HYPOTHALAMIC PEPTIDYL-GLYCINE α -AMIDATING MONOOXYGENASE: PRELIMINARY CHARACTERIZATION¹

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Abstract

An enzymatic activity capable of converting mono-[¹²⁵I]-D-Tyr-Val-Gly into mono-[¹²⁵I]-D-Tyr-Val-NH₂ was identified in a crude mitochondrial/synaptosomal preparation from rat hypothalamus. Further subcellular fractionation studies localized a majority of this enzymatic activity to fractions enriched in synaptic vesicles. The α -amidation activity demonstrated optimal activity at pH 7.5 to 8, was stimulated by the presence of copper ions and reduced ascorbate and required the presence of molecular oxygen. Endogenous α -amidation activity was inhibited by the addition of ascorbate oxidase. Kinetic analyses demonstrated Michaelis-Menten type kinetics for D-Tyr-Val-Gly as the varied substrate with the values of K_m and V_{max} increasing as the ascorbate concentration in the reaction increased. A variety of peptides possessing carboxyl-terminal glycine residues were potent inhibitors of the reaction, while peptides lacking a carboxyl-terminal glycine residue were not, suggesting that many glycine-extended peptides may serve as substrates in the α -amidation reaction. The characteristics of hypothalamic α -amidation activity are similar to those previously reported for the α -amidation activity in rat pituitary and mouse corticotropin tumor cells suggesting the presence of closely related enzymes in these tissues.

A number of neuroendocrine peptides contain an α -amide moiety at their carboxyl terminal. In general, the presence of such an α -amide group is essential for optimal biological activity. The occurrence of this post-translational modification is so characteristic of bioactive peptides that Tatemoto (1982) has purified several novel bioactive peptides based solely on the presence of such an amino acid α -amide group. The hypothalamus is a particularly rich source of peptides with α -amide groups including oxytocin, vasopressin, α -melanocyte-stimulating hormone (α -MSH),² growth hormone- and corticotropin-releasing factors, as well as luteinizing hormone- and thyrotropin-releasing hormones (for review see Mains et al., 1983).

The amino acid sequences of many precursors to α -amidated peptides have been elucidated over the past several years (Nakanishi et al., 1979; Amara et al., 1982; Land et al., 1982; Tatemoto, 1982; Yoo et al., 1982). In every case, the amino acid which ultimately bears the α -amide moiety in the final product

is followed by a glycine residue in the precursor molecule. This observation has led investigators to speculate that the amino nitrogen of the glycyl residue in the precursor serves as the source for the amide nitrogen in the final product. Additional evidence in support of this premise has come from the identification of what appears to be incompletely processed, glycine-extended forms of pancreatic polypeptide and α -MSH in cultured cells (Schwartz et al., 1980; Paquette et al., 1981; Eipper et al., 1983a).

Bradbury et al. (1982) designed a synthetic substrate (D-Tyr-Val-Gly), based on the carboxyl terminus to the presumed immediate precursor for α -MSH, to detect a peptide α -amidation activity in porcine pituitary secretory granules. Substrate synthesized with [¹⁵N]glycine was used to demonstrate that the amide nitrogen of the product is derived from the amino nitrogen of the glycine residue present in the precursor.

Recently, Eipper et al. (1983b) have described a peptidyl-glycine α -amidating activity in secretory granule fractions from rat anterior, intermediate, neural and bovine intermediate pituitary lobes as well as a mouse corticotropin tumor cell line (AtT-20) (Mains et al., 1984). This activity was stimulated by the presence of copper ions and ascorbate and requires the presence of molecular oxygen. Current data suggest that the substrate specificity for pituitary α -amidation activity is very broad (Bradbury et al., 1982; Bradbury and Smyth, 1983; Landymore-Lim et al., 1983), and similar enzymatic activities have been found in other tissues (Husain and Tate, 1983; Kizer et al., 1984; Myers et al., 1984; Wand et al., 1984).

The biosynthetic processes involved in the formation of a majority of hypothalamic peptide hormones are just beginning to be elucidated. The majority of the proteolytic cleavages that

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² The abbreviations used are: bis-Tris, (bis(2-hydroxyethyl)imino)Tris(hydroxymethyl)methane/Tris(hydroxymethyl)aminomethane; DDC, diethyldithiocarbamate; DMPH₄, 6,7-dimethyl-5,6,7,8-tetrahydropterine; α -MSH, α -melanocyte-stimulating hormone; Na TES, N-[Tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid.

produce α -MSH and β -endorphin from pro-ACTH/endorphin in the intermediate lobe of the pituitary do not occur until the prohormone has moved into the secretory granules (Glembotski, 1981, 1982). A similar sequence of processing events for these hormones has also been postulated in the hypothalamus (Barnea et al., 1981). Thus, any α -amidation activity involved in the production of hypothalamic hormones or releasing factors might be expected to exist within the secretory granules (synaptic vesicles). Since the hypothalamus is responsible for the biosynthesis of many peptides which possess α -amide groups, the presence of a peptide α -amidation activity in this tissue was examined.

Materials and Methods

Subcellular fractionation

Crude fractionation. All procedures were carried out at 4°C. Whole brains from male Sprague-Dawley rats (100 to 125 gm) were rapidly removed following decapitation. Hypothalamic tissue (~25 mg/hypothalamus) was dissected and homogenized by hand in 10 vol (w/v) of buffered, isotonic sucrose (0.32 M sucrose/2 mM MgCl₂/10 mM Tris-HCl (Tris(hydroxymethyl)aminomethane), pH 7.4) with four strokes utilizing a Potter-Elvehjem glass homogenizer with Teflon pestle (0.25 mm clearance). The homogenate was centrifuged at 1,000 $\times$ g for 10 min to sediment nuclei and cellular debris (P1) (Whittaker, 1969). The volume of the 1,000 $\times$ g supernatant fraction (S1) was adjusted to 10 ml with homogenization buffer, and the supernatant was centrifuged at 20,000 $\times$ g for 30 min in a fixed-angle rotor (Beckman, type 50 Ti). The resulting 20,000 $\times$ g pellet (P2), representing the crude mitochondrial/synaptosomal fraction (Gray and Whittaker, 1962), was resuspended in homogenization buffer, frozen and thawed five times, and used as a source of α -amidation activity in further experiments.

Subsynaptosomal fractionation. Hypothalamic tissue from four to six rats was obtained as described above and homogenized in 10 vol (w/v) of homogenization buffer (0.32 M sucrose/10 mM Tris-HCl, pH 7.0) in a Potter-Elvehjem glass homogenizer with Teflon pestle (0.125 mm clearance) with 12 strokes at 840 rpm. The homogenate was fractionated by differential centrifugation as described above with the exception that the P1 pellet was washed once by resuspension and centrifugation. The 1,000 $\times$ g supernatant fluids (S1) were then pooled and centrifuged at 20,000 $\times$ g for 30 min. The pellet (P2) was gently resuspended in distilled water (2 ml/gm of original tissue) by trituration. This suspension was further fractionated on a discontinuous sucrose density gradient modified from Whittaker et al. (1964). The gradient was obtained by layering 800- μ l volumes of 1.2, 1.1, 1.0, 0.90, 0.80, and 0.32 M sucrose into a 5-ml polyallomer tube approximately 1 hr before use. All sucrose solutions were buffered with 10 mM Tris-HCl, titrated to pH 7.0 with HCl. The sample was placed upon the 0.32 M sucrose layer and the gradient was centrifuged at 100,000 $\times$ g for 1 hr utilizing a swinging bucket rotor (Beckman, type SW55 Ti). The gradient was collected into 200- μ l fractions, from least to most dense.

