Regulatory Peptides in Endocrine Cells and Autonomic Nerves

Electron Immunocytochemistry

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Advances in immunocytochemistry, particularly at the electron microscope level, have enabled us to establish further details of the ultrastructural appearance of endocrine cells and autonomic nerves of the gastrointestinal tract.

Its contribution can be summarised as follows:

a) Validation of previously recognised endocrine cell types.
b) Recognition of sub-groups within endocrine cells and autonomic nerve cell types previously included within one single type (e.g. D_1 cells and p-type autonomic nerves).
c) Discrimination of molecular forms now known to be stored in morphologically distinguishable secretory granules or parts thereof (e.g. pro-glucagon and glucagon in A cells and gut and antral gastrin-producing cells).

Advances in the techniques and the accurate quantification of the end products will enable us to recognise changes in gastrointestinal tract diseases in man.

Key-words: Autonomic nerves; electron microscopy; endocrine cells; gut; regulatory peptides

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Since the discovery of secretin in 1902 (2), more than 25 regulatory peptides have been shown to be present in the gastrointestinal tract, localised to endocrine cells and/or autonomic nerves (14). It is known that most of these regulatory peptides are stored in distinct types of endocrine and neural cells which is in agreement with earlier ultrastructural observations that the gastrointestinal tract contains a wide variety of cell types (22). The different cells are distinguished by the presence of electron-dense secretory granules varying in their shape, their size and the form of their limiting membrane (see Solcia et al., this issue). Until recently, the functional classification of the various endocrine cell types was based mainly in the parallel distribution of a predominating cell type and a particular regulatory peptide (see Solcia et al., this issue).

As a result of advances in immunocytochemical methods it has now been possible, in many instances, (e.g. antral G cells) to validate earlier classifications. In addition, sub-groups of cells previously considered to be of one type (e.g. D_1 cells) have been recognised as, indeed, have sub-groups of autonomic, peptide-containing (p-type) nerves (23, 24). The refined methods have even been able to reveal the localisation of various molecular forms (pro-hormones—hormone) in different areas of the secretory granules (e.g. pro-glucagon and glucagon in the A cells of the pancreatic islets) (19).

ELECTRON IMMUNOCYTOCHEMICAL METHODS

The introduction of peroxidase labelling of antibodies for immunocytochemistry (25) and the

realisation that the end-product of the reaction could be made electron-dense by osmium tetroxide has led to the development of several other electron immunocytochemical methods (26). The most popular techniques at present are the peroxidase anti-peroxidase (PAP) method and a variety of gold-labelling procedures including protein A gold (20), gold-labelled immunoglobulin (10) and the directly labelled antigen procedure (11) (GLAD *G*old *L*abelled *A*ntigen *D*etection method) (Fig. 1).

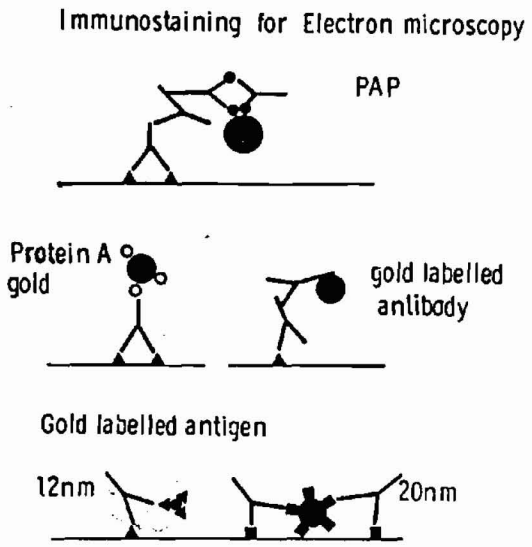


Fig. 1. Diagrammatic representation of the immunostaining methods commonly used in electron microscopy. The top diagram shows the end product of the peroxidase anti-peroxidase method. The middle diagram shows, on the left protein A-conjugated colloidal gold and on the right, colloidal gold-labelled immunoglobulin. The final diagram shows the antigen linked to colloidal gold and also demonstrates the use of two different sizes of gold particles.

We shall now discuss the contributions made by electron immunocytochemistry to a number of problems of particular interest to gastroenterologists.

A) *Validation of earlier ultrastructural classifications*

Electron immunocytochemical procedures have been instrumental in confirming many earlier suggestions that distinct cell types were responsible for the production of particular gut peptides. Examples include the gastrin-producing G cell of the antral mucosa, the secretin-producing S cell and the CCK-producing I cell of the small intestine.

B) *Subclassification of the D_1 cells (small granuled) and L cells (large granuled)*

Small granuled cells were first described in 1965 (21) and were termed D_1 in 1972 (6). These cells were present in all areas of the gut (stomach, small and large intestine and pancreas). A separate population of large granuled cells (L) was found mainly in the ileum and large intestine.

No particular peptide product was identified within the D_1 cells but Solcia and co-workers suggested that the L cells were responsible for the production of gut glucagon (enteroglucagon) (27).

i) *D_1 cell subclassification*

The first indication that the D_1 cells were a more heterogenous group than was originally suspected came in 1979 (15). At this time comparative light microscopical immunocytochemistry and electron microscopical morphology (serial semithin/thin method) revealed the presence of gastrin-like immunoreactivity (gastrin of intestinal origin) in a subpopulation of D_1 cells thereafter termed IG (intestinal gastrin) cells (4). This discovery was soon followed by the localisation of motilin-like immunoreactivity to another subpopulation of D_1 cells (15, 16), separate from that responsible for the production of intestinal gastrin, and now internationally recognised as the Mo cells (23, 24) (Figs. 2 and 3).

ii) *L cell subclassification*

In 1977 we, as well as others, reported the electron immunocytochemical localisation of a newly discovered 'brain and gut' regulatory peptide, neurotensin. Neurotensin was found to be produced by a subpopulation of large granuled (L) cells of the ileal mucosa (Figs. 4 and 5), thus establishing the subclassification of the L cells of the lower gut, originally considered to be a single cell type containing gut glucagon (13).

