Peptides, Vol. 3, pp. 259–272, 1982. Printed in the U.S.A.

Immunogold Staining Procedure for the Localisation of Regulatory Peptides

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VARNDELL, I. M., F. J. TAPIA, L. PROBERT, A. M. J. BUCHAN, J. GU, J. DE MEY, S. R. BLOOM AND J. M. POLAK. *Immunogold staining procedure for the localisation of regulatory peptides.* PEPTIDES 3(3) 259–272, 1982.—The use of protein A- and IgG-conjugated colloidal gold staining methods for the immuno-localisation of peptide hormones and neurotransmitters at light- and electron microscope level are described and discussed. Bright-field and dark-ground illumination modes have been used to visualise the gold-labelled antigenic sites at the light microscope level. Immunogold staining procedures at the ultrastructural level using region-specific antisera have been adopted to localise specific molecular forms of peptides including gastrin (G17 and G34), glucagon and pro-glucagon, insulin and pro-insulin, in normal tissue and in tumours of the gastroenteropancreatic system. Similar methods have been used to demonstrate the heterogeneity of p-type nerves in the enteric nervous system. Vasoactive intestinal polypeptide (VIP) has been localised to granular sites (mean±S.D. granule diameter=98±19 nm) in nerve terminals of the enteric plexuses and in tumour cells of diarrhoeogenic VIP-producing neoplasias (mean±S.D. granule diameter=126±37 nm) using immunogold procedures applied to ultraviolet-cured ultrathin sections. Co-localisation of amines and peptides in carotid body type I cells and in chromaffin cells of normal adrenal medulla and phaeochromocytomas has also been demonstrated. Advantages of the immunogold procedures over alternative immunocytochemical techniques are discussed.

Immunogold staining methods Regulatory peptides Light microscopy Electron microscopy
Protein A-gold Molecular forms P-type heterogeneity Co-localisation

THE number of peptides isolated and characterised from animal tissues, and for which regulatory functions have been proposed, has increased dramatically in recent years. It is well recognised that most of these regulatory peptides are stored in distinct types of endocrine and neural cells which are distinguished from other cell types and from each other by the presence of electron-dense secretory granules varying in their shape, size and the form of their limiting membrane [39]. Most peptides have also been found to exist, both in blood and tissue, in multiple molecular forms, the ratios of which vary according to the dynamic state of the cell.

Evidence is accumulating to implicate abnormalities of peptide synthesis and release in disease states in man and other mammals [2]. Therefore, knowledge of the precise localisation of peptides in various tissues in health and disease is important and immunocytochemistry has played a key role in these investigations for many years.

The classical immunofluorescence [6] and peroxidase [26,40] techniques have been modified and further developed into a variety of procedures so that immunocytochemistry is now a very precise and sensitive tool [43]. One revolutionary step was the application of immunoperoxidase [26] and peroxidase-antiperoxidase [40] techniques at the electron microscope level, which revealed, conclusively, a granular localisation for regulatory peptides. Unfortunately, certain drawbacks inherent to the peroxidase techniques are now becoming apparent. Of particular concern in this respect is the homogeneous deposition of the electron-dense final reaction product over the antigenic structures. It is increasingly evident from ultrastructural studies that the enormous heterogeneity of secretory granule structure observed among neuronal and endocrine cell types may have some functional importance with respect to the peptides they contain. Therefore, it is important to be able to investigate the ultrastructure of immunoreactive organelles. The use of the semi-thin serial sectioning technique partly overcame the problem of antigenic site obfuscation, however, with increasing evidence for the co-existence of peptides and other antigens in single organelles, a more precise electronimmunocytochemical technique for the ultrastructural localisation of tissue-bound antigens was sought.

In 1971 Faulk and Taylor [11] conjugated colloidal gold to

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immunoglobulins and suggested the value of this technique in immunological procedures at the electron microscope level. Successive modifications [9, 13, 18, 34] have produced a highly efficient immunogold technique for the localisation of antigens at both light- and electron microscope level. In parallel, colloidal gold conjugated to a staphylococcal protein, protein A, which has a marked reactivity with the Fc component of IgG molecules, was developed as a highly specific immunocytochemical technique [31, 33, 35].

Using these techniques, we have been able to localise a wide variety of regulatory peptides in nerves and endocrine cells in health and disease at both light- and electron microscope levels.

METHOD

Experimental animals used in this study included cats, piglets, horses, guinea pigs and albino rats (Sprague-Dawley).

Adult cats, weighing between 1.8 and 2.0 kg, were anaesthetised by an intraperitoneal overdose of sodium pentobarbitone ("Euthatal", May and Baker Ltd., 200 mg/ml; 1 ml per kg) and were perfused, via the ascending aorta, according to the procedure detailed below. In addition, guinea pigs and rats were anaesthetised by "Penthrane" (Abbott Labs.) inhalation and perfusion-fixed as follows.

Tissue Preparation

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}).

Small blocks of tissue (carotid body, adrenal medulla, pancreas, regions of gut from stomach to colon) were excised and fixed by immersion in the latter solution for a total time of two hours. Half of the blocks were rinsed in buffer and osmicated (1% osmium tetroxide in Millonig's buffer, pH 7.2 for one hour at 4°C), whereas the other half were merely rinsed in buffer.

Some of the non-osmicated blocks were dehydrated and embedded in a hydrophilic resin (Lemix; EMscope Labs., U.K.). The remaining blocks were dehydrated in a graded series of ethanols and infiltrated with Araldite epoxy resin. Some of the Araldite blocks were cured by ultraviolet irradiation (10 days to 3 weeks; room temperature).

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, eight guinea pigs, six rats, and six neonatal piglets, aged one day to three weeks, were killed by pentobarbitone overdose and dissected fresh. In addition, pieces of tissue were excised from the adrenal glands and gut of five horses (two normal, three with grass sickness). Small blocks from each organ under investigation were fixed either in the above fixative or in 3% buffered glutaraldehyde and processed for electron microscopy. Larger blocks of fresh tissue were snap-frozen in melting Arcton-12, freeze-dried, and fixed in *p*-benzoquinone vapour. The final blocks were then embedded in Ralwax, sectioned at 3–7 μm, and mounted on poly-L-lysine-coated glass slides.

Finally, normal and pathological tissue was removed from human subjects at surgery or autopsy and fixed for light- or 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.

Preparation of Gold Reagents

Colloidal suspensions of gold particles were prepared by reduction of tetrachloroauric acid with white phosphorus (particle diameter 5–12 nm), according to the method of Faulk and Taylor [11], with modifications [17, 34, 38], or with *tri*-sodium citrate [12,38] (particle diameter 16–20 or 30–40 nm). Goat anti-rabbit immunoglobulins were linked to colloidal gold [9], according to the principles of Geoghegan and Ackerman [13]. The protein A-gold complex was prepared as described by Roth and co-workers [35] and Slot and Geuze [38] and uniformly sized subfractions of gold reagents were prepared as described by the latter authors.

Antisera

The antisera used were raised in rabbits against synthetic peptides and, in the case of dopamine-β-hydroxylase, against purified bovine enzyme [36]. Characteristics of the antisera used are presented in Table 1. Antiserum specificity was verified by the lack of staining in adjacent sections which had been incubated in antisera preabsorbed with the specific antigen (nmol/ml of the diluted antiserum).

