Immunohistochemical investigations on the pyloric glands of the ruin lizard (*Podarcis sicula campestris* de Betta)

Domenico Ferri and Giuseppa Esterina Liquori

Institute of Zoology and Comparative Anatomy, University of Bari, Via Amendola 165/A, 70126 Bari, Italy

Summary

Mucous cells and enteroendocrine cells of the pyloric region of the ruin lizard (*Podarcis sicula campestris* De Betta) have been examined by lectin histochemical and immunohistochemical methods. Binding to five plant lectins (*Canavalia ensiformis*, Con A; *Triticum vulgare*, wheat germ, WGL; *Lotus tetragonolobus*, winged pea, WPL; *Glycine max*, soybean, SBL; *Arachis hypogaea*, peanut, PNL) was performed to characterize glycoconjugates in the secretory products of superficial and glandular mucous cells. Lectin histochemistry revealed the presence of N-acetyl-D-galactosamine, D-galactose and N-acetyl-D-glucosamine in the pyloric superficial cells. Mucous glandular cells mainly contained neutral glycoproteins with terminal residues of galactose, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine. These cells did not react with Con A after periodate oxidation-sodium borohydride reduction (Paradoxical Con A staining). In the pyloric glands three different types of endocrine cells were identified immunohistochemically: gastrin-, serotonin- and somatostatin-immunoreactive cells; VIP-, bombesin- or cholecystokinin-immunoreactive cells have not been found in the pyloric mucosa.

Key words: enteroendocrine cells — glycoconjugates — pyloric glands — ruin lizard — immunohistochemistry — lectin histochemistry

Introduction

The digestive tract of higher vertebrates has been extensively studied at histological, histochemical and ultrastructural levels. In reptiles, this tract has been less well analysed and, in particular, little attention has been paid to the comparative histochemistry of the mucosal glycoconjugates (Luppa, 1976; Giraud et al., 1979; Suganuma et al., 1981) or immunocytochemistry of the enteroendocrine cells (Larsson and Rehfeld, 1977; Masini et al., 1990).

Therefore, mucous cells and enteroendocrine cells of the pyloric glands of the ruin lizard *(Podarcis sicula campestris)* were examined by immunocytochemical methods. Binding to five plant lectins (ConA, WGL, WPL, SBL, PNL) conjugated with horseradish peroxidase was performed to characterize glycoconjugates in the secretory product of the glands.

Enteroendocrine cells were identified by the peroxidase-anti-peroxidase (PAP) method (Sternberger et al., 1970) and indirect immunofluorescence method of Sloan et al. (1979).

Materials and Methods

The stomachs were obtained from male and female specimens of *Podarcis sicula campestris*, fixed in 10% formaldehyde with 2% calcium acetate. *Bouin*'s fluid or *Carnoy*'s solution, dehydrated through graded alcohols, and embedded in paraffin.

Mucosubstance histochemistry. Serial paraffin sections were cut at 5 μm and stained by the Periodic acid-Schiff (PAS) method (Mowry and Winkler, 1956) or with Alcian blue (AB) at pH 2.5 (Lev and Spicer, 1964). *Lectin histochemistry.* Binding to five plant lectins conjugated with horseradish peroxidase (Table 1) was performed to determine the nature and the distribution of glycosidic residues in the mucous cells of the pyloric region.

Table 1. Characteristics of the plant lectins utilized

Lectin	Source	Hapten sugars	Concentration (mg/ml)
Con A	*Canavalia ensiformis*	D-mannose D-Glucose	0.05
WGL	*Triticum vulgaris*	N-acetyl-D-glucosamine sialic acid	0.02
SBL	*Glycine max*	N-acetyl-D-galactosamine D-galactose	0.02
PNL	*Arachys hypogaea*	D-galactose	0.06
WPL	*Lotus tetragonolobus*	L-fucose	0.1

Binding of peroxidase-labelled lectins was carried out as reported previously (Ferri and Liquori, 1992). Briefly, rehydrated sections were exposed to 3% hydrogen peroxide (H_2O_2) for 10 min to block endogenous peroxidase activity and incubated with peroxidase-labelled lectins for 30 min at room temperature. Horseradish peroxidase was localized with a 3,3'-diaminobenzidine (*DAB*)-H_2O_2 medium (Graham and Karnowsky, 1966). Finally, the sections were dehydrated, cleared, and mounted with DPX. The control tests included a) substitution of the respective peroxidase-labelled lectin with PBS, b) incubation with the peroxidase-labelled lectin in the presence of the appropriate competing sugar to confirm the specificity of lectin staining, and c) incubation of untreated sections with DAB-H_2O_2 to control the endogenous peroxidase activity.

