PHYLOGENY OF THE GUT HORMONES FROM AMPHIBIA TO MAN

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ABSTRACT

The distribution, tissue localisation and actions of the gut peptides have been comprehensively studied solely in mammals. The aim of this study was to expand the information to include the lower vertebrates (in this case the amphibians and reptiles) by establishing the phylogenetic development of the regulatory peptide system.

The work involved the localisation of the gut peptides by immunocytochemical techniques using well characterised antisera to the mammalian peptides. The gut and pancreas from twenty-five species was investigated. The species were drawn from the following families -

Reptilia - Emydidae, Chelydridae, Crocodylidae, Agamidae, Gekkonidae, Boidae, Colubridae and Viperidae,

Amphibia - Salamandridae, Discoglossidae, Ranidae, Bufonidae, Atelopodidae and Hylidae.

The peptides studied included gastrin, cholecystokinin, insulin, glucagon (pancreatic and enteric), somatostatin, pancreatic polypeptide, gastric inhibitory polypeptide, motilin, secretin, neurotensin, vasoactive intestinal polypeptide, substance P, enkephalin and bombesin.

The results show that in the amphibia the dual localisation to mucosal endocrine cells and nerve fibres and cell bodies, as seen in the mammals, was already well established. Of the peptides/.....

peptides investigated only cholecystokinin, motilin, gastric inhibitory polypeptide and secretin were undetected in the amphibians. Even in mammalian tissues there are species-specific problems in detecting motilin and gastric inhibitory polypeptide, therefore as the investigation was limited to the use of mammalian antisera the absence of immunoreactivity cannot exclude the presence of the peptides in amphibian material. In the reptilia all the peptides investigated were detectable.

The study has demonstrated that the regulatory peptide system is, in comparative terms, well established in vertebrate evolution. It will also show that, especially in some Anurans and Serpentes, further work will provide excellent experimental models which may give considerable information as to the actions of some of the regulatory peptides.

ABBREVIATIONS

Ins	-	Insulin
Gluc	-	glucagon
Gas	-	gastrin
CCK	-	cholecystokinin
SP	-	substance P
BN	-	bombesin
NT	-	neurotensin
SOM	-	somatostatin
GIP	-	gastric inhibitory polypeptide
VIP	-	vasoactive intestinal polypeptide
MOT	-	motilin
SEC	-	secretin
PP	-	pancreatic polypeptide
ENK	-	enkephalin
NSE	-	Neurone specific enolase
IMF	-	immunofluorescence
PAP	-	peroxidase anti-peroxidase
PBS	-	Phosphate buffered saline

TABLE OF CONTENTS

Aknowledgements			1
Abstract			2
Abbreviations			4
Table of Contents			5
List of Tables			10
List of Diagrams			12
Chapter 1			
1.1	Introduction		13
1.2	Histology		14
1.3	APUD concept		16
1.4	Peptide-containing Autonomic Nerves		18
1.5	Physiology		19
1.6	Pathology		20
1.7	Chemistry		20
1.8	Antisera		23
1.9	Immunocytochemistry		23
1.10	Outline of Thesis		25
Chapter 2 -	Information on the relevant anatomy and histology of the gastrointestinal tract and pancreas		
2.1	Amphibia		30
	2.1.1	Phylogeny	30
	2.1.2	Peptides	30
	2.1.3	Conventional Histology and Anatomy	31
		a. Pancreas	33
		b. Stomach	34
		c. Intestine	35
2.2	Reptilia		
	2.2.1	Phylogeny	36
	2.2.2	Conventional Histology and Gross Anatomy	38
		a. Pancreas	
		b. Stomach	
		c. Intestine	
Chapter 3 -	Material		
3.1	Amphibia		40
3.2	Reptilia		41

Chapter 4/.....

Chapter 4 - Methodology

4.1 Fixation 44

4.2. Immunocytochemistry 45

4.3 Antisera 47

Chapter 5 - Results - Light microscopy and Gross Anatomy

5.1 Legend of Tables 52

5.2 Amphibia 54

5.2.1 Histology 54

5.2.2 Urodela 54

a. Immunostaining 57

i. Salamandra salamandra 57

ii. Ambystoma mexicanum 62

iii. Cynops honkongensis 63

b. Summary - Urodela 66

5.2.3 Anura 66

a. Pancreas 69

i. Discoglossidae - Bombina bombina 69

ii. Alytes obstetricans 70

iii. Ranidae Rana temporaria 74

iv. Rana esculenta 82

v. Bufonidae Bufo bufo 87

vi. Atelopodidae Atelopus oxyrhynchus 105

vii. Hylidae Hyla arborea 106

viii. Hyla crepitans 114

b. Summary - Anura 119

5.3 Reptilia

5.3.1 Histology 120

5.3.2 Testudinata

a. Immunostaining 124

i. Emys orbicularis 128

ii. Pseudemys scripta 129

iii. Chelydra serpentia 135

b. Summary - Testudinata 143

5.3.3 Squamata

a. Lacertilia

i. Hemidactylus brookii 143
ii. Agama agama 145
b. Summary - Lacertilia 157
c. Serpentes
i. Boidae - Python recticularis 157
ii. Colubridae - Thamnophis sirtalis 163
iii. Natrix natrix 171
iv. Nerodia rhombifera 172
v. Viperidae - Agkistrodon piscivora 179
vi. Crotalus atrox 180
d. Summary - Serpentes 180
5.3.4 Crocodilia
a. Crocodylidae 183
i. Alligator mississipiensis
ii. Caiman crocodylus crocodylus 198
b. Summary - Crocodylidae 199
Chapter 6 - Discussion - Light Microscopy
6.1 Pancreas 202
a. Islet Structure 202
b. Distribution of islet cells 203
c. Regional variations 203
d. Individual Peptides 204
i. Pancreatic Polypeptide
ii. Glucagon
iii. Insulin and Somatostatin
e. Innervation 208
f. Summary 210
6.2 Stomach 210
a. Fundus
i. Neurotensin
ii. Glucagon
iii. Substance P/bombesin
iv/.....

iv. Somatostatin
v. VIP
vi. Substance P
vii. Bombesin
viii. Hibernation
b. Antrum 216
i. Neurotensin
ii. Gastrin/CCK
iii. Somatostatin
iv. VIP
6.3 Intestine - Duodenum 220
a. Peptides of limited distribution 221
b. VIP 221
c. Other peptides 225
i. Endocrine cells
d. Hibernation 225
e. Neural Elements 227
i. VIP
ii. Substance P
iii. Endogenous opiates
iv. Bombesin
v. Other peptides
vi. NSE

Chapter 7 - Scanning Electron Microscopy
7.1 Note 237
7.2 Results
a. Anura Rana temporaria 237
b. Alligator mississipiensis 237
c. Rattus rattus 249
d. Summary 249

Chapter 8 - Electron Microscopy - Transmission
8.1 Results 252
8.2 Urodela - Cynops hongkongensis 253
8.3 Anura - Hyla arborea 260
8.4 Testudinata - Cheldra serpentia 267
8.5 Serpentes - Agkistrodon piscivora 280
8.6 Crocodylidae - Alligator mississipiensis 295

Chapter 9/.....

Chapter 9 - Discussion - Transmission Electron Microscopy 317

Chapter 10 - Evaluation 326

Appendices 335

References 340

LIST OF TABLES

1. Wiesbaden Classification.

2. Revised Lausanne Classification.

3. Regulatory Peptides and their main actions.

4. Pathological Disorders.

5. Species Studied.

6. Antisera 1 - Gastrin/CCK.

7. Antisera 2 - VIP.

8. Antisera 3 - Region Specific Antisera.

9. Antisera 4 - Non-region specific antisera.

10. Salamandra salamandra.

11. Ambystoma mexicanum.

12. Cynops hongkongensis.

13. Bombina bombina.

14. Alytes obstetricans.

15. Rana temporaria.

16. Rana esculenta.

17. Bufo bufo (hibernating).

18. Bufo bufo (re-fed).

19. Atelopus oxyrhynchus.

20. Hyla arborea.

21. Hyla crepitans.

22. Emys orbicularis.

23. Pseudemys scripta.

24. Chelydra serpentia.

25. Hemidactylus brookii.

26. Agama agama.

27. Python recticularis.

28. Thamnophis sirtalis.

29. Natrix natrix.

30. Nerodia rhombifera.

31. Agkistrodon piscivora.

32. Crotalus atrox.

33. Alligator mississipiensis.

34. Caiman crocodylus crocodylus.

35. Secretory granule diameter in the pancreatic islet cells.

36. Summary of the information collected during this study on the presence of the gut peptides in Amphibia and Reptilia.

DIAGRAMS

1. Sequence similarities Gastrin and CCK.
2. Sequence similarities Secretin family.
3. Sequence similarities Substance P/bombesin.
4. Sequence similarities Neurotensin and Xenopsin
5. Phylogenic relationships Amphibia.
6. Phylogenic relationships Reptilia.
7. Location of the reptilian pancreas.
8. Serpentes intestine and pancreas.
9. Alligator stomach.
10. Proposed model of nerve action.

Chapter 1

1.1. Introduction

The term "Gut Hormone" refers to the biologically active peptides isolated from mammalian intestine. It has been suggested that these peptides form a major regulatory system controlling a number of gastrointestinal functions including motility, blood flow and secretion (Polak and Bloom 1980). In the strict sense of the word the gut peptides should not be termed hormones as many of the criteria for their recognition as such have as yet to be fulfilled. The precise physiological action of most of these peptides is unknown, although there is growing evidence from pharmacological studies that specific actions do exist.

Due to the wide variety of physiological processes involved, the control of gut function is a complex system. Functions such as the release of the digestive enzymes, acids and mucins as well as muscle contraction and the normal balance of nutrient absorption must be regulated, at least in part by the proposed system.

The original Pavlovian view that all functions are ultimately under neural control was transiently reversed by Bayliss and Starling at the beginning of the century. They isolated an active, humoral factor from pig duodenum which, when administered to dogs with a denervated stomach, elicited a dose related release of pancreatic juice; this factor was subsequently/.....

subsequently named secretin (Bayliss and Starling 1902). Their work was taken, then, to have disproved Pavlov's theory of neural control, leading to an almost exclusive concentration of effort on purification and identification of further humoral (peptide) factors from the intestine. Much of this work has been carried out by Mutt and co-workers at the Karolinska Institute.

1.2. Histology

As these peptides were being isolated from intestinal tissue, it was thought that they were synthezised and secreted from this region. Feyrter had, in 1938, demonstrated the existence of a system of clear cells 'helle Zellen' distributed throughout the intestine, which appeared to be unstained with common histological methods (Feyrter 1938). Feyrter suggested that these cells might have a local (paracrine) action on the surrounding tissue.

Subsequent ultrastructural work showed that the cells contain characteristic secretory granules, varying from one cell type to another. A classification of cell types based on the appearance of the cells and their characteristic secretory granules was introduced in 1970 (Creutzfeld 1970) (see Table 1) in order to provide a uniform nomenclature. There was at that time no direct evidence available(from localisation work at the ultrastructural level) that these cells did in fact produce the biologically active peptide found in the gut, although the antral G-type cells were/.....

were linked with the production of gastrin (Solcia et al 1969) as the distribution of these cells paralleled the results found in immunocytochemical work using antisera to gastrin with light microscopy (McGuigan 196?).

Table 1 - Wiesbaden Classification

Cell type		Secretory granules	Localisation	Hormone
I (serotonin)		polymorphic electronopaque		
	a	big	cardia to jej.	serotonin
	b	small	cardia to jej.	
	c	nibbled	stomach	
II (A cells)		round, electronopaque		
	a	big	cardia, fundus	?
	b	dense core	fundus	?
	c	small	small intestine	secretin pancreozy
III (D cells)		pale, round		
	a	variable electrondensity	fundus	?
	b	big	pylorus	?
IV (X cells)		round, electrondense		
	a	big	stomach to jej.	?
	b	irregular, clear core	fundus	?
V (gastrin cell)		round, variable electrondensity	pylorus	gastrin

The further study of these cells proceeded at the same time as the biochemical purification of gut peptides. When pure samples of the peptides were available it was possible to produce specific antibodies to the peptides and to determine their amino acid compositions.

The/.....

The development of specific antisera to the individual peptides was necessary in order to demonstrate their cellular origin. When these antisera were available techniques such as immuno-cytochemistry were modified as required for studies of these peptides by both light and electron microscopy.

The result of all this has been to show that the cells of the 'helle Zellen' system are in fact the source of some of the intestinal peptides. The up-dated classifications produced at Lausanne (Solcia et al 1978) and Cambridge (Solcia et al 1981) (see Table 2) summarise the present knowledge of this system, the majority of the cell types no longer being defined purely on morphological grounds but by the peptide produced. However, it must be noted that the information used to create the classification is confined to mammals with special emphasis on man.

1.3. APUD concept

As early as 1968, a theory was put forward linking the intestinal endocrine cells with those of the endocrine glands. This was the APUD theory (Pearse 1968) based on certain common cytochemical characteristics, principally the presence of intrinsic biogenic amines or the ability to take up and decorboxylate their amino acid precursors. APUD is in fact an acronym of this ability, ie Amine Precursor Uptake and Decarboxylation.. It was originally proposed that all members of the APUD group, regardless of spatial location, were derived from the neural crest. In the light of more recent/.....

Table 2 Revised Lausanne Classification of Human Gastroenteropancreatic Endocrine Cells.

CELL	MAIN PRODUCT	PANCREAS	STOMACH Oxyntic	STOMACH Antral	INTESTINE Small Upper	INTESTINE Small Lower	INTESTINE Large
P	?	a	+	+	+		
D_1	?	f	+	f	f	f	f
EC	5HT, Peptides	r,b	+	+	+	+	+
D	Somatostatin	+	+	+	+	f	f
B	Insulin	+					
A	Glucagon	+	a,b				
X	?		+				
ECL	?(b,histamine)		+				
G	Gastrin			+	f		
IG	Gastrin			b	+	r	
TG	C-terminal G/CCK			b	+	b	
I	CCK				+	f	
S	Secretin				+	f	
K	GIP				+	f	
Mo	Motilin				+	f	
N	Neurotensin				r	+	r
L	GLI				f	+	+

a = foetus or newborn b = animals f = few r = rare

recent evidence this has been modified to include specialised neural and placodal ectoderm (Pearse and Takor Takor 1976).

1.4. Peptide-containing Autonomic nerves

A peptide-containing component of both the peripheral and central nervous systems has recently been demonstrated by immunocytochemistry. Peptides originally thought to be produced in endocrine cells have been localised in intestinal nerve plexuses, such as substance P (Nilsson et al 1975, Pearse and Polak 1975) and vasoactive intestinal polypeptide (VIP) (Bryant et al 1976, Larsson et al 1976), both the myenteric and the sub-mucous. On the other hand enkephalin, first isolated from brain tissue (Hughes et al 1975) has been found in a population of enteric nerves (Alumets et al 1978).

These findings provide circumstantial evidence in support of the APUD theory.

An extension of the APUD theory which links the morphological appearance of the individual elements with the possible mode of secretory activity has been suggested. In relation to this it has been proposed that all the secretory elements, whether nerves or endocrine cells, are merely a refinement of an original secretory cell (Fujita & Kobayashi 1979). In this way the regulatory peptides may show a spectrum of potential actions. For convenience these can be divided into four general groups:-

1. Classical/.....

1. Classical neurotransmitters, released from a nerve terminal at a synaptic junction, fast acting and of short duration.
2. Neuromodulators, released from nerve terminals but not necessarily at a synaptic junction, slow in onset and of longer duration.
3. Paracrine (locally acting) cells, releasing their product into the intercellular spaces and thus controlling the function of surrounding cells.
4. Endocrine cells releasing their product into the circulation and affecting their target organs which may be quite distant.

In effect it would seem as if both Pavlov and Bayliss and Starling were correct in that the control of gut function is accomplished by an interaction between humoral (endocrine) and neural elements with a common factor being the presence of peptides as the proposed active agents.

1.5. Physiology

At the same time as their morphological localisation was being established, physiological studies were being initiated to establish the action of each of the purified peptides. There are at present 15 recognised biologically active peptides isolated from the gut. Their actions show considerable similarities but presumably each responds to different stimuli. In Table 3 the peptides and actions, as established to date, are given. It must be remembered that/.....

Table 3 Regulatory Peptides and their main actions.

PEPTIDE	ACTIONS
Gastrin	Gastric acid release Trophic
CCK	Pancreatic enzyme release Gall bladder contraction
Secretin	Pancreatic bicarbonate release
Motilin	Upper intestinal motility
GIP	Insulin release
Neurotensin	Stimulates glycogenolysis
Enteroglucagon	Trophic
Insulin	Hypoglycaemic
Glucagon	Hyperglycaemic
Somatostatin	Inhibitory
PP	Inhibits pancreatic enzyme release
Substance P	Vasodilation smooth muscle contraction
VIP	Vasodilation smooth muscle relaxation
Enkephalin	Inhibits gastric emptying
Bombesin	pH dependant gastrin release Stimulates glucagon and PP release.

that much of this work has involved the use of pharmocological doses and therefore its exact biological significance is difficult to assess.

1.6. Pathology

The regulatory peptides have been shown to be altered in a number of pathological conditions such as diabetes, duodenal ulceration, automomic neuropathy and infant nesidioblastosis (Table 4, Polak and Bloom 1980) Of these only the role of insulin in diabetes is well understood; for the remainder it is not known whether the changes in the peptides are causative or secondary to an unknown primary factor.

As a means of establishing the precise role of the individual peptides significant information can be obtained both from studies of their localisation in normal human and mammalian tissues and from their phylogenetic development. Once the contribution of each of these peptides to the normal regulatory system is understood, a further evaluation of their status in pathological situations can be undertaken.

1.7. Chemistry

As the peptides were purified, protein chemists determined the amino acid sequences. It was found that the peptides could be grouped according to sequence similarities. Thus gastrin and cholecystokinin share a C-terminal pentapeptide sequence (Diagram 1). Secretin, VIP, gastric inhibitory polypeptide (GIP), pancreatic and enteric glucagon have numerous common sequences (Diagram 2) and substance P and bombesin share two C-terminal residues (Diagram 3). At present it appears as if somatostatin, motilin, insulin, neurotensin and pancreatic polypeptide are not members of any related family group. There are indications from some preliminary data concerning the isolation of a peptide PYY which has N-terminal sequence similarities/.....

TABLE 4 - PATHOLOGICAL DISORDERS

Syndrome	Peptide	Increase	Decrease
Diabetes	Ins		x
Gastrinoma	Gas	x	
Vipoma	VIP	x	
Crohn's	VIP	x	
Chagas	VIP		x
Nesioblastosis	SOM		x
Glucagonoma	Gluc	x	
Insulinoma	INS	x	

similarities to neurotensin and C-terminal similarities to pancreatic polypeptide (Tatemoto and Mutt 1980) that these two peptides may be related in some way. In addition, neurotensin is related to the amphibian peptide, xenopsin (Diagram 4).

1.8. Antisera

These shared sequences have led to problems in raising specific antisera to the related peptides. For example antisera to gastrin that bind to the C-terminal region of gastrin will also bind the C-terminal region of cholecystokinin (CCK); this multi-specificity is most unsatisfactory in immunocytochemical studies. In order to overcome this problem, antisera to the N-terminal of gastrin have been raised which therefore do not cross react with CCK (Vaillant et al 1979). The raising of specific antisera to CCK was also a problem because of the same dual binding. However, synthesis of a partial sequence (9-20) of the molecule allowed the production of antibodies which did not bind to gastrin (Polak et al 1977).

Similar problems have been found with glucagon and GIP (Buchan et al 1978a) and with bombesin and substance P. In all immunocytochemical work great care must be taken in order to eliminate false results produced by inadequately characterised antisera.

1.9. Immunocytochemistry

All immunocytochemical methods rely on the production of antibody/antigen complexes which are then visualised by a variety of techniques. There are two main problems to be faced/.....

Diagram 1 C-terminal sequence similarities Gastrin and CCK.

CCK-8 -ASP-TYR(HSO_3)-Met-GLY-TRP-MET-ASP-PHE-NH_2

Gastrin -Tyr(HSO_3)-GLY-TRP-MET-ASP-PHE-NH_2

Caerulein -Gln-ASP-TYR(HSO_3)-Thr-GLY-TRP-MET-ASP-PHE-NH_2

Diagram 2 Sequence similarities Secretin Family

Secretin HIS-SER-ASP-Gly-THR-PHE-THR-SER-Glu-Leu-SER-ARG-LEU-ARG-

VIP HIS-SER-ASP-Ala-Val-PHE-THR-Asp-Asn-Tyr-Thr-ARG-LEU-ARG-

Glucagon HIS-SER-Gln-GLY-THR-PHE-THR-SER-ASP-TYR-SER-Lys-Tyr-Leu-

GIP Tyr-Ala-Glu-GLY-THR-PHE-Lke-SER-ASP-TYR-SER-Ile-Ala-Met-

Secretin -ASP-Ser-Ala-ARG-Leu-GLN-Arg-Leu-LEU-GLN-Gly-LEU-Val-NH_2

VIP -Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-LEU-Asn-Ser-Ile-LEU-Asn-NH_2

Glucagon -ASP-Ser-Arg-ARG-Ala-GLN-ASP-PHE-VAL-GLN-TRP-LEU-Met-Asn-Thr---

GIP -ASP-Lys-Ile-ARG-Gln-GLN-ASP-PHE-VAL-Asn-TRP-LEU-LEU-Ala-Gln---

Diagram 3 Sequence similarities Substance P and Bombesin

Substance P Arg-Pro-Lys-PRO-Gln-Gln-PHE-Phe-GLY-LEU-MET-NH_2

Bombesin Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-LEU-MET-NH_2

Physalaemin Pyr-Ala-Asp-PRO-Asn-Lys-PHE-Tyr-GLY-LEU-MET-NH_2

Diagram 4 Sequence similarities Neurotensin and Xenopsin

Neurotensin -Lys-Pro-Arg-ARG-PRO-Tyr-ILE-LEU-NH_2

Xenopsin -Glu-Gly-Lys-ARG-PRO-Trp-ILE-LEU-NH_2

(C-terminal octapeptide).

faced when undertaking such work. Firstly the fixation employed must give an acceptable morphological preservation whilst also retaining the antigencity of the peptides. Secondly, the antisera employed must be specific for a single peptide, which in these studies is complicated as the antisera are applied to tissue sections and can bind to any of the available tissue antigens. It follows from this that an antiserum can only be considered as specific if the antigenic determinants detected are not shared with any other peptide in the tissue. The problems inherent in immunocytochemical work have been extensively reported in two recent publications (Petrusz et al 1980, Van Noorden and Polak 1981).

An exciting new development has been the production of monospecific antisera by the use of cloning selective hybridoma cell lines (Milstein et al 1979). These monoclonal antisera may prove to be the answer to the immunocytologists prayer, as all non-specific antibody interactions will be eliminated and there will be an unlimited supply of the antisera.

Immunocytochemical studies are essential in order to establish the tissue components responsible for the production and storage of the individual regulatory peptides. It is only by such studies that a full understanding of the dynamic processes of peptide release, as demonstrated by the secretory cycle of the individual cells and nerves, can be obtained.

1.10. Outline of Thesis

It is generally accepted that the evolution of different species/.....

species has proceeded by means of natural selection, whereby only those species best adapted for the prevailing conditions will thrive, ie 'survival of the fittest'.

The framework of this thesis rests on the major assumption that the ancestral, aquatic, vertebrate stock gave rise first to a primitive partially terrestrial amphibian-like creature which through continued speciation gave rise to both the amphibians as we know them today and the reptiles. The original common ancestors presumably shared many common characteristics which may still exist in the present day species. Adaptive radiation within the two groups has produced increased specialisation such as the secondary development of the hind limbs in the frogs and the total loss of even vestigal limbs in the advanced snakes.

As a basis for the work to be presented, it is suggested that during this adaptive radiation the regulatory peptide system would have evolved in parallel with the differentiating species and would become increasingly complex with increasing specialisation of the animals.

As this work proceeds the above hypothesis will be tested and either endorsed or refuted. Comparative studies are also of interest in establishing the chemical and morphological antecedants of the regulatory peptide system. By use of such an approach various aspects can be shown to be either intrinsic to the organisation of the system from its origin or innovations limited to specialised animals. For example the phylogentic development of the intestinal neuroendocrine system with its dual localisation to endocrine cells and nerves has been investigated, in order to/.....

to give information as to whether the cellular localisation pre-dated the neural or the neural the cellular.

In addition, pre-mammalian species are often easier to study as their metabolism tends to be simpler and therefore the regulatory mechanisms may be relatively uncomplicated. By studying such systems it should be possible to see how the various components of the regulatory peptide system interact. If such information is available it can be a valuable basis on which to plan further mammalian studies. However, such further work can only be contemplated if the existence of the full regulatory peptide system (or a reasonable part thereof) has been established in the lower vertebrates.

Although there are indications of the presence of some of the intestinal biologically active peptides in primitive vertebrates (cyclostomes, Van Noorden et al 1972; teleosts, Van Noorden & Patent 1980, Dockray 1974, Klein & Van Noorden 1980; invertebrates (ascidians, Fritsch et al 1979; Thorndyke and Bevis 1980; Lumbricus, Sundler et al 1977; molluscs, Fritsch et al 1976; insects, Kramer et al 1977;) there is no detailed study of the existence of this system as a unit.

Initially it was thought that problems might have been encountered in detecting the non-mammalian peptides, as the antisera used in the staining procedures were mostly raised to mammalian (porcine) antigens in rabbits. In practice it has been shown that most mammalian antisera successfully bind to pre-mammalian peptides. This may be due to the fact that the antibodies present in the antisera are usually/.....

usually directed to the active portion of the antigen, ie the sequence involved in receptor binding. This is the region that would be expected (and indeed has been shown in insulin (Peterson et al 1974, Steiner et al 1973)) to remain most stable throughout evolutionary development, provided that the peptide retains a similar biological action.* If this is so it would pre-suppose that the amphibian and reptilian peptides which cross-react with antisera to mammalian peptides interact with receptors in the same locations and have the same effects as their mammalian counterparts. If this is correct it emphasises how useful these animals would be as experimental models. Enthusiasm must however be tempered with caution as physiological studies on glucose homeostasis in amphibians and reptiles have already shown that mammalian insulin and glucagon do exert the same effects as in mammals, but onset is slower and the duration of the effect is much increased (Penhos and Ramey 1973, Epple et al 1980, Miller 1960). Whether the same results would be obtained with native amphibian/reptilian peptides has yet to be determined.

If individual peptides are not detected in the amphibian and reptilian material using mammalian antisera this alone cannot eliminate the possibility that a related peptide with a modified structure is present.

Whenever possible, different region-specific antisera have been used in order to show which regions of the peptides are present in the non-mammalian species. This is the only way in which an idea of peptide composition can be obtained/.....

* see note on page 29

obtained using immunocytochemical methods.

It is possible that some peptides will remain undetected in the less advanced amphibian species, irrespective of antisera limitations. If this does occur it will be of great interest to see if the first immunologically detectable demonstration can be uncovered during the investigation of the increasingly advanced amphibian and reptilian species.

It is also possible that the distribution pattern of the intestinal peptides may show inter-species, inter-familial or inter-order changes which may give additional information on phylogenetic relationships.

* However from studies of the action of insulin in lower vertebrates, it has been demonstrated that this peptide has a much wider spectrum of actions than in mammals, including an effect on osmoregulation and amino acid metabolism. This in turn indicates that the receptors available for insulin-binding in these animals are more widespread and therefore in studies of the function of the regulatory peptides using lower vertebrates as experimental models those actions retained in mammalian metabolism must be clearly distinguished from those peculiar to the lower vertebrates.

Chapter 2 - INFORMATION ON THE RELEVANT ANATOMY AND HISTOLOGY OF THE GASTROINTESTINAL TRACT AND PANCREAS

2.1. Amphibia

Amphibians were the first vertebrates to live the majority of their life on land, only returning to water to spawn. All amphibia lay eggs which are coated in a gelatinous substance which cannot withstand dessication. The young, immature amphibians are fully aquatic, undergoing a metamorphosis from the larval (tadpole) stage to the adult land living form. Even the adults are not fully adapted for terrestrial life as the skin remains fully permeable, restricting them to habitats with easy access to water or a very high humidity.

2.1.1. Phylogeny. There are three orders of amphibia - Urodela, Anura and Apoda. The Urodela (salamanders and newts) are the most primitive, having remained anatomically unchanged from the first identified fossil amphibia (Kingsley Noble 1954).

The most prolific order is the Anura, the highly specialised frogs and toads. The Apoda (caecilians), a specialised group adapted for a burrowing existence and having secondarily lost all but vestigal limbs, are much less common than the Anurans. The phylogenetic relationships as at present understood are shown in Diagram 5.

2.1.2. Peptides. An interesting feature was the finding of several biologically active peptides in sub-cutaneous glands of many Anurans (frogs) (Erspamer and Melchiorri 1973). The function of these peptides is uncertain. They may possibly act as deterrants to predators,/.....

predators, as they are excreted on to the skin surface if the animals are handled (Dockray & Hopkins 1975). Several of these peptides have been isolated and sequenced and shown to be either identical (as in the case of bombesin) or related (as in physalaemin and substance P) to mammalian gut peptides (see Diagram 3). It will be of interest to see if the peptides present in the skin are also present in the intestines of the same species. Bombesin, a 14 amino acid peptide was originally isolated from the skin of Bombina bombina (Anastasi et al 1971) and although bombesin-like immunoreactivity has been reported in the stomach of Rana temporaria (Lechago et al 1976) it remains to be detected in the intestine of Bombina.

2.1.3. Conventional Histology and Gross Anatomy

a. Pancreas. Amphibian pancreas consists of columns of acinar cells with interspersed endocrine islets. The islets are found throughout the exocrine pancreas, being clearly separated by a capsule of connective tissue (Penhos and Ramey 1973). Unlike some fishes and reptiles there is no aggregation of islet tissue to the splenic lobe (tail). The islets are thought to be budded off from the ductular epithelium as the pancreas matures (Gerlovin 1976, Falkmer et al 1977). The amphibia are known to have three main islet cells: A, B and D (Epple and Lewis 1973) although there are reports suggesting the presence of relatively few A cells, the islets being made up of/.....

of mostly B cells (Lange 1973). In the primitive Urodeles it was originally suggested that there was a total lack of A cells (Falkmer and Marques 1972) but this has since been disproved by more advanced cytochemical staining.

Anuran islets consist of a central core of B cells with the A and D cells at the periphery (Lange 1973). In several species there are also scattered cells in amongst the acinar tissue. This pattern is similar to that found in the mammals, especially man (Orci and Unger 1975).

The information presented above is mainly based on ultrastructural and cytochemical work with a small number of immunocytochemical studies mostly confined to the Anura (Falkmer 1979).

There is no mention in the literature of the presence of the fourth mammalian pancreatic hormone, pancreatic polypeptide, although some studies refer to the presence of a fourth cell type which has yet to be linked to the production of any peptide.

Work on the innervation of the amphibian pancreas has shown that there are both cholinergic and adrenergic fibres in the exocrine and endocrine tissue, no peptidergic component has yet been identified.

The pancreatic hormones insulin and glucagon are known, in mammals, to be involved in maintaining glucose homeostasis. Amphibia have been shown to have relatively low blood sugar levels, except during/.....

during the mating season. Insulin-induced hypoglycaemia in frogs (caused by injection of mammalian insulin) is slow in onset and of prolonged duration (Penhos and Ramey 1973). Whether this mimics the effect of amphibian insulin is unknown, the purification of amphibian insulin is required before this can be clarified. However the pancreas in the amphibia is not the only site of glucose control, the pituitary, thyroid and inter-renal glands are also involved (Kingsley Noble 1954).

As the vast majority of the physiological work has involved the use of mammalian insulin and glucagon the precise importance of these hormones in regulating amphibian glucose levels remains uncertain.

b. Stomach. The stomach can, histologically, be divided into two areas - upper (fundic) and lower (antral). In both regions the mucosa is made up of numerous glands, those in the fundus being deeper than those in the antrum. The cells comprising these glands are mostly mucin-containing, those of the fundus producing acid polysaccharides and those of the antrum, neutral polysaccharides. Endocrine cells have been identified in both regions. In the Ranidae (Anura) some of the endocrine cells have been linked with the production of bombesin (Lechago et al 1978) and of a gastrin/cholecystokinin-like peptide (Larsson and Rehfeld 1977).

There/.....

There are well developed circular and longitudinal muscle layers, with a rich cholinergic and adrenergic innervation, the circular muscle coat having a denser innervation, a similar situation to mammals (Rogers and Burnstock 1966, Campbell 1969).

In the amphibia the stomach functions as a sack for the storage and lengthy digestion of the ingested food (mostly live insects and worms). The well developed muscle layers are probably related to holding the initially alive prey. They are also presumably involved in the movement of the digested food into the foregut (duodenum).

It is known that both hibernation and seasonal variations occur in the thickness of the muscle and mucosal layers of the stomach, possibly related to the difference in general metabolic rate in amphibians (Geuze 1971 a, b). Further studies of possible changes in the regulatory peptide system would be of great interest. A thorough investigation was considered to be outside the scope of this study.

c. Intestine. The intestine is lined either by a simple or stratified epithelium, depending on the species. There are no true villi and crypts such as are found in mammals. The absorptive surface is increased by gut folds which superficially resemble villi.

Although there is no direct proof of a peptidergic component/.....

component to the peripheral innervation, physiological studies showing the presence of an atropine-resistant vagal response are suggestive of its presence (Carlson and Luckhard 1921).

The colon of these amphibians has an additional function as a bladder-like organ in that it re-absorbs significant amounts of water prior to excretion of the faeces (Carter 1975).

2.2. Reptilia

The reptiles were the first truly terrestrial animals. With the development of an impermeable, scale covered skin they were no longer limited to areas near water. Some reptiles are extremely well adapted for life in the most arid conditions, such as the desert wastes. The young are laid as eggs (oviparous) or born live (viviparous).

2.2.1. Phylogeny. There are four living orders of reptiles - Testudinata, Crocodilia, Rhynochephalia and Squamata. The Testudines comprise the turtles and tortoises, all distinguished by the possession of a hard boney carapace enclosing the body. The Crocodilia consist of crocodiles, alligators/caiman and gharials. Of these the alligators are the oldest, being recognisable in the Jurassic (150 million years ago) (Young 1950). The crocodiles are an emergent form which have replaced the alligator in most areas apart from the Southern United States and China. The caiman is the alligators' answer to the crocodile; being smaller and more aggressive, and it is gradually invading the territories/.....

territories taken over by the crocodiles. The gharials (or gavil) are the fish eating crocodiles with greatly elongated, tapering snouts which are thought to be advantageous for fish catching. The exact relationship of this family to the other members is at present uncertain.

The order - Rhynochephalia - has been created for a single species, Sphenodon, which is confined to a few islands around the coast of New Zealand. Due to the rarity of this species, it has been impossible to include this fascinating animal in the present study.

The Squamata includes the most abundant sub-orders - the Lacertilia (lizards) and Serpentes (snakes). The lizards and snakes have undergone extensive adaptive radiation, being found from temperate zones to the arid desert zones.

The phylogenetic relationships of the various reptilian orders and the sub-orders are shown in Diagram 8.

2.2.2. Conventional Histology and Gross Anatomy. Although the pancreas has been studied in several species, there is in general a lack of information on the histology of the intestinal tract in the reptiles. The following summary will of necessity be confined to the species described in the literature.

a. Pancreas. Although the anatomical site of the pancreas varies in relation to the spleen and stomach it is always attached to the duodenum in all the species so far described (Miller and Lagios 1970).

In/.....

In the Testudines and the majority of Lacertilia the arrangement of the pancreas is similar to that in amphibia and mammals. The endocrine islets are found throughout the exocrine tissue. Only two cell types investigated by cytochemical techniques are at present recognised in those species. B cells are found in the centre of the islets and A cells at the periphery (Miller 1962).

In the Serpentes and some Lacertilia the pancreatic anatomy differs, especially in the primitive snakes (Thomas 1942) and the Varanidae (lizards) where there is a reversion to the fish pattern of a concentration of islet tissue to the splenic lobe ie the Brockmann bodies, or sometimes to a single 'giant' islet as in the Varanidae.

There are also reports of islets occurring actually within the spleen, being totally separated from the rest of the pancreas (Hellerstrom and Asplund 1966). In these animals the arrangement of the cells within the pancreas differs from the mammalian, with the A cells being found in the centre and the B cells at the periphery (Hellerstrom and Asplund 1966). This was presumed to be a primitive characteristic; however, similar findings were also reported in members of the Viperidae, the most advanced of the snake families.

The situation in the Crocodilia is confusing - some reports stating that there is a splenic concentration of/.....

of the islets (Miller 1962) whereas others have described islets distributed throughout the pancreas (Penhos and Ramey 1973). As no definitive description is available this will be investigated in the present study.

b. Stomach. The stomach was divided into two areas - fundic and antral - the mucosa of both regions consists of glands, deeper in the fundus than in the antrum. The glands are made up of two types of cells, mucous and serous (Gabe and Saint Girons 1972) The mucous cells produce either acid or neutral mucopolysaccharides, the serous cells produce zymogen-like enzymes. Endocrine cells detected by both the argentaffin and argyrophyl silver staining methods have been identified in the mucosa (Ferri et al 1974). One of the cell types has been suggested as the site of gastrin-production by histochemical methods but this has not as yet been proved by immunocytochemistry (Gabe 1972).

c. Intestine. The longest intestines (relative to body length) are found in the Testudines and the shortest in the snakes (Luppa 1977). The mucosa lining the intestine may be either simple or stratified. The Lacertilia, Crocodilia and Testudinata, all have pseudo-crypts in that there are small glandular depressions in which cell divisions have been detected but there are no true villi and the majority of cell divisionsstill take place at t basal lamina (Wurth and Musacchia 1964). The snakes /.....

snakes have been shown to be totally lacking pseudo-crypts, the gut being lined by simple folds (Reis and Lyons 1943).

Four types of cells are distinguished in the intestine ie Paneth, mucous, absorptive and endocrine (Luppa 1977). Two types of endocrine cells have been described - argentaffin enterochromaffin (EC) cells (Vialle 1929) and argyrophil, spindle-shaped cells (Gabe 1973). Although no quantitative data is available it is thought that there are more endocrine cells in the lizard intestine than in that of the snake (Vialli 1966 In the colon there is usually a single cell type - mucous - producing both acidic and neutral mucins (Gabe 1971). However, the advanced Colubrid snakes have both mucin and goblet cells (Junqueira et al 1966). In general the muscle layers are much better developed in the colon than in the upper intestine.

Although a peptidergic innervation has not been detected there is a considerable body of work on autonomic innervation of the intestine which suggests that the peptidergic system may be present (Burnstock 1969).

Chapter 3 - MATERIAL

3.1. Amphibia

In all, 72 animals from 11 species were collected. The species studied were chosen to cover both the primitive and highly specialised members of the phylum.

The primitive ancestral types are found in the Urodela, therefore three species were chosen for study. These were the adult forms Salamandra salamandra and Cynops hongkongensis, Salamandra being a mostly terrestrial species and Cynops mostly aquatic. In addition, Ambystoma mexicanus, the permanent larval form of the salamander, was investigated to see if the pre-metamorphosed animals shared the adult characteristics.

In the Anura, eight different species were studied ranging from the unspecialised Discoglossid frogs to the highly specialised Hylidae (tree frogs). The species were chosen either as representative of different stages in adaptive radiation or because if they were known to contain biologically active peptides in the sub-cutaneous glands. Thus, from the Discoglossidae, Bombina bombina was chosen as its skin is known to contain the peptide bombesin and Alytes obstetricans because its skin contains physalaemin which is related to substance P.

I was exceptionally lucky in being able to study a member of the divergent family Atelopodidae, which is confined to the South American rain forests. This was made possible by the kind gift of three Atelopus oxyrhynchus from Dr Scorza during a visit to our laboratory.

The full list of species studied and the number of specimens is/.....

is given in Table 5 while Diagram 5 shows the relationships between the different species.

3.2. Reptilia

A total of 52 animals from 14 species were investigated. Again, the species studied were chosen as being representative of the different orders.

Testudinata - The most primitive reptilian order still extant, this order can be roughly divided into the terrestrial tortoises and the aquatic terrapins and turtles.

Four families of the aquatic animals were studied - the European pond tortoise, Emys orbicularis, the North American fresh water turtles, Pseudemys (two species) and the snapping turtle Chelydra serpentia.

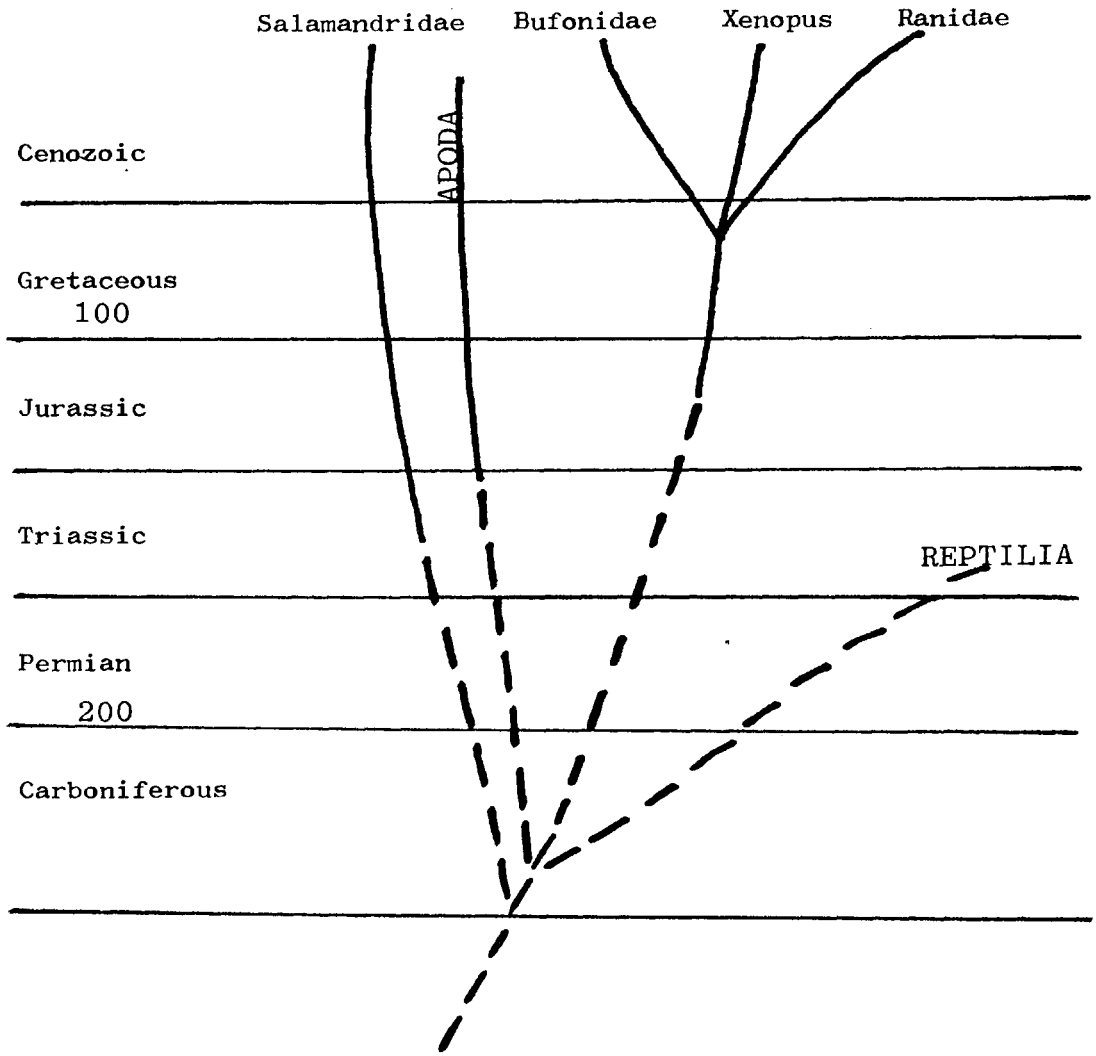
Squamata - The Squamata is divided into two sub-orders - the snakes Serpentes and lizards Lacertilia Of these, two species of lizard were studied. From the snakes members of the Boidae, Colubridae and Viperidae families were included.

Crocodilia - Originally, it was thought that due to difficulties in obtaining specimens, the work would have to be confined to the sub-species - Caiman - a recent offshoot from the Alligatorinae. However, due to collaboration with Dr Lance at Louisiana State University it has been possible to obtain several fully adult wild specimens of the Mississippi alligator. As the alligators are the ancient relic form of the Crocodilia, it was therefore of especial interest to be able to study this species.

Table 5 shows the exact species and numbers of animals studied, while Diagram 6 shows their phylogenetic relationships.

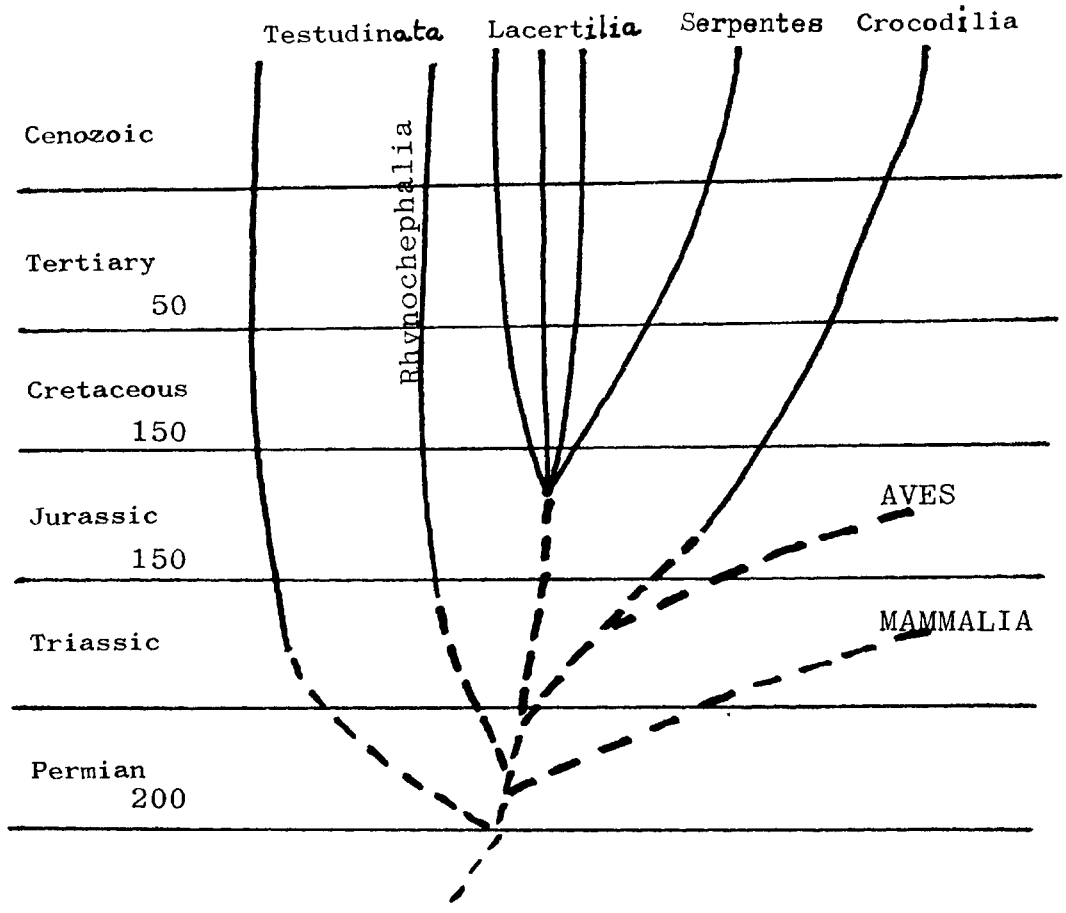
Sampling/.....

DIAGRAM 5 - PHYLOGENTIC RELATIONSHIPS - AMPHIBIA



(After J.Z.Young)

DIAGRAM 6 - PHYLOGENIC RELATIONSHIPS - REPTILIA



(After J.Z.Young)

Sampling Procedure -

Material was taken from the following areas -

Pancreas (whenever possible divided into head, body and tail regions)

Stomach
- Fundus
- Antrum

Duodenum

Small intestine 1 (jejunum)

Small intestine 2 (ileum)

Large intestine (colon)

The animals were all kept in the laboratory for a minimum of 5 days before being killed.

The majority of the frogs were killed by simple decapitation as were the lizards and turtles. The snakes were anaesthetised prior to decapitation to prevent excessive muscle spasms, which made the dissection difficult.

The alligators were shot through the base of the spine prior to dissection.

To check the effect of anaesthetics on the detection of the regulatory peptides a controlled experiment using two sets of frogs, one anaesthetised, one normal were immunostained. No difference in the immunostainings was detected in the two groups. It was therefore decided that anaesthetisation was acceptable.

The animals kept in the laboratory were fed on mealworms, earthworms, crickets and daphnia depending on species.

TABLE 5

SPECIES STUDIED

Order	Family	Species	Number
Amphibia			
Urodela	Salamandridae	Salamandra salamandra	6
		Ambystoma mexicanum	4
		Cynops hongkongensis	5
Anura	Discoglossidae	Bombina bombina	15
		Alytes obstetricans	4
	Ranidae	Rana temporaria	10
		Rana esculenta	8
	Bufonidae	Bufo bufo	10
	Atelopodidae	Atelopus oxyrhnchus	3
	Hylidae	Hyla arborea	4
		Hyla crepitans	3
Reptilia			
Testudinata	Emydidae	Emys orbicularis	3
		Pseudemys scripta	6
		Pseudemys picta	3
	Chelydridae	Chelydra serpentia	5
Crocodilia	Crocodylidae	Alligator mississipiensis	11
		Caiman crocodylus crocodylus	1
Squamata			
Lacertilia	Agamidae	Agama agama	3
	Gekkonidae	Hemidactylus brooki	3
Serpentes	Boidae	Python recticularis	1
	Colubridae	Natrix natrix	3
		Nerodia rhombifera	8
		Thamnophis sirtalis	3
	Viperidae	Agistrodon piscivora	1
		Crotalus atrox	1

TOTAL NUMBER OF SPECIES - 25

TOTAL NUMBER OF ANIMALS - 124

Chapter 4 - METHODOLOGY

4.1. Fixation

Light Microscopy - Samples from each area were immediately quenched in Arcton, freeze-dried overnight, vapour fixed in p-benzoquinone dissolved in toluene (Pearse and Polak 1975) for three hours and then embedded in paraffin wax. Five micron sections were cut for the immunostaining.

Transmission Electron Microscopy - The fixation of the samples for electron microscopy presented two problems, namely that the tissue had to be preserved to a sufficiently high standard to given an acceptable ultrastructural appearance but at the same time the antigencity of the peptides had to be retained to allow for identification of the peptide-containing structures by immunocytochemical staining.

During the course of this study successively improved fixations have been developed, with the result that material from the first animals collected, although adequately fixed, is not of as high a standard as the samples from the last animals studied.

Conventional Electron Microscopy - Material for purely morphological study was fixed in 3% glutaraldehyde in 0.1M phosphate buffer pH7.1 for two hours at room temperature followed by post-fixation in 1% osmium tetroxide in Millonig's buffer (phosphate) for one hour at 4°C.

This material normally cannot be used for immunocytochemical stainings because the presence of osmium masks the antigenic determinants preventing successful binding. However, in the last year (1980) a method of removing the osmium by oxidation with periodic acid has been optimised and a limited number of peptides can now be detected in the osmicated material.

Immunoctyochemistry for electron microscopy - Two main fixations have been employed. Firstly, 2.5% vacuum-purified glutaraldehyde in 0.05M phosphate buffer pH7.2 for 10-15 minutes at 4°C (Buchan et al 1978) Secondly a modified Karnovsky's fixative consisting of one part 5% vacuum-purified glutaraldehyde in 0.2M phosphate buffer pH7.2 and nine parts of 2% paraformaldehyde in 0.2M phosphate buffer pH7.2, samples were fixed for two hours at 4°C. In both cases the material was not post fixed in osmium tetroxide.

In/.....

In all cases the samples after fixation were washed in phosphate buffer, de-hydrated in alcohol and finally embedded in araldite at 60°C. Details of the immunostaining performed on the material for electron microscopy are given on page 46.

For observation in the electron microscope, 60nm sections were cut on a Reichert Ultratome or an LKB III ultratome. The sections were collected on either copper or nickel grids. The copper grids were subsequently counterstained with 5% uranyl acetate in methanol and Reynolds lead citrate. The sectionson the nickel grids were used for the immunostaining procedure as outlined on page 336 . In all cases the grids were examined in an AEI 6B microscope.

Scanning Electron Microscopy - Tissue samples of $2cm^3$ were fixed by the conventional routine as given on page 44 , then dehydrated through to 100% ethanol, subjected to critical point drying and finally coated with gold palladium to a thickness of 2mm.

The samples were mounted on to platinum stubs and observed in a Cambridge Stereoscan S4-10.

4.2. Immunocytochemistry

Light Microscopy -

The wax was removed from the 5 micron sections by immersion in xylene followed by either petroleum ether or inhibisol.

The two main methods used were the indirect immunofluorescence technique (IMF) (Coons et al 1955) and the peroxidase-antiperoxidase method (PAP) of Sternberger (1979). In special cases the double staining technique developed by Nakane (1968) was also used.

The antisera were applied at their optimal dilutions (see page 49) and incubated at 4°C for between 24 and 48 hours.

For/.....

For the IMF technique the sections were rinsed in phosphate buffered saline (PBS) pH7.2 then a second layer of fluorescein conjugated goat anti-rabbit globulin (IgG) (Miles) was applied at a dilution of 1:150 for one hour at room temperature. The sections were again rinsed in PBS and coverslipped in PBS/glycerine (9:1) and screened with a Leitz orthoplan microscope.

The sections stained by the PAP method were rinsed by PBS and incubated with an unconjugated goat antirabbit globulin (IgG) (Dakopats) at the dilution of 1:150 for 30 minutes, rinsed in PBS and incubated with a peroxidase antiperoxidase complex (Miles) at a dilution of 1:130 for 30 minutes.
After a further rinse in PBS the complex was visualised by development in a solution of diaminobenzidene (25mg per 100 ml) and hydrogen peroxide (50ul per 100mls) for 5-10 minutes. After this the sections were stained with haematoxylin, de-hydrated and coverslipped in DPX.

If the double staining method was to be employed, instead of staining with haematoxylin the whole staining procedure was repeated with a second antiserum, excepting the final step where the PAP complexes were visualised by the use of 4-chlorol-naphthol which produces a blue end colour contrasting with the brown of the diaminobenzidene.

Where antisera raised in guinea pigs were used the IMF schedule was altered by the use of a fluorescein conjugated rabbit anti-guinea pig IgG at a dilution of 1:100 for one hour at room temperature. The PAP technique could not be performed; instead, a two step peroxidase method was used, with a second layer of peroxidase conjugated rabbit anti-guinea pig IgG at a dilution of 1:100 for 30 minutes at room temperature; the development was identical to that for the PAP method.

Immunostaining for electron microscopy -

Two types of immunostaining were used during this study. Firstly a technique making use of a combination of light and electron microscopy - the semithin/thin method (Polak et al 1976). In this, serial semithin (500nm) and thin (80nm) sections/.....

sections are cut. The 500nm sections are mounted on glass slides and air dried whereas the 80nm sections are picked up on copper grids (200 mesh). The 500nm sections, after removal of the araldite by immersion in a saturated solution of Na OH in ethanol (Lane and Europa 19[illegible]), are used for the immunostaining. The protocol for this is identical to that described above for light microscopy, both IMF and PAP methods were used.

The second immunostaining method was to stain directly on the thin sections. For this the 60nm (thin) sections collected on the nickel grids (300 mesh) were used.

The immunostaining involved floating the grids on successive drops of reagents, the full sequence is given in Appendix II. The marker in this case was the electron dense colloidal gold linked either to protein A (from Staphylococcus aureus) which binds specifically to IgG, to goat antirabbit IgG or to the parent antigen (Roth et al 1978, Larsson 1979). The gold particles were either 12nm or 20nm in diameter. After the immunostaining was completed the grids were thoroughly washed and then counterstained for a short period to enhance the background tissue detail.

4.3. Antisera

During the course of this study a total of 32 different antisera to 18 different peptides were used. As it was essential to know, as far as possible, the precise region of the molecule detected by each antiserum, the specificity controls were extremely important.

With those peptides already successfully synthesized smaller component fragments were available which were indispensible for/.....

for the precise characterisation of the antisera.

The peptides for which fragments were available included gastrin, motilin, CCK, bombesin, substance P and VIP.

For the remaining peptides, studies of the competitive binding by related peptides and a check to ensure that the antisera were specific for the parent antigen showing no crossreaction with other peptides, were the only means of monitoring the specificity of the antisera.

The characterisation of the antisera was performed by two methods; firstly, the well established pre-absorption with nanomolar quantities of the individual peptides and the available fragments; secondly, as a further check, the newly developed ELISA method (Voller et al 1976) was used as previously described (Buchan et al 1978a). This involves the solid phase absorption of the peptide to a polystyrene plate to which the antisera are added. Those antisera bound to the absorbed peptide are detected by an alkaline phosphatase enzyme reaction. The ELISA method was very useful when checking for crossreactivity between the antisera and the full range of available gut peptides. Tables 6, 7, 8 and 9 give details of the individual antisera and their working dilutions for the IMF and PAP techniques.

