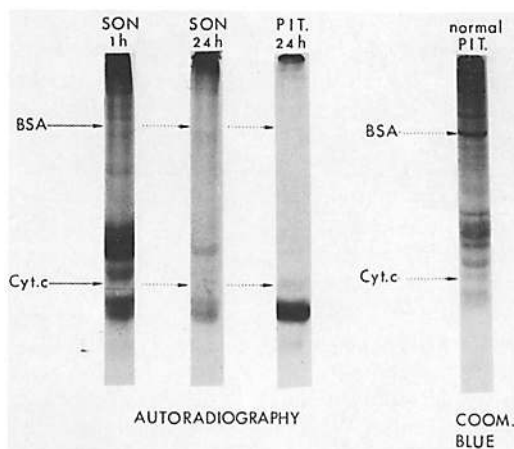


FIGURE 4 Autoradiographic and Coomassie Blue (COOM. BLUE)-staining patterns of SON and pituitary (PIT) proteins electrophoresed on 15% acid-urea slab gels. The radioactive proteins were obtained from extracts of tissues from salt-treated female rats (5 days), whereas the Coomassie Blue-stained gel contained posterior pituitary proteins from a normal (control) female rat. The Coomassie Blue band corresponding to the labeled peak generally appears less densely stained on slab gels than on disc gels shown in Fig. 3. Arrows designate migration positions of bovine serum albumin (BSA) and cytochrome *c* (Cyt. *c*) marker proteins co-run on the same slab gel.

of radioactivity (Fig. 5 A), with migration rates much lower than that of the labeled neurophysin peak arriving in the pituitary at 24 h, i.e., peak *e* in Fig. 5 C. Note that two other minor components, *f* and *g*, could also be detected in the 24-h posterior pituitary using autoradiographic techniques. Hence, after 1 h, only 8.4% of the total radioactivity in the SON appeared in the rapidly migrating peaks, i.e., peaks *d* and *e*, whereas 72.6% of the cpm resided in peaks *a* and *b*. As can be seen in Fig. 5 B and C, the relative radioactivity in peaks *a* and *b* continued to decrease, while that of peaks *d* and *e* increased with time. In comparison, there appeared to be relatively little change in peak *c* with time. The above-described events appeared to occur in an identical fashion in both male and female rats which were induced to increase their neurohypophysial activity by drinking 2% NaCl (unpublished data).

The apparent decrease in radioactivity in peaks *a* and *b* simultaneous with the increase in relative radioactivity of peaks *d* and *e* in the SON, in such a "pulse" label paradigm, is consistent with a precursor-product relationship between these pro-

teins. Since neurophysins are known to be synthesized by neurons in the SON (22, 44), and appear to correspond to the labeled peak *e* in the SON and pituitary after 24 h (Fig. 5), then peaks *a* and *b* may represent the protein precursors of the oxytocin-related and vasopressin-related neurophysins. Since peak *d* is only found in the SON at short times after the injection of ^{35}S -cysteine, it is possible that this may be a short-lived intermediate in the conversion of the precursor proteins to neurophysin. In order to determine whether the labeled peaks (*a* and *b*) were greater in molecular weight than the presumed product (peak *e*), labeled proteins extracted from the SON and posterior pituitary 1 and 24 h post-injection, respectively, were electrophoresed on acid-urea gels. Peaks *a + b*, *c*, and *e* were located on the gels, cut out, eluted, and evaluated for their apparent molecular weights by three different methods: Sephadex G-75 chromatography, PAGE in SDS, and Ferguson plot analysis (9, 24, 39) of acid-urea gels. The results of all three methods were in close agreement (Table II), showing the molecular weights of the putative precursors (peaks *a* and *b*) and neurophysins (peak *e*) to be 19,000–20,000 mol wt and 12,000–13,000 mol wt, respectively.

