An identical cytochrome \( b_{561} \) is present in bovine adrenal chromaffin vesicles and posterior pituitary neurosecretory vesicles*

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An identical cytochrome \( b_{561} \) was found to be an integral component of both chromaffin vesicles from adrenal medulla and neurosecretory vesicles from posterior pituitary by spectrophotometric and immunological techniques. The neurosecretory vesicles had \( 6.8 \mu g \) of cytochrome/mg of membrane protein versus \( 69 \mu g/\)mg in chromaffin vesicles. This cytochrome was also immunologically detected in various regions of bovine brain and was immunologically distinct from the cytochrome found in serotonin-containing vesicles from platelets. Dopamine \( \beta \)-hydroxylase involved in the biosynthesis of catecholamines was not present in neurosecretory vesicles, suggesting an alternative functional role for the cytochrome in these vesicles. Neurosecretory vesicles do contain a mixed function oxidase (peptidyl \( \alpha \)-amidase) which appears to be involved in \( \alpha \)-amidation of the carboxyl termini of vasopressin and oxytocin. We suggest that cytochrome \( b_{561} \) in the two vesicles may be functionally associated with different ascorbic acid-dependent, copper-containing mixed function oxidases: dopamine \( \beta \)-hydroxylase and peptidyl \( \alpha \)-amidase.

Cytochrome \( b_{561} \) is a major component of the chromaffin vesicle membranes from bovine adrenal medulla. This cytochrome was reported to be the only heme-containing protein in the vesicles and was shown to have a high midpoint oxidation reduction potential of +140 mV (1). The molecular weight of cytochrome \( b_{561} \) has been calculated to be 30,000 (2), and it has been chemically and immunologically distinguished from mitochondrial and microsomal cytochromes (2, 3). The function of cytochrome \( b_{561} \) is not known but it has been suggested to be responsible for a transmembrane electron transport which could supply electrons for intragranular dopamine \( \beta \)-hydroxylase (4, 5). Therefore, it is of interest to question whether or not cytochrome \( b_{561} \) is always co-localized with dopamine \( \beta \)-hydroxylase when either protein is present in a particular tissue.

A cytochrome \( b_{561} \) has also been spectrophotometrically identified in noradrenergic storage vesicles from bovine splenic nerves (6) and in the membranes of serotonin dense core vesicles in porcine platelets, which have an embryologically distinct origin from the adrenal catecholamine-synthesizing cells (7). An earlier immunological study on the tissue distribution of chromomembrin B, which has recently been identified as cytochrome \( b_{561} \), detected the presence of this protein or a related antigen in posterior and anterior (8) lobes of the pituitary and in other tissues. The presence of cytochrome \( b_{561} \) in either platelets or pituitary would suggest that this protein may not be functionally associated only with dopamine \( \beta \)-hydroxylase since the latter should not be localized in these tissues.

Neurosecretory vesicles in the nerve endings of the posterior pituitary are intracellular organelles which contain in their matrix stores of the hormones vasopressin and oxytocin. Like a number of other neuropeptides and peptide hormones, both vasopressin and oxytocin have an \( \alpha \)-amide moiety at their carboxyl termini. This amidation of the peptides occurs post-translationally within neurosecretory vesicles during processing of the prohormones (9, 10). Similarly, \( \alpha \)-melanocyte-stimulating hormone is amidated during biosynthesis in the cells of the intermediate lobe of the pituitary. Recently, an \( \alpha \)-amidating enzyme activity was recognized in porcine pituitary homogenates (11, 12) and in secretory vesicle fractions from all three lobes of the pituitary (13). This enzyme activity was dependent on \( Cu^{+} \) ions, molecular oxygen, and ascorbic acid (13). In these properties, this amidating enzyme is strikingly similar to dopamine \( \beta \)-hydroxylase in chromaffin vesicles. Hence, a cytochrome \( b_{561} \) could function in the neurosecretory vesicle to supply electrons for intravesicular \( \alpha \)-amidase in a manner similar to its putative role with dopamine \( \beta \)-hydroxylase in the chromaffin vesicles.

