ENDOCRINE CELLS IN THE GASTROINTESTINAL TRACT OF SHEEP

A thesis presented in partial fulfilment of
the requirements for the
Degree of Doctor of Philosophy
in Histology
at Massey University

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1985

To the memory of my father

Arthur Peter Gurnsey

25-11-1921 to 26-9-1984

"One man in his time plays many parts"

William Shakespeare

ABSTRACT

Previous investigations have shown that the digestive activities of the mammalian GI tract are controlled, in part at least, by biologically active compounds released from endocrine cells in the mucosa of the GI tract itself. Despite this, comparatively few studies have been made of the endocrine cells in the GI tract of sheep. There is also a paucity of information about the suitability and reliability of histochemical and immunohistochemical methods for the identification of GI endocrine cells in sheep.

The aims of this study were to: (a) establish reliable techniques for identifying endocrine cells in the GI tract of sheep, (b) use these techniques to investigate the effects of age on the distribution and densities of various GI endocrine cells, and (c) investigate possible changes in endocrine cell densities due to infection with the helminth parasite Trichostrongylus colubriformis.

Initially, various histochemical and immunohistochemical staining techniques were investigated for their suitability for identifying endocrine cells in mucosal samples from reticulum, rumen, body and antral regions of the abomasum, three duodenal sites, ileum, colon and caecum, as well as the pancreas, of adult animals. As a result, the De Grandi technique was selected to estimate argyrophilic cell densities, EC cells were identified by the fast garnet technique, and ECL cells by their silver staining and morphological characteristics. The PAP immunohistochemical technique was used to identify G, S, and A cells, using antisera to gastrin, secretin, and pancreatic glucagon, respectively.

No endocrine cells of any type were found in the reticulum or rumen. Argyrophilic cell densities were greatest in the abomasal body and proximal duodenum, then decreased distally. EC cell densities were highest in the duodenum, although, like argyrophilic cells, they were found throughout the abomasum and intestines. In contrast, ECL cells were confined to the abomasal body. Greatest densities of G cells occurred in the abomasal antrum and proximal duodenum; they were absent from the abomasal body, ileum and large intestine. S cells were

confined in their distribution to the small intestine.

Pancreatic islets of Langerhans contained A cells, as well as cells with slight argyrophilia; the identity of the latter cells was not determined. A cells were also found in exocrine acini, but these were the only cells in the exocrine portion of the pancreas that were stained by any of the histochemical or immunohistochemical techniques used. No A cells were identified in the mucosa of the GI tract.

These studies also clearly established that ovine G cells are not argyrophilic. This finding is in contrast to those reported for most mammalian species with a simpler form of stomach.

The effects of age on endocrine cell densities were studied using the tissues from 100 - 110 day old foetuses, 2 week and 24 week old lambs, and adult sheep. All endocrine cell types identified in adult sheep were also present at the other ages. However, in the foetuses, endocrine cell densities were lower than in other age groups. The most notable age-related trend was that antral G cell densities increased with increasing age. In contrast, from 2 weeks of age, there was a decrease in intestinal G cell densities with increasing age. It was also clear that D cell densities were much higher in 2 week old lambs than for any other age group. Possible explanations for these age-related changes in endocrine cell densities are discussed.*

The effects on endocrine cell densities of an experimental infection with 40,000 T. colubriformis larvae was investigated in 40 week old lambs. Although the resultant infestation was mild, there was a significant ($P<0.001$) increase in argyrophilic cell densities in the proximal small intestine. Specific identification of the argyrophilic cell type(s) which had increased was not possible, however, the most likely candidates were D_1, X and K cells.

It was concluded from these studies that endocrine cells, similar in morphology and staining characteristics to those of other mammalian species, occur within the mucosa of the abomasum, small and large intestine of sheep. Greatest densities of endocrine cells occur in the

Footnote * D (somatostatin containing) cells were located throughout the abomasum and intestines of all nonadult animals and in pancreatic islets of 2 and 24 week old lambs.

abomasum and proximal duodenum. Cell types identified in the GI tract included EC, ECL, G, S and D cells, while D and A cells were identified in pancreatic islets. It was demonstrated that endocrine cell densities change with age and that significant changes in cell densities can occur in mild trichostrongylosis.

ACKNOWLEDGEMENTS

Special gratitude is due to Drs K.R. Lapwood and G.W. Reynolds for their continued encouragement and the guidance so willingly given throughout the term of this project.

I also wish to thank Professor R.E. Munford for his role as supervisor and for making available the facilities of the Department of Physiology and Anatomy.

Special thanks are also due to Mr M.J. Birtles and Dr W.A.G. Charleston for the many helpful discussions and the expert advice given.

Massey University sheep farm supervisor, Mr P.H. Whitehead, is thanked for provision and management of sheep used in this study.

I sincerely thank Messrs R.N. Ward, I. Denby, Mrs B.G. Batchelar, Miss P.J. Wylie and S. Thomas for the skilled technical assistance so generously given, Mr K. Korndorffer for assistance in preparation of figures, Mr T.J. Law for photographic advice and Mrs G. Shaw for her patient typing of this thesis.

Dr J. Hansky is thanked for generously providing gastrin antiserum (As'74) and Dr R.V. Brunsdon is thanked for making available the *T. colubriformis* larvae used in this study.

I am indebted to Dr R.W. Bailey and the D.S.I.R. for enabling me to undertake this study.

Finally I wish to acknowledge the moral support and encouragement given by my wife Catherine during the period of this study.

TABLE OF ABBREVIATIONS

Abbreviation	
Ab	Abomasum
APUD	Amine precursor uptake and decarboxylation
As'74	Antiserum '74
BLI	Bombesin-like immunoreactivity
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
$CaCl_2$	Calcium chloride
CCK	Cholecystokinin
Chr	Chromaffin
CI	Colour Index
Cl^-	Chloride ions
cm	centimetre
$c.mm^{-2}$	cells per square millimetre
CNS	Central nervous system
CTMC	Connective tissue mast cell
DAB	Diaminobenzidine tetrahydrochloride
DF	Degrees of freedom
DG	De Grandi
Duo	Duodenum
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscope
emg	electromyogram
epg	eggs per gram
FB	Fast Black
FG	Fast Garnet
Fig.	Figure
g	gram
Gas	Gastrin
GEP	Gastro-entero-pancreatic
GI	Gastrointestinal

GIP	Gastric inhibitory polypeptide
GL	Glucagon-like immunoreactivity
GRP	Gastrin releasing peptide
H and E	Haematoxylin and eosin
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate ions
Histo	Histochemical
hr(s)	hour(s)
5-HT	5-hydroxytryptamine (serotonin)
IgG	Immunoglobulin G
Immuno	Immunohistochemical
IR	Immunoreactive
K^+	Potassium ions
KCl	Potassium chloride
K_2CrO_4	Potassium chromate
$K_2Cr_2O_7$	Potassium dichromate
kg	kilogram
KIU	Kallikrein inhibitor unit
ℓ	litre
LI	Large intestine
m	metre
M	Molar
mg	milligram
Mg^{2+}	Magnesium ions
MH	Masson-Hamperl
min	minute
ml	millilitre
mm	millimetre
MMC	Mucosal (or Migrating) mast cell
mmol	millimole
mol	mole
Mol. wt.	Molecular weight
m.p.	melting point
µl	microlitre
µmol	micromole

n	number/group
N	Normal
Na^+	Sodium ions
No	Animal number
PAP	Peroxidase anti-peroxidase
PBS	Phosphate buffered saline
pers. com.	personal communication
pg	picogram
PHI	Porcine heptacosapeptide
pmol	picomole
PO_4^{3-}	Phosphate ions
PP	Pancreatic polypeptide
%	percent
RIA	Radioimmunoassay
Sec	Secretin
SEM	Standard error of the mean
SI	Small intestine
SM	Sevier-Munger
Som	Somatostatin
Tris	Tris(hydroxymethyl)aminomethane
VFA	Volatile fatty acid
VIP	Vasoactive intestinal polypeptide
vs	versus
wks	weeks
yrs	years

Abbreviations used for specific endocrine cell types are given in Table 1.1.

Abbreviations used for sites from which mucosal samples were taken are given in Tables 2.2 and 7.3.

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Regulation of GI secretion and motility is achieved by integrated action of neural and hormonal mechanisms. Bayliss and Starling (1902), with the discovery of secretin, were the first to demonstrate the existence of hormonal control over a GI function. Subsequently many crude extracts from the gut mucosa, with effects on GI secretion or motility, were prepared and claimed to contain specific hormones. There resulted a proliferation of putative GI hormones, only a small number of which have met the strict criteria (see Buchanan, 1979) for acceptance as true hormones.

A major source of the hormones, bioactive peptides and amines found in the GI tract are endocrine or endocrine-like cells (Solcia et al., 1981a). These cells, first recognized in the GI tract more than 100 years ago by Heidenhain (1870), are scattered through the mucosa of almost the entire digestive tract, making the GI tract the largest and most diffuse endocrine organ in the body (Johnson, 1980). Peptide and/or amine products are released by these cells into the circulation (endocrine), gut lumen (exocrine) or interstitial fluid (paracrine) in response to stimulation by nervous, hormonal, luminal and inter-cellular factors (see Track et al., 1980).

Although endocrine cells have been demonstrated in the GI mucosa of sheep (Tehver, 1930), and some specific endocrine cell types have been identified (see Section 1.8.4), there is a general paucity of information on the types and distribution of endocrine cells in the ovine GI tract.

In ruminant animals such as sheep, a major feature of the GI system is that fermentative digestion due to bacteria and protozoa occurs on a large scale in the forestomach. This fermentative digestion, necessary for the breakdown of the ingested plant structural carbohydrates,

cellulose and hemicellulose, precedes digestion by the animal's own digestive enzymes which occurs in the abomasum and intestines.

This literature review surveys the published data on the structure, development, function and regulation of the ovine GI tract. Where such information is scarce or nonexistent, particularly with regard to endocrine cells, reference has been made to data obtained from other species.

1.2 Anatomy of the Ovine Stomach and Intestines

The anatomical nomenclature used in this thesis is based on that adopted in Nomina Anatomica Veterinaria and Nomina Histologica (1983).

This brief outline will be confined to the anatomy of the stomach and intestines as far as, and including, the descending colon. For more detailed descriptions of these and other regions of the ovine GI tract see Habel (1975), Barone (1976) and Schummer and Nickel (1979).

1.2.1 Gross Anatomy

1.2.1.1 Stomach

The compound stomach of the adult sheep is divided into four compartments. These are, in aboral sequence, the reticulum, rumen, omasum (which together are referred to as the forestomach) and abomasum (see Fig. 1.1).

1.2.1.1.1 Forestomach

Reticulum

The reticulum, which is the most cranial compartment of the stomach, has three openings: the ruminoreticular opening, the cardia and the reticulo-omasal orifice. The reticulum communicates with the cranial sac of the rumen via the ruminoreticular opening and with the

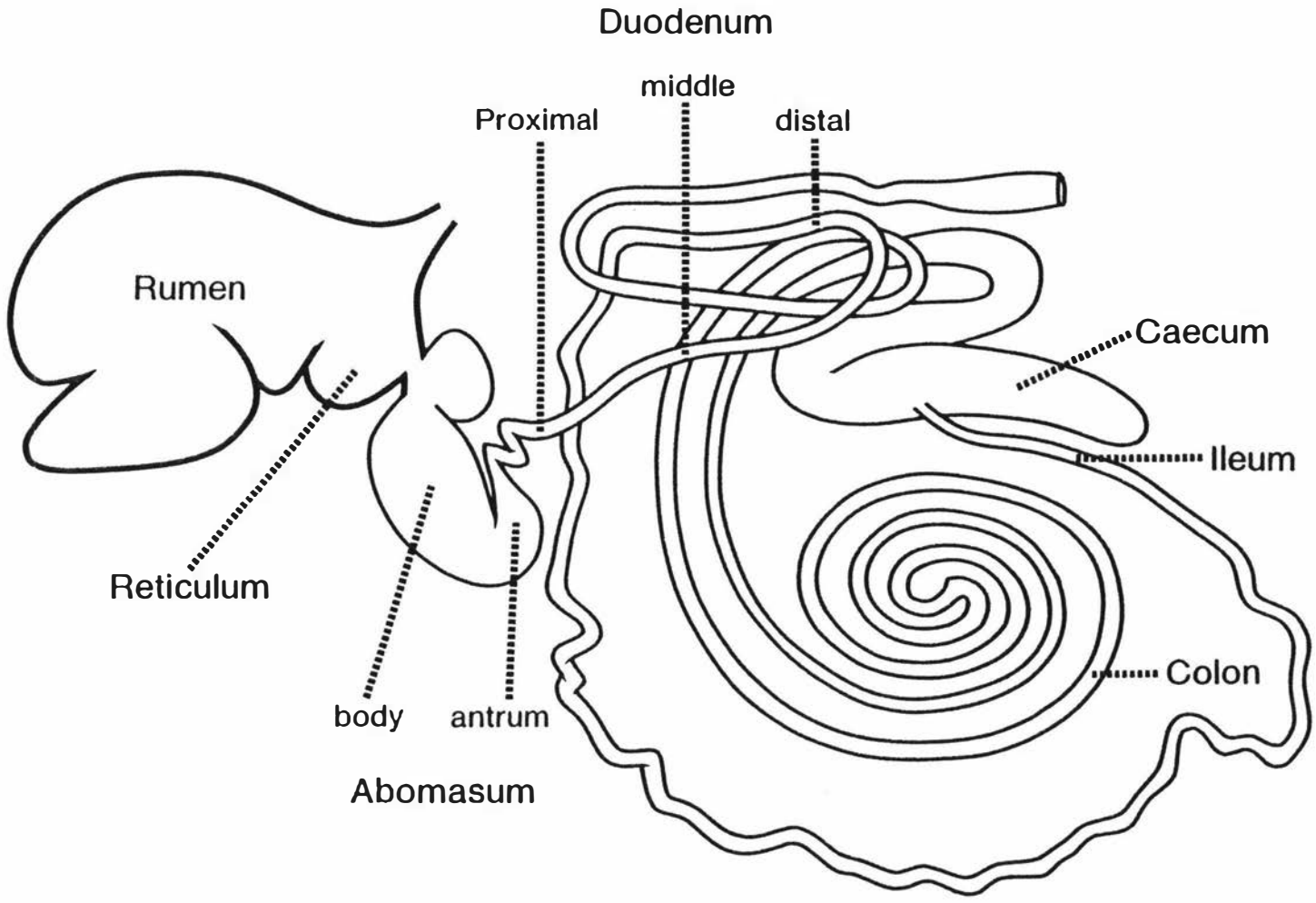


Fig. 1.1: Diagrammatic representation of the ovine GI tract

omasum through the reticulo-omasal orifice. The oesophagus opens into the reticulum at the cardia.

The mucosal surface of the reticulum has a honeycomb-like appearance due to an hexagonal arrangement of many low (2 - 8 mm) folds, called reticular ridges. Numerous small conical papillae arise from the mucosal surface of the reticulum, thus greatly increasing the surface area.

Rumen

The rumen is the largest compartment of the sheep's stomach. In the adult it accounts for up to 85% of total stomach volume (Warner and Flatt, 1965).

Several muscular pillars partially divide this compartment into a number of sacs: cranial, dorsal and ventral sacs, and caudodorsal and caudoventral blind sacs. The surface area of the mucosa is increased by the presence of numerous papillae which vary in size, shape (generally spatulate) and density from region to region.

Omasum

The omasum is the smallest of the three compartments of the forestomach. It has two openings: the reticulo-omasal orifice and the omasoabomasal orifice which opens into the abomasum.

The most notable feature of the omasum is the large number (72 - 80) of omasal laminae which arise from the greater curvature and project into the lumen. Each omasal lamina consists of a thin sheet of smooth muscle covered by mucous membrane from which numerous papillae arise.

1.2.1.1.2 Abomasum

The abomasum is analogous in both form and function to the stomach of species such as cat, dog and man. In adult sheep the abomasum represents about 12% of total stomach volume (Warner and Flatt, 1965). It is divided, according to gland type, into cardiac, body and antral regions.

The mucous membrane lining the body region is characterized by large, spirally arranged folds which commence at the omasoabomasal orifice and extend as far as the angular incisure. Hill (1968) estimated that the mucosal surface area of the abomasal body is increased more than 7 times by these folds.

A few irregular mucosal folds are present in the antral region.

1.2.1.2 Small Intestine

In adult sheep, the small intestine (Fig. 1.1) is 18 - 35 m long and is divided in aboral sequence into duodenum, jejunum and ileum.

The duodenum consists of cranial, descending and ascending parts. At the duodenojejunal flexure the duodenum is continued as the jejunum which is the longest part of the ovine small intestine. The jejunum is arranged in numerous close coils surrounding the spiral loop of the ascending colon. The ileum, which in sheep is very short, extends from the border of the ileocaecal fold to its termination at the ileal orifice.

The mucosal surface of the small intestine is characterized by permanent circular folds of the mucous membrane (Plicae circulares) and by villi, which greatly increase the absorptive area of the mucosa.

1.2.1.3 Large Intestine

In adult sheep the large intestine is 4 - 8 m long and is divided in aboral sequence into caecum, colon, rectum and anal canal.

The caecum is a slightly sigmoid-shaped, sacculated blind tube which in the adult sheep is 25 - 35 cm in length and 5 - 7 cm in diameter (Ulyatt et al., 1975). It is continuous with the colon at the caecocolic junction.

The colon has ascending, transverse and descending parts. In ruminants the ascending colon is elongated and coiled on itself to form the spiral loop of the colon. In sheep the spiral loop consists of three centripetal and an equal number of centrifugal turns and is continuous with the transverse colon. The descending colon is continuous with the rectum.

The mucous membrane of the large intestine has no villi, however, the surface is penetrated by the openings of the many crypts of Lieberkuhn.

1.2.2 Histology

In sheep, as in animals with a simpler form of stomach, the wall of the stomach and intestines consists of four main layers: from the outside in these are the Tunica serosa, Tunica muscularis externa, Tunica submucosa and Tunica mucosa.

The serosa consists of areolar connective tissue and a single layer of squamous mesothelial cells.

In most parts of the GI tract, two layers of smooth muscle make up the muscularis externa. These are an inner layer, which generally is the thicker and circularly arranged, while the outer layer is longitudinally disposed. In the forestomach the arrangement of muscularis externa is more complex. For example, the longitudinal smooth muscle layer of the forestomach divides at the cardia into two parts. Furthermore, a third smooth muscle layer of internal oblique fibres is found in the dorsal, ventral and blind sacs of the rumen. For a detailed description see Pernkopf (1930).

The submucosa is made up of loose connective tissue containing elastic fibres, blood vessels and the submucosal (Meissner's) nerve plexus. Submucosal (Brunner's) glands are found in first 60 - 70 cm of the duodenum where they augment the secretions of the epithelium. The glands open via short ducts into the base of the duodenal crypts.

The mucous membrane is made up of three layers: Lamina muscularis mucosae, Lamina propria mucosae and Lamina epithelialis.

Two thin muscle layers, an inner circular and a longitudinal outer layer, make up the muscularis mucosae. In sheep, there is no muscularis mucosae in the rumen, while in the reticulum it is represented only as bands of muscle reinforcing the free borders of the reticular ridges. In the omasum, the muscularis mucosae extends into the laminae where it forms terminal thickenings near their free borders.

The lamina propria consists of connective tissue containing many capillaries, lymphatics, nerve fibres and a few smooth muscle fibres. Cellular components of the lamina propria include CTMC's (Toledo et al., 1981) and connective tissue fibroblasts (see Kaye et al., 1968). In the ovine forestomach the lamina propria and submucosa merge with no distinct boundary separating them (Wardrop, 1961a). The lamina propria, throughout the entire length of the intestines, contains numerous tubular intestinal glands (crypts of Lieberkuhn).

The epithelium is separated from the lamina propria by the basal lamina. In the reticulum, rumen and omasum, the epithelium is a stratified, squamous, keratinizing epithelium in which four cell layers can be distinguished: Stratum basale, Stratum spinosum, Stratum granulosum and Stratum corneum (Dobson et al., 1956). At the omasoabomasal orifice there is a change in epithelium to a columnar mucous border. In the abomasum the epithelium is invaginated to form gastric pits which in the cardiac and body regions are short, while those of the antral region are long. Gastric pits are lined by surface epithelial cells. One or more tubular glands open into the bottom of each pit.

Three different types of abomasal gland have been described: cardiac, gastric and antral.

Cardiac glands are confined to a narrow rim of mucosa at the margin of the omasoabomasal orifice (Sommerville, 1956). The glands are highly coiled and contain only cells which secrete a viscous mucus (M.J. Birtles, pers. com.)

Gastric glands are found throughout the body region of the abomasum and occupy about two thirds of the mucosal thickness. They are straight tubules which may be divided into three equal parts: isthmus, neck and base. Murray (1970) described five cell types in the abomasal epithelium of cattle: surface epithelial, mucous neck, parietal, zymogenic and endocrine.

Surface epithelial cells are found intermingled with parietal cells in the isthmus of gastric glands (Murray, 1970). Much of the supranuclear cytoplasm is occupied by membrane-bound granules containing mucus. The nucleus, which is located in the base of the cell, is most often oval or pear-shaped.

Mucous neck cells are common in the middle region of gastric glands, but may also be found in the basal region (Murray, 1970). They are relatively small, columnar cells although their shape may be quite variable due to deformation by larger neighbouring cells. The nucleus, located near the base of the cell, is irregular in shape and frequently indented by granules. Membrane-bound granules containing mucus occupy most of the supranuclear cytoplasm.

Parietal cells are found intermingled with other cell types throughout the gastric glands but are most common in the neck and isthmus regions. They are large cells, oval to pyramidal in shape with a broad base which often protrudes into the lamina propria. They have a round, centrally placed nucleus and the eosinophilic cytoplasm lacks secretory granules (Murray, 1970).

In the sheep, parietal cell density varies throughout the body region; the largest numbers are found close to the cardia and frequency decreases with increasing distance from the cardiac orifice (Sommerville, 1956).

Zymogenic (or chief) cells are most common in the base of the gastric glands but are also found in the neck regions. They are columnar cells with a centrally placed nucleus. The cytoplasm is characterized by the presence of abundant rough endoplasmic reticulum in the base of the cell and numerous membrane-bound zymogen granules in the

apical region. The zymogen granules in man have been shown to contain pepsinogens (Hirschowitz, 1967).

Zymogenic cells are usually the predominant cell type at the base of gastric glands in the cranial part of the body of the abomasum, but their distribution may show considerable variability and they are frequently absent from glands close to the antral region (Sommerville, 1956).

Endocrine cells are the smallest, least numerous cells in the gastric mucosa and usually occur as single cells scattered among other cells, particularly in the basal region of the glands. Details of the morphology and distribution of specific endocrine cell types are described in Sections 1.8.2 and 1.8.4.

The glands of the antral mucosa are coiled and contain four cell types: surface epithelial, antral gland, endocrine and parietal cells. The surface epithelial cells are identical to those found in the body region. Antral gland cells, which morphologically resemble mucous neck cells of gastric glands, contain mucus in their apical cytoplasm (Murray, 1970). Although parietal cells have been identified in antral glands of both sheep (Sommerville, 1956) and cattle (Murray, 1970), Sommerville found that they differed in both staining reaction and morphology from parietal cells found in gastric glands. He therefore regarded them as 'abnormal'.

Because of the paucity of published data on the histology of the ruminant small intestine, the description that follows is taken from species with a simpler form of stomach (Trier and Madara, 1981).

The epithelium of the small intestine contains several different cell types, the most common of which are the absorptive cells. These are highly polarized, tall columnar cells with prominent striated borders consisting of many closely packed microvilli (see Trier and Madara, 1981).

Undifferentiated crypt cells are the most common cell type found in the crypts of Lieberkuhn. These cells proliferate rapidly, migrate up the wall of the crypt and differentiate into absorptive, Paneth, endocrine and goblet cells (Cheng and Leblond, 1974).

Intestinal goblet cells are found scattered throughout the epithelium but increase in relative frequency distally. They are frequently goblet shaped, lack well developed microvilli and the apical portion of the cell is distended by many mucinogen granules.

Paneth cells are most common in the base of crypts of Lieberkuhn and increase in number from duodenum to ileum. Although Paneth cells have been shown to be active secretory cells and contain lysozymes and immunoglobulins, their function in the small intestine is unknown.

Endocrine cells are found scattered through the epithelium of the small intestine, but are most numerous near the base of crypts in the duodenum. Small numbers of endocrine cells are also found in the glands of Brunner.

Glands of Brunner, which are more extensive in herbivora than in carnivora (Cooke, 1968), are composed of branched tubules into which acini open. The ducts open into the base or sides of the crypts of Lieberkuhn. The principal cell type is one secreting mucus although in some species serous cells have also been identified (see Friend, 1965). Endocrine, Paneth and goblet cells may also be found near the duct openings into the crypts (Friend, 1965). The gland secretion contains considerable quantities of glycoprotein and bicarbonate and small amounts of pepsin and mucinase activity (see Cooke, 1968).

Nodules of diffuse lymphoid tissue, which in the ileum are called Peyer's patches, occur in the submucosa but may penetrate the mucosa to come into direct contact with the epithelium.

1.3 Development of the Ovine GI Tract

1.3.1 Embryological Development

Much of the description that follows is derived from Pernkopf (1931) and Wardrop (1961a). The description of histological changes is confined to those changes observed in the epithelium of the GI tract of the foetal lamb.

1.3.1.1 Stomach

Pernkopf (1931) demonstrated that the bovine forestomach develops from a simple spindle-shaped enlargement of the primitive digestive tube. Up to the 7 mm stage of embryonic development, the gastric spindle is similar to that in animals with a simpler form of stomach. In sheep, by the 16 mm stage (approximately 37 days), all parts of the adult compound stomach can be distinguished (see Warner and Flatt, 1965). Subsequent development of the foetal stomach consists of differential growth of the forestomach and abomasum. At 56 days gestational age, the rumen represents approximately 45% of total stomach weight, while at 140 days it represents only 26% (Wallace, 1948). Over the same period, the reticulum and omasum also show relative declines in weight (14 to 10% and 24 to 8%, respectively) while the abomasum undergoes a marked increase in relative weight (from 17 to 56%).

The mucous membrane of the reticulum displays a small amount of folding in 70 day old foetuses and reticular ridges are obvious in 100 day old foetuses. At birth, the honeycomb structure, typical of the reticulum, is well developed with small, rounded and conical papillae on and between each reticular ridge (Wardrop, 1961a).

The epithelium lining the reticulum of 46 day old foetuses is a stratified, cuboidal type, about eight cells in depth. It consists of a basal layer, about three cells thick, of densely packed columnar cells and several layers of large, less densely packed, roughly hexagonal cells. By 100 days the basal layer is folded to form the developing reticular ridges. Above this layer is a fine network of swollen cells which become flattened towards the folded free surface (Wardrop, 1961a).

The histological appearance of the ruminal epithelium of 46 day old foetuses is similar to that of the reticulum at the same age (see above). In 100 day old foetuses the rumen mucosal surface appears smooth, however, at birth, the numerous short, conical papillae are obvious, and the epithelium is similar to that of the rumen of the adult (i.e. keratinized, stratified, squamous type).

The omasal mucosal surface is folded in 46 day foetuses and from 70 days onwards the omasal laminae are prominent. The mucosa of these laminae consist of a single basal layer of densely packed, columnar cells. Above this is a network of swollen cells approximately six cells thick. At birth no stratum granulosum can be identified and the stratum corneum consists of a thin layer of flattened cells (Wardrop, 1961a).

Abomasal mucosal development has been described by Hill (1956). The epithelium of 20 day old foetuses consists of a single layer of columnar cells which gradually become folded so that by 40 days the developing mucosal folds project into the abomasal lumen. By 60 - 75 days the epithelium of the entire abomasum is formed into shallow pits from which develop abomasal glands. Chief cells are evident in 75 - 85 day foetuses although zymogen granules are not numerous until day 119.

1.3.1.2 Intestines

In what is apparently the only paper on the subject, Toofanian (1976b) described the histological development of the small intestine in ovine foetuses. In foetuses estimated to be 24 days old, the primitive small intestine consists of a simple epithelium surrounded by a mesenchymal layer. By the 26th day the epithelium has become stratified and at 33 days the characteristic coiling of the intestine is evident. Rudimentary villi are seen as outgrowths of the small intestinal epithelium in 39 day old ovine foetuses (Toofanian, 1976b). These consist of a central core of mesenchyme covered in a stratified epithelium. However, by 46 days gestational age these duodenal epithelial outgrowths are elongated and take on the appearance of true villi, with a core of lamina propria surrounded by a simple epithelium. Toofanian (1976b) also reported the first appearance of intestinal glands in the duodena of 56 day old ovine foetuses. The two layers of the muscularis externa of the intestine are discernable by 63 days gestational age, and all intestinal structures are present at 101 days gestational age. Throughout foetal life, development is more advanced in the proximal parts of the small intestine than in the more distal portions. Toofanian concluded that the development of the small intestinal mucosa in lambs is similar to that described in other vertebrate species (Deren, 1968; Toofanian, 1976a).

1.3.2 Postnatal Development

After birth there is considerable growth of the compound stomach relative to the entire GI tract, so that it increases in proportion from 22% of the total weight of the GI tract at 1 day of age, to 49% in the adult (Wardrop and Coombe, 1960). Changes also occur in the relative size of the various compartments of the stomach. At birth the rumino-reticulum accounts for 31%, the omasum 8% and the abomasum 61% of the total weight of the stomach, whereas in the adult, these regions account for 69, 5 and 23% respectively (Wardrop and Coombe, 1960).

The rapid postnatal development of the ruminoreticulum is associated with the ingestion of dry feed (Warner and Flatt, 1965). Warner et al., (1956) demonstrated that an important stimulus to forestomach development is the presence and/or production in the forestomach of VFA's, the end products of fermentative digestion. In lambs fed solely on milk, development of the forestomach is retarded to about the level found in three week old grazing lambs (Wardrop, 1961b).

After birth the basal epithelial layers of the ruminoreticulum become more folded and a distinct stratum granulosum forms. Growth continues, particularly in length of reticular ribs and papillae, so that at 77 days of age the mucosa is similar in appearance to that of the adult (Wardrop, 1961a).

During the first three days of postnatal life, parietal cell numbers increase about ten fold in the body region of the abomasum (Hill, 1956). After three days the appearance of gastric glands is similar to that found in the adult, except that they continue to increase in length and degree of coiling for some weeks after birth.

In milk fed lambs papillae in the ruminoreticulum are smaller and the stratum granulosum and stratum corneum less well developed than in lambs fed lucerne chaff (Wardrop, 1961b). When lambs are maintained on a milk diet for 7 weeks after birth, the histological appearance of the omasum remains similar to that of the newborn lamb. Omasal laminae remain short and closely packed together, muscle layers are often poorly developed and the epithelium poorly keratinized (Wardrop, 1961b).

1.4 Functions of the Ovine GI Tract and its Secretions

1.4.1 Saliva

Sheep secrete saliva continuously and during the course of a day may produce from 5 (Somers, 1957) to 16 (Kay, 1960) litres. The ruminoreticular contents are buffered by the secretion in saliva of large quantities of sodium and potassium salts. In addition to its role in lubricating the bolus of feed, thus aiding mastication and swallowing, the saliva of ruminants has some enzymic activity. Although ruminant saliva does not contain amylase activity it does contain pregastric esterase (Ramsey et al., 1960; Ramsey, 1962) and lipase activity (Grosskopf, 1965). Saliva also provides nutrients for rumen microorganisms, particularly mucin, urea, PO_4^{3-}, Mg^{2+} and Cl^- (Kay, 1960; Hungate, 1966).

1.4.2 Forestomach

1.4.2.1 Ruminoreticulum

The ruminoreticulum provides a large chamber within which microorganisms break down feeds by fermentative digestion. Breakdown of feed may be aided by mechanical abrasion resulting from complex mixing movements of the ruminoreticulum (Ulyatt et al., 1985). Ruminoreticular movements are also involved in regurgitation of digesta which then undergoes further mechanical disruption during remastication of the regurgitated bolus (Ulyatt et al., 1985).

Other functions of the ruminoreticulum include absorption of water (Engelhardt, 1970), VFA's (Barcroft et al, 1944; Danielli et al., 1945; Kiddle et al., 1951; Masson and Phillipson, 1951; Pfander and Phillipson, 1953) and some ions including Na^+, K^+, Cl^- (Parathasarathy and Phillipson, 1953)* and Mg^{2+} (Care et al., 1984). Nitrogenous compounds which are absorbed across the rumen epithelium include ammonia (Hogan, 1961; Bloomfield et al., 1963), urea (see Church, 1973), lysine (Lewis and Emery, 1962), glycine, serine, threonine, methionine sulphoxide, aspartic acid, glutamine, isoleucine, leucine (Cook et al., 1965) and B complex vitamins (see Church, 1973).

* (Keynes and Harrison, 1970)

1.4.2.2 Omasum

The reticulo-omasal orifice regulates the flow of digesta from reticulum into the omasum (Bost, 1970) where fermentative digestion continues. In the omasum, water (Engelhardt and Hauffe, 1975), VFA's (Barcroft et al., 1944), ammonia (McDonald, 1948), Na^+ and K^+ (Engelhardt and Hauffe, 1975) and Mg^{2+} (Edrise and Smith, 1979) are absorbed, while Cl^- (Bost, 1970; Engelhardt and Hauffe, 1975) is secreted. Engelhardt and Hauffe (1975) concluded that omasal absorption of water, Na^+, K^+ and ammonia is about 20 - 30% of that in the ruminoreticulum but, absorption of VFA's is only 10 - 15% of that in the ruminoreticulum.

1.4.3 Abomasum

The abomasum is the site of acid digestion in the ovine GI tract. The pH of digesta entering the abomasum is about 6, whereas the abomasal contents are usually at a pH of between 1.6 and 4.2 (Weston and Hogan, 1968). This low abomasal pH is due to the secretion of HCl by parietal cells in the gastric glands of the abomasal body. Hydrogen ion concentration in abomasal secretion ranges from 20 (Hill, 1968) to 150 (McLeay and Titchen, 1970a) mmol/ℓ. Estimates of the volume of abomasal secretion range from 4 - 6 ℓ/24 hrs (Masson and Phillipson, 1952; Hill, 1960) to 10 - 12 ℓ/24 hrs (McLeay, 1971).

The proteolytic activity of abomasal secretion, in the adult animal, is due to the enzyme pepsin (Hill, 1956). Pepsinogen, the precursor and inactive form of pepsin, is produced by the chief cells which are confined to the mucosa of the body region of the abomasum (Murray, 1970). At pH's lower than 6.0, pepsinogen is converted into pepsin.

A small amount of pepsin activity is also found in secretions from pouches of the antral region of the abomasum (Harrison and Hill, 1962; McLeay and Titchen, 1975).

Mucus, which protects the mucosa from physical damage by the digesta, also provides a barrier to autodigestion of the epithelium by HCl and pepsin. The antral mucosa produces a mucus rich secretion of pH 7.5-9.0 (Harrison and Hill, 1962; McLeay and Titchen, 1975), however, as the amount secreted is small (Ash, 1961a; Harrison and Hill, 1962; McLeay and Titchen, 1975) it has been suggested that it performs a local protective function.

In the young milk fed animal, rennin is secreted in the gastric juice during feeding (Fomin, 1939: cited by Berridge, 1951). Rennin, which is produced by the chief cells (Edkins, 1906), is responsible for the rapid clotting of milk within the abomasum (Grosskopf, 1959). In the newborn, rennin is the only enzyme present in abomasal secretions (see Hill, 1968). In calves maintained on a pure milk diet it remains the most important proteolytic enzyme secreted by the abomasum (Berridge et al., 1943), although small amounts of pepsin are also produced (Henschel et al., 1961). In addition to its secretory activity, the abomasum has absorptive functions. Williams et al., (1968) have shown that VFA's are absorbed from the abomasum at rates per unit surface area similar to those in the ruminoreticulum. Ammonia may also be absorbed across the abomasal mucosa (see Church, 1973).

1.4.4 Intestines

The small intestine is the major site of proteolysis (Armstrong and Hutton, 1975) and lipolysis (Leat and Harrison, 1975). It is also the primary site for absorption of most organic compounds other than VFA's, including amino acids, fatty acids and some ammonia. In addition it is an important region of absorption and secretion of water and many minerals (Kay and Pfeffer, 1970).

Fermentative digestion also occurs in the caeca of ruminants (Barcroft et al., 1944). Between 5 and 30% of digestible cellulose is digested in the large intestine of ruminants fed a wide range of feeds including fresh herbage, hay, silage and cereals (Ulyatt et al., 1975). The major products of fermentation are VFA's which are readily absorbed across the caecal epithelium (Barcroft et al., 1944). Estimates of the

amounts of VFA's produced by caecal fermentation range from 8 to 16% of total production (Ulyatt et al., 1975).

The caecum is particularly efficient at water absorption; approximately 90% of water entering the caecum is normally absorbed (Goodall and Kay, 1965; Ulyatt and MacRae,1974). In sheep the large intestine is also a major site of Na^+ (Goodall and Kay, 1965; Bruce et al., 1966; Grace et al., 1974) and Cl^- (Goodall and Kay, 1965; Bruce et al., 1966) absorption, while small amounts of K^+, Mg^{2+}, Ca^{2+} and PO_4^{3-} are also absorbed in this region (Bruce et al., 1966; Pfeffer et al., 1970; Grace et al., 1974).

1.4.5 Bile

In sheep 500 - 1500 ml of bile is produced per day. It contains water and electrolytes, mainly Na^+, K^+, Cl^- and HCO_3^-, which contribute to the buffering of the acidified digesta entering the duodenum from the abomasum (Caple and Heath, 1972; 1975). Bile also contains conjugated bile salts, particularly sodium taurocholate (Peric-Golia and Socic, 1968) and fatty acid containing lipids, especially phospholipids (Adams and Heath, 1963). The latter, in particular phosphatidylcholine, assist bile salts to render soluble other lipids in the intestinal contents (Heath and Hill, 1969; Leat and Harrison, 1969).

1.4.6 Pancreatic Juice

In sheep 300 - 400 ml of pancreatic juice are secreted per day. It contains water and electrolytes, mainly Na^+, K^+, Cl^- and HCO_3^- (Caple and Heath, 1972), together with amylytic, proteolytic and lipolytic enzymes (Taylor, 1962; Reynolds and Heath, 1981). Pancreatic HCO_3^- aids in the buffering of duodenal contents, while the enzymes hydrolyze carbohydrates, proteins and lipids.

1.5 Innervation of the GI Tract

1.5.1 Extrinsic Nerve Supply

In sheep the GI tract is innervated by both divisions of the autonomic nervous system. The description that follows is taken largely from Habel (1956) and Comline et al. (1968).

1.5.1.1 Parasympathetic

The parasympathetic contribution is derived from the dorsal vagal nuclei of the medulla oblongata. Fibres travel in the left and right vagi which anastomose in the thorax, then divide to form the dorsal and ventral vagal trunks associated with the oesophagus.

After receiving a communicating branch from the ventral trunk, the dorsal trunk divides to innervate the caudal surface of the reticulum, rumen, omasum, and the visceral (Habel, 1956) and parietal surfaces of the abomasum (Comline et al., 1968). In addition, the dorsal vagal trunk supplies branches to the coeliac plexus. Many vagal fibres pass through this plexus and follow branches of the cranial mesenteric artery to innervate the intestines as far as the left colic flexure.

The ventral vagal trunk innervates the cranial surface of the reticulum, ventral surface of the omasum and the parietal surface of the abomasum. The long pyloric nerve, a branch of the ventral trunk, gives off branches to the hepatic plexus, duodenum and antral region of the abomasum.

Parasympathetic innervation to the descending colon and rectum is derived from the sacral outflow and travels in the pelvic splanchnic nerves.

1.5.1.2 Sympathetic

Sympathetic nerves leave the spinal cord of the thoracolumbar region in spinal nerves and pass via the white rami communicantes to the paravertebral sympathetic ganglion chain. Preganglionic fibres pass

through the ganglion chain without synapsing, emerge in the splanchnic nerves and terminate in the prevertebral sympathetic ganglia: coeliac, cranial and caudal mesenteric. The postganglionic fibres emerge from these ganglia as mesenteric nerves which accompany blood vessels to the GI tract. Each artery is accompanied by a network of small paravascular nerves which supply adrenergic perivascular fibres to these blood vessels.

1.5.2 Intrinsic Nerve Supply

The intrinsic innervation of the GI tract includes the myenteric (Auerbach's) and submucosal (Meissner's) plexuses.

1.5.2.1 Myenteric Plexus

In species with a simpler form of stomach, the myenteric plexus, which is located between the two layers of the muscularis externa, is coextensive with the smooth musculature, without discontinuities, from oesophagus to anal canal (Gabella, 1981). This plexus is also found in the ovine forestomach and abomasum (Habel, 1956).

1.5.2.2 Submucosal Plexus

The submucosal plexus is found throughout the entire length of the ruminant intestine, whereas the only part of the ruminant stomach that possesses a true submucosal ganglion plexus is the abomasum (Habel, 1956). Many small nerve fibres are found in the submucosa of the non-glandular parts of the stomach, but ganglion cells are rare (Habel, 1956). The submucosal plexus is made up largely of non-myelinated postganglionic sympathetic fibres, but also contains a few terminal ganglia of the parasympathetic system.

Although there have been no reports of the identification of nerve fibres synapsing with specific epithelial cells of the GI tract of the sheep, there have been a few such reports in other species.

Morphological studies in rats indicate vagal fibres directly innervate parietal (Kalahanis et al., 1974) and ECL cells (Kalahanis et al., 1976). Newson et al. (1979) demonstrated a close relationship between endocrine cells of the rat terminal ileum and nerve terminals which contained vesicles suggestive of adrenergic or peptidergic nerves (Nyhus, 1982). A close association between EC cells and cholinergic and adrenergic nerve terminals has also been shown to exist in the guinea pig (Lundberg et al., 1978). These findings provide histological evidence of the possible role of the nervous system in the regulation of activity of some endocrine cell types in the GI tract, at least in species with a simpler form of stomach.

1.6 Regulation of GI Secretory Activity

Major exocrine organs contributing digestive secretions to the lumen of the GI tract of sheep include the salivary glands, abomasum, pancreas, liver and intestines. With the exception of the salivary glands, the secretory activities of these organs are influenced by both neural reflexes and hormones or peptides released from endocrine-like cells in the mucosa lining the GI tract. Secretory activities of the salivary glands are controlled exclusively by neural mechanisms. For a review of the regulation of salivary secretion in sheep, see Kay (1966).

1.6.1 Regulation of Abomasal Secretion

Although secretion of acid and pepsin by the abomasum of the adult ruminant is continuous (Babkin, 1950), this is not due to an innate ability of the glands to secrete spontaneously (Hill, 1955). Instead, it is due to specific stimuli which may either excite or inhibit(Hill, 1965) abomasal secretions. Endogenous chemicals considered to be important in the physiological excitation of abomasal secretions are gastrin, acetylcholine, histamine and bombesin.

1.6.1.1 Gastrin

Gastrin has been shown to occur in a number of molecular forms:

(a) Minigastrin (G14) with 14 amino acid residues (Gregory and Tracy, 1974).

(b) Little gastrin (G17) with 17 amino acid residues (Bentley et al., 1966; Gegory and Tracy, 1964).

(c) Big gastrin (G34) with 34 amino acid residues (Yalow and Berson, 1970).

(d) Big big gastrin (BBG) Mol. Wt. approximately 20,000 (Yalow and Berson, 1972).

(e) Component I (C-1) size intermediate between G34 and BBG (Rehfeld and Stadil, 1973).

(f) NH_2 - terminal fragment of G17 (NT13) with 13 amino acid residues (Gregory, 1974).

(g) Tetragastrin (G/CCK-4), the C-terminal tetrapeptide fragment of gastrin or CCK (Rehfeld et al., 1980).

In sheep only the G17 (Agarwal et al., 1968; Shulkes et al., 1981a, b)and the G34 (Lichtenberger et al., 1981; Shulkes et al., 1981a, b) forms have been found in GI tissues. Ovine G17 has been characterized (Agarwal et al., 1968) and shown to have an amino acid sequence similar to that of human, porcine, feline and canine gastrins, except for 1 or 2 substituted residues in the middle portion of the molecule. These differences are considered to have little biological significance as the active part of the molecule is the C-terminal tetrapeptide (Tracy and Gregory, 1964) which is identical in all biologically active forms of gastrin (Kenner and Sheppard, 1973). Ovine G34 has been found in the mucosa of the GI tract of lamb foetuses (Lichtenberger et al., 1981; Shulkes et al., 1981a, b) and 6 day old lambs (Lichtenberger et al., 1981). Whether it also occurs in the tissues of adult sheep remains to be determined.

Substantial amounts of bioassayable gastrin-like activity have been found in the mucosa of the abomasal antrum (Anderson et al., 1961, 1962; Olowo-okorun, 1975; Jury and McLeay, 1977), the abomasal body (Anderson et al., 1962; Jury and McLeay, 1977) and forestomach (Jury and McLeay, 1977) of sheep. By contrast, no immunoreactive gastrin has been found

in mucosal extracts of the forestomach of this species (Reynolds et al., 1984). The major site of immunoreactive gastrin production in sheep is the abomasal antrum with lesser concentrations being found in the duodenum (Reynolds et al., 1979a; 1980; 1984). This latter distribution of gastrin in sheep is in accord with the distribution of G cells which are responsible for the synthesis, storage and release of gastrin (Bunnett and Harrison, 1979a; Bunnett, 1984), and the distribution of gastrin and G cells in animals with a simpler form of stomach (Bloom and Polak, 1978).

The principal physiological action of gastrin in sheep, as in animals with a simpler form of stomach (see Walsh, 1981)*, is thought to be stimulation of gastric (abomasal) acid secretion. The importance of antral gastrins to the maintenance of gastric acid secretion in the sheep was demonstrated by McLeay and Titchen (1975) who found that both the volume and concentration of acid secreted by abomasal pouches was substantially reduced following removal of the major source of gastrin by antrectomy. Intravenous administration of either synthetic gastrin (Onapito et al., 1978) or the gastrin analogue, pentagastrin, (Carr et al., 1970; McLeay and Titchen, 1970b; 1975; 1977b) stimulates abomasal secretion of acid.

The regulation of gastrin release in sheep depends on chemical stimuli acting at either the luminal or basal surface of G cells. Intraluminal factors which stimulate gastrin release include protein or its breakdown products (van Bruchem, 1977) and possibly VFA's (McLeay and Titchen, 1971). Perfusion of antral pouches in sheep with acid solutions is thought to inhibit release of gastrin from the antral mucosa (McLeay and Titchen, 1977b). Thus, in sheep, as in other species (Walsh and Grossman, 1975; Walsh et al., 1975), gastrin release is regulated by a negative feedback loop involving acid secretion from gastric glands.

