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An Immunohistochemical Study of the Endocrine Cells in the Gastrointestinal Mucosa of the *Caiman latirostris*[*]

Junzo YAMADA[1], Valêncio J. M. CAMPOS[2], Nobuo KITAMURA[1], Arli C. PACHECO[2], Tadayuki YAMASHITA[1] and Noboru YANAIHARA[3]

Department of Veterinary Anatomy (Prof. T. YAMASHITA)[1], Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan ; Department of Morphology (Prof. V. J. M. CAMPOS)[2], São Paulo State University, Botucatu (SP), Brazil; and Laboratory of Bioorganic Chemistry (Prof. N. YANAIHARA)[3], Shizuoka College of Pharmacy, Oshika, Shizuoka, Japan

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Summary. **Twelve endocrine cell types immunoreactive for either 5-hydroxytryptamine (5-HT), somatostatin, gastrin, motilin, neurotensin, bovine pancreatic polypeptide (BPP), avian pancreatic polypeptide (APP), pancreatic glucagon, enteroglucagon, glicentin, secretin or cholecystokinin (CCK) were found in gastrointestinal mucosa of *Caiman latirostris.***

Moderate numbers of enteroglucagon-immunoreactive cells, a few 5-HT-, somatostatin- and motilin-immunoreactive cells and rare pancreatic glucagon-immunoreactive cells were found in the fundic stomach. Numerous gastrin-immunoreactive cells and moderate numbers of somatostatin- and motilin-immunoreactive cells were seen in the pyloric stomach. Moderate numbers of 5-HT-, gastrin-, motilin- and enteroglucagon-immunoreactive cells, a few somatostatin-, neurotensin- and BPP-immunoreactive cells, and rare APP-, pancreatic glucagon-, glicentin-, secretin- and CCK-immunoreactive cells were observed in the proximal intestine. Moderate numbers of 5-HT-immunoreactive cells, small to moderate numbers of neurotensin- and enteroglucagon-immunoreactive cells and occasional somatostatin-, motilin- and BPP-immunoreactive cells were seen in the distal intestine. Moderate numbers of neurotensin-immunoreactive cells and a few 5-HT-immunoreactive cells were found also in the cloaca. Cells immunoreactive for gastrin releasing polypeptide, bombesin and gastric inhibitory peptide were not observed in the caiman gastrointestinal epithelium.

The differences in endocrine cell types between the caiman and alligator are discussed in terms of their topographic distribution.

Though gastrointestinal regulatory peptides and amines in various submammalian vertebrate classes have been intensively studied, immunohistochemical studies on reptilian species are surprisingly scarce (SEINO et al., 1979 ; REINECKE et al., 1980 ; EL-SALHY and GRIMELIUS, 1981 ; MASINI, 1986). The crocodile family is positioned at a very important location in the phylogenetical tree ; nervertheless, only one immunohistochemical study in available concerning the regulatory peptides in the gut of *Alligator mississippiensis* (BUCHAN et al., 1983).

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Significant differences between pancreatic endocrine cells in *Alligator mississippiensis* and *Caiman latirostris* have been reported (YAMADA et al., 1986b). The purpose of this study is to use immunohistochemical methods to examine the gut endocrine cells of the caiman, *Caiman latirostris,* for regulatory peptides and an amine.

MATERIALS AND METHODS

Specimens of *Caiman latirostris* (53-140 cm in body length) were captured in October, 1983, around Botucatu (SP), Brazil. Five animals of both sexes were used in this study. Seven regions of the digestive tract and the cloaca were dissected out (Fig. 1) and fixed with Bouin's fluid. After paraffin embedding, serial sections were cut either at a 4 μm or 1.5-2 μm thickness. The peroxidase-antiperoxidase (PAP) (STERNBERGER, 1979) and bridge (MASON et al., 1969) methods were used in this study to identify specific endocrine cells. Details of the antisera used are shown in Table 1. The specificity of each immunohistochemical reaction was determined as recommended by STERNBERGER (1979) and included replacement of the specific antiserum with the antiserum preincubated with the corresponding antigen. The antisera and control sera were diluted in 0.01M phosphate buffered with 0.5M saline (pH 7.3) to prevent nonspecific binding of immunoglobulins by ionic interaction (GRUBE, 1980). To prevent nonspecific staining, the sections were incubated with non-immunized goat or rabbit serum prior to incubation with specific antisera.

After immunohistochemical staining, the sections were lightly counter stained with Mayer's hematoxylin, dehydrated, cleared in xylene, and mounted. Immunostained sections were examined and photographed with normal and Nomarski optics. The frequency and distribution of immunoreactive endocrine cells were investigated in 4 μm-thick serial sections. The immunoreactive cells were graded subjectively into five groups according to their relative frequency as confirmed by light microscopy. The relationships among the endocrine cells immunoreactive to the different antisera were examined in thin serial sections cut at a 1.5-2 μm thickness. Immunoreactivity was not observed in control specimens.