Although we have endeavoured to ensure the specificity of all the antisera employed, cross-reaction with a closely related, but so far unidentified antigen cannot be discounted.

Electronimmunocytochemistry

The grid-mounted sections of unosmicated tissue were dried overnight and then etched in 10% hydrogen peroxide for ten minutes. This step was later found not to be always necessary. Thorough washing of the grids in 0.05 M Tris-buffered saline (TBS), pH 7.3, was followed by incubation in normal goat serum (NGS) for 30–60 min at room temperature. Normal goat serum was not applied to grids subjected to the protein A-gold technique. The NGS was drained from the grids, each of which were then incubated in 10 μl of primary antiserum for 20–24 hr at 4°C. The antisera were diluted with TBS containing 1% bovine serum albumin (TBS/BSA) pH 7.2.

Control sections were incubated in normal (pre-immune) rabbit serum at the same dilution as the primary antiserum or in first layer antisera preabsorbed with pure antigen.

After thorough washing in TBS/BSA (pH 8.2) buffer the grids were transferred to the gold-labelled solutions for one hour at room temperature. The final dilutions of the gold-labelled solutions in comparable conditions are given in Table 2. This incubation was followed by thorough washing in large volumes of TBS/BSA buffer. Finally, the grids were rinsed in Millipore-filtered distilled water, dried, counterstained as for electron microscopy with uranyl acetate and lead citrate and viewed with a Zeiss 10CR electron microscope operating at 60 kV.

Light Microscope Immunocytochemistry

Araldite-embedded tissue: Semi-thin (0.5–2.0 μm) sections of Araldite-embedded tissue were mounted on poly-L-

TABLE 1

CHARACTERISTICS OF THE ANTISERA USED

Antiserum raised against	Region specificity	Dilution	Concentration of antigen used for absorption (nmol/ml diluted antiserum)	Ref.*
Gastrin	C-term.	1:2000	0.1	[15]
Glucagon	N-term.	1:2000	2	
Glicentin	N-term.‡	1:1000	2	[3]
Insulin	—	1:800	2	[3]
C-peptide	—§	1:400	5	
Met-enkephalin	C-term.	1:4000	1	[43]
Substance P	Whole molecule	1:8000	3	[29]
VIP	C-term.	1:6000	5	[29]
Somatostatin	—	1:6000	1	[15]
Secretin	—	1:6000	1	[15]
Serotonin	Amino side chain	1:2000	6.10³	[15]
Dopamine-β-hydroxylase	Bovine enzyme	1:2000	0.5†	[36,43]

*References cited in text.

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

‡From Dr. A. J. Moody, Novo Research Institute, Denmark.

§From Prof. N. Yanaihara, Shizuoka School of Pharmacy, Japan; this antiserum (R2303) is known to cross-react extensively with pro-insulin.

lysine-coated glass slides and dried overnight at room temperature. The sections were de-resinated in sodium ethoxide and washed in a large volume of PBS. Wax-embedded tissue: Sections, 3–7 μm in thickness, mounted on glass slides, were de-waxed and hydrated to PBS.

An immunogold staining technique was applied to the slide-mounted sections, based on the on-grid method described above. Gold particles (40 nm) conjugated to goat anti-rabbit sera were used for the light microscope visualisation of tissue-bound antigens. The gold-labelled antiserum was applied for one hour at room temperature and the final preparations were viewed by dark-ground (semi-thin) and bright-field (thick) microscopy with a Reichert-Jung Polyvar (British American Optical Co.).

The peroxidase-antiperoxidase (PAP) procedure (after Sternberger, [40]) was applied to sections of wax- and resin-embedded tissue [32]. The reaction product was developed either in a filtered solution of 0.05% 3,3′-diaminobenzidine (Aldrich) in phosphate-buffered saline pH 7.2, containing 0.06% hydrogen peroxide, or in a solution of 0.03% 4-chloro-1-naphthol, pH 7.1, containing 0.03% hydrogen peroxide, as previously modified by us from the method of Nakane [15,26].

RESULTS

We have used the protein A- and the immunoglobulin-conjugated colloidal gold methods for the immunolocalisation of regulatory peptides at both the light- and electron microscope level and some of our results are summarised below.

Light Microscope Level

The immunofluorescence [6] and a variety of peroxidase techniques, including the peroxidase-antiperoxidase (PAP) method [40] have been used routinely in a large number of laboratories for the immunohistochemical localisation of regulatory peptides. Recently, however, we have applied immunogold staining procedures for the immunohistochemical localisation of peptide hormones and neurotransmitters at the light microscope level [8, 9, 15] and some of our results are presented below.

TABLE 2

DILUTION OF COLLOIDAL GOLD-LABELLED SOLUTIONS CORRELATED WITH GOLD PARTICLE SIZE*

Particle size (nm)	Gold conjugated to Protein A	Immunoglobulin
5	1:50–1:80	1:20
12	1:20	1:8
20	1:8–1:20	1:4
40	—	1:1

*Dilution factors based on high granule:background ratio staining of cat pancreatic α-granules by glucagon antiserum 499 (see Table 1).

Immunoreactive sites in thick (5–7 μm) sections, from wax-embedded tissue, appear red when viewed by bright-field microscopy following immunogold staining. The sequential use of the PAP and the immunogold staining methods [15] has facilitated the development of double- and multiple staining techniques for the simultaneous localisation of tissue-bound antigens. Gold-labelled immunoreactive material in semi-thin sections, however, is not usually visible by bright-field microscopy. Observation of such preparations by dark-ground illumination reveals the gold-labelled structures. Figure 1 exhibits a semi-thin section of a cat pan-