Factors which may act at the basal surface of G cells to influence gastrin release include blood borne factors, paracrine secretions and neurotransmitter substances. Blood borne peptides which, when administered exogenously, have been shown to suppress gastrin release and gastric acid secretion in both man and dog, include

* also Nilsson (1980)

secretin, glucagon, VIP, GIP and somatostatin (see Walsh and Grossman, 1975; Dockray, 1979; Konturek, 1980b).*

In calves, administration of secretin or glucagon reduces abomasal secretion of acid (Bell and McLeay, 1980), however, their effect on circulating gastrin levels is unknown. The physiological role of any or all of the above mentioned peptides in the regulation of gastrin release in sheep remains to be determined.

1.6.1.2 Acetylcholine

The secretion of acid and pepsin by the abomasum is stimulated by administration of acetylcholine or the cholinomimetic agent carbachol (Hill, 1968; McLeay and Titchen, 1975). Acetylcholine, which is released from intramural postganglionic parasympathetic fibres, is thought to act directly on parietal cells to stimulate acid production (see Sachs and Berglindh, 1981). This effect is blocked by atropine indicating that cholinergic receptors on parietal cells are muscarinic (Grossman, 1967; Berglindh, 1977; Soll, 1980). Acetylcholine may also have an indirect effect on acid secretion by parietal cells by potentiating the direct stimulatory effects of histamine or histamine and gastrin together (Soll, 1981).

Acid secretion by parietal cells may also be stimulated indirectly by acetylcholine-mediated gastrin release. In sheep, acid and pepsin secretion from pouches of the body region of the abomasum increase following perfusion of an antral pouch with acetylcholine or carbachol (McLeay and Titchen, 1977a), indicating that acetylcholine may stimulate release of gastrin from the antrum. However, the enhancement of gastrin release in dogs, following vagotomy, in response to food or intravenous infusion of bombesin (Schafmayer et al., 1978) is interpreted as indicating that there are also cholinergic pathways which inhibit gastrin secretion (see Walsh, 1981).

Footnote * GRP is thought to be a parasympathetic neurotransmitter which, in the case of the pig, stimulates antral gastrin output (Knutsen et al., 1984).

1.6.1.3 Histamine

Histamine, which occurs in ECL cells in the body region of the stomach in rats (Rubin and Schwartz, 1979a; Soll et al., 1981; Kubota et al., 1984) is also found in mast cells in the lamina propria of the GI tract in both man and dog (Soll et al., 1979). Systemic histamine stimulates gastric acid secretion in sheep (Hill, 1968; McLeay and Titchen, 1975) as well as in animals with a simpler form of stomach (Grossman, 1967).

Current evidence supports the hypothesis that parietal cells have separate receptors for histamine, gastrin and acetylcholine (see Konturek, 1980a). Histamine acts via H_2-receptors which can be blocked by H_2-receptor antagonists such as cimetidine and ranatidine (see Sachs and Berglindh, 1981; Soll, 1981). Several studies have indicated potentiating interactions between secretagogues. For example, the presence of histamine potentiates the actions of acetylcholine and gastrin when administered separately or in combination (see Soll, 1981). It has been suggested that potentiating interactions involving endogenous histamine and acetylcholine may be important in modulating parietal cell sensitivity to superimposed stimulation. However, in ruminants, no evidence is available on the relationship between endogenous histamine and abomasal secretory activity.

1.6.1.4 Bombesin

Bombesin, originally isolated from the skin of the frog Bombina bombina (Erspamer et al., 1972) also occurs in the CNS (Brown et al., 1978; Walsh et al., 1979), myentric and submucosal nerve plexuses (Dockray et al., 1979), and endocrine cells in the GI tract of mammals (Polak et al., 1976). It is structurally related to porcine GRP (McDonald et al., 1979), in that the minimal sequence of amino acids required for biological activity is the same for both peptides (Bloom et al., 1983).

Bombesin is a potent stimulus for gastric acid secretion in humans, dogs and cats, but not the rat (see Walsh, 1981). Its effect is

indirect, resulting from increased release of gastrin. While intravenous infusion of low doses of bombesin stimulates gastrin release (Bertaccini et al., 1974; Hirschowitz and Molina, 1983), high doses may inhibit acid secretion (Varner et al., 1980; 1981), probably as the result of somatostatin release (Hirschowitz and Molina, 1983). The stimulatory effects of bombesin on gastrin release are partially inhibited by antral acidification, abolished by antrectomy, and not affected by atropine (Impicciatore et al., 1974). In contrast, Bloom et al., (1983) have demonstrated that while intravenous infusion of GRP in calves resulted in increased plasma concentrations of pancreatic glucagon, PP, insulin and somatostatin, there was no change in plasma gastrin concentration.

1.6.1.5 pH

Although it is well established that lowering pH inhibits gastrin release and gastric acid secretion in dogs (Woodward et al., 1954), the mechanism(s) involved are not clearly understood. It is not known whether acid directly suppresses G cells or whether it releases an inhibitor of gastrin release from the antral mucosa. Konturek (1980a) has suggested that inhibition of gastrin release is a direct effect of acid on G cells.

Some workers have postulated that lowering of antral pH results in release of antral chalone which inhibits parietal cell secretory activity (see Grossman, 1974). Most current evidence, however, raises doubts about the existence of antral chalone (see Glass, 1980).

Considerable evidence, however, exists for the presence within the mucosa of the antrum in man and the antrum and duodenal bulb of the dog, of an as yet uncharacterized substance called gastrone (see Glass, 1980). When administered intravenously to dogs and rats, this glycopeptide inhibits gastric acid secretion (see Glass, 1980).

Somatostatin (see below), a potent inhibitor of gastrin release and gastric acid secretion (Konturek et al., 1976a), increases markedly in concentration in blood draining the stomach and pancreas of dogs

following antral or duodenal acidification (Schusdziarra et al., 1978). This suggests that somatostatin may play a role in regulation of gastric acid secretion, acting as a mediator in antral and duodenal inhibitory mechanisms (see Arimura et al., 1978).

1.6.1.6 Adrenaline

Catecholamine-containing fibres are found in the human abdominal vagus (Lundberg et al., 1976) and the adrenergic neurotransmitter released from postganglionic fibres of the sympathetic branch of the autonomic nervous system is noradrenaline (see Burnstock, 1981). There is evidence that both in man (Stadil and Rehfeld, 1973) and rat (Lundberg et al., 1976) beta-adrenergic mechanisms stimulate the release of gastrin. Ash (1961b) demonstrated that in sheep, exogenous adrenaline inhibits abomasal pouch secretion of acid in response to introduction of buffered VFA's into the abomasum. At present, however, it is not possible to conclude whether adrenergic mechanisms play a significant physiological role in regulation of gastrin release.

1.6.1.7 Somatostatin

Somatostatin, a cyclic tetradecapeptide originally isolated from ovine hypothalamic tissue (Brazeau et al., 1973; Burgus et al., 1973), has also been identified in other parts of the CNS (Hokfelt et al., 1978; Epelbaum et al., 1977), in the vagus nerve (Uvnas-Wallensten et al., 1978), myenteric plexus (Hokfelt et al., 1978), endocrine D cells in the pancreatic islets (Luft et al., 1974; Dubois, 1975) and throughout much of the mucosa of the GI tract (Polak et al., 1975b).

The major effect of exogenous somatostatin on gastric function is to inhibit acid and pepsin secretion stimulated by exogenous or endogenous gastrin, acetylcholine or histamine (see Konturek, 1980a, b; Walsh, 1981; Chew, 1983). Recent data (Chew, 1983) indicates that somatostatin acts directly on parietal cells to inhibit histamine activation of H^+ secretion. It also acts indirectly to inhibit the acid secretory response to gastrin, probably by blocking the gastrin-

stimulated release of histamine from paracrine or endocrine-like cells present in gastric mucosa (Chew, 1983). Somatostatin also inhibits the release of gastrin (Konturek et al., 1976a; Bolman et al., 1978).

The possibility that somatostatin is involved in regulation of gastric acid secretion via a paracrine pathway was suggested following the finding that some D cells, in rat gastric mucosa, have long cytoplasmic processes that terminate close to: (a) G cells in the antrum, and (b) parietal cells in the body region of the stomach (Larsson et al., 1979). Further evidence of a possible paracrine role for somatostatin in the regulation of gastric secretion was provided by Chiba et al. (1981), who demonstrated that although intravenous injection of antisomatostatin gamma-globulin, in rats, did not affect circulating gastrin levels, when rat antral mucosa was incubated with the antiserum, basal gastrin release increased. These results were interpreted as indicating that circulating somatostatin does not play a major role in the regulation of gastrin release, but that the effect of the peptide is largely at a local level (i.e. it has a paracrine effect).*

Recent evidence (Schubert et al., 1982; Schubert and Makhlouf, 1982; Saffouri et al., 1984) indicates that somatostatin release from rat stomach, is regulated by cholinergic (atropine-sensitive) inhibitory neurons and non-cholinergic (atropine-resistant) stimulatory neurons. The stimulatory neurotransmitter probably is bombesin or VIP. These peptides are present in gastric mucosal nerves (see Hokfelt et al., 1980) and both are known to stimulate somatostatin secretion (Chiba et al., 1980a,b; DuVal et al., 1981; Saffouri et al., 1981).

In animals with a simpler form of stomach, exogenous somatostatin has also been shown to inhibit the release of secretin (Boden et al., 1975; Konturek et al., 1976b), CCK (Konturek et al., 1976b), motilin (Mitznegg et al., 1977) and PP (Kayasseh et al., 1978), and to inhibit bicarbonate and enzyme secretion by the pancreas, and pepsin secretion by the stomach (see Konturek, 1980b).

Bell et al., (1981a,b) have postulated a role for somatostatin in the regulation of abomasal emptying and acid secretion in the calf.

Footnote * Further, paracrine release of somatostatin is probably the mediator of the inhibitory effect of criculating GIP on gastrin release (Holst et al., 1983).

Bell et al. (1981b) demonstrated that inhibition of gastric emptying following duodenal acidification coincided with high levels of circulating somatostatin and further, that the inhibition was maintained following sectioning of both the splanchnic nerves and vagi together (Bell et al., 1981a). These results indicate that the inhibitory process emanating from duodenal acid chemoreceptors is at least in part an endocrine mechanism. Somatostatin is the most likely mediator of this mechanism. However, the role of somatostatin in the regulation of GI tract secretory functions in the sheep, has not yet been investigated.

1.6.2 Regulation of Pancreatic Exocrine Function

Pancreatic secretion in sheep is continuous (Taylor, 1962) and fluid secretory rate relatively constant with only a slight increase in response to increased flow of digesta into the duodenum (Harrison and Hill, 1962). However, when abomasal contents were prevented from entering the duodenum, the rate of pancreatic secretion decreased rapidly to about 30% of the normal rate, suggesting that the frequent passage of digesta from the abomasum to the duodenum is a major factor in the regulation of the continuous flow of pancreatic juice (Taylor, 1962).

Duodenal pH is important in regulation of pancreatic secretion (Magee, 1961). Intraduodenal infusion of acid stimulates pancreatic secretion (Taylor, 1962; Caple and Heath, 1972; Reynolds and Heath, 1981), however, the response is small compared with that observed in dogs and pigs (Magee, 1965). In contrast, intraduodenal infusion of peptone solutions causes little or no change in pancreatic flow or output of enzymes (Reynolds and Heath, 1981). In sheep, intravenous administration of short-chain fatty acids, particularly butyrate, results in a rapid increase in flow of pancreatic juice and amylase and protein output (Harada and Kato, 1983). These workers concluded that short-chain fatty acids produced stimulatory effects by direct action on pancreatic acinar cells. Thus, VFA's resulting from fermentative digestion in the ruminoreticulum, when absorbed into the venous system may have a direct effect on the flow and composition of pancreatic juice.

Cholinergic fibres in the vagus nerve play an important part in regulation of flow rate and composition of pancreatic juice (Taylor, 1962; Caple and Heath, 1975). Vagal nerve stimulation results in a large (5 to 10 fold) increase in pancreatic enzyme output and a smaller (2 to 3 fold) increase in fluid and electrolyte secretion. The neurotransmitter involved is thought to be acetylcholine (see Gorelick and Jamieson, 1981; Reynolds and Heath, 1981). Recently, Pierzynowski and Barej (1984) have suggested that insulin potentiates the stimulatory action of the vagus nerve on pancreatic secretion.

Peptides which may play a role in the regulation of pancreatic secretion include secretin (Chey et al., 1984), CCK (You et al., 1983; Chey et al., 1984), VIP (see Chey, 1980; Holst et al., 1984), PP (Beglinger et al., 1984), somatostatin (see Walsh, 1981), glucagon (Nakajima and Magee, 1970), GRP (Bloom et al., 1983, 1984), PYY (Tatemoto, 1982; Szecowka et al., 1983b; Chen et al., 1984), PHI (Szecowka et al., 1983a) and motilin (Magee and Naruse, 1984b).

1.6.2.1 Secretin

Secretin, which is a linear peptide containing 27 amino acid residues (Mutt et al., 1970), is structurally similar to glucagon, VIP and GIP. Secretin-like activity has been extracted from the duodena of sheep (Taylor, 1962; Reynolds and McLeay, 1980) and the extracted peptide shown to stimulate pancreatic secretion in anaesthetized rats and to react with antibodies raised against porcine secretin (Reynolds and McLeay, 1980). The extract was distinguished from CCK activity by its failure to cause contraction of sheep gall bladder in vitro. In all species so far studied, secretin is confined in its distribution to the upper small intestine where it is synthesized, stored and released from S cells (Bussolati et al., 1971; Polak et al., 1971b).

The principal physiological action of secretin is to stimulate the pancreas to produce large volumes of bicarbonate rich secretion (Bayliss and Starling, 1902). When administered on its own, secretin has little effect on pancreatic enzyme secretion (see Mutt, 1980a), however, it may

potentiate the effects of endogenous or exogenous CCK on pancreatic enzyme secretion (see Grossman, 1973).

In sheep, although secretin infusion stimulates secretion of water and electrolytes from the pancreas, the effect is not as marked as the response of the liver (Caple and Heath, 1972). Indeed, the volume of bile and the quantity of bicarbonate secreted may be 3 - 5 times higher than for pancreatic juice. In addition, the responsiveness of the sheep pancreas to exogenous secretin is less than that observed in the dog. Thus, it is estimated that for comparable doses of secretin, the flow of pancreatic juice in the dog is 5 - 10 times more than in sheep, and that bicarbonate output is 6 times greater than from sheep pancreas (see Fawcett, 1970).

In animals with a simpler form of stomach, the main stimulus to endogenous secretin release is the presence of acid in the duodenum (Schaffalitzky de Muckadell and Fahrenkrug, 1978; Kim et al., 1979). A similar mechanism also exists in sheep, as Ho and Hansky (1979) reported a rapid rise in circulating immunoreactive secretin concentrations following instillation of acid into the duodena of anaesthetized sheep.

1.6.2.2 CCK

CCK was originally shown to have 39 or 33 amino acid residues (Mutt and Jorpes, 1971; Mutt, 1976). Since then, CCK-12, CCK-8 and CCK-4 have been isolated (see Rehfeld, 1981) and all these forms have been found in intestinal and brain tissue. CCK-like activity has been extracted from the mucosa of the duodenum of sheep (Ivy et al., 1929; Reynolds and McLeay, 1980), but the structure of ovine CCK has not been elucidated. However, CCK-8 has been isolated from sheep brain and shown to have the same amino acid sequence as porcine CCK-8 (Dockray et al., 1978).

The cells that synthesize and store CCK in the GI tract are the I cells of the upper small intestine (Polak et al., 1975a; Buffa et al., 1976).

Although CCK was originally discovered because of its action on the gall bladder (Ivy and Oldberg, 1928), several other actions were soon described (see Mutt, 1980). One of the main physiological effects of CCK is its action on pancreatic secretion (see Grossman, 1977).

Exogenous CCK is a strong stimulant of pancreatic enzyme secretion (Harada et al., 1982) and may also stimulate de novo synthesis of zymogens (see Mutt, 1980). It does not, however, stimulate secretion of all pancreatic enzymes (Nakajima et al., 1973).

CCK has also been shown, in rats (Heatley, 1968) and dogs (Debas and Grossman, 1973), to have a weak stimulatory effect on pancreatic secretion of water and bicarbonate. However, endogenous CCK greatly potentiates the effect of secretin in stimulating pancreatic secretion of water and bicarbonate (Meyer et al., 1971). Current evidence indicates that, in man and dog, interaction between CCK, secretin and cholinergic nerves is essential for the stimulation of pancreatic secretion during digestion (see Chey, 1980).

1.6.2.3 PP

This peptide which was isolated during purification of insulin from chickens (Kimmel et al., 1975) and insulin and glucagon from pigs and cattle (Chance et al., 1975), has also been isolated from pancreatic extracts of several mammalian species including sheep and cattle (Floyd et al., 1977). PP-producing cells, identified by immunohistochemical procedures, are found mainly in the pancreas, but have also been found in the antral region of the stomach of dogs (Nilsson et al., 1975) and rats (Sundler et al., 1977), and the small (Paulin and Dubois, 1978) and large (Buffa et al., 1978) intestines of humans.

PP both stimulates and inhibits pancreatic secretion in dogs (Lin et al., 1977). At very low doses it acts as a weak agonist on pancreatic water and electrolyte secretory mechanisms. More commonly, however, it inhibits pancreatic fluid, bicarbonate and enzyme output (see Lin, 1980). PP also inhibits pancreatic fluid, bicarbonate and enzyme secretion stimulated by secretin and CCK (Lin et al., 1977; Taylor et al., 1978).

The physiological function of PP in sheep, however, is yet to be determined.

1.6.2.4 Somatostatin

In both man (Hanssen et al., 1977) and dog (Konturek et al., 1976b) somatostatin is a potent inhibitor of pancreatic bicarbonate secretion stimulated by exogenous secretin or endogenous secretin released by duodenal acidification. Somatostatin also inhibits pancreatic enzyme secretion stimulated by CCK (Dollinger et al., 1976).

Evidence suggests that the inhibitory effects of somatostatin on pancreatic exocrine function may be due to a number of mechanisms, including:

(a) competitive inhibition of pancreatic secretin receptors (Konturek et al., 1976b),
(b) inhibition of the release of CCK from intestinal mucosa (Konturek et al., 1976b), or
(c) inhibition of the release of secretin from intestinal mucosa (Boden et al., 1975) although the dose of somatostatin used to elicit this effect probably was pharmacological.

Although other peptides have been shown to have inhibitory effects on pancreatic exocrine secretion, (see Chey, 1980) only PP and somatostatin appear to be involved in the physiological inhibition of pancreatic secretion.

1.6.3 Regulation of Bile Flow

In sheep (Heath et al., 1970), as in other mammalian species (Bergstrom and Danielsson, 1968), the rate of transport of bile salts by liver cells is an important regulator of the secretion of the bile acid dependent fraction of bile. However, bile acid independent secretion of the liver is probably largely under hormonal control (see Chey, 1980). Vagal nerve stimulation has little or no effect on bile flow in sheep

(Pass and Heath, 1976). Heath (1970) has suggested that secretin is an important physiological stimulus to bile secretion in sheep. Secretin stimulates bile flow and causes an increase in its bicarbonate concentration in several species (see Mutt, 1980) including sheep (Caple and Heath, 1972). Chloride concentration also increases but bile salt output, in dogs, is unaffected by secretin adminstration (Jones et al., 1971).

In animals with a simpler form of stomach, choleretic responses have also been observed following administration of CCK (Jones and Grossman, 1970; Gardiner and Small, 1976), gastrin (Gregory and Tracy, 1964; Zaterka and Grossman, 1966; Jones and Grossman, 1970), glucagon (Morris et al., 1967; Dyck and Janowitz, 1971; Jones et al., 1971) or VIP (Makhlouf et al., 1974; Thulin and Hellgren, 1976; Makhlouf et al., 1978). The potency of these peptides is always considerably less than for secretin and whether or not they have a physiological role in the regulation of bile flow and composition has yet to be established.

1.6.4 Regulation of Intestinal Secretion

Intestinal secretion is the net result of absorption and secretion that occur concurrently and continuously across the intestinal mucosa. The regulation of intestinal secretion involves endocrine, paracrine and neural factors (see Field, 1981).

The involvement of the nervous system in the regulation of intestinal secretion in sheep has not been elucidated. However, Hubel (1976) demonstrated that in dogs cholinergic agonists stimulate intestinal secretion and that the effect can be blocked with atropine. Such results suggest that the parasympathetic nervous system influences intestinal secretion.

Peptidergic nerves in the intramural plexuses may also have a role in the regulation of intestinal secretion. Possible neurotransmitters include VIP, substance P, bombesin, neurotensin, somatostatin, enkephalins and 5-HT. For a review of the distribution of these peptides in nerves and their effects on intestinal secretions, see Field (1981).

In sheep, peptidergic nerve fibres with immunoreactivity to VIP (Harrison and Wathuta, 1980), bombesin or substance P (Harrison and Wathuta, 1982a) have been located in the intramural plexuses of almost the entire GI tract. The physiological role that these or any other peptidergic nerves may play in the regulation of intestinal secretion in sheep is yet to be established.

The peptide GIP, which is found in K cells (Buchan et al., 1978) predominantly in the crypts of the duodenum and jejunum (Buffa et al., 1975; Bunnett and Harrison, 1979a), has been shown to stimulate intestinal secretion in dogs (Barbezat and Grossman, 1971; Barbezat, 1973). Exogenous GIP has also been shown to inhibit net water absorption in the intestine of man (Helman and Barbezat, 1976; 1977) and to induce secretion of chloride ions (Helman and Barbezat, 1977). However, more recently Barbezat (1981) has stated that in some cases the dose of GIP produced plasma concentrations outside the normal physiological range. Further, doses of GIP which produced plasma concentrations within the physiological range, had no effect on net movements of water and electrolytes in the small intestine thus casting doubt upon the role of GIP in intestinal secretion.

VIP, a peptide containing 28 amino acid residues with structural similarities to secretin, glucagon and GIP (Said, 1980), stimulates intestinal secretion. In dogs (Barbezat and Grossman, 1971) and humans (Krejs et al., 1980), infusion of VIP results in decreased absorption of water and sodium ions from the jejunum. However, the physiological role of VIP in regulation of intestinal secretion is as yet unknown. It has been suggested that the effects of VIP on net secretion of water and sodium ions in the small intestine are due to a generalized cardiovascular effect and a localized effect on release of acetylcholine (Mailman, 1978). Infusion of VIP results in decreased intestinal blood flow due to increased resistance to blood flow at the absorption site. These inhibitory effects of VIP are blocked by atropine (Mailman, 1978).

Although somatostatin does not appear to affect jejunal fluxes when given alone (Barbezat and Reasbeck, 1981), it is capable of inhibiting prostaglandin-stimulated secretion in rats (Dharmsathaphorn et al., 1980) and glucagon-stimulated secretion in dogs (Barbezat and Reasbeck, 1981).

Other peptides which have been shown to stimulate intestinal secretion in dogs include glucagon and pentagastrin (Barbezat, 1973), while in man similar responses have been noted for secretin (Hicks and Turnberg, 1972), CCK (Moritz et al., 1973) and glucagon (Hicks and Turnberg, 1974). However, in many instances the dosages of peptides used in these studies were in the pharmacological range. Recent evidence (Kirkegaard et al., 1984) demonstrates that secretin, at very low plasma concentrations, stimulates secretion of bicarbonate, protein and mucus from Brunner's glands of rats. Glucagon, however, was without effect. The physiological role of any of these peptides in regulation of ovine intestinal secretions is yet to be established.

1.7 Regulation of motility of the Ovine GI Tract

This section will briefly review the regulation of the motility of the ruminoreticulum, omasum, abomasum, intestines and gall bladder by neural and humoral factors.

1.7.1 Ruminoreticulum

Orderly, synchronized movements of the ruminoreticulum are required for the mixing of digesta, regurgitation of boli of digesta for rumination, eructation of rumen gases, and movement of digesta through the reticulo-omasal orifice. For reviews of ruminoreticular movements, eructation and rumination see Dougherty (1968); Stevens and Sellers (1968); Titchen (1968); Phillipson (1977); and Wyburn (1980).

1.7.1.1 Reflex Control

Ruminoreticular movements have been obtained in decerebrate preparations under conditions indicating the responses are reflex in nature (see Titchen, 1968). Evidence indicates the reflex centres are situated in the medulla oblongata. Reflex responses are evoked by stimulation of various parts of the GI tract and such reflexes

depend on the integrity of the vagi. Orderly sequential movements of the ruminoreticulum cease following section of the vagi (Duncan, 1953; Habel, 1956), although spontaneous reticular contractions may reappear (Phillipson, 1977).

Mechanical stimulation of the buccal (Borgatti and Matscher, 1958), oesophageal (Clark and Weiss, 1952; Kay and Phillipson, 1959; Sellers and Titchen, 1959), reticular (Titchen, 1958), ruminal (Ash and Kay, 1959; Reid, 1963), omasal (Titchen, 1958) and abomasal (Titchen, 1958) mucosa has been shown to increase the frequency of ruminoreticular contractions. Inhibition of ruminoreticular contractions has also been reported following severe distension of the oesophagus (Sellers and Titchen, 1959), reticulum (Reid, 1962), rumen (Dougherty, 1940), omasum (Schalk and Amadon, 1928), abomasum (Phillipson, 1939; Weiss, 1953; Titchen, 1958; 1960) and intestine (Phillipson and Ash, 1965).

Tension receptors involved in the reflex regulation of ruminoreticular motility have been demonstrated in various parts of the GI tract including: the medial wall of the reticulum around the reticular groove, the cranial sac of the rumen (Leek, 1969), the omasal canal and abomasum (Leek and Harding, 1975), and duodenum (Leek and Harding, 1975; Cottrell and Iggo, 1984a).

Mucosal receptors sensitive to both mechanical and chemical stimulation are found in the reticulum and cranial sac of the rumen (Iggo and Leek, 1970; Leek and Harding, 1975), abomasum and duodenum (Leek and Harding, 1975; Cottrell and Iggo, 1984b).

1.7.1.2 Hormonal Control

Several GI hormones or peptides have been shown to influence motility of the ruminoreticulum. Exogenous administration of pentagastrin (Carr et al., 1970; McLeay and Titchen, 1970b; Ruckebusch, 1971; Grovum and Chapman, 1982) inhibits ruminoreticular motility. Infusion of an impure preparation of secretin has been shown to reduce both the frequency and amplitude of rumen movements. More recently, Grovum (1981) demonstrated that intravenous infusion of secretin reduces the

frequency of reticular contractions but has no effect on their amplitude. In sheep, CCK has a marked inhibitory effect on the frequency of reticular contractions (Wilson et al., 1976; Grovum, 1981; 1982) and causes an increase in the strength of rumen contractions (Wilson et al., 1976). What role, if any, these hormones play in the regulation of ruminoreticular motility has not been established. Grovum (1981), however, has suggested that elevated plasma levels of gastrin and CCK may be responsible for the ruminal stasis frequently observed in intestinal parasitism (see Section 1.9).

1.7.2 Omasum

Omasal motility is regulated at least in part by reflex control mechanisms. This is demonstrated by:

(a) contractions of the omasal canal in the calf are stimulated following rapid emptying of the abomasum (Stevens et al., 1960),
(b) the rate of omasal contractions increases during feeding and rumination, (i.e. in periods of increased motility of the ruminoreticulum (Ohga et al., 1965)), and
(c) in the calf distension of either the ruminoreticulum or abomasum inhibits omasal movements (Stevens et al., 1960)

The role, however, that GI hormones or peptides may play in regulation of omasal motility is yet to be established. Ruckebusch (1971) has shown that exogenous pentagastrin stimulates the myoelectrical activity and motility of the omasum. The physiological significance of this observation is not clear.

1.7.3 Abomasum

Abomasal activity and emptying is largely regulated by the effects of digesta upon duodenal receptors sensitive to volume and chemical stimuli (see Bell, 1980).

Abomasal emptying is stimulated when digesta are diverted away from the duodenum and inhibited, temporarily, by the return of contents to the duodenum (Hogan and Phillipson, 1960).

Intraduodenal infusion of isotonic bicarbonate or sodium chloride stimulates abomasal emptying, while dilute HCl, KCl, $CaCl_2$ or hypertonic solutions of mono- and disaccharides inhibit emptying (see Bell, 1980). In sheep abomasal emptying is also inhibited by intraduodenal infusion of VFA's, particularly butyric acid (Bolton et al., 1976).

There is a parasympathetic component in the regulation of abomasal motility as shown by the changes observed following section of the vagi. Bell et al. (1977) demonstrated that in the calf, abomasal motility, particularly of the body region, ceases and emptying is impaired following bilateral cervical vagotomy. These workers found that motility and emptying resumes 7 - 29 days after vagotomy, probably as a result of re-establishment of intrinsic nervous control.

In ruminants several GI hormones or peptides have been shown to influence abomasal motility. Exogenous pentagastrin has been shown to inhibit the myoelectric activity of the abomasum of sheep (Ruckebusch, 1971). A similar response has also been demonstrated in calves (Bell et al., 1977). Secretin also has an inhibitory effect on abomasal motility in the calf (McLeay and Bell, 1980). In calves Bell et al. (1981b) demonstrated that intravenous injection of somatostatin causes a dose-dependent reduction in abomasal emptying and abolishes antral emg's for as long as plasma somatostatin levels remain above 200 pg/ml. This action of somatostatin may be of physiological importance as these workers demonstrated that intraduodenal infusion of acid caused plasma somatostatin levels to rise to an average of 286 $\pm$ 16 pg/ml and this concentration was maintained throughout the period of infusion. McLeay and Bell (1980) demonstrated that in the calf intravenous infusion of CCK reduces abomasal emg activity and inhibits abomasal emptying. It was earlier suggested (Bell and McLeay, 1978) that CCK is involved in the inhibition of abomasal emptying observed following intraduodenal infusion of fat. Such a mechanism could be of considerable importance to the suckling animal to ensure that milk protein and fat was retained in the abomasum until gastric digestion was complete.

1.7.4 Intestines

Little information is available on the neural regulation of intestinal motility in sheep. In species with a simpler form of stomach, parasympathetic stimulatory effects on both small and large intestine have been reported (for a recent review of control of intestinal motility see Roman and Gonella, 1981). Inhibitory effects of parasympathetic nerve stimulation have also been reported, the effect being mediated by intramural purinergic or peptidergic (i.e. non-cholinergic, non-adrenergic) neurons.

It is well established that the sympathetic innervation to both small and large intestine is noradrenergic and exerts a tonic inhibitory effect on intestinal motility (see Roman and Gonella, 1981).

Intrinsic neural regulation of intestinal motility is complex. In addition to intramural cholinergic and adrenergic fibres a large number of peptidergic fibres have been described (see Gabella, 1981; Wood, 1981). However, the role of such peptidergic nerves in sheep has yet to be established.

1.7.5 Gall Bladder

The gall bladder of sheep displays rhythmic patterns of contraction (Caple and Heath, 1971) similar to those observed in other animals (Ivy, 1934). The frequency and amplitude of contractions increase rapidly during feeding and decrease during fasting (Caple and Heath, 1971). Contraction of the gall bladder during feeding or duodenal infusion of acid is inhibited by intravenous injection of atropine, thus demonstrating a cholinergic involvement in regulation of motility of the gall bladder (see Caple and Heath, 1975). The principal factor regulating gall bladder motility and emptying, however, is the hormone CCK (Caple and Heath, 1971). Recent evidence (Magee and Naruse, 1984a) indicates that in animals with a simpler form of stomach the vagus nerve plays a more important role in the regulation of gall bladder emptying than had hitherto been thought.

1.8 Endocrine Cells of the GI Tract

This section will briefly outline the methods of demonstration and identification of GI endocrine cells, their general morphological characteristics, classification, origins and growth. Discussion of the distribution of GI endocrine cells is largely confined to studies on sheep.

1.8.1 Methods of Demonstration and Identification of Endocrine Cells

Techniques for demonstration and identification of endocrine cells fall into four main groups: histochemical, immunohistochemical, electron microscopy and autoradiography.

One of the earliest histochemical techniques used to demonstrate endocrine cells was the chromaffin method used to demonstrate the presence of chromaffin cells in the adrenal medulla and various abdominal paraganglia (see Lillie, 1965). Subsequently, this technique has been used to demonstrate enterochromaffin (EC) cells in a variety of tissues including the stomach (Heidenhain, 1870), intestine (Schmidt, 1905) and duodenum (Falck et al., 1959). This method generally involves fixation of tissue in formalin-dichromate mixtures or chromation in mixtures of $K_2Cr_2O_7$ and K_2CrO_4. Under appropriate conditions, EC cell granules yield a yellowish-brown colour, thought to be due to formation of a tetrahydro-4-carboline reaction product (Smuckler et al., 1960).

EC cells may also be demonstrated by the azo coupling reaction described by Cordier and Lison (1930). During fixation in formaldehyde or glutaraldehyde, 5-HT, which is contained in EC cell secretory granules, is converted into a β-carboline compound (Barter and Pearse, 1955). In alkaline conditions, this compound couples with a diazonium salt to yield an insoluble azo dye, the colour of which depends on the diazonium salt used (Lillie et al., 1961).

A variety of silver staining techniques have been employed to demonstrate argentaffin or argyrophil reactions in GI endocrine cells. Cells which demonstrate a positive argentaffin reaction possess the

ability to reduce silver salts in the dark, without the addition of any external reducing agent. In cells that contain 5-HT, it is thought that β-carboline is responsible for the reduction of silver salts to metallic silver (Dawson, 1978). It has also been suggested that the argentaffin reaction of formalin fixed tissues is due to some phenolic compounds in the granules (Thompson, 1966). The most commonly used argentaffin methods are the Masson-Hamperl (Hamperl,1927), Masson-Fontana (Pearse, 1972) and a modified Masson-Hamperl technique (Singh, 1964).

Cells which are argyrophilic may be impregnated with silver, but light or an external reducing agent is required to produce the black deposit of metallic silver (Nichols et al., 1974). The precise basis for this reaction has not been established, however, evidence presented by Vassallo et al. (1971a) suggests that in EC cells, 5-HT may be involved in the reaction and that in pancreatic A cells, glucagon is not involved. The most commonly employed argyrophilic techniques include those described by Grimelius (1968), variations of that technique (De Grandi, 1970), and the Sevier and Munger (1965) method.

Cells which are argentaffinic are also argyrophilic, but the converse is not necessarily true, thus argyrophilic cells are generally more numerous (Nichols et al., 1974). Because many GI endocrine cell types stain with silver techniques, (see Tables 2.1 and 2.2) these methods have been used for screening tissues for the presence of endocrine cells.

Immunohistochemical techniques for the demonstration of endocrine cells depend upon an immune reaction involving a specific antibody and the antigen to be identified. Firstly, an antibody, raised against the peptide to be identified, is applied to tissue sections containing the antigen under study. The subsequent steps in immunohistochemical techniques involve identification of the sites at which the binding of antibody and antigen have occurred. Two of the most commonly used immunohistochemical methods are the indirect peroxidase conjugate method (see Sternberger, 1979; Taylor, 1978) and the PAP method (see Sternberger, 1979; Taylor, 1978). A recent development in this field is the use of the streptavidin-biotin system (Bonnard et al., 1984).

In the indirect method, following application of a primary antiserum, a second antiserum is applied to the tissue section. This secondary antiserum, raised in a third species (i.e. not the species under study, nor the species in which the primary antiserum was produced), is directed against the immunoglobulin components of the primary antiserum and therefore will bind specifically to the antigen-primary antibody complex. In this technique, the secondary antiserum is conjugated with horseradish peroxidase. The location of bonded complex is then demonstrated using a suitable chromagen to stain the peroxidase. Diaminobenzidine (Novikoff and Goldfischer, 1969; Novikoff et al., 1971) or tetramethylbenzidine (Mesulam, 1978) are commonly used for this purpose.

The PAP technique differs from the indirect method described above, in that it uses three antisera: a primary antiserum with antibodies which bind with the antigen to be identified, a secondary antiserum which binds with the immunoglobulin components of both the primary and tertiary antisera, and a tertiary antiserum-complex. The antiserum in this complex is raised in the same species as the primary antiserum and contains antibodies to horseradish peroxidase which are then complexed with horseradish peroxidase antigen. The location of bound antigen-complex is revealed by use of a suitable chromagen such as used in the indirect method.

These techniques have been used extensively and have contributed greatly to the identification of endocrine-like cells and neurons producing biologically active peptides. The great advantage of these techniques is that they are generally specific. Despite this, hormone homology may interfere with this specificity. For example CCK and gastrin have identical C-terminal pentapeptide sequences (see Buchan et al., 1979; Rehfeld, 1981), thus a single antibody may bind with both CCK and gastrin. However, because the antigen binding site is small, averaging 3 to 8 amino acids in length (see Larsson, 1981), it is possible to raise antibodies against a portion of the molecule which is unique to either CCK or gastrin.* Use of such region specific antisera has contributed greatly to the identification of specific endocrine

* (Larsson and Rehfeld, 1977)

cell types, for example, the TG cells of the duodenum (Larsson and Rehfeld, 1979; Larsson et al., 1981).

Electron microscopic identification of GI endocrine cells has largely centred on differences in the ultrastructural characteristics of the peptide storage granules (Grube and Forssmann, 1979). Criteria for identification have included variations in granule size, shape, electron density and matrix structure. Within any species these characteristics are unique for each endocrine cell type, however, a universal GI endocrine cell classification system does not seem possible because of species variations in granule characteristics (Grube and Forssmann, 1979).

Electron microscopy has also been particularly valuable in locating the presence of multiple peptide products within a single cell. For example, rat pancreatic A cells exhibit immunoreactivity for an endorphin as well as pancreatic glucagon (see Grube and Aebert, 1981). Electron microscopy has demonstrated that both immunoreactivities are located within the same secretory granules (Grube et al., 1978a).

Electron microscopic autoradiography has been used, in particular, to identify pathways of amine synthesis in gastric endocrine cells (see Rubin and Schwartz, 1981). Using tritium labelled amine precursors, Rubin et al. (1971) demonstrated that all endocrine cell types found in the mouse stomach are able to take up amine precursors and decarboxylate them (i.e. they are members of the APUD series of cells: Pearse, 1969). In particular, Rubin and Schwartz (1979a) have shown that all rat gastric EC cells can readily synthesize appreciable amounts of 5-HT from tryptophan. These workers also demonstrated that in the rat, ECL cells can synthesize and store 5-HT, but the quantity involved is not normally enough to be detected by conventional argentaffin or amine-fluorescent techniques.

Organ culture studies, using rat stomach and tritiated histidine, followed by electron microscopic autoradiography has revealed that ECL cells in this species synthesize appreciable quantities of histamine

(Rubin and Schwartz, 1979b). This finding has not yet been confirmed in other species.

All the techniques outlined have provided valuable information concerning types, distribution, secretory products and possible functions of GI endocrine cells. In general, no one technique provides complete answers but rather the methods are seen as complementary. In this thesis, histochemical and immunohistochemical techniques have been used.

1.8.2 General Morphology

Endocrine cells in the GI tract are found scattered in the epithelium lining the stomach, intestinal crypts, glands of Brunner and intestinal villi. Typically the nuclei of these endocrine cells are round and vesicular in appearance, while the cytoplasm is refractory to most routine histological stains. This characteristic led Feyrter (1938; 1953) to describe them as 'clear' cells. However, some 'clear' cells have been shown to be agranular, poorly differentiated cells (see Solcia et al., 1975a) and thus are not endocrine cells.

Most endocrine cells lie directly on the basal lamina of the epithelium. In the cardiac and antral regions of the stomach and in the intestine, the majority of these cells reach the gut lumen and hence are called 'open' type endocrine cells (Fujita and Kobayashi, 1973). These cells are frequently pyramidal or teardrop in shape with the narrow pole of the cell reaching the gut lumen. The luminal surface is covered with microvilli which are considered to serve a sensory function (Solcia et al., 1967; Fujita and Kobayashi, 1978). The supranuclear cytoplasm of these cells contains coated vesicles, a centriole, Golgi complex and elongated cisternae (see Fujita and Kobayashi, 1977). Characteristically, these cells have secretory granules concentrated in the infranuclear portion of the cell. Thus 'open' type endocrine cells display a degree of cell polarity (see Solcia et al., 1981a).

In contrast, cells which lack luminal contact show little evidence of polarity (see Solcia et al., 1975a). Such endocrine cells, called 'closed' type by Fujita and Kobayashi (1973), are most common in the body region of the stomach in mammals with a simpler form of stomach (see Solcia et al., 1975a). Most 'closed' type endocrine cells lie directly on the basal lamina, are frequently oval or flattened in shape and have secretory granules scattered throughout their cytoplasm (see Solcia et al., 1981a).

A small proportion of endocrine cells are characterized by basal cytoplasmic processes (Larsson et al., 1979). These cytoplasmic processes have been observed passing between the basal lamina and the bases of neighbouring cells, to terminate close to other epithelial cells some distance from the endocrine cell (Larsson et al., 1979). Such cells were largely confined in distribution to the stomach where 'targets' for such processes included parietal cells and antral G cells. Both 'open' and 'closed' type endocrine cells with cytoplasmic processes have been identified but as yet such cells have not been found in the GI tract of sheep.

1.8.3 Classification of Endocrine Cells

Several attempts have been made to classify the endocrine cells of the human GI tract using results of histochemical, ultrastructural and immunohistochemical studies. The latest of these attempts, the Santa Monica (1980) classification (Solcia et al., 1981b), is shown in Table 1.1.

While such classifications have proved to be extremely useful, attempts to classify endocrine cells on a functional basis have been fraught with difficulties. For example, although it has been a widely held concept that each GI endocrine cell produces only one peptide product, there is mounting evidence that some may produce a number of secretory products. Examples include:

(a) EC cells which produce 5-HT (Barter and Pearse, 1953; Facer et al., 1979) may also contain substance P (Nilsson et al.,

Table 1.1 Santa Monica classification of human GEP endocrine cells, their distributions and secretory products (Solcia et al., 1981b)

Cell Type	Distribution						Product(s)
	Pancreas	Stomach		Small Intestine		Large Intestine	
		Oxyntic	Pyloric	Upper	Lower		
A (α cell)	+	+[ab]	-	-	-	-	Glucagon
B (β cell)	+	-	-	-	-	-	Insulin
D (δ cell)	+	+	+	+	+	+	Somatostatin
D_1	+	+	+	+	+	+	?
EC	+[a]	+	+	+	+	+	Serotonin + various peptides
ECL	-	+	-	-	-	-	? + histamine
G	+[ab]	-	+	+	-	-	Gastrin
I	-	-	-	+	+	-	CCK
IG	-	-	-	+	+	-	Gastrin
K	-	-	-	+	+	-	GIP
L	-	-	-	+	+	+	Glucagon-like immunoreactivity
Mo	-	-	-	+	+	-	Motilin
N	-	-	-	-	+	-	Neurotensin
P	+[b]	+	+	+	-	-	?
PP	+	-	+[a]	-	-	-	Pancreatic polypeptide
S	-	-	-	+	+	-	Secretin
TG	-	-	+[a]	+	+[a]	-	C-terminal gastrin immunoreactivity
X	-	+	+[a]	-	-	-	?

a found in other animals; only exceptionally in man

b found in foetus or newborn; only exceptionally in adults

\+ cells present

\- cells absent

1975) and leu-enkephalin (Alumets et al., 1978).

(b) Antral G cells which produce gastrin also demonstrate immunoreactivity for met-enkephalin (Polak et al., 1977; Larsson and Stengaard-Pedersen, 1981), β-endorphin (Forssmann et al., 1977; Larsson and Stengaard-Pedersen, 1981; Polak et al., 1978) and growth hormone (Hakanson et al., 1978).

(c) Pancreatic A cells demonstrate CCK (Grube et al., 1978a) and endorphin (Grube et al., 1978b) immunoreactivity as well as immunoreactivity to glucagon.

Some of these multiple specificities may be due to nonspecific binding of antisera in immunohistochemical techniques (Grube and Aebert, 1981). Support for such an explanation was provided by Buffa et al. (1979a, b) who demonstrated that their growth hormone antiserum reacted with G, A and D cells in a non antigen-antibody mechanism. This reaction was mediated by the C_{1q} fraction of complement. How extensive such non-immune reactions are is not known.

Larsson and Jorgensen (1978) have demonstrated multiple secretory products in some endocrine cells in human and rat foetuses. These workers, using region specific antisera, found that some duodenal G cells contain both gastrin and CCK. In addition, S cells, 25 - 40% of duodenal G cells, and up to 65% of I cells contain 5-HT. Such findings led Larsson and Jorgensen (1978) to suggest some endocrine cells of the foetal duodenum are not fully differentiated and are able to synthesize and store several different biologically active products.

An additional difficulty is that for some cell types which are well characterized morphologically, no peptide secretory product has yet been identified. For example, ECL cells have been identified in ultrastructural studies and have been shown, in rats, to synthesize histamine (Rubin and Schwartz, 1979a; Soll et al., 1981; Rubin and Schwartz, 1981; Kubota et al., 1984). However, no peptide secretory product has yet been identified.

Finally, considerable interspecies variations in both ultrastructural appearance and distribution of some GI endocrine cells may preclude the establishment of an acceptable universal classification (see Grube and Forssmann, 1979; Solcia et al., 1981b).

To date, workers describing endocrine cells in the GI tract of sheep have classified such cells on the basis of systems similar to that shown in Table 1.1. A similar classification will be followed in this thesis.

1.8.4 Distribution of Endocrine Cells and Hormones

The distribution of endocrine cells producing, storing and releasing gastrin, secretin, CCK, PP, GIP, motilin, enteroglucagon, somatostatin or neurotensin is shown in Table 1.1 (Solcia et al., 1981b). A more detailed study of the gastric mucosa, with some references to species differences, was presented by Solcia et al. (1975a).

In species with a simpler form of stomach, including man, rat, cat, dog and baboon, the regions of the gut with highest total numbers of endocrine cells are the stomach and duodenum, and thereafter, numbers decrease distally along the intestines (Bloom and Polak, 1978). Some endocrine cell types are widely distributed throughout the GI tract (e.g. EC cells; Sjolund et al., 1983) while others are limited in distribution (e.g. ECL cells are limited to the body region of the stomach; see Table 1.1). Most endocrine cells are found in the middle and/or lower thirds of the thickness of the mucosa.

Little work has been published on the distribution of endocrine cells in the GI tract of the sheep. Tehver (1930) demonstrated argentaffin cells in the abomasum where they are common in the body region, but rare in the antrum. These findings were confirmed by Sommerville (1956). Tehver (1930) also found argentaffin cells in the crypts of Lieberkuhn in the duodenum.

Endocrine cells showing immunoreactivity to a porcine gastrin/CCK antiserum have been found in the ovine GI tract (Bunnett and Harrison 1979 a, b; Bunnett, 1984). These immunoreactive cells are most numerous in the abomasal antrum, but also occur in the villi and crypts of the upper small intestine, and a few are found in the submucosal glands of Brunner. In the abomasal antral mucosa these cells are characteristically pyramidal in shape, of the 'open' type and occur throughout the entire mucosal thickness. These workers concluded that such antral cells were probably exclusively G cells as I cells have not been found in the stomachs of other mammalian species (see Table 1.1). Recent electron microscopic studies (Bunnett and Harrison, 1981a) reveal that antral G cells have microvilli on their apical borders and the secretory granules are generally basally located, about 180 nm in diameter and have an electron dense core. This morphological description is similar to that of human antral G cells (Lechago and Weinstein, 1978). Because their gastrin antiserum also cross-reacts with CCK, Bunnett and Harrison (1979a) suggested that the immunoreactive cells they found in the small intestine represent a mixture of G and I cells.