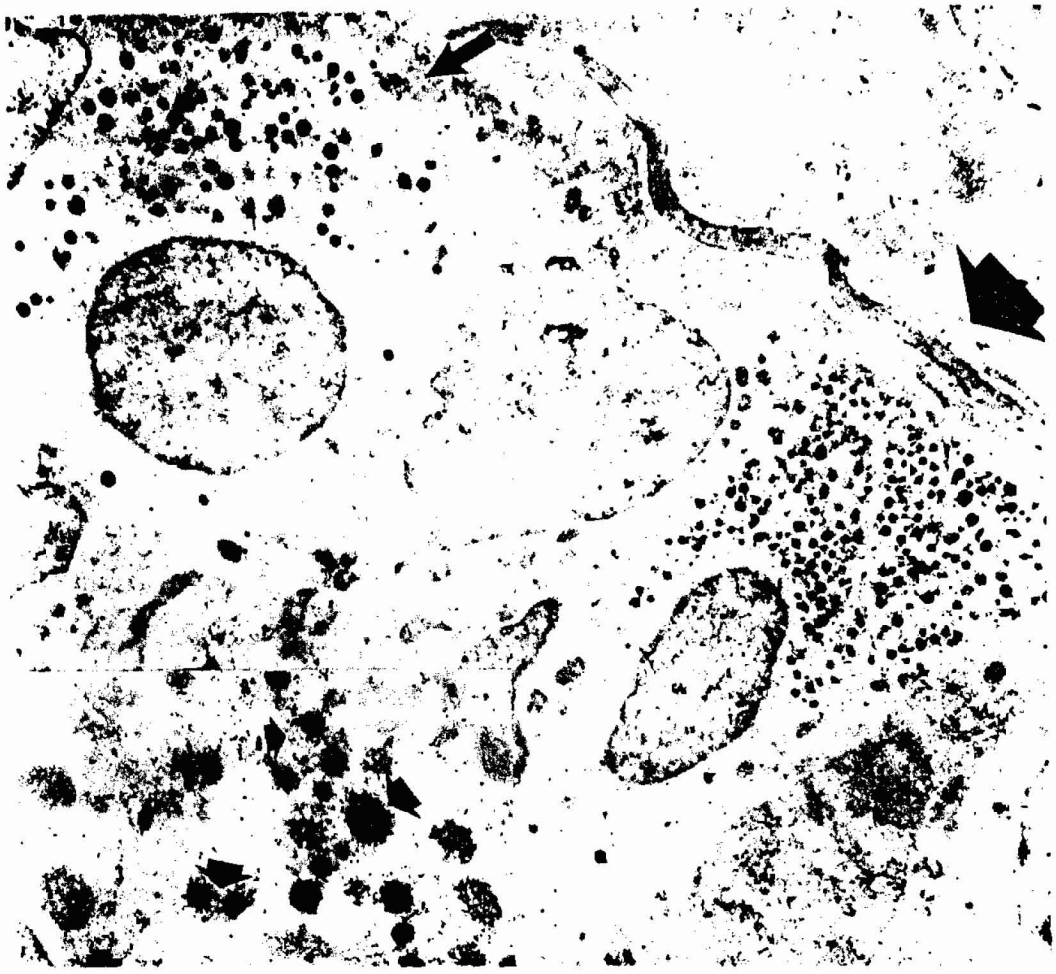


Fig. 2. 80 nm section of human duodenum showing a gastrin-containing cell (large arrow) labelled by 12 nm colloidal gold and an unlabelled endocrine cell (small arrow). × 10,000
The insert shows the labelled secretory granules. Note the concentration of gold particles over the granules (arrows) with unlabelled mitochondria and cytoplasm. × 30,000

C) *Electron immunocytochemistry of variant forms of peptide molecules*

It is now well recognised that most, if not all, gut regulatory peptides are present in both the tissue and the circulation in a variety of molecular forms of differing sizes (8) (Fig. 6). Usually these differing molecular forms suggest the existence of precursors or pro-hormones, capable of giving rise, by biosynthetic processes to increasingly bioactive smaller fragments (see Fig. 6 for illustrative examples). The fact that antibodies can be raised to specific regions of a peptide molecule has permitted the immunocytochemical identification of the site of production of different molecular forms of a single peptide. Two examples illustrate this point.

i) The localisation of antral gastrin and of intestinal gastrin to two types of endocrine cells containing distinctly different secretory granules has recently been achieved by the use of region specific antibodies to gastrin 17 and gastrin 34. The antral G cells, known to store mainly the smaller molecular form of gastrin, G17, are characterised by mostly large (average size 340 ±