Characterization of subsynaptosomal fractionation. Fumarase activity of sucrose gradient fractions was assessed by a modification of the spectrophotometric procedure of Racker (1950). An aliquot of each fraction (5 to 50 μ l) was incubated at 25°C in the presence of 100 mM sodium phosphate and 0.015% Triton X-100 at pH 7.4 (Glembotski, 1982). The reaction was started by the addition of sodium L-malate (final concentration, 43 mM). Changes in absorbance were measured at 240 nm.

For immunoassays, aliquots of each fraction (40 μ l) were diluted 2-fold into 50 mM sodium phosphate buffer, pH 7.4, which contained 0.1% Triton X-100 and assayed for 16,000-dalton fragment immunoreactivity using antiserum Georgie as previously described (Eipper and Mains, 1978).

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Aliquots of each fraction were assayed for α -amidation activity in the presence of [¹²⁵I]-D-Tyr-Val-Gly, 0.5 μ M D-Tyr-Val-Gly, 12.5 μ M CuSO₄, 0.625 mM ascorbic acid, 100 μ g/ml of catalase (Boehringer Mannheim no. 106810; 65,000 units/mg), and 67 mM bis-Tris (bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane)/67 mM Tris-HCl, pH 7.5, as described below.

Amidation assays

The amidation assay used was adapted from that of Eipper et al. (1984). Assays were carried out in duplicate at 37°C in 1.5-ml microfuge tubes with a final volume of either 20 or 40 μ l. Results presented are the average of assay duplicates which usually agreed within 5% of their mean. Unless otherwise indicated, assay tubes contained 10,000 to 20,000 cpm of mono-[¹²⁵I]-D-Tyr-Val-Gly; the indicated concentrations of D-Tyr-Val-Gly (Bachem), ascorbic acid, CuSO₄, 100 μ g/ml of catalase, and either crude mitochondrial/synaptosomal or synaptic vesicle-associated protein. The standard stock assay buffer was either 75 mM bis-Tris/75 mM Tris-HCl, pH 7.5, or 150 mM Na TES (N-[Tris(hydroxymethyl)methyl-2-amino]ethanesulfonic acid), pH 7.0, as noted in the figure legends. In the final assay solution, the stock buffer was usually diluted by about 20%.

The reaction was allowed to proceed for 3 to 6 hr and was stopped by the addition of 1.3 ml of 10 mM sodium phosphate, pH 5.0. The diluted samples were then applied to a 1.0-ml column of SP-Sephadex-C25-120 equilibrated with 10 mM sodium phosphate, pH 5.0. After washing the column with 9 ml of 10 mM sodium phosphate, pH 5.0, to remove unreacted substrate, the reaction product was eluted with two 1.7-ml aliquots of 0.5 M NaCl in 10 mM sodium phosphate, pH 5.0. Samples were assayed for radioactivity in a gamma scintillation counter (75% efficiency). Reaction velocities were calculated as previously described (Eipper et al., 1983b). Specific assay conditions are described in the figure legends. Columns were recycled for use in subsequent assays by washing the ion-exchange resin with the following: 5 ml of 100 mM NaOH, 10 ml of H₂O, 5 ml of 100 mM H₃PO₄, 10 ml of H₂O, 5 ml of 200 mM sodium phosphate, pH 5.0, and 5 ml of 10 mM sodium phosphate, pH 5.0; results obtained from columns regenerated in this manner were indistinguishable from results obtained with previously unused ion-exchange resin.

Results

Initial identification of α -amidation activity in hypothalamic extracts. Preliminary experiments involving the incubation of [¹²⁵I]-D-Tyr-Val-Gly with whole hypothalamic homogenates or crude subcellular fractions obtained by differential centrifugation were unable to detect the presence of α -amidation activity in this tissue. Previous studies on α -amidation activity in rat pituitary secretory granules (Eipper et al., 1983b) demonstrated the occurrence of potent inhibitor(s) of such activity in both whole pituitary homogenates and various Percoll gradient subcellular fractions; the effect of these inhibitors was largely overcome by the addition of exogenous copper (Glembotski et al., 1984). In addition, ascorbic acid was shown to be a potent stimulator of pituitary α -amidation activity (Eipper et al., 1983b, 1984; Glembotski et al., 1984). When copper and ascorbic acid were added to incubations with whole hypothalamic homogenates or crude subcellular fractions, it was possible to detect a peptide α -amidation activity. Addition of optimal concentrations of ascorbic acid (0.625 mM) to crude mitochondrial/synaptosomal fractions resulted in a 43-fold increase in hypothalamic α -amidation activity. There was no detectable activity in the absence of exogenous copper, so that the fold-stimulation upon addition of optimal concentrations of copper (25 μ M) was undefined.

Subcellular fractionation of hypothalamic tissue revealed that approximately 40% of the total α -amidation activity could be recovered in a crude mitochondrial/synaptosomal pellet (P2). The majority of the remaining activity (50%) was isolated in the pellet representing nuclei and cellular debris (P1). Attempts to further homogenize the nuclear pellet, in order to release additional activity, resulted in little increase of α -amidation activity in the crude mitochondrial/synaptosomal fraction and generally caused the solubilization of such activity as it moved from the nuclear pellet to the postmicrosomal supernatant (S3).

To establish the incubation times in which steady product formation was occurring, a time course was established using the α -amidation activity derived from a crude mitochondrial/

synaptosomal pellet (P2). The formation of D-Tyr-Val-NH₂ was linear in time for at least 6 hr after an initial lag of approximately 30 min; a similar lag has previously been described for bovine intermediate pituitary and AtT-20 α -amidation activity (Eipper et al., 1984; Mains et al., 1984). When the amount of protein from a crude mitochondrial/synaptosomal pellet was varied from 1.25 to 22.5 μ g, a linear increase in the production of product was observed. Enzyme that was boiled for 5 min before incubation in the α -amidation reaction showed no detectable activity (less than 0.1% conversion).

To determine whether the radioactivity, which eluted from the cation exchange resin (SP-Sephadex) at high ionic strength (0.5 M NaCl), upon application of the hypothalamic reaction product, represented [¹²⁵I]-D-Tyr-Val-NH₂, such reaction products were analyzed by reversed phase high performance liquid chromatography (Fig. 1). The radiolabeled product of such α -amidation assays co-eluted with synthetic mono-[¹²⁵I]-D-Tyr-Val-NH₂, thereby justifying the use of this ion-exchange procedure as an effective means of separating product from unreacted substrate as well as confirming the identity of the hypothalamic α -amidation product.

Since a crude preparation of synaptosomes contains a significant amount of other subcellular organelles, it was necessary to further fractionate the crude preparation into its component organelles to remove possible inhibitor(s) to the α -amidation reaction and to focus on the enzymatic activity involved in the biosynthetic processing of hypothalamic peptide hormones.

