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Neurophysin biosynthesis in normal rats and in rats with hereditary diabetes insipidus

(vasopressin/oxytocin/neurohypophysis/supraoptic nucleus)

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ABSTRACT **When [^{35}S]cysteine was injected adjacent to the supraoptic nucleus (SON) in rats, it was rapidly incorporated into proteins in the SON. The [^{35}S]cysteine-labeled proteins extracted from the SON were separated by isoelectric focusing on polyacrylamide gels. Twenty minutes after the injection of [^{35}S]cysteine, two major labeled peaks (pI = 5.4 and 6.1) were found in the SON of normal rats; Brattleboro rats had only one major labeled peak (pI = 5.4). One hour after the injection, four major radioactive peaks were found in the SON of normal animals (pI = 5.1, 5.4, 5.6, and 6.1). Animals with diabetes insipidus had only two major labeled proteins (pI = 5.1 and 5.4). Twenty-four hours after normal rats were injected with [^{35}S]-cysteine, all of the labeled peaks described above, except for the one with pI = 5.1, had decreased markedly in size and a small amount of labeled protein with pI about 4.8 was present in the SON. After 24 hr the posterior pituitary of normal animals contained two [^{35}S]cysteine-labeled proteins with pI = 4.6 and 4.8. The pituitaries of Brattleboro rats had only the pI = 4.6 labeled protein. These pulse-chase data, with data we have presented elsewhere, indicate that the vasopressin- and oxytocin-neurophysins are synthesized as parts of separate precursors (pI = 6.1 and 5.4, respectively). These precursors are converted into at least two intermediates (pI = 5.6 and 5.1) which, in turn, yield the vasopressin-neurophysin (pI = 4.8) and the oxytocin-neurophysin (pI = 4.6).**

Neurons in the supraoptic nucleus (SON) and paraventricular nucleus synthesize two peptide hormones (oxytocin and vasopressin) as well as their "carrier" proteins (neurophysins) (1–3). The oxytocin, vasopressin, and neurophysins are transported, in axons of the neurons that synthesize them, to the posterior pituitary via the median eminence. Sachs and his colleagues (4–8) have hypothesized that vasopressin and its neurophysin come from a common precursor protein that is synthesized in the hypothalamus by a ribosomal mechanism. We have already presented some evidence consistent with this hypothesis (9, 10). We have found that, within 1 hr, [^{35}S]cysteine injected adjacent to the SON is incorporated into two proteins with molecular weights (M_r) of about 20,000 and pI of 5.4 and 6.1 (9, 10). It appears that, in time, these two proteins give rise to the two neurophysins (M_r 12,000). The conversion of the precursors into the neurophysins seems to take place mainly during axonal transport of the proteins to the posterior pituitary and to proceed via two approximately 17,000 M_r intermediate species (pI = 5.1 and 5.6) (9, 10).

In order to establish better that the pI 5.4 and 6.1 protein species are precursors for the labeled proteins that appear later, we undertook a more detailed study of the patterns of incorporation of [^{35}S]cysteine into proteins as a function of time. In addition, we used Brattleboro rats—i.e., animals that lack vasopressin and its associated neurophysin (11, 12) and hence have diabetes insipidus—to determine which of the putative precursors, intermediates, and neurophysins are related to vasopressin and which to oxytocin.

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MATERIALS AND METHODS

Animals and Operative Procedures. Female Osborne–Mendel (225–250 g) and Brattleboro (130–150 g) rats were used in these studies. Some of the normal Osborne–Mendel rats were given 2% (wt/vol) saline to drink for 1 week prior to use in order to stimulate vasopressin and neurophysin synthesis; others were given water ad lib as were the Brattleboro animals.