Table 6 Antisera 1 Gastrin /CCK

Antiserum			Antigens				
Region detected	No.	Diln.	Gastrin G4	Gastrin G17	Gastrin G34	CCK CCK8	CCK 1-36
9-20 CCK	280	1/400	X	X	X	X	-
C-term CCK	198	1/200	-	-	-	-	-
C-term Gas.	278	1/200	-	-	-	-	-
N-term G17	1295	1/200	X	-	X	X	X
N-term G34	L33	1/100	X	X	-	X	X

Amount of antigen added = 5nM per ml of diluted antiserum.

- = Absorption successful X = Absorption negative.

Antisera 1295 and L33 were kindly donated by Dr. J. Walsh and Dr. G. Dockray respectively.

TABLE 7 Antisera 2 VIP

Antiserum			Antigens - VIP fragments				
Region detected	No.	Diln.	1-28	1-6	1-18	15-28	18-28
mid-Cterm.	324	1/2000	-	X	X	-	X
whole	V9	1/500	-	X	X	X	X
C-term	V7	1/500	-	X	X	-	-
N-term	V22	1/500	-	-	-	X	X

- = Absorption successful X = Absorption negative.

Table 8 Antisera 3 Region specific antisera

Antiserum Region detected	No.	Diln.	Antigens PGlu	Glic	BN	MOT 1-17	MOT 7-22	SP	PP
C-term Gluc.	RCS5	1/100	-	X					X
N-term Gluc	38	1/1000	-	-					X
Glicentin	575	1/600	X	-					X
HPP	319	1/400	X	X					-
BPP	317	1/600	±	ND					-
APP	623	1/500	X	X					-
N-term Mot	M4	1/200				-	X		
C-term Mot	GP71	1/500				X	-		
C-term BN	627	1/400			-			-	
N-term BN	377	1/200			-			X	

- = Absorption successful ± = Partial absorption

X = Absorption negative. ND = Not done

All absorptions were performed with the other antigens at a concentration of 10nM per ml of diluted antiserum whereas the parent antigen was added at 1-2nM per ml.

The following antisera were kindly donated from various sources - 575 from Dr. A. Moody, RCS5 from Dr. S.R.Bloom, 623 from Dr. J.Kimmel, GP71 from Dr. J. Brown and 377 from Dr. J. Walsh.

Table 9 **Antisera** 4 Non-region specific

Antiserum	Diln.	Antigens									
		Sec	Gluc	VIP	GIP	NT	SP	ENK	INS	END	BN SOM
Secretin	1/1000	-	X	X	X						
GIP	1/800	X	X	X	-						
Neurotensin	1/400		X			-					
Substance P	1/2000						-	X			X
Insulin	1/400								-		
Somatostatin	1/800										-
Endorphin	1/200							X		-	
Enkephalin	1/400							-		X	

- = Absorption successful X = Absorption negative

The absorption controls involved addition of related peptides and those occurring in the same area. In all cases the absorptions with the other antigens were performed at a concentration of 10nM per ml of diluted antiserum, whereas the absorption with the parent antigen involved the use of 1-2nM per ml of the diluted antiserum.

Negative Control

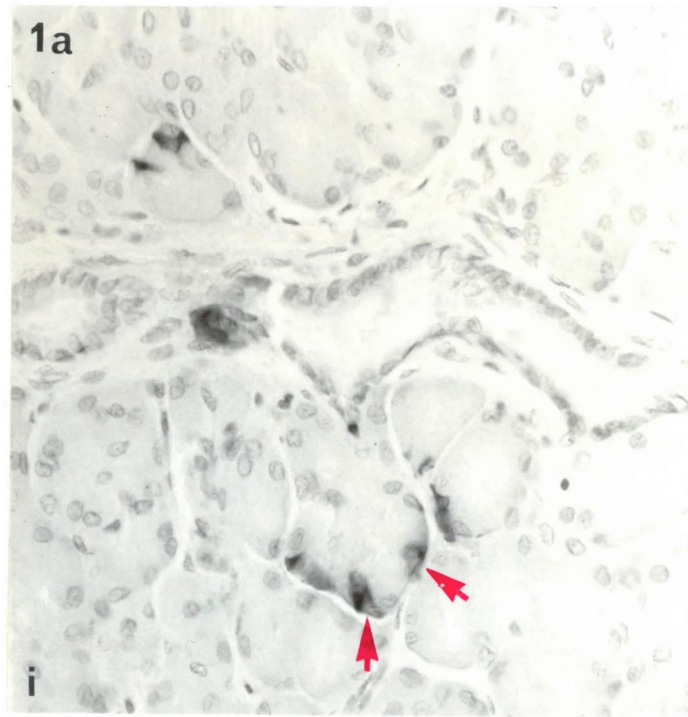
Figure 1a

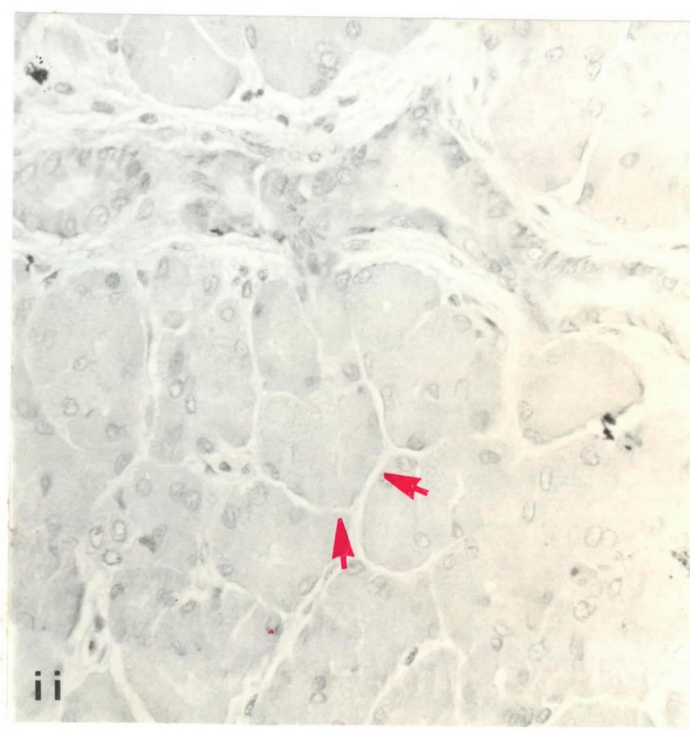
i 5 micron section of alligator pancreas stained with antibodies to pancreatic glucagon, showing several positive cells (arrows).

x 250

ii The same area in a serial section stained with the pancreatic glucagon antiserum pre-absorbed with glucagon (0.5nm/ml of diluted antiserum), showing the absence of any immunoreactivity.

x 250


1a
i


ii

5.1. Legend of the Tables given for each individual species.

Antisera to the peptides as available during the course of the study are shown along the horizontal axis of the Table, the anatomical areas of the gut and pancreas are given by the vertical axis.

The abbreviations are as follows:-

Areas - Panc - Pancreas in those species where the pancreas cannot be divided.

Panc. H - Pancreas head region.

Panc. B - Pancreas body.

Panc. T - Pancreas tail region.

Duod. - Duodenum.

Jej. - Jejunum.

Peptides (antisera) -

Ins - anti insulin.

Gluc. - anti glucagon detecting both the pancreatic and enteric forms.

Gli. - antiglicentin detecting the large molecular weight enteric form.

PGlu. - anti pancreatic glucagon.

Som. - anti somatostatin.

APP - anti avian pancreatic polypeptide.

BPP - anti bovine pancreatic polypeptide.

HPP - anti human pancreatic polypeptide.

G/CCK - anti C-terminal gastrin which also detects CCK.

G17 - anti gastrin specific for the gastrin 17 form.

G34 - anti gastrin specific for the gastrin 34 form.

CCK - anti CCK specific for the mid-portion (9-20) of the molecule.

Bn1/.....

Bn1 - anti bombesin detecting the C-terminal region.

Bn2 - anti bombesin detecting the N-terminal region.

SP - anti substance P.

NT - anti neurotensin.

Mot 1 - anti motilin detecting the C-terminal region.

Mot 2 - anti motilin detecting the N-terminal region.

GIP - anti GIP.

Sec - anti secretin.

VIP 1 - anti VIP detecting the C-terminal region.

VIP 2 - anti VIP detecting the N-terminal region.

VIP 3 - anti VIP detecting the whole molecule of VIP.

Enk. - anti enkephalin specific for the met-enkephalin form.

Prl. - anti prolactin.

ACTH - anti ACTH.

NSE - anti neurone-specific enolase*.

* Neurone specific enolase is an isoenzyme isolated from rat brain which was found earlier to occur exclusively in nerve axons and cell bodies in brain tissue (Marangos et al 1979). Recent work has shown that this isoenzyme is also present in the gut mucosal endocrine cells as well as in the nerve plexuses. It was therefore included in the study as a possible marker of the components of the neuroendocrine system.

The/.....

The results table is split into sections, the largest part refers to the mucosal endocrine cells with the second half confined to those peptides found within the nerve fibres. Those peptides specifically immunostained will be indicated by '+' - the more abundant peptides will be indicated by '++'. In the case of those peptides which showed positive immunostaining which was later shown to be non-specific the inability to prove the specificity of the immunostaining will be indicated by 'x'.

5.2. Amphibia

5.2.1. Histology. In general the structure of the gut in the Urodela and Anura was very similar. The stomach was lined by a glandular epithelium in which the most numerous cell types were those containing mucin. The intestines had no true villi and the epithelium was lined by a single stratified layer (Figure 1). The thickness of the muscle coat varied from region to region being more developed in the stomach and colon compared to the rest of the gut.

5.2.2. Urodela.

Pancreas. The pancreas was found attached to the duodenum as a thin lobulate mass, extending dorsally along the intestine, varying between 1 - 2 cms in length.

Stomach. Two macroscopically distinct regions were seen in the stomach. The fundus was distinguished by a lighter colour and irregular folds and the antrum was distinguished by a light colour and regular horizontal folds.

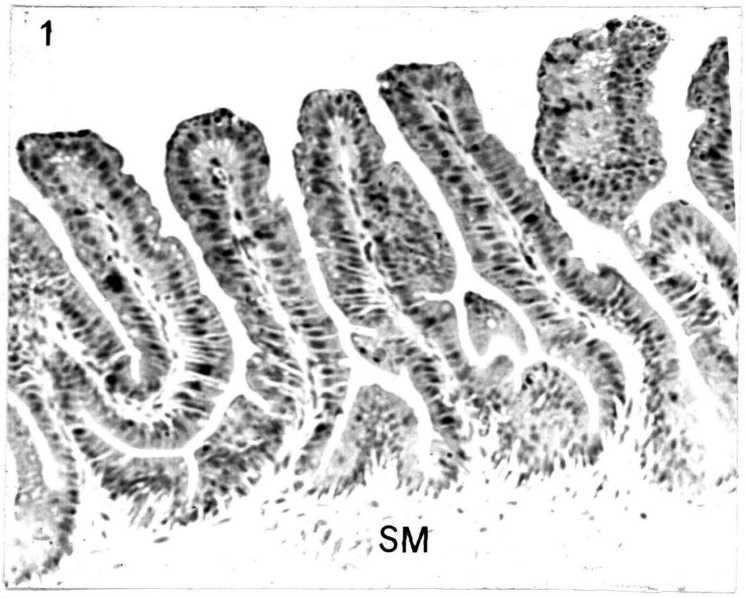
Intestine./.....

Figure 1

A 5 micron section through the duodenum of Rana temporaria, showing both the stratified epithelium and lack of crypt development.

SM = sub mucosa

x 120


1
SM

Intestine - The duodenum was defined as the region to which the pancreas was attached. The small intestine was divided into two regions, upper jejunal and lower ileal.

5.2.2.a. Immunostaining. In all cases only those immunostainings that have been successfully absorbed with 1-5nM/ml of diluted antiserum of the parent antigen (and no other) will be referred to as specific, the term non-specific will refer to those stainings which were unabsorbed in the control experiments.
In all the species identical results were obtained whether the immunostaining was performed by the peroxidase-antiperoxidase (PAP) or the immunofluorescence (IMF) method.

i. Salamandra salamandra -

Pancreas. Insulin-, glucagon-, somatostatin- and pancreatic polypeptide-immunoreactive cells were found either as scattered elements or in small clusters. No large, well-defined islets were seen in any specimen. There did not appear to be any distinct distribution pattern of the cell types to a particular region of the pancreas. Glucagon-immunoreactivity was detected with both the N- and C- terminal specific antisera but the glicentin antisera showed no staining. Although the pancreatic polypeptide (BPP) antiserum stained numerous cells, these were identical to those immunostained by/.....

by the glucagon antiserum. However, cross-absorptions with glucagon failed to remove the BPP staining, indicating that there may be a dual peptide content in these cells. Neither the APP or HPP antisera showed any detectable immunoreactivity.

An attempt to localise peptide-containing nerves in pancreatic tissue failed. Antisera to VIP, substance P, bombesin and met-enkephalin gave no positive staining.

Table 10 Salamandra salamandra European

	INS	GLU	PGLU	SOM	BPP		G/CCK	NT	BN1	BN2	SP	NT					VIP1	VIP2	VIP3	END
Panc.	+	+	+	+	+															
Fundus		+		+					+		+	x					+			
Antrum		+					+										+			
Duod.				+					+		+	+					+			+
Jej.		+							+		+	+					+			
Ileum		+		+					+			+					+			
Colon		+		+					+		+	+					+			

Stomach/.....

Stomach - Fundus

Endocrine cells were demonstrated with antisera to bombesin, glucagon, neurotensin, somatostatin and substance P. Of these, those cells stained by the bombesin, substance P and glucagon antisera were the most numerous. Absorption controls showed that the majority of the stainings were specific, except that obtained with the neurotensin antiserum. The immunostaining was not removed by the addition of either neurotensin or the related amphibian peptide, xenopsin.

Nerve fibres were only detected using the anti-VIP serum (Figure 2); enkephalin, substance P, bombesin and gastrin/CCK antisera did not stain any recognisable fibres.

Stomach - Antrum

Antisera to gastrin/CCK, glucagon and somatostatin detected muscosal endocrine cells. Somatostatin cells often displayed long cytoplasmic processes either projecting along the basal lamina of the gland or inter-digitating with other epithelial cells (Figure 3).

As in the fundus, nerve fibres were only stained by the VIP antiserum (see Table 10).

Intestine - Duodenum

The immunostained endocrine cells were found at all levels of the gut folds. In general the/.....

Figure 2

VIP-immunoreactive nerve fibres in the circular muscle of the stomach.

x 400

Figure 3

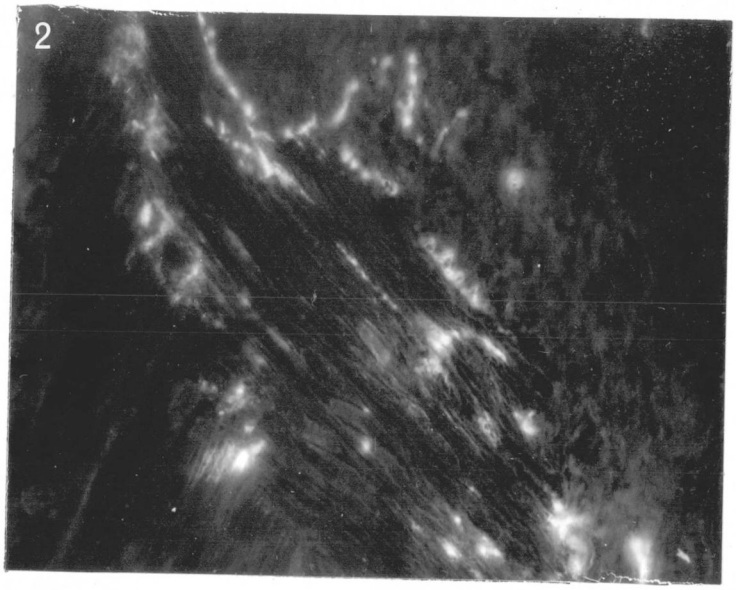
Somatostatin-immunoreactive cell in the antral mucosa with two cytoplasmic processes(arrows).

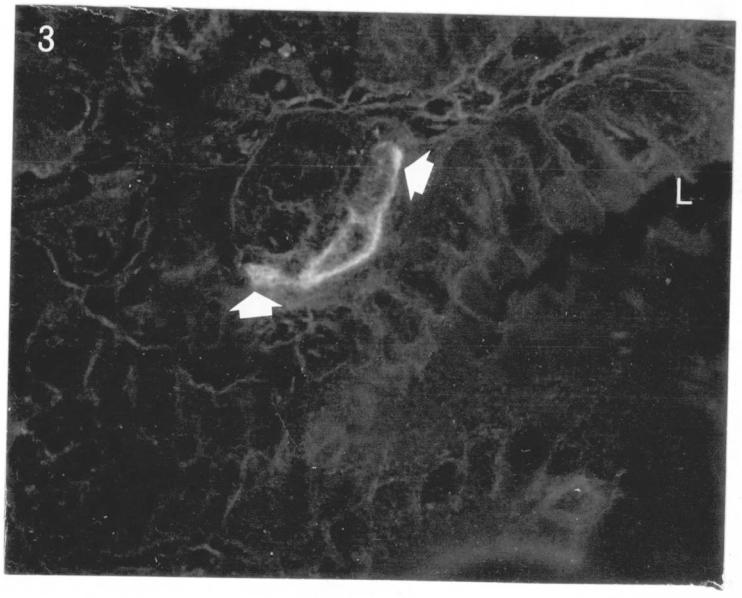
L = Lumen x 400

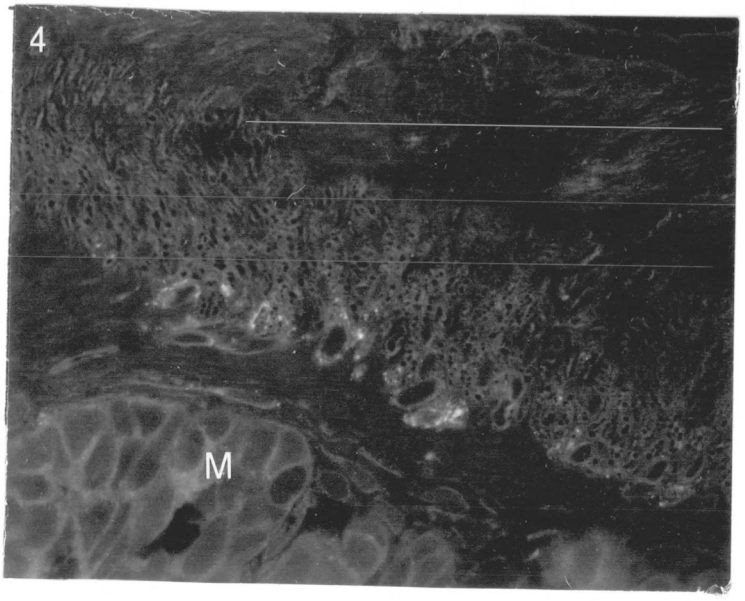
Figure 4

β endorphin-positive fibres at the edge of the circular muscle, in a position corresponding to the deep muscular plexus in mammals.

x400


2


3
L


4
M

the cells had large central nucleii with the immunostaining confined to the outer edges. An interesting finding was the presence of B-endorphin-immunoreactive cells and fibres in the lamina propria of the duodenum (Figure 4). These cells stained specifically for B-endorphin, being unstained by ACTH, enkephalin or corticotrophin-like intermediate peptide (CLIP) antisera. Of the peptides found in neural tissue in mammals, only VIP was found in nerve fibres in the salamander gut.

The immunostaining pattern in the ileum and colon was essentially the same as found in the duodenum (see Table 10) except that the number of immunostained endocrine cells was fewer.

ii. Ambystoma mexicanum

The results with this species were similar to those in the adult salamander, with the exception of the finding of motilin-like immunoreactivity in the duodenum. The motilin-containing cells were only detected with the C-terminal antiserum (Figure 5). It was interesting to find that the neurotensin antiserum again stained non-specifically cells in the stomach and intestine. In the intestine the gastrin/CCK antiserum detected numerous cells in the duodenal mucosa (Figure 6). Of the neuropeptides again only VIP-immunoreactive fibres and cell bodies were detected. The cell bodies/.....

bodies were confined to the myenteric plexus with the majority of the positive fibres occurring in the circular muscle layer (Figure 7) For a summary of the results see Table 11 below.

Table 11 Ambystoma mexicanum European

	INS	GLU	PGLU	SOM	BPP		G/CCK	NT	BN1	BN2	SP	MOT1	ENK				VIP1	VIP2	VIP3	END
Panc.	+	+	+	+	+															
Fundus		+		+													+			
Antrum				+				X									+			
Duod.				+			+	+			+	+	+				+			+
Jej.				+			+				+		+				+			
Ileum				+			+						+				+			
Colon		+						+									+			

iii. Cynops hongkongensis

In this species the neurotensin antiserum only detected non-specific cells within the stomach. Of the neuropeptides, only VIP was found in intestinal nerve fibres. For a summary the results see Table 12 overleaf.

Figure 5

Motilin-immunoreactive cells in the duodenal mucosa, stained by the C-terminal antiserum.

x 200

Figure 6

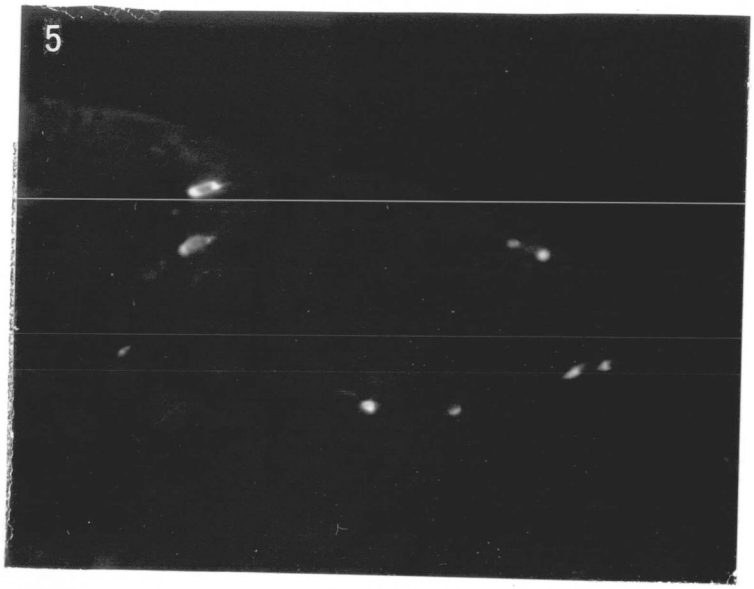
Gastrin/CCK-immunoreactive cells in the duodenum, note the long cytoplasmic extentions (arrows).

L = Lumen x 200

Figure 7

VIP-immunoreactive fibres in the myenteric plexus and circular muscle layer. Note the immunoreactive cell body (arrow).

S = Serosa x 200


5


6
L

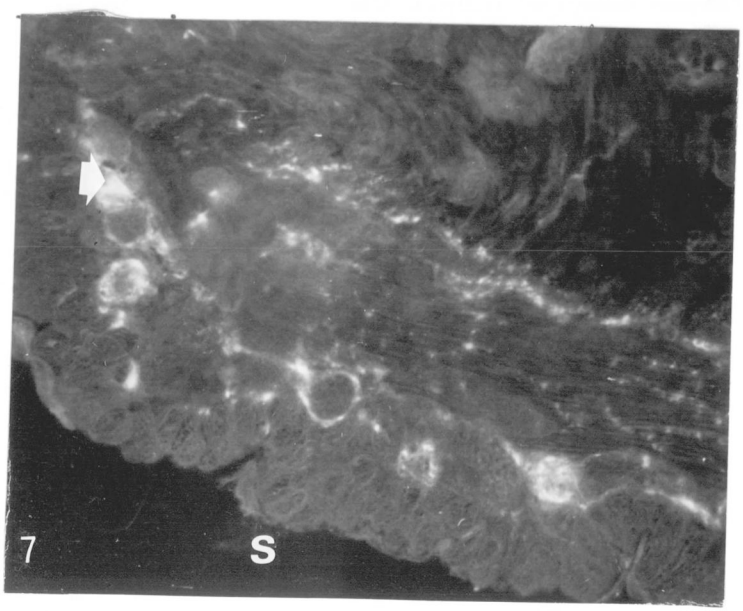

7
S

Table 12 Cynops hongkongensis European

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP					VIP1	VIP2	VIP3	
Panc.	+	+	+	+	+													
Fundus				+			×	+							+			
Antrum				+			×								+			
Duod.		+ +		+		+				+					+			
Jej.		+		+											+			
Ileum										+					+			
Colon										+					+			

5.2.2.b. Summary - Urodela

The endocrine cells are found scattered amongst other cell types in both the gut and pancreas. Immunostaining, with the antisera to GIP, the N-terminal region of motilin, secretin, mid-portion CCK and neurone specific enolase (NSE) were all negative.

The peptide-containing nerves were concentrated mainly in the circular muscle layers with lesser amounts in the myenteric plexus (Figure 8).

5.2.3. Anura

Below is given an outline of the families and their phylogenetic relationships as understood -

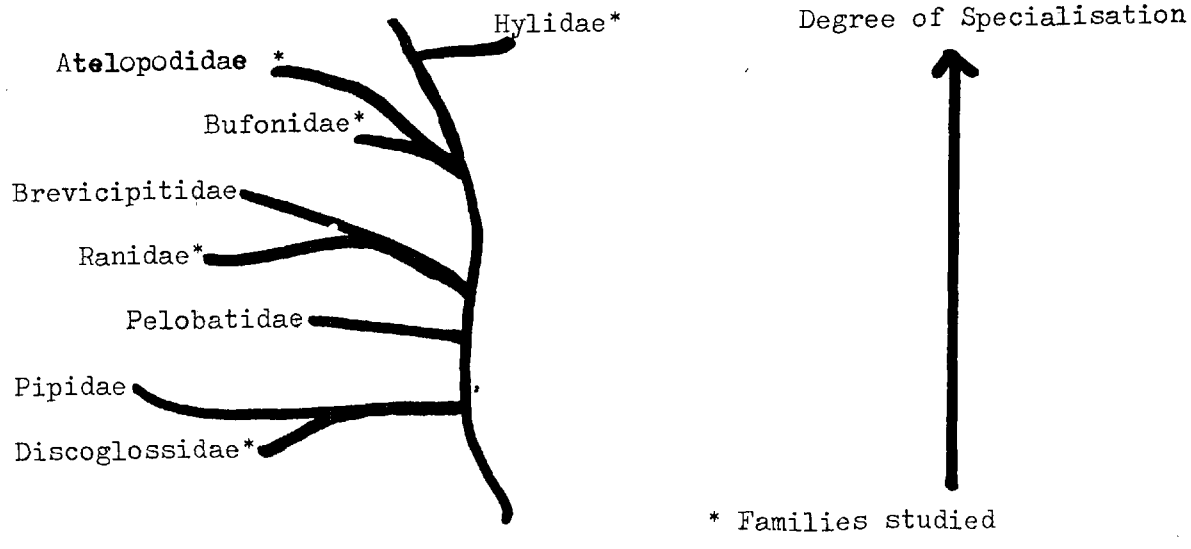
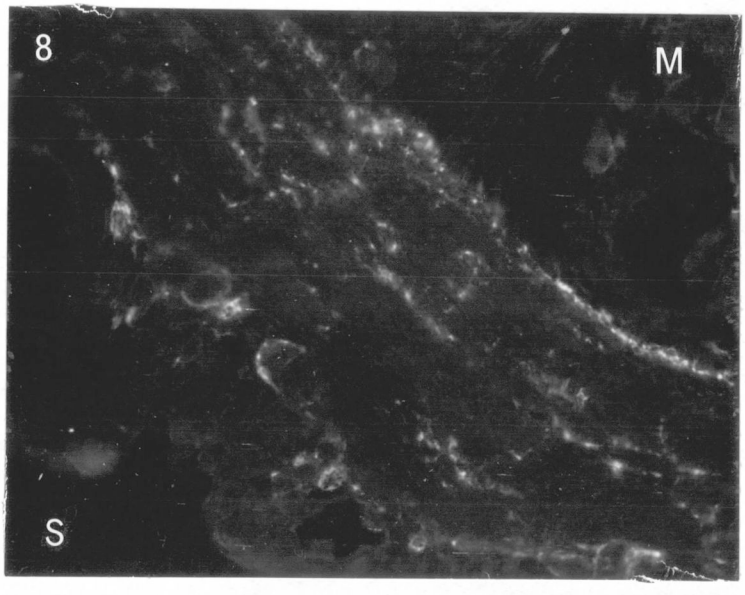


Figure 8

VIP-immunoreactive fibres in the circular muscle layer and myenteric plexus in the antrum of Ambystoma mexicanum.

S = Serosa M = Mucosa x 120


8
M
S

5.2.3.a. Pancreas. The pancreas was always found as a small lobulate mass attached to the duodenum at one end and extending to the spleen at the other. As it was extremely difficult to divide the small mass of pancreas into different regions the whole piece was treated as one.

Stomach. The stomach was divided into two regions - fundus and antrum, as in the Urodela.

Intestines. The intestine could be divided more accurately than in the Urodela, as there were clear macroscopic differences in the appearance of the mucosa at different levels. The differences were not identical in all the species studied but in general the duodenal mucosa was thicker and of a darker colour than the lower intestine. The upper small intestine (jejenum) could be distinguished from the lower intestine by changes in the pattern of the mucosal folding. The colon was easily distinguished as a greatly distended region at the end of the gut.

Immunostaining

i. Discoglossidae - Bombina bombina

The results of the immunostaining are given in Table 13 overleaf.

VIP was the only neuropeptide present in the autonomic nerves of the gut, being concentrated in the stomach. No staining was seen in the pancreas.

Table 13 Bombina bombina European

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP						VIP1	VIP2	VIP3	
Panc.	+	+	+	+	+														
Fundus				+				+	+							+			
Antrum						+										+			
Duod.				+		+										+			
Jej.				+		+										+			
Ileum		+					+									+			
Colon																+			

All four pancreatic hormones were immunostained and the positive cells were either grouped into small islet structures or scattered amongst the exocrine tissue. Again the BPP was the only positive PP antiserum and the cells detected were also immunostained with the antiglucagon serum.

Somatostatin cells were found in the pancreas, stomach and upper intestine but not in the colon and ileum. Specific neurotensin-immunoreactive cells were found in the ileum.

Numerous bombesin-like immunoreactive cells were found in the mucosa of the fundus and antrum, immunostained with both the N- and C-terminally directed to bombesin antisera (Figure 9). The substance P antiserum was unreactive.

No immunostaining for the mid-sequence of CCK, secretin, motilin (N- or C-terminal) or GIP was detected in this species.

ii. Alytes obstetricans /.....

Table 14 Alytes obstetricans European

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP	VIP1	VIP2	VIP3	VIP1	VIP2	VIP3	SP
Panc.	+	+	+	+	+												
Fundus		+		+				+	+					+	+	+	+
Antrum				+		+		+						+	+	+	
Duod.		+		+		+	+				+	+	+	+	+	+	
Jej.				+										+	+	+	
Ileum		+		+		+								+	+	+	
Colon														+	+	+	

The staining pattern for the pancreas was similar to that found in Bombina bombina with well established islet-like structures and scattered cells throughout the exocrine tissue (Figure 10).

The most interesting finding was the existence of numerous VIP-containing endocrine cells within the duodenal mucosa. These cells were found at all levels of the gut folds, the immunostaining was shown to be specific as the pre-addition of 1-0.1 nmol quantities of VIP (pure porcine) completely removed the staining while the addition of the related peptides secretin, GIP and glucagon even at higher concentrations had no effect on the immunostaining. VIP-immunoreactive nerve fibres were also seen throughout the entire length /.....

Bombina bombina

Figure 9

Bombesin-immunoreactive cells in the fundic mucosa (arrows).

x 200

Alytes obstetricans

Figure 10

Insulin-containing cells in the pancreas, mostly in islet-like structures with some scattered cells (arrows).

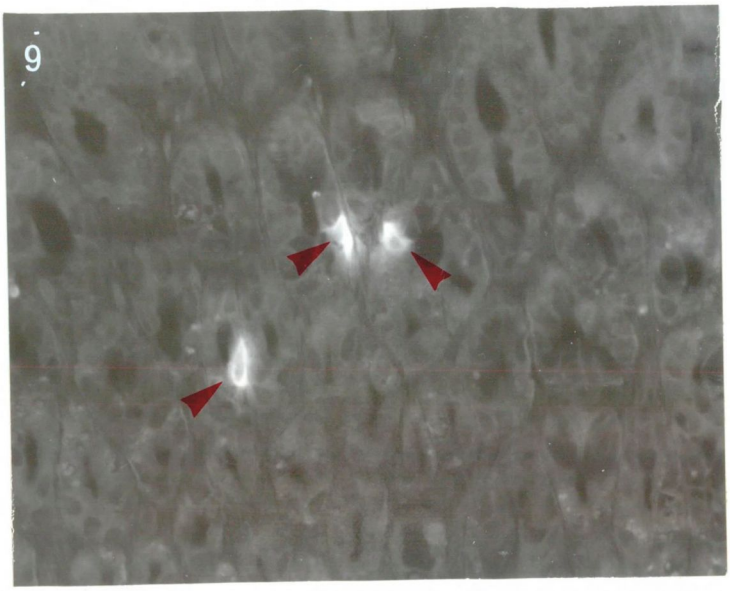
x 120

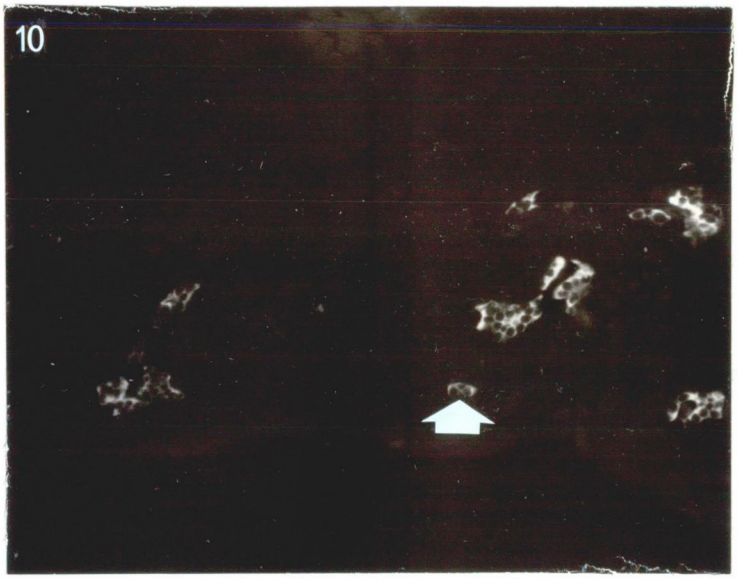
Figure 11

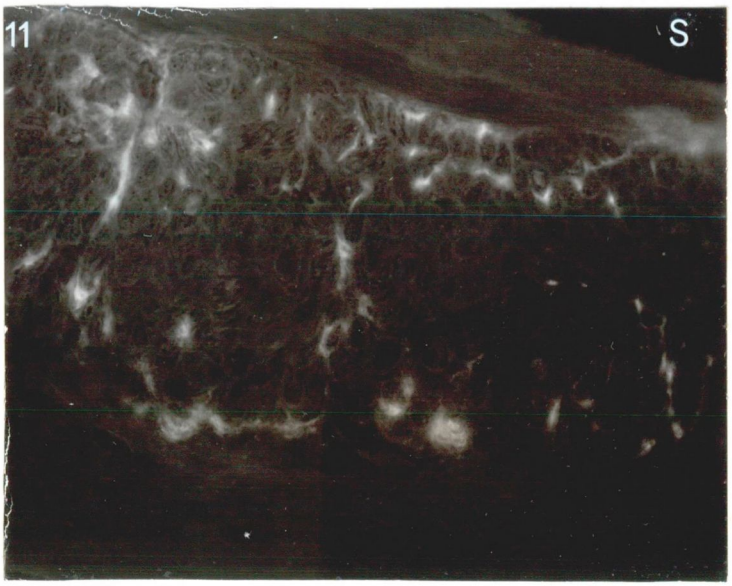
VIP-immunoreactive fibres in the circular muscle layer of the duodenum.

S = Serosa

x 400


9


10


11
S

length of the gut, but were absent from the pancreas (Figure 11).

In order to see if the VIP-like peptide in the endocrine cells was similar to that detected in the nerves, a series of different region-specific antisera were used (see Table 7).

Both the mucosal endocrine cells and autonomic nerves were equally reactive with the various region specific antisera to VIP available during this investigation (Figure 12).

Of the other neuropeptides only substance P was detected in nerve fibres, confined to the fundic region. Bombesin and somatostatin were found only in mucosal endocrine cells, while neither met-or leu-enkephalin could be detected in any region.

The antisera to the peptidesGIP, secretin, motilin and CCK did not give any detectable immunostaining.

iii. Ranidae - Rana temporaria

Table 15 Rana temporaria European

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP	VIP1	VIP2	VIP3	END	ENK	VIP1	VIP2	VIP3	SP
Panc.	+	+	+	+	+														
Fundus		+		+			×	+	+							+		+	+
Antrum		+		+		+	×	+	+						+	+		+	
Duod.		+		+		+	+				+	+	+	+		+		+	
Jej.		+		+		+	+									+		+	
Ileum		+		+												+		+	
Colon		+		+												+		+	

Figure 12

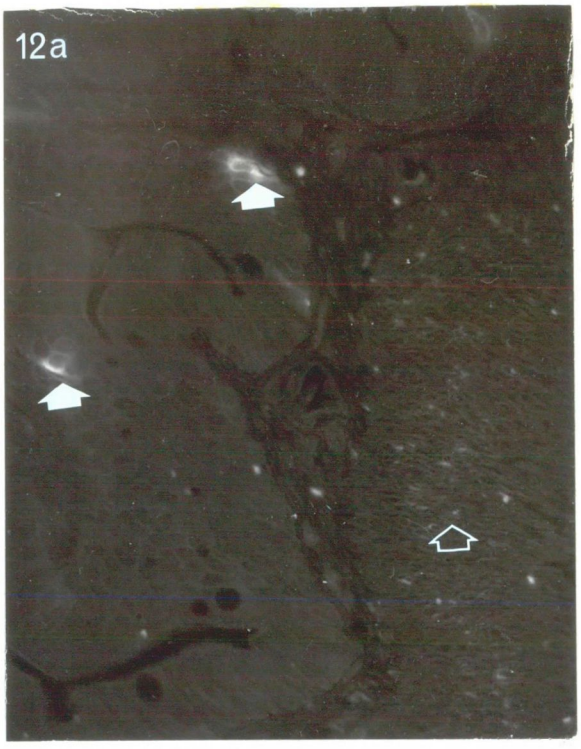
a - 5 micron section of the duodenal mucosa immunostained with the C-terminal VIP antiserum, showing positive endocrine cells (large arrows) and nerve fibres (small arrows).

x 120

b - 5 micron section of the duodenal mucosa immunostained with the N-terminal antiserum, showing a positive endocrine cell and nerve fibres (arrow).

x 200

Note that in both of these examples the staining of the mucosal endocrine cell is blurred, this is caused by overstaining due to a long incubation in order to allow recognition of the positive nerve fibres in a low magnification photograph.


12a


b

Figure 12

c - 5 micron section of the duodenal region showing a positive endocrine cell and nerve fibres as stained by a whole molecule specific antiserum to VIP.

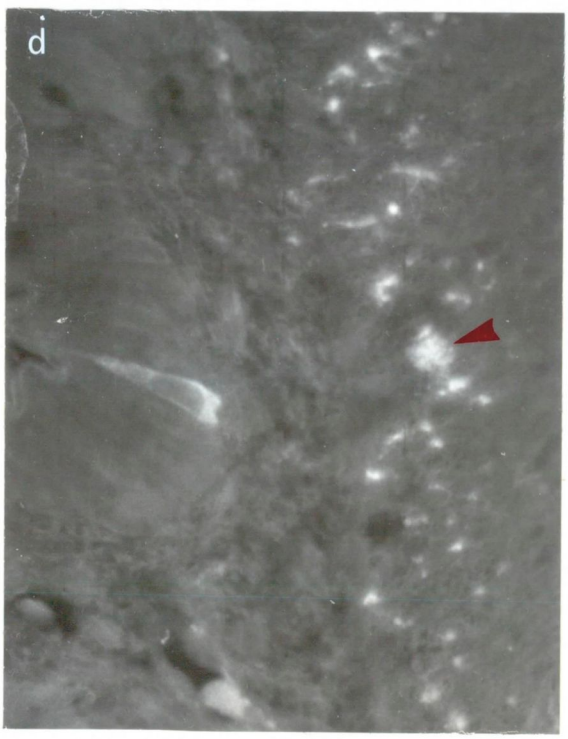
x 400

d - 5 micron section of the duodenum showing a positive endocrine cell and nerve fibres (arrow) as stained by a mid-portion specific antiserum.

x 400

In these examples the overstaining of the endocrine cells was not seen but this meant that the photographs had to be taken at a higher magnification in order to show the immunostained nerve fibres


12c


d

VIP-containing cells were found within the duodenal mucosa and nerve fibres in all areas of the gut but not the pancreas. VIP-immunoreactive cell bodies were found within the myenteric plexus with little or no staining in the sub-mucous plexus (Figure 13).

Somatostatin was present in endocrine cells from the pancreas and lower small intestine. The cells often showed long basal processes and some appeared to have bulbous projections at the basal lamina which may extend into the lamina propria (Figure 14).

The pancreas again contained both scattered endocrine cells and islet-like structures. The PP-immunostained cells were positive with both the anti-BPP and anti-glucagon sera.

In the intestine and stomach scattered nerve fibres were seen with the antisera to substance P, enkephalin(met) and endorphin, in no case were cell bodies observed. Endorphin-immunoreactive fibres were also seen in the duodenal region with immunoreactivity confined to the base of the circular muscle layer (Figure 15).

Neurotensin was present in endocrine cells in both the stomach and duodenal region. However, only the staining found in the duodenal mucosa was specific.

iv./.....

Figure 13

VIP-immunoreactive nerves in the myenteric plexus

x 400

Figure 14

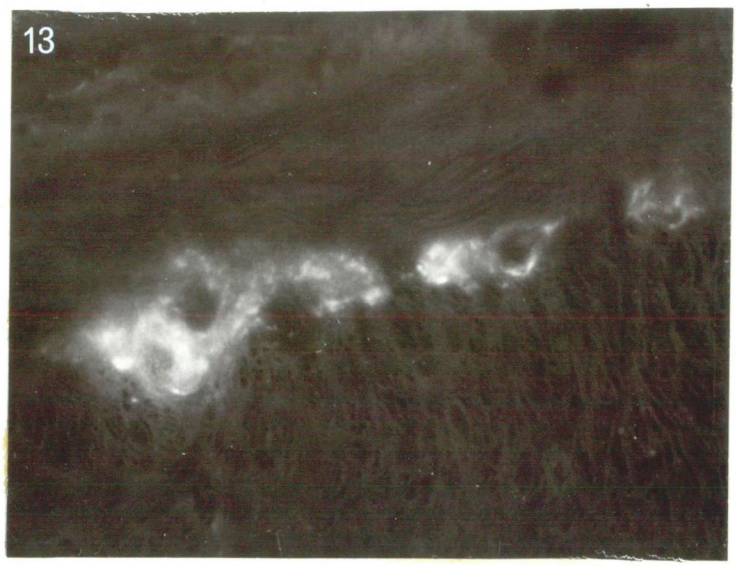
Somatostatin-immunoreactive cell in the intestine, with a basal projection (arrow).

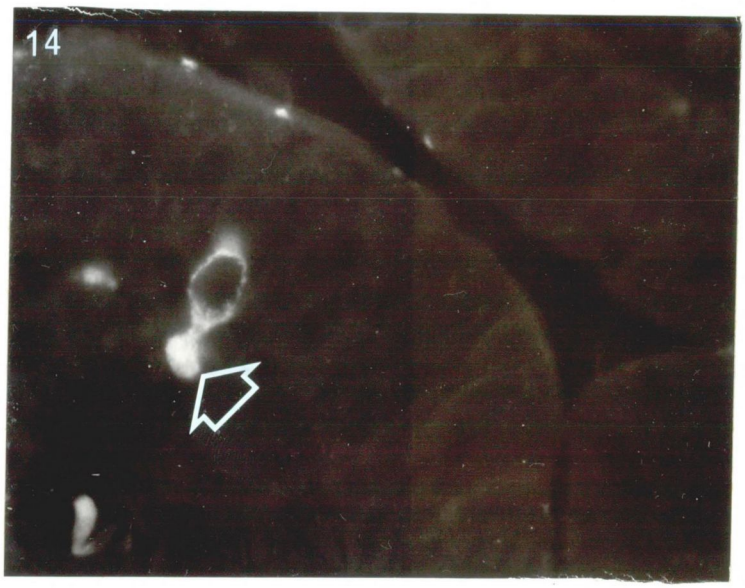
x 400

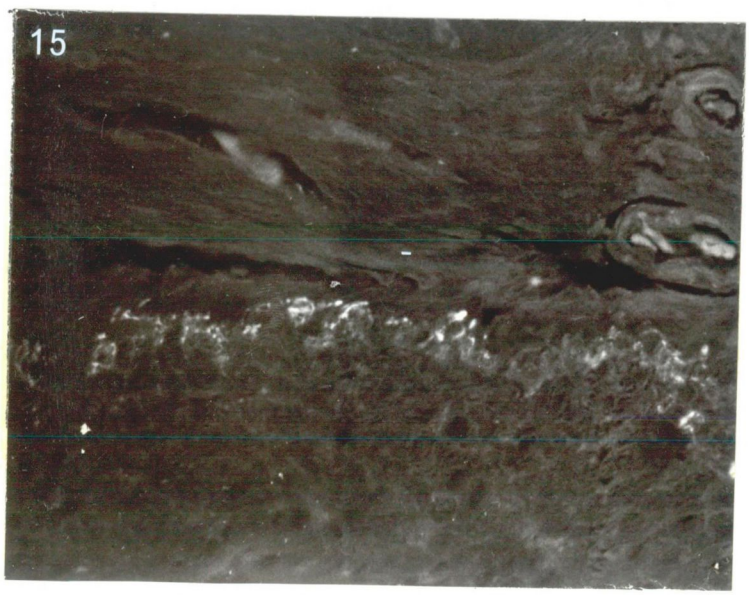
Figure 15

β endorphin-immunoreactive fibres in the duodenum confined to the edge of the circular muscle.

x 400


13


14


15

iv. Ranidae - Rana esculenta

Table 16 Rana esculenta European

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP					VIP1	VIP2	VIP3	
Panc.	+	+	+	+	+													
Fundus		+		+											+	+	+	
Antrum				+		+									+	+	+	
Duod.				+											+	+	+	
Jej.				+			+								+	+	+	
Ileum				+											+			
Colon				+											+			

The pancreatic endocrine tissue was found almost exclusively in medium-sized islets, the insulin cells forming the central core with peripheral glucagon/PP- and somatostatin-containing cells (Figure 16). These islets did not appear to have any peptidergic innervation. The VIP-immunoreactive fibres were confined to the intestine and stomach. In the gut the most numerous endocrine cell types in this species were somatostatin- and gastrin-containing. These somatostatin cells occurred from the stomach throughout the intestine, whereas the gastrin/CCK cells were confined to the antral region of the stomach (Figure 17).

Apart /.....

Figure 16

a - Insulin-containing cells forming the centre of a pancreatic islet.

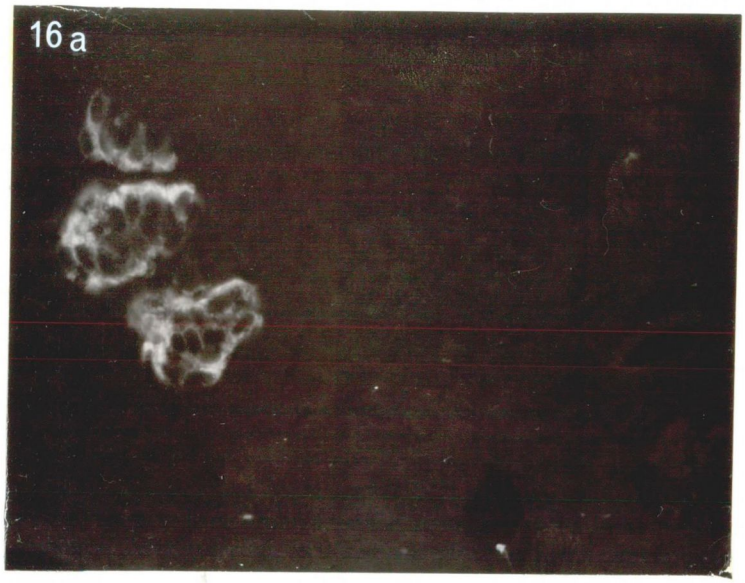
x 400

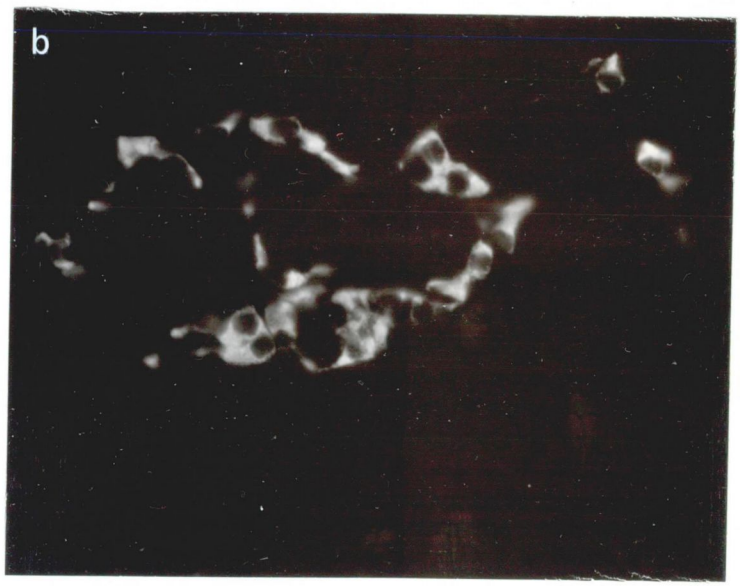
b - Peripheral somatostatin-containing cells surrounding the central insulin cells.

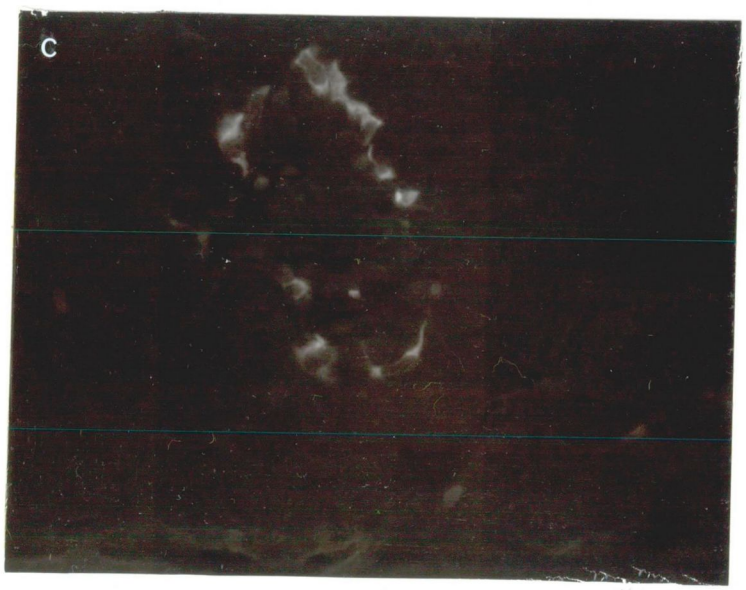
x 400

c - Glucagon-containing cells surrounding the insulin-cells seen in a.

x 400


16 a


b

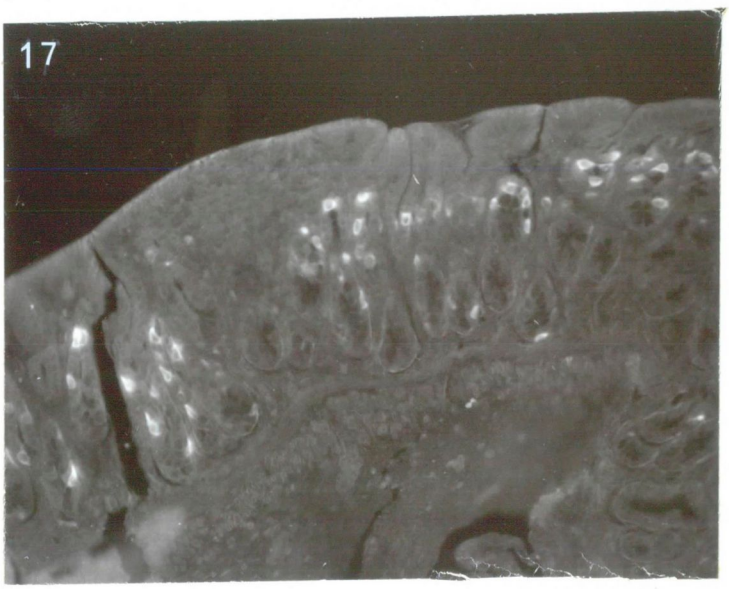

c

Rana esculenta

Figure 17

Somatostatin-containing cells in the antral mucosa.

x 120


17

Apart from the VIP-immunoreactive fibres no other peptide-containing nerves were seen in any area of the intestine.

v. Bufonidae - Bufo bufo

Material from two different groups of Bufo bufo were collected, the first group were killed whilst in hibernation at the beginning of February and the second group were killed in May after approximately four weeks of feeding after the hibernation period. The results of the immunostainings obtained with the two groups are given below and show several differences.

Table 17 Bufo bufo - hibernating European

	INS	GLU	PGLU	SOM	BPP		G/CCK	NT	BN1	BN2	SP	VIP1	VIP2	VIP3			VIP1		VIP3	SP
Panc.	+	+	+	+	+															
Fundus									+								+		+	+
Antrum		+		+				X	+								+		+	+
Duod.		+		+								+	+	+			+	+	+	
Jej.		+															+		+	
Ileum		+															+			
Colon		+															+			

Table 18 Bufo bufo - Post hibernation European

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP	VIP1	VIP2	VIP3		VIP1	VIP2	VIP3	SP
Panc.	+	+	+	+	+													
Fundus								+	+						+	+	+	+
Antrum		+		+			X	+	+						+	+	+	+
Duod.				+ +			+				+	+	+		+	+	+	
Jej.		+		+			+				+	+	+		+	+	+	+
Ileum		+		+			+								+	+	+	
Colon		+		+											+	+	+	

Pancreas. In the hibernating animals although the four pancreatic hormones were always detectable the staining was greatly reduced and the islets appeared to be reduced in size (Figure 18). The islets in the post-hibernation toads were particularly well developed and showed the typical distribution of cell types, the insulin cells occurring in the centre with the somatostatin and glucagon/PP- immunoreactive cells at the periphery. The major difference from mammalian pattern was that the insulin cells appeared to occur in ribbon-like structures rather than the well-defined islets as seen in mammals. Of the PP antisera available only the BPP antisera gave

positive/.....

Bufo bufo

Figure 18

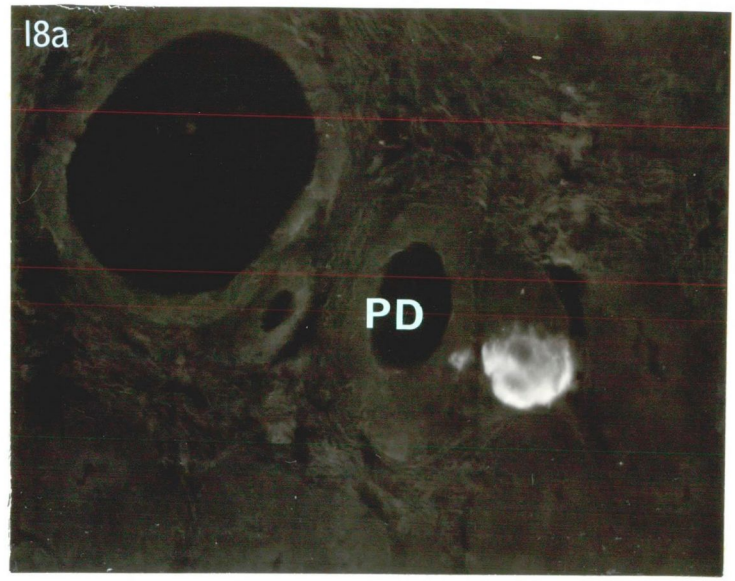
a - Insulin-containing cells in close association with the pancreatic ducts (PD) in a hibernating toad.

x 400

b - Insulin-containing cells in a typical ribbon-like arrangment as seen in a post hibernation toad.

x 400

Note the increased size of the islet seen in the post hibernation toad.


18a
PD


b

positive immunostaining, giving an identical pattern to that seen using the anti-glucagon serum.