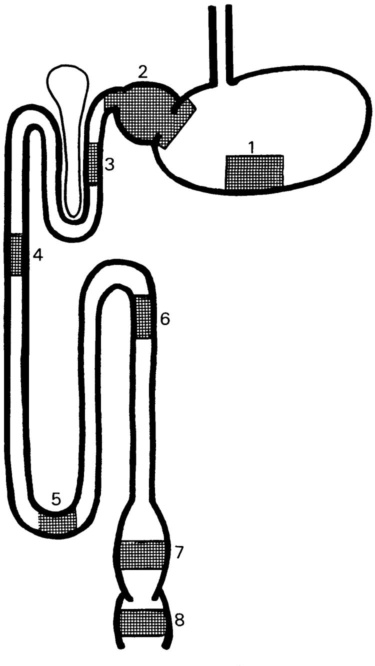

Fig. 1. Sampling portions.
1 fundic region, *2* pyloric region, *3* initial region of the intestine, *4* middle region of proximal half of the intestine, *5* middle region of whole length of the intestine, *6* middle region of distal half of the intestine, *7* ampulla (terminal region of the intestine), *8* cloaca.

The relative frequency and distribution of immunoreactive cells in the gastrointestinal mucosa are summarized in Table 2. In this study, glucagon-immunoreactive cells were divided into two subtypes: cells detected by an antiserum (GL-5, produced by N. YANAIHARA) specific for pancreatic glucagon were classified as pancreatic glucagon-immunoreactive cells, and cells detected by another antiserum (RPN. 1602, purchased

Table 1. Antisera used

Antisera raised to[a]	Code	Specificity	Dilution
Insulin[b]	47291	–	1: 1,000
Synthetic human cyclic somatostatin[c]	–	–	1: 3,000
Synthetic porcine glucagon[j]	GL-5	Reacts with pancreatic glucagon	1: 3,000
Porcine glucagon[d]	RPN. 1602	Completely cross-reacts with pancreatic and intestinal glucagon	1: 1,000
Synthetic porcine glicentin (49-69)[j]	R-4804	Reacts with the C-terminal	1: 3,000
APP[e]	Lance-10/ 5/81 Bleed	No cross-reaction with glucagon	1: 10,000
BPP[i]	615-R-110-146-17	Cross-reacts with human pancreatic polypeptide	1: 12,000
5-HT[b]	Lot. 16302	–	1: 10,000
Synthetic porcine motilin[j]	R-1104	Reacts against entire molecules	1: 100 to 1: 4,000
Synthetic porcine motilin[j]	R-1106	Reacts primarily with the C-terminal	1: 100 to 1: 4,000
Synthetic human gastrin[j]	GP-1304	No cross-reaction with CCK-8	1: 5,000
GIP[g]	G/R/34-III D	No cross-reaction with glucagon	1: 10,000
Natural porcine CCK-33[h]	–	Reacts with CCK 11-20; no cross-reaction with gastrin	1: 3,000
CCK-8[b]	72812	Cross-reaction with gastrin	1: 1,000
Synthetic porcine secretin[j]	R-801	Reacts with the C- and N-terminals	1: 1,000
Synthetic bovine neurotensin[j]	R-3501	–	1: 1,000
Leucine-enkephalin[f]	I671	–	1: 80,000
Synthetic substance P[j]	R-2404	No cross-reaction with GRP	1: 1,000
Synthetic porcine VIP[j]	R-502	Reacts against entire molecules	1: 2,000
Synthetic porcine GRP[j]	R-6902	Reacts primarily with the C-terminal; no cross-reaction with substance P	1: 1,000
Bombesin[b]	27070	Cross-reaction with GRP	1: 3,000

[a] All antisera were raised in rabbits except those against insulin and gastrin which were raised in guinea pigs. [b,d,f,g] These antisera were purchased from Immunonuclear Corp., Stillwater; Amersham International pl., Amersham; UCB-Bioproducts, Bruxelles; Guildhay, Surrey, respectively. [c,d,g,i] These antisera were kindly donated by Dr S. ITO, Niigata; Dr J. R. KIMMEL, Kansas City; Prof. D. GRUBE, Hannover; Dr R. E. CHANCE, Indianapolis, respectively. [j] These antisera were produced by N. YANAIHARA.

Table 2. Distribution and frequency of gastrointestinal endocrine cells in the caiman

	Stomach		Intestine					Cloaca
	Fundic	Pyloric	Initial region	Middle of proximal half	Middle of whole length	Middle of distal half	Ampulla	
5-HT	+	−	++	++	++	++	++	−
Somatostatin	+	++	+	+	+	+	±	−
Gastrin	−	+++	++	++	+	−	−	−
Motilin	±	++	++	++	+	±	−	−
Neurotensin	−	−	+	+	+	+	++	++
BPP	−	−	±	±	±	−	−	−
APP	−	−	−	±	−	−	−	−
Pancreatic glucagon	±	−	±	±	±	−	−	−
Enteroglucagon	++	−	++	++	++	+	±	−
Glicentin	−	−	±	±	±	−	−	−
Secretin	−	−	±	−	−	−	−	−
CCK-33	−	−	±	−	−	−	−	−
GRP and Bombesin	−	−	−	−	−	−	−	−
GIP	−	−	−	−	−	−	−	−

− absent, ± rare (not detected in every animal), + few (detected in every animal but not in every section), ++ moderate, +++ numerous.

from Amersham, completely cross-reacted with pancreatic- and entero-glucagons) were classified as enteroglucagon-immunoreactive cells.