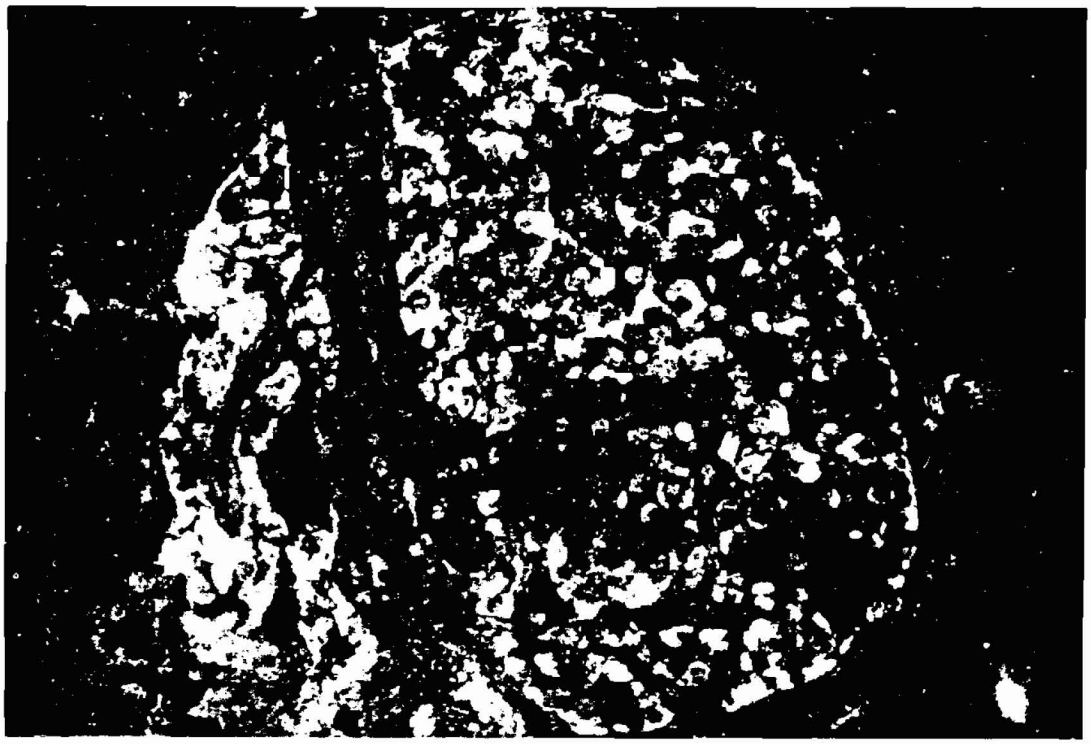

FIG. 1. Semi-thin (2 μm) section of a cat pancreatic islet immunostained for glucagon with the immunogold technique and visualised with dark-ground illumination. ×300.

creatic islet immunostained for glucagon with an immunogold staining procedure and photographed with dark-ground illumination.

Electron Microscope Level

The immunogold staining procedures have considerable advantages over alternative techniques when applied at the electron microscope level for the immunocytochemical localisation of regulatory peptides, as discussed later. In this section we describe some results of our investigations on the ultrastructural localisation of regulatory peptides in endocrine cells and nerve fibres in health and disease.

Immunocytochemical demonstration of peptide molecular forms. It is now well established that most of the regulatory peptides occur in a variety of molecular forms [10]. This suggests the existence of precursor molecules or prohormones which can be distinguished from their bioactive catabolites by the application of antibodies raised to specific regions of the peptide (for reviews see [10,22]). The use of region-specific antisera has permitted the immunocytochemical identification of the sites of production and storage of different molecular forms of a single peptide. Examples of this technology in combination with the immunogold staining procedures include:

(1) The localisation of gastrin to two types of endocrine cells containing different secretory granule populations has been achieved by the use of region-specific antisera directed to the N-terminals of gastrin 17 and gastrin 34. G cells in the antrum, which is known to store mainly the smaller molecular form of gastrin, G17, are characterised by their large (mean±SD, 340±54 nm) electron-lucent secretory granules. In contrast, G cells in the intestine, which is known, at least in man, to store predominantly the larger molecular form of gastrin, G34, are characterised by small (mean±SD, 175±21 nm) spherical, electron-dense secretory granules [3]. Thus, it would appear that the predominance of a particular molecular form determines the structure of the secretory granules [28]. This phenomenon is also evident in tumours, as cells from the rare gastrinomas known to produce predominantly gastrin 17 contain a preponderance of the antral gastrin type of secretory granules. Conversely, secretory granules in tumours producing gastrin 34 as the predominant molecular form are mostly small and electron-dense (Fig. 2a and b) resembling those of the intestinal gastrin cells.

(2) Using the protein A-immunogold staining method in combination with antibodies to glicentin (proglucagon) and glucagon, Ravazzola and Orci [30] first localised both molecular forms to different areas of the pancreatic A cell granule. We have recently provided further evidence to corroborate this finding using both the protein A- and IgG-immunogold staining methods [28]. Glicentin (proglucagon) is localised in the outer portion of the secretory granules (Fig. 3a) and glucagon in the core (Fig. 3b). The halo of the α-granules had previously been distinguishable from the inner portion by its distinct reactivity to the Grimelius silver impregnation technique [14]. This apparent topographic separation of molecular forms within one granule indicates the existence of post-translational enzymatic processes involved in the conversion of proglucagon into the smaller bioactive peptide, glucagon. However, no evidence has been obtained from immunocytochemistry to determine whether these enzymatic processes have taken place prior to granule formation in the Golgi apparatus or following release from that organelle. Molecular forms of glucagon are not only found in normal pancreatic cells but also in tumours which frequently

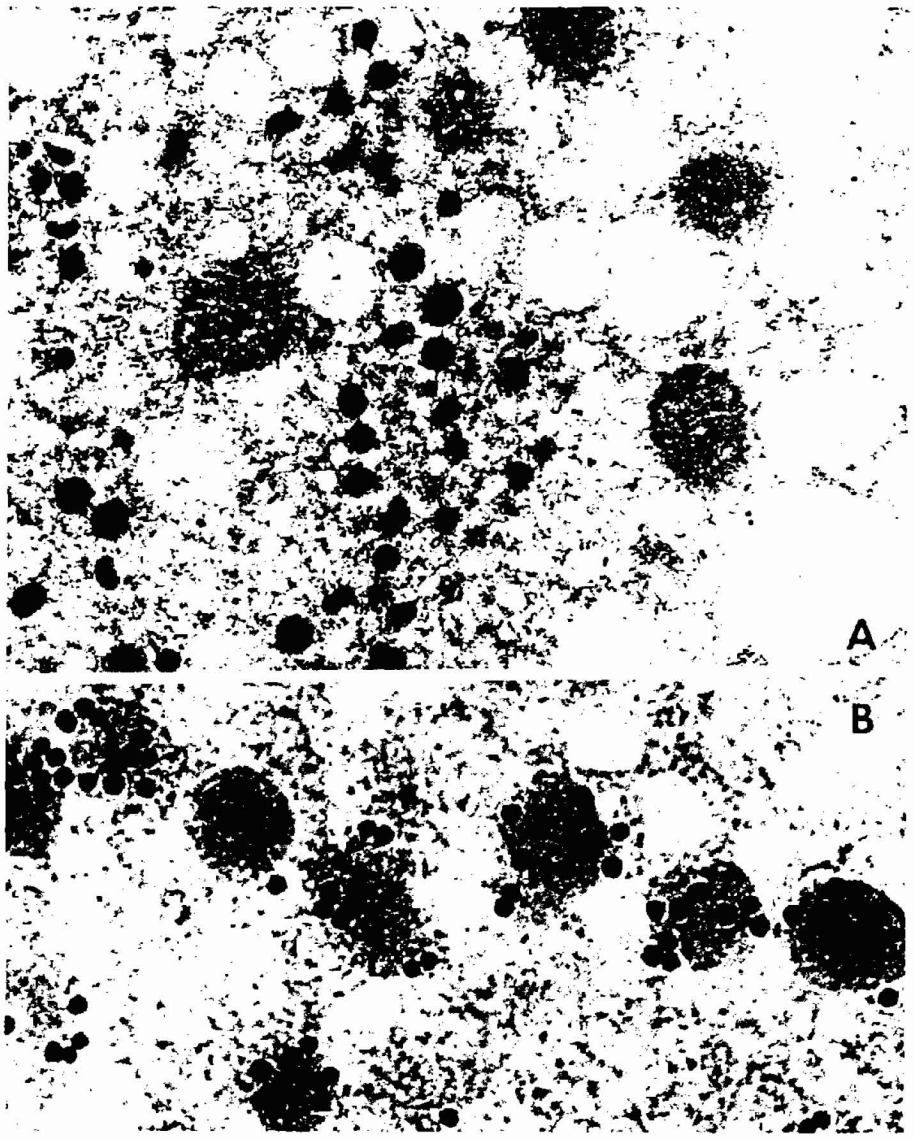

FIG. 2. Secretory granules in a tumour producing predominantly intestinal gastrin (G34). Immunostained using the immunogold technique. a, ×30,000; b, ×150,000.