No evidence of a peptidergic innervation could be seen even in the post-hibernation animals with large islets.

Stomach and Intestines. Comparison of the haematoxylin and eosin-stained sections of the hibernating and post-hibernation animals showed clear differences in the thickness and development of the mucosa.

In the stomach, the post-hibernation toads showed less epithelial depth (Figure 19) whereas in the duodenum and upper small intestine the reverse was found, with the duodenal folds being greatly enlarged both in height and complexity (Figure 20).

In parallel with the changes in morphology the gut peptides were found to be altered. The four peptides showing the greatest changes were bombesin, VIP, somatostatin and substance P. Substance P in the hibernating toads was present in fine nerve fibres only in the stomach, in the post-hibernation toads these nerves were increased both in numbers and in distribution being found also in the upper small intestine (Figure 21).

Somatostatin was present in endocrine mucosal cells of the intestine and pancreas in both groups./.....

Figure 19

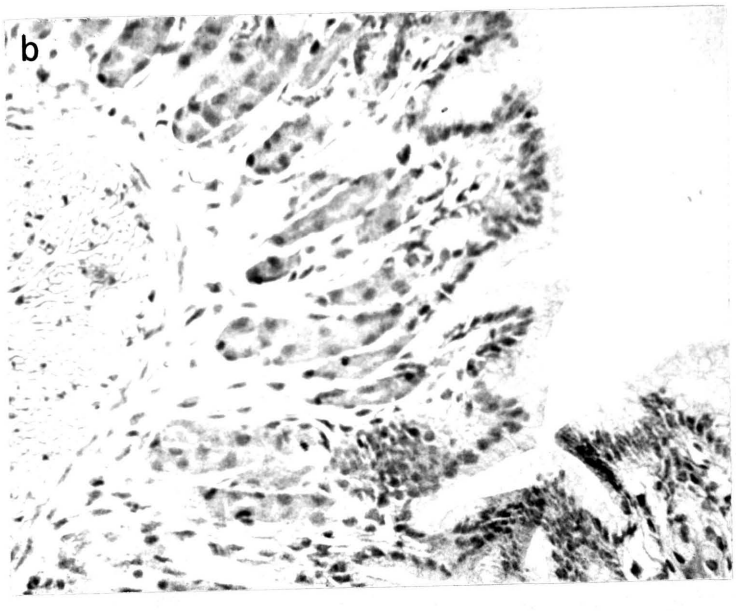
a - Section through the fundus of a hibernating toad showing an increased mucosal depth as compared to b.

x 120

b - Section through the fundus of a post-hibernating toad.

x 120


19a


b

Bufo bufo

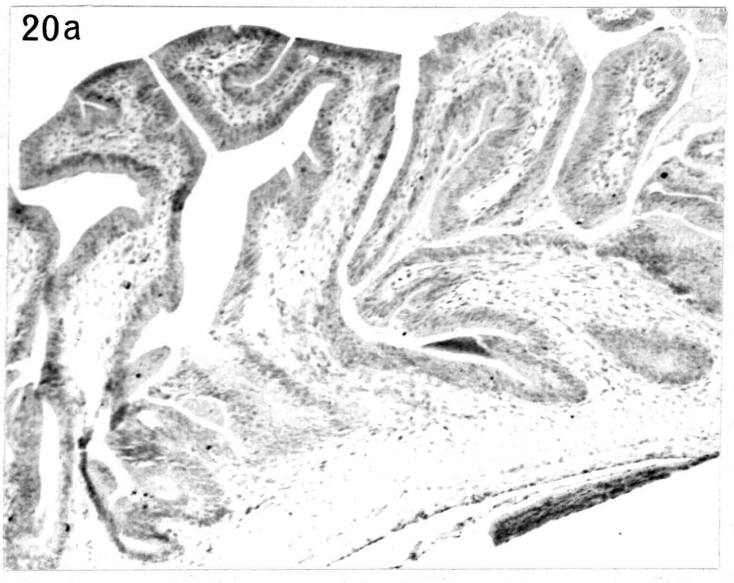
Figure 20

a - Shows the duodenal folds in a hibernating toad.

x 120

b - A similar area in the post-hibernation toad showing the increased development of the folds.

x 120


20a

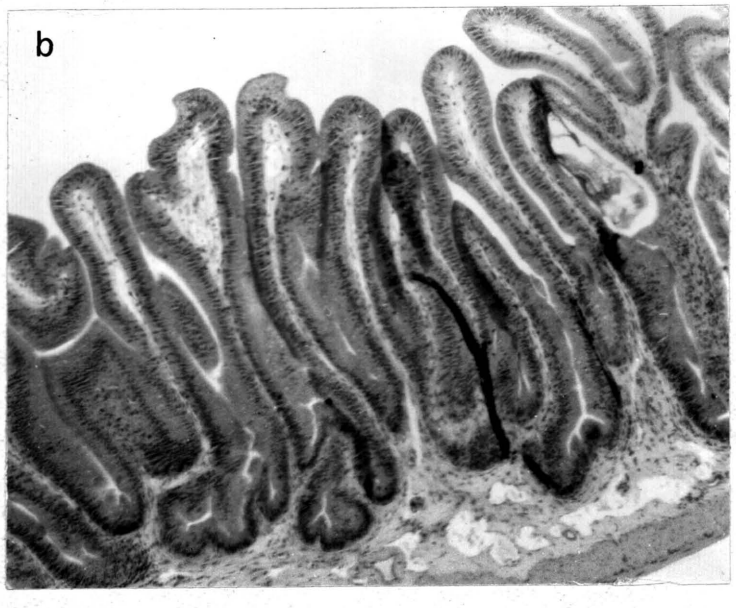

b

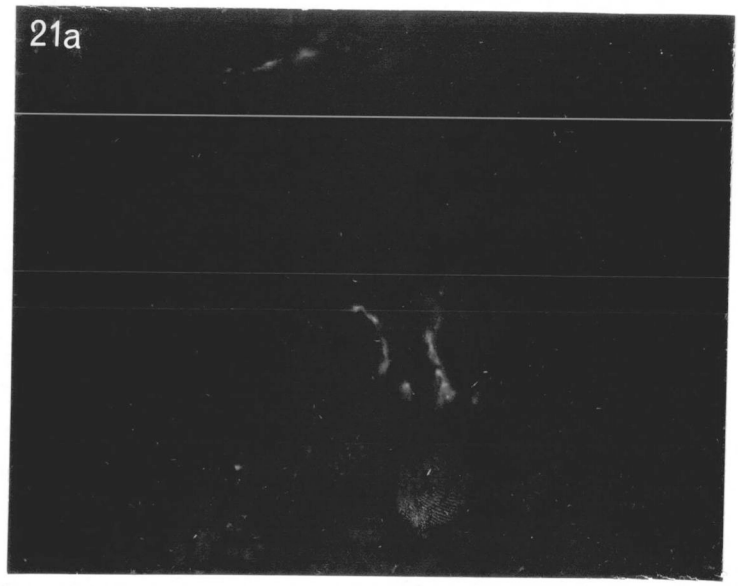
Figure 21

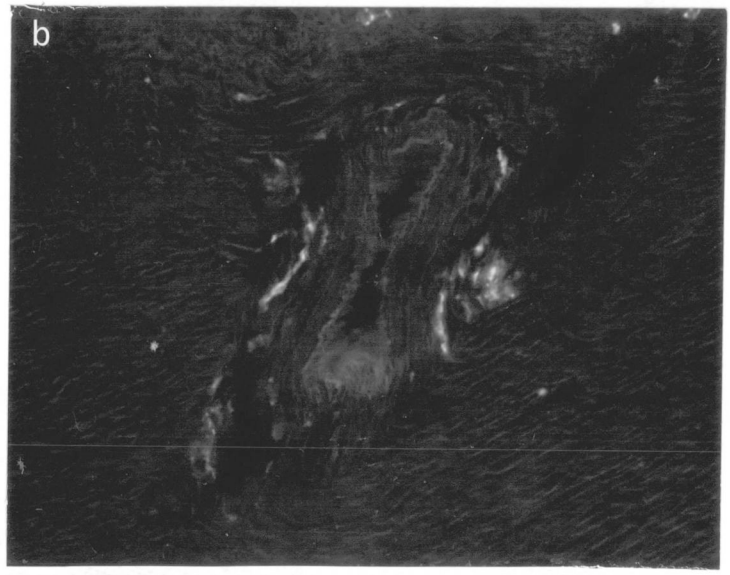
a - Substance P-immunoreactive fibres around a blood vessel in the stomach.

x 400

b - Similar area in a post hibernation frog showing an increase in the number of fibres detected.

x 400


21a


b

groups. Although the distribution of the cells did not alter, in the post-hibernation toads (Figure 22), the cells were more numerous with conspicuous basal processes.

Bombesin was only present in the antral region of both hibernating and post-hibernating animals - however, a vast increase in the number of cells was detected in the post-hibernating animals (Figure 23).

In both groups VIP was found in intestinal endocrine cells and nerve fibres throughout the intestine and stomach. In the post-hibernation toads the distribution of the endocrine cells was increased, being confined to the duodenum during hibernation but later extending into the jejunal region (Figure 24).

No change was detected in numbers of glucagon and neurotensin cells. The gastrin/CCK antisera were uniformly negative, although a slight staining was observed in the duodenal mucosa of both groups.

The peptides GIP, CCK, motilin and secretin were all undetectable.

vi./.....

Figure 22

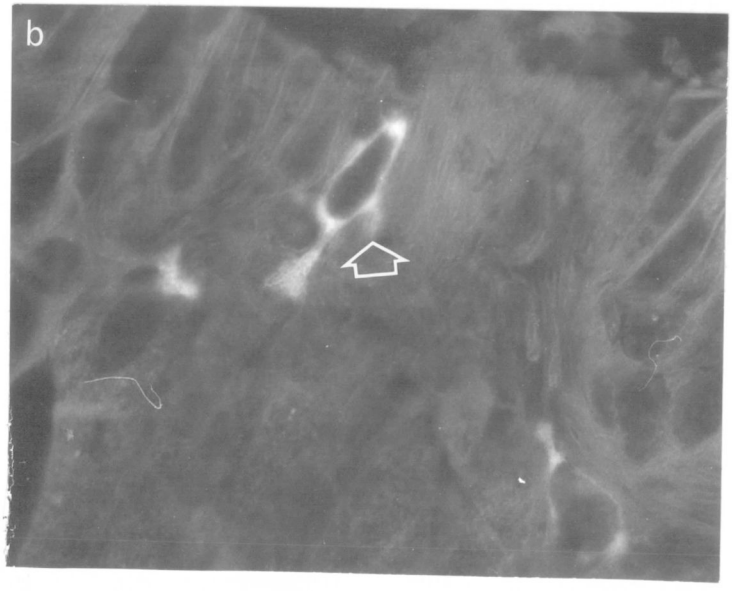
a - Somatostatin-immunoreactive cell in the duodenal mucosa of a hibernating toad.

x 400

b - Somatostatin-immunoreactive cell in the duodenal mucosa of a post-hibernating toad - note the cytoplasmic process (arrow).

x 400


22a


b

Bufo bufo

Figure 23

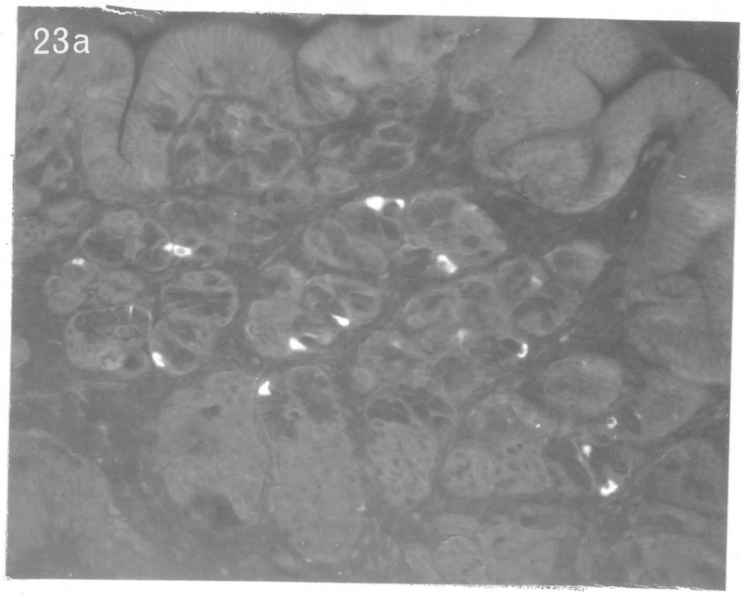
a - Bombesin-immunoreactive cells in the fundus of a hibernating toad.

x 120

b - Bombesin-immunoreactive cells in the fundus of a post-hibernation toad - note the increased number of cells detected.

x 200

Photograph a. is at a slightly lower magnification in order to give a better idea of the distribution of the cells detected in the hibernating animals.


23a

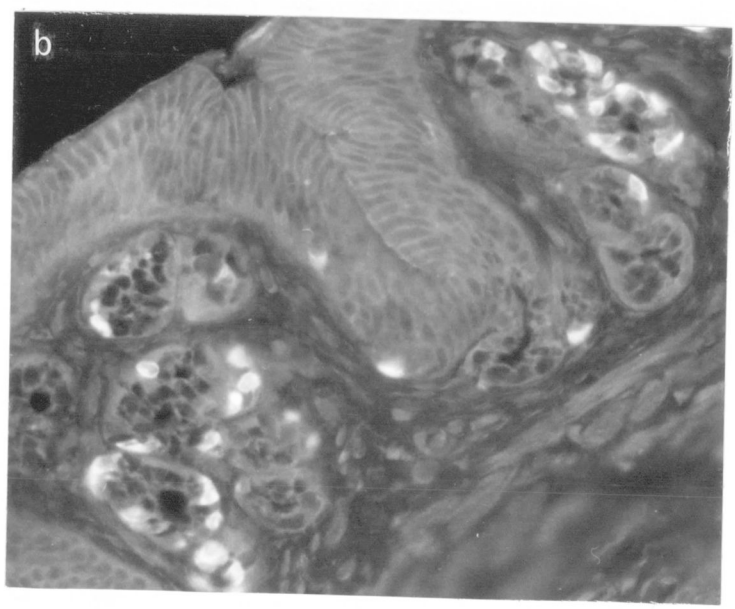

b

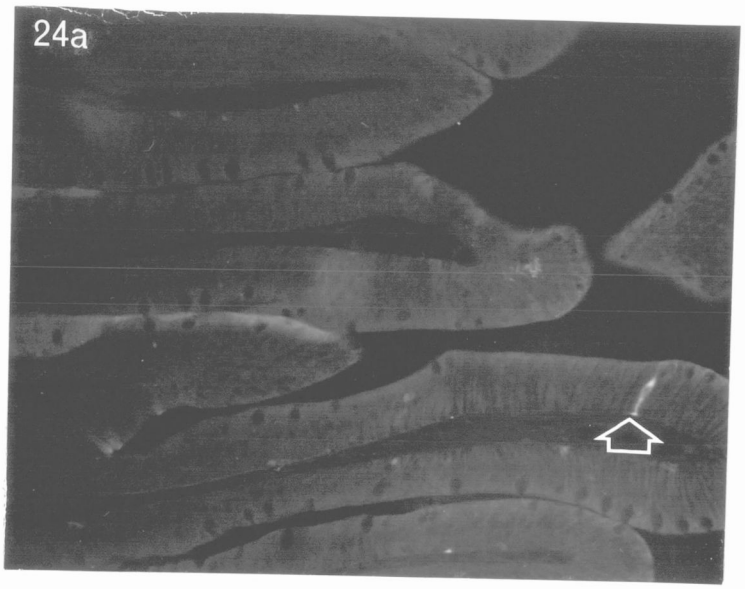
Figure 24

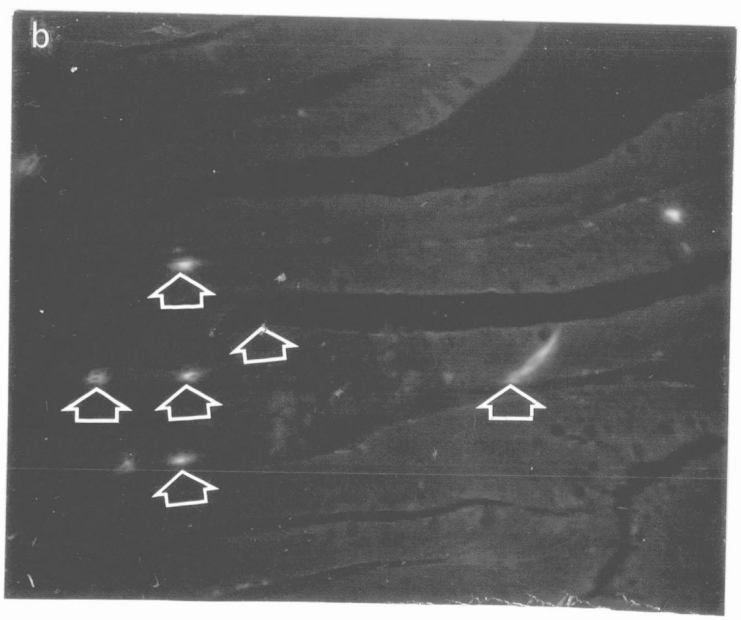
a - VIP-immunoreactive cell (arrow) in the duodenum of a hibernating toad.

x 120

b - VIP-immunoreactive cells in the duodenum of a post-hibernation toad.

x 120


24a


b

vi. Atelopodidae - Atelopus oxyrhynchus

Table 19 Atelopus oxyrhynchus - South American

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP	VIP1	VIP2	VIP3	PRL	VIP1	VIP2	VIP3	SP
Panc.	+	+ +	+	+	+													
Fundus		+		+			X			+								+
Antrum		+		+			X			+				+				+ +
Duod.		+		+		+ +					+	+	+	+	+	+	+	
Jej.											+	+	+	+	+	+	+	
Ileum		+		+			+							+	+	+	+	
Colon										+					+	+	+	

Pancreas. Both islets and scattered endocrine cells were seen. The scattered cells were mostly immunostained with glucagon/PP antisera. The islets showed a central core of insulin-immunoreactive cells with an outer layer of glucagon/PP and somatostatin cells. No immunostaining was observed with any of the antisera to the neuropeptides.

Stomach and Intestine. In the antrum there were numerous substance P-immunoreactive fibres, seen both in the circular muscle and myenteric and sub-mucous plexuses (Figure 25). No immunostaining was seen with the antisera to VIP and gastrin/CCK. However, gastrin/CCK cells/.....

cells were especially numerous in the duodenal mucosal folds.

VIP-immunoreactivity was present in both mucosal endocrine cells and the intestinal nerve plexuses. The VIP-immunoreactive endocrine cells were confined to the mucosa of the duodenal and jejunal regions whereas the nerve fibres were found throughout the intestine.

Substance P-immunoreactive endocrine cells were also detected in the colon of this species.

In addition the antiserum to prolactin detected numerous cells in the antrum and small intestine (Figure 26).

vii. Hylidae - Hyla arborea

Table 20 Hyla arborea European

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP	VIP1	VIP2	VIP3	VIP1	VIP2	VIP3
Panc.	+	+	+	+	+									+	+	+
Fundus				+										+	+	+
Antrum				+		+	X							+	+	+
Duod.				+		+	+				+	+	+	+	+	+
Jej.		+		+		+	+			+				+	+	+
Ileum														+	+	+
Colon														+	+	+

Figure 25

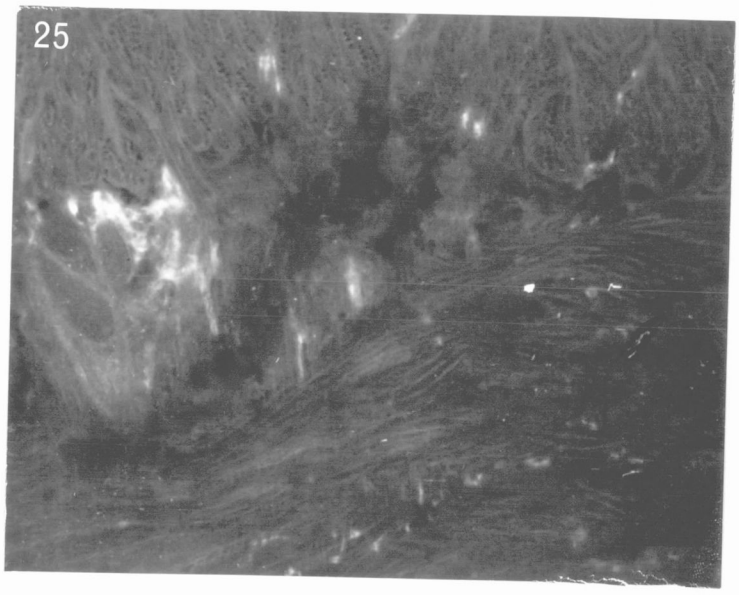
Substance P-immunoreactive fibres in the myenteric plexus of the antrum.

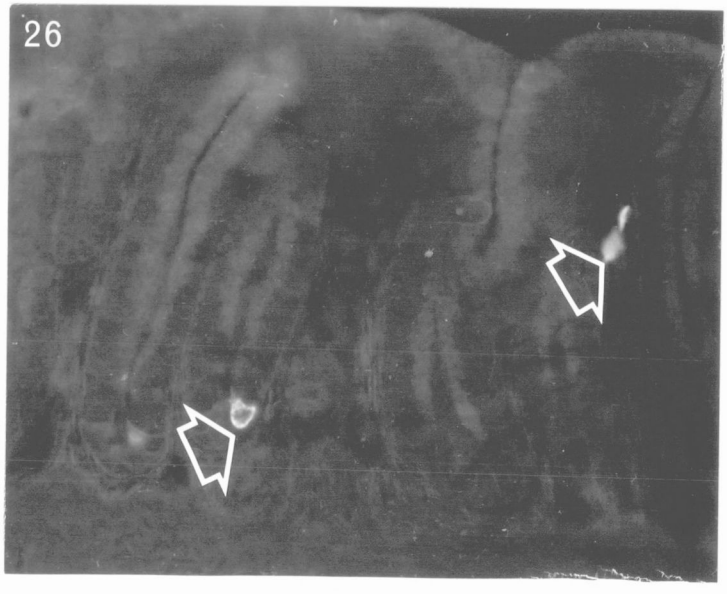
x 400

Figure 26

Prolactin-immunoreactive cells in the antral mucosa.

x 400


25


26

Pancreas. The endocrine tissue was mostly present in islets showing the classical mammalian organisation (Figure 27). The usual immunostaining pattern was seen with the glucagon/BPP antisera staining the same cell population. Indications of a peptidergic component of the pancreatic innervation was also demonstrable (Figure 28). VIP-immunoreactive nerve fibres were seen in the exocrine tissue but not in association with the endocrine tissue. No other neuropeptides were demonstrable in the pancreatic tissue.

Stomach and Intestine. VIP-immunoreactivity was present in nerve fibres from stomach to large intestine and in endocrine cells of the duodenal mucosa. Gastrin/CCK, glucagon and neurotensin were all present in mucosal endocrine cells. Neurotensin-immunoreactivity was present in both the stomach and the upper small intestine but only the cells of the upper intestine were specifically immunostained (Figure 29). Substance P was present, not in nerve fibres or in cells of the stomach, but in numerous mucosal cells in the jejunum (Figure 30).

The peptides GIP, CCK, motilin, secretin and enkephalin were all undetectable in this species.

viii./.....

Hyla arborea

Figure 27

a - Insulin-containing cells in the centre of an islet.

x 400

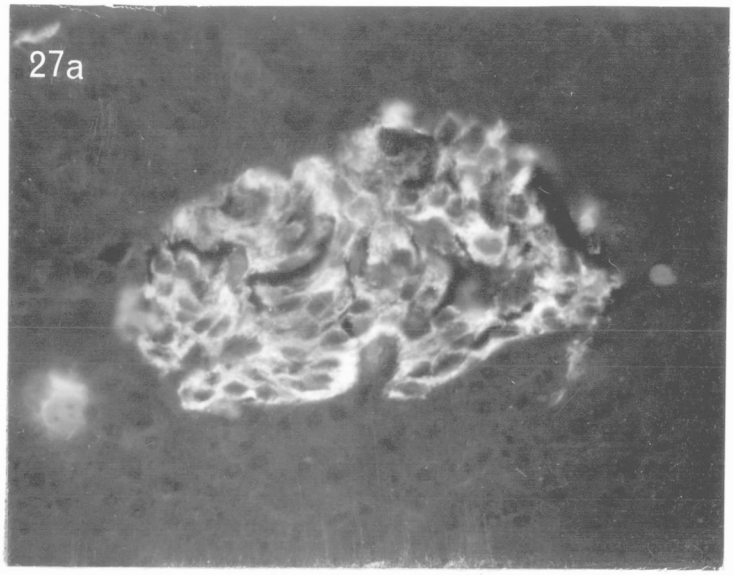
b - A serial section showing the glucagon-containing cells surrounding the central insulin-containing cells.

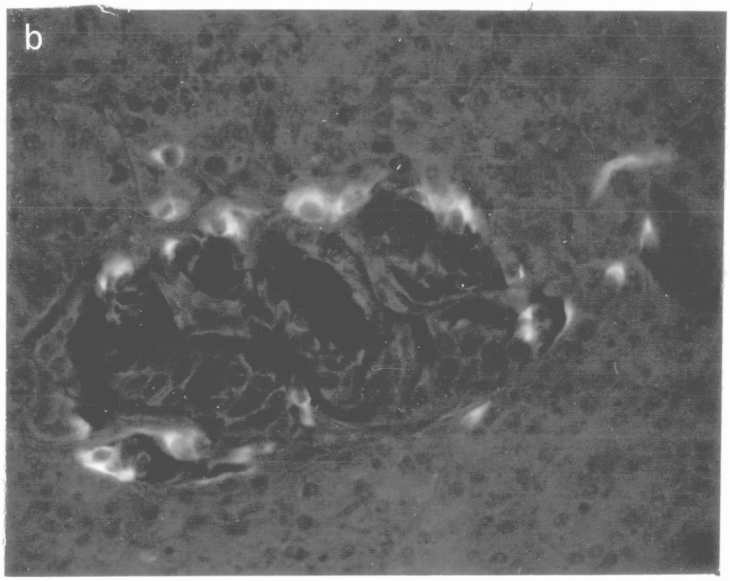
x 400

c - A third serial section showing the somatostatin-containing cells also at the periphery of the islet.

x 400

NB. Unfortunately a complete series of the three immunostainings was not obtained in optimally fixed material, thus the morphological preservation, especially in the second of the three sections, was not perfect.


27a


b

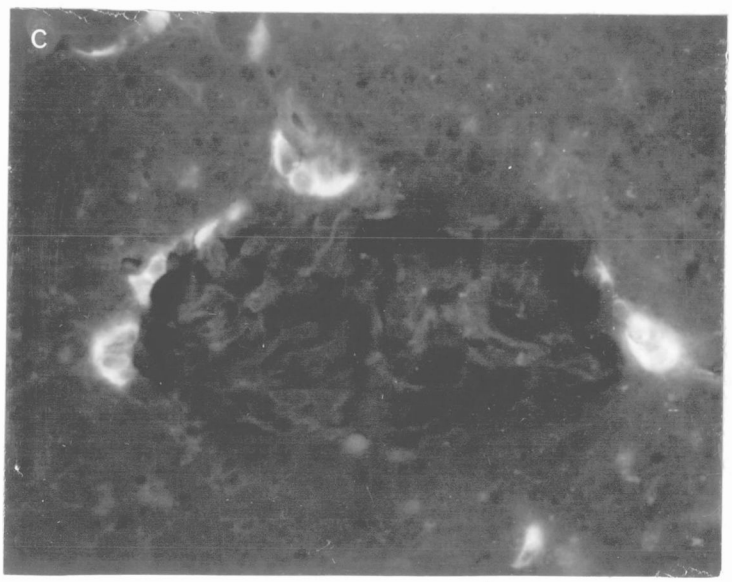

c

Figure 28

Fine nerve fibre running in the exocrine pancreas (arrow).

x 400

Figure 29

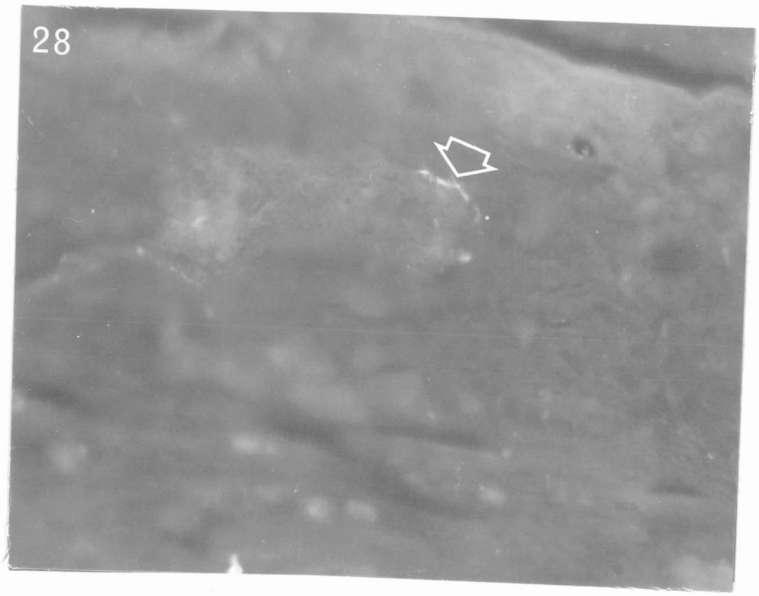
A neurotensin-immunoreactive cell in the small intestinal mucosa.

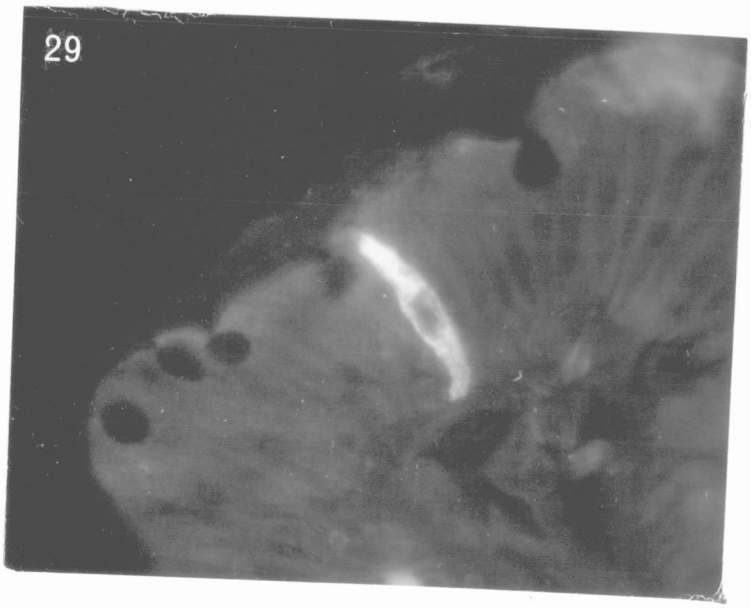
x 400

Figure 30

Substance P-immunoreactive cells in the jejunal mucosa.

x 250


28


29


30

viii. Hyla crepitans

Table 21 Hyla crepitans South American

	INS	GLU	PGLU	SOM	BPP	G/CCK	NT	BN1	BN2	SP	VIP1	VIP2	VIP3	ENK	PRL	VIP1	VIP2	VIP3
Panc.	+	+	+	+	+													
Fundus		+	+	+			×									+	+	+
Antrum		+	+	+		+	×	+	+					+	+	+	+	+
Duod.		+		+		+	+				+	+	+	+		+	+	+
Jej.		+		+												+	+	+
Ileum		+		+												+	+	+
Colon		+		+												+	+	+

Pancreas. The majority of the endocrine tissue was found as large islets, although the glucagon/BPP cells were also found scattered throughout the exocrine tissue (Figures 31 and 32). There was no indication of a peptidergic innervation in any area of the pancreas.

Stomach and Intestine. Somatostatin and glucagon immunostained cells and VIP immunoreactive nerve fibres were seen throughout the length of the intestines and in the stomach. Neurotensin-immunostained cells were seen in the stomach and duodenum but only those in the duodenum were specifically stained. Scattered VIP-immunostained endocrine cells were seen only in the duodenal mucosa. Met-enkephalin containing/.....

Figure 31

a - Insulin-containing cells forming the centre of a large islet.

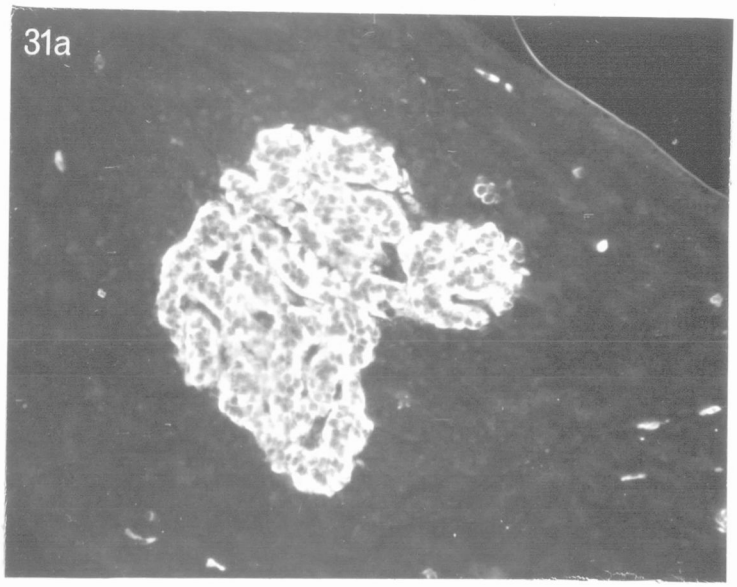
x 250

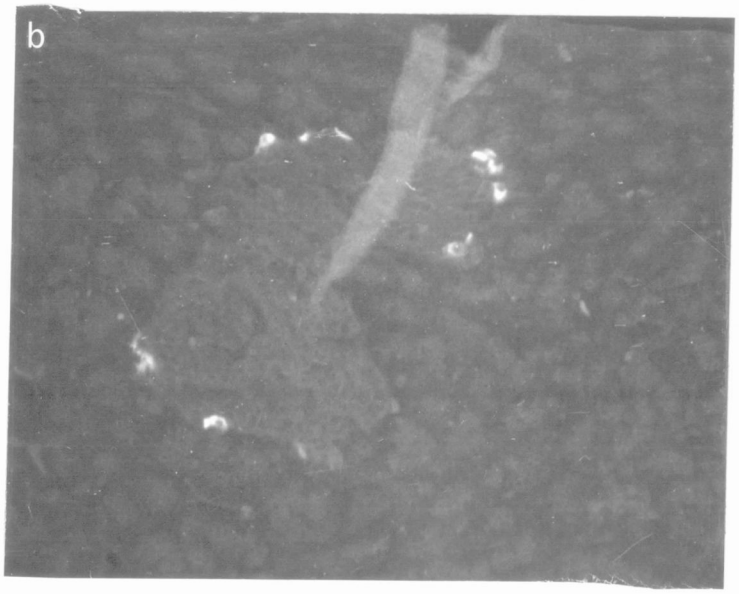
b - Same region in a serial section showing the peripheral somatostatin-containing cells.

x 250

c - The same area in a third serial section showing the scattered glucagon-containing cells (arrows).

x 250


31a


b

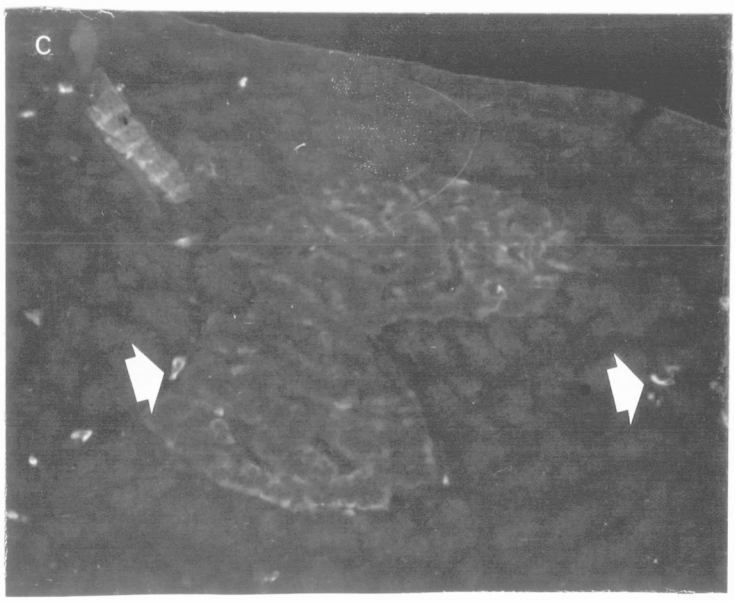

c

Figure 32

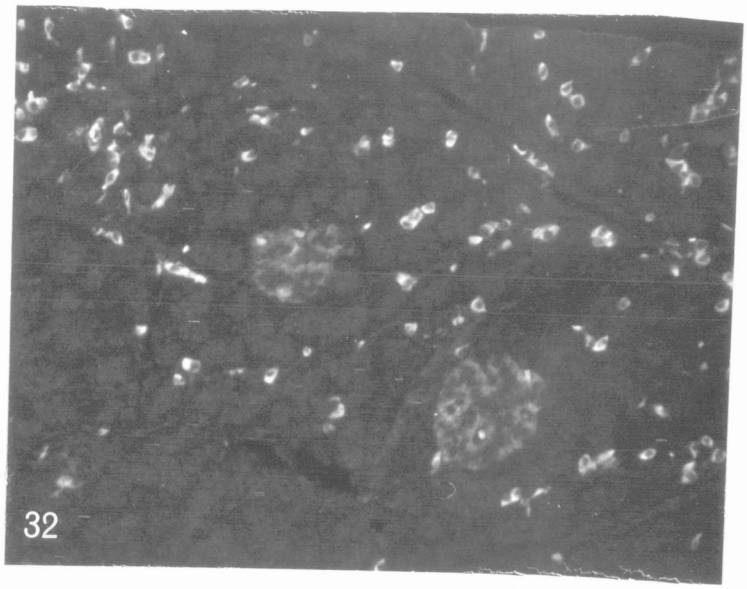
Glucagon-immunoreactive cells scattered throughout the exocrine tissue.

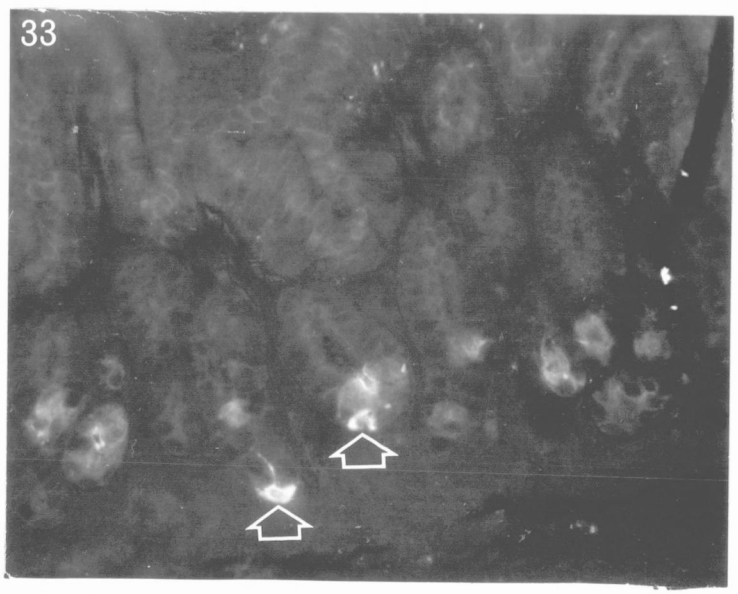
x 250

Figure 33

Prolactin-immunoreactive cells in the antral mucosa (arrows).

x 120


32


33

containing cells were found in the antrum and duodenum of this frog. Prolactin-immunoreactive cells were present but restricted to the antral mucosa (Figure 33).

5.2.3.b. Summary - Anura

The pancreatic islets were well developed especially in the more specialized species. Double staining was observed with the glucagon and BPP antisera. VIP-immunoreactivity was found in the pancreatic tissue although only in one of the most advanced species (Hyla arborea). In the intestines VIP-immunoreactivity was found in nerve fibres and cell bodies and also in mucosal endocrine cells of the upper intestine. Substance P-immunoreactivity was also found in both mucosal endocrine cells and nerve fibres but the dual localisation was not seen within the same area in the same animal. There was also a double staining of the bombesin and substance P antisera of certain cells within the stomach of several species; this, like the glucagon/BPP staining, cannot be fully removed by the pre-absorption of the antisera with the crossreacting peptide. The results with the neurotensin antisera showed that the immunostainings in the stomach was non-specific whereas that in the intestine were specific.

Of the neuropeptides only, VIP and substance P were detected with any frequency in the nerve plexuses, the/.....

the other peptides only being found on occasion (met-enkephalin) or never (somatostatin).

The peptides secretin, CCK, GIP and motilin were undetectable in this material with all the antisera available during this study.

5.3. Reptilia

Light Microscopy.

5.3.1. Histology. The basic structure of the gut did not vary greatly between orders. The stomach invariably consisted of glands lined by mucin cells and some endocrine cells. The intestine showed no clearly recognisable villus and crypt regions although complex gut folding was observed.

The mucosa was composed of enterocytes, mucin cells and scattered endocrine cells.

The greatest differences were found in the pancreas both in location and size of the organ (see Diagram 7). The intestinal muscle layers also varied both in size and consistency - these will be discussed later.

In the reptilia the macroscopic appearance of the intestine varied tremendously. In the Testudinata the structure was simple, resembling that of the Anura, with a long, looped undifferentiated gut. In the Squamata the Laecertilia had a short, compact gut, which appeared relatively thick in comparison to the Anura and Testudinata. The snakes showed two arrangements of the gut (Diagram 8). In all species/.....

DIAGRAM 7 LOCATION OF THE REPTILIAN PANCREAS

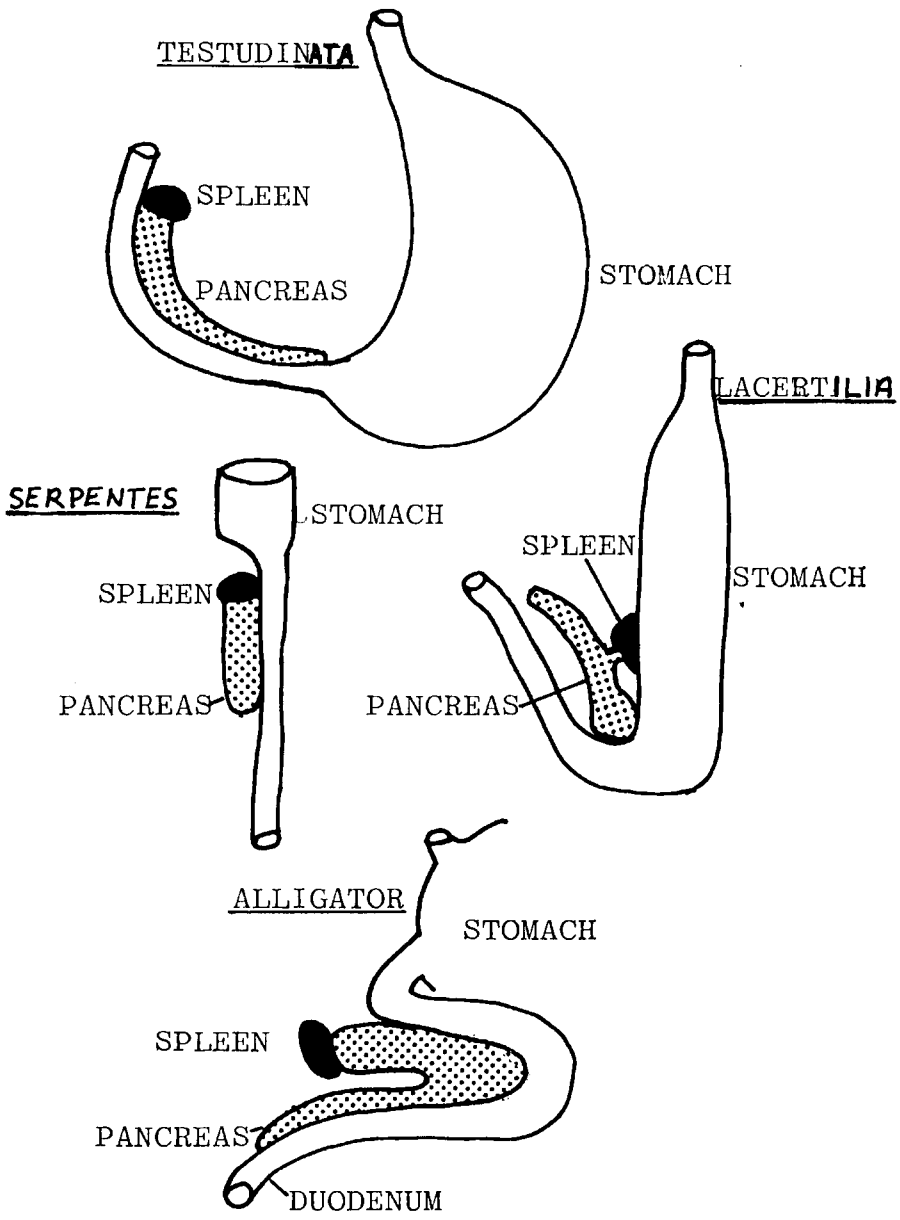
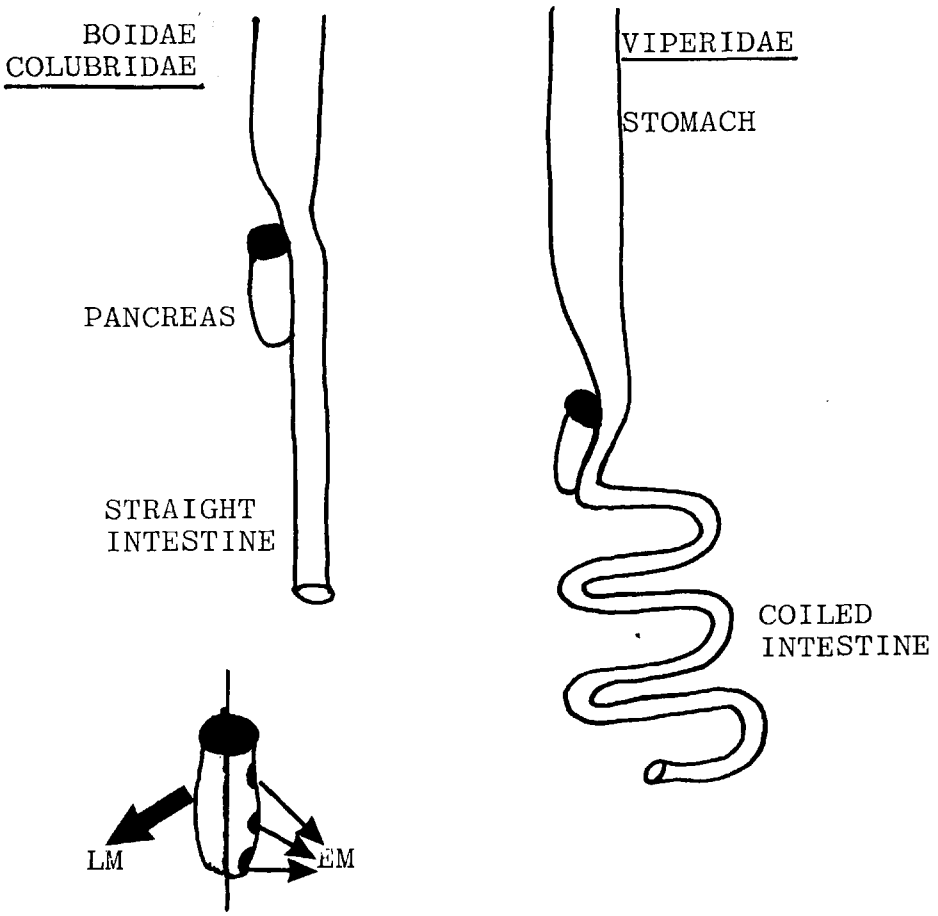


Diagram 8 SNAKE Intestine and Pancreas



DIVISION OF
PANCREAS

species except the Vipera, the gut was a long straight tube which could only be sub-divided after opening the gut to expose the inner surface. The Vipera, however, had an extremely long oesophagus and stomach extending almost 2/3 of the length of the animal and the remainder of the intestine was found coiled laterally in small loops. In the Crocodilia the differences, as observed in the alligator, were probably associated with the size and feeding habits of the animals. The gut was looped inside the body cavity, but was relatively short compared to the size of the animal. A number of outstanding features were seen on the inner mucosal surface of the gut. The intestine had had three easily identifiable regions, the duodenum with diamond-shaped patterns of gut folds, the small intestine with a zig-zag pattern of folding and the lower intestine with plain longitudinal folds. Another feature of the alligator intestine was the extreme development of the muscle layers which were almost cartilaginous in nature. The precise difference in composition could not be fully investigated but in wax sections the appearance was totally different from any other muscle seen during the study (Figure 34).

The stomach was constructed as a bag, with the oesophageal and duodenal openings being next to one another. The main part of the bag was composed of the fundic area/.....

area with numerous acid secreting cells. The antral region was found just proximal to the duodenum and partly separated from the fundic region by a sphincter (see Diagram 9). The thickness of the mucosal layers did not alter but in the fundic 'bag' region the muscle coat was greatly increased in the area directly opposite the oesophageal and duodenal entrances (see Diagram 9).

Examination of haematoxylin and eosin stained sections of the duodenal region showed an exceptional organisation of the mucosa which was not observed in any other species. The gut folds appeared to occur in groups. The sub-mucous plexus was thus carried up into the folds (Figure 35). In the other regions of the intestine the general histological appearance was similar to that seen in other species. In contrast to the appearance of the duodenal region in the alligator, the snake gut mucosa was much simpler with long, thin folds which looked very similar to the villi as seen in mammals (Figure 36).

5.3.2. Testudinata

5.3.2.a. Immunostaining

i. Emys orbicularis

Pancreas. The pancreas was found attached to the duodenum, extending in a short ventral process and a longer dorsal process with the spleen attached to one end. The pancreas when dissected was relatively easy to divide into head, body and tail regions, the head being the/.....

Diagram 9 Alligator Stomach

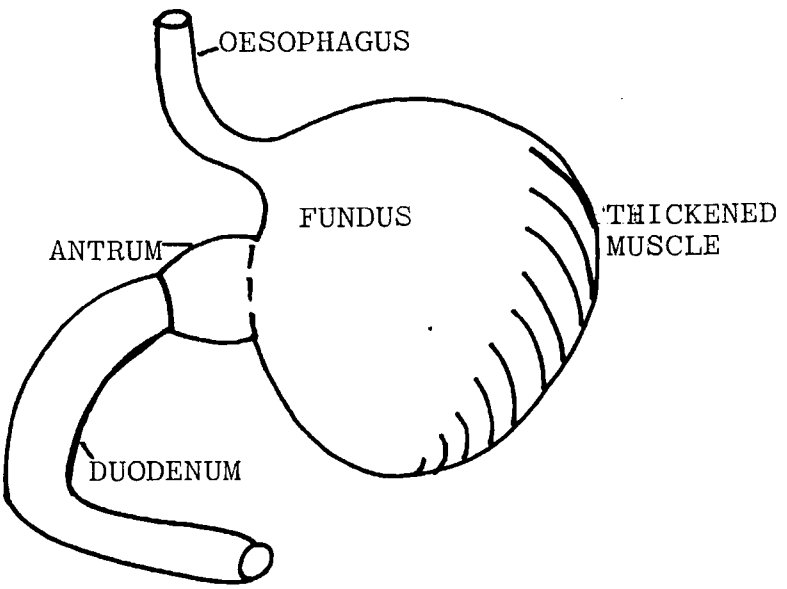


Figure 34

Alligator muscle in the duodenal circular muscle layer - note the jagged appearance of the individual fibres.

x 400

Figure 35

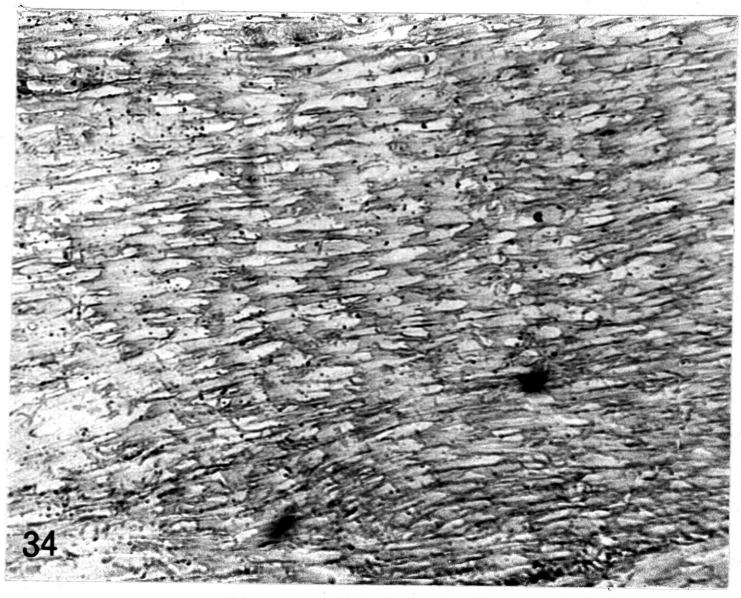
Alligator duodenum showing the complex gut folds - note that there is still no crypt development.

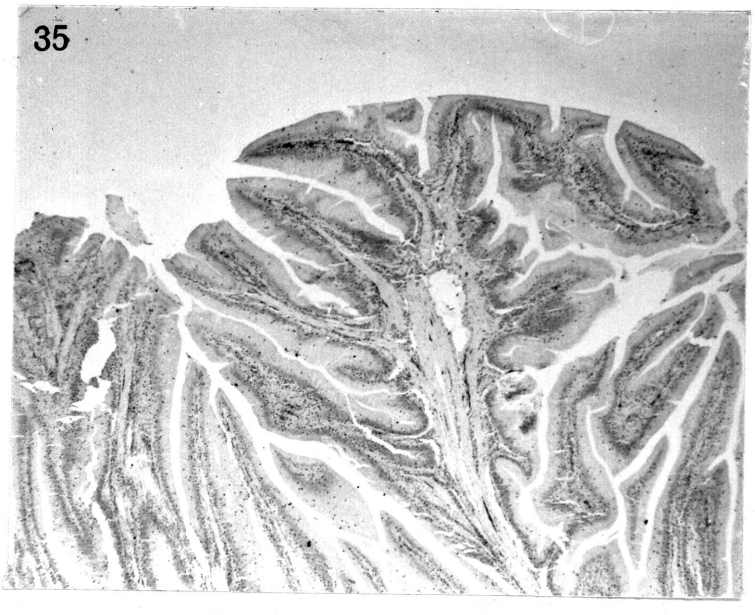
x 120

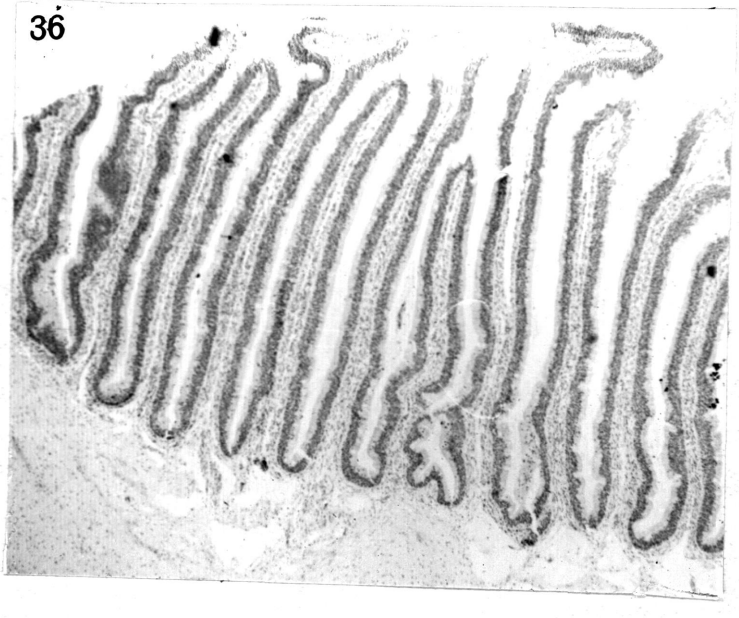
Figure 36

Nerodia rhombifera duodenum showing the long villus-like folds with no crypt region.

x 120


34


35


36

the ventral portion, the body central and the tail the part attached to the spleen.

All four pancreatic hormones were found in the pancreatic tissue. No aggregation of the endocrine tissue was found in any area including the tail region. The PP-immunoreactive cells appear to form a separate group to the glucagon-containing cells. The cells were only immunostained with the BPP antiserum and not by the APP and HPP.

In the pancreas VIP-immunoreactivity was found around both blood vessels and pancreatic ducts but not in either the endocrine or exocrine tissue. The other neuropeptides were all negative.

