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Electron Immunocytochemical Localization of Enkephalin-like Material in Catecholamine-containing Cells of the Carotid Body, the Adrenal Medulla, and in Pheochromocytomas of Man and other Mammals

IAN M. VARNDELL, FELIX J. TAPIA,[1] JAN DE MEY, ROBERT A. RUSH, STEPHEN R. BLOOM, and JULIA M. POLAK

Histochemistry Unit (Department of Histopathology) (I.M.V.; F.J.T.; J.M.P.) and Department of Medicine (S.R.B.), Royal Postgraduate Medical School, Hammersmith Hospital, London W12 OHS, U.K., Laboratory of Oncology (J.D.M.), Department of Life Sciences, Janssen Pharmaceutica, 2340 Beerse, Belgium, and Department of Physiology (R.A.R.), Flinders Medical Center, Bedford Park 5042, South Australia

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Enkephalin-like immunoreactivity has been localized to electron-dense secretory granules of cat and piglet carotid bodies and adrenal medullae, horse adrenal medulla, and also to human adrenal medulla and pheochromocytomas using a gold-labeled antibody technique performed at the electron microscopic level. The same granules were also demonstrated to exhibit dopamine-β-hydroxylase-like immunoreactivity, which suggests a granular colocalization of amines and peptides in catecholamine-storing cells.
KEY WORDS: Electron immunocytochemistry; Methionine enkephalin-like immunoreactivity, localization of; Gold-labeled antibody technique; Carotid body; Adrenal medulla; Pheochromocytoma; Cat; Pig; Horse; Man.

Introduction

Type I glomus cells of the carotid body and both chromaffin cell types of the adrenal medulla are designated as members of the APUD series (Pearse, 1969) and are considered to be intimately related on morphologic, embryologic, and histochemical grounds (Böck, 1980). The type I and adrenomedullary gland cells have been demonstrated to contain catecholamines (Böck and Gorgas, 1976; Dreyer et al., 1976; Winkler 1976), while the catecholamine-synthesizing enzymes tyrosine hydroxylase, dopamine-β-hydroxylase and phenylethanolamine-*N*-methyltransferase have been visualized at the electron microscope level in the adrenal medullae of several species, including frog, rat, and cow (Nagatsu and Kondo, 1974; Thomas et al., 1974; Nagatsu et al., 1979).

Recently, a number of regulatory peptides have been localized within cells and nerves of the feline carotid body. Lundberg et al. (1979) and Wharton et al. (1980) reported leucine and methionine enkephalin from the type I cells, in addition to demonstrating substance P- and vasoactive intestinal polypeptide (VIP)-like immunoreactive fibers innervating the clusters of glomus cells. In addition, Wharton et al. (1980) demonstrated that leucine and methionine enkephalin-like material occurred in considerable quantities in cat carotid body extracts. Methionine enkephalin-like immunoreactivity has also been reported from the adrenomedullary gland cells of the cat, rat, and guinea pig (Schultzberg et al., 1978a,b), while multiple molecular forms of methionine enkephalin have been detected in bovine adrenal glands (Yang et al., 1980a). In addition, enkephalin-like immunoreactive material has been reported to occur in human peripheral neuroendocrine tumors, for example, adrenal ganglioneuromas (Sullivan et al., 1978) and pheochromocytoma (Sullivan et al., 1978; Lungberg et al., 1980).

A granular localization was attributed to the antigenic site by Schultzberg et al. (1978b), and corroborative evidence for this suggestion was provided by Viveros et al. (1979) and Wilson et al. (1980), who demonstrated that opiate-like peptides were present in the adrenal medullae of many species, including man, with the highest specific activity recorded in the purified chromaffin granule fraction.

Although Yang et al. (1980b) emphasized the significance of the coexistence of catecholamines and enkephalin-like pep-

[1]Visiting fellow in the Histochemistry Unit, on leave from Institute Nacional de Dermatologia (Caracas), supported by a fellowship from Fundacion Gran Mariscal de Ayacucho (Venezuela).

tides in understanding opioid modulation of catecholamine release, only one brief communication on the ultrastructural localization of enkephalin-like immunoreactive material, solely in the rat adrenal medulla, has been published to our knowledge (Hervonen et al., 1980). Furthermore, no systematic study of the ultrastructural localization of enkephalin-like immunoreactivity in amine-containing tissues, for example, carotid body, adrenal medulla, and pheochromocytoma, of man and other mammals has been reported to date. In the present study we report on the intracellular localization of methionine enkephalin- and dopamine-β-hydroxylase-like immunoreactivity in the carotid body and adrenal medulla of several mammals, including man. The electron microscopic localization of enkephalin-like immunoreactivity in human pheochromocytomas is also presented. Finally, further evidence to support the concept of costorage of amines and peptides in single cells has been obtained using the immunogold staining procedure applied at the electron microscope level, and is reported here.

