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Endocrine cells of the gastric mucosa of *Rana temporaria* L.

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Summary. The endocrine cells of the gastric mucosa of *Rana temporaria* have been studied according to the ultrastructure, the staining properties of the granules with Masson Fontana's and Grimelius' silver methods, silver impregnation of Davenport on deplasticised semithin sections and immunocytochemical techniques. Seven different types of endocrine cells have been described. Six were regarded as belonging to known types: G, A, EC, ECL, D and P cells. One type was considered as unclassifiable.

Key words: Gastric mucosa -Frog -Electron microscopy- Endocrine cells -Immunocytochemistry

Introduction

The first light microscope studies on endocrine cells of the gastrointestinal tract are those by De Filippi (1930), Citterio (1935) and Camisani (1941), who identified enterochromaffin cells (EC) in some species of anurans and urodeles. Gabe (1972) described, histochemically, four types of endocrine cells in the gastroduodenal mucosa of 16 species of Amphibia, *Rana temporaria* among them. Two cell types were found in the stomach, enterochromaffin-like (ECL) cells and gastrin (G) cells, and two others in the duodenum, enterochromaffin (EC) cells and «non-EC» cells.

Ultrastructural studies on endocrine cells are scarce. Welsch and Löwe (1973) described 10 types of endocrine cells in the gastro-intestinal tract of *Rana temporaria:* four in the stomach and six in the small intestine. Kataoka (1974) characterized 10 types in the gastro-intestinal tract, six of which were in the stomach of *Rana nigromaculata nigromaculata,* according to the ultrastructure of the secretory granules. Five of these were identified by comparing them with already known mammalian types: enterochromaffin cells (EC), enterochromaffin-like (ECL) cells, glucagon (A) cells, gastrin (G) cells and secretin (S) cells. Giraud and Yeomans (1981) have identified three types of endocrine cells in the stomach of *Bufo marinus.* In spite of the extensive bibliography regarding the application of immunocytochemical techniques to the study of the enteroendocrine system in mammals, we were able to find only a few works on Amphibia. Larsson and Rehfeldt (1977) have reported in *Ranidae* the presence of a gastrin/cholecystokinin-like peptide in the stomach. Lechago and colleagues (1978), also working on *Ranidae,* have reported the presence of bombesin-like immunoreactive cells in the gastric mucosa. Buchan et al. (1980a), using antisera against a variety of mammalian gut peptides, have identified cells showing bombesin-, gastrin-, somatostatin-, substance P-, and glucagon-like immunoreactivity in the gut of the urodele *Salamandra salamandra.* Vasoactive intestinal polypeptide (VIP)-like immunoreactivity was identified in the anuran intestine by Buchan et al. (1980b, 1981). Somatostatin was demonstrated in fundus and antrum of *Xenopus laevis* by Hacker et al. (1983) and serotonin in esophageal epithelium of frog by Nada et al. (1984).

In the present work, endocrine cells of the gastric mucosa of *Rana temporaria* are classified according to their response to silver staining and the immunocytochemical and ultrastructural characteristics of their endocrine granules, although other characteristics were also taken into account.

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Table 1. Survey of antisera used

Antiserum[1] Peptide	Dilution	Source	Catalog N.°
Gastrin 1-17	1:3,000	Immunonuclear	05H2T
Glucagon	1:1,000	"	33H2T
Serotonin	1:2,000	"	43H2T
Somatostatin	1:2,000	"	20H2T
Vasoactive intestinal polipeptide	1:500	"	39H2T
Bombesin	1:400	"	30H2T

1. All the antisera were raised in rabbits.

Materials and methods

Thirty specimens of *Rana temporaria,* collected between the months of December and February, were used. The animals were maintained at 4°C in buckets of water until sacrificed.

Immunocytochemistry

Paraffin sections were used for an indirect antibody method for detection and localization of gastrin/CCK, glucagon, bombesin, serotonin, somatostatin and the vasoactive intestinal polypeptide (VIP), using the peroxidase anti-peroxidase (PAP) technique (Sternberger, 1979).

After removing the paraffin, the intrinsec peroxidase was blocked by treatment of the sections with 0.3% H_2O_2 for 30 min, followed by thorough washing in phosphate buffered saline (PBS) pH 7.2. The background blocking was performed with 1:30 normal goat serum for 30 min, prior to the incubation with the specific antiserum (gastrin/CCK, glucagon, serotonin, somatostatin, vasoactive intestinal polipeptide and bombesin), which was carried out for 16-20 h at 4°C. Details of the antisera used are given in Table 1. After rinsing in PBS, the sections were incubated in goat anti-rabbit IgG (Miles) at a dilution of 1:10 for 30 min. Following a secondary rinse in PBS, the sections were incubated in PAP for 30 min. After a final rinse, horseradish peroxidase was visualized with diaminobenzidine (50 mg per 100 ml) and H_2O_2 (60 µl per 100 ml); development time being 1-3 min.

Immunohistochemical controls included:

1) The use of normal rabbit serum, diluted 1:30, in place of the antiserum.
2) Omission of the first layer.
3) Absorption of antisera prior to immunostaining: the diluted antisera were incubated with 10-100 µg of the corresponding antigens.

