AN IMMUNOCYTOCHEMICAL STUDY OF SEVERAL TYPES OF GUT ENDOCRINE CELLS IN CHICK EMBRYOS

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Gastrin/CCK-immunoreactive cells in the pyloric region at 21 days of incubation

ABSTRACT

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The time of first appearance and distribution of several types of peptide-storing cells were studied in the gastrointestinal tract of chick embryos between 12 days of incubation and hatching. Immunocytochemical methods have been employed to demonstrate cells immunoreactive for gastrin/cholecystokinin (CCK), neurotensin, somatostatin, glucagon and avian pancreatic polypeptide (APP) in the proventriculus, gizzard, pyloric region, duodenum, upper and lower ileum, caecum and rectum.

No immunoreactive cells were found in any region at 11 days of incubation. Immunoreactive cells which first appeared at 12 days were: somatostatin- and neurotensin-immunoreactive cells in the

proventriculus, pyloric region and duodenum, neurotensin-immunoreactive cells in the rectum; at 13 days, APP-immunoreactive cells in the duodenum and upper ileum, glucagon-immunoreactive cells in the proventriculus; at 14 days, somatostatin- and neurotensin-immunoreactive cells in the upper and lower ileum, gastrin/CCK-immunoreactive cells in the small intestine, APP-immunoreactive cells in the proventriculus and lower ileum, glucagon-immunoreactive cells in the pyloric region and small intestine; at 16 days, gastrin/CCK-immunoreactive cells in the pyloric region.

Subjective assessment showed an increase in frequency of all five cell types until the numbers at hatching were approximated, a few days before hatching. No immunoreactive cells of any type were detected in the gizzard; only neurotensin-immunoreactive cells were detected in the caecum and rectum.

When these endocrine cells first appeared, the surface epithelium of the gastrointestinal tract was relatively undifferentiated. Cells of all five types were found in glands once morphogenesis had begun.

The coexistence of APP- and glucagon-like immunoreactivity in the same cells was studied in the proventriculus and small intestine, and of gastrin/CCK- and

neurotensin-like immunoreactivity in the pyloric region and duodenum. Consecutive sections of each region were examined at 17½-, 19- and 21-days of incubation. For practical reasons, earlier stages, when cell numbers were small, were not studied. However, the trend shown in the proventriculus and ileum suggests that most, if not all, the relevant cells may contain APP- and glucagon-like peptides from the time they first appear. No dual immunoreactivity was detected in the duodenum. The proportion of cells deronstrating dual immunoreactivity for gastrin/CCK and neurotensin showed no clear change in either region.

DECLARATION

I declare that this dissertation is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Signature: _____

Barbara Clare Alison

_____ day of _____, 19__.

Animal Ethics Clearance Certificate Number: 89/80/1

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CHAPTER 1

INTRODUCTION

Endocrine cells are widely scattered throughout the length of the gastrointestinal tract of vertebrates and are also found in many invertebrates. Between them, these cells contain a wide range of regulatory peptides and serotonin. All show the presence of secretory granules. Many, if not all, types of gut endocrine cells have the ability to take up amine precursors and decarboxylate them with the formation of a biogenic amine for which characteristic feature Pearse (1968) coined the term APUD. While the main action of these cells is believed to be endocrine, evidence of a paracrine function has also been presented; for example, somatostatin-immunoreactive cells have processes which terminate on gastrin-producing G cells (Larsson, 1985).

In general terms the functions of gut endocrine cells concern the local regulation of digestive processes (see Grube, 1986). The products of some of these cells may play an important role in the control and maintenance of growth of gastrointestinal tissues: for example, gastrin may have a trophic effect (Johnson, 1976) and somatostatin, an inhibitory

peptide, has had antitrophic actions attributed to it (Lehy et al., 1979, Lehy, 1984).

A number of different methods have been used to demonstrate gut endocrine cells. Techniques which utilize silver impregnation are still used. These demonstrate cells which are argyrophil but not argentaffin, and enterochromaffin (EC) cells which typically are both argyrophil and argentaffin. EC cells are characterized by their serotonin content and some have been reported to contain motilin (Polak et al., 1975; Heitz et al., 1977) and others substance P (Pearse and Polak, 1975b). Not all argyrophil non-EC cells respond in the same way to silver impregnation. A wide variety, but not every type of gut endocrine cell is demonstrated by the Grimelius silver technique. Whereas, for example, gastrin-immunoreactive cells are impregnated by this method but not by that of Hellerström and Hellman, the opposite applies to somatostatin-immunoreactive cells (Larsson et al., 1974c).

Early electron microsopic studies demonstrated marked differences in the morphology of the storage granules from one cell type to another, resulting initially in ultrastructural classification of gut endocrine cells (Solcia et al., 1981). With the development of immunocytochemistry, regulatory peptides were

localized in specific cells i.e. endocrine cells (and also neurones). Methods such as the demonstration of neurone specific enolase (Bishop et al., 1982) allow detection of all gut endocrine cells types; chromogranin, a protein initially detected in the adrenal medulla, is now widely used as a marker for the demonstration of endocrine cells of the gastrointestinal tract (Lloyd and Wilson, 1983).

In the last three decades an explosion of information has resulted from the isolation, sequencing and chemical characterization of regulatory peptides. As a consequence at least twenty regulatory peptides have been identified, ranging from the well-established ones such as secretin and gastrin to the newer ones such as polypeptide YY (PYY) (Tatemoto, 1982) and pancreastatin (Schmidt et al., 1985). It is now clear that some of the peptides occur in multiple forms i.e. fragments of various length of the precursor molecule which all contain the biologically active part. These forms may occur in the same or in different cells; for example, cells immunoreactive for glucagon itself and others immunoreactive for its larger molecular forms have been detected in the gut of mammals (see for example, Larsson et al., 1975b; Grimelius et al., 1976; Tsutsumi, 1984).

Several families of structurally similar peptides have also been recognised: gastrin/CCK, glucagon and pancreatic polypeptide (PP) families.

With regard to terminology for gut endocrine cells demonstrated by immunocytochemical techniques, cells which stain after application of an antiserum raised against a given peptide, are named after that peptide for the sake of convenience. For example, cells stained with anti-somatostatin serum are generally said to show "somatostatin-like immunoreactivity" or to be "somatostatin-immunoreactive". This is done with the full knowledge that the sequence recognised by the antiserum may also occur in a totally different peptide and therefore what has been demonstrated in the cell is not necessarily somatostatin even if the requisite control procedures have been carried out.

The demonstration of the affinity of the same gut endocrine cells for antisera raised to different peptides has suggested that some cells produce more than one peptide. Such dual immunoreactivity may be the result of the origin of the peptides from the same precursor molecule or from different precursor molecules. The coexistence of PP- and glucagon-related substances, for example, appears to be common through-out vertebrate species (see, for example,

Sjölund et al., 1983a; Ali-Rachedi et al., 1984). The phenomenon of dual immunoreactivity has been studied extensively in mammals but far less information is available with regard to avian species. In birds dual immunoreactivity for avian pancreatic polypeptide (APP)- and glucagon-related substances as well as for gastrin/cholecystokinin (CCK)- and neurotensin-like substances has been reported (see Sundler et al., 1977; Rawdon et al., 1983).

Extensive immunocytochemical studies have been carried out on the distribution of gut endocrine cells in a wide variety of invertebrates and vertebrates, and especially in mammals (see for example, Pufener et al., 1975; Sundler et al., 1977; Alumets et al, 1977; Helmstaedter et al., 1977c; Seino et al., 1979). Some studies on avian gut have been conducted at hatching (Rawdon and Andrew, 1981a) and thereafter in young birds (for instance, Larsson et al., 1974c) and adults (for example, Yamada et al., 1979; El-Salhy et al., 1982d).

Immunoreactive cells of various types have also been demonstrated during prenatal life, for example, in rat and human foetal gut (Larsson et al., 1974a, 1975a; Dubois et al., 1976 a,b; Larsson et al., 1977; Helmstaedter et al., 1977b; Dupouy et al.,

1983; Kataoka et al., 1985a,b). There is however much less published work on embryonic avian material; that of Sundler et al. (1977) and Salvi and Renda (1986) reports on the distribution and ontogeny of neurotensin-, somatostatin-, gastrin/CCK-immunoreactive cells in chick embryos at different stages of development with somewhat differing results. Yet other immunocytochemical studies have been carried out on the ontogeny of cells showing pancreatic polypeptide (PP)-like substances (Larsson et al., 1974b; Alumets et al., 1978), PYY (El-Salhy et al., 1982b) VIP (vasoactive intestinal polypeptide) (Sundler et al., 1979a) and bombesin (D'Este et al., 1984) in the same species. No information seems to be available on the ontogeny of cells demonstrating glucagon-like immunoreactivity in chickens except at hatching (Rawdon and Andrew, 1981a).

Aims of the present study

The present study was undertaken to determine the distribution and time of first appearance of several types of gut endocrine cells in different regions of the gastrointestinal tract in chick embryos.

The cell types selected show immunoreactivit, for somatostatin, neurotensin, gastrin/CCK, APP and

glucagon; they were sought between 11- and 21- days of incubation.

A second aim was to determine whether two peptides are stored in the same cell when they first appear or whether the peptides stored by the cells are initiall present in separate cells. For this purpose, dual immunoreactivity for APP and glucagon and for gastrin/CCK and neurotensin was sought in selected regions of the gastrointestinal tract of chick embryos. The proportions of cells exhibiting dual immunoreactivity in the selected regions were studied to determine whether any changes occured with increasing age of the embryo.

CHAPTER 2

BACKGROUND INFORMATION ON THE REGULATORY PEPTIDES, HISTOLOGY OF THE GASTROINTESTINAL TRACT OF CHICK EMBRYOS AND IMMUNOCYTOCHEMICAL PROCEDURES

To provide background information on the regulatory peptides dealt with in this study an overview of the relevant literature is given with regard to the primary structure of the peptides, distribution of the peptide-storing cells and the physiological roles of the peptides, with particular reference to birds. Further, a description of the histology of the gastrointestinal tract of developing chickens from 11 or 12 days until 21 days of incubation is presented and the methodology of immunocytochemical procedure in general is outlined.

2.1 Regulatory Peptides

2.1.1 Gastrin

Gastrin was the first peptide hormone to be localized immunocytochemically in entero-endocrine cells (McGuigan, 1968) having previously been isolated and chemically characterized as G-17-I and G-17-II by Gregory and Tracy (1964) in hog intestinal mucosa. Subsequently gastrin

heptadecapeptides have been isolated from other mammals (Kenner et al., 1973, see Nilsson, 1980) including man (Bentley et al., 1966). In the past few years it has become evident that in mammals, at least, gastrin exists in several other molecular forms differing in peptide chain lengths (Walsh, 1981) and that a larger form is frequently a biosynthetic precursor of a smaller biologically active form (Tager and Steiner, 1973). Following the introduction of radioimmunoassay (RIA) methods a peptide consisting of 34 amino acid residues, big gastrin, was purified and characterised from porcine intestine and from gastrinomas (Gregory and Tracy, 1972). Thus "big" gastrin G-34 is the immediate precursor of G-17. Since then Noyes et al. (1979) have shown that the partial nucleotide sequence for gastrin mRNA predicts that G-34 is further generated from an even larger peptide "big, big gastrin" through trypsin-like cleavage. Other components with gastrin-like immunoreactivity have been identified, i.e. component I (Rehfeld and Stadil, 1973) and a molecular form of 14 amino acids (minigastrin) has been isolated in small amounts from gastrinomas (Gregory and Tracy, 1974). These related forms of gastrin exist in serum as well as in tissue extracts, G-17 being the

predominant form in the pyloric mucosa whereas G-34 is the most abundant circulating form.

The gastrin molecule shares its terminal five amino acids with the pentapeptide sequence molecule of CCK (Mutt and Jorpes, 1971). This fragment has all the chemical and biological actions of the two hormones, supporting the concept that gastrin and CCK have evolved from a common anchestral molecule (Larsson and Rehfeld, 1977; Rosenquist and Walsh, 1980). The tyrosine residue in gastrin at position six may be either sulphated (gastrin II) or unsulphated (gastrin I) counting unconventionally from the carboxyl terminal. In CCK the tyrosine residue at position seven is always unsulphated (Barrington and Dockray, 1976).

CCK was first isolated from porcine intestinal mucosa as a 33-amino acid residue peptide by Mutt and Jorpes (1968). Since then evidence has accummulated suggesting the existence of other forms with 4, 8, 12, 39 and 58 residues (see Maton et al., 1984). The common pentapeptide shared by CCK and gastrin constitutes a highly immunogenic portion of the molecule; hence many antisera raised against one hormone react also with the other. The distinction between gastrin

and CCK has been made possible by the use of region specific antisera directed towards the NH_2-terminal or mid-region of the gastrin molecule. However Larsson and Rehfeld (1977) found that cells in the avian pyloric region failed to react with antisera specific for these regions of the gastrin molecule, supporting the idea that a close homology between avian and mammalian gastrin exists only at the COOH-terminal.

Rawdon and Andrew (1981b) have presented immunocytochemical findings suggesting that cells in the avian pyloric region contain a gastrin-like peptide while those in the small intestine contain a peptide related to CCK. It seems that this peptide resembles mammalian CCK at the COOH-terminus and also show some sequence homology with it outside this region.

In all species investigated (among mammals, birds and reptiles) the antral mucosa in general contains the greatest number of gastrin cells whereas CCK cells are most numerous in the duodenum and proximal jejunum but absent from the ileum (Buffa et al., 1976). Several studies have indicated that a large proportion of CCK

present in the small intestine is CCK 8 with CCK 33 being less significant. In mammals, birds and reptiles, too, gastrin- and CCK-like molecules occur in separate cell types, whereas in amphibians and bony fish the same cell type seems to be responsible for the production of gastrin and CCK (Larsson and Rehfeld, 1977). Besides occurring in the gut, both gastrin- and CCK-like peptides have been found in the central nervous system and minute quantities of gastrin have been detected in the mammalian pancreas (see Nilsson, 1980).

In mammals the physiological actions of gastrin include stimulation of gastric acid and pepsinogen secretion and the secretion of water and electrolytes (Guyton, 1987). It also exerts a trophic action on certain tissues of the gut and together with CCK, also on the pancreas (Johnson, 1976).

CCK opposes the stimulatory effects of gastrin, as well as stimulating gallbladder contraction and pancreatic enzyme secretion. It also inhibits gastric emptying (see Nilsson, 1980). The pattern of activity of gastrin and CCK is governed by the structural features outside the tetrapeptide, in particular, the tyrosine

residue and whether it is sulphated or not. For typical CCK-like actions (high potency on gallbladder and pancreas and a relatively low potency on acid secretion) the tyrosine residue must be sulphated and at position 7 from the COOH- terminus whereas for gastrin-like activity there is no requirement for sulphation of the tyrosine (Dockray, 1977).

2.1.2 Neurotensin

Neurotensin was originally isolated from extracts of bovine hypothalamus by Carraway and Leeman (1973) and was characterized shortly after its discovery as a tridecapeptide (Carraway and Leeman, 1975). Subsequently neurotensin was isolated from both bovine and human intestine (Kitabgi et al., 1976; Hammer and Leeman, 1981).

The known biological actions of the peptide reside almost exclusively in the COOH-terminal portion of the molecule; evidence suggests that this terminus of neurotensin has been conserved during evolution (Carraway et al., 1982).

The character of neurotensin-related peptides has been studied in extracts of chicken intestine by means of chromatography (Carraway and Bhatnager, 1980; Carraway and Ferris, 1983). Of these, one was shown to be identical to bovine neurotensin except for three amino acid substitutions located within the NH_2-terminal half of the molecule; another was a hexapeptide referred to as LANT-6, a natural variant of neurotensin having only six residues, four of which are identical with those in neurotensin.

By means of immunocytochemistry and RIA, neurotensin has been found in man (Helmstaedter et al., 1977a,c; Sjölund et al., 1983b) and mammals to be localized in the epithelium of the jejunum and ileum and to a lesser extent in the colonic and duodenal mucosa (Orci et al., 1976; Polak et al., 1977a; Frigerio et al., 1977; Helmstaedter et al., 1977a, b; Buchan et al., 1978b; Krauze et al., 1985, 1986). The peptide has occasionally been detected in human endocrine tumours, e.g. of the appendix and rectum (O'Brian et al., 1982; Yang et al., 1983). Cells containing neurotensin have as wide a distribution in non-mammalian vertebrates as in mammalian vertebrates, occurring throughout the

gastrointestinal tract (Reineke et al., 1980) except the gizzard, of chickens (Sundler et al., 1977, 1983; Rawdon and Andrew, 1981a). Neurotensin-immunoreactive cells are particularly numerous in the chicken antrum where they occur intermingled with somatostatin- and gastrin-immunoreactive cells which are also numerous here (Larsson et al., 1974c; and Alumets et al., 1977).

The pharmacological actions of neurotensin in mammals include the induction of hypotension, gut contraction, inhibition of insulin release as well as induction of the release of glucagon (Carraway et al., 1976). Neurotensin has a powerful histamine-releasing action on mast cells (Buchanan and Shaw, 1986) and has an inhibitory effect on canine gastric secretion stimulated by pentagastrin but not by histamine (Andersson et al., 1976; Brown et al., 1978). Although the precise physiological activities of neurotensin remain to be elucidated it appears to have two roles. It is active as a peptidergic transmitter in neural tissue (Buchan et al., 1978b) and a hormonal role has also been postulated (Buchan et al., 1978b; Blackburn and Bloom, 1981).

2.1.3 Somatostatin

In 1973 a substance isolated from ovine hypothalami was fully characterized as a tetradecapeptide (Brazeau et al., 1973; Burgus et al., 1973). This substance, somatostatin (SS 14), was found to block the secretion of growth hormone. Somatostatin was the first bioactive peptide the localization of which was confirmed in both the neuron and the endocrine cell; not only has it been detected in the brain, (e.g. Finley et al., 1978) but also in endocrine cells of the gastrointestinal tract and pancreas of mammals (Arimura et al., 1975; Pearse et al., 1977; King and Millar, 1979; Shultzberg et al., 1980 and Patel et al., 1981) and birds (Rawdon and Andrew, 1979; Seino et al., 1979; Alumets et al., 1977), thyroid parafollicular cells (Parsons et al., 1976) and chicken thymus (Sundler et al., 1978) by RIA and immunocytochemistry.