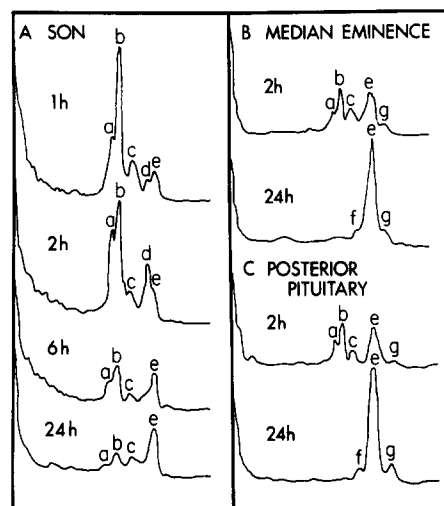


FIGURE 5 Densitometric tracings of autoradiographs of 15% acid-urea slab gels on which ^{35}S -labeled proteins from the SON, median eminence, and posterior pituitary were electrophoresed. Normal male rats were injected with ^{35}S -cysteine into the SON, and tissues were taken for analysis at various times postinjection (1–24 h). The letters *a–g* shown over the tracings correspond to distinct radioactive peaks in the autoradiographs.

TABLE II
Molecular Weight and Isoelectric Point Determinations of the Putative Precursors and Neurophysins in the Rat*

Protein	G-75	Ferguson plot	SDS	IEP§
		<i>mol wt</i> ($\times 10^{-3}$)‡		
Precursors (peaks <i>a</i> + <i>b</i>)	19	20	20	5.4, 6.1
Intermediates (peak <i>c</i>)	15	—	15	5.1, 5.6
Neurophysins (peak <i>e</i>)	12	13	12	4.6, 4.8

* Labeled proteins were extracted from the SON and posterior pituitary of salt-treated rats after 1 and 24 h postinjection, respectively, and electrophoresed on acid-urea gels. The labeled peaks from the SON (represented by peaks *a*, *b*, and *c* in Fig. 5 A) and one labeled peak from the pituitary (peak *e* in Fig. 5 B) were eluted from the gels and analyzed.

‡ Molecular weights of the isolated labeled peaks *a*, *b*, *c*, and *e* were determined by three independent methods: (a) chromatography using Sephadex G-75, (b) Ferguson plot analysis of acid-urea gels, and (c) SDS gel electrophoresis.

§ Isoelectric points (IEP) of the isolated proteins were determined by isoelectric focusing of peaks *a*, *b*, *c*, and *e* on polyacrylamide gels. Each peak eluted from the acid-urea gel run appeared to be heterogeneous. Two major peaks of radioactivity were found each for the precursor (*a* + *b*), the intermediate (*c*), and the neurophysin (*e*).

Therefore, these data are consistent with the proposal that peaks *a* and *b* represent precursors of the labeled proteins in peak *e*. As pointed out earlier, peak *e* actually contains two major, distinctly labeled proteins as revealed by IEF (pI 4.6 and 4.8; Table II), and peaks *a* and *b* also run as two independent labeled peaks with pI values of 5.4 and 6.1 on IEF gels (Table II). The data represent necessary but not sufficient evidence to prove the identity of a precursor, and further experiments (see Discussion) are clearly necessary for this purpose. However, we have observed, in the SON, a biosynthetic process consistent with the requirements of a precursor-product relationship, and tentatively propose that peaks *a* and *b* represent the precursors to the neurophysins found in peak *e*.

