IN : MEM (Magazine for Electron Microscopists), Zeiss Information, Volume 1, pp 39-44, 1982.

# The Ultrastructural Localisation of Regulatory Peptides in Endocrine Cells and Nerves of the Diffuse Neuroendocrine System by a Combined Immunogold Staining Procedure and Image Analysis.

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Received 16 April 1982

## Introduction

The number of bioactive peptides extracted mostly from the brain and gut, but now known to be present in most organ systems, is continuously increasing [1]. These peptides include: vasoactive intestinal polypeptide (VIP), substance P, the enkephalins, the endorphins (endogenous morphines), gastrin, cholecystokinin (CCK), motilin, gastric inhibitory polypeptide (GIP) bombesin, somatostatin, neurotensin, glucagon, pancreatic polypeptide, insulin and enteroglucagon. Immunocytochemistry has been instrumental in determining the precise localisation of regulatory peptides in endocrine cells and also in central and peripheral neurons [2]. The advances in accurate tissue localisation of regulatory peptides by immunocytochemistry have been mainly due to the production of specific (on many occasions monoclonal) antibodies of high titre and good avidity, and to the development of useful modifications of the original methods to improve their sensitivity, reliability and specificity [3]. Application of immunocytochemistry at the ultrastructural level has demonstrated the sites of intracellular peptide synthesis, storage and possibly release. A variety of gold-labelled immunostaining reagents have been particularly useful in this work [4]. In this paper we refer to our experience with one of the marker methods using colloidal gold particles, the immunogold staining method [5] applied at the ultrastructural level for the intracellular localisation of regulatory peptides in a variety of peripheral tissues. In addition, details of an image analysis technique using an IBAS II computerised image analyser equipped with morphometric and densitometric capabilities in combination with a Zeiss 10 C transmission electron microscope fitted with a 35 mm camera are given.

## Material and methods.

### Electron Immunocytochemistry by the Immunogold Staining Method (IGS).

Immunocytochemistry was carried out on resin-sections of tissue obtained from several species of mammals including man (normal and diseased) as described in previous publications from our group [4, 6]. Tissue was taken from carotid bodies, adrenal glands, pancreas and gastrointestinal tract. The antibodies were raised in rabbits and guinea pigs to a variety of pure natural and synthetic peptides and catecholamines (or their specific converting enzymes). Details of the antisera used are given in Table 1.

Tab 1. Characteristics of the antisera used.

| Antiserum raised against | Region specificity | Dilution | Concentration of antigen used for absorption (nmol/ml diluted antiserum) |
|---|---|---|---|
| Gastrin | C-term. | 1 : 2000 | 0.1 |
| Glucagon | N-term. | 1 : 2000 | 2 |
| Glicentin | N-term.[1] | 1 : 1000 | 2 |
| Insulin | – | 1 : 8000 | 2 |
| C-peptide | –[2] | 1 : 4000 | 1 |
| Methionine enkephalin | C-term. | 1 : 4000 | 1 |
| Substance P | Whole molecule | 1 : 8000 | 3 |
| Vasoactive Intestinal Polypeptide | C-term. | 1 : 6000 | 5 |
| Somatostatin | – | 1 : 6000 | 1 |
| Serotonin | Amino side chain | 1 : 2000 | $6 \cdot 10^{-5}$ |
| Dopamine-β-hydroxylase | Bovine enzyme[3] | 1 : 2000 | 0.5[4] |

[1]) From Dr. A. J. Moody, Novo Research Institute Denmark;
[2]) From Prof. W. Creutzfelt (Germany) and Dr. L. Heding, Novo Research Institute Denmark;
[3]) From Dr. R. A. Rush, Flinders Medical Centre, S. Australia;
[4]) Refers to enzyme units (Sigma: type III) required per ml of diluted antiserum.

The immunogold staining method makes use of the electron density of gold particles combined with their ability to adsorb macromolecules such as antibodies.

the controlled reduction of gold salt solutions. Details of the gold-labelling procedure and of the immunostaining method employed are given in Tables 2 and 3.

Table 2. Procedure for the preparation of the gold-labelled antisera.
(Adapted from De Mey et al., ref. [5]).