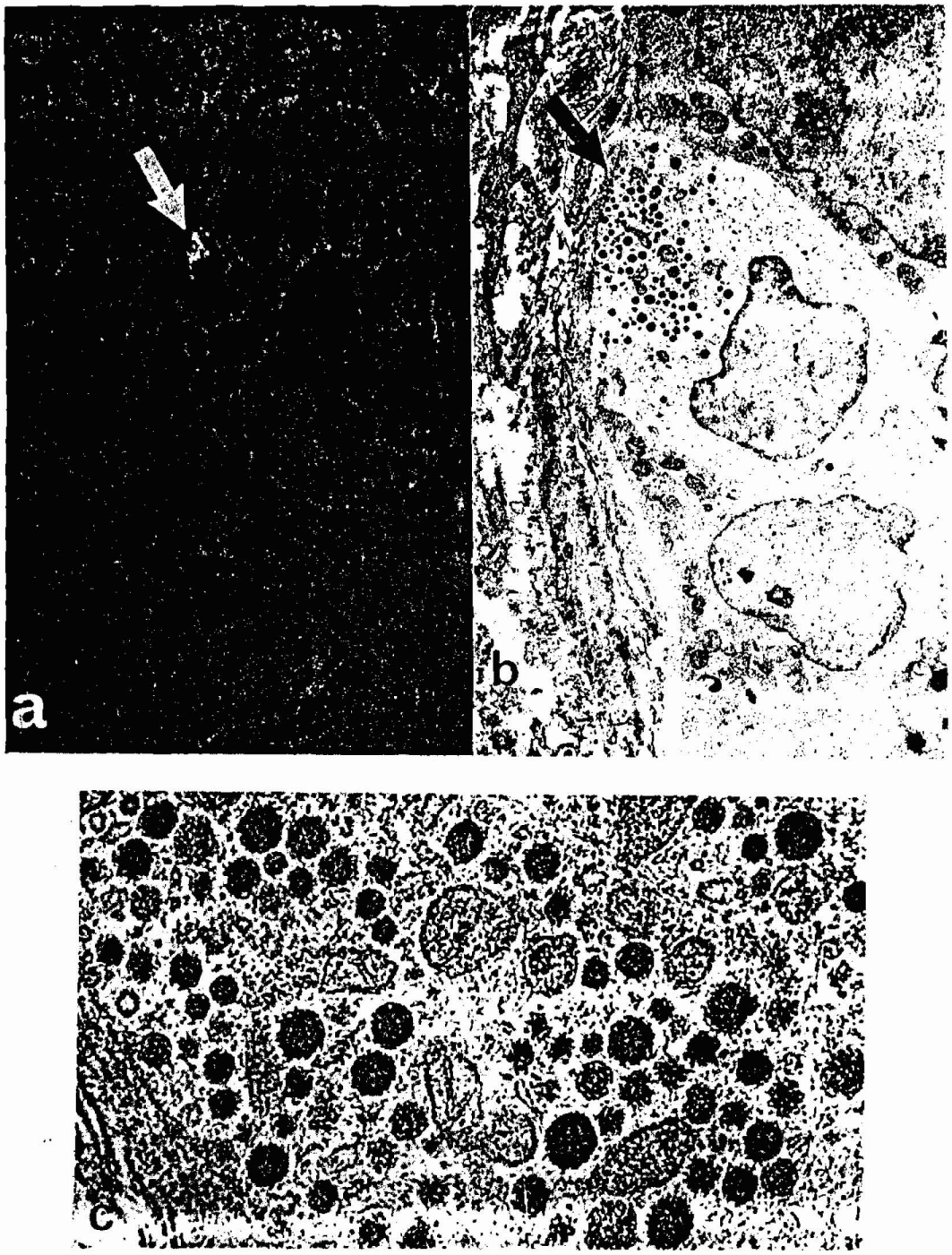


Fig. 3. a) Semithin (500 nm) section of duodenum showing a motilin-containing cell (arrow) detected using a C-terminal directed antiserum by the indirect immunofluorescent method. × 500
b) The same cell identified in a serial thin (60 nm) section (arrow). × 5,000
c) At higher magnification details of the characteristic secretory granules are visible. × 30,000

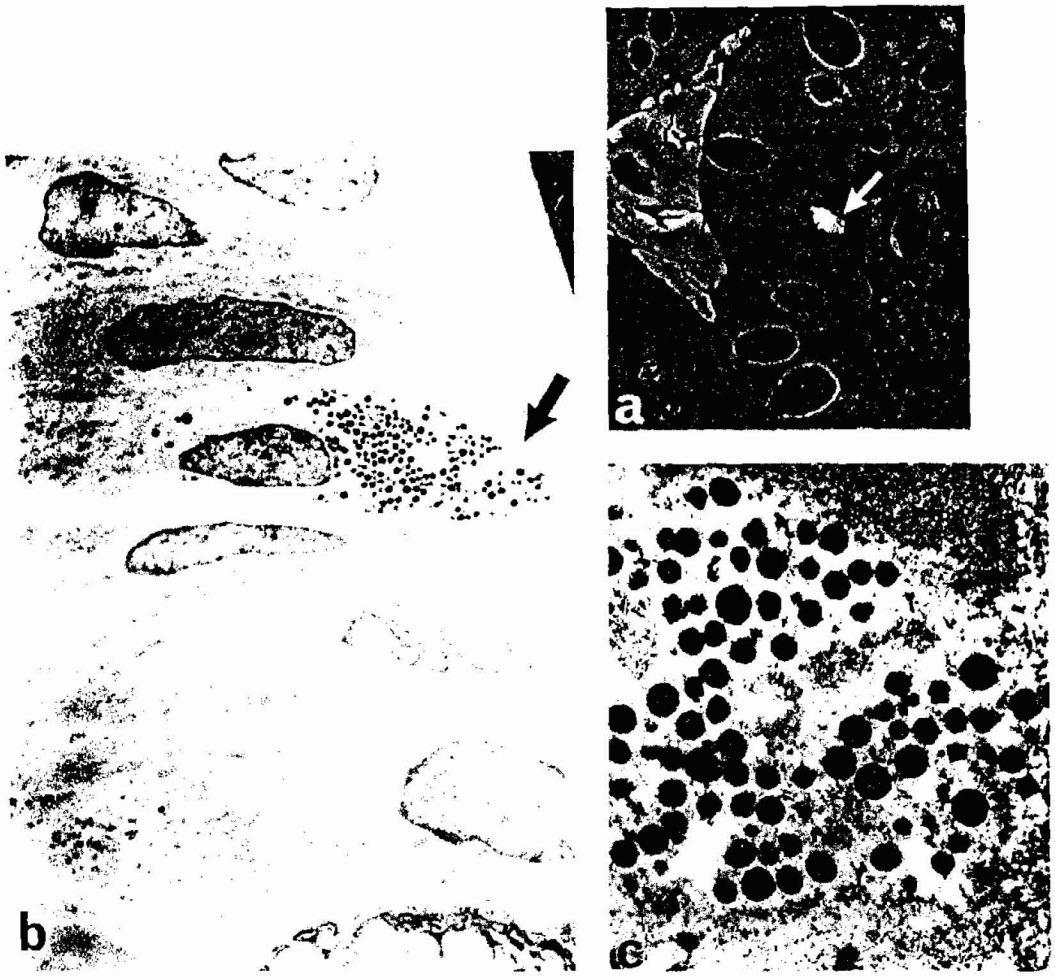


Fig. 4. a) A semithin (500 nm) section showing a neurotensin-immunoreactive cell (arrow). × 500
b) A serial thin (60 nm) section of the same area, with the immunostained cell indicated by the arrow. × 5,000
c) Higher magnification of the cell, revealing details of the secretory granules (compare with Fig. 5c). × 30,000