Electron microscopy

Small fragments of tissue were fixed in 4% glutaraldehyde, followed by fixation in 1% osmium tetroxide, and embedded in Epon-812 (Luft, 1961). The embedding Epon was entirely removed with sodium methoxide (Mayor et al., 1961). From this material, semithin sections, 1 µm in thickness, were made and stained with methylene blue borated and lead hematoxylin (Solcia et al., 1969). Silver impregnation after Grimelius (Grimelius, 1968) and Davenport (Hellerström and Hellman, 1960) for argyrophilia and Masson-Fontana (Masson, 1923) for argentaffinity was carried out.

Grimelius and Masson-Fontana techniques were accurately modified to apply them to semithin sections of Epon-embedded material (López et al., 1983).

From inspection of semithin sections, suitable fields were selected for ultrathin sections which were stained with uranil-acetate and lead hydroxide, and observed with a Siemens Elmiskop 1A electron microscope.

In order to correlate ultrastructural findigns with light microscope observations, serial sections were used; one for silver impregnation, the other for electron microscopy. Six hundred endocrine cells of the gastric mucosa were studied.

Results

The mucosa is made up by simple tubular glands. The glands are composed of mucous cells, oxyntic-peptic cells and endocrine cells. Mucous cells are restricted to the upper region of the glands. The oxyntic-peptic cells occupy the rest of the gland. The transition between both regions is abrupt (Fig. 1). Endocrine cells are preferentially

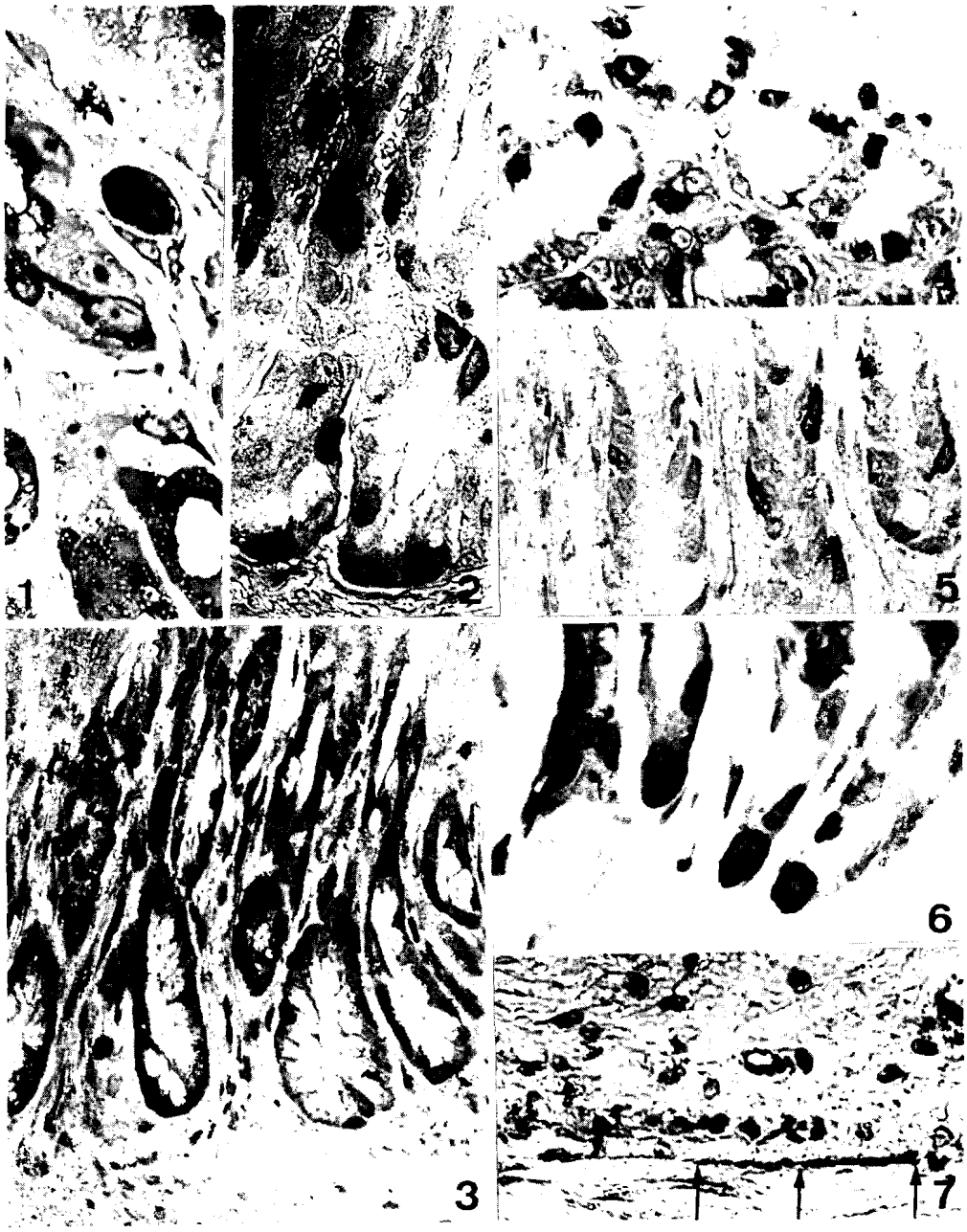


Fig. 1. Panoramic view of a semithin section of the mucosa stained with the Masson-Fontana argentaffin technique. x 600

