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Immunocytochemical and ultrastructural characterization of endocrine cells in chicken proventriculus

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Summary. The endocrine cells of the chicken proventriculus were investigated immunocytochemically, using the peroxidase-antiperoxidase technique on paraffin and semithin sections for light microscopy, and immunogold staining in osmium-fixed material for electron microscopy. The fixation procedure also allowed a detailed ultrastructural investigation. Twenty-three antisera were tested and 7 immunoreactive cell-types were identified: D-cells containing somatostatin-like peptide; EG-cells immunoreactive to anti-glucagon, anti-GLP1 and anti-neurotensin; NT-cells labelled only with anti-neurotensin; BN-cells containing bombesin-like material; ENK-cells showing met-enkephalin immunoreactivity; EC-cells reactive to anti-serotonin; and APP-cells positive to anti-avian pancreatic polypeptide. In addition, enterochromaffin-like (ECL) cells, were also detected by electron microscopy. The presence of ENK-cells and the ultrastructure of these and NT-cells are described for the first time in chicken proventriculus, and glucagon, GLP1 and neurotensin are shown to be colocalized in the EG-cells.

Key words: Proventriculus – Endocrine secretory cells – Secretory granules – Peptide hormones – Colocalization – Immunocytochemistry – Colloidal gold – Chicken

During the past few years the endocrine cells of the chicken proventriculus have been studied both by light (Vaillant et al. 1979; Rawdon and Andrew 1981; Yamada et al. 1985; Alison 1989) and electron microscopy (Alumets et al. 1977; Andrew et al. 1982; Usellini et al. 1983; Rawdon 1984). Six hormones have so far been identified in this tissue using immunohistochemical techniques namely, glucagon (Rawdon and Andrew 1981; Usellini et al. 1983), somatostatin (Alumets et al. 1977; Rawdon and Andrew 1981; Usellini et al. 1983; Rawdon 1984), bombesin (Timson et al. 1989; Vaillant et al. 1979; Rawdon and Andrew 1981; Usellini et al. 1983), serotonin (Usellini et al. 1983), avian pancreatic polypeptide (Alumets et al. 1978; Rawdon and Andrew 1981), and neurotensin (Rawdon and Andrew 1981; Usellini et al. 1983). However, the ultrastructural characterization of only two cell-types have been reported to date (Timson et al. 1979; Usellini et al. 1983).

In the present study, light and electron microscopy, combined with immunocytochemical and immunogold techniques in osmium tetroxide fixed material, have been used to investigate the ultrastructure of the endocrine cells in chicken proventriculus, in an attempt to improve our knowledge of the peptide-hormone-producing cells and their ultrastructural characteristics in birds.

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Materials and methods

Thirty-five newborn chicks from a local farm, were used. The animals were anesthetized with ether. Their proventriculi were then removed and immersed in fixative for subsequent investigation by light and electron microscopy.

Paraffin embedding (light microscopy)

For conventional microscopic study, the proventriculi of 10 animals were cut into small pieces which were then fixed in Bouin's fluid for 24 h, and then placed in 70% ethanol. The pieces were subsequently dehydrated by means of a graded ethanol series and embedded in paraffin. Sections were stained with hematoxylin-eosin, PAS, and Masson trichromic. In addition, some sections were subjected to the peroxidase-antiperoxidase (PAP) immunocytochemical technique according to the method used by Sternberger (1979).

Resin embedding (light microscopy and electron microscopy)

Small pieces (1 mm^3) of the proventriculus of 18 specimens were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer,

Table 1. Antisera used in incubations

Antisera	Source	Code	Result
Somatostatin 14	Immunonuclear	20-H2T	+
Somatostatin 28	Professor Polak	1198	−
Glicentin	Milab	B37–100	+
Glicentin	Professor Polak	468	−
Glucagon	Immunonuclear	33-H2T	+
GLP1	Professor Polak	1642	+
GLP2	Professor Polak	1482	−
GIP	Milab	B35-1	+
Neurotensin	Milab	B44–100	+
Human PP	Miles Sci.	64-711-1	+
Avian PP	Professor Kimmel		+
Bombesin	Immunonuclear	30-H2T	+
Met-enkephalin	Immunonuclear	18-H2T	+
Serotonin	Immunonuclear	43-H2T	+
Substance P	Immunonuclear	17-H2T	−
Motilin	Professor Polak	932	−
Gastrin 17	Immunonuclear	05-H2T	−
Gastrin 34	Immunonuclear	55-H2T	−
CCK 10–20	Milab	B38–100	−
CCK 8	Immunonuclear	42-H2T	−
VIP	Immunonuclear	39-H2T	−
Secretin	Milab	B33–100	−
Histamin	Peninsula	61069	−

pH 7.2, at 4° C for 5 h. The material was postfixed in 1% phosphate-buffered osmium tetroxide, pH 7.2, at 4° C for 2 h. Pieces of proventricular tissue from a further 7 specimens were fixed in 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer for 2 h (Priestley 1984).

All the pieces, both osmicated and nonosmicated, were then dehydrated with an ethanol series, washed in propylene oxide, then embedded in Epon 812.