Materials and Methods

Twelve adult cats, weighing between 1.8 and 2.0 kg, were anesthetized by an intraperitoneal overdose of sodium pentobarbitone (Euthatal, 200 mg/ml, 1 ml per kg) and perfused, via the ascending aorta, according to the following procedure. Exsanguination was achieved with 150 ml of ice-cold 0.1 M sodium phosphate buffer, pH 7.3, (50 ml/min^{-1}) and was followed immediately by fixation with an ice-cold solution of 1% formaldehyde (prepared from its *para* polymer) plus 2% glutaraldehyde in 0.075 M sodium phosphate buffer (pH 7.3; 720 mOsm) for 15 min (50 ml/min^{-1}). Carotid bodies (n = 14) and adrenal glands (n = 12) were excised, cut into small blocks, and fixed by immersion in the latter solution for a total time of 2 hr. Half of the blocks were rinsed in buffer and osmicated (1% osmium tetroxide in Millonig's buffer, pH 7.2, for 1 hr at 4°C), whereas the other half were merely rinsed in buffer, before conventional processing and embedding in Araldite.

Silver to silver-grey sections were cut on a Reichert-Jung Ultracut and were collected on uncoated 300-mesh nickel grids. Semithin (0.5–1.0 μm) sections were also cut, mounted on clean glass slides, and stained with a solution of Azure II and methylene blue in borax buffer.

A second group of twelve adult cats (weighing between 1.6 and 2.0 kg) and six neonatal piglets, aged 1 day to 3 weeks, were killed by pentobarbitone overdose and dissected fresh. The adrenal medulla was separated from each adrenal gland from each animal and the pieces were processed for electron microscopy as described above. In addition, blocks of tissue excised from the adrenal medullae of three horses were fixed and processed for electron microscopy as described above.

Finally, normal adrenal medulla and pheochromocytoma tissue was removed from human subjects at adrenalectomy or autopsy and fixed for electron microscopy according to the methods recorded above. Only human material showing good morphological preservation when studied by conventional transmission electron microscopy was investigated immunocytochemically.

Electron immunocytochemistry. Localization of methionine enkephalin- and dopamine-β-hydroxylase-like immunoreactivities were obtained using a modified on-grid immunogold staining method (De Mey et al., 1981; Gu et al., 1981) utilizing a goat anti-rabbit second layer antiserum linked directly to colloidal gold, originally introduced by Romano et al. (1974). Briefly, the on-grid staining procedure was followed as outlined below. The grid-mounted sections of unosmicated tissue were dried overnight and then etched in 10% hydrogen peroxide for 10 min. This latter step was later found not to be essential and the routine use of hydrogen peroxide was discontinued. Thorough washing of the grids in phosphate-buffered saline (PBS), pH 7.3, was followed by incubation in normal goat serum (NGS) for 30–60 min at room temperature. The NGS was drained from the grids, each of which was then incubated in 10 μl of first layer antiserum for 20–24 hr at 4°C. The antisera were diluted with a Tris-bovine serum albumin (BSA) buffer (pH 7.2) to final titers of 1:4000 (rabbit anti-methionine enkephalin) and 1:2000 (rabbit anti-bovine dopamine-β-hydroxylase). Characteristics of the antisera are presented in Table 1. Control sections were incubated in normal (preimmune) rabbit serum or in first layer antisera preabsorbed with pure peptide (5 nmol/ml) or with extracted enzyme (Sigma type III; Sigma London Chemical Co., Poole, U.K.).

After thorough washing in Tris-BSA buffer the grids were transferred to the gold-labeled antiserum (dilution 1:8) for 1 hr at room temperature. This incubation was followed by thorough washing in large volumes of Tris-BSA buffer. Finally, the grids were rinsed in Millipore-filtered distilled water, dried, counterstained for conventional electron microscopy, and viewed with a Zeiss 10C electron microscope operating at 60 kV.

In addition, the Masson-Fontana argentaffin reaction (Pearse, 1972) was employed for the ultrastructural demonstration of specific catecholamines.

Mean secretory granule diameter was calculated from measurements of 100 of the largest granule profiles. The assumptions were made that the granules are spherical and that granule profile size is randomly distributed and described by a unimodal curve. The mean number, with 95% confidence limits attached, of immunoreactive sites for each immunocytochemical staining reaction were calculated from a minimum of 12 micrographs.

Table 1. *Preparation and characterization of antisera for electron immunocytochemistry*

Antiserum	Ref.	Raised in/against	Titer	Absorption (1–40 nmol per ml)[a]				
				Met-enk	Leu-enk	β-end	β-LPH	DβH
Met-enkephalin	497	Rabbit/synthetic peptide	1:4000	−(1)	−(40)	−(40)	+	+
Met-enkephalin	507	Rabbit/synthetic peptide	1:4000	−(1)	−(10)	−(40)	+	+
Dopamine-β-hydroxylase	677	Rabbit/purified bovine enzyme	1:2000	+	+	+	+	−(0.5)[b]

[a]Controls included the application of antiserum preabsorbed with methionine enkephalin (Met-enk), leucine enkephalin (Leu-enk), β-endorphin (β-end), β-lipotropin (β-LPH) or dopamine-β-hydroxylase (DβH). + = no change in the intensity of the immunostaining with 40 nmol peptide per ml of diluted antiserum; − (nmol/ml^{-1}), minimum amount of peptide required to abolish the immunostaining.