This contention prompted us to undertake the present study where we have used spectrophotometric and immunological techniques to demonstrate the presence of cytochrome \( b_{561} \) in highly purified fractions of neurosecretory vesicles. Furthermore, we have determined the distribution of cytochrome \( b_{561} \) in different regions of bovine brain and its apparent absence in a number of other tissues.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals used for polyacrylamide gel electrophoresis and molecular weight standards were purchased from Bio-Rad. Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody and peroxidase-conjugated goat anti-mouse IgG antibody were obtained from Boehringer Mannheim. Nitrocellulose sheets (0.45-μm pore size) were from Schleicher & Schuell. Goat serum was from Gibco Laboratories. 4-Chloro-1-naphthol was from Sigma. Rabbit antisera against bovine soluble dopamine \( \beta \)-hydroxylase was generously supplied by Liz McHugh from the laboratory of one of us (P. J. F.). Monoclonal antibody against bovine cytochrome \( b_{561} \) was a gift from Dr. Rebecca Fruea, National Institutes of Health.

**Preparation of Membrane Fractions from Bovine Tissues**—Chromaffin granule membranes were isolated from the adrenal medulla using a previously described procedure (14). Neurosecretory vesicles

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from the posterior lobe of the pituitary were purified by Percoll density gradients (18). Secretory vesicles from intermediate lobes were prepared using a Metrizamide/sucrose gradient. A crude preparation of anterior pituitary secretory granules was obtained by centrifugation of tissue homogenate at 800 x g followed by 25,000 x g in 0.3 M sucrose and collection of the second pellet.

Crude membrane extracts were prepared from pineal and the following areas of bovine brain: median eminence, hypothalamus, spinal cord, hippocampus, substantia nigra, caudate nucleus, cerebellum, locus coeruleus, medulla oblongata, cortex, and pons. The pituitary gland was dissected into anterior, intermediate, and posterior lobes. The peripheral tissues were selected as follows: adrenal gland (cortex and medulla), heart, kidney, pancreas, spleen, and liver.

After the dissection, each tissue was minced finely and suspended (0.2 g/ml) in 10 mM HEPES, pH 7.4, phenylmethylsulfonyl fluoride (25 mg/liter). These tissues were disrupted in a Polytron homogenizer (Brinkmann Instruments). The homogenates were centrifuged in a type 50.1 Ti rotor at 26,000 x g for 15 min and then at 105,000 x g for another 45 min. The membrane pellets were resuspended in 10 mM HEPES, frozen and thawed, and centrifuged again at 105,000 x g for 60 min. Each membrane fraction was extracted in 1% Triton X-100 at 4°C for 30 min and centrifuged at 105,000 x g to remove nonsolubilized materials. The supernatants were stored in aliquots at -20°C.

Porcine and bovine platelet membranes were prepared from 1 unit of blood freshly obtained in the presence of citrate (16). Red cells were removed by centrifuging the blood sample at 1,000 x g for 10 min. The platelet-rich plasma supernatant was then centrifuged at 5,000 x g for 10 min to obtain a platelet pellet. Platelets were resuspended in phosphate-buffered saline, identified microscopically, homogenized with a Polytron homogenizer, and frozen and thawed twice. The platelet membranes were collected by centrifuging at 105,000 x g and extracted in Triton as described.

Electrophoretic Blotting Procedure and Immunodetection of Proteins or Nitrocellulose—Proteins were first separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and transferred to nitrocellulose sheets according to Towbin et al. (17). A voltage gradient of 60 V was applied for 2 h. At the end of the transblotting run, the nitrocellulose sheets were soaked in 10% goat serum in saline (0.8% NaCl, 10 mM TRIS-HCl, pH 7.4) at 4°C overnight. The sheets were incubated in primary antiserum appropriately diluted in 10% goat serum overnight at 4°C. At the end of incubation, they were washed in saline for 1 h at room temperature and transferred into a solution of peroxidase-conjugated secondary antibody and incubated for 2 h at room temperature followed by an extensive wash with saline. The peroxidase activity was developed in a freshly made solution of 105 mg of 4-chloro-1-naphthol in 30 ml of methanol mixed with 175 ml of saline and 0.01% H2O2. The reaction was terminated by washing with water, and the sheets were dried between filter paper before storage.

