Neurosecretory granule lysate from bovine posterior pituitary was shown to contain both carboxypeptidase B and amidating activities. The former sequentially releases COOH-terminal basic residues from the oxytocin biosynthetic precursor fragment oxytocinyl-GKR (CYIQNCPLGKR) to form oxytocinyl-GK and then oxytocinyl-G. The amidating enzyme converts the resulting oxytocinyl-G into oxytocin (CYIQNCPLG-NH₂). The carboxypeptidase B was separated from a less specific carboxypeptidase present in granule lysate by gel filtration on Sephacryl S-300. Percoll density gradient centrifugation (after preliminary differential centrifugation) also yielded granule fractions enriched in the specific carboxypeptidase B activity. The carboxypeptidase B which converts the oxytocinyl peptides showed a fairly sharp pH dependence with an optimum of 5.5–6, was activated by cobalt ion, and was inhibited by cupric ion, EDTA, and a thiol protease inhibitor, p-chloromercuribenzoate. The amidating activity which converts oxytocinyl-G to oxytocin was competitively inhibited by degradation due to proteases and/or peptidases present in lysate of Percoll gradient-derived granules. Oxytocinyl-GKR was shown by analytical affinity chromatography to bind noncovalently to neurophysin with an affinity close to that of mature oxytocin. This binding activity and the observation of the specific carboxypeptidase B activity in the presence of large concentrations of neurophysin are consistent with the view that the exoproteolytic processing and amidation steps which occur after initial endoproteolysis of pro-oxytocin/neurophysin likely take place on oxytocin intermediate peptides which are bound in noncovalent complexes with the neurophysin domain from the precursor.

The neurohypophysial peptide hormones vasopressin and oxytocin are synthesized in the hypothalamus as parts of large precursors, which are post-translationally converted to noncovalent complexes of the mature hormones and neurophysins during axonal transport to the posterior pituitary (1–3). The primary structures of the precursors have been deduced from the cDNA sequences derived from specific hypothalamic mRNAs (Fig. 1) (4, 5). As in other endocrine and neuronal peptide precursors, a typical proteolytic processing signal, Lys-Arg, exists in the precursors of both oxytocin and vasopressin, in these cases between the hormone and neurophysin domains. Through studies on processing of proinsulin and other biologically active peptides, it has been suggested that cleavage at dibasic residues involves both trypsin-like endopeptidase and carboxypeptidase B-like exopeptidase activities (6). There have been several studies on endoproteolytic processing enzymes for proinsulin (7, 8), proglucagon (8), proinsulin (9), proenkephalin (10–12), proopiomelanocortin (13), and proparathyroid hormone (14, 15). For exopeptidases, a few carboxypeptidase B activities have been reported as processing enzymes for insulin (16) and enkephalin-containing peptides (17, 18, 37). For both the endoproteases and exopeptidases, however, the catalytic properties vary within each class, suggesting that different enzymes may act on different subsets of precursors. The presence of processed products or intermediates with either a COOH-terminal or an NH-terminal basic residue (e.g., Refs. 19 and 20) as well as intact dibasic residues within isolated mature peptides suggests that both dibasic residues could be processed through more than one pathway and that factors in addition to sequence control which dibasic residues serve as major cleavage sites.

In order to understand more fully the post-translational mechanisms for synthesis of biologically active peptides, it has become increasingly compelling to isolate and characterize the processing enzymes involved and to determine the factors which control their actions on biosynthetic precursors and intermediates. Further, comparison of the properties and distribution of specific processing enzymes in multiple sites of synthesis of a given set of peptides would allow an improved understanding of the relationship between molecular events occurring in these sites. In the present study we have synthesized peptides deduced to be intermediates, or closely related to these intermediates, in the formation of oxytocin from its biosynthetic precursor and examined the processing of these peptides by enzymes in the neurosecretory granules of bovine posterior pituitaries. We have detected the presence of a carboxypeptidase activity specific for the COOH-terminal basic residues of the oxytocin-containing peptides oxytocinyl-Gly-Lys-Arg and oxytocinyl-Gly-Lys. The activity was stimulated by cobalt ion and inhibited by a thiol protease inhibitor, p-chloromercuribenzoate and EDTA. The properties of the enzyme are similar to those reported (18) for the carboxypeptidase B which is active in enkephalin precursor fragments.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Amidation of the oxytocin-Gly product of carboxypeptidase cleavage also was detected.3