Figs. 2-7. Immunocytochemical staining with peroxidase-antiperoxidase technique. Figs. 2, 3, 4, 5, and 6. Cells of gastric glands showing gastrin-(2), glucagon-(3), serotonin-(4), somatostatin-(5) and bombesin-(6) immunoreactivity. VIP-immunoreactive fiber —arrows– (figure 7) in myenteric plexus of the stomach. 2, 3, 6, x 650; 4, 5, 7, x 400

located in two regions along the glands. The majority of them are located in the transitional zone between mucous and oxyntic-peptic cells; a quite large number inhabit the floor of the glands, between the oxyntic-peptic cells and a small number are scattered between the mucous cells.

Antisera to gastrin/CCK, glucagon, somatostatin, serotonin and bombesin stained different types of gastric endocrine cells (Figs. 2-6). In the stomach VIP-immunoreactivity was found only in nerve fibers (Fig. 7).

Seven different cell types have been established according to the immunostaining properties of the granules and other ultrastructural characteristics.

G cells containing gastrin/CCK were found only in the antral region of the stomach (Fig. 2). They are elongated or oval cells whose long axis lies parallel to the basal membrane, although some cells are seen obliquely oriented (Figs. 8, 9). The nucleus is deeply indented, as is easily seen under light microscopy. The cells react with the Grimelius' stain, although the reaction is weaker than that of other cell types, as shown in figure 9. When semithin sections are stained with methylene blue, the secretory granules are strongly stained (Fig. 8A). These were round granules, with an average diameter of 280 nm and displayed an electron-dense, homogeneous content. They are membrane-bound, a feature that can only be

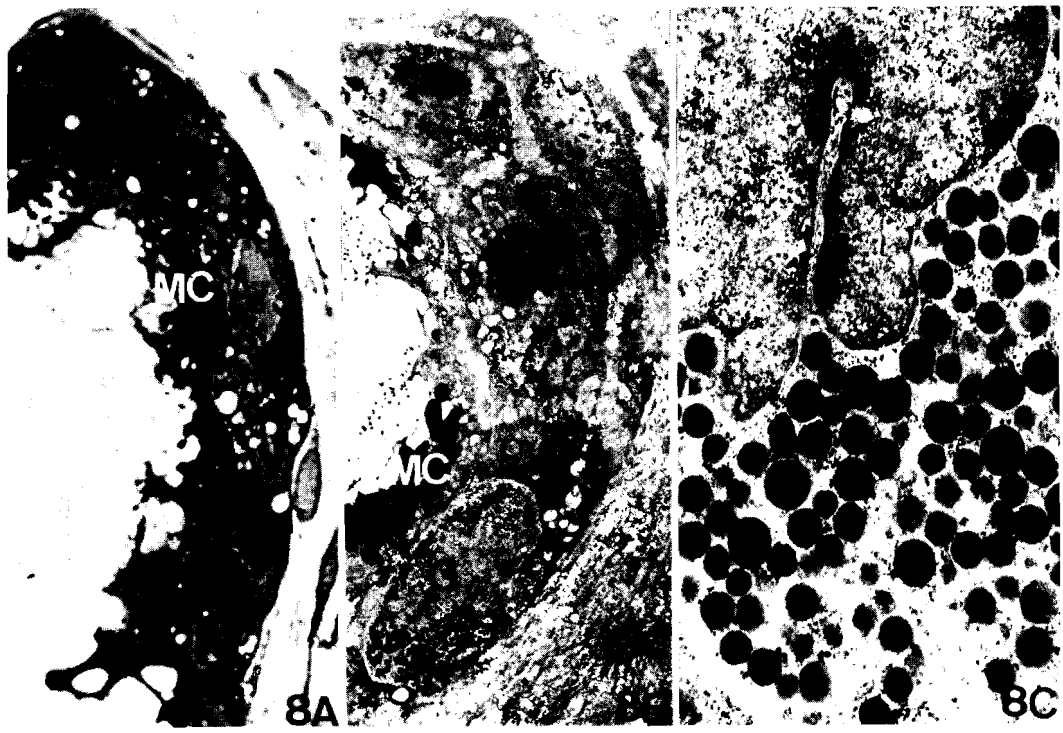


Fig. 8. Serial sections. **A.** Semithin section stained with methylene blue. A G cell containing deeply stained secretory granules is seen. x 1,500. **B.** Electron micrograph of the same endocrine cell seen in A. MC: mucosecretory cell. x 2,700. **C.** Detail of the granules. x 21,600

seen at high magnification in those granules whose content is of low density (Fig. 8C).

EC cells, serotonin cells (Fig. 4), have polymorphic, predominantly elongated granules which are osmiophilic, argentaffin and intensively Grimelius-reactive (Fig. 9). The average size of the granules is 310 x 120 nm (Fig. 10). Lipid droplets are frequently present (Fig. 10B). These cells are often found in the transition zone between mucous and oxyntic-peptic cells of the gland. The nuclei are indented.