Semithin 1 μm-thick, sections, were cut and transferred onto glass slides, and the Epon entirely removed with sodium methoxide (Mayor et al. 1961) or, in those intended for immunocytochemistry, with aged saturated sodium hydroxide in ethanol (Lane and Europa 1965). Some deplasticized sections were stained with borated methylene blue, and others were used for the PAP technique.

For the ultrastructural investigation, suitable ultrathin sections were selected, then double stained with uranyl acetate and lead hydroxide and examined with a Zeiss EM10CR electron microscope.

Immunocytochemistry

Paraffin sections were treated according to the PAP technique (Sternberger 1979), using a variety of antisera (Table 1). Background blocking was performed with normal goat serum (Table 2), prior to incubation with the specific antiserum (Table 1). Incubation was carried out for 16–20 h at 4° C. After rinsing in TRIS buffered saline (TBS), the sections were incubated in goat anti-rabbit IgG. Following a secondary rinse in TBS, the sections were incubated in rabbit-PAP and after a final rinse, were visualized with diaminobenzidine (50 mg per 100 ml) and H_2O_2 (60 μl/100 ml); development time was 0.5–2 min.

The semithin Epon-embedded sections, after deplasticization according to Lane and Europa (1965), were rinsed in absolute ethanol, then hydrated in graded ethanol and washed twice in TBS 0.1 M at pH 7.6. The sections were incubated for 1 h in saturated sodium metaperiodate in double-distilled water and TBS. The PAP technique was then applied. The primary antisera were used at dilutions ranging from 1:500 to 1:3000.

Table 2. Antigens and reagents

	Source	Code
Antigens		
Somatostatin	Sigma	S-9129
Glucagon	Sigma	G-4250
GIP	Sigma	G-5512
Neurotensin	Peninsula	7351
Human PP	Sigma	P-9903
Bombesin	Sigma	B-5508
Met-enkephalin	Sigma	M-6638
Serotonin	Sigma	H-5755
Other reagents		
Normal goat serum	University of Navarra	NG-1
Goat anti rabbit	University of Navarra	GB-1
Rabbit PAP	Dakopatts	Z113
Bovine serum albumin	Sigma	B-2518
Goat anti rabbit IgG-gold, 15 nm	E.Y. Labs.	GAF-012

Immunogold staining

The ultrastructural immunolabeling was carried out according to De Mey et al. (1981). Thin sections of osmicated material were mounted on nickel grids and incubated in saturated sodium metaperiodate for 1 h, followed by 3 washes in double-distilled water. Then, sections were exposed to a 1% bovine serum albumin (BSA) in TBS for 30 min, followed by incubation at 4° C with the primary antisera for 24 h. Subsequent steps, all at room temperature, included rinses in 1% BSA-TBS; incubation with goat anti-rabbit IgG-gold colloidal particles, 15 nm (Table 2) for 45 min; rinses with 1% BSA-TBS and double-distilled water, and double staining for 15 min in 7% aqueous uranyl acetate and 10 min in lead hydroxide.

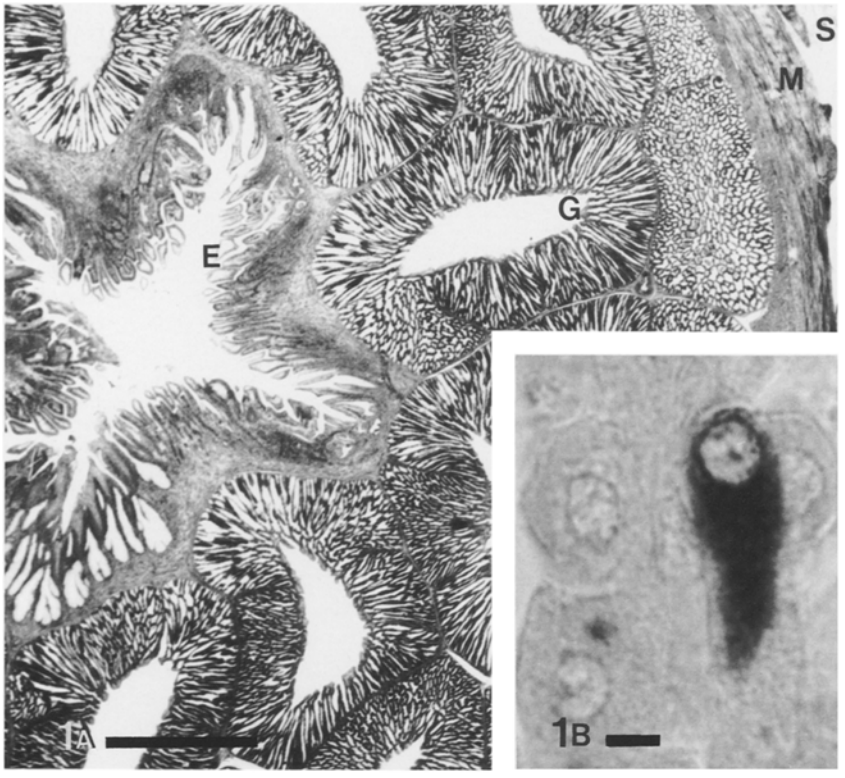
Controls

Immunocytochemical controls included: (a) Replacement of the anti-serum by nonimmune rabbit serum in the incubation medium; (b) Positive control with mammalian pancreas, gut and brain; (c) Preabsorption of the antibody with an excess of the corresponding antigen (homologous or heterologous), 1–10 nmol per ml of diluted antiserum for 18 h at 4° C, prior to the immunocytochemical labeling; (d) Omission of the first stage; and (e) Staining using DAB/H_2O_2 substrate alone, and colloidal-gold labels alone.