RESULTS

The stomach

The caiman has an avian-like stomach which consists of a large gizzard-like stomach and a small pyloric caecum (KENT, 1978). The former has thick muscular walls and is lined with gastric glands, while the latter is a small chamber situated between the gizzard-like stomach and the duodenum, being lined with pyloric glands. The two compartments are clearly distinguished by the presence of a constriction having a small connecting segment. In this study the former component shall be termed the fundic stomach and the latter the pyloric stomach. The gastric glands of the fundic stomach open into overlying gastric pits. The gastric glands are composed of a single cell type, the oxynticopeptic cell, which corresponds to both chief (pepsinogen-producing) and parietal (acid-producing) cells in the mammalian stomach. The surface epithelium and mucous neck cells in the fundic stomach are stained in purple with the alcian blue-PAS staining method. The length of the pyloric glands is shorter than that of the fundic glands. They consist of cells that resemble the surface epithelium and mucous neck cells of the fundic stomach.

Cells immunoreactive for 5-hydroxytryptamine (5-HT), somatostatin, enteroglucagon, pancreatic glucagon and motilin were observed in the glands of the fundic stomach. They arranged in number from few to rare, with the exception of enteroglucagon-immunoreactive cells (Table 2 and Fig. 2A, B). Most of these immunoreactive cells were distributed throughout the fundic stomach. In contrast, motilin-immunoreactive cells were restricted in location to the small connecting segment between the fundic and pyloric stomachs, and were confined primarily to the basal half of the glands. The majority of endocrine cells in this region were round or oval in shape and did not have luminal contact via an apical cytoplasmic process. However, some endocrine cells did reveal their basal cytoplasmic processes as coursing along the basal lamina (Fig. 2B). Only on rare occasions were the open-type endocrine cells observed, these having established contact with a gland lumen via singler apical cytoplasmic process (Fig. 2A). Gastrin releasing peptide (GRP)- and bombesin-immunoreactivities were not observed in endocrine cells of the fundic glands, but were seen in the nerve fibers located in the surrounding muscle layers (Fig. 2C).

