not a sine qua non as has been advocated by so many investigators in the field of atherosclerosis. Thus, the observation of Zilversmit *et al.* (*5*) that certain lipids (phospholipids) may be synthesized in atherosclerotic lesions assumes a renewed importance.

The hypertensive syndromes of pregnancy may be the outcome of several conditions; all of these may be setting a stage for an injury to the uterine smooth muscle cells comparable with respect to severity and other parameters to that prevailing in the arterial wall at the inception of the atherosclerotic process (*6*). It is believed by most investigators that in hypertensive disorders of pregnancy there is a reduced uteroplacental perfusion (*7*) with resultant hypoxia. Since hypoxia has been also considered to represent a factor in the etiology and pathogenesis of atherosclerotic lesions (*6*), it may play a causative role in fatty metamorphosis of both the uterine and arterial smooth muscle cells.

It should be of interest to extend the reported studies to assess the extent of changes with respect to the entire thickness of the uterus (hysterectomy specimens); to other pathological pregnancies (for example, diabetes mellitus, dysmaturity); and to other organs containing smooth muscle cells (for example, gastrointestinal tract, peribronchial tissues) under a variety of pathological conditions. Notwithstanding the results of any such studies, the observations reported herein imply that the view that the arterial smooth muscle cells are biologically (metabolically) different than are those elsewhere in the body is no longer tenable, at least not with respect to cytoplasmic lipid accumulations.

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Neurophysin Biosynthesis: Conversion of a Putative Precursor During Axonal Transport

Abstract. *[^{35}S]Cysteine injected adjacent to the supraoptic nucleus of the rat is rapidly incorporated into a 20,000-dalton protein that, in time, is converted to a 12,000-dalton labeled protein, neurophysin. This putative precursor of neurophysin appears to be synthesized in the supraoptic nucleus and transformed to neurophysin and related peptides during axonal transport to the neurohypophysis.*

Neurophysins represent a group of cysteine-rich proteins that are synthesized by neurons in the supraoptic and paraventricular nuclei of the hypothalamus and transported to nerve terminals in the posterior pituitary, where they are released together with their associated peptides, oxytocin and vasopressin (*1*). Sachs and colleagues (*2*) have hypothesized that the neurohypophysial peptides (such as vasopressin) and neurophysin derive from the posttranslational cleavage of a common precursor protein. Although indirect evidence supports this hypothesis (*2*, *3*), no biosynthetic evidence identifying this postulated precursor has been reported. In this report, biosynthetic evidence for the existence of a precursor to neurophysin and for its posttranslational modification during axonal transport is presented.

Female rats of the Osborne-Mendel strain (225 to 250 g) were studied. The heads of ether-anesthetized animals were fixed in a stereotaxic instrument (5 deg, nose down) and their brains were exposed. 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 μc of L-[^{35}S]cysteine (50 c/mmole, New England Nuclear) in 0.9 percent NaCl and 10 m*M* dithiothreitol was injected through each needle over a period of 10 minutes. The tips of the needles were found by histological examination of the brains to have been just above and lateral to the supraoptic nuclei. Following the injection, the needles were left in position for 10 minutes, and were then removed from the brain. The scalp was closed with wound clips, and the animals awoke 10 minutes after surgery. At various times after injection, the animals were killed by decapitation, and their brains and pituitaries were quickly removed and frozen on Dry Ice. Serial frontal sections (300 μm thick) of the brain were cut in a cryostat at −9°C. Representative regions of neuronal perikarya in the supraoptic nucleus and of axons in the median eminence were dissected from these frozen sections by the Palkovits punch technique (*4*), and the posterior pituitary was isolated as a sample of the nerve terminal region. The isolated tissues were homogenized in 0.1*N* HCl in order to destroy degradative enzymes (*5*) and were stored at −70°C. The proteins in the tissues were extracted with 1 percent Triton X-100 in 8*M* urea and separated by acid-urea polyacrylamide gel electrophoresis (*6*). The gels were sliced and radioactivity was counted by conventional techniques.