In sheep, GIP immunoreactive cells occur throughout the small intestine, but are most numerous in the duodenum (Bunnett and Harrison, 1979a). Typically these cells are teardrop in shape, of the 'open' type and localized in the deeper parts of the crypts of Lieberkuhn. These workers also extracted a peptide from sheep small intestine which, when infused into sheep or rats, inhibits pentagastrin stimulated gastric acid secretion (Bunnett and Harrison, 1981b). Because this peptide extract, which may contain GIP, does not cause contraction of isolated sheep gall bladder, it was concluded that the extract does not contain CCK.

Glucagon-like immunoreactive material is present in mucosal extracts of the ovine GI tract (Bassett and Madill, 1978). Highest concentrations of such material occur in the distal jejunum and ileum, while small or undetectable quantities are found in the abomasum, duodenum and proximal jejunum.

Bunnett (1980) using an antiserum raised against pancreatic glucagon, demonstrated numerous glucagon immunoreactive cells in the

distal small intestine and pancreata of sheep. Similar cells also occur in the mucosa of the abomasum, proximal small intestine and large intestine, but are absent from the forestomach. The distribution of these endocrine cells is similar to the pattern of distribution of glucagon like immunoreactive material in lambs as reported by Bassett and Madill (1978). This suggests that the material extracted from the mucosa by Bassett and Madill (1978) was the same material that Bunnett (1980) located in endocrine cells.

Immunocytochemical techniques have been used to demonstrate the presence of substance P-like and bombesin-like reactivity in the ovine GI tract (Harrison and Wathuta, 1982a). Substance P-like immunoreactivity is found in 'open' type endocrine cells in both the small and large intestines, and intramural nerve fibres. Bombesin-like activity, however, is confined to nerve fibres and nerve cell bodies in the GI tract (Harrison and Wathuta, 1982a).

VIP immunoreactivity in sheep (Harrison and Wathuta, 1980), as in other species (Bishop et al., 1980), is confined to nerve fibres and does not occur in any discrete endocrine cells in any region of the GI tract.

5-HT, a product of EC cells (Dey and Hoffpauir, 1984), is found in mucosal extracts of the abomasum, small and large intestines, but is absent from the forestomach of sheep (Faustini, 1955). This pattern is similar to the distribution of EC cells, demonstrated using histochemical techniques, in both sheep and cattle (Rizzotti et al., 1976; Rizzotti and Domeneghini, 1976). Rizzotti and co-workers found that EC cells are most numerous in abomasal gastric glands, the duodenum and rectum.

1.8.5 Origin and Development of Endocrine Cells

Although Pearse (1981) has presented evidence to suggest that GI endocrine cells arise from the neuroectoderm, their origin remains controversial (Andrew et al., 1983). Pearse (1981) claims that the presence of the enzyme neuronal specific enolase in neurones and APUD

cells (Schmechel et al., 1978), but not other cell types, indicates that APUD cells and neurones have a common embryological origin. In contrast, Andrew (1982) suggests that the presence of neuronal specific enolase in neurones and APUD cells may merely indicate a similarity in metabolism rather than a common embryonic origin. Alternatively, Andrew (1982) claims that any common source of APUD cells is "too far back in development to be of any significance". Evidence from experiments using a "biological marker" system has been interpreted as supporting the hypothesis that GI endocrine cells are derived from endoderm (see Le Douarin, 1978). Thus the debate on the origin of GI endocrine cells remains unresolved.

There has also been debate about the ability of GI endocrine cells to divide postnatally. Chang and Leblond (1971) were unable to demonstrate mitotic figures or ^{3}H-thymidine labelling of argentaffin cells in mouse colon and therefore concluded that these cells did not have the capacity to divide. In contrast, Cheng and Leblond (1974), using the electron microscope, observed endocrine cells in the mouse small intestine which were undergoing mitosis. These cells were located at the crypt base and resembled neighbouring columnar cells from which they were thought to arise. As these endocrine like cells acquired more basal granules they lost the ability to divide. They then migrated up the wall of the crypts towards the extrusion zone of the villus. Lehy and Willems (1976) demonstrated that antral G cells of mice were capable of cell division, but were not able to show that new G cells arose exclusively by this means.

Endocrine cell turnover times have been estimated from kinetic studies of the incorporation of ^{3}H-thymidine. In the mouse, turnover times vary from region to region; being in the duodenum and jejunum, 3.9 - 4.0 days (Ferreira and Leblond, 1971; Cheng and Leblond, 1974), in the descending colon 23.3 days (Tsubouchi and Leblond, 1979), and for antral G cells as long as 2 - 4 months (Lehy and Willems, 1976). Kinetic studies of turnover rates of GI endocrine cells have not been reported for sheep.

Studies of changes in serum hormone levels may be a useful indication of GI endocrine cell development. For example, Shulkes and co-workers

(Shulkes et al., 1981a, b; Shulkes and Hardy, 1982) demonstrated the development of hypergastrinaemia in sheep foetuses from 15 - 20 days prior to parturition and which continued for up to 14 days after birth. Compared with adult values (21 ± 5 pmol/ℓ for nonpregnant ewes), foetal plasma gastrin levels are low at 101 - 105 days (7 ± 1 pmol/ℓ), however, from 130 days gestation foetal plasma gastrin levels are greater than adult values, rising to a peak of 47 ± 5 pmol/ℓ at 141 - 145 days. There is a sharp rise in plasma gastrin levels in the immediate postpartum period (90 ± 13 mol/ℓ at 1-5 days) falling to 38 ± 5 pmol/ℓ at 66 - 10 days postpartum. Similar results have been reported by Lichtenberger et al. (1981). Shulkes et al., (1982) demonstrated that gastrin found in foetal plasma was of foetal origin and that the hypergastrinaemia observed did not result from a failure of metabolic clearance of gastrin.

Although Lichtenberger et al. (1981) were able to detect gastrin in serum at 107 days gestation, they were unable to locate immunoreactive G cells in the abomasum until 120 - 130 days gestation. These workers reported an increase in the number of G cells throughout the remainder of pregnancy and the first 2 weeks postpartum. This increase is due largely to an elongation of the antral glands, hence total G cell numbers, rather than any change in G cell density. The increase in antral G cell numbers closely parallels the rise in serum gastrin levels. These workers did not, however, report any observations on the development of intestinal G cells.

The apparent discrepancy between the ages at which serum gastrin and antral G cells are first detected, may be due to two factors. Firstly, RIA and immunocytochemical methods are of different sensitivities and secondly, the serum gastrin detected in 107 day old foetuses may have been derived from intestinal G cells and not antral G cells.

Although the development of other endocrine cell types has not been studied in sheep, the presence of PP and GLI has been reported in sheep foetuses. Measurable concentrations of PP are found in foetal plasma at 101 days gestation but values are only about 4% of adult values (Shulkes and Hardy, 1982). These workers found that concentrations remain low

until the week before birth when there is a sharp increase to about 30% of adult levels. Then, in the first week postpartum plasma PP rises to 221 ± 38 pmol/ℓ, which is within the normal range for adult sheep. They suggested that foetal plasma PP is of foetal origin and probably secreted by pancreatic PP cells. Although PP cells have been reported to occur in GI tract mucosa (see Lin, 1980), particularly in the antrum, they have not been identified in the ruminant.

GLI, measured by RIA, is found in the intestinal mucosa of foetal lambs from 55 days of gestation (Bassett & Madill, 1978). Highest concentrations were detected in the terminal ileum with lesser concentrations in the abomasum and proximal small intestine. These workers found that the concentration of GLI in the distal one third of the small intestine increased from 55 days to term, when levels are comparable to those found in adult sheep. The endocrine cell responsible for the synthesis of GLI in the ovine foetus remains to be identified.

It is clear from this discussion that little information has been published on the endocrine cell types found in GI mucosa of the foetal lamb or on the manner in which such cells develop. The functions of GI hormones in the ovine foetus also remain to be elucidated.

1.9 Pathophysiological Effects of Parasites in the GI Tact

This review is largely confined to the effects of the nematode Trichostrongylus columbriformis on GI physiology in sheep. One other parasite, Ostertagia circumcincta, will also be discussed because of its profound effect on abomasal secretion in sheep.

Recently, evidence has been obtained indicating GI parasites cause severe perturbations in the normal mechanisms regulating secretion of GI hormones (see Titchen, 1982). Thus some of the clinical effects of parasitic infestation may be due to disturbances of the GI endocrine system. Such disturbances may be due to effects on the synthesis and/or release of hormone, number and/or distribution of endocrine cells, or alteration of the sensitivity of target cells. Possible mechanisms by which intestinal parasites may cause such effects remain largely unexplored.

Trichostrongylus colubriformis

This intestinal nematode, commonly found in domestic ruminants, inhabits the upper small intestine where they are characteristically found in 'tunnels' in the epithelium (Barker, 1973).

Infection may cause occasional diarrhoea, inappetence, weight loss and emaciation accompanied by decreased protein digestion, poor utilization of calcium and phosphorus, anaemia, hypoproteinaemia and hypoalbuminaemia; death may follow many months after the initial infection (Horak et al., 1968).

In sheep depression of feed intake is common following the establishment of a T. colubriformis infection, the degree of depression being related to the severity of the infestation. Symons and Hennessy (1981) suggested that the effect of T. colubriformis infection on feed intake was due to an increase in plasma CCK levels. Using a bioassay, these workers demonstrated the presence in plasma of a factor they concluded was CCK, the concentration of which was elevated in more heavily infected animals. In addition, they demonstrated that intravenous infusion of CCK octapeptide depresses feed intake in sheep. Other workers have also proposed that CCK operates as a satiety factor (see Mutt, 1980b). Evidence for such a role in sheep includes the finding that cerebral ventricular injection of CCK inhibits feeding (Della-Fera and Baile, 1979), while prolonged administration of an antiserum to CCK into the lateral ventricles results in increased food intake (Della-Fera et al., 1981). Grovum (1981) has shown that intravenous administration of CCK, as well as pentagastrin and secretin, inhibits feed intake. However, since the doses of CCK and pentagastrin required to achieve this effect were high, the role CCK plays in the physiological regulation of feed intake of normal sheep remains to be determined. In parasitized animals, however, elevated plasma CCK levels may account for the reduced feed intakes.

Although reduced feed intake is an important contributor to impaired production, there are components of impaired growth which cannot be explained on this basis. Part of the reduction in efficiency of feed conversion is due to increased rates of blood protein turnover

(see Dargie, 1980) resulting from the loss of up to 220 ml of plasma per day into the GI tract (Barker, 1973; Yakoob et al., 1983). Intestinal parasitism also results in impaired protein deposition (Sykes and Coop, 1976), due largely to an increase in urinary nitrogen excretion (Roseby, 1973), and an increase in urea synthesis accompanied by an increased and irreversible loss of urea nitrogen (Roseby and Leng, 1974).

Mucosal morphology of both the upper small intestine (Barker, 1973; 1974) and abomasum (Barker and Titchen, 1982) are altered during infection with T. colubriformis. The predominant duodenal mucosal histopathological feature of infected lambs is villus atrophy (Barker, 1974), characterized by shortening of the villi and the presence of long, straight, often dilated crypts containing an abnormally large number of cells with mitotic figures. Many epithelial cells are of a rounded, low columnar type rather than the normal columnar type (Barker, 1975a). Small erosions of the epithelial surface are occasionally observed while eosinophils and polymorphonuclear cells are seen in the gut lumen. The lamina propria is heavily infiltrated by mononuclear cells, particularly plasma cells, and in some areas neutrophils are observed in the subepithelial connective tissue (Barker, 1975a).

Barker (1974) also described a more extreme form of villus atrophy characterized by a complete absence of villi in the upper small intestine. Crypt openings project into the lumen and are surrounded by a ring of epithelial cells. Surface epithelial cells are frequently cuboidal in shape and the epithelium may have many discontinuities through which eosinophils and neutrophils leak into the gut lumen. Sometimes the entire surface between crypt openings is devoid of epithelial cells, the boundary between lumen and mucosa being a layer of connective tissue.

Barker (1975b) has demonstrated increased permeability of capillaries and venules in the small intestinal lamina propria of infected sheep. Increased permeability is limited to infected portions of the GI tract. The severity of the leakage is related to the worm density within the upper small intestine. It was suggested that leakage resulted from the development of transient or discontinuous gaps in endothelial junctions.

The only gross changes observed in the abomasa of infected sheep were occasional areas of reddening on rugae (Barker and Titchen, 1982). These workers observed histological changes in the mucosa of the body region of the abomasum of infected sheep, particularly in those animals in which abomasal pH was elevated. In those animals many parietal cells appeared poorly differentiated and EM studies indicated that these cells did not secrete acid. In severely affected animals, mucous neck cells extended into the depths of gastric glands thereby possibly reducing parietal cell mass. The abomasal mucosa of infected animals remained substantially intact although small erosions occurred at the edges of the rugae. A moderate infiltration of the lamina propria by mononuclear inflammatory cells was frequently seen in infected sheep. T. colubriformis worms were only rarely seen in epithelial tunnels in the abomasal antrum (Barker and Titchen, 1982).

Abomasal secretion of acid is inhibited by infection with T. colubriformis (Barker and Titchen, 1982). These workers reported a reduction in volume and concentration of acid secreted, such that more than half of the infected animals failed to maintain abomasal pH at the same level as pair-fed controls or controls fed ad libitum. Evidence suggests that the mechanism of acid secretion inhibition is an indirect one (i.e. the parasites do not have a direct inhibitory effect on parietal cells). Firstly, very few of these parasitic worms become established in the abomasum (Barker, 1974) and those which do are found mainly in the antral mucosa. Secondly, profound inhibition of acid secretion is also observed in animals in which the parasites are confined to the small intestine (see Titchen, 1982). The mechanism by which T. colubriformis infestations inhibit acid secretion has yet to be established. Inhibition of gastrin release from TG or IG cells in the duodenal mucosa does not seem a likely explanation as plasma gastrin levels do not change significantly following infection (Titchen and Anderson, 1977). Barker and Titchen (1982) suggested, rather, that the inhibition was due to the release from the parasitized small intestine of a systemically acting gastric inhibitory factor, the identity of which is as yet unknown. While somatostatin is a possible candidate, particularly because of its established role in the inhibition of acid secretion, no information is currently available on the effect of T. colubriformis infection on circulating levels of this hormone.

Infestation of the abomasum by Ostertagia circumcincta causes a gastritis accompanied by reduced acid secretion (McLeay et al., 1973). The parietal cells of the abomasa of sheep exposed to infection for 24 days display histological characteristics of inactive (i.e. non-secreting) parietal cells. McLeay et al. (1973) postulated that a factor released from the parasites or from the parasitized mucosa, directly inhibits the parietal cells.

Ostertagiasis is accompanied by a profound hypergastrinaemia (Anderson et al., 1976b, 1981). Serum gastrin levels increase within eight days of oral administration of larvae and reach a peak, in the seventh week of infection, of 14 - 65 times that in control animals (Reynolds et al., 1979a). These workers found that the change in circulating gastrin concentration was directly related to the severity of the infections. It is clear that the hypergastrinaemia observed in ostertagiasis results from an increase in gastrin secreted by the abomasal antrum (see Titchen, 1982). In the more severe infections, abomasal mucosal gastrin concentrations were also significantly higher than in control animals, while duodenal mucosal gastrin concentrations were depressed.

Secretion of both acid and pepsin from non-infected abomasal pouches increases markedly in animals in which the rest of the abomasal mucosa is exposed to O. circumcincta (McLeay et al., 1973; Anderson et al., 1976a). These effects are due to an increase in circulating levels of gastrin (Anderson et al., 1976b; 1981) and are abolished by antrectomy (Anderson et al., 1981) which removes the main source of endogenous gastrin. The involvement, if any, of hypergastrinaemia in the development of this syndrome is obscure.

Although there have been a few direct measurements of changes in plasma hormone and tissue hormone levels during parasitic GI infections, the mechanism whereby this occurs has not been elucidated. No information has been published on changes in density, distribution or secretory activity of GI endocrine cells of the sheep during intestinal parasitic infection.

1.10 Aims of the Experiments Described in this Thesis

The experiments described herein were designed to:

(a) investigate the suitability of a range of histochemical and immunohistochemical techniques in demonstrating endocrine cells in the GI tract of sheep,

(b) identify specific endocrine cell types in the gut mucosa,

(c) determine the distribution of endocrine cells throughout the GI tract and establish specific endocrine cell densities in the ruminoreticulum, abomasum, duodenum, ileum, large intestines and pancreas,

(d) investigate age related changes in both endocrine cell distributions and densities in animals ranging in age from 100 - 110 day old foetuses to mature adults, and

(e) study the possible effects of a *T. colubriformis* infection on the distribution patterns and densities of endocrine cells in order to determine whether patho-physiological effects of infection might be mediated by disturbances in the GI endocrine system.

CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Ages, weights, sexes and diets for the Romney-cross sheep used in these investigations are given in Table 2.1. Specific details of the treatment of animals before the collection of tisues are given in the appropriate chapters of this thesis.

Food, but not water, was withheld from all animals for approximately 24 hours before tissue sampling.

2.2 Collection of Tissue Samples

Regions of the GI tract from which samples were collected for studies described in Chapter 3 and 6 are listed in Table 2.2, while those sampled for experiments described in Chapters 4, 5 and 7 are given in Sections 4.2, 5.2 and 7.2, respectively. The tissue samples were collected after the animals were anaesthetized with intravenous pentobarbitone sodium ('Nembutal', Abbott Laboratories Pty Ltd., Australia: 30 mg/kg body weight), or following exsanguination after stunning with a percussion gun (see Table 2.1).

For anaesthetized animals, samples were collected *via* a laparotomy incision while the viscera remained *in situ*. Special care was taken to minimize handling of tissues and to keep them free of contaminants (blood and digesta). Exposed viscera were kept warm and moist with 0.9% saline (37°C) during the period of collection.

For the exsanguinated animals, the samples were collected after the viscera had been removed. This method of collecting samples proved to be rapid and obviated the use of an anaesthetic agent. Care was taken to ensure that sites sampled corresponded as closely as possible to those of the anaesthetized animals.

Table 2.1: Sexes, body weights, ages and diets of animals and sample collection methods used in the studies described in this thesis

Animal number	Sex[1]	Body weight (kg)	Age	Diet	Collection[2] method	Chapter
1	E	40.5	4 yrs	pasture	S	3
2	W	38.5	5 "	"	S	"
3	E	33.3	3 "	"	A	"
4	E	36.3	3 "	"	A	"
5	E	-	Foetus	-	A	4
6	E	-	"	-	A	"
7*	E	-	"	-	A	"
8	R	-	"	-	A	"
9*	R	-	"	-	A	"
10	E	6.8	2 wks	milk	A	5
11	E	7.3	"	"	A	"
12	R	7.3	"	"	A	"
13	R	8.3	"	"	A	"
14	E	4.0	"	"	A	"
15	R	28.5	24 wks	pasture	A	6
16	W	26.0	"	"	A	"
17	E	28.5	"	"	A	"
18	E	22.8	"	"	A	"
19	W	20.5	"	"	A	"
20	R	20.5	"	"	A	"
21	R	15.5	"	"	A	"
22	R	34.0	40 wks	lucerne nuts and chaff	S	7
23	E	34.5	"	"	S	"
24	E	28.8	"	"	S	"
25	E	32.0	"	"	S	"
26	R	32.5	"	"	S	"
27	E	26.8	"	"	S	"
28	E	28.3	"	"	S	"
29	R	33.0	"	"	S	"

* Twins

1. E : ewe, R : ram, W : wether
2. S : stunned and exsanguinated, A : anaesthetized
   foetuses were not weighed

Table 2.2: Regions of the digestive system from which tissues were collected (Chapters 3 and 6)

Region	Site	Abbreviation
Reticulum	fundus	Re
Rumen	ventral sac	Ru
Abomasum	body : 20 - 22 cm proximal to pylorus	Ab_b
Abomasum	antrum : 1.5 - 2.5 cm proximal to pylorus	Ab_a
Duodenum	proximal : 2 - 4 cm distal to pylorus	Duo_p
Duodenum	middle : 12 - 14 cm distal to pylorus	Duo_m
Duodenum	distal : 30 - 32 cm distal to pylorus	Duo_d
Ileum	10 - 12 cm proximal to ileocaecal junction	Il
Colon	15 - 20 cm distal to ileocaecal junction	Co
Caecum	2 - 4 cm from end of blind sac	Cae
Pancreas	head	Pan

Samples of tissues from the first two animals (Nos. 1 and 2) were longitudinal sections. For all subsequent animals only samples of reticulum, rumen and abomasal body were longitudinal sections, all other samples (abomasal antrum, small and large intestines) were cross-sections.

At the completion of sampling the anaesthetized animals were killed with pentobarbitone sodium ('Pentobarb 300', South Island Chemicals Ltd., Christchurch).

2.3 Sample Preparation

Tissue samples were trimmed of extraneous tissues and, where easily and quickly accomplished, the muscularis externa removed. Each sample was then divided into three parts approximately 0.5 cm wide. Cross-sectional rings of intestine were opened longitudinally and formed into a 'Swiss roll' by winding round an orange stick. Each part was then placed on a separate piece of light card, the orange stick removed, and the card plus tissue immersed in one of three fixatives (see Section 2.5). In the case of intestinal samples from foetuses and two week old lambs, 'Swiss roll' preparations were not made as cross-sectional samples were sufficiently small to be placed, intact, on card before fixation. 'Swiss roll' preparations of reticulum and ventral rumen were found to be unsatisfactory because of contraction of the muscularis externa. Thus these samples were simply immersed in fixative.

2.4 Chemicals and Stains

All chemicals and stains used in histological processing and staining are listed in Appendix 1.

2.5 Fixation and Processing

A sample from each site was divided into three and each part fixed by one of the following methods, for which fixatives and fixation times used were:

(a) Bouin's fluid (Culling, 1974), tissue immersed for up to 24 hrs,

(b) Zenker-formol fixative (Culling, 1974), tissue immersed for up to 24 hrs,

(c) 6% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 (Solcia et al., 1968), tissue immersed for 24 hrs.

After 1 - 2 hrs fixation the samples were removed from their cards and returned to the fixative. Following fixation, samples fixed in Zenker-formol or glutaraldehyde were washed in tap water for 6 - 8 hrs. After fixation all samples were stored in 70% ethyl alcohol until processed.

Most histochemical and all immunohistochemical staining techniques were applied to tissue samples fixed in Bouin's fluid. Exceptions were the chromaffin techniques (applied to Zenker-formol fixed tissues) and the xanthydrol and diazonium techniques (applied to glutaraldehyde fixed tissues).

An automatic tissue processor (Shandon Elliott, Liverpool, U.K.) was used to dehydrate, clear and wax impregnate the tissues according to the schedule shown in Table 2.3. The paraffin wax blocks were trimmed and mounted on small wooden chucks. Sections 5 - 6 µm thick were cut using a Reichert (Vienna, Austria) sliding microtome, floated on warm water, transferred to albuminized 3" x 1" glass slides and air dried at 60°C overnight.

Before staining, all sections were dewaxed and 'brought to water' by immersing them in xylene (2 baths of 5 minutes each) followed by brief immersion in absolute ethyl alcohol, 70% ethyl alcohol, then tap water.

2.6 Histochemical Staining Techniques

The first requirement of this project was to evaluate stains, selected on the basis of their use in species with a simpler form of stomach, for their suitability to stain endocrine cells in the ovine GI

Table 2.3 Schedule for dehydration, clearing and impregnation of tissues using a Shandon Elliott automatic tissue processor (Birtles, 1981).

Process	Reagent	Time
Dehydration	70% ethyl alcohol	1 hr
	95% " "	"
	Change 1. 100% " "	"
	" 2. 100% " "	"
	" 3. 100% " "	"
	" 4. 100% " "	2 hrs
	Chloroform	1 hr
Clearing	Change 1. Xylene	"
	" 2. Xylene	
Impregnation	Change 1. Paraffin wax m.p. 56°C	2 hrs
	" 2. " " " "	"

tract. Lists of endocrine cell types and their staining reaction with commonly used histochemical staining methods are given in Tables 2.4 and 2.5. TG, IG and P cells have been omitted from these tables as no histochemical staining results have yet been reported for these cell types.

Four silver staining methods were evaluated: the Grimelius, De Grandi, Sevier-Munger and Masson-Hamperl techniques. The Grimelius technique (Table 2.7) has been used extensively to identify argyrophilic cells (see Section 1.8.1) and for this reason was the method of first choice in the present study. When this technique failed to give satisfactory results (see Section 3.3.1) it was replaced by the De Grandi argyrophilic technique (Table 2.7). Another argyrophilic method, the Sevier-Munger technique (Table 2.8), was used because it stains a slightly different range of endocrine cells: it does not stain D, G or L cells (see Tables 2.4 and 2.5). The Masson-Hamperl argentaffinic technique (Table 2.9) stains yet another range of endocrine cell types: it does not stain ECL or K cells (see Tables 2.4 and 2.5).

As described in section 1.8.1, EC cells may be identified using a chromaffin technique (Table 2.10) which forms a yellow reaction product with the secretory granules of EC cells. Because the method did not give satisfactory results (see Section 3.3.1), another set of slides was stained using a modification of this method, in which the dichromate solution was replaced by a mixture of 100 ml of 5% aqueous potassium dichromate and 10 ml of 5% aqueous potassium chromate (Hillarp and Hokfelt, 1955). When this modified method also gave unsatisfactory results, a further set of slides was stained using a solution of 2.5% potassium dichromate in 0.1 M acetate buffer, pH 4.0 and containing 1% sodium sulphite (Jaim-Etchevery and Zieher, 1968), in place of the dichromate solution used in the method described in Table 2.10. This modification also proved unsatisfactory. Discussion of the reasons for the unsatisfactory chromaffin staining results is given on p 139.

EC cells may also be identified by the coloured products resulting from the azo coupling reaction produced by diazonium staining techniques (see Section 1.8.1). Such techniques make it possible to distinguish, by the colour of the reaction product, between 5-HT and several other

Table 2.4 Endocrine cell types found in gastric mucosa and their response to various histochemical stains. Numbers indicate references as listed in Table 2.6.

Staining Method and Reference	Endocrine Cell Type							
	A	D	D_1	EC	ECL	G	PP	X
Grimelius (8)	+8	+23	+26	+23	+23	-14,+23	+7	-16,+23
De Grandi (5)								
Sevier-Munger (19)		-29	+26	+29	+29	-29		-29
Masson-Hamperl (20)		-26		+29	-16	-29		-29
Davenport (4)	+9	+22	±26	+29		+22		±26,-29
Hellerstrom-Hellman (9)		+14				-11,+25	-7	-12
Bodian (1)	+22	+22		+22	+26	-14,+21		
Lead haematoxylin (23)	+23	+23	+26	+29	+23	-15,+29		-16,±23,+26
Chromaffin (13)				+29				
Diazonium (24)				+22		-11		-29
Fast Garnet (24)				+24	±29			
Fast Black (24)				+24	±29			
Xanthydrol (24)	+23	-29		+24	-29	±18		+29
Toluidine Blue (22)		+22				-29		+29
HCl-Toluidine Blue (22)	+22	+22	+26	+22	-16,±23	+22		+23
Phosphotungstic Acid (3)	+26	-26	+26	+29				+26
Phosphotungstic-haematin (28)	+23	-29			-29	-29	-7	+26

+ : stained - : not stained ± : weakly stained or few cells stained

Table 2.5 Endocrine cell types found in intestinal mucosa and their response to various histochemical stains. Numbers indicate reference as listed in Table 2.6

Staining Method and Reference	Endocrine Cell Type									
	D	D_1	EC	G	I	K	L	Mo	N	S
Grimelius (8)	+23	+26	+23	-14,+23	-3	+27	+3		±6	+27
De Grandi (5)							+17			+17
Sevier-Munger (19)	-29	+26	+29	-29	±2	+2	-27			±27
Masson-Hamperl (20)	-26		+29	-29	-3	-2	-3	-10	-6	-3
Davenport (4)	+22	±26	+29	+22						
Hellerstrom-Hellman (9)	+14			-11,+25						
Bodian (1)	+22		+22	-14,+21						
Lead haematoxylin (23)	+23	+26	+29	-15,+29			+27			+17
Chromaffin (13)			+29							
Diazonium (24)			+22	-11		-2	-27			-27
Fast Garnet (24)			+24							
Fast Black (24)			+24							
Xanthydrol (24)	-29		+24	±18			+17			-29
Toluidine Blue (22)	+22			-29						
HCl-Toluidine Blue (22)	+22	+26	+22	+22			+29			
Phosphotungstic Acid (3)	-26	+26	+29							+3
Phosphotungstic-haematin (28)	-29			-29			+17			+17

+ : stained - : not stained ± : weakly stained or few cells stained

Table 2.6: Key to reference numbers and species used in publications cited in Tables 2.4 and 2.5

	Reference (Details in Bibliography)	Species
1	Bodian (1936)	-
2	Buffa et al. (1975)	dog, human, pig
3	Bussolati et al. (1971)	dog
4	Davenport (1929)	-
5	De Grandi (1970)	cattle, dog, guinea pig, human, pig, rabbit, sheep
6	Frigerio et al. (1977)	dog
7	Gepts et al. (1978)	-
8	Grimelius (1968)	human
9	Hellerstrom and Hellman (1960)	rat
10	Helmstaedter et al. (1979)	human, monkey
11	Larsson et al. (1974)	cat, human, pig, rabbit, rat
12	Lazarus and Shapiro (1970)	dog
13	Lillie (1965)	-
14	McGuigan and Greider (1971)	pig
15	McGuigan et al. (1972)	human, pig, rabbit, rat
16	Polak et al. (1971a)	human
17	Polak et al. (1971b)	human
18	Polak et al. (1972)	human
19	Sevier and Munger (1965)	cat, dog, guinea pig, human monkey, opposum, rat
20	Singh (1964)	-
21	Solcia et al. (1967)	dog, guinea pig, horse, human, rabbit, rat
22	Solcia et al. (1968)	dog, guinea pig, hamster, horse, monkey
23	Solcia et al. (1969a)	cat, dog, guinea pig, horse, human monkey, mouse, rabbit, rat
24	Solcia et al. (1969b)	dog, guinea pig, hamster, horse, human, monkey, mouse, rabbit, rat sheep
25	Solcia et al. (1969c)	cat, dog, guinea pig, human, monkey, mouse, rabbit, rat
26	Solcia et al. (1975a)	dog, pig
27	Solcia et al. (1975b)	dog, human, pig
28	Terner et al. (1964)	-
29	Vassallo et al. (1969)	cat

- species not given

Table 2.7: The Grimelius (Grimelius, 1968) and De Grandi (De Grandi, 1970) argyrophilic methods.

Step	Procedure
1	Sections incubated in freshly prepared silver nitrate solution.*
2	Sections drained and reduced for 1 minute in freshly prepared solution of 1% hydroquinone and 5% sodium sulphite at 45°C.
3	Rinsed in distilled water.
4	Fixed for 2.5 minutes in 5% sodium thiosulphate
5	Rinsed in tap water
6	Counter-stained with acetic light green+.
7	Rinsed in tap water.
8	Dehydrated, cleared and mounted in DPX.

* For Grimelius method: 0.03% silver nitrate in 0.1 M acetate buffer, pH 5.6, at 37 - 40°C for 24 hrs.

For De Grandi method: 0.05% silver nitrate in 0.02 M acetate buffer, pH 5.6, at 60°C for 3 hrs.

\+ 2% Light green (yellowish) in 1% acetic acid.

Table 2.8: The Sevier-Munger argyrophilic method (Sevier and Munger, 1965)

Step	Procedure
1	Sections incubated for 15 minutes in 20% aqueous silver nitrate solution at 60°C in the dark.
2	Rinsed in 2 changes of tap water.
3	Sections developed for 5 - 30 minutes in ammoniacal silver solution*, until golden brown.
4	Rinsed in several changes of tap water.
5	Fixed for 2 minutes in 5% sodium thiosulphate.
6	Rinsed in water.
7	Counter-stained with acetic light green (Table 2.7).
8	Rinsed, dehydrated, cleared and mounted in DPX.

* Cold ammonium hydroxide (28 - 30%) was added drop by drop to 50 ml of a 10% silver nitrate solution until the dark brown precipitate first formed had almost disappeared. Then 0.5 ml of sodium carbonate solution (10%) was added, followed by 25 drops of ammonium hydroxide (28-30%). The mixture was shaken well then filtered. Immediately before use 10 drops of 2% formalin were added to the staining solution.

Table 2.9: The Masson-Hamperl argentaffin method, as modified by Singh (1964)

Step	Procedure
1	Sections impregnated for 15 - 30 minutes with freshly prepared ammoniacal silver* solution in the dark at 60°C.
2	Rinsed in distilled water.
3	Fixed for 30 seconds in 1% sodium thiosulphate.
4	Counter-stained with acetic light green (Table 2.7).
5	Rinsed in tap water.
6	Dehydrated, cleared and mounted in DPX.

* Ammonium hydroxide (28-30%) was added to a 10% aqueous silver nitrate solution, drop by drop, until the precipitate first formed had redissolved. Then 10% silver nitrate solution was added, drop by drop, until a slight opalescence persisted. The solution was diluted to 1 in 10 with distilled water and heated to 60°C immediately before use.

Table 2.10: Chromaffin method (Lillie, 1965)

Step	Procedure
1	Sections immersed for 5 minutes in Lugol's iodine (Culling, 1974) solution.
2	Washed in running tap water.
3	Immersed for 5 minutes in 5% sodium thiosulphate
4	Rinsed in tap water.
5	Immersed for 48 hrs in 5% aqueous potassium dichromate solution (modifications of this solution are given on p 65).
6	Rinsed in tap water.
7	Counter-stained with Mayer's haemalum (Culling, 1974) according to steps 3 - 6 of Table 2.11.
8	Dehydrated, cleared and mounted in DPX.

biogenic amines: secretory granules containing 5-HT stain red with fast garnet and black with fast black, while tryptamine, dopa, dopamine, adrenaline and noradrenaline produce a yellow or yellow-brown reaction product with fast garnet and a red product with fast black (Solcia et al., 1968). The fast garnet technique, which was used in all experiments, and the fast black technique, which was used only with the 6 month old animals, are described in Tables 2.11 and 2.12, respectively.

A xanthydrol method (Table 2.13) which demonstrates indoles including 5-HT (Solcia et al., 1969b), was selected to identify A cells, on the basis that glucagon, a product of A cells, stains blue-gray with xanthydrol, 5-HT stains blue-green, while lysozyme and tryptophan stain purple-violet (Solcia et al., 1968).

A toluidine blue technique (Table 2.14) which demonstrates metachromasia (Solcia et al., 1968) was used to identify MMC's which might otherwise be mistaken for endocrine cells (Morales et al., 1980). Because of the water solubility of this stain, post-staining rinsing and dehydration were carried out rapidly.

The alcian blue/haematoxylin and eosin method (Table 2.15) was selected to demonstrate the general histological structure of the GI tract, and 'clear cells' (Feyrter, 1938).

At low pH (<1.0), alcian blue stains highly sulphated compounds (e.g. heparin in mast cells: Morales et al., 1980). Thus an alcian blue technique (Table 2.16) was used to assist in identifying non-endocrine mucosal cells that might be mistaken for endocrine cells.

Lead haematoxylin has been shown to stain a wide variety of endocrine cells including A, D, D_1, EC, ECL and S cells (see Tables 2.4 and 2.5). In addition, G (Vassallo et al., 1969; McGuigan et al., 1972) and X (Solcia et al., 1969a; Polak et al., 1971a) cells may be stained by this method. In view of the wide range of cells stained, a lead haematoxylin technique (Table 2.17) was used in initial attempts to estimate total endocrine cell densities (see Section 3.3.1).

Table 2.11: Fast garnet diazonium method (Solcia et al., 1969b)

Step	Procedure
1	Sections stained for 2 minutes in fast garnet solution* at room temperature.
2	Rinsed in tap water.
3	Counter-stained for 10 minutes with Mayer's haemalum (Culling, 1974).
4	'Blued' for 2 minutes in Scott's tap water (Scott, 1912).
5	Dehydrated, cleared and mounted in DPX.

* 0.1% of fast garnet in 0.1 M phosphate buffer, pH 7.8.

Table 2.12: Fast black diazonium method (Solcia et al., 1969b)

Step	Procedure
1	Sections stained for 2 minutes in fast black solution* at room temperature.
2	Rinsed in tap water.
3	Counter-stained with acetic light green (Table 2.7) or Mayer's haemalum (Table 2.11).
4	Rinsed in tap water.
5	Dehydrated, cleared and mounted in DPX.

* 0.1% fast black in 0.75% aqueous sodium bicarbonate.

Table 2.13 Xanthydrol method (Solcia *et al.*, 1969b)

Step	Procedure
1	Sections stained in 3% solution of xanthydrol in glacial acetic acid/concentrated hydrochloric acid (9:1).
2	Rinsed in tap water.
3	Counter-stained with acetic light green (Table 2.7).
4	Dehydrated, cleared and mounted in DPX.

Table 2.14: Toluidine blue stain (Disbrey and Rack, 1970)

Step	Procedure
1	Sections stained for 2 minutes in 0.5% toluidine blue in acetate buffer, pH 4.5.
2	Rinsed rapidly in tap water.
3	Rapidly dehydrated, cleared and mounted in DPX.

Table 2.15: The alcian blue/haematoxylin and eosin method (Birtles, 1981)

Step	Procedure
1	Sections stained for 10 minutes in 0.3% alcian blue in 3% acetic acid, pH 2.5.
2	Rinsed in tap water.
3	Sections stained for 10 minutes in Mayer's haemalum (Culling, 1974).
4	Rinsed in tap water.
5	'Blued' for 2 minutes in Scott's tap water (Scott, 1912).
6	Rinsed in tap water.
7	Sections stained for 2 minutes in 1% aqueous eosin.
8	Rinsed in tap water.
9	Dehydrated, cleared and mounted in DPX.

Table 2.16: Alcian blue haematoxylin and eosin low pH method (Culling, 1974)

Step	Procedure
1	Sections stained for 10 minutes in 0.3% alcian blue in 0.1 N HCl, pH 1.0.
2	Continue with staining procedure as outlined in Table 2.15, steps 2-9.

Table 2.17: Lead-haematoxylin method (Solcia *et al.*, 1969*a*)

Step	Procedure
1	Sections stained for 6 hrs with freshly prepared lead-haematoxylin solution* at room temperature.
2	Rinsed in tap water.
3	Counter-stained with acetic light green (Table 2.7).
4	Rinsed in tap water.
5	Dehydrated, cleared and mounted in DPX.

* 0.2 g of haematoxylin in 1.5 ml ethyl alcohol (95%) and 10 ml distilled water was added to 10 ml of 'stabilized lead solution'+. The mixture was stirred repeatedly, filtered after 30 minutes and made up to 75 ml with distilled water.

\+ 'Stabilized lead solution': 50 ml of saturated ammonium acetate was added to 50 ml of aqueous lead nitrate (5%), the mixture was filtered and 2 ml of 40% formaldehyde added.

2.7 Immunohistochemical Staining Techniques

Immunohistochemical staining was initially carried out using 4 commercially produced primary antisera (gastrin, pancreatic glucagon, secretin and somatostatin) and Hansky's gastrin antiserum (As'74). These primary antisera were used with two immunohistochemical techniques: the indirect peroxidase-conjugate method (Taylor, 1978) and the PAP method (Sternberger, 1979). The former was chosen because of its simplicity and the latter because of its greater sensitivity (see Section 1.8.1).

The indirect peroxidase-conjugate method used in these studies (Table 2.18) was a modification of that described by Taylor (1978). Modifications were:

(a) The preliminary endogenous peroxidase blocking step was found to have little or no effect on the amount of background staining, which was already minimal and thus this step was omitted.

(b) The antiserum was diluted with PBS (Kruse and Patterson, 1973) containing 2% BSA, according to the supplier's instructions. For consistency, 0.01 M PBS, pH 7.4 was used in all washing steps in place of the Tris buffer (Pearse, 1968) originally specified by Taylor (1978).

(c) Non-specific background staining was suppressed by applying 2% BSA in PBS to the sections prior to application of the primary antiserum. In Taylor's (1978) description, porcine serum was specified.

Because of the poor staining results (see Section 3.3.1) obtained using the Calbiochem-Behring gastrin antiserum with the above method, this antiserum was not used after preliminary testing. At the same time it was decided to opt for the PAP technique to take advantage of its greater sensitivity (see Section 1.8). Thus the PAP technique (Sternberger, 1979) was used with each of the primary antisera, with the exception of the Calbiochem-Behring gastrin antiserum.

Table 2.18: Indirect peroxidase conjugate method (modified from Taylor, 1978)

Step	Procedure
1	Sections equilibrated for 10 minutes in PBS.
2	Sections covered for 10 minutes with PBS containing 2% BSA.
3	Incubated with primary antiserum (for details see Table 2.20).
4	Washed for 15 minutes in 3 changes of PBS.
5	Incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG serum diluted with PBS (for details see Table 2.20).
6	Repeat step 4.
7	Peroxidase stained for 3 - 8 minutes with freshly prepared solution of 0.05% DAB in 0.05 M Tris buffer, pH 7.6, containing 0.01% hydrogen peroxide.
8	Rinsed with PBS.
9	Counter-stained in acetic light green (Table 2.7).
10	Dehydrated, cleared and mounted in DPX.

Note: a. All reactions were carried out at room temperature.

b. Incubations were performed in an enclosed Petri dish containing a filter paper soaked in PBS.

c. Between steps 2 and 3, and 4 and 5, excess buffer was shaken off and slides carefully blotted without allowing sections to dry out.

The PAP technique used in these studies (Table 2.19) was a modification of that described by Sternberger (1979). Modifications were:

(a) The preliminary endogenous peroxidase blocking step was omitted.

(b) The primary antisera, antiserum to rabbit IgG and PAP were diluted with PBS. For consistency PBS was used for all washing steps rather than Tris buffer as described in the original method.

(c) Prior to application of the primary, secondary and tertiary antisera, the sections were covered with PBS buffer containing 2% BSA. This step suppressed non-specific background staining due to the affinity of the proteins (antibodies) in low concentrations, to surfaces, including that of the glass slide.

2.8 Antisera

Sources, dilutions and incubation times for antisera used with the immunohistochemical methods described above are shown in Table 2.20. All primary and tertiary antisera were raised in rabbits.

Data provided by the suppliers concerning the cross-reactivity of the primary antisera with various peptides are given in Table 2.21. (Specific data concerning the cross-reactivity of somatostatin antiserum were not available, however, the supplier was 'not aware' of any cross-reactivity with other gastrointestinal peptide hormones (T. Boenisch, Dako Corp., Santa Barbara, USA; pers. comm., 1982)).

Gastrin antiserum As'74, raised against synthetic human gastrin, cross-reacts with ovine G17-II gastrin (C. Soveny, pers. com., 1983). In RIA this antiserum gives comparable standard curves with synthetic human and ovine G17-II gastrins and binds equally with sulphated and nonsulphated forms of G17 and G34 (Anderson et al., 1981). In RIA As'74

Table 2.19: Peroxidase-antiperoxidase (PAP) method (modified from Sternberger, 1979)

Step	Procedure
1	Sections equilibrated for 10 minutes in PBS.
2	Sections covered for 10 minutes with PBS containing 2% BSA.
3	Incubated with primary antisera (for details see Table 2.20).
4	Washed for 15 minutes in 3 changes of PBS.
5	Repeat step 2.
6	Incubated with approximately 30 µl of swine anti-rabbit IgG serum diluted with PBS containing 2% BSA (for details see Table 2.20).
7	Repeat step 4.
8	Repeat step 2.
9	Incubate with approximately 30 µl of rabbit PAP diluted with PBS containing 2% BSA (for details see Table 2.20).
10	Repeat step 4.
11	Peroxidase stained for 3 - 8 minutes with freshly prepared solution of 0.05% DAB in 0.05 M Tris buffer, pH 7.6, containing 0.01% hydrogen peroxide.
12	Rinsed in Tris buffer.
13	Counter-stained in acetic light green (Table 2.7)
14	Dehydrated, cleared and mounted in DPX.

Note: a. All reactions were carried out at room temperature.

b. Incubations were performed in an enclosed Petri dish containing a filter paper soaked in PBS.

c. Between steps 2 and 3, 5 and 6, and 8 and 9 excess buffer was shaken off and slides carefully blotted without allowing sections to dry out.

Table 2.20: Sources, dilutions and incubation times of antisera used in the indirect peroxidase and PAP methods

Type of Antisera	Antiserum to	Dilution	Incubation Time
Primary	Gastrin[1] (synthetic I α II)	1:1-1:5	24 hrs
	Gastrin[2] (Antiserum As'74)	1:400	24 hrs
	Secretin[1] (synthetic porcine)	1:20	24 hrs
	Somatostatin[3] (human)	1:400	1 hr
	Glucagon[1] (bovine pancreatic)	1:20	24 hrs
Secondary	Horse radish peroxidase conjugated - swine anti-rabbit IgG[4]	1:20	30 min
	Swine anti-rabbit IgG[3]	1:20	30 min
Tertiary	Peroxidase-antiperoxidase complex (PAP)[3]	1:100	30 min

Sources:
1. Calbiochem-Behring, La Jolla, U.S.A.
2. Dr J. Hansky, Prince Henry's Hospital, Melbourne, Victoria, Australia
3. Dako Corporation, Santa Barbara, U.S.A.
4. Miles-Yeda Ltd., Rehovot, Israel

Table 2.21 Cross-reactivities (%) of gastrin, secretin and glucagon antisera as determined by RIA by the supplier (Calbiochem-Behring, La Jolla, U.S.A.)

Peptide	Cross-reactivities		
	Gastrin	Secretin	Glucagon
Gastrin I, synthetic	100	-	-
Gastrin II, synthetic	100	-	-
Secretin, synthetic porcine	<0.01	100	<0.0025
Motilin, synthetic porcine	<0.01	<0.5	<0.0025
VIP, synthetic porcine	<0.01	<0.5	<0.0025
Insulin, bovine pancreatic	<0.01	-	-
Glucagon, bovine pancreatic	<0.01	<0.5	100
C-peptide, synthetic human	-	<0.5	<0.0025
GLI	-	-	<1

- : no information available

is 1,000 times more sensitive to gastrin than CCK (C. Soveny, pers. comm., 1983).

2.9 Reconstitution and Dilution of Antisera

Freeze dried primary antisera, with the exception of gastrin antiserum As'74 which was reconstituted with sterile normal saline, were reconstituted in 1 ml of sterile, distilled water, divided into 15 µl aliquots, snap frozen in liquid nitrogen, and stored at -20°C. Before use the antisera were thawed at room temperature and diluted as follows:

(a) Gastrin (both antisera), somatostatin, swine anti-rabbit IgG and PAP antisera were diluted with PBS containing 2% BSA, 0.05 M EDTA and 0.01 M thimerosal.