Numerous gastrin-immunoreactive cells and moderate numbers of somatostatin-

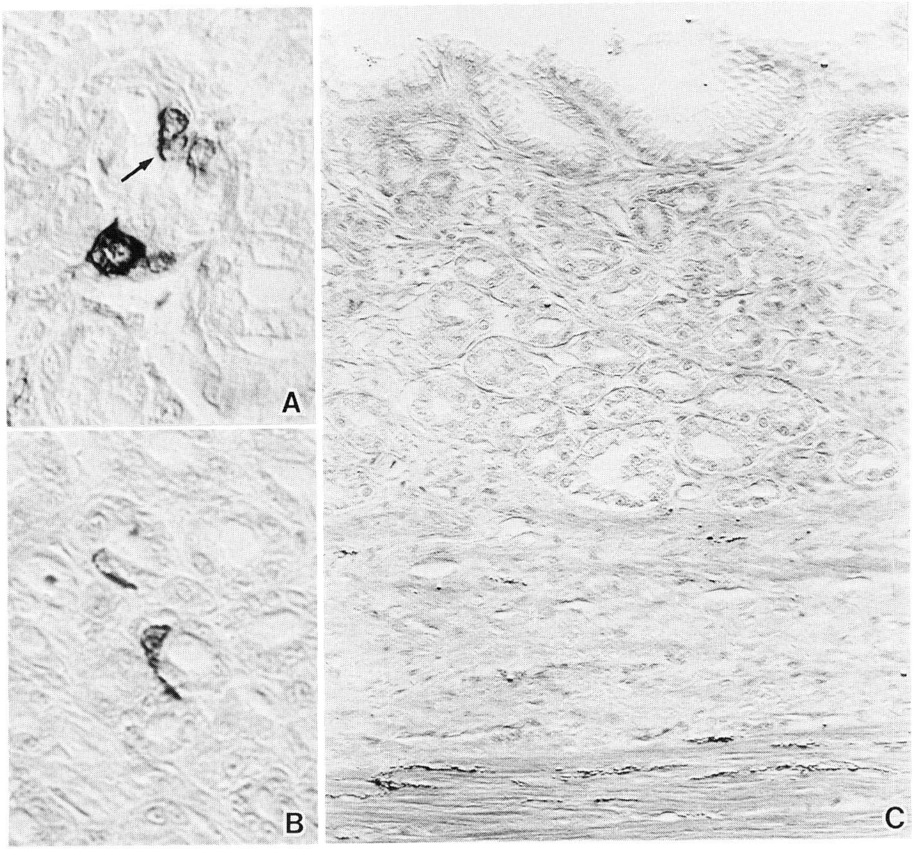

Fig. 2. **A.** Pancreatic glucagon-immunoreactive cells in the fundic glands. One cell appears to reach the lumen of the gland with an apical process (arrow). **B.** Motilin-immunoreactive cells in the fundic glands. These cells seem to be of the closed type. **C.** GRP-immunoreactivity is found in nerve fibers in the smooth muscle layer of the fundic stomach but not in cells of the fundic glands. PAP method. A, B : ×360, C : ×180

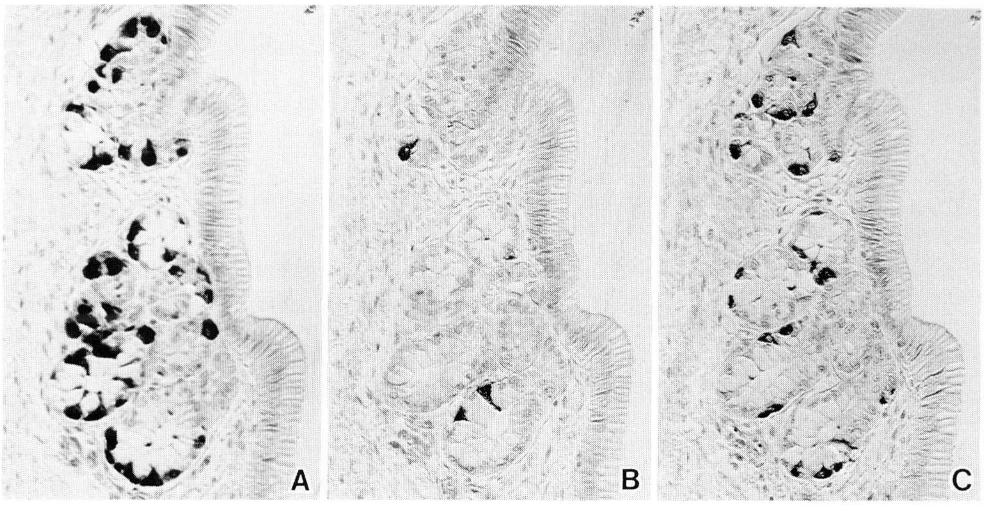

Fig. 3. Gastrin- (**A**), motilin- (**B**) and somatostatin- (**C**) immunoreactive cells in serial sections of the pyloric glands. Motilin-immunoreactive cells do not show cross-reactivity with either gastrin or somatostatin. A: bridge method, B, C: PAP method. ×180

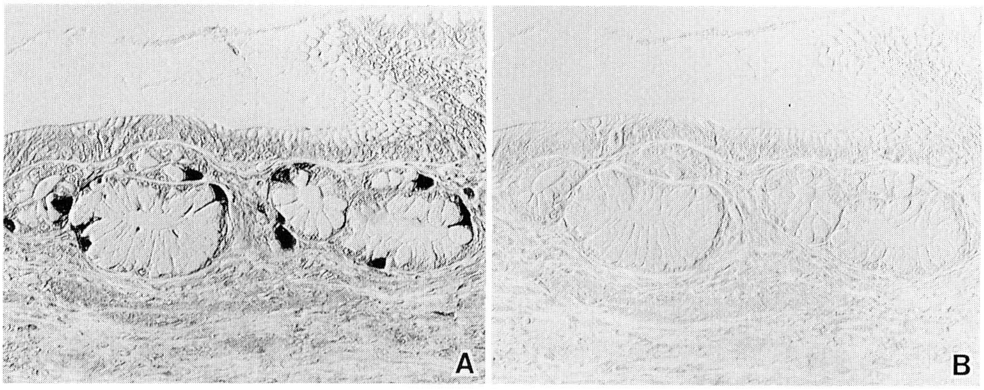

Fig. 4. Two serial sections stained with anti-gastrin (**A**) and anti-CCK-33 (**B**) sera. Gastrin-immunoreactive cells are not stained with anti-CCK serum. A: bridge method, B: PAP method. ×180