Subsequently, somatostatin 28 (SS 28), consisting of 28 amino acid residues, was isolated from porcine intestine and ovine hypothalamus (Schally et al., 1980; Esch et al., 1980). This peptide is believed to be a precursor of SS

14 as its COOH-terminal tetradecapeptide is identical to the full sequence of SS 14. Iwanaga et al (1985), for example, have found it to be a NH_2-terminally extended form of somatostatin 14. The wide distribution of SS 28-like immunoreactivity in the gastro-entero-pancreatic endocrine system of man and rat (Ito et al., 1982a,b) and in the rat hypothalamus (Iwanaga et al., 1983) supports the view that SS 28 is a precursor of SS 14. According to Patel et al., (1981) higher molecular weight forms of somatostatin immunoreactants, including SS 28, predominate in the intestinal mucosa rather than in the stomach mucosa and pancreas. Region-specific antisera to [Tyr 14]-SS 28 (1-14) were employed by Baskin and Ensinck (1984) to identify cells with immunoreactivity of the 1-14 fragment of SS 28 in rat gastric and intestinal epithelial cells. Evidence presented by these authors suggests that in the pancreas and antral mucosa, the SS 28 molecule is processed into two fragments, the SS 28 (1-14) and the SS 28 (15-28) portions, whereas in the intestine SS 28 remains intact. After trypsin treatment the results of Baskin and Ensinck (ibid.) indicated that the intestinal epithelial cells contain SS 28 (1-14) as part of a larger peptide in which

the antigenically reactive sites of the 1-14 fragments are masked and that in this region SS 28 is not normally processed to smaller peptides.

In mammals somatostatin has a wide variety of biological actions all of which are inhibitory (Thomas, 1980). It is known to suppress the release of gastrin, insulin and glucagon by direct action on their respective secretory cells (Alberti et al., 1973; Konturek et al., 1976; Patel et al., 1981), and also to inhibit the release of other gastrointestinal hormones such as secretin (Boden et al., 1975), CCK (Konturek et al., 1976), motilin (Mitznegg et al., 1977) and pancreatic polypeptide (Kayasseh et al., 1978). Somatostatin also inhibits nutrient absorption in the intestine and gut mobility (Krejs, 1986).

2.1.4 Avian Pancreatic Polypeptide

This straight chain peptide of 36 amino acids was discovered by Kimmel and his co-workers in 1968 as a major contaminant when they were purifying chicken insulin. Since then PP has been completely sequenced in several species; human

PP (HPP), bovine PP (BPP) and avian PP (APP) (Lin and Chance, 1974; Kimmel et al., 1975; Lin et al., 1977). APP shares 16 of its 36 amino acid residues with BPP.

APP was first localized in the pancreas by Langslow et al. (1973), further localized to a specific secretory cell in that organ by Larsson et al. (1974b) and subsequently demonstrated by RIA in chicken plasma by Kimmel et al., (1975).

Extrapancreatic PP-like immunoreactivity has been recognized in a wide variety of mammals, for example, man, dog, opossum (Larsson et al., 1976; Baetens et al., 1976a), foetal rat (El-Salhy et al., 1982c) and human foetal tissue (Leduque et al., 1983) distributed in various regions of the gut. In adult man and other mammals PP cells appear to be confined to the colon and rectum (Buffa et al., 1978; Christina et al., 1978; Sjolund et al., 1983b; El-Salhy et al., 1983).

PP-immunoreactive cells are more wide-spread in the gut of birds than in that of man and mammals. In adult birds APP-immunoreactive cells are found throughout the small intestine and colon/rectum and in addition, in the proventriculus in

young birds (Alumets et al., 1978) and chicks at hatching (Alumets et al., ibid; Rawdon and Andrew, 1981a).

In 1982 Tatemato sequenced two new peptides from hog intestine, peptide YY (PYY) and brain neuropeptide Y (NPY). PYY has an NH_2-terminal and a COOH-terminal tyrosine. The PYY molecule, like PP, contains 36 amino acids. Moreover the PP and PYY molecules have the same COOH-terminus and many other amino acid residues in common (Tatemoto and Mutt, 1980; Tatemoto, 1982). PYY-immunoreactive cells are numerous in mammalian small intestine (Lundberg et al., 1982; El-Salhy et al., 1983; Böttcher et al., 1984) and in the proximal part of the small intestine only, in adult birds and chick embryos (El-Salhy et al., 1982b,d,). According to El-Salhy et al. (1982d) APP and PYY occur in separate cells in the gut of the domestic fowl.

Functionally, APP has been found to be an effective glycogenolytic agent. Unlike glucagon it causes hypoglycerolaemia and has no effect on plasma glucose levels (Hazelwood et al., 1973). Both amplitude and frequency of contraction of the turkey gizzard are inhibited by APP (Duke et al., 1978) and APP is a potent stimulator of

proventricular secretion of acid and pepsin (Kimmel et al., 1971; Haze'wood et al., 1973).

2.1.5 Glucagon

In the present study the term "pancreatic glucagon" or simply 'glucagon', is used for the single chain polypeptide composed of 29 amino acid residues found in the pancreas, and for molecules identical to glucagon derived from the pancreas, i.e. the pancreatic glucagon found in the gastric mucosa of several species of mammals (Larsson et al., 1975b; Baetens et al., 1976; Ito and Kobayashi, 1976; Sundler et al., 1976; Kitemura et al., 1982) and human foetuses (Ravazolla et al., 1981; Buchan et al., 1982; Stein et al., 1983) but not in adult man (Ito et al., 1981). Larger molecular forms of glucagon occurring in the enteral region are referred to as glucagon-like immunoreactants (GLI's).

The primary structure of all mammalian glucagons is identical with the possible exception of guinea pig glucagon (Sundby et al., 1976). Avian glucagon differs slightly from the mammalian form; chicken and turkey glucagon differ from each other by one amino acid residue at position 28 and duck glucagon has a further

substitution at position 16 (Markussen et al., 1972; Hazelwood et al., 1973). There is evidence for the existence of two large "glucagon" species, previously implicated in the biosynthesis of glucagon by avian islets (Tung et al., 1975).

Pancreatic glucagon provokes the formation of two main types of antibodies (Moody and Thim, 1983). The more common type of glucagon antibody reacts with both mammalian and avian glucagon and GLI's of enteral origin. These antisera are directed towards the NH_2-terminus or the mid portion of the glucagon molecule Yanaihara et al. (1981) have proposed that the NH_2-terminal immunoreactant includes the sequence 11-16. The other type of glucagon antiserum reacts only with glucagon itself and is directed towards the COOH-terminal end of the molecule (Assan and Slusher, 1972). Heding et al. (1976) have shown that the COOH-terminal immunoreactant of glucagon lies in the sequence 24-29.

The gut GLI's contain the full sequence of glucagon. Most of them differ chemically, biologically and immunochemically from glucagon. Of a variety of GLI's, two dominating species have

been recorded, one with a molecular weight of approximately 10 000 and the other with a molecular weight of about 4 000 daltons. In birds the bulk of the gut GLI's have a high molecular weight (Moody and Thim, 1983); duck gut GLI consists of a single molecular species with a molecular weight of between 6 000 and 13 000 daltons (Krug and Miahle, 1971).

The amino acid sequence of one of the larger mammalian forms, glicentin, has been determined by Moody and Thim (1981). The peptide consists of 69 amino acid residues (not 100 as first reported by Sundby et al., 1976) and contains the entire glucagon sequence at positions 33-61 with extensions at both the NH_2- and COOH-terminal ends. Glicentin does not react with antibodies to glucagon 24-29 because the C-terminal glucagon immunoreactant is masked by the extension of the glucagon moiety at this COOH-terminal. The NH_2-terminal portion of glicentin (1-30) is identical to the sequence of porcine glicentin-related pancreatic polypeptide (GRPP) (Moody and Thim, 1981; Thim and Moody, 1982). The amino acid sequence of one of the smaller molecular forms of glicentin oxyntomodulin or glicentin-37 has been isolated and

characterized from extracts of porcine small intestine (Bataille et al., 1982a,b).

The highest concentration of gut GLI in mammals is found in the lower intestine and colon (Bryant and Bloom, 1975) and also the greatest number of GLI-immunoreactive cells has been found in these regions (Larsson et al., 1975b). Glicentin-immunoreactive cells have been found in the proventriculus in young birds (Usellini et al., 1983) and pancreatic glucagon-like immunoreactive cells in the same region in adult chickens (Yamada et al., 1985) whereas Rawdon and Andrew (1981a) have revealed cells immunoreactive with both NH_2-terminal and COOH-terminal antisera in the proventriculus and upper ileum of chicks at hatching.

One of the main functions of avian glucagon concerns the regulation of lipid metabolism (Sitbon and Miahle, 1980). Avian adipose tissue is particularly sensitive to glucagon: in birds, unlike mammals, insulin apparently has no antilipolytic effect. The glycaemic state of birds is far more dependent on glucagon than on insulin (Hazelwood et al., 1973; Epple et al., 1980 - see Falkmer and Van Noorden, 1983; Sitbon and Miahle, 1980).

2.2 The Histology of the Digestive Tract of Chick Embryos

The gastrointestinal tract of birds has certain distinctive features which distinguish it from that of mammals and reptiles.

In the oesophagus of birds is a localized dilatation, the crop. The stomach consists of two parts which differ both morphologically and physiologically. The glandular part or proventriculus which leads from the oesophagus is mainly secretory but also functions as a storage organ. The muscular part or gizzard is highly specialized as a "masticatory" organ although some digestion does take place there (Andrew and Hickman, 1974). A narrow transitional zone, the pyloric region (so-called by Rawdon and Andrew, 1981a) exists between the gizzard and the duodenum. This region is referred to as the gizzard-duodenal junction by Larsson et al. (1977) and Sundler et al. (1977) or antrum by Larsson et al. (1974c) and Sundler et al. (1982). Extending from the pyloric region the duodenum forms a loop around the pancreas. The duodenum becomes continuous distally with the longest and most highly convoluted division of the tract referred to by some authors as the jejunum and

ileum (Larsson et al., 1974c; Alumets et al., 1977; Sundler et al., 1982) but as the upper and lower ileum in this study (as by Rawdon and Andrew, 1981a). The terms "large intestine" or "colon" have been used to describe the caudal region of the gastrointestinal tract (Alumets et al., 1977). In this study 'rectum' refers to the composite colon and rectum (colo-rectum) which terminates in an expansion, the *coprodeum* (Romanoff, 1960).

The junction of the small intestine and rectum is marked by the presence of bilateral caeca. These are blind pockets which can be, according to Calhoun (1954 - see Romanoff, 1960) and Lim and Low (1977) divided into three distinct regions after 16 days of incubation. In this study, from 16 days onwards only the middle region of the caeca was examined.

A brief description of personal observations on the histology of the mucosa of those parts of the tract of chick embryos which were studied in this project is given here. Observations of Aitken (1958); Andrew (1959); Romanoff (1960); Andrew and Hickman (1974); Lim and Low, (1977); Altamirano et al. (1984); Avila et al. (1986) and of Pacini et al. (1979); Pacini and Bryk (1979) and Ishizuya (1980) who used scanning electron microscopy (SEM) and

transmission electron microscopy (TEM) have been integrated into the description.

2.2.1 Proventriculus

At 11- and 12-days of incubation the surface epithelium varies from pseudostratified columnar to simple columnar. A few compound glands with small secretory lobules are present in the lamina propria (Fig. 1a). Ducts of the glands have a large central lumen which open onto the free surface. A few simple tubular glands develop by division of the superficial epithelium which results in the formation of folds between the openings of the compound glands (Romanoff, 1960; Lim and Low, 1977). Two days later the proventricular lumen is lined entirely by a simple columnar epithelium which extends into the lamina propria in the form of a few definitive short simple tubular glands. The compound glands have increased in complexity, consist of a large number of lobules and show considerable development compared with that of the simple glands (Altamirano et al., 1984). From 16 days of incubation until hatching the number of secretory lobules further increase in the compound glands. Numerous lobules radiate from a central duct lined by a simple columnar

Fig. 1: a. Proventricular mucosa lined by a simple columnar or pseudostratified columnar epithelium at [illegible] days of incubation. Compound glands are present in the lamina propria.
Benzoquinone vapour fixation; haematoxylin x390

b. Gizzard mucosa lined by a 3-4 layered pseudo-stratified columnar epithelium at 12 days of incubation. Fine basophilic granules are present in the middle region (arrow) L = *Lumen*.
Benzoquinone vapour fixation; haematoxylin x390

c. Pyloric mucosa lined by a pseudostratified columnar epithelium at 12 days of incubation. No glands are present at this stage.
Benzoquinone vapour fixation; haematoxylin x390

epithelium; the compound glands extend throughout the lamina propria. The mucosal surface of the proventriculus is thrown into folds or plicae of varying heights and the simple tubular glands between them increase in number and length. Romanoff (1960) has described the appearance of granular argentophilic (endocrine) cells in the superficial epithelium on the 8th day of incubation and in the compound glands on the 12th day. According to Avila et al. (1986), with the appearance of argentophil cells at 15 days of incubation morphological differentiation of the proventriculus is completed.

2.2.2 Gizzard

At 11-12 days of incubation the gizzard is lined by a relatively flat 3-4 layered pseudostratified epithelium. Secretory "blebs" are evident on the surface epithelial cells and those cells in the middle region of the epithelium contain fine basophilic granules in sections counterstained with haematoxylin (Fig 1b). By 13 days of incubation a few simple tubular glands have developed. The glands extend for a short distance into the lamina propria. The surface epithelium is still pseudostratified at this

stage. Two days later the simple tubular glands have increased in length extending throughout the depth of the lamina propria. They are uncoiled tubules lined by a low simple columnar or cuboidal epithelium. Towards the open ends of the glands the cells become taller and over the free surface of the gizzard the epithelium is simple columnar. The lumen of the glands is usually filled with secretion which extends over the luminal surface of the gizzard to form a thick layer. According to Aitkin (1958) argentaffin endocrine cells are absent from the gizzard of chick embryos.

2.2.3 Pyloric region

At 11-12 days of incubation this region is lined by a pseudostratified epithelium continuous with, and indistinguishable from, that lining the gizzard. No glands are present at these stages (Fig. 1c). The pyloro-duodenal junction is well marked by the presence of previllous ridges in the duodenum (Fig. 1d). At 14 days a few short simple tubular glands extend into the lamina propria. Two days later the glands have increased in length and are lined by an epithelium of tall columnar cells. The glands have a narrow lumen. From 17½ days of incubation

Fig. 1: (cont)

d. Mucosa of *pyloro*-duodenal junction at 12 days of incubation. The presence of a previllous ridge in the duodenum demarcates the pyloric region (left) from the duodenum (right).
Benzoquinone vapour fixation; haematoxylin x390

e. Mucosa of the small intestine lined by a pseudostratified or simple columnar epithelium without goblet cells at 12 days of incubation.
Benzoquinone vapour fixation; haematoxylin x390

f. Rectal mucosa lined by a simple columnar epithelium without goblet cells at 12 days of incubation.
Benzoquinone vapour fixation; haematoxylin x370

onwards the glands are closely packed. The tall .icae are lined by a simple columnar epithelium which is mucus-secreting. The presence of villi in the duodenum now clearly demarcate the pyloric region from the duodenum.

2.2.4 Small intestine

At 11-12 days of incubation the luminal surface of the duodenum, upper and lower ileum is lined by a pseudostratified or simple columnar epithelium (Fig. 1e). At 14 days of incubation the epithelium has differentiated into simple columnar throughout the small intestine. No goblet cells are present in any of the three regions at this stage.

At 11 days of incubation the mucosal surface of the duodenum has developed four longitudinal previllous ridges (Lim and Low, 1977). At 14 to 15 days a zigzag pattern of longitudinal ridges develops (Pacini et al., 1979). A double row of villi will form from each zigzag ridge (Lim and Low, 1977; Pacini et al., 1979). The base of each developing duodenal villus is broad at this stage. Pacini et al. (1979) report the presence of goblet cells in the duodenal epithelium at 15 days of incubation. In this study goblet cells

were seen for the first time at 19 days of incubation. On the 16th day, according to Romanoff (1960), the first intestinal glands invaginate between the ridges. As the ridges become more acutely folded, mounds demarcate the site of the developing villi. Definitive villi are seen in the duodenum only at 18-19 days of incubation and the openings of goblet cells are detected at the tips of the villi (Lim and Low, 1977). Villi in the duodenum are numerous and leaf-like.

In the ileum the same basic mechanisms of villus formation from the zigzag pattern of ridges is present and has already developed by 12 days of incubation : the previllous ridges are thicker and fuller than those of the duodenum and the bases of the folds narrower. At 17 days the ileal ridges are very folded and the sites of villous formation are marked by mounds at each bend of the folds (Lim and Low, 1977). In the ileal portion villi are shorter and have narrower bases than those of the duodenum. Intestinal glands form between the villi: the glands occupy most of the lamina propria between the bases of the villi.

2.2.5 Rectum

Villus-formation in the rectum is less well organized than that in the small intestine. At 12 days of incubation villous folds are randomly scattered throughout the mucosa (Lim and Low, 1977). The epithelium is simple columnar without goblet cells (Fig. 1f). Definitive ridges only appear at 17 days of incubation. In general when villi appear at 18 days they are lower, broader and less numerous than those of the small intestine (Lim and Low, 1977). In the present study goblet cells were seen in the rectum for the first time at 19 days of incubation and were particularly numerous at hatching. According to Simard and Van Campenhout (1932) argentaffin endocrine cells are present in the large intestine on the 14th day of incubation.

2.2.6 Caecum

In the caecum, too, villus-formation shows no definite arrangement. According to Romeo et al (1960), by 11 days of incubation a few irregular folds are present and by 14 days small protrubences resembling low villi are

appeared. According to Lim and Low (1977), however, by 16 days the mucosa is thrown up into ridges, each ridge consisting of numerous transverse folds. At 18 days short villi are present in the middle portion of the caecum only.