The animals were anesthetized with ether, their heads were fixed in a stereotaxic device (5°, nose down), and a flap of bone was removed from their skulls. The 30-gauge stainless steel needles were positioned 7 mm rostral to the interauricular line (6.8 mm for Brattleboro rats), and 2.5 mm on each side of the midline (2 mm for Brattleboro animals). The needles were lowered 8.6 mm (8.2 mm for Brattleboro animals) beneath the dural surface, and 1 μl of a solution containing 20 μCi of [^{35}S]cysteine (50 Ci/mmol; New England Nuclear) in 0.9% NaCl and 10 mM dithiothreitol was injected through each needle over a 4-min period. After the injection, the needles were left in place for 10 min and then removed. At various times after the injections the rats were killed by decapitation, and their brains and pituitaries were quickly removed and frozen on dry ice. The hypothalamus of each brain was serially sectioned in the coronal plane in a cryostat (−9°C). Samples of the supraoptic nuclei and median eminence were then dissected from the thick frozen sections with hollow stainless steel needles (13). The posterior lobe was cut freehand from the frozen pituitary. The tissue samples pooled from three to six animals were homogenized in 0.1 M HCl and the homogenates were stored at −70°C until they were analyzed.

Polyacrylamide Gel Electrophoresis. Isoelectric focusing in polyacrylamide gels (14) was used to separate ^{35}S-labeled proteins. Ten percent (wt/vol) trichloroacetic acid was added to the 0.1 M HCl tissue extract, and the precipitated proteins were separated from the soluble peptides by centrifugation. The precipitated material was washed extensively with ether to remove the trichloroacetic acid and then was redissolved in sample buffer containing 8 M urea, 1% (vol/vol) Triton X-100, and 2% (wt/vol) ampholytes (pH 3–10 or pH 4–6 nominal ranges, Bio-Rad). The sample was loaded at the anodal end of the gel. After isoelectric focusing according to the method of O'Farrell (14), the radioactive patterns on the gels were determined by conventional slicing and counting techniques.

In one experiment, samples were subjected to isoelectric

Abbreviations: SON, supraoptic nucleus; M_r, molecular weight; NaDodSO$_4$, sodium dodecyl sulfate.

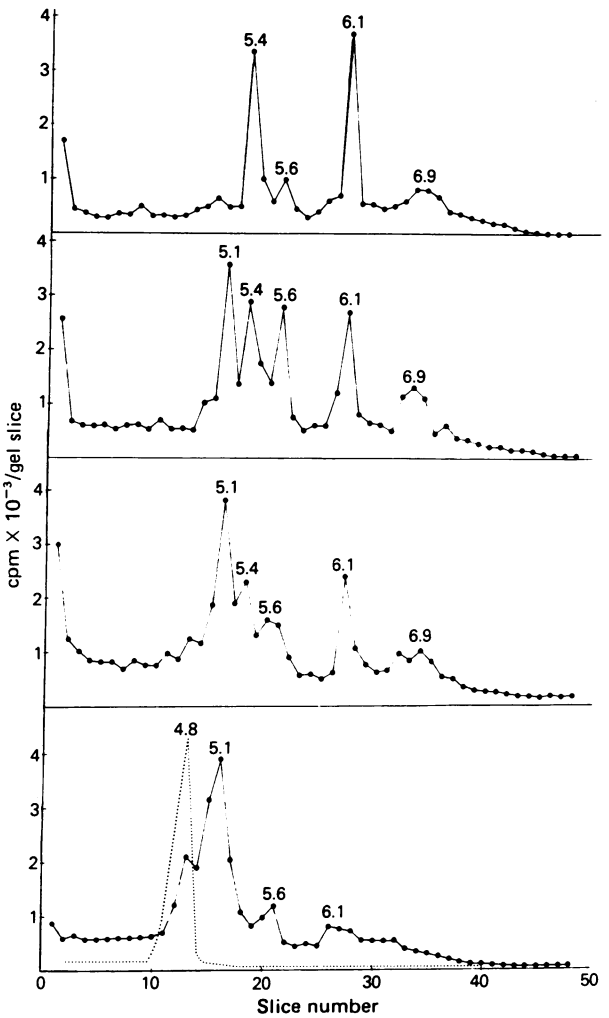

FIG. 1. Isoelectric focusing (pH 3–10 range ampholytes) of ^{35}S-labeled proteins extracted from SON of normal rats at various times after microinjection of [^{35}S]cysteine in the SON. From top to bottom: 20 min; 1 hr; 6 hr; 24 hr. The pI values of the proteins are indicated above each peak. The position of a rat neurophysin standard (pI = 4.5) is shown by the dotted line on the 24-hr plot.