The *A cell* cytoplasm (Fig. 3) contains round granules having a size (190 nm) smaller than that of G cells. The granules are electron-dense with homogeneous contents and have a conspicuous membrane (Fig. 11). They do not react with argyrophilic techniques (Fig. 9).

ECL cells are neither argyrophilic (Fig. 9) nor argentaffin. They show vesicular granules with an osmiophilic and generally excentric core. The average diameter of the granules is 340 nm (Fig. 12). These cells are elongated, and their axes are parallel to that of the gland. They are found between the mucous and oxyntic-peptic cells.

D cells react positively with Davenport's silver impregnation (Fig. 13) and do not react with Grimelius (Figs. 9, 14) and Masson-Fontana techniques. This procedure, when applied to 1 µm sections, disclosed fine, long cytoplasmic processes in which there were granules identical to those in the rest of the cytoplasm (Fig. 13). Somatostatin-immunoreactivity is present in these cells (Fig. 5). Under electron microscopy the granules are ovoid, with a closely applied membrane, and show moderate osmiophilia (Fig. 14).

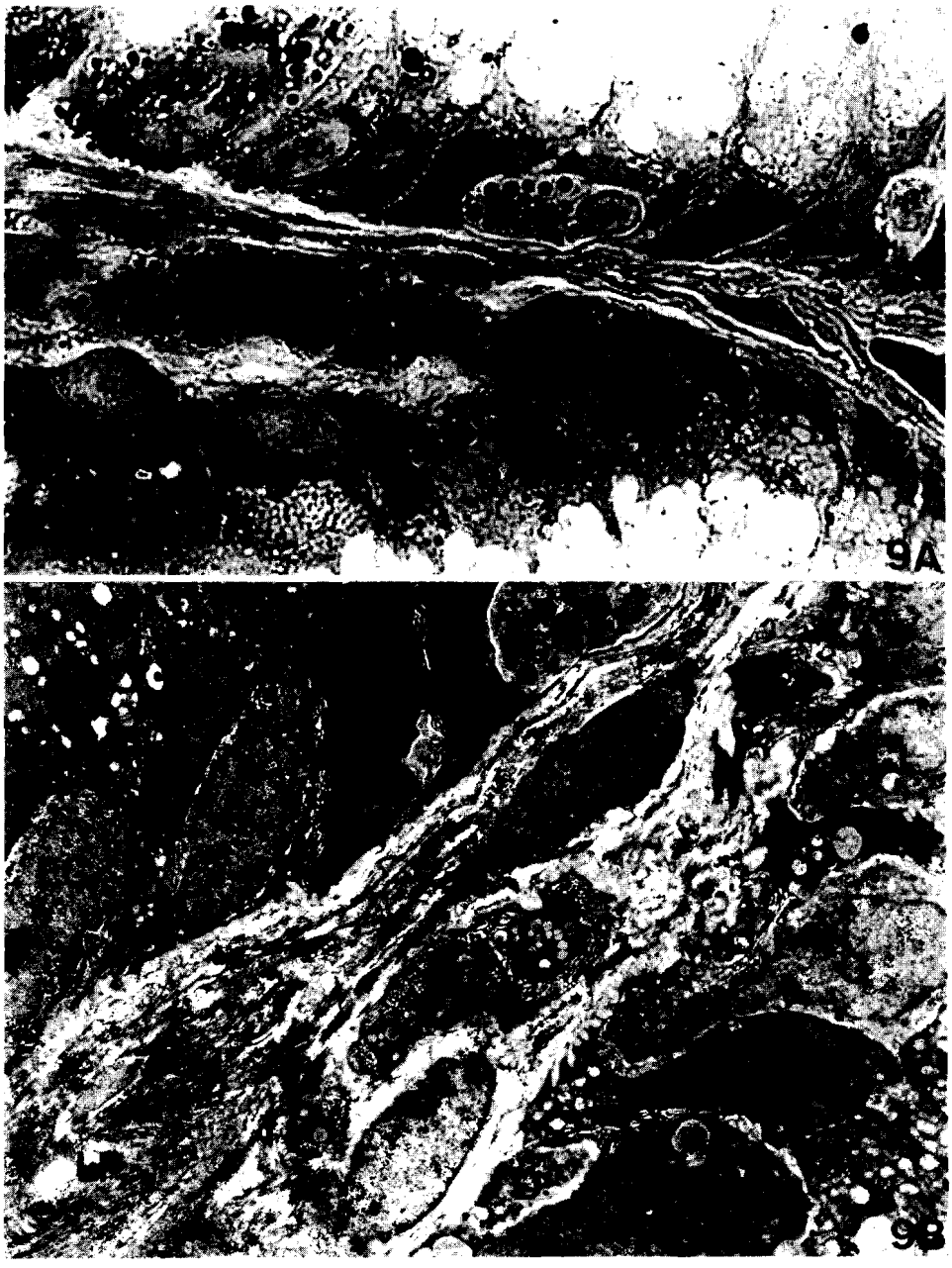
Bombesin-immunoreactivity is found in *P cells* (Fig. 6). They are mainly characterized by having the smallest granules of all the endocrine cells here described (170 nm). These granules are round and osmiophilic, with a sharp, visible membrane (Fig. 15C). These clear cells have an elongated, pyramidal or oval shape and are found frequently at the deepest part of the glands (Figs. 6, 15). In semithin sections they appear separated from the oxyntic-peptic cells by a clear space (Fig. 15). A large number of filaments surround the central nucleus. The nucleus shows one or two deep indentations. The chromatin is loose and the internal dense lamina associated with the nuclear membrane is prominent (Fig. 15C). These cells are Grimelius (Fig. 15A) and Masson-Fontana negative.