2.3 Immunocytochemistry

2.3.1 Immunocytochemical procedures

Immunocytochemistry involves the use of labelled antibodies as specific reagents for the localization of tissue constituents (antigens) in situ (van Noorden and Polak, 1983). The original direct method, in which the specific antibody is labelled, has largely been replaced by more sensitive and economical methods such as the indirect and peroxidase-antiperoxidase (PAP) methods. In both, the specific unlabelled antiserum is first applied to the tissue section. The indirect (two-step) method differs from the PAP (three-step) method in that in the former a labelled antibody raised to the immunoglobulin of the species in which the first antibody was raised, is bound directly to the primary antibody whereas in the more sensitive

PAP procedure, the labelled antibody (PAP complex) is bound to an unconjugated second layer antibody.

Initially antibodies were labelled with fluorescent dyes to visualize antigenic sites. Since then enzymes such as peroxidase, alkaline phosphatase and glucose oxidase have been used as labels and appropriate histochemical techniques applied to demonstrate the enzyme reaction. Other methods rely on the labelling of antibodies with colloidal gold particles. The gold labelling may be further enhanced with silver (Springall et al., 1984). Modifications of the indirect and PAP methods have been introduced involving the use of protein A/ colloidal gold whereby the protein A can bind to the Fc portion of the immunoglobulin and also to colloidal gold particles. The protein A/colloidal gold complex is used in a second step in the procedure. Similarly an avidin/ biotin complex can be used in the second layer of an immunocytochemical stain (see Polak and van Noorden, 1984). This technique uses a primary antibody followed by a biotinylated second antibody directed against the first antibody. The third layer is an avidin-biotinylated peroxidase complex which may be developed with the 3,3' diaminobenzidine

tetrahydrochloride (DAB) reaction (see Graham and Karnovsky, 1966) or other technique.

The indirect method has been used in this study with an enzyme in preference to a fluorescent dye as a label because permanent preparations result whereas fluorescence fades. Furthermore this method is well-established in this laboratory.

2.3.2 Indirect peroxidase method

This is a two-step procedure which involves the application of an unlabelled specific first layer antibody which serves as the antigen for the labelled second antibody. Incubation of the section with the first layer antiserum allows the primary antibodies to interact with the antigen (in this case a peptide) in the fixed tissue. Unbound antiserum is then rinsed off and the second, peroxidase-labelled antibodies, raised in another species against the immunoglobulin of the species ("donor species") in which the primary antiserum had been raised, is then applied.

Sections are subsequently treated by the DAB reaction to demonstrate peroxidase activity.

The DAB acts as an electron donor to the peroxidase complex and undergoes oxidative polymerization forming an intensely brown coloured insoluble polymer at the antigenic site (see Sternberger, 1979). The exposure time of the tissue to the DAB solution used to demonstrate the enzyme label should be critically controlled. If the tissue is exposed to the DAB for too long background staining is enhanced more than specific staining.

Pretreatment of tissue sections with trypsin (as used, for example, by D'Este et al., 1984, 1986) is advocated to free masked immunoreactive sites, but was not used in this study in case it were to result in non-specific staining.

2.3.3 Immunocytochemical controls

As non-specific reactions between antibodies and tissue components can also occur certain control procedures are necessary.

2.3.3.1 Specificity of the primary antiserum

This must be tested by absorbing the primary antiserum with excess homologous

antigen prior to immunocytochemical staining. If the antiserum is specific for the antigen under investigation, no staining should occur as all the reactive sites of the antibody should be occupied by antigen. As Grube (1980) has pointed out, absence of staining after preabsorption of the primary antiserum with its homologous antigen does not discriminate between specific and nonspecific binding of immunoglobulins: any non-specific antibodies in the antiserum that combine with the antigen in the tissue by ionic bonds will result in staining; these nonspecific antibodies will also combine with antigen in the test-tube and so no staining will occur in a section treated with the preabsorbed antiserum. The result may be taken to show that the antibody is specific for the antigen in question when only non-specific immunoglobulins are present in the serum. Elimination of this problem can be achieved by the use of diluent/rinses containing 0.5M NaCl, the salt competing favourably with the antibodies for the ionised sites in the tissue. Therefore if there are reservations with regard to the specificity

of an antiserum, "high" salt treatment should be used.

The specificity of the primary antiserum must also be tested by preabsorbing the primary antiserum with structurally related antigens, i.e. those which share amino acid sequences with the peptide in question, to ascertain whether cross-reactivity with the related peptide has occurred. The use of region-specific antisera raised to unshared portions of the molecule will provide good evidence that the specified peptide is being demonstrated. The primary antiserum must also be preabsorbed with a range of structurally unrelated antigens such as may be present in the tissue being studied. *This procedure should not affect staining.*

Contaminating antibodies known to be present in an antiserum can be preabsorbed with their corresponding antigens (see 3.5.1).

2.3.3.2 Method controls

These are an essential part of an immunocytochemical procedure and have been

successful when no staining due to mechanisms other than the antibody-antigen reaction results (van Leeuwen, 1986).

Background staining by non-specific attachment of serum immunoglobulins to tissue sections may be reduced by the application of a solution of proteins prior to staining with the primary antibody, e.g. non-immune serum from the species donating the second antiserum, bovine serum albumin or gelatine. The protein will block sites which would be stained non-specifically by antibodies in the antiserum and it will not bind to or interfere with the specific attachment of antibodies.

High background staining resulting from the non-specific attachment of antibody to tissue components by ionic bonds should be reduced by using the "high" salt treatment of Grube (1980) i.e. by using buffers and/or diluent containing 0.5M NaCl.

Background staining is reduced by using primary antibodies at their optimal dilution i.e. the minimal concentration of antibody required to give acceptable

specifi ing with low background. Antibo . at their optimal dilution also reduces the concentration of any unwanted antibodies present.

Method control includes replacing the primary antiserum with the diluent used to prepare the primary antiserum. No staining should occur. If any tissue components do stain, endogenous peroxidase present in them has reacted with the DAB solution (van Noorden and Polak, 1983). Staining can be blocked by prior treatment of the section with hydrogen peroxide.

To test for non-specific staining of particular tissue components by the primary antiserum, the primary antiserum is replaced by non-immune serum from the species in which the primary antiserum was raised. No staining should occur. If staining does take place this is due to the non-specific uptake of immunoglobulins from the primary or second antiserum (van Noorden and Polak, 1983).

A standard section known to contain the antigen under investigation and known to stain with the procedure to be used, must be included with each batch of slides stained, to check that the entire staining procedure has been successfully carried out on that occasion.

2.3.4 Fixation, embedding and sectioning of tissues and mounting of sections

Various methods of tissue fixation appropriate for immunocytochemistry are available. Tissues must be preserved immediately upon removal from the embryos to preserve the antigen. They may be rapidly frozen at an extremely low temperature and freeze-dried. Fixation in liquids, e.g., parabenzoquinone, glutaraldehyde or in different forms of formaldehyde such as Bouin's fluid or buffered picric-acid formalin, followed by dehydration and wax embedding or even vapour fixatives have proved satisfactory for light microscopy (Gosselin et al., 1986). The method of choice in this laboratory for gut peptides is vapour fixation in parabenzoquinone vapour which preserves the antigenicity of numerous gut peptides besides retaining the morphological detail of the tissue (Pearse and

Polak, 1975a; Facer et al., 1980). Embedding in Epon Araldite allows the preparation of semi-thin sections which give a clearer image than thicker wax sections. A disadvantage is that only small pieces of tissue can be embedded in resin. Although heat can be deleterious to antigens, the immunoreactivity of antigens in endocrine cells is unaffected by drying mounted tissue sections at 60°C (Grube and Kusumoto, 1986).

CHAPTER 3

MATERIALS AND METHODS

3.1 Tissue Processing

Chick embryos of the Black Australorp strain obtained from reliable sources were incubated at 37.5-38°C at a relative humidity of 50%. On removal from the eggs, the embryos were staged according to the normal table of Hamburger and Hamilton (1951). After killing the embryos by decapitation, pieces of gut, to be referred to as "specimens", were rapidly dissected out from eight regions of the tract (see Fig. 2(i), (ii)) at each of the following stages: 11 (stage 37), 12 (stage 38), 13 (stage 39), 14 (stage 40), 16 (stage 42), 17½ (stages 43-44) and 19 days of incubation and at hatching. Because the gut is shorter in smaller embryos, specimens at 11-14 days of incubation (Fig. 2(i)) are more representative of the region than those taken from older embryos (Fig. 2 (ii)). To preclude tissue autolysis a maximum of four specimens was taken from each embryo. All specimens were slit longitudinally and cut into longitudinal 1 mm-wide strips of tissue in order to include a more representative length than would be provided by a transverse strip. Since the

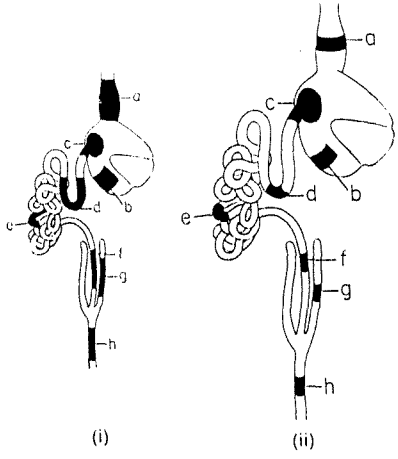

Figure 2 : Gastrointestinal tract of the chick embryo *indicating the regions a-h studied*

(i) 11-14 days of incubation; (ii) 16 days incubation - hatching
(a) Proventriculus; (b) Gizzard; (c) Pylorus; (d) Duodenum
(e) Upper ileum; (f) Lower ileum; (g) Caecum; (h) Rectum

pyloric region is not easily recognisabl macroscopically, a piece of gut stretching from the distal end of the gizzard to the cranial end of the duodenum was dissected out and slit longitudinally from the duodenum craniad. The specimen was opened out, mucosal surface uppermost and viewed with the aid of a stereomicroscope. The pyloric region is distinguishable as a pale, relatively smooth area situated between the rougher surface of the gizzard cranially and the duodenal villi caudally. Longitudinal 1mm-wide strips of tissue were cut to include a small area of gizzard, the whole length of the pyloric region and a small area of duodenum. It was important to include duodenal villi in the sections as positive identification of the pyloric region in younger embryos was dependent upon locating the pyloro-duodenal junction.

Sufficient specimens were accumulated from 2 to 5 embryos at each stage of incubation for every region, in order to study the distribution of a given endocrine cell type. Specimens were quenched in melting methyl butane (Merck) for 30 seconds and freeze-dried overnight at -40°C at $10^{-2,5}$ Torr.

3.2 Fixation and Embedding

Specimens were fixed in parabenzoquinone vapour (see Pearse and Polak, 1975a) prepared from re-crystallized parabenzoquinone (Merck), for 3 hours at 60°C and infiltrated with Epon-Araldite under vacuum for 3 hours. They were positioned in resin-filled silicone rubber moulds so that sectioning would be perpendicular to the mucosal surface. The resin was cured for 48 hours at 60°C.

3.3 Sectioning of Specimens and Storage of Sections

Preliminary one micron sections of each tissue block were cut on an ultramicrotome and counterstained with toluene blue to visualize the morphology of the region. Sets of four consecutive one micron sections were mounted, one per slide, in wells on PTFE-coated slides (see Rawdon, 1978). Sections were subjected to brief drying on a hot plate at 60°C, dried overnight at the same temperature and stored at 4°C.

3.4 Selection of Stages and Regions for Study

Initially cell types to be studied were sought in all eight regions of the gut at 21 days of incubation

i.e. after hatching, in order to verify or otherwise the results of Rawdon and Andrew (1981a) and other workers (Alumets et al., 1977; Sundler et al., 1977) for chicks at hatching, and also at 19 days of incubation to detect any differences in the frequency and distribution of immunoreactive cells. As the earliest age/stage at which endocrine cells have been detected by other workers (Salvi and Renda, 1986) is 11 days of incubation, immunoreactive cells of all five types were sought at this stage and thereafter at 13-, 14-, 16- and 17½-days of incubation in regions where cell types were expected on the basis of observations made at 12-, 19- and 21 days. Because none of the five cell types studied were detected in the gizzard and none except neurotensin in the rectum and caecum at 19- or 21- days of incubation, immunoreactive cells were not consistently looked for in these regions at earlier stages. Neurotensin-immunoreactive cells were sought in the rectum and also in the caecum as occasional cells have been reported to occur in the rectum at 18 days of incubation, and they were rarely found in the caecum (Sundler et al., 1977).

3.5 Localization of Gut Endocrine Cells

As it became apparent that few immunoreactive cells

were present at 12 days and none detected at 11 days of incubation, sections from embryos at the latter stage were stained by the Grimelius method (1968). This silver technique demonstrates the presence of a wide range of endocrine cell types in the gastrointestinal tract. Several sections, 20u apart, from the proventriculus, pyloric region and duodenum of two different embryos from each region were impregnated in this way.

Dual immunoreactivity was sought in cells immunoreactive for neurotensin and gastrin in the pyloric region and duodenum at 17½-, 19- and 21-days of incubation and for APP and glucagon in the proventriculus, duodenum, upper and lower ileum at the same stages. One to five sets of four consecutive sections from each of two specimens from each region in which relevant immunoreactive cell types had already been detected, were treated. The first and third sections of each set were stained with neurotensin antiserum and the second and fourth sections with gastrin/CCK antiserum; APP and glucagon antisera were used following the same procedure. Only if a cell stained in the first and third sections or in the second and fourth sections was it certain that the same cell was present, whether immunoreactive or not, in the intervening section treated with the other antiserum; only such

cells were scored. The distribution of these immunoreactive cells was recorded on camera lucida drawings of the sections (see Appendix C, pp. 191-195). The number of cells stained for both antigens and the numbers staining for only one or other antigen were counted in each section.

3.5.1 Immunocytochemical procedure

Sections were deplasticized in sodium ethoxide. The first and third sections of each set of four were stained with primary antiserum. If a cell had stained in the first and third sections it was then known that it was present (although unstained) in the intervening section which was used for absorption control purposes, i.e. preabsorption of the primary antiserum with its homologous antigen. In alternate sets of four slides the fourth section was incubated either with normal rabbit serum or Tris saline as a substitute for the primary antiserum.

As already mentioned (2.1.2), the indirect immunoperoxidase method (for particular procedure see Pawdon and Andrew, 1979) was used to detect sites of antigen-antibody interaction in cells containing gut peptides. Details of the primary antisera (all polyclonal) used are

listed in Table 1. All antisera had been raised in rabbits. The specificity had been tested on embryonic gut from the same strain of chicken as used here and the optimum dilution determined previously by Rawdon and Andrew (1979) who also preabsorbed each with a range of structurally related and unrelated gut peptides including the COOH-terminal pentapeptide of gastrin/CCK (CCK 5), COOH-terminal octapeptide of CCK (CCK 8), CCK-7-1, secretin (synthetic porcine), VIP (natural porcine), substance P (synthetic bovine), bombesin (synthetic amphibian), motilin (natural porcine), met-enkephalin (synthetic porcine) and insulin (natural porcine) besides those listed in Table 2. No cross reactivities had been detected.

As antiserum L48 is directed against the shared COOH-terminal pentapeptide of gastrin and CCK, this antiserum does not distinguish between cells with gastrin- and CCK-like immunoreactivity. Cells showing immunoreactivity with this antiserum are hence collectively referred to as gastrin/CCK-immunoreactive cells. Antiserum YY59, raised against natural porcine pancreatic glucagon, has NH_2-terminal regional specificity and therefore stains both pancreatic and the larger molecular forms of mammalian glucagon.

Table 1. Antisera used

Antiserum raised to:	Code	Specificity	Dilution	Source
Cholecystokinin (CCK 8-11)	L48	COOH-terminal	1 : 8000	G J Dockray. Liverpool[b]
Neurotensin (fragment 8-13) (synthetic bovine)	R94	COOH-terminal	1 : 1000	P Emson, Cambridge[a]
Somatostatin (synthetic cyclic ovine)	195-8-11-8-76	-	1 : 8000	M P Dubois, Nouzilly[b]
Glucagon, pancreatic (natural porcine)	YY59	NH_2-terminal	1 : 2000	K O Buchanan, Belfast[b]
Pancreatic polypeptide (Natural avian) (APP)	-	-	1 : 8000	J R Kimmel, Kansas City[b]

a - I gratefully acknowledge this gift.
b - I gratefully acknowledge the use of these gifts made to Professors A Andrew and B B Rawdon.

Table 2. Antigens used for absorption purposes

Antigen	Concentration in ug/ml diluted antiserum	Sources
CCK 39	20 ug/ml	V. Mutt, Stockholm[a]
Neurotensin (synthetic bovine)	20 ug/ml	Sigma Chemical Co.
Somatostatin (synthetic toad)	20 ug/ml	Sigma Chemical Co.
Glucagon (synthetic bovine/ porcine)	20 ug/ml	Research Plus Labs.
Pancreatic polypeptide (avian - APP - II - 117)	10 ug/ml	J R Kimmel, Kansas city[a]

a - I gratefully acknowledge the use of these gifts made to Professors A Andrew and B B Rawdon

If similar forms exist in chicks, both should stain with antiserum YY59. The APP antiserum used does not distinguish between APP and peptide YY (PYY); thus cells showing immunoreactivity with the APP antiserum could contain either APP or PYY or both. Contaminating antibodies known to be present in the APP antiserum, (see Cowap, 1985) were routinely absorbed out with insulin and glucagon.

All antisera were diluted with 0.05M Tris buffer, pH 7.6, containing 0.95% NaCl (Tris saline) and 1% non-immune swine serum. The latter prevents the antiserum from attaching to the wall of the vessel (van Noorden and Polak, 1983) and also blocks sites responsible for non-specific staining (see 2.3.3.2).

Tris saline was routinely used for rinsing and washing sections. The wash following incubation with the primary antiserum also contained non-immune horse serum at a final concentration of 1%. (Horse serum was used here instead of the more expensive swine serum.)