show mixed populations of secretory granules. In addition to classical α granules which are immunoreactive to glucagon antisera (mean±SD, 250±25 nm) some glucagonoma cells contain granules resembling those of the intestinal L cells which contain enteroglucagon (mean±SD, 140±35 nm) (Figs. 4a and b). In these tumours approximately 60% of the cells are also immunoreactive to pancreatic polypeptide (Fig. 4c). These cells contain granules of similar morphology to those containing glicentin. Using the immunogold technique we can now distinguish between the different peptide-containing cells within these tumours.

(3) Molecular forms of insulin have also been localised to B cells of normal pancreas and also to different types of granule found within cells of insulin-producing tumours (insulinomas). The bioactive peptide, insulin, is enzymatically catabolised from its precursor molecule, proinsulin, with the liberation of a 29 amino acid molecule, C-peptide. It is now well established [21] that two distinct types of secretory granule are present in normal pancreatic B cells, one type possessing a crystalline core with a distinct limiting membrane and one type with a homogeneous core. The ratio between granule types observed within pancreatic B cell populations varies considerably between species. Both granule types are immunoreactive to insulin antisera (Fig. 5), but only the granule type with a homogeneous core is immunoreactive to pro-insulin (C-peptide) antisera (Fig. 6). It is interesting to note that only granules with a homogeneous core are observed in the Golgi apparatus, thus the immunogold staining methods have been instrumental in demonstrating a putative maturation process of granules within the cytoplasm following release from the Golgi apparatus. In our studies we have studied four insulinomas which displayed immunoreactivity to insulin, pro-insulin (C-peptide) and pancreatic polypeptide antisera. In addition to the typical β-granules (crystalline and dense, homogeneous core), some insulinomas are composed of atypical cells immunoreactive

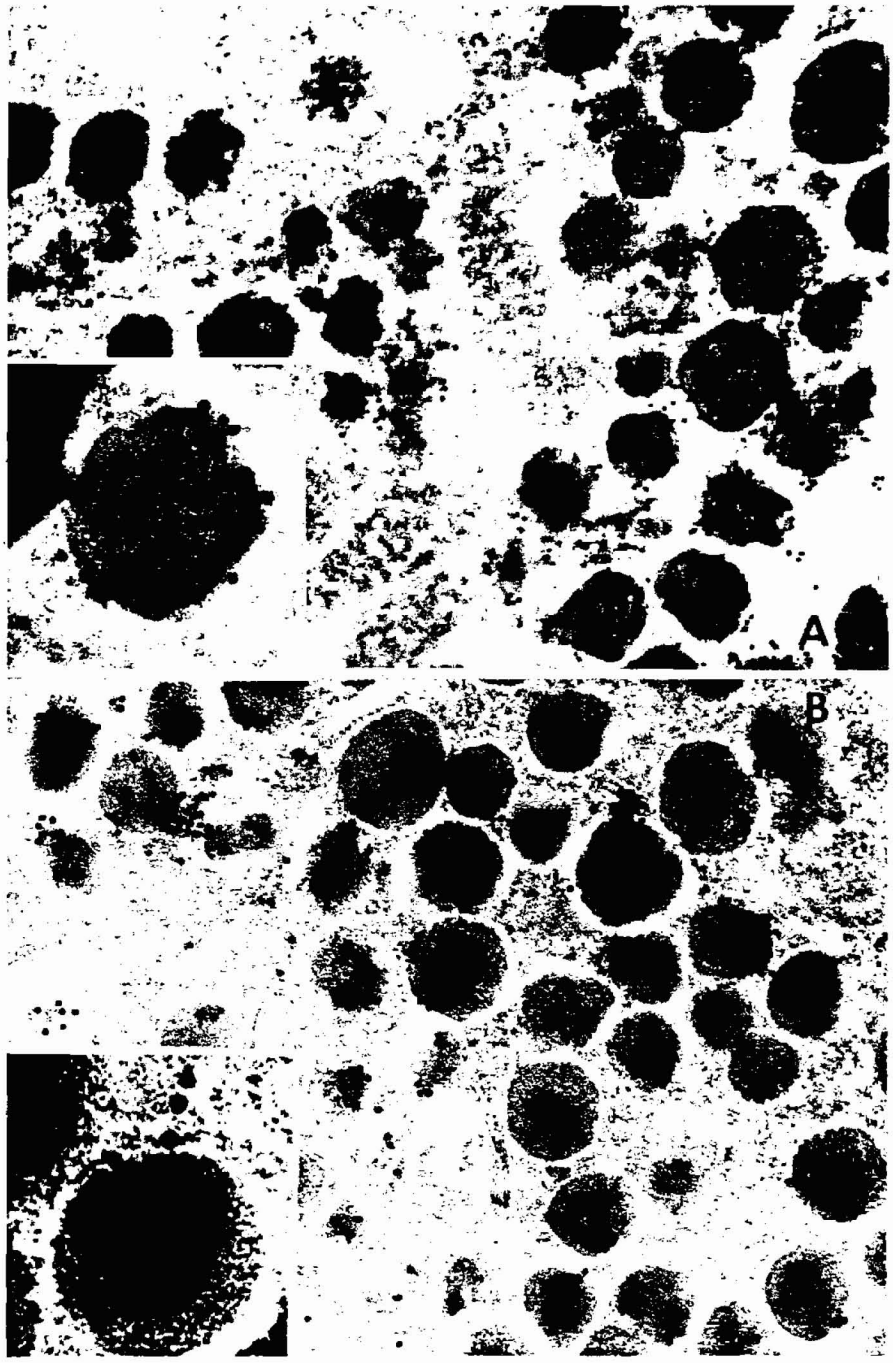

FIG. 3. A-cell granules from human pancreas immunostained by the immunogold technique for, a, glicentin (immunoreactivity is localised to the halo of the granules); b, pancreatic glucagon (immunoreactivity is localised to the core of the granules). ×60,000; inset ×136,000.

to insulin or are a combination of typical and atypical insulin cells [7]. These atypical cells have secretory granules (mean±SD, 148±33 nm) morphologically similar to those containing pancreatic polypeptide (PP) (mean±SD, 145±26 nm) which are also very common in many insulinomas. Using the immunogold techniques we can now distinguish between cells containing PP-immunoreactivity and those containing insulin-immunoreactivity.