A variant of the Concanavalin A-horseradish peroxidase method, the Paradoxical Concanavalin A staining (*PCS*; periodate oxidation-borohydride reduction-Concanavalin A sequence) was also carried out for further characterization of mucosubstances (Katsuyama and Spicer, 1978).

Immunohistochemistry. Enteroendocrine cells were identified by the peroxidase-anti-peroxidase (PAP) method (Sternberger et al., 1970) and indirect immunofluorescence method of Sloan et al. (1979).

The primary antibodies utilized are listed in Table 2.

For the PAP method rehydrated sections were incubated for 30 min at 37 °C, for each step in a) primary antibodies, b) goat antirabbit IgG (Sigma), c) peroxidase-anti-peroxidase complex (Sigma). The immunoreaction was revealed using a 3,3'-diaminobenzidine (*DAB*)-H_2O_2 medium (Graham and Karnowsky, 1966). The sections were then rinsed with water, dehydrated, cleared, and mounted in DPX.

For indirect immunofluorescence, after incubation with the primary antisera and goat anti-rabbit IgG-FITC (30 min at 37 °C for each step), the sections were mounted with Mounting medium (Sigma) and examined with an epifluorescence microscope. Control stainings were performed by replacing each primary antibody by rabbit preimmune serum or incubating the sections in each antiserum containing the specific antigen.

Results

In the ruin lizard the stomach is subdivided into a corpus, or fundus, and pars pylorica which occupies a restricted area. The stomach was lined by a thick mucosa with a few longitudinal folds. The *lamina propria* of the gastric mucosa contained numerous tubular glands of the single or branched type, comprising fundic and pyloric glands that both emptied

Table 2. Characteristics of the primary antibodies utilized

Rabbit antibody	Antigen	Company	Working dilutions	
			PAP	IF
Anti-gastrin	Synthetic human gastrin-I	Chemicon	1:200	1:40
Anti-somatostatin	Synthetic somatostatin	ICN	1:2000	1:40
Anti-serotonin	Synthetic serotonin	ICN	1:2000	1:20
Anti-bombesin	Synthetic bombesin	ICN	1:500	1:40
Anti-vasointestinal peptide (VIP)	Synthetic VIP	ICN	1:500	1:40
Anti-cholecystokinin	Synthetic cholecystokinin	Chemicon	1:500	1:500

PAP = peroxidase-anti-peroxidase method, IF = immunofluorescence

into gastric pits. The luminal surface of the pylorus and the pyloric pits were lined by a single layer of mucous-secreting columnar cells. The pyloric glands were shorter and less branched than those of the fundus and contained only mucous and enteroendocrine cells. Oxinticopeptic cells, typical for the fundic glands, were not present in the pyloric glands.

The superficial and foveolar mucous cells showed a strong PAS-reactivity (Fig. 1 A), while they did not stain for AB at pH 2.5. The secretory product of these cells reacted moderately with PNL (Fig. 1 B) and SBL (Fig. 1 C). A widespread reactivity for WGL was present in the pyloric mucosa (Fig. 1 D). No binding sites were found for Con A and for WPL.

The mucous glandular cells showed an intense PAS staining (Fig. 1 A), but did not react with AB. They also showed a strong reaction with PNL (Fig. 1 B) and stained with SBL (Fig. 1 C). Some binding sites were also found for WGL (Fig. 1 D) but not for WPL. Even after periodate oxidation-borohydride reduction, the mucous cells did not react with Con A.

In the ruin lizard the pyloric region is the richest source of enteroendocrine cells. By immunohistochemical methods we have identified three types of endocrine cells in the pyloric glands: gastrin-, somatostatin- and serotonin-immunoreactive cells.

Gastrin-immunoreactive cells (Fig. 2 A) were well represented and predominantly located in the lower third of the pyloric glands. They were large and ovoidal or pyramidal in shape. The immunoreactivity was present all over the cytoplasm.

Somatostatin-immunoreactive cells were numerous in the pyloric glands (Fig. 2 C). They were flask-shaped cells which extended from the basement membrane up the lumen of the glands, and belonged to the open type (Fig. 2 D).

Serotonin-immunoreactive cells (Fig. 2 B) were also present in the pyloric region. They were large and filled with numerous secretory granules.

VIP-, bombesin- or cholecystokinin-immunoreactive cells were absent in the pyloric mucosa.