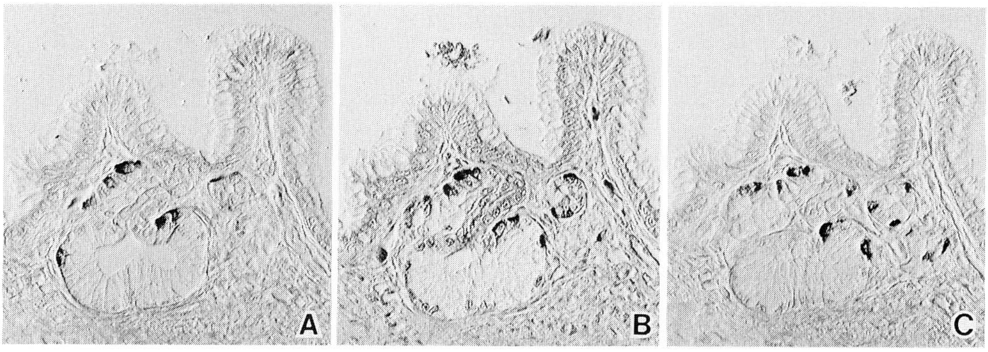

Fig. 5. Three serial sections stained with anti-CCK-8 (**A, C**) and anti-gastrin (**B**) sera. All gastrin-immunoreactive cells are also stained with anti-CCK-8 serum. A, C: PAP method, B: bridge method. A–C: ×720

and motilin-immunoreactive cells were distributed throughout the pyloric glands of the pyloric stomach (Table 2 and Fig. 3). These endocrine cells were pyramidal in shape and often established contact with the lumen via slender, elongated, apical cytoplasmic processes (Fig. 3-5). When utilizing the thin serial sections, motilin immunoreactivity was never observed to coexist in the same cell with the other two peptides found in this region (Fig. 3). The gastrin-immunoreactive cells were stained with the anticholecystokinin (CCK)-8 serum (Fig. 5), but did not cross-react with the anti-CCK-33 serum specific for CCK-11-20 (Fig. 4).

The intestine

Because the caiman lacks a caecum, an obvious macroscopic subdivision of the intestinal tract into small and large components is difficult. Therefore, intestinal tissues from five different portions were examined as shown in Figure 1 and Table 2. Although the cloaca is not considered to be a portion of the digestive tract in most instances, it was examined in this study. Intestinal folds, not villi, were found along the entire length of the intestine with exception of the terminal region or ampulla. The mucosa of the intestine was lined with a pseudostratified epithelium and the number of goblet cells increased distally toward the cloaca. Structures equivalent to the crypts of Lieberkühn were not observed, although invaginations were seen at the basal portions of the intestinal folds.

In the intestine, 12 types of immunoreactive cells were observed and are listed in Table 2. They were long pyramidal and long spindle in shape, and reached the intestinal lumen with a long apical process (Fig. 6, 7). Cells immunoreactive for 5-HT, somatostatin, enteroglucagon and neurotensin were distributed along the entire length of the intestine. Table 2 summarizes the distribution of other type of immunoreactive cells. Cells containing 5-HT-immunoreactivity were the most abundant of the endocrine cells observed in the intestine. Somatostatin- and enteroglucagon-immunoreactive cells were found in moderate numbers in the proximal intestine but decreased in number distally. In contrast, neurotensin-immunoreactive cells were found in relatively small numbers in the proximal intestine, increasing in number in the distal intestine. Gastrin- and motilin-immunoreactive cells were found in the proximal half and in the proximal four-fifths of the intestine, respectively. They were moderate in number in the proximal half of the intestine, and decreased in number from moderate to rare in the distal intestine. A few bovine pancreatic polypeptide (BPP)-immunoreactive cells were found in the proximal four-fifths of the intestine, while avian pancreatic polypeptide (APP)-immunoreactive cells were found on occasion in the middle portion of the proximal half of the intestine. Pancreatic glucagon- and glicentin-immunoreactive cells were rarely found in the proximal half of the intestine. Occasionally, a few secretin- and CCK-immunoreactive cells were detected in the initial portion of the intestine. No other types of immunoreactive cells could be detected in the caiman intestine.

A small number of 5-HT-immunoreactive cells and moderate numbers of neurotensin-immunoreactive cells were also a regular observation in the cloacal mucosa.

DISCUSSION

The results of this study confirm and further extend the information available concern-