Fig. 1. A Section of chicken proventriculus. *E* Superficial epithelium; *G* compound glands in submucosa; *M* double muscle layer; *S* serosa. Masson trichromic. ×20. *Bar*: 1.0 mm. **B** Oxyntic-peptic cells lining a tubular gland. An APP-immunoreactive cell in contact with the basal lamina is observed. PAP. ×1500. *Bar*: 5.0 μm

Fig. 2. Ultrastructure of bombesin-containing cell: *G* Golgi complex; *M* mitochondria; *R* rough endoplasmic reticulum; *C* centriole; *H* hormone-containing granules. ×11700. *Bar*: 1.0 μm

Fig. 3. A D-cell delivering the contents of a granule to the basal membrane. ×6030. *Bar*: 2.0 μm. **B** Detail of **A**. ×28800. *Bar*: 0.5 μm. **C** Somatostatin-reactive cell in a semithin section. ×1500. *bar*: 5.0 μm. **D** The same cell as in **C**, in a serial section labeled with anti-somatostatin immunogold. ×9270. *Bar*: 1.0 μm. **E** Detail of **D**. ×28800. *Bar*: 1.0 μm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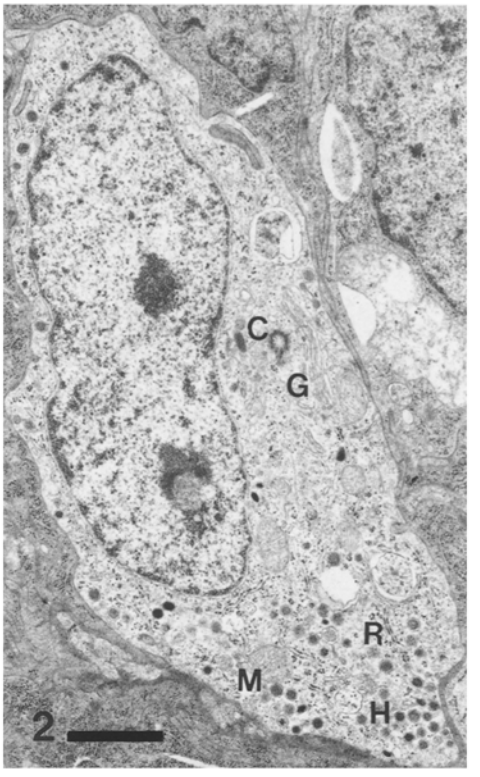
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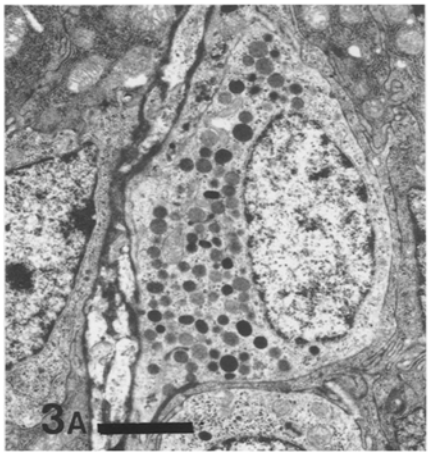
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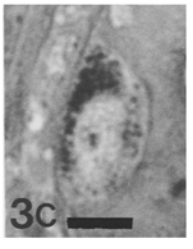
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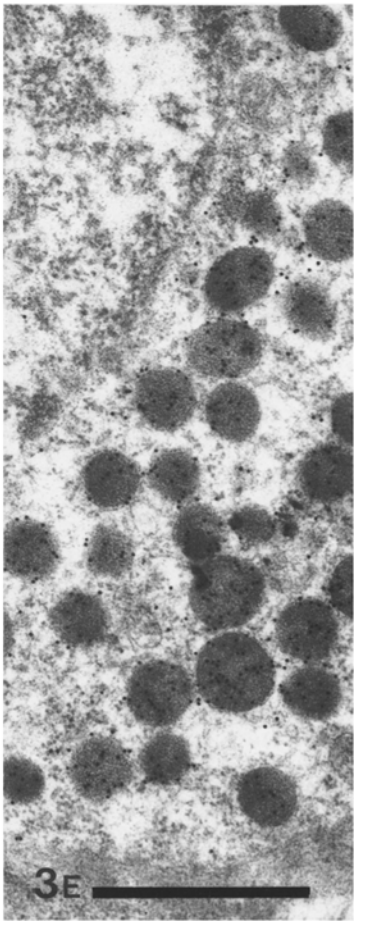
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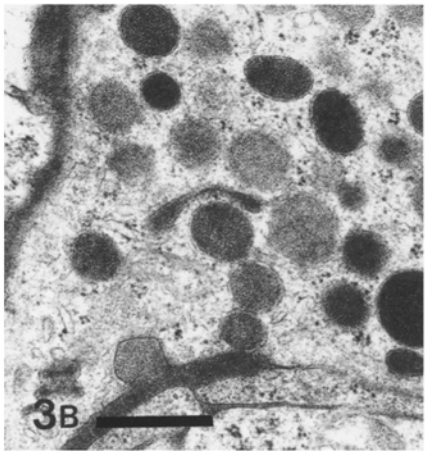