[b]Refers to enzyme units (Sigma; Type III) required per ml of diluted antiserum.

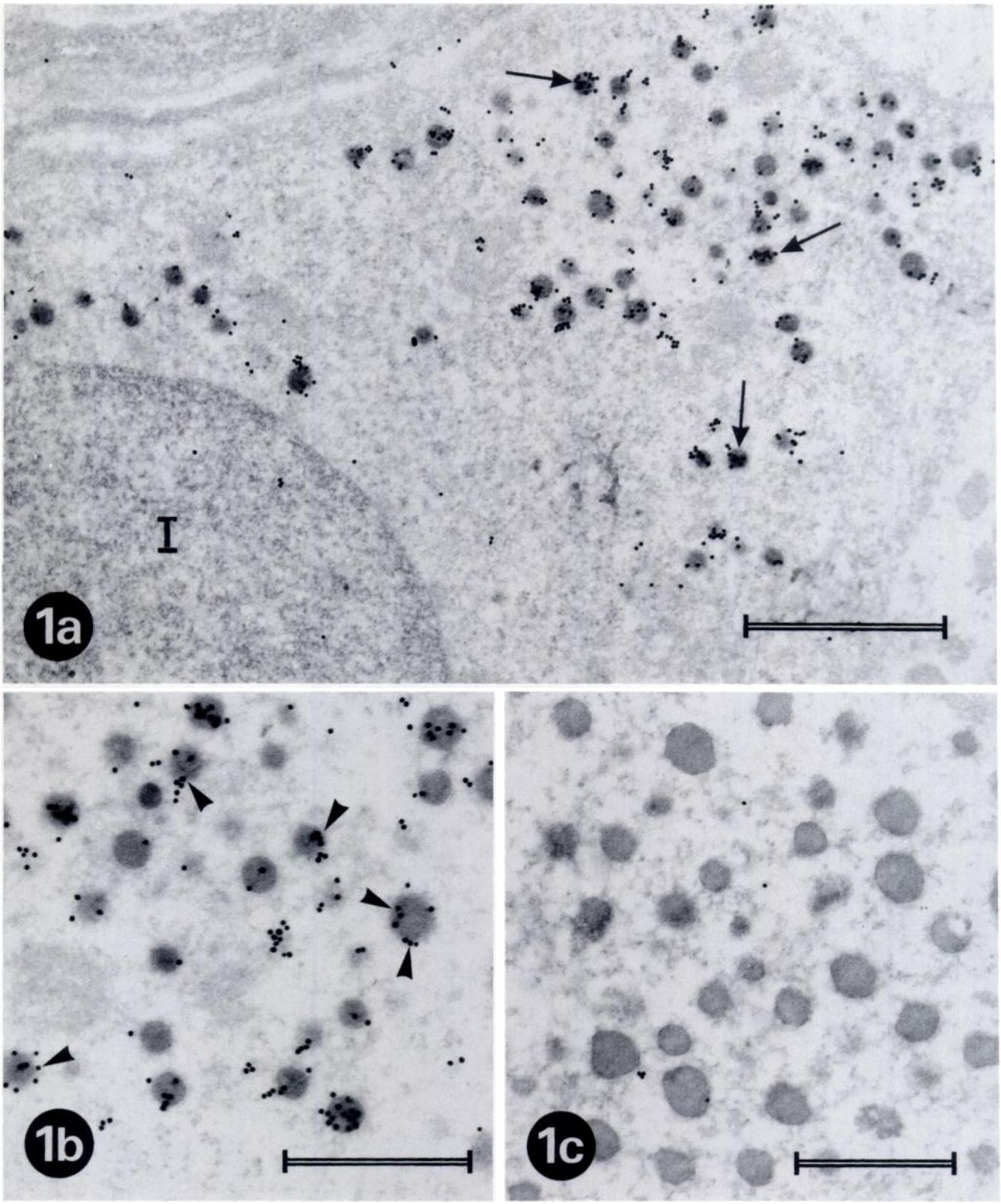


Figure 1. (a) Methionine enkephalin-like immunoreactivity localized to electron-dense granules (arrows) in a type I cell (I) of the cat carotid body. Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate and lead citrate. Original magnification ×34,300. Bar = 1.0 μm. (b) Higher magnification of the cat carotid body type I cell granules exhibiting methionine enkephalin-like immunoreactivity. Much of the immunoreactive material appears to be localized to the periphery of the granules (arrowheads). Original magnification ×65,000. Bar = 0.5 μm. (c) Cat adrenal medulla immunostained with methionine enkephalin antiserum preabsorbed with 1 nmol/ml pure synthetic peptide. Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate and lead citrate. Original magnification ×55,000. Bar = 0.5 μm.