The time course of appearance of labeled proteins in the various regions of the hypothalamo-neurohypophyseal system following [^{35}S]cysteine injection in the supraoptic nucleus was similar to that in previous reports (*7*). Labeled proteins first appeared at the level of the median eminence after about 1 hour, while in the posterior pituitary labeled proteins appeared about 1.5 hours after injection. These values were consistent with the expected axonal transport rates of the neurohypophyseal proteins (*7*), and intraventricular injection of colchicine completely prevented the appearance of labeled proteins in the median eminence and posterior pituitary with little or no effect on incorporation in the supraoptic nucleus (*8*).

The profiles of the labeled proteins separated on acid-urea gels from three regions of the hypothalamo-neurohypo-

physeal system at various times after injection of [^{35}S]cysteine in the supraoptic nucleus are shown in Figs. 1 and 2. One hour after injection, the supraoptic nucleus labeling profile is dominated by a large peak of radioactivity (slice 31, Fig. 1A) with a much lower migration rate than the labeled rat neurophysin peak which ultimately appears in the posterior pituitary (Fig. 2C, slice 43). The arrow designated "pituitary peak" in Fig. 1A corresponds to this neurophysin peak of radioactivity. After 1 hour, very little labeled protein in the supraoptic nucleus comigrates with the pituitary peak. The slower peak in the supraoptic nucleus decreases in radioactivity with time relative to the pituitary peak, so that after 24 hours (Fig. 1D) there is virtually no radioactivity in the slower peak while the pituitary peak has become dominant. The molecular weights of the slowly migrating peak in Fig. 1A (slice 31) and the pituitary peak (neurophysin) in Figs. 1D and 2C were estimated by three different methods to be about 20,000 and 12,000 daltons, respectively (*9*). Thus, we have observed, in the supraoptic nucleus, a biosynthetic process consistent with the conversion of a larger precursor protein to a smaller protein product transported to the posterior pituitary.

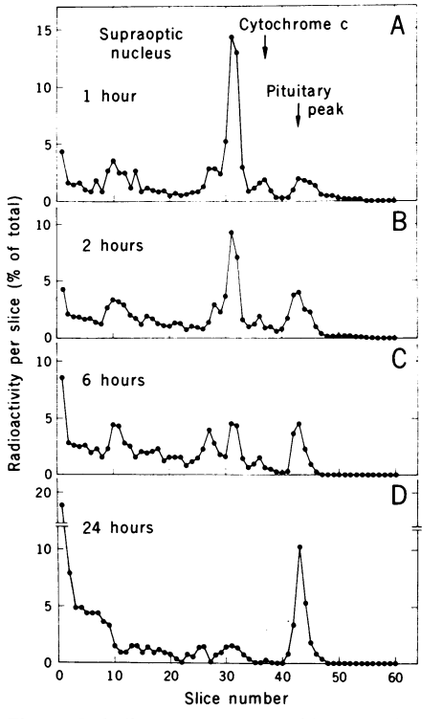

Fig. 1. Labeling patterns on acid-urea polyacrylamide gels (15 percent acrylamide) of proteins extracted from regions of rat supraoptic nucleus at various times after microinjection of [^{35}S]cysteine above and lateral to the supraoptic nucleus. The major radioactive peak after 1 hour (A) was around slice 31; this peak decreased with time (B and C) relative to a radioactive peak (slice 43) that was dominant after 24 hours (D). The latter peak comigrates with the major radioactive peak found in the posterior pituitary after 24 hours (pituitary peak arrow; see also Fig. 2C). Cytochrome c was used as an internal marker protein in each gel run. The radioactivity in each gel slice is plotted as percentage of the total radioactivity on the gel. Each gel pattern represents data from a separate rat.