Unclassifiable cells, not included in any of the previous groups and not similar to any other cell type described in vertebrates, are gathered under this label.

Discussion

Seven types of endocrine cells have been identified in the mucosa. Of these, six types have characteristic features of endocrine cells previously described in Amphibia (Welsch and Löwe, 1973; Kataoka, 1974; Lechago et al., 1978; Buchan et al., 1980a; Crawford and Lechago, 1981; Giraud and Yeomans, 1981; El-Sahly et al., 1981; Hacker et al., 1983; Nada et al., 1983) and in mammals (Vasallo et al., 1969; Solcia et al., 1975, 1978).

Among the G cells we include those giving gastrin/CCK immunoreactivity, containing round granules, averaging 280 nm in diameter. They were found in the transitional zone of the antral glands. They were weakly argyrophil. The presence of gastric-containing cells in



Fif. 9. Serial sections. **A.** Semithin section stained with Grimelius silver nitrate. x 1,500. **B.** Thin section. x 2,800. In **A**, a G cell is weakly argyrophil (dotted line). EC cells are strongly argyrophil; D and ECL do not stain with this technique. Es: eosinophil. F: fibroblast of the lamina propia (LP). x 2,800

the antrum of Amphibia has been described by Larsson and Rehfeldt (1977) in the frog, and Buchan et al. (1980a) in the salamander.

The size of the granules of the G cells observed by us in *Rana temporaria* is smaller than that (390-440 nm) described by Welsch and Löwe (1973) in the same species and larger than that (180 nm) reported by Buchan et al. (1980a) in *Salamandra salamandra.* The core of the granules of the G cells of the frog are more electron-dense than in mammals; a feature also described by other authors (Solcia et al., 1967; Forssman et al., 1969; Vasallo et al., 1972). In mammals, G cells were described extending to the lumen by narrow cytoplasmic processes, a feature that we have also observed in *Rana.* As is known, gastrin is a polypeptide whose major target is the parietal cell, stimulating HCl secretion.

The EC cells, are very abundant and account for the strong reactivity with silver techniques (argyrophilia and argentaffinity) observed in the transitional zone of the glands. They were the first observed in the gastric mucosa (Heidenhain, 1870). EC cells were earlier described in the gut of Amphibia (De Filippi, 1930; Vialli and Erspamer, 1933; Citterio, 1935). Although De Filippi (1930) and Vialli and Erspamer (1933) stated that these cells were found in the duodenum but were absent from the stomach, they were described in the stomach of *Rana temporaria* (Welsch and Löwe, 1973) and *Rana nigromaculata nigromaculata* (Kataoka, 1974). EC cells

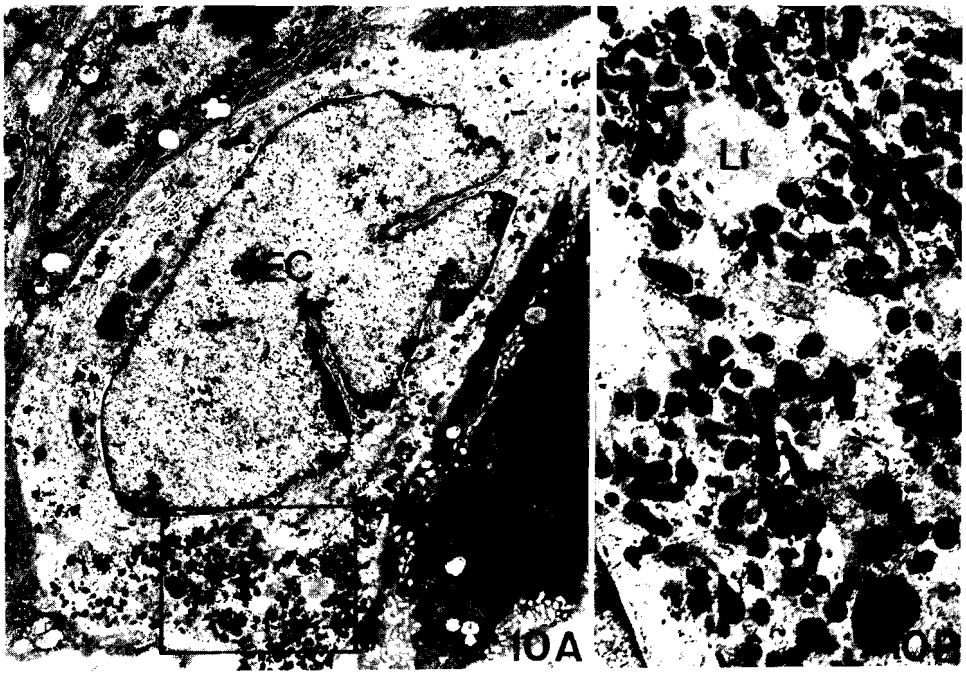


Fig. 10. A. Electron micrograph of an intestinal EC cell (EC). x 6,600.
B. Detail of the area corresponding to the rectangle of **A.** Variability of granule morphology is seen, with elongated forms predominating. Li: lipid droplets. x 21,600