Light microscope immunocytochemistry. Semithin (0.2–0.5 μm) sections of Araldite-embedded tissue were mounted on poly-*L*-lysine-coated glass slides and dried overnight at room temperature. The sections were de-resinated in a saturated solution of sodium hydroxide in absolute methanol and washed in a copious volume of PBS. An immunogold staining technique was applied to the sections, based on the on-grid method described above. The slides were kept in humidity chambers to prevent drying of the incubation media. The gold-labeled antiserum was applied for 1 hr at room temperature and the final preparation was viewed by dark-field microscopy. No reaction product deposits are visible using conventional bright-field microscopy due to the low density of gold labeling on the semithin sections.

Results

Carotid Body

Two major types of glomus cells, type I and type II, are readily recognized in the feline and porcine carotid bodies. Several varieties of type I cells are also distinguished based on size, opacity, and number of granules (Chen et al., 1976). Methionine enkephalin-like immunoreactivity was localized predominantly to the periphery of the granules (mean diameter ± 95% confidence limits = 114.5 ± 7.5 nm) distributed throughout the cytoplasm of each type I cell variety using the gold-labeled antibody method (Figure 1a and b). Specific staining was abolished after preabsorption of the first layer antiserum with pure peptide and no gold labeling was observed after incubation in preimmune rabbit serum (Figure 1c).

Application of the immunogold staining procedure to serial sections of the type I cells revealed dopamine-β-hydroxylase-like immunoreactivity associated with many (mean ± 1.96 SEM = 77.2 ± 5.1%; n = 314) of the secretory granules (Figure 2). Likewise, this activity, which was also localized to the periphery of the granules, was abolished after application of the controls.

Type II cells, which are characterized by irregularly shaped nuclei and the absence of dense-cored cytoplasmic granules, were not immunoreactive to enkephalin or dopamine-β-hydroxylase antisera.

Adrenal Medulla

In the adrenal glands of the mammalian species studied, the noradrenergic (N-cell) and adrenergic (A-cell) chromaffin cell types are readily differentiated on morphological grounds. The secretory granules in the N-cells are less regularly shaped, relatively larger, and more electron dense than the spherical granules characteristic of the A-cells (Figure 3a). The majority (mean ± 1.96 SEM = 94.7 ± 2.3%; n = 500) of the granules of each cell type exhibit methionine enkephalin-like immunoreactivity (Figure 3b) and also dopamine-β-hydroxylase-like immunoreactivity (mean ± 1.96 SEM = 79.4 ± 4.4%; n = 175) (Figure 4). Both immunoreactivities have been localized to single granules using the gold-labeled antibody technique applied to serial thin sections (Figure 5a and b).

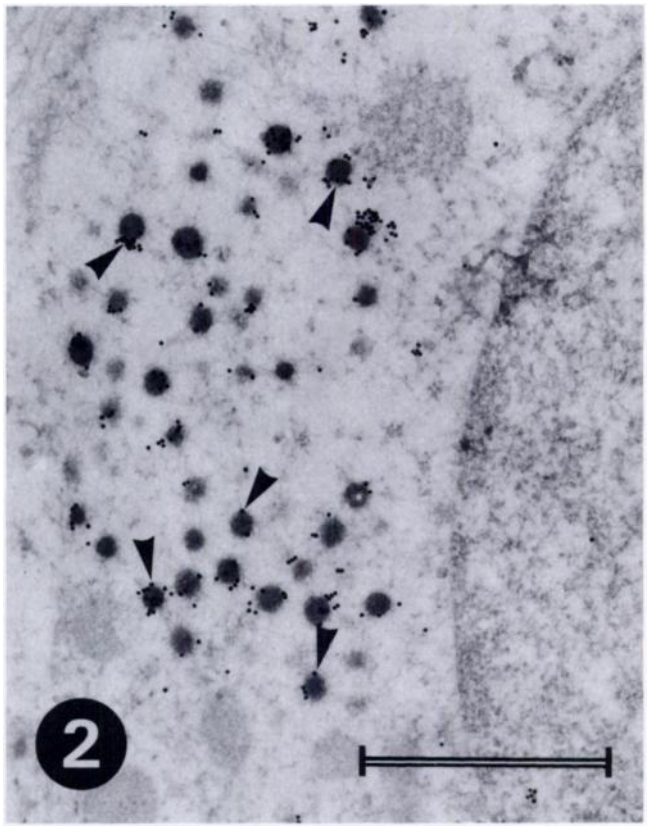


Figure 2. Dopamine-β-hydroxylase-like immunoreactivity localized to the periphery of the electron-dense granules (arrowheads) in a type I cell of the cat carotid body. Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate and lead citrate. Original magnification ×33,100. Bar = 1.0 μm.