(b) Secretin antiserum was diluted as in (a), but thimerosal was replaced by 500 KIU Trasylol/ml.

(c) Glucagon antiserum was diluted with 0.05 M barbital buffer, pH 8.6 containing 2% BSA, 0.035% EDTA, and 500 KIU Trasylol/ml.

2.10 Microscopy and Photomicroscopy

Slides were examined for the presence of endocrine cells at 64, 125, 160, 400 and 1000 times magnifications using a Leitz Ortholux microscope (Ernst Leitz, Wetzlar, Germany) with a tungsten light source. Photomicrographs were taken on Agfachrome 50L (Agfa Gaevert N.Z. Ltd., Auckland) film using a Leica 35 mm camera (Ernst Leitz, Wetzlar, Germany) with exposure times determined using a Microsix L (Ernst Leitz, Wetzlar, Germany) exposure meter. Colour prints in this thesis were reproduced by Elmar Studios Ltd. (Palmerston North, N.Z.), from 35 mm colour transparencies.

Most photomicrographs presented in this thesis are orientated so that the mucosal surface is either towards the top or the right of the photomicrograph.

2.11 Cell Counting Methods

For most sections endocrine cell densities were estimated by counting the number of nucleated endocrine cells in the epithelium of the entire mucosal thickness of 10 randomly selected fields of view. For counting purposes, a 12.5^{X} or 16^{X} objective lens and a Leitz Periplan GF10^{X} eyepiece (Ernst Leitz, Wetzler, Germany) with a counting graticule was used. The graticule, which had been calibrated with an Olympus objective micrometer (Olympus Optical Co. Ltd., Tokyo, Japan), was aligned with the muscularis mucosa and the thickness of the mucosa recorded. The area of mucosa counted could then be calculated.

When the tissue sample was very small (e.g. foetal samples) or when cell densities were very low, counting was undertaken using a 40^{X} objective lens and the number of endocrine cells in the epithelium of the entire section was counted. The total area of mucosa surveyed was calculated from the number of fields counted and the average thickness of the mucosa.

All results are expressed as the mean number of endocrine cells per square millimetre of mucosa (or submucosa in the case of endocrine cells in Brunner's glands) $\pm$ one standard error of the mean (c.mm^{-2} $\pm$ SEM).

2.12 Statistical Methods

Endocrine cell densities for Brunner's glands were not included in the analyses of variance which were only performed on mucosal, not submucosal, cell data. For this reason they were also excluded from all histograms presented.

All estimates of endocrine cell densities were transformed prior to statistical analyses using the formula: transformed endocrine cell density = $\sqrt{(x+1)}$, where x is the endocrine cell density in c.mm^{-2}. This transformation was used to stabilize the variance prior to statistical analyses (Snedecor and Cochran, 1969).

The significance of main effects in each experiment was examined by analysis of variance and tested by examining contrasts for individual degrees of freedom, based on orthogonal and non-orthogonal coefficients (Cochran and Cox, 1960). The orthogonal contrasts were constructed *a priori* to test specific hypotheses. Thus, to evaluate distributions of endocrine cells the following orthogonal contrasts were examined: abomasal body *vs* antrum, proximal duodenum *vs* mid-duodenum, proximal + mid-duodenum *vs* distal duodenum, duodenum *vs* ileum, colon *vs* caecum, abomasum *vs* small intestine, and abomasum + small intestine *vs* large intestine. Similarly to evaluate differences between staining methods the following orthogonal contrasts were examined: De Grandi *vs* fast garnet, anti-gastrin *vs* anti-secretin, anti-gastrin + anti-secretin *vs* anti-somatostatin, and histochemical *vs* immunohistochemical methods. Non-orthogonal contrasts were constructed to test additional hypotheses which were not possible within the constraints of orthogonality.

The sampling sites x staining techniques interaction also was partitioned into single degree of freedom contrasts using orthogonal polynomials produced by cross multiplication of the matrices used to partition each main effect.

Levels of significance in analysis of variance tables are denoted thus:

*	$P<0.05$
**	$P<0.01$
***	$P<0.001$

CHAPTER 3

EVALUATION OF STAINING METHODS AND IDENTIFICATION OF ENDOCRINE CELLS IN THE GI TRACT OF ADULT SHEEP

3.1 Introduction

As discussed in Section 1.8 there is little information available concerning morphology, staining characteristics, or distribution of endocrine cells in the GI tract of sheep.

Although endocrine cells have long been identified in the GI tract of sheep (Tehver, 1930; Sommerville, 1956; Rizzotti et al., 1976), the only specific endocrine cell types identified so far are G (Bunnett and Harrison, 1979a, b; Lichtenberger et al., 1981; Bunnett, 1984), EC (Rizzotti et al., 1976), GIP (Bunnett and Harrison, 1979a) and cells which contain substance P (Harrison and Wathuta, 1982a).

In sheep, the only endocrine cell types for which cell densities have been determined are G (Bunnett and Harrison, 1979a; Bunnett, 1984) and GIP (Bunnett and Harrison, 1979a) cells.

Here, experiments are described in which the staining characteristics, morphology and distribution of endocrine cells in the GI tract of adult sheep have been studied. Further, an attempt has been made to identify non-endocrine cells which might, because of their staining characteristics and location, be mistaken for endocrine cells.

3.2 Methods

Four adult Romney-cross sheep (Table 2.1) were used in this study. They were brought indoors approximately 24 hrs before sample collection, and food, but not water, withheld.

Tissue samples were collected from the sites listed in Table 2.2, as described in Section 2.2. Samples were prepared, fixed and processed in the manner described in Sections 2.3 and 2.5, respectively.

All the histochemical techniques, with the exception of the fast black method, described in Section 2.6 were used in this study. The indirect peroxidase-conjugate method (Table 2.18) was used with the Calbiochem gastrin antiserum, while the PAP immunohistochemical method (Table 2.19) was used with the following antisera: gastrin (As'74), secretin and glucagon (Table 2.20).

Suitable dilutions for each of the primary antisera (Table 2.20) were obtained from suppliers information or from preliminary experiments to establish the techniques. Secretin, glucagon and Calbiochem gastrin antisera were found to be of low titre. In order to obtain reasonable G cell staining with the indirect peroxidase-conjugate method, it was found necessary to use the Calbiochem gastrin antiserum at dilutions of 1:5 or less. Both secretin and glucagon antisera gave satisfactory staining results with the PAP immunohistochemical method, when used at dilutions of 1:10 to 1:20.

Because no data on the immunohistochemical staining characteristics of gastrin antiserum As'74 were available, a dilution study was carried out using the PAP technique. Dilutions of IgG and PAP antisera were kept constant at 1:20 and 1:100, respectively, (suppliers recommendation), while dilutions of gastrin antiserum (1:40, 1:400, 1:800, 1:1600, 1:3200, 1:6400) were applied to serial sections of abomasal antrum. Results of this dilution study are given in Table 3.2.

To supplement the RIA cross-reactivity data provided by the suppliers of the primary antisera, the cross-reactivities of antisera, with the exception of the Calbiochem gastrin, were tested by incubating each of the primary antisera for 24 hours at 4°C with each of the peptides listed in Table 3.1, at the concentrations shown. Following absorption, antisera were brought to room temperature and the PAP technique used in the normal manner to stain serial sections of abomasal antrum, mid-duodenum and pancreas.

Testing for non-specificity of the secondary and tertiary antisera and DAB was carried out as follows: serial sections of abomasal antrum, mid-duodenum and pancreas were stained using the PAP technique and the antisera,

Table 3.1 Peptides and peptide fragments used in cross-reactivity studies (Table 3.3)

Peptide	Concentration (µg/ml of diluted antiserum)
Gastrin 2-17 (human)*	100
Secretin penta-acetate*	50
Glucagon*	50
Carboxyterminal tetrapeptide* of gastrin/CCK	25
Carboxyterminal octapeptide* of CCK	25
Somatostatin‡	50

* Research Plus Inc., Bayonne, New Jersey, U.S.A.

‡ Calbiochem-Behring, La Jolla, U.S.A.

with the exception of the Calbiochem gastrin antiserum, listed in Table 2.20. The following modifications were made to the PAP technique (Table 2.19), for each antiserum and each tissue sample:

(a) the primary antiserum (Step 2) was omitted, and replaced by normal rabbit serum,
(b) the antiserum to rabbit IgG (Step 5) was omitted,
(c) the PAP antiserum complex (Step 8) was omitted, and
(d) the DAB (Step 10) was omitted.

Modification (a) was employed routinely as a test for non-specific staining in all subsequent immunohistochemical staining.

Day to day variation of staining results for the De Grandi and fast garnet techniques, the two histochemical techniques used with all animals, and the PAP technique, using gastrin antiserum As'74 as the primary antiserum, was checked in the following manner:

(a) De Grandi: tissue sections from the abomasal body region of 11 animals (Nos 1 to 4 and 15 to 21, inclusive) were stained in a single batch. A further series of sections from the mid-duodenal sites of the same animals, were also stained in a single batch.

(b) Fast garnet: tissue sections from the mid-duodenum of each of the above 11 animals were stained in a single batch.

(c) PAP technique: tissue sections from the abomasal antrum of each of the above 11 animals were stained on the same day using gastrin antiserum As'74 as the primary antiserum.

Endocrine cell counting was carried out in the manner described in Section 2.11. Following this, endocrine cell densities for each of the batch staining methods described above, were compared with the results obtained when sections were stained on different days (i.e. in the course of routine staining of tissue from these adult and 6 month old animals). These latter data were used in the analyses of variance summarized in Tables 3.6 and 6.1. In each case the variance for data

obtained when slides were stained in a single batch clearly contains components of animal variation, while the variance for data obtained on different days will in addition contain components of day to day variation. Thus the ratio of these variances will test whether day to day variation in staining results is significantly greater than that due to animal to animal variation. To further illustrate that variations in endocrine cell densities were substantially due to animal to animal (and presumably site to site variations), the linear regressions (see Figs. 3.11 to 3.14) between single and separate batch staining results were calculated by the method of least squares (Snedecor and Cochran, 1969). The correlation coefficients for these linear regressions were calculated according to the method described by Snedecor and Cochran (1969).

3.3. Results

3.3.1 Performance of Staining Techniques

Endocrine cells were intensely and reliably stained by the De Grandi (Fig. 3.1) and Sevier-Munger (Fig. 3.2) techniques. No endocrine cells were stained by the Grimelius technique. Slight non-specific background staining, frequently observed with the Sevier-Munger, but not the De Grandi technique, did not cause any problems in identifying endocrine cells.

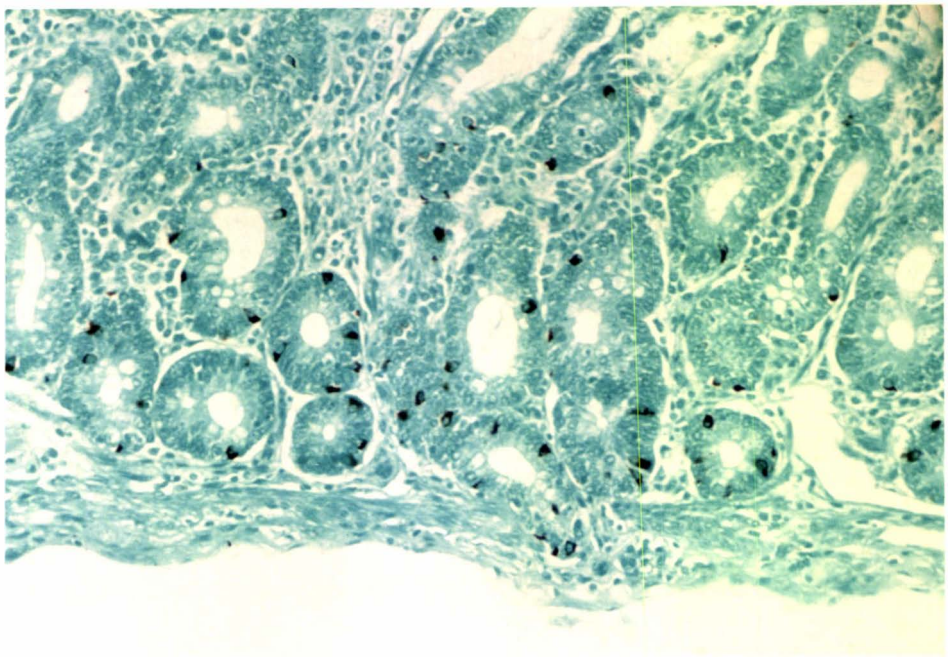
The Singh (1964) modification of the Masson-Hamperl technique gave variable results. Occasionally endocrine cells were intensely stained, but often such cells were found together with poorly stained cells (Fig. 3.3). More frequently all argentaffin cells were very pale (Fig. 3.4), making them difficult to identify and hence count. When staining results were poor, the procedure was repeated on additional sections from the same tissue block, but without any noticeable improvement. In the case of one animal (No. 2), no endocrine cells were stained with this technique.

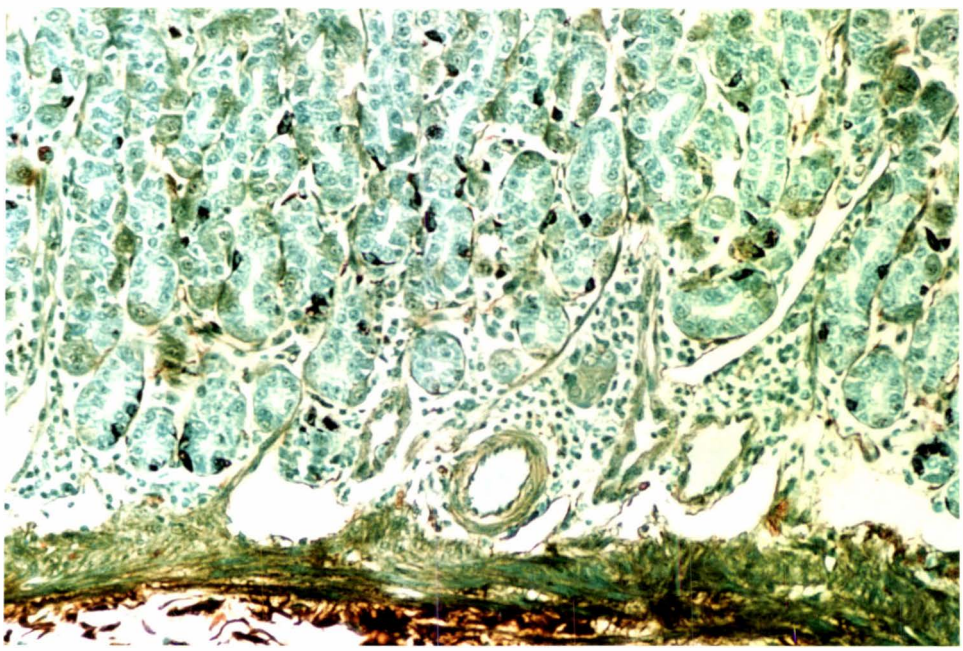
The diazonium technique, using fast garnet, was found to be reliable and satisfactorily stained endocrine cells in intestinal mucosal samples (Fig. 3.5). However, endocrine cells in both the body

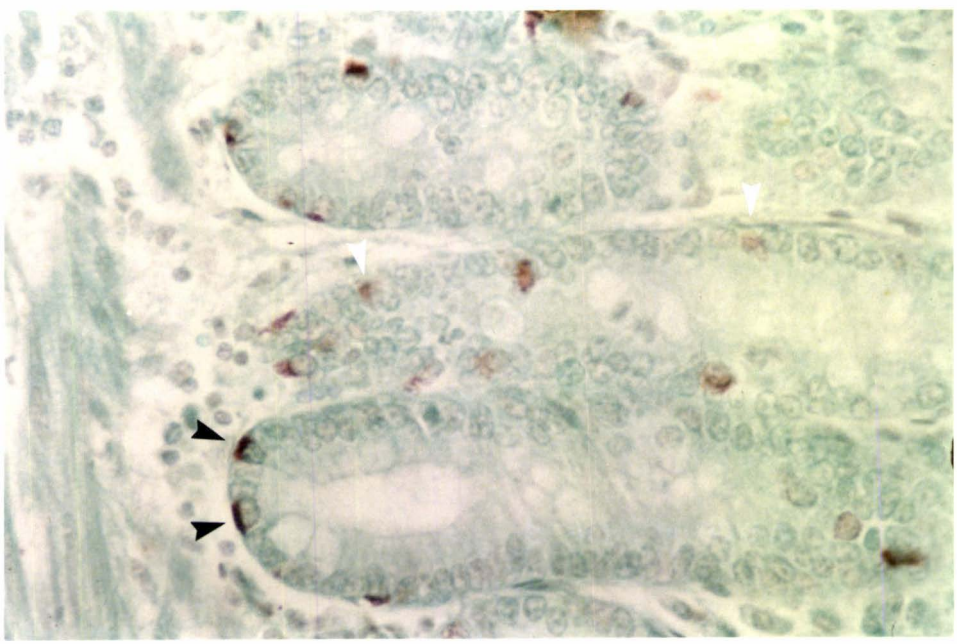
Figure 3.1: Argyrophilic cells (black) near bases of crypts of Lieberkuhn. Distal duodenum; De Grandi method; magnification x 300.

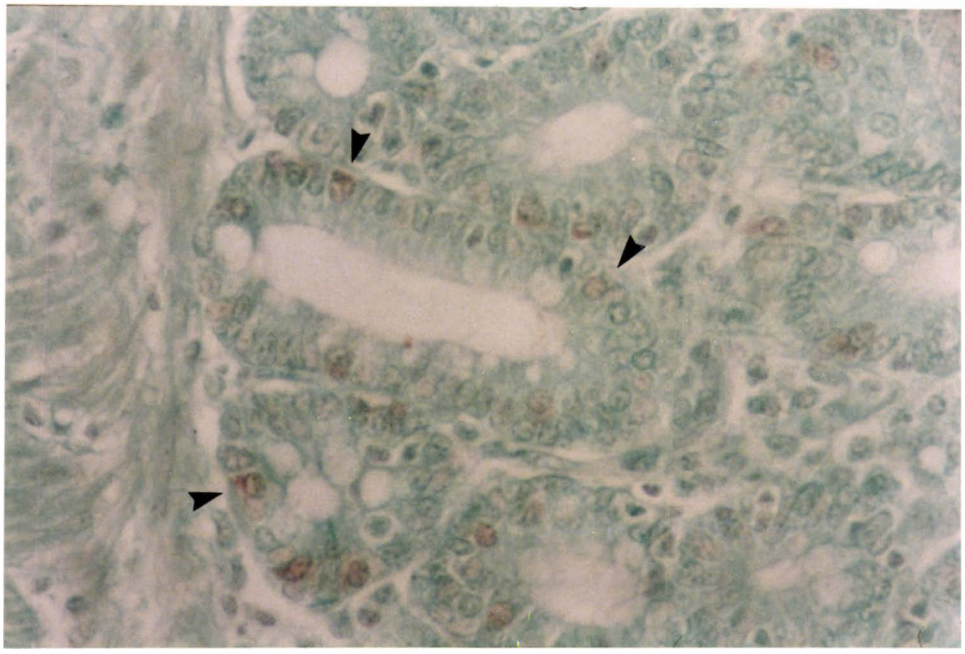
Figure 3.2: Argyrophilic cells (black) near bases of gastric glands. Abomasal body; Sevier-Munger method; magnification x 300.

Figure 3.3: Argentaffin cells, some well stained (black arrows) others poorly stained (white arrows). Mid-duodenum; Masson-Hamperl method; magnification x 740.









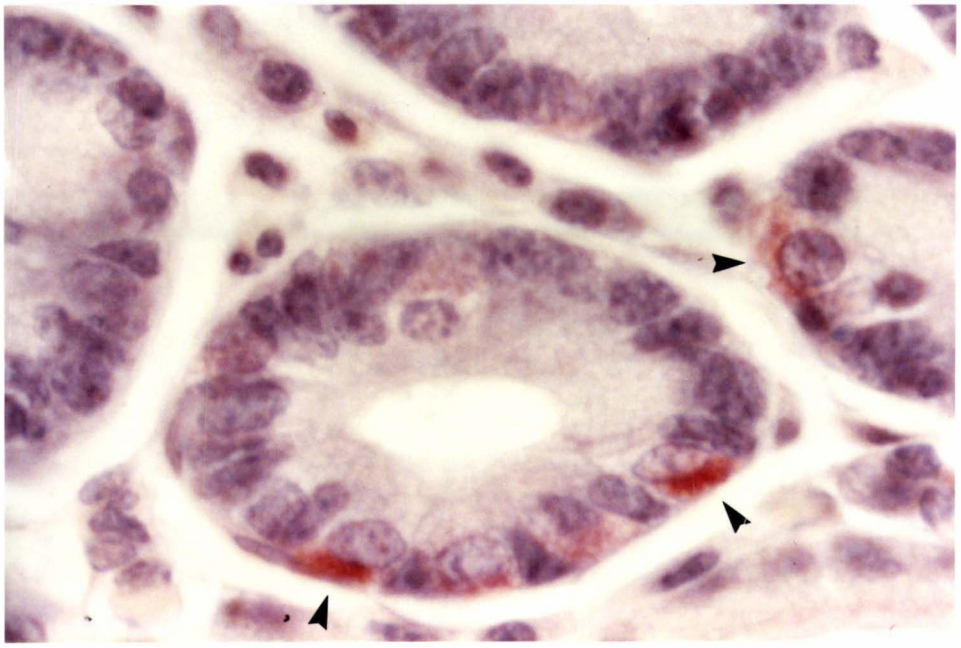


Figure 3.4: Poorly stained argentaffin cells (arrows) near bases of crypts of Lieberkuhn. Mid-duodenum; Masson-Hamperl method; magnification x 740.

Figure 3.5: EC cells (arrows) near bases of crypts of Lieberkuhn. Proximal duodenum; fast garnet method; magnification x 1850.

(Fig. 3.6) and antral regions of the abomasum were poorly stained. The cytoplasm of some non-endocrine epithelial cells was stained yellow (see Fig. 3.22), while in some connective tissue cells it stained red and in others yellow (see Fig. 3.23).

As outlined in Section 2.6, chromaffin techniques were generally found to be unsatisfactory. Thus, although the cytoplasm of EC cells was occasionally well stained (Fig. 3.7, 3.8) by chromaffin methods, more frequently staining was very pale (Fig. 3.9). There was no discernible difference in staining intensity between the three chromaffin methods tried, and no improvement was observed with any of these methods when staining time was increased from 2 to 7 days. In the case of one animal (No. 1) no EC cells were found at any of the sampling sites using chromaffin staining techniques.

The alcian blue/H and E, alcian blue and low pH, and toluidine blue methods all produced intense staining and such results were repeatable (see Figs. 3.31 to 3.42). Detailed presentation of these results is given in Section 3.3.3.

The lead- haematoxylin and xanthydrol techniques failed to give positive results in any of the tissues studied.

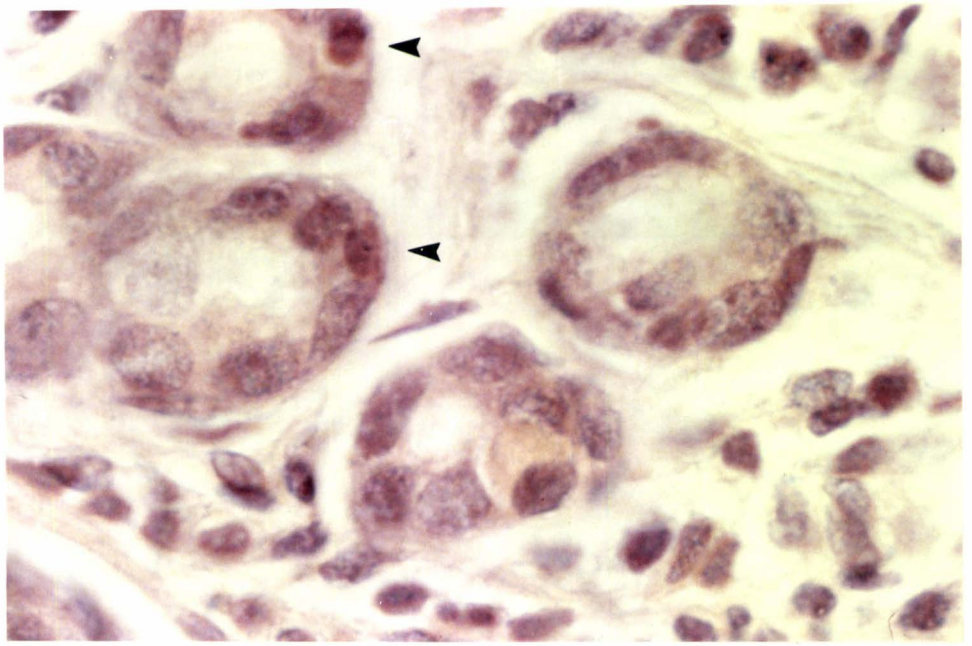
Immunohistochemical staining using the PAP technique with the antisera listed in Table 2.18 gave good and consistent staining intensities (e.g. Fig. 3.10). Occasional failure of the technique was attributed to the use of diluted antisera that had been stored at 4°C for more than a week. No failures occurred when freshly diluted antisera were used.

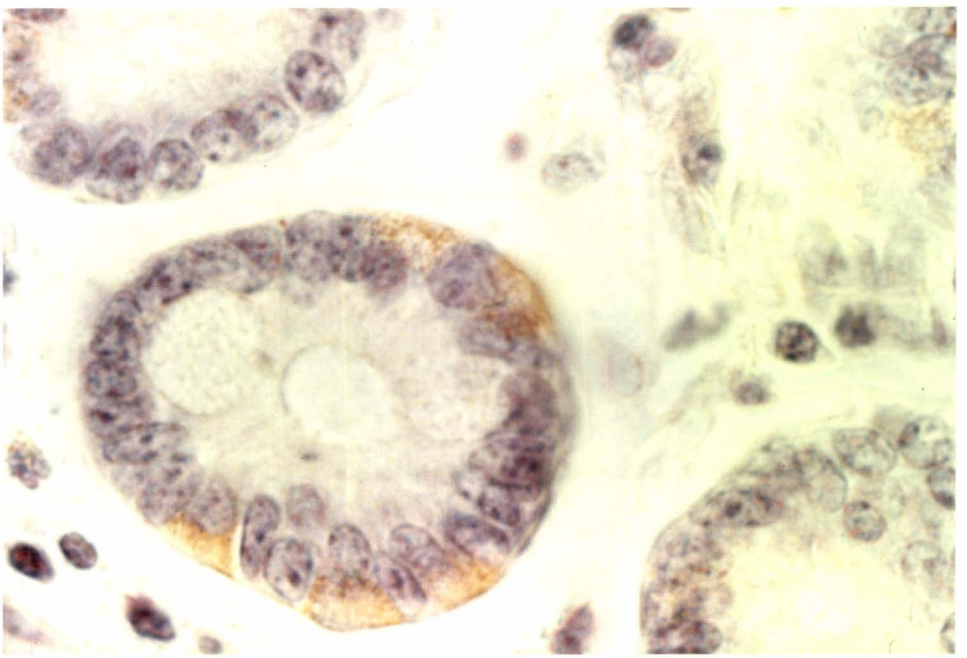
Staining of G cells using the indirect peroxidase-conjugate method with the Calbiochem gastrin antiserum (dilution range 1:1 - 1:5) was generally poor. This was attributed to the low titre of the gastrin antiserum and low sensitivity of the technique. For G cell staining this method was abandoned in favour of the PAP technique using Hansky's As'74.

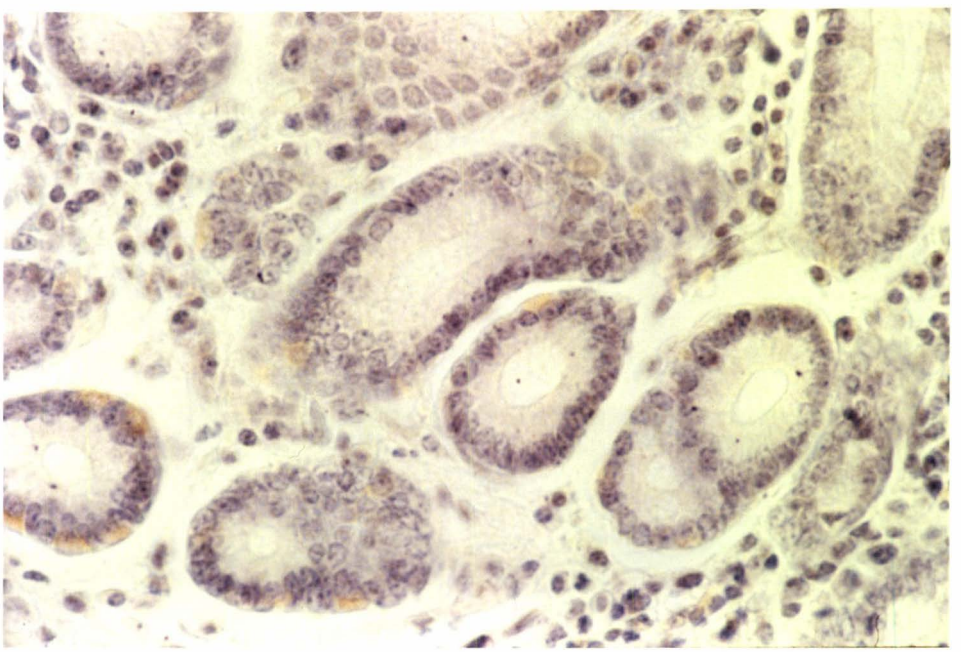
Figure 3.6: Poorly stained EC cells (arrows) near bases of gastric glands. Abomasal body; fast garnet method; magnification x 1850.

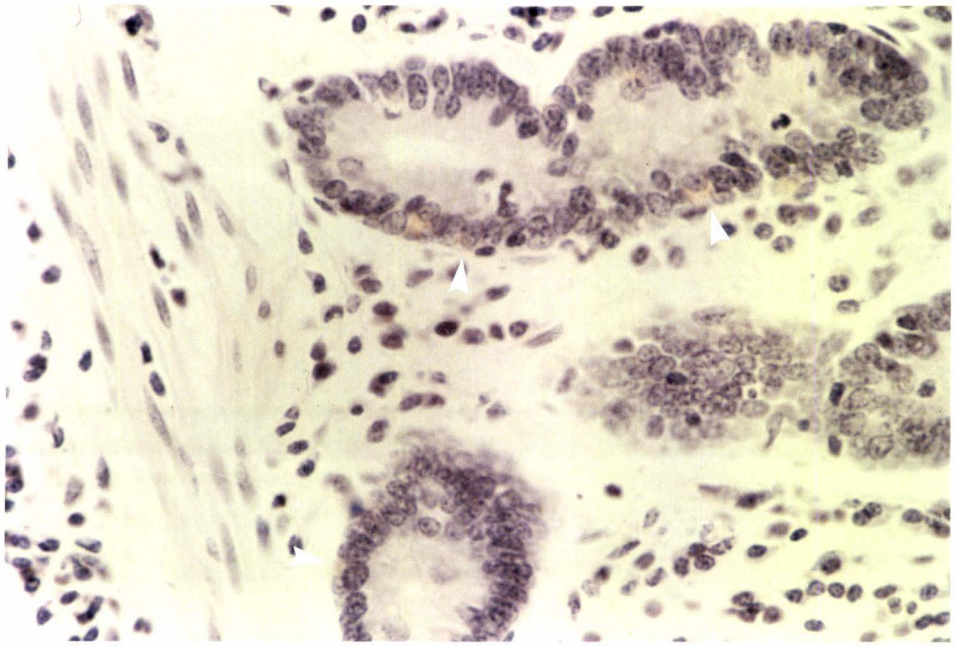
Figure 3.7: EC cells (yellow) with well stained basal granules, near bases of crypts of Lieberkuhn. Proximal duodenum; chromaffin method (Jaim-Etchevery and Zieher, 1968); magnification x 1850.

Figure 3.8: Well stained EC cells (yellow) near bases of crypts of Lieberkuhn. Proximal duodenum; chromaffin method (Jaim-Etchevery and Zieher, 1968); magnification x 300.









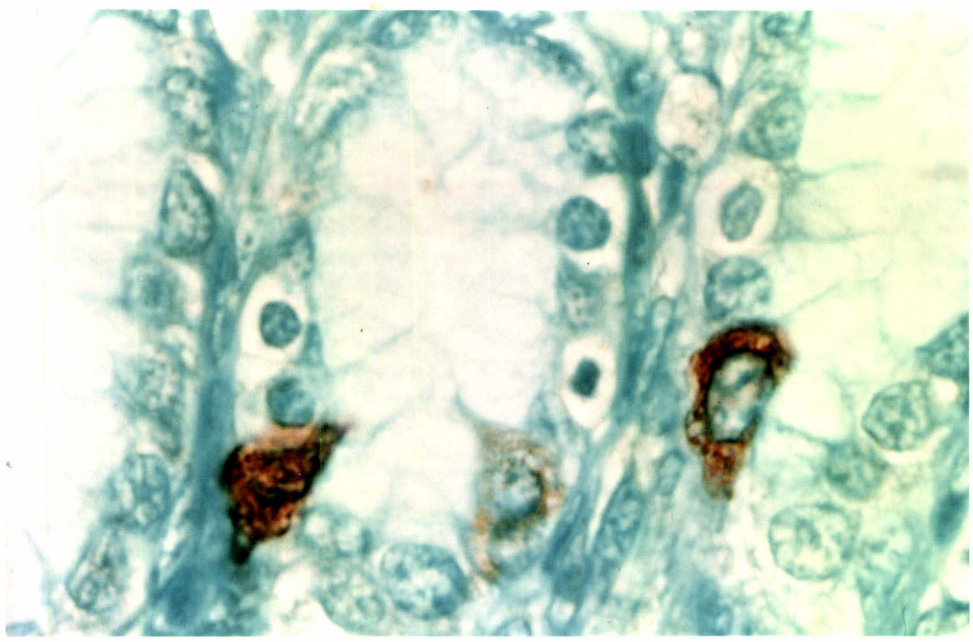


Figure 3.9: Poorly stained EC cells (arrows) near base of crypt of Lieberkuhn. Distal duodenum; chromaffin stain (Jaim-Etchevery and Zieher, 1968); magnification x 300.

Figure 3.10: Immunoreactive G cells (brown) in antral glands. Abomasal antrum; gastrin antiserum As'74/PAP method; magnification x 1850.

Results of the dilution study using the PAP technique with gastrin As'74 are given in Table 3.2.

Table 3.2: Effects of dilution of gastrin antiserum As'74 on G cell counts in abomasal antral mucosa (sheep No. 4; n = 10)

Dilution of gastrin antiserum	1:40	1:400	1:800	1:1600	1:3200	1:6400
G cell density ($c.mm^{-2}$)	134	139	128	125	60	0
SEM	9.5	10.4	7.9	8.9	7.8	0

There were no significant differences between G cell densities for gastrin antiserum dilutions of 1:40 to 1:1600, however, at the 1:3200 dilution G cell counts were significantly ($P<0.001$) lower than for lesser dilutions. No cells stained with the 1:6400 dilution.

At a dilution of 1:40, G cell staining intensity was excellent, however non-specific background staining was undesirably prominent. At a dilution of 1:800, G cell staining was fairly pale and somewhat variable over the section. Staining results at 1:400 dilution were excellent with well stained G cells and little or no non-specific background staining and therefore this dilution was used routinely for gastrin antiserum As'74.

When the primary, secondary or tertiary antisera were omitted from the PAP technique, no endocrine cells were stained and no background staining occurred apart from slight staining of red blood cells. These results indicate that only specific staining was obtained with each of the antisera used in this study.

The results of incubation of the primary antisera with a range of synthetic peptides (Table 3.1) prior to use in the PAP technique are shown in Table 3.3. Results indicate that staining with all the primary antisera was completely inhibited when each was incubated, at dilutions shown in Table 2.20, for 24 hrs at 4°C, with the peptide/peptide fragment against which they were raised. With the exception of As'74, none of the antisera cross-reacted with any other peptide fragments. In the case of As'74, there was evidence of slight cross-reactivity with CCK C-terminal tetra- and octapeptides. This inhibition took the form of paler endocrine cell staining, however, endocrine cell densities were not significantly depressed. G cell densities observed when the primary antiserum was gastrin antiserum alone, or gastrin antiserum which had been preincubated with either CCK C-terminal tetra- or octapeptides were: 146 ± 18, 139 ± 21 and 154 ± 24 c.mm^{-2}, respectively.

F values for day to day variation compared to single batch staining, calculated as outlined in Section 3.2, for the argyrophilic, fast garnet and PAP techniques, were all less than 1 (range 0.55 - 0.82 with 99 degrees of freedom for both the numerator and denominator), hence not statistically significant.

The mean endocrine cell densities, for the three methods mentioned above, obtained from slides stained on one day and slides stained on different days, are plotted in Figs. 3.11 to 3.14, inclusive. Equations for the linear regressions shown in these figures are:

(a) for argyrophilic cells in the abomasal body region

$Y = 1.015X - 4.99$

with a correlation coefficient of 0.9697,

(b) for argyrophilic cells in the mid-duodenum

$Y = 1.109X - 13.58$

with a correlation coefficient of 0.9918,

(c) for EC cells in the mid-duodenum

$Y = 1.43X - 50.82$

with a correlation coefficient of 0.9663, and

(d) for G cells in the abomasal antrum

$Y = 0.87X + 15.13$

with a correlation coefficient of 0.9185

Table 3.3 Specificity of primary antisera as indicated by inhibition of staining, following incubation of primary antisera with peptides or peptide fragments

Peptide/peptide fragment	Primary antisera			
	Gastrin	Secretin	Somatostatin	Glucagon
Gastrin (human 2-17)	+	-	-	-
Secretin (penta-acetate)	-	+	-	-
Somatostatin *	-	-	+	-
Glucagon (synthetic)	-	-	-	+
CCK tetrapeptide	±	-	-	-
CCK octapeptide	±	-	-	-

\+ complete inhibition

± Partial inhibition

\- no inhibition

* see Chapter 4

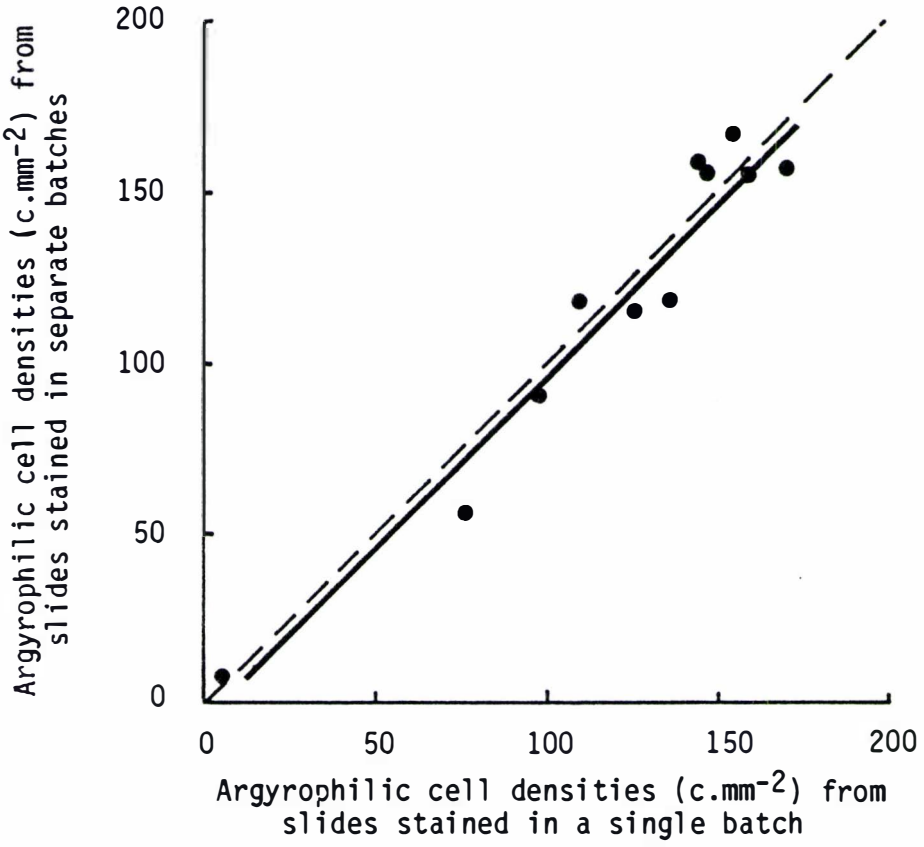


Figure 3.11: Argyrophilic cell densities from slides of abomasal body mucosa, stained in single or separate batches. Linear regression (b = 1.015) is given by solid line. Broken line is a 45° line through the origin.

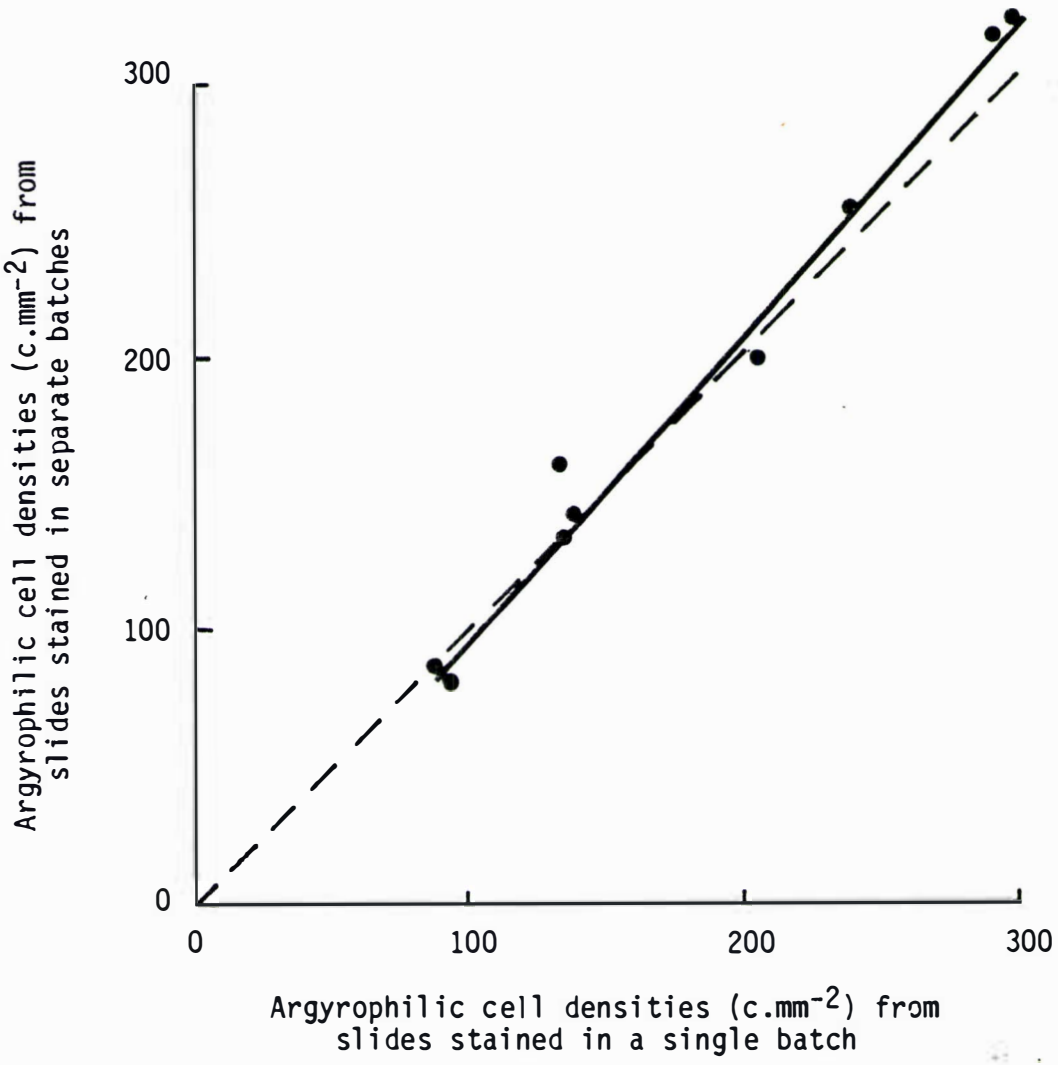


Figure 3.12: Argyrophilic cell densities from slides of mid-duodenal mucosa, stained in single or separate batches. Linear regression (b = 1.109) is given by the solid line. Broken line is a 45° line through the origin.

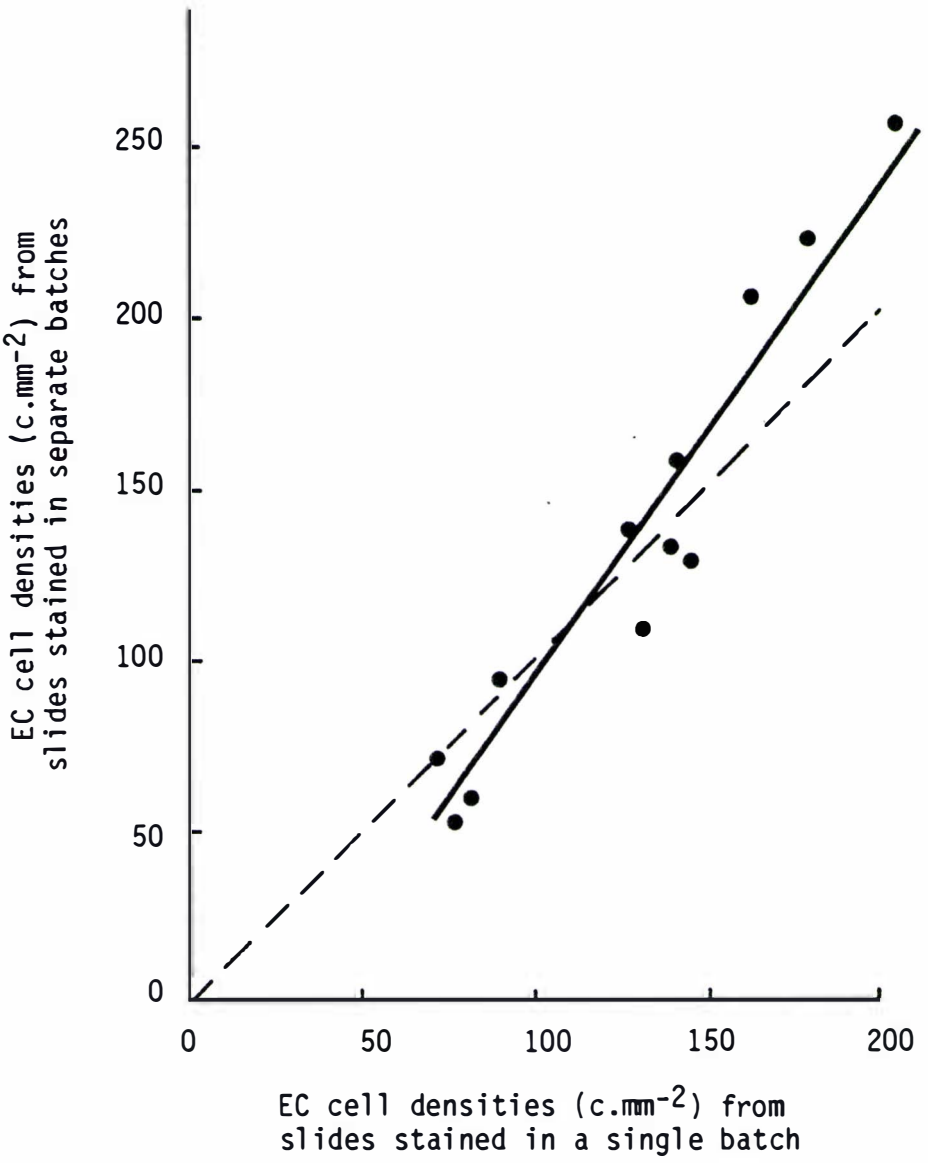


Figure 3.13: EC cell densities from slides of mid-duodenal mucosa, stained in single or separate batches. Linear regression (b = 1.43) is given by solid line. Broken line is a 45° line through the origin.