Characterization of synaptic vesicle-associated α -amidation

Figure 1. Product identification. Synthetic [¹²⁵I]-D-Tyr-Val-Gly and [¹²⁵I]-D-Tyr-Val-NH₂ (Bachem) were analyzed both separately and together by reversed phase high performance liquid chromatography on a Waters C₁₈ μ Bondapak column equilibrated with 4% CH₃CN in 0.1% trifluoroacetic acid and eluted with a linear gradient to 20% CH₃CN in 0.1% trifluoroacetic acid over 60 min. The elution position of D-Tyr-Val-Gly, used as an internal standard in each analysis, was determined by monitoring A₂₂₀. The elution positions for the synthetic standards are designated by arrows. The [¹²⁵I]-labeled hypothalamic reaction product analyzed was obtained from an assay containing [¹²⁵I]-D-Tyr-Val-Gly, 25 μ M CuSO₄, 0.625 mM ascorbic acid, 100 μ g/ml of catalase, and 122 mM Na TES, pH 7.0. The assay was incubated for 5 hr with 72 μ g of crude synaptosomal-associated protein (50% conversion). The hypothalamic reaction product was bound to and then eluted from an SP-Sephadex column as described. The overall recovery of radioactivity for the hypothalamic reaction product was 98% with 88% of the recovered counts eluting with the retention time of synthetic [¹²⁵I]-D-Tyr-Val-NH₂.

activity. Discontinuous sucrose density gradient centrifugation was used to prepare subcellular fractions enriched in synaptic vesicles from hypotonically lysed crude mitochondrial/synaptosomal preparations (Whittaker et al., 1964; Tucek, 1966; Whittaker and Barker, 1972; Parker et al., 1978). Gradient fractions were characterized by radioimmunoassay for pro-ACTH/endorphin-derived 16,000-dalton fragment (a marker for synaptic vesicles), fumarase activity (a marker for mitochondria), protein concentration (Fig. 2), and electron microscopy. Two major peaks of fumarase activity were observed in the dense region of the gradient (fractions 18 to 22 and fraction 25; bottom). These two regions possess the sedimentation characteristics of subcellular fractions containing partially lysed synaptosomes (fractions 18 to 22) and mitochondria (fraction 25) (Whittaker et al., 1964; Parker et al., 1978). Pro-ACTH/endorphin-derived 16,000-dalton fragment immunoreactivity (Fig. 2, top) was found in three regions corresponding to subcellular fractions containing soluble proteins (fractions 1 to 3), synaptic vesicles (fractions 7 to 17), and partially lysed synaptosomes (fractions 18 to 22). These three peaks contained 15, 65, and 17% of the total recovered 16,000-dalton fragment immunoreactivity, respectively. The presence of 16,000-dalton fragment immunoreactivity at the top of the gradient suggests

Figure 2. Sucrose gradient subcellular fractionation of rat hypothalamus. Hypothalami from six male Sprague-Dawley rats were homogenized and fractionated on a discontinuous sucrose gradient, and fractions were assayed for fumarase activity (mitochondria, □), 16,000-dalton fragment immunoreactivity (synaptic vesicles, ●), α -amidation activity (○), and protein content (■) as described under "Materials and Methods;" the recoveries of fumarase activity and 16,000-dalton fragment immunoreactivity were in excess of 90% when compared to the crude mitochondrial/synaptosomal pellet. The left side of the figure represents the top of the centrifuge tube (lowest density).

the breakage of a portion of synaptic vesicles resulting from hypotonic treatment of intact synaptosomes or from subsequent fractionation procedures. Electron microscopic analyses of sucrose gradient fractions confirmed the identity of these subcellular fractions and were consistent with radioimmunological and enzymatic markers.

The distribution of hypothalamic α -amidation activity paralleled that of 16,000-dalton fragment immunoreactivity (Fig. 2, top). The majority of the recovered α -amidation activity was associated with the synaptic vesicle fraction (59%) with lesser amounts in the soluble (12%) and synaptosome-associated (19%) regions. The specific activity of α -amidation activity (picomoles per microgram of protein per hour) in the synaptic vesicle region was 3.3- and 7.3-fold greater than the specific activity of α -amidation activity from the soluble and synaptosomal regions, respectively. A closer comparison of the distribution of α -amidation activity and 16,000-dalton fragment immunoreactivity indicates that α -amidation activity is more prevalent in the region of less dense synaptic vesicles (e.g., fraction 9), whereas 16,000-dalton fragment immunoreactivity is relatively enriched in the region of more dense synaptic vesicles (e.g., fraction 15). Those fractions containing the greatest levels of α -amidation activity (fractions 9 to 13) were pooled, frozen, and thawed five times and designated as the synaptic vesicle fraction for use in further experiments.

A time course for the formation of D-Tyr-Val-NH₂ utilizing the synaptic vesicle-associated α -amidation activity was linear for at least 7 hr after an initial lag of approximately 30 min. When the amount of synaptic vesicle-associated protein was varied from 0.5 to 16 μ g, a linear increase in the production of product was observed. Synaptic vesicle fractions that were boiled for 5 min before incubation in the α -amidation reaction showed no detectable activity. When the synaptic vesicle-associated α -amidation reaction product (26% conversion) was analyzed by reversed phase high performance liquid chromatography, 99% of the recovered counts eluted the retention time of synthetic [¹²⁵I]-D-Tyr-Val-NH₂ (Overall recovery was 89%; similar to Fig. 1).

The pH optimum of the synaptic vesicle-associated α -amidation activity was observed to be between 7.5 and 8.0 (Fig. 3). While the enzymatic activity was totally eliminated at pH 5.5, the velocity of the reaction was decreased by only 53% at pH 10.0.

Copper dependence. The stimulatory effects of copper on pituitary α -amidation activity have been described previously (Bradbury and Smyth, 1983; Eipper et al., 1983b, 1984; Glembofski et al., 1984), and the addition of copper was essential to the detection of α -amidation activity in whole hypothalamic homogenates or crude subcellular fractions. Whereas the presence of an α -amidation activity in hypothalamic synaptic vesicle fractions can be detected without the addition of copper, such activity is inhibited by the addition of diethyldithiocarbamate (DDC), a divalent cation chelator. The ability of a variety of cations to reverse the inhibitory effect of DDC on rat hypothalamic α -amidation activity was determined (Table I). Of those metals tested, only copper was capable of reversing the inhibitory effect of DDC. The concentration of copper resulting in maximal stimulation of hypothalamic α -amidation activity was determined in several experiments and found to be 25 μ M, utilizing a crude synaptosomal preparation as an enzyme source. With purified synaptic vesicles the dose response curve for copper tended to plateau between 5 and 50 μ M CuSO₄ with approximately 30% more activity at 400 μ M CuSO₄; assays were routinely performed with 12.5 μ M CuSO₄ to avoid the potential inhibitory effects of higher copper levels (Fig. 4). Optimal copper concentrations varied slightly between crude synaptosome and purified synaptic vesicle preparations, indicating the necessity to optimize copper levels for each individual

Figure 3. pH dependence of hypothalamic α -amidation activity. Assays contained [¹²⁵I]-D-Tyr-Val-Gly, 0.5 μ M D-Tyr-Val-Gly, 12.5 μ M CuSO₄, 0.625 mM ascorbic acid, 100 μ g/ml of catalase, and 57 mM bis-Tris/57 mM Tris-HCl. Stock buffers were all made with 75 mM bis-Tris/75 mM Tris-HCl titrated to pH with either NaOH or HCl. Assays were incubated for 5 hr with 2 μ g/assay tube of synaptic vesicle-associated protein. Maximal velocity was 0.26 pmol/ μ g/hr at pH 7.5.

TABLE I

Effect of exogenous metal addition on α -amidation activity in rat hypothalamus

Assays contained DDC (12.5 μ M) to inhibit endogenous α -amidation activity, [¹²⁵I]-D-Tyr-Val-Gly, 0.5 μ M D-Tyr-Val-Gly, 0.625 μ M ascorbic acid, 100 μ g/ml of catalase, 62 mM bis-Tris/62 mM Tris-HCl, pH 7.5, as well as the metals listed (12.5 μ M). Assays were incubated for 4.5 hr with 2 μ g/assay tube of synaptic vesicle-associated protein. Data are expressed as percentage of maximal velocity (0.19 pmol/ μ g/hr in the presence of 12.5 μ M CuSO₄).