Axonal Transport and Post-Translational Processing of the Putative Precursor

Although peak *e* in the SON increases in radio-

activity as peaks *a* and *b* decrease (Fig. 5 A), peak *e* never attains the level of radioactivity expected if there were simply a conversion of *a* and *b* to *e* in the SON, i.e., at 1 h *a* and *b* = 72.6%, whereas the maximal value attained in the SON (at 24 h) for peak *e* = 28.5%. The data illustrated in Fig. 5 A suggest that a significant amount of peaks *a* and *b* is transported out of the SON, presumably into the axons, before conversion takes place. Indeed, on the basis of other data, Sachs and colleagues (44) had hypothesized that the conversion of the precursor takes place in the axon during axonal transport. This hypothesis is supported by the data illustrated in Fig. 5 B and C. At early times postinjection (e.g., 1–2 h), when the putative precursor is heavily labeled in the SON (Fig. 5 A), one might expect that the labeled proteins in the axon (derived from axonal transport of proteins from the neuronal somata in the SON) should contain significant labeled precursor. However, at later times, i.e., 24 h postinjection, when virtually only labeled neurophysin is present in the SON, the axonally transported proteins should contain principally labeled neurophysin. These expectations are borne out by the data shown in Fig. 5 B. 2 h postinjection, the labeling profile in the median eminence, an area rich in axons from the SON, indicates that about 40% of the radioactivity is in the precursors (Fig. 5 B, peaks *a* and *b*). If one considers the time delay between synthesis in the SON and transport to the axons in the ME, it is apparent that the labeled proteins arriving after 2 h in the ME (Fig. 5 B) must have been derived from the population of labeled proteins in the SON after about 1 h (Fig. 5 A). Hence, the precursor to product conversion process, resulting in the labeling pattern seen in Fig. 5 B (2 h), must have occurred intra-axonally during transport. 24 h postinjection, the ME labeling profile displays virtually only neurophysin (Fig. 5 B), consistent with the pattern seen in the SON at that time (Fig. 5 A). A similar sequence of events appears to be evident for the axon terminals in the posterior pituitary (Fig. 5 C).

Peptides Transported to the Posterior Pituitary

If one assumes that the 19,000–20,000-mol wt putative precursor is converted to a 12,000–13,000-mol wt neurophysin during axonal transport, one might expect to find other peptide products (in addition to the 1,100-mol wt vasopressin and oxytocin) as a result of the post-translational

cleavage process. Consequently, we have examined the diversity of ^{35}S -cysteine-labeled peptides which are synthesized in the SON and transported to the posterior pituitary.

Fig. 6 depicts the results of Sephadex G-25 chromatography of the TCA-soluble fraction from a posterior pituitary 24 h after injection of ^{35}S -cysteine in the SON. The labeled peptides are represented by a broad peak corresponding to molecular weights ranging from 700 to 2,500 mol wt. Note that there is no ^{35}S -label which comigrates with the riboflavin marker (RIBO, Fig. 6), which indicates that no free ^{35}S -cysteine or its oxidized by-products are transported to the pituitary. Similar labeling profiles were obtained from the TCA-soluble fractions from the SON (1 and 24 h postinjection) after Sephadex G-25 separation.

The labeled peptides from the G-25 separations were lyophilized and spotted on a cellulose (TLC) plate and subjected to two-dimensional TLC. The results of the TLC separations are illustrated in Fig. 7. Fig. 7 shows a peptide map of ^{35}S -labeled peptides found in either the SON or the posterior pituitary. 18 distinct spots containing ^{35}S -label could be detected in the hypothalamo-neurohypophysial system. One of these spots (spot 8) appears to be more heavily labeled in the posterior pituitary than either oxytocin (spot 13) or arginine vasopressin (spot 11). That is, of the 103,722 cpm in labeled peptides from the 24-h posterior pituitary spotted on the TLC plate, the labels con-

tained (expressed as a percentage of total cpm) in spots 4, 5, 6, 8, 11, 13, and 14 were 3.2, 8.2, 0.7, 33.0, 25.6, 14.0, and 4.7%. Although the functional significance of these spots and their relative abundance are still undetermined, it is apparent from these data that many more peptides, other than the known neurohypophysial hormones oxytocin and vasopressin, are synthesized in the SON and transported to the posterior pituitary.

A similar chromatographic analysis of the labeled peptides found in the SON 1 and 24 h postinjection was made. The distribution of label in the 1-h SON peptides was 7.3, 12.6, 1.9, 56.1, 6.1, 1.4, and 0.5% (expressed as a percentage of 121, 102 cpm spotted) for spots 4, 5, 6, 8, 11, 13, and 14, respectively; whereas for the 24-h SON peptides, the distribution of label was 7.5, 6.1, 2.6, 52.7, 11.6, 1.6, and 0.1% (102,950 cpm spotted). Thus, the ^{35}S -labeled spots numbered 4, 5, 8, and 11 (Fig. 7) contained substantial radioactivity in the SON at both 1 and 24 h. Since both arginine vasopressin (AVP) and oxytocin (OXY) are found in the SON (49), it is of interest that spot 11 (AVP) increased in radioactivity, whereas spot 13 (OXY) did not increase in radioactivity in the SON after 24 h (as compared to 1 h postinjection). However, in the posterior pituitary (24 h), spot 13 contained a substantial amount of the label. The appearance of substantial radioactivity in vasopressin (spot 11) but not oxytocin (spot 13) in the SON (after 24 h) is consistent with previous