Pheochromocytomas

The granules of both chromaffin cell types, which compose a markedly heterogeneous population, exhibited intense methionine enkephalin-like immunoreactivity (Figure 6a and b). In addition, dense deposits of silver particles were observed, associated with the granules of the N-cell type (Figure 7), following application of the Masson-Fontana procedure. It is important to note, however, that although the Masson-Fontana argentaffin reaction is widely used to demonstrate the presence of noradrenalin, it is not specific for this amine as other tissue components, notably dopamine, also reduce silver solutions and are, therefore, visualized using this procedure (Pearse, 1972).

Discussion

Immunocytochemistry at the light microscope level has revealed the coexistence of enkephalin-like immunoreactive material in catecholamine-containing cells of the mammalian carotid body (Lundberg et al., 1979; Wharton et al., 1980), adrenal medulla (Schultzberg et al., 1978a,b), and pheochro-

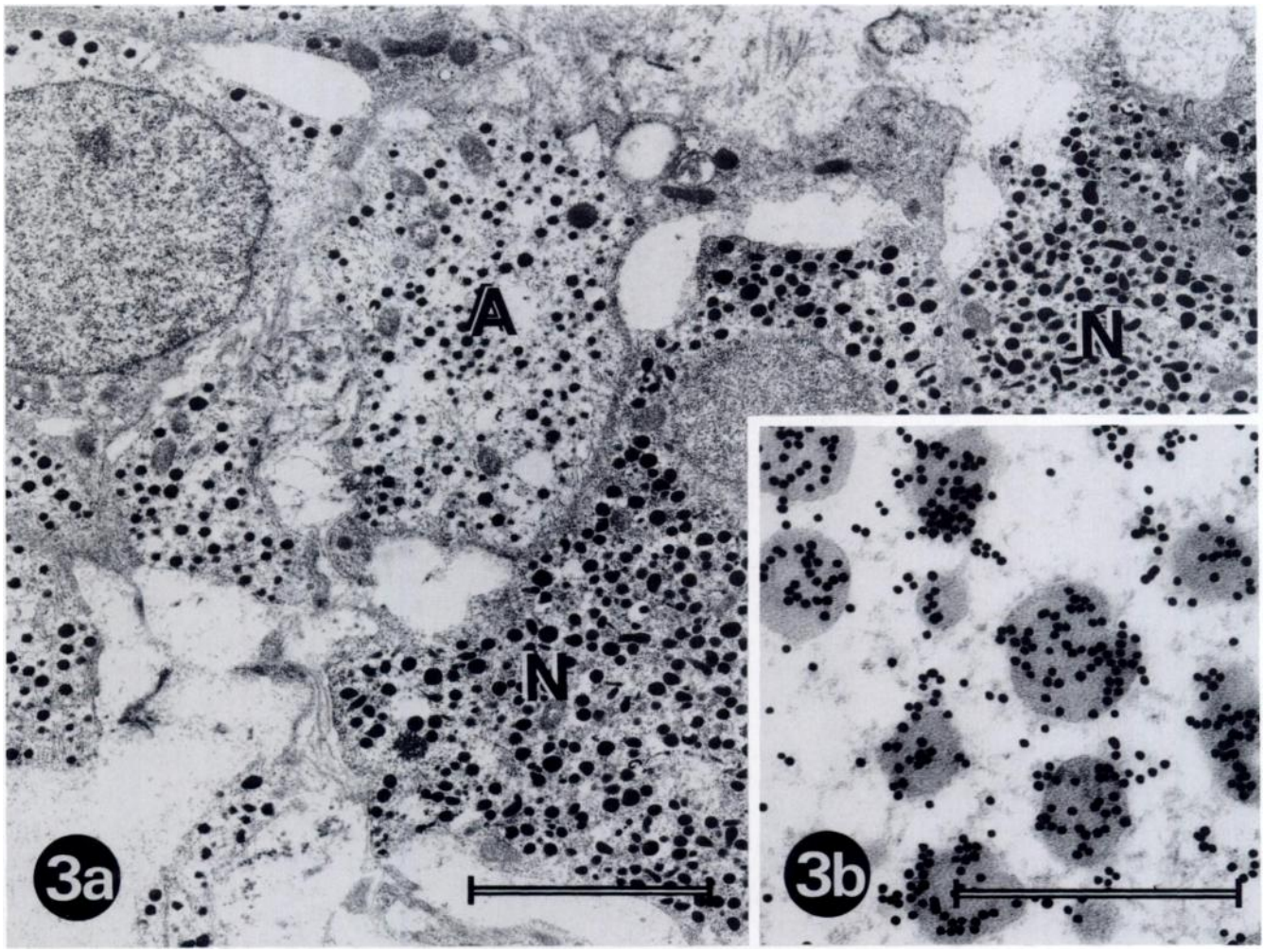


Figure 3. (a) Electron-dense granules of both (A, adrenergic; N, noradrenergic) chromaffin cell types of the horse adrenal medulla are immunostained for methionine enkephalin-like material. The highly pleomorphic nature of the noradrenalin granules is evident. Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate and lead citrate. Original magnification ×8,200. Bar = 4 μm. (b) High magnification of the cat adrenomedullary chromaffin cell granules exhibiting methionine enkephalin-like immunoreactivity. Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate. Original magnification ×68,750. Bar = 0.75 μm.