Swine serum at a concentration of 10% was applied to tissue sections prior to the incubation of tissue sections with primary

antiserum. Because the swine serum binds to tissue sites it prevents non-specific attachment of the primary or secondary antibodies (see 2.3.3.2).

Immunocytochemical controls included replacement of the primary antiserum with primary antiserum preabsorbed for 22 hours at 4°C with its homologous antigen (see 2.3.3.1) at the concentration shown in Table 2, replacement of the primary antiserum with Tris saline to test for endogenous peroxidase (see 2.3.3.2) and replacement of the primary antiserum with non-immune rabbit serum at the same dilution as the primary antiserum, to test for non-specific staining by the primary antiserum (see 2.3.3.2).

The "high salt" procedure of Grube (1980) was used with the neurotensin and glucagon antisera in an attempt to reduce non-specific background staining (see 2.3.3.2).

3.6 Mounting, Counterstaining and Viewing of Sections

Routinely the first of each set of four consecutive sections, used to demonstrate the frequency and

distribution of immunoreactive cells, was mounted in veronal buffered glycerol, pH 8.6; the other three were weakly counterstained with haematoxylin and mounted in DPX. Sets of sections stained to demonstrate dual immunoreactivity were either mounted in veronal buffered glycerol or counterstained with haematoxylin and mounted in DPX. All sections were studied by both phase contrast and bright field microscopy. Interference contrast and bright field microscopy were used for photographic purposes.

CHAPTER 4

RESULTS

4.1 Immunocytochemical Controls

Controls for specificity of the antisera used, as well as all method controls, gave satisfactory results (see Figs. 3a-c, 4a-c). Thus staining of endocrine cells was eliminated when the primary antisera were preabsorbed with their homologous antigens, indicating that the primary antisera were likely to be specific for the antigens in question. Also as there was no staining when the primary antiserum was replaced by non-immune serum of the species in which the primary antiserum was raised, this source of possible non-specific staining was not operative. Furthermore, because no staining occurred when the primary antiserum was replaced by Tris saline endogenous peroxidase had not interfered with the results.

The "high" salt procedure of Grube (1980) sometimes, but not always, reduced non-specific background staining by the neurotensin and glucagon antisera; cells of both types were nevertheless distinguishable in all preparations.

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The "high" salt procedure of Grube (1980) sometimes, but not always, reduced non-specific background staining by the neurotensin and glucagon antisera; cells of both types were nevertheless distinguishable in all preparations.

Fig. 3: Consecutive sections of the pyloric region at 17½ days of incubation demonstrating

a. somatostatin-immunoreactive cells; counterstained

c. somatostatin-immunoreactive cells; interference contrast

b. lack of staining after absorbtion of antisomatostatin with somatostatin; counterstained

Benzoquinone vapour fixation x890

Fig. 4: Consecutive sections of the pyloric region at 14 days of incubation demonstrating

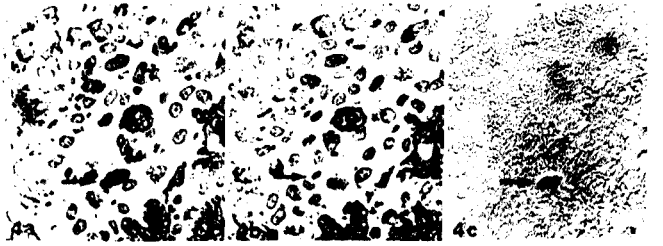
a. AFP-immunoreactive cell (arrow); counterstained

c. APP-immunoreactive cell (arrow); interference contrast

b. Lack of staining after absorbtion of anti-APP with APP; (position of cell indicated by arrow); counterstained

Benzoquinone vapour fixation x890


3a
3b
3c


4a
4b
4c

4.2 Time of First Appearance, Distribution and Frequency of Endocrine Cells

The early appearance, increase in frequency and distribution of somatostatin-, neurotensin-, gastrin/CCK-, APP- and glucagon-immunoreactive cells in the epithelium of the gastrointestinal tract of chick embryos is illustrated by photomicrographs in Figs. 7-16. Block diagrams, Figs. 5 and 6 record their time of first appearance, frequency and distribution at serial ages between 11-days (not reflected in Fig. 6) and 21 days of incubation.

In these diagrams the frequency of the different types of immunoreactive cells in various regions of the tract has been subjectively graded into six categories. Endocrine cell numbers are recorded separately for all eight regions for each embryo at all stages of incubation studied, in Tables A1-A5 (see Appendix B, pp. 186-190); only averages are presented in Figs. 5 and 6. It should be noted that numbers and frequencies, i.e. in Figs. 5 and 6 and Tables A1-A5, are not directly comparable from one specimen of gut to another as sections vary in length and depth of the mucosa.

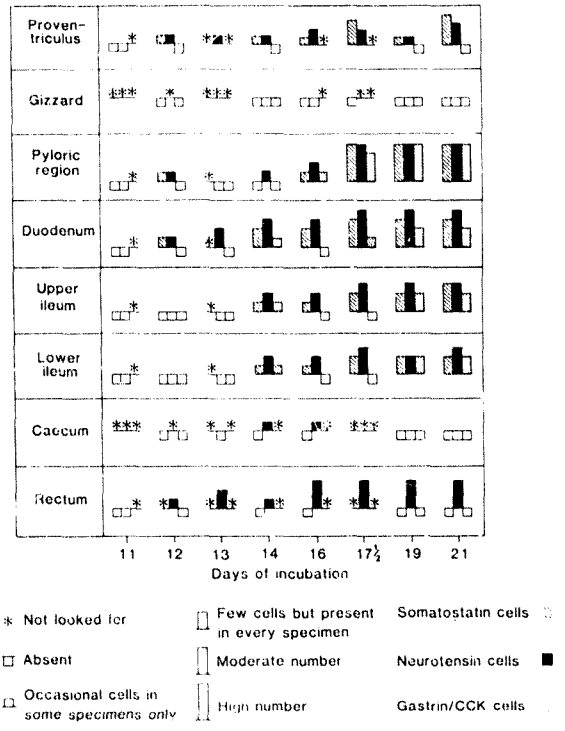


Figure 5: Block diagram to demonstrate the first appearance, distribution and frequency of cells showing immuno-reactivities for somatostatin, neurotensin and gastrin/CCK in the gastrointestinal tract of chick embryos

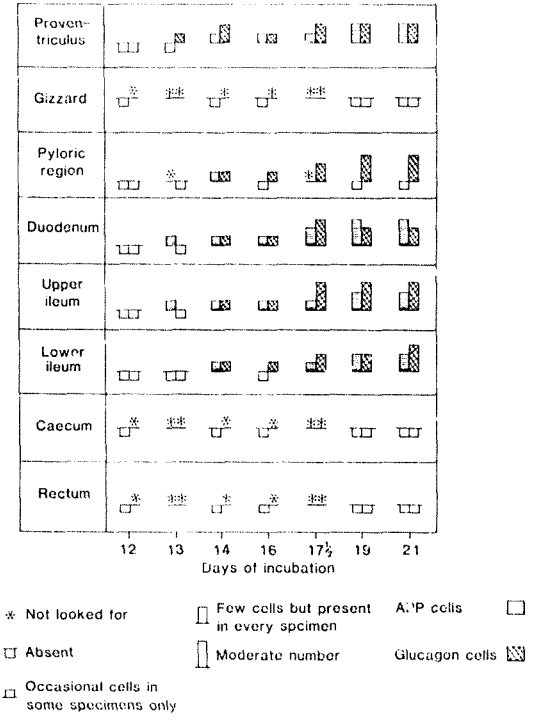


Figure 6: Block diagram to demonstrate the first appearance, distribution and frequency of cells showing immunoreactivities for APP and glucagon in the gastrointestinal tract of chick embryos.

(Additional copies of Figs. 5 and 6 have been included in the Appendix, pp. 184-185. These figures fold out to facilitate correlation of the Block Diagrams with the following text.)

4.2.1 Time of first appearance of endocrine cell

No cells immunoreactive for any of the five antisera used were detected at 11 days of incubation, the earliest stage at which immunoreactive cells were sought. At this stage only four cells in each of two well-spaced sections of the proventriculus from the same embryo were impregnated by the Grimelius method, none being detected in either the pyloric region or the duodenum (see 3.5).

Figs. 5 and 6 show that the first immunoreactive cells appeared at 12 days of incubation. They were very sparsely distributed, some being well granulated (Fig. 9b) while others only contained a few granules (Fig. 13d). Somatostatin- and neurotensin-immunoreactive cells first appeared at this stage in almost all the same regions of the tract: the proventriculus, pyloric region and duodenum (Figs. 9a,b,c,

7a,b,c); neurotensin-immunoreactive cells were detected in the rectum, too, at this stage.

APP- and glucagon-immunoreactive cells first appeared a day later than somatostatin- and neurotensin-immunoreactive cells, i.e. on the 13th day of incubation. Cells immunoreactive for APP were found in the duodenum (Fig. 13b) and upper ileum, glucagon-immunoreactive cells in the proventriculus (Fig. 15a).

On the 14th day of incubation cells containing a gastrin- or CCK-like peptide appeared for the first time; this was in all parts of the small intestine (Fig. 11b). At this stage APP-immunoreactive cells made their first appearance in the proventriculus (Fig. 13a), pyloric region and lower ileum (Fig. 13d) and cells immunoreactive for glucagon in the pyloric region (Fig. 15b) and all parts of the small intestine (Fig. 15c).

In the pyloric region gastrin/CCK-immunoreactive cells were not detected before 16 days of incubation (Fig. 11a); this was the latest stage at which immunoreactive cell types tested for, appeared in any region examined.

4.2.2 Distribution of endocrine cells

Figs. 5 and 6 illustrate the distribution of the five types of endocrine immunoreactive cells studied, in the various regions of the embryonic chick gut. Immunoreactive cells of all these types were found to be located mainly in the gastric region, except for the gizzard where no immunoreactive cells were found, and in the small intestine. Endocrine cells in other regions, i.e. the caecum and rectum were sparsely distributed.

For the reasons given in 3.4 only neurotensin-immunoreactive cells were consistently looked for in the caecum and rectum. This cell type was found in the rectum from 12 days to 21 days of incubation; in the caecum a single neurotensin-immunoreactive cell was detected at 14 days and another at 16 days of incubation. No other immunoreactive cell types were found in the caecum or rectum and no immunoreactive cells at all were found in the gizzard at any stage examined.

An unexpected observation was the detection of an APP-immunoreactive cell in the pyloric region at 14 days of incubation (Figs. 4a,c); no other

cells of this type were detected here in the period studied.

When immunoreactive cells of a given type were first detected they were sparsely distributed and were not necessarily seen at every successive stage or in every specimen at the same early stage, e.g. cells with gastrin/CCK-like immunoreactivity although detected in the small intestine on day 14 and from day 19 onwards, were not seen on day 16 and only in the duodenum on day 17½; neurotensin-immunoreactive cells, although seen in the pyloric region on days 12 and 14 were not detected on day 13; somatostatin-immunoreactive cells were detected in the pyloric region on day 12 and from day 16 onwards but not on day 14; and APP-immunoreactive cells, although seen in the lower ileum at 14 days and from 17½ days onwards were not detected at 16 days of incubation.

4.2.3 Frequency and increase in number of endocrine cells

The changes in frequency of the five different types of immunoreactive cells in the various regions of the tract at successive stages are represented in Figs. 5 and 6 and illustrated in

Fig 7-16. From 12 to 14 days of incubation, except for neurotensin-immunoreactive cells in the duodenum, immunoreactive cells were sparsely distributed in all regions.

Subjective assessment of the numbers of immunoreactive cells of each cell type (see Figs. 5 and 6) showed that, as a rule from 16 days of incubation and onwards, the numbers increased fairly steadily in all regions in which they were present. Hence by 16 days - (for neurotensin-immunoreactive cells in some regions), and more particularly by 17½ days - (for somatostatin-, glucagon- and gastrin/CCK-immunoreactive cells; neurotensin-immunoreactive cells in other regions) and 19 days- (for APP-immunoreactive cells) of incubation, numbers had mounted, especially in the regions where immunoreactive cells were numerous at hatching. Thus the frequency of cells at hatching generally appeared to be attained by 17½ days of incubation for cells with neurotensin-, somatostatin- and glucagon-like immunoreactivity but only at 19 days for cells immunoreactive for gastrin/CCK and for APP.

Fig. 7: Early appearance of neurotensin-immunoreactive cells in:

a. a proventricular gland at 12 days of incubation (arrow)
b. the pyloric epithelium at 12 days of incubation
c. the duodenal epithelium at 12 days of incubation
d. the rectal epithelium at 14 days of incubation

Benzoquinone vapour fixation; unstained, interference contrast x890

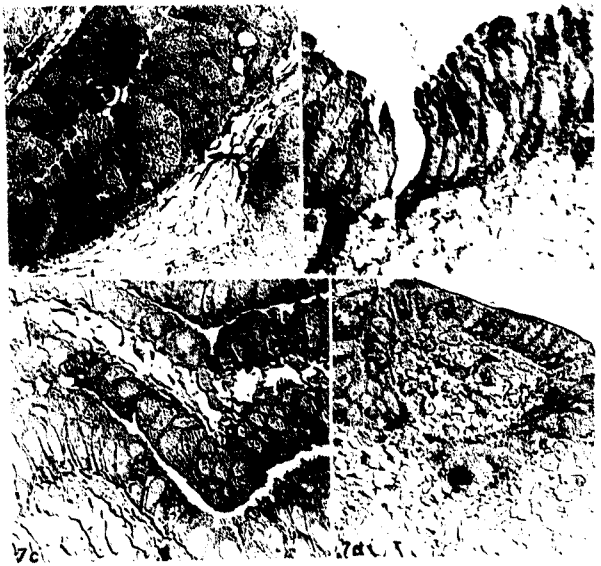
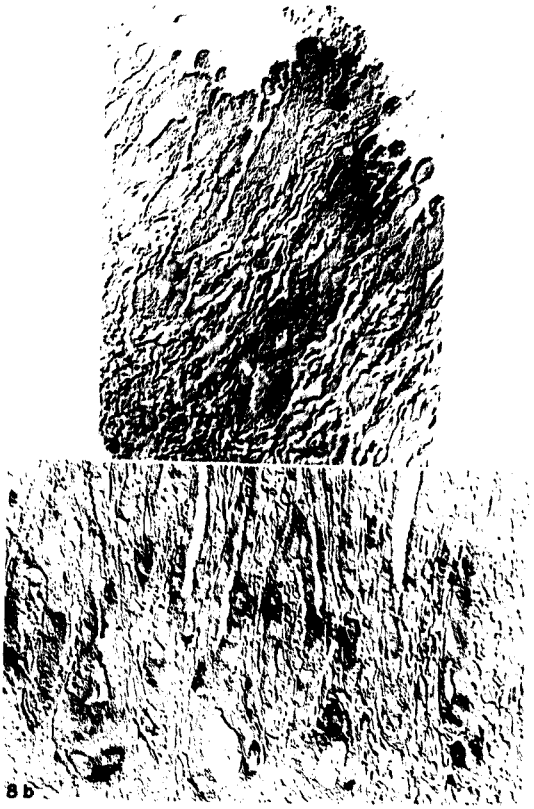


Fig. 5: Neurotensin-immunoreactive cells in the pyloric region at:

a. 16 day of incubation - one immunoreactive cell is seen

b. 17½ days of incubation - the number of neurotensin-immunoreactive cells has increased.