1. Colloidal gold sols are prepared by the reduction of chloroauric acid.
   Reducing agent: White phosphorus = 3 – 6 nm gold particles.
   *Tri*-sodium citrate = 20 – 80 nm gold particles.
2. Goat anti-rabbit or anti-guinea pig IgG prepared from serum by antigen affinity chromatography.
3. Antibodies dialysed against 2 mM borax buffer (pH 9.0), centrifuged at 100,000 × g for 1 hr at 4° C just before use.
4. Gold sol adjusted to pH 9.0 with 0.2 M $K_2CO_3$.
5. 10 – 40 µg antibody/ml gold sol mixed.
6. Bovine Serum Albumin (BSA; Sigma fraction V) added to final concentration of 1%.
7. Centrifugation to remove unbound or loosely bound antibodies and insufficiently stabilised gold granules.
8. Gold conjugated antibodies resuspended in 20 mM Tris-buffered saline (TBS) containing 1% BSA plus $2.10^{-2}$ M sodium azide.
9. Gold immunostaining solutions stored at 4° C.

Table 3. Double immunogold staining method for the simultaneous ultrastructural localisation of two antigens in a single tissue section.

1. Small blocks of tissue should be fixed in conventional aldehyde-based solutions but not osmicated. The tissue should be dehydrated and embedded in Araldite, Epon or Spurr's.
2. Silver-grey to pale gold sections – mount on uncoated 300-mesh nickel grids and dry overnight.
3. Etch sections in 10% hydrogen peroxide for 10 minutes.
4. Wash in several changes of PBS (pH 7.2).
5. Expose sections (by floating the grids) on 1:10 – 1:30 normal goat serum (NGS) for 30 minutes at room temperature.
6. Drain off excess NGS but do not wash.
7. Incubate sections in primary antisera. For the double immunogold staining procedure one antiserum should be raised in rabbits and the other in an unrelated species. The mixture should contain optimal titres of each antiserum. Incubate for 1 hr at room temperature to avoid the possibility of cross-reactivity.
8. Wash in several changes of PBS.
9. Wash in TBS (see Table 2) containing 0.2% BSA.
10. Incubate in a mixture of gold-conjugated antisera, e.g. goat anti-rabbit (5 or 40 nm gold particle size) and goat anti-guinea pig (20 nm gold particle size) for 30 – 60 minutes at room temperature.
11. Wash in TBS, PBS and distilled water.
12. Dry, and counterstain with aqueous uranyl acetate and lead citrate.
13. Observe in transmission electron microscope.

## Immunogold staining and image analysis.

This was carried out using one of the following photographic techniques.

a) Direct reversal panchromatic film (Kodak® Precision Line LPD 4) exposed, developed in Kodak DG 10 developer (1 + 4) and directly linked to the IBAS II image analyser computerised system via an Illumitran® microphotographic reproduction unit or via a wide aperture low power objective lens fitted to an ocular microscope.

b) Electron micrographs printed from Eastman® Fine Grain Release Positive or Kodalith® orthochromatic films were entered directly into the image analyser using an on-line television camera.

We have carried out a variety of investigations for the demonstration of intracellular antigens. The investigations, details of which are given later on, included: –

a) Determination of granular to cytoplasmic immunolabelling (the ratio of gold particles localised to secretory granules to those randomly distributed throughout the cell). The method proved especially helpful,
i) in areas of strong immunoreaction, such as adrenal medulla or phaeochromocytoma granules stained for enkephalin, where the abundance of gold particles makes non-automated counting laborious and inaccurate,
and ii) when it is necessary to demonstrate minimal labelling (e.g. of peptides in the early stages of synthesis) and to differentiate it from unspecific background "noise". This newly-devised quantitative method has been particularly useful in the analysis of very active (stimulated) endocrine cells (see later).

b) Intragranular distribution of different molecular forms of a single peptide. By labelling antibody to a larger form of a molecule (prohormone) with gold particles of one size (e.g. 20 nm diameter) and antibody to a smaller, bioactive form of the molecule with particles of another size (e.g. 40 nm diameter), we have been able to produce accurate quantitative data on the topographic segregation of different molecular forms of a peptide within a single granule.