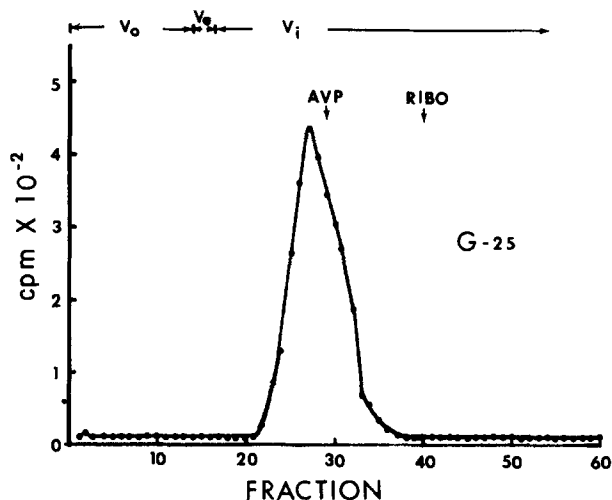


FIGURE 6 Sephadex-G25 separation of TCA-soluble, labeled peptides in the posterior pituitary 24 h after injection of ^{35}S -cysteine into the SON. V_0 , V_e , and V_i represent the void, excluded, and included volumes, respectively. Arrows show the fractions corresponding to arginine vasopressin (AVP) and riboflavin (RIBO).

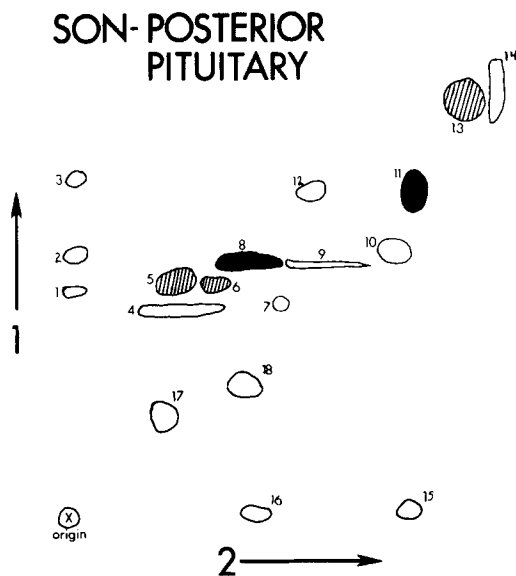


FIGURE 7 Labeled peptide map obtained by two-dimensional TLC of TCA-soluble, labeled peptides in the posterior pituitary 24 h postinjection. Spots numbered 11 and 13 correspond to arginine vasopressin and oxytocin, respectively. See text.

suggestions that the precursor to vasopressin is processed more rapidly than that to oxytocin (23, 53).

Release of Labeled Peptides from the Posterior Pituitary

The diversity of labeled peptides found in the pituitary after ^{35}S -cysteine injection in the SON raises the question whether these peptides are released from the posterior pituitary by depolarization of the nerve terminals in the presence of extracellular calcium. In these experiments, ^{35}S -cysteine was injected into the SON of normal rats, as opposed to salt-treated rats, in order to minimize the release of neurohypophysial principals *in vivo*. 24 h postinjection, the posterior pituitaries were removed from the rats and incubated in saline containing normal levels of K^+ and Ca^{++} (see Materials and Methods). These pituitaries were then transferred and incubated serially in the various media in the following sequence: *a*) 5.4 mM K^+ , 1.3 mM Ca^{++} (basal release media), *b*) 5.4 mM K^+ , 0 mM Ca^{++} (+ 1 mM EDTA), *c*) 80 mM K^+ , 1.3 mM Ca^{++} . The TCA-precipitable and -soluble cpm released from the pituitaries into the various media were determined and expressed as a

fraction of the release from the same tissue in the basal release medium. The data are illustrated in Fig. 8. There was no increase in TCA-soluble or -precipitable cpm released over basal release by pituitaries depolarized by 80 mM K^+ in the absence of Ca^{++} . However, there was a twofold increase in TCA-soluble cpm and a threefold increase in TCA-precipitable cpm when Ca^{++} (1.3 mM) was added to the medium containing 80 mM K^+ (Fig. 8 A and B). Between 2 and 5% of the total cpm in the posterior pituitary was released by the calcium-dependent, potassium depolarization of the terminals.