54 nm) electron-lucent secretory granules, whereas intestinal gastrin cells, known (at least in man) to store predominantly the larger molecular form of gastrin, G34, are characterised by small (average size 175 ± 21 nm) round, electron-dense secretory granules (4). These granules are quite distinct from those of the I cells in the same area which secrete a chemically related peptide, CCK, but have larger (300 ± 28 nm) secretory granules (3). Thus it would appear that the predominance of a particular molecular form (gastrin 17 or gastrin 34) determines the structure of the secretory granules (Fig. 7). This phenomenon is also noticeable in tumours. The cells of the rare gastrinomas found to produce predominantly gastrin 17, contain mainly the antral gastrin type of secretory granules, whereas the granules of tumors which produce gastrin 34 as the main molecular form are mostly small and electron-dense resembling those of the 'intestinal gastrin cells' (Fig. 8).

ii) Glicentin or 'glucagon of intestinal origin', is a newly discovered regulatory peptide (12). It has now been found to be chemically identical

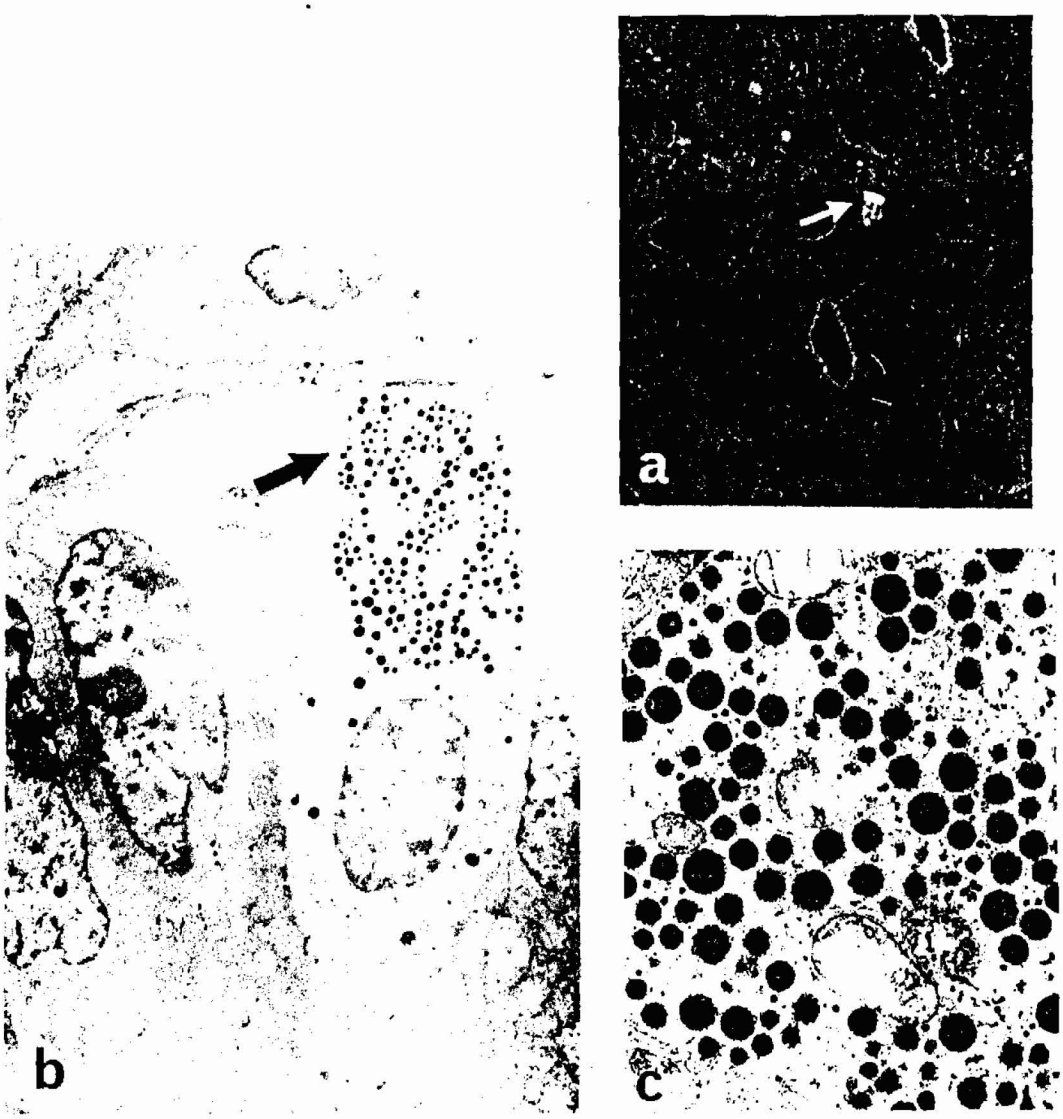


Fig. 5. a) Semithin (500 nm) section of ileal mucosa immunostained using highly specific antibodies to glicentin, showing an immunoreactive cell (arrow). × 500
b) Electron micrograph of a serial thin (60 nm) section showing the same cell (arrow). × 5,000
c) Details of the secretory granules present in the glicentin-immunoreactive cell (compare to Fig. 4c). × 30,000

MOLECULAR FORMS OF GASTRIN AND GLUCAGON

Gastrin
G34 QLGPQGPHSLVADPSKKQGPWLEEEEEAYGWMDF
G17 QGPWLEEEEEAYGWMDF

Glucagon
Glicentin RRAQKFVQWLMNTKRNKNNIA—
HSQGTFTSDYDKYLDSRRAQDFVQWLMNTKRNKNNIA
Glucagon HSQGTFTSDYSKYLDSRRAQDFVQWLMNT

Fig. 6. Gastrin 34 and Gastrin 17 share the whole of their common C-terminal sequence whereas glicentin has both an N-terminal and a C-terminal extension to the glucagon molecule. The whole of the glicentin sequence cannot be given as it has yet to be completely sequenced.