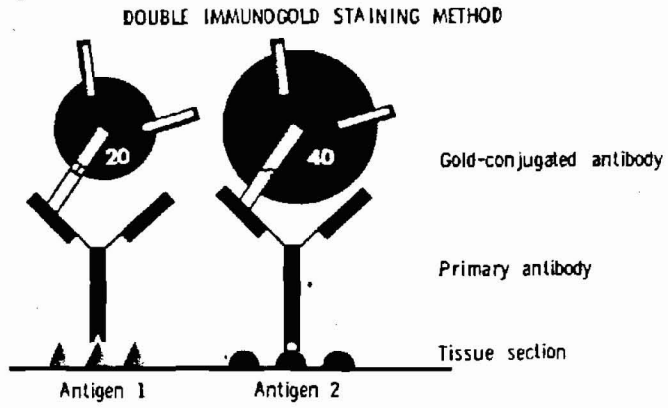
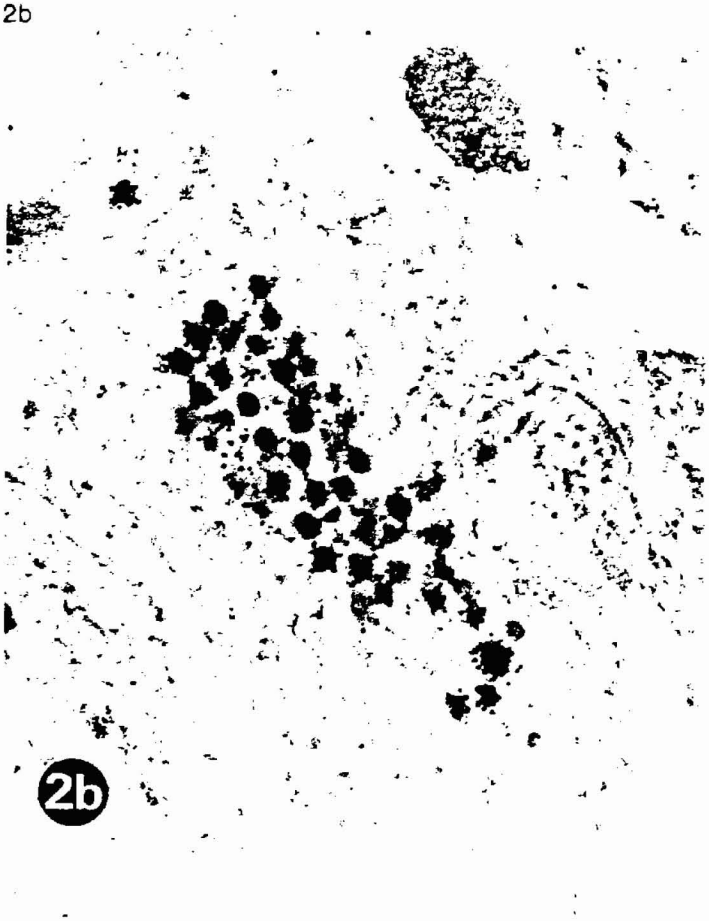



*Fig. 1: Diagram of the double immunogold staining method for the simultaneous ultrastructural localisation of two antigens in a single tissue section. The gold particles (20 and 40 nm) are not drawn to the same scale as the antibodies.*

*Fig. 2: Ultrathin sections of guinea pig myenteric plexus showing nerve terminals:*
*a) Immunostained with substance P antiserum (resin polymerised at 60° C).*
*b) Immunostained with vasoactive intestinal polypeptide antiserum (resin polymerised at 18° C with ultraviolet light). Immunogold staining procedure with 20 nm gold particles, counterstained with uranyl acetate and lead citrate. Magnification × 58,000.*

2a


2b




## Results.

The experiments we have carried out by using this technique are grouped for clarity into three major areas: –

1. Simultaneous localisation of separate peptide antigens in a single tissue section. A single tissue section may contain several endocrine or neural cell types producing different regulatory peptides. The secretory granules in which the peptides are stored are sometimes difficult to distinguish from each other on morphological grounds. Thus, in the classification of gut endocrine cells by conventional electron microscopy, there is a tendency to include several cellular subtypes with very similar secretory granules within one single type. For example, following immunocytochemical staining at the electron microscopical level, the $D_1$ cell of the upper small intestine was re-classified into at least two separate cell types producing intestinal gastrin and motilin [7] and the L cell of the lower small intestine was shown to accommodate two different cell types, the N (neurotensin) cell and the EG (enteroglucagon) cell [2]. In order to immunostain multiple antigens simultaneously in a single tissue section, we have employed antibodies raised in separate species and labelled with different sizes of gold particles [8] (see Figure 1).