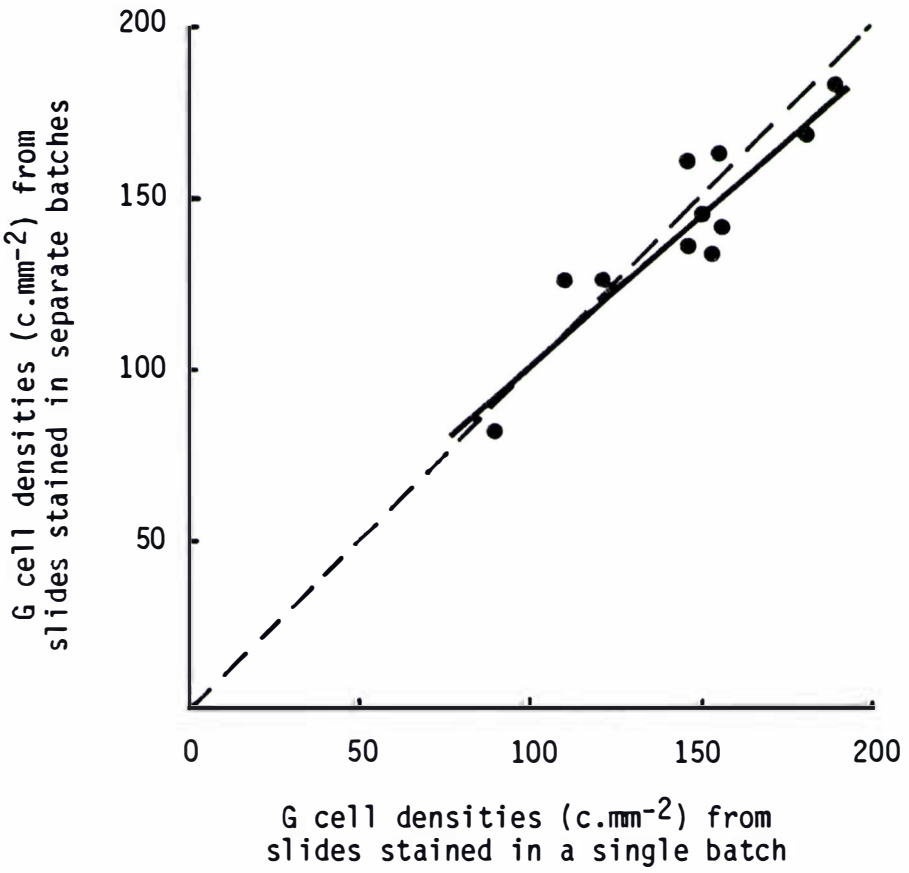


Figure 3.14: G cell densities from slides of abomasal antral mucosa, stained in single or separate batches. Linear regression (b = 0.870) is given by solid line. Broken line is a 45° line through the origin.

where Y = endocrine cell density from slides stained in separate batches and X = endocrine cell density from slides stained in a single batch. For these regressions the proportions of the variance of Y that could be attributed to its linear regression on X were: 94.1%, 98.4%, 93.4% and 84.4%, respectively.

3.3.2 Comparison of Histochemical Staining Methods

Because of the unsatisfactory results obtained with the chromaffin and Masson-Hamperl techniques (see above), data for endocrine cell densities were incomplete for sheep Nos. 1 and 2, respectively. For this reason comparisons of the chromaffin with diazonium, and of the Masson-Hamperl with De Grandi and Sevier-Munger techniques, were analysed separately (Tables 3.4 and 3.5, respectively). Results from chromaffin and Masson-Hamperl staining methods were excluded from the main analysis of variance (Table 3.7).

Analysis of variance (Table 3.4) of the data shown in Fig. 3.55 revealed a significant ($P<0.001$) overall difference between the fast garnet and chromaffin staining techniques. The former method gave higher cell counts at all sites, particularly in the duodenum (interaction component D(ii), Table 3.4).

Analysis of variance of the data shown in Fig. 3.50 did not reveal any significant overall difference between the number of cells stained by the De Grandi and Sevier-Munger methods (Table 3.5). The absence of any significant component in the site x staining method interaction for these two techniques indicates that the results were similar for each of the sampling sites. There was, however, a significant difference ($P<0.001$) between the two argyrophilic and the argentaffin techniques. This was due to the generally low counts obtained with the argentaffin method.

There was a significant difference in the distribution of cells demonstrated by these three techniques as shown by the significant interaction components Di-iii (Table 3.5), all of which involved comparisons in which the abomasum was included. This was due to the low

Table 3.4: Summary of analysis of variance of data obtained using the chromaffin α fast garnet staining methods‡

Source of Variation			DF	Variance Ratios
A.	Animals		2	1.84
B.	Sites		7	
	(i)	Duo_p + Duo_m vs Duo_d	1	4.65*
	(ii)	Duo vs Il	1	30.14***
	(iii)	Ab vs SI	1	45.51***
	(vi)	Ab + SI vs LI	1	16.36**
		Remainder	3	0.61
C.	Staining methods		1	35.73***
D.	Interaction: sites x staining methods		7	
	(i)	Duo vs Il x Chr vs FG	1	8.33*
	(ii)	Ab vs SI x Chr vs FG	1	15.63**
		Remainder	5	0.91
	Residual mean square		14	4.36

‡ Data for sheep No 1 not included

Table 3.5: Summary of analysis of variance of data obtained using three silver staining methods (De Grandi, Sevier-Munger and Masson-Hamperl)‡

Source of Variation			DF	Variance Ratios
A.	Animals		2	2.92
B.	Sites		7	
	(i)	Ab_b vs Ab_a	1	232.44***
	(ii)	Duo_p + Duo_m vs Duo_d	1	92.70***
	(iii)	Duo vs Il	1	276.04***
	(iv)	Ab vs SI	1	131.71***
	(v)	Ab + SI vs LI	1	271.96***
		Remainder	2	0.71
C.	Staining Methods		2	
	(i)	DG vs SM	1	1.11
	(i)	DG + SM vs MH	1	126.18***
D.	Interaction: sites x staining methods		14	
	(i)	Ab_b vs Ab_a x DG + SM vs MH	1	79.96***
	(ii)	A_b vs SI x DG + SM vs MH	1	14.34***
	(iii)	Ab + SI vs LI x DG + SM vs MH	1	8.50**
		Remainder	11	0.91
	Residual mean square		46	1.13

‡ Data for sheep No. 2 not included

density (<8 $c.mm^{-2}$) of argentaffin cells in the abomasal body. The distribution of argyrophilic and argentaffin cells is described in detail in Section 3.3.4.

3.3.3 Morphology of Endocrine Cells

Argyrophilic cells in the body region of the abomasum were typically small, irregularly shaped and of variable staining intensity (Fig. 3.15). Many were of the 'closed' type, with no apparent cell polarity (i.e. cytoplasmic staining was not confined to any particular region of the cell). Some argyrophilic cells in the body region of the abomasum had basal cytoplasmic projections containing granules (Fig. 3.16). Such cells were commonly found next to parietal cells. The basal cytoplasmic process of some cells was observed to pass between the basal lamina and the base of other cells before terminating close to a parietal cell (Fig. 3.17).

Argyrophilic cells in the antral region of the abomasum were always of the 'closed' type. A few had basal cytoplasmic processes.

Most intestinal argyrophilic cells were of the 'open' type, and polarized, the intensity of cytoplasmic staining being greatest in the infranuclear part of the cell. Cell size, although not measured directly, appeared variable. Argyrophilic cells were commonly pyramidal or teardrop in shape (Fig. 3.18), whereas neighbouring non-endocrine (i.e. non-argyrophilic) cells were columnar.

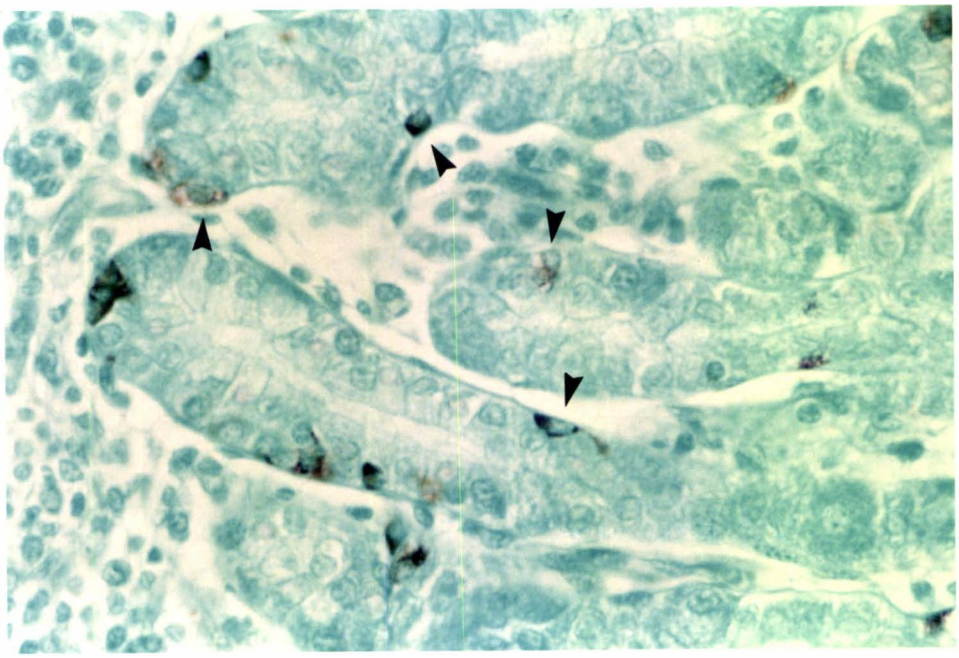
Endocrine cells lightly stained by the De Grandi technique, were found throughout many pancreatic islets. The secretory granules of such cells were particularly fine, giving the cytoplasm a sandy appearance (Fig. 3.19). Some islets, however, did not contain any positively stained cells and overall, the majority of islet cells were not stained. No Sevier-Munger positive cells were identified in the pancreas.

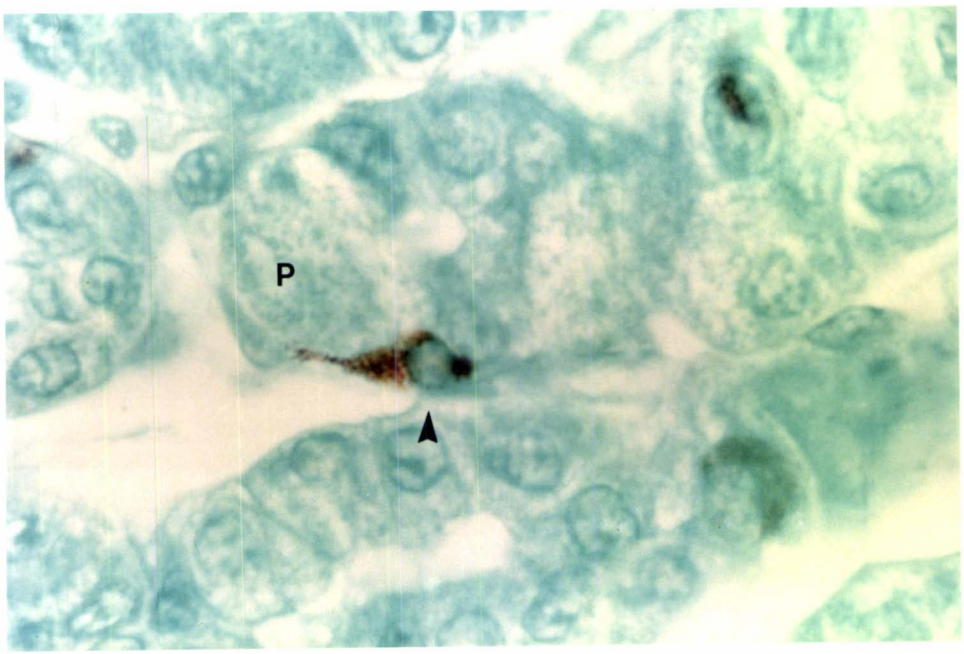
In any given region of the GI tract, the number, distribution and morphology of the argyrophilic cells stained by the De Grandi technique were indistinguishable from those stained by the Sevier-Munger

Figure 3.15: Argyrophilic cells (arrows) of variable shape and staining intensity near bases of gastric glands. Abomasal body; De Grandi method; magnification x 740.

Figure 3.16: Argyrophilic cell (arrow), with basal cytoplasmic process, adjacent to parietal cell (P). Abomasal body; De Grandi method; magnification x 1850.

Figure 3.17: Argyrophilic cell (arrow), with basal cytoplasmic process terminating on distant parietal cell (P). Abomasal body; De Grandi method; mangification x 1850.

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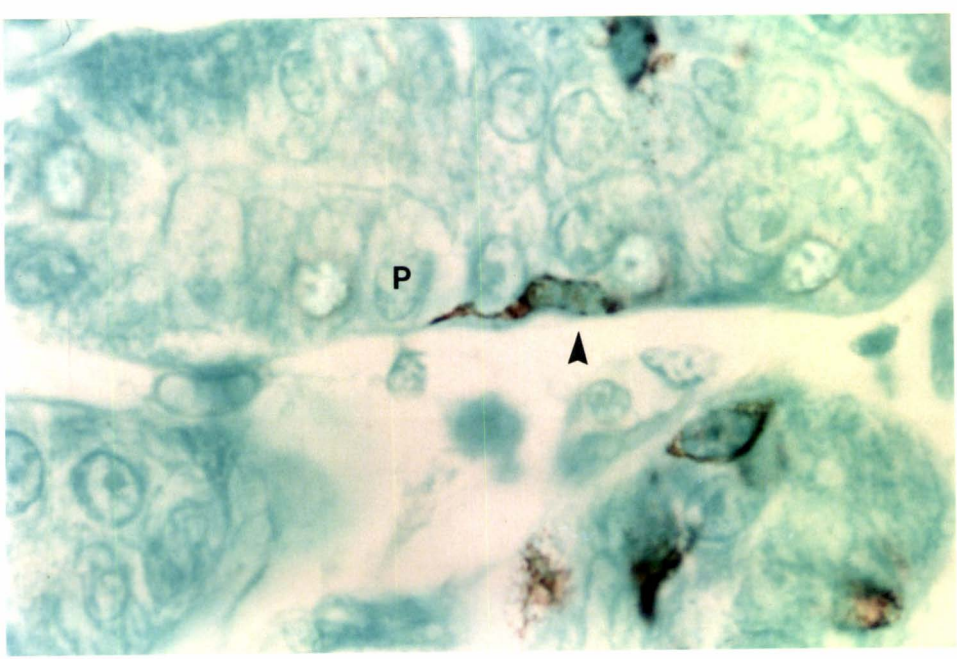
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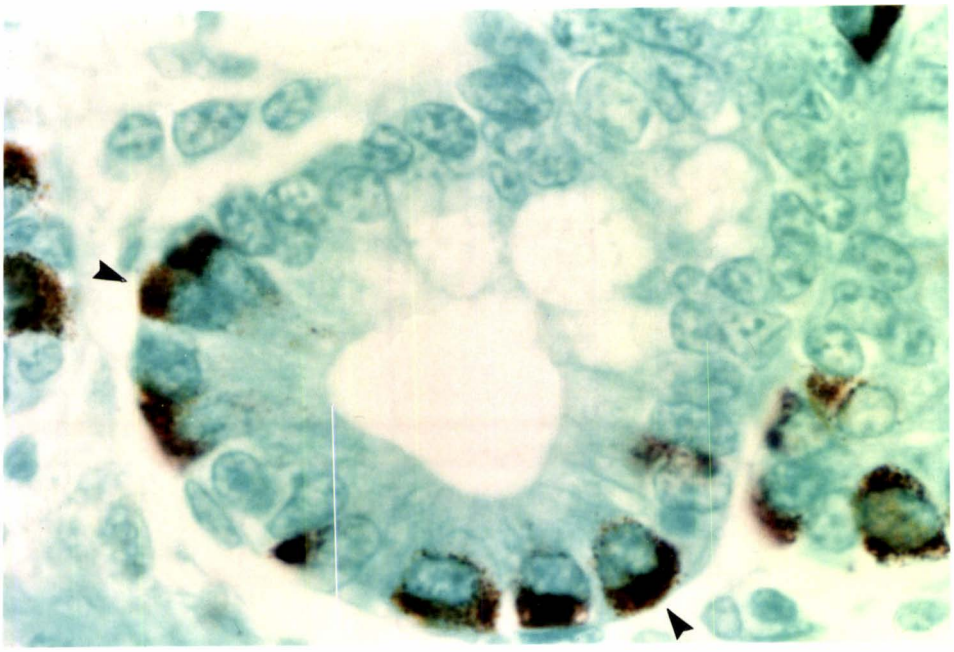


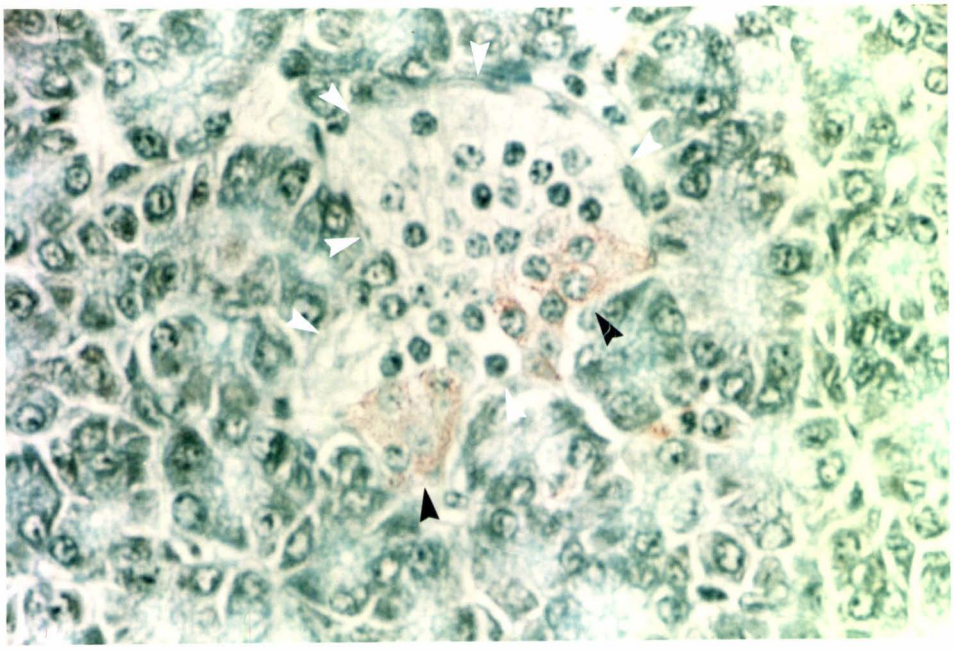


Figure 3.18: Pyramidal shaped argyrophilic cells (arrows), with infra-nuclear cytoplasmic granules (black), in crypt of Lieberkuhn. Mid-Duodenum; De Grandi method; magnification x 1850.

Figure 3.19: Groups of argyrophilic (black arrows) and non-argyrophilic (white arrows) endocrine cells within islet of Langerhans. Pancreas; De Grandi method; magnification x 740.

technique. For this reason, and because the latter technique frequently produced considerable non-specific background staining, the majority of photomicrographs used to demonstrate argyrophilic cells are of sections stained by the De Grandi technique.

Argentaffin cells (i.e. those stained by the Masson-Hamperl method) in the body region of the abomasum were of the 'closed' type and contained pale staining infranuclear granules (Fig. 3.20). In the intestinal mucosa and glands of Brunner, argentaffin cells were of similar morphology to the argyrophilic cells in these regions.

In the abomasum four different cell types were distinguished using the fast garnet technique. A summary of their staining characteristics, type, distribution and the probable identity of these cells is given in Table 3.6. The reasons for classifying these cells in this manner are discussed on p145-147.

All abomasal EC cells were poorly stained (Fig. 3.21) by the fast garnet technique. With this technique yellow staining of the cytoplasm made red granules difficult to demonstrate in photomicrographs, even though they were visible upon careful microscopic examination. Thus, although red cytoplasmic granules were difficult to see they were clearly observed in most cells when examined at 1000x magnification.

MMC's (Fig. 3.22) were small, frequently round in shape with fine cytoplasmic granules most of which stained yellow, while the remainder stained orange-red with the fast garnet technique.

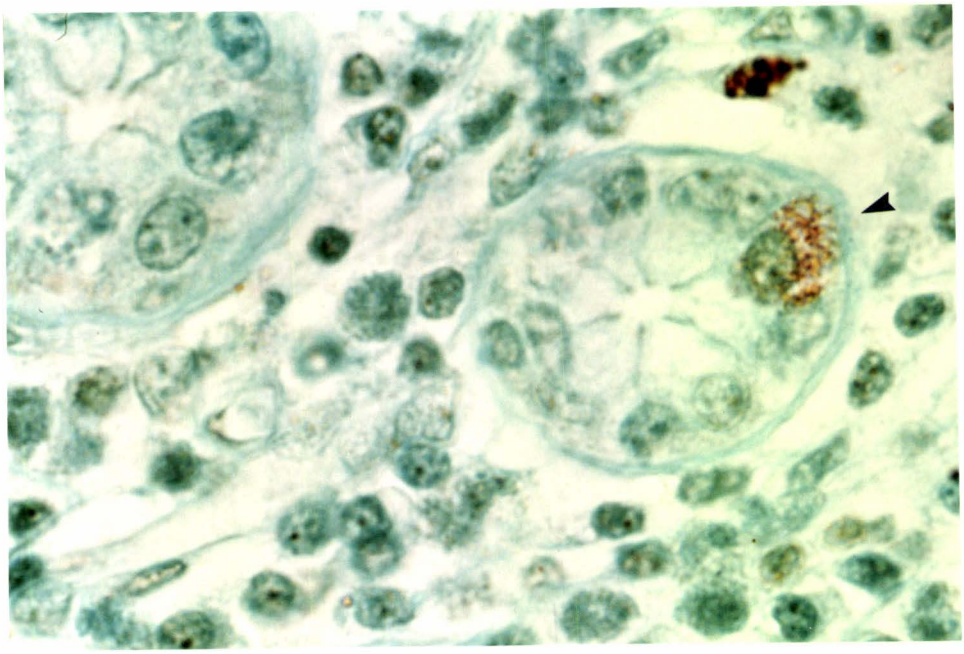
CTMC's (Fig. 3.23) generally were of variable size and shape, some containing predominantly red and others mainly yellow or yellow-brown staining cytoplasmic granules. Such cells were found in connective tissue at all sites examined within the ovine GI tract.

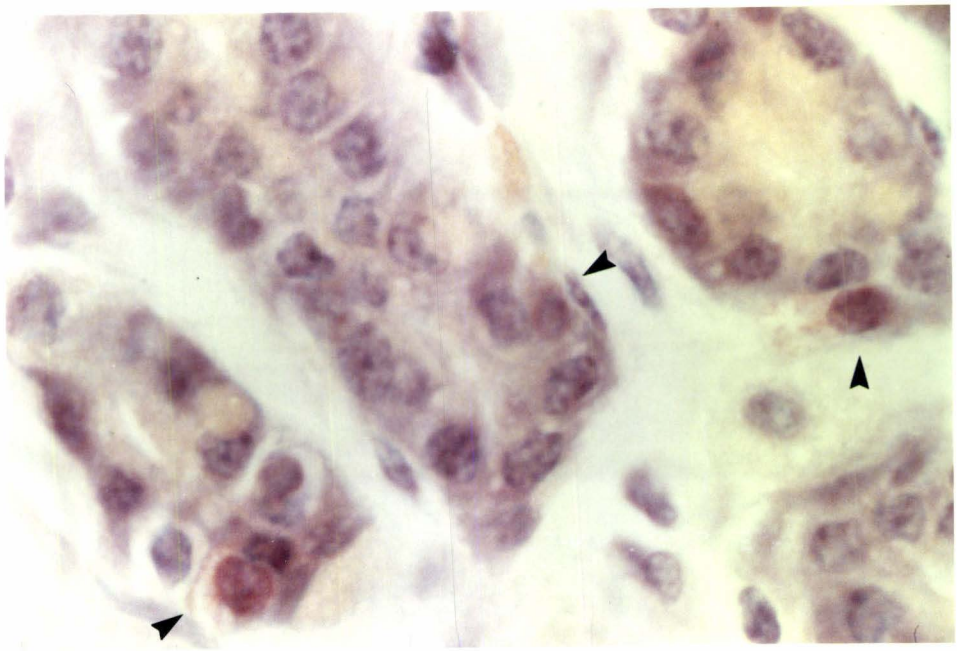
Globular leucocytes (Fig 3.30) were readily distinguished from the three cell types described above by the presence of very large cytoplasmic granules which stained yellow or yellow-brown with the fast garnet technique. Frequently these granules were difficult to demonstrate in photomicrographs (Fig. 3.24).

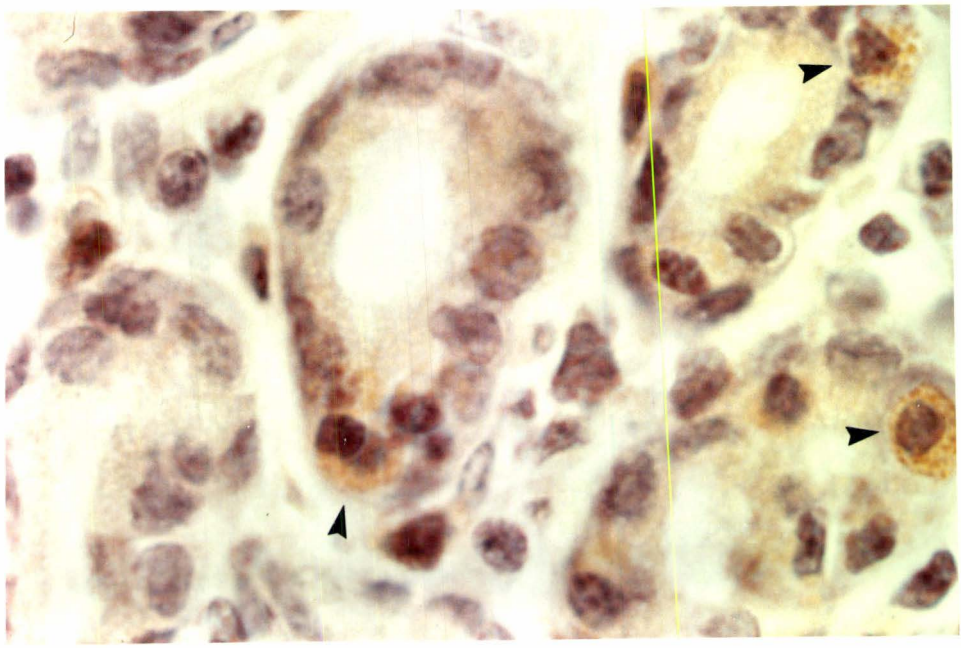
Figure 3.20: Argentaffin cell (arrow) near base of gastric gland. Abomasal body; Masson-Hamperl method; Magnification x 1850.

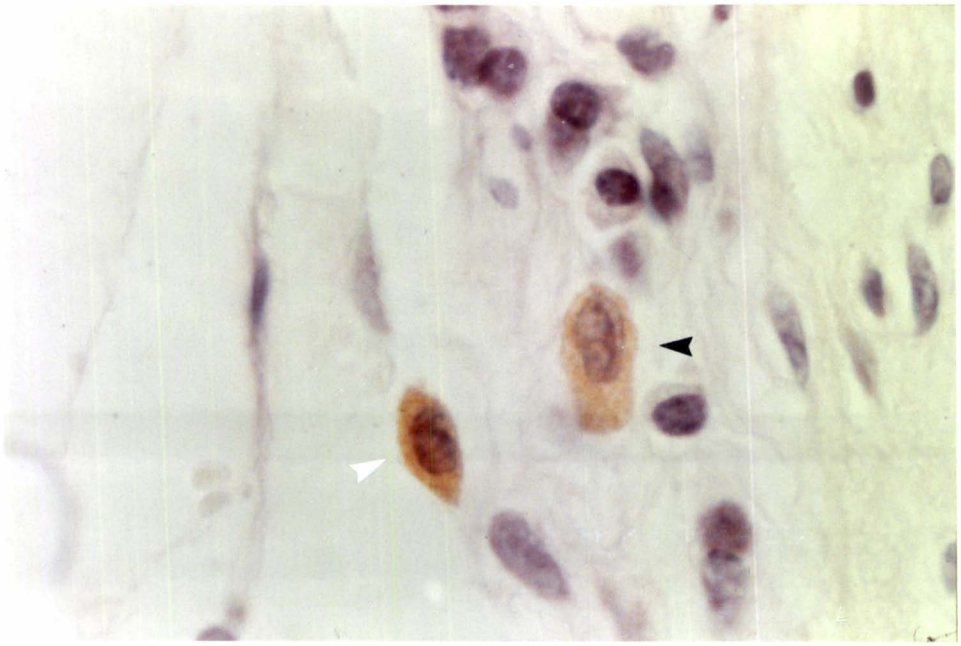
Figure 3.21: Poorly stained EC cells (arrows) near bases of gastric glands. Abomasal body; fast garnet method; magnification x 1850.

Figure 3.22: MMC's (arrows), in gastric glands, containing predominantly fine, yellow cytoplasmic granules. The large granules are red. Abomasal body; fast garnet method; magnification x 1850.









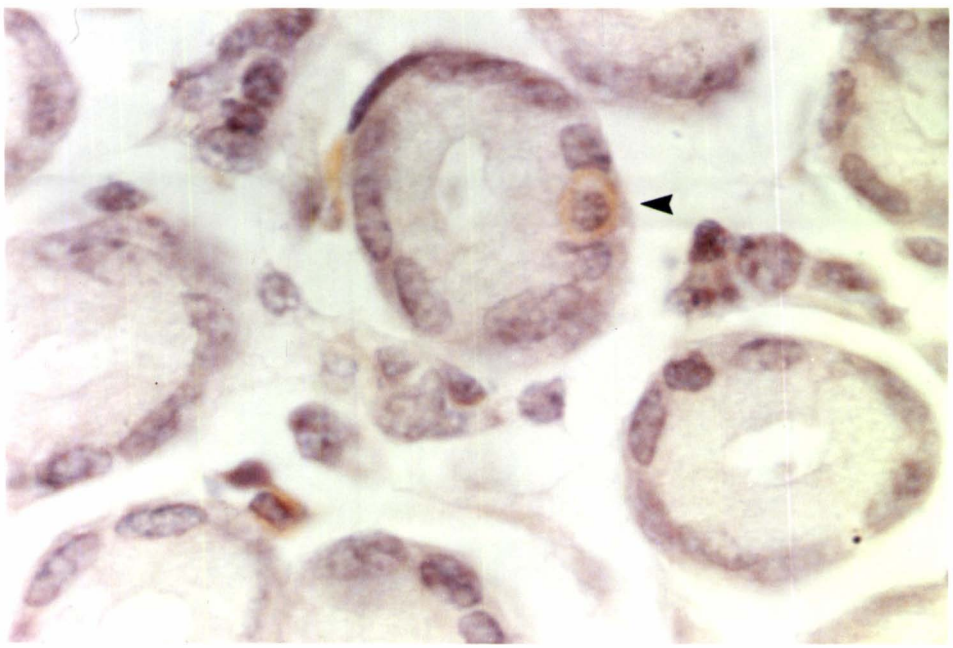


Figure 3.23: CTMC's, one containing yellow (black arrow) the other yellow and red (white arrow) cytoplasmic granules, in abomasal submucosal connective tissue. Abomasal antrum; fast garnet method; magnification x 1850.

Figure 3.24: Globular leucocyte (arrow) with yellow cytoplasmic staining, in antral gland. Abamasal antrum; fast garnet method; magnification x 1850.

Table 3.6 Cytoplasmic staining characteristics, type, distribution and probable identity of cells demonstrated within the abomasal mucosa using the fast garnet staining technique

Cytoplasmic staining characteristics	Type	Distribution within the mucosa	Probable cell identity	Fig.
pale pink, uneven staining	'closed'	epithelium, near base of gastric glands	EC	3.21
fine, yellow granules some coarser, orange-red granules	'closed'	epithelium, throughout glands	MMC	3.22
fine red or yellow to yellow-brown granules or a mixture	-	throughout connective tissue	CTMC	3.23
coarse yellow or yellow-brown granules	'closed'	epithelium, near base of glands	globular leucocyte	3.24

- Classification inappropriate

The vast majority of intestinal EC cells were of the 'open' type and typically pyramidal in shape, with many intensely stained red cytoplasmic granules located in the basal portion of the cell (Fig. 3.25). These EC cells were most common near the base of crypts of Lieberkuhn, however a few were also found in the depths of the glands of Brunner (Fig. 3.26). Also found in the glands of Brunner were cells similar in appearance to abomasal EC cells (i.e. of the 'closed' type with an uneven, pale pink staining cytoplasm (Fig. 3.27)), but these cells were rare.

Brunner's glands also contained oval shaped cells, considerably smaller than the neighbouring columnar epithelial cells, with the nucleus located at one pole of the cell and the cytoplasm staining yellow (Fig. 3.28). Such cells were most common in the epithelium of the duct of Brunner's glands, particularly around the level of the muscularis mucosa. These cells may be globular leucocytes, but their identity is by no means certain (see p146).

Cells thought to be MMC's (Fig. 3.29) and globular leucocytes (Fig. 3.30) were found in the depths of the glands of Brunner. The cytoplasm of both cell types stained yellow with fast garnet. MMC's were small, frequently round in shape with very fine cytoplasmic granules, whereas globular leucocytes were characterized by very large cytoplasmic granules.

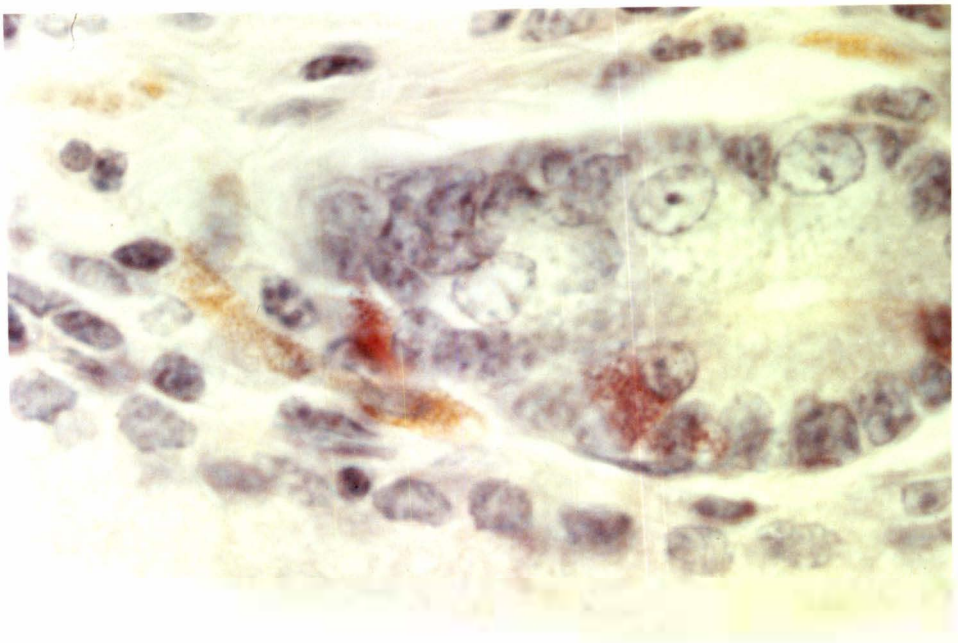
Many 'clear' cells were identified in the abomasal body region by the alcian blue/haematoxylin and eosin staining method. They were small, frequently oval or pyramidal in shape with a round, centrally placed nucleus and clear (i.e. unstained) cytoplasm (Fig. 3.31). Many such cells were observed to lie directly on the basal lamina of the glands and were of the 'closed' type. They were found throughout the epithelium, but were most common in the lower third of the gastric glands where they occurred as single cells. Frequently they were in close proximity to parietal or chief cells.

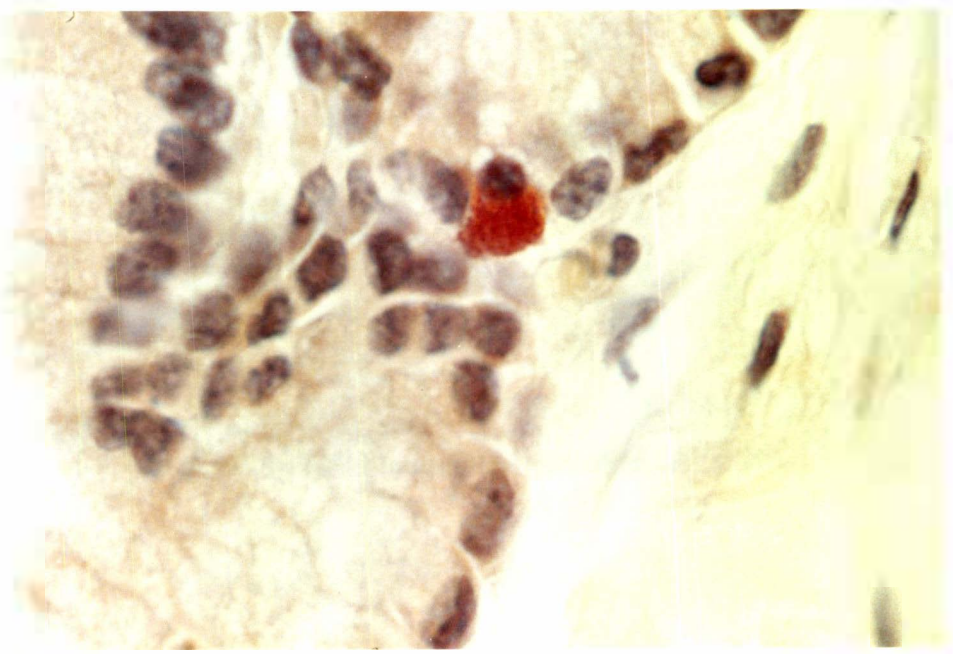
'Clear' cells in the abomasal antrum were more difficult to identify than those of the body region because of slight cytoplasmic basophilia (Fig. 3.32). Most of the endocrine cells seen were of the

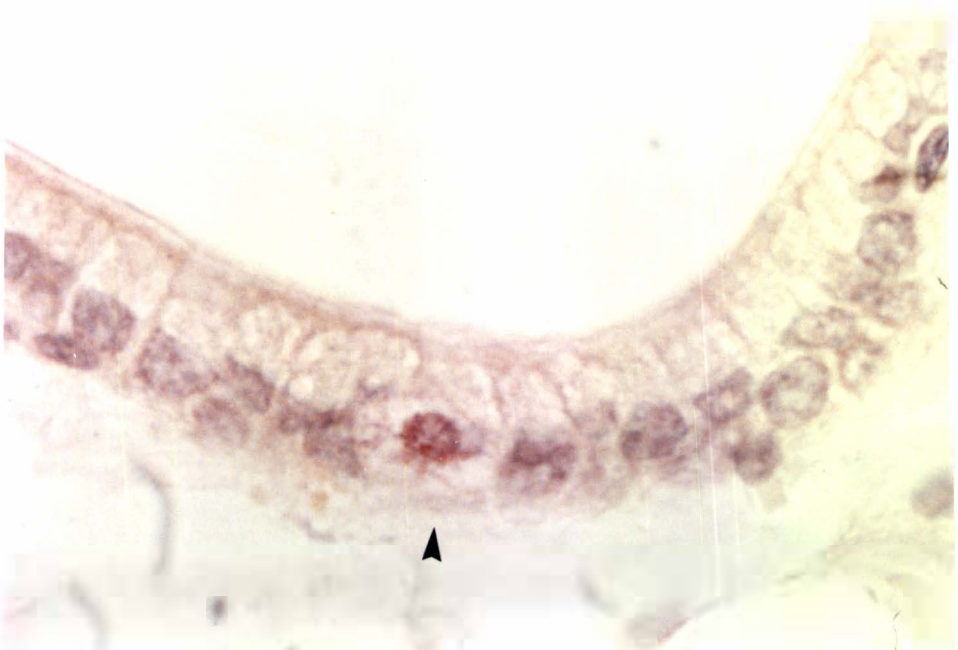
Figure 3.25: Intestinal EC cells (red granules) and CTMC's (yellow granules) near base of crypt of Lieberkuhn. Proximal duodenum; fast garnet method; magnification x 1850.

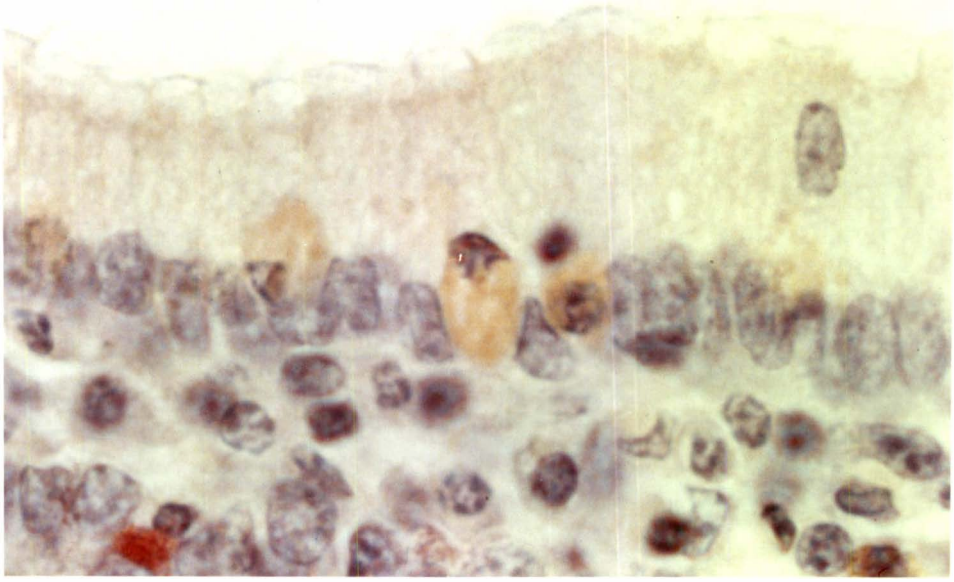
Figure 3.26: EC cell (red granules) with intense infranuclear staining, in depths of Brunner's gland. Proximal duodenum; fast garnet method; magnification x 1850.

Figure 3.27: Poorly stained 'closed' type EC cell (arrow) in depths of Brunner's gland. Mid-duodenum; fast garnet method; magnification x 1850.









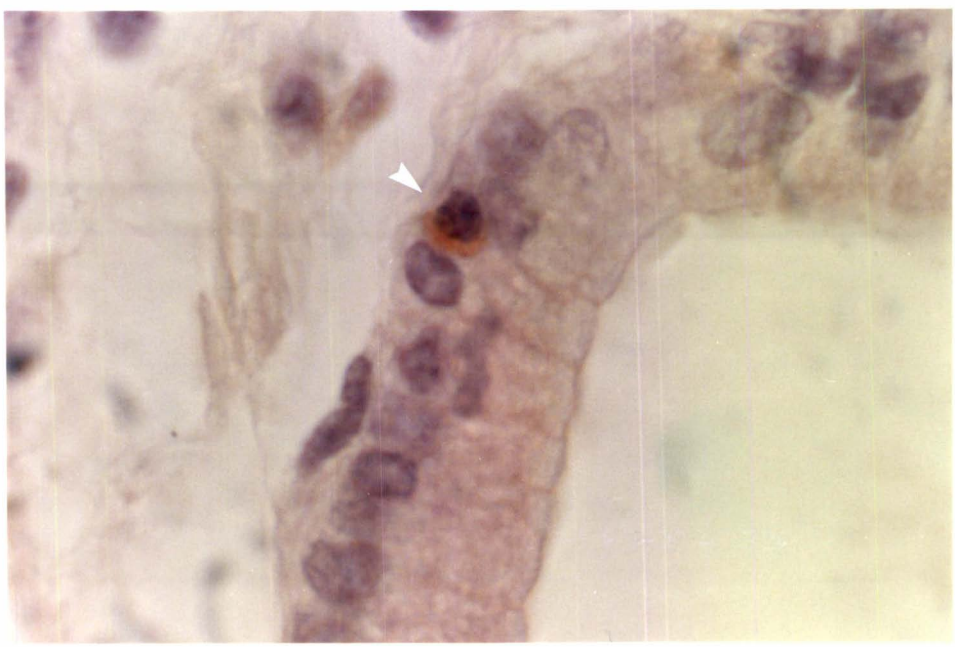


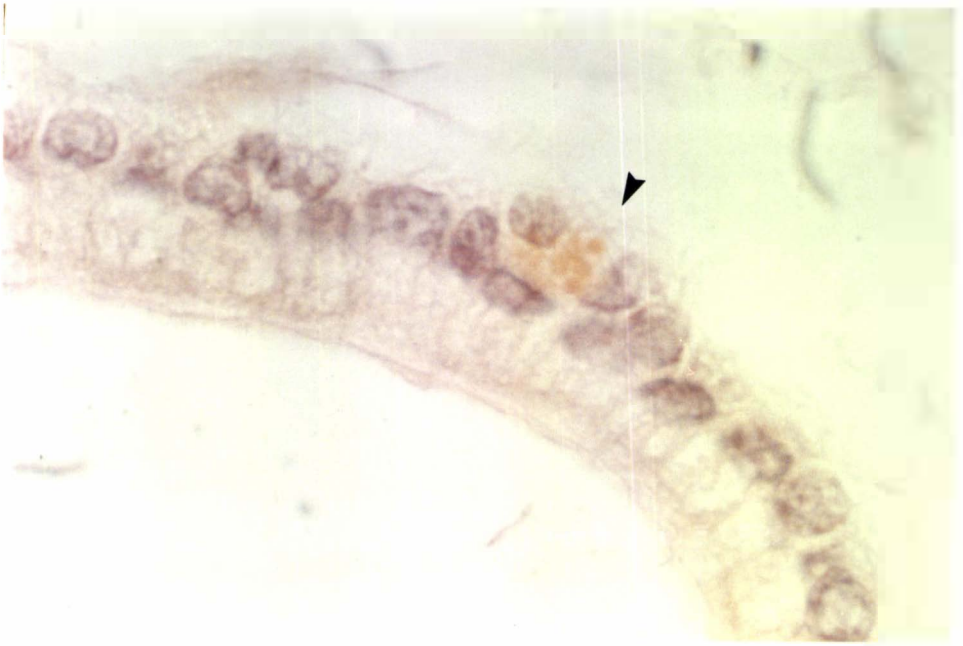
Figure 3.28: Diazonium positive (yellow cytoplasmic staining) cells, possibly globular leucocytes, in epithelium of duct of Brunner's gland. Proximal duodenum; fast garnet method; magnification x 1850.