Treatment	Percentage of Maximal Velocity
No addition	3.3
DDC (12.5 μ M)	1.4
Metal (12.5 μ M) + DDC (12.5 μ M)	
CuSO ₄	100
CaCl ₂	0.3
MgCl ₂	0.9
ZnCl ₂	0.8
MnCl ₂	1.1
CoCl ₂	1.1
BaCl ₂	1.1
NiSO ₄	0.9
FeSO ₄	0.8
CdCH ₃ COOH	2.1
CrCl ₃	0.5
(NH ₄) ₆ Mo ₇ O ₂₄	1.4
Na ₃ VO ₄	0.8
Na ₂ SeO ₃	0.6
Na ₂ SiO ₃	1.0

Figure 4. Effect of exogenous copper addition on α -amidation activity from rat hypothalamus. Assays utilizing crude synaptosomal-associated protein (●) contained the indicated concentrations of CuSO_4 , [^{125}I]-D-Tyr-Val-Gly, 2.43 μM D-Tyr-Val-Gly, 0.625 mM ascorbic acid, 100 $\mu\text{g}/\text{ml}$ of catalase, and 121 mM Na TES, pH 7.0. Assays were incubated for 4 hr with 8 μg of protein/assay tube. Reaction velocity in the absence of exogenous copper was not detectable; maximal velocity was 0.08 pmol/ $\mu\text{g}/\text{hr}$ at 25 μM CuSO_4 . Assays utilizing synaptic vesicle-associated protein (○) contained the indicated concentrations of CuSO_4 , [^{125}I]-D-Tyr-Val-Gly, 0.83 μM D-Tyr-Val-Gly, 0.625 mM ascorbic acid, 100 $\mu\text{g}/\text{ml}$ of catalase, and 125 mM Na TES, pH 7.5. Assays were incubated for 6.2 hr with 1.9 μg of protein/assay tube. Reaction velocity in the absence of exogenous copper was 0.012 pmol/ $\mu\text{g}/\text{hr}$; maximal velocity was 0.23 pmol/ $\mu\text{g}/\text{hr}$ at 400 μM CuSO_4 .

preparation. Similar variations in copper optima have previously been described for both pituitary α -amidation activity (Eipper et al., 1983b; Mains et al., 1984) and dopamine β -hydroxylase (Duch et al., 1968; Orcutt and Molinoff, 1976).

Oxygen dependence. A number of copper-requiring enzymes are involved in interactions with molecular oxygen (Ullrich and Duppel, 1975; Cass and Hill, 1980; Jameson, 1981). Based upon the oxygen requirement of the pituitary α -amidation activity (Eipper et al., 1983b; Mains et al. 1984), the rate of hypothalamic α -amidation was determined in the presence of air (control) or in the presence of a humidified stream of argon or nitrogen (Table II). When utilizing both crude synaptosomal and synaptic vesicle-associated protein as the source of enzymatic activity, the rate of the reaction in the oxygen-depleted assay tubes dropped to 10 to 20% of the control rate. Hypothalamic peptide α -amidation activity must therefore be dependent upon the presence of molecular oxygen.

Ascorbate dependence. The fact that hypothalamic α -amidation activity showed a dependence on copper and molecular oxygen, similar to pituitary α -amidation activity, suggested that ascorbic acid might play an important role in the hypothalamic reaction as it does in the pituitary reaction (Eipper et al., 1983b, 1984; Glembotski et al., 1984; Mains et al., 1984). Various reduced and oxidized flavin, pyridine, and pteridine nucleotides, as well as ascorbate and reduced glutathione, were tested for their ability to stimulate hypothalamic α -amidation (Table III). Of those cofactors tested, ascorbate appeared to have the greatest stimulatory effect when utilizing either synaptic vesicle (2- to 7-fold stimulation) or crude synaptosomal-associated protein

TABLE II

Dependence of hypothalamic α -amidation activity on molecular oxygen

Assays utilizing crude synaptosomal-associated protein as an enzyme source contained [^{125}I]-D-Tyr-Val-Gly, 0.42 μM D-Tyr-Val-Gly, 25 μM CuSO_4 , 0.625 mM ascorbic acid, 100 $\mu\text{g}/\text{ml}$ of catalase, and 133 mM Na TES, pH 7.0. Assays were incubated for 3 hr with 18.3 μg of protein/assay tube under an atmosphere of air or a humidified stream of argon. Assays utilizing synaptic vesicle-associated protein as an enzyme source contained [^{125}I]-D-Tyr-Val-Gly, 0.5 μM D-Tyr-Val-Gly, 12.5 μM CuSO_4 , 0.625 mM ascorbic acid, 100 $\mu\text{g}/\text{ml}$ of catalase, and 69 mM bis-Tris/69 mM Tris-HCl, pH 7.5. Assays were incubated for 3 hr with 2 μg of protein/assay tube under an atmosphere of air or a humidified stream of nitrogen.

Enzyme Source	Reaction Velocity			Activity remaining in N_2 or Argon
	Air	N_2	Argon	
	pmol/ $\mu\text{g}/\text{hr}$			%
Crude synaptosome	0.081		0.009	11
Synaptic vesicle	0.114	0.022		19

TABLE III

Effect of exogenous cofactor addition on α -amidation activity in rat hypothalamus

Assays contained the indicated concentrations of the cofactors listed, [^{125}I]-D-Tyr-Val-Gly, 0.5 μM D-Tyr-Val-Gly, 12.5 μM CuSO_4 , 100 $\mu\text{g}/\text{ml}$ of catalase, and 61 mM bis-Tris/61 mM Tris-HCl, pH 7.5. Assays were incubated for 5 hr with 2 $\mu\text{g}/\text{assay tube}$ of synaptic vesicle-associated protein. Data are expressed as percentage of maximal velocity (0.20 pmol/ $\mu\text{g}/\text{hr}$ in the presence of 0.625 mM ascorbic acid).

Cofactor	Maximal Velocity
mM	%
Ascorbate (0.625)	100
Control (no addition)	15
Ascorbate	
1.0	85
0.1	71
NADPH	
1.0	9
0.1	11
NADP	
1.0	13
0.1	11
NADH	
1.0	12
0.1	14
NAD	
1.0	10
0.1	13
FAD	
1.0	15
0.1	14
FMN	
1.0	16
0.1	11
DMPH ₄	
1.0	43
0.1	63
Reduced glutathione	
1.0	5
0.1	14

(43-fold stimulation), respectively. The concentration of ascorbate resulting in maximal stimulation of hypothalamic α -amidation activity was determined in several experiments and was found to be 0.625 mM (Fig. 5). Addition of ascorbate alone, in the absence of catalase, resulted in a dose-dependent inhibition

Figure 5. Effect of exogenous ascorbic acid addition on α -amidation activity from rat hypothalamus. Assays utilizing crude synaptosomal-associated protein (●) contained the indicated concentration of ascorbic acid, [125 I]-D-Tyr-Val-Gly, 1.87 μ M D-Tyr-Val-Gly, 25 μ M CuSO_4 , and 121 mM Na TES, pH 7.0. Assays were incubated for 4 hr with 8 μ g of protein/assay tube. Control velocity in the absence of exogenous ascorbate was 0.003 pmol/ μ g/hr; maximal velocity was 0.14 pmol/ μ g/hr in the presence of 0.625 mM ascorbic acid. Assays utilizing synaptic vesicle-associated protein (O) contained the indicated concentration of ascorbic acid, [125 I]-D-Tyr-Val-Gly, 0.5 μ M D-Tyr-Val-Gly, 12.5 μ M CuSO_4 , 100 μ g/ml of catalase, and 66 mM bis-Tris/66 mM Tris-HCl, pH 7.5. Assays were incubated for 5.5 hr with 2 μ g of protein/assay tube. Control velocity in the absence of exogenous ascorbate was 0.09 pmol/ μ g/hr; maximal velocity was 0.25 pmol/ μ g/hr in the presence of 0.625 mM ascorbic acid.