2. Heterogeneity within the large p- (peptidergic) type neurosecretory granules of the enteric nervous system. The existence of large, dense-cored neurosecretory granules in nerve terminals of the enteric nervous system, quite distinct from the nerve terminals containing the classical neurotransmitters, acetylcholine and adrenaline, was recognised as early as 1965 by Taxi [9] and termed p- (peptidergic) type by Baumgarten [10]. Using the immunogold staining method at the ultrastructural level with specific antibodies to substance P and VIP [11], we have been able to separate at

least two sub-classes of neurosecretory vesicle, distinguished by their peptide products and by their morphology (Figure 2). Substance P-containing neurosecretory vesicles are mostly spherical in shape while VIP-containing vesicles often appear more irregular, these terminals containing a mixed population of spherical and elongated vesicles. Quantitative estimation of the average size of the granules was carried out using the unpaired Student's t-test which showed that VIP neurosecretory granules are significantly ($p < 0.01$) larger (mean $\pm$ S. D. = 98 $\pm$ 19 nm) than substance P (mean $\pm$ S. D. = 85 $\pm$ 15 nm) (Figure 3). The other subclasses of p-type neurosecretory granules remain to be correlated with their specific storage products.

3. Peptide/amine co-existence. A number of regulatory peptides (e.g. the enkephalins, somatostatin, neurotensin) have recently been shown to be present in a variety of structures of the central nervous system, e.g. Raphe nucleus of the medulla oblongata of the brain, and in peripheral tissues reputed to contain, produce and release amines (e.g. adrenal medulla, carotid body and sympathetic chain). Electron immunocytochemistry with the IGS method has demonstrated, in most instances, the co-localisation of a regulatory peptide with an amine [4, 6]. The example given here (Figure 4)