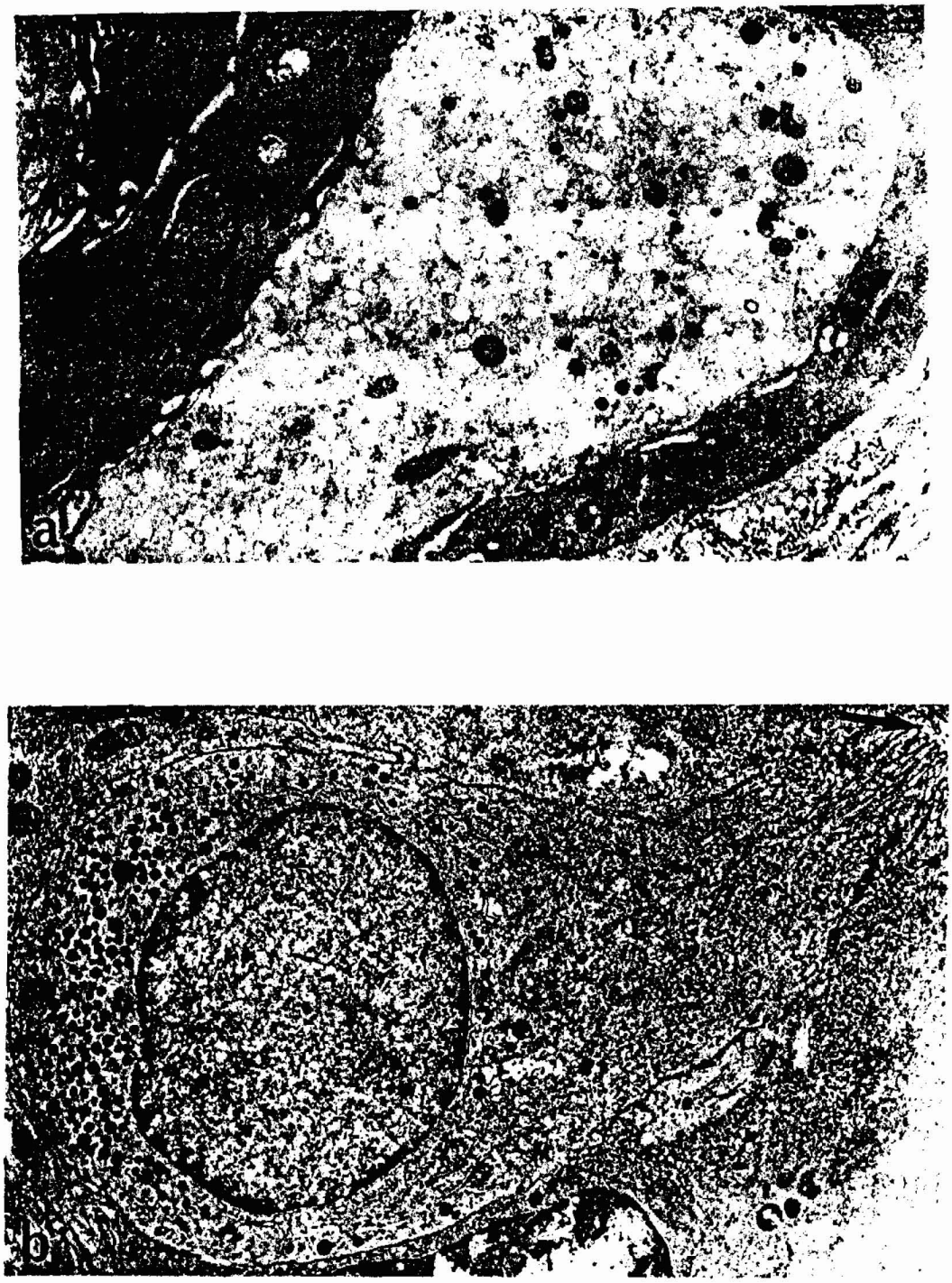


Fig. 7. (a) Antral G cell, Note the numerous electron-lucent vesicles. × 12,000
(b) Intestinal gastrin D_1 type cell. Note connection with the lumen (arrow). × 12,000