Figure 6. Effect of ascorbate oxidase addition on endogenous α -amidation activity from rat hypothalamus. Assays contained the indicated concentrations of ascorbate oxidase (Sigma A-2769; 130 units/mg), [125 I]-D-Tyr-Val-Gly, 0.5 μ M D-Tyr-Val-Gly, 12.5 μ M CuSO_4 , 100 μ g/ml of catalase, and 56 mM bis-Tris/56 mM Tris-HCl, pH 6.5. Assays were performed at pH 6.5 due to the disparity between the pH optima for the hypothalamic α -amidation activity (pH 7.5) and ascorbate oxidase (pH 5.6). Assays were incubated for 7 hr with 1.9 μ g/assay tube of synaptic vesicle-associated protein. Control velocity in the absence of ascorbate oxidase was 0.067 pmol/ μ g/hr.

of α -amidation activity, as seen for dopamine β -hydroxylase (Friedman and Kaufman, 1965; Diliberto and Allen, 1980). When catalase concentrations in reaction tubes were varied between 0.4 and 2700 μ g/ml, a dose-dependent increase in α -amidation activity was observed with optimal levels occurring at 300 μ g of catalase/ml. In the case of dopamine β -hydroxylase, catalase is thought to act by preventing both the auto-oxidation of ascorbate and damage to the enzyme caused by superoxide radical production during such an auto-oxidation event (May et al., 1981; Wong et al., 1981; Scarpa et al., 1983).

When utilizing a crude synaptosomal fraction as an enzyme source, ascorbate was the most potent stimulator of the α -amidation reaction, but several other cofactors, including flavin adenine dinucleotide, flavin mononucleotide, and 6,7-dimethyl-5,6,7,8-tetrahydropterin (DMPH₄), were also effective in stimulating the reaction significantly above control values (data not shown). Except for ascorbic acid, the only cofactor capable of stimulating hypothalamic α -amidation when utilizing purified synaptic vesicles as an enzyme source was the synthetic bioppterin analogue DMPH₄. The concentration of DMPH₄ resulting in maximal stimulation of hypothalamic α -amidation activity (1.7-fold) was determined in several experiments and was found to vary between 200 and 400 μ M at pH 7.5.

The ability of increasing concentrations of ascorbate oxidase to diminish synaptic vesicle-associated α -amidation activity in the absence of exogenous cofactor was assessed (Fig. 6). As the concentration of ascorbate oxidase in the reaction tubes was increased, the rate of endogenous α -amidation activity was decreased to 5% of the control value in the absence of ascorbate

oxidase. When the rate of hypothalamic α -amidation activity in the presence of DMPH₄ was determined with increasing amounts of ascorbate oxidase, a graded reduction in α -amidation activity was also seen. Concentrations of ascorbate oxidase which resulted in a maximal decrease of α -amidation activity in the absence of exogenous cofactor decreased the pteridine-induced fold-stimulation by approximately 60%.

Substrate specificity. All known precursors to α -amidated peptides contain a glycine residue immediately following the amino acid which ultimately bears the α -amide moiety (Mains et al., 1983); the occurrence of this common amidation "signal" raises the possibility that a single enzymatic activity could account for the α -amidation of a great variety of α -amidated peptides from various tissues.

In order to investigate the substrate specificity requirements of the peptide α -amidation activity found in rat hypothalamus, various peptides (100 μ M) were assayed for their ability to inhibit the conversion of [125 I]-D-Tyr-Val-Gly into [125 I]-D-Tyr-Val-NH₂ (Table IV); peptides which can serve as potential substrates of the α -amidation activity instead of D-Tyr-Val-Gly would be expected to demonstrate such an inhibitory effect. Peptides possessing carboxyl-terminal glycine residues were potent inhibitors of the hypothalamic α -amidation reaction. Even those glycine-extended molecules which are not thought to serve as relevant physiological substrates (e.g., ACTH(1-10); Tyr-Gly-Gly) were capable of such inhibition. Removal of the carboxyl-terminal glycine residue (ACTH(1-13)) or extension of the peptide beyond the glycine moiety (Tyr-Gly-Gly-Phe) greatly diminished the inhibitory effects of these peptides. A variety of small peptides with carboxyl-terminal α -amides (thyrotropin-releasing hormone (pGlu-His-Pro-NH₂); Pro-Leu-

Gly-NH₂; D-Tyr-Val-NH₂; Trp-Met-Asp-Phe-NH₂) were not effective at inhibiting the production of [¹²⁵I]-D-Tyr-Val-NH₂ at the concentrations tested. Addition of 100 μ M α -MSH (α -N-acetyl-ACTH(1-13)NH₂) did have an inhibitory effect on hypothalamic α -amidation.

Kinetic studies. Kinetic studies were carried out to determine the effect of increasing concentrations of D-Tyr-Val-Gly on reaction velocities for both the crude synaptosomal and synaptic vesicle-associated α -amidation activities. As the concen-

tration of D-Tyr-Val-Gly was increased, the reaction velocity increased in a saturating manner (Fig. 7). Both Lineweaver-Burk and Eadie-Hofstee (Fig. 7, *inset*) analyses of these data demonstrated Michaelis-Menten type kinetics with D-Tyr-Val-Gly as the varied substrate. When assayed under optimal conditions, the apparent K_m for peptide substrate was approximately 2- to 3-fold higher for the crude mitochondria/synaptosomal activity than the apparent K_m when using the synaptic vesicle-associated α -amidation activity. V_{max} values for the crude activity were 5-fold less than the corresponding values obtained for the synaptic vesicle-associated α -amidation activity. The addition of increasing concentrations of ascorbic acid to pituitary secretory granule fractions resulted in an increase in α -amidation reaction velocity at all concentrations of peptide substrate (Eipper et al., 1983b, 1984). The effects of varied concentrations of exogenous ascorbate on the kinetic parameters of the hypothalamic crude synaptosomal α -amidation activity were determined (Table V). A 4-fold increase in the concentration of ascorbate was accompanied by an 8-fold increase in V_{max} and a 4-fold increase in K_m .

TABLE IV

Effect of exogenous peptide addition on α -amidation activity in rat hypothalamus

Assays contained [¹²⁵I]-D-Tyr-Val-Gly, 0.5 μ M D-Tyr-Val-Gly, 12.5 μ M CuSO₄, 0.625 mM ascorbic acid, 100 μ g/ml of catalase, 65 mM bis-Tris/65 mM Tris-HCl, pH 7.5, and the peptides listed (100 μ M). Added peptides were dried into the reaction tubes by vacuum centrifugation prior to incubation. Assays were incubated for 6 hr with 2.4 μ g/assay tube of synaptic vesicle-associated protein. Data are expressed as percentage of control (0.26 pmol/ μ g/hr).