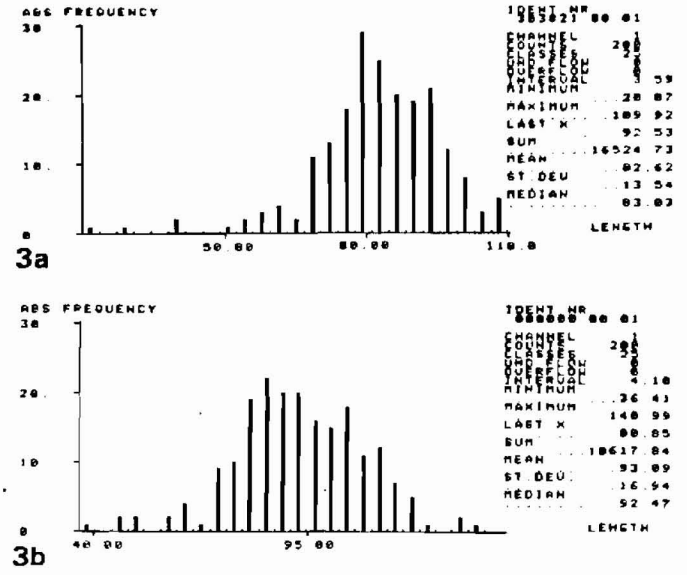


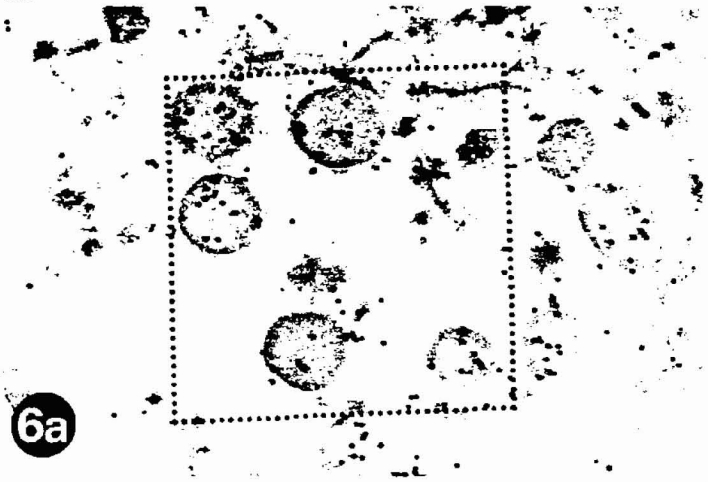

*Fig. 3: Print-out traces from the IBAS computerised image analyser.*
*a) Distribution curve of substance P-containing vesicle diameter with a series of statistics for diameter range, mean and standard deviation of the mean.*
*b) Distribution curve of vasoactive intestinal polypeptide-containing vesicle diameter with similar values displayed.*
*Each curve displays the result of 200 individual vesicle counts.*


*Fig. 4: 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 pleiomorphic nature of the noradrenalin granules is evident. Immunogold staining method with 20 nm gold particles, counterstained with uranyl acetate and lead citrate. Scale 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. Scale bar = 0.75 µm.*

**determine with certainty the intracellular sites of peptide synthesis and storage. Advanced image analysis procedures allow the estimation of synthesis relative to storage. These parameters can in addition be correlated with precise quantitative information obtained by the measurement of the amount of the peptide in the tissue and in the circulation.**

6a




6b

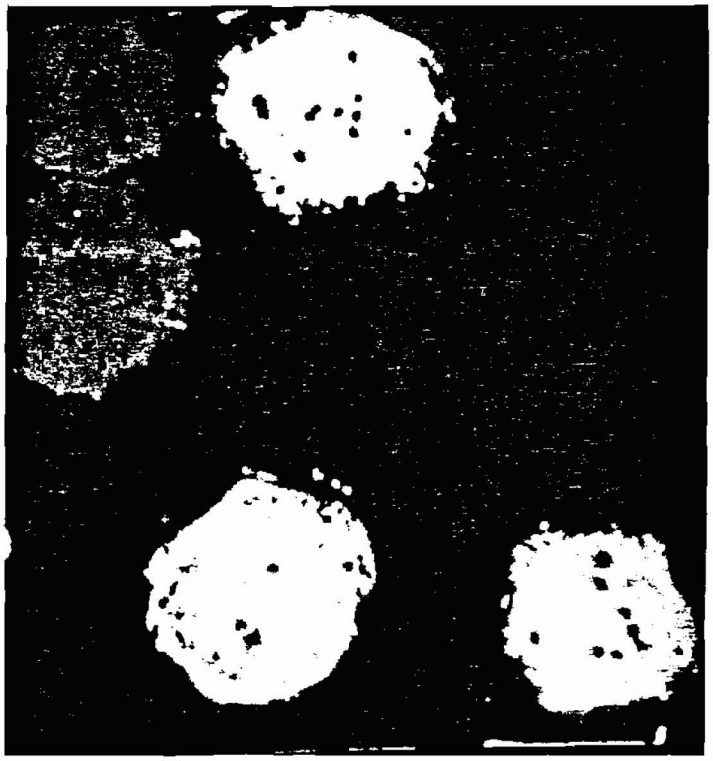


*Fig. 6: a) Neurotensin-like immunoreactivity localised to electron-dense pleiomorphic granules in the cat adrenal medulla following application of the immunogold staining procedure with 20 nm gold particles. The area delineated by the dotted lines has been analysed using the IBAS II and the result is shown in Figure 6 b below. Uranyl acetate counterstain; magnification = × 35,000.*

*b) Image of the area indicated in 6a above following densitometric analysis with the IBAS II computerised system. Differences in granule density are detected by the IBAS and colours are generated in response to the specific densities according to a pre-defined grey-level programme. This enables the observer to express gold particle distribution in terms of granule density and thus recognise vesicle subtypes within heterogeneous populations.*

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