Table 22 Emys orbicularis European

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP	MOT1	MOT2			BN	VIP1	VIP2	VIP3	SP
Panc.H	+	+	+	+		+											+	+	+	
Panc.B	+	+	+	+		+											+	+	+	
Panc.T	+	+	+	+		+											+	+	+	
Fundus		+		+				X	+	+	+						+	+	+	
Antrum		+		+			+	X	+	+	+						+	+	+	
Duod.		+ +		+			+	+				+	+				+	+	+	+
Jej.		+		+				+			+	+	+			+	+	+	+	
Ileum		+		+				+			+					+	+	+	+	
Colon		+		+				+									+	+	+	

Stomach and Intestine. Non-specific neurotensin-immunostained cells were detected in the stomach although specifically stained cells were seen in the lower intestine. In the stomach there was a double staining of a single cell population by the antisera to substance P and bombesin, the results of the absorption controls were identical to those in the amphibia (Figure 37). Bombesin and substance P-immunostained nerve fibres were also found, substance P-positive fibres in the duodenum and ileum with the bombesin-positive fibres confined to a separate group of fibres in the ileum.. The duodenum villus-like structures were well developed with scattered glucagon-immunoreactive cells found along their length (Figure 38). Of the remaining peptides, motilin-like immunoreactivity was detected with both the N- and C-terminal antisera (Figure 39) but GIP, secretin and CCK were undetectable.

ii. Pseudemys scripta

Table 23 Pseudemys scripta North American

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP					VIP1	VIP2	VIP3
Panc.H	+	+	+	+	+	+										+	+	+
Panc.B	+	+	+	+	+	+										+	+	+
Panc.T	+	+	+	+	+	+										+	+	+
Fundus		+		+					+	+						+	+	+
Antrum		+		+					+	+						+	+	+
Duod.				+												+	+	+
Jej.				+												+	+	+
Ileum				+												+	+	+
Colon				+												+	+	+

Figure 37

Bombesin-immunoreactive cells in the fundic mucosa.

x 250

Figure 38

Glucagon-immunoreactive cells, as detected with the N-terminal antiserum, in the duodenal mucosa.

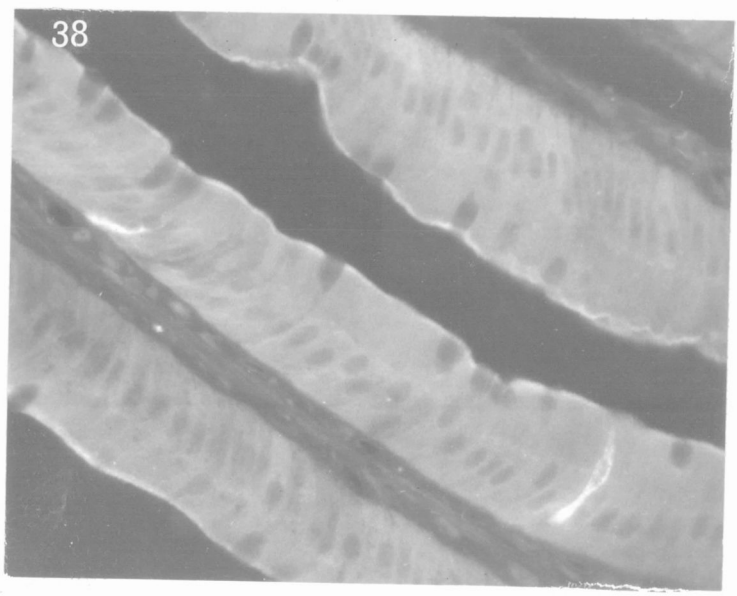
x 400

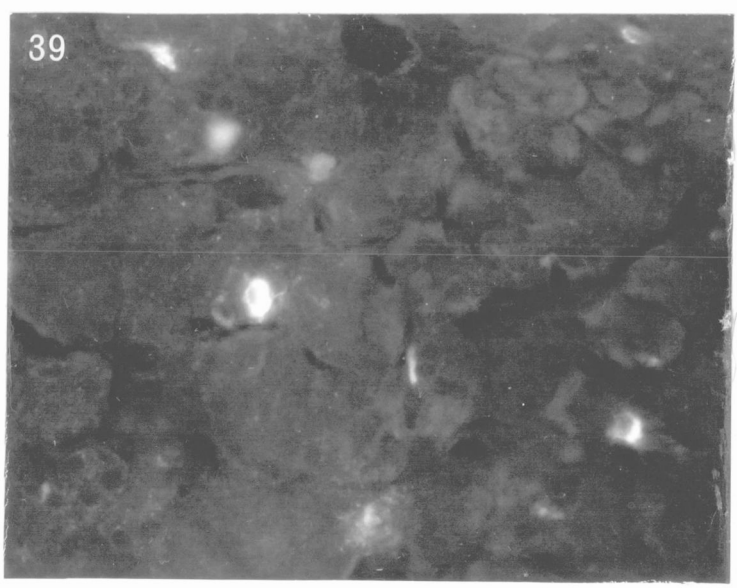
Figure 39

Motilin-immunoreactive cells in the duodenal mucosa detected with the N- and C-terminal antisera.

x 400


37


38


39

Pancreas. The pancreas was found in an identical location to that of Emys orbicularis and was sub-divided in the same way. Again there was no increase in the amount of endocrine tissue seen in the tail region. The structure of the islets differed from that found in the mammals and advanced amphibia. No central core of insulin cells surrounded by an outer peripheral aggregation of other cell types was demonstrable. The islet-like structure in these animals resembled the ribbon-like structures seen in the Bufonidae, in which all four cell types apparently occur with little or no further organisation (Figure 40). An interesting finding was that the insulin- and somatostatin-containing cells were much more numerous than the glucagon- and PP-containing cells. Also interesting was the finding that the PP cells reacted with antisera to both BPP and APP. VIP-immunoreactive fibres and cell bodies were detected in the pancreas but only in association with blood vessels and the muscle surrounding the pancreatic ducts.

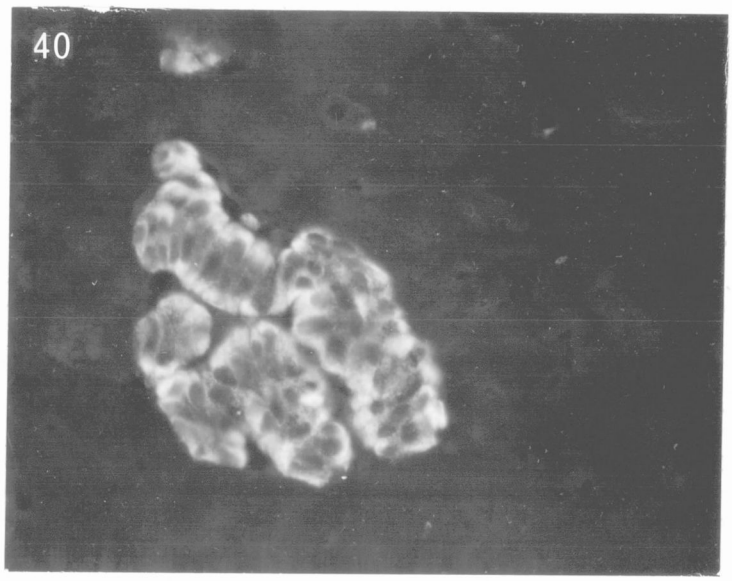
Stomach and Intestine. Endocrine cell staining in these areas was relatively poor. In the stomach cells containing somatostatin, glucagon and bombesin were identified as well as nerve fibres immunostained by the VIP antiserum. The remainder of the antisera gave negative/.....

Pseudemys scripta

Figure 40

Insulin-containing cells in a typical islet-like structure composed of ribbons of mostly rectangular cells.

x 250


40

negative results. In the intestine somatostatin cells and VIP-immunoreactive nerves were present.

iii. Chelydra serpentia

Table 24 Chelydra serpentia North American

	INS	GLU	PGLL	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP	SEC					VIP1	VIP2	VIP3	
Panc.H	+	+	+	+	+	+														
Panc.B	+	+	+	+	+	+														
Panc.T	+	+	+	+	+/−	+/−														
Fundus		+	+	+					+	+	+						+	+	+/−	
Antrum		+	+	+			+	X	+	+	+						+	+	+	
Duod.		+		+				+				+					+	+	+	
Jej.																				
Ileum																				
Colon																				

Pancreas. The location of the pancreas was identical to the other two species and there was no aggregation of the endocrine tissue in the tail region. The pancreas in this species showed most clearly that the endocrine tissue in the Testudinata does not occur in discrete mammalian-type islets. The endocrine cells were found in long ribbons, often associated with both exocrine tissue and the pancreatic ducts (Figures 41 and 42). No infiltration of the splenic/.....

Figure 41

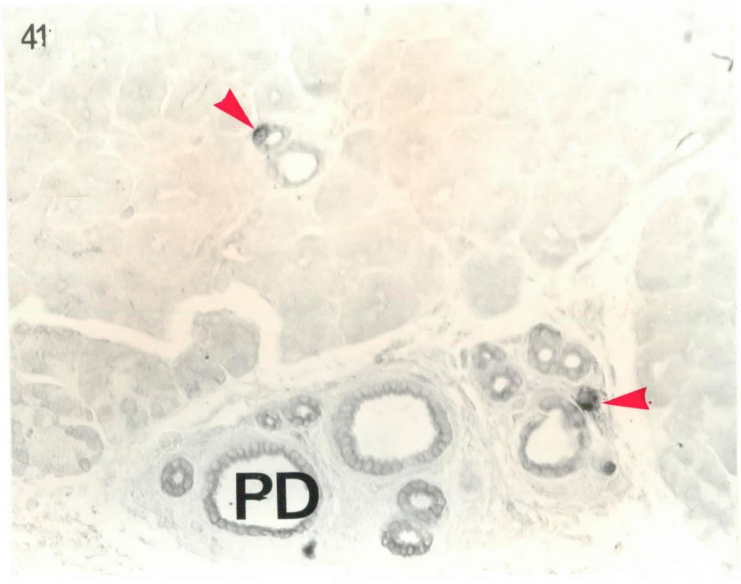
Somatostatin-containing cells (arrows) lining the pancreatic ducts.

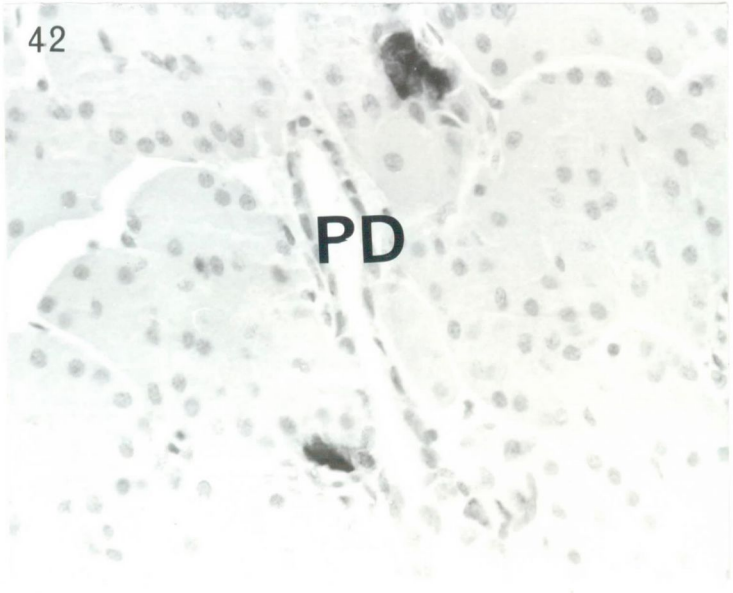
x 120

Figure 42

Insulin-containing cells beside a pancreatic duct.

x 230


41
PD


42
PD

splenic tissue by endocrine cells was seen in any specimen examined.

The PP-immunoreactive cells were immunostained with both the BPP and APP antisera. Of interes was the finding that the distribution of the endocrine cells varied with the area of the pancreas. Most striking were the PP-immunoreactive cells, which were commonest in the head region and almost completely lacking in the splenic lobe (Figure 43).

VIP-immunoreactive fibres were not detected within the pancreatic tissue.

Stomach and Intestine. In the stomach, the fundus contained numerous substance P/bombesin - immunoreactive cells, as well as somatostatin and glucagon cells. In the antral region in addition to the above, numerous gastrin/CCK reactive cells and also some displaying the non-specific neurotensin-immunoreactivity were detected. VIP-immunoreactivity was present in numerous nerve fibres and cell bodies of the underlying muscularis mucosae, the cell bodies being mostly located in the sub-mucosal plexus (Figure 44).

In the intestine, secretin-containing cells were detected. These cells were not stained by the glucagon, GIP or VIP anti-sera, nor was the immunostaining affected by the addition of any peptide other than secretin (Figure 45)./.....

Figure 43

Single PP-containing cell (arrow) in the tail region of the pancreas.

x 200

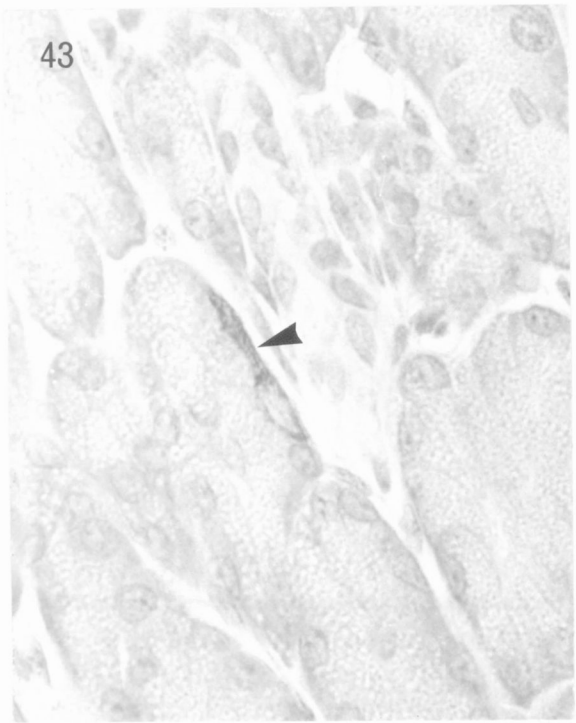

43

Figure 44

VIP-immunoreactive fibres in the muscle coat of the stomach - note the concentration of fibres in the sub-mucous plexus (SMP).

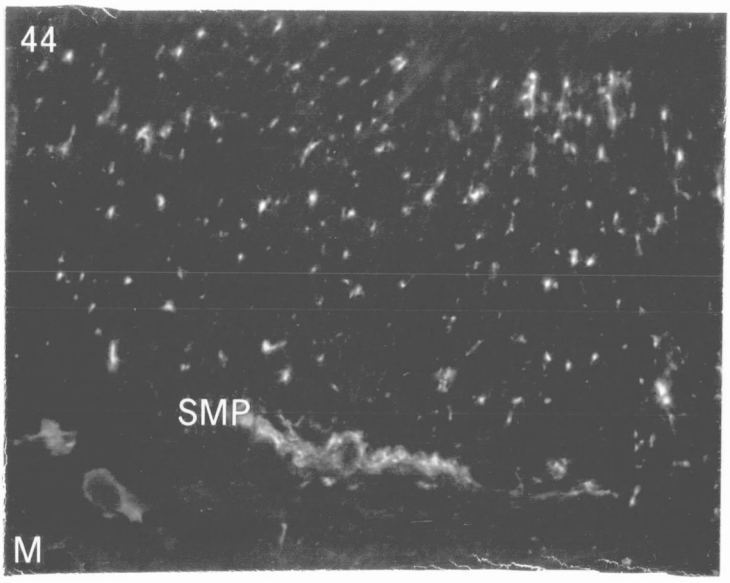
S = Serosa M = Mucosa

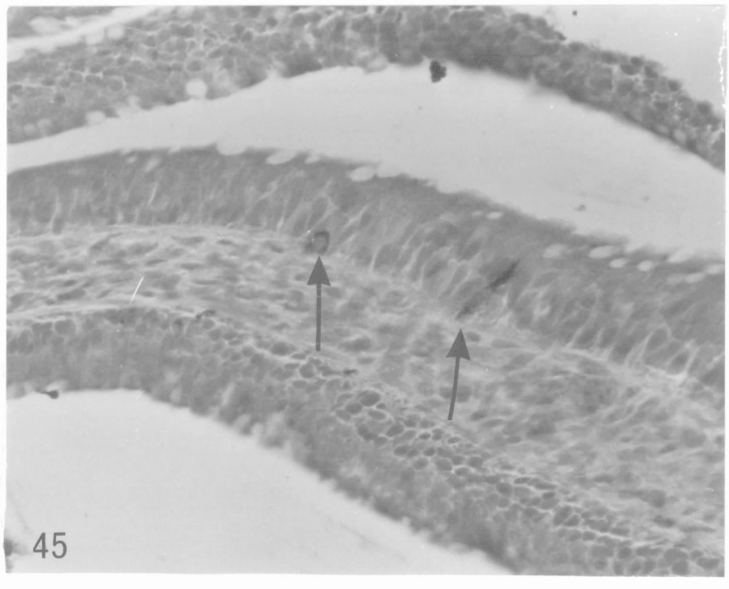
x 120

Figure 45

Secretin-containing cells (arrows) in the duodenum mucosa.

x 400


44
SMP
M


45

(Figure 45). The staining of the intestinal neurotesin-immunoreative cells was specific .

5.3.2.b. Summary - Testudinata

The pancreas can be easily subdivided into head, body and tail regions as defined in mammals. Although it appeared that the distribution of the constituent endocrine cells varies with the region, the endocrine islets were not concentrated in any particular region. VIP-immunoreactive nerves were detectable in all regions of the gut as well as the pancreas, although they were confined to the exocrine tissue and pancreatic ducts. In this family secretin and motilin were detected within the intestinal mucosa. CCK and GIP were still undetectable in any region.

5.3.3. Squamata

5.3.3.a. Lacertilia

i. Hemidactylus brookii

Pancreas. The pancreas was identified as a small discrete organ attached to the duodenum with no obvious attachment to the spleen. It was therefore taken as a whole and not subdivide into the three regions as in the Testudines. The endocrine tissue in small clusters and as single cells was evenly distributed throughout the pancreas.

All four pancreatic hormones were present, thoug insulin or somatostatin were the predominant cell types. The glucagon cells, although relatively/.....

relatively few in number, were distinct from the PP cells. The PP cells were detected by both APP and BPP antisera.

VIP was found in pancreatic nerve fibres actually running within the exocrine tissue (Figure 46). Enkephalin and substance P were both negative.

Stomach and Intestine. In the fundic region, cells containing neurotensin- and somatostatin-like immunoreactivity were detected, those detected with the somatostatin antiserum being specific while those by the neurotensin antiserum were non-specific. VIP-containing nerve fibres were also detected.

Table 25 Hemidactylus brookii European

	INS	GLU	PGLU	SOM	BPP	APP	G/CCK	NT	BN1	BN2	SP					SP	VIP1	VIP2	VIP3	BN
Panc.	+	+	+	+	+	+											+	+	+	x
Fundus				+				x									+	+	+	
Antrum				+			+	x	+	+							+	+	+	x
Duod.				+				+	+	+							+	+	+	
Jej.				+				+									+	+	+	
Ileum				+				+									+	+	+	
Colon				+	+		+									+	+	+	+	

In the antral region bombesin, gastrin/CCK and somatostatin cells were detected (Figure 47) as were the non-specific neurotensin cells. Numerous VIP-immunoreactive fibres and cell bodies were present in this region.

In the upper intestine specific immunostaining for bombesin, neurotensin and somatostatin cells as well as VIP nerves and cell bodies was observed (Figure 48).

No GIP, secretin, motilin or CCK immunoreactivity was detectable in this species.

In the large intestine, gastrin and PP immunoreactive cells were detected as well as numerous substance P and VIP nerve fibres (Figure 49).

ii. Agama agama

Table 26

	Agama agama										European							
	INS	GLU	PGLU	SOM	BPP	APP	G/CCK	NT	BN1	BN2	SP	MOT1	GIP			VIP1	VIP2	VIP3	SP
Panc.	+	+	+	+	+	+	X					X				+	+	+	
Fundus		+		+				X	+	+						+	+	+	
Antrum				+			+	X	+	+						+	+	+	
Duod.				+			+	+	+	+			X			+	+	+	
Jej.				+			+	+								+	+	+	
Ileum		+		+				+								+	+	+	+
Colon				+				+								+	+	+	+

Figure 46

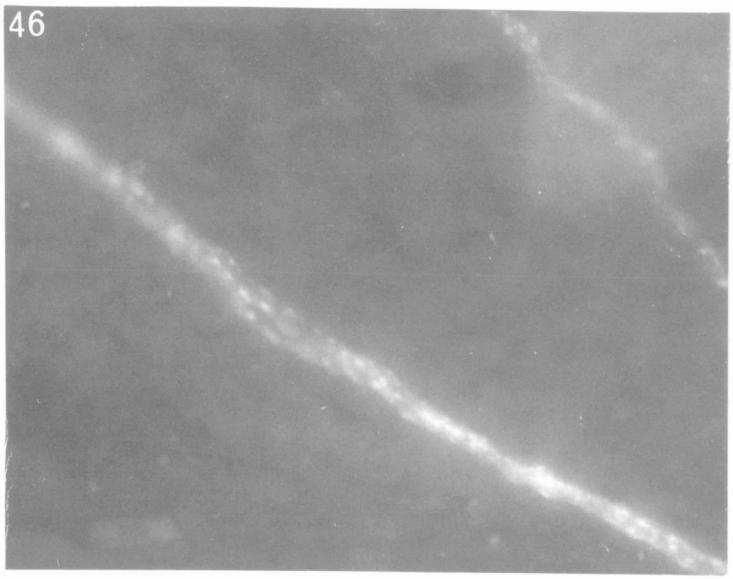
VIP-immunoreactive fibre in a 10 micron section of pancreas.

x 400

Figure 47

Somatostatin-containing cells in the antral mucosa.

x 120


46

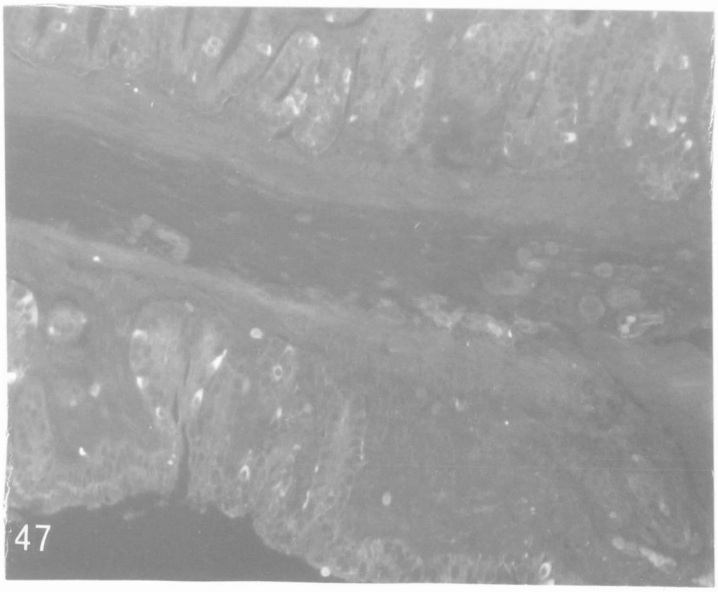

47

Figure 48

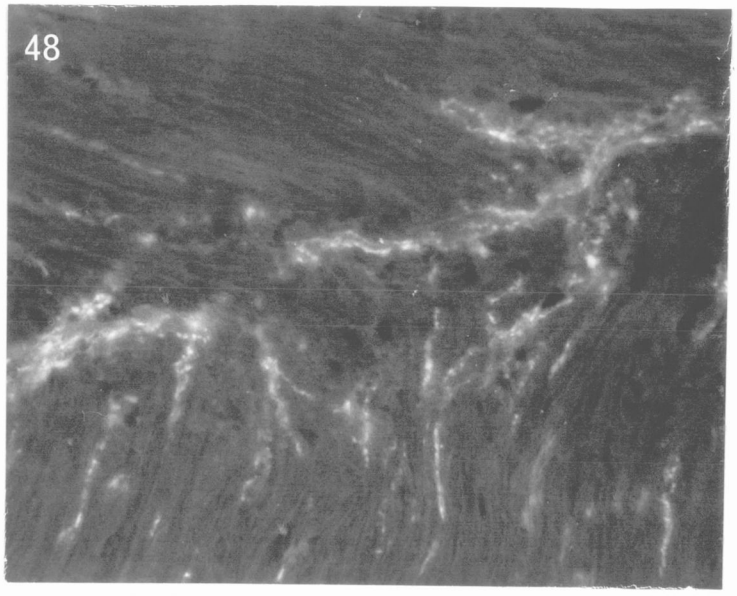
VIP-immunoreactive nerve fibres in the duodenal circular muscle.

x 400

Figure 49

PP-immunoreactive cells in the colonic mucosa (arrow).

x 400


48


49

Pancreas. The pancreas was taken as one piece, as there was no obvious means of separating the various regions. The endocrine tissue was evenly distributed throughout the pancreas. The four pancreatic hormones were present in ribbon-like islets (Figure 50). VIP-containing nerves were found mostly around blood vessels with occasional fibres in the exocrine tissue.

Stomach and Intestine. In the stomach the fundic region contained somatostatin (Figure 51), bombesin and glucagon (Figure 52) cells, the neurotensin staining was non-specific. In the antral region additional gastrin/CCK cells were detected.

VIP-immunoreactivenerve fibres and cell bodies were found in the nerve plexuses and muscularis mucosae throughout the entire length of the gut, including the stomach.

In the small intestine, cell immunoreactive with bombesin, gastrin/CCK, glucagon, neurotensin (Figure 53), BPP and somatostatin (Figure 54) were detected. The antisera to motilin, secretin and CCK all gave negative results.

In the lower intestine (colon) very few endocrine cells immunostained by the somatostatin neurotensin antisera were detected.

Substance P-immunoreactive nerve fibres were also present alongside the VIP-containing fibres (Figure 55).

Agama agama

Figure 50

a - Glucagon-containing cells in the pancreas stained by the C-terminal antiserum.

x 120

b - Somatostatin-containing cells occurring either in small groups or as single cells.

x 120

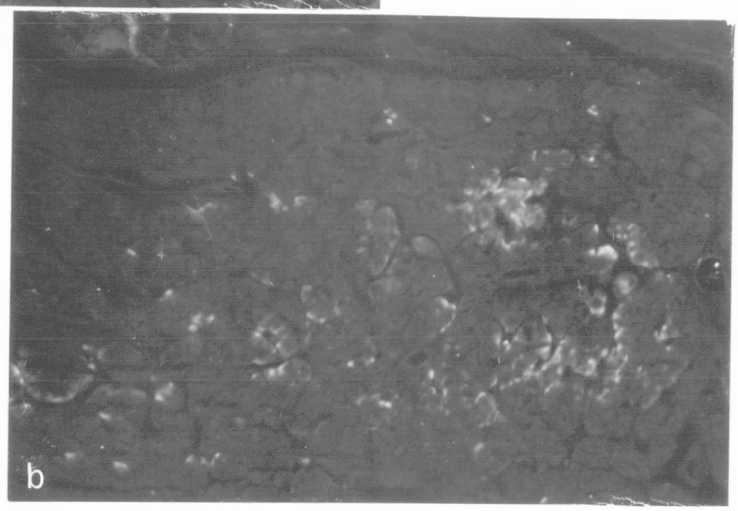
c - Insulin-containing cells seen in small clusters and in ribbon-like structures.

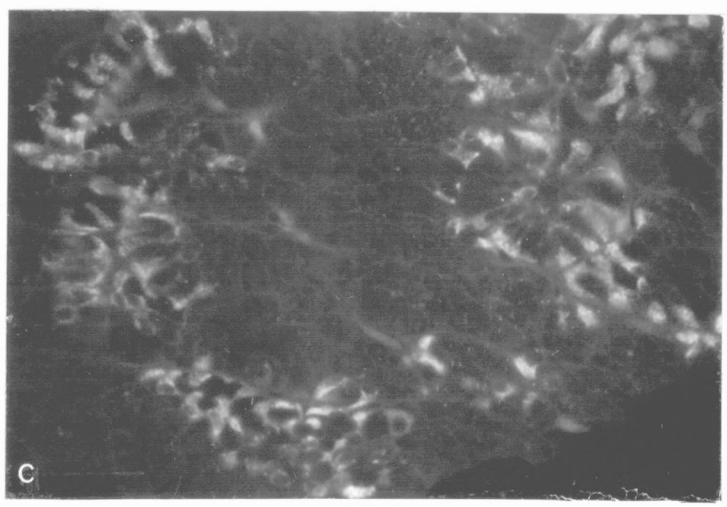
x 400

d - PP-containing cells stained by the BPP antiserum.

x 120


50a


b


c

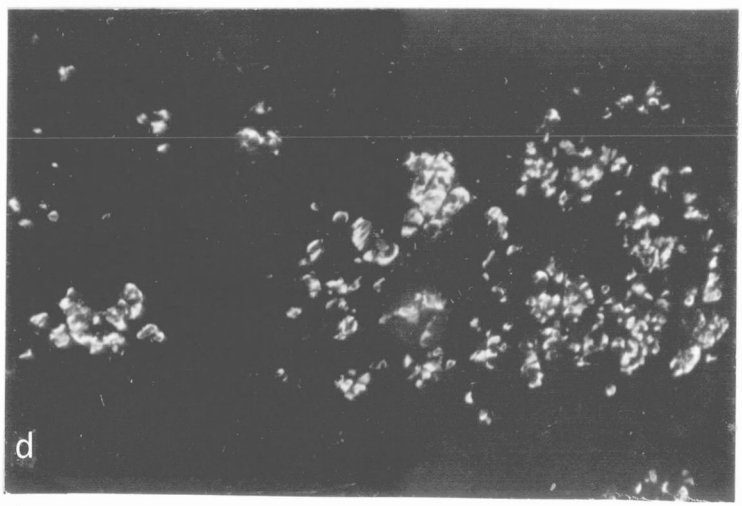

d

Figure 51

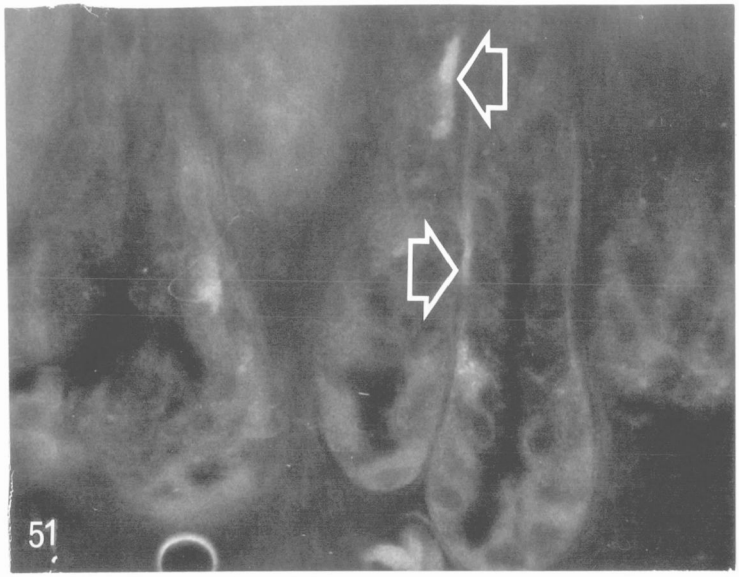
Somatostatin-containing cells in the fundic mucosa - note the cytoplasmic extensions (arrows).

x 250

Figure 52

A glucagon-immunoreactive cell in the fundic mucosa with a well developed cell process.

x 400


51

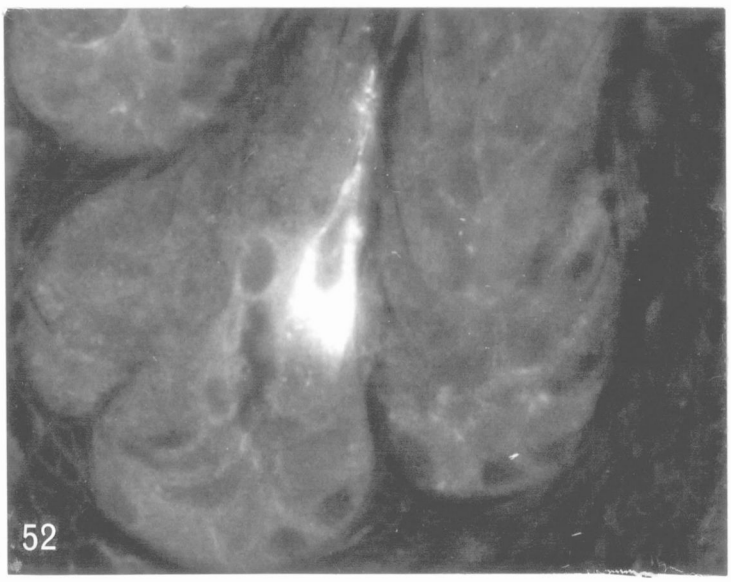

52

Figure 53

A neurotensin-immunoreactive cell in the duodenal mucosa.

x 250

Figure 54

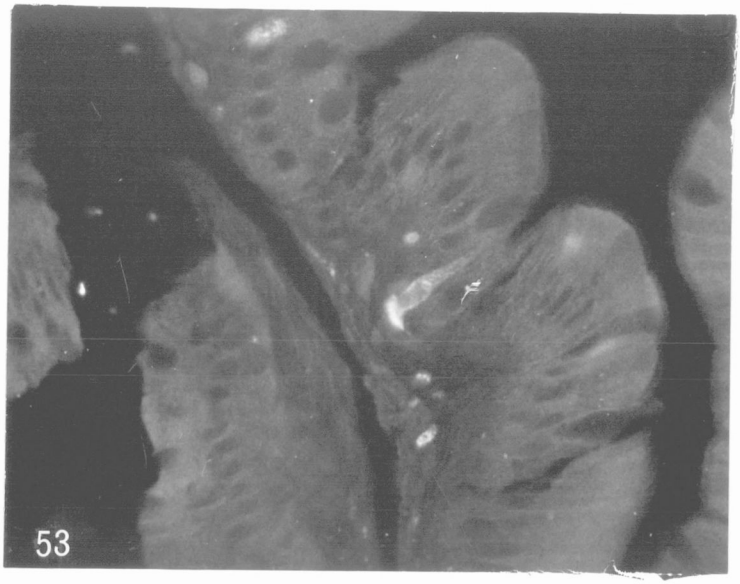
A somatostatin-immunoreactive cell in the jejunal mucosa - note the extension at the basal lamina.

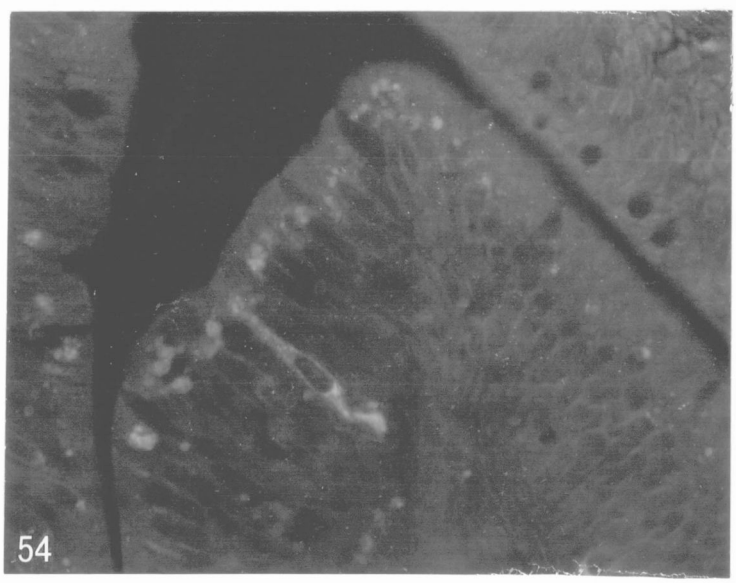
x 250

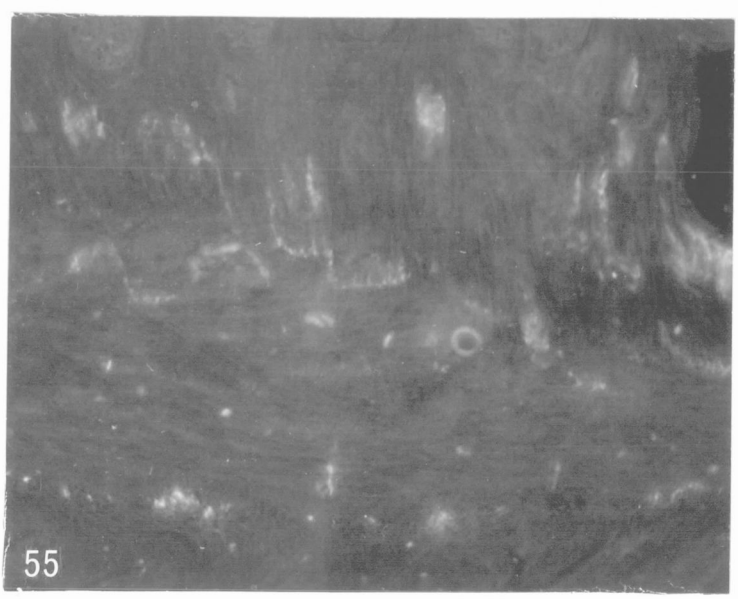
Figure 55

Substance P-immunoreactive fibres in the circular muscle layer of the colon.

x 120


53


54


55

5.3.3.b. Summary - Lacertilia

The pancreas was undivided and the endocrine tissue was evenly distributed throughout. There did not appear to be any difference in the distribution of the endocrine cells. The majority of the cells were found in the ribbon-like islets although some scattered PP and somatostatin cells were also found.

Of the neuropeptides both VIP and substance P were found in intestinal nerves with VIP-stained nerves being by far the most numerous.

The neurotensin staining in the stomach was again non-specific although the intestinal staining was specific.

GIP, motilin, secretin, CCK, enkephalin and endorphin were all undetectable.

5.3.3. c. Serpentes

In all the snakes studied the pancreas was found as a discrete lobular mass attached to the duodenum with the small round spleen adhering to one end. It was impossible to distinguish different regions therefore the whole pancreas was divided into two and each half embedded (see Diagram 8).

In this way the region surrounding the spleen was termed the tail region, the central zone was taken as equivalent to the body and the part furthest from the spleen as the head region.

i. Boidae - Python recticularis

Table 27 Python recticularis European

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP		GTP	GCK			VIP1	VIP2	VIP3	NSE
Panc.H	+	+	+	+													+	+	+	+
Panc.B	+	+	+	+													+	+	+	+
Panc.T	+	+	+	+													+	+	+	+
Fundus				+																
Antrum				+			+													
Duod.		+		+			+	+					+	+						
Jej.				+			+													
Ileum				+			+													+
Colon		+																		+

Pancreas. The endocrine tissue was found in extremely large islets in all regions of the pancreas. The islets consisted of a central region of glucagon-immunoreactive cells with peripheral insulin and somatostatin cells (Figure 56). No PP cells were found with the BPP, APP or HPP antisera.

The VIP innervation of the pancreas was striking, the VIP-immunoreactive nerves being concentrated in the endocrine islets as well as infiltrating the exocrine tissue (Figure 57).

Neurone-specific enolase (NSE) was also positive in the pancreatic tissue (Figure 58).

Stomach and Intestine. In the stomach there was/.....

Figure 56

5 micron section of the pancreas stained with the insulin antiserum showing the positive cells mainly at the periphery (large arrows). The cells are stained with 4-chloronapthol which when photographed under Nomarski optics gives a dark green colour. The brown reaction product seen at the edges of the cells are the VIP-containing nerve terminals demonstrated by the diaminobenzidene reaction (small arrows).

x 400

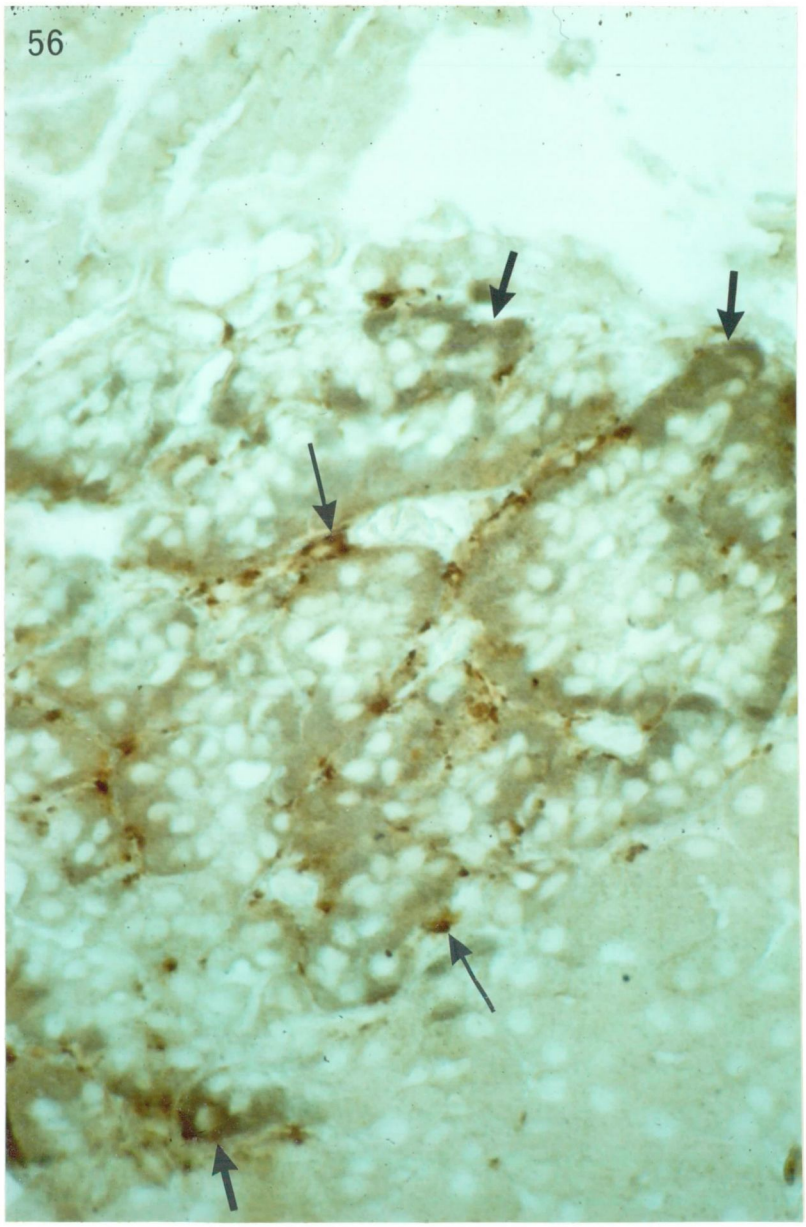
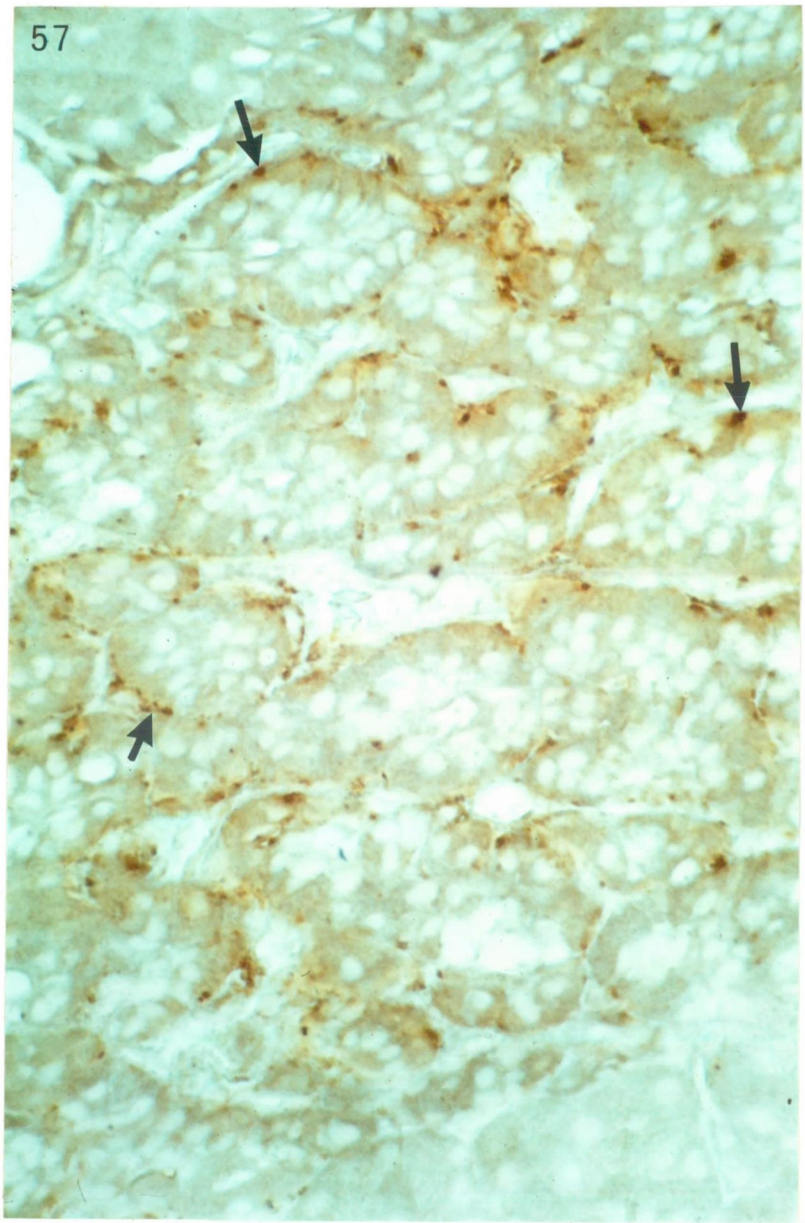

56

Figure 57

5 micron section of a pancreatic islet stained by the pancreatic glucagon antiserum (localised by the 4-chloronapthol reaction) showing that the majority of the cells are positive. The section has also been incubated with C-terminal VIP antiserum and localised with diaminobenzidene. The VIP-ergic terminals were found in close association with the endocrine cells shown in this example (arrows).

x 400


57

was a complete absence of immunostained innervation. Somatostatin and gastrin/CCK were the only endocrine cell types detected. The lack of immunoreactive nerves was also seen in the intestine where VIP-immunoreactive fibres were completely absent. However in the upper small intestine CCK and GIP immunoreactive cells were specifically detected (Figures 59 and 60).

ii. Colubridae - Thamnophis sirtalis

Table 28 Thamnophis sirtalis European

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP					VIP1	VIP2	VIP3	SP
Panc.H																			
Panc.B																			
Panc.T	+	+	+	+															
Fundus				+				X	+	+									
Antrum		+		+			+	X	+	+									+
Duod.				+			+	+											+
Jej.				+			+	+											+
Ileum		+		+			+	+											+
Colon				+				+											+

Pancreas. The endocrine tissue was found as large islets confined to the tail region consisting of a central core of glucagon-immunoreactive cells with peripheral insulin- and/.....

Figure 58

NSE-immunostained fibres around a pancreatic blood vessel.

x 400

Figure 59

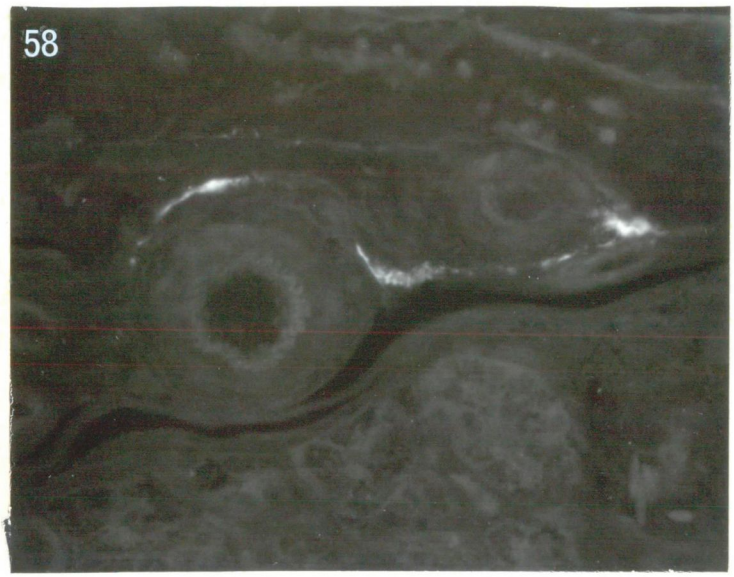
CCK-containing cells in the duodenum (arrows) stained by the mid-portion (9-20) specific antiserum.

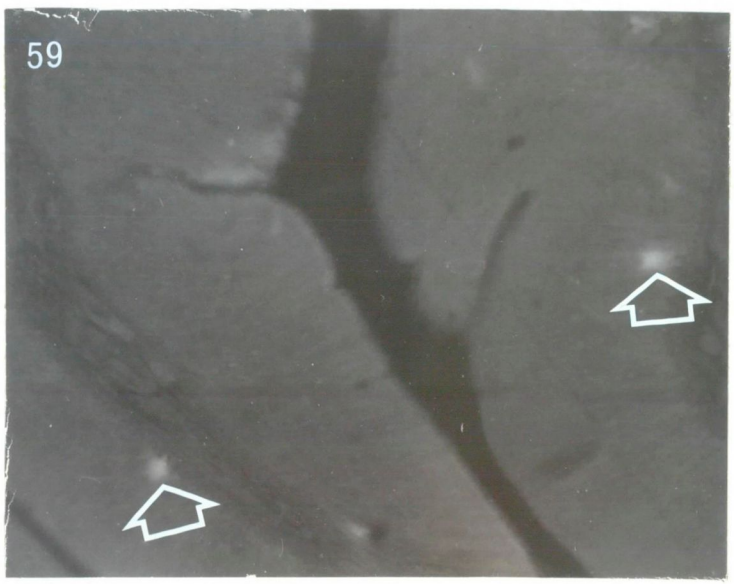
x 250

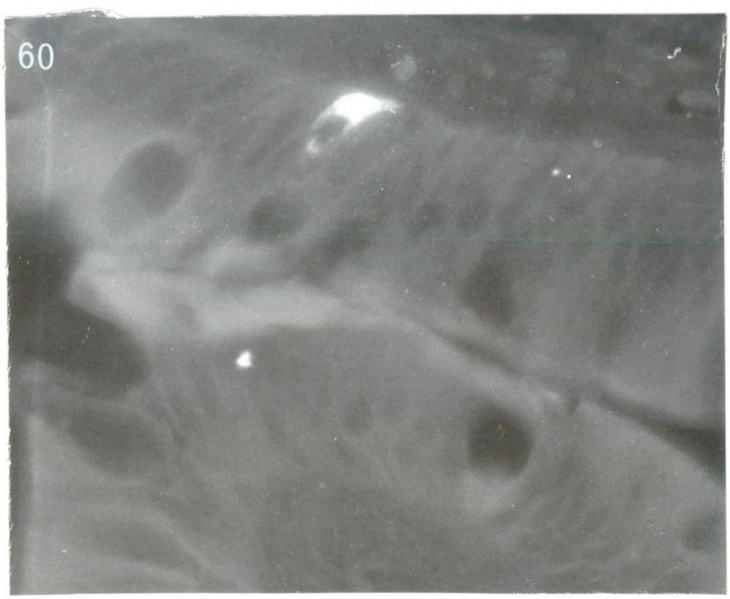
Figure 60

GIP-immunoreactive cell in the duodenum mucosa.

x 400


58


59


60

and somatostatin-containing cells. In this species there was a total lack of VIP-immunoreactivity both in the pancreas and the intestine. This was consistent no matter which antiserum was used (C-terminal, N-terminal or whole molecule specific).

Stomach and Intestine. The fundus showed immunoreactive bombesin and somatostatin cells and also non-specific neurotensin cells. Cells containing bombesin-, gastrin/CCK-, glucagon- and somatostatin-like immunoreactivity were detected in the antral mucosa. In addition numerous substance P-immunoreactive nerve fibres were seen in the muscularis mucosae (Figure 61).

In the intestine, endocrine cells were present reacting with neurotensin-, somatostatin- and gastrin/CCK antisera, the most numerous being the gastrin/CCK cells (Figure 62).

In the lower intestine (colon) only a very few endocrine cells were detected with the neurotensin and somatostatin antisera (Figure 63) Throughout the length of the intestine numerous substance P-immunoreactive nerve fibres were detected in the muscle coat and cell bodies were present in the myenteric plexus (Figure 64).

iii. Natrix natrix/.....

Figure 61

Substance P-immunoreactive fibres in the myenteric plexus of the antrum.

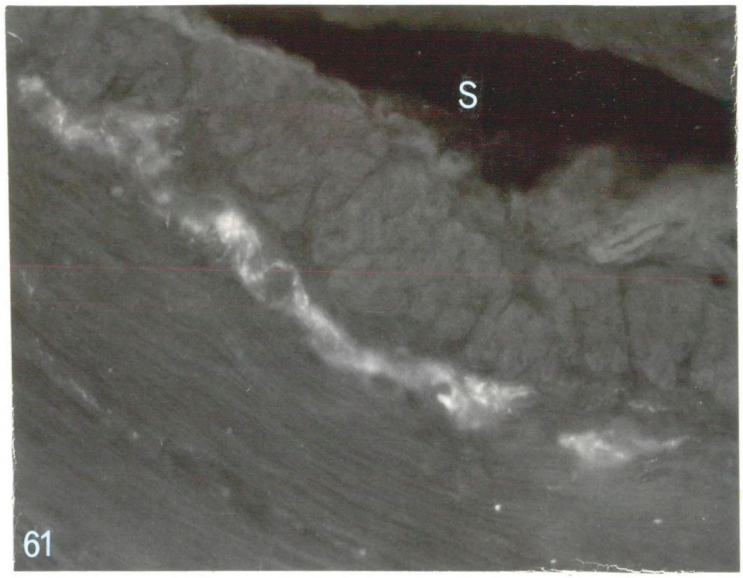
S = Serosa

x 250

Figure 62

Gastrin/CCK - immunoreactive cells in the duodenal mucosa.

x 250


S
61

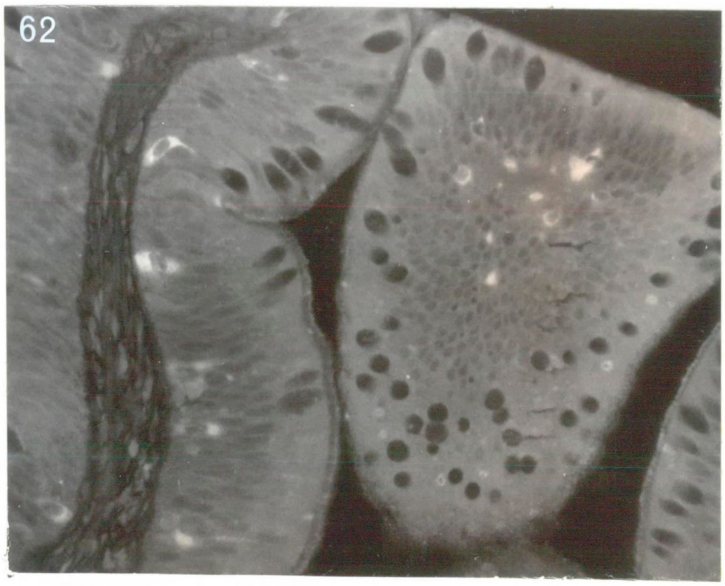

62

Figure 63

a - Somatostatin-immunoreactive cells in the colonic mucosa (arrows).

x 400

b - A neurotensin-immunoreactive cell in the colonic mucosa.