Peptide (100 μ M)	Percentage of Control
Control (no addition)	100
D-Tyr-Val-Gly	14
Ac-ACTH (1-14)	18
Tyr-Gly-Gly	58
γ_2 -MSH	21
ACTH(1-10)	5
ACTH(1-13)	94
Tyr-Gly-Gly-Phe	89
α -MSH	79
Trp-Met-Asp-Phe-NH ₂	93
TRH (pGlu-His-Pro-NH ₂)	94
D-Tyr-Val-NH ₂	95
Pro-Leu-Gly-NH ₂	93

TABLE V

Effect of ascorbic acid concentration on kinetic parameters for D-Tyr-Val-Gly

Assays contained [¹²⁵I]-D-Tyr-Val-Gly; 25 μ M CuSO₄; 100 μ g/ml of catalase; 119 mM Na TES, pH 7.0; either 0.3125, 0.625, or 1.25 mM ascorbic acid; and varying concentrations of D-Tyr-Val-Gly. Assays were incubated for 3.5 hr with 8 μ g/assay tube of crude synaptosomal-associated protein. Kinetic parameters were calculated by Lineweaver-Burk transformation of the data in which best fit lines were obtained using the unweighted least squares method (Cleland, 1979).

Ascorbate	V_{max}	K_m
mM	pmol/ μ g/hr	μ M
0.3125	0.24	20
0.625	0.68	26
1.25	2.0	90

Figure 7. Saturation and Eadie-Hofstee plot of hypothalamic α -amidation activity with D-Tyr-Val-Gly as the varied substrate. Assays contained [¹²⁵I]-D-Tyr-Val-Gly, 12.5 μ M CuSO₄, 0.625 mM ascorbic acid, 100 μ g/ml of catalase, 57 mM bis-Tris/57 mM Tris-HCl, pH 7.5, and varying amounts of D-Tyr-Val-Gly. Assays were incubated for 6 hr with 1.66 μ g/assay tube of synaptic vesicle-associated protein. Best fit lines for the Eadie-Hofstee plot were obtained using the unweighted least squares method (Cleland, 1979).

Discussion

The hypothalamus represents an important neuroendocrine tissue involved in the biosynthesis of many bioactive peptides with essential carboxyl-terminal α -amide groups. The synthetic substrate [125 I]-D-Tyr-Val-Gly (Bradbury et al., 1982) has been used to detect and study the subcellular localization and characteristics of an α -amidation activity from rat hypothalamus. The results of these studies have demonstrated the presence of a synaptic vesicle-associated peptide α -amidation activity that is stimulated by the presence of copper ions and ascorbic acid, requires the presence of molecular oxygen, and appears to have a broad substrate specificity for peptides possessing a carboxyl-terminal glycine residue. The characteristics of hypothalamic α -amidation activity are similar to those previously described for the pituitary α -amidation activities from several species (Bradbury et al., 1982; Eipper et al., 1983b, 1984; Husain and Tate, 1983; Glembotski et al., 1984; Mains et al., 1984). Based upon what is known about the characteristics of the hypothalamic α -amidation activity and the presumed reaction mechanism, the suggested systematic name for this activity is peptidyl-glycine, ascorbate:oxygen oxidoreductase (α -amidating); the name recommended for common use is peptidyl-glycine α -amidating monooxygenase (PAM).

Evidence from a variety of studies supports the idea that hypothalamic peptide hormones are processed from precursor molecules within secretory vesicles both in the neuronal perikarya and during subsequent axonal transport (Sachs and Takabatake, 1964; Gainer et al., 1977; North et al., 1977; Russell et al., 1980; Barnea et al., 1981; Loh and Chang, 1982; Moore et al., 1982). The localization of a peptide α -amidation activity along with 16,000-dalton fragment immunoreactivity in hypothalamic synaptic vesicle fractions is consistent with the proposed subcellular location of hypothalamic post-translational processing events.

The average density of synaptic vesicles containing 16,000-dalton fragment immunoreactivity (Fig. 2, top) was slightly greater than that seen for vesicles containing α -amidation activity; this observation suggests the presence of such enzymatic activity in a population of vesicles unrelated to the ACTH/endorphin system. Comparisons of the sedimentation characteristics of hypothalamic vesicles containing ACTH/endorphin and other hypothalamic α -amidated peptides have yet to be determined. Alternatively, such vesicular heterogeneity may be the result of processes involved in vesicular maturation (Nordmann et al., 1979; Nordmann and Morris, 1984). Although the pro-ACTH/endorphin-derived immunoreactive content of the hypothalamus is approximately 100-fold lower than that of the intermediate lobe of the pituitary, the ratio of α -amidation activity to pro-ACTH/endorphin-derived immunoreactivity in the hypothalamus exceeds that value obtained for the pars intermedia by a factor of 30; this observation further suggests the presence of this enzymatic activity in cells other than those producing pro-ACTH/endorphin.

Ascorbic acid has been shown to be of widespread importance in a number of key biochemical processes including the hydroxylation of dopamine to form norepinephrine (Levin et al., 1960; Friedman and Kaufman, 1965) and the conversion of *p*-hydroxyphenylpyruvate to homogentisate in liver (Lewin, 1976); ascorbic acid is also required for normal collagen (Barnes and Kodicek, 1972; Holloway and Rivers, 1981) and cholesterol (Murad et al., 1981) biosynthesis. Essentially all of the endogenous α -amidation activity present within synaptic vesicle fractions can be abolished by the addition of ascorbate oxidase to the α -amidation reaction mixture (Fig. 6), suggesting that the actual cofactor used by the α -amidation activity *in vivo* is ascorbic acid. Whereas the synaptic vesicle-associated α -amidation activity is relatively stable when stored below 0°C, the fold-stimulation induced by exogenously administered ascor-

bate increases with time in storage (data not shown), suggesting that an endogenous ascorbate pool becomes oxidized upon storage. The inhibition of hypothalamic α -amidation at supraoptimal concentrations of ascorbate in the presence of copper ions may be a direct result of the copper-induced auto-oxidation of ascorbic acid or may be the result of enzymatic inactivation caused by free radical production induced by ascorbate and superoxide in the presence of copper (Lewin, 1976; Scarpa et al., 1983; Shinar et al., 1983).

In the adrenal medulla, where ascorbate serves as a reducing agent in the production of catecholamines, chromaffin granules contain approximately 20 mM ascorbate (Ingebrechtsen et al., 1980). Ascorbic acid appears to be present in all animal tissues (Hornig, 1975) and exists at a relatively high concentration in almost all parts of the mammalian brain; hypothalamic concentrations of ascorbate are about 2mM (Milby et al., 1982). Recent studies have demonstrated that depolarizing stimuli such as high potassium induce the secretion of ascorbate from cultured adrenal chromaffin cells (Levine et al., 1983) and purified synaptosome preparations (Milby et al., 1981), and *in vivo* electrical stimulation of the rat medial lemniscus causes an increase in ascorbate efflux from somatosensory cortex (Milby et al., 1981). These results argue that some cellular ascorbic acid is stored within secretory vesicles which, upon depolarization, release their contents into extracellular space.

The ability of DMPH₄ to stimulate hypothalamic α -amidation activity could either reflect the ability of the α -amidation activity to utilize directly the reduction potential of this pteridine cofactor or be the result of enzymatic or chemical regeneration of endogenous ascorbate pools. Hydroxylation of proline residues by prolyl hydroxylase continues in the face of extremely low levels of ascorbic acid and, thus, ascorbate presumably is being replaced by other endogenous reductants (Basu and Schora, 1982). Hypothalamic concentrations of tetrahydropterin are twice as great as total brain levels, and approximately 25% of the total bipterin content exists within synaptosomes (Hennings and Rembold, 1982).