Several lines of evidence have suggested that the conversion of precursor to product may be occurring in the secretory granule during axonal transport (*10*). The data in Fig. 2 are consistent with this hypothesis. Two hours after injection, the labeling profile in the median eminence contains two major peaks, the putative precursor peak and the neurophysin peak (Fig. 2A). If the time delay between synthesis in the supraoptic nucleus and axonal transport to the median eminence is considered, it is apparent that the labeled proteins arriving in the median eminence after 2 hours must have been derived from the population of labeled proteins in the supraoptic nucleus after about 1 hour (Fig. 1A). Hence, the precursor-to-product conversion process seen in Fig. 2A must have occurred intraaxonally during transport. Twenty-four hours after injection, almost all the radioactivity in the median eminence profile is in the neurophysin peak (Fig. 2B); the same is true for the posterior pituitary (Fig. 1C). The labeling profiles of proteins transported to the posterior pituitary at 6, 12, and 24 hours resembled that in Fig. 2C, whereas the 2-hour pattern was similar to that in the median eminence at 2 hours (Fig. 2A). Thus, the major stored product in the pituitary is the smaller 12,000-dalton labeled protein. We have demonstrated that, like neurophysin, this labeled protein is released from the posterior pituitary by calcium-dependent potassium depolarization (*11*). The lack of heterogeneity of [^{35}S]cysteine-labeled proteins transported to the posterior pituitary (Fig. 2C and ^{35}S radioautographic inset in Fig. 2) stands in contrast to the Coomassie blue protein staining pattern of the same gel (Fig. 2) (*12*).

Since it appears that the labeled 20,000-dalton peak is converted into the 12,000-dalton neurophysin peak in the rat supraoptic nucleus in vivo (Fig. 1), it seems likely that the former is a precursor (or a stable intermediate state of the precursor) of the neurophysin. Data indicating that only these two labeled peaks are transported to the median eminence and the posterior pituitary and that only the neurophysin peak is present at times later than 2 hours (Fig. 2) provide further evidence for the precursor-product relationship between these peaks and suggests that the conversion process is occurring during axonal transport. The above biosynthetic data, showing that a labeled polypeptide of higher molecular weight is first synthesized and then disappears as the lower molecular weight labeled peptide (or peptides) is formed, represents the initial step in identification of a precursor. A definitive demonstration of the precursor nature of the 20,000-dalton protein peak also requires immunological, peptide mapping, and amino acid sequencing studies. We have demonstrated that the labeled precursor proteins were immunoreactive with antibodies to rat neurophysin (*13*), which suggests significant sequence homology between the putative precursor and neurophysin.

Assuming that the 20,000-dalton peak described above contains the common precursor for neurophysin (12,000 daltons) and vasopressin (1,100 daltons) postulated by Sachs *et al.* (*2*), then about 7,000 daltons of the putative precursor still remain unaccounted for. Since the posttranslational conversion of the precursor appears to be occurring in the se-

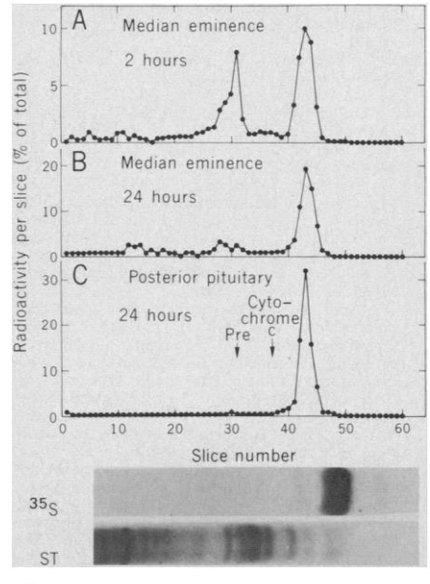

Fig. 2. Labeling patterns on 15 percent acid-urea polyacrylamide gels of proteins transported to the median eminence at 2 hours (A) and 24 hours (B) and to the posterior pituitary at 24 hours (C) after injection of [^{35}S]cysteine in the supraoptic nucleus. A major radioactive peak (*Pre*), which comigrates with the major peak in Fig. 1A, is found in the median eminence 2 hours after injection. The insets are radioautograph (*^{35}S*) and Coomassie blue staining patterns (*ST*) of gel containing labeled proteins in the posterior pituitary 24 hours after injection. Each gel pattern represents data from a separate rat.