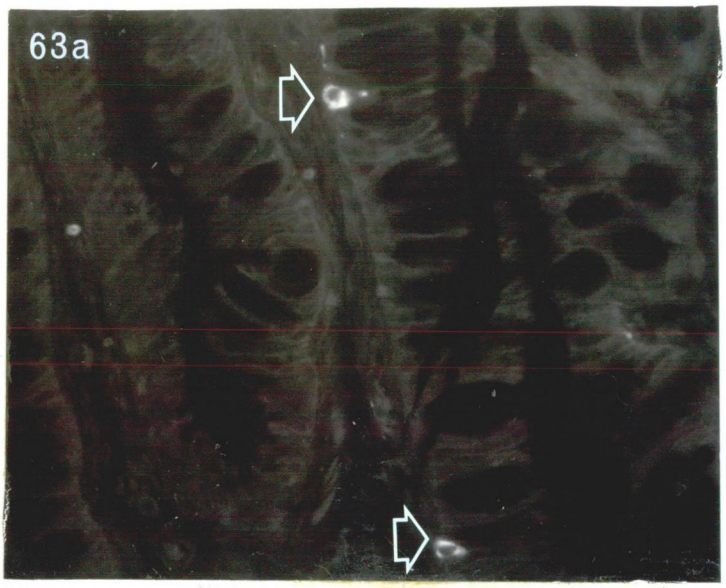
x 400

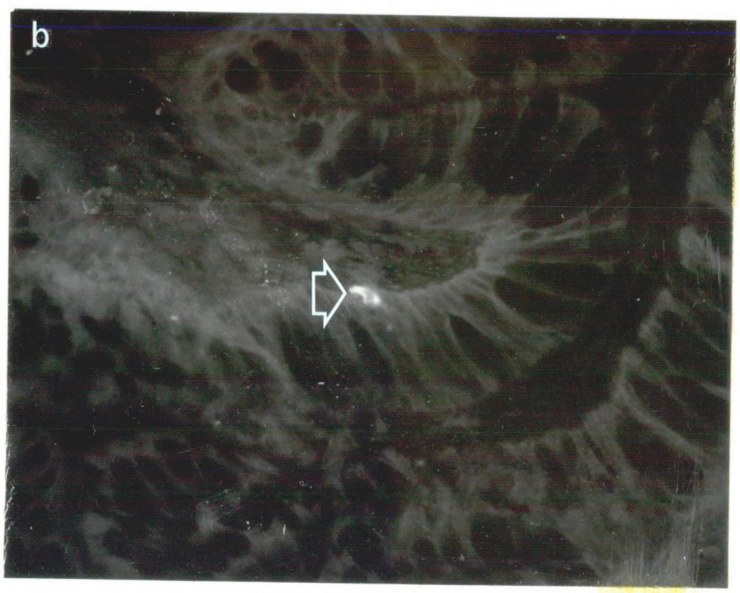
Figure 64

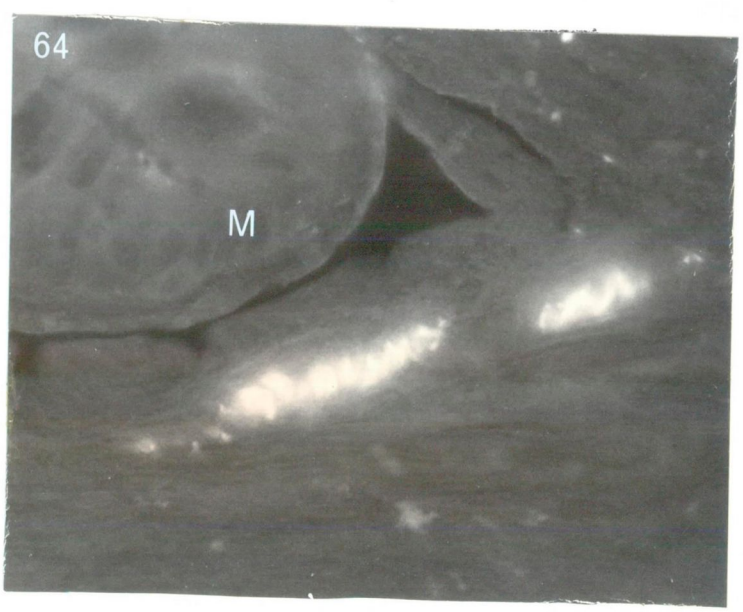
Substance P-immunoreactive fibres in the sub-mucous plexus.

M = Mucosa

x 400


63a


b


64
M

iii. Natrix natrix

Table 29 Natrix natrix European

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP					VIP1	VIP2	VIP3	NSE
Panc.H																			
Panc.B																			
Panc.T	+	+	+	+												+	+	+	
Fundus				+															+
Antrum				+															
Duod.				+			+												+
Jej.				+			+												+
Ileum				+			+	+											+
Colon				+															+

Pancreas. The endocrine tissue was confined to the tail region in several large islets. The vast majority of the cells were glucagon-containing with fewer insulin- and somatostatin-containing cells. Although the staining was not as pronounced as in the python there was an indication that there were VIP-containing nerve fibres around the outside of the islets but little or no reactivity was seen in the exocrine tissue.

Stomach and Intestine. Again there was a total lack/.....

lack of VIP-immunoreactivity in all areas. The only positive immunostaining of nerve fibres was seen with the NSE antiserum, this was seen in all areas of the intestine and the stomach but not in the pancreas.

There were relatively few endocrine cells seen in the stomach and intestine. Those detected contained either somatostatin or gastrin/CCK with a few glucagon immunostained cells occurring in the lower gut.

iv. Nerodia rhombifera

Table 30 Nerodia rhombifera North American

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP					VIP1	VIP2	VIP3	
Panc.H																			
Panc.B	+	+	+	+															
Panc.T	+ +	+ +	+ +	+ +															
Fundus		+		+					+	+									
Antrum		+		+			+	+											
Duod.				+			+	+											
Jej.				+			+	+											
Ileum				+				+											
Colon																			

Pancreas. The endocrine tissue was found concentrated in the tail region, with a few cells occurring in the body. The islets seen in/.....

in the tail region were composed mainly of glucagon-containing cells with some insulin- and somatostatin-containing cells (Figure 65). There was no indication of the present of endocrine tissue in the spleen although the large islets were very close to the limits of the pancreas adjoining the spleen (see Figure 66). No evidence for PP cells could be found with any of the PP antisera available, nor was there any indication of the presence of VIP-immunoreactive nerves.

Stomach and Intestine. In the stomach the usual cell types were present such as bombesin and somatostatin. No evidence of a peptidergic component of the innervation could be seen with either the VIP or substance P antisera. Gastrin/CCK containing cells were the most abundant cell type. A few somatostatin, neurotensin and glucagon cells were also detected. In the lower intestine (colon) no endocrine cells were detected, the whole of the mucosa being composed of mucin producing cells.

v. Viperidae - Agkistrodon piscivora

Figure 65

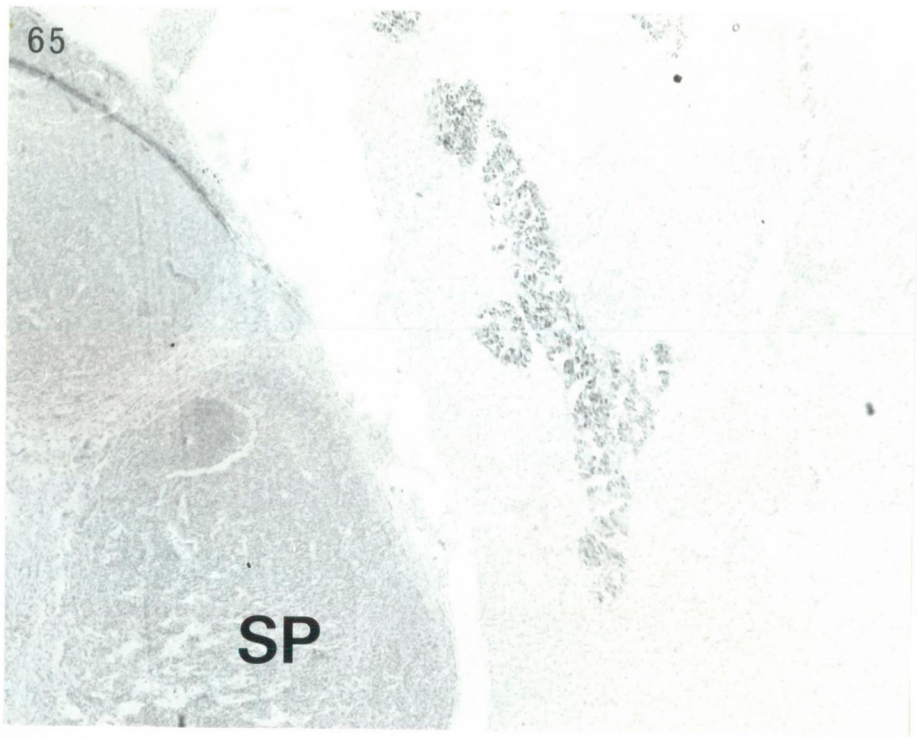
Glucagon-containing cells in a large islet close to the spleen (SP) - note that the glucagon A cells form the major component of the islet.

x 120

Figure 66

A serial section showing the somatostatin-containing cells in the same islet. The cells are found throughout the structure but are not as numerous as the glucagon-containing cells.

x 120


65
SP

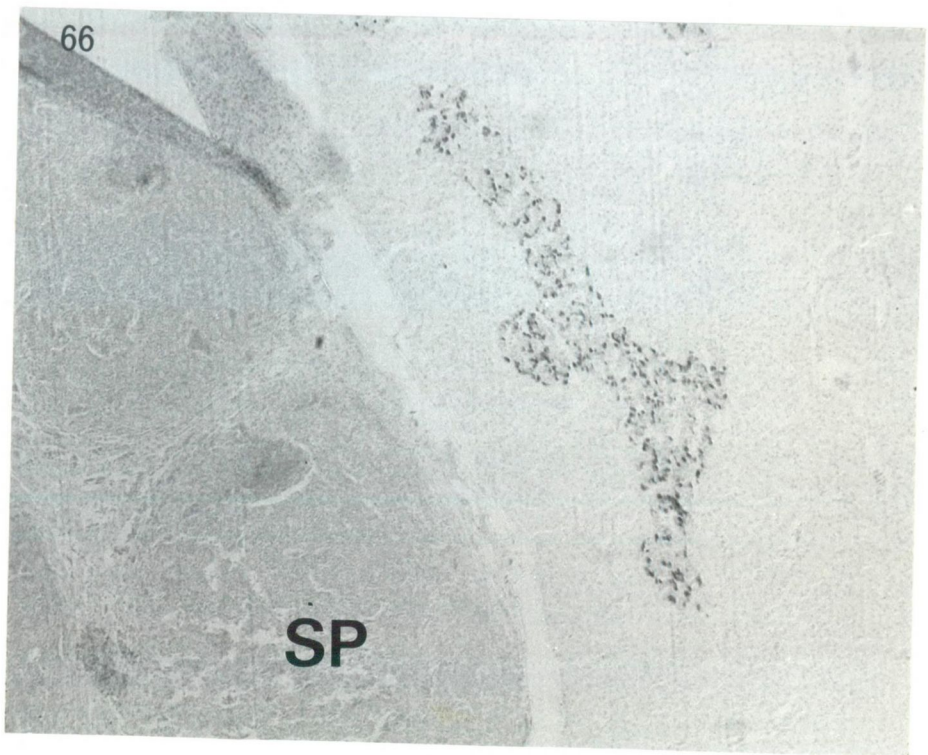

66
SP

Table 31 — Agkistrodon piscivora — North America

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP					VIP1	VIP2	VIP3
Panc.H																		
Panc.B																		
Panc.T	+	+	+	+												+	+	+
Fundus		+	+	+												+	+	+
Antrum		+	+	+				X								+	+	+
Duod.				+			+	+								+	+	+
Jej.				+			+	+								+	+	+
Ileum				+												+	+	+
Colon				+												+	+	+

Pancreas. The pancreatic islets were concentrated at the splenic pole and consisted of glucagon-, insulin- and somatostatin-containing cells. There were no PP cells demonstrable with the BPP, APP or HPP antisera. VIP-immunoreactive fibres were seen in both the endocrine and exocrine tissue.

Stomach and Intestine. Somatostatin-containing cells and VIP-containing nerves were detected in the stomach (Figure 67). The upper small intestine contained numerous gastrin/CCK cells as well as a few somatostatin, neurotensin and glucagon cells and several VIP-immunoreactive fibres (Figure 68).

The colonic region was again devoid of any endocrine cells although a few VIP-immunoreactive nerve fibres were observed.

vi. Crotalus atrox/.....

Figure 67

A somatostatin-containing cell (arrow) in the duodenal mucosa.

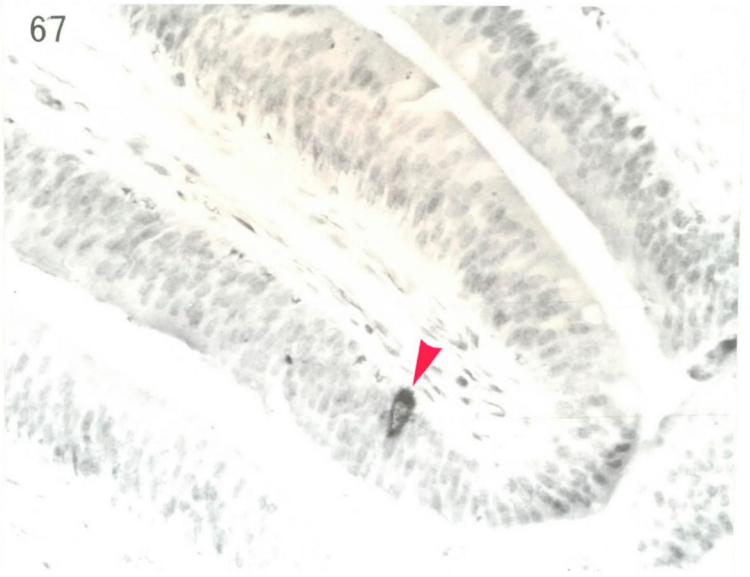
x 400

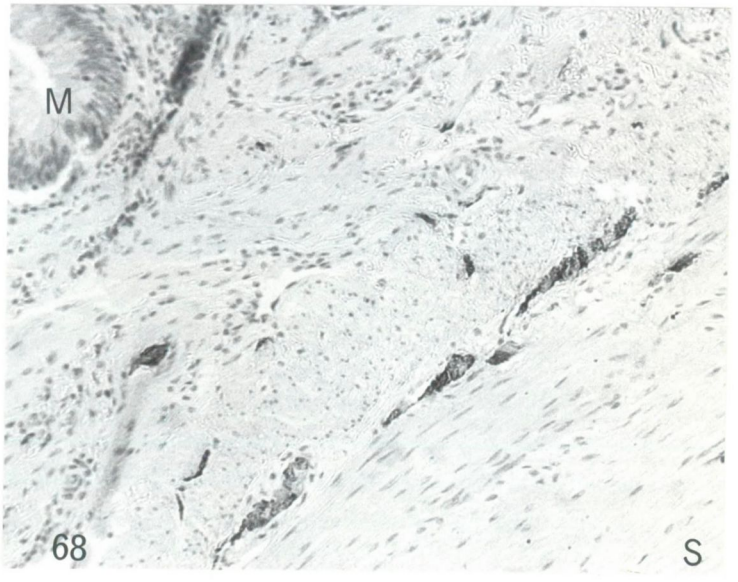
Figure 68

VIP-immunoreactive fibres in the myenteric plexus of the duodenum.

M = Mucosa S = Serosa

x 120


67


M
68
S

vi. Crotalus atrox

Table 32 Crotalus atrox North American

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP					VIP1	VIP2	VIP3	
Panc.H																			
Panc.B																			
Panc.T	+	+	+	+												+	+	+	
Fundus																			
Antrum																			
Duod.		+		+			+	+								+	+	+	
Jej.																			
Ileum																			
Colon																			

Pancreas. The pancreatic endocrine islets were found concentrated in the region next to the spleen (tail) and several islets were observed actually in the spleen (Figure 69). Insulin-, somatostatin- and glucagon-immunostained cells were present but no PP. A small number of VIP-immunoreactive fibres were also seen but these were confined to the exocrine tissue.

Stomach and Intestine. In the duodenum numerous neurotensin- and glucagon-containing cells were present as well as some gastrin/CCK and somatostatin cells. VIP-immunoreactive nerve fibres were also seen - these were confined/.....

confined to the sub-mucous plexus (Figure 70).

5.3.3.d. Summary - Serpentes

In most species (except the python) the pancreatic islets were concentrated at the tail region next to the spleen. The islets consisted of glucagon-, somatostatin- and insulin-immunoreactive cells with the glucagon cells being the most numerous. No evidence for the existence of PP-immunoreactivity was found in any species. VIP-immunostained nerve fibres were commonly found in both the pancreatic islets and exocrine tissue. However, with the exception of the Viperidae, VIP-immunoreactive nerve fibres and cell bodies were completely absent from the intestine and stomach. In the Natricine snakes there appeared to be an increase in the abundance of the substance P-immunoreactive fibres in the intestine, whether this was related to the absence of the VIP-containing fibres could not be established especially as this was not seen in the python or garter snake (Thamnophis sirtalis).

5.3.4. Crocodilia

5.3.4.a. Crocodylidae

i. Alligator mississipiensis

Figure 69

Insulin-containingcells in an islet actually within the spleen.

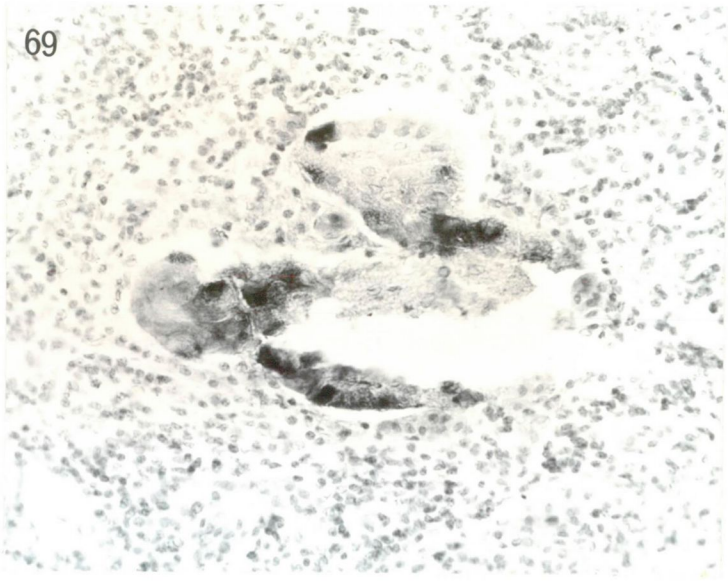
x 400

Figure 70

VIP-immunoreactive fibres in a 10 micron section of the duodenum.

S = Serosa

x 250


69

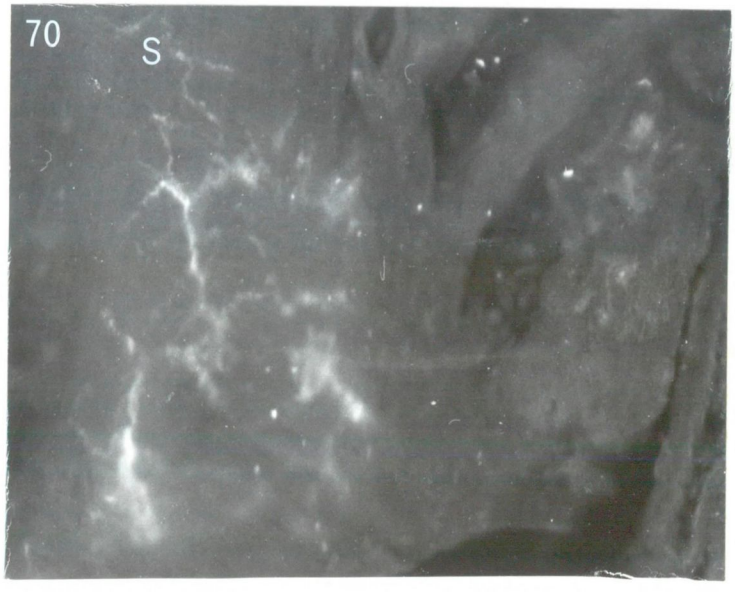

70
S

Table 33 — Alligator mississipiensis — North America

	INS	GLU	PGLU	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP	SEC			NSE	BN	VIP1	VIP2	VIP3	SP
Panc.H	+	+	+	+	+										+		+	+	+	
Panc.B	+	+	+	+	+										+		+	+	+	
Panc.T	+	+	+	+	+										+		+	+	+	
Fundus		+	+	+				X							+		+	+	+	
Antrum		+	+	+			+	X	+	+					+		+	+	+	
Duod.		+		+				+				+			+		+	+	+	
Jej.		+		+			+	+							+		+	+	+	+
Ileum		+		+				+							+	+	+	+	+	+
Colon		+		+				+							+	+	+	+	+	+

Pancreas. The pancreas in these animals was found embedded in fat in a loop of the duodenum (see Diagram 7). Three areas of the pancreas were distinguished. The head region was a long process ventral to the main body of the pancreas and the tail (splenic) portion lay adjacent to the attached spleen.

The endocrine tissue was found in all three areas. Discrete islets were not found, the cells being either grouped in clusters bordering ductular-like structures (Figure 71) or found in groups or single cells amongst the exocrine tissue (Figure 72). In some of the sections excellent examples of the budding of endocrine cells from the ductular epithilium were seen, especially insulin- and somatostatin-containing cells (Figure 73).

All four pancreatic hormones were distinguished including PP, which was only detected with the APP/.....

Figure 71

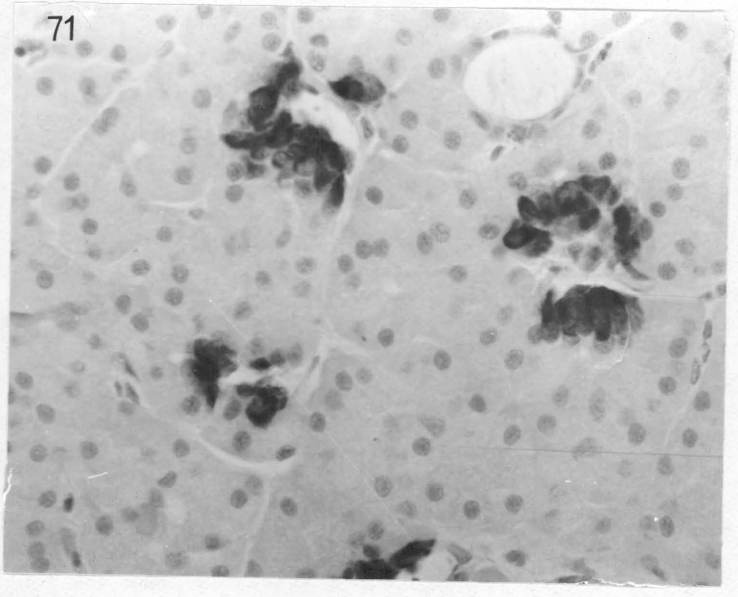
Insulin-containing cells bordering the exocrine tissue.

x 350

Figure 72

Insulin-containing cells in a small group surrounded by exocrine tissue.

x 400


71

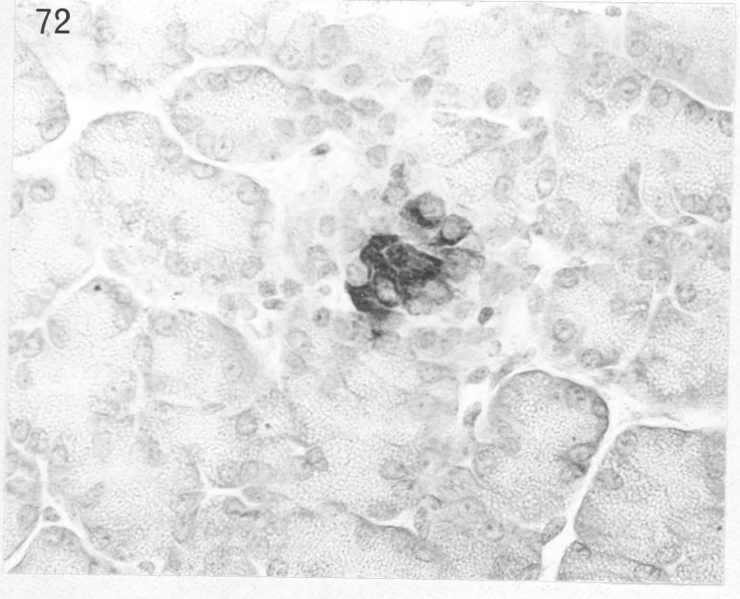

72

Figure 73

Insulin cells budding off from a pancreatic duct (PD).

x 600


73
PD

APP antiserum.

The peptidergic innervation was represented by numerous VIP-containing fibres in both the exocrine and endocrine tissue (Figure 74). None of the other neuropeptides were detected in the pancreatic tissue.

There was no evidence of an aggregation of either the endocrine tissue or the VIP-immunoreactive fibres to the tail region, the distribution appeared similar in all areas.

An interesting finding was the presence of ganglia containing VIP-immunoreactive cell bodies in the tissue surrounding the main pancreatic ducts (Figure 75).

Stomach and Intestine. In this species no bombesin/substance P-immunoreactive cells were detected in the fundic mucosa, although a few cells were observed in the antrum. Non-specific neurotensin cells were again detected in the fundus.

The antrum contained relatively more endocrine cells than the fundus, which were usually found towards the base of the glands. The glucagon and somatostatin cells showed long cytoplasmic processes (Figure 76).

Numerous VIP-immunoreactive nerves were present in both the circular muscle and sub-mucosa at the top of the glands (Figure 77).

VIP-immunoreactive cell bodies were found in the/.....

Figure 74

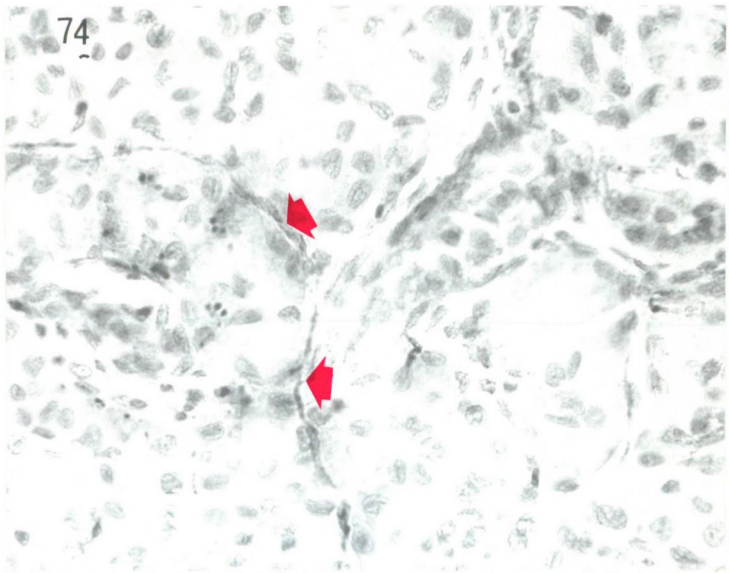
VIP fibres (arrows) within the exocrine pancreas.

x 400

Figure 75

VIP-immunoreactive cell bodies (arrows) in a ganglion outside the main pancreatic tissue, surrounding the main pancreatic duct.

x 400


74

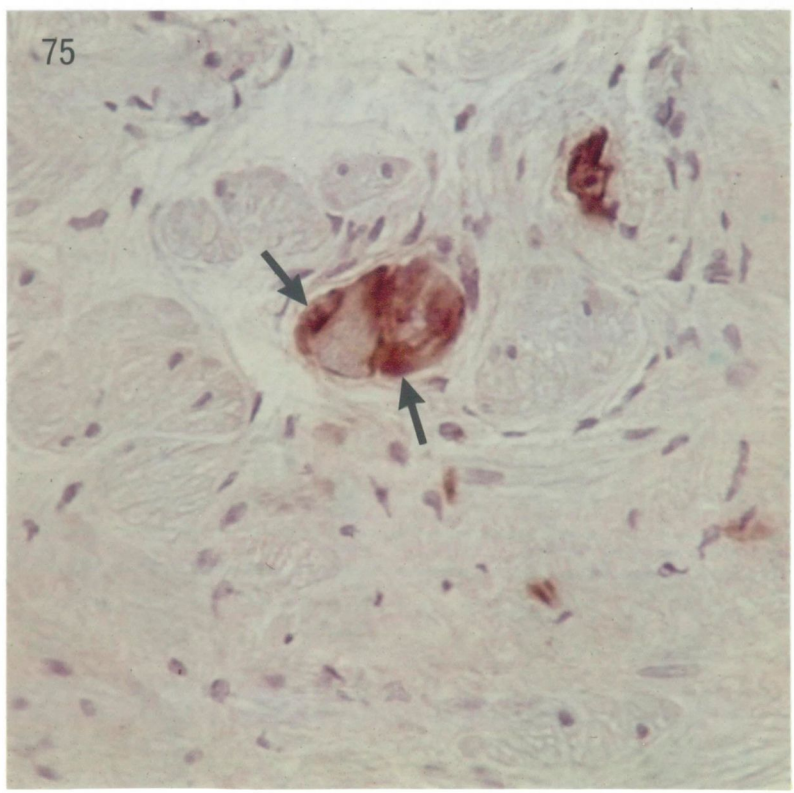

75

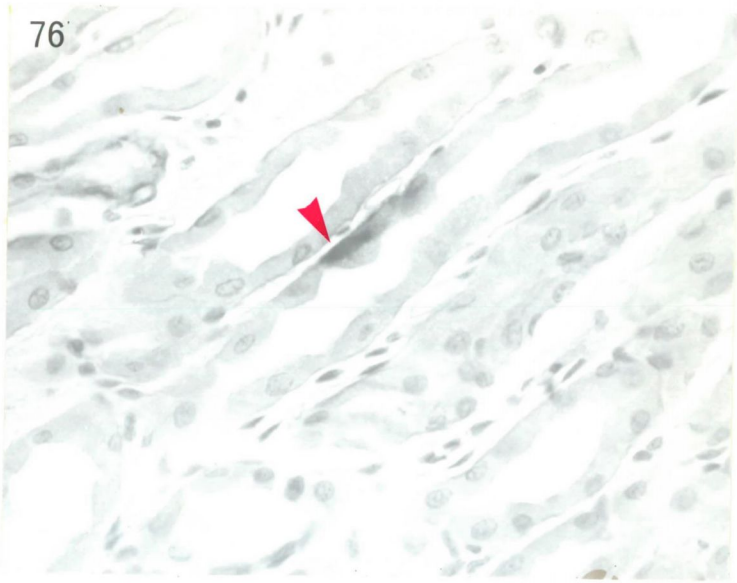
Figure 76

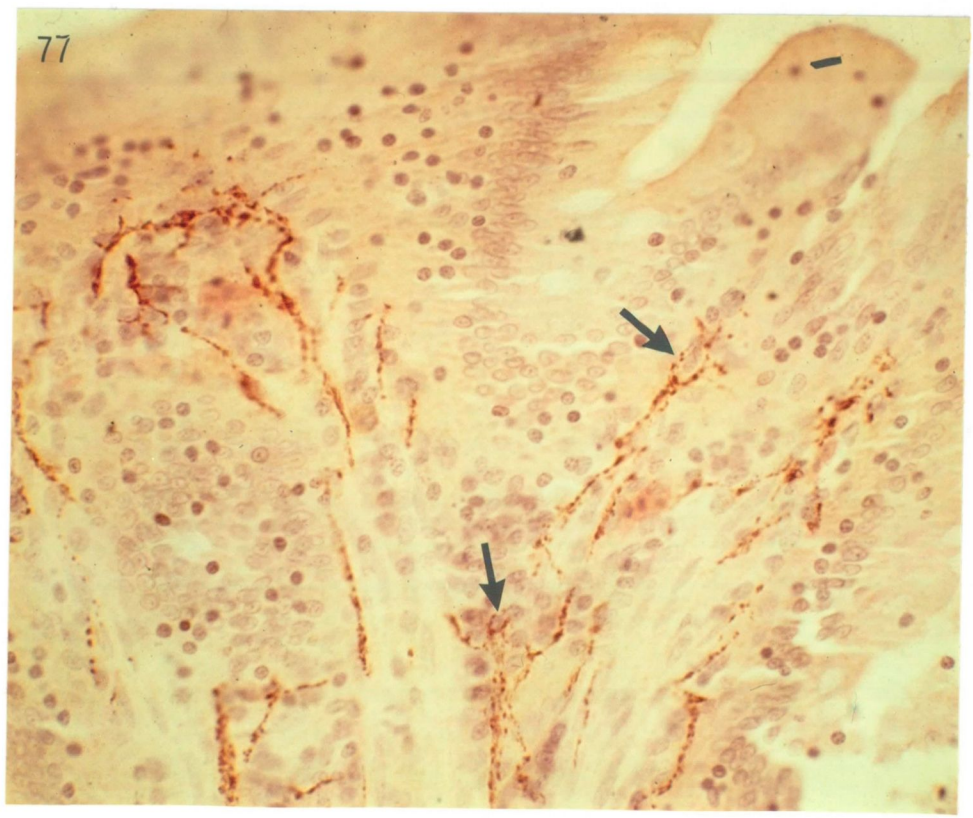
Glucagon-immunoreactive cell in the fundic mucosa with a cell process (arrow).

x 400

Figure 77

VIP-immunoreactive fibres at the top of the gut folds in a 5-micron section of the duodenum. The PAP stained section has been counterstained with haematoxylin and eosin to show the individual cells. Note the close association of the VIP-containing fibres and the mucosal cells. In some cases the VIP nerves appear to surround individual cells (arrows).


76


77

the sub-mucous plexus. No other neuropeptides were found in the nerve plexuses.

In the upper, small intestine, secretin-immunoreactive cells were detected, usually at the top of the gut folds (Figure 78). No gastrin/CCK cells were found in the duodenal region although they were numerous in the jejunum.

The NSE antiserum stained the nerve plexuses and sub-mucosal nerve fibres in all areas of the intestine (Figure 79) but no mucosal endocrine cells were detected. VIP-immunoreactive nerve fibres were found throughout the intestine, the fibres were confined to the sub mucosa, sub-mucosal plexus and the circular muscle, no fibres were found in the longitudinal muscle layers or myenteric plexus.

In addition to the VIP-immunostained fibres several bombesin-immunoreactive fibres were also detected. The distribution of the bombesin- and VIP-containing fibres was clearly distinct (Figure 80).

In the colonic region the number of endocrine cells was greatly reduced, the only cells detected being neurotensin and glucagon.

ii. Caiman crocodylus crocodylus

Figure 78

A secretin-immunoreactive cell (arrow) in the duodenal mucosa.

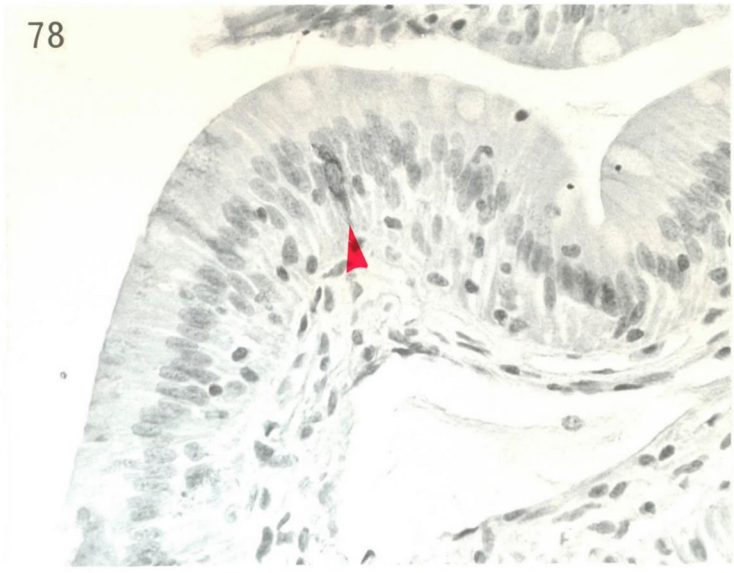
x 400

Figure 79

NSE-immunoreactive fibres in the sub-mucous plexus of the jejunum.

M = Mucosa

x 250


78

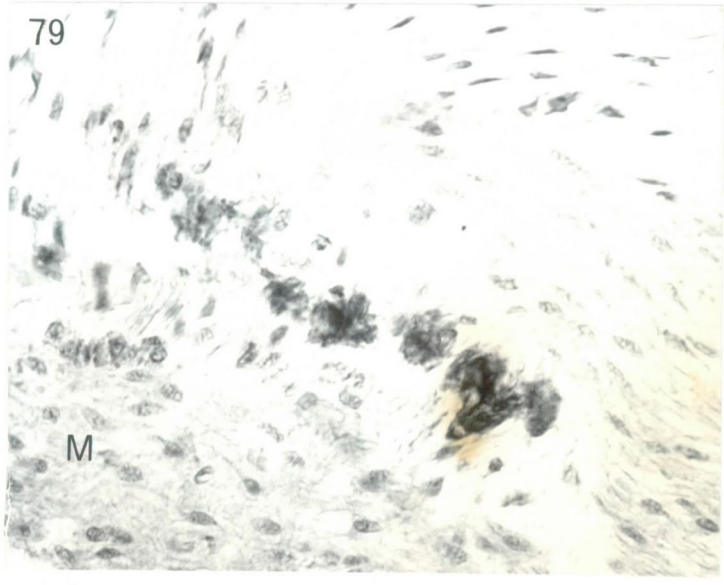

79
M

Figure 80

a - VIP-immunoreactive fibres in the sub-mucous plexus of the jejunum - note the positive cell body (arrow).

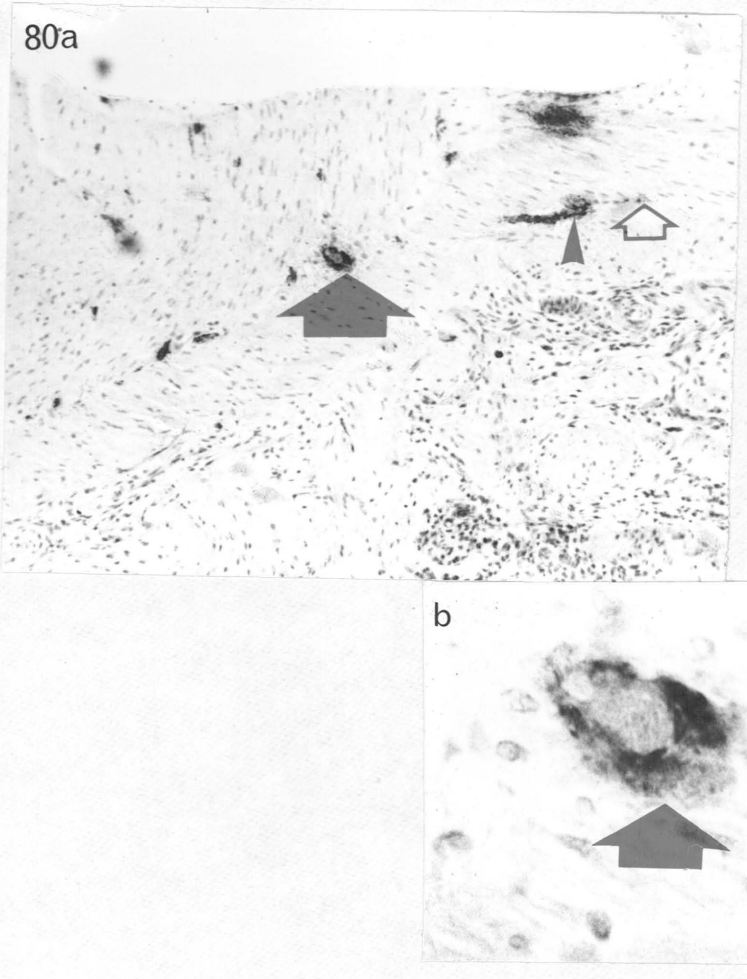
x 120

b - Detail of the positive cell body.

x 600

c - Bombesin-immunoreactive fibres in a serial section - note the different distribution of the nerve fibres as compared to a.

x 120


80a
b


c

ii. Caiman crocodylus crocodylus

Table 34 Caiman crocodylus crocodylus European

	INS	GLU	PSLI	SOM	APP	BPP	G/CCK	NT	BN1	BN2	SP	MOT1	MOT2	SEC			VIP1	VIP2	VIP3
Panc.H	+	+	+	+	+														
Panc.B	+	+	+	+	+														
Panc.T	+	+	+	+	+														
Fundus				+				✘			+						+	+	+
Antrum				+			+	✘									+	+	+
Duod.		+		+			+	+				+	+	+			+	+	+
Jej.		+		+				+									+	+	+
Ileum		+		+													+	+	+
Colon		+		+													+	+	+

Pancreas. The pancreatic endocrine tissue was found mostly in large islets composed of all four pancreatic hormones. There was some indication that as in the Vipera, some endocrine tissue was also present in the spleen (Figure 81) but this would require further investigation of more individuals, which were not available. The PP cells were again only detected by the APP antiserum. In this specimen there was no evidence for VIP-containing nerve fibres but this may be due to the fixation (Bouin).

Stomach and Intestine. VIP-immunoreactive nerve fibres were detected in the sub-mucous plexus in all regions of the intestine. In the/.....

the intestine secretin- and motilin-containing cells were detected in the duodenal mucosa, the motilin-containing cells being immunostained by both the N- and C-terminal antisera (Figure 82).

The number of immunostained cells decreased lower down the gut with a corresponding increase in the number of mucin-producing cells.

5.3.4.b. Summary - Crocodylidae

GIP, CCK and enkephalin were all undetectable in these animals. Those cells detected were usually found throughout the intestines, the exceptions being secretin and motilin (confined to the duodenum) and bombesin (confined to the stomach). Of the neuropeptides, only VIP and bombesin were found in the nerve fibres and cell bodies NSE immunoreactive autonomic nerves were demonstrated although the endocrine cells remained unstained.

Figure 81

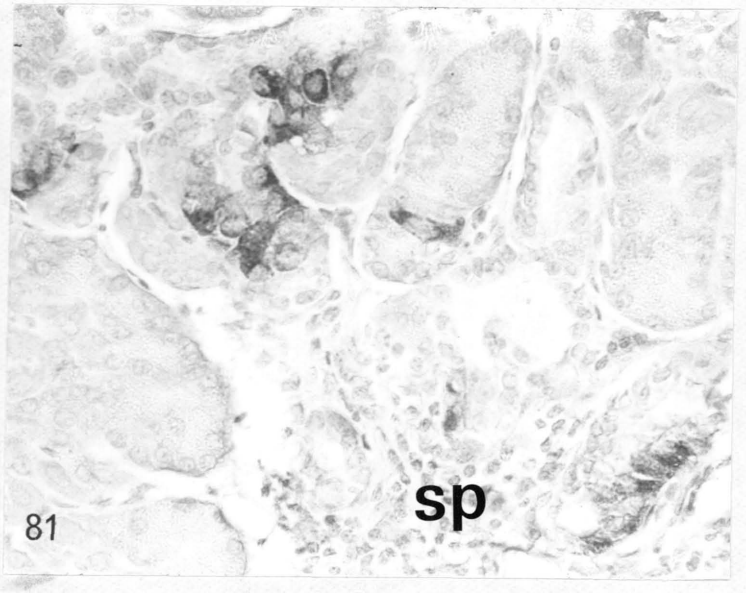
Glucagon-containing cells on the edge of the spleen (SP).

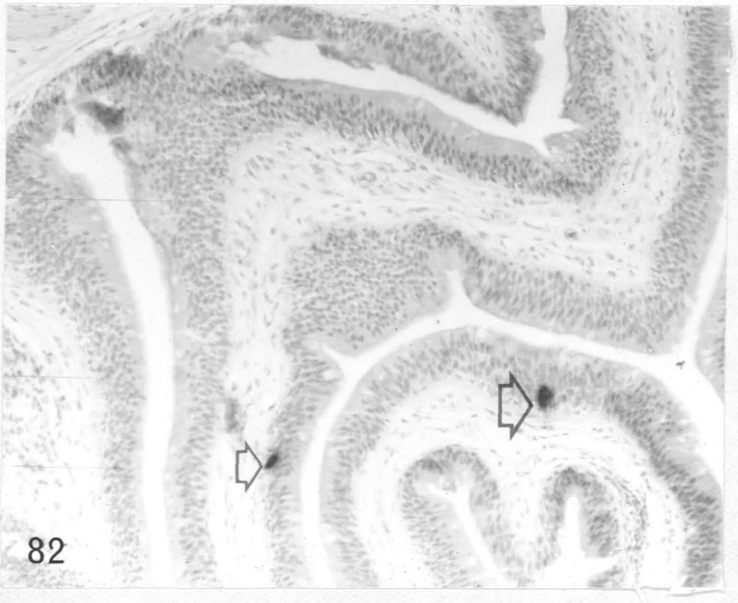
x 400

Figure 82

Motilin-immunoreactive cells in the duodenal mucosa (arrows), stained with the N-terminal antiserum.

x 250


sp
81


82

6.1. Pancreas

a. Islet structure.

The cells of the pancreatic islets are probably the most intensively studied components of the regulatory peptide system.

Considerable differences were seen between the mammalian and the amphibian/reptilian islets in size, composition and cell characteristics.

It was noted that in the more 'primitive' ancestral-type animals, such as the salamander, turtle and alligator the islets were ribbon-like, occurring along the edge of the acinar tissue, whereas in the more advanced species, such as the tree frogs and snakes, mammalian-type islets were present.

In the reptiles all four pancreatic hormones were detected with the available antisera but in the amphibia the presence of PP was equivocal.

An additional feature of the snake pancreas was the concentration of islet tissue to the splenic (tail) region, with large islets occurring right next to the spleen. The most advanced of the snake families, the Viperidae, have taken this one stage further, as islets were found actually within the spleen. The finding of splenic islets in members of the Viperidae agrees with a previous report of their presence in the species Crotalus viridis helleri and Vipera berus, which are both members of the Viperidae (Hellerstrom and Asplund 1966)./.....

1966). The significance of these splenic islets is at present unknown.

A concentration of islet tissue near the spleen has also been reported in several groups of fishes where islet tissue is concentrated in discrete 'Brockmann' bodies.

b. Distribution of Islet Cells.

In mammals the existence of a differential distribution of the islet cells has been well established (Orci and Unger 1975). A more recent finding was the distinct regional localisation of PP in several mammals (Orci et al 1976, Orci et al 1978) and also teleosts (Stefan and Falkmer 1979).

Of the more advanced species investigated the difference in composition of the islets was most striking in the snakes. In these animals the glucagon-containing cells were the major component as compared to the insulin-rich islets of mammals.

In other groups such as the frogs the typical mammalian pattern of central insulin with peripheral glucagon and somatostatin cells was demonstrated.

c. Regional Variations.

The pancreas in the amphibians and some reptiles was difficult to sub-divide into head, body and tail regions with any accuracy. It was thought that in view of the reported regional variation in the occurrence of PP cells this might have been used as a marker for the head and tail regions. In practice this was impossible, as/.....

as in all species the distribution showed no marked gradation from area to area.

However a differential localisation of PP cells was seen in Chelydra serpentia where no PP cells were present in the tail region. This was identical to the reported distribution pattern in mammals and some fishes.

d. Individual Peptides.

i. Pancreatic Polypeptide.

Of the four recognised pancreatic hormones PP was the only one not to be specifically detected within the amphibian pancreas.

It appeared from the immunostaining of the amphibian pancreas that, regardless of species, those cells reacting to the PP antiserum also reacted with the glucagon antisera. Further, of the available PP antisera, only the BPP antiserum gave positive results, the immunostainings with the APP and HPP antisera were totally negative. The control incubations showed that although the addition of glucagon significantly diminished the level of the immunoreaction there was still a demonstrable level of binding to the pancreatic cells. Recent work on the cross-reaction of GIP antisera with mammalian pancreatic glucagon cells showed that the common determinant was present in the glicentin (Moody et al 1978) molecule not in the pancreatic glucagon sequence (Larsson and Moody 1980). It may be therefore that the reaction seen/.....

seen with the BPP antiserum in the pancreas of these amphibians was the result of the binding of the BPP antibodies to an amphibian equivalent of glicentin: until this problem can be fully studied by the purification of the pancreatic glucagons from these species the question of whether the BPP staining was purely a crossreactivity with glucagon-like peptides cannot be answered.

Work on purification of glucagon from the fishes has already shown that in these species considerable sequence homologies exist between the glucagon and mammalian PP which would tend to support the idea that if an amphibian PP does exist it was not detected by the present antiserum (Steiner personal communication 1980).

Immunostaining of the reptilian pancreas has shown that even in the 'primitive' members, ie turtles, there was a clear distinction between the cells reacting with the glucagon antisera (pancreatic and N-terminal) and those reacting with the APP and BPP antisera, thus indicating that at this level of organisation PP-like peptide(s) with sufficient sequence similarities to bind both sets of PP antisera but not the HPP antiserum, were already established. It was interesting to note that in the Crocodylidae only the APP antiserum detected the PP cells, which, as the alligators are supposed to derive from the same ancestral line from which the Aves have diverged, may suggest that the avian peptide/.....

peptide was established earlier in evolutionary development.

Of further interest was the finding that in snakes, from the primitive python to the highly advanced rattlesnake, there appeared to be a complete lack of PP-immunoreactivity. As has already been mentioned, the lack of immunoreactivity cannot exclude the existence of a related peptide; therefore once again, the extraction and purification of the pancreatic hormones from snakes will be necessary to establish conclusively whether PP really is absent in these species.

If the lack of PP is confirmed by further work, these animals may be an ideal experimental model in which to study the effects of exogenous administration of PP. This is especially attractive in view of the present lack of information as to a precise biological role for PP in mammals.

It has been suggested that PP may be involved in the regulation of food intake and digestion (Gates and Lazarus 1977, Malaisse-Lague et al 1977); such a function would be supported by the morphological finding of PP-rich areas in the head of the pancreas which is in close relation to the intestine (Falkmer 1979).

ii. Glucagon

Immunocytochemistry showed the presence of a glucagon-like peptide in all the species investigated.

Of/.....

Of the three types of region specific antisera available, those detecting the N-terminal to mid-region of glucagon(which also detect the enteric forms)and the C-terminally directed pancreatic glucagon-specific antiserum gave positive results. In no species was immunoreactivity detected using the glicentin specific antiserum. These results indicate that the sequence of pancreatic glucagon has remained stable for a considerable period, or at least those areas detected by the antisera to the C- and N-terminal regions, whereas the larger enteric form(s) has probably changed considerably between the amphibians/reptiles and mammals.

It was noticeable that in the majority of species the glucagon-containing cells were not confined to the islets as in mammals, but were found scattered within the exocrine tissue. In those species with a concentration of glucagon cells to the islets they usually formed the major constituent of the islet.

As already mentioned glucagon in these lower species appears to have the same role in glucose homeostasis as seen in the mammals.

iii. Insulin and somatostatin

With both these peptides, immunoreactive cells were seen in every species investigated. The insulin and somatostatin cells were often found in close association, a finding which supports the contention that these two peptides have long been related functionally./.....

functionally.

Previous evolutionary studies have suggested that the first endocrine cells to migrate from the gut into the developing pancreas were those producing insulin. These were then followed by somatostatin, glucagon and finally PP-producing cells (Falkmer et al 1977).

From a functional standpoint, in vitro work with isolated fish islets has shown that application of somatostatin to cultured islets completely abolished the release of insulin. This effect could be reversed in incubation of the same islets in a somatostatin-free medium (Stewart et al 1978). Thus somatostatin presumably acts primarily to inhibit the release of insulin in the pancreas while insulin retains its usual hyperglycaemic effect.

e. Innervation.

In mammals both VIP-containing nerves (Larsson et al 1978, Bishop et al 1980) and tetragastrin-containing nerves (Larsson 1979b). have been described in association with the endocrine islets. In none of the species investigated in this study were nerve fibres detected with any of the antisera to the C-terminal region of gastrin.

The results of the present study showed that in the amphibians only the most specialised tree frogs (Hyla arborea) had recognisable VIP-containing fibres in the pancreas suggesting the presence of a peptidergic component of the autonomic innervation.

Immunostaining/.....

Immunostaining of the reptilian material showed that most species had VIP-containing fibres. The fibres were mostly confined to the exocrine pancreas and around the pancreatic ducts, except in the Ophidia and alligator where a rich innervation of the pancreatic islets was also demonstrated.

In the python the use of the PAP double staining technique showed that the VIP-immunoreactive nerve fibres were in close association with both insulin- and glucagon-containing cells. Recent work in a related species Elaphe quadrivirgata (Boidae) has also shown a rich VIP-innervation of the region around the islet, although a direct relationship with the islet cells was not established (Fujii et al 1980).

It has been demonstrated in mammals that VIP releases insulin, glucagon, somatostatin and PP (Schebalin et al 1977, Ipp et al 1978, Adrian et al 1978). The close association of the VIP-containing terminals with the insulin and glucagon cells suggests that VIP may influence the release of at least these two peptides. Clearly further physiological studies are required to establish the exact effect of the endogenous VIP.

Although immunoreactive cell bodies were seen in the plexus surrounding the main pancreatic duct no positive cell bodies were seen within the pancreatic tissue therefore the VIP-fibres may prove to be extrinsic. Alternatively it may be that the cell bodies were not detected in the material available due to low amounts of peptide. Treatment of further animals with agents such/.....

such as colchicine before sacrifice may render the ganglion cells within the pancreas detectable.

f. Summary.

The results confirm the presence of insulin-, glucagon-, and somatostatin-like peptides in the pancreas of all the species investigated.

A PP-like peptide certainly occurs in the majority of the reptiles with the exception of the snakes. In the amphibia the present evidence indicated that if a PP-like peptide exists, it does not contain determinants detectable by any of the available antisera.

The presence of a VIP-ergic component of the pancreatic innervation has been established although its functional significance remains unclear. Further work using the snake pancreas may be of importance in answering this problem.

No evidence of a further peptidergic component was found, neither was there any evidence for the presence of other peptide-containing cells, accepting the limitations of the use of the mammalian based antisera.

6.2. Stomach

a. Fundus

At present mucosal endocrine cells containing somatostatin bombesin, serotonin and glucagon have been demonstrated in mammals. The glucagon cells, which were seen only in canine fundus, were found to be identical to the pancreatic glucagon cell (Grimelius et al 1976).

In/.....

In the muscle coat and nerve plexuses, several neuropeptides have been identified including substance P, VIP, enkephalin and bombesin (Polak and Bloom 1979 , Schultzberg et al 1980).

The fundic region of the amphibians and reptiles invariably contained glucagon, bombesin/SP, somatostatin and neurotensin immunoreactive cells.

i. Neurotensin

The neurotensin immunoreactivity in the fundic mucosal cells was found to be non-specific in that it was un-absorbed by the pre-addition of either neurotensin or the related amphibian peptide xenopsin. However, it is of interest that in recent studies using region-specific antisera to neurotensin, it has been demonstrated that with the C-terminal specific antisera several related peptides can be detected in the stomach of both mammalian and non-mammalian species. On the other hand, the N-terminal specific antiserum only detected 'true' neurotensin which was not present in the stomach of the non-mammalian species (Carraway 1981). In addition in previous investigations with frogs and lizards a similar pattern of a non-absorbable immunostaining in the stomach was reported.

It would appear from the present information that the peptide(s) detected by the neurotensin antisera available during this study correspond to members of the "neurotensin family" which all share a common/.....

common C-terminal sequence with differing N-terminal determinants (Carraway and Bhatnagar 1980).

A second explanation involves the newly identified peptide PYY. This peptide has been shown to have an N-terminal identity with neurotensin, therefore it may be related to the peptide detected in the stomach of these animals. Further biochemical work on the extraction and purification of this substance(s) will help to clarify the question .

ii. Glucagon

The glucagon-containing cells were present throughout the fundic glands usually occurring close to the basal lamina. These cells were detected by both the N- and C- terminal antisera as were the pancreatic glucagon-containing cells, suggesting that the same situation exists as reported for canine stomach. The function of this extra-pancreatic glucagon is unknown.

iii. Substance P/bombesin

In several species, staining of identical mucosal endocrine cells was detected with both antisera to bombesin (N- and C-terminal) and substance P (C-terminal). The pre-absorption of antisera with the other antigen (ie anti-bombesin with substance P and vice versa) demonstrated that the resultant immunostain was not removed by the cross-absorption. In some other species bombesin-

or/

or substance P immunoreactive nerve fibres were also seen but these were not double-stained ie where present the fibres were only detected with one group of antisera.

For several reasons it was unlikely that the double staining seen by immunocytochemistry was caused by the presence of the two identical amino acids at the C-terminal region. For instance, the cross absorptions should have blocked the binding to that particular region and further the use of the N-terminal bombesin antiserum gave the same immunostaining pattern and it lacks any reactivity with the common C-terminal region.

In addition the immunocytochemical localisation of bombesin to the fundic mucosa has been confirmed by radioimmunoassay, where high levels of bombesin-like immunoreactivity have been demonstrated (Appendix III)by an assay system which does not detect substance P to any significant degree (Ghatei in preparation).

Therefore it may be that in those species where the double staining occurs a related peptide is responsible, which has available binding sites for both the substance P and bombesin.

iv. Somatostatin

Somatostatin cells were found to be especially numerous in the deeper crypt regions of the glands. These cells frequently showed long processes reaching along the basal lamina. These processes have also been seen in several mammalian cell types/.....

types which include somatostatin and it has been suggested that these processes are related to the postulated paracrine function of this peptide (Larsson et al 1979).

Of the neuropeptides detected in mammalian tissues, VIP, bombesin and substance P nerves were seen in the fundic region of most species.

v. VIP

In mammals the VIP-immunoreactive fibres were found in all species studied except most of the Serpentes and the frog Atelopus oxyrhynchus. The VIP-innervation was usually well developed. In the amphibians the majority of the fibres were seen in the myenteric plexus and circular muscle with only a few fibres in the lamina propria. In the reptiles the situation differed from species to species. In the Testudinata the staining pattern was similar to that in the amphibians with little or no immunostaining being found in the lamina propria, in the agamids and gekko the innervation showed an increasing development in the sub-mucous plexus although the majority was still in the muscle layers and the myenteric plexus. In the alligator there was an almost total reversal with little immunoreactivity being found in the myenteric plexus. Almost all the staining was found in the sub-mucous plexus and in the lamina propria where there was a close connection between the cells at the basal lamina and the positive nerve fibres./.....

fibres.

vi. Substance P

In some species, in addition to the VIP innervation, substance P positive fibres were also detected.

In the amphibia the substance P nerves were usually found around the blood vessels, only rarely being seen in the myenteric plexus and never in the muscular layers.