Analysis of the TCA-precipitable cpm released into the 80 mM K^+ , 1.3 mM Ca^{++} medium after electrophoresis on acid-urea gels revealed a pattern identical to that shown in Fig. 2 B. The TCA-soluble cpm in the 80 mM K^+ , 1.3 mM Ca^{++} medium and pituitaries were spotted for two-dimensional TLC as described earlier. The results of this analysis showed that the labeled peptides which were released into the medium were comparable to those found in the posterior pituitary,

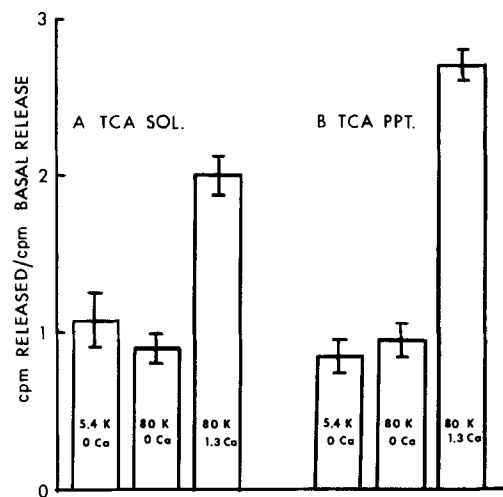


FIGURE 8 Release of labeled peptides by calcium-dependent, potassium depolarization of the posterior pituitary (24 h after injection of ^{35}S -cysteine into the SON of normal rats). (A) TCA-soluble (SOL.) peptides. (B) TCA-precipitable (PPT.) peptides. Data are expressed as cpm released per cpm basal release, i.e., basal release refers to the cpm appearing in the medium in the presence of 5.4 mM K^+ , and 1.3 mM Ca^{++} . See text for details of experiments. The K^+ and Ca^{++} concentration of each medium is shown within the bars in the figure. The data are expressed as the average value \pm SEM. of 13 experiments.

i.e., they corresponded to the pattern shown in Fig. 7. The principal released peptides corresponded to the spots numbered 4, 5, 8, 11, 13, and 14, which contained 2.7, 9.3, 24.5, 14.8, 19.0, and 14.6%, respectively, of the total label spotted (1,200 cpm). Therefore, the labeled peptides transported to the posterior pituitary appear to be accessible to the release mechanism in the nerve terminals.

DISCUSSION

Biosynthetic Evidence for a Precursor of Neurophysin

The initial step in the identification of a precursor is the demonstration in a pulse label paradigm that a labeled polypeptide of higher molecular weight is first synthesized and then decreases in radioactivity in time as the lower molecular weight labeled peptide (or peptides) is formed. Since the biosynthetic process for neurophysin and neurohypophysial hormones is located in the hypothalamic nuclei, we analyzed the labeling profiles of proteins extracted from the SON at various times after injection of ^{35}S -cysteine by PAGE. 1 h after injection of ^{35}S -cysteine, the SON labeling profile was dominated by two closely migrating peaks of radioactivity, i.e., peaks *a* and *b*, Fig. 5 A, corresponding to proteins of about 20,000 mol wt (Table II). These labeled peaks decreased in radioactivity with time, as the 12,000 mol wt neurophysin peak (peak *e*, Fig. 5 A) relatively increased. As was pointed out earlier, the decline in radioactivity in the putative precursor peaks in the SON was probably due to two factors: (a) the conversion of the precursor peaks to neurophysin and (b) the axonal transport of the precursor out of the neuronal perikarya in the SON (the evidence for this comes from the observation of precursor peaks in the regions of the axons, i.e., the ME and posterior pituitary (Fig. 5 B and C). Thus, we have observed in the SON, a biosynthetic process consistent with the expectations of a precursor-product relationship between peaks *a* and *b* and peak *e* (which upon IEF reveals two labeled peaks). Because of these data, we propose that peaks *a* and *b* represent the putative precursors for the neurophysins, and presumably the neurohypophysial peptides.