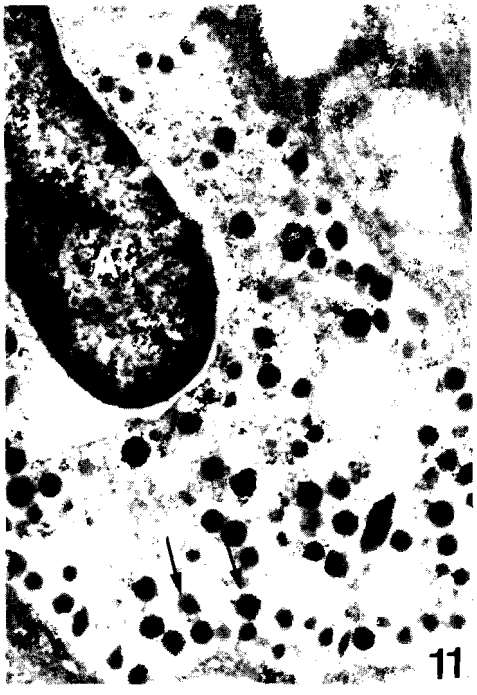


Fig. 11. Cell type A. Some granules display a dense core (arrows). x 21,600

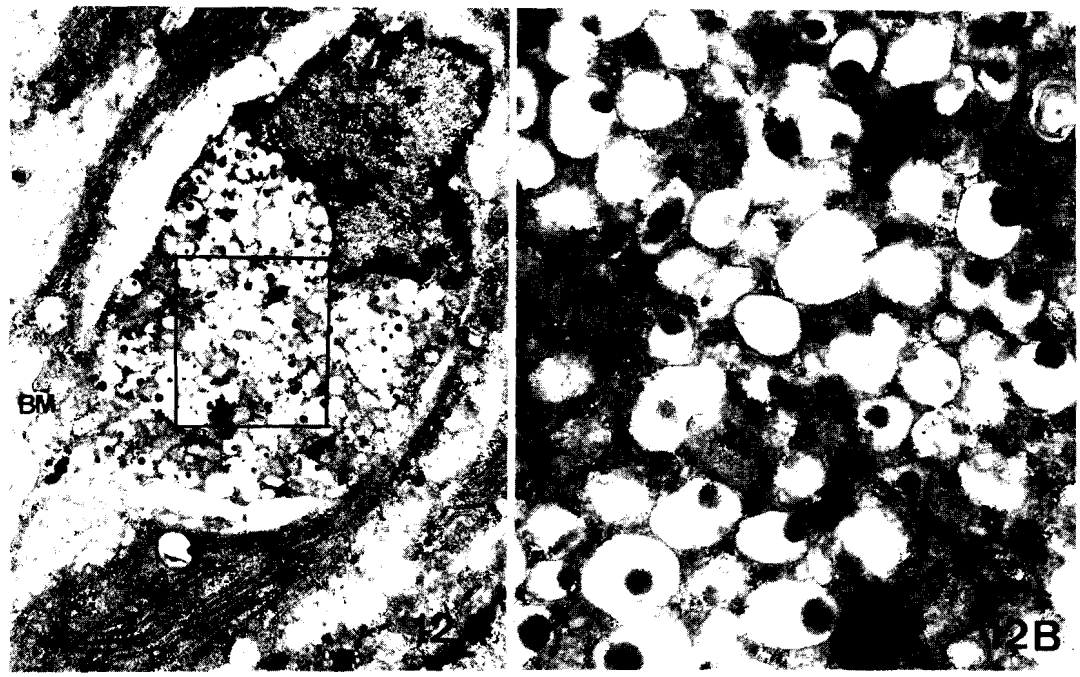
are considered to be engaged principally in the synthesis of 5HT (Facer et al., 1979; Inokuchi et al., 1982).

The cells with glucagon-like immunoreactivity, labeled as type A, are located predominantly in the transitional zone of the glands. They display the same ultrastructural features as those of *Salamandra salamandra* (Buchan et al., 1980a). El-Salhy et al. (1981) have demonstrated glucagon immunoreactive cells in gut of *Bufo regularis.* A cells of the frog are similar to the A cells of the mammals, a feature already stressed by Polak et al. (1971), Larsson et al. (1975) and Buchan et al. (1980a); but the core of the secretory granules is less dense. The A cells of the frog do not give an argyrophil reaction with Grimelius technique. They are reported as strong argyrophil in man, but weak in the rat and dog (Grimelius et al., 1978). In the frog, they do not reach the gland lumen, as is seen in mammals (Grimelius et al., 1978).