Benzoquinone vapour fixation; unstained, interference contrast x890



8D

Fig. 9: Early appearance of somatostatin-immunoreactive cells in the;

a. proventriculus at 12 days of incubation
b. pyloric region at 12 days of incubation
c. duodenum at 12 days of incubation
d. upper ileum at 14 days of incubation (arrow)

Benzoquinone vapour fixation; unstained, interference contrast x890

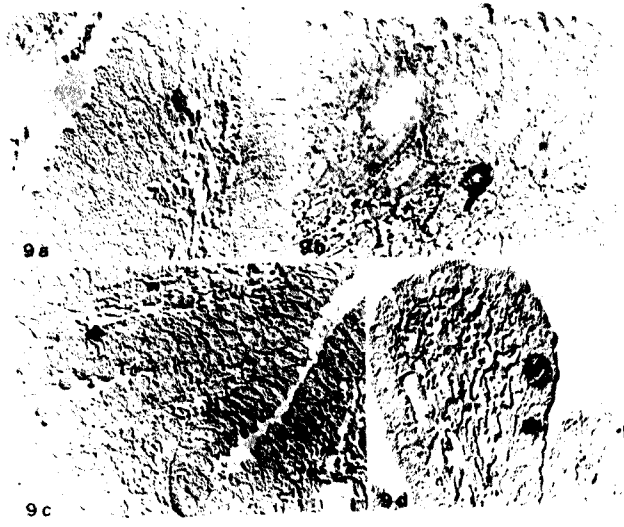

9a
9b
9c
9d

Fig. 10: Somatostatin-immunoreactive cells in the pyloric region at;

a. 16 days of incubation - few immunoreactive cells are present

b. 17½ days of incubation - note the increase in the number of somatostatin-immunoreactive cells

Benzoquinone vapour fixation; unstained, interference contrast x890

10b

Fig. 11: First appearance of gastrin/CCK-immunoreactive cells in the;

a. pyloric region at 16 days of incubation

b. duodenum at 14 days

Benzoquinone vapour fixation; unstained, interference contrast x840

11b

Fig. 12: Gastrin/CCK-immunoreactive cells in the pyloric region at:

a. 17½ days of incubation - few immunoreactive cells are present

b. 19 days of incubation - immunoreactive cells are numerous

Benzoquinone vapour fixation: unstained, interference contrast x890

12/b

Fig. 13: Early appearance of APP-immunoreactive cells in the:

a. proventriculus at 14 days of incubation
b. duodenum at 13 days of incubation
c. upper ileum at 14 days of incubation
d. lower ileum at 14 days of incubation (arrow)

Benzoquinone vapour fixation; unstained, interference contrast x890

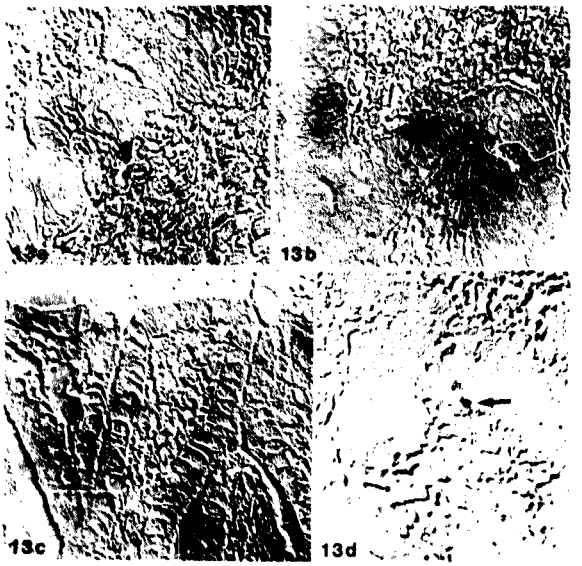

13a
13b
13c
13d

Fig. 14: APP-immunoreactive cells in the duodenum at;

a. 14 days of incubation

b. 16 days of incubation

Immunoreactive cells are sparsely distributed in a and b

c. 17½ days of incubation - APP-immunoreactive cells have increased in number (arrows)

Benzoquinone vapour fixation; unstained, interference contrast x890

Fig. 15: First appearance of glucagon immunoreactive cells in the;

a. proventriculus at 13 days of incubation
b. pyloric region at 14 days of incubation
c. duodenum at 14 days of incubation

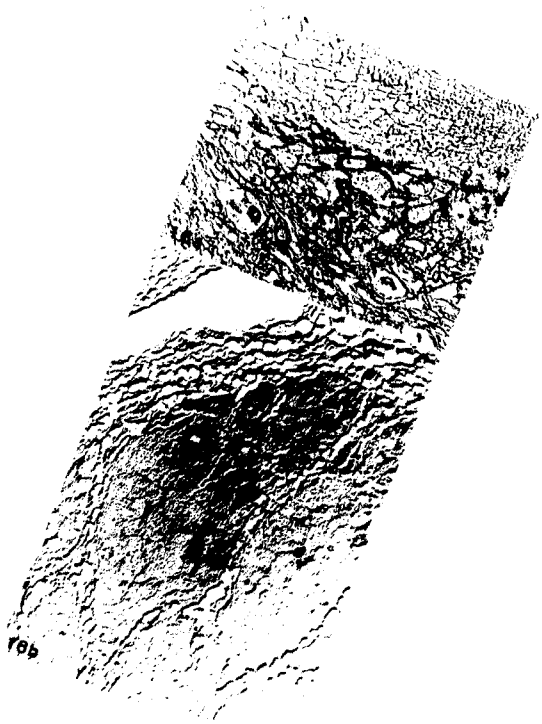
Benzoquinone vapour fixation; unstained, interference contrast x890

Fig. 16: Glucagon-immunoreactive cells in the proventriculus at;

a. 14 days of incubation - one immunoreactive cell is present

b. 17½ days of incubation - immunoreactive cells have increased in number

Benzoquinone vapour fixation; unstained, interference contrast x890



A variation in staining intensity was noted in the neurotensin-immunoreactive cells from 17½ days of incubation onwards.

4.3 Morphology of the Gut at the Time of First Appearance of Endocrine Cells

The morphology - i.e. the degree of differentiation - of the gastrointestinal tract in relation to the time at which endocrine cells first appear is worthy of note. When neurotensin- and somatostatin-immunoreactive cells first appeared in the proventriculus (Figs. 7a, 9a), they were confined to the surface epithelium which was relatively undifferentiated, i.e. pseudostratified, and in the simple glands only, although compound glands with a few acini were present. When glucagon-immunoreactive cells appeared one day later in the same region they were found only in the surface epithelium (Fig. 15a). By 14 days of incubation when APP-immunoreactive cells first appeared in the proventriculus (Fig. 13a) they were found together with sparsely distributed neurotensin-, somatostatin- and glucagon-immunoreactive cells in the simple columnar surface epithelium and also in the fairly well-developed compound glands; no immunoreactive cells of any type were detected in the short simple tubular glands at this time. At 16 days of

incubation and onwards all five types of immunoreactive cells were seen in the well-developed compound glands, in the surface epithelium and in the simple tubular glands which had increased in length.

In the pyloric region when somatostatin- and neurotensin-immunoreactive cells first appeared at 12 days of incubation, (Figs. 9b, 7b) the surface epithelium here too, was pseudostratified with no sign of gastric pits or glands. At 14- and 16-days of incubation when both of these immunoreactive cell types together with glucagon-immunoreactive cells (Fig. 15b) were present, all three cell types were sparsely distributed in the short simple tubular glands. At 17½-days and onwards, gastrin/CCK, neurotensin- and somatostatin-immunoreactive cells were plentiful, (Figs. 12b, 8b, 10b) and glucagon-immunoreactive cells occurred in moderate numbers in the now well-developed simple tubular glands.

In the small intestine, too, when a few neurotensin- and somatostatin-immunoreactive cells were first detected at 12 days of incubation (Figs. 7c, 9c), and one day later when APP-immunoreactive cells were found in the same region (Fig. 13b), the surface epithelium was pseudostratified or simple columnar and only longitudinal previllous ridges had formed.

By 14 days of incubation when cells showing gastrin/CCK-like immunoreactivity appeared for the first time (Fig. 11b), all five immunoreactive cell types were present in the newly-formed zigzag previllous folds.

By 17½ days when villi and intestinal glands were beginning to differentiate, gastrin/CCK-, neurotensin-, somatostatin-, APP- (Fig. 14c) and glucagon-immunoreactive cells had become fairly numerous in all three parts of the small intestine.

When neurotensin immunoreactive cells were detected in the rectum at 12 days of incubation the epithelium was simple columnar and previllous ridges were poorly developed. Neurotensin-immunoreactive cells were seen in the rectal epithelium at 14- (Fig. 7d), 16- and 17½-days of incubation in the previllous ridges and at 19 days of incubation and at hatching in the definitive low villi. In the caecum when a single neurotensin-immunoreactive cell was detected at 14- and another at 16-days of incubation in the simple columnar epithelium, no definitive villi had differentiated.

4.4 Topographical Distribution of Endocrine Cells

Once a certain number of immunoreactive cells had appeared in a given region, they demonstrated a specific topographical distribution. In the proventriculus, neurotensin-, somatostatin-, APP- and glucagon-immunoreactive cells, although always few in number, generally occurred more frequently in the compound glands than in the simple tubular glands or the surface epithelium. In the pyloric region, neurotensin-, somatostatin-, gastrin/CCK- and glucagon-immunoreactive cells were more concentrated in the deeper halves of the glands and more sparsely distributed in the upper portions. Immunoreactive cells of all five types showed a random distribution in both the villous folds/villi and intestinal glands of the small intestine; neurotensin-immunoreactive cells were similarly distributed in the caecum and rectum.

4.5 Degree of Granulation of Endocrine Cells

When some immunoreactive cells of a given type were first observed in any region, their secretory granules were few in number, tending to be located towards the bases of the cells (Fig. 9c). Other cells were fairly heavily granulated (Fig. 9b).

Later in development, when the epithelium had undergone morphogenesis and differentiation and resembled the epithelium of chicks at hatching, as a rule immunoreactive granules filled the endocrine cells (Fig. 12b).

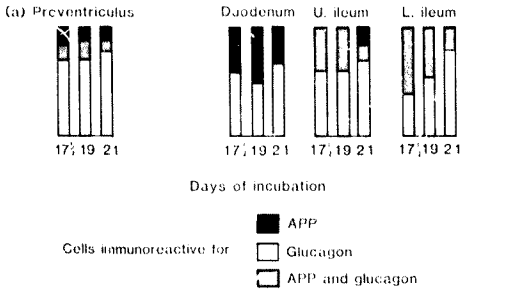
4.6 Coexistence of Peptides

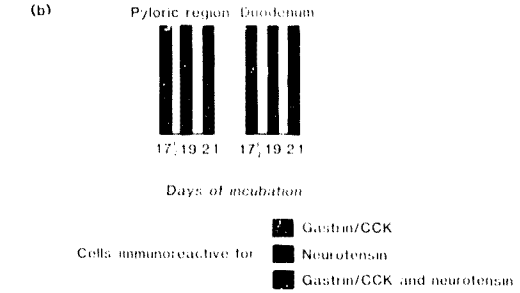
Table 3 (p. 86) shows the occurrence of dual immunoreactivity for APP and glucagon in the same cells in chick embryos at 17½-, 19- and 21-days of incubation in selected regions of the gastrointestinal tract; data for gastrin/CCK and neurotensin are set out in Table 4 (p. 91). Because gastrin/CCK-immunoreactive cells occur only in the pyloric region and duodenum, these two regions were selected to demonstrate dual immunoreactivity for these two peptides; the occurrence of dual immunoreactivity for APP and glucagon in the same cells was sought in the proventriculus, duodenum, upper and lower ileum in both cell types occur in all four regions. Dual immunoreactivity was not sought at or before 16 days of incubation for any of the peptides since at this stage so few gastrin/CCK- immunoreactive cells were detected in the pyloric region and none in the small intestine; only occasional APP- and glucagon-immunoreactive cells were detected in the proventriculus

and small intestine and not in all specimens at this stage. The paucity of cells at these ages made it impractical to test for dual immunoreactivity.

Numbers of cells exhibiting dual immunoreactivity and of cells showing immunoreactivity for only one of the peptides in question are recorded. The numbers of sets of sections examined varied as in some regions immunoreactive cells were few in number and those demonstrating the coexistence of peptides were even sparser, for example, at 17½ days of incubation gastrin/CCK-immunoreactive cells in the duodenum (see Table 4) and APP-immunoreactive cells in the upper and lower ileum (see Table 3). Fig. 17a (p. 84) illustrates the proportion of cells demonstrating dual immunoreactivity for APP and glucagon as well as separate populations of APP- and glucagon-immunoreactive cells at 17½, 19 and 21 days of incubation. Data for gastrin/CCK and neurotensin are set out in Fig. 17b (p. 84). Proportions were calculated in order to enable comparison, even though the numbers in some cases were so small.

Fig 17 Proportions of cells exhibiting dual immunoreactivity for (a) APP and glucagon and (b) gastrin/CCK and neurotensin in various regions of the gastrointestinal tract at 17½, 19 and 21 days of incubation





4.6.1 Coexistence of APP- and glucagon-like immunoreactivity

APP-immunoreactive cells were always fewer than those immunoreactive for glucagon in all regions examined. Whereas in the proventriculus (Fig. 18) and upper (Fig. 19) and lower ileum (Fig. 20) there were at all stages some cells which exhibited dual immunoreactivity, none were present in the duodenum at any of the stages examined. Except for three cells immunoreactive for APP only - all in the same embryo at 21 days of incubation - all other APP-immunoreactive cells in the upper and lower ileum were also glucagon-immunoreactive. A population of cells demonstrating only glucagon-immunoreactivity (e.g. Fig. 18) occurred at all stages in all regions examined.

Fig. 17a is suggestive of changing proportions of cells with dual immunoreactivity but the trends should be viewed with circumspection since the numbers of cells involved are low in some cases. In the lower ileum at least, the proportion of dual immunoreactive cells decreases during the last days before hatching and the proportion of cells demonstrating only glucagon-like immunoreactivity increases.

Table 3. Dual immunoreactivity for APP and glucagon

Region	Days of incubation	No. of embryos	No. of sets of sections	Cell/s immunoreactive for: APP and glucagon	APP only	Glucagon only
Proventriculus	21	2	2	1	1	8
	19	2	3	2	1	7
	17½	2	4	1	1	4
Duodenum	21	2	2	0	6	12
	19	2	4	0	7	6
	17½	2	4	0	6	8
Upper ileum	21	2	5	3	3	14
	19	2	2	6	0	10
	17½	2	5	3	0	5
Lower ileum	21	2	4	3	0	15
	19	2	3	4	0	5
	17½	2	5	3	0	2

Fig. 18: Consecutive sections demonstrating immunoreactive cells in the proventriculus at 19 days of incubation

a) and c) stained with glucagon antiserum

b) stained with APP antiserum

Note: two cells (arrows) exhibit dual immunoreactivity for glucagon and APP. One cell (arrowhead) is immunoreactive for glucagon only.

Benzoquinone vapour fixation; unstained, interference contrast x900

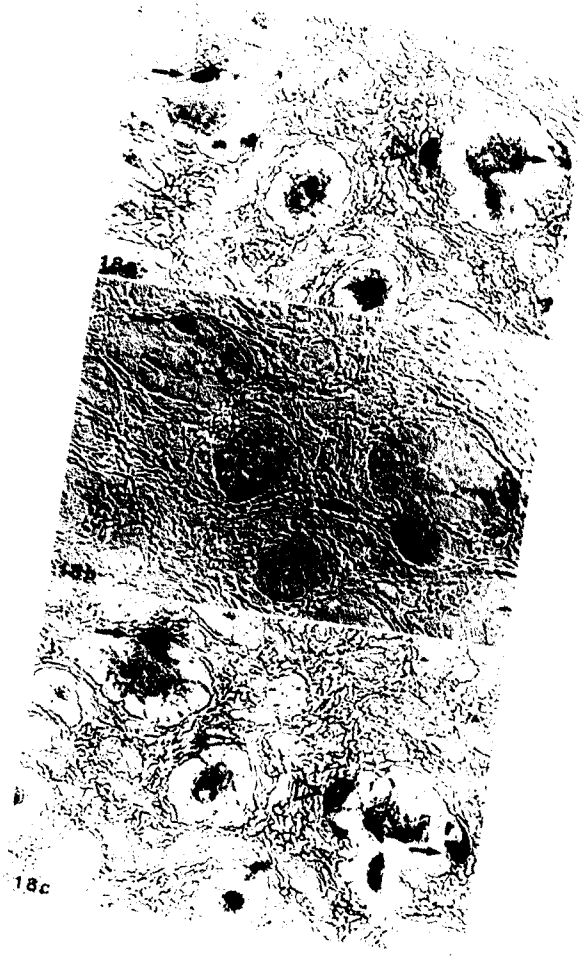

18a
18b
18c

Fig. 19: Consecutive sections demonstrating immunoreactive cells in the upper ileum at 21 days of incubation

a) and c) stained with glucagon antiserum
b) stained with APP antiserum

Note: two cells (arrows) exhibit dual immunoreactivity for APP and glucagon. One cell (arrowhead) is immunoreactive for APP only.

Benzoquinone vapour fixation; unstained, interference contrast x890

19c

Fig. 20: Consecutive sections demonstrating a cell with dual immunoreactivity for APP and glucagon in the lower ileum at 17½ days of incubation

a) and c) stained with glucagon antiserum
b) stained with APP antiserum

Benzoquinone vapour fixation; unstained, interference contrast x890

200a
200b
200c

4.6.2 Coexistence of gastrin/CCK- and neurotensin-like immunoreactivity

Neurotensin-immunoreactive cells were far fewer than cells immunoreactive for gastrin/CCK in the pyloric region; conversely in the duodenum gastrin/CCK-immunoreactive cells were much sparser than cells immunoreactive for neurotensin (see Table 4). In the pyloric region large numbers of cells exhibited dual immunoreactivity (Figs. 21, 22), even more only gastrin/CCK-immunoreactivity and few cells demonstrated only neurotensin- immunoreactivity whereas in the duodenum (Fig. 23) nearly all gastrin/CCK-immunoreactive cells were also immunoreactive for neurotensin.

There are no clear changes in the proportions of the cells in question at any of the stages examined (Fig.17b). As previously mentioned (see 4.2) a variation in staining intensity is demonstrated by the neurotensin-immunoreactive cells; it is noted here that both light and dark cells are among those that exhibit dual immunoreactivity for neurotensin and gastrin/CCK.

Table 4. Dual immunoreactivity for Gastrin/CCK and neurotensin

Region	Days of incubation	No. of embryos	No. of sets of sections	Cell/s immunoreactive for: Gastrin/CCK and neurotensin	Gastrin/CCK only	Neurotensin only
Pyloric region	21	2	3	23	31	5
	19	2	3	17	30	8
	17½	2	4	31	36	5
Duodenum	21	2	4	5	0	47
	19	2	4	10	1	52
	17½	2	6	8	1	42

Fig. 21: Consecutive sections demonstrating immunoreactive cells in the pyloric region at 19 days of incubation

a) stained with neurotensin antiserum

b) stained with gastrin/CCK antiserum

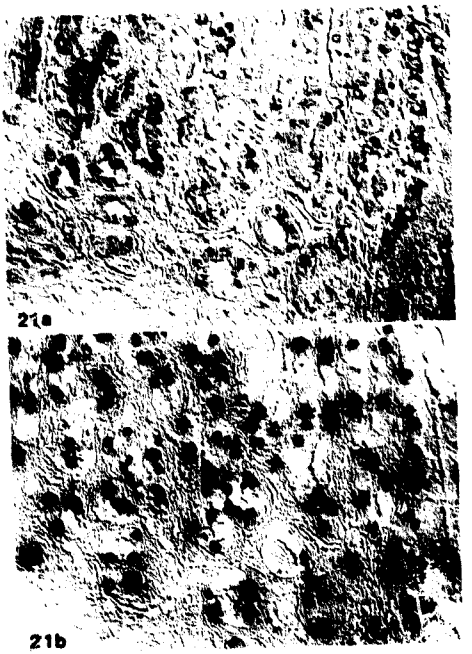
Note on the overlay:

Green - cells showing neurotensin-immunoreactivity only

Red - cells showing gastrin/CCK-immunoreactivity only

Purple - cells showing dual immunoreactivity for gastrin/CCK and neurotensin

Benzoquinone vapour fixation; unstained, interference contrast x400



21a

21b

Fig. 22: Consecutive sections demonstrating immunoractive cells in the pyloric region at 21 days of incubation

a) and c) stained with gastrin/CCK antiserum

b) stained with neurotensin antiserum

Note: three cells (arrows) are gastrin/CCK-immunoreactive only. Two cells (arrowheads) are neurotensin-immunoreactive only. Other immunoreactive cells (unmarked) exhibit dual immunoreactivity for gastrin/CCK and neurotensin.