In the snakes the substance P fibres were very much more numerous (Colubridae) and the myenteric plexus was particularly rich in nerve fibres. Whether the increase in substance P content could be related to the absence of VIP in these animals in this region is unknown.

vii. Bombesin

Bombesin-immunoreative fibres were by far the least common, only occurring in a few amphibian species and the alligator.

viii. Hibernation

An interesting result was seen in the comparison of the fundus in the hibernating Bufo bufo as compared to those re-fed for a period of about one month.

The depth of the fundic glands appears to be decreased in the fed toads, whether this was related to stretching of the mucosa due to excessive food intake is unknown.

These results are the opposite to those obtained during a study of the effect of hibernation on the gastric mucosa of the frog Rana esculenta (Geuze 1971 a and b)/.....

(Geuze 1971 a and b) where the fundic glands were reported to be shorter in the hibernating animals. An explanation for this discrepency would be that the toads in the present study were only re-fed for one month whereas the frogs were killed in mid-summer. It is possible that a further change in the mucosa may occur after a prolonged period of feeding. Therefore the effect of the 'stretching' would be compensated for and a further cell proliferation might occur which would produce the deeper crypts seen in the mid-summer frogs. Changes in the endocrine cell content and nerve fibre density were also observed in these animals with the substance P-containing fibres becoming more numerous around the blood vessels and the bombesin cells being increased in the fed toads. The effect of hibernation on the regulatory peptides will be discussed further on page 225.

b. Antrum

The structure of the antral region was essentially similar to that seen in the fundus.

Of the endocrine cells demonstrated in the fundus only those containing somatostatin and neurotensin were a constant feature in the antral mucosa of the majority of the sub-mammalian species. In addition to these, cells detected by the gastrin/CCK antisera were always present. The neural component was represented by VIP, substance P and enkephalin.

i. Neurotensin/.....

i. Neurotensin

The same situation was found as in the fundus, except the cells were confined to the mucosa being found in the lamina propria only in salamanders.

ii. Gastrin/CCK

Several antisera to gastrin and CCK were available. Some, detecting the common C-terminal region were grouped together as G/CCK specific, as with these there was no possibility of distinguishing between gastrin and CCK at least by immunocytochemical methods. The other antisera were either to Gastrin 34, Gastrin 17 or CCK alone (see Table 6 page49).

As in no case were the antral cells stained by any other than the G/CCK antisera this suggested that 'true' gastrin was not present in these animals. However, this obviously assumes that the mammalian antisera to Gastrin 17 and Gastrin 34 would detect their amphibian equivalent.

There has been some conflicting evidence in the literature concerning the presence of gastrin and CCK-like peptides in lower species. One report using a bioassay system indicates the presence of a true gastrin-type peptide in the stomach of Rana berlandieri. Similar findings in extracts of the salmon intestine and stomach led the author to suggest that the gastrin- and CCK-like peptides diverged at the level of the chondrichthyeans (Vigna 1979)./.....

(Vigna 1979). In addition, significant amounts of gastrin were detected by radioimmunoassay in the stomach of the amphibians Rana catesbeiana and Necturus maculosa. Gel permeation chromatography on G50 columns demonstrated that a G34-like peptide was the predominant form although some of a G17-like size was also present (Gibson et al 1976).

In contrast to the above findings, and in agreement with the present observations, work using region - specific antisera to gastrin and CCK has shown that in frog antral extracts the molecular forms present were equivalent to a large CCK, CCK-33 and the decapeptide caerulin, with no detectable gastrin (Larsson and Rehfeld 1977). In addition the antral endocrine cells were detected by both C-terminal gastrin and CCK antisera, indicating that the frog does not have separate gastrin and CCK peptides.

Clearly, in order to solve the present dilemma, the purification and full characterisation of the peptides by amino acid analysis will be required. If it can be shown that the region specific antisera are capable of binding to the peptides from lower species the present data would support the belief that CCK and gastrin as separate entities are only recognisable at the reptilian level of organisation.

An interesting observation was that although the region specific antisera to G17, G34 and CCK were almost always negative, if the C-terminal gastrin antisera/.....

antisera were pre-absorbed by the addition of gastrin pentapeptide, the immunostaining seen was not removed , whereas if the antiserum was absorbed with G17 no immunostaining was detected. This may indicate the existence of a second antibody population in the C-terminal antiserum directed to an antigenic determinant at the C-terminal region which differs from that detected by the specific G17 antisera. Further, this determinant was present in the antral and duodenal mucosa of several of the species investigated (the full results of the work completed at present are given in Appendix IV).

It follows from this that the peptide detected in these areas by the use of the C-terminal antiserum is nearer to the gastrin-type peptides than the CCK-like. Although more advanced studies must be initiated to finally solve the question, there is an indication from these results that the use of region-specific antisera to areas of the molecule that are probably non biologically active (such as the mid-region of CCK) may give rise to false negatives, as amino acid substitutions probably occur more often in these areas thus causing a negative result.

iii. Somatostatin

These cells were relatively less numerous than the gastrin/CCK cells in the majority of the species studied. Morphologically they were identical to those/.....

those seen in the fundic mucosa.

iv. VIP

Nerve fibres and cell bodies containing a VIP-like peptide were detected in all of the species investigated except the majority of the Serpentes and the frog Atelopus oxrhynchus.

The VIP-fibres were numerous in the muscle layers especially in the circular muscle. In mammals VIP has been shown to modulate smooth muscle relaxation (Kachelhoffer et al 1976). In view of the highly localised distribution to the muscle layers it may be that the amphibian/reptilian peptide has a similar action on the muscle fibres.

The VIP-containing cell bodies were confined to the myenteric plexus in the amphibians, whereas in the reptiles the vast majority of the cells were found in the sub-mucous plexus, similar to the situation reported in mammals (Schultzberg et al 1980, Jessen et al 1980).

6.3. Intestine - Duodenum

In general the greatest numbers of endocrine cells were seen in this region. The cell types varied greatly from species to species, as did the morphological appearance of the duodenal mucosa.

Although the actual structure and the cell types detected altered, the distribution of the cells within the mucosa was fairly/.....

fairly constant.

a. Peptides of limited distribution

Several peptides were only detected sporadically such as GIP, secretin, CCK and motilin. In every case, when these peptides were detected the immunostained cells occurred in the duodenal mucosa. It was interesting to note that the detection of GIP and motilin presents considerable difficulties even in some mammals(ie rat, mouse and dog) indicating that this was not an unusual occurrence.

The lack of consistent detection of secretin immunoreativity was interesting as this peptide has been demonstrated in avian intestine (Nilsson 1974) and there has been no difficulty encountered with species to species cross-reactivity in mammals. However, in the Crocodylidae ,both the alligator and the caiman contained secretin immunoreactive cells in the duodenal mocosa. As previously mentioned, the

have been suggested to be the ancestral stock of the Aves. The fact that secretin was not easily detected in most of the lower species and yet was present in these animals can be added to the APP reactivity in the pancreas as another link between the two groups.

The lack of immunoreactivity found with the CCK antiserum has already been discussed (see Page 217)

b. VIP

The results of the VIP-staining, especially in the amphibia were quite remarkable. In the Anura there appeared to be two groups, the terrestrial species

(Rana/.....

(Rana temporaria, Alytes obstretricans, Bufo bufo, Atelopus oxrhynchus, Hyla arborea and Hyla crepitans) with both VIP-containing mucosal endocrine cells and VIP-ergic nerve fibres and cell bodies, and the aquatic frogs (Bombina bombina and Rana esculenta) which lacked immunoreactive endocrine cells. In an attempt to analyse whether the immunoreactive material detected in the cells was of a similar chemical nature to that in the nerve fibres, a group of three types of region specific antisera were employed for immunocytochemical staining, each detecting different regions of the molecule (see Table 7 , Page 49). The results showed an identical staining pattern with all the antisera, indicating that not only was the peptide in both locations very similar but also that it probably has considerable structural identity with the mammalian VIP used to produce the antisera. The immunocytochemical results were supported by the findings obtained on VIP-immunoreactivity in radioimmunoassay of extracts of Anuran duodenum from both groups of frogs (in co-operation with Dr S. R. Bloom's laboratory, using the assay developed by M. G. Bryant - see Bryant and Bloom 1979).

The results of the assay are shown in Table Page 337 (in Appendix III). From the levels detected, it was clear that the duodenal extracts from the terrestrial frogs had a higher VIP content than those of the aquatic species. A further investigation into the molecular form of the VIP in these extracts was undertaken using gel permeation chromatography/.....

chromatography in combination with the aforementioned assay.

The profiles produced showed that the majority of the VIP detected eluted in a position identical to porcine VIP, although two minor peaks were also detected (see Page 338 , in Appendix III).

Considerable technical difficulties, due to the thinness of the Anuran gut, were encountered in separating the mucosa from the muscle coat. Therefore the chromatographic profile from an area with cells and nerves was compared to an area containing only nerves Page 338 ,in Appendix III). The profile was found to be identical, lending further support to the immunocytochemical finding of a single peptide with a dual localization.

The finding of VIP-immunoreactive cells and nerves in the same area was confined to the Anura, and not seen in any of the Urodele or Reptilian species studied.

In these species, VIP was only demonstrable in nerve fibres and cell bodies which, as they were immunostained by each of the antisera types, indicated that the VIP-like peptide had remained remarkably stable throughout these later evolutionary developments.

The significance of the presence of VIP-immunoreactive cells only in the terrestrial Anuran species is not understood at present. There is a report of a similar finding in chicken intestine (Sundler et al 1979) and it is interesting that this was not paralleled by the finding of such a localisation in the Crocodylidae during/.....

during this study.

The only common factor between the Anuran species was their predominantly terrestrial habitat. VIP, in the mammals, was originally isolated due to its vasodilatory action (Said and Mutt 1970). Whether the increased quantities of VIP in the amphibia reflected an osmoregulatory control of water intake by controlling blood flow merits investigation. If this was the case, then the lack of cells in the aquatic frogs and Urodeles would be explained by the fact that these animals have no need for a strong control of water intake as there would be no lack of water. Equally, the reptiles can dispense with this as they possess an impermeable skin which cuts off excessive water loss. An alternative explanation could be that as these species lack a recognisable secretin-like peptide, the VIP-like peptide has a secretin-like effect on the pancreas. In birds not only has VIP been shown to have such an effect (Dockray 1973) but the structure of avian VIP has been established (Nilsson 197[illegible]).

Unfortunately, although the idea is quite attractive, the absence of both secretin and VIP cells in the Urodeles, aquatic frogs and the majority of the reptiles suggests that additional cross-reactivity problems may also be influencing the results.

Clearly, further physiological studies on the function of these peptides in the Anura and Reptilia is required in order to clarify the problem.

c. Other Peptides/.....

c. Other Peptides

i. Endocrine Cells

Neurotensin-, somatostatin-, glucagon- and gastrin, CCK-immunoreactive cells were detected in most species.

Neurotensin. Unlike that in the stomach, in all the species the immunoreactivity found in the intestine was specific. The presence of neurotensin in frog (Rana temporaria and Xenopus laevis) and lizard (Lacerta viridis) has recently been reported (Reinecke et al 1980) by use of region specific antisera to C- and N-terminal fragments of the peptide. The cells were shown to be most numerous in the pyloric region, the numbers decreasing in the distal intestine. In a further communication the same group have reported that the immunoreactivity found in the stomach was caused by non-specific binding as encountered in the present study. In general the agreement between the two sets of data was reasonable except that the concentration of cells to the pyloric region as reported by the German group was not confirmed by the results of this investigation.

d. Hibernation

As the amphibians during hibernation do not eat any food adaption of the intestine might be expected to parallel that of mammals during prolonged fasting.

In mammals experiments have shown that during fasting the depth of the mucosa decreases, on re-feeding there is an initial hyperplasia of the mucosa which gradually returns/.....

returns to normal.

In Bufo bufo the adaption of the gut to the fasting (Hibernating) and re-fed states differed according to the area studied.

Stomach. In addition to the histological changes the immunostaining of two peptides showed marked changes. Bombesin-containing cells within the mucosa and substance P-containing nerve fibres were both increased in the re-fed toads.

Bombesin in mammals has been shown to potentiate the release of gastrin from the antirum and therefore indirectly influences the release of gastric acid (Erspamer and Melchiorri 1973). Whether bombesin would have the same effect in amphibia has to be proven but if so this would be an explanation of the increase seen after feeding.

Substance P in mammalian intestine has been shown to increase motor activity (Von Euler and Gaddum 1931). If the amphibian peptide has a similar action then the increase after feeding could be in or to stimulate intestinal muscle contractions to move food through the gut.

Small intestine. There was clearly an hyperplasia of the intestinal mucosa in the re-fed toads as compared with the hibernating specimens. The hyperplasia was most marked in the duodenal region where differences in the content of the VIP cells (increased number) and somatostatin cells (number of cellular processes). VIP-containing/....

VIP-containing cells were increased in number in the fed animals, it has already been suggested that in the amphibia VIP may play a role in the control of the output of pancreatic bicarbonate and it may be that the increase in the detectable VIP cells reflects increased pancreatic activity.

Although the number of somatostatin-containing cells did not alter, the staining intensity and abundance of cellular processes were increased. Somatostatin in mammals acts primarily to regulate the release of the other hormones from the gut. If somatostatin also affects the release of the possible trophic factors from the gut the increase in the cell processes may reflect a means of preventing excessive mucosal hyperplasia in the fed toads.

It is hoped to increase the information available on the effects of hibernation on the regulatory peptides in a future study.

e. Neural Elements

Several peptides were detected in nerve fibres and cell bodies of the myenteric and sub-mucous plexuses. The peptides concerned included VIP, bombesin, substance P, enkephalin, and endorphin as well as the enzyme NSE.

i./.....

i. VIP

VIP-immunoreactive nerve fibres and cell bodies were detected in every species investigated with the sole exception of the snake Nerodia rhombifera.

Amphibia. In all of the amphibian species a VIP-like peptide was detected from stomach to colon, the majority of the immunoreactive fibres were seen in the myenteric plexus and circular muscle with little or no immunoreativity being detected in either the sub-mucous plexus or the lamina propria. The VIP-containing cell bodies were found to be completely confined to the myenteric plexus, which was the opposite of the mammalian findings, where VIP has been shown to occur in cell bodies of the sub-mucous plexus (Jessen et al 1980, Schultzberg et al 1980).

The finding of the cell bodies in the myenteric plexus does, however, agree with comparative studies on the development of the autonomic innervation. It has been shown that in the Amphibia no ganglion cells were present in the sub-mucous plexus (Gunn 1951) and that those of the myenteric plexus were scattered amongst the fibres of the plexus instead of being aggregated into the large ganglia as seen in mammals (Burnstock 1969). In addition, it has been demonstrated that the innervation of the circular muscle was greater than any other region of the gut wall (Rogers and Burnstock 1966).

It/.....

It is interesting to note that this pattern is also seen in the gut of human foetuses. A study of the developing nervous system in human foetuses showed that initially all the ganglion cells were confined to the myenteric plexus and migrated through to the sub-mucous plexus around the third month of gestation (Okamoto and Ueda 1967). This correlates well with the finding of VIP-immunoreactive fibres and cell bodies initially in the myenteric plexus of 12 - 16 week old foetuses and then in the sub-mucous plexus of foetuses over 20 weeks of gestation (Buchan et al 1980).

In view of the localisation of these VIP-containing fibres predominantly to the circular muscle layer it appears likely that the action of VIP in the Amphibia is linked to an effect on the smooth muscle fibres. In mammals VIP has been shown to relax smooth muscle fibre contraction (Kachelhoffer 1976, Said and Mutt 1972) and to produce increased movement through the pyloric sphincter (Edin et al 1979) and has been suggested as a mediator in atropine-resistant gastric relaxation (Fahrenkrug et al 1978).

The high concentration of VIP-fibres seen in the stomach coupled with the high quantities seen by radioimmunoassay suggest that VIP may be involved in the control of food transit into the upper intestine. Whether there was an increased VIP content in the pyloric sphincter as has been reported/.....

reported in some mammals (Alumets et al 1979) has not been established.

The potent action of VIP as a vasodilator, seen in mammals (Said and Mutt 1970) may not be of importance in amphibians as VIP-ergic nerves were only rarely seen innervating blood vessels.

Reptilia. In the Testudinata and Lacertilia VIP was found throughout the intestinal tract, including the stomach. The distribution of the immunoreactive fibres and cell bodies was similar to that seen in the amphibia, although some positive cell bodies were seen in the sub-mucous plexus. The VIP-containing fibres were seen predominantly in association with the circular muscle, therefore, as in the amphibians, its action is probably associated with smooth muscle relaxation.

In the snakes a variety of results were found, in the majority of species (boid and natricine) the VIP-immunoreactivity was confined to the pancreas, except in Nerodia rhombifera where no VIP-immunoreactivity at all was detected. Nerodia rhombifera is a water snake whereas the other snakes investigated in the present study are terrestrial. Whether there is any significance in the reduction in VIP-content and an aquatic existence is at present unknown.

In the Viperidae, VIP-immunoreactive fibres and cell bodies were seen throughout the intestines and stomach. The intestine in the Viperidae was morphologically/.....

morphologically simple with a thin gut wall and long tapering gut folds. The immunoreative VIP fibres were confined to the gut wall, never being detected in the gut folds.

The finding of VIP-immunoreactive fibres and cell bodies in the gut only of the Viperidae was interesting as in this family the intestine was found in loops and not as a straight tube as in the other snakes (see Diagram 8 , Page 122). Clearly, the looping of the gut in the Viperidae will restrict the passage of food from duodenum to colon, requiring increased peristaltic movement, and it may be that the presence of the VIP-containing fibres was associated with this.

In the Crocodylidae several differences were observed. As previously mentioned the gut wall in these animals was exceptionally thick which may have some significence on the results of the immunostaining. The two enteric plexuses were well delineated being separated by a thick circular muscle layer. The VIP-containing fibres and cell bodies in the gut wall were mostly found in the sub-mucous plexus and the upper layers of the circular muscle. It is possible that further fibres and cell bodies may have been present in the lower circular muscle and the myenteric plexus but were not localised due to the problems in the collection and fixation of the whole thickness specimens.

An/.....

An exceptional finding was seen in the duodenal mucosa where the VIP-containing nerves were found not only in the muscle coat and sub-mucous plexus but also in the complex gut folds. It appeared as if, in addition to the two enteric plexuses a third plexus occurred just underneath the mucosal folds. A similar plexus has been reported in teleost fishes (Burnstock 1959) but not in other vertebrates. From the VIP-immunostaining it appeared that the plexus contained occasional intrinsic cell bodies, therefore differing from the amphibian sub-mucous plexus which was only a loose network of fibres. In addition to the third plexus, this region also showed an exceptionally rich innervation of the villus-like folds. The immuno-reactive fibres did not appear to be in contact with the blood capillaries but were in close association with the mucosal epithelium (see Figure 77, Page 192) It may be that in this situation VIP is involved in the control of the gut secretion.

In general, from the results of the use of the region-specific antisera it appears as if the VIP-like peptide detected in all the reptilian species is very similar to the mammalian (porcine) form as seen with the amphibians.

ii. Substance P

Amphibia. In the amphibians the substance P-containing fibres were confined to the stomach occurring/.....

occurring around the blood vessels.

The two main actions originally attributed to substance P were vasodilation and smooth muscle contraction (Von Euler and Gaddum 1931, Pernow 1953). From the anatomical localisation it appears likely that in these amphibians, substance P is involved in vasodilation.

However the frog Atelopus oxyrhynchus contained numerous substance P immunoreactive fibres predominantly in the myenteric plexus, although no fibres were seen in the circular muscle layer. As the fibres were absent from the muscle layer it is unlikely that substance P is influencing smooth muscle contraction.

Recent work on the ultrastructural localisation of substance P in mammalian gut has shown the presence of numerous terminals around the cell bodies of the myenteric plexus, several of which form synaptic contact with the cell bodies (Probert et al 1980). The same phenomenon may be occurring in this frog, in which case the substance P may be affecting the function of the ganglion cells.

Reptilia. In the reptiles, substance P-containing fibres were seen in the intestines, with varying distributions, occurring either in the duodenum or in the ileum and colon.

In the Testudinata, Lacertilia and Crocodylidae, the fibres were found in both the myenteric plexus and the circular muscle layer. It is possible therefore that/.....

that in these animals substance P has an effect on both the contraction of smooth muscle and on the control of the ganglion function.

In the snakes (except the Viperidae) a similar staining pattern to that seen in Atelopus oxyrhynchus was seen, with the fibres occurring in the myenteric plexus, suggesting that its role is confined to the control of the ganglion cells.

During this study several antisera to substance P were used but they were all specific for the C-terminus of the molecule. Therefore from the immunocytochemical studies there can be no firm indication as to whether the peptide detected in this study is identical to the mammalian form.

In a recent report, using analysis of parallelism of dilution curves, it was suggested that the peptide detected in frog and pigeon intestine with a C-terminal antiserum was not identical to the mammalian peptide. However, the peptide detected in frog skin and frog and pigeon brain was identical to the mammalian peptide (Creagh et al 1980). Clearly, further biochemical work is necessary to evaluate the data correctly.

iii. Endogenous Opiates

Both enkephalin and endorphin were found in the enteric nerve fibres of the amphibians. The enkephalin-immunoreactivity was confined to the antrum with the endorphin-like peptide being detected in the duodenum.

The/.....

The enkephalin-immunoreactive fibres were seen in the myenteric plexus only, no fibres were seen in the muscle layers.

The known actions of enkephalin in the mammalian gut, include inhibition of smooth muscle contraction (Waterfield et al 1977), and inhibition of neuronal activity (North and Williams 1976, Sakai et al 1978). With the present information indicating that the enkephalin-like peptide is limited to the myenteric plexus,it is likely that its action will involve regulation of the ganglion cells.

Endorphin-immunoreactive fibres were seen in the duodenal mucosa in the sub-mucous plexus. The action of endorphin in the gut is thought to be linked to increased transit time, whether this effect is common to the frog gut remains to be elucidated.

iv. Bombesin

Bombesin-containing fibres were seen in several amphibian and reptilian species. In view of the present problems encountered in ensuring that the staining is truly specific, no comment on the possible significance of these fibres can be made.

v. Other peptides

No evidence for nerve fibres containing gastrin/CCK, somatostatin or neurotensin could be demonstrated, although these have been reported in mammals (Rehfeld et al 1980 , . Costa et al 1977, Hokfelt /.....

Hökfeldt et al 1977, Schultzberg et al 1980).

vi. NSE

An interesting finding was that NSE-immunoreactivity when observed was confined to nerve fibres and cell bodies, in no case were positive endocrine cells detected. This is in contrast to the findings in mammalian tissue where both nerve fibres and mucosal endocrine cells have been detected (Marangos et al 1979, Facer et al 1980).

It is, of course, possible that the iso-enzyme in the pre-mammalians differs from that in mammals, and thus only those structures with a high content are detectable. Clearly further work on this aspect is required.

Chapter 7 - SCANNING ELECTRON MICROSCOPY

7.1. Note

The findings from this part of the study were not directly linked to the localisation of the regulatory peptides but the study was undertaken to give an indication of the changes in structure of the intestines between amphibians, reptiles and mammals. It was also an attempt to introduce the concept of the three dimensional structure of the samples which can only be represented in two dimensions by the other techniques employed in the present work. Further, it may be that the gross morphological appearance of the samples will be of importance in assessing the significance of the immunocytochemical and ultrastructural results.

The scanning electron microscopy was only carried out on intestinal samples from the species Rana temporaria, Alligator mississipiensis and for comparison Rattus rattus.

7.2. Results

a. Anura - Rana temporaria

In Rana temporaria, material from the duodenum and small intestine was examined, in order to establish the surface appearance of the gut folds. In all regions the mucosa was found to be relatively flat with some lateral folding (Figure 83). At higher magnification the surface could be seen to contain numerous mucous producing glands (Figure 84) each being surrounded by cells with well developed microvilli (Figures 85 & 86).

b. Alligator mississipiensis.

As previously observed in the material collected for light/.....

Figure 83

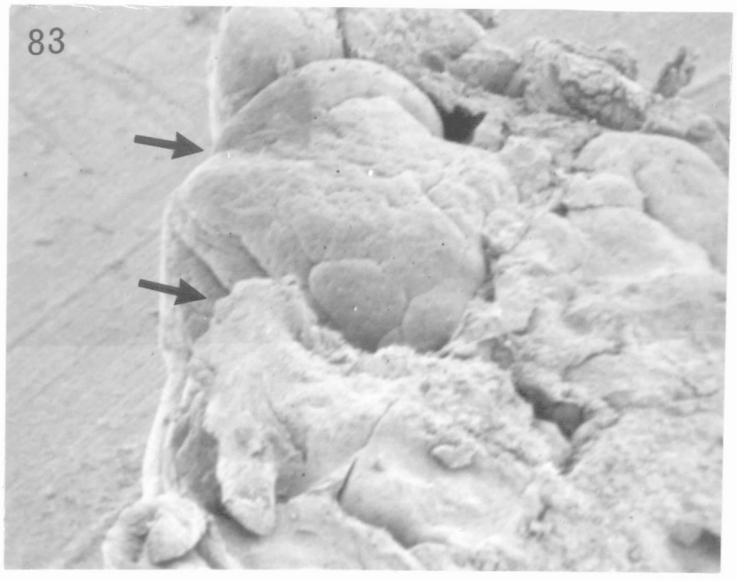
Duodenal mucosa showing the gut folding (arrows).

x 20

Figure 84

At higher magnification the borders of the individual cells can be seen (arrows).

x 500


83

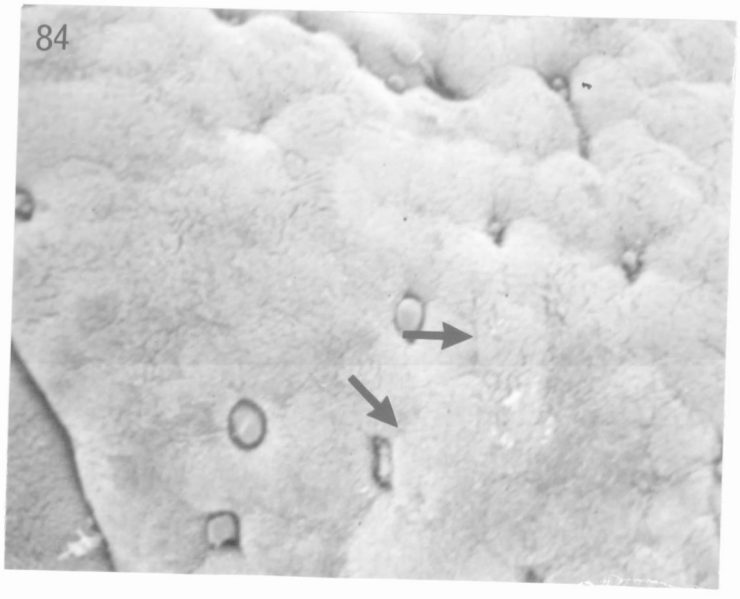

84

Figure 85

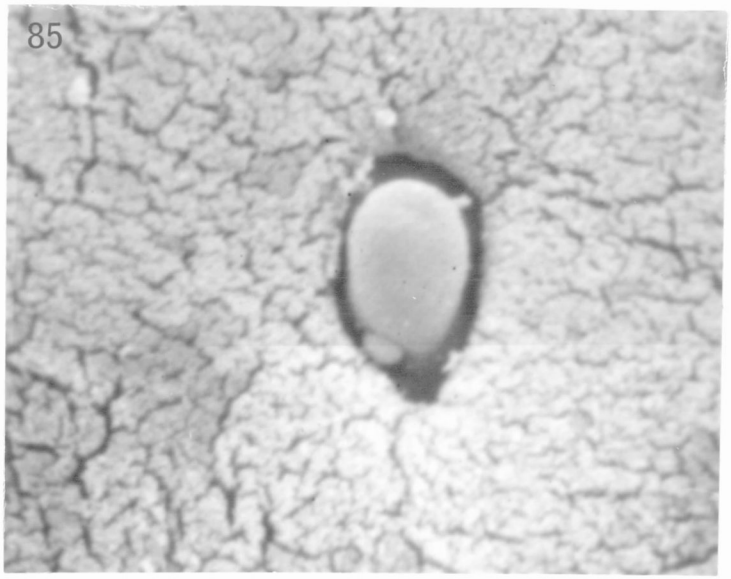
Higher magnification showing the microvilli surrounding a mucin gland.

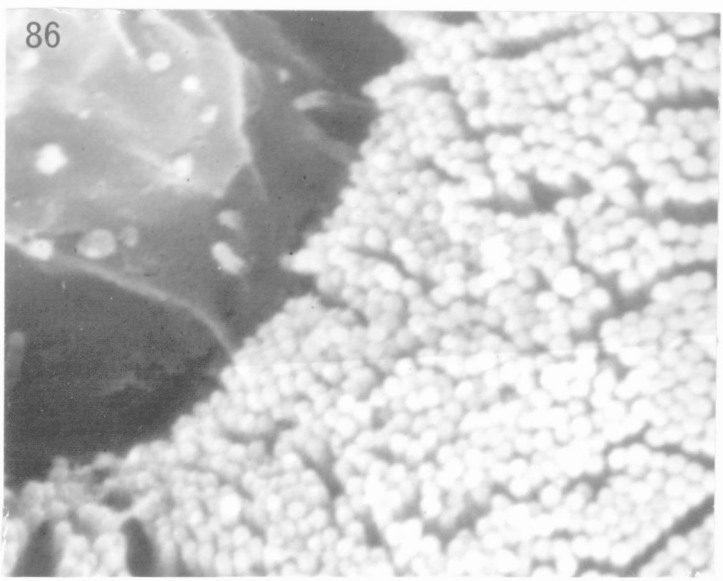
x 2,000

Figure 86

Detail of the microvilli around the mucin gland.

x 5,000


85


86

light microscopy, the structure of the gut varied with the region of the intestine. By use of the scanner, details of these differences could be examined further. The macroscopic appearance of the duodenal and small intestinal surfaces was clearly identified at low magnification. Figure 87 shows the characteristic diamond pattern of raised folds in the duodenum which contrasts with Figure 88 showing the small intestinal surface where only one half of the diamond pattern was present, giving the appearance of a zig-zag pattern. A comparison of higher magnification pictures showed that the ridges or folds in both regions were composed of the same type of tissue (Figure 89). At higher magnification the surface epithelium closely resembles that seen in the frog duodenum (compare Figure 90 with Figure 84).

It has also been previously mentioned that in the alligator the gut wall was extremely thick which made collection of full thickness samples difficult to achieve and made evaluation of the immunostaining a problem.

To give an idea of the depth of the gut wall in this species a montage of two low magnification pictures has been made showing the duodenal area from mucosa through to the serosa (Figure 91). As can be seen from this illustration, the gut of this animal was exceptionally difficult to handle as the mucosa formed approximately one fourth of the total depth of the gut thus making the collection of flat cross-sectional samples a considerable challenge.

c./.....

Figure 87

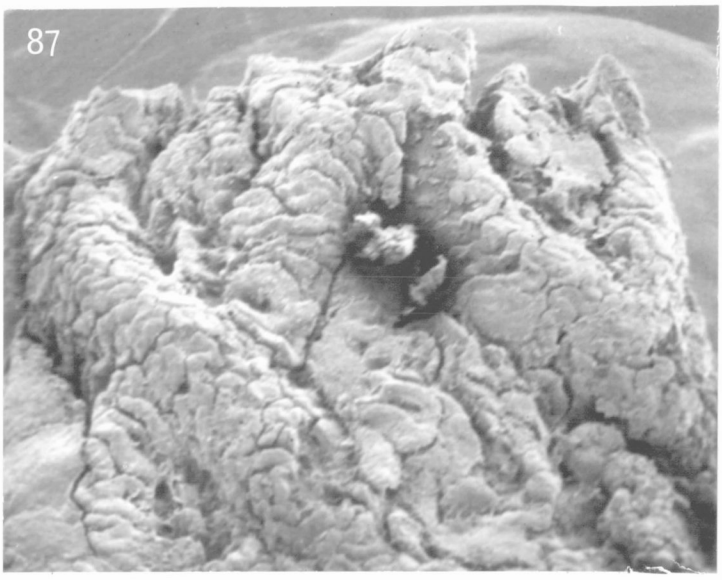
At low magnification the diamond shape of the raised duodenal folds can be seen.

x 20

Figure 88

In the small intestine the raised folds give a zig-zag pattern.

x 20


87


88

Figure 89

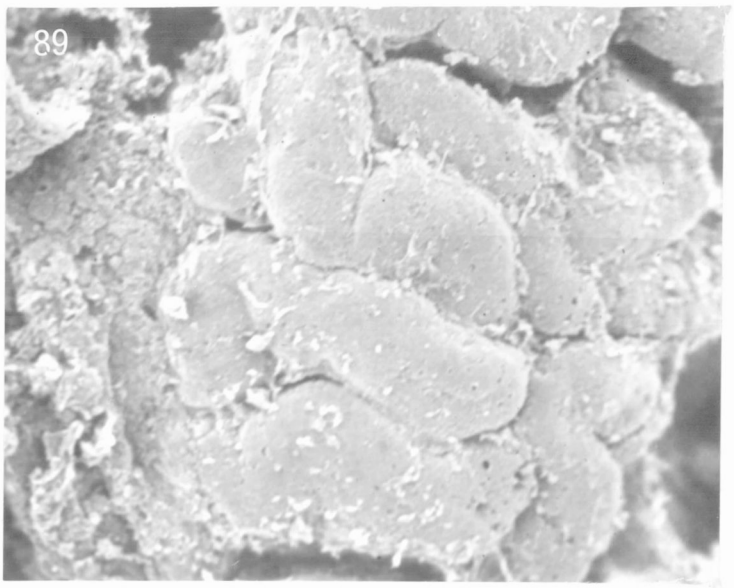
Detail of the arrangement of the duodenal folds in one of the ridges.

x 200

Figure 90

At higher magnification the features of the mucosa resemble those in Rana temporaria (see Figure 84).

x 500


89

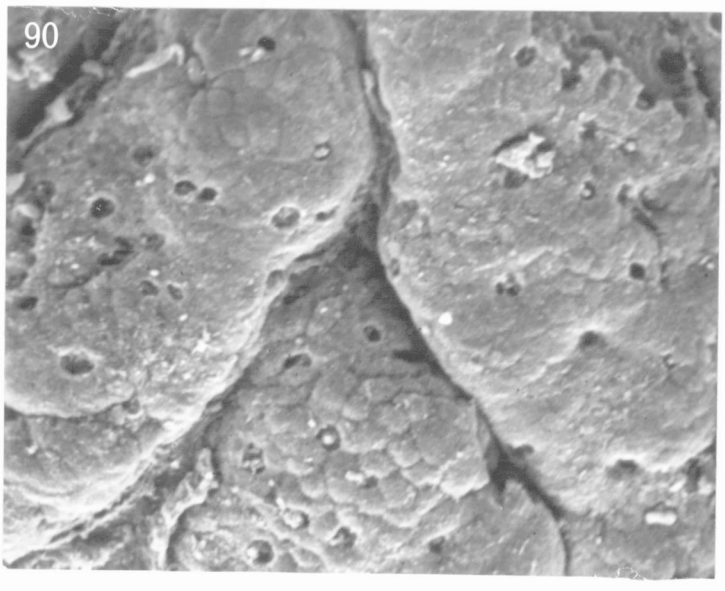

90

Figure 91

Composite photograph showing a cross-section through the alligator duodenum.

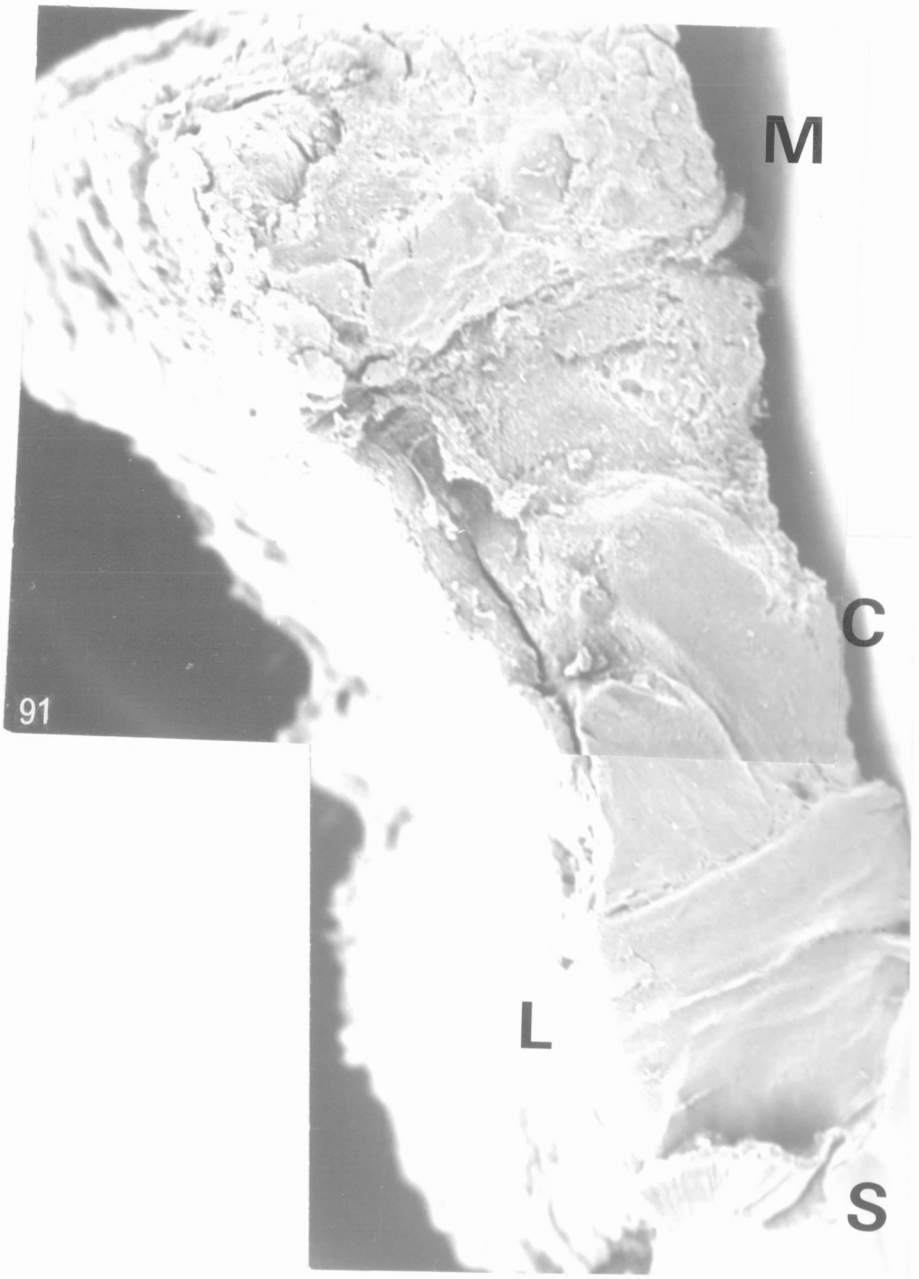
M = Mucosa

C = Circular muscle

L = Longitudinal muscle

S = Serosa

x 20


M
C
91
L
S

c. Rattus rattus

At the lower magnification the rat duodenum was seen to consist of numerous villous structures which were totally lacking in the frog or alligator duodenum (Figure 92). At high magnification it became obvious that the scale of the villus structures in the rat greatly increased the surface area available for absorption (Figure 93) whereas in the frog and alligator it was the individual microvilli which were better developed in order to increase the absorptive area. It was impossible to photograph the individual microvilli in the rat as the resolution of the scanning microscope gave insufficient detail.

d. Summary

The results of the scanning electron microscopy were of interest in order to show the changes in the development of the intestinal surface between the different species.

It is intended to have a further look at the alligator material from all regions of the intestine as the structure appears to vary greatly from any others seen during this study.

Figure 92

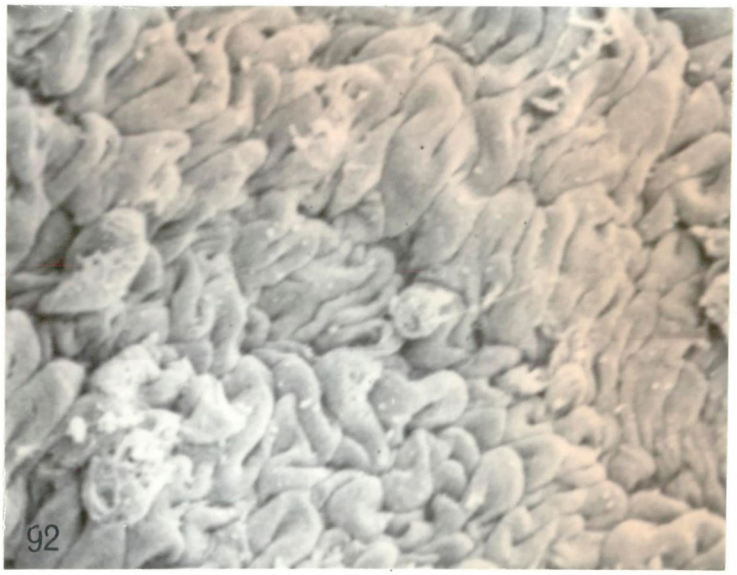
At low magnification the duodenal villi superficially resemble elaborate gut folds.

x 20

Figure 93

At higher magnification the individual villi can be discerned.

x 200


92


93

Chapter 8 - ELECTRON MICROSCOPY - TRANSMISSION

8.1. Results

Note:

The original aim for the electron microscopical study was to establish whether there was any relationship between the ultrastructural morphology of the peptide-containing endocrine cells in the amphibians and reptiles as compared to mammals, in addition to establishing the individual ultrastructural characteristics of the immunoreactive cells.

It became evident in the early stages of this work that there was little or no similarity between the cells as identified in the pre-mammalian species and those in mammals.

It was therefore decided that rather than a description of the species by species characterisation of the individual cell types that one interesting area would be chosen and the results of representative species reported to show the types of ultrastructural appearance detected.

The region chosen was the pancreas, as the pancreatic islet cells are the best characterised of the mammalian cells, being described in several species, so that a comparison with the pre-mammalian species was of great interest. Also, the insulin cells are reportedly easily recognisable in lower species by virtue of the presence of a crystalline core in many/.....

many of the granules, again the possibility of either confirming or refuting this idea was of interest.

For the purpose of the comparison, the following species were chosen:-

Urodela	-	Cynops hongkongensis
Anura	-	Hyla arborea
Testudinata	-	Chelydra serpentia
Serpentes	-	Agkistrodon piscivora
Crocodilia	-	Alligator mississipiensis

The size of the secretory granules was established by measuring the diameter of a mimimum of 50 granules $\pm$ SEM.

8.2. Urodela - Cynops hongkongensis

The cell types were identified in material fixed in glutaraldehyde and with osmium tetroxide post-fixation by the semithin/thin method.

Insulin. The insulin-immunoreactive cells were commonly found occurring in ribbons on the edges of the exocrine tissue in close association with blood vessels and capillaries. The cells contained numerous large (407 $\pm$ 19nm) irregular granules some of which at higher magnification were shown to have bar shaped crystalline inclusions (Figure 94). In several cells exocytotic profiles were seen between the cell and the lumen of the blood capillaries (Figure 95 and 96).

Glucagon. The glucagon cells were far less numerous than the insulin or somatostatin cells. The cells were detected by/.....

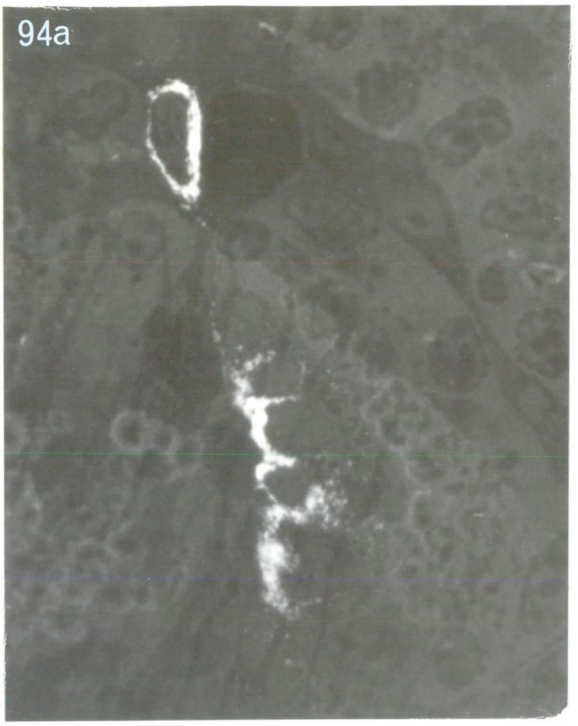
Figure 94

a - Semithin (500nm) section showing several insulin-immunoreactive cells. The cells identified in the serial thin (60nm) section are indicated by the arrow.

x 400

b - Insulin-containing cells identified in serial thin section on the edge of a blood vessel (BV).

x 6,000


94a

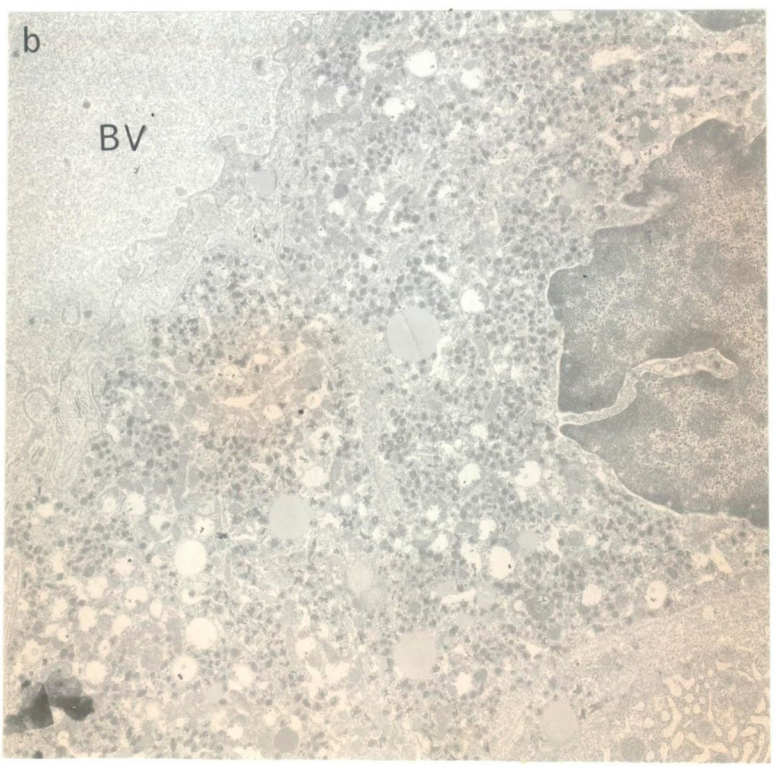

b
BV

Figure 94c

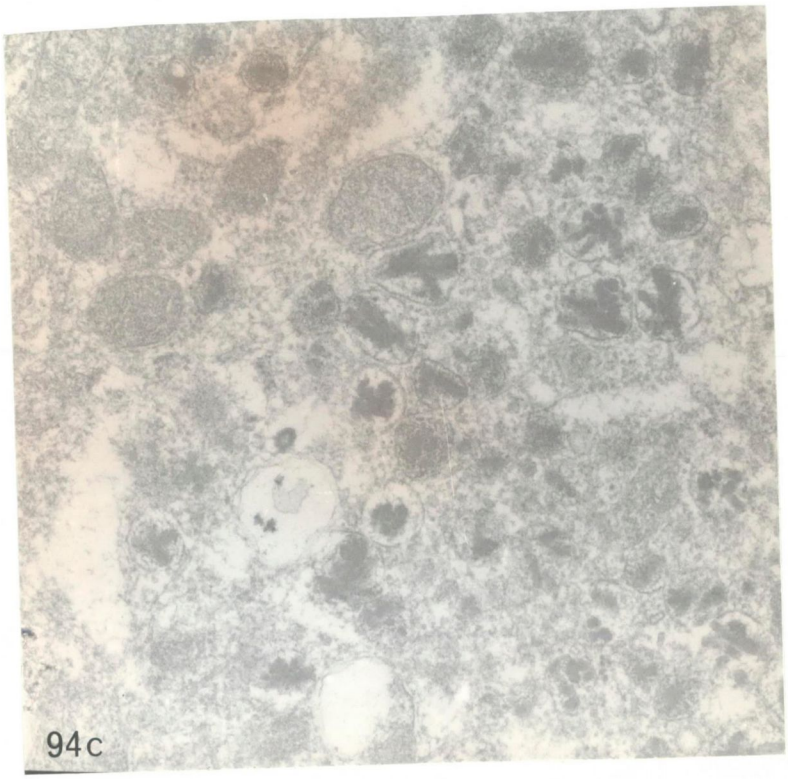
c - Detail of the secretory granules showing the typical bar-shaped inclusions.

x 40,000

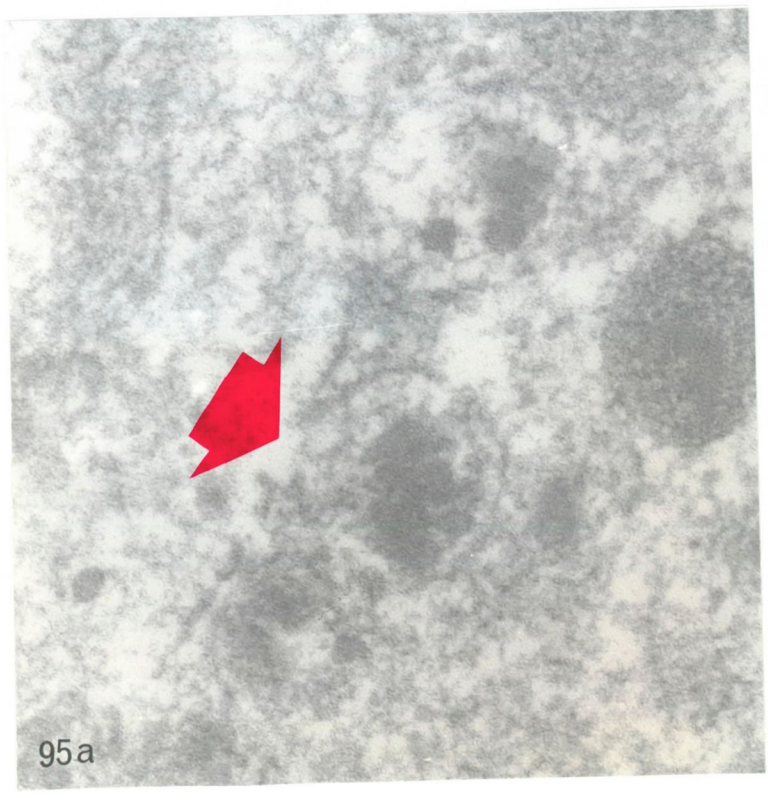
Figure 95

a - Exocytotic granule showing the secretory granule membrane fused to the plasmalemma (arrow).

x 80,000



94c



95a

Figure 95b

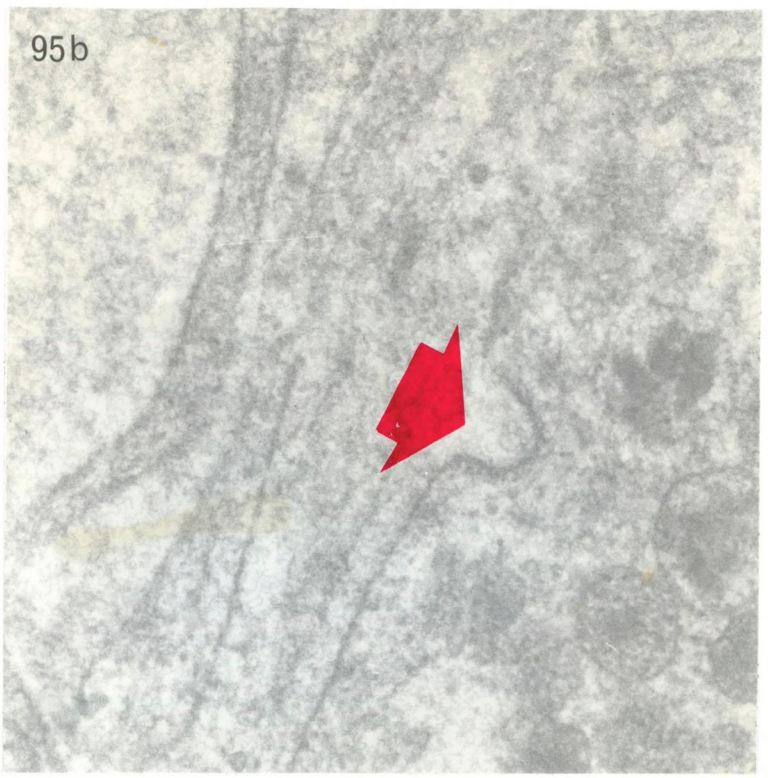
b - Typical exocytotic profile after the release of the granule content (arrow).

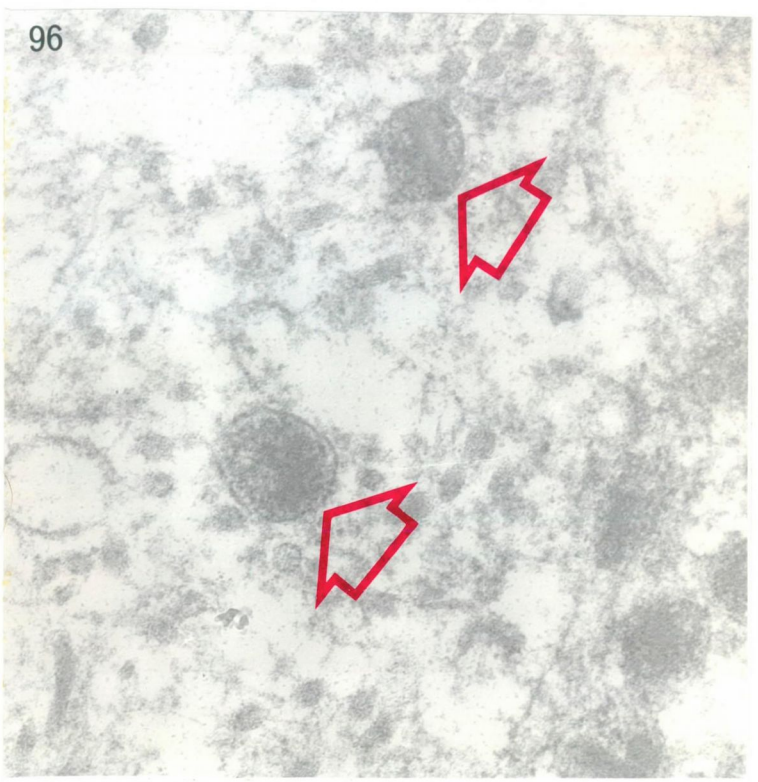
x 80,000

Figure 96

c - Two insulin-like secretory granules (arrows) actually within the blood capillary.

x 80,000


95b


96

by both the specific pancreatic glucagon (C-terminal) and the cross-reacting glucagon (N-terminal) antisera. The cells were also seen in close association with the capillaries, containing numerous, irregular, medium sized (229 ± 8nm) electron-dense secretory granules with a clear limiting membrane (Figure 97).

Somatostatin. These cells were found scattered throughout the pancreatic tissue, not necessarily associated with either other endocrine cells or the blood capillaries. The secretory granules were uniformly round and electron-dense with an average diameter of 131 ± 6nm (Figure 98).

An additional, unidentified cell type was also seen in this material. The secretory granules present in the cytoplasm were fairly small (100 ± 4nm), very irregular and of varying electron-density. None of the antisera used in this study detected these cells (Figure 99).

8.3. Anura - Hyla arborea

The cells were identified in modified Karnovsky-fixed material by the semithin/thin method.

Insulin. The insulin cells were usually found in small to large cell clusters, closely surrounded by the exocrine tissue. The secretory granules were either small (110 ± 4nm), uniformly round and electron-dense or contained several small, bar-shaped inclusions surrounded by a clear halo/.....