Data such as those presented above represent necessary but not sufficient proof for the identity of a precursor. Further immunological, peptide

mapping (after limited proteolysis) and ultimately amino acid sequencing studies are clearly required, and are currently underway in our laboratories. In a recent report (27), specific antibodies to vasopressin and its associated neurophysin in the rat were used to measure the content of these molecules in the neurohypophysis and hypothalamus. In contrast to the neural lobe which had a neurophysin:vasopressin ratio of about 1, the hypothalamic ratio was >2 . The authors concluded that this finding would be compatible with the presence of a common precursor protein which had a higher affinity for neurophysin than vasopressin antiserum. In recent immunoprecipitation experiments, we found that the putative labeled precursors (peaks *a + b*), intermediates (peak *c*), and neurophysins (peak *e*) are all specifically immunoprecipitated by antibodies to rat neurophysin.¹ Thus, there appears to be significant amino acid sequence homology between the putative precursors, intermediates, and neurophysin, thereby strengthening the case for identification of the precursors. Furthermore, the apparent heterogeneity in the labeled precursor peak (*a + b*) revealed by IEF (Table II) has been clarified by our recent experiments on rats homozygous for diabetes insipidus. Pulse-labeling experiments in these vasopressin-deficient rats have shown that only the labeled 5.4 pI precursor, 5.1 pI intermediate, and 4.6 pI neurophysin were synthesized (see Table II for pI values found in normal rats). Thus, it would appear that the precursor for the oxytocin-related neurophysin has a pI = 5.4, whereas the vasopressin-related precursor has a pI = 6.1.

Post-Translational Modification during Axonal Transport

The data illustrated in Fig. 5 B and C show that the earliest arriving labeled proteins in the median eminence and pituitary contain significant amounts of labeled putative precursor. However, at later times, only labeled neurophysins were found. This suggests that the precursors are packaged in a translocatable compartment (e.g., the neurosecretory granules) and therefore, may undergo post-translational cleavage to the products intragranularly during axonal transport. A hypothetical model of this phenomenon is presented in Fig. 9.

¹ Brownstein, M. J., A. G. Robinson, and H. Gainer. Manuscript in preparation.