EXPERIMENTAL PROCEDURES

Synthesis of Oxytocin Dodecapeptide (OT-GKR'), Undecapeptide (OT-GKR), and Decapeptide (OT-G) - Oxytocin-dodecapeptide substrate was synthesized in conventional solid-phase synthesis from phenylacetic acid triethylammonium chloride resin (details described elsewhere (38)). Briefly, N\text{-}t	ext{-}butylxycarbonyl-arginyl-oxymethyl-phenylacetic acid triethylammonium chloride resin by the stepwise method. Resin cleavage and deprotection were by hydrogen fluoride at 0 °C for 1 h. Formation of the disulfide bridge between \( \text{Cys} \) residues 1 and 6 in the isolated synthetic peptide was carried out with potassium ferricyanide (22).

The residues of individual fractions were dissolved in constant boiling HCl (1 ml) and hydrolyzed at 110 °C for 24 h. The peptide was purified on Sephadex G-15 gel chromatography (1.7 cm, in 30% acetonitrile) with, respectively, immobilized trypsin (Pierce) and soluble trypsin in 200 pl of 0.1 M ammonium bicarbonate (pH 8.6) at 37 °C for 18 h. The peptide was purified by RP-HPLC on Zorbax CN (cyanopropylsilyl, Dupont, 0.94 cm, in 30% acetonitrile at 20 min. For amino acid analyses of degraded products, 0.5-min (0.4-ml) HPLC fractions were collected and dried in vacuo. The residues of individual fractions were dissolved in constant boiling HCl (1 ml) and hydrolyzed at 110 °C for 24 h.

Assay of Amidation by Reverse-phase HPLC — Aliquots of the granule lysate and fractions further purified on Sephacryl S-300 were incubated with the peptide substrates in 0.1 M sodium acetate or 0.1 M phosphate buffer, at 37 °C. The concentrations of the substrates and other conditions are specified under “Results.” After removal of insoluble materials by centrifugation, a aliquot of the supernatant was applied to the cyanopropylsilyl column. Unless otherwise noted, peptides were eluted with a linear gradient, 0.8 ml/min, from 98% TEAP/7% acetonitrile at zero time to 70% TEAP/30% acetonitrile at 20 min. For amino acid analyses of degraded products, 0.5-min (0.4-ml) HPLC fractions were collected and dried in vacuo. The residues of individual fractions were dissolved in constant boiling HCl (1 ml) and hydrolyzed at 110 °C for 24 h.

Assay of Proteolytic Activity by Reverse-phase HPLC — Aliquots of the granule lysate and fractions further purified on Sephacryl S-300 were incubated with the peptide substrates in 0.1 M sodium acetate or 0.1 M phosphate buffer, at 37 °C. The concentrations of the substrates and other conditions are specified under “Results.” After removal of insoluble materials by centrifugation, a aliquot of the supernatant was applied to the cyanopropylsilyl column. Unless otherwise noted, peptides were eluted with a linear gradient, 0.8 ml/min, from 98% TEAP/7% acetonitrile at zero time to 70% TEAP/30% acetonitrile at 20 min. For amino acid analyses of degraded products, 0.5-min (0.4-ml) HPLC fractions were collected and dried in vacuo. The residues of individual fractions were dissolved in constant boiling HCl (1 ml) and hydrolyzed at 110 °C for 24 h.

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ammonium acetate, pH 5.0, and injected onto the Zorbax ODS column for RP-HPLC fractionation as above for radiolabeled OT-G and OT. Eluted fractions were counted using a Beckman gamma 4000 counter. RP-HPLC elutions also were carried out for separated 125I-OT-G and 125I-OT, and elution positions of these were used to assign substrate and product elution positions for chromatograms of reaction mixture aliquots. Calculations of the amount of counts in peaks corresponding to 125I-OT-G and 125I-OT involved subtraction of base-line counts in both cases and, for 125I-OT product, subtraction of a small contribution of counts from a peak, eluting close to the 125I-OT position, which was observed in the elution of starting 125I-OT-G substrate.