3B

Characterization of the secretory granules

Electron micrographs of the same magnification (×16000) were used to determine the size of the hormone-containing granules. The mean diameter of the biggest granules in each cell was calculated and then multiplied by the appropriate correction factor (Martínez et al. 1989). The number of granules measured depended on the amount of granules present in the cell; an eighth of the total if the cell had enough granules or a fourth if the number was smaller.

Results

The chicken proventriculus consists of 4 layers (Fig. 1A), namely, a superficial columnar epithelium composed of mucosecretory cells lining the luminal surface and crypts, a submucosa with numerous tubular compound glands which open into a central secretory space and which are lined by oxyntic-peptic cells, an underlying double muscle layer, and finally, a serosa layer covering the organ.

The endocrine cells appear in close contact with the basal lamina among the mucous and oxyntic-peptic cells (Fig. 1B), and in many cases show long cytoplasmic processes. They are more abundant deep in the tubular glands and crypts. The endocrine cells belong to the "closed" type since contact with the lumen has not been observed in any case.

Ultrastructurally, the endocrine cells are identified by the presence of numerous specific hormone-containing granules in a basal location, an ovoid nucleus with one or two small nucleoli, a well-developed Golgi complex, some mitochondria and profiles of rough endoplasmic reticulum. Usually a centriole is present (Fig. 2).

According to the structure and immunostaining properties of the secretory granules, seven immunoreactive cell-types can been characterized (Table 3):

D-cells (Fig. 3). These contain somatostatin-like peptide and are positive to anti-somatostatin 14 but do not react with specific anti-somatostatin 28. They have the largest secretory granules, 360 nm in diameter. The granules are round or lightly polyhedric with a variable electron-dense, finely granular core.

EG-cells (Figs. 4–6). The granular content reacts with anti-pancreatic glucagon, anti-glicentin (Milab), anti-GIP, anti-GLP1 and anti-neurotensin. The absorption controls show that anti-glicentin (Milab) and anti-GIP crossreact with the glucagon molecule, since the incubation of antisera with pancreatic glucagon precludes the staining (a more specific anti-glicentin provided by Professor Polak, does not react with this cell type); conversely anti-neurotensin and anti-GLP1 are not blocked by pancreatic glucagon and viceversa (Fig. 4). Homologous absorptions with all the antisera tested fail to give a positive result in any cell.

The secretory granules are round or irregular, 280 nm in diameter, with a homogeneous, thin-haloed, electron-dense content. Moreover, numerous mitochondria and a Golgi complex with swollen components are present in these cells.

Table 3. Relative concentrations of immunoreactive cell-types in different areas of the chicken proventriculus. Region I: luminal epithelium. Region II: apical zone of the tubular glands. Region III: bottom of the tubular glands. +++, Very numerous; ++, moderately numerous; +, few; + −, very small quantity; −, absent

Cell-type	Region I	Region II	Region III
D-cells	+++	+++	++
EG-cells	++	++	+++
NT-cells	++	++	+++
BN-cells	+++	++	+
ENK-cells	+	+	+ −
EC-cells	+	+	+ −
APP-cells	−	+ −	+ −

NT-cells (Fig. 7). Anti-neurotensin labels these cells (Fig. 7B) as well as EG-cells, nevertheless anti-glucagon never stains them. The cytoplasm is filled with granules, 200 nm in diameter, that contain an heterogeneous, electron-dense material and display an electron-lucent halo which separates the contents from the limiting membrane.

BN-cells (Fig. 8). PAP and immunogold staining show immunoreactivity with anti-bombesin. These cells appear more frequently in the crypts, but occasionally, are also found in the glands. Long cytoplasmic processes can be seen in these cells (Fig. 8A). The granules, 150 nm in diameter, are the smallest ones found in chicken proventriculus endocrine cells; they are round, thin-haloed, and contain a moderately electron-dense matrix.

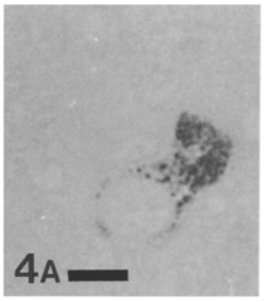
ENK-cells (Fig. 9). These give a positive reaction to anti-met-enkephalin serum, and are found only in the mucosecretory epithelium. The granules are round, small (190 nm) and contain electron-dense material.