Heterogeneity of peptidergic nerves. In 1965 Taxi [42] described enteric nerve profiles which were ultrastructurally distinct from the adrenergic and cholinergic types. The nerves were characterised by the presence of large, granular, secretory vesicles similar to those containing vasopressin and oxytocin in the posterior pituitary, which led Baumgarten and co-workers [1] to describe them as p-type (peptidergic) neurones. Subsequently, morphological heterogeneity

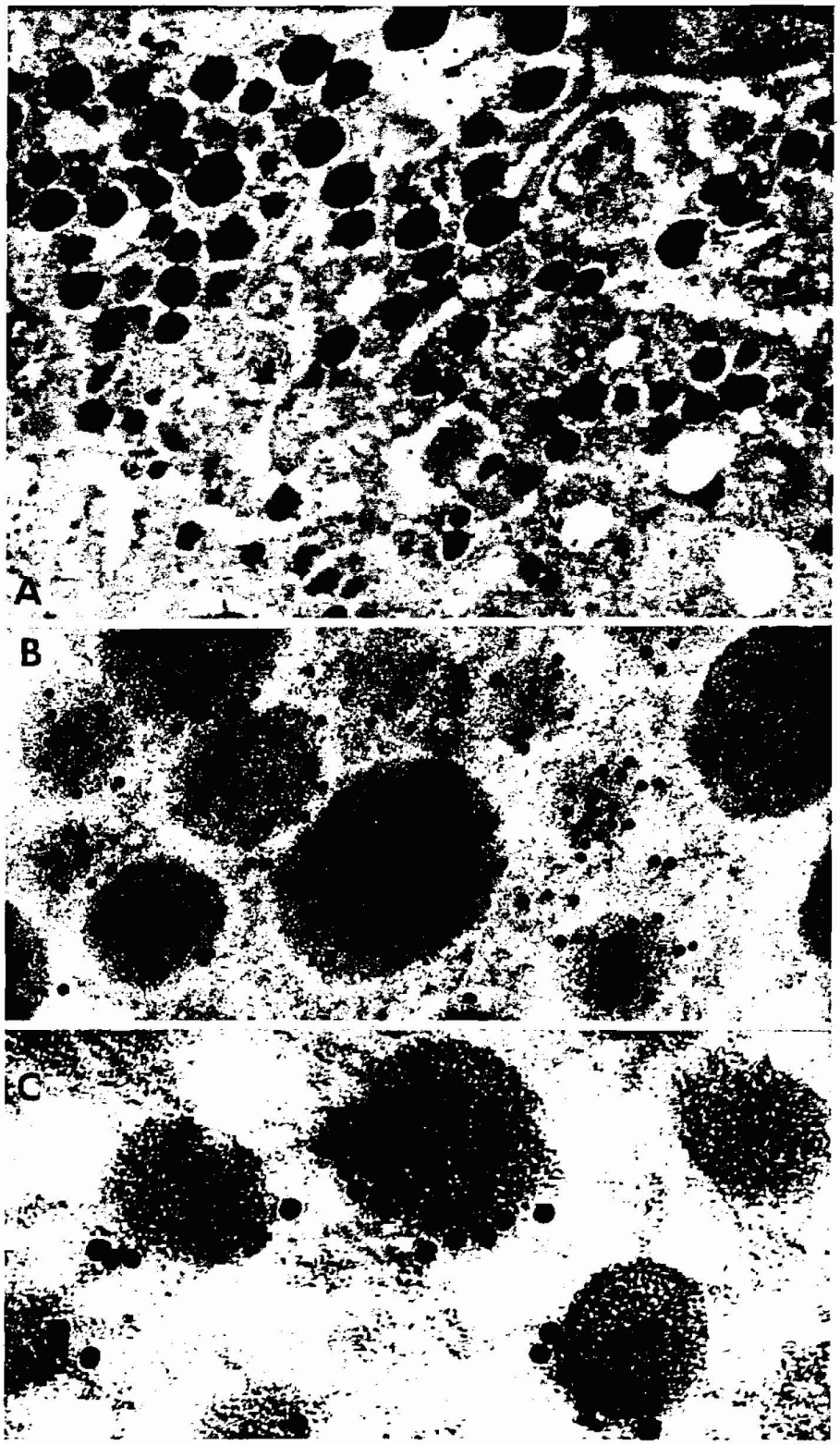

FIG. 4. A glucagonoma containing, a and b. glicentin-immunoreactive granules (a, ×34,500; b, ×138,000); c, PP-immunoreactive granules ×138,000.

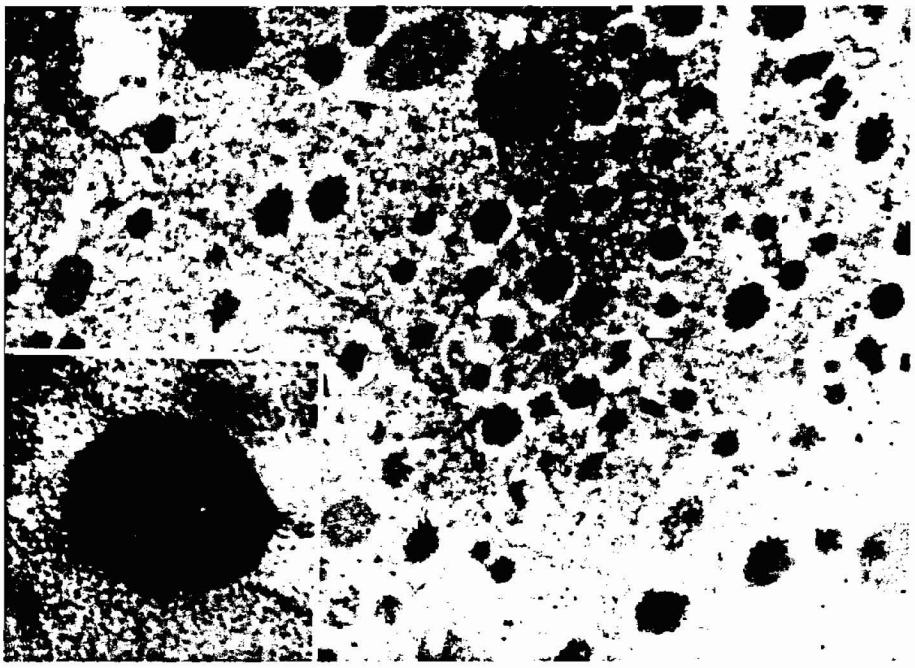

FIG. 5. An insulinoma cell containing β-granules immunoreactive for insulin. ×30,000; inset ×125,000.

within this extensive non-adrenergic, non-cholinergic group was described, based on the electron microscope appearance of these secretory vesicles [4,5]. Our observations using immunogold staining methods at the electron microscope level corroborate the claim of a marked heterogeneity among the p-type components of the autonomic nervous system. We have been able to localise substance P and vasoactive intestinal polypeptide (VIP) to distinct sub-populations of p-type neurones in the enteric nervous system [29]. Details of the electronimmunocytochemical localisation of VIP are discussed below. Morphologically, the separate neurone populations can be distinguished by the size (mean±SD, substance P: 85±15 nm, VIP: 98±19 nm) [29], and appearance of their granular secretory vesicles (Fig. 7a and b). Analysis of serial ultrathin sections immunostained for substance P and VIP reveals a significant proportion of distinct p-type nerves which remain unlabelled with the immunogold procedures. Our findings provide circumstantial evidence to corroborate immunohistochemical investigations which have shown that at least six neuropeptides, other than substance P and VIP, are found in nerve fibres in the enteric nervous system [19], including bombesin, cholecystokinin, enkephalin, neurotensin, somatostatin and thyrotropin releasing hormone (TRH).