RESULTS

Interaction of Oxytocinyl Dodecapeptide with Immobilized Neurophysin—The neurophysin-binding properties of oxytocin dodecapeptide (OT-GKR) were examined by affinity chromatography using [neurophysin-II]Sephrose. As shown in Fig. 2, OT-GKR was found to bind to [neurophysin-II]Sephrose in 0.4 M ammonium acetate, pH 5.7, and to be eluted with 0.2 M acetic acid. This behavior is similar to that found for authentic oxytocin, Met-Tyr-Phe-NH2, a tripeptide analogue of the NH2-terminal sequences of oxytocin (and vasopressin) which is known to bind to neurophysin at the hormone-binding site but with a 1 order of magnitude lower affinity than for oxytocin (2), is retarded more weakly by [neurophysin-II]Sephrose and can be eluted with pH 5.7 buffer. Net retardation of the mobile interactant (observed elution volume minus unretarded (void) volume) is proportional to its binding affinity (23). The results indicate that OT-GKR, a putative processing intermediate, can bind to immobilized neurophysin with an affinity at least 10-fold that for Met-Tyr-Phe-NH2. The affinity of OT-GKR is at least the same order of magnitude as that of oxytocin.

Conversion of Oxytocinyl Peptides OT-GKR and OTK-G with Neurosecretory Granule Lysate—The degradation of OT-GKR was followed analytically by RP-HPLC. Upon incubation with the neurosecretory granule lysate for a relatively short time, two new peaks with greater retention times than OT-GKR were observed (Fig. 3). Comparison of the elution positions of these peaks, b and c in Fig. 3, with those for authentic OT-GK and OT-G, respectively, allowed identification of peak b as OT-GK and peak c as OT-G. The degradation of OT-GKR to OT-G appeared to take place sequentially at the COOH terminus, suggesting that this conversion is due to the action of a carboxypeptidase. OT-GK was confirmed to be a substrate for pituitary carboxypeptidase B by separately incubating this peptide and detecting OT-G as a product (see below). The conversion of OT-GKR to OT-GK appeared faster than that of OT-GK to OT-G. Additional peaks were observed by RP-HPLC after prolonged incubation with secretory granule lysate. These peaks appear to be more extensively degraded forms including OT acid (nonapeptide with free carboxyl terminus), OT(1-8) and OT(1-7), as judged by amino acid analysis. No degradation of the ring portion (residues 1-6 with disulfide formed by half-cystines 1 and 6) was observed. A pH optimum of 5-6 was observed for the granule lysate activity which degrades OT-GKR into OT-GK and OT-G. Under the conditions of the above experiment, no detectable UV-absorbing peak of oxytocin was formed from either OT-GKR or OT-G.

Comparative OT-GKR conversion assays similar to those in Fig. 3 were carried out for neurosecretory granule lysate in the presence versus the absence of bovine neurophysin I. Reverse-phase HPLC assay of these conversion reactions utilized a linear gradient of 100% TEAP at zero time to 60% TEAP/40% acetonitrile at 20 min or sequential linear gradients of 90% TEAP/10% acetonitrile at zero time to 80% TEAP/20% acetonitrile at 20 min to 60% TEAP/40% acetonitrile at 25 min. For this experiment, 0.7 mM OT-GKR was reacted at 37°C in 0.1 M sodium phosphate buffer, pH 5.5, containing 200 mM NaCl, with or without 6.75 mM neurophysin I. Assuming a hormone-neurophysin Kd of ~4 x 10^-6 M, most (~96%) of OT-GKR would be expected to be in the form of peptide-neurophysin complex. Under the lysate reaction conditions used, the extent of conversion of OT-GKR to OT-GK in the absence of neurophysin was 60% at 2 h and 95% at 8 h. By comparison, conversion in the presence of neurophysin was 25% at 2 h, 40% at 8 h, and about 90% at 27 h. At 96 h, the proportion of product in the form of OT-G was 30% in the absence of neurophysin, about 15% in its presence. The significant although reduced rate of carboxypeptidase B conversion in the presence of neurophysin is consistent with the probability that, in vivo, carboxypeptidase B reaction could occur with peptides bound noncovalently to the neurophysin domain of the original biosynthetic precursor.

In order to visualize the amidation of OT-G to form oxytocin, OT-G was radioiodinated, and micro amounts of the radiolabeled peptide reacted with large amounts of granule lysate. A chromatography assay was established for separation of 125I-OT-G and 125I-OT (see "Experimental Procedures"). When 125I-OT-G was reacted with a lysate of neurosecretory granule fractions 4 + 5 (see "Experimental Procedures") obtained by Percoll density gradient centrifugation of total posterior pituitary granules, conversion was sufficiently strong and nonspecific degradation sufficiently minimized to detect 125I-OT (Fig. 4). Eventually, however, substrate and product both were degraded to a set of earlier eluting, likely proteolytically degraded forms (Fig. 4, A and B). The granule fractions in which amidating enzyme was detected also were found to contain OT, vasopressin, and the major neurophysins (see Fig. 7B below).