focusing on gels and then the gels were cut longitudinally into two halves. One half was sliced and its radioactive profile was ascertained; the other half was stored at −70°C. When the location of the radiolabeled proteins had been determined, the slices containing the peaks were individually incubated in sodium dodecyl sulfate ($NaDodSO_4$) (14) and analyzed by re-electrophoresis on a $NaDodSO_4$ gel. $NaDodSO_4$ gel electrophoresis was done with the buffer system described by Neville (15). The running pH was 9.5; the sample buffer contained 1% $NaDodSO_4$ and 2% 2-mercaptoethanol (in the upper gel buffer.) Bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 29,000), cytochrome *c* (M_r 12,000), and insulin α and β subunits (M_r 2500 and 3500) were used as marker proteins.

Analysis of Radioactivity. Polyacrylamide gels were stained with Coomassie blue, destained, sliced by conventional methods, and processed for measurement of their radioactivity as described elsewhere (16).

RESULTS

[^{35}S]Cysteine-Labeled Proteins in SON of Normal Rats. Fifteen to 30 min after [^{35}S]cysteine was injected next to the SON of normal animals (given water or 2% saline to drink), two major labeled proteins were found in acid extracts of the SON (Fig. 1). These had pI of 5.4 and 6.1, and their molecular weights (as determined by $NaDodSO_4$/gel electrophoresis) were approximately 20,000–22,000 (Fig. 2). At 1 hr after injection, there were four major radioactive peaks instead of two; the two new peaks had pI of 5.1 and 5.6 (Fig. 1) and their M_r were between 15,000 and 17,000 (Fig. 2). As the time after injection of cysteine increased, the original proteins (pI 5.4 and 6.1) gradually disappeared from the SON as did the pI 5.6 protein.‡ Thus, at 6 hr after injection the pI 5.4, 5.6, and 6.1 peaks were relatively small and at 24 hr after injection a significant amount of only the pI 5.1 peak remained (Fig. 1). In addition to the pI 5.1 peak, however, there appeared to be some material with a pI of about 4.8 (presumably neurophysin) in the SON 24 hr after injection (Fig. 1).

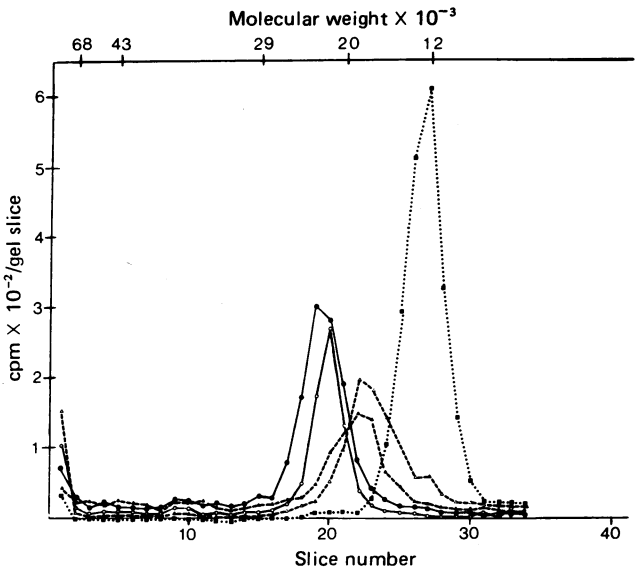

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis (11% gels), eluted isoelectric focused proteins. The pI 5.4 (○—○) and 6.1 (●—●) precursors have apparent M_r of 20,000–22,000; the pI 5.1 (△ - - △) and 5.6 (▲ - - ▲) intermediates have M_r 15,000–17,000; and the rat neurophysin (■ ··· ■) has M_r of about 12,000.