Preservation of antigenicity by low temperature processing. By far the majority of regulatory peptides, against which antisera have been raised, can be stained with either of the immunogold procedures described here when applied to ultrathin, grid-mounted sections of aldehyde-fixed, non-osmicated tissue. However, some regulatory peptides, notably VIP, are heat-labile in the presence of resin components and consequently have proved difficult to visualise at both light- and electron microscope levels. One particular example is the localisation of VIP in VIP-producing tumours of the pancreas. It was assumed that the lack of immunocytochemical staining in such tumours was due to the dynamic state of the VIP-producing cells, releasing peptide without storage. However, considerable amounts of extractable VIP were measured by radioimmunoassay in some of these tumours. We have now been able to localise VIP to dense-cored granules (mean±SD, 126±37 nm; Tapia and co-workers, [41]) within tumour cells in VIPomas embedded in Araldite epoxy resin which was cured with ultraviolet irradiation at room temperature (Fig. 8).

Co-storage of peptides with other bioactive molecules. Several reports of the co-existence of peptides and classical neurotransmitters have been published in recent years [16, 24, 25, 37], based mainly on observations made at the light microscope level. One of the most interesting reports suggests the co-existence of VIP with acetylcholine [24]; this is corroborated by the electron microscopical identification of agranular cholinergic-type vesicles in VIP-immunoreactive nerves of the submandibular gland [20] and gut (Polak and Probert, 1981, unpublished observations). Double staining immunocytochemical techniques at the electron microscope level, necessitating the use of the pre-embedding PAP method, are required to investigate this proposed co-existence in more detail.

Recently, Pelletier, Steinbusch and Verhofstad [27] demonstrated the co-localisation of substance P and serotonin in the same secretory granules of raphe nuclei and in the dorsal horn of the spinal cord. Using the immunogold staining procedures we have obtained ultrastructural evidence to support the concept of co-localisation of peptides and amines by visualising enkephalin-like immunoreactive material in dense-cored granules of catecholamine-containing cells [44]. Dopamine-β-hydroxylase-like and methionine enkephalin-like immunoreactivities have been localised to small (mean±SD, 115±5 nm), electron-dense granules found in the type I cells of cat and piglet carotid bodies (Fig. 9) and also to the heterogeneous population of large, (mean±SD, 250±50

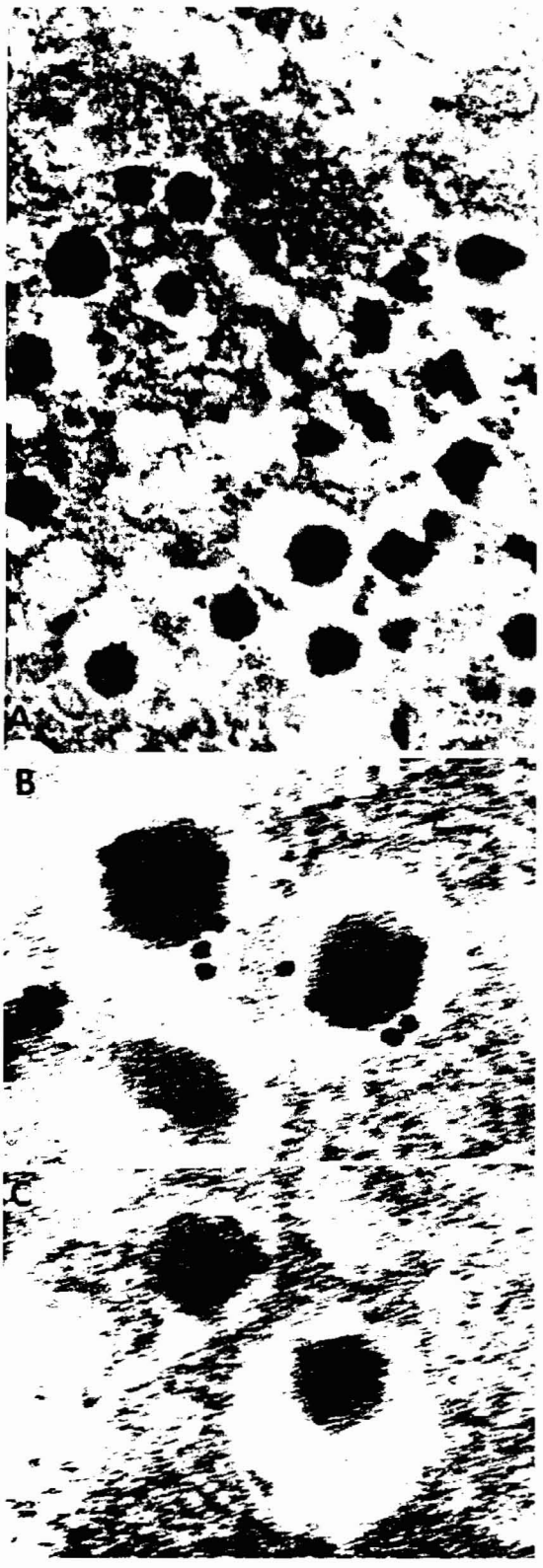

FIG. 6. Insulinoma cell showing pro-insulin C-peptide immunoreactivity within β-granules. a, ×51,000; b, immunoreactive granules with crystalline core, ×106,250, and c, non-immunoreactive homogeneous core granules, ×106,250.

nm), electron-dense granules characteristic of both chromaffin cell types of the adrenal medulla (Fig. 10) of several mammalian species, including man. Enkephalin-like immunoreactivity has also been localised to the pleiomorphic granules characteristic of phaeochromocytoma cells.

DISCUSSION

For many years the peroxidase-antiperoxidase method, modified from Sternberger [40], has been the technique of choice for the immunocytochemical localisation of tissue-bound antigens. The standard procedure involves the formation of several immunological complexes and the peroxidase-mediated deposition of an amorphous, electron-opaque oxidation product of 3,3'-diaminobenzidine (DAB), which in the presence of osmium tetroxide undergoes cyclic polymerisation to the electron-dense osmium black [23]. Apart from the toxicity of DAB, one drawback of the procedure is that the fine structure of the immunostained organelles is largely obliterated by the homogeneous deposition of final reaction product. Furthermore, conventional counterstaining with heavy metal salts cannot be performed profitably on the immunostained sections which prejudices the precise interpretation of tissue morphology.