The copper-induced stimulation of hypothalamic α -amidation places this enzymatic activity in a category of cuproenzymes which includes dopamine β -hydroxylase, phenol *o*-monooxygenase (tyrosinase), cytochrome *c* oxidase, superoxide dismutase, amine oxidase, and lysyl oxidase (Cass and Hill, 1980). The effects of dietary or genetic copper deficiency on the activities of these enzymes vary widely from species to species (Underwood, 1977), but neurological dysfunction is invariably apparent (Hunt, 1980). Considering the stimulatory effects of copper on hypothalamic α -amidation activity and the widespread distribution of α -amidated peptides throughout the central nervous system (Snyder and Innis, 1979; Krieger, 1983), altered α -amidation of a variety of neuropeptides may play a significant role in the etiology of copper-deficient neurological and endocrinological disorders.

Note added in proof. According to the current IUPAC/IUB guidelines for the classification and nomenclature of enzymes (Biochim. Biophys. Acta 429: 1–45 (1976)), "The direct attachment of -ase to the name of the substrate will indicate that the enzyme brings about hydrolysis." The name "amidase," for an enzymatic activity which serves to produce a carboxyl-terminal α -amide group on peptides, is therefore inappropriate.

References

- Amara, S. G., V. Jonas, M. G. Rosenfeld, E. S. Ong, and R. M. Evans (1982) Alternative RNA processing in calcitonin gene expression generates mRNAs encoding different polypeptide products. *Nature* 298: 240–244.
- Barnea, A., G. Cho, N. S. Pilote, and J. C. Porter (1981) Regional differences in the molecular weight profile of corticotropin and α -melanotropin in the hypothalamus. *Endocrinology* 108: 150–156.

- Barnes M. J., and E. Kodicek (1972) Biological hydroxylations and ascorbic acid with special regard to collagen metabolism. *Vitam. Horm.* 30: 1-43.
- Basu, T. K., and C. J. Schora (1982) *Vitamin C in Health and Disease*, Avi Publishing Co., Westport, CT.
- Bradbury, A. F., and D. G. Smyth (1983) Substrate specificity of an amidating enzyme in porcine pituitary. *Biochem. Biophys. Res. Commun.* 112: 372-377.
- Bradbury, A. F., M. D. A. Finnie, and D. G. Smyth (1982) Mechanism of C-terminal amide formation by pituitary enzymes. *Nature* 298: 686-688.
- Cass, A. E. G., and H. A. O. Hill (1980) Copper proteins and copper enzymes. *Ciba Found. Symp.* 79: 71-84.
- Cleland, W. W. (1979) Statistical analysis of enzyme kinetic data. *Methods Enzymol.* 63: 103-138.
- Diliberto, E. J., and P. L. Allen (1980) Semidehydroascorbate as a product of the enzymic conversion of dopamine to norepinephrine: Coupling of semidehydroascorbate reductase to dopamine- β -hydroxylase. *Mol. Pharmacol.* 17: 421-426.
- Duch, D. S., O. H. Viveros, and N. Kirshner (1968) Endogenous inhibitors in adrenal medulla of dopamine β -hydroxylase. *Biochem. Pharmacol.* 17: 255-264.
- Eipper, B. A., and R. E. Mains (1978) Existence of a common precursor to ACTH and endorphin in the anterior and intermediate lobes of the rat pituitary. *J. Supramol. Struct.* 8: 247-262.
- Eipper, B. A., C. C. Glembotski, and R. E. Mains (1983a) Selective loss of α -melanotropin-amidating activity in primary cultures of rat intermediate pituitary cells. *J. Biol. Chem.* 258: 7292-7298.
- Eipper, B. A., R. E. Mains, and C. C. Glembotski (1983b) Identification in pituitary tissue of a peptide α -amidation activity that acts on glycine-extended peptides and requires molecular oxygen, copper, and ascorbic acid. *Proc. Natl. Acad. Sci. U. S. A.* 80: 5144-5148.
- Eipper, B. A., C. C. Glembotski, and R. E. Mains (1984) Bovine intermediate pituitary α -amidation enzyme: Preliminary characterization. *Peptides* 4: 921-928.
- Friedman, S., and S. Kaufman (1965) 3,4-Dihydroxyphenylethylamine β -hydroxylase: Physical properties, copper content, and role of copper in the catalytic activity. *J. Biol. Chem.* 240: 4763-4773.
- Gainer, H., Y. Sarne, and M. J. Brownstein (1977) Neurophysin biosynthesis: Conversion of a putative precursor during axonal transport. *Science* 195: 1354-1356.
- Glembotski, C. C. (1981) Subcellular fractionation studies on the post-translational processing of pro-adrenocorticotrophic hormone/endorphin in the rat intermediate pituitary. *J. Biol. Chem.* 256: 7433-7439.
- Glembotski, C. C. (1982) Acetylation of α -melanotropin and β -endorphin in the rat intermediate pituitary. *J. Biol. Chem.* 257: 10493-10500.
- Glembotski, C. C., B. A. Eipper, and R. E. Mains (1984) Characterization of a peptide α -amidation activity from rat anterior pituitary. *J. Biol. Chem.* 259: 6385-6392.
- Gray, E. G., and V. P. Whittaker (1962) The isolation of nerve endings from brain: An electron microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat.* 96: 79-87.
- Hennings, G., and H. Rembold (1982) Regional and subcellular distribution of bipterin in the rat. *Int. J. Vitam. Nutr. Res.* 52: 36-43.
- Holloway, D. E., and J. M. Rivers (1981) Influence of chronic ascorbic acid deficiency and excessive ascorbic acid intake on bile acid metabolism and bile composition in the guinea pig. *J. Nutr.* 111: 411-424.
- Hornig, D. (1975) Distribution of ascorbic acid, metabolites and analogues in man and animals. *Ann. N. Y. Acad. Sci.* 258: 103-118.
- Hunt, D. M. (1980) Copper and neurological function. *Ciba Found. Symp.* 79: 247-266.
- Husain, I., and S. S. Tate (1983) Formation of the COOH-terminal amide group of thyrotropin releasing factor. *FEBS Lett.* 152: 277-281.
- Ingebrechtsen, O. C., O. Terland, and T. Flatmark (1980) Subcellular distribution of ascorbate in bovine adrenal medulla. *Biochim. Biophys. Acta* 628: 182-189.
- Jameson, R. F., (1981) Copper proteins. In *Metal Ions in Biological Systems*, H. Sigel, ed., Vol. 12, pp 1-30, Marcel Dekker, Inc., New York.
- Kizer, J. S., W. H. Busby, Jr., C. Cottle, and W. W. Youngblood (1984) Glycine directed peptide amidation: Presence in rat brain of two enzymes which convert P-Glu-His-Pro-Gly-OH into P-Glu-His-Pro-NH₂ (thyrotropin-releasing hormone). *Proc. Natl. Acad. Sci. U. S. A.* 81: 3228-3232.
- Krieger, D. T. (1983) Brain peptides: What, where and why? *Science* 222: 975-985.
- Land, H., G. Schutz, H. Schmale, and D. Richter (1982) Nucleotide sequence of cloned cDNA encoding bovine arginine vasopressin-neurophysin II precursor. *Nature* 295: 299-303.
- Landymore-Lim, A. E. N., A. F. Bradbury, and D. G. Smyth (1983) The amidating enzyme in pituitary will accept a peptide with C-terminal D-alanine as substrate. *Biochem. Biophys. Res. Commun.* 117: 289-293.
- Levin, E. Y., B. Levenberg, and S. Kaufman (1960) The enzymatic conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. *J. Biol. Chem.* 235: 2080-2086.
- Levine, M., A. Asher, H. Pollard, and O. Zinder (1983) Ascorbic acid and catecholamine secretion from cultured chromaffin cells. *J. Biol. Chem.* 258: 13111-13115.
- Lewin, S. (1976) *Vitamin C: Its Molecular Biology and Medical Potential*, Academic Press, Inc., New York.
- Loh, Y. P., and T. L. Chang (1982) Pro-opioid corticotin converting activity in rat intermediate and neural lobe secretory granules. *FEBS Lett.* 137: 57-62.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Mains, R. E., B. A. Eipper, C. C. Glembotski, and R. M. Dores (1983) Strategies for the biosynthesis of bioactive peptides. *Trends Neurosci.* 6: 229-235.
- Mains, R. E., C. C. Glembotski, and B. A. Eipper (1984) Peptide α -amidation activity in mouse anterior pituitary AtT-20 cell granules: Properties and secretion. *Endocrinology* 114: 1522-1530.
- May, S. W., R. S. Phillips, P. W. Mueller, and H. H. Herman (1981) Dopamine β -hydroxylase: Demonstration of the enzymatic ketonization of the product enantiomer, S-octopamine. *J. Biol. Chem.* 256: 2258-2261.
- Milby, K. H., I. N. Mefford, W. Chey, and R. N. Adams (1981) *In vitro* and *in vivo* depolarization coupled efflux of ascorbic acid in rat brain preparations. *Brain Res. Bull.* 7: 237-242.
- Milby, K., A. Oke, and R. N. Adams (1982) Detailed mapping of ascorbate distribution in rat brain. *Neurosci. Lett.* 28: 169-174.
- Moore, G. J., Y. C. Kwok, E. M. Ko, D. L. Severson, and J. C. Rosenior (1982) Extended chain analogs of [arginine⁸]vasopressin as model prohormones: Investigation of precursor-processing enzymes in extracts of rat hypothalamus and neural lobe. *Endocrinology* 111: 1626-1631.
- Murad, S., A. Sivarajah, and S. R. Pinnell (1981) Regulation of prolyl and lysyl hydroxylase activities in cultured human skin fibroblasts by ascorbic acid. *Biochem. Biophys. Res. Commun.* 101: 868-875.
- Myers, A., R. Mains, and B. Eipper (1984) Identification of enzymatic activity producing α -amidated peptides in rat tissues. VII Int. Congr. Endocrinol. Abstr., abstr. no. 2104, p. 1312.
- Nakanishi, S., A. Inoue, T. Kita, M. Nakamura, A. C. Y. Chang, S. N. Cohen, and S. Numa (1979) Nucleotide sequence of cloned cDNA for bovine corticotropin- β -lipotropin precursor. *Nature* 278: 423-427.
- Nordmann, J. J., F. Louis, and S. J. Morris (1979) Purification of two structurally distinct populations of rat neurohypophyseal secretory granules. *Neuroscience* 4: 1367-1379.
- Nordmann, J. J., and J. F. Morris (1984) Method for quantitating the molecular content of a subcellular organelle: Hormone and neurophysin content of newly formed and aged neurosecretory granules. *Proc. Natl. Acad. Sci. U. S. A.* 81: 180-184.
- North, W. G., H. Valtin, J. F. Morris, and F. T. LaRochelle, Jr. (1977) Evidence for metabolic conversions of rat neurophysins within neurosecretory granules of the hypothalamo-neurohypophyseal system. *Endocrinology* 101: 110-118.
- Orcutt, J. C., and P. B. Molinoff (1976) Endogenous inhibitors of dopamine- β -hydroxylase in rat organs. *Biochem. Pharmacol.* 25: 1176-1184.
- Paquette, T. L., R. Gingerich, and D. Scharp (1981) Altered amidation of pancreatic polypeptide in cultured dog islet tissue. *Biochemistry* 20: 7403-7408.
- Parker, C. R., Jr., W. B. Neaves, A. Barnea, and J. C. Porter (1978) Studies on the subsynaptosomal localization of luteinizing hormone-releasing hormone and thyrotropin-releasing hormone in the rat hypothalamus. *Endocrinology* 102: 1167-1175.
- Racker, E. (1950) Spectrophotometric measurements of the enzymatic formation of fumaric and *cis*-aconitic acids. *Biochim. Biophys. Acta* 4: 211-214.