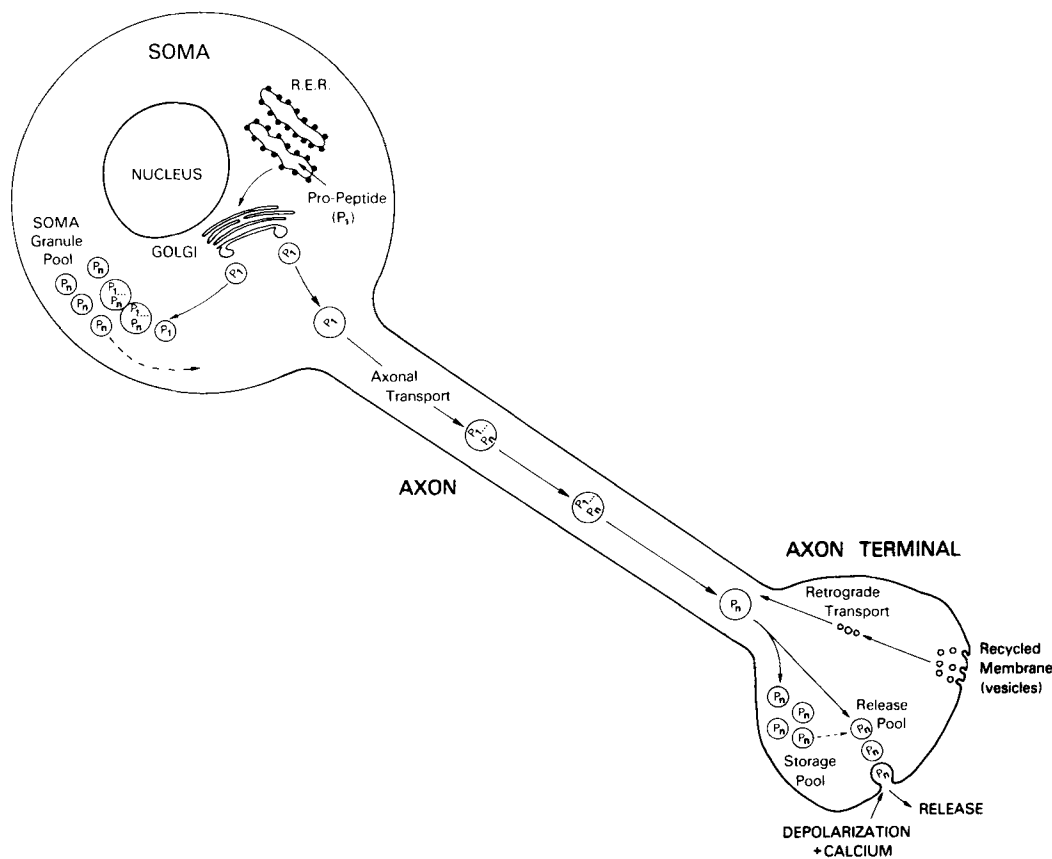


FIGURE 9 Hypothetical model of biosynthesis, translocation, processing, and release of peptides in a peptidergic neuron. Translation of mRNA occurs on the rough endoplasmic reticulum (R. E. R.) in the neuron soma yielding a propeptide or precursor protein molecule (P_1) in the cisternal space of the R. E. R. The packaging of P_1 into secretory granules occurs in the Golgi body. The secretory granule represents the site of post-translational processing to smaller peptide products ($P_1 \dots P_n$) which can occur either in the neuron soma or in the axon during axonal transport. The peptide products (P_n) are released from the neuron terminals by depolarization in the presence of extracellular calcium.

In addition to this evidence, several other lines of evidence suggest that the processing of the precursor occurs intragranularly during axonal transport. Sachs (41) found that the vasopressin content of granules in the neurohypophysis is five-fold greater than in the hypothalamus, and suggested that the generation of vasopressin from the precursor occurs principally in a region in between the perikarya in the hypothalamus and the neural lobe, i.e., in the axon. More recently, morphological evidence in support of intragranular processing has been reported. Using a triple aldehyde fixative, Morris and Cannata (28) found that the dense core or neurosecretory granules in the neural lobe was best preserved at pH 5.0-6.0, but that a marked loss of density of the granule core

occurred at pH 8.0 (only 5% of all granules in the neural lobe remained dense cored at pH 8.0). In contrast, the granules in the perikarya in the SON and PVN showed a high degree of preservation of the dense core at pH 8.0 (7). The authors suggest that this difference in response to fixation at pH 8.0 between hypothalamus and neural lobe may reflect "maturation" changes in the granule during axonal transport. This maturation presumably reflects the precursor-to-product conversion process.

Diversity of Peptides Transported to the Posterior Pituitary

The posterior pituitary gland is known to contain a wide variety of peptides relative to other

tissues (46, 51). The biosynthesis-axonal transport paradigm employed in this study has allowed us to examine which of these peptides were synthesized in the hypothalamus, i.e., the SON in our experiments, ultimately for release by the posterior pituitary. Thus, by this approach, we have used two properties of the peptidergic neuron as "biological filters" to elucidate which peptides may be "functionally relevant": a) the axonal transport mechanism and b) the release mechanism at the nerve terminal. For the latter, the criterion of calcium-dependent release (12) was used.

At least six substantially labeled peptides, in addition to oxytocin and vasopressin, were detected in the SON and the posterior pituitary, and were released by calcium-dependent, potassium depolarization of the pituitary. It should be pointed out that this represents a minimum estimate since only ³⁵S-cysteine-containing peptides could be detected in these experiments. The use of other labeled amino acid precursors in future experiments may allow the resolution of still other peptides which are synthesized in the SON and transported to the neurohypophysis. The functional significance of these peptides, other than oxytocin and vasopressin, is unclear. Whether they are simply degradative by-products of the post-translational cleavage of the precursor or are mediators of some still unknown hormonal functions (possibly trophic) of the posterior pituitary remains to be determined.

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