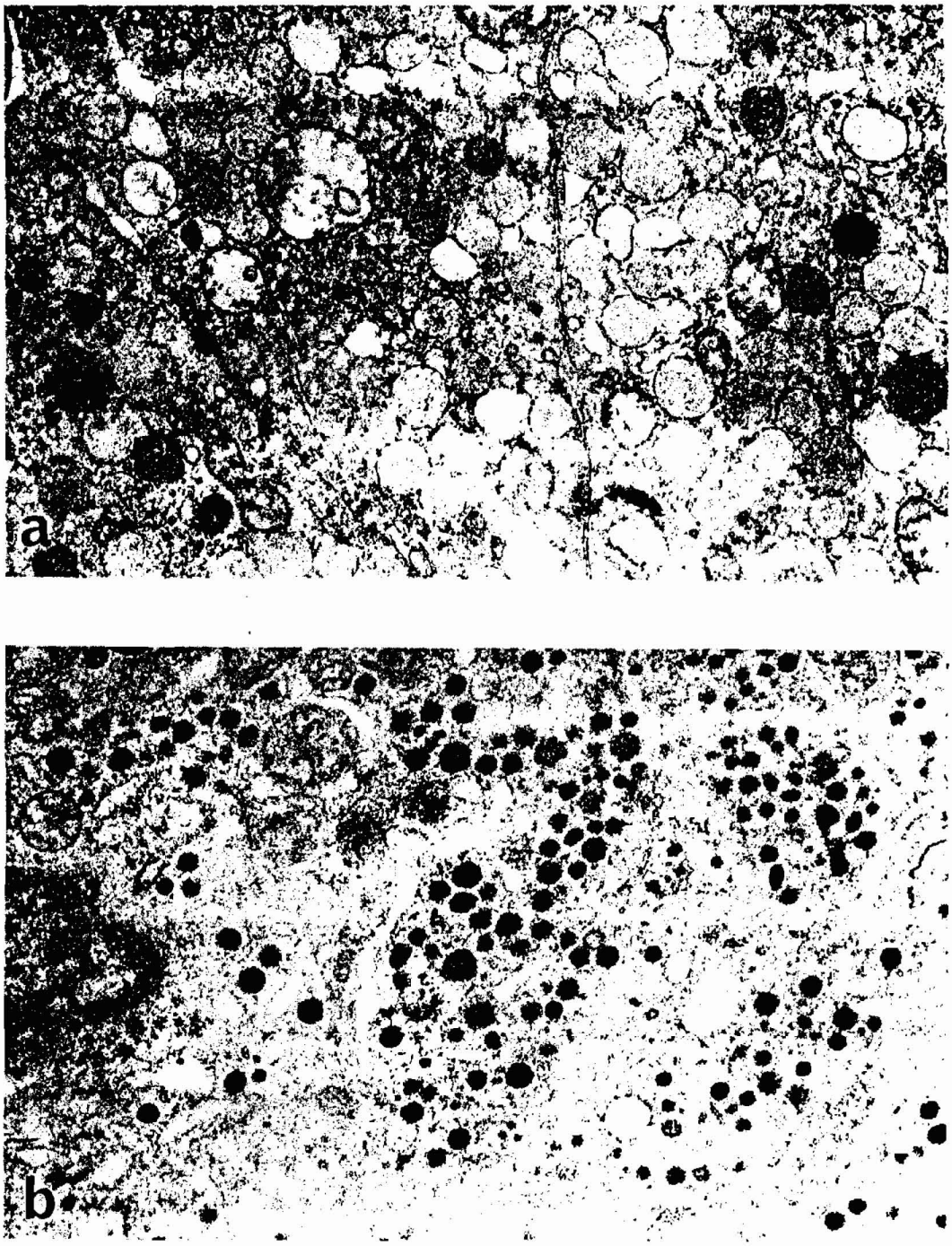


Fig. 8. (a) Secretory granules in a tumour producing predominantly gastrin 17. × 30,000
(b) Secretory granules in a tumour producing predominantly gastrin 34. × 30,000

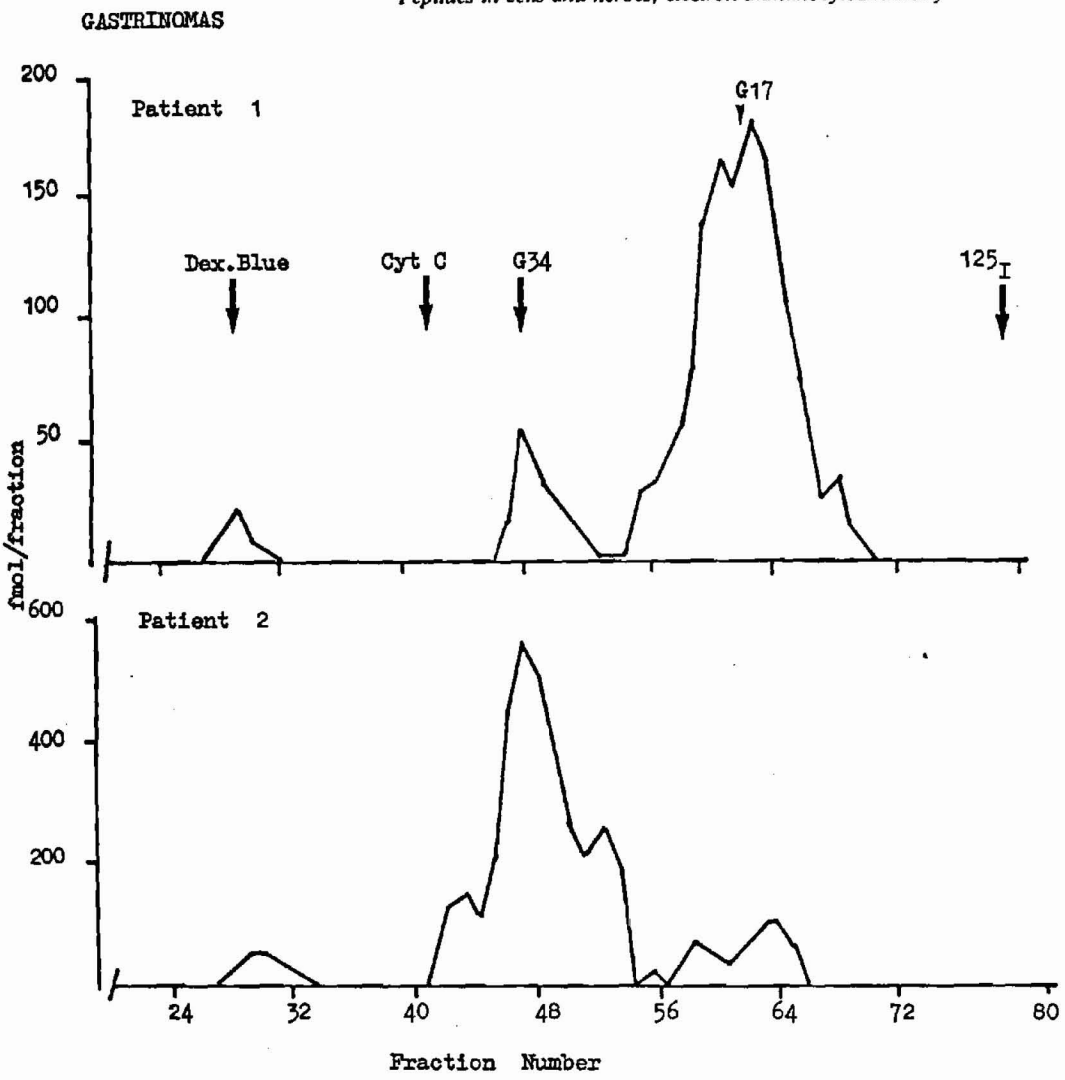
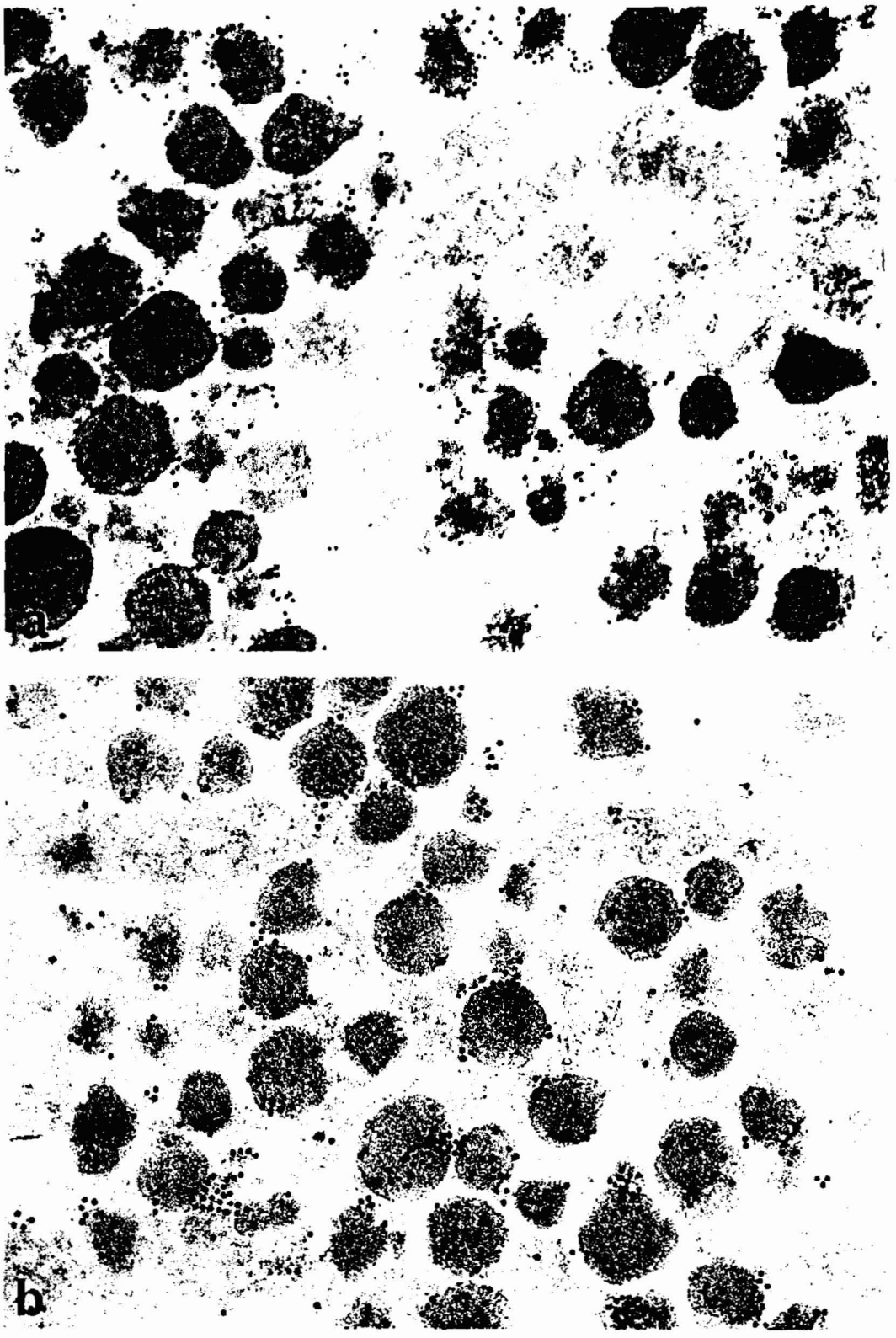