Discussion

In the ruin lizard, the superficial epithelial cells and the mucous glandular cells of the pyloric mucosa probably produce neutral glycoproteins, as revealed by the positive PAS reaction and negative AB staining at pH = 2.5.

As in most reptiles (Lehman and Smith, 1975) the secretory products of these cells in the fundic region of this lacertid seem to contain mainly neutral glycoconjugates (Ferri and
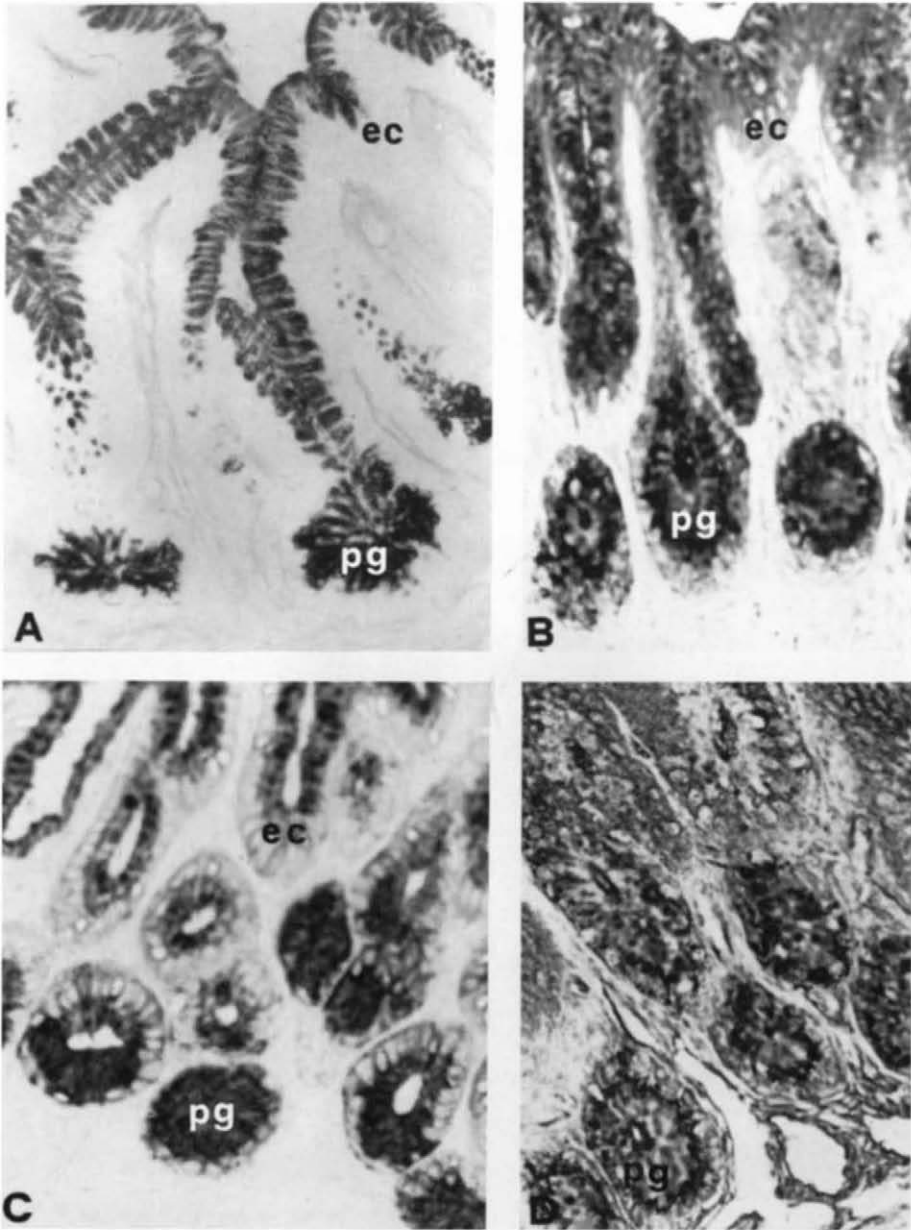
Fig. 1. Pyloric mucosa of the ruin lizard *(Podarcis sicula campestris* De Betta) stained with different histochemical methods. A. Periodic acid-Schiff (PAS). Pyloric glands (pg) and superficial epithetial cells (ec) are intensely stained by the PAS method. × 400. B. Peanut lectin (PNL). The mucous cells of the pyloric glands (pg) are intensely stained, while the superficial