Fig. 4A–F. Absorption controls in semithin serial sections demostrating the coexistence of glucagon and neurotensin immunoreactivity in the same cell. **A–C** The same cell stained with (**A**) anti-glucagon, (**B**) anti-neurotensin, and (**C**) anti-neurotensin preincubated with glucagon. **D–F** Another cell stained with (**D**) anti-glucagon, (**E**) anti-glucagon preincubated with neurotensin, and (**F**) anti-neurotensin. ×1500. *Bar*: 5.0 μm

Fig. 5. A EG-cell reactive to anti-pancreatic glucagon. ×650. *Bar*: 20 μm. **B** The same cell labeled by the same antiserum. The *arrow* points to a centriole with an outstanding rootlet. ×6030. *Bar*: 2.0 μm. **C** Detail of **B**. ×28800. *Bar*: 0.5 μm. **D** EG-cell labeled by anti-neurotensin. ×72000. *Bar*: 0.1 μm

Fig. 6A, B. Serial sections stained with anti-GLP1 (**A**) and anti-glucagon (**B**). The same cell is present in both sections ×600. *Bar*: 20.0 μm

Fig. 7. A NT-cell containing numerous secretory granules with an electron-lucent halo. ×9270. *Bar*: 1.0 μm. **B** Same granules labeled with anti-neurotensin immunogold. ×72000. *Bar*: 0.1 μm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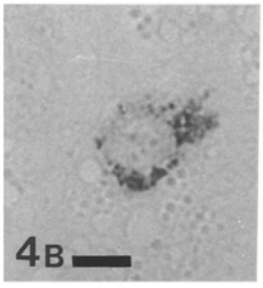