We report here the application of two immunogold staining methods for the light and electron microscopic localisation of peptide hormones and neurotransmitters. Both the staphylococcal protein A- and the immunoglobulin-conjugated gold methods have considerable advantages over the alternative techniques in that they are composed of fewer immunological steps with less toxic reagents which are easy and cheap to prepare. Antigenic sites are not obliterated, therefore organelle ultrastructure is observable and, in addition, conventional counterstaining can be performed on colloidal gold immunostained sections. These factors have proved to be crucial when the topographic distribution of two distinct molecular forms of one peptide within a single granule, for example glucagon and glicentin (proglucagon) in pancreatic α-granules, are considered. The core of the α-granule, now shown by immunogold staining to contain glucagon-like immunoreactive material, is distinguishable by conventional uranyl acetate and lead citrate staining from the outer portion by a marginally greater affinity for these heavy metal salts. Conventional ultrastructural morphology in combination with immunocytochemical staining for specific peptide molecules has also proved instrumental in the investigation of p-type neurone heterogeneity. Co-existence of amines and peptides within single granules from catecholamine-containing cells of the carotid body and adrenal medulla has been demonstrated using the immunogold technique. In this context it is expedient to introduce one further advantage of the immunogold staining methods which is the development of double- and multiple staining methods at the electron microscope level utilising the different sizes of gold particles available. At present there are some technical problems to be overcome before two or more antigenic sites within one cell, or even one granule, can be visualised. The potential applications of double-labelling techniques are limitless.

To date, most regulatory peptides against which specific antibodies have been raised, have been visualised with the on-grid immunogold staining procedures; the localisation of heat-labile peptides, for example VIP, requiring only minor modifications to the preparative procedure (ultraviolet-cured resin, [29]).

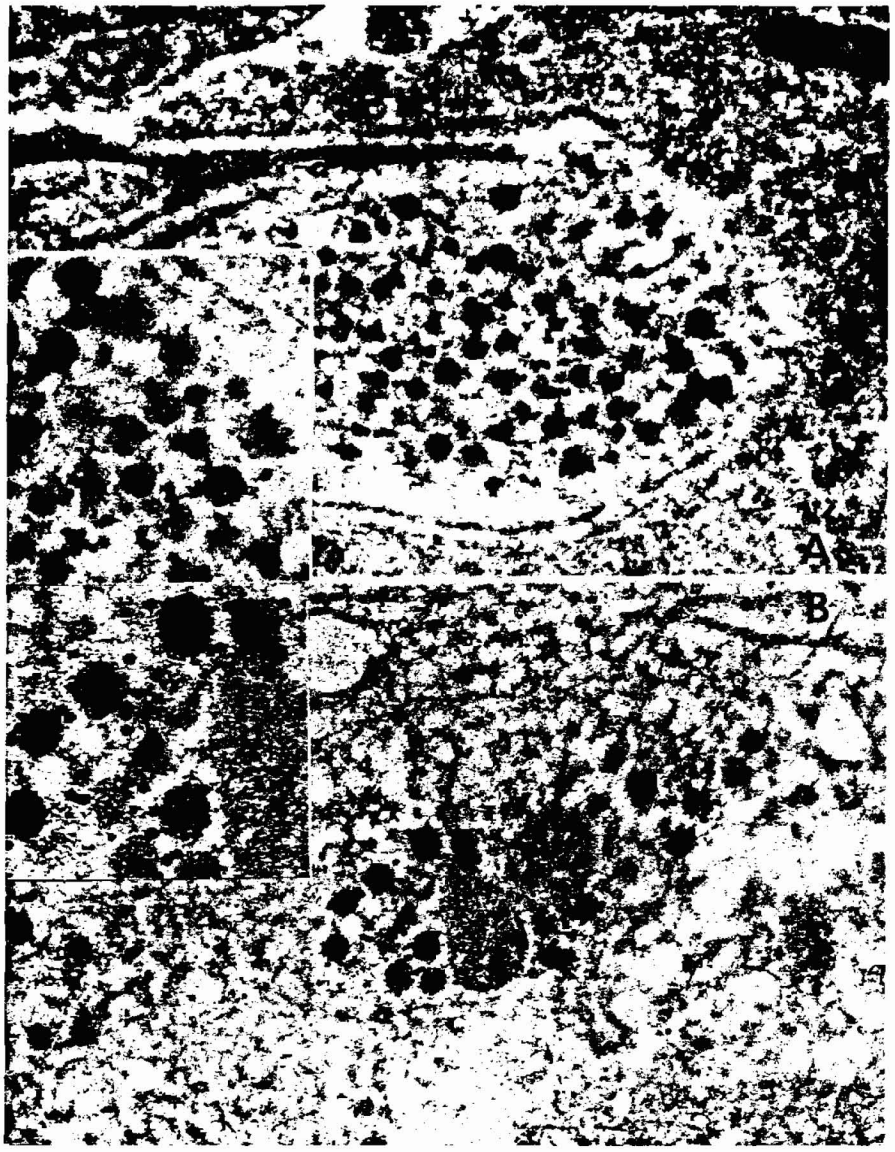

FIG. 7. Ultrathin sections of guinea-pig myenteric plexus showing nerve terminals a, immunostained with Substance P, ×50,000; inset ×75,000, b, immunostained with VIP, ×50,000; inset ×75,000. Note the specific gold labelling over the large, granular, p-type vesicles present in the nerve terminals shown.

By far the most important advantage of the immunogold staining procedures over the alternative techniques is the suitability of punctate labelling for quantification purposes. Ratios of granular immunoreactive sites to background sites can now be calculated by simple point-counting techniques and expressed in statistical terms; thus subjective criteria need not be used when describing the specificity of immunoreactions.

The application of immunogold staining methods at the light microscope level [15] have demonstrated the advantages of these techniques in double- and multiple staining procedures using bright field illumination. A new application for the colloidal gold methods, based on the observation of immunostained semi-thin sections by dark-ground illumination, has provided a very important link between light and electron microscope immunocytochemistry and further illustrates the advantages of gold-labelling techniques.

ACKNOWLEDGEMENTS

F. J. T. is a visiting fellow in the Histochemistry Unit, on leave from Instituto Nacional de Dermatologia (Caracas), supported by a fellowship from Fundacion Gran Mariscal de Ayacucho (Venezuela). L. P. is supported by a studentship from the Medical Research Council. J. G. is a visiting scholar from Peking Medical College, The Peoples Republic of China. The authors wish to thank Mr. P. Flecknell (Northwick Park Hospital, London, U.K.) and Dr. N. P. Hodson (Royal Veterinary College, London and Potters Bar, U.K.) for making available some of the animal tissue used in this study. Generous gifts of C-peptide antisera were received from Dr. L. G. Heding (Novo Research, Denmark) and Prof. N. Yanaihara (Shizuoka College of Pharmacy, Japan) and dopamine-β-hydroxylase antisera from Dr. R. A. Rush (Flinders Medical Center, Australia).