This particular field selected for photography, does not represent the proportions of dual and singly immunoreactive cells in the entire section.

Benzoquinone vapour fixation; unstained, interference contrast x400

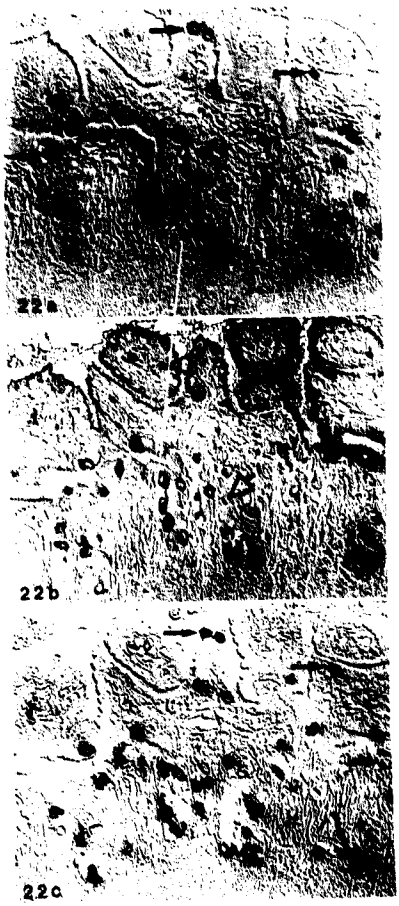

22a
22b
22c

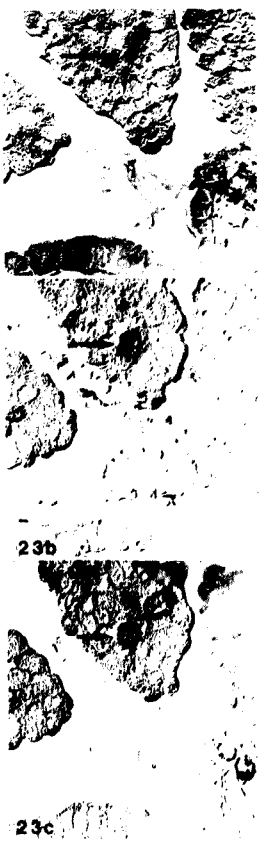
Fig. 23: Consecutive sections demonstrating immuno reactive cells in the duodenum at 21 days of incubation

a) and c) stained with neurotensin anti- : um

b) stained with gastrin/CCK antiserum

Note: of the four neurotensin-immuno-reactive cells, one (arrow) demon-strates dual immunoreactivity for gastrin/CCK.

Benzoquinone vapour fixation: interference contrast x890



23b

23c

CHAPTER 5

DISCUSSION

5.1 Limitations to the Approach Used

5.1.1 Sampling and natural variation

In order to detect the first appearance of endocrine cells in chick embryonic gut in the present study, many specimens were used, particularly at the early stages where samples were taken on consecutive days instead of on alternative days as for older embryos. Not many immunoreactive cells would be expected at early stages and sampling lessens the chances of detecting them. Natural variation in the time of first appearance of endocrine cells may explain the apparent absence or scarcity of immunoreactive cells in some specimens in the early stages of incubation, i.e. occasional cells were seen in only one of two or more specimens studied. It is probable that sampling, rather than individual variation, also accounts for the apparent interruption in the occurrence of cells of a particular type at certain times, particularly since this was observed in regions where immunoreactive cells

were sparse (see 4.2.2). Hence the first cells may appear even earlier than those detected here.

In a few cases the numbers of immunoreactive cells present were unexpectedly low in a population otherwise gradually increasing in number with time/age, e.g. at 19 days of incubation fewer somatostatin- and neurotensin-immunoreactive cells were detected in the proventriculus than at 17½ days. Sampling or individual variation may account for such observations, as thereafter, endocrine cells generally increased in number in those regions.

Gastrin/CCK-immunoreactive cells were found to occur so infrequently in the pyloric region at 16 days of incubation that a larger number of specimens was examined. Despite this only one of the four specimens studied showed the presence of gastrin/CCK-immunoreactive cells in this region at this age.

Exposure of sections to trypsin prior to the immunocytochemical procedure was not carried out for the reason given earlier (2.3.2). If this treatment had been implemented, it is possible that immunoreactive cells would have

been demonstrated somewhat earlier than they were in the present study.

Although it is possible that the very first immunoreactive cells of the cell types studied here may appear a little earlier than reported, the results nevertheless give a clear indication of the time and specific regions in which they may first be expected.

5.1.2 Immunocytochemical limitations

Some antisera raised to synthetic antigens and some raised to natural antigens were used for immunocytochemistry in the present study. Synthetic antigens are pure and the chances are that the antiserum is more likely to be specific than if the antigen is a natural (perhaps contaminated) peptide. Non-specific staining is always a possibility in immunocytochemistry and no controls provide absolute certainty that the peptide in question has been demonstrated. However, since staining of immunoreactive cells was in all cases quenched if the antiserum had previously been absorbed with its corresponding antigen and since all other controls gave satisfactory results, the antisera used in this study were likely to be

specific for the peptides in question. Antisera with different regional specificities for the avian peptides sought, were not available.

Immunocytochemistry has the advantage of being a technique which will detect cells with only a few granules present in the cytoplasm. It is desirable to use complementary evidence obtained from other methods, for example RIA, to support the results of immunocytochemistry. Tissue extracts for this technique are only suitable if adequate quantities of the peptide are present in the tissue. In chick embryos RIA may be applicable in the pyloric region at the later stages of incubation when large numbers of immunoreactive cells are present but when immunoreactive cells first appear and at later stages where they are sparsely distributed, for example, in the small intestine, the peptide content of the tissue would be extremely low.

5.2 First Appearance of Endocrine Cells

In the present study no immunoreactive cells were detected at 11 days of incubation in any region of the gut examined. A method for argyrophilia was

applied (see 3.5) because a large number of endocrine cell types respond to this silver method, and so a better opportunity is thereby provided for detecting endocrine cells, of whatever type, earlier on than by immunocytochemistry. If cells were detected at 11 days they could be of some of the cell types stained for in the present study. If not, then the evidence presented for the time of first appearance of endocrine cells is probably correct. In fact a few argyrophilic cells were demonstrated at 11 days of incubation in the proventriculus only. Hence cells immunoreactive for neurotensin first detected in the proventriculus by immunocytochemistry at 12 days, may be present at 11 days. However, bombesin-immunoreactive cells are known to appear at 11-12 days and could be responsible for the argyrophilia. Argyrophilic cells are described in the proventricular surface epithelium of chick embryos as early as 8 days of incubation and in the compound glands a few days later (see Romanoff, 1960) but immunocytochemistry has not to date provided an indication of their identity. The fact that APUD cells have been detected in chick intestinal epithelium from 12-13 days of incubation (Andrew, 1975) is in line with the findings of the present study that endocrine cells are present in the small intestine from these stages.

As no argyrophilic cells were seen in the pyloric region or duodenum at 11 days, it is plausible that both neurotensin and somatostatin-immunoreactive cells indeed make their first appearance in these regions one day later, as demonstrated by immunocytochemistry.

5.3 Regions with Few Immunoreactive Cells: Caecum and Rectum

The earliest reports of gastrin-immunoreactive cells in the colorectal region of chickens and quails by Polak et al. (1974) has not been confirmed by subsequent studies. Since then other immunoreactive cell types have however been detected in the caecum and rectum (Sundler et al., 1977). Neurotensin-immunoreactive cells were found in the rectum in the present study which confirmed the results of Sundler et al. (1977) in chick embryos and Rawdon and Andrew (1981a) in chicks at hatching. However the times at which these cells have been reported to appear do not coincide. Neurotensin-immunoreactive cells were detected as early as 12 days of incubation in the present study whereas Sundler et al. (1977) reported their first appearance at 18 days in this region. According to these workers neurotensin-immunoreactive cells develop much later than any other endocrine

cell type and reach their adult frequency of occurrence a few days after hatching. In the present study these cells appeared to reach hatching frequency at 17½ days, two days earlier than cells had even been detected by Sundler et al. (ibid).

The occurrence of neurotensin-immunoreactive cells in the caecum at 14 and 16 days of incubation in the present study was rare as noted also by Sundler et al. (1977) in chick embryos and young birds; Rawdon and Andrew (1981a) detected none in chicks at hatching.

Although neurotensin-, glicentin-, PP- and PYY-immunoreactive cells occur frequently in the hind gut of mammalian adults and foetuses (Buffa et al., 1978; El-Salhy et al., 1982c; El-Salhy and Grimelius, 1983; Leduque et al., 1983), of those studied here, the only immunoreactive cell type, other than neurotensin, reported in the colon/rectum of chicks is the PP-immunoreactive cell (Alumets et al., 1978). PP-immunoreactive cells were sought by these workers in chick embryos over a wide range of ages but were detected in the colon/rectum only on the 21st day of incubation. PP-immunoreactive cells were not found in the rectum at any embryonic age in the present study.

5.4 Region Lacking Immunoreactive Cells: Gizzard

Previous studies have reported differing results with regard to the presence or absence of endocrine cell types in the gizzard of avian embryos and of adult birds. While Polak et al. (1974), Okamoto et al. (1980) and Yamada et al. (1986) described gastrin-immunoreactive cells in the gizzard of adult chickens, quails and ducks, earlier authors have found neither gastrin-like biological activity nor gastrin-like immunoreactivity in the gizzard of adult chickens (Ketterer et al., 1973; Larsson et al., 1974c). Yamada et al. (1986) have found, in addition to gastrin-immunoreactive cells, other varieties of endocrine cells, i.e. somatostatin-, APP-, motilin-, serotonin-, and gastrin-releasing peptide (GRP)-immunoreactive cells in several adult avian species including the chicken. All these cell types were found particularly in the cranial and caudal diverticulae of the gizzard while few immunoreactive cells were detected in other regions. Since these endocrine cell types, found by Yamada et al. (1986), with the exception of GRP, occurred so infrequently and in so few specimens these authors have suggested that these immunoreactive cells may be ectopic and of no functional significance. Yet another immunoreactive cell type found in the gizzard of adult birds is bombesin, reported by Timson et al. (1979)

of Vaillant et al. (1979). As bombesin and GRP are very similar these bombesin-immunoreactive cells are likely to be the same GRP-immunoreactive cells as Yamada et al. (1986) found.

It therefore appears that a wide variety of endocrine cell types, albeit sparsely distributed, are present in the adult avian gizzard in particular regions. This contrasts with the situation in young birds and chicks at hatching. Occasional bombesin/GRP- and motilin-immunoreactive cells were found by Rawdon and Andrew (1981a) in the gizzard of hatched chicks. No somatostatin-immunoreactive cells (Sundler et al. (1977) or APP-immunoreactive cells (Alumets et al., 1978) were found in the gizzard of young birds. However Alumets et al. (1978) did report the presence of APP-immunoreactive cells in the gizzard of chicks at hatching but these cells had disappeared one week later. Despite the fact that the middle region of the gizzard and the pyloro-gizzard junction were sampled both in the present study and by Rawdon and Andrew (1981a) in chicks at hatching, no immunoreactive cells of any of the five types sought were detected. No immunoreactive cells of these types were detected in any of the earlier stages examined either.

It seems likely that endocrine cells generally differentiate in the gizzard after hatching However, the facts that the regional distribution of endocrine cells in the gizzard of adult birds is by no means uniform and that the regions of embryonic gizzard sampled in the present study differed from those of the adult gizzard sampled by Yamada et al. (1986), might possibly account for the differences in the results of the two studies. Hence more comprehensive sampling of the embryonic avian gizzard may reveal a variety of endocrine cells not detected here.

5.5 Regions in which Immunoreactive Cells Appeared Consistently: Proventriculus, Pyloric Region and Small Intestine

5.5.1 Gastrin/CCK-immunoreactive cells

The first attempts to detect gastrin in avian gut employed bioassay methods; Olowa-Okorun and Amure (1973) reported gastrin-like bioactivity in the chicken proventriculus but not in the duodenum whereas Ketterer et al. (1973) by RIA, detected gastrin-like immunoreactivity in the

duodenum only, although other regions were examined. However, immunocytochemical studies have demonstrated no gastrin-like immunoreactive cells in the proventriculus of adult chickens (Larsson et al., 1974c; Polak et al., 1974; Usellini et al., 1983) or chickens at hatching (Rawdon and Andrew, 1981a). In the present study too, no gastrin-like immunoreactive cells were detected in the chicken proventriculus. Here also, in chicks at hatching gastrin-immunoreactive cells were found to be concentrated in the pyloric region and some were distributed throughout the small intestine. These immunoreactive cells were demonstrated with a COOH-terminal antiserum which is not specific for gastrin but will stain CCK as well. No specific antisera are as yet available for avian gastrin (see 5.1.2). The question then arises as to whether the cells stained in the pyloric region and small intestine in the present study are gastrin- or CCK-immunoreactive cells.

One of the first reports of gastrin-like immunoreactivity in the gastrointestinal tract of birds was that of Polak et al. (1974) who reported in an immunocytochemical study, gastrin/CCK-like immunoreactive cells from the

gizzard to the rectum in adult chickens and quails. Other workers have found gastrin/CCK-like immunoreactive cells to be less widespread in avian gut (Larsson et al., 1974c; Rawdon and Andrew, 1981a); the distribution is similar to that found in man and mammals where gastrin-like immunoreactive cells are largely confined to the antrum and small intestine (Tobe et al., 1974; Larsson et al., 1975a, 1977; Dubois et al., 1976a; Kataoka et al., 1985a,b).

Subsequent RIA studies have shown that a gastrin-like factor is situated in the pyloric region and small intestine in adult birds. In 1974 Larsson et al. discovered the important antral region which had been overlooked by previous workers. These authors demonstrated a gastrin-like factor similar in molecular size to mammalian G-17 in this region. In 1984 Vigna confirmed the presence of a gastrin-like peptide in the chicken antrum by RIA and bioassay. Dockray (1978) identified a similar factor in the turkey antrum with COOH-terminal antisera but not with antisera specific for intact mammalian G-17 or its NH_2-terminus, demonstrating that the avian gastrin-like factors differ from mammalian gastrin. Thus

avian and mammalian gastrin are of comparable molecular size but resemble each other closely only at the functionally critical COOH-terminus. Larsson et al. (1974c) have suggested that this may explain why avian gastrin is undetectable in some RIA's. It also explains only antisera raised to the COOH-terminus of mammalian gastrin can be used to demonstrate gastrin-like immunoreactivity in birds as in the present study.

Such antisera have revealed gastrin/CCK-immunoreactive cells in the pyloric region and small intestine of adult chickens (Larsson et al., 1974c) and quails (Yamada et al., 1979) and in chicks at hatching (Rawdon and Andrew, 1981a). Thus RIA and immunocytochemical evidence presented by previous workers supports the view that gastrin-like immunoreactivity is present in the pyloric antrum. Since in mammals the antral cells have been shown to contain gastrin, in chickens, the cells stained with the COOH-terminal antiserum in this region in the present study are likely to be gastrin-immunoreactive cells.

CCK-like peptides have been detected in the avian small intestine and appear to be

different from mammalian CCK (Rawdon and Andrew, 1981b). Several studies have examined the forms of CCK-like peptides in the intestinal mucosa of mammals. It is evident that a large proportion of CCK present in the upper small intestine in mammals is CCK 8 (Dockray, 1977). Dockray (1979) has detected a factor closely resembling the COOH-terminal octapeptide of porcine CCK in extracts of turkey jejunum. The immunocytochemical study of Rawdon and Andrew (1981b) demonsrates that although COOH-terminal antisera stain chick intestinal cells, the use of antisera to different regions of mammalian CCK does not allow specific distinction of CCK cells in this species. They conclude that outside the COOH-terminus the avian peptide in chick gut differs from the mammalian . Since there is a CCK-like peptide in chick gut, it seems that at least some of the cells which demonstrated immunoreactivity with the COOH-terminal antiserum in the small intestine in the present study are likely to contain a CCK-like peptide.

With regard to the time of first appearance of gastrin-immunoreactivity in the present study, despite examination of two specimens of the pyloric region and small intestine at each of

12, 13 and 14 days and four at 16 days of incubation, gastrin/CCK-immunoreactive cells were first detected only on the 14th day of incubation in the small intestine and on the 16th day in the pyloric region. Even then the cells were very sparse. However, Salvi and Renda (1986) have reported the rare occurrence of such cells at 11-12 days of incubation in the pyloric region and duodenum. A COOH-terminal antiserum was used by these authors but all sections were routinely submitted to trypsinization prior to the immunohistochemical procedure. It is worthy of note that while Salvi and Renda (ibid.) make no further reference to this treatment with regard to gastrin/CCK-immunoreactive cells, all sections of chick embryonic proventriculus had to be submitted to trypsinization up to 15 days of incubation in order to demonstrate cells immunoreactive for bombesin; thereafter this step became unnecessary (D'Este et al., 1984). Although the use of trypsin is a recognised procedure, care should be taken with the interpretation of results, since sites in proteins other than those stained for, being exposed, may stain, thereby giving a false positive result. It is possible that Salvi and Renda (ibid.) may be demonstrating precursor

molecules of the peptides, the immunoreactive site of the peptide itself being masked in the precursor and then freed by trypsin for reaction with the antiserum.

It is of interest that Salvi and Renda (1986) report the first appearance of endocrine cells in the pyloric region of their chick embryos at 11-12 days of incubation when the first buds of glands appear in the mucosa. Since no indications of gland development was detected in the present study at this stage, the findings of Salvi and Renda (ibid.) suggest that differentiation of the gut in their study is more advanced than at the same incubation age in the present study. This, too, may account for the fact that these authors were able to demonstrate the presence of immunoreactive cells at an earlier stage.