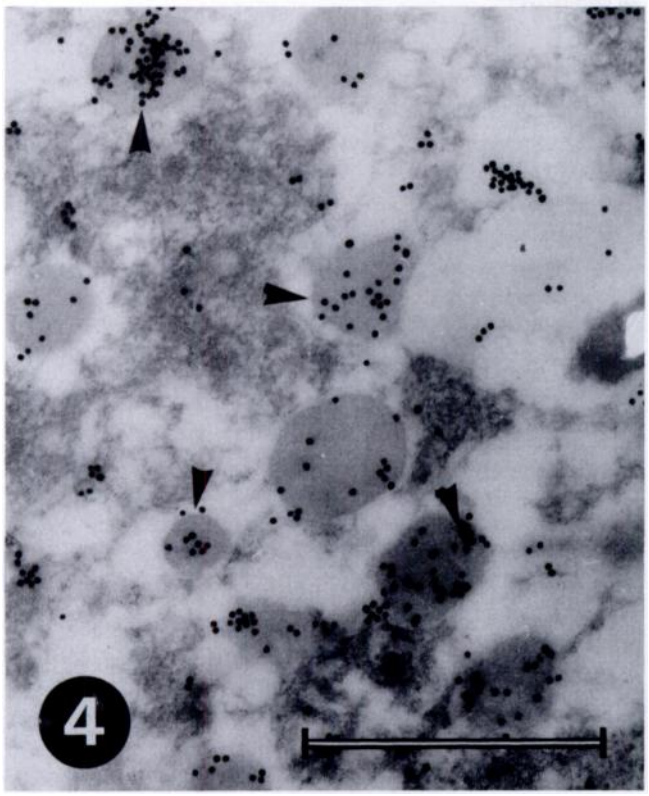


Figure 4. Dopamine-β-hydroxylase-like immunoreactivity (arrowheads) localized to electron-dense chromaffin cell granules of the piglet adrenal medulla. Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate and lead citrate. Original magnification ×42,000. Bar = 0.8 μm.

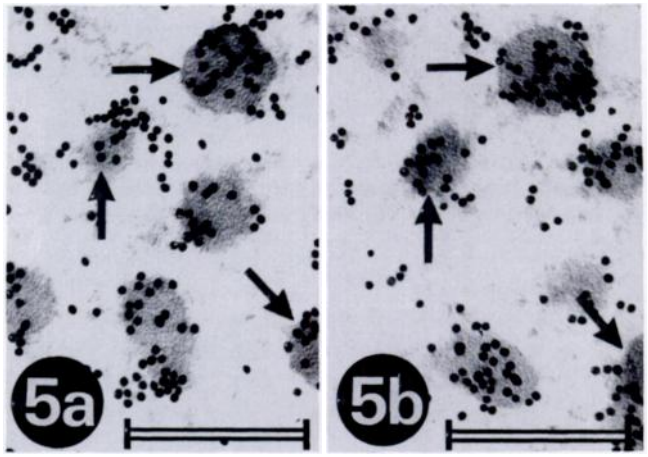
mocytoma (Sullivan et al., 1978; Lundberg et al., 1980). Moreover using quantitative biochemical assay techniques, Viveros et al. (1979) demonstrated the highest specific enkephalin activity in the purified granule fraction of bovine adrenal medullary chromaffin cells. Using the immunogold staining procedure at the electron microscope level we have demonstrated methionine enkephalin-like immunoreactive material and dopamine-β-hydroxylase-like immunoreactivity in the same secretory granules of the type I cells of the cat and piglet carotid bodies, the adrenomedullary gland cells of all the species studied, and in human pheochromocytomas. In the carotid body a considerable proportion of the gold particles were localized to sites on the periphery of the granules. Whether this reflects the actual antigenic site for the enzyme and peptide or whether this, apparently similar, topographic distribution is induced during processing and immunostaining cannot be established from this study. Previous workers have demonstrated a differential localization of antigens in single granules, for example, glucagon in the core and glicentin in the halo of pancreatic α-granules (Ravazzola and Orci, 1980), and tyrosine hydroxylase on the membrane and dopamine-β-hydroxylase in the granules of rat adrenomedullary cells (Nagatsu et al., 1979). The latter finding is of interest in the present study as both methionine enkephalin-like and dopamine-β-hydroxylase-like immunoreactivities have been visualized over the ground substance of the chromaffin granules and were not found to be restricted exclusively to the limiting membrane.