Other Procedures—Polyacrylamide electrophoresis was performed on slab gels (8 cm long, 0.75 mm thick). The discontinuous sodium dodecyl sulfate/Tris buffer system, described by Laemmli (18), was employed with 4.5% acrylamide stacking gels and 12% acrylamide separating gels. Assay for dopamine β-hydroxylase activity was performed according to Kato et al. (19). Protein determination was performed according to the procedure of Peterson (20) with bovine serum albumin as standard. Preparation of rabbit anti-cytochrome b65, antisera has been described previously (3). Optical spectra were recorded on an Aminco DW-2 spectrophotometer.

RESULTS

In an attempt to identify heme-containing proteins in the neurosecretory vesicle membranes, a dithionite-reduced minus ferricyanide-oxidized spectrum of the vesicle membranes was obtained. A characteristic cytochrome spectrum was observed with absorbance maxima at 428, 530, and 561 nm (Fig. 1B). The amount of cytochrome b65 in neurosecretory vesicle membrane was calculated from this spectrum to be 8.8 μg/mg of membrane protein (ε = 26,880 mm⁻¹ cm⁻¹) at 561-570 nm (21). A reduced minus oxidized spectrum of chromaffin vesicle membranes measured under identical conditions is shown in Fig. 1A for comparison. The two spectra are virtually superimposable. However, the amount of cytochrome present on chromaffin vesicle membrane was calculated to be 19 μg/mg of membrane protein, which is 10 times greater than in neurosecretory vesicles. Ascorbic acid (1 mM) was able to reduce more than 90% of the spectrally observed cytochrome in both neurosecretory and chromaffin vesicle membranes (data not shown).

Cytochrome b65 is the only spectrophotometrically resolvable cytochrome in the membranes of adrenal chromaffin vesicles and neurosecretory vesicles from posterior pituitaries. These experiments suggested that the neurosecretory vesicle membranes like the chromaffin vesicles possess cytochrome b65, although in smaller amounts. Studies on the distribution of marker enzymes published elsewhere (15) clearly indicate that only very minimal mitochondrial and lysosomal contamination is present in neurosecretory vesicles prepared on Percoll gradients. The relative specific activities (specific activity in the fraction/specific activity in the original ho-
mogenate) of vasopressin, monoamine oxidase (mitochondria marker), and β-glucuronidase (lysosomal marker) in this vesicle fraction were 10.6, 0.31, and 0.14, respectively, suggesting that a 10-fold enrichment of the neurosecretory vesicles over the homogenate was achieved. The contaminants were substantially removed since these vesicles are purified 34- and 76-fold over mitochondria and lysosomes, respectively. Lactate dehydrogenase (cytosolic marker) was undetectable, indicating the absence of pinched off nerve endings.

**Immunological Identity of Cytochrome b₅₆₁**—Having spectrophotometrically identified the presence of cytochrome b₅₆₁ on neurosecretory vesicle membranes, a specific antisera against chromaffin vesicle cytochrome b₅₆₁ was used in order to check for immunological identity. An antiserum against dopamine β-hydroxylase was also used to verify the absence of this enzyme in neurosecretory vesicle membranes. Preliminary experiments were carried out using adrenal tissue to determine the sensitivity of the immunological detection on transblots. The results are presented in Fig. 2. With the Western blotting procedure, visible bands can be observed with 20 ng of purified cytochrome b₅₆₁ and 10 ng of purified soluble dopamine β-hydroxylase on immunostaining.

Previously, the purified cytochrome b₅₆₁ was observed as a diffuse single band on polyacrylamide gels (2, 3). In this study, the cytochrome was resolved into a doublet when the proteins were separated with constant voltage instead of constant current and the electrophoretic period prolonged to 5 h. The characteristic doublet of cytochrome b₅₆₁ was also observed by Hunter et al. (21). We obtained evidence that the lower molecular weight band is a modified product of the native cytochrome which is formed upon isolation or storage. As shown in Fig. 2, A and B, only the upper band of cytochrome b₅₆₁ was observed in freshly prepared extracts from adrenal medullary tissue. As the chromaffin vesicle membranes were isolated, the lower molecular weight product appeared. In a purified preparation of cytochrome b₅₆₁, which was frozen and thawed several times, the lower molecular weight product became the major component. Upon storage, the cytochrome was often aggregated into a dimer with an apparent molecular weight of 44,000 on sodium dodecyl sulfate-polyacrylamide gels. The 44,000-dalton band and the doublet near 28,000 daltons were shown to be cytochrome b₅₆₁ since all three bands were decorated by a monoclonal antibody against the bovine cytochrome b₅₆₁ (Fig. 2B). As expected, dopamine β-hydroxylase was found in both chromaffin vesicle membranes and vesicle lysate (Fig. 2C), whereas the cytochrome was not detected in the lysate.