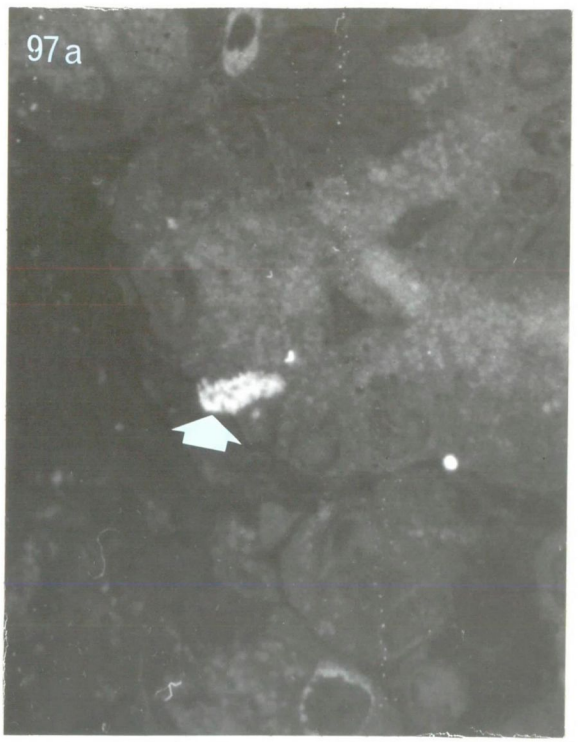
Figure 97

a - Semithin (500nm) section stained with the C-terminal glucagon antiserum showing a positive cell (arrow).

x 400

b - Serial thin (60nm) section showing the same cell, bordering a capillary (c).

x 6,000


97 a

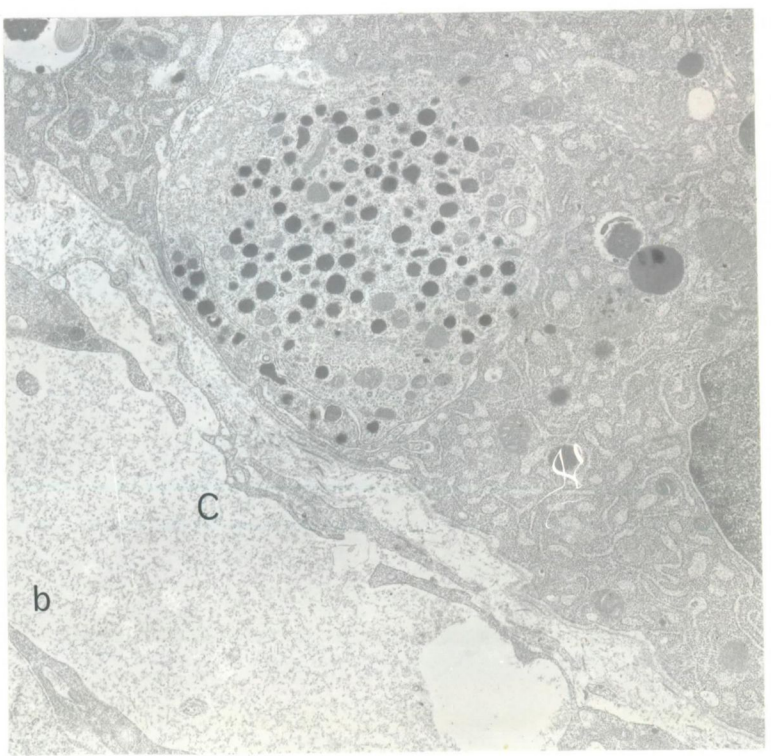

C
b

Figure 97

c - Detail of the secretory granules which appear to be cylindrical in shape (arrow).

x 30,000

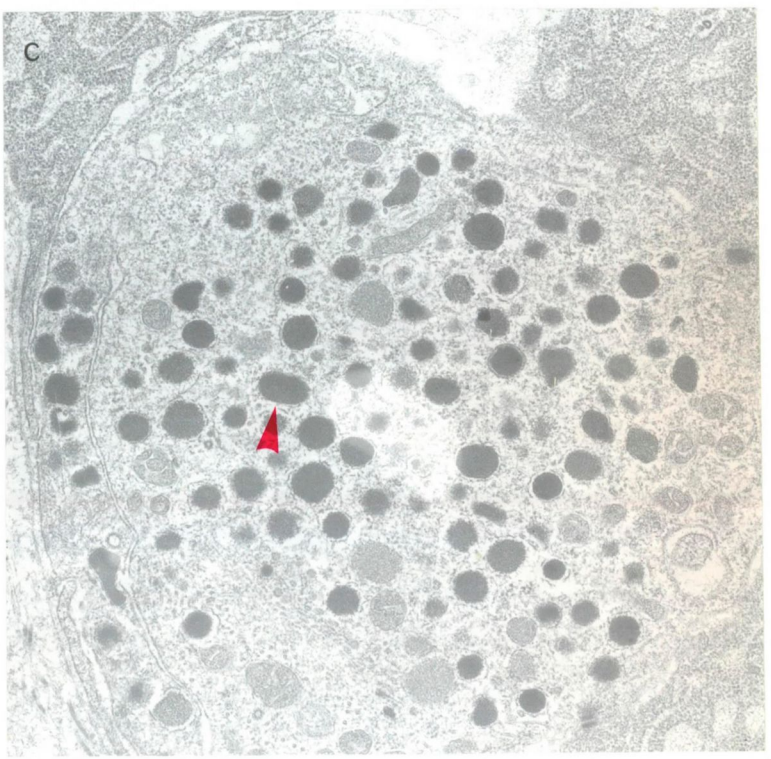

C

Figure 98

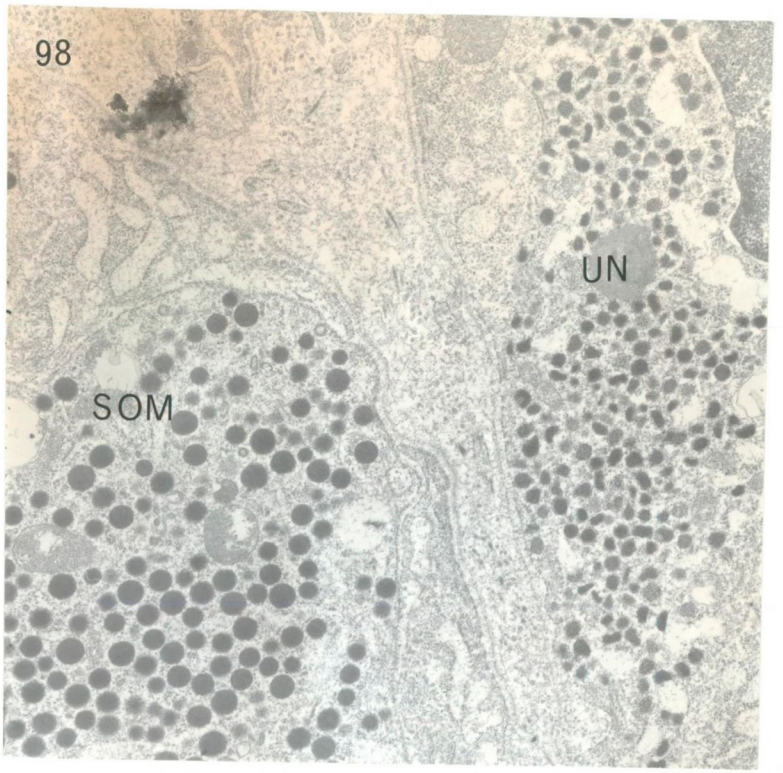
Somatostatin-containing cell (SOM) and unreactive cell (UN).

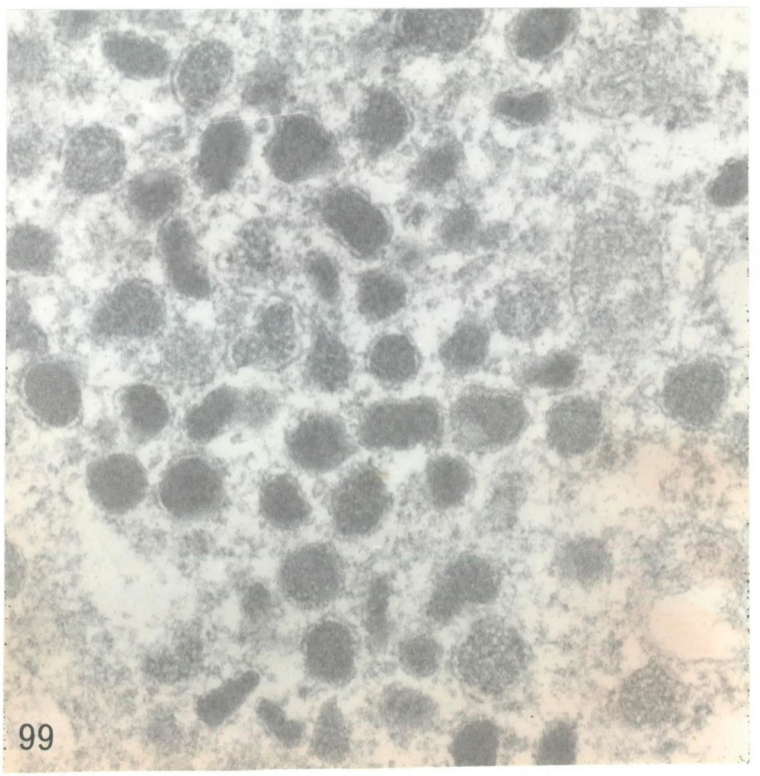
x 20,000

Figure 99

Detail of the secretory granules seen in the cell which did not react with any of the available antisera.

x 60,000


98
UN
SOM


99

halo (295 ± 12nm) (Figure 100).

Glucagon. These cells were either found as small clusters or as single cells. They were characterised by irregular, bar shaped granules which had a mean cross-sectional diameter of 186 ± 8nm) with a length of 315 ± 11nm (Figure 101).

Somatostatin. Somatostatin cells were most often found in association with the insulin cells. They were characterised by small (161 ± 7nm), round, relatively electron-lucent granules (Figure 102).

8.4. Testudinata - Chelydra serpentia

The cells were identified in glucaraldehyde and osmium post-fixed material by the semithin/thin method.

Insulin. The cells were usually found in small clusters. The secretory granules were obviously crystalline in nature, with a variety of different inclusions, ranging through from simple rods to star shaped clusters. In every case the inclusions were surrounded by a clear limiting membrane. The size of the granules, calculated as the diameter of the membrane bound area, was 333 ± 12nm (Figure 103).

Glucagon. The granules found in these cells were slightly irregular, with a mean diameter of 210 ± 5nm, electron-dense with/.....

Figure 100

a - Semithin (500nm) section showing positive insulin cells.

x 400

b - Serial semithin section showing positive glucagon cells.

x 400

c - The same area identified in a serial thin (60nm) section.

x 3,000

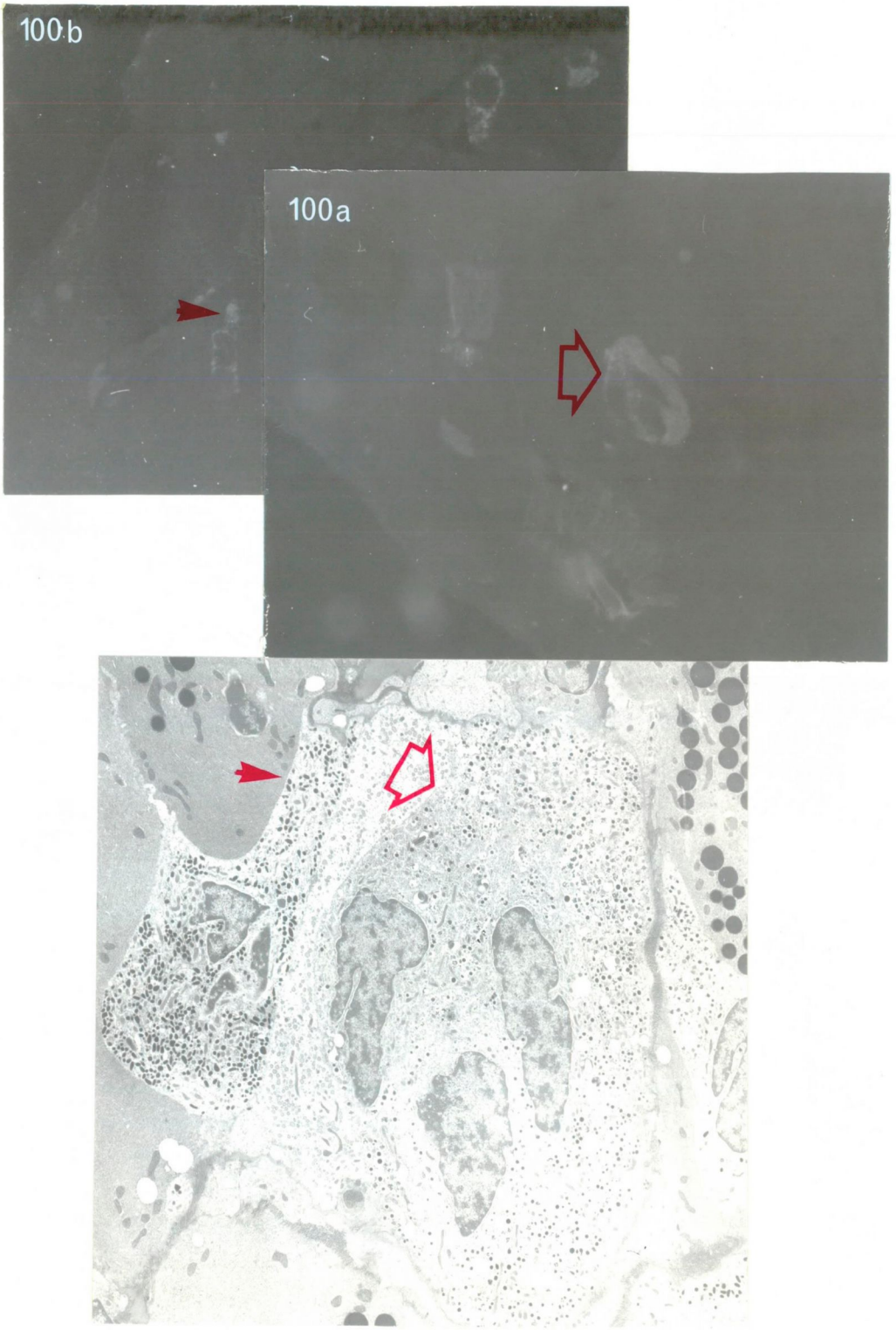

100b
100a

Figure 100d

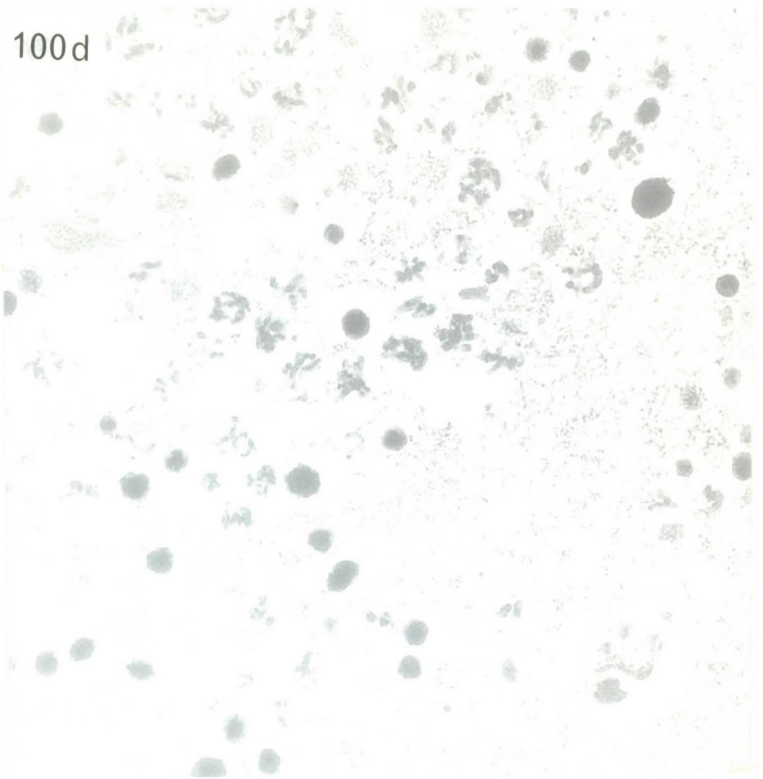
a - Detail of the secretory granules in the insulin cell.

x 10,000

Figure 101

- Detail of the secretory granules in the glucagon cell.

x 10,000


100d

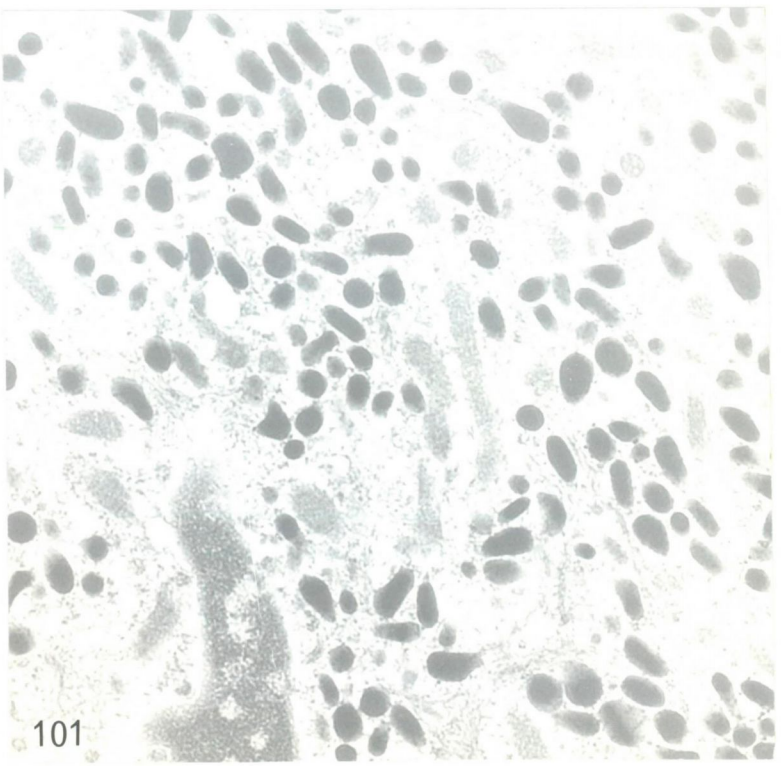

101

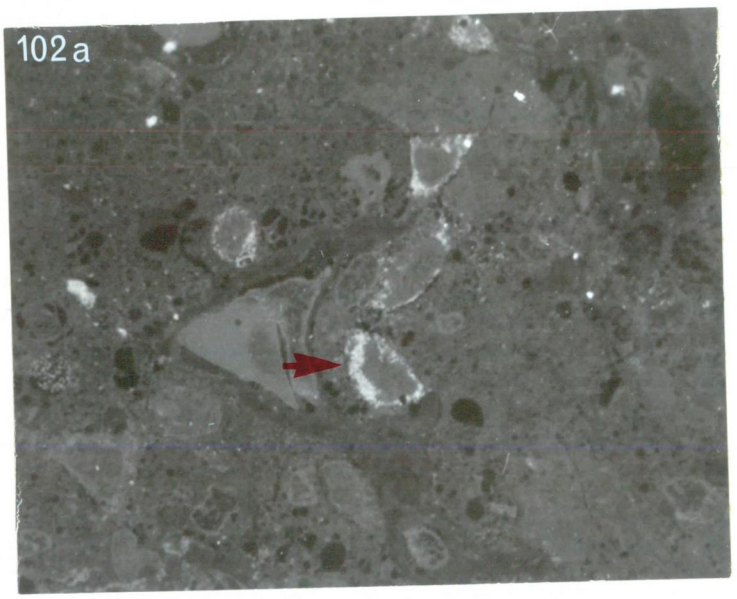
Figure 102

a - Semithin (500nm) section stained with the somatostatin antiserum showing a positive cell (arrow).

x 400

b - The same cell identified in the serial thin (60nm) section (arrow).

x 3,000


102 a


b

Hyla arborea

Figure 102

c - Detail of the somatostatin-containing cell showing the characteristic secretory granules.

x 10,000

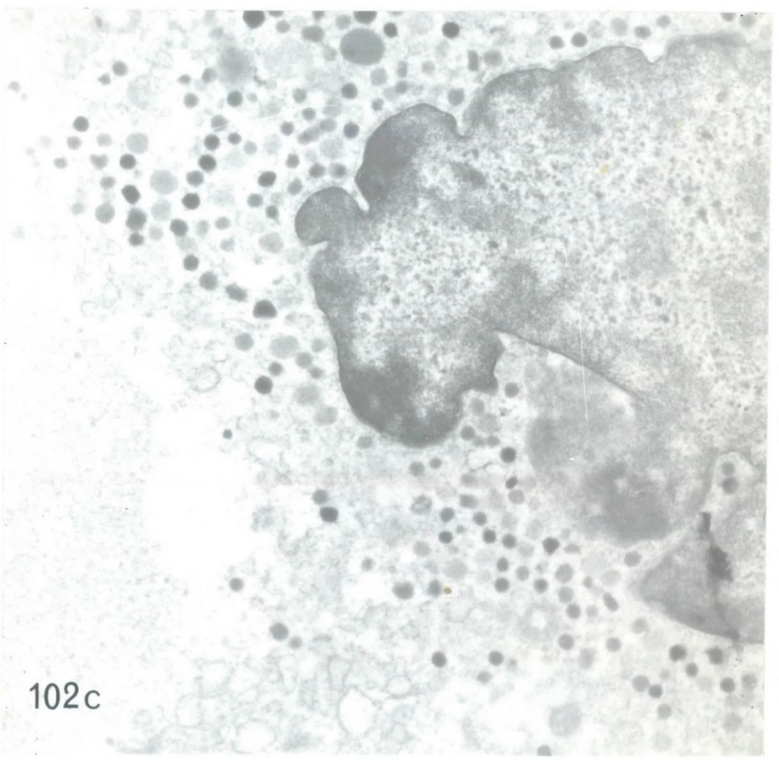

102c

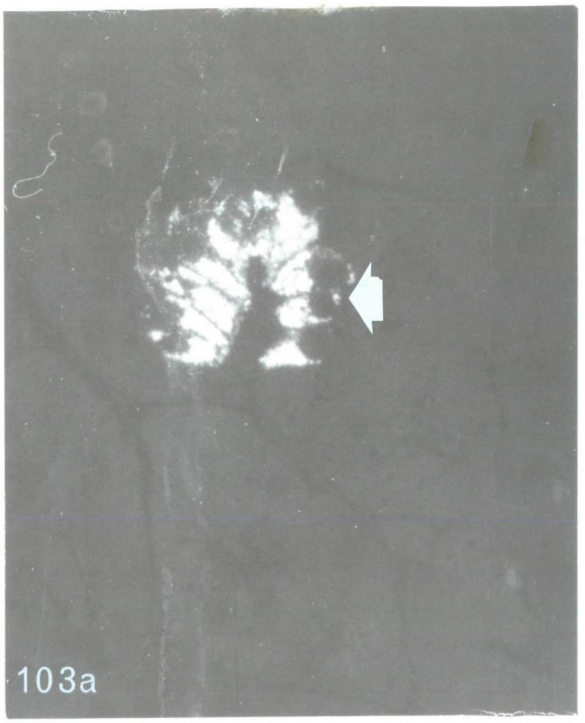
Figure 103

a - Semithin (500 nm) section showing a group of insulin-containing cells. The cells identified in the serial thin section are shown by the arrow.

x 400

b - The cells are identified in the serial thin section.

x 6,000


103a

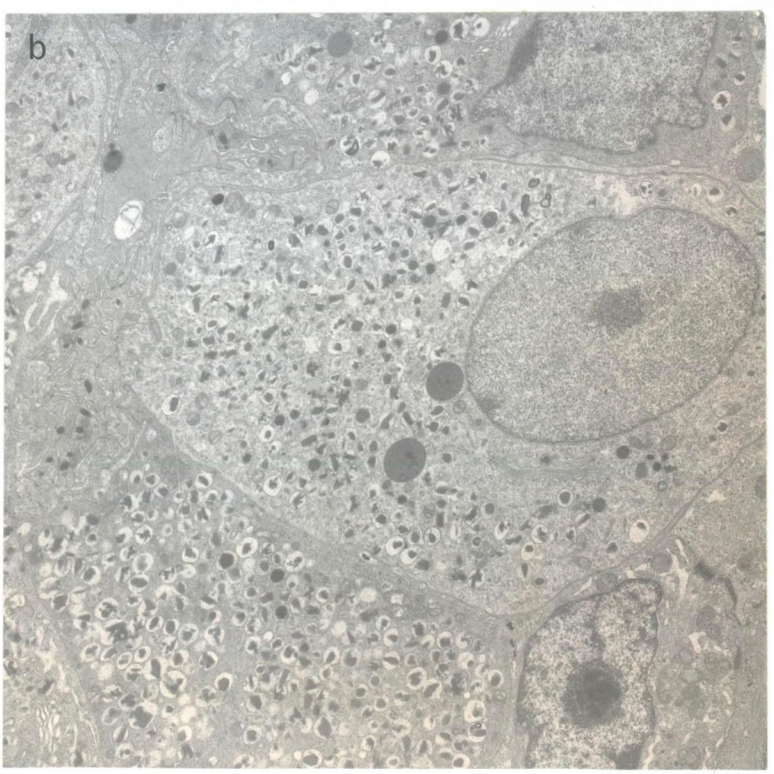
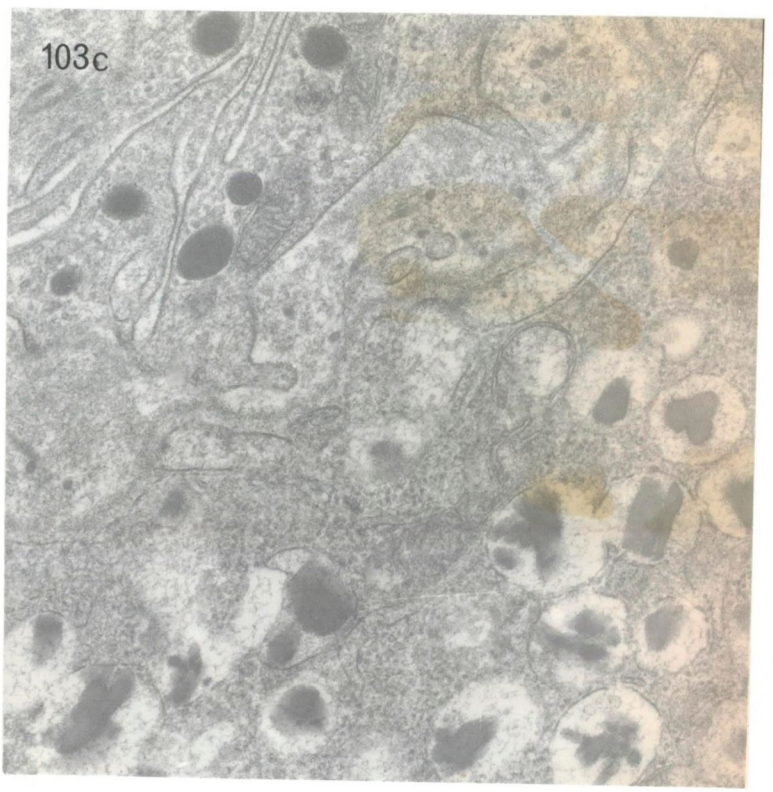

b

Figure 103

c - Detail of the secretory granules present in the cell.

x 40,000


103c

with a clear limiting membrane (Figure 105).

Somatostatin. They were found as either single cells or small groups in association with the insulin cells. They contained fairly large (236 ± 7nm), round, floccular granules with a clear limiting membrane (Figure 104).

PP. These cells were often found as single elements amongst the exocrine tissue. They contained predominantly round, medium sized granules (225 ± 8nm) of uniform electron density and showing a clear limiting membrane (Figure 106).

8.5. Serpentes - Agkistrodon piscivora

The cells were identified in glutaraldehyde and osmium post fixed material by either the semithin/thin or the gold labelled anti-rabbit IgG methods.

Insulin. Insulin cells were found throughout the large islet structures. These contained large (495 ± 27nm) secretory granules, which varied from round and homogeneous, round with a central core to large and empty with a small eccentric core (Figure 107).

Glucagon. Glucagon cells were predominant in this tissue. The cells contained extremely large granules (553 ± 27nm), round and of varying electron-density with a clear limiting membrane (Figure 108).

Somatostatin/.....

Chelydra serpentia

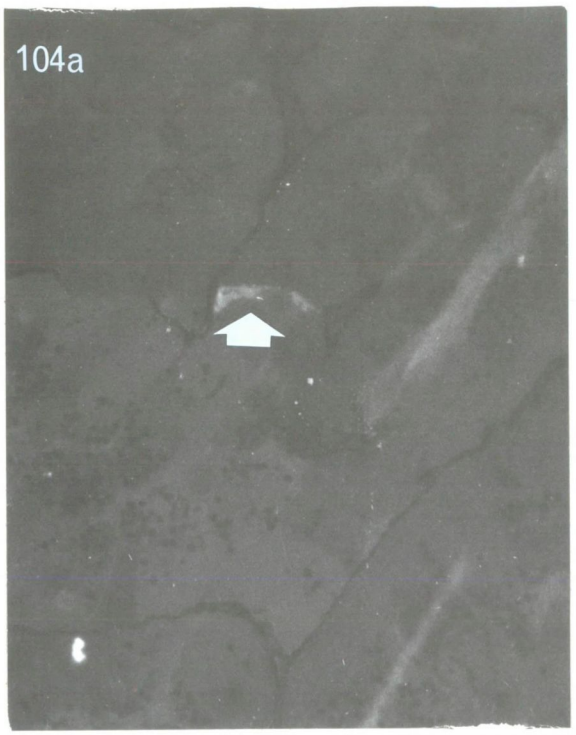
Figure 104

a - Semithin (500nm) section showing an immunoreactive - somatostatin cell (arrow).

x 400

b - The same cell as identified in a serial (60nm) section.

x 6,000


104a

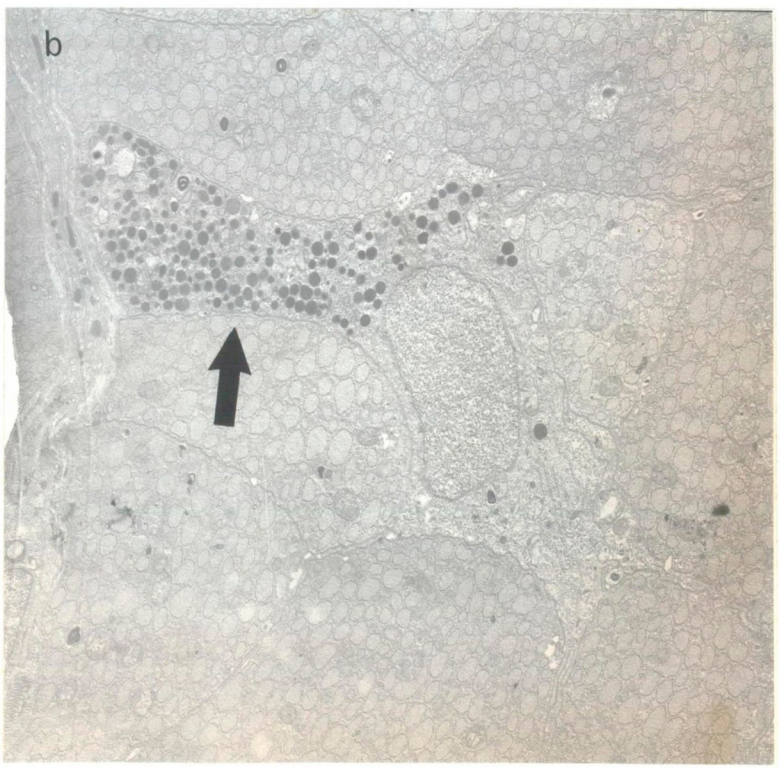

b

Figure 104

c - Detail of the secretory granules present in the somatostatin-containing cell.

x 40,000

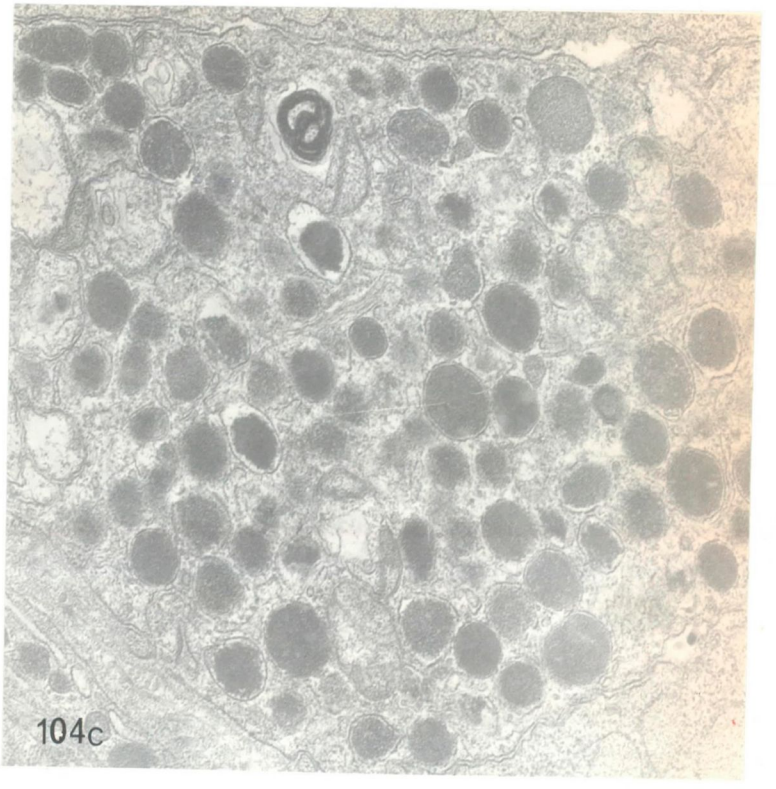

104c

Figure 105

Micrograph showing both the glucagon- and PP-containing cells.

GL = glucagon

PP = pancreatic polypeptide

x 6,000

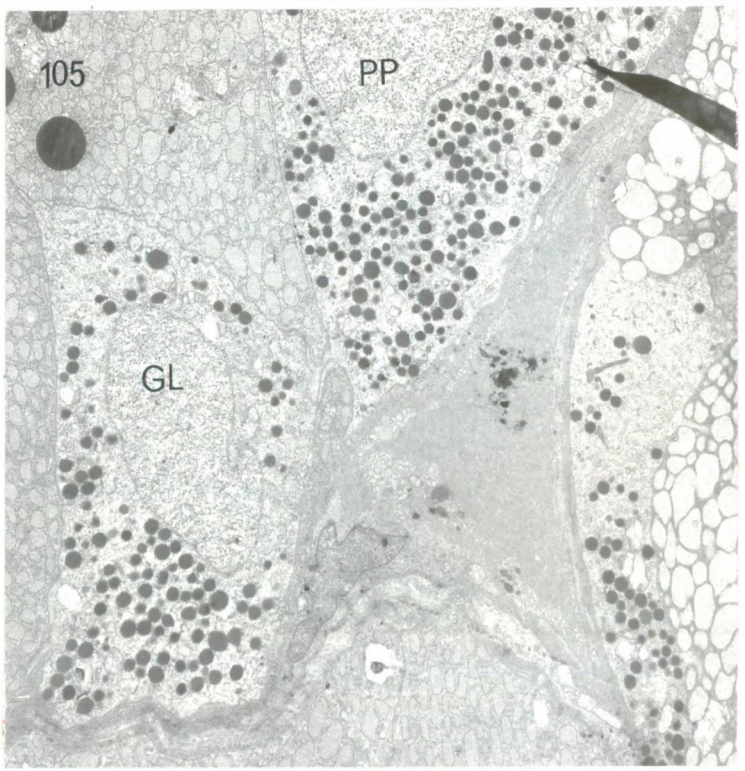

105
PP
GL

Figure 105

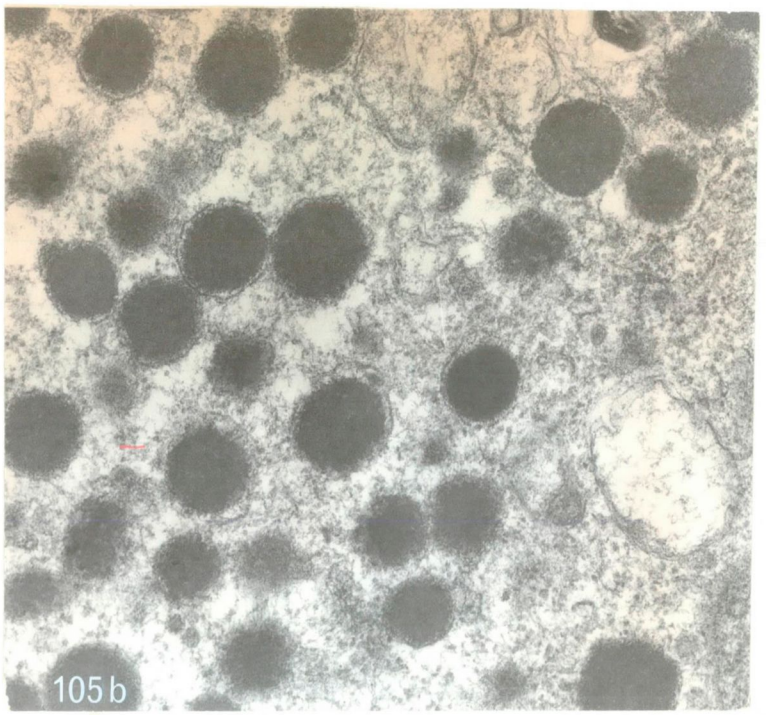
b - Detail of granules in the glucagon-containing cell.

x 60,000

Figure 106

- Detail of the granules in the PP-containing cell.

x 60,000


105 b

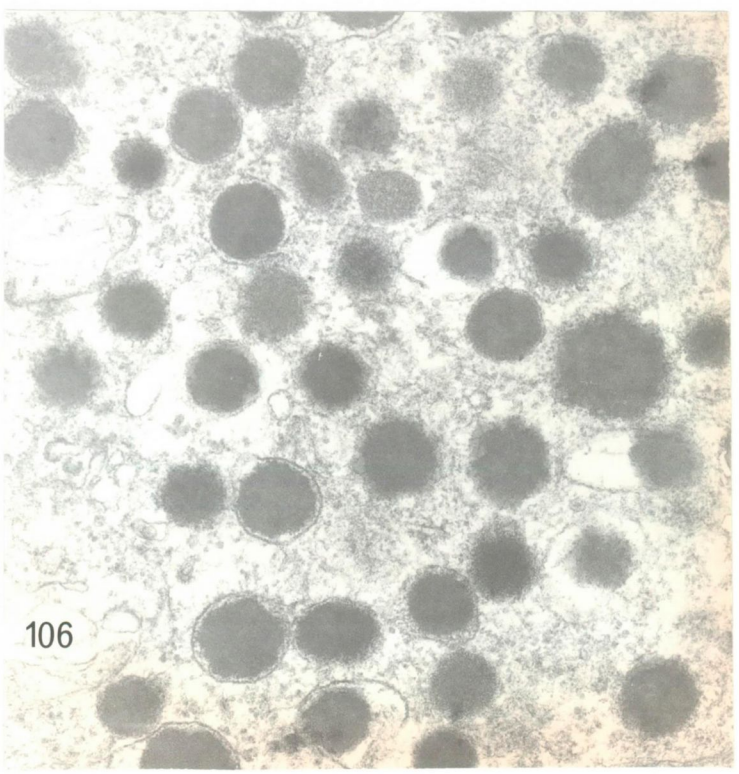

106

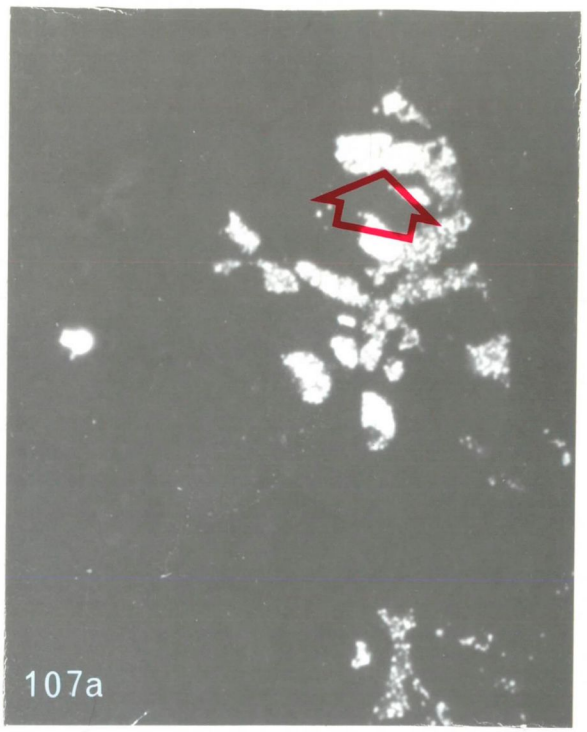
Figure 107

a - Semithin section showing numerous insulin-containing cells, the cells identified in the serial thin section are indicated by the arrow.

x 400

b - The insulin-containing cells (arrows) as identified in the serial thin section.

x 6,000


107a

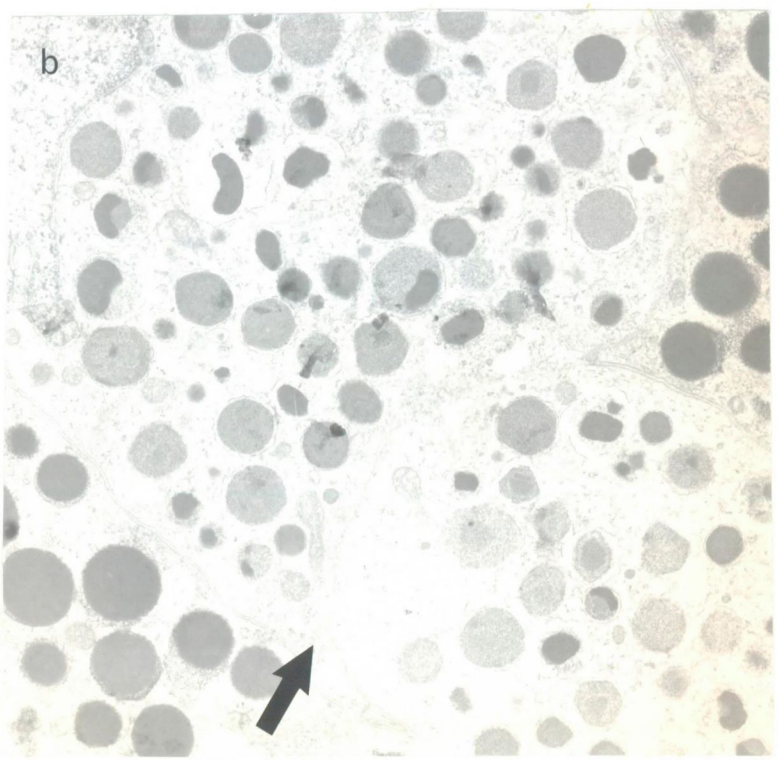
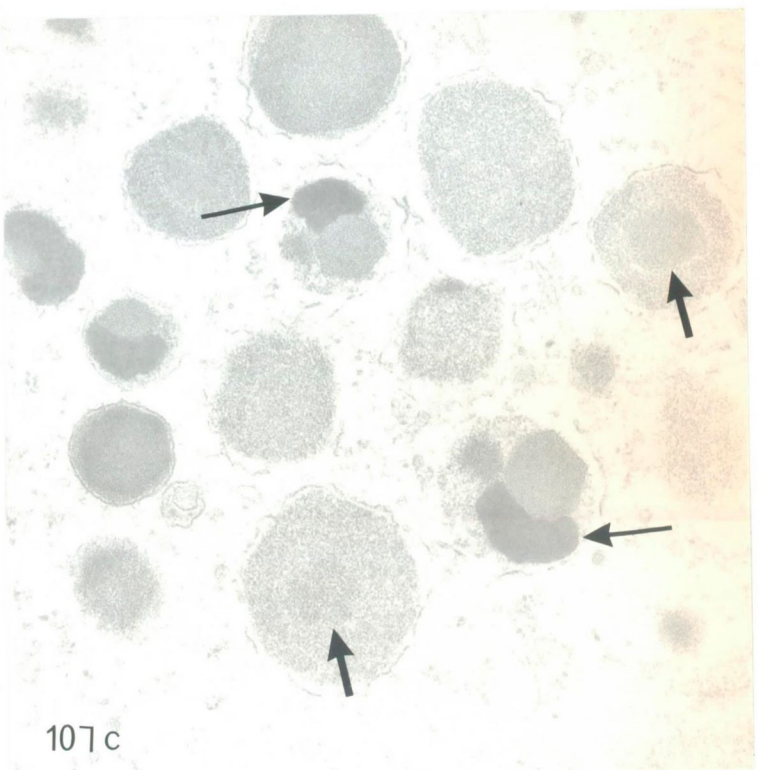

b

Figure 107

c - Detail of the secretory granules, note the presence of the acentric inclusions (small arrows) and the dense core in the larger granules (large arrows).

x 40,000



107 c

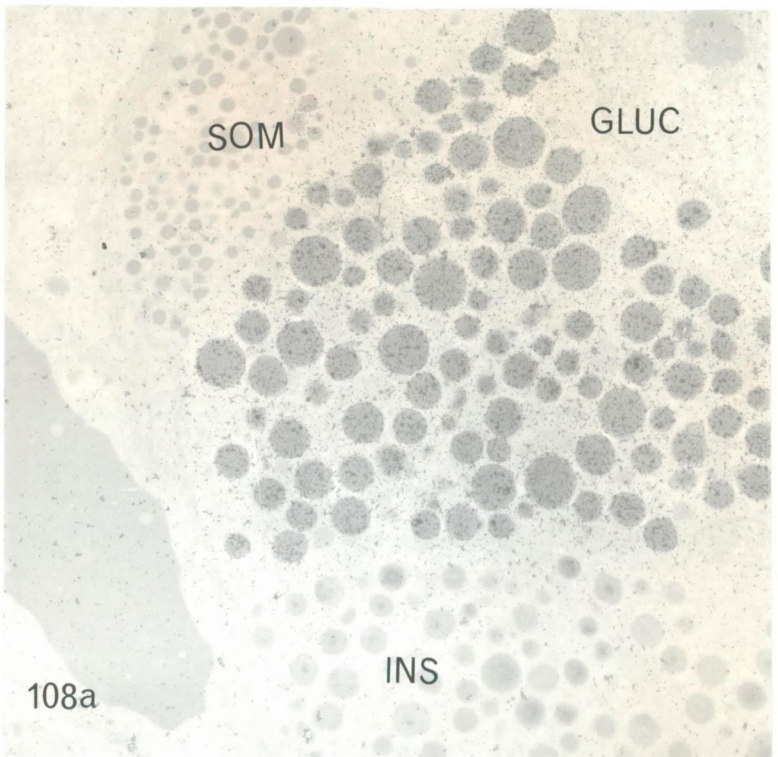
Figure 108

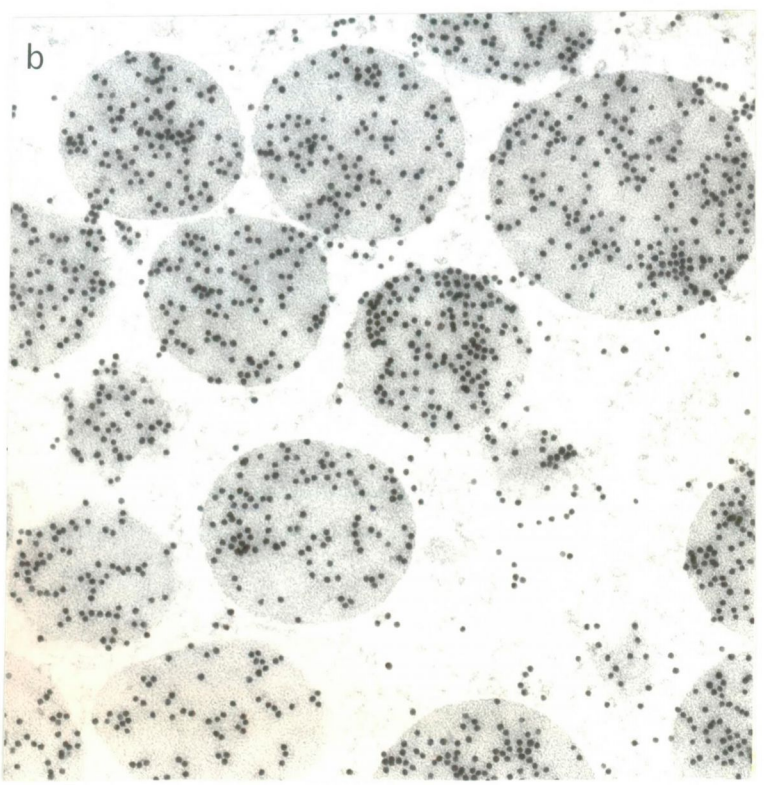
a - Thin (60nm) section immunostained with the N-terminal glucagon antiserum then labelled with colloidal gold (12nm) conjugated to goat-antirabbit IgG globulin. The colloidal gold spheres are concentrated over the glucagon-containing cell (gluc).

x 6,000

b - Detail of the glucagon-containing cell showing the colloidal gold spheres over the secretory granules.

x 60,000


SOM
GLUC
INS
108a


b

Somatostatin. These cells contained uniformly round secretory granules of medium size (325 ± 13nm) with a closely applied membrane (Figure 109).

An additional feature of this pancreas was the presence of numerous nerve terminals containing large electron-dense vesicles (Figure 110). The terminals were often found in close association with endocrine cells in the islets (Figure 111). Occasionally profiles were seen with synaptic junctions between the nerve terminal and the endocrine cells (Figure 112). These terminals clearly contained a mixed population of vesicles as can be seen in Figure 113 where numerous small (34 ± 3nm), agranular vesicles were found alongside the large (101 ± 4nm) dense cored vesicles.

8.6. - Alligator mississipiensis

The cells were identified in both tissue fixed in glutaraldehyde with osmium tetroxide post fixation and without the osmium treatment. Three methods of localisation were used: the semithin/thin, the gold labelled anti IgG, and gold labelled protein A.

Insulin. These were found in islet-like structures. The secretory granules showed many variations in appearance from large (187 ± 6nm), round and electron-dense with a clear halo between the core and the limiting membrane to single or many bar shaped inclusions enclosed by a membrane (113 ± 12nm) (Figure 114).

Glucagon/.....