Partial Purification and Characterization of Carboxypeptidase Activity—The soluble fraction of neurosecretory granule lysate was partially purified by gel chromatography on a Sephacryl S-300 column (1 x 58 cm) (Fig. 5). The enzyme activity of each fraction was measured at pH 6.0 using OT-GKR and OT-G as substrates in order to examine the differ-

![Graph](image-url)
Enzymatic Conversion of Oxytocin Precursor Intermediates

FIG. 4. RP-HPLC analysis of conversion of \(^{125}\)I-OT-G to COOH-terminal amidated \(^{125}\)I-OT and proteolytically degraded forms upon reaction with neurosecretory granule lysate. A, RP-HPLC profile of aliquot of reaction mixture taken at 135 min after addition of lysate. Reaction using Percoll gradient fractions (4 + 5) and chromatographic separation conditions are described under "Experimental Procedures." S is \(^{125}\)I-OT-G (substrate); P is \(^{125}\)I-OT (product). Peaks centered at 4 min (breakthrough volume) and 15 min increase with time of reaction and are assumed to arise from nonspecific proteolytic degradation of S, P, or both. B, time course of decrease in S and transient increase followed by decrease in P. Counts/min values in peaks were calculated as defined under "Experimental Procedures."

FIG. 5. Gel filtration chromatography of oxytocinyl carboxypeptidase B from neurosecretory granule lysate. The soluble fraction of neurosecretory granule lysate (two bovine tissues) was fractionated on Sephacryl S-300 (1 × 58 cm) with 50 mM sodium phosphate (pH 6.0) containing 100 mM NaCl. A 50-ml aliquot of each fraction (total volume 2 ml) was incubated with 11 nmol of OT-GKR (open bar) and OT-G (closed bar) at 37 °C for 3 and 24 h, respectively. The per cent conversion was analyzed by RP-HPLC (monitored at 280 nm) and defined as follows: (sum of the peak areas for products)/(total peak area); detectable products are OT-G and OT-GK from OT-GKR, and OT(1–7) and OT(1–8) from OT-G.

We characterized both the pH dependence and sensitivity to inhibitors and activators of the Sephacryl-fractionated carboxypeptidase B activity. As shown in Fig. 6, the purified activity has a sharp pH dependence, with an optimum at pH 5.5–6. The efficiency of conversion of OT-GKR was greater in acetate than in phosphate buffer. As shown in Table I, the activity was stimulated by cobalt ion and mercaptoethanol and was not affected by N\(^{\text{\textsuperscript{5}}}\)-p-coumaramide chloromethyl ketone and calcium ion. In contrast, EDTA, cupric ion, and p-chloromercuribenzoate completely inhibited the activity. The properties found for the partially purified carboxypeptidase B described here and in a preliminary report (21) are similar to those recently reported elsewhere for crude enzyme (28). The cobalt stimulation was verified to be due to the carboxypeptidase B activity by the finding that degradation products upon cobalt stimulation were, as expected, OT-GK and OT-G, as shown by amino acid analysis (in which \(^{\text{\textsuperscript{5}}}\)Cys was not determined). OT-GK: Asp (0.99), Glu (0.94), Pro (0.98), Gly (2.70), Ile (1.02), Leu (1.00), Tyr (0.61), Lys (1.02), Arg (0.33). OT-G: Asp (1.03), Glu (1.00), Pro (0.99), Gly (2.02), Ile (0.97), Leu (1.02), Tyr (0.50), Lys (0.22), Arg (0.60). Further evidence that the cobalt-stimulated conversion of OT-GKR to OT-GK was due to a carboxypeptidase and not to an endoprotease with trypsin-like specificity was obtained by assay with isolated OT-GK as a substrate (Table I). The conversion of OT-GK to OT-G was slower than that of OT-GKR to OT-GK by 7-fold. However, both of the conversion rates were enhanced to the same degree, about 3-fold, by the addition of cobalt chloride. The data indicate that a single carboxypeptidase B enzyme is involved in both steps of the sequential degradation process of OT-GKR to OT-G.