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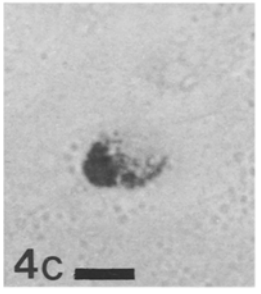
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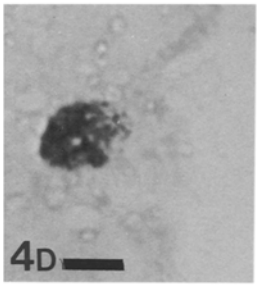
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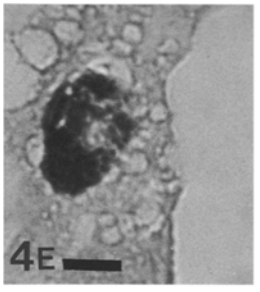
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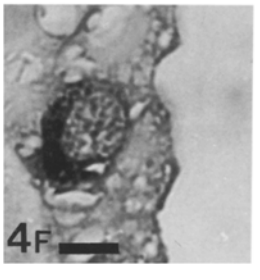
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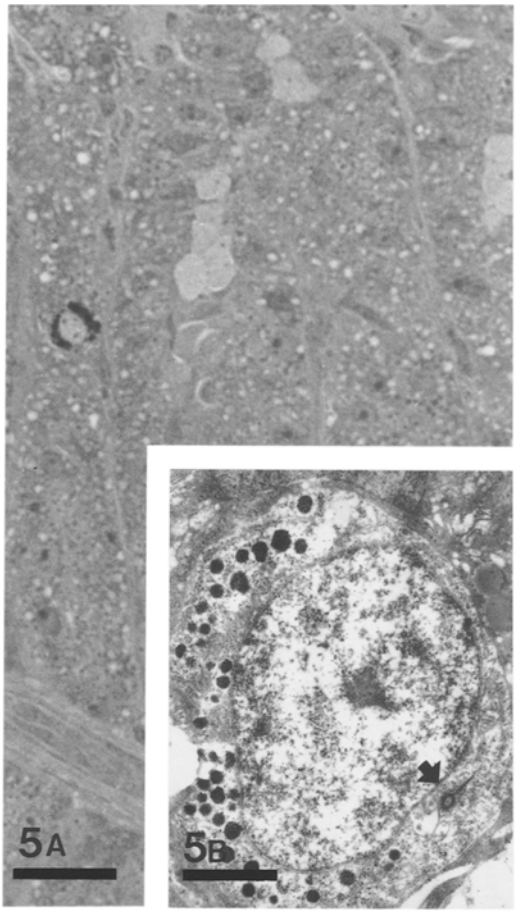
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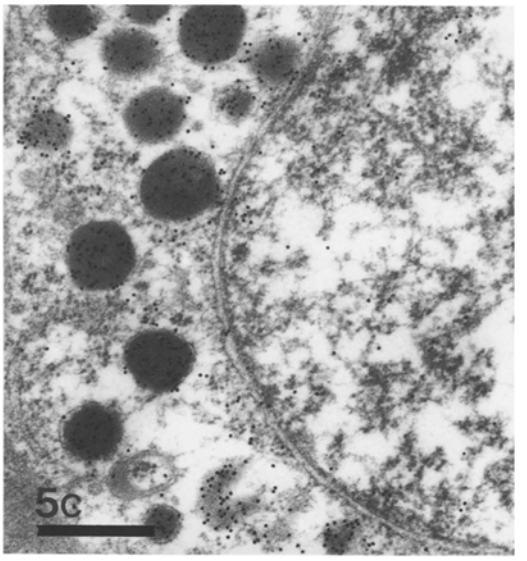
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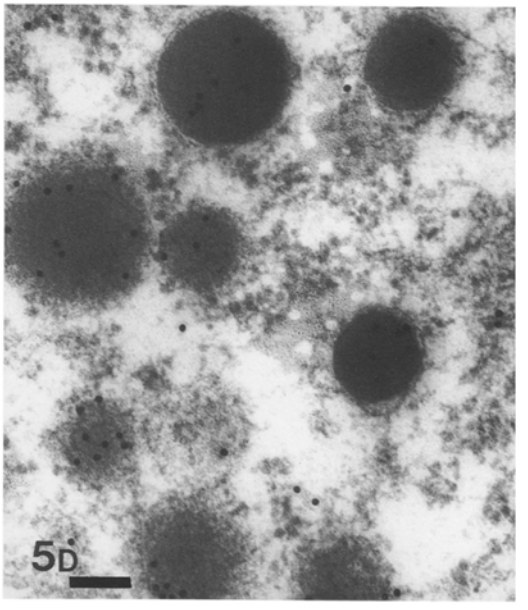
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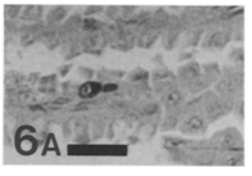
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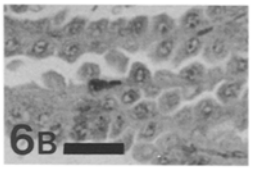
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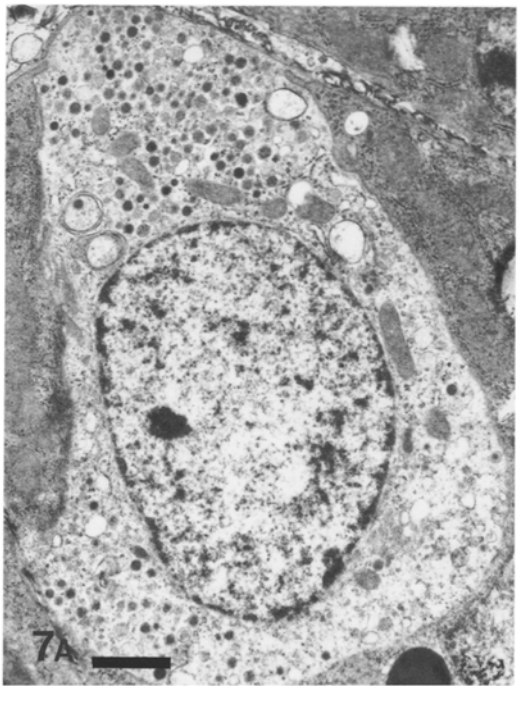
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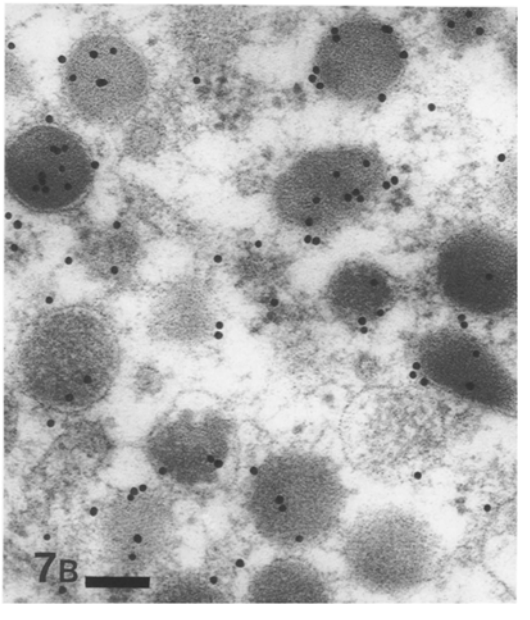
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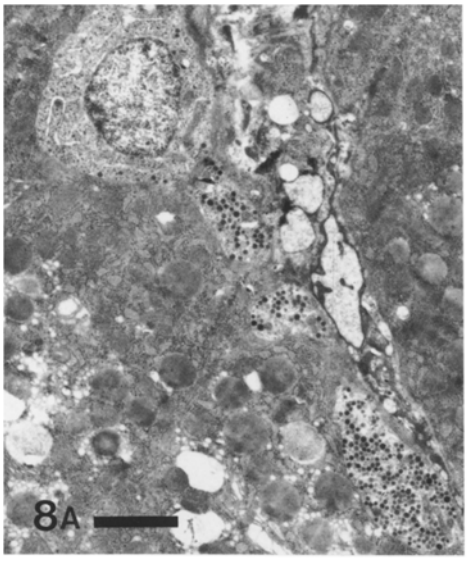