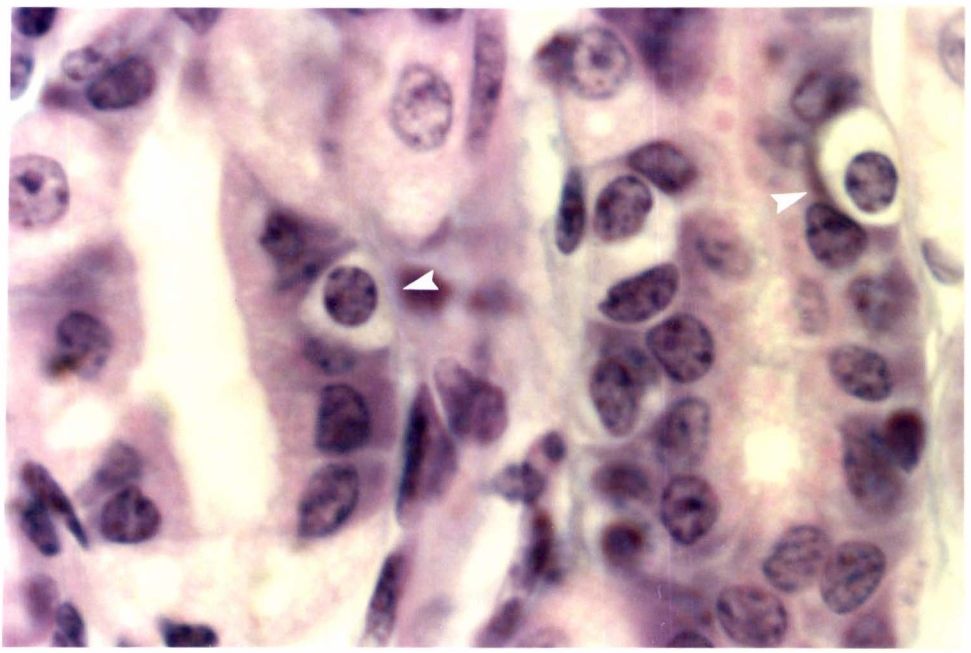
Figure 3.29: MMC (arrow) with yellow cytoplasmic staining, in Brunner's gland. Mid-duodenum; fast garnet method; magnification x 1850.

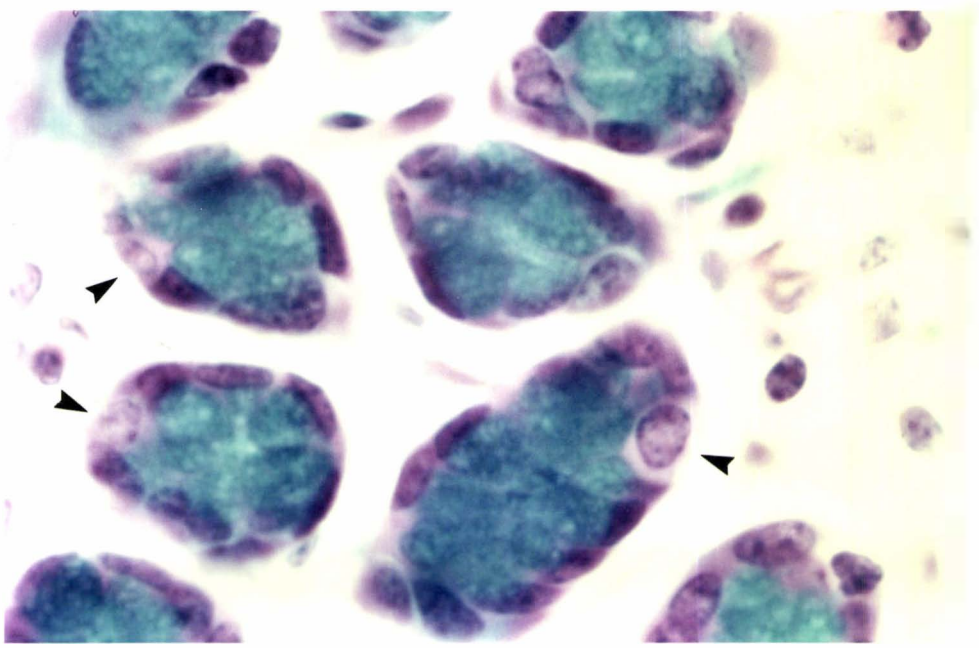
Figure 3.30: Globular leucocyte (arrow), with large, yellow stained cytoplasmic granules, in depths of Brunner's glands. Mid-duodenum; fast garnet method; magnification x 1850.

Figure 3.31: 'Clear' cells (arrows), with very pale or unstained cytoplasm, in gastric glands. Abomasal body; alcian blue/H and E method; magnification x 1850.

Figure 3.32: 'Clear' cells (arrows), with lightly stained cytoplasm, in antral glands. Abomasal antrum; alcian blue/H and E method; magnification x 1850.







'closed' type and were most numerous in the lower half of the antral glands.

In both body and antral regions of the abomasum, alcian blue positive cells were observed in the connective tissue and epithelium. Three different cell types were distinguished:

(a) Round or oval cells, of the 'closed' type and found as single cells scattered throughout the gland epithelium. Cytoplasmic staining intensity varied between cells and, in any one cell frequently was uneven (Fig. 3.33). It was concluded that these cells were MMC's.

(b) Elongated cells with flattened nuclei found closely associated with gastric (Fig. 3.34) and antral glands. It was not possible to determine whether these cells lay within or outside the basal lamina as they appeared to be part of the lamina. There were many cells of similar appearance and location whose cytoplasm did not stain with alcian blue (Fig. 3.35). Both the stained, but more particularly the unstained cells were common near the bases of gastric and antral glands and appeared to form a sheath surrounding the bases of the glands. Both the stained and the unstained cells were most likely fibroblasts, forming the subepithelial fibroblastic sheath.

(c) Connective tissue cells of variable shape but frequently of flattened oval appearance. Their nuclei were also of variable shape but the cytoplasm was evenly and intensely stained (Fig. 3.36). These cells probably were CTMC's.

Toluidine blue revealed three definite metachromatic cell types in the GI mucosa:

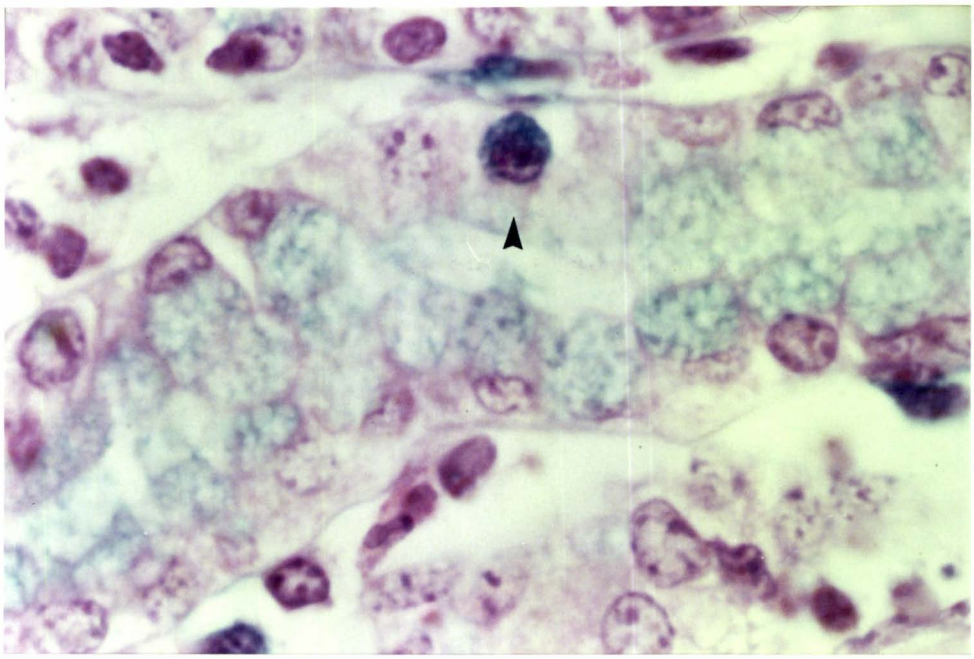
(a) Cells thought to be MMC's were found at all sites. Generally they were round or oval cells, of the 'closed' type, with cytoplasmic granules which were stained a bluish-purple (Fig. 3.37).

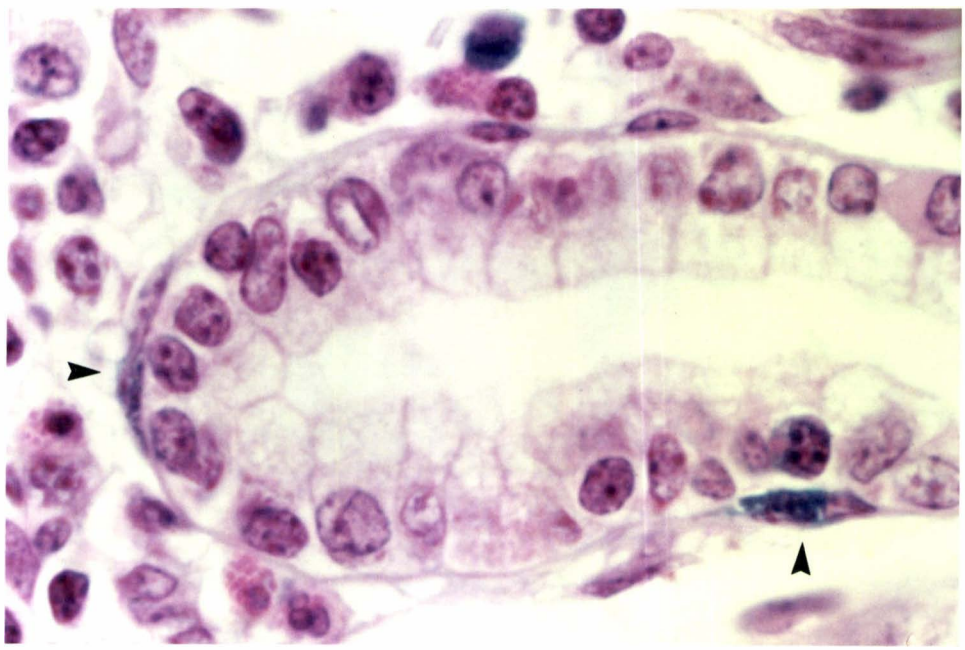
(b) A flattened and often very elongated cell type was closely associated with the gastric glands, particularly near their base.

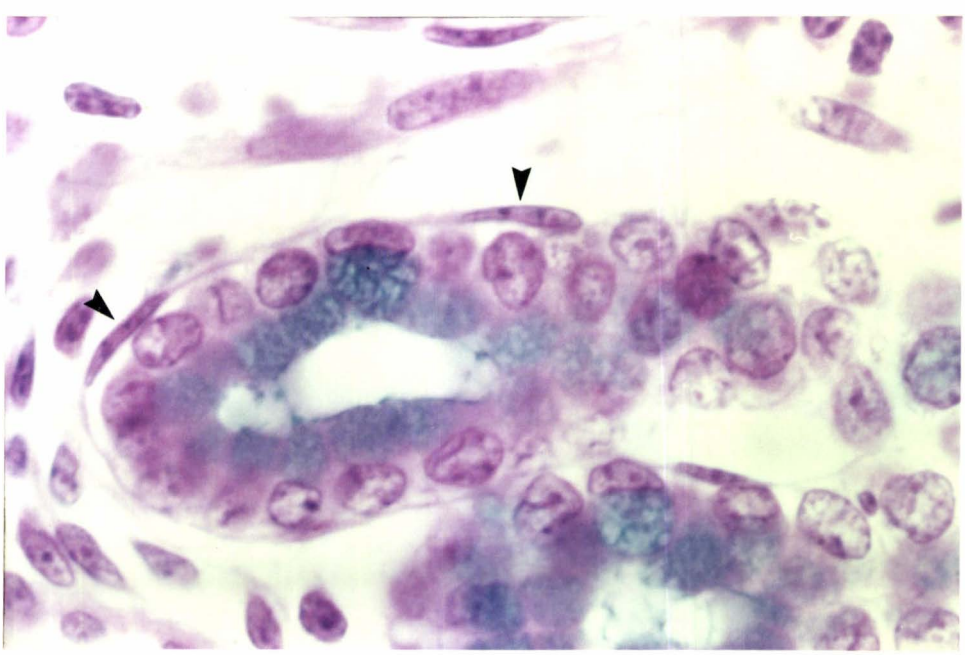
Figure 3.33: MMC (arrow) with alcian blue positive cytoplasm, in gastric gland. Abomasal body; alcian blue/H and E method; magnification x 1850.

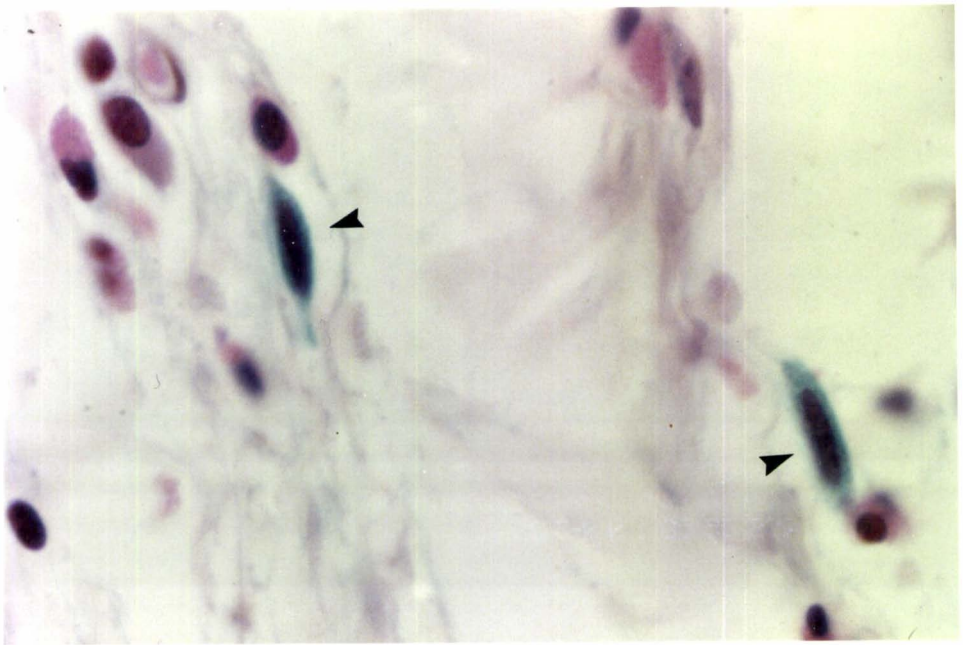
Figure 3.34: Alcian blue positive (arrows) fibroblasts of subepithelial fibroblastic sheath. Abomasal body; alcian blue/H and E method; magnification x 1850.

Figure 3.35: Alcian blue negative (arrows) fibroblasts of subepithelial fibroblastic sheath. Abomasal antrum; alcian blue/H and E method; magnification x 1850.









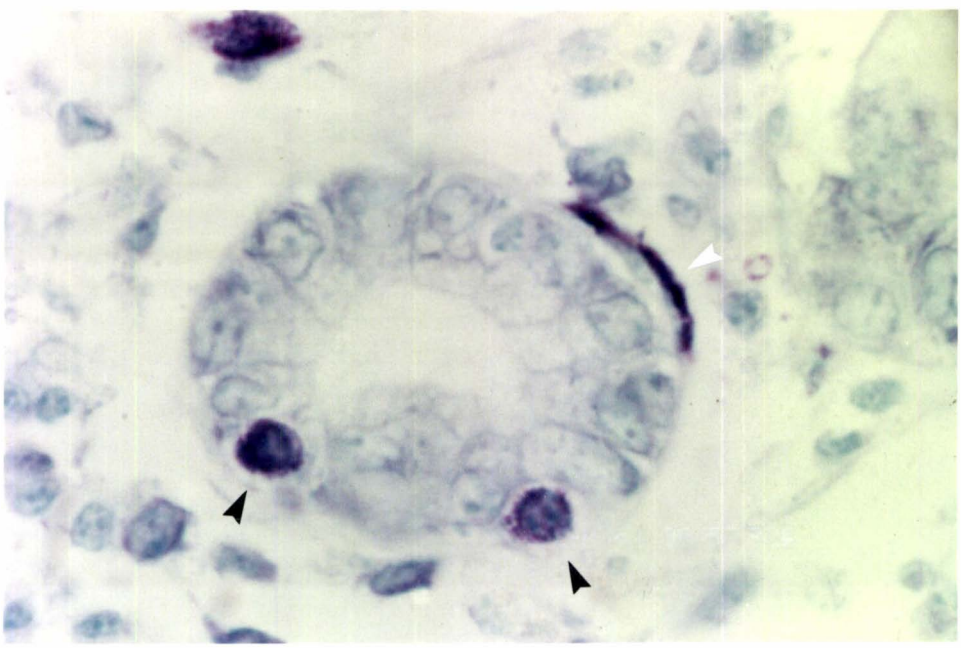


Figure 3.36: CTMC's (arrows) with alcian blue positive cytoplasm in abomasal submucosal connective tissue. Abomasal body; alcian blue/H and E method; magnification x 1850.

Figure 3.37: MMC's (black arrows) and subepithelial fibroblast (white arrow) near base of gastric gland. Abomasal body; toluidine blue method; magnification x 1850.

They appeared to lie within (Fig. 3.38), to be part of, or lie outside the basal lamina (Figs. 3.37, 3.39). It was concluded that these cells were subepithelial fibroblasts.

(c) Globular leucocytes were found in all samples of mucosa examined and were distinguished by the presence of very large metachromatic cytoplasmic granules (Fig. 3.40). These cells were variable in size and shape and were found scattered throughout the depth of mucosa.

Other small 'closed' type cells containing metachromatic granules were found in the glands of Brunner. In the depths of the glands these cells were oval or bean-shaped, with their long axis oriented along the basal lamina of the gland (Fig. 3.41). In the ducts of the glands these cells were oval in shape with an eccentrically placed nucleus and with the long axis of the cell at right angles to the basal lamina of the duct (Fig. 3.42). It is likely that these cells were either MMC's or globular leucocytes.

Antral G cells were of the 'open' type and although cell shape was variable, pyramidal and teardrop shapes (Fig. 3.43) were common. Secretory granules were found throughout the cell cytoplasm (Fig. 3.44), but staining was generally most intense in the basal portion of the cell which was in contact with the basal lamina.

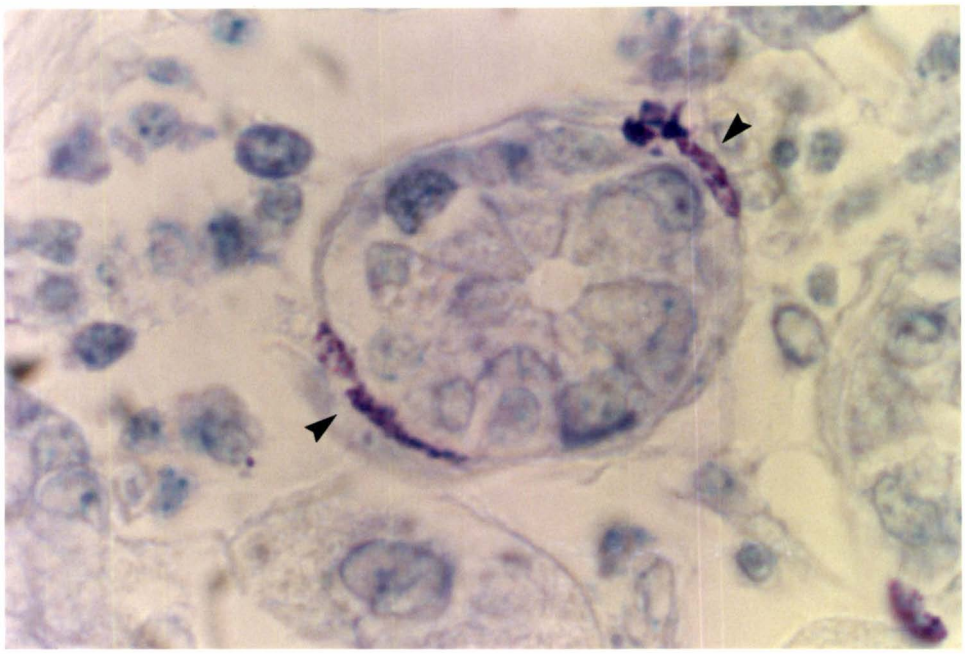
Intestinal G cells were generally teardrop in shape (Fig. 3.45), of the 'open' type with secretory granules concentrated in the infranuclear region. The few G cells found near the tips of villi differed in morphology from those described above in that they were tall, columnar cells with flattened nuclei (Fig. 3.46). Cells similar in morphology to those found in intestinal crypts were observed in the glands of Brunner (Fig. 3.47). In all intestinal G cells, peroxidase staining was observed throughout the cell cytoplasm.

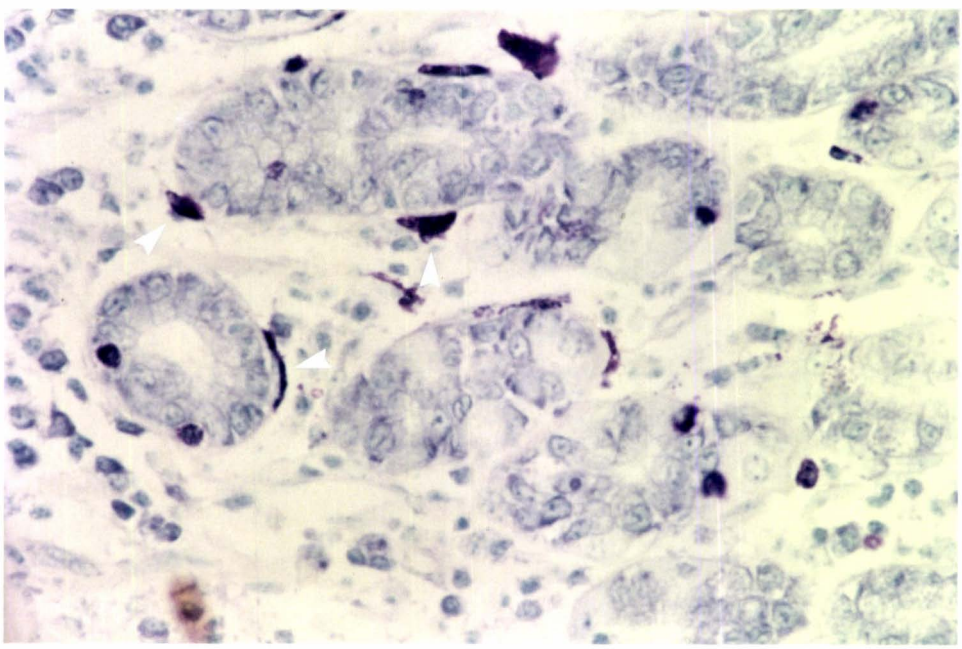
Immunoreactive S cells were of the 'open' type and were densely stained throughout their cytoplasm. S cells in the intestinal crypts were pyramidal or tear-drop in shape (Fig. 3.48) whereas those in the intestinal villi were tall, columnar cells (Fig. 3.49).

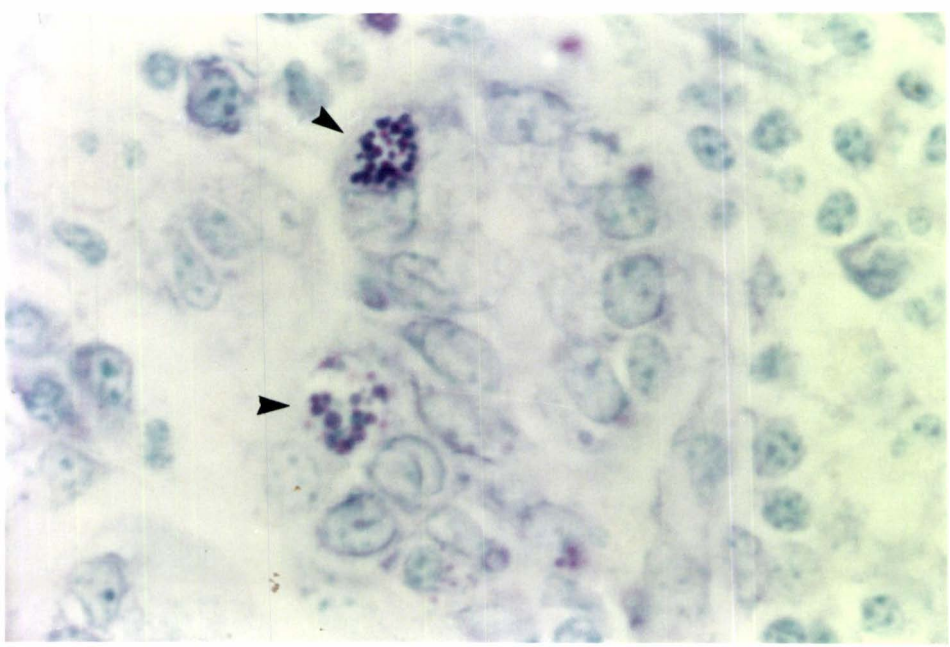
Figure 3.38: Subepithelial fibroblasts (arrows), within basal lamina of gastric gland. Abomasal body; toluidine blue method; magnification x 1850.

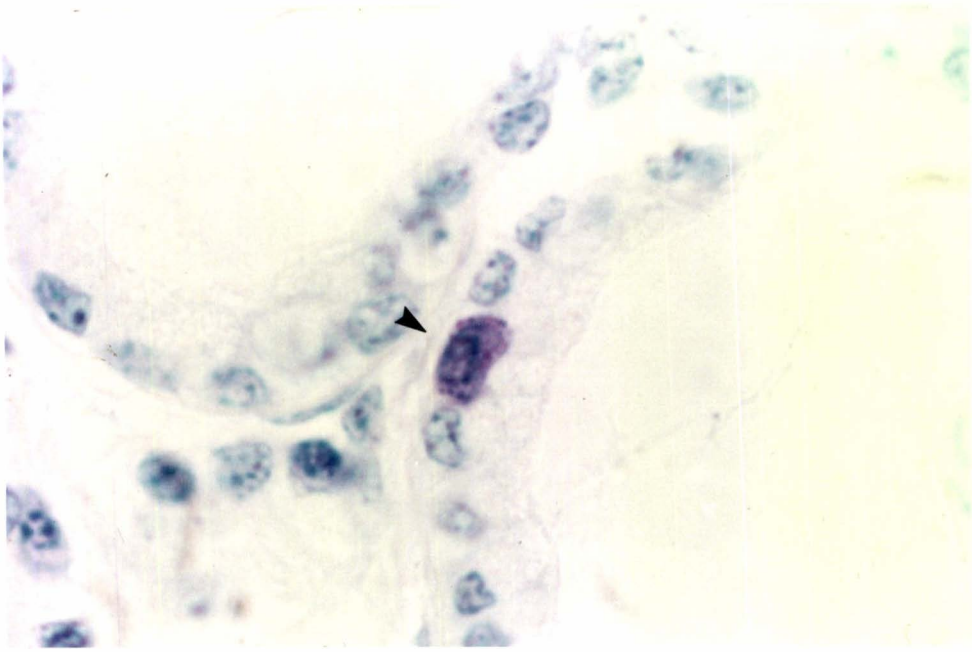
Figure 3.39: Subepithelial fibroblasts (arrows) outside basal lamina of gastric glands. Abomasal body; toluidine blue method; magnification x 740.

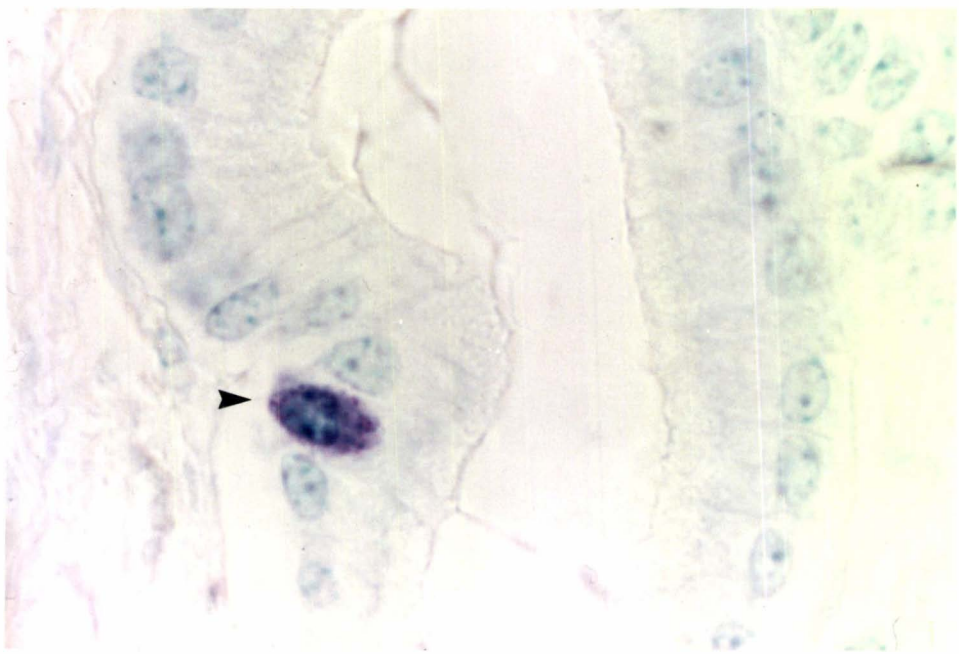
Figure 3.40: Globular leucocytes (arrows) with large, metachromatic cytoplasmic granules, in gastric glands. Abomasal body; toluidine blue method; magnification x 1850.











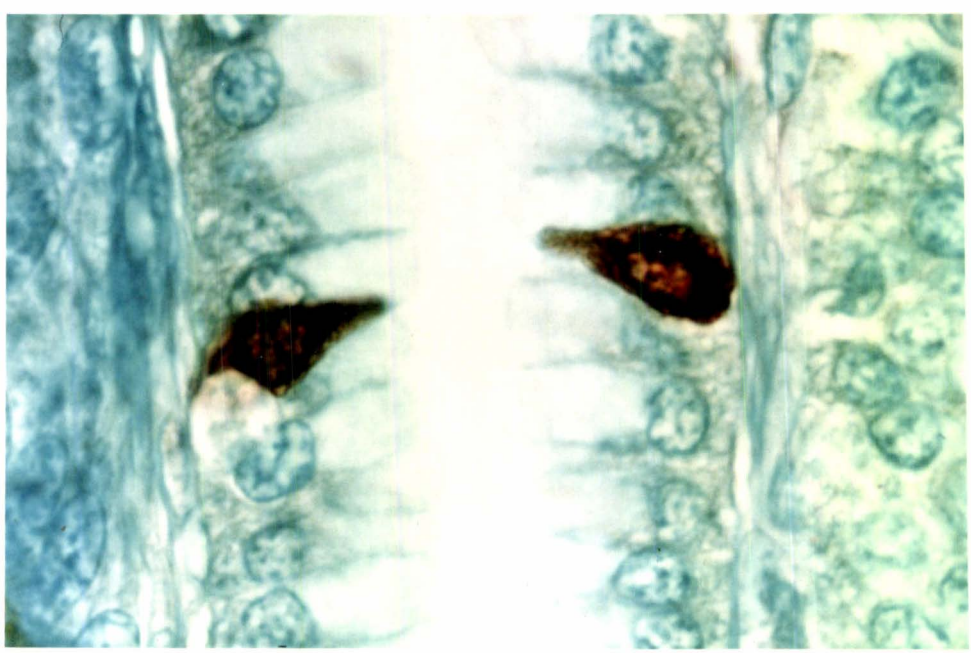


Figure 3.41: 'Closed' type cell (arrow) with metachromatic cytoplasmic staining, in depths of Brunner's gland. Proximal duodenum; toluidine blue method; magnification x 1850.

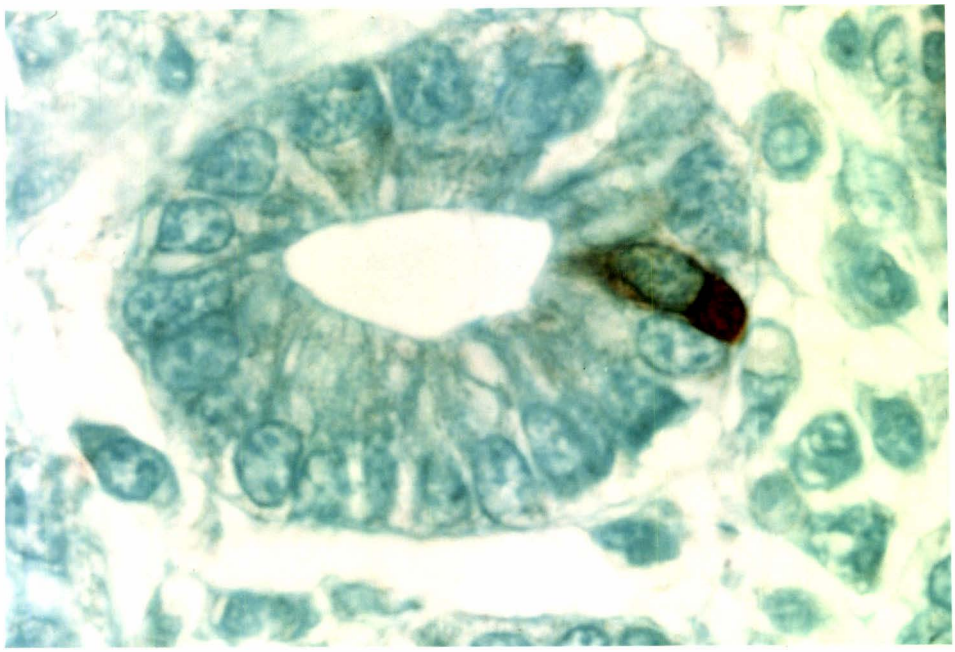
Figure 3.42: 'Closed' type cell (arrow) with metachromatic cytoplasmic staining, in duct of Brunner's gland. Proximal duodenum; toluidine blue method; magnification x 1850.

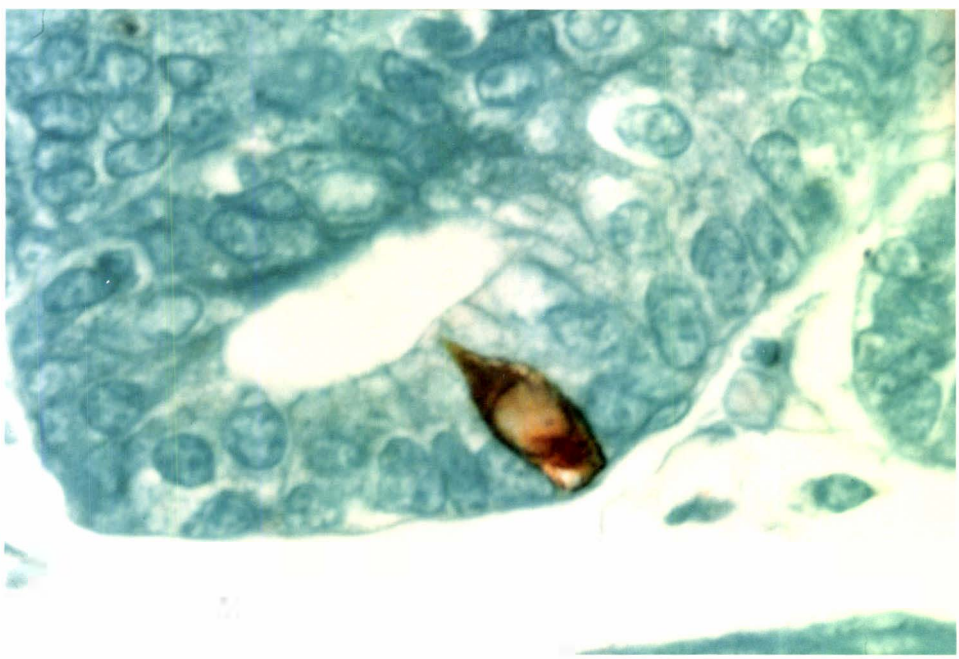
Figure 3.43: Intensely stained (brown) antral G cells, 'teardrop' or 'pyramidal' in shape, in antral gland. Abomasal antrum; gastrin antiserum As'74/PAP method; magnification x 1850.

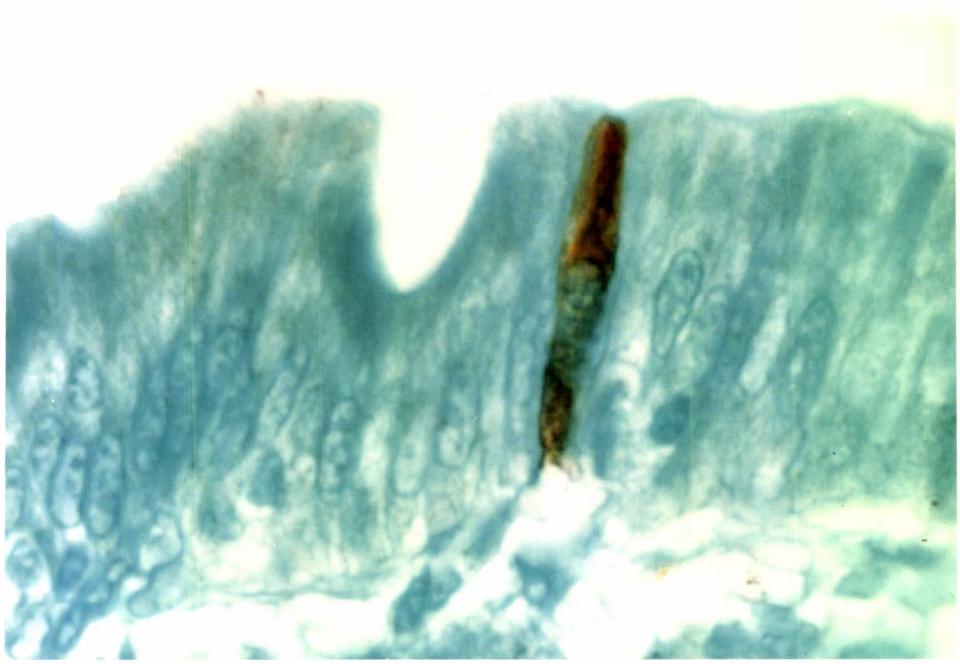
Figure 3.44: Antral G cell (brown) with sectory granules concentrated in infranuclear cytoplasm. Abomasal antrum; gastrin antiserum As'74/PAP method; magnification x 1850.

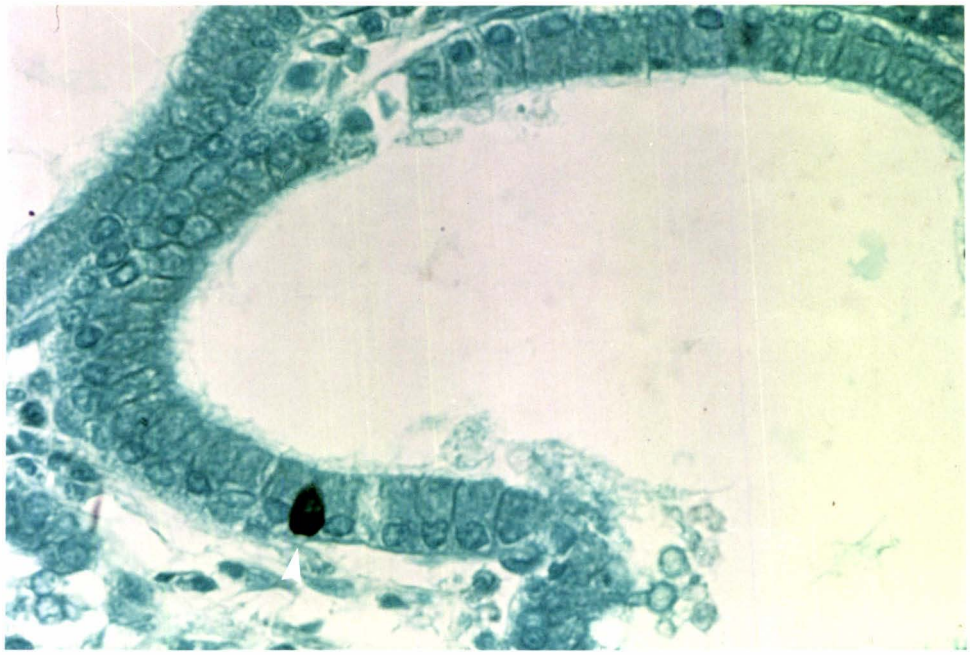
Figure 3.45: Intestinal G cell (brown), showing typical teardrop shape, near base of crypt of Lieberkuhn. Proximal duodenum; gastrin antiserum As'74/PAP method; magnification x 1850.

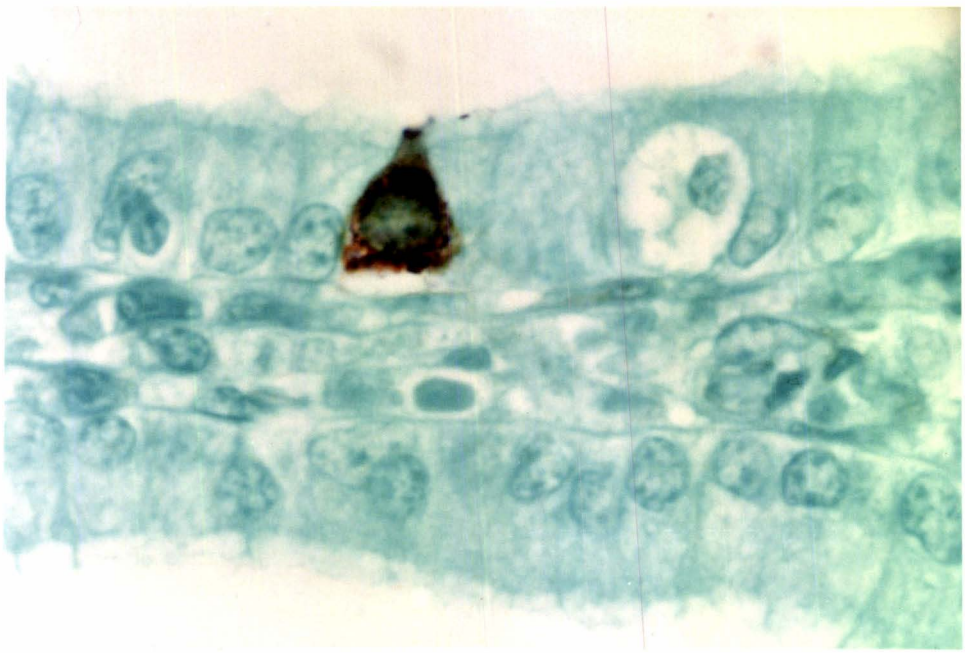
Figure 3.46: Intestinal G cell (brown) near tip of villus, showing typical columnar shape. Mid-duodenum; gastrin antiserum As'74/PAP method; magnification x 1850.











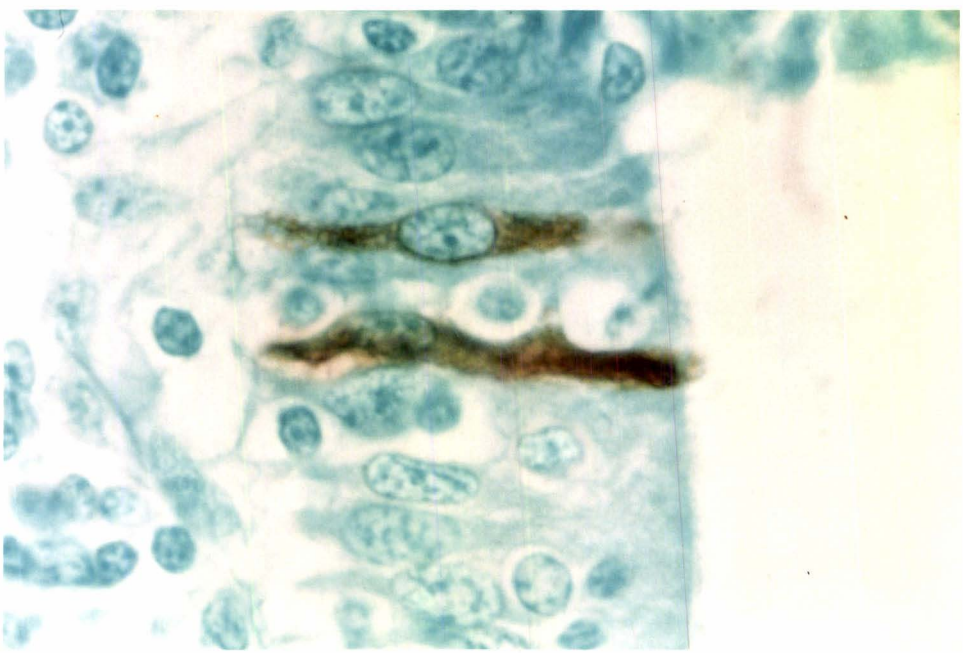


Figure 3.47: Immunoreactive G cell (arrow) in depths of Brunner's gland. Proximal duodenum; gastrin antiserum As'74/PAP method; magnification x 740.

Figure 3.48: Intensely stained (brown), pyramidal shaped S cell in crypt of Lieberkuhn. Proximal duodenum; secretin antiserum/PAP method; magnification x 1850.

Figure 3.49: Columnar shaped intestinal S cells (brown) near tip of villus. Mid-duodenum; secretin antiserum/PAP method; magnification x 1850.

3.3.4 Distribution of Endocrine Cells

The distributions of argyrophilic and argentaffinic cells are shown in Fig. 3.50.

Large numbers of argyrophilic cells were revealed by the De Grandi and Sevier-Munger techniques in the abomasal body (119 ± 35 and 153 ± 6 $c.mm^{-2}$), proximal (146 ± 50 and 122 ± 36 $c.mm^{-2}$) and mid-duodenum (152 ± 8 and 149 ± 8 $c.mm^{-2}$), with decreasing densities in more distal sites. Argyrophilic cells were rare (<5 $c.mm^{-2}$) in the abomasal antrum and glands of Brunner.

In the abomasal body, argyrophilic cells were commonly found scattered in the epithelium of the lower half of the gastric glands (Fig. 3.51), while in the antral region they occurred only in the depths of the glands. Argyrophilic cells were found scattered throughout the intestinal epithelium, but were most numerous in the lower half of the crypts of Lieberkuhn (Fig. 3.52) where they often occurred in small groups. The argyrophilic cells of the glands of Brunner occurred as single cells in the depths of the glands and gland ducts (Fig. 3.53), or in small groups (Fig. 3.54).