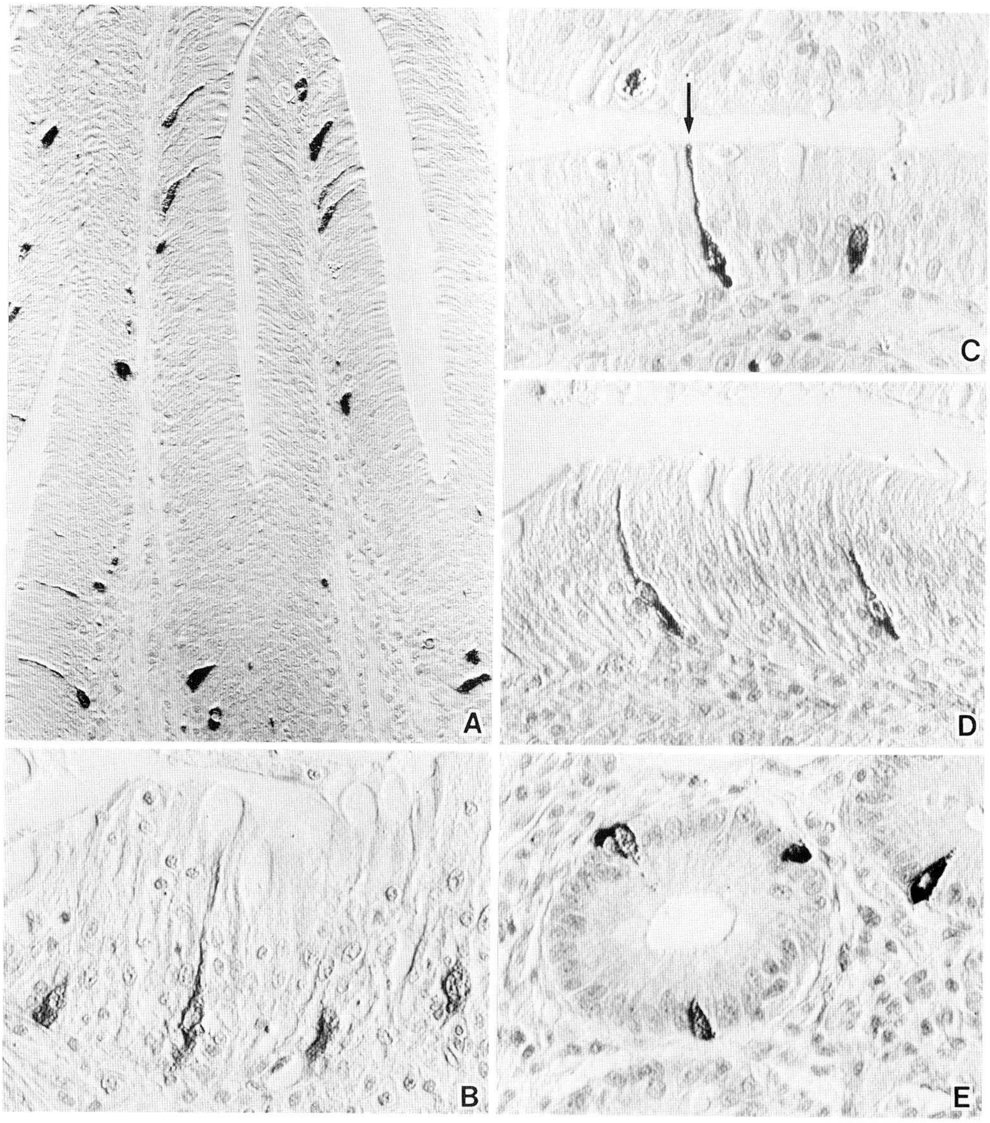

Fig. 6. **A.** 5-HT-immunoreactive cells in the intestinal epithelium in the initial region of the intestine. ×180. **B.** Four gastrin-immunoreactive cells in an epithelium in the initial region of the intestine. ×360. **C** and **D.** Two somatostatin- (**C**) and motilin- (**D**) immunoreactive cells in the epithelium in the middle region of the proximal half of the intestine. One somatostatin-immunoreactive cell shows contact with the lumen via an apical cytoplasmic process (arrow). ×360. **E.** Neurotensin-immunoreactive cells in the intestinal crypts of the ampulla. A, C-E : PAP method, B : bridge method. A-E : ×360

ing the reptilian gut endocrine cells. In an earlier study by GABE (1973) that examined reptilian species, including the crocodiles (*Crocodylus nitoricus* and *Osteolaemus tetraspis*), only two types of endocrine cells were identified using cytochemical and histochemical methods. Recently, BUCHAN et al. (1983) reported seven types of