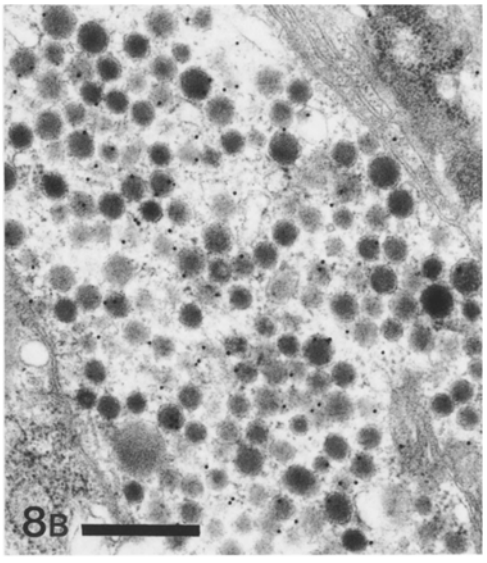
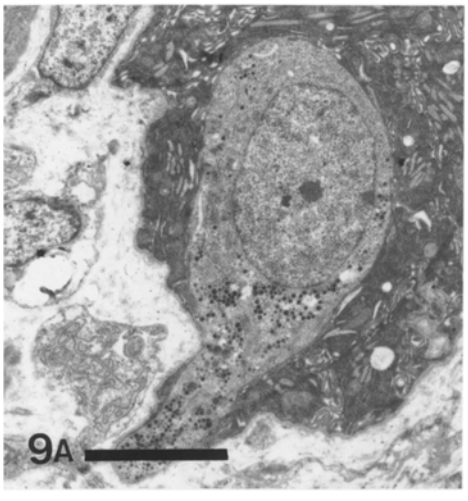


Fig. 8. A BN-cell possessing a long cytoplasmic process. Granules are reactive to antibombesin serum. ×5300. *Bar*: 2 μm. **B** Detail of **A**. ×28800. *Bar*: 0.5 μm



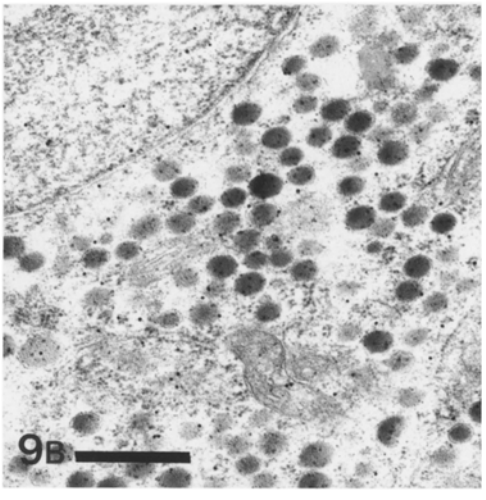
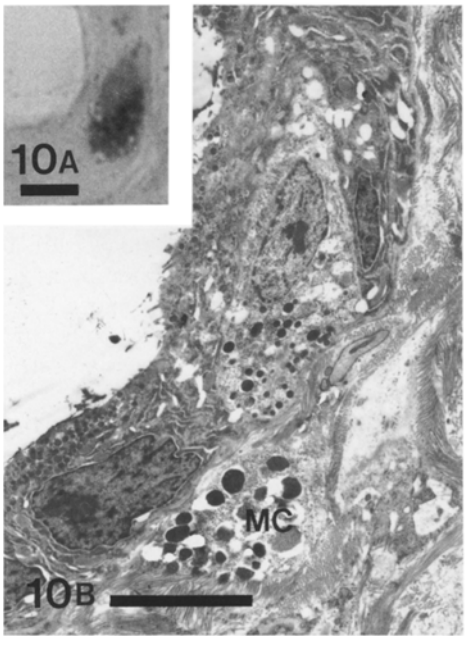


Fig. 9. A Met-enkephalin-immunoreactive cell in close contact with the basal membrane. ×3780. *Bar*: 5.0 μm. **B** Detail of the secretory granules labeled with gold particles. ×28800. *Bar*: 0.5 μm



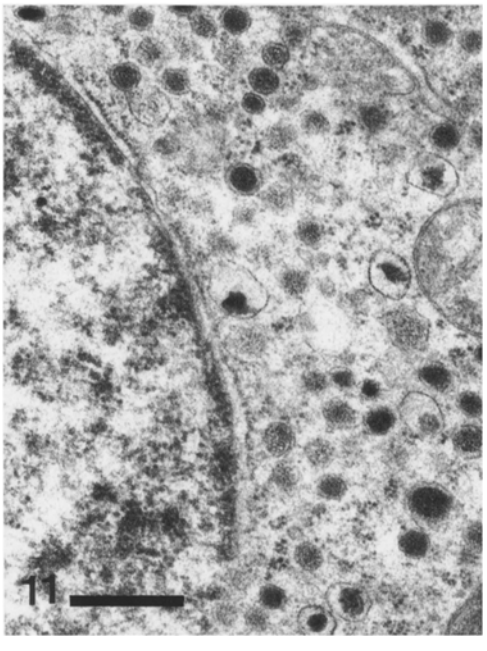


Fig. 10. A Anti-serotonin-positive cell in a crypt. ×1500. *Bar*: 5.0 μm. **B** The same cell in a thin section. Characteristic EC-granules can be seen in the cytoplasm. *MC* Mast cell. ×3780. *Bar*: 5.0 μm

Fig. 11. ECL-cell with the cytoplasm filled with vesicular granules. ×28800. *Bar*: 0.5 μm

EC-cells (Fig. 10). These serotonin-immunoreactive cells have been identified in semithin-thin sections, but do not show a clear result with the immunogold technique. Their strongly osmiophilic secretory granules are predominantly round and have a diameter of 300 nm, but some ovoid or elongated granules are also present.