Fig. 9. Gel chromatography of tissue extracts from two gastrinomas. Patient 1. 10% gastrin 34 and 90% gastrin 17 (see Fig. 8a). Patient 2. 90% gastrin 34 and 10% gastrin 17 (see Fig. 8b). Column calibration
Dex. Blue = Dextran Blue
Cyt. c = Cytochrome c
125_I = Iodine 125.

with pro-glucagon, the precursor hormone of pancreatic glucagon. Using electron immunocytochemistry with the immunogold method and antibodies to glicentin (pro-glucagon) and glucagon, these two different molecular forms of the peptide have been localised to different areas of the pancreatic A cell granule (19). Glicentin is present in the outer (halo) portion of the secretory granules and glucagon, in the core (Fig. 10). This may indicate that some of the post-transitional enzymatic processes involved in converting the pro-hormone (glicentin) into the smaller more active form (pancreatic glucagon) have taken place prior to granule packaging.

The core of the glucagon granule had previously been distinguishable from the outer halo by its distinct reactivity to the Grimelius silver impregnation but the underlying difference in peptide content of the two areas was not understood (9).


a
b

D) *Functional classification of p (peptidergic)-type autonomic neurosecretory granules*

In 1970 Baumgarten and his co-workers described autonomic nerves of a new class in the gastrointestinal tract (1). The nerves were characterised by the presence of ultrastructurally distinct neurosecretory granules, which were larger and more electron-dense than the vesicles associated with the classical neurotransmitters, acetycholine and noradrenalin.

Baumgarten termed these granules p-type because of their resemblance to the peptidergic neurosecretory granules of the posterior pituitary which contained the peptides vasopressin and oxytocin. At the time of their description Baumgarten had little suspicion of the imminent discovery of a massive peptidergic component of the autonomic nervous system which occurred from 1976 onwards.

Baumgarten's description was followed by the ultrastructural observations of Cook and Burnstock of significant differences between the newly recognised p-type nerves (7). At least 3 types were then recognised. Our recent observations using immunocytochemistry at the electron microscopial level fully support Burnstock's claim of a marked heterogeneity among the non-adrenergic, non-cholinergic (p-type) components of the autonomic nervous system. For example, substance P is an eleven amino acid peptide which is known to be a powerful regulator of gastrointestinal functions. Using the immunogold 'on grid' staining procedure we have been able to localise substance P to a sub-population of p-type nerves in the guinea pig colon (17). These substance P-containing nerves are characterised by the presence of round neurosecretory granules of medium electron density with a distinct halo between the core and the limiting membrane, which were classified by Cook and Burnstock as Type 5b (Fig. 11).

Preliminary observations seem to indicate that vasoactive intestinal polypeptide (VIP), a twenty-eight amino acid brain and gut peptide, is present in a different sub-population of p-type nerves (18). These are characterised by a preponderance of small, agranular vesicles intermingled with large, dense round granules (Type 5c of Cook and Burnstock), that are specifically labelled by VIP antibodies using the peroxidase anti-peroxidase procedure carried out on vibrotome sections before embedding.

Systematic analysis of ultrathin sections immunostained for substance P and VIP shows a significant proportion of distinct p-type nerves which remain unstained by either substance P or VIP antibodies (18). These findings are in keeping with the widely accepted view that there are many peptides, other than VIP and substance P, in the p-type nerves of the gut, e.g. enkephalin, TRH, CCK, bombesin, neurotensin and somatostatin.