cretory granule (*10*) during axonal transport, one might expect to find previously unreported labeled peptide products transported to the pituitary as well. Indeed, we have detected, in addition to labeled oxytocin and vasopressin, at least four major ^{35}S-labeled peptides (ranging between 700 and 2,500 daltons) that are transported to, stored in, and released by the posterior pituitary (*11*). The release of these peptides, which appear to be derived from the putative precursor, raises the question of whether they mediate some still unknown function of the posterior pituitary.

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6. The acid-urea polyacrylamide gels were prepared as described by Y. P. Loh and H. Gainer [*Brain Res.* **92**, 181 (1975)] except that the separating gel contained 15 percent acrylamide and 0.1 percent Triton X-100. Electrophoresis was toward the cathode, and the running *p*H of the gel was 2.7.
7. The appearance of labeled proteins in the posterior pituitary 1.5 hours after injection of [^{35}S]cysteine in the supraoptic nucleus of the rat is consistent with the time course found by A. Norström and J. Sjostrand [*J. Neurochem.* **18**, 29 (1971)] after supraoptic nucleus injection and by B. T. Pickering and C. W. Jones [in *Subcellular Organization and Function in Endocrine Tissues*, H. Heller and K. Lederis, Eds. (Cambridge Univ. Press, New York, 1971), pp. 337–351)] after intracisternal injection of the radioactive amino acid. In addition, the kinetics of appearance of labeled protein in the supraoptic nucleus and median eminence (*11*) are consistent with the axonal transport rates of protein found in the rat hypothalamo-neurohypophyseal system [tabulated by H. Valtin, J. Stewart, and H. W. Sokol, in *Handbook of Physiology*, E. Knobil and W. H. Sawyer, Eds. (American Physiological Society, Washington, D.C., 1974), sect. 7, vol. 4, pp. 131–171].
8. Twelve hours before bilateral injection of [^{35}S]cysteine into the rat supraoptic nucleus, approximately 100 μg of colchicine dissolved in 0.9 percent NaCl was injected intraventricularly. This treatment completely prevented the transport of labeled proteins from the supraoptic nucleus to the median eminence and posterior pituitary. Similar effects of colchicine were demonstrated by A. Norström [*Ann. N.Y. Acad. Sci.* **248**, 46 (1975)] and H. Sachs, D. Pearson, and A. Nureddin (*ibid.*, p. 36).
9. The apparent molecular weights of the putative precursor and neurophysin peaks were calculated to be 20,000 ± 2,000 and 12,000 ± 2,000 daltons, respectively. The three methods used for these determinations included sodium dodecyl sulfate (SDS) gel electrophoresis, Ferguson plot analysis of acid-urea gels, and Sephadex G-75 gel chromatography (*11*).
10. The fact that the vasopressin content of secretory granules is fivefold greater in the neurohypophysis than in the hypothalamus [H. Sachs, *J. Neurochem.* **10**, 289 (1963)] led to the suggestion that this peptide is generated from the precursor protein in the axonal region between the neuron perikarya in the hypothalamus and the nerve terminals in the neurohypophysis. Morphological observations indicating a "maturation" of the secretory granule during axonal transport have been reported by M. A. Cannata and J. F. Morris [*J. Endocrinol.* **57**, 531 (1973)] and J. F. Morris and M. A. Cannata (*ibid.*, p. 517). The possible relationship between this morphological "maturation" and an intragranular conversion of precursor protein to neurophysin and peptides is discussed by B. A. Cross, R. E. Dyball, R. G. Dyer, C. W. Jones, D. W. Lincoln, J. F. Morris, and B. T. Pickering (*Recent Prog. Horm. Res.* **31**, 243 (1975)].
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12. The Coomassie blue staining pattern of proteins extracted from the neurohypophysis and separated on acid-urea gels reveals a number of stained bands; the most rapidly migrating of these bands corresponds in mobility to the labeled peak (insets, Fig. 2). This rapidly migrating stained band is the one which dramatically decreases in intensity when rats are given 2 percent saline to drink for 5 days (*11*). This indicates that it is this protein (or proteins) which is released by the pituitary during osmotic stress. The labeled neurophysin, which consistently migrates as a single peak on acid-urea gels (and SDS gels), may in fact be heterogeneous, since separation of the labeled posterior pituitary proteins by isoelectric focusing revealed two major peaks of radioactivity with isoelectric points of 4.6 and 4.8 (*11*). Using another gel system, G. D. Burford and B. T. Pickering [*Biochem. J.* **128**, 941 (1972)] reported the transport of two major peaks of labeled neurophysin to the rat neurohypophysis after intracisternal injection of [^{35}S]cysteine.
13. The labeled putative precursors and products (corresponding to peaks in slices 31 and 43, respectively) in Figs. 1 and 2 were specifically precipitated by antibodies to rat neurophysin (antibodies were obtained from Dr. A. G. Robinson). The labeled proteins of higher molecular weight (such as those in slice 10 in Fig. 1) were not precipitated by antibody.