A co-occurrence of carboxypeptidase B activity and OT/arginine vasopressin/neurophysin species was observed with lysates from Percoll density gradient fractions of bovine pituitary neurosecretory granules. Granule fractions from the presence of the carboxypeptidase activities of each fraction. Two types of activity were separated by the gel chromatography, one centered at about 20-ml elution volume and the second at about 30 ml. Both of these are capable of degrading OT-GKR to OT-GK and OT-G. However, the earlier peak, which eluted at the void volume, showed an activity for converting OT-G to more extensively degraded forms (1–9 acid, 1–8, and 1–7). This was similar to the conversion, as described above, found with extended incubation of OT-GKR with the crude granule lysate. In contrast, the second peak (fractions 13–18, centered at 30 ml in Fig. 5), converted substantial amounts of OT-GKR to OT-G sequentially in a 3-h incubation but showed only slight activity to convert OT-G to shorter forms even after 24 h of incubation. This result suggests that the major carboxypeptidase activity, corresponding to the second peak, is specific for COOH-terminal basic residues. It is this latter enzyme, a carboxypeptidase B, which has characteristics suggesting its involvement in the post-translational processing of pro-OT/neurophysin.

FIG. 6. pH dependence of partially purified carboxypeptidase B enzyme activity. OT-GKR (25 nmol) in 50 ml of 0.2 M acetate or phosphate buffer at various pH values was incubated with 10 ml of the combined Sephacryl fractions (13–18), which had been concentrated to 1 ml by ultrafiltration with PM-10 filter (Millipore), at 37 °C for 1 h. The per cent conversion was analyzed as described in the legend to Fig. 5. Only OT-GK was formed under the reaction conditions used.
TABLE I

Effects of activators and inhibitors on posterior pituitary carboxypeptidase B activity

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Effector</th>
<th>Incubation time</th>
<th>Conversion*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>OT-GKR</td>
<td>None</td>
<td>0.5 h</td>
<td>18</td>
</tr>
<tr>
<td>I</td>
<td>OT-GKR</td>
<td>CoCl₂</td>
<td>2.0 h</td>
<td>31</td>
</tr>
<tr>
<td>I</td>
<td>OT-GKR</td>
<td>β-2-Mercaptoethanol</td>
<td>0.5 h</td>
<td>57</td>
</tr>
<tr>
<td>II</td>
<td>OT-GKR</td>
<td>None</td>
<td>0.5 h</td>
<td>12</td>
</tr>
<tr>
<td>II</td>
<td>OT-GKR</td>
<td>CoCl₂</td>
<td>0.5 h</td>
<td>47</td>
</tr>
<tr>
<td>II</td>
<td>OT-GKR</td>
<td>CoCl₂</td>
<td>1.5 h</td>
<td>5</td>
</tr>
<tr>
<td>II</td>
<td>OT-GKR</td>
<td>CoCl₂</td>
<td>4.5 h</td>
<td>9</td>
</tr>
<tr>
<td>II</td>
<td>OT-GKR</td>
<td>CoCl₂</td>
<td>4.5 h</td>
<td>12</td>
</tr>
<tr>
<td>II</td>
<td>OT-GKR</td>
<td>CoCl₂</td>
<td>4.5 h</td>
<td>27</td>
</tr>
</tbody>
</table>

*The conversion was calculated as described in the legend to Fig. 5. Only OT-GK was detected under the conditions used when OT-GKR was substrate. OT-G was the product of conversion with OT-GK as substrate.

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<td>II</td>
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<td>CoCl₂</td>
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<tr>
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<td>CoCl₂</td>
<td>4.5 h</td>
<td>27</td>
</tr>
</tbody>
</table>

*Reactions were carried out in 0.2 M acetate buffer, pH 5.5, containing 1 mM of effector (except 0.1 mM for p-chloromercuribenzoate) at 37 °C.*

*p-chloromercuribenzoate.