CONCLUSIONS

Early ultrastructural studies using conventional methods revealed the existence in the gut of numerous endocrine cell types characterised by the presence of distinct secretory granules, suggesting the production of a wide variety of active peptides. Thus, electron microscopists stimulated the successful search for peptide hormones and their chemical characterisation. To date more than 25 regulatory peptides have been identified. Immunocytochemistry has become the 'obligatory' tool for investigating their cellular localisation. Advances in the technique, in particular its successful use at the electron microscopial level have led to the more functional classification of the endocrine cells of the gut. Increasing emphasis is placed on establishing the type of peptide produced rather than relying solely on the size and shape of the intracellular secretory granules.

Electron immunocytochemistry has, in addition, contributed to the recognition of new endocrine cell types previously included as part of a poorly understood group of cells (e.g. intestinal gastrin and motilin cells were previously grouped together under the general term of D_1 cell).

Although in its infancy, the classification of the

Fig. 10. A-cell granules from human pancreas immunostained using gold-labelled antibodies.
(a) Gut glucagon (glicentin). The immunoreactivity is localised to the halo of the granules. × 60,000
(b) Pancreatic glucagon. The immunoreactivity is localised to the core of the granules. × 60,000

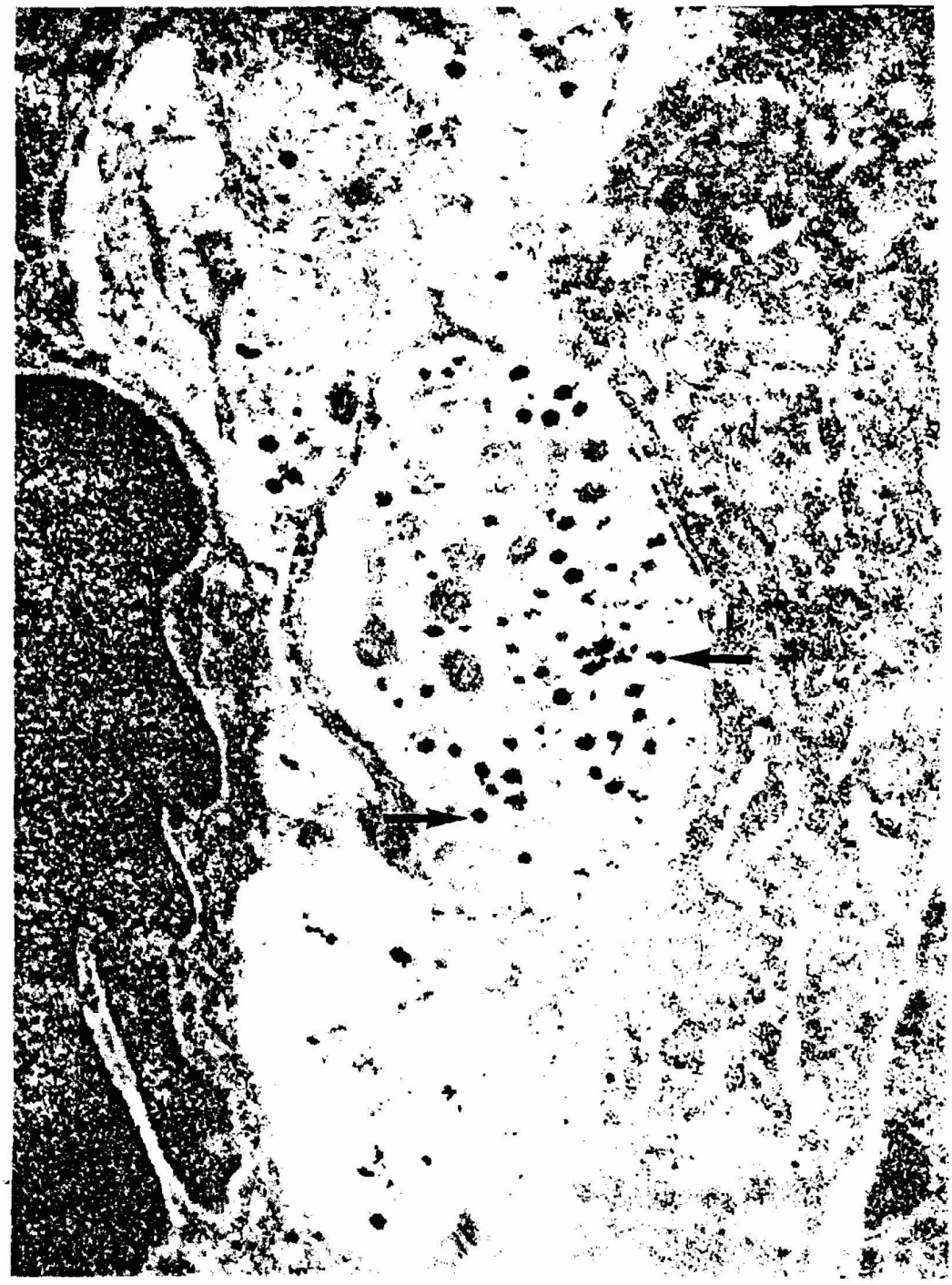


Fig. 11. Nerve terminal in the myenteric plexus of the guinea pig colon, showing substance P-like immunoreactivit Antibodies were labelled with colloidal gold particles of 20 nm diameter. Note the concentration of the gold lab over the granular secretory vesicles. × 35,000

autonomic nerves of the gut, especially of the p-type, into distinct groups is beginning to be a possibility. Substance P-, in particular, and VIP-containing terminals can now be distinguished from the rest of the p-type peptidergic nerves which have yet to be identified. The future looks exceedingly promising. The availability of highly specific 'monoclonal' antibodies seems a near reality and with it the opportunity for different groups of scientists to compare their findings. The use of antibodies labelled with colloidal gold particles of different sizes will allow the accurate demonstration of separate antigens (peptides in cells and/or nerves) in a single tissue section.

Quantification of immunocytochemical staining at the ultrastructural level is advancing at a rapid pace. We shall soon be able to obtain precise information on the amount of hormone release from a cell as well as the amount stored. The years to come promise exciting new vistas.

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