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Glutamine Synthetase: Glial Localization in Brain

Abstract. *Light microscopy immunohistochemical techniques were used to examine the distribution of glutamine synthetase in rat brain. Glutamine synthetase was found to be localized in the glial cells. Neuronal cell bodies, endothelial cells, and choroid epithelium contained no enzyme. The findings indicate that glia have a crucial role in glutamic acid, γ-aminobutyric acid, and ammonia metabolism in brain.*

Glutamic acid participates in a number of important metabolic processes in brain (*1*) and may also serve as an excitatory neurotransmitter (*2, 3*). It is generally accepted that glutamate metabolism in brain is compartmentalized (*4*). A large compartment has been linked with energy metabolism, whereas a small compartment with a fast turnover has been associated with glutamine synthesis, ammonia detoxification, and metabolism of the putative inhibitory neurotransmitter, γ-aminobutyric acid (GABA). The site of these metabolic compartments has not been established, although indirect studies suggest that glia may contain the small glutamate compartment (*5*).

Glutamine synthetase (E.C. 6.3.1.2) is a key enzyme of the small glutamate compartment which catalyzes the amidation of glutamate to glutamine (*1*). If the location of this enzyme could be determined, then this would provide strong evidence for the site of the small compartment. Glutamine synthetase is present chiefly in the microsomal fraction of brain (*6*), although a small amount has also been found in the synaptosomal fraction (*7*). Attempts to determine the location of glutamine synthetase more precisely by bulk cell isolation techniques have been unsuccessful (*8*). By using immunohistochemical techniques specific for glutamine synthetase, we have found that the enzyme is localized in the glial cells of rat brain.

Three adult albino rabbits were immunized by weekly subcutaneous injections of 0.1 mg of glutamine synthetase (purified from sheep brain, Sigma G-6632, lot 35C-8650, 120 unit/mg) dissolved in 0.5*M* phosphate-buffered saline, *p*H 7.4, and mixed with Freund's adjuvant (1 : 1 by volume). Serum was collected weekly, and at the end of 8 weeks the rabbits were killed. A rat brain extract containing glutamine synthetase antigen was obtained from the supernatant fraction by homogenization in sodium barbital buffer, *p*H 8.6, containing 1 percent Triton X-100, and subsequent centrifugation at 40,000*g* for 1 hour. The rabbit antiserum was characterized against both sheep glutamine synthetase and the rat brain extract by double immunodiffusion and immunoelectrophoretic studies (*9*). By immunodiffusion, both antigens (from sheep and rat) gave a single fused precipitin line indicating antigenic identity (Fig. 1A). Immunoelectrophoresis demonstrated the monospecificity of the