Notably, in the cat carotid body, some secretory granules were found to be unlabeled with gold particles following immunostaining for both methionine enkephalin and dopamine-β-hydroxylase. One interpretation is that the antigenic sites are not exposed during the immunocytochemical procedure or, more likely, that the antigenic structure of the majority of the enzyme or peptide molecules is modified during fixation or tissue processing to give compounds which are not recognized by the specific antibodies applied. Alternatively, or in addition, the unlabeled secretory granules may represent a subpopulation containing a precursor, product, or variant of the molecule against which the specific antibodies applied are directed.

It is clearly evident that methionine enkephalin-like immunoreactivity is localized to a heterogeneous granule population. Furthermore, it appears that noradrenergic granules which also exhibit dopamine-β-hydroxylase-like immunoreactivity but are distinguished from other granule types by the argentaffinity of the stored amine, also compose a markedly heterogeneous granule population. This precludes the possibility of designating different populations of electron-dense granules as enkephalin-containing purely on the basis of size.

Recently, Pelletier et al. (1981) demonstrated the colocalization of substance P and serotonin in the same secretory granules of raphe nuclei and dorsal horn of the spinal cord. Our findings provide ultrastructural evidence to support the concept of colocalization of peptides and amines in the dense-cored granules of catecholamine-containing cells.

Figure 5. (a) Methionine enkephalin-like immunoreactivity (arrows) localized to dense-cored chromaffin granules of the piglet adrenal medulla. Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate. Original magnification ×78,750. Bar = 0.3 μm. (b) Serial thin section to Figure 5a, immunostained to demonstrate dopamine-β-hydroxylase-like immunoreactivity (arrows). Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate. Original magnification ×78,750. Bar = 0.3 μm.