APP-cells (Fig. 1 B). These are positive to anti-avian pancreatic polypeptide and, very slightly, to anti-human PP. They are observable only in the paraffin sections owing to their small quantity.

ECL-cells (Fig. 11). Those are very similar to the enterochromaffin-like cells observed in mammals, and represent only a small proportion of the proventricular endocrine cell population. Their characteristic vesicular granules allow their identification only by electron microscopy. Their granular contents range from loose and flocculent material to a core of varying density, often eccentrically located within the vesicle. The core is 132 nm in diameter and the vesicle can be as much as 290 nm.

Discussion

In this study the ultrastructural identity of several endocrine cell-types has been demonstrated in chicken proventriculus using the immunogold staining technique applied to osmium-fixed material. Furthermore, met-enkephalin-like immunoreactivity has been shown for the first time in this organ, and has been matched to a specific cell-type; the coexistence of glucagon-, GLP1- and neurotensin-like material in the same cell-type has also been described.

Six hormones have, to date, been identified in the chicken proventriculus, but only two have been correlated with specific cell-types: bombesin-like (Timson et al. 1979) and enteroglucagon-like cells (Usellini et al. 1983). The present observations confirm these descriptions, and additionally, provide evidence demonstrating the presence of further cell-types associated with the remaining hormones.

D-cells have the largest secretory granules and are the most abundant cell-type in the proventriculus, as is also the case in the mammalian stomach (Rawdon 1984). This abundance is due to their important role in gastric physiology; somatostatin is known to inhibit gastric acid secretion in mammalian (Barros D'Sa et al. 1975), probably acting as a modulator of oxyntic-peptic cells. Furthermore, a close similarity has been found between avian and mammalian somatostatin (King and Millar 1979; Crim and Vigna 1983). The results of both antisera suggest that in the avian proventriculus only somatostatin 14 is produced.

The ultrastructure of EG-cells is similar to that observed in mammals (Larsson et al. 1975). They contain at least 3 peptides in their granules; glucagon-, GLP1- and neurotensin-like. This first report of colocalization of these 3 peptides in proventricular-EG-cells is in agreement with the previously known coexistence of neurotensin and gastrin in the chicken pylorus (Rawdon and Andrew 1981). Kirkegaard et al. (1982) have found that glucagon and other related peptides act as powerful inhibitors of gastric acid secretion and this fact could explain the abundance of EG-cells in the proventriculus. The presence of larger molecular weight forms of the glucagon family has been suggested in the chick (Rawdon 1984), and chromatographic studies have revealed 3 neurotensin molecular forms (Carraway and Bhatnagar 1980), but the physiological role of this hormone is not well known.

Although neurotensin appears in EG-cells, it is also found exclusively in the granules of the NT-cells. This hormone has been described in chicken proventriculus (Rawdon and Andrew 1981) and also in other birds (Yamada et al. 1985), but it is remarkable that this immunoreactivity can be detected in two different cell-types. Sundler et al. (1977) have described the ultrastructure of the neurotensin-immunoreactive cells in chicken antrum, but their secretory granules are bigger than those in the proventricular cells noted in the present study.

BN-cells have the smallest granules among the proventricular endocrine cells, and their ultrastructure, as seen in the present study, confirms that described in previous reports (Timson et al. 1979) using semithin-thin section techniques.

In mammals, bombesin-like immunoreactivity is located principally in nerves (Polak et al. 1978), but in avian and amphibian gut bombesin is linked with endocrine cells (Rawdon 1984). A similar pattern is also observed for other peptides (Anderson and Campbell 1988). Thus, the present results demonstrate that met-enkephalin immunoreactivity occurs in the proventricular tissue. Previously, King and Millar (1980) reported the presence of this hormone in chicken intestine, and the present data is the first report of its presence in the proventriculus. In mammalian EC-cells, enkephalin has been found in addition to serotonin (Alumets et al. 1978), but in our results these two regulatory peptides appear in different cell-types.

EC-cells are immunoreactive to anti-serotonin and this hormone has been reported in some avian species (Yamada et al. 1985). In the chicken this cell-type is numerous in the intestine but is scarce in the proventriculus (Rawdon 1984). In mammals, in addition to serotonin, EC-cells may also contain substance P, enkephalin or motilin (Rawdon 1984), but in the present study we did not find such mixed immunoreactivity; substance P and motilin were not present and the localization and ultrastructure of serotonin- and enkephalin-containing cells were very different.

APP-cells are found in the pancreas and intestine, and their ultrastructure in pancreas has been described by Alumets et al. (1978). Ultrastructural characterization of these cells in the present study on the proventriculus was not possible because of their sparsity and dispersion, even though they could be identified in the wax-embedded tissue. ECL-cells, on the other hand, could easily be characterized by the ultrastructural appearance of their cytoplasmic granules. These latter cells have been proposed as the source of histamine (Hakanson et al. 1986), but we have found no immunoreactive cells

to this antiserum. Usellini et al. (1983) describe a striking variation in the number of these cells; ECL-cells are present in very low quantities at hatching, but they increase to the point of being the predominant endocrine cell-type in the 21-day-old chicken. These authors relate the late development of ECL cells with the poor acid secretion in the proventriculus of newly hatched chicks.

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