- Russell, J. T., M. J. Brownstein, and H. Gainer (1980) Biosynthesis of vasopressin, oxytocin, and neurophysins: Isolation and characterization of two common precursors (propressophysin and prooxyphysin). *Endocrinology* 107: 1880–1891.
- Sachs, H., and Y. Takabatake (1964) Evidence for a precursor in vasopressin biosynthesis. *Endocrinology* 75: 943–948.
- Scarpa, M., R. Stevanto, P. Viglino, and A. Rigo (1983) Superoxide ion as active intermediate in the autoxidation of ascorbate: Effect of superoxide dismutase. *J. Biol. Chem.* 258: 6695–6697.
- Schwartz, T. W., R. L. Gingerich, and H. S. Tager (1980) Biosynthesis of pancreatic polypeptide. *J. Biol. Chem.* 255: 11494–11498.
- Shinar, E., T. Navok, and M. Chevion (1983) The analogous mechanisms of enzymatic inactivation induced by ascorbate and superoxide in the presence of copper. *J. Biol. Chem.* 258: 14778–14783.
- Snyder, S. H., and R. B. Innis (1979) Peptide neurotransmitters. *Annu. Rev. Biochem.* 48: 755–782.
- Tatemoto, K. (1982) Neuropeptide Y: Complete amino acid sequence of the brain peptide. *Proc. Natl. Acad. Sci. U. S. A.* 79: 5485–5489.
- Tucek, S. (1966) On subcellular localization and binding of choline acetyltransferase in the cholinergic nerve endings of the brain. *J. Neurochem.* 13: 1317–1327.
- Ullrich, V., and W. Duppel (1975) Iron- and copper-containing monooxygenases. In *The Enzymes*, P. D. Boyer, ed., Vol. 12B, pp. 253–297, Academic Press, Inc., New York.
- Underwood, E. J. (1977) *Trace Elements in Human and Animal Nutrition*, Ed. 4, pp. 56–108, Academic Press, Inc., New York.
- Wand, G., S. Baylin, R. Ney, and E. Eipper (1984) Peptide α -amidation activity in human plasma and tissues. VII Int. Congr. Abstr., abstr. no. 2676, p. 158.
- Whittaker, V. P. (1969) The synaptosome. In *Handbook of Neurochemistry*, A. Lajtha, ed., Vol. 2, pp. 327–364, Plenum Press, New York.
- Whittaker, V. P., and L. A. Barker (1972) The subcellular fractionation of brain tissue with special reference to the preparation of synaptosomes and their component organelles. In *Methods of Neurochemistry*, R. Fried, ed., pp. 1–52, Marcel Dekker, Inc., New York.
- Whittaker, V. P., I. A. Michaelson, and R. J. A. Kirkland (1964) The separation of synaptic vesicles from disrupted nerve-ending particles ("synaptosomes"). *Biochem. J.* 90: 293–303.
- Wong, D. L., S. J. Masover, and R. D. Ciaranello (1981) Regulation of dopamine β -hydroxylase synthesis and degradation: Ascorbic acid stabilization of the enzyme against tryptic proteolysis. *J. Biol. Chem.* 256: 695–700.
- Yoo, O. J., C. T. Powell, and K. L. Agarwal (1982) Molecular cloning and nucleotide sequence of full-length cDNA coding for porcine gastrin. *Proc. Natl. Acad. Sci. U. S. A.* 79: 1049–1053.