Hortnagl et al. (8) have previously observed the presence of chromomembrin B or a related antigen in anterior and posterior hypophysis by microcomplement fixation technique. Since Western blotting technique reveals the identity of the antigen based on its apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gels, we employed this technique to re-examine the cross-reactivities of the anti-cytochrome b₅₆₁ antibody with the membranes from anterior, intermediate, and posterior pituitaries. Cytochrome b₅₆₁ was clearly demonstrated to be present in the membrane extracts from the intermediate and posterior lobes (Fig. 3A). Furthermore, cytochrome b₅₆₁ was shown to be located in the neurosecretory vesicle membranes and α-melanocyte stimulating hormone-containing secretory vesicles from the intermediate lobe of the pituitary (Fig. 4A). The amounts of cytochrome b₅₆₁ in these vesicles, however, were significantly lower than in chromaffin vesicles which is consistent with the spectrophotometric measurements presented earlier. These experiments show that the cytochrome b₅₆₁ on the neurosecretory vesicles is identical to the chromaffin vesicle cytochrome immunologically and spectrophotometrically and in electrophoretic mobility.

The cross-reacting material with an apparent molecular weight of 55,000 observed in hypophyseal membranes was not further characterized. A faint immunostain of cytochrome b₅₆₁ was observed with 100 µg of crude membrane proteins from anterior pituitary, and staining increased only slightly when 200 µg were used (Fig. 3A). When membranes from a crude preparation of anterior lobe vesicles were tested, a positive but faint immunostain for the cytochrome was observed. The amount of cytochrome in anterior lobe vesicles was therefore much lower than in vesicles from intermediate or posterior lobes. Dopamine β-hydroxylase was not detected in the crude membrane fractions of the three lobes from pituitary glands (Fig. 3B) or in the vesicle membrane fractions of the intermediate and posterior lobes (Fig. 4B). Dopamine β-hydroxylase activity was also assayed in the pituitary lobes. No detectable activity was found in anterior, intermediate, or posterior lobes. With our assay conditions, the lower limit of detection for enzyme activity was 0.014 nmol/min/mg of protein and crude membrane extract from bovine adrenal medulla had a hydroxylase activity of 71 nmol/min/mg.

A general survey of the distribution for cytochrome b₅₆₁ in bovine tissue is listed in Table I. With the electroblotting and immunostaining techniques, the cytochrome was shown to be absent in adrenal cortex, heart, kidney, pancreas, spleen, liver, and platelet membranes. However, the cytochrome was present in low concentration in various bovine brain areas. In cerebellum and locus coeruleus, this antigen was particularly concentrated (Fig. 5).

**DISCUSSION**

The experiments presented in this paper show that neurosecretory vesicles from the posterior pituitary possess a cytochrome b₅₆₁ which is identical to the cytochrome on chromaffin vesicles from adrenal medulla by three different criteria. 1) Both membranes have identical reduced minus oxidized absorbance spectra. 2) The electrophoretic mobility of the cytochromes is the same in both cases. 3) Cytochromes from both vesicles are immunologically identical.

The functional role of cytochrome b₅₆₁ in chromaffin vesicles...
A rabbit anti-cytochrome \( b_{561} \) antiserum was used with samples of crude membrane extracts from the following tissues: lane 1, 50 \( \mu \)g of adrenal medulla; lane 2, 200 \( \mu \)g of anterior pituitary; lane 3, 100 \( \mu \)g of intermediate pituitary; lane 4, 100 \( \mu \)g of posterior pituitary. An identical gel was stained with rabbit antiserum against dopamine \( \beta \)-hydroxylase.