epithelial cells (ec) are weakly reactive. × 400. C. Soybean lectin (SBL). Mucous cells of the pyloric glands (pg) and superficial epithelial cells (ec) stained moderately with this lectin. × 400. D. Wheat germ lectin (WGL). A widespread reactivity for this lectin was present in the pyloric mucosa. Binding sites were particularly evident in the mucous cells of the pyloric glands (pg). × 400.
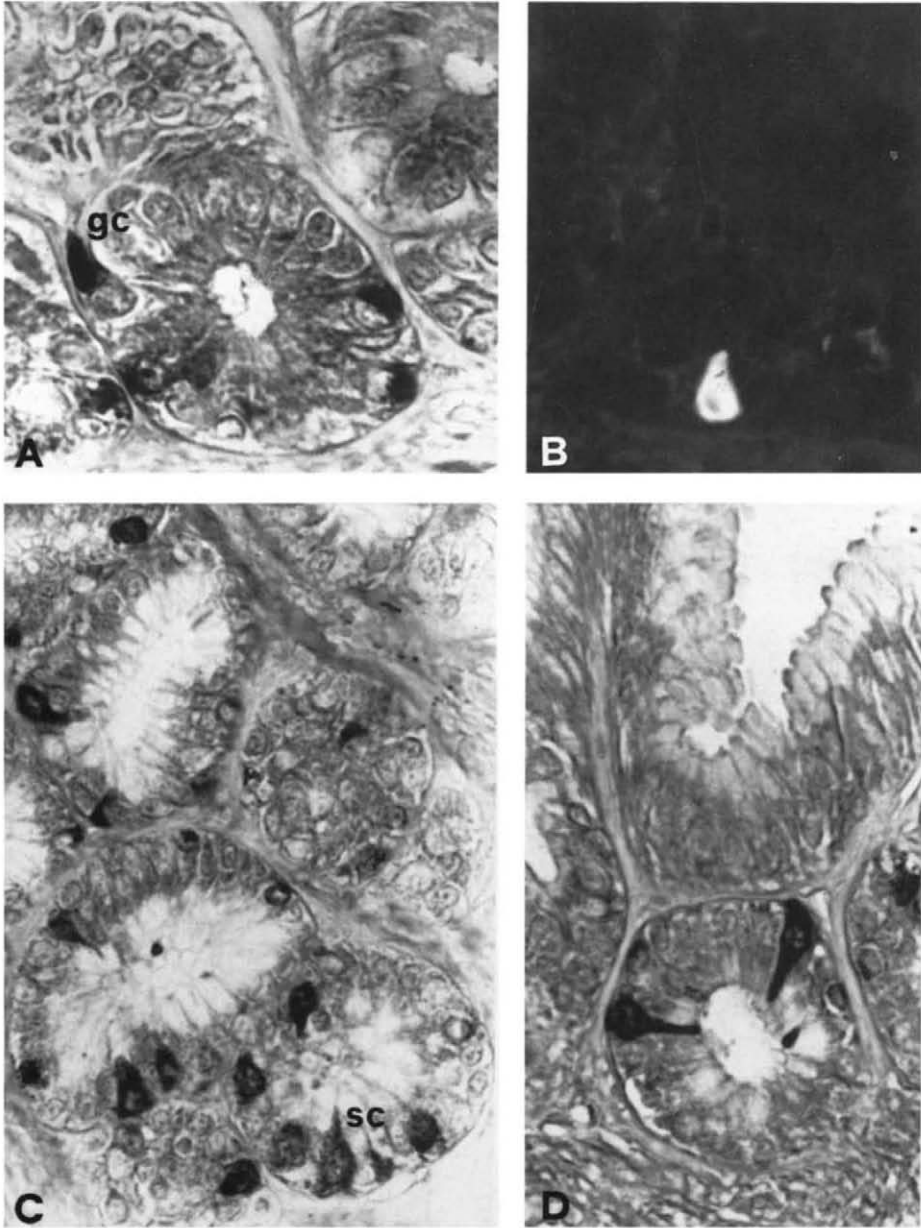
Fig. 2. Endocrine cells in the pyloric glands of the ruin lizard *(Podarcis sicula campestris* De Betta). A. Gastrin-immunoreactive cells (gc). PAP, × 400. B. Serotonin-immunoreactive cell. Indirect immunofluorecence, × 400. C. Somatostatin-immunoreactive cells (sc) are numerous in the pyloric glands. PAP, × 400. D. Somatostatin-immunoreactive cells are clearly of the open type. PAP, × 600.