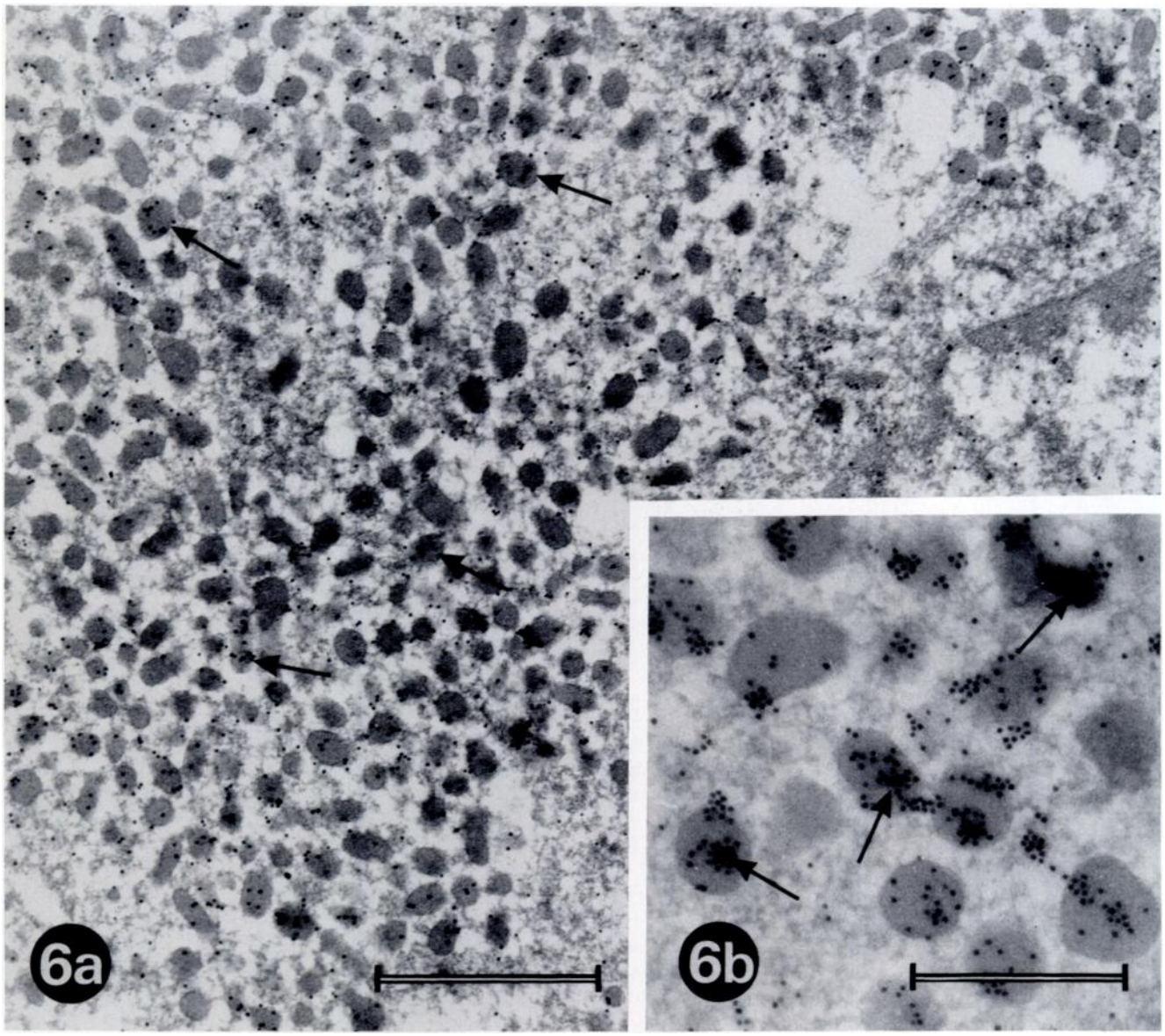


Figure 6. (a) Human pheochromocytoma immunostained to demonstrate the granular localization of methionine enkephalin-like immunoreactivity (arrows). Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate and lead citrate. Original magnification ×32,500. Bar = 1.0 μm. (b) Methionine enkephalin-like immunoreactive material localized to chromaffin cell granules in a human pheochromocytoma by the dense deposits of 20 nm gold particles (arrowed) after the application of the immunogold staining procedure. Counterstained with uranyl acetate and lead citrate. Original magnification ×63,000. Bar = 0.5 μm.

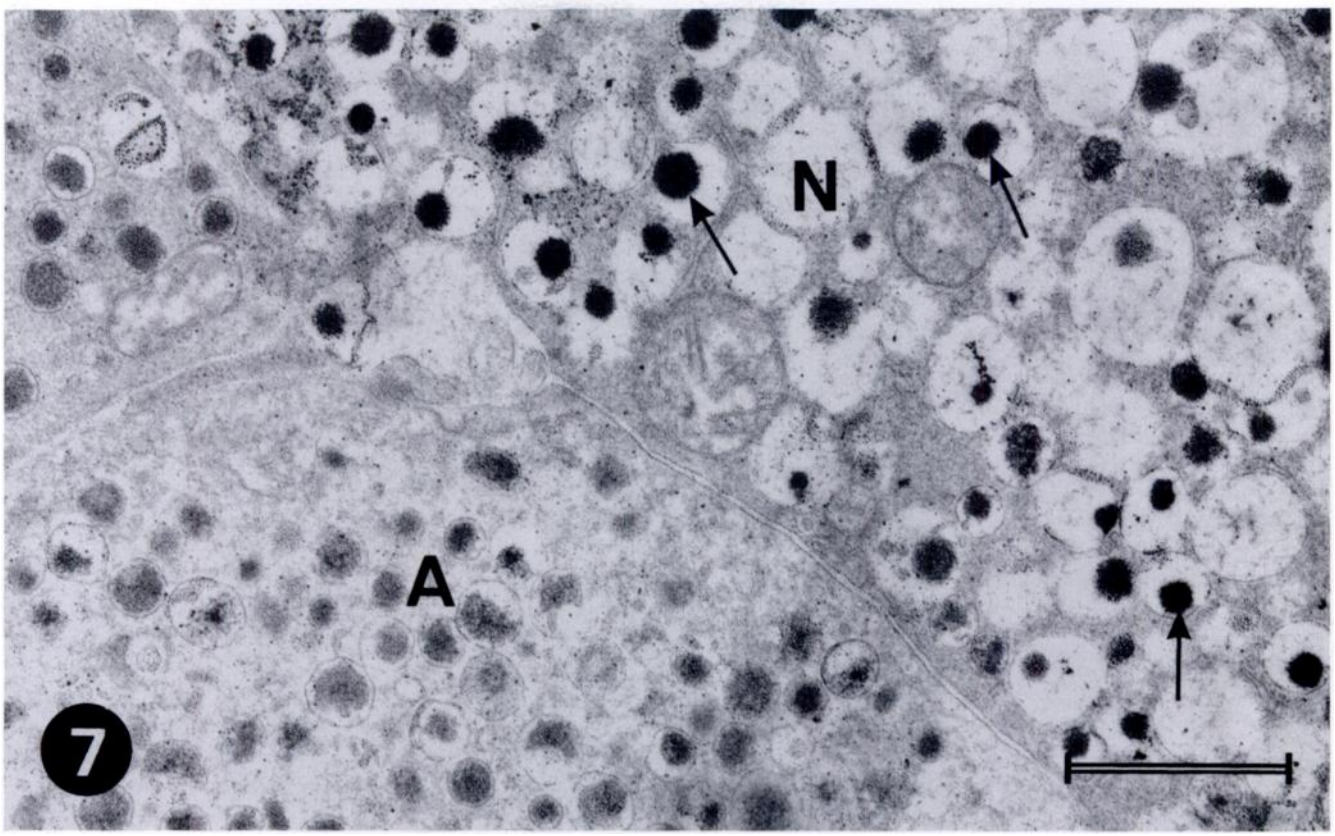


Figure 7. Reduced silver deposits (arrows) over the secretory granules of a noradrenergic (N) cell from a human pheochromocytoma after the application of the Masson-Fontana technique. The adrenergic (A) granules are only lightly stained. Original magnification × 28,500. Bar = 1.0 μm.

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