**Reactions were carried out by incubating OT-GKR or OT-GK (40 nmol) in 44 μl of 0.1 M sodium acetate, pH 5.5, with 10 μl of combined Sephacryl fraction (13-18). Other conditions were as described in the legend to Fig. 6.*

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**Discussion**

In this study, we have identified and isolated a carboxypeptidase from bovine posterior pituitary which is specific for COOH-terminal basic residues. This enzyme can sequentially convert the putative oxytocin processing intermediate, OT-GKR, to OT-GK and OT-G. Characterization of the carboxypeptidase B activity, after separation from a less-specific carboxypeptidase, allows comparison with other precursor-processing carboxypeptidases reported previously, including those proposed to be involved in enkephalin processing in chromaffin granules of adrenal medulla (17, 18). The pH optimum of the presently obtained carboxypeptidase B, pH 5.5-6, is consistent with the internal pH of neurosecretory granules, 5.7. The complete inhibition with EDTA differentiates this activity from lysosomal cathepsin B (18, 29) as well as from the chromaffin granule carboxypeptidase activity proposed by Hook et al. (17) to be a processing enzyme for synthesis of enkephalin. Recently, Fricker and Snyder (18) also isolated a carboxypeptidase from adrenal chromaffin granules which converts [Leu]enkephalin-Arg to [Leu]enkephalin. The properties of this latter enkephalin convertase are very similar to the carboxypeptidase B found in the present study. Both are stimulated by cobalt ion and inhibited by EDTA, cupric ion, and thiol inhibitors. Furthermore, substrates with a COOH-terminal arginine residue are converted at a greater rate by the enzyme than are substrates with a
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COOH-terminal lysine residue. Fricker and Snyder (18) also have found the enkephalin-converting activity in pituitary glands. These and the present results suggest that oxytocin and enkephalin processing involve the same or closely related carboxypeptidases in adrenal and pituitary tissues. It is of interest to note that Met-enkephalin and dynorphin have been found to co-exist in some oxytocin- and vasopressin-producing neurons, respectively (30, 31) and, therefore, could enlist common proteolytic activities for conversion from precursors to active forms.

The detection of enzymatic amidation of oxytocin-Gly to form oxytocin has proven to be problematic in the present study due largely to nonspecific proteolytic degradation of both substrate and product. Amidation of X-Gly substrates to X-amides has been reported previously, mainly for small peptides, with the enzyme responsible being detected in several sites including the pituitary and found to be activated by several substances, including copper ion and ascorbate (32, 33). In the present work, formation of oxytocin as the expected last step in sequential conversion of nonradio-labeled OT-GK by granule lysate could not be detected to any significant extent, when the reaction was carried out with pituitary granule lysate in the presence of high concentrations of bovine neurophysin I. Noncovalent binding between hormonal and neurophysin domains during processing could impose steric constraints which, to degradation products such as OT acid and OT(1-8). However, when discrete subfractions of posterior pituitary neurosecretory granules were incubated with tracer amounts of 125I-OT-G, the amidated product, 125I-OT, could be detected (Fig. 4). Nonetheless, the product was relatively short-lived and, as with substrate, ultimately was converted to apparently degraded forms by prolonged reaction due to contaminating proteases. Given the susceptibility of extended polypeptides such as oxytocin to the latter proteases, efficient conversion of OT-G to oxytocin likely will require a significantly purified preparation of amidation enzyme; preliminary data suggest that the latter can be achieved by using alternative granule subfractions from Percoll density gradient centrifugation.

In this study, we have shown that oxytocinyl-Gly-Lys-Arg can bind to neurophysin, with an affinity close to that of intact oxytocin. Assuming that OT-GK and/or closely related forms by prolonged reaction due to contaminating proteases. Amidation of X-Gly substrates, describing amidating enzyme in Percoll gradient fractions of neurosecretory granules along with OT, vasopressin, and the major neurophysins (Figs. 4 and 7); (b) the correspondence of pH optimum of carboxypeptidase B conversion with the internal pH of secretory granules (Fig. 6); and (c) the ruling out of both carboxypeptidase B steps as lysosomal as judged by Co2+ stimulation (Table I, Fig. 7). Nonetheless, as stated earlier, the conversion reactions would occur intragranularly in a concentrated milieu in which substrates are parts of cooperative noncovalently interacting complexes with neurophysins and in which unprocessed precursors as well as hormone-neurophysin complexes coexist which could interact to form higher order aggregates. Thus, while the description of the molecular mechanisms of enzymatic hormonogenesis can be improved by in vitro study of isolated enzymes and isolated substrates, describing in vivo mechanisms likely will require a fuller evaluation of the impact of molecular order of peptide/protein complexes. In addition, the possible effect of intragranule membranes on molecular organization of enzymes as well as substrates remains to be evaluated.

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