Argentaffin cells (i.e. those cells stained by the Masson-Hamperl technique) were most common in the proximal (102 ± 14 $c.mm^{-2}$), mid (119 ± 20 $c.mm^{-2}$) and distal (39 ± 11 $c.mm^{-2}$) duodenum. Few (<10 $c.mm^{-2}$) argentaffin cells were found in the abomasal body, while in the abomasal antrum, ileum, colon and caecum they were rare.

Although argentaffin cells were found scattered throughout the mucosal thickness in the abomasal body and intestine, they were most numerous in the lower half of the mucosa. In the abomasal antrum they were confined to the deeper parts of the antral glands. In the duodenal mucosa argentaffin cells commonly occurred in groups while at other sites, including the glands of Brunner, they generally occurred as single cells in the depths of the glands or in the epithelium lining the ducts of the glands.

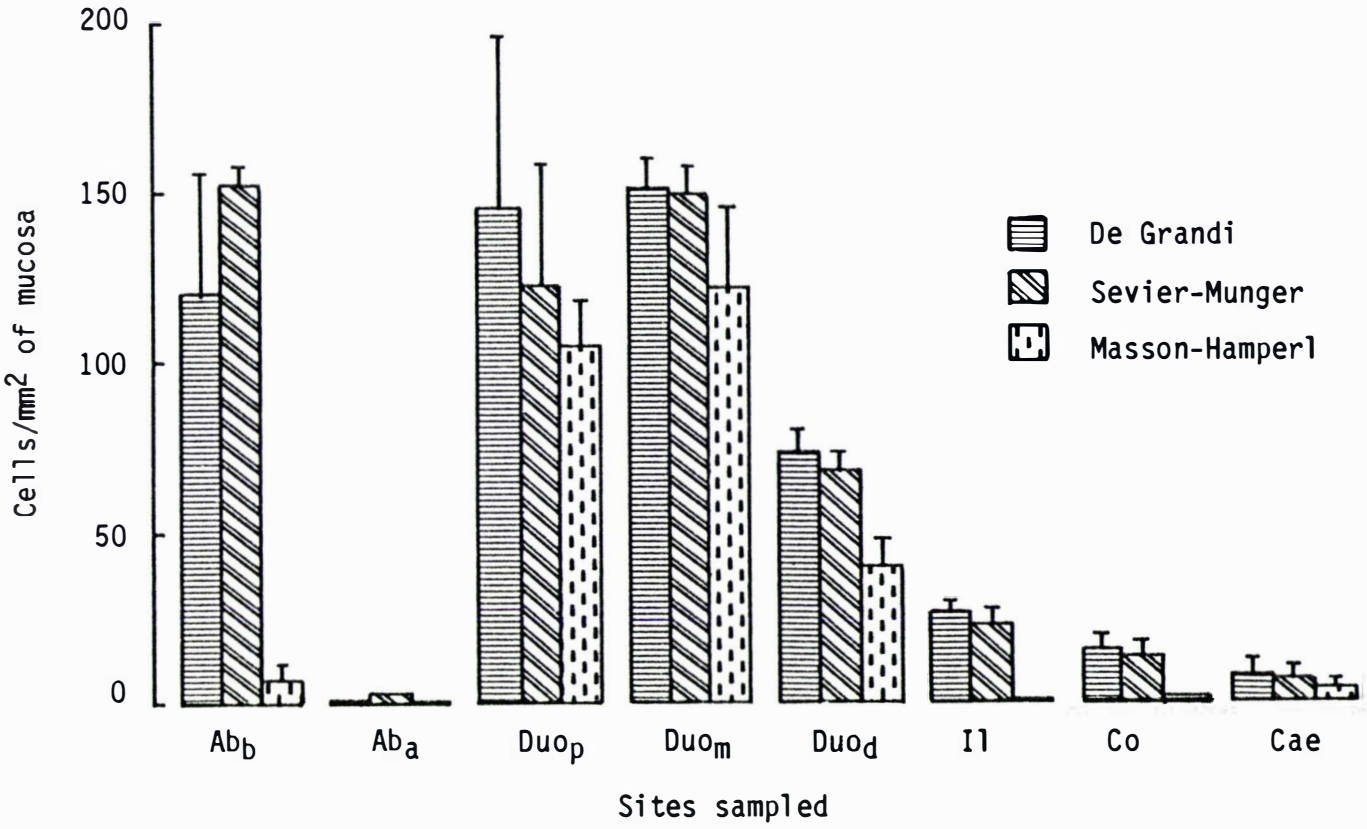
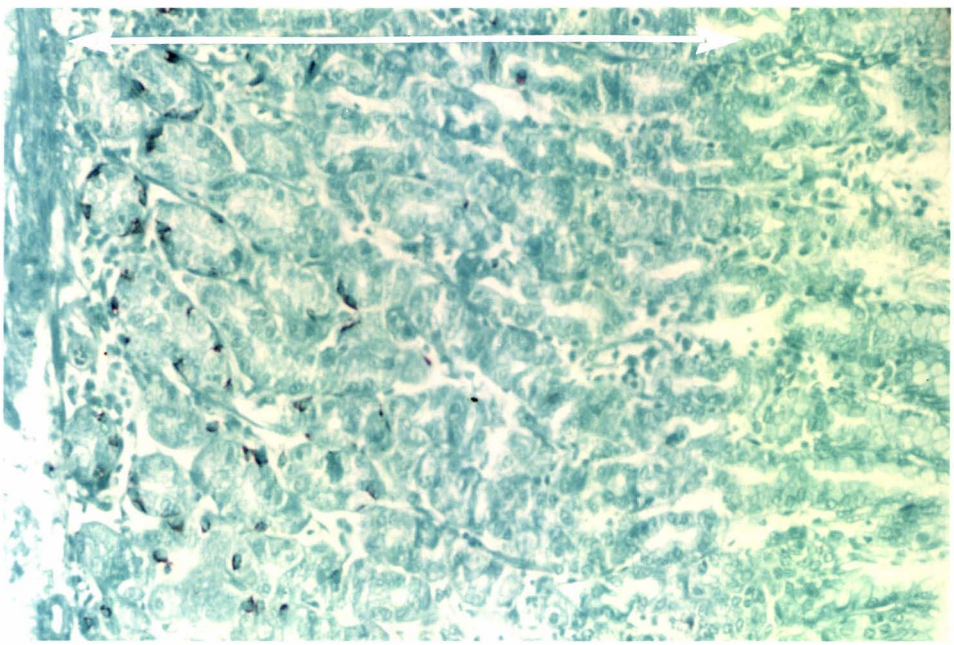
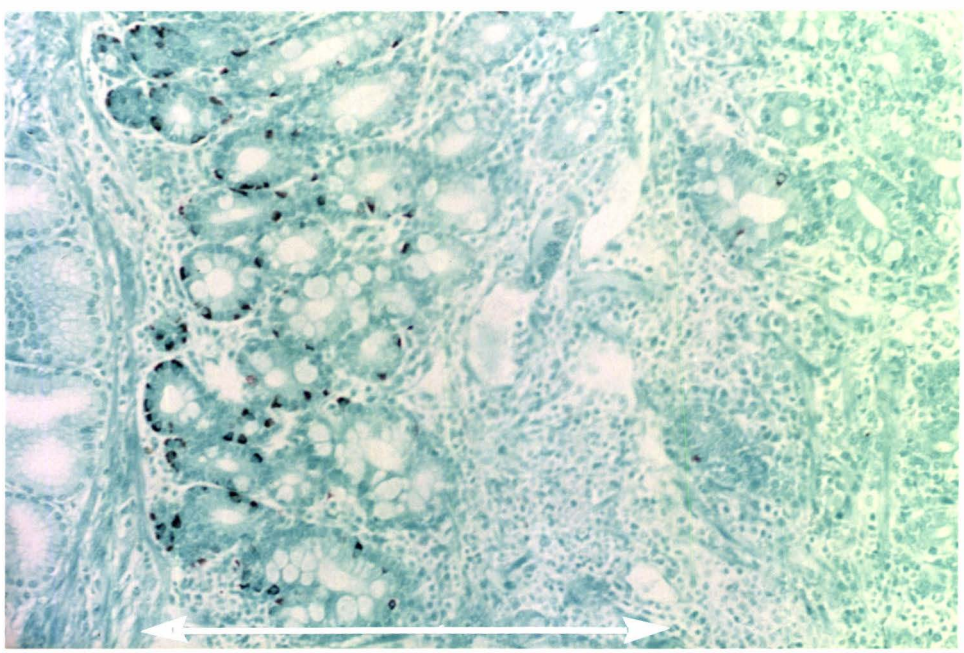


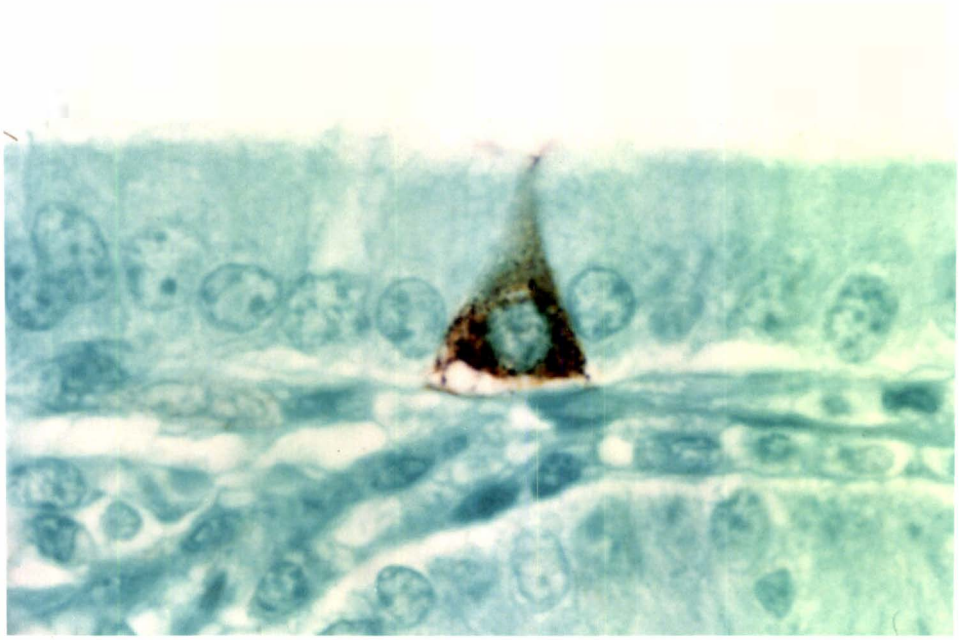
Figure 3.50: Mean (+SEM) density of argyrophilic and argentaffinic cells in the gastrointestinal tract of adult sheep (n = 4) as demonstrated by the De Grandi, Sevier-Munger and Masson-Hamperl techniques.

Figure 3.51: Argyrophilic cells (black) in deep half of gastric glands. Depth of glands indicated by arrows. Abomasal body; De Grandi method; magnification x 300.

Figure 3.52: Argyrophilic cells (black) in deep half of crypts of Lieberkuhn. Depth of crypts indicated by arrows. Proximal duodenum; De Grandi method; magnification x 300.







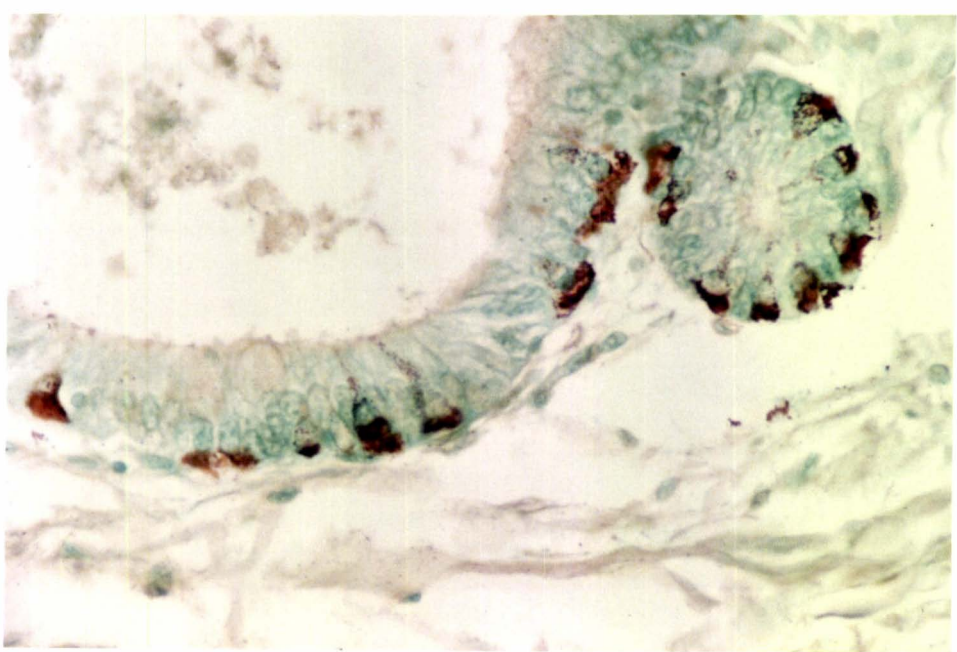


Figure 3.53: Pyramidal shaped argyrophilic cell (brown) in duct of Brunner's gland. Proximal duodenum; De Grandi method; magnification x 1850.

Figure 3.54: Small groups of argyrophilic cells (brown) in depths of Brunner's gland. Proximal duodenum; De Grandi method; magnification x 740.

EC cells, stained by the fast garnet technique, were most common in the duodenum where they ranged in density from 182 ± 37 $c.mm^{-2}$ in the mid-duodenum to 79 ± 13 $c.mm^{-2}$ in the distal duodenum (Fig. 3.55). They were uncommon in the abomasal body, ileum, colon and caecum and rare in the abomasal antrum. No EC cells were identified in the pancreas.

In the body of the abomasum, EC cells generally occurred singly and were most common in the lower half of the gastric glands. Intestinal EC cells were most common in the depths of the crypts of Lieberkuhn, where they frequently occurred in small clusters (Fig. 3.56). A few EC cells were identified in the glands of Brunner.

Immunoreactive G cells occurred in highest densities in abomasal antral mucosa (155 ± 11 $c.mm^{-2}$). G cells were also common in the duodenum, particularly the proximal and mid-duodenum, but were less numerous (<40 $c.mm^{-2}$) than in the abomasal antrum (Fig. 3.57). No G cells were found in the abomasal body, ileum, colon, caecum or pancreas.

Antral G cells, although found scattered throughout the epithelium of the glands, were most common in the middle and upper regions of the glands (Fig. 3.58). Intestinal G cells (Fig. 3.59) were most common in the deeper parts of the duodenal crypts of Lieberkuhn. A small number of G cells were observed in the depths of the glands of Brunner (Fig. 3.60).

Immunoreactive S cells were restricted in their distribution to the small intestine. Here they were most common in the proximal duodenum (34 ± 12 $c.mm^{-2}$) and decreased in density in more distal mucosal samples (Fig. 3.57).

Although S cells were found scattered throughout the depth of the small intestinal crypts and villi, they were most common in the upper parts of the crypts. There were no S cells in the glands of Brunner.

Cells that stained with the antiserum to pancreatic glucagon were identified in both pancreatic islets and pancreatic exocrine acini. In the islets they were frequently irregular in shape and occurred mainly near the periphery of the islet (Fig. 3.61). Immunoreactive cells in

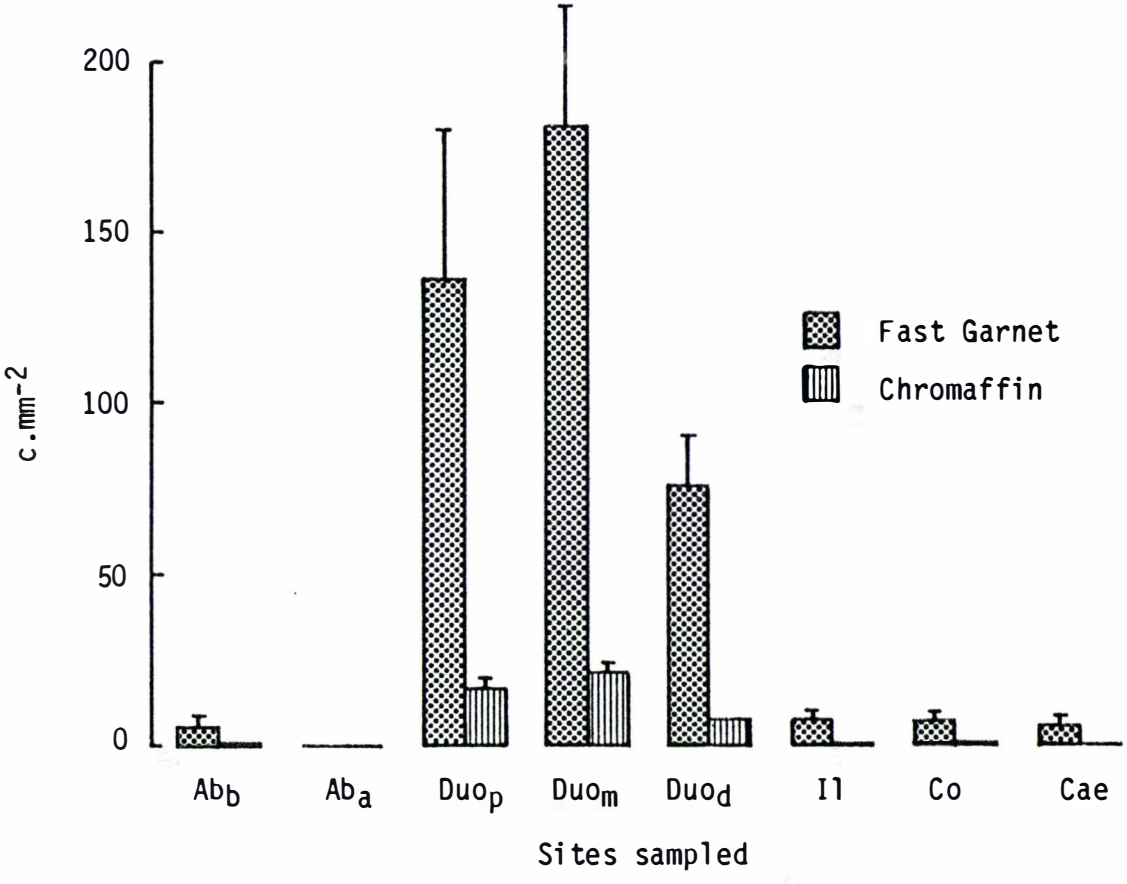


Figure 3.55: Mean (+ SEM) density of EC cells in the gastrointestinal tract of adult sheep (n = 4) as demonstrated by the diazonium and chromaffin techniques.

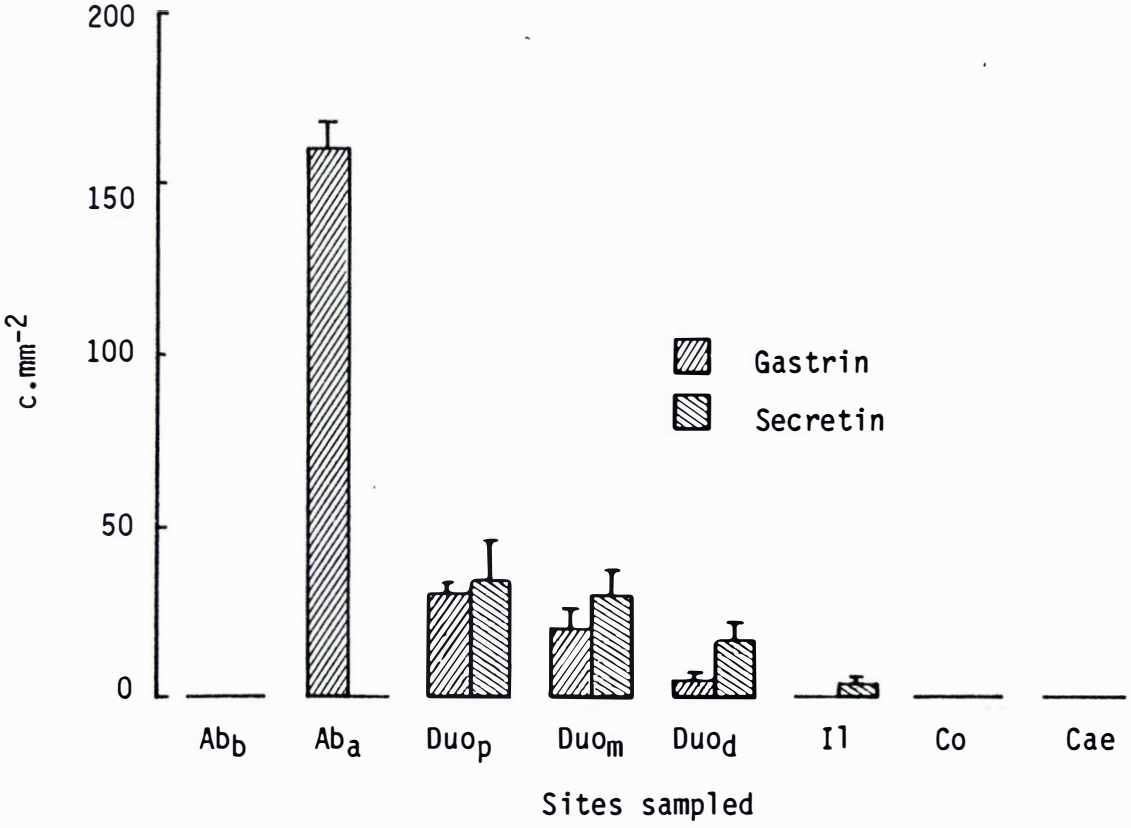
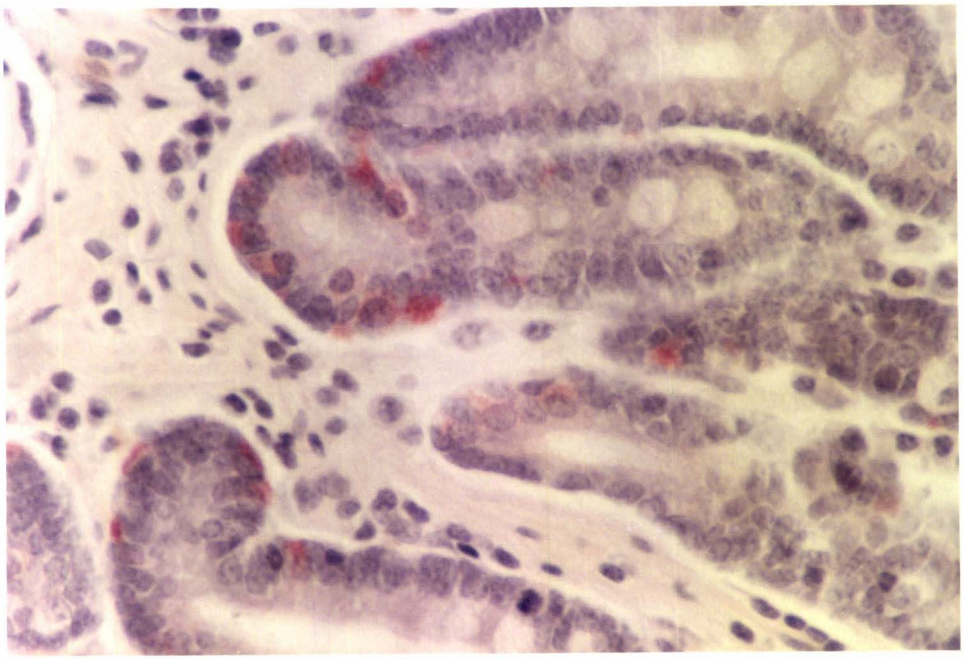


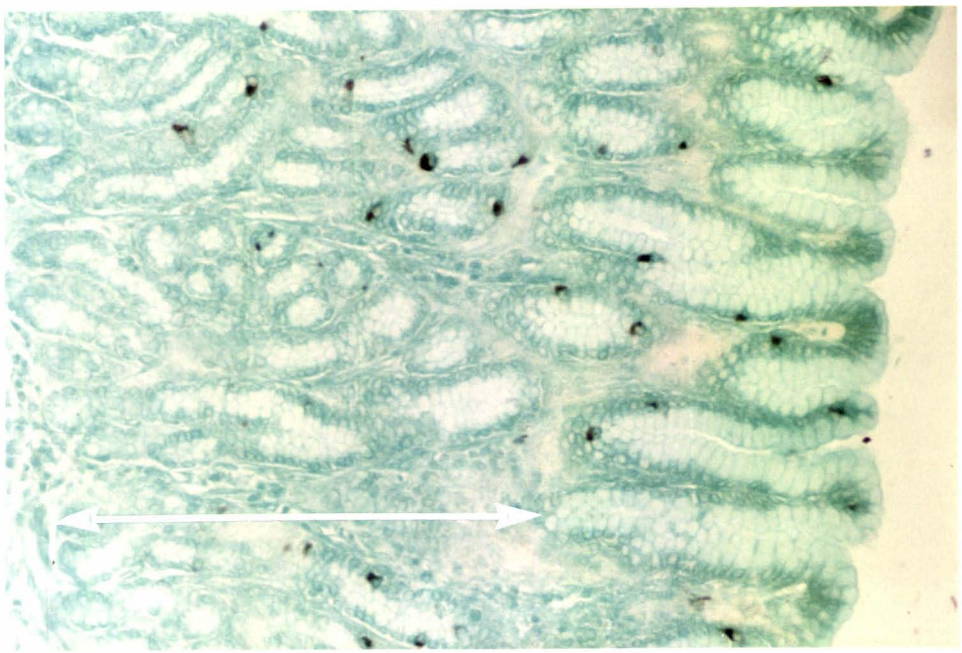
Figure 3.57: Mean (+ SEM) density of gastrin and secretin containing cells in the gastrointestinal tract of adult sheep (n = 4)

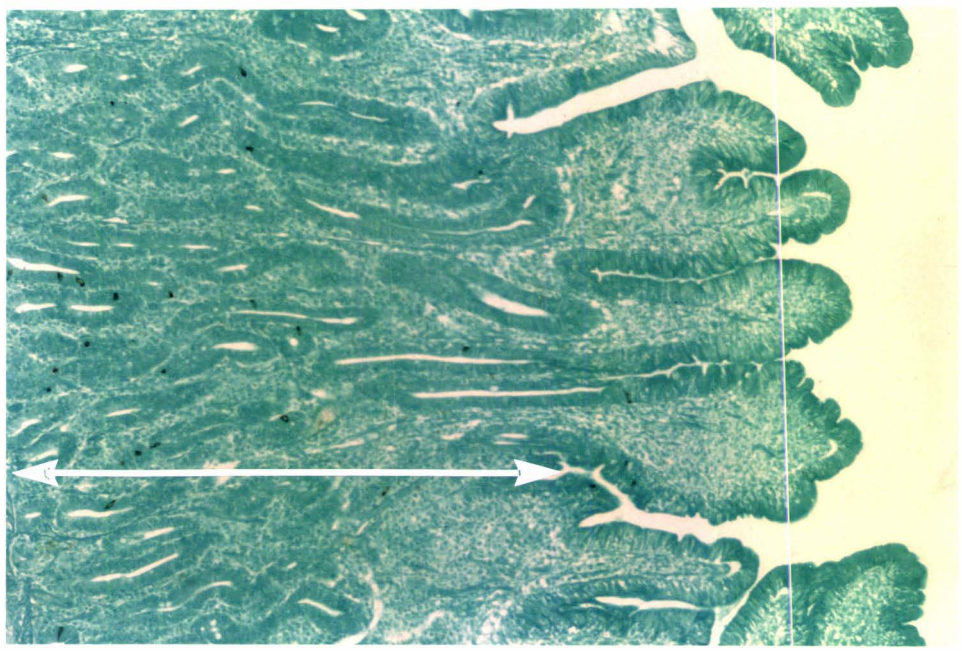
Figure 3.56: Small clusters of intestinal EC cells (red) near bases of crypts of Lieberkuhn. Proximal duodenum; fast garnet method; magnification x 740.

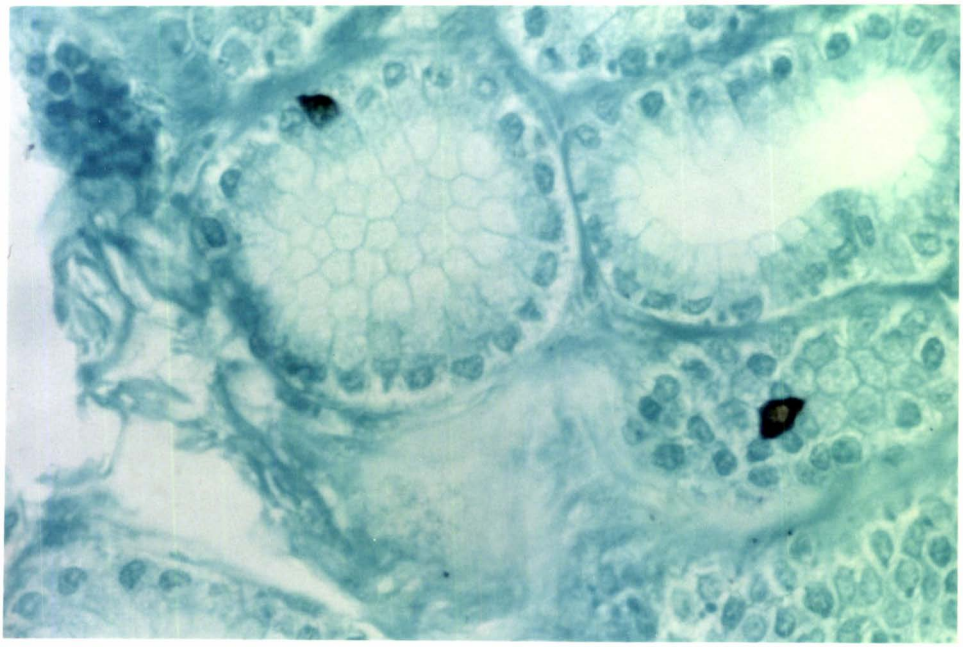
Figure 3.58: Antral G cells (black) mainly in upper two thirds of antral glands. Depth of glands indicated by arrows. Abomasal antrum; gastrin antiserum As'74/PAP method; magnification x 300.

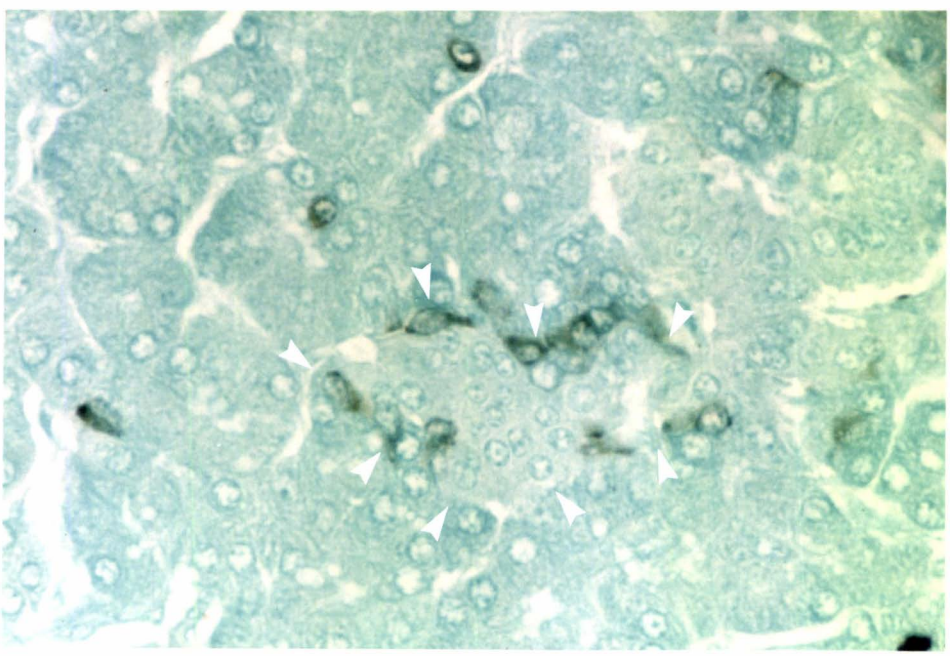
Figure 3.59: Intestinal G cells (black) mainly in lower two thirds of crypts of Lieberkuhn. Depth of crypts indicated by arrows. Proximal duodenum; gastrin antiserum As'74/PAP method; magnification x 230.











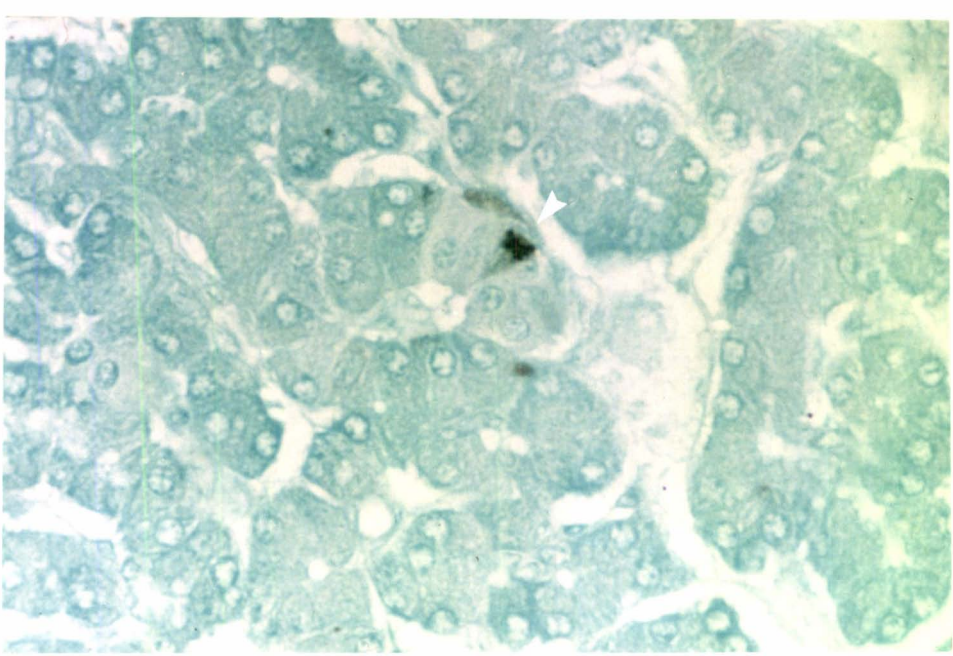


Figure 3.60: Immunoreactive G cells (black) in Brunner's glands. Proximal duodenum; gastrin antiserum As'74/PAP method; magnification x 740.

Figure 3.61: Immunoreactive A cells (black), of variable shape, mainly near periphery (indicated by arrows) of islets of Langerhans. Pancreas; glucagon antiserum/PAP method; magnification x 740.

Figure 3.62: Immunoreactive A cell (arrow) in pancreatic exocrine acinus. Pancreas; glucagon antiserum/PAP method; magnification x 740.

the exocrine acini were pyramidal in shape with most of the staining in the basal part of the cell, adjacent to the vascular supply (Fig. 3.62).

No pancreatic glucagon immunoreactive cells were found in the mucosa of the GI tract.

No endocrine cells were identified, using any of these staining techniques, in mucosal samples from the reticulum or rumen.

Analysis of variance (Table 3.7) demonstrated a significant ($P<0.001$) overall difference in cell density between animals. This was largely due to low counts obtained from two sites in sheep No. 2. Thus, low endocrine cell counts were obtained for the abomasal body region using the De Grandi technique, and for the proximal duodenum by the De Grandi, Sevier-Munger and fast garnet techniques. These low counts undoubtedly contributed to the large SEM for these two sites (see Figs. 3.50 and 3.55).

Overall, there were significantly ($P<0.001$) fewer EC cells, as demonstrated by the fast garnet method, than argyrophilic cells (Table 3.7, component C(ii)). The cell densities and distribution patterns of EC and argyrophilic cells within the duodenum were similar (Figs. 3.50 and 3.55), whereas there were significantly ($P<0.001$) more argyrophilic than EC cells in the abomasal body region (Table 3.7, interaction component D(i)).

The distribution of S cells differed from those of both G cells (Table 3.7, interaction components D(iv) and (v)) and argyrophilic cells (Table 3.7, interaction components D(xiv-xvi)) in that they were found only in the small intestine. Overall S cells also were less numerous ($P<0.05$) than G cells (Table 3.7, component C(iii)) and much less numerous ($P<0.001$) than argyrophilic cells (Table 3.7, component C(vi)).

Table 3.7: Summary of analysis of variance of data for staining methods which demonstrate argyrophilic, EC, G and S cells

Source of Variation		DF	Variance Ratios
A. Animals		3	9.39***
B. Sites		7	
(i)	Ab_b vs Ab_a	1	10.86**
(ii)	Duo_p + Duo_m vs Duo_d	1	36.58***
(iii)	Duo vs Il	1	134.11***
(iv)	Ab vs SI	1	59.10***
(v)	Ab + SI vs LI	1	164.09***
	Remainder	2	1.88
C. Staining methods		4	
(i)	DG vs SM	1	0.07
(ii)	DG + SM vs FG	1	13.27***
(iii)	Gas vs Sec	1	5.70*
(iv)	Histo vs Immuno	1	161.36***
Non-orthogonal contrasts			
(v)	DG + SM vs Gas	1	86.56***
(vi)	DG + SM vs Sec	1	145.10***
D. Interaction: sites x staining methods		28	
(i)	Ab_b vs Ab_a x DG + SM vs FG	1	33.45***
(ii)	Duo vs Il x DG + SM vs FG	1	4.44*
(iii)	Duo vs Il x DG + SM vs FG	1	25.44***
(iv)	Ab_b vs Ab_a x Gas vs Sec	1	49.10***
(v)	Ab vs SI x Gas vs Sec	1	43.98***
(vi)	Ab_b vs Ab_a x Histo vs Immuno	1	137.78***
(vii)	Duo_p vs Duo_m x Histo vs Immuno	1	5.55*

Table 3.7 continued

(viii)	Duo vs Il x Histo vs Immuno	1	14.61***
(ix)	Ab vs SI x Histo vs Immuno	1	44.62***
(x)	Ab + SI vs LI x Histo vs Immuno	1	8.47**
	Remainder	18	2.04
	Non-orthogonal contrasts		
(xi)	Ab_b vs Ab_a x DG + SM vs Gas	1	216.75***
(xii)	Duo vs Il x DG + SM vs Gas	1	4.28*
(xiii)	Ab vs SI x DG + SM vs Gas	1	50.79***
(xiv)	Ab_b vs Ab_a x DG + SM vs Sec	1	43.97***
(xv)	Duo vs Il x DG + SM vs Sec	1	4.95*
(xvi)	Ab + SI vs LI x DG + SM vs Sec	1	11.31**
	Residual mean square	117	2.73

3.4 Discussion

3.4.1 Reliability and Specificity of Staining Techniques

It is clear from the results presented in Section 3.3 that the De Grandi, fast garnet and PAP immunohistochemical methods were reliable. Valid comparisons of data obtained on different days was possible as it was established that day to day variations in results were less than the between animal variances (see Section 3.3.1).

The cross-reactivity study established that the gastrin antiserum (As'74) was the only primary antiserum to show immuno-reactivity with any peptide or peptide fragment, other than the peptide against which it had been raised. As the partial inhibition of As'74 took the form of lighter staining of cells and not a reduction in endocrine cell count, it is concluded that this antiserum contains a high proportion of antibodies which bind with a portion of the gastrin molecule other than the C-terminal tetrapeptide. However, the antiserum probably also contains very small amounts of antibodies that are specific for the C-terminal tetrapeptide of gastrin/CCK. As the duodenal endocrine cells stained by this antiserum were all intensely stained, it is concluded that they were duodenal G cells and not I cells.

3.4.2 Factors Affecting Staining Performance

In view of the reliability of the De Grandi and Sevier-Munger techniques, the reasons for the poor results obtained using the Grimelius and modified Masson-Hamperl techniques are not obvious. Other workers (Holmes, 1943; Romanes, 1950; Singh, 1964) have alluded to the capricious nature of silver staining techniques in general. Factors which have been implicated in failures of these techniques include: purity of the water and reagents used, temperature of solutions, concentration of reagents, and pH of solutions (see Young, 1969). Throughout the studies described in this thesis, the source of distilled water and sources of reagents remained unchanged. Care was taken to ensure that the concentration of reagents, pH and temperature at which staining was carried out was exactly as specified in the original methods. When it

had been demonstrated that the De Grandi technique was thoroughly reliable, reasons for the failure of other silver staining techniques were not pursued.

The fast garnet technique gave excellent staining results in the intestine, however, abomasal cells were poorly stained, probably due to the lower content of 5-HT in gastric EC cells (see Solcia et al., 1975a).

It is apparent that the chromaffin methods used in this study were unreliable when applied to the mucosa of the ovine GI tract. Although tissues were fixed in Zenker-formol fluid, which contains approximately 2% formaldehyde, and a post-fixation chromation step was carried out, staining intensity was generally poor. Even when the chromation step was buffered at pH 4.0 (Jaim-Etchevery and Zieher, 1968) the intensity of staining did not differ from that obtained using unbuffered dichromate or a chromate/dichromate mixture.

Previous studies (see Lillie, 1965) indicate that the chromaffin reaction is sometimes unreliable, however, no satisfactory explanation has been advanced for this. While there is some evidence that the presence of formalin or low pH (Hillarp and Hokfelt, 1955) inhibits the chromaffin reaction, Lillie (1985) noted that chromaffin staining was good following fixation of tissues in either Spuler, Orth or Kose's fluids, all of which contain approximately 4% formaldehyde. The reason(s) for the unsatisfactory chromaffin staining results in the present study remain unknown.

At all sites sampled, the chromaffin reaction was found to be less sensitive than the diazonium reaction in identifying EC cells. The number of chromaffin positive cells was always less than the number of diazonium positive cells and, in the abomasum in particular, chromaffin positive cells were frequently very poorly stained. It has been observed (see Solcia et al., 1975a) that the chromaffin method is not as sensitive as other techniques, specifically the argentaffin and diazonium methods, for demonstrating EC cells. In particular, gastric EC cells may escape detection by the chromaffin method because they frequently store less 5-HT than intestinal EC cells (Faustini, 1955).

Results presented here, however, demonstrate that the chromaffin method is less sensitive than the diazonium method at all sites studied and many intestinal EC cells also escape detection with the chromaffin method.

3.4.3 Staining Characteristics and Identification of Endocrine Cells

Many endocrine cells were demonstrated in the duodenum using the De Grandi, Sevier-Munger and fast garnet techniques. Cell densities in this region were similar with each of these staining methods indicating these three methods stained the same endocrine cell types(s). It is therefore concluded that the bulk of the argyrophilic cells found in the ovine duodenum are EC cells. This is supported by evidence (Solcia et al., 1975a) that in other species EC cells are also argyrophilic.

In the abomasal body region, however, EC cells represent only about 5% of the argyrophilic cells at this site. The question therefore arises as to what cell types this large population of argyrophilic, non-argentaffinic, diazonium negative cells represents. Many of these cells were of the 'closed' type, were most numerous in the deeper parts of the gastric glands and some had basal cytoplasmic processes. Cells which exhibit one or more of these characteristics include the A, D, D_1, ECL and X cells.

Evidence which suggests that a large proportion of the abomasal argyrophilic, non-argentaffin cells identified in these sheep represent ECL cells includes:

(a) In man (Solcia et al., 1975a) and rats (Hakanson et al., 1976) the majority of the argyrophilic, non-argentaffin cells of the fundic mucosa have been identified as ECL cells.

(b) In most mammalian species, but not the cat (Vassallo et al., 1969), ECL cells have been shown to be Grimelius positive (Solcia et al., 1969a), Sevier-Munger positive (Vassallo et al., 1969), but Masson-Hamperl negative (Polak et al., 1971a).

(c) In all species so far investigated, ECL cells have been found almost exclusively in the mucosa of the acid secreting portion of the stomach (Solcia et al., 1978; 1980; 1981a, b).

(d) ECL cells have been found to be of the 'closed' type (Capella et al., 1969; Vassallo et al., 1969), were most common in the deeper parts of the gastric glands and sometimes exhibited basal cytoplasmic processes (see Solcia et al., 1975a).

It is unlikely that either A, D, D_1 or X cells make up any significant part of the population of the argyrophilic, non-argentaffin cells of the abomasal body for the following reasons:

(a) Rizzotti et al., (1980b) were unable, in an EM study, to identify A cells in bovine abomasal mucosa. This confirmed the findings of their earlier histochemical studies (Rizzotti et al., 1976), showing A cells were absent from the abomasa of both sheep and cattle.

(b) In other species D cells have been shown to be consistently unreactive with the Grimelius (see Solcia et al., 1975a), Sevier-Munger (Vassallo et al., 1969; Capella and Solcia, 1972) and Masson-Hamperl (Solcia et al., 1975b) techniques. Similar staining characteristics have been reported for bovine antral D cells (Rizzotti et al., 1980a).

(c) Although Rizzotti et al., (1979) have demonstrated that D_1 cells found in bovine rectal mucosa were Grimelius positive and Masson-Hamperl negative, D_1 cells were scarce in bovine gastric mucosa (Rizzotti et al., 1980b).

(d) X cells, in other species, are both Sevier-Munger and Masson-Hamperl negative (Vassallo et al., 1969), however, their reactivity with the Grimelius technique varies with species (see Solcia et al., 1969a; Polak et al., 1971a).

It is now generaly agreed that the component of EC cell secretory granules that gives a positive (i.e. red) diazonium reaction with fast garnet, is 5-HT (Hakanson et al., 1970). Cells other than EC cells

which have been shown to contain 5-HT include ECL cells in the cat and rabbit, A cells of the porcine pancreas, and CTMC's in the rat, mouse, horse and dog (see Solcia et al., 1969b). However, ovine ECL cells contain little or no 5-HT, for although there may be considerable numbers of ECL cells in the abomasal body, there is little 5-HT present there as judged by diazonium staining.

Data presented in Figs. 3.50 and 3.57 indicate that ovine antral G cells are non-argyrophilic. This conclusion follows from the observation that G cell densities are high while argyrophilic cell densities are particularly low at this site. These results are at variance with those presented by Rizzotti et al. (1976) who showed that bovine antral G cells are Grimelius positive. While the majority of workers are agreed that antral G cells, in animals with a simpler form of stomach, are argyrophilic (see Solcia et al., 1975a), McGuigan and Greider (1971) claimed porcine G cells were non-argyrophilic. Such discrepancies have not been adequately explained and largely have been ignored.

It is not clear from comparisons of argyophilic and G cell densities whether duodenal G cells are also non-argyrophilic. Because no accurate assessmment of the number of argyrophilic, non-EC cells in the duodenum can be derived from the data presented here, no valid comparison can be made between the densities of this population and that of G cells in the duodenum. Duodenal argyrophilic cells could not be identified, using the techniques described in this thesis, as belonging to any specific endocrine cell type. Thus, in order to determine whether duodenal G cells had histochemical staining characteristics similar to those of antral G cells would require the use of dual or sequential staining techniques on the same tissue section, or the identification of G cells by electron microscopy. Recent studies have demonstrated that G cells found in the small intestine (i.e. IG and TG cells) differ morphologically from antral G cells (see Solcia et al., 1981), and may be distinguished from them using region specific antisera (Larsson and Rehfeld, 1979; Buchan et al., 1979). Whether these morphological and immunohistochemical differences are reflected in differences in histochemical staining characteristics, or function, has yet to be determined.