[^{35}S]Cysteine-Labeled Proteins in SON of Brattleboro Rats. Shortly (15–30 min) after the injection of [^{35}S]cysteine adjacent to the SON of Brattleboro rats, only one major labeled protein appeared (Fig. 3). This protein had a pI of 5.4. At 1 hr after injection, a second significant peak with a pI of 5.1 was found in the SON (Fig. 3). The large pI 5.6 and 6.1 peaks and the smaller pI 6.9 peak observed in normal rats (Figs. 1 and 3) were never seen in rats with diabetes insipidus (Brattleboro rats).

[^{35}S]Cysteine-Labeled Proteins in Posterior Pituitaries of Normal and Brattleboro Rats. Twenty-four hours after [^{35}S]cysteine was injected into the SON of normal animals, two radioactive proteins were found in their posterior pituitaries (Fig. 4). These had pI of 4.6 and 4.8 and M_r of about 12,000 (Fig. 2). Consistent with the SON analyses (Fig. 3), the pituitaries of Brattleboro rats contained only one ^{35}S-labeled protein and it had a pI of 4.6 (Fig. 4).

The patterns of protein labeling in the SON and posterior pituitary described above were remarkably constant from sample to sample.

‡ The large labeled proteins seemed to disappear from the SON more rapidly in rats drinking 2% saline than in rats drinking water (unpublished data).

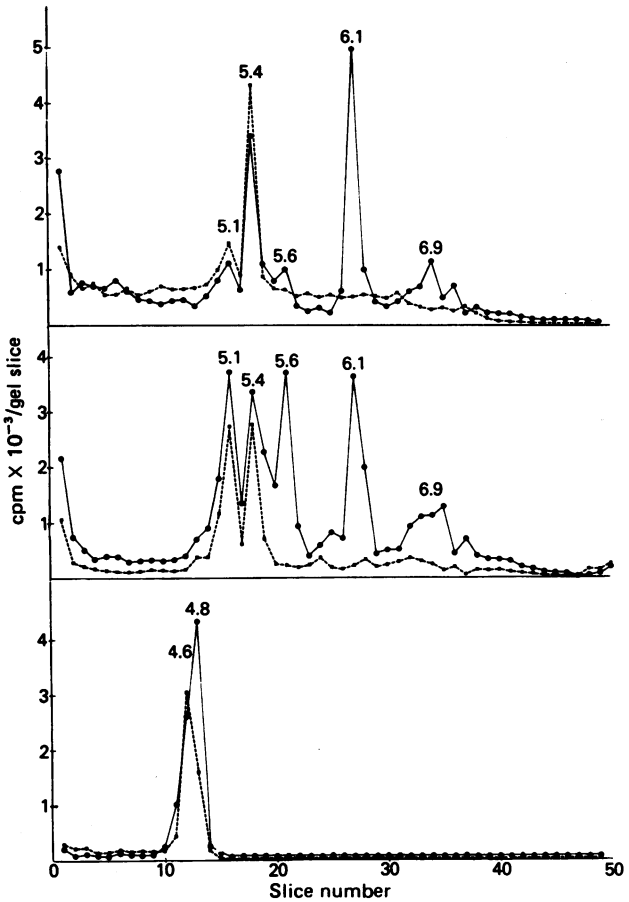

FIG. 3. Isoelectric focusing (pH 3–10 range ampholytes) of ^{35}S-labeled proteins extracted from SON and posterior pituitaries of 2% saline-treated Osborne–Mendel rats (solid lines) and Brattleboro rats (broken lines) after injecting [^{35}S]cysteine into the SON. The pI values of the protein peaks are indicated above them. The Brattleboro rats lack the pI 5.6, 6.1, and 6.9 peaks. They also lack the pI 4.8 neurophysin (as shown more clearly in Fig. 4). (*Top*) SON at 20 min. (*Middle*) SON at 1 hr. (*Bottom*) Posterior pituitary at 24 hr.