ECL cells are found predominantly in the oxyntic mucosa, but they are also present in less proportion in the antral mucosa. They are located in the transitional zone proximal to the mucous cells. ECL cells were described by Gabe (1972) under the light microscope in several amphibian species. This cell type corresponds closely to the type II cells of *Rana nigromaculata nigromaculata* (Kataoka, 1974), *Bufo marinus* (Giraud and Yeomans, 1981) and mammals (Solcia et al., 1978).

According to Polak et al. (1971) and Solcia et al. (1978) the granules of these cells in mammals are argyrophil but not argentaffin. In the frog they are neither argentaffin nor argyrophil.

The D cell granules in *Rana* display medium electron density. They were identified by their ultrastructure which closely resembles that of the mammal cells. Our findings agree with data reported by Buchan et al.



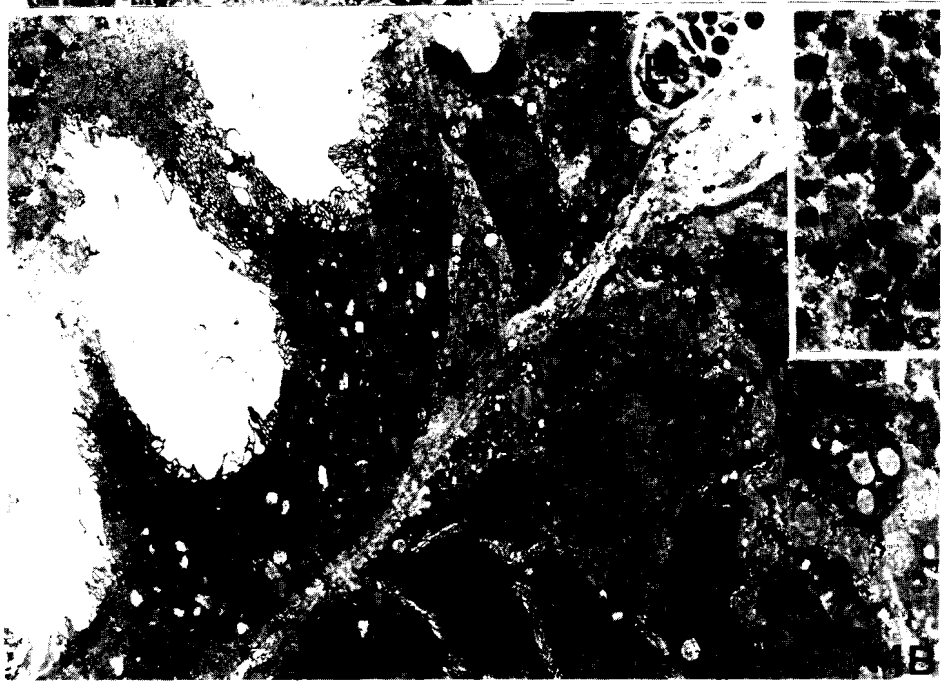


Fig. 12. A. ECL cell. BM: basement membrane. x 7,200. **B.** Detail of the area corresponding to the square. x 25,200

Fig. 13. Semithin section stained with the Davenport technique showing a positively reacting D cell. x 1,500

Fig. 14. Serial sections. **A.** Semithin section stained with the Grimelius technique. x 1,500. **B.** Thin section. D, A and ECL cells (dotted line) do not react with the Grimelius technique. x 2,700. Es: eosinophil. Inset: Detail of the granules of the D cell. x 21,600