Liquori, 1992) although in some species, small amounts of sialo- and sulfomucins can also be present (Suganuma et al., 1981). Lectin histochemistry reveals some differences between the mucins produced by the mucous cells of the different areas of the stomach of the ruin lizard for the distribution pattern of glycosidic residues. Binding of SBL, PNL and WGL demonstrates

the presence of the glycosidic residues of N-acetyl-D-galactosamine, D-galactose and N-acetyl-D-glucosamine in the secretory products of superficial and foveolar cells of the pyloric region. The mucins produced by the same cells of the fundic region are similar, but in addition contain D-glucose and D-mannose (Ferri and Liquori, 1992).

The mucous cells of the pyloric glands have glycoconjugates with glycosidic residues of N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and, especially, D-galactose, while those of the fundic glands produce neutral glycoproteins with mainly D-galactose and some L-fucose (Ferri and Liquori, 1992).

The mucins formed by the mucous glandular cells of the two regions of the stomach differ mainly with regard to their affinity to Con A after periodate oxidation-borohydride reduction. The reactivity is strong in the fundic glands (Ferri and Liquori, 1992) and therefore they contain stable class III-reactive mucosubstances which characterize mucous neck cells, pyloric glands and Brunner's glands in mammals (Katsuyama and Spicer, 1978), while the pyloric glands of the ruin lizard show a negative staining using the paradoxical-Con A method.

Our results differ from those reported for the turtle *Clemmys japonica* and for the snake *Elaphe climacophora* (Suganuma et al., 1981) in which the mucous cells of the pyloric glands contain stable class III-mucosubstances.

Suganuma et al. (1981) hypothesized that the neck cells of the gastric glands, mucous cells of the pyloric glands and cells of the Brunner's glands constitute only one group and have a similar embryonal origin in vertebrates. We suggest that there might be a specialization of these different cellular types with a differentiation in the various vertebrate groups during phylogenesis.

The microheterogeneity of the mucins between the pyloric and fundic regions of the stomach of the ruin lizard revealed by lectin histochemistry can be attributed to the different role of the mucus in these two regions. In the fundus the main function of the mucus is the defense of the gastric epithelium against the action of hydrochloric acid and pepsin. The pyloric glands produce mucus which probably is important for the lubrication of the contents of the stomach before its passage into the duodenum.

In the ruin lizard the pyloric region is the richest one in endocrine cells. In this area we have immunohistochemically identified three different types of endocrine cells: gastrin-, serotonin- and somatostatin-immunoreactive cells.

The large pyramidal gastrin (G)-immunoreactive cells constitute a considerable population in this lacertid. Gabe and Saint Girons (1972) and Giraud et al. (1979) presented histological evidence for the existence of cells in different species of amphibians and reptiles resembling the mammalian G cells. Larsson and Rehfeld (1977) identified G cells in the stomach of the chelonian *Testudo graeca* by immunofluorescence. The location of gastrin-immunoreactive cells in the stomach of amphibians and reptiles is similar to that observed in mammals. These cells, absent in the fundic regions, appear in the pyloric area and are the most numerous just before the beginning of the duodenum. In the pylorus of the ruin lizard cholecystochinin immunoreactivity was absent. The gastrin cells of this lizard differ from those of amphibians where they also react for this peptide. Therefore, in the ruin lizard, as in birds and mammals, there is a specialization of two different types of endocrine cells for the production of the two different hormones.

Somatostatin-immunoreactive cells have been observed in the stomach of various vertebrates, from cartilaginous fishes (Faraldi et al., 1986) to mammals (Solcia et al., 1981). These cells, that have been previously found by Masini et al. (1990) along the digestive tract of the ruin lizard, are numerous in the pyloric glands of this lacertid and are of the open type as in other vertebrates.

In the pyloric mucosa of the ruin lizard we have also found numerous serotonin-immunoreactive cells. They are similar to the enterochromaffin (EC) cells of the pyloric glands of mammals which contain 5-hydroxytryptamine (5HT). Reports on the presence of EC cells in the gastric mucosa of lower vertebrates, particularly of reptiles, are scarce and vague.

Ferri et al. (1974) briefly described argentaffin cells in the stomach of the snake *Xenodon merremii*. Cells resembling the typical mammalian EC cells have been found in the blue-tongued lizard *Tiliqua scincoides* by traditional histochemical and ultrastructural methods (Giraud et al., 1979).

VIP-, bombesin- or cholecystokinin-immunoreactive cells were not found in the pyloric mucosa.