DISCUSSION

In Brattleboro rats only one labeled protein was found in the SON 20 min after the injection of [^{35}S]cysteine into the SON. This protein had a M_r of about 20,000 and a pI of 5.4. An hour after the [^{35}S]cysteine injection, two labeled proteins were found in the SON instead of one. The new protein had a M_r of 15,000–17,000 and a pI of 5.1. A single 12,000 M_r labeled protein (pI 4.6) was present in the posterior pituitary of the Brattleboro rat at 24 hr after the injection of [^{35}S]cysteine. The pI 4.6, 5.1, and 5.6 molecules all have been shown to react with a specific antiserum against rat neurophysin (17). From their sequential appearance in pulse-chase experiments, their M_r, and their immunological properties, we have concluded that the pI 5.4, 5.1, and 4.6 proteins are precursor, intermediate, and product, respectively. Because these proteins are found in Brattleboro rats, we have concluded that they are oxytocin-associated molecules. The cells of the hypothalamus-neurohypophysis system of normal animals synthesize four distinct proteins (pI 5.6, 6.1, 6.9, and 4.8) in addition to those found in the Brattleboro animals. Therefore, we think that these additional proteins are related to vasopressin production.

The largest and initially formed vasopressin-associated protein has a M_r of 20,000 and a pI of 6.1; the intermediate has a M_r of 17,000 and a pI of 5.6; the neurophysin has a M_r of

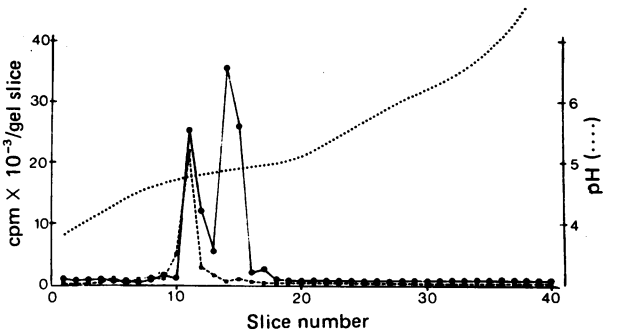

FIG. 4. Isoelectric focusing (pH 4–6 range ampholytes) of proteins extracted from the posterior pituitaries of normal (solid line) and Brattleboro (broken line) rats at 24 hr after injection of [^{35}S]cysteine into the SON. The Brattleboro rats have only one of the two labeled neurophysins (pI 4.6).

12,000 and a pI of 4.8. All of these proteins bind to antibodies against rat neurophysin (17). The fourth protein, which is found in normal rats but not in rats with diabetes insipidus, has a pI of 6.9. A small amount of this labeled pI 6.9 protein was present in the SON at every time point examined. This pI 6.9 material did not exhibit the same kinetic behavior as the other peaks, and the relationship of this material to the pI 6.1, 5.6, and 4.8 molecules is not clear at present. Too little of this protein was present in SON extracts for us to determine unequivocally whether it binds to anti-neurophysin antibodies.

It is noteworthy that the two precursor molecules (pI 5.4 and 6.1) are more basic (i.e., have higher isoelectric points) than their respective intermediates and the two neurophysins. This indicates that the portions of the precursors and intermediates that are removed in order to generate the neurophysins are basic peptides. Because arginine-vasopressin and oxytocin are quite basic (pI >10), and because we have detected several other basic peptides synthesized in the SON and transported to and released by the posterior pituitary (unpublished data; ref. 10), our data are consistent with Sach's hypothesis (4–8) of a common precursor for the neurohypophysial hormones and their associated neurophysins. Removal of the basic peptide moieties (e.g., oxytocin or vasopressin) would then leave the precursor molecules more acidic. Whether vasopressin and oxytocin are in fact parts of their respective neurophysins' precursors can now be tested directly in limited proteolysis and peptide mapping studies, and endogenous proteases that may be responsible for the maturation in the precursors *in situ* can be sought. Whether the precursors are glycoproteins, as are the precursors of corticotropin (ACTH) (18), also can be investigated. These studies are essential in laying the foundation for subsequent analyses of the physiological and pharmacological regulation of oxytocin, vasopressin, and neurophysin synthesis.

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