The function of gastrin during foetal life appears to be uncertain. Since significant quantities are present, Larsson et al. (1977) have suggested that it has a trophic role in the early development of the gastrointestinal tract. Evidence from studies on foetal (Larsson et al., 1974a) and neonatal rats (Braaten et al., 1976) indicates that the early

strin produced is secreted into the blood stream, and Aynsley-Green et al. (1979) have shown an increase in plasma gastrin levels after oral feeding in human neonates. Muller et al. (1980) has suggested the possibility that increased gastrin levels in foetal mammals may prime upper gastrointestinal growth in readiness for the onset of oral feeding. In adult fed and fasted mammals, gastrin has been implicated as a trophic agent, necessary for the maintenance of the functional and structural integrity of the gastrointestinal tract (Johnson et al., 1976). Majumder (1984) has found it to be a trophic agent during certain stages of development.

Although gastrin cells have been shown to appear later than other endocrine cell types in the pyloric region in the present study, they do nevertheless occur in significant numbers well before hatching; it is conceivable that they perform the same functions as their mammalian counterparts.

5.5.2 Neurotensin-immunoreactive cells

Avian neurotensin-immunoreactive cells react with antisera to the COOH-terminus of mammalian neurotensin (Sundler et al., 1977; Reineke et al., 1980; Rawdon and Andrew, 1981a) and are more widespread in avian than mammalian gut (Sundler et al., 1977). In the present study, at 21 days of incubation such cells were detected throughout the gastrointestinal tract from the proventriculus to the rectum, except for the gizzard, in accordance with the results of Rawdon and Andrew (1981a) in chicks at this time. Findings of other workers with regard to the presence or absence of neurotensin-immunoreactive cells in the proventriculus vary considerably. Whereas these cells have been detected as early as 12 days of incubation in the proventriculus in the present study and are numerous at hatching, they were not observed there by Sundler et al. (1977) from 10 days of incubation onwards, in chicks at hatching or in young birds; neither have these cells been observed in the proventriculus of adult quails by Reineke et al. (1980) or adult chickens by Yamada et al. (1986). Nevertheless the latter authors have detected rare neurotensin-immunoreactive cells in this region of the gut in

other species of adult birds including the quail.

In the present study neurotensin-immunoreactive cells were one of the earliest endocrine cell types found in the gut of chick embryos - they were detected very much earlier than by previous workers. These cells were observed in the proventriculus, pyloric region, duodenum and rectum at 12 days of incubation whereas Sundler et al. (1977) reported the first appearance of neurotensin-immunoreactive cells in the colon/rectum only at 18 days of incubation and in the small intestine and pyloric region two days later, i.e. respectively 6 and 8 days later than they were first detected in the present study.

5.5.3 Somatostatin-immunoreactive cells

In the chick embryos studied here somatostatin-immunoreactive cells, like neurotensin-immunoreactive cells, appeared in some specimens at 12 days of incubation and in the same regions (proventriculus, pyloric region and duodenum) except for the rectum, whereas Alumets et al. (1977) reported them to be confined to the

proventriculus at 12 days. Wium (pers. commun.) too, has detected somatostatin-immunoreactive cells in the proventriculus at this stage. At 14 days they were found in addition in the upper and lower ileum in the present study. In the pyloric region Salvi and Renda (1986) too, found cells with somatostatin-like immunoreactivity at 12 days and in the duodenum rare somatostatin-immunoreactive cells one day earlier. The number of specimens that they studied is not indicated but all specimens were subjected to trypsinization prior to immunohistochemical procedures.

In the present study somatostatin-immunoreactive cells were found, in addition, in the rest of the small intestine in some specimens at 14 days and by 16 days they were present in every specimen of the duodenum. These findings are contrary to those of Alumets et al. (1977) who reported the first appearance of somatostatin-immunoreactive cells in the duodenum only at 16 days of incubation. Furthermore, in the present study somatostatin-immunoreactive cells were already numerous in the pyloric region at 17½ days of incubation whereas Alumets et al. (ibid.) detected such cells in this region for the first time at 19 days of

incubation. They reported somatostatin-immunoreactive cells to be virtually absent from the jejunum and ileum; in contrast the present study revealed the presence of few to moderate numbers of somatostatin-immunoreactive cells in the upper and lower ileum in at least two specimens at each stage of incubation from 17½ days onwards until hatching.

In human foetal gut somatostatin-immunoreactive cells appear as early as 8 weeks in both the stomach and small intestine according to some authors (Track et al., 1979; Stein et al., 1981, 1983); these findings are contrary to those of Dubois et al. (1976); Chayvaille et al. (1980) and Buchan et al. (1981) in which somatostatin-immunoreactive cells are reported to appear a few weeks later in the stomach than in the ducdenum. Conflicting results have been reported too in avian gut where in the present study somatostatin-immunoreactive cells appeared simultaneously and early in both of these regions whereas according to Alumets et al. (1977) they appear at different ages in the stomach and duodenum.

In the present study the distribution of somatostatin-immunoreactive cells at 21 days of

incubation is the same as that found in chicks at hatching by Rawdon and Andrew (1981) and in young chickens by Alumets et al. (1977). The detection of somatostatin-immunoreactive cells in the duodenum of adult quails (Seino et al., 1979) appears to be the only report of these cells in adult birds.

The fact that somatostatin-immunoreactive cells are present in the gut of chick embryos, concentrated particularly in the pyloric region before hatching, suggests that they may be involved functionally in embryonic life. Since in adult mammals somatostatin-immunoreactive cells have an inhibitory effect on secretion by other endocrine cell types it is conceivable that they may perform the same function in the embryo.

5.3.4 Avian pancreatic polypeptide-immunoreactive cells

The present findings regarding the distribution of APP-immunoreactive cells at hatching in general correspond well with those of Alumets et al. (1978) and Rawdon and Andrew (1981a). The former workers, but not the latter, reported APP-immunoreactive cells in the

gizzard and colon/rectum at this stage. These cells appear to be transitory as they were not detected in the gizzard or colon/rectum by Alumets et al. (ibid.) prior to 21 days of incubation and they had disappeared a week later. In the present study, however, no APP-immunoreactive cells were detected in the gizzard, caecum or rectum at hatching or at any of the preceding stages examined.

The earliest age at which APP-immunoreactive cells were detected in the present study was at 13 days of incubation, one day later than that at which neurotensin- and somatostatin-immunoreactive cells are reported to appear for the first time in chick embryos in the present study and two days later than somatostatin-immunoreactive cells were reported in the pyloric region and small intestine by Salvi and Renda (1986). The results of the present study indicate that in embryonic avian tissue APP-immunoreactive cells appear considerably earlier than reported by other workers: examination of at least two specimens at each stage of incubation revealed APP-immunoreactive cells for the first time in the duodenum and upper ileum as early as 13 days of incubation, i.e. four days earlier than found were by

Alumets et al. (1978), three days earlier than by Larsson et al. (1974b) in the duodenum and six days earlier than by Alumets et al. (ibid.) in the rest of the small intestine. Furthermore cells of this type were identified in the proventriculus at 14 days of incubation, i.e. 7 days earlier than by Alumets et al. (ibid.). From their observations on the order of appearance of APP-immunoreactive cells in the various parts of the gut, these workers have suggested that they invade the gut cranially and caudally from a point of origin in the duodenum. The results of the present study do not support this view, as APP-immunoreactive cells were detected in the proventriculus and throughout the small intestine between 13 and 14 days of incubation and thereafter increased in number only in the same regions.

APP-immunoreactive cells are not normally present in the pyloric region of the chick gastro-intestinal tract (see Larsson et al., 1974b; Rawdon and Andrew, 1981a). Hence the APP-immunoreactive cell detected in this region at 14 days of incubation may be regarded as ectopic. Andrew et al. (1988) have reported the occurrence of ectopic gut endocrine cells in chorioallantoic grafts of experimental

material and Yamada et al. (1986) have considered several endocrine cell types in adult avian gizzard to be ectopic and of no functional significance (see 5.4).

As already mentioned, according to El-Salhy et al. (1982d) PP and PYY do not occur in the same cells in adult chickens. Using an antiserum specific for PYY on chick embryonic tissue fixed in Bouin's fluid, El-Salhy et al. (1982b) demonstrated PYY-immunoreactive cells in the duodenum only; this was at hatching and at 18 days of incubation but no earlier. Unfortunately El-Salhy's PYY antiserum has failed to demonstrate immunoreactive cells in parabenzoquinone vapour-fixed tissue in any region of the gastrointestinal tract of chicks at hatching (Rawdon, pers. commun.). PYY-immunoreactive determinants not stained by this antiserum may nevertheless be present in tissue fixed in parabenzoquinone vapour and be reactive with APP antiserum. With the same APP antiserum, Alumets et al. (1978) and the present author have demonstrated immunoreactive cells in the proventriculus and small intestine much earlier than PYY-immunoreactive cells were detected by El-Salhy et al. (1982b). If there are indeed no PYY cells in the proventriculus

and ileum before hatching, then the cells stained there with the anti-APP serum could be PP rather than PYY cells. The same applies to the cells demonstrated in the duodenum before 18 days. If, however, PYY cells are scarce and so were missed by El-Salhy et al. (ibid.) in sampling these regions, the cells stained in the present study in the proventriculus, duodenum and ileum may include either or both of PP or PYY cells. The likelihood of at least some cells in the ileum containing PYY is suggested by El-Salhy's demonstration of PYY-immunoreactive cells in the ileum after hatching - also, in mammals such cells occur mainly in the distal small intestine and large intestine (Lundberg et al., 1982). Clearly the identity of the PP-immunoreactive cells in chick embryos can only be solved by application of specific PYY antiserum to suitably fixed material.

5.5.5 Glucagon-immunoreactive cells

For chicks at hatching, the present findings regarding the distribution of glucagon-immunoreactive cells correspond well with those of Rawdon and Andrew (1981a). The present study appears to be the only one undertaken so far on

the ontogeny of glucagon-immunoreactive cells in chick embryonic gut. Cells immunoreactive for glucagon appeared in the proventriculus at the same time as APP-immunoreactive cells appeared in the duodenum and upper ileum, i.e. at 13 days of incubation, one day later than somatostatin- and neurotensin-immunoreactive cells were detected in the same region in the proventriculus, pyloric region and duodenum. At 14 days of incubation glucagon-immunoreactive cells had appeared throughout the small intestine together with the other four endocrine cell types studied here.

As already mentioned (see 3.5.1), antiserum YY59, having NH_2-terminal specificity, stains pancreatic glucagon as well as its larger molecular forms: therefore, whether the cells stained with the antiserum in the present study contain pancreatic glucagon or a GLI is uncertain. Some gut endocrine cells which, like pancreatic endocrine cells, stain with specific anti-pancreatic glucagon sera (COOH-terminus specific) occur in certain mammals where they are confined to the stomach. In the mammalian small intestine, immunoreactive GLI's stain with NH_2-terminal glucagon and

specific glicentin antisera but not with COOH-terminal glucagon antisera (Polak et al., 1971; Baldisera and Holst, 1984). However, in chicks at hatching, Rawdon and Andrew (1984b) have reported that both NH_2-terminal and COOH-terminal glucagon antisera stained the same cells in the proventriculus, upper ileum and pancreas of chicks at hatching; they suggest that either specific pancreatic glucagon occurs in all these immunoreactive cells or that any GLI present has an unmasked COOH-terminus. These authors were unable to demonstrate staining with a specific glicentin antiserum in their material, processed as in the present study (pers. commun.). In young chickens, however, Usellini et al. (1983) reported that only non-COOH-terminal glucagon antisera stained cells in the proventriculus, which indicates that at this age the glucagon sequence is present here only in a GLI.

5.6 Regional Distribution and Frequency of Immunoreactive Cells

According to the present findings, the regional distribution of neurotensin-, APP- and glucagon-immunoreactive cells in the chick gastrointestinal

tract is established as early as 14 days of incubation, that for somatostatin-immunoreactive cells by two days later and for gastrin/CCK-immunoreactive cells only by 19 days of incubation. Immunoreactive cells of all five type studied showed a general increase in number during development until the numbers at hatching was established. Subjective assessment of the frequency of these cells at hatching appears to be reached by gastrin/CCK- and APP-immunoreactive cells about two days before hatching and by somatostatin-, neurotensin- and glucagon-immunoreactive cells a day or two earlier. It may be that the trend changes shortly after hatching as has been shown for example by Alumets et al. (1978) for APP-immunoreactive cells (see 5.5.4). Further changes may well occur in young birds to attain the adult pattern. In general, endocrine cells appear to be less wide spread in adult birds than in young birds and those at hatching (cf. Yamada et al., 1986) .

5.7 Morphology of the Gut at the Time of First Appearance of Endocrine Cells

The degree of differentiation of the gut in relation to the time of first appearance of immunoreactive cells is worth considering. It is significant that

cells immunoreactive for neurotensin, somatostatin, APP and glucagon make their first appearance when the epithelium and glands of the gastrointestinal tract are relatively undifferentiated. This shows that differentiation of endocrine cells is not dependent on the presence of well-differentiated epithelial cells of other types.

On the other hand goblet cells appear only when the gut is reasonably well-differentiated. They were detected for the first time in the present study at 19 days of incubation in the small and large intestine and were abundant at hatching. Romanoff (1960) too, found goblet cells at 19 days of incubation. The earliest stage at which goblet cells have been detected in chick embryonic gut is in SEM studies. Pacini et al. (1979) found goblet cells in the small intestine at 15 days while Lim and Low (1977) described goblet cell openings at the tip of each villus in the duodenum, when definitive villi were first seen, at 18-19 days of incubation. In quail embryos, (which hatch at 16 days of incubation), the mouths of goblet cells were recognised at 12 days of incubation also at the time when definitive crypts and villi were seen (see Ishizuya, 1980). Since the morphogenesis of the intestine and cytodifferentiation of goblet cells occur simultaneously in the quail, Ishizuya (ibid.) has suggested that

some relationship may exist between them. The same could well apply to the findings of Lim and Low (1977) in the chick embryo.

5.8 Coexistence of Peptides

Evidence has accumulated, for mammals, for the occurrence in the same cell of two or more related forms of a peptide, for example, pancreatic glucagon and glicentin (Larsson and Moody, 1980; Grimelius et al., 1976; Orci et al., 1983; Colony et al., 1982) and also of unrelated peptides, for example, glicentin and PYY (El-Salhy et al., 1983; Ali-Rachedi et al., 1984; Böttcher et al., 1984, 1986; see Solcia et al., 1987) and gastrin together with immunoreactivity for the following: ACTH (Larsson, 1977a, 1978a,b), αMSH (Larsson and Rehfeld, 1981), growth hormone (Sundler et al., 1979b) and enkephalin (Polak et al., 1977b). PP- and PYY-like peptides are reported to coexist in teleost gut (Abad et al., - see Rombout et al., 1987) although not in mammals (El-Salhy and Grimelius, 1983) or birds (El-Salhy et al., 1982d).

In chicks at hatching and in adult birds dual immunoreactivity for unrelated peptides, namely APP and glucagon (Rawdon and Andrew, 1981a), gastrin/CCK and neurotensin (Sundler et al., 1983; Rawdon

et al., 1983) and for bombesin and serotonin (D'Este et al., 1986) have been reported.

The present study, besides aiming to confirm or otherwise these reports of dual immunoreactivity for APP and glucagon and for gastrin/CCK and neurotensin in chicks at hatching, was extended to determine whether these dual immunoreactivities are present before hatching.

5.8.1 APP- and glucagon-like immunoreactivity

Before discussing the results of the present study, it is necessary to review the relevant literature with regard to APP and glucagon and their related forms, PYY and glicentin, in gut endocrine cells. This is done in order, firstly, to determine if possible the likelihood of the peptides dealt with here being PP itself or PYY, pancreatic glucagon or glicentin and secondly, to assess the situation with regard to the coexistence of these peptides in other species.

The coexistence of PP- and glucagon-like peptides has been reported to occur in the pancreata of mammals (e.g. Grube et al., 1982) and chick embryos (Cowap, 1985) and also

repeatedly in the gut of various mammals, for example in human colorectal cells (Solcia et al., 1979, 1985), in the ileum and colon of cats (Ravazolla and Orci, 1980) as well as in the proventriculus and upper ileum of chicks at hatching (Rawdon and Andrew, 1981a).

Initially the antisera which were available to localize endocrine cell types did not distinguish between different forms of the peptides under discussion. It appeared that the coexistence of PP- and glucagon-like substances was common along the length of the gut of vertebrate species. With the development of specific anti-PYY and anti-glicentin sera it has been shown that cells exhibiting dual immunoreactivity in the intestine of mammals contain PYY- and glicentin- rather than PP- and glucagon-like immunoreactants (El-Salhy et al., 1983; Ali-Rachedi et al., 1984; Böttcher et al., 1984, 1986; Barbosa et al., 1987). Ali-Rachedi et al. (ibid.) reported that the majority of cells immunoreactive for PYY were also glicentin-immunoreactive ; hence a separate population of PYY-immunoreactive cells must have been present. Since PYY is not contained in the glucagon/glicentin precursor sequence (Bell et al., 1983a,b), Sundler et al.

(1985) have proposed that PYY and glicentin are derived from two separate precursor molecules. Results have furthermore indicated the coexistence of PYY- and glicentin-like immunoreactivity in the same secretory granules (Böttcher et al., 1986) in certain mammals. These findings differ from those of El-Salhy et al. (1983) in which PYY-immunoreactivity in adult human intestinal cells did not occur together with PP- or 'enteroglucagon'-like immunoreactivity. In the adult monkey, on the other hand, PYY- and glicentin-immunoreactivity were found in the same cells (El-Salhy and Grimelius, 1983).

A related finding is that PP and PYY almost always are found in separate cells. PP occurs mostly in the pancreas. When specific PP-immunoreactivity coexists with immunoreactivity for the icosapeptide fragment of the PP precursor, as in the pancreata and stomach of certain mammalian species, the immunoreactive cells have been referred to as "true PP" cells by Sundler et al. (1984). PYY occurs mostly in the gut (Solcia et al., 1985) being located in the ileum, colon and rectum in mammals (Lundberg et al., 1982).