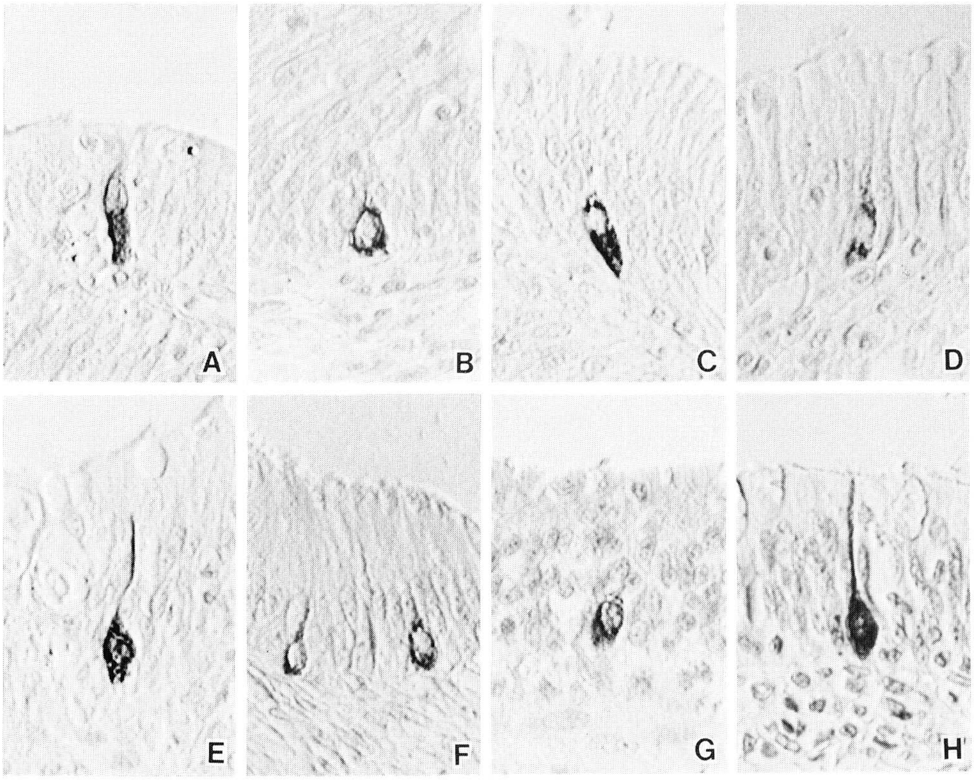

Fig. 7. Eight types of endocrine cells immunoreactive for BPP (**A**), neurotensin (**B**), secretin (**C**), CCK-33 (**D**), APP (**E**), pancreatic glucagon (**F**), glicentin (**G**) and enteroglucagon (**H**) immunoreactive cells from the intestinal folds in the initial region (**A, B**), from the middle region of the proximal half (**B, C, D, E**), from the middle region of the whole length (**F, G**) and from the middle region of the distal half of the intestine (**H**). Note the open type of the endocrine cells. PAP method. ×360

endocrine cells immunoreactive for gastrin, somatostatin, pancreatic glucagon, enteroglucagon, neurotensin, motilin and bombesin in the gut of the alligator, *Alligator mississippiensis*.

In the present study, twelve types of endocrine cells immunoreactive for 5-HT, somatostatin, gastrin, motilin, neurotensin, BPP, APP, pancreatic glucagon, enteroglucagon, glicentin, secretin and CCK were identified in the gastrointestinal mucosa of the caiman. Although the caiman and the alligator are classified in the same subfamily, *Alligatorinae*, some differences were found to exist with regard to the types and distribution of the gut endocrine cells between these two species.

It is noteworthy that bombesin-immunoreactive endocrine cells were not found in the fundic stomach of the caiman, while they were numerous in the same region of the stomach of the alligator (BUCHAN et al., 1983). Bombesin-immunoreactive endocrine cells have been reported in the stomachs of amphibians (LECHAGO et al., 1978 ; BUCHAN et al., 1980 ; EL-SALHY et al., 1981) and birds (TIMSON et al., 1979 ; VAILLANT et al., 1979). Although cells immunoreactive for bombesin and gastrin releasing polypeptide (GRP) were not observed in the fundic glands of the caiman, a small population of nerve fibers immunoreactive for both peptides was observed in the muscle layers. The

GRP molecule contains 27 amino acid residues as compared with 14 residues found in bombesin and nine of the carboxyl terminal amino acids occupy identical positions in the two molecules. Because of the sequence similarity in the carboxyl-termini of both peptides, the GRP antiserum used consistently cross-reacted with the bombesin antiserum (YANAIHARA et al., 1981; BUFFA et al., 1982). GRP-immunoreactive cells have also been reported in the avian proventriculus (BUFFA et al., 1982; YAMADA et al., 1985) and gizzard (YAMADA et al., 1986a). In reptiles, bombesin-immunoreactive cells have been described in the grass lizard stomach (EL-SALHY and GRIMELIUS, 1981). The phylogenetical line from amphibians to birds via reptiles is well established, and crocodiles and early birds are considered to have evolved from the same ancestral branch of reptiles. This relationship suggests the presence of bombesin- or GRP-immunoreactive cells in the caiman stomach. However, bombesin immunoreactivities were not observed in cells of the fundic glands and were found only in the intramural nerve fibers as reported in the mammalian stomach (DOCKRAY et al., 1979). This results suggests that the caiman stomach may more closely resemble the stomach of mammals than that of other reptiles and birds.