In conclusion, the distribution pattern of endocrine cells in the stomach of the ruin lizard is similar to that of mammals although in some of them VIP endocrine cells have also been found (Buffa et al., 1977; Kitamura et al., 1982; Yanaihara et al., 1980).

References

Buffa R, Capella C, Solcia E, Frigerio B, and Said S (1977) Vasoactive intestinal peptide (VIP) cells in the pancreas and gastrointestinal mucosa. Histochemistry **50**: 217−227

Faraldi G, Farina L, Tagliafierro G, and Vacchi M (1986) Distribution of somatostatin- and glucagon-like cells in the gastric mucosa of some cartilaginous fish. Bas Appl Histochem **30** (suppl): 88

Ferri S, Madeiros LO, and Stipp AC (1974) Gastric wall histological analysis and cellular types distribution in *Xenodon merremii* Wagler, 1824 (Ophidia). Morph Jb **120**: 627−637

Ferri D, and Liquori GE (1992) Characterization of secretory cell glycoconjugates in the alimentary tract of the ruin lizard (*Podarcis sicula campestris* De Betta) by means of lectin histochemistry. Acta histochem **93**: 341−349

Gabe M, and Saint Girons H (1972) Contribution à l'histologie de l'estomac des lepidosauriens (Reptiles). Zool Jb **89**: 579−599

Giraud AS, Yeomans ND, and St John DJB (1979) Ultrastructure and cytochemistry of the gastric mucosa of a reptile, *Tiliqua scincoides*. Cell Tiss Res **197**: 281−294

Graham RC, and Karnowsky MJ (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem **14**: 291−302

Katsuyama T, and Spicer SS (1978) Histochemical differentiation of complex carbohydrates with variants of the concanavalin A-horseradish peroxidase method. J Histochem Cytochem **26**: 233−250

Kitamura N, Yamada J, Yamashita Y, and Yanaihara N (1982) Endocrine cells in the gastrointestinal tract of the cat. Biomed Res **3**: 612−622

Larsson L-I, and Rehfeld JF (1977) Evidence for a common evolutionary origin of gastrin and cholecystochinin. Nature **269**: 335−338

Lehman S, and Smith AA (1975) Regional differentiation in the stomach of the green anole. Anat Rec **220**: 364−368

Lev R, and Spicer SS (1964) Specific staining of sulfated groups with alcian blue at low pH. J Histochem Cytochem **5**: 445−458

Luppa H (1976) Histology of the digestive tract. In: Gans C, and Parsons TS (Eds) Biology of the reptilia. Academic Press, London, pp 225−302

Masini MA, Napoli L, Devecchi M, and Uva B (1990) Gut regulatory peptides and their distribution in *Podarcis sicula* Raf. Atti 53° Cong UZI, pp 368−369

Mowry R, and Winkler CH (1956) The coloration of acidic carbohydrates of bacteria and fungi in tissue sections with special reference to capsules of *Cryptococcus neoformans, Pneumococci* and *Staphilococci*. Am J Path **32**: 628−629

Sloan JM, Buchanan KD, McFarland RJ, Titterington P, and Sanford JC (1979) A histological study of the effect of chronic gastritis on gastrin cell distribution in the human stomach. J clin Path **32**: 201−207

Solcia E, Creutzfeldt W, Falkmer F, Fujita T, Grieder MH, Grossmann MI, Grube D, Hakanson R, Larsson L-I, Lechago J, Lewin K, Polak JM, and Rubin W (1981) Human gastroenteropancreatic endocrine-paracrine cells: Santa Monica 1980 classification. In: Grossmann MI, Brazier MAB, and Lechago J (Eds) Cellular basis of chemical messangers in the digestive system. Academic Press, New York, pp 159−165

Sternberger LA, Hardy PM, Cuculius JJ, and Meyer HG (1970) The unlabelled antibody enzyme method of immunohistochemistry, preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in the identification of *Spirochaetes*. J Histochem Cytochem **18**: 315−333

Suganuma T, Katsuyama T, Tsukahara M, Tatematsu M, Sakakura Y, and Murata F (1981) Comparative histochemical study of alimentary tracts with special reference to the mucous neck cells of the stomach. Amer J Anat **161**: 219–238

Yanaihara C, Sakagami M, Mochizuki T, Sato H, Yanaihara N, Iwanaga T, Fujii S, and Fujita T (1980) Immunoreactive VIP (vasoactive intestinal peptide) in canine intestinal mucosa and muscle. Biomed Res **1**: 449–455