With regard to the presence of glucagon-like substances in mammalian gut endocrine cells, pancreatic glucagon-immunoreactivity is found in cells of the pancreas and gastric mucosa (see, for example, Baetens et al., 1976b; Sundler et al., 1976), whereas glicentin-immunoreactivity is located mainly in cells of the lower small intestine (see, for example, Larsson et al., 1975b).

In birds glicentin has been demonstrated only in the proventriculus of young chickens and of chicks at hatching (Usellini et al., 1983). By analogy with mammals this is an unexpected observation: pancreatic glucagon, rather than glicentin, would be thought to occur in this gastric region.

With regard to PYY, this peptide is less widely distributed in birds than in mammals; it is confined in adult chickens to the duodenum and jejunum (El-Salhy et al., 1982d) the same distribution as in amphibians and reptiles (El-Salhy et al., 1982a). These authors have suggested that because the frequency and distribution of PP- and PYY-immunoreactive cells differ in the gut of the domestic fowl, these two polypeptides must occur as two independent

cell types. The icosapeptide in the PP precursor was not tested for but specific anti-PP and anti-PYY sera were used to demonstrate the cells.

In the present study dual immunoreactivity for APP and glucagon has been exhibited by cells in the proventriculus, upper and lower ileum. As explained earlier (3.5.1), the antisera used do not distinguish between PP and PYY or between pancreatic glucagon and glicentin, and specific antisera have not been successfully applied to this material. Since dual immunoreactivity in mammalian cells concerns PYY and glicentin, the same situation is likely to pertain in birds. This possibility is discussed below in the light of available data.

In embryonic chick gut, PYY-immunoreactive cells have been demonstrated only in the duodenum and only from 18 days of incubation (El-Salhy et al., 1982b). It therefore appears that only those APP-immunoreactive cells demonstrated in the present study in the duodenum at and after 18 days might be PYY-immunoreactive cells. However this is the one region where no dual immunoreactivity for PP and glucagon was found. Of the APP-immunoreactive cells

demonstrated in the proventriculus and ileum, a fair proportion showed glucagon-like immunoreactivity; hence the possibility that these APP-immunoreactive cells contain PYY-immunoreactants should be re-examined.

In the present study, by analogy with mammals, some or all of the glucagon-immunoreactive cells in the small intestine could be expected to be glicentin-immunoreactive cells. However, no evidence has yet been found for specific glicentin-immunoreactivity in cells of the small intestine (see Rawdon and Andrew, 1981a) for chicks at hatching. It is hence at present unclear whether PP- and glucagon-like immunoreactivity in embryonic chick gut is attributable to PYY and glicentin or not. The problem can only be resolved if PYY and glicentin - perhaps an avian form - PP itself and pancreatic glucagon can be successfully demonstrated in chick gut.

The results of the present study for the coexistence of glucagon- and APP-like substances in chicks at hatching differ somewhat from those of Rawdon and Andrew (1981a). Whereas in the present study in the proventriculus and upper ileum only, some APP-immunoreactive cells

were also glucagon-immunoreactive, Rawdon and Andrew (ibid.) found that in these regions all APP-immunoreactive cells were immunoreactive for glucagon. These workers used a different fixative but the same antisera and the same breed of domestic fowl (pers. commun.). In the lower ileum, a region not sampled by Rawdon and Andrew (1981a), all APP-immunoreactive cells were glucagon-immunoreactive. Surprisingly no dual immunoreactivity was found in the duodenum at any of the stages studied despite the fact that a large number of sections was examined. Rawdon and Andrew (ibid.) did not look for dual immunoreactivity in the duodenum.

Despite an extensive search for cells showing dual immunoreactivity for APP and glucagon, the cell numbers are in some cases, low. However, changes in the proportion of such cells and also of cells showing immunoreactivity for each of the peptides separately are indicated between 17½- and 21-days of incubation in certain regions. In the lower ileum, at least, the proportion of cells exhibiting dual immunoreactivity appears to decrease with time and the proportion of those cells immunoreactive for glucagon only, increases. As explained already (3.5), dual immunoreactivity was not

sought at the time when APP- and glucagon-immunoreactive cells first appear, but if the above trends are extrapolated backwards and forwards in time it may be speculated that when APP- and glucagon-immunoreactive cells first appear, which is at approximately the same time, all stainable cells, in the lower ileum even if nowhere else, may store both peptides; if this trend were extended forward, cells exhibiting dual immunoreactivity would be seen to decrease even further in the weeks after hatching. However, changes in proportion may fluctuate, as do those reported by D'Este et al. (1986). These authors found that cells exhibiting dual immunoreactivity for serotonin and bombesin in chick embryonic proventriculus were very few initially, reached a peak at 17-18 days of incubation and had decreased markedly by hatching.

It would be interesting to extend this study to seek confirmation or otherwise for the apparent trend in the proportion of dual immunoreactive cells demonstrated here. Stages of particular interest would be the time of first appearance, the postnatal period and adulthood.

D'Orte et al. (1986) have reported that the cells demonstrating the coexistence of serotonin- and bombesin-like substances become less frequest in the post-hatcing and adult stages.

5.8.2 Gastrin/CCK- and neurotensin-like immunoreactivity

Sundler et al. (1977) and Rawdon and Andrew (1981a, Rawden et al., 1983) reported the presence of dual immunoreactivity for gastrin/CCK and neurotensin in the pyloric region of chicks at hatching: the present study confirms these reports for this stage and also shows dual immunoreactivity for the two peptides in the duodenum. At 19- and 17½-days of incubation the proportion of cells exhibiting dual immunoreactivity for these peptides in the pyloric region was virtually the same as that at hatching. In the duodenum the same lack of a clear change in the proportion of dually immunoreactive cells is evident.

Since in the pyloric region neurotensin-immunoreactive cells were present for the first time at 12 days of incubation, four days earlier than gastrin/CCK-immunoreactive cells, according to

the present study, it appears that neurotensin-immunoreactive cells are initially a separate population of cells. However, Salvi and Renda (1986) detected gastrin/CCK-immunoreactive cells, albeit after trypsin treatment, at 11-12 days of incubation. Hence, dual immunoreactivity for gastrin/CCK and neurotensin may occur at this stage. On the other hand, according to the findings of D'Este et al. (1986), cells demonstrating dual immunoreactivity later on, do not necessarily do so when they first appear. These authors found that serotonin-immunoreactive cells first appeared in the proventriculus at 8 days of incubation, bombesin-immunoreactive cells 3-4 days later and dual immunoreactivity of the two peptides was only demonstrated two days after this. Hence, here again further study, seeking neurotensin-immunoreactivity in cells stainable for gastrin after trypsin treatment, should be profitable, as would be an analysis of the situation after hatching and in the adult.

5.9 Conclusion

The present study has provided a survey of the regional distribution of endocrine cell types with immunoreactivities indicative of the presence of the peptides somatostatin, neurotensin, gastrin/CCK, APP and glucagon. It has shown further that dual immunoreactivity for APP and glucagon and for gastrin/CCK and neurotensin does occur in the embryo from an early stage, the pattern of dual immunoreactivity varying from one region to another. These findings form the basis for planning of further work on the initiation of the coexistence of gut peptides in the same cells in embryonic chick gut.

This study has also demonstrated that the five cell types studied make their first appearance in various regions of chick embryonic gut at ages between 12 and 16 days of incubation. Although it is possible that the very first cells of the types studied here may appear a little earlier than reported, the results give a clear indication of the ages and specific regions in which they may first be expected. The observations also show when any are certain to be present, in reasonable numbers, in a given region the gastrointestinal tract. This information is of particular value in the planning of experiments for

instance, on the differentiation of gut endocrine cells. In the latter experiments, it is important that the duration of embryonic gut culture is sufficient to allow for the differentiation of endocrine cells. The experiments of Wiedtz-Hoessels et al. (1987) on the role of the notochord in gut differentiation, of Andrew et al. (1988) on the role of mesenchyme and of Wium (in progress) on the role of hormones in endocrine cell differentiation are cases in point.

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APPENDIX A

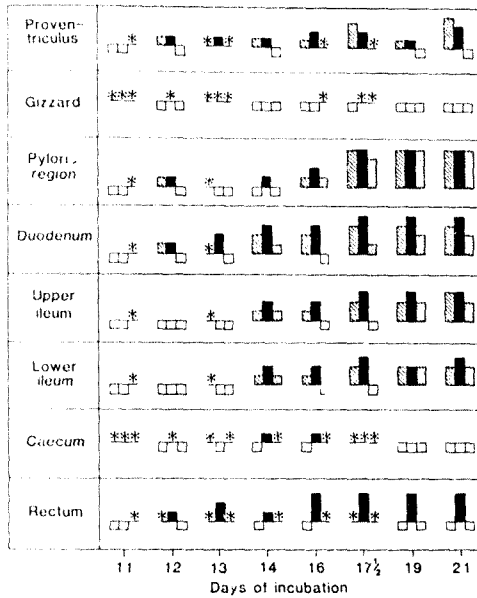


Figure 5: Block diagram to demonstrate the first appearance, distribution and frequency of cells showing immunoreactivities for somatostatin, neurotensin and gastrin/CCK in the gastrointestinal tract of chick embryos.

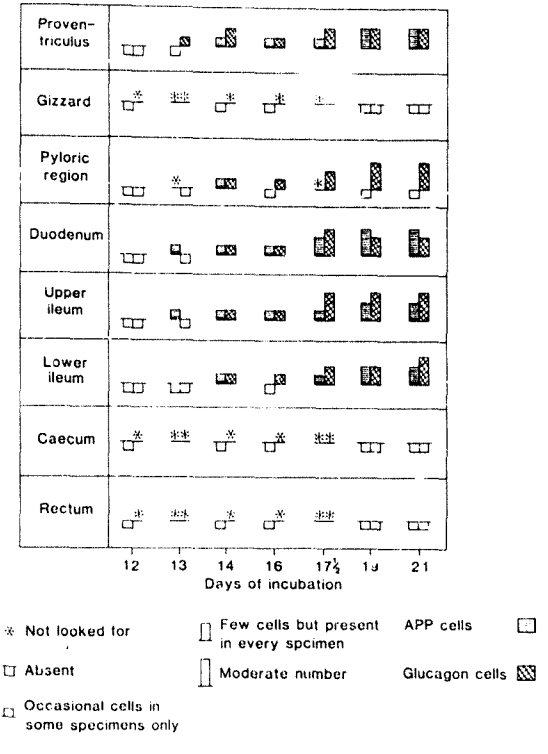


Figure 6: Block diagram to demonstrate the first appearance, distribution and frequency of cells showing immunoreactivities for APP and glucagon in the gastrointestinal tract of chick embryos.

APPENDIX B

TABLE A1. Numbers of somatostatin-immunoreactive cells found in each specimen

Days of incubation	No. of specimens	Proventriculus	Gizzard	Pyloric region	Duodenum	Upper ileum	Lower ileum	Caecum	Rectum
11	2	–	*	–	–	–	–	*	–
		–	*	–	–	–	–	*	–
12	3	2	–	1	1	–	–	–	*
		–	–	–	–	–	–	–	*
		1	–	1	–	–	–	–	*
13	0	*	*	*	*	*	*	*	*
14	2	4	–	–	10	4	2	–	–
		–	–	–	1	–	–	–	–
16	4	4	–	2	2	–	1	–	–
		–	–	–	2	–	–	–	–
		–	–	1	2	2	–	–	–
		2	–	3	2	–	–	–	–
17½	2	10	–	17	13	1	2	*	*
		6	–	23	18	3	10	*	*
19	2	–	–	21	3	1	4	–	–
		3	–	19	15	3	3	–	–
21	2	10	–	24	5	7	6	–	–
		20	–	18	16	13	5	–	–

* = not looked for – = absent

TABLE A2. Numbers of neurotensin-immunoreactive cells found in each specimen

Days of incubation	No. of specimens	Proven-triculus	Gizzard	Pyloric region	Duodenum	Upper ileum	Lower ileum	Caecum	Rectum
11	2	– –	* *	– –	– –	– –	– –	* *	– –
12	3	1 – –	* * *	1 – –	– – 2	– – –	– – –	* * *	– – 1
13	2	1 –	* *	– –	2 1	– –	– –	– –	1 1
14	2	3 –	– –	1 –	15 7	12 3	4 1	1 –	1 –
16	3	3 1 2	– – –	2 3	7 19 4	3 7 6	3 2 4	– 1 –	1 24 5
17½	2	2 3	* *	30 15	20 30	6 14	10 30	* *	4 20
19	2	2 –	– –	17 23	30 13	14 3	2 4	– –	7 15
21	2	8 9	– –	19 29	21 16	3 20	14 7	– –	14 17

* = looked for – = absent

TABLE A3. Numbers of gastrin/CCK-immunoreactive cells found in each specimen

Days of incubation	No. of specimens	Proventriculus	Gizzard	Pyloric region	Duodenum	Upper ileum	Lower ileum	Caecum	Rectum
11	0	*	*	*	*	*	*	*	*
12	2	- -	- -	- -	- -	- -	- -	- -	- -
13	2	* *	* *	- -	- -	- -	- -	*	*
14	2	- -	- -	- -	- 1	1 -	1 -	* *	* *
16	4	* * * *	* * * *	- - - 3	- - - -	- - - -	- - - -	* * * *	* * * *
17½	2	* *	* *	10 14	3 -	- -	- -	* -	* *
19	2	- -	- -	30 27	5 1	2 2	1 2	- -	- -
21	2	- -	- -	21 36	3 0	3 4	1 3	- -	- -

* = not looked for - = absent

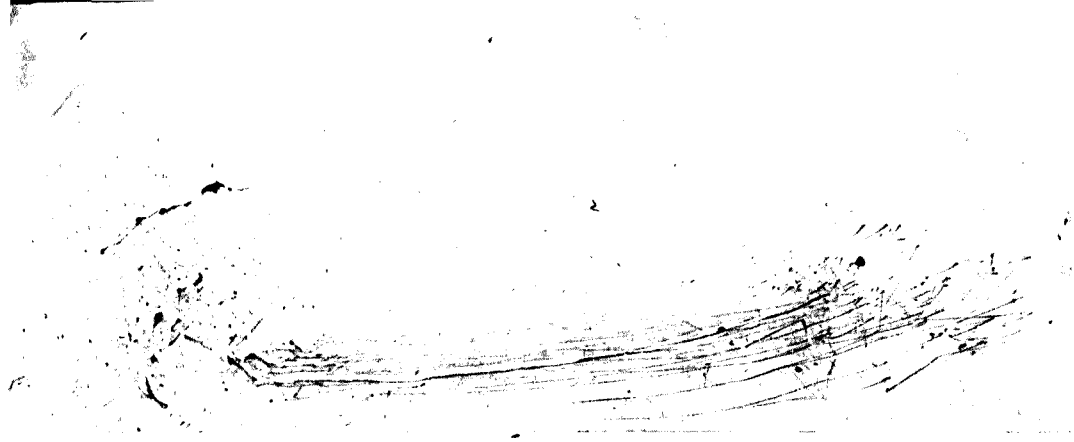


TABLE A4. Numbers of APP-immunoreactive cells found in each specimen

Days of incubation	No. of specimens	Proven-triculus	Gizzard	Pyloric region	Duodenum	Upper ileum	Lower ileum	Caecum	Rectum
12	2	–	–	–	–	–	–	–	–
		–	–	–	–	–	–	–	–
13	2	–	*	*	–	–	–	*	*
		–	*	*	1	1	–	*	*
14	5	–	–	1	–	–	–	–	–
		–	–	–	5	3	–	–	–
		2	–	–	2	–	1	–	–
		1	–	–	–	–	–	–	–
		1	–	–	–	1	–	–	–
16	4	1	–	–	–	–	–	–	–
		–	–	–	1	–	–	–	–
		–	–	–	–	–	–	–	–
		–	–	–	3	1	–	–	–
17½	2	1	*	*	1	2	1	*	*
		–	*	*	3	–	–	*	*
19	2	3	–	–	9	7	8	–	–
		1	–	–	11	2	6	–	–
21	2	2	–	–	10	6	6	–	–
		3	–	–	12	4	2	–	–

* = not looked for – = absent

TABLE A5. Numbers of glucagon-immunoreactive cells found in each specimen

Days of incubation	No. of specimens	Proventriculus	Gizzard	Pyloric region	Duodenum	Upper ileum	Lower ileum	Caecum	Rectum
12	2	- -	* *	- -	- -	- -	- -	* *	* *
13	2	1 -	* *	- -	- -	- -	- -	* *	* *
14	4	2 1 2 3	* * * *	1 1 - 1	2 - 2 2	- 1 - 1	- - 1 1	* * * *	* * * *
16	3	6 - -	* * *	- 1 -	1 1 -	2 1 -	2 3 -	* * *	* * *
17½	2	4 5	* *	5 1	11 13	14 7	7 6	* *	* *
19	2	7 5	- -	14 11	2 4	14 9	2 3	- -	- -
21	2	5 6	- -	10 12	7 4	10 13	10 11	- -	- -

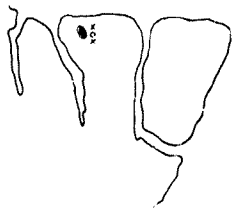
* = not looked for - = absent

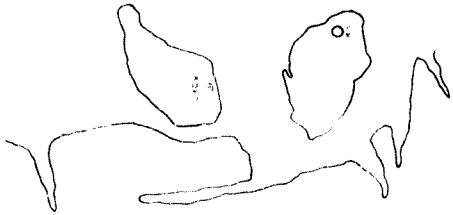
APPENDIX C

Camera lucida drawings

Dual immunoreactivity for APP and glucagon in four consecutive sections. Upper ileum at 19 days of incubation - two fields.

Section no.	1.	red x	= glucagon-immunoreactive cells
	2.	green o	= APP-immunoreactive cell
	3.	red x	= glucagon-immunoreactive cells
	4.	green o	= APP-immunoreactive cell





Dual immunoreactivity for gastrin/CCK and neurotensin in four consecutive sections. Pyloric region at 21 days of incubation - two fields.

Section no. 5. red o = neurotensin-immunoreactive cells

6. green x = gastrin/CCK-immunoreactive cells

7. red o = neurotensin-immunoreactiv cells

8. green x = gastrin/CCK-immunoreactive cells