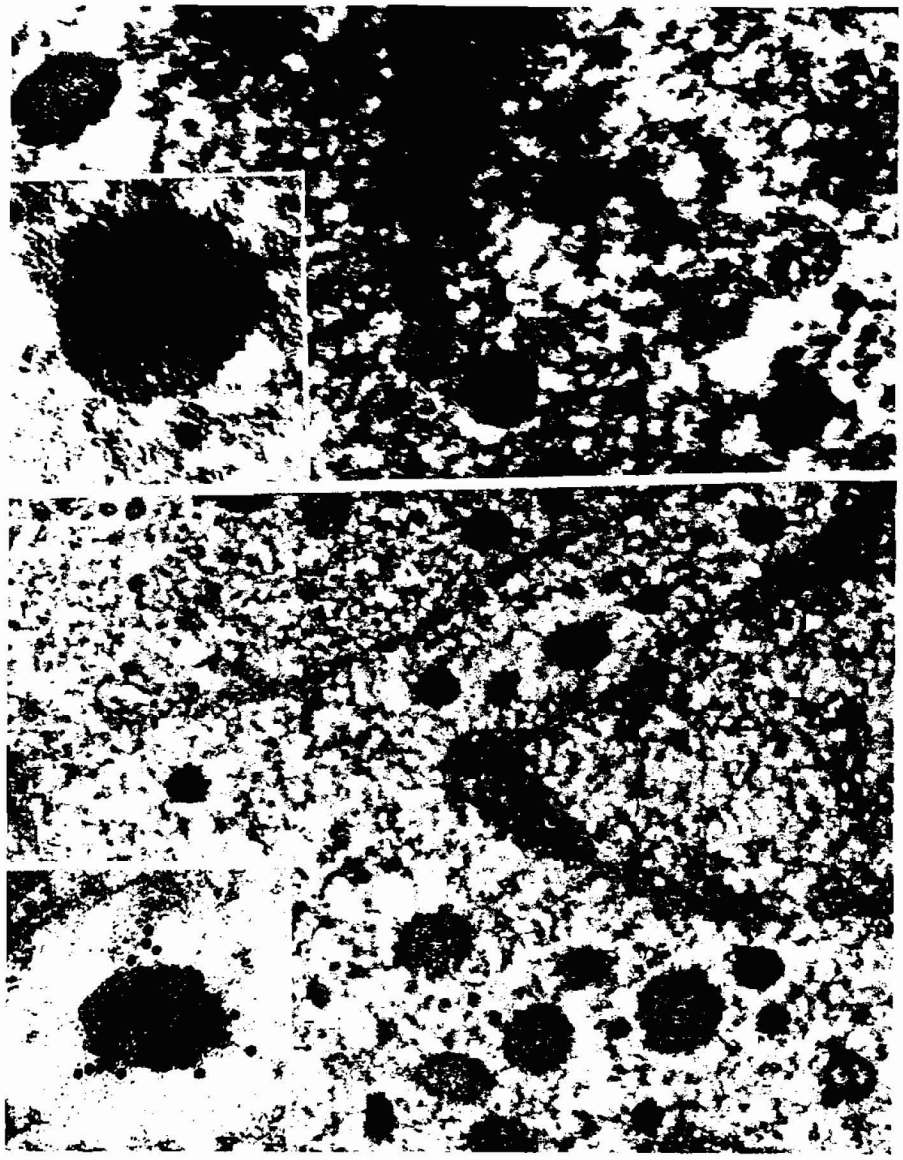

FIG. 8. a. VIP-immunoreactive granules in a pancreatic diarrhoeogenic tumour. ×56,000; inset, ×200,000. b. PP-immunoreactive granules in the same tumour. ×36,000; inset ×128,000.

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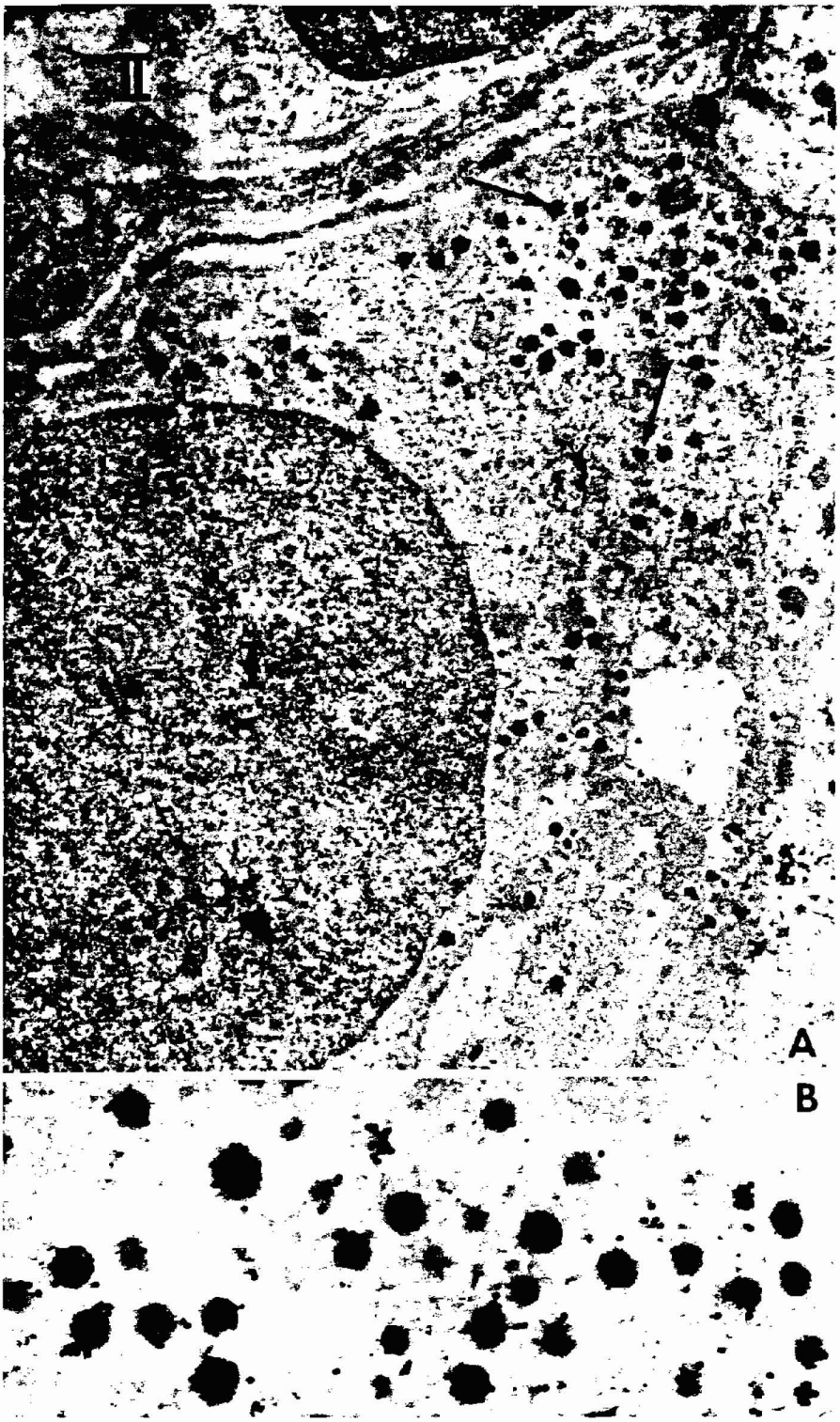

FIG. 9. a. Met-enkephalin-like immunoreactivity localised to electron-dense granules (arrows) in a type I cell (I) of the cat carotid body. Part of a type II glomus cell (II) is visible in the upper half of the micrograph. ×15,000. b. Higher magnification of the cat carotid body type I cell granules immunoreactive to met-enkephalin. ×46,000.

FIG. 10. a. The electron-dense granules of both (A, adrenergic; N, noradrenergic) chromaffin cell types of the horse adrenal medulla are immunostained for met-enkephalin using the immunogold technique. ×7,000. b. Higher magnification of a. ×75,000.

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