Another interesting observation in the caiman gut mucosa was the presence of motilin-immunoreactive cells. Motilin-immunoreactive cells were found in the fundic and pyloric stomachs as well as in the intestine of the caiman. They were not observed in the alligator gastrointestinal tract (BUCHAN et al., 1983) nor in the small intestine of snakes (MASINI, 1986), and attempts to demonstrate motilin-immunoreactive cells in eleven teleost fishes have failed (LANGER et al., 1979). In addition, motilin-immunoreactive cells have been observed in the duodenum of the axolotl, *Ambystom mexicanum* (BUCHAN 1986), but not in any of the other amphibian species examined (BUCHAN et al., 1980; EL-SALHY et al., 1981; FUJITA et al., 1981; BUCHAN, 1986). Immunoreactive cells have been demonstrated in the small intestine of only one reptilian species, the grass lizard (EL-SALHY and GRIMERIUS, 1981). Although motilin-immunoreactive cells are considered to be one of the major endocrine cell types in the mammalian small intestine, they have not been detected in several mammalian species including the rat, vampire bat and field vole (SMITH et al., 1981; YAMADA et al., 1984; OHARA et al., 1986). It is not yet clear whether these results may be due to the absence of the endocrine cell type in a specific species or to the high species specificity of motilin antisera (BLOOM and POLAK, 1978). In the caiman, small to moderate numbers of motilin-immunoreactive cells were observed in the fundic and pyloric stomachs. The coexistence of motilin-like immunoreactivity with other peptides and an amine was not observed in the same cell type in the stomach. The intravenous infusion of motilin is known to induce migrating contractions from the stomach to the intestine during the interdigestive state in dogs (ITOH, 1981) and endogeneous motilin also has been shown to stimulate pepsin secretion in dogs (KOCH et al., 1976; KONTURECK et al., 1976). Based on information available from mammalian species, it seems reasonable to suggest that motilin-immunoreactive cells in the caiman stomach may also play a role in gastric secretion and emptying.

The distribution and relative frequency of somatostatin-, gastrin- and secretin-immunoreactive cells in the caiman gastrointestinal tract are similar to those reported in the alligator (BUCHAN et al., 1983). Neurotensin-immunoreactive cells in the caiman were scattered not only throughout the length of the intestine but also in the cloaca. They were observed, however, only in the small intestine of the alligator (BUCHAN et al., 1983). The functional significance of the wide distribution of neurotensin-immunoreactive cells in the caiman is not known. Since 5-HT and pancreatic polypeptides (PP) have not been investigated in the alligator (BUCHAN et al., 1983), the present results on 5-HT and PP of the caiman can not be compared with those of the alligator. Interestingly, 5-HT-immunoreactive cells, which are common in mammals, were not observed in the caiman pyloric stomach.

According to BUCHAN et al., (1983), CCK-like peptide, present in the reptile intestine, has a regional similarity to the CCK octapeptide region (25-32) of porcine CCK but lacks the mid-portion (9-20) sequence as demonstrated by the results of the immunostainings. In the caiman, however, very small numbers of CCK-immunoreactive cells could be identified using antiserum reactive to CCK 11-20 which has no cross-reactivity with gastrin. In the alligator, the pancreatic glucagon specific antiserum has demonstrated numerous mucosal endocrine cells in the fundus but could not detect any cells in the intestine (BUCHAN et al., 1983). In the snake, no immunoreactivity was found with pancreatic glucagon specific antiserum (MASINI, 1986). In the present study, however, pancreatic glucagon-immunoreactive cells were found only rarely in the fundic stomach and proximal half of the intestine. Glicentin-immunoreactive cells were seldom found in the caiman intestine, and were not detected at all in the alligator (BUCHAN et al., 1983). The antiserum used for glicentin in the present study was carboxyl-terminal specific, which may be different from that of BUCHAN et al. (1983). The absence of GIP-immunoreactive cells in the caiman intestine corroborates observations reported in the alligator (BUCHAN et al., 1983). However, the intestine of the grass lizzard (EL-SALHY and GRIMERIUS, 1981), the teleost fishes (FALKMER et al., 1980) and the cartilaginous fish (EL-SALHY, 1984) do contain GIP-immunoreactive cells.

Pancreatic hormones of the alligator are reported to be more closely related to avian than mammalian species (BUCHAN et al., 1983 ; LANCE et al., 1984). LANCE et al., (1984) also have shown that the amino acid sequence of alligator insulin has more similarities to avian insulin than to insulin from snakes. These observations support the evolutionary evidence for the development of birds and certain crocodilians from a common branch of the reptilian group. In the caiman pancreas (YAMADA et al., 1986b), however, pancreatic polypeptide containing cells were detected by both APP- and BPP-antisera. Alligator PP-containing cells were detected by APP-antiserum only (BUCHAN et al., 1982). Bombesin- or GRP-immunoreactivity was demonstrated only in the intramural nerve fibers, a situation that also occurs in mammalian species. In the alligator, both types of immunoreactivities were identified in endocrine cells of the fundic glands as well as in the intramural nerves, as also holds true in the case of birds (BUCHAN et al., 1983). Although the alligator and caiman are placed in the same subfamily, *Alligatorinae*, they seem to be significantly different with regard to the type and distribution of gastroenteropancreatic regulatory peptides. The present data suggest that the caiman may be a type of the crocodilian more closely alinged to mammalian forms, whereas the alligator type of crocodilian may be more closely alinged to avian forms.

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山田純三
〒080 帯広市稲田町
帯広畜産大学畜産学部
家畜解剖学教室

Dr. Junzo YAMADA
Department of Veterinary Anatomy
Obihiro University of Agriculture and Veterinary Medicine
Inada-cho, Obihiro, 080 Japan