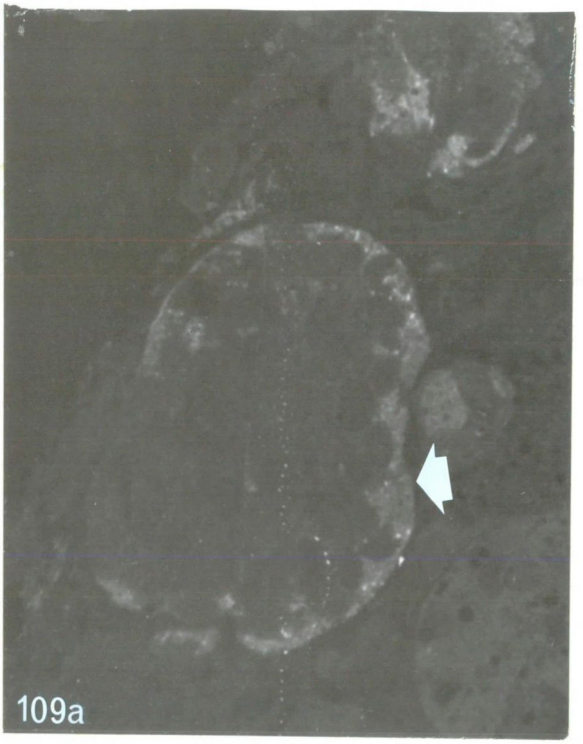
Figure 109

a - Semithin (500nm) section stained with the somatostatin antiserum showing numerous immunoreactive cells. The cell identified in the serial thin section is shown by the arrow.

x 400

b - Somatostatin-containing cell (arrow) next to an insulin-cell (INS) Small arrow indicates nerve terminal containing P-type vesicles.

x 6,000


109a

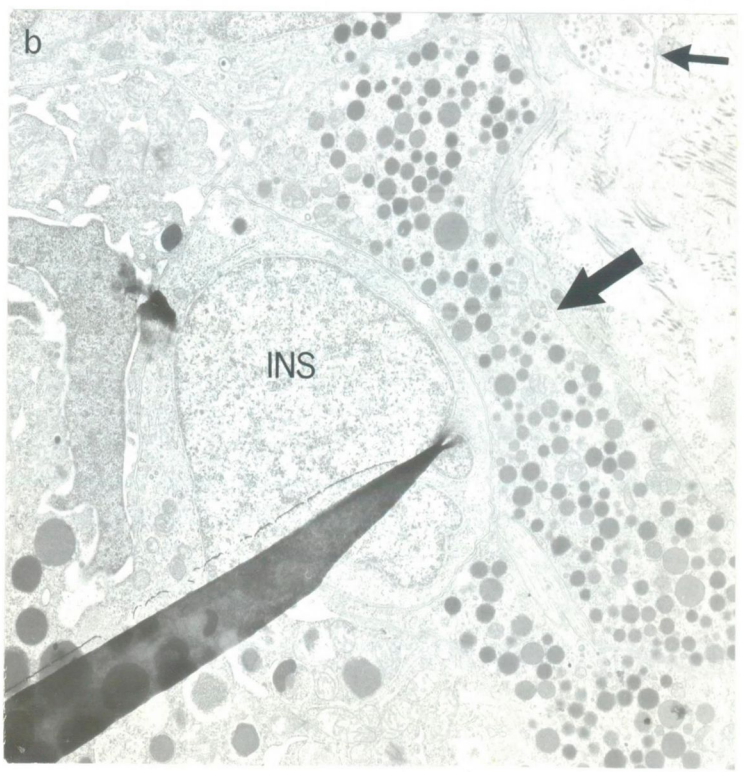

b
INS

Figure 109

c - Detail of the secretory granules seen in the somatostatin cell.

x 60,000

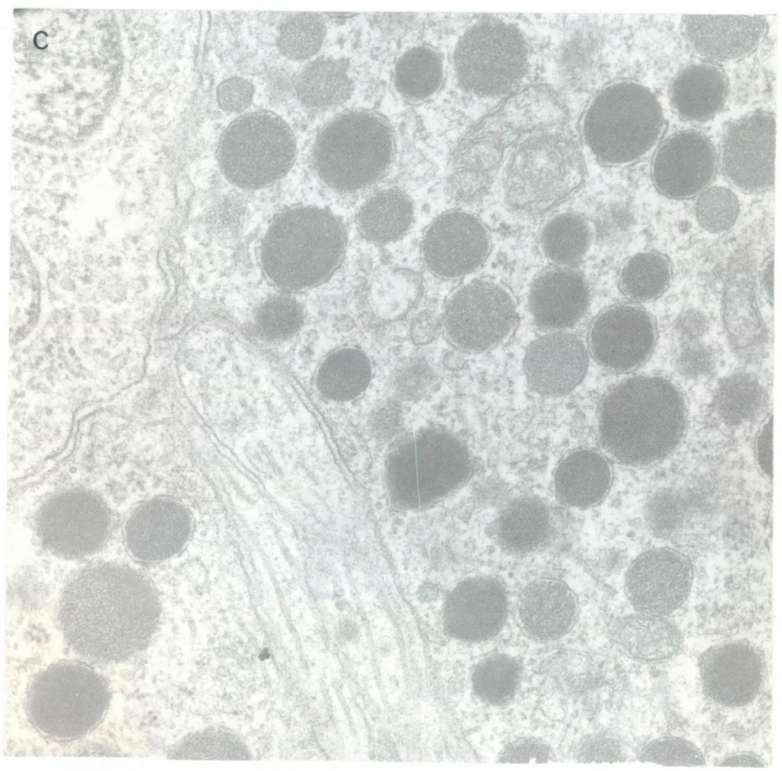

C

Figure 110

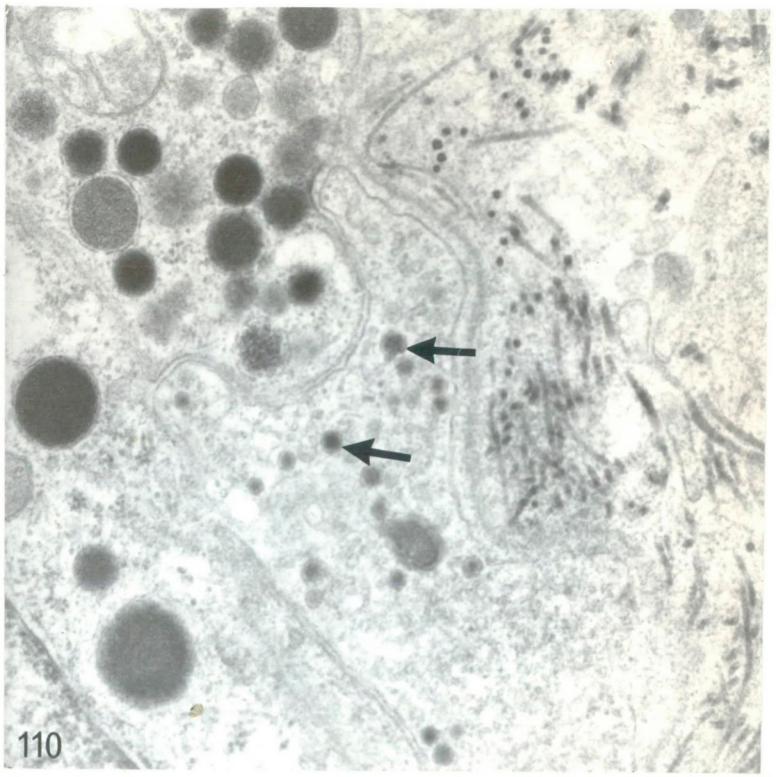
A nerve terminal on the edge of a pancreatic islet in close association with a glucagon-containing cell. Note the dense cored vesicles (arrows).

x 6,000

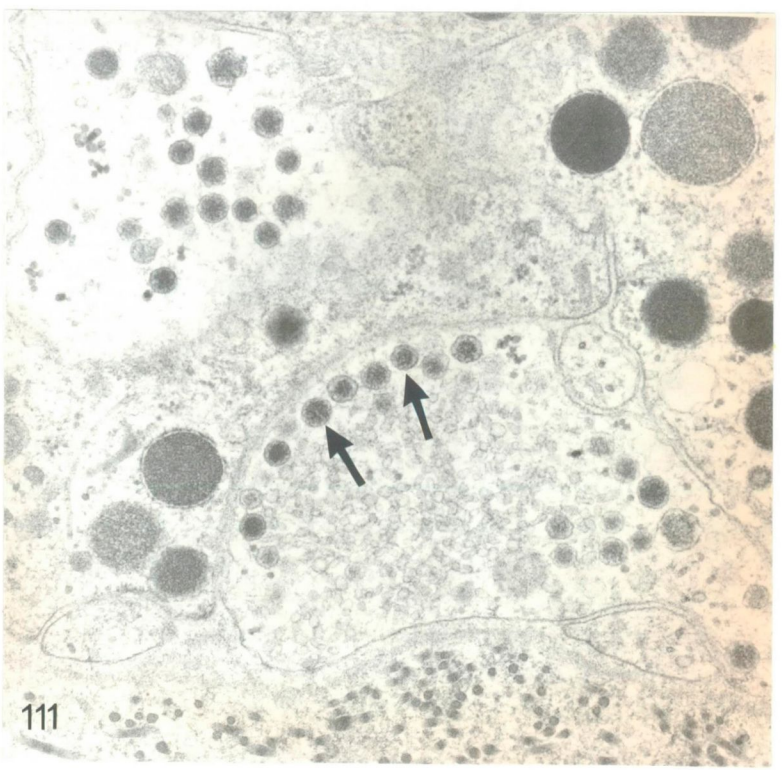
Figure 111

A nerve terminal in close association with two glucagon-containing cells. Note the congregation of the dense-core vesicles along the plasmalemma in close proximity to the glucagon cells (arrows).

x 6,000



110



111

Figure 112

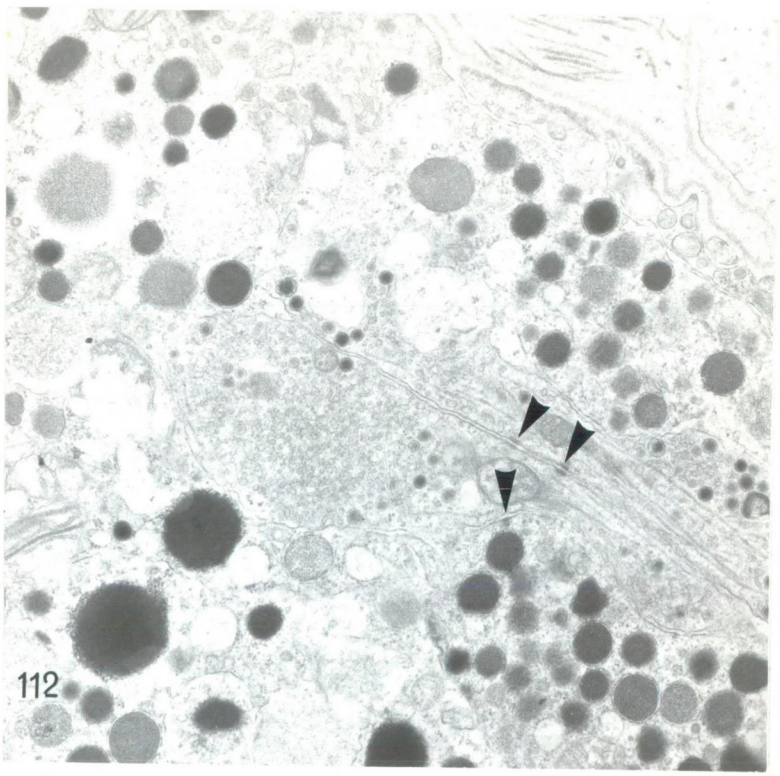
Nerve terminal actually within the pancreatic islet surrounded by glucagon- and somatostatin-containing cells. Arrows indicate synapses between two nerve terminals and an islet cell.

x 6,000

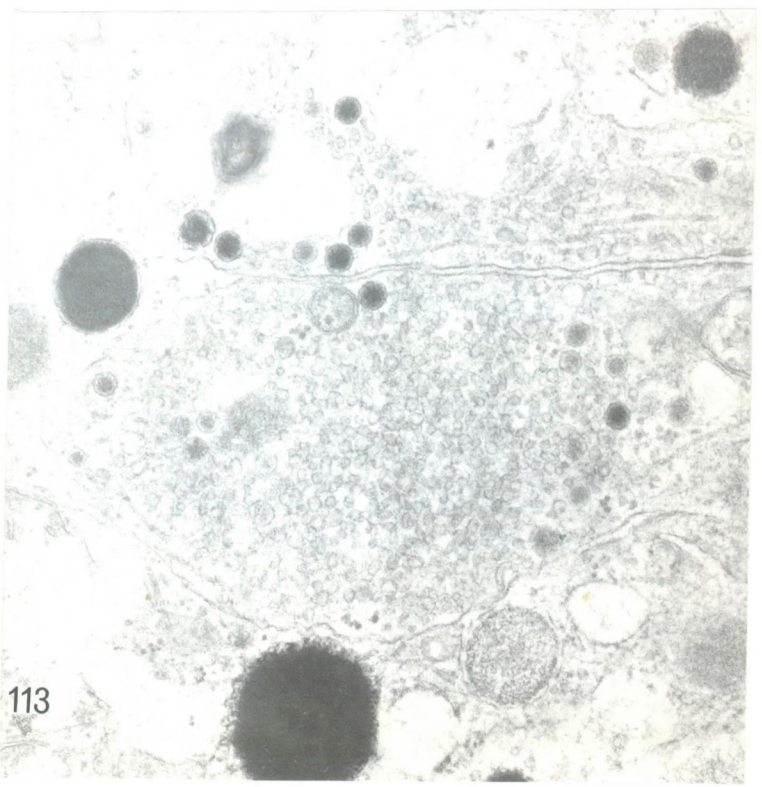
Figure 113

Detail of a typical nerve terminal showing the two types of vesicles - large, dense core and the small clear vesicles.

x 60,000



112



113

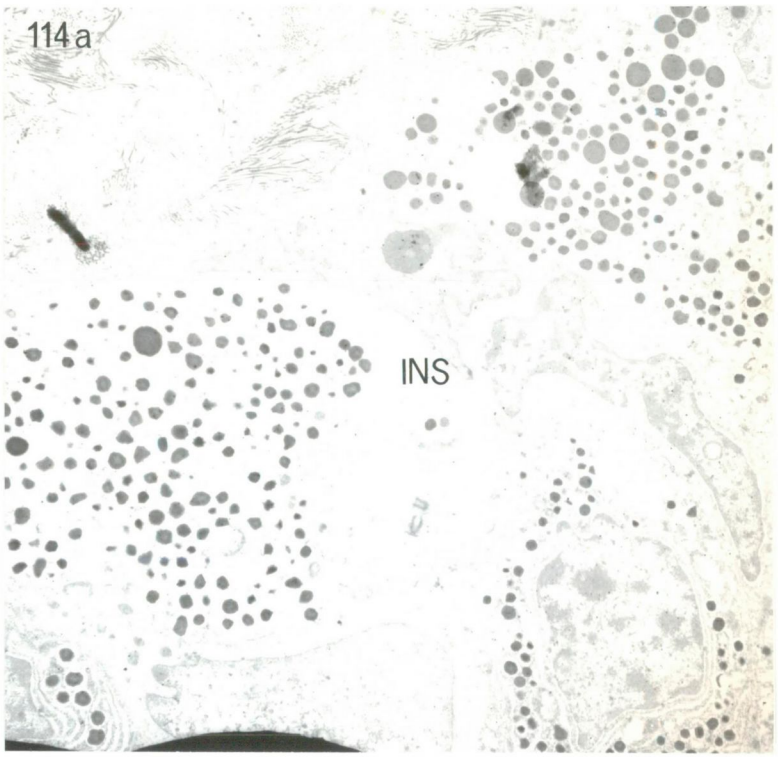
Figure 114

a - Section of pancreas immunostained with the anti-insulin serum then localised with colloidal gold conjugated to protein A.

x 6,000

b - The insulin-containing granules showing the decreased labelling density characteristic of the protein A technique.

x 60,000


114 a
INS

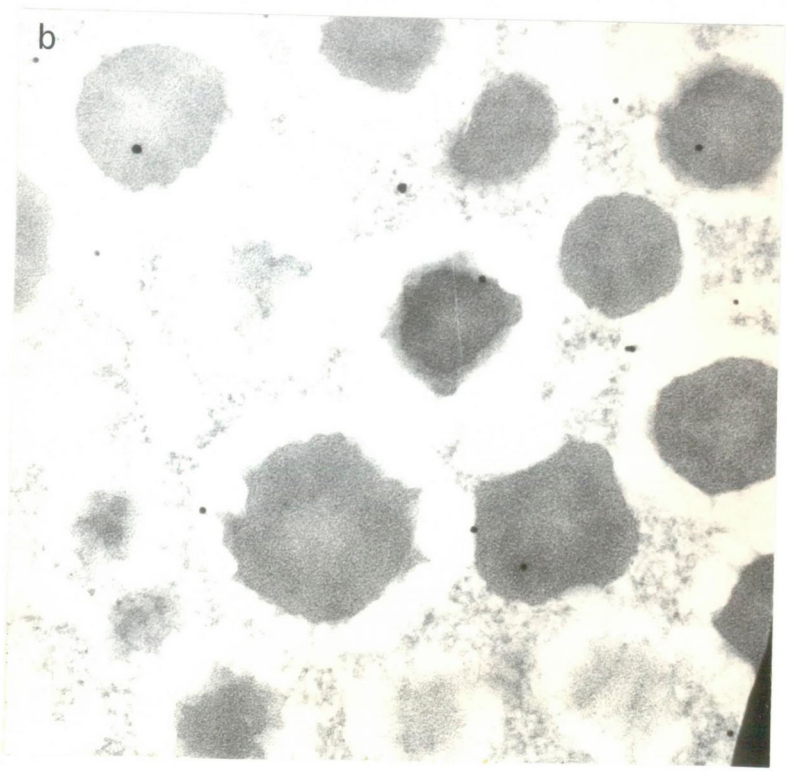

b

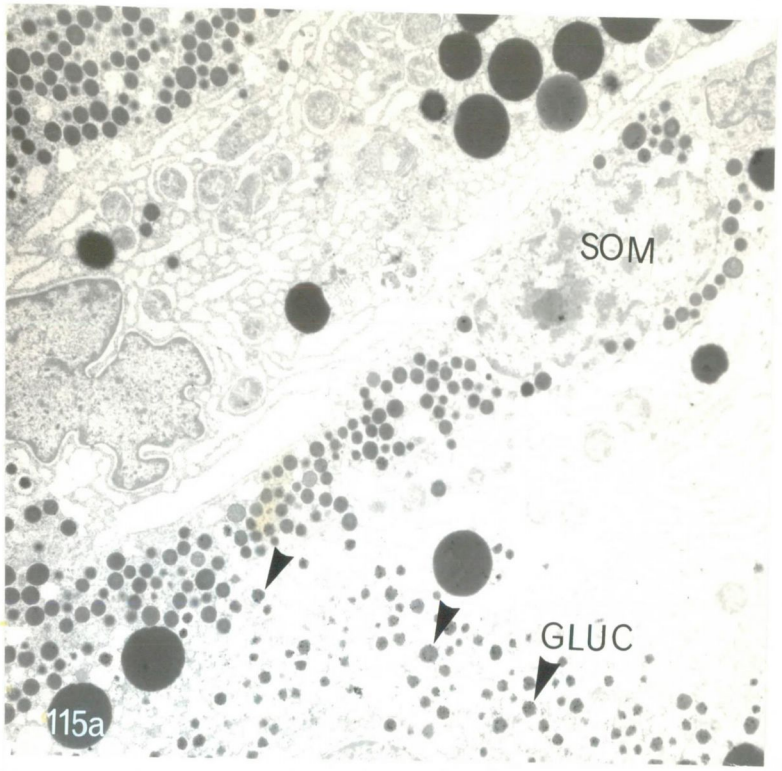
Figure 115

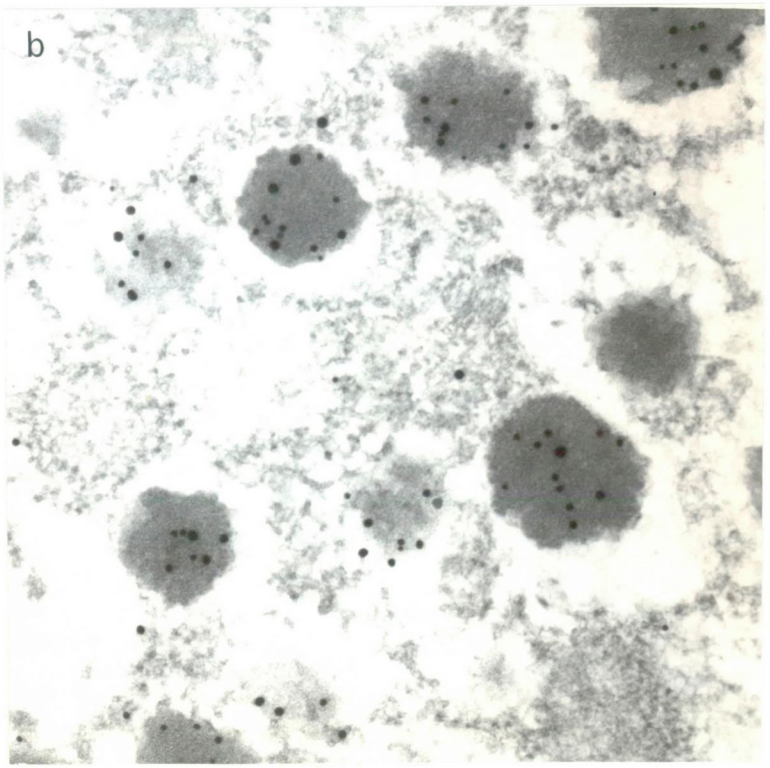
a - Thin section stained with the C-terminal glucagon antiserum localised by colloidal gold conjugated to protein A. Note that the granules in the somatostatin cell (SOM) are not labelled whereas those in the glucagon cell (gluc) are (arrows).

x 6,000

b - Detail of the labelling seen over the glucagon-containing granules.

x 60,000


SOM
GLUC
115a


b

Glucagon. Glucagon cells contained medium sized (224 ± 11nm) granules, round to irregular with a reasonable electron-density (Figure 115).

Somatostatin. Somatostatin cell granules were large (296 ± 11nm), uniformly round of medium electron-density and with a clear limiting membrane (Figure 116).

APP. The secretory granules present in these cells were similar to those seen in the glucagon cells but were smaller being 190 ± 7nm in diameter (Figure 117).

Nerve terminals. Around the pancreatic islets and in the exocrine tissue, P-type vesicles were detected, the dense cored vesicles had a diameter of 110 ± 8nm, these terminals also contained small agranular vesicles of 41 ± 3nm in diameter (Figure 118).

Table 35 - Secretory granule diameter in the pancreatic islet cells (mean ± SEM)

Species	Insulin	Glucagon	Somatostatin	PP
Cynops h.	407 ± 19	229 ± 8	131 ± 6	-
Hyla a.	110 ± 4	315 ± 11	161 ± 7	-
Chelydra s.	333 ± 12	210 ± 5	236 ± 5	225 ± 8
Agkistrodon p.	495 ± 27	553 ± 27	325 ± 13	-
Alligator m.	187 ± 6	224 ± 11	296 ± 11	190 ± 7
Man	270 ± 9	250 ± 6	400 ± 24	210 ± 4

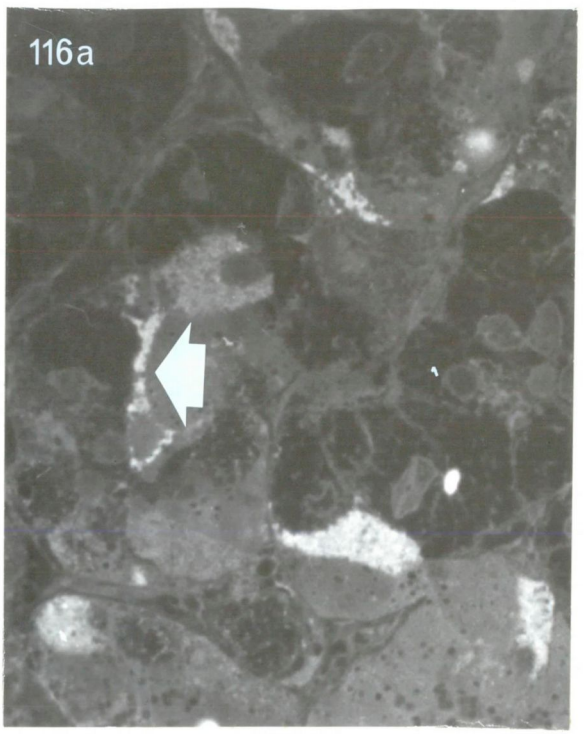
Figure 116

a - A semithin section immunostained by the somatostatin antiserum showing several positive cells. The cell identified in the serial thin section is shown by the arrow.

x 400

b - The somatostatin cell (arrow) as seen in the serial thin section.

x 6,000


116a

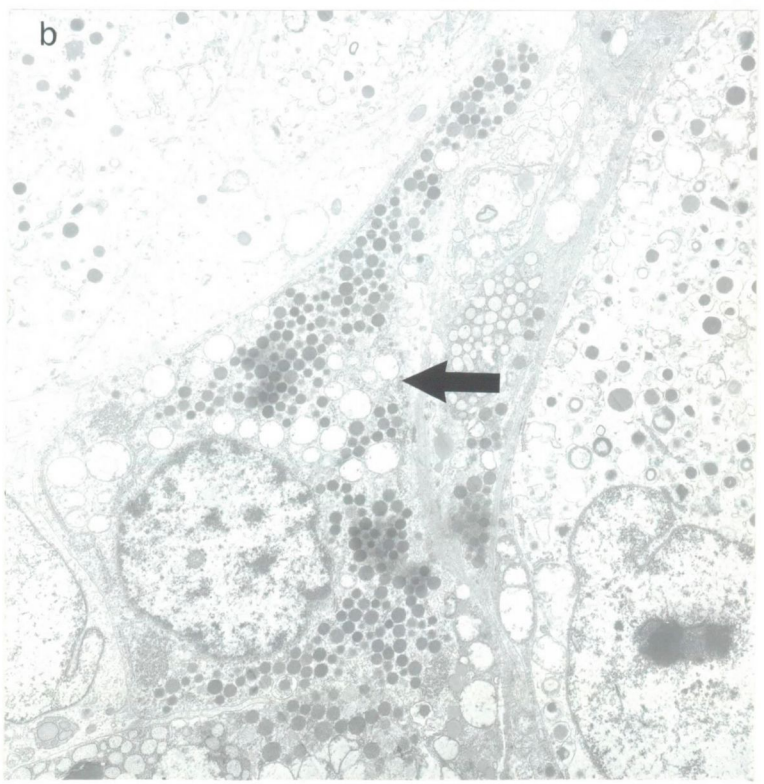

b

Figure 116

c - Detail of the secretory granules seen in the cell.

x 60,000

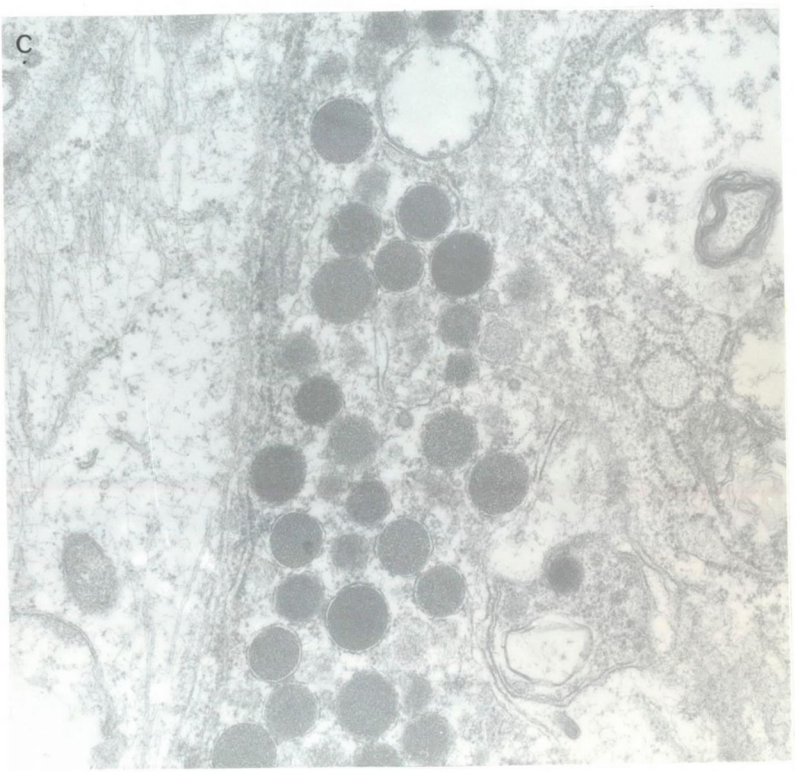

C

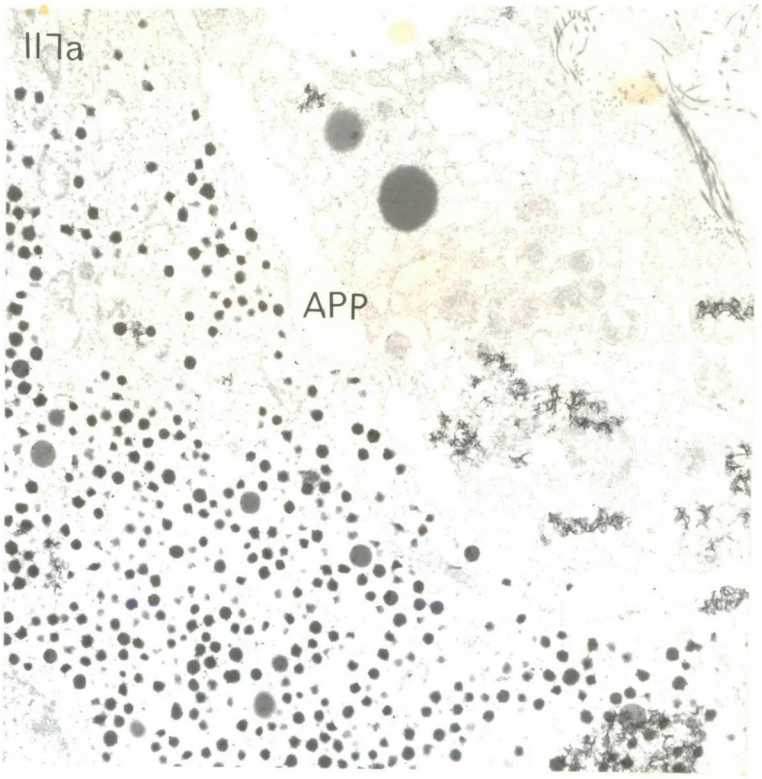
Figure 117

a - Thin section immunostained by the APP antiserum localised by colloidal gold conjugated to protein A gold.

x 6,000

b - Detail of the secretory granules showing the labelling with the colloidal gold spheres (20nm).

x 60,000


II Ia
APP

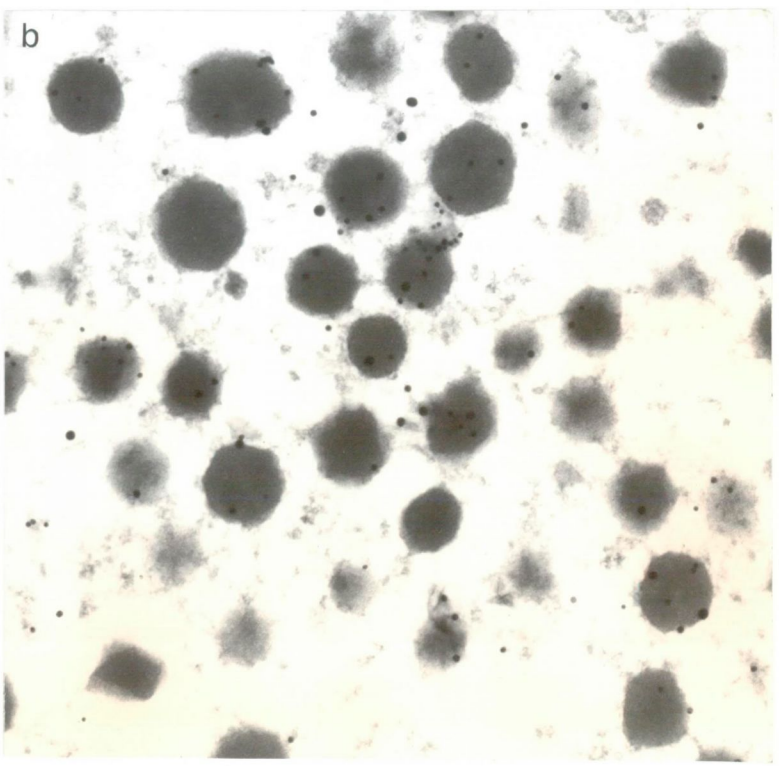
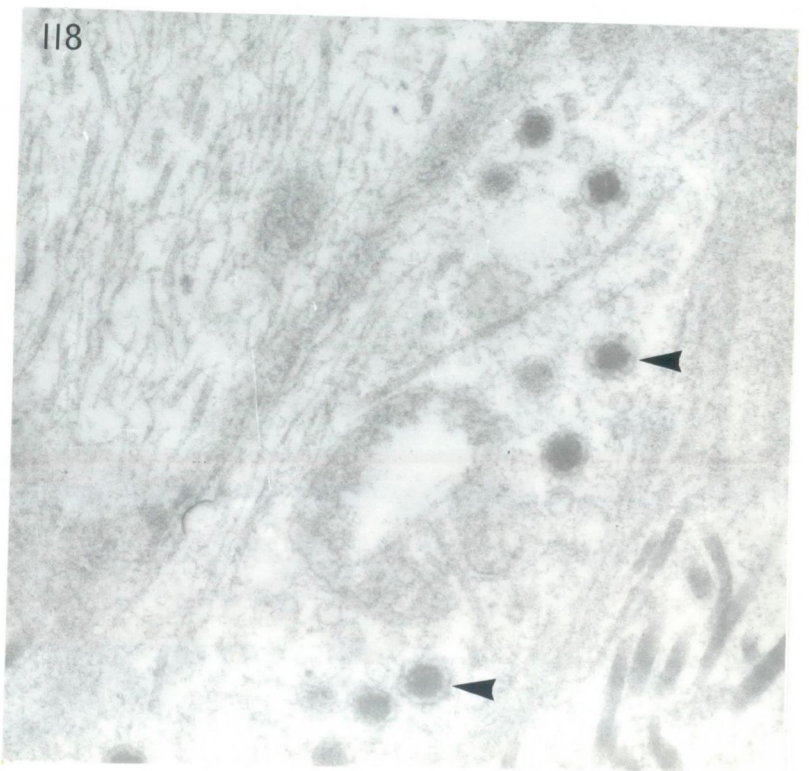

b

Figure 118

Typical nerve terminal found around a pancreatic islet - note the dense core vesicles (arrows).

x 60,000


118

Chapter 9 - DISCUSSION - TRANSMISSION ELECTRON MICROSCOPY

Comparison with the mammalian islet cells:-

It was clear from the results obtained, that the intention to provide a comparative assessment of the ultrastructural appearance of the sub-mammalian islet cells, as contrasted with those in mammals, was unreasonable as the variation between species was too great.

Table 35 shows a comparison of the values obtained for the mean diameter of the secretory granules from the individual cell types, including those of man. There was no pattern visible in the changes of granule size with species.

Comparison with related species:-

There are many purely ultrastructural studies on the structure of the islet cells in lower vertebrates (see Sato et al 1966, Epple and Lewis 1973, Patent 1976, Magnier 1976). In some cases, a similarity between the profiles reported in this study and those of related species in the literature can be seen. For example, in the islets of Rana ridibunda, the Type II cell (Lange 1967) was similar to the somatostatin cells found in Hyla arborea , even the granule diameters were similar with the somatostatin cell (Hyla a.) 162 $\pm$ 6nm and the Type II cell (Rana r.) 160 $\pm$ 8nm.

A previous study has shown the ultrastructural appearance of the islet cells in the West African crocodile, Osteoleomus tetraspis (Adesanya et al 1976). A comparison of those cells and the types as detected in Alligator missipiensis in the present study was undertaken. Obvious similarities/.....

similarities were seen between the insulin (N) cells of the two species but the cells identified as A (Glucagon) and D (somatostatin) in the crocodile bore little resemblance to those identified by immonocytochemistry in the alligator. Whether the identification of the cell types by ultrastructural morphology would be confirmed by immunocytochemical work in the crocodile remains to be elucidated.

Of the other species studied and reported in the literature little similarity could be seen between their cells and those identified during the present study. These findings emphasise the importance of clearly establishing the exact species investigated, as even species from the same family vary tremendously, eg the alligator and crocodile - both members of the Crocodylidae.

Cell Type Identification

Several other factors must be considered if the identification of cell type is to be based on the size and shape of the intracellular secretory granules.

For example, the physiological state of the animal may produce variations. Studies on the ultrastructural appearance of islet cells in lizards after glucose-loading and chronic insulin administration (to produce hypoglycaemia) showed marked alterations in the secretory granule profiles.

In the glucose-loaded animals the changes involved the B cells which showed a decrease in granules size and type of pleomorphic inclusion found within the granules. The hypoglycaemic animals showed a degranulation of the A cells and a decrease in the size of the remaining secretory granules (Rhoten 1971, 1973). The changes were not confined to the secretory granules but these are the alterations most relevant to the present discussion.

In/.....

In addition there is evidence of seasonal variation in the islet cells of snakes , where both the size and numbers of the individual cells were altered (Moscona 1954).

From these reports and the great heterogeneity of the secretory granule profiles, as demonstrated in the different species, it becomes evident that further criteria, such as immunocytochemical detection must be considered when identifying cell types in any species.

PP

Amphibia. The immunocytochemical localisation of PP in the amphibians posed a problem. In all the amphibian species studied the immunostaining with the BPP antiserum detected exactly the same cells as those reacting with the antiglucagon sera. The ultrastructural investigation revealed that only one type of cell was being detected by both of the antisera. The reasons for doubt about the immunostainings seen with the BPP antiserum were several.

Firstly, the staining produced by the BPP antiserum could be significantly reduced although not completely removed by the addition of pancreatic glucagon. This may be due to binding to glicentin rather than pancreatic glucagon as previously discussed (page 204). Another possibility is that the BPP antiserum may be contaminated with a sub-population of glucagon antibodies (Larsson personal communication 1980)

Secondly, during the screening of the pancreatic material, unstained cells were detected in several species. Whether these un-stained cells are responsible for the production of an amphibian PP undetected by the BPP, APP or HPP antisera remains to be determined.

Finally, in both the reptiles (as demonstrated in the present study) and teleost fishes (Van Noorden personal communication 1980), the PP-containing cells were found to be a totally distinct population from the glucagon-containing cells and were stained by both the APP and BPP antisera.

Taken together the present results indicate that an amphibian pancreatic polypeptide may exist but that it cannot be detected by the antisera available.

Serpentes A rather different situation exists in the snakes where no immunoreactivity was detected with any of the antisera. In addition a thorough screening of the pancreatic tissue from these amimals does not reveal the presence of a distinct cell type unstained by antisera to the other pancreatic hormones.

In a previous, cytochemical and ultrastructural study the existence of four cell types in Natrix natrix was reported (Trandaburu and Caligareau 1969). However, in another study the presence of only three cell types was reported in exactly the same species (Titlbach 1967).

The present results support the initial report of only three cell types in the snakes.

If/.....

If the absence of PP from the snake pancreas can be substantiated, further study of the interelationship between the remaining islet cells in these animals will be of great interest.

Innervation

The ultrastructural studies showed that in both the alligator and snake there were numerous nerve endings found in close association with the endocrine islets.

The appearance of these terminals was very similar to the P-type nerves as described in mammalian and sub-mammalian species (Baumgarten et al 197C Cook and Burnstock 1976).

In the snake especially these terminals demonstrated several striking features. Firstly the vesicular content was not homogeneous. A mixture of large, dense cored, P-type vesicles and small, clear vesicles, reminiscent of the mammalian cholinergic vesicles were found in the same terminals (see Figure113, Page 303). It has already been suggested that peptide-containing terminals may also contain cholinesterase activity in mammals (Hokfelt et al 1980). Furthermore as early as 1974, cholinesterase activity had been reported to occur in the snake Natrix natrix (Trandaburu 1974) in nerve terminals also containing dense cored vesicles.

The results of the light microscopical immunostaining strongly suggests that these dense cored vesicles store the neuropeptide VIP. Unfortunately, all attempts to stain for VIP in the material collected for electron-microscopy have failed, therefore this awaits further direct/.....

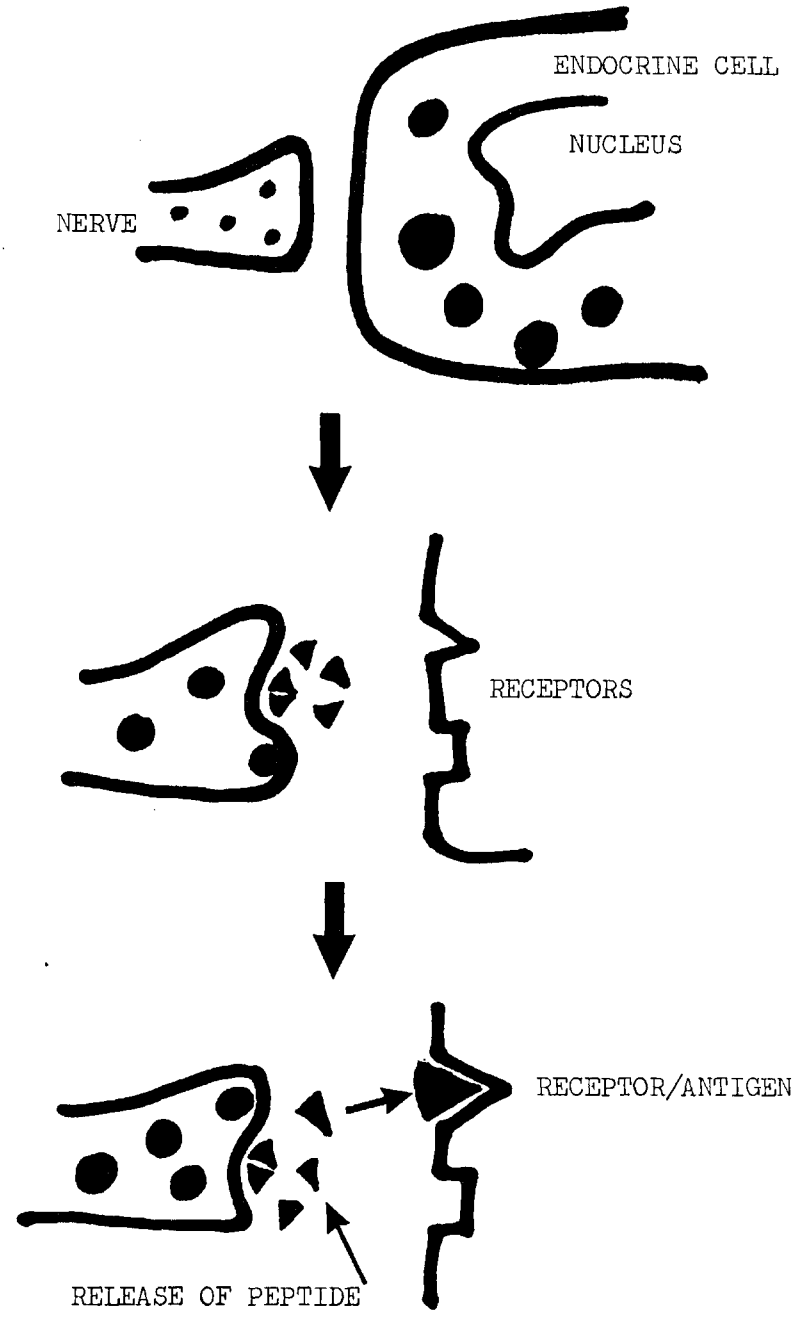
direct confirmation. Indirect confirmation that these terminals may well contain a peptide such as VIP comes from investigations in mammals, where, in both the central and peripheral nervous system substance P has been shown to be located in dense-cored vesicles (Pickel et al 1977, Probert et al 1980) and VIP has been localised in large, dense-cored vesicles in the intestinal nerve plexuses (Larsson 1977).

A second interesting finding was in the organisation of the dense cored vesicles within the terminals. In many of the nerve endings the dense cored vesicles were found aligned along the plasmalemma in contact with the opposing endocrine cell membrane (see Figure 111, Page 301) but in these regions no synaptic junctions could be seen although these were detected in other regions (see Figure 112, Page 303). On the contrary, profiles resembling those reported as indicative of exocytotic release were occasionally observed. The absence of synaptic junctions was not due to inadequate fixation as junctions were observed in other areas.

It may be that the way in which these peptide neuroendocrine substances act is not by true synaptic release but by exocytosis as seen in the endocrine cell and that the action is completed by uptake by specific receptors on the target cell membrane (see Diagram 10).

Clearly, to investigate this suggestion further, time-related observations on the release pattern after nerve stimulation must be undertaken. The extremely rich innervation seen in the snakes, coupled with the limited location of the islets within the pancreas presents an attractive experimental model for the proposed study. It is hoped to take the pancreas from further snakes, isolate the islets and set up cultures in which the response of these nerves to selected stimuli/.....

DIAGRAM 10 PROPOSED MODEL OF NERVE ACTION



● PEPTIDE-CONTAINING GRANULES

▼ INDIVIDUAL PEPTIDE(ANTIGEN)

stimuli can be monitored.

Two hypotheses have been put forward suggesting a function for this rich innervation.

In teleosts, a similar innervation of the pancreatic islets has been reported (Epple and Lewis 1975), and it was suggested that this innervation was related to the additional osmoregulatory function of the pancreas in fishes.

Alternatively the innervation may be related to the need of a particularly fine control over the release of the pancreatic hormones in these species. Some support for this from mammalian studies is indicated by the stimulation of the release of glucagon, somatostatin, PP and insulin seen after administration of exogenous VIP (Adrian et al 1978).

As well as investigating the release pattern of the peptides from the terminals, cultures of the islets from the snakes could be used to study the effect of VIP on the release of the other pancreatic hormones.

General comments

It was interesting that in the majority of the animals studied except the snake and alligator the islet cells were found close to the capillaries. In some of the sections it appeared that not only was the content of the granule released but also the secretory granule's limiting membrane. This would contradict the present theory of exocytosis whereby the granule membrane is supposed to fuse with the plasma membrane and then to be re-absorbed into the cell as an empty pinocytotic vesicle./.....

vesicle. However, the present findings may be an artefact created by fixation damage during collection of the material. Further studies are clearly required to solve this problem.

Future

An extension of the work completed on the ultrastructural variations between the peptide-containing cells and nerves in the pancreas will be of interest mainly on comparative terms. Additional studies on the dynamic changes within the cells and nerves may be of greater interest with respect to related problems in the mammalian regulatory peptide system.

Chapter 10 - EVALUATION

The aims of this study has previously stated, were as follows:-

1. To investigate the existence of the intestinal regulatory peptide system in reptiles and amphibians.
2. To demonstrate, where possible, the molecular form of the peptides detected by the use of region specific antisera.
3. To establish whether there were peptides undetected in the amphibians investigated, which could be detected in the reptiles.
4. To evaluate the relevance of species-specificity in relation to the use of mammalian antisera.
5. To look at the question of whether the gut hormones are evolutionary relics.
6. To show the ultrastructural characteristics of the peptide-producing cells in the non-mammalian pancreas and compare them with the cells as identified in man.
7. To indicate areas for further research.

Point 1. The largest part of the work presented comprises a report on the detection of regulatory peptides by immunocytochemistry in the gastro-entero-pancreatic tract of a variety of amphibian and reptilian species using antisera to the mammalian peptides. A total of 25 species, 11 amphibian and 14 reptilian were investigated.

Peptides detected:-

VIP-, neurotensin-, glucagon (pancreatic and enteric)-, insulin-, somatostatin-, gastrin/CCK-, bombesin- and substance P-like immunoreactivity were always present.

Met- enkephalin, prolactin and β endorphin were demonstrable in a limited number of species, which included the primitive amphibians.

Secretin-/.....

Secretin-, motilin- and CCK-like peptides were only detected in some reptiles and in none of the amphibians investigated.

The immunostaining observed with the available antisera to GIP was limited to a single species of reptile. It is intended to raise more antisera, if possible to different regions, in order to establish firmly the presence of lack of a GIP-like peptide in these species.

A PP-like peptide has been detected in the reptiles but the situation with respect of the amphibians requires further clarification.

Localisation of peptides:-

This study has demonstrated that both peptide-containing cells and nerves were already well established in the amphibians. Of the peptides detected in intestinal nerve fibres in mammals, only VIP was consistently found in neural tissue in all of the animals studied. Substance P, bombesin and met-enkephalin were also detected in nerve fibres of the intestine but not as frequently. Neurotensin, somatostatin and gastrin/CCK containing nerve fibres were not detected in any species. This is of particular interest as the frequency of detection in the different species paralleled the abundance of the different types of peptide-containing fibres in mammals, VIP being the most abundant, followed by substance P, bombesin and met-enkephalin. Only a few neurotensin, somatostatin, and gastrin/CCK fibres have been detected (Schultzberg et al 1980).

Clearly the equivalent of a major part of the mammalian regulatory peptide system is present in the amphibians and reptiles investigated.

Table/.....

Table 36 outlines the additional information on the presence of the gut hormones in amphibia and reptiles found during this study.

Point 2. Region specific antisera to gastrin/CCK, VIP, bombesin, motilin and glucagon were available during this study. Of the results obtained those seen with VIP and glucagon antisera were of most interest. Studies with the VIP antisera indicated that in those frogs showing a dual localisation in cells and nerves, the peptide detected was immunologically identical in both locations. In addition, the equal reactivity of the three antisera indicated that the peptide detected must be very similar to mammalian VIP. This suggestion has been re-inforced by the radioimmunoassay data but cannot be proved until the actual peptide is isolated and purified.

The results obtained with the glucagon antisera demonstrated that the peptide detected in the pancreas and stomach was similar to mammalian pancreatic glucagon, while that in the lower intestine was not like either pancreatic glucagon or the mammalian enteric glucagon, in this case represented by glicentin.

With the antisera to motilin and bombesin, immunoreactivity was present with both the N- and C-terminal specific antisera, suggesting that the peptides detected may have a reasonable resemblance to the mammalian form used to raise the antisera.

Of the region specific antisera to gastrin only the C-terminally directed antisera produced convincing results, which limits any comment/.....

Table 36 - SUMMARY OF THE INFORMATION COLLECTED DURING THIS STUDY ON THE PRESENCE OF THE GUT PEPTIDES IN AMPHIBIA AND REPTILIA

Peptide	ORDER					
	Urodela	Anura	Testudinata	Lacertilia	Serpentes	Crocodilia
G/CCK	P	P(C)	P	P	P	P
INS	P	P(C)	P	P(C)	P	P
PGluc	P	P	P	P	P	P
Gluc	P	P(C)	P	P(C)	P	P
PP	A?	A?	P	P	A	P
SOM	P	P(C)	P	P(C)	P	P
VIP	P	P(R)	P	P	P	P
NT	P	P(C)	P	P(C)	P	P
BN	P	P(C)(R)	P	P	P	P
SP	P	P	P	P	P	P
ENK	P	P	A	A	A	A
9-20 CCK	A	A	A	A	P	A
GIP	A	A	A	A	P	A
SEC	A	A	A	A	A	P

P = present detected by immunocytochemistry only.

P(C) = present and detected by more than one group.

P(R) = present and detected also by radioimmunoassay

A? = absent but doubt about antisera

A = absent with all antisera available at present.

comment on the molecular identity of the peptide detected.

Although these results to show that the use of region specific antisera can give an idea of peptide structure there is clearly a need for the production of a larger range of these antisera, from which it should be possible to deduce considerable information on the types and molecular forms of the peptides detected in these species. However it must be remembered that the production of region specific antisera to mammalian peptides is still no guarantee that they will detect the non-mammalian peptides has has been clearly demonstrated with the gastrin and CCK antisera in the present study. Hopefully, as the isolation and purification of these non-mammalian peptides continues it should be possible to raise region specific antisera that will be of use in phylogenetic studies.

Point 3. Among the peptides undetectable in the amphibian material, but found in certain reptile species were secretin and motilin. The presence of immunoreactivity with the available antisera to these peptides was sporadic, in that only some of the snakes , Testudinata and Crocodylidae showed any immunostaining. However, when immunoreactivity was detected it was a constant finding within all members of the species studied. At the moment any definite comment on the evolutionary development of these peptides is impossible. A much wider survey of the reptiles and possibly also monotremes and marsupials will be required before anything other than pure speculation can be attempted.

Point 4. When evaluating the usefulness of mammalian antisera, the results of this study give support for the theory that the antisera to the biologically active region of the mammalian peptides will be of/.....

of greater importance than those to the non-active area in investigations of non-mammalian species. For instance the antisera to the N-terminal regions of glicentin and gastrin 34 gave negative results with all of the species examined. This may be taken to indicate that similar actions and even receptors may be expected to occur in these animals as the biologically active antigenic determinants have remained stable.

Point 5. It has been claimed that the gut hormones are evolutionary relics and that the regulatory peptide system in the intestine is of little physiological importance in mammals, the peptides being simply vestiges from earlier evolutionary stages.

The case in favour of a significant role for the gut peptides is strengthened by the finding that although a considerable number of the regulatory peptides have been demonstrated in the amphibians and reptiles the system as a whole was not as extensive as that present in mammalian intestine. It appears as though both the peptide-containing cells and nerves have undergone further differentiation in mammals. This differentiation applies both to the range of peptides detectable and the site of production. In mammals, peptides such as GIP, secretin and motilin are well represented in endocrine cells of the upper intestinal mucosa and on the neural side neurotensin, somatostatin, and gastrin/CCK are found in nerve fibres throughout the length of the gut. It seems unlikely that the gut peptides can be considered as merely evolutionary relics as the mammalian system would appear to have expanded considerably from that found in even the most advanced of the reptiles.

The/.....

The data presented here would seem to agree with the initial assumption that the regulatory peptide system did evolve in parallel with the emerging species.

Point 6. The ultrastructural investigation of the pancreatic islet cells clearly demonstrated the existence of inter-species variation. The different ultrastructural profiles, as defined by the size, shape and electron-density of the introcellular secretory granules could not be predicted with any accuracy.

A good example of this was seen with the somatostatin-containing cells. In the amphibians the granules were small, in the snakes they were slightly larger and whilst in the alligator they were the largest of those seen in all the cell types identified - Table 35 (page 308) shows a comparison of granule size with species (including man for comparative purposes).

It is obvious that the size or shape of the intracellular granules can no longer be taken to be indicative of the peptide-product and that all ultrastructural studies must employ immunocytochemical localisation of the peptides (whether by serial sectioning or directly staining the thin sections), if any definite conclusion as to granule product is to be drawn.

The fact that there is tremendous species variation, must not be taken to indicate that ultrastructural studies are of no value. Electron microscopical work on the alterations in secretory granules morphology may be used as an index of how individual cells respond to different physiological stimuli. However, the necessity of well controlled, accurate identification of the cell types/.....

types cannot be sufficiently stressed.

Point 7. Future work. The present work has demonstrated that the study of the lower vertebrates can be extremely interesting and of relevance to similar studies in mammalians. There is clearly a great lack of the physiological and biochemical knowledge required to define the precise actions and structure of these non-mammalian peptides.

Apart from further morphological studies in a greater range of species, there are many areas in which morphological techniques can be used; for example, functional studies such as a thorough investigation of the changes seen with seasonal variation (between hibernating and non-hibernating stated). The quantitiative techniques employed at present to assess differences in the peptide-containing cells in mammalian experimental models can be adapted to give similar information in these animals.

The work on the snake pancreas and gut will be extended. In the pancreas the innervation of the pancreatic islets was particularly striking and presents an exciting model for studying the interaction of the peptidergic nerves and endocrine cells. If cultures of the isolated pancreatic islets with the attendant nerve fibres can be set up, then the interaction of the two systems can be monitored in response to several different stimuli. Physiological studies will be set up to investigate the effect of VIP on food transit time in the straight gut snakes and the vipers. It is hoped that both of these studies can be undertaken in the near future.

CONCLUSION

It appears that the regulatory peptide system is an integral part of the/.....

the intestinal organisation, at least in vertebrates. It remains to be determined whether, although the complexity of the system has increased in mammals, the basic actions of the peptides have remained unaltered.

Appendix 1 Fixation experiments

Light microscopy

Fixative	Composition	PH	Temperature	Time	Comments
Vapour					
Benzoquinone	Benzoquinone+ Toluene	-	60°C	3hrs	Immunostain Morphology Successful
Diethylpyro-carbonate(DEPC)	DEPC + Alcohol	-	60°C	3hrs	Immunostain Limited
Liquid					
Bouin	75% Picric Acid 25% Formalin 1% Acetic Acid	4-5	25°C	2hrs	Immunostain Limited
Benzoquinone	1% + 0.1MPhosp. buffer	7.2	25°C	1hr	reasonable morphology
	4% + "	7.2	25°C	1hr	Immunostain for nerves
	10% "	7.2	4°C	2hr	excellent but not optimal for cells.

Electron microscopy

Fixative	Composition	pH	Temp	Time	Comments
Glutaraldehyde	3% + 0.1M PO_4 buffer, post-fixed in Os O_4	7.2	4°C	2hrs	Ultrastructural morphology excellent Immunostain limited.
Glutaraldehyde	2% + 0.1 PO_4 buffer	7.2	25°C	1hr	Morphology acceptable, Immunostain limited.
Karnovsky's fixative modified	9ml 2% para-formaldehyde 1ml 5% purified glutaraldehyde in 0.2M PO_4	7.3	4°C	2hrs	Morphology acceptable, Immunostaining successful.
Purified glutaraldehyde	2.5% + 0.05M PO_4 buffer	7.1	4°C	15min	Morphology acceptable Immunostain optimal.

Appendix 2

Gold-labelling Method

80nm sections mounted on 300 mesh nickel grids.

1- Etch for 10 minutes in 10% hydrogen peroxide.

2- Wash 3 x 5 minutes in distilled water.

3- incubate in 30% normal goat serum for 30 minutes

4- incubate in primary antiserum either

a overnight at room temperature with the antisera at the following dilutions -

glucagon	1 / 1,000
insulin	1 / 2,000
PP	1 / 2,000
Som.	1 / 600

b for 48 hours at 4°C with the anti sera at the following dilutions -

glucagon	1 / 5,000
insulin	1/ 3,000
PP	1/ 6,000
Som.	1/ 4,000

5- wash 3 x 5minutes in 0.1M phosphate buffer (PBS)

6- wash in PBS with 0.5mg per ml of polyethyleneglycol(PEG) molecular weight 20,000.

7- incubate in either protein A conjugated to colloidal gold diluted 1:1 with PBS containing PEG,

or rabbit anti-IgG conjugated to colloidal gold, diluted 1:20 in PBS containing PEG.

both incubations for 30 minutes at room temperature.

8- wash in PBS containing PEG (gold buffer), 3x 5minutes

9- wash in PBS alone, 1 x 5 minutes

10- wash in distilled water 1 x 10 minutes

11- counterstain with uranyl acetate and lead citrate and examine in an electron microscope.

Appendix 3 Radioimmunoassay Results

a VIP in the Anura

Species	Areas					
	Fundus	Antrum	Duodenum	Jejunum	Ileum	Colon
Bombina b.	ND	ND	56	39	ND	ND
Alytes o.	32	41	112	56	51	59
Rana t.	19	28	36	37	27	41
Rana e.	32	30	45	51	27	35
Bufo b.	52	40	68	51	66	83
Hyla a.	36	36	41	31	22	43
Hyla c.	62	46	71	50	43	61

All the results are expressed as pmol / gram wt weight.

ND = not done.

b Bombesin in the Anura

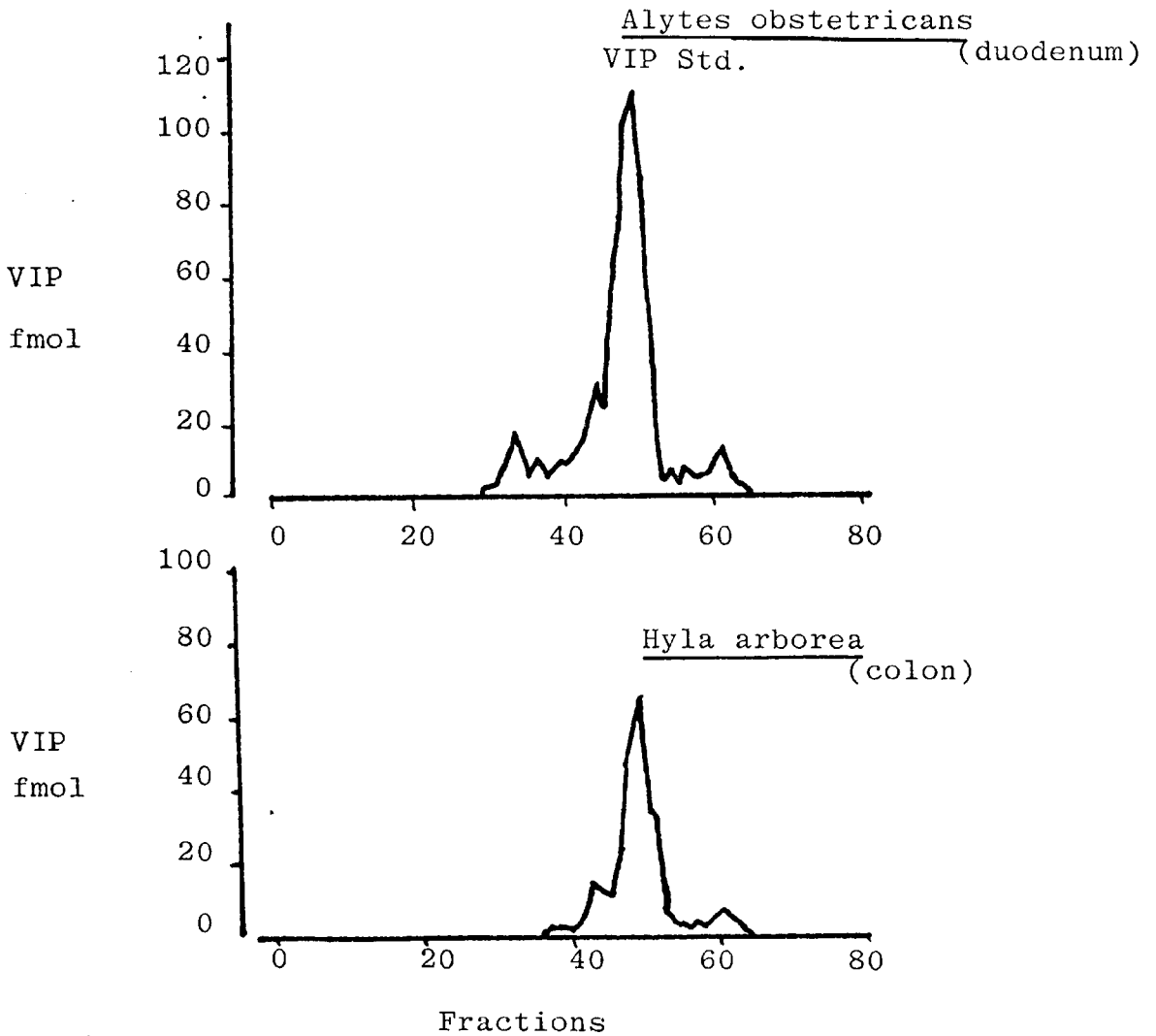
	Fundus	Antrum	Duodenum	Jejunum	Colon
Rana e.	38	50	15	9	0
Rana t.	29	36	20	6	0
Bufo b.	53	62	22	7	4
Hyla a.	35	41	16	2	0

All the results are expressed as pmol/gram wet weight.

Appendix 3

Results - Column Chromatography

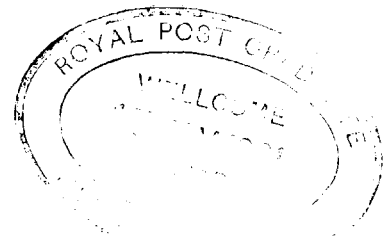
VIP



Appendix IV

	anti-gastrin	anti-gastrin + G4
Ambystoma m.	+++	+
Bufo b.	+++	+++
Hyla a.	+++	+++
Nerodia r.	++	+
Chelydra s.	++	++

G4 is the C-terminal tetrapeptide of gastrin which contains the sequence homology with CCK. All the pre-absorptions were performed by the addition of 5nanomoles of G4 to the diluted C-terminal gastrin antiserum. In all cases the absorption of the antiserum with G4 did not completely remove the immunostaining, indicating that there is further immunological similarity between the peptide detected and gastrin, than soley the C-terminal region.



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