In 1971, Flatmark et al. (22) reported that cytochrome \( b_{561} \) was reduced by NADH via an activity resembling NADH:(acceptor) oxidoreductase activity. However, the same group later found no NADH-linked cytochrome \( b_{561} \) reduction in highly purified vesicle membranes (23). In unpublished experiments, we have not been able to demonstrate a reduction of cytochrome \( b_{561} \) by NADH or NADPH using various types of chromaffin vesicle membrane preparations. An NADH:(acceptor) oxidoreductase has been purified from the chromaffin vesicle membranes and was shown to be a transmembrane protein (24), but apparently it is unable to donate to cytochrome \( b_{561} \). However, it is known that ascorbic acid will reduce cytochrome \( b_{561} \) (22). Since the cytochrome is exposed to the cytoplasmic surface of the chromaffin vesicle membrane (3), cytoplasmic ascorbic acid must be considered as a potential physiological electron donor to cytochrome \( b_{561} \).

It has been suggested that the cytochrome supplies reducing equivalents to dopamine \( \beta \)-hydroxylase (4). In the presence of an electron donor and molecular oxygen, dopamine \( \beta \)-hydroxylase catalyzes the formation of norepinephrine from dopamine. Even though ascorbic acid is apparently the best electron donor for the hydroxylation reaction in vitro, it has not been proven to be the physiological electron donor for this enzyme. Recently, only dehydroascorbate was found to be transported across the vesicle membrane (25). Thus, if ascorbate is the physiological electron donor to dopamine \( \beta \)-hydroxylase, there must exist a system for reducing dehydroascorbate to ascorbate inside the chromaffin granule. Cytochrome \( b_{561} \) is an obvious candidate for this ascorbate-regenerating system. In fact, the cytochrome may function by transferring electrons from cytoplasmic ascorbic acid to intragranular dehydroascorbate (or semidehydroascorbate) and thus function to catalyze a transmembrane electron flow (4).

In order to obtain evidence for the functional association of cytochrome \( b_{561} \) and dopamine \( \beta \)-hydroxylase, we looked for a physical association of these two proteins in terms of their respective tissue distributions. The data presented here are consistent with a role for cytochrome \( b_{561} \) in providing electrons to dopamine \( \beta \)-hydroxylase but also suggest a wider functional role for the cytochrome. This study has demonstrated the presence of cytochrome \( b_{561} \) in neurosecretory vesicles from neurohypophysis and secretory vesicles from the intermediate lobe of the pituitary, where no dopamine \( \beta \)-hydroxylase activity was detected, which suggests an alternative role for cytochrome \( b_{561} \) in these tissues. As pointed

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Table I

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cytochrome cross-reactivity</th>
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<tr>
<td>Adrenal cortex</td>
<td>-</td>
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<tr>
<td>Heart</td>
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<td>Kidney cortex</td>
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<td>Kidney medulla</td>
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<td>Pancreas</td>
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<td>Liver</td>
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<td>Platelets</td>
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<td>Bovine</td>
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<td>Porcine</td>
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<td>Brain</td>
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<tr>
<td>Pineal</td>
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<td>Median eminence</td>
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<td>Hypothalamus</td>
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<tr>
<td>Spinal cord</td>
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<tr>
<td>Hippocampus</td>
<td>-</td>
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<tr>
<td>Substantia nigra</td>
<td>+</td>
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<tr>
<td>Caudate nucleus</td>
<td>+</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>+++</td>
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<tr>
<td>Locus coeruleus</td>
<td>+++</td>
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<tr>
<td>Medulla oblongata</td>
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<td>Cortex</td>
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<td>Pons</td>
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*In each case 100 \( \mu \)g of crude membrane protein extract was tested, except that up 800 \( \mu \)g of bovine and 400 \( \mu \)g of porcine platelet membrane protein was tested.

*One plus sign (+) indicates barely detectable immunostaining and three plus signs indicate intense immunostaining of the cytochrome. A minus sign (−) indicates no detectable immunostaining.

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a reduction of cytochrome \( b_{561} \) by NADH or NADPH using various types of chromaffin vesicle membrane preparations. An NADH:(acceptor) oxidoreductase has been purified from the chromaffin vesicle membranes and was shown to be a transmembrane protein (24), but apparently it is unable to donate to cytochrome \( b_{561} \). However, it is known that ascorbic acid will reduce cytochrome \( b_{561} \) (22). Since the cytochrome is exposed to the cytoplasmic surface of the chromaffin vesicle membrane (3), cytoplasmic ascorbic acid must be considered as a potential physiological electron donor to cytochrome \( b_{561} \).