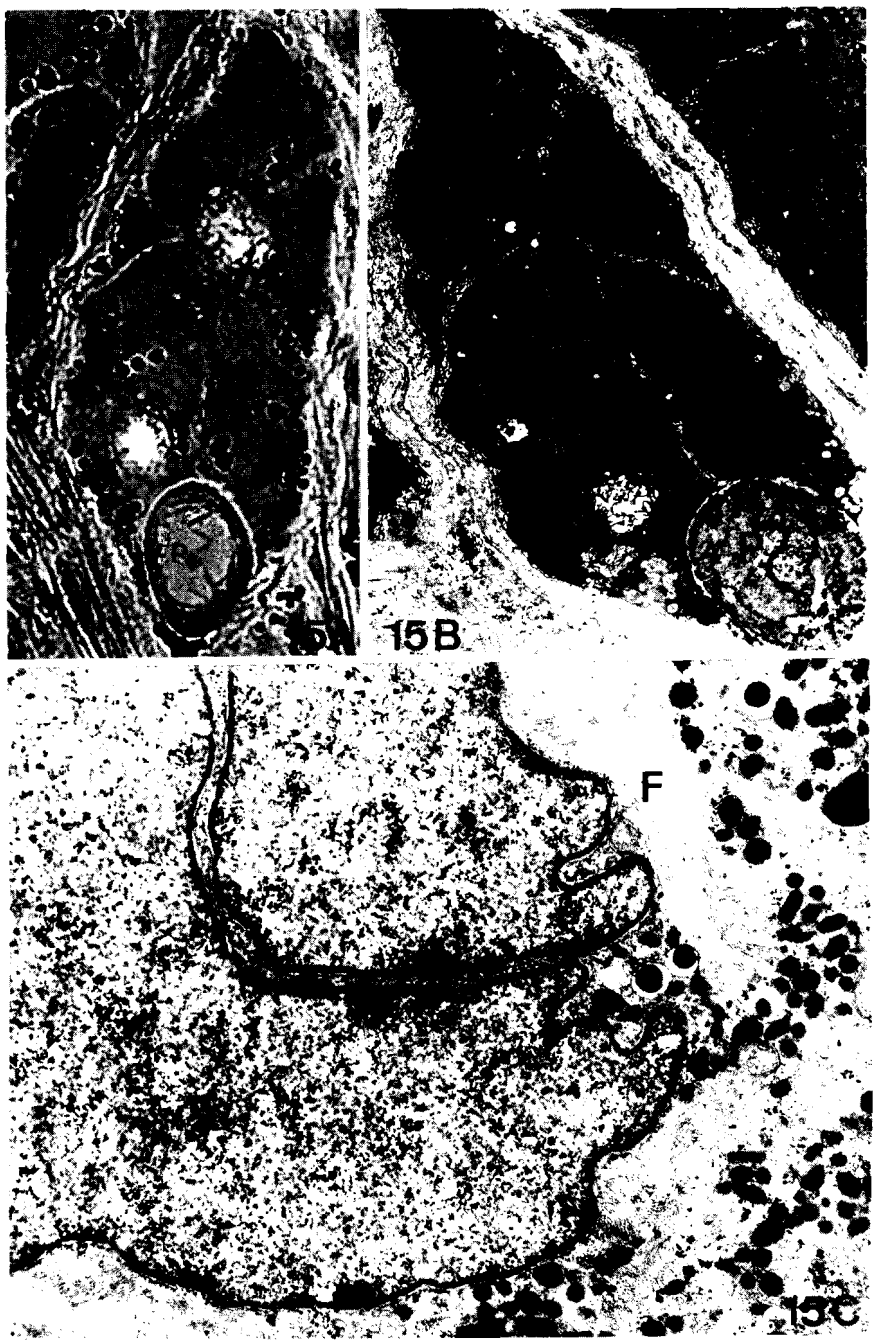


Fig. 15. A. Semithin section stained with Grimelius technique. A Grimelius negative P cell is seen at the floor of the gland. Its nucleus exhibits a very deep indentation. x 1,500. **B.** Electron micrograph of the same field seen in A. x 1,900. **C.** Detail of **B.** A well developed dense internal lamina of the nuclear membrane is present. Round and elongated granules are seen. In some granules a clear halo is observed between boundary membrane and central core. F: filaments. x 21.600

(1980a) in the salamander. In mammals, somatostatin (D cells) is thought to act in a paracrine (local) way (Bloom and Polak, 1978; Polak and Bloom, 1979), and it has been suggested that the long processes found on these cells indicate that the individual cells act directly, having an inhibitory effect on several surrounding cells (Pearse et al., 1977; Larsson et al., 1979; Solcia et al., 1980). In Amphibia, a similar mechanism for these cells has been suggested by Buchan et al. (1980a), who observed immunoflurescent D cell processes with anti-somatostatin serum. We also observed such processes in this cell type, both with the Davenport technique and the PAP technique. Most of D cells are closed. Open cells are scarce. Hacker et al. (1983) have observed open D cells in gut of *Xenopus laevis.*

P cells are believed to secrete bombesin, a polypeptide isolated for the first time from the skin of the frog *Bombina bombina* (Erspamer and Melchiorri, 1973). They contain the smallest granules (170 nm in diameter) of all of the endocrine cells studied here, a result which agrees with Crawford and Lechago (1981). Cells with similar features, giving bombesin-

immunoreactivity, have been described in the frog gastric mucosa by Lechago et al. (1978), and in the salamander gut (from stomach to the colon) by Buchan et al., (1980a). It is possible that this cell type is analogous to the type III described by Kataoka (1974) in *Rana nigromaculata nigromaculata*. P cells were also described in the mammalian gastro-intestinal tract (Solcia et al., 1975; Buchan and Polak, 1980). Our findings, with respect to location, agree with the description of Lechago et al. (1978). The P cells are always located in the floor of the fundus of the gland where there is a peculiar halo around them, a finding which agrees with those of Lechago et al. (1978), Polak et al. (1976, 1978b) and Timson et al. (1979).

It has been pointed out that P cell granules stain very weakly with the Grimelius technique in mammals (Hage, 1974; Capella et al., 1978; Polak et al., 1978a). We found that in frog they are neither argentaffin nor argyrophil, a result which agrees with that of Lechago et al. (1978).

A striking feature of P cells is the large amount of perinuclear, cytoplasmic filaments, which were also seen by Kataoka (1974) in type III cells of *Rana nigromaculata nigromaculata*, by Crawford and Lechago (1981) in *Rana catesbiana* and by Solcia et al. (1980) en mammals.

In the frog stomach, VIP-immunoreactivity was confined to the myenteric plexus, which innervates the muscle layer, suggesting that this peptide may play a role in muscle relaxation (Buchan et al., 1980B; Reinecke et al., 1981).

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