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Le T. Duong, P. J. Fleming, and J. T. Russell, unpublished experiments.
out earlier, these secretory vesicles contain an α-amidase activity presumably responsible for amidating the carboxyl termini of secretory peptides, e.g. vasopressin, oxytocin, and α-melanocyte-stimulating hormone (13). In vitro, this enzyme also requires Cu²⁺ ions, ascorbic acid, and molecular oxygen (13). Thus it is possible that the cytochrome b₅₆₁ on neurosecretory vesicles and α-melanocyte-stimulating hormone-containing intermediate lobe vesicles is involved in regenerating ascorbic acid by transferring electrons from a cytoplasmic donor. This hypothesis is supported by the following observations. 1) Cytochrome b₅₆₁ on neurosecretory vesicles can be completely reduced by ascorbic acid. 2) In pituitary intermediate lobe cells in primary culture, the newly synthesized α-melanocyte-stimulating hormone is not amidated when grown in the absence of ascorbic acid in the medium and is amidated when ascorbic acid is provided (26). 3) The neurosecretory vesicles have 1–3 mM ascorbic acid in their matrix. 4) Consistent with this hypothesis is our finding that cytochrome b₅₆₁ is present in very low concentrations in the anterior pituitary lobe where the peptide hormones are not α-amidated even though the α-amidase enzyme level is high (13). Our results with the anterior and posterior pituitary lobes agree qualitatively with those of Hortaing et al. (8), who found in this tissue an antigen (chromomembrin B) subsequently identified as cytochrome b₅₆₁ (27). It should be noted that the adenohypophysis is enriched in another protein found in chromaffin vesicles, chromogranin A, in contrast to cytochrome b₅₆₁ (28).

The presence of cytochrome b₅₆₁ in posterior pituitary neurosecretory vesicles adds to the list of similarities between these vesicles and chromaffin vesicles. Both neurosecretory vesicles and chromaffin vesicles maintain an acidic intravesicular pH and transmembrane potential (29, 30) apparently due to a proton-translocating ATPase present on each vesicle membrane. Furthermore, both types of secretory vesicles contain proteolytic enzymes involved in the post-translational processing of prohormones to biologically active peptides (31, 32). With the recent discovery of an amidated opioid peptide in the adrenal medulla (33), it is likely that chromaffin granules also contain the α-amidase activity as in the neurosecretory vesicles. However, because of the large amounts of catecholamines in the chromaffin vesicle, it is most likely that dopamine β-hydroxylase is the major ascorbate-utilizing enzyme in these vesicles.

Johnson and Scarpa (7) have spectrophotometrically identified a cytochrome b₅₆₁ in the brown pineal granules of porcine platelets, and these authors have also suggested that the respiratory chain of the chromaffin granules may not be related to the electron transfer to dopamine β-hydroxylase. The antisera against bovine chromaffin vesicle cytochrome b₅₆₁ which we employed, however, does not cross-react with bovine or porcine platelet membranes (Table I). In unpublished experiments, we found that this antisera cross-reacts with porcine chromaffin vesicle cytochrome b₅₆₁ with apparent excellent avidity. Therefore, the cytochrome observed in serotonin dense granules is apparently not the same as cytochrome b₅₆₁ in the chromaffin vesicle and neurosecretory vesicle membranes. It has also been reported that no cytochrome b₅₆₁ could be detected by spectral analysis of serotonin dense granules from rabbits (34).

A recent study on the localization of mRNA for dopamine β-hydroxylase in rat brain has found that RNA from every brain area tested showed some hybridization with mRNA for dopamine β-hydroxylase, but that locus coeruleus, hypothalamic, and cerebellum were particularly enriched (35). This observation is in agreement with our finding that cytochrome b₅₆₁ is present in most bovine brain regions but more abundant in locus coeruleus and cerebellum.

In summary, this study has demonstrated co-localization of cytochrome b₅₆₁ with either one of the ascorbate-dependent enzymes, dopamine β-hydroxylase or peptide α-amidase. This observation therefore supports the hypothesis that cytochrome b₅₆₁ supplies electrons for regeneration of ascorbic acid utilized by these enzymes in their respective secretory vesicles. Further experiments are necessary in order to obtain direct evidence for the coupling of electron transport to the enzymatic activities.

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