Observations presented here indicate that ovine S cells have morphology and distribution patterns similar to those described for other species. In the present study, however, no attempt was made to determine the histochemical staining characteristics of ovine S cells. To do so would have required the use of double staining techniques or the use of electron microscopy for ultrastructural identification of S cells. Such techniques were not used in the current study. However, in other species S cells have been found to be Grimelius positive (Vassallo et al., 1971), Masson-Hamperl negative (Bussolati et al., 1971), while results were somewhat equivocal with the Sevier-Munger technique (Solcia et al., 1975b).

The absence of any endocrine cells in the mucosa of the GI tract which cross-reacted with the pancreatic glucagon antiserum indicates that A cells are absent from the mucosa of the ovine GI tract. However, L cells, which contain GLI, a peptide structurally related to pancreatic glucagon (see Sundby and Moody, 1980; Moody and Thim, 1983), have been identified in the mucosa of the ovine colon and caecum (Rizzotti et al., 1979). These workers demonstrated that ovine L cells were Masson-Hamperl negative but weakly stained by the Grimelius technique. L cells were not found in the present studies probably because of the high specificity of the antiserum used, indeed, information provided by the supplier indicated that in RIA this antiserum was specific for pancreatic glucagon and did not cross-react with gut glucagon. In order to demonstrate L cells an N-terminally directed glucagon antiserum should be used (see Orci and Perrelet, 1981).

3.4.4 Distribution of Endocrine Cells

Results presented here demonstrate that the ovine abomasum and duodenum are richly endowed with endocrine cells, and cell densities decrease in more aboral samples. The forestomach, however, is devoid of endocrine cells. These findings are supported by those of Rizzotti et al., 1976).

Argyrophilic cell densities were greatest in the abomasal body and duodenum. Very few argyrophilic cells were found in the abomasal

antrum. This finding is at variance with those of other workers (see Solcia et al., 1975a) who found large numbers of argyrophilic cells in the antral region of the stomach of other species. The reason for this discrepancy is that ovine G cells, unlike those of most other species, are non-argyrophilic (see Section 3.4.3).

EC cell distribution followed a similar pattern to that for argyrophilic cells, because EC cells are also argyrophilic and at most sites examined make up the bulk of the argyrophilic cells present. The notable exception was the abomasal body where most of the argyrophilic cells are ECL cells. EC cells are not common in the ovine abomasum whereas in species with a simpler form of stomach they are generally numerous (see Solcia et al., 1975a). It is not clear from results presented here whether EC cells are truly uncommon in the abomasum or whether low cell densities observed with the fast garnet technique resulted from poor staining due to the low 5-HT content of abomasal EC cells. Such uncertainties could be resolved by identification of EC cells by electron microscopy or by the use of serotonin specific antisera (Facer et al., 1979; Buffa et al., 1980; Inokuchi et al., 1982).

Recently, EC cells have been identified in human intestinal mucosal samples using a rabbit serotonin antiserum and the PAP technique (Inokuchi et al., 1983). These workers reported that this technique was more sensitive than either the Masson-Hamperl or Grimelius silver impregnation techniques. It is therefore likely that results reported in this thesis underestimate EC cell densities, particularly in regions where levels of stored 5-HT are low.

Data presented here, while not conclusive, indicate that ECL cells form a large part of the endocrine cell population of the abomasal body. While these results do not exclude the possibility that ECL cells are found at other sites within the GI tract, it is clear that they do not form a large proportion of endocrine cells at any other site. In other species ECL cells are confined in their distribution to the body region of the stomach (see Solcia et al., 1975a).

G cells were most numerous in the antrum, but are also found in the upper small intestine. S cells, which are confined in distribution to the small intestine are most numerous in the proximal duodenum and decrease in density in more distal samples. Distribution of these two cell types is similar to that described for other species (see Bloom and Polak, 1978).

3.4.5 Identification of Non-endocrine Cells

Several different non-endocrine cell types within the mucosa of the GI tract were found to give positive staining reactions with the fast garnet, alcian blue and toluidine blue methods. Cell types stained by these methods include mast cells (both CTMC's and MMC's) and globular leucocytes.

The connective tissue cells stained by fast garnet were morphologicaly similar to those connective tissue cells stained an intense blue by acidic alcian blue. These cells are probably ovine CTMC's. The present observations suggest that there is considerable variation in the amount of 5-HT contained in ovine CTMC's: some contain very little 5-HT and therefore their cytoplasm appears predominantly yellow or yellow-brown, while those containing larger quantities of 5-HT have red staining cytoplasmic granules. Mast cells, however, were never observed to stain with the same intensity of red as intestinal EC cells, indicating that mast cells have a lower 5-HT content. The yellow or yellow-brown cytoplasmic staining described here in ovine CTMC's is due to the presence of dopamine (Falck *et al.*, 1964).

It was concluded that the small, generally round, epithelial cells observed in the abomasum, intestine and glands of Brunner, that were stained yellow with fast garnet, were ovine MMC's. Cells of similar morphology and distribution, and which therefore also were MMC's, were stained with alcian blue and toluidine blue. This conclusion is supported by the evidence of Morales *et al.* (1980) who demonstrated the existence of MMC's in the epithelium of the bovine gall bladder. Studies by Toledo *et al.* (1981) have shown that in ruminants MMC's are both alcian blue and intensely toluidine blue positive.

The evidence presented above indicates that in sheep MMC's contain little or no 5-HT, while many CTMC's contain detectable quantities of 5-HT. This result is in accord with similar findings in animals with a simpler form of stomach (Enerback, 1981; Bienenstock et al., 1982).

The large cells found scattered throughout the epithelium of the abomasum and intestine, and which were characterized by the presence of very large cytoplasmic granules, were globular leucocytes. Their granules stained yellow or yellow-brown with fast garnet and deep blue with toluidine blue. These staining characteristics indicate the probable presence of a monoamine, but the absence of 5-HT, a conclusion supported by the fluorescence studies of Murray et al. (1968). Further, the staining characteristics and morphology of the cell type described here are similar to the ovine globular leucocytes described by the above workers.

The cells, (Fig. 3.28) found in the ducts of Brunner's glands, with staining characteristics similar to those described above for globular leucocytes, may also be globular leucocytes, although the possibility that they were MMC's cannot be excluded. Cytoplasmic staining was more even than that of most globular leucocytes and the very large cytoplasmic granules, typical of globular leucocytes, were not seen.

The cells identified, in acid alcian blue/haematoxylin and eosin stained sections, as flattened cells, forming a continuous sheath, one cell thick and closely associated with the basal lamina of gastric pits, are not endocrine cells but fibroblasts of the subepithelial fibroblast sheath. While many of these cells demonstrated a pale pink cytoplasmic staining, some contained alcian blue positive cytoplasmic granules. The toluidine blue positive cells found in the same region and with similar morphology represent the same cell type. Marsh and Trier (1974) described a similar cell type in the jejunum of the mouse. They identified these cells as fibroblasts and demonstrated that they formed a continuous, thin, subepithelial, fibroblast sheath which was closely associated with the basal lamina, particularly in the depths of the crypts of Lieberkuhn. These cells were described as containing a few deeply stained, toluidine blue positive, cytoplasmic granules which were thought to contain lysozymes. Although a subepithelial fibroblast

sheath has also been described in the colon of man (Kaye et al., 1968) and rabbits (Pascal et al., 1968; Kay et al., 1968), this is the first description of a subepithelial fibroblast sheath in the abomasa of sheep.

3.4.6 Conclusion

The De Grandi and fast garnet techniques gave excellent staining intensity and repeatable results. The De Grandi method demonstrates several types of endocrine cell, however, not all endocrine cells are argyrophilic. In the present study it gave the best estimate of total endocrine cell densities. A better estimate could be obtained with an immunohistochemical technique using an antiserum to neuronal specific enolase as the primary antiserum (Schmechel et al., 1978; Bishop et al 1982). The diazonium technique, using fast garnet, stained EC cells and some non-endocrine cells, however, EC cells were always readily distinguished from other cell types on the basis of differences in morphology.

The alcian blue and toluidine blue techniques were included in this study primarily to assist in identifying non-endocrine cells which might be mistaken for endocrine cells. In this respect they proved most useful in demonstrating CTMC's, MMC's, globular leucocytes and subepithelial fibroblasts. Having established the morphology and distributions of such cells, and demonstrated that they could readily be distinguished from endocrine cells, these methods were discontinued.

Although the Sevier-Munger technique gave reliable results they did not differ significantly from those obtained with the De Grandi method. It had been hoped that when used in combination with the De Grandi technique it would be possible to distinguish between ECL and G cells. This was not possible because ovine G cells proved to be non-argyrophilic thus, as the Sevier-Munger technique did not add any new information to that obtained with the De Grandi method, it was not used in subsequent experiments.

The PAP immunohistochemical technique was found to work well and with the appropriate primary antisera, was found to specifically stain

G, S and A cells. The technique was continued in all subsequent studies.

Because of the general unsatisfactory results obtained with the Masson-Hamperl, chromaffin, lead-haematoxylin and xanthydrol methods, they were not used in later studies.

CHAPTER 4

ENDOCRINE CELLS IN THE GI TRACT OF OVINE FOETUSES

4.1 Introduction

Few endocrine cell types have been identified in the mucosa of the GI tract of the ovine foetus: only EC (Tehver, 1930), G (Lichtenberger et al., 1981) and endocrine cells containing substance P (Harrison and Wathuta, 1982b) in the abomasum and small intestine have been described (see Section 1.7.5).

This scant information has been supplemented by papers describing the presence of GI hormones in gut mucosal extracts and plasma of foetal lambs. That literature, relating only to gastrin, GLI and PP is reviewed in Section 1.8.5 of this thesis.

No data has been published on the densities of endocrine cells in foetal sheep.

The purpose of this experiment was to undertake a systematic study of GI endocrine cells in foetal lambs, in particular, to establish the endocrine cell types present, as well as their densities, distribution patterns and histochemical staining characteristics. *

4.2 Methods

Foetuses (Table 2.1) from four pregnant Romney-cross ewes, aged 5 - 6 years and weighing 64 - 70 kg, were used in this study. Foetal age, determined from conception dates and crown-rump length measurements, was between 100 and 110 days of gestation.

Ewes were anaesthetized, as described in Section 2.2, and the foetus delivered via a hysterotomy. The umbilical cord remained intact and patent throughout the collection of tissues and the foetuses kept warm and moist with 0.9% saline (37°C).

Footnote * In addition the distribution of D cells was investigated using the PAP technique in conjunction with somatostatin antiserum.

Foetal tissue samples were removed at laparotomy as described in Section 2.2. Tissue was collected from the fundus of the reticulum, ventral sac of rumen, body of the abomasum (35 - 45 mm proximal to the pylorus), abomasal antrum (2 - 10 mm proximal to the pylorus), proximal duodenum (5 - 15 mm distal to the pylorus), terminal ileum (20 mm proximal to ileocaecal junction) and caecum (5 - 15 mm from pole). Tissue samples were prepared as described in Section 2.3 and fixed and processed as previously described (Section 2.5).

Two histochemical staining techniques were used: De Grandi (Table 2.7) and fast garnet (Table 2.11). The PAP immunohistochemical method (Table 2.19) was used with primary antisera to gastrin, secretin, pancreatic glucagon and somatostatin (see Table 2.20). Endocrine cell densities were determined as described in Section 2.11.

With the De Grandi, fast garnet and anti-gastrin methods, staining reactions for some samples were inadequate for reliable endocrine cell counting, even despite repeated staining. In these circumstances endocrine cells were not counted, instead values for these missing data were calculated by the method of Goulden (1952).

4.3 Results

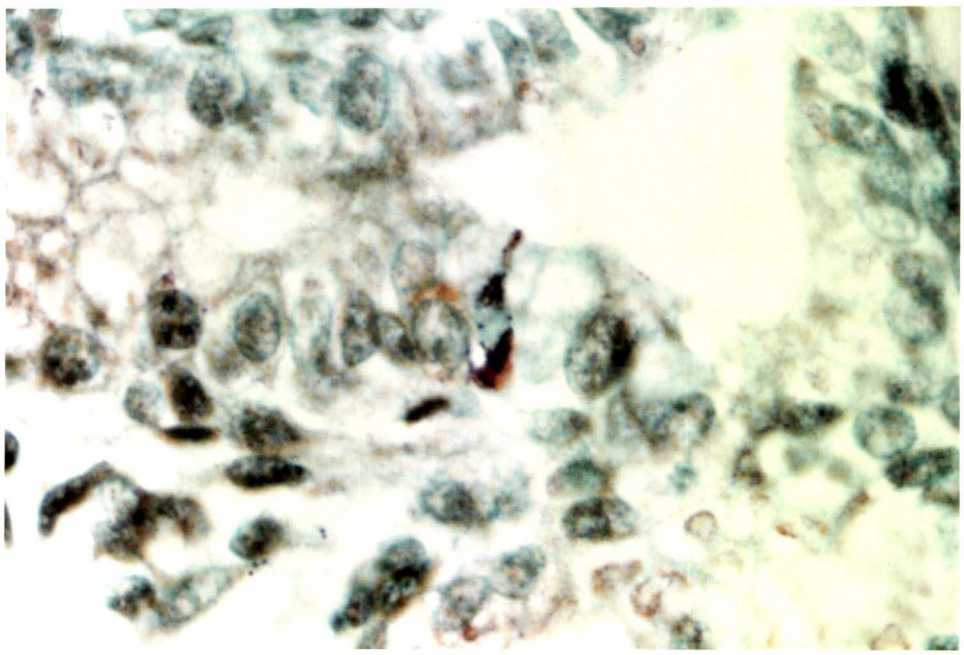
A notable characteristic of this study was the considerable non-specific background staining with all staining techniques, except fast garnet. Such staining was particularly obvious with the De Grandi technique. Connective tissue, in particular, appeared to contain many fine, brown to black, silver granules (Fig. 4.1).

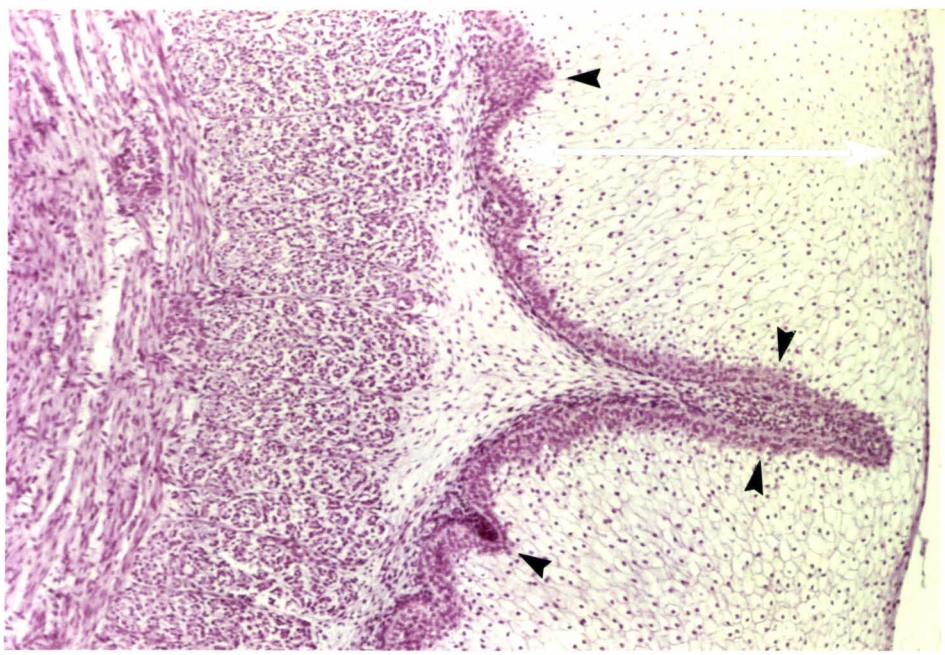
4.3.1 General Morphology

Foetal gut mucosal thickness in the forestomach and abomasum was approximately 40 - 50% of that found in adults, while in the small intestine and large intestine it was 30 - 40% and 20 - 30%, respectively, of adult values. The reticular epithelium of the foetus was characterized by a folded basal layer above which were more than 20 layers of swollen cells (Fig. 4.2). The ruminal epithelium was also characterized by a

Figure 4.1: Fine silver granules (brown) mainly throughout connective tissue. Proximal duodenum; De Grandi method; magnification x 1850.

Figure 4.2: Reticular mucosa showing folded basal epithelial layer forming developing reticular ridges (black arrows) and many layers of swollen epithelial cells (white arrows). Reticulum; alcian blue/H and E method; magnification x 120





folded basal layer, forming the connective tissue core of developing ruminal papillae, above which were 10 - 20 layers of large, vesicular cells (Fig. 4.3). Gastric (Fig. 4.4) and particularly antral (Fig. 4.5) pits were shallow compared with those of adults. Abomasal glands were short and demonstrated little sign of coiling. Intestinal villi were shorter than those of adults and crypts were short but showed signs of some coiling. There were no submucosal glands in upper duodenal mucosal samples (Fig. 4.6).

Evidence of an oral-aboral difference in the rate of mucosal development was apparent within the small intestine: crypts of Lieberkuhn were deeper and demonstrated more coiling in the proximal duodenum (Fig. 4.6) than in the ileum (Fig. 4.7).

4.3.2 Distribution of Endocrine Cells

Argyrophilic cells (Fig. 4.8) were most common in the body of the abomasum (82 $\pm$ 15 c.mm^{-2}) and proximal duodenum (79 $\pm$ 18 c.mm^{-2}), and successively less frequent in the ileum (38 $\pm$ 3 c.mm^{-2}), caecum (16 $\pm$ 3 c.mm^{-2}) and abomasal antrum (<8 c.mm^{-2}).

Greatest numbers of EC cells (Fig. 4.8) were found in the proximal duodenum (76 $\pm$ 12 c.mm^{-2}). EC cell density was lower in mucosal samples from the ileum, caecum and abomasal body region (<20 c.mm^{-2}). EC cells were rare in the antral region, being undetectable in this region in three foetuses.

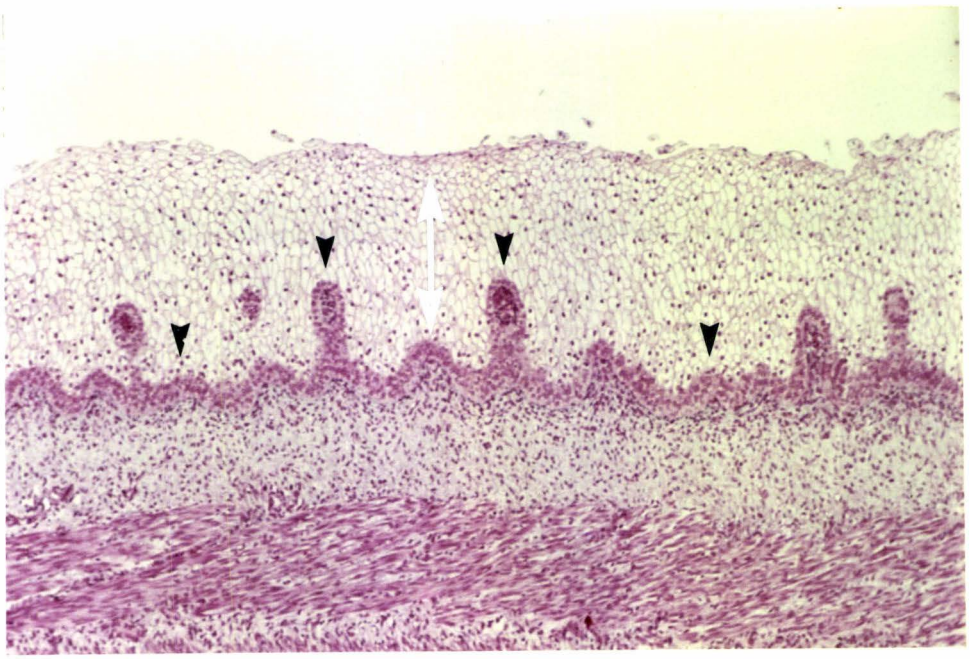
G cells (Fig. 4.9) were most numerous in abomasal antral glands (50 $\pm$ 16 c.mm^{-2}), while in the proximal duodenum their density was about half that value (23 $\pm$ 4 c.mm^{-2}). Only one immunoreactive G cell was found in the samples of ileal mucosa which were examined, while none were seen in the abomasal body or caecum.

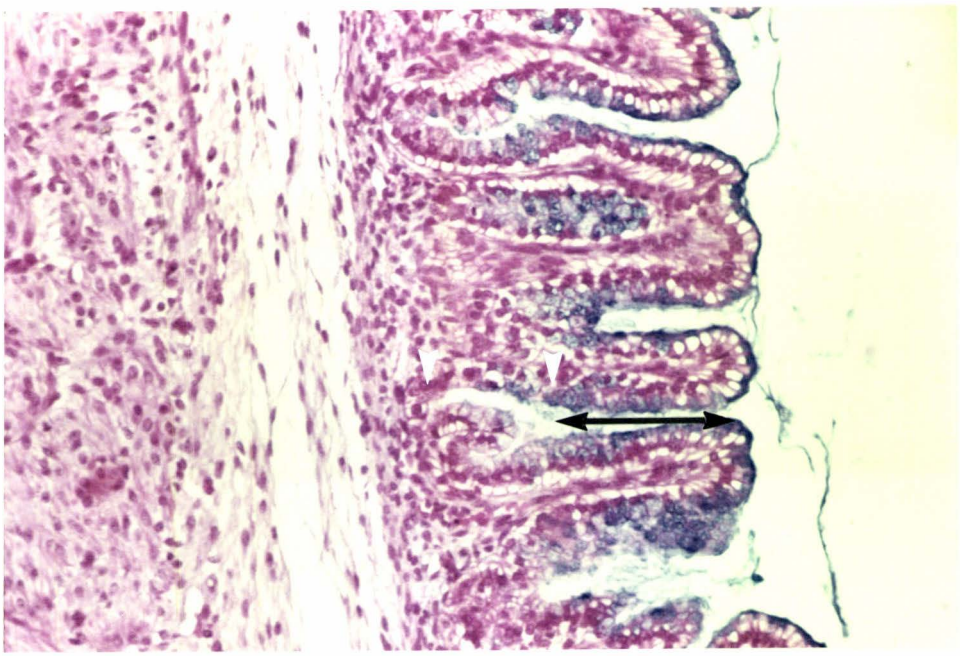
Immunoreactive S cells (Fig. 4.9) were most common in the proximal duodenum (22 $\pm$ 4 c.mm^{-2}), rare (<1 c.mm^{-2}) in the ileum, and absent from the abomasum and caecum.

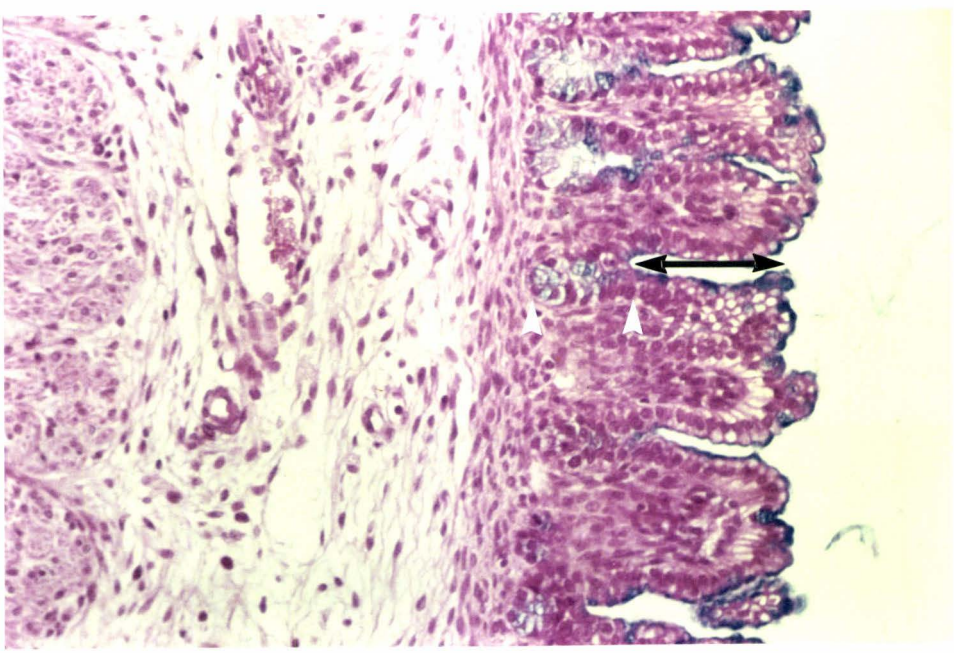
Figure 4.3: Ruminal mucosa showing folded basal epithelial layer forming developing ruminal papillae (black arrows) and many layers of swollen vesicular epithelial cells (white arrows). Rumen; alcian blue/H and E method; magnification x 120.

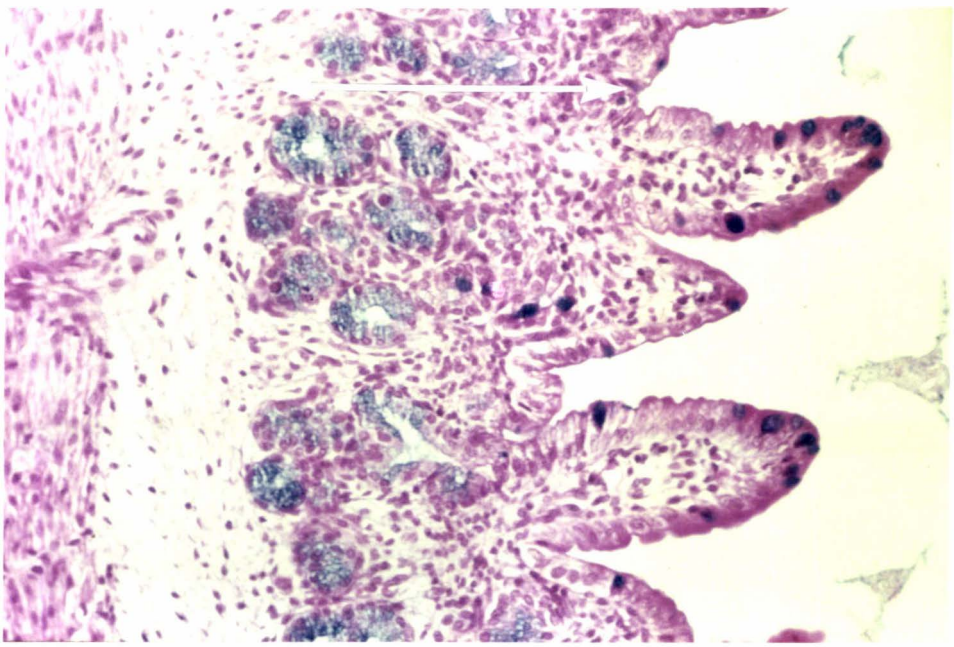
Figure 4.4: Shallow gastric pits (depth indicated by black arrows) and short gastric glands (white arrows) in foetal abomasum. Abomasal body; alcian blue/H and E method; magnification x 300.

Figure 4.5: Shallow gastric pits (depth indicated by black arrows) and short antral glands (white arrows) in foetal abomasum. Abomasal antrum; alcian blue/H and E method; magnification x 300.









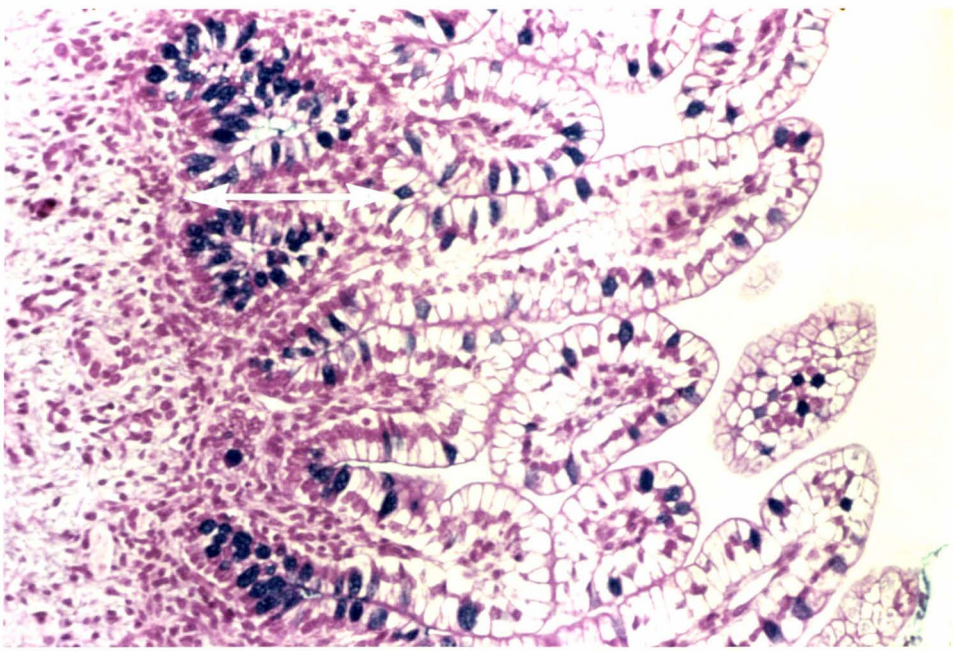


Figure 4.6: Duodenal crypts (depth indicated by arrows) showing evidence of coiling. No Brunner's glands present. Proximal duodenum; alcian blue/H and E method; magnification x 300.

Figure 4.7: Shallow intestinal glands (depth indicated by arrows) showing little evidence of coiling. Ileum; alcian blue/H and E method; magnification x 300.

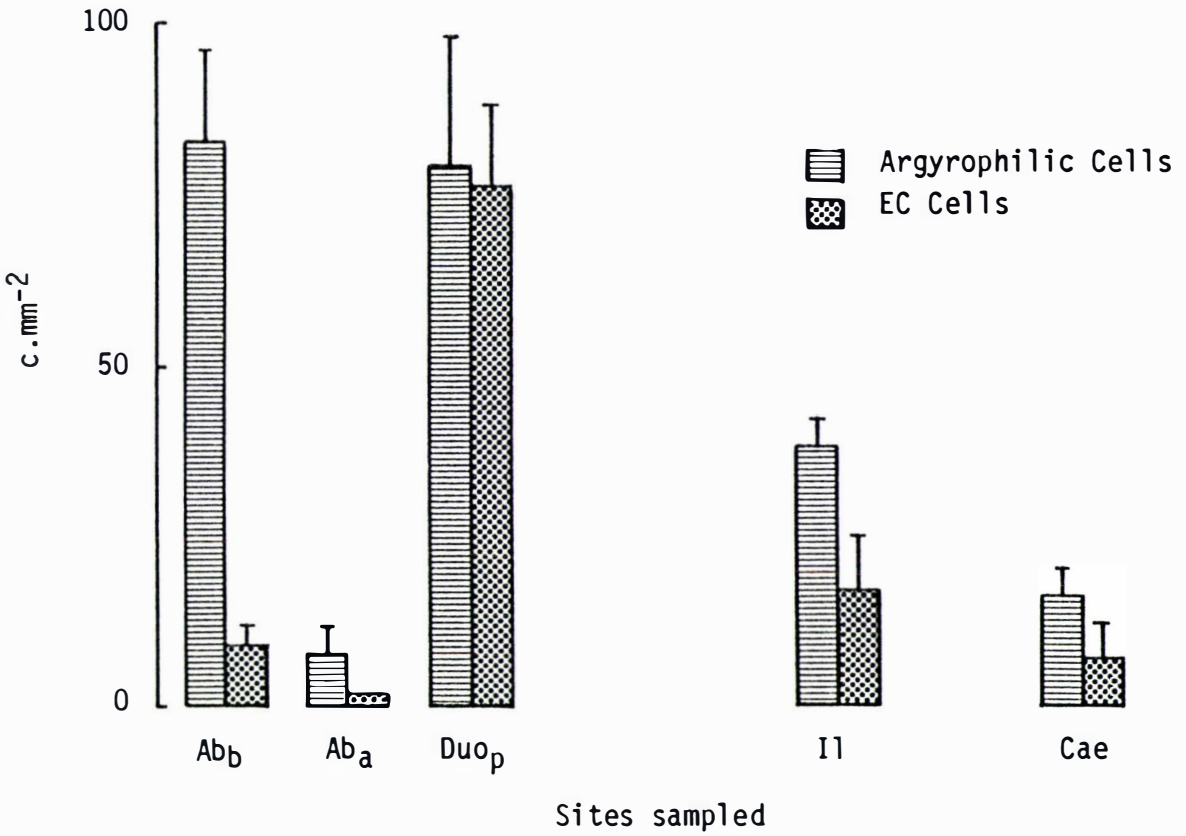


Figure 4.8: Distribution (mean + SEM) of argyrophilic and EC cells in the GI tract of 100-110 day old foetuses (n = 5).

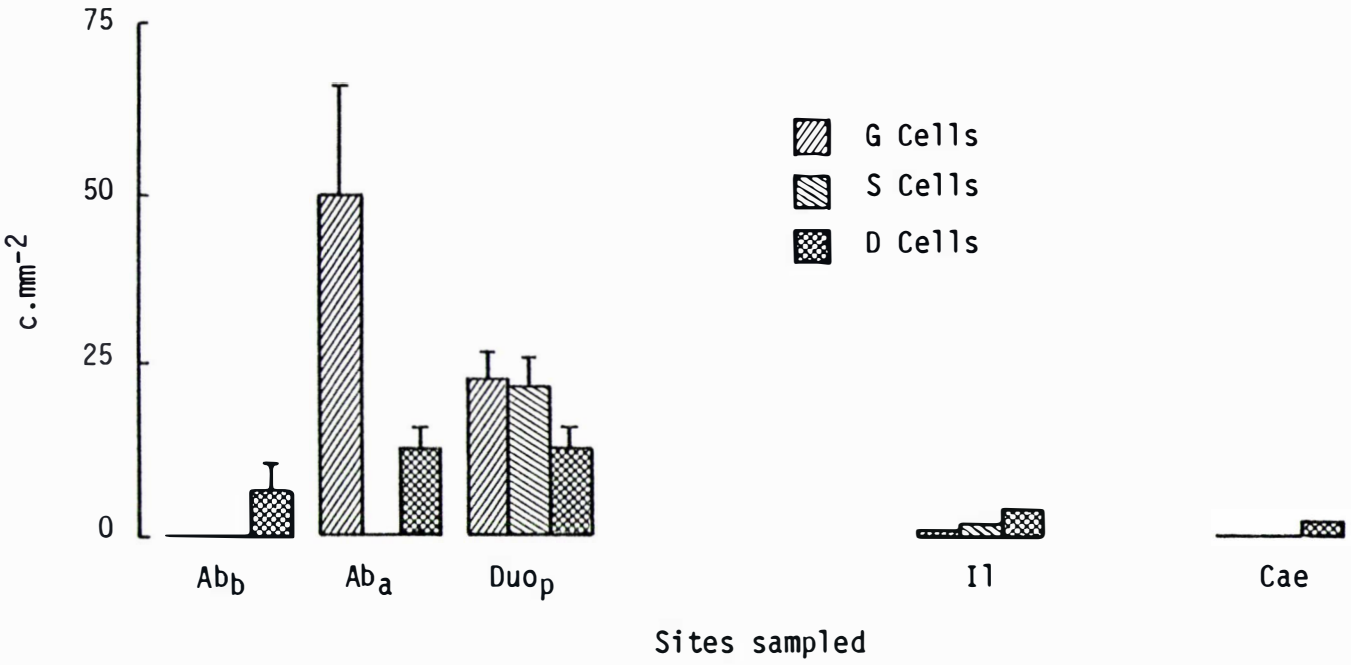


Figure 4.9: Distribution (mean + SEM) of G, S and D cells in the GI tract of 100-110 day old foetuses (n = 5).

Highest D cell densities (Fig. 4.9) were found in the proximal duodenum (12 ± 4 c.mm^{-2}) and abomasal antrum (13 ± 3 c.mm^{-2}), while in the abomasal body their density was about half these values (7 ± 2 c.mm^{-2}). D cells were rare in the ileal and caecal mucosa of foetal lambs.

No endocrine cells were identified, using any of the histochemical or immunohistochemical techniques described, in mucosal samples of reticulum or rumen. Further, no endocrine cells were identified in the GI tract of these foetuses using a pancreatic glucagon antiserum.

Analysis of variance (Table 4.1) revealed that overall, argyrophilic cell densities were significantly ($P<0.001$) higher than those for EC, G, S and D cells (Table 4.1, components C(i) and (v-vii)). It also showed (Table 4.1, interaction component D(iii)) that although EC cells constituted a large proportion of the argyrophilic cells found in the small intestine, in the abomasum they formed only a small part of the argyrophilic cell population.

G cells, as well as being more numerous, overall, ($P<0.001$) than S cells (Table 4.1, component C(ii)), differed in their distribution. The significant ($P<0.001$) interaction components (Table 4.1, D(v-vi)) for G and S cells in the abomasum and small intestine reflect the absence of S cells in the abomasum and the high density of G cells in the antrum.

Overall, D cells were less numerous ($P<0.001$) than EC cells (Table 4.1, component C(x)). Within the abomasal antrum, however, D cell densities were greater than those for EC cells, giving rise to a significant ($P<0.01$) interaction component (Table 4.1, D(xxii)) in the analysis of variance. The analysis also revealed a significant ($P<0.001$) interaction component (Table 4.1, D(xvi)) for argyrophilic and D cell densities within the abomasum, resulting from the finding that the highest densities of argyrophilic cells occurred in the abomasal body, while D cells were most numerous in the antrum. The pattern of distribution of D cells (Fig. 4.9) within the intestines, however, generally was similar to those for argyrophilic and EC cells (Fig. 4.8).

Table 4.1: Summary of analysis of variance of data obtained using methods which demonstrate argyrophilic, EC, G, S and D cells

Source of Variation		DF		Variance Ratios
A. Animals		4		1.14
B. Sites		4		
(i)	Ab_b vs Ab_a		1	0.65
(ii)	Duo vs Il		1	116.76***
(iii)	Ab vs SI		1	39.14***
(iv)	Ab + SI vs LI		1	63.40***
Non-orthogonal contrast				
(v)	SI vs LI		1	96.50***
C. Staining methods		4		
(i)	DG vs FG		1	49.14***
(ii)	Gas vs Sec		1	14.00***
(iii)	Gas + Sec vs Som		1	177.96***
(iv)	Histo vs Immuno		1	0.76
Non-orthogonal contrasts				
(v)	DG vs Gas		1	110.18***
(vi)	DG vs Sec		1	202.73***
(vii)	DG vs Som		1	134.88***
(viii)	FG vs Gas		1	12.15***
(ix)	FG vs Sec		1	52.24***
(x)	FG vs Som		1	21.20***
(xi)	Sec vs Som		1	6.89*
D. Interaction: sites x staining methods		28		
(i)	Ab_b vs Ab_a x DG vs FG		1	25.65***
(ii)	Duo vs Il x DG vs FG		1	4.02*
(iii)	Ab vs SI x DG vs FG		1	14.20***
(iv)	Ab + SI vs LI x DG vs FG		1	5.27*

Table 4.1 continued

(v)	Ab_b vs Ab_a x Gas vs Sec	1	39.46***
(vi)	Ab vs SI x Gas vs Sec	1	22.09***
(vii)	Ab_b vs Ab_a x Gas + Sec vs Som	1	5.53*
(viii)	Duo vs Il x Gas + Sec vs Som	1	4.35*
(ix)	Ab_b vs Ab_a x Histo vs Immuno	1	110.73***
(x)	Ab vs SI x Histo vs Immuno	1	34.07***
	Remainder	18	42.73
Non-orthogonal contrasts			
(xi)	Ab_b vs Ab_a x DG vs Gas	1	172.77***
(xii)	Ab vs SI x DG vs Gas	1	14.20***
(xiii)	SI vs LI x DG vs Gas	1	4.16*
(xiv)	Ab_b vs Ab_a x DG vs Sec	1	47.10***
(xv)	Ab + SI vs LI x DG vs Sec	1	5.85*
(xvi)	Ab_b vs Ab_a x DG vs Som	1	63.48***
(xvii)	Ab vs SI x DG vs Som	1	7.87**
(xviii)	SI vs LI x DG vs Som	1	6.71*
(xix)	Ab_b vs Ab_a x FG vs Gas	1	65.28***
(xx)	Ab vs SI x FG vs Gas	1	56.88***
(xxi)	Ab vs SI x FG vs Sec	1	8.07**
(xxii)	Ab_b vs Ab_a x FG vs Som	1	8.43**
(xxiii)	Duo vs Il x FG vs Som	1	10.27**
(xxiv)	Ab vs SI x FG vs Som	1	43.20***
(xxv)	SI vs LI x FG vs Som	1	4.13*
(xxvi)	Ab_b vs Ab_a x Gas vs Som	1	26.80***
(xxvii)	Duo vs Il x Gas vs Som	1	5.29*
(xxviii)	Ab vs SI x Sec vs Som	1	13.92***
	Residual mean square	88	1.12

4.3.3 Morphology and Mucosal Location of Endocrine Cells

All argyrophilic cells found in the mucosa of the abomasal body appeared to be of the 'closed' type, they were small, irregularly shaped and some possessed basal cytoplasmic projections (Fig. 4.10). The majority of argyrophilic cells occurred singly near the base of the gastric glands, but a few were found near the pit surface, or in small groups near the gland base (Fig. 4.11).

In the abomasal antral region, argyrophilic cells of both 'open' and 'closed' types were observed, although the latter predominated. 'Open' type cells were small, pyramidal (Fig. 4.12) or teardrop shaped with well stained granules scattered throughout the cytoplasm. 'Closed' type cells (Fig. 4.13) in this region were similar in morphology to those found in the body of the abomasum. Both 'open' and 'closed' type argyrophilic cells occurred singly, scattered throughout the epithelium of the abomasal antrum.

Argyrophilic cells in the intestinal mucosa were found mainly in the villi (Fig. 4.14). They were 'open' type cells but differed from those observed in the abomasum in that they were columnar in shape. 'Closed' type argyrophilic cells were observed near the base of crypts in the caecum (Fig. 4.15). These cells were small, irregularly shaped and with relatively few stained cytoplasmic granules.

All EC cells in the abomasum were oval, of the 'closed' type (Fig. 4.16) and their cytoplasm lightly and unevenly stained. In both body and antral regions of the abomasum EC cells occurred singly in the deeper parts of the developing gastric and antral glands.

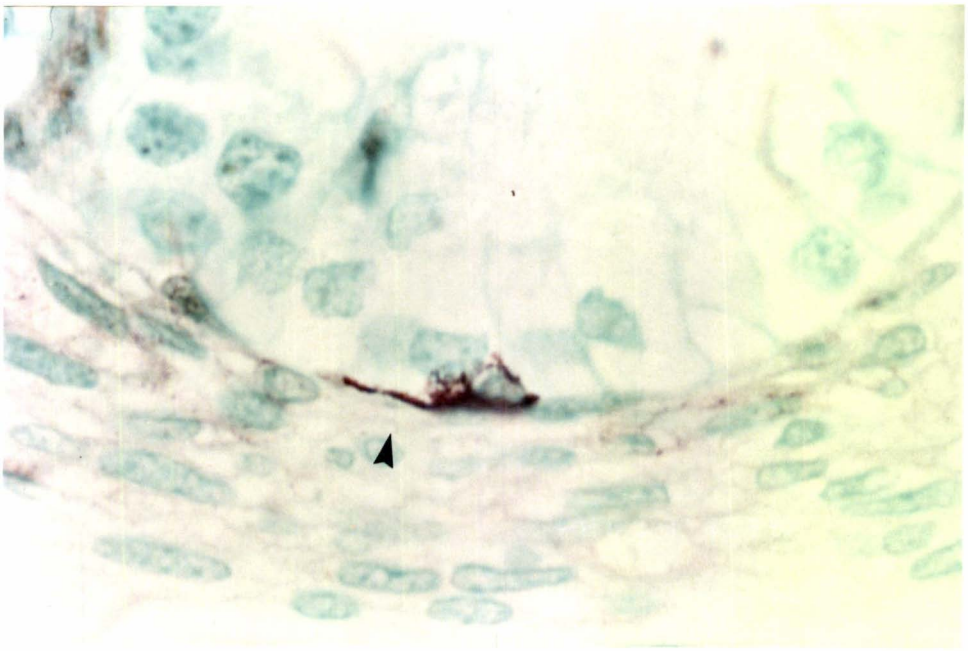
In the intestine three cell types contained cytoplasmic granules that stained red with the fast garnet technique:

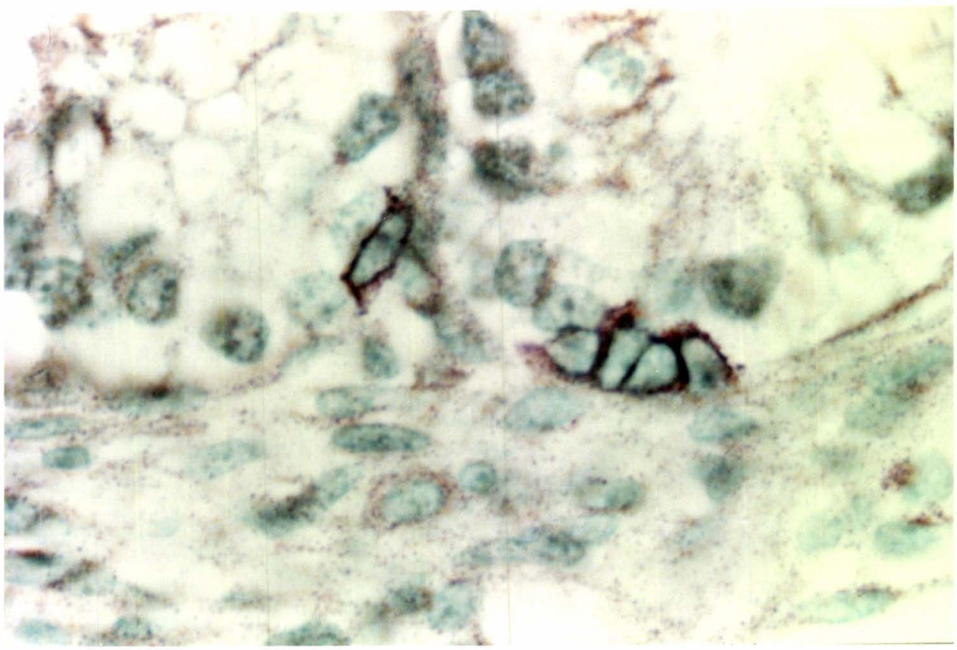
(a) EC cells, all of which were of the 'open' type. Some EC cells were pyramidal in shape, with intensely stained infranuclear cytoplasm (Fig. 4.17) and were found near the base of developing intestinal crypts. Others were columnar in shape with well stained granules throughout their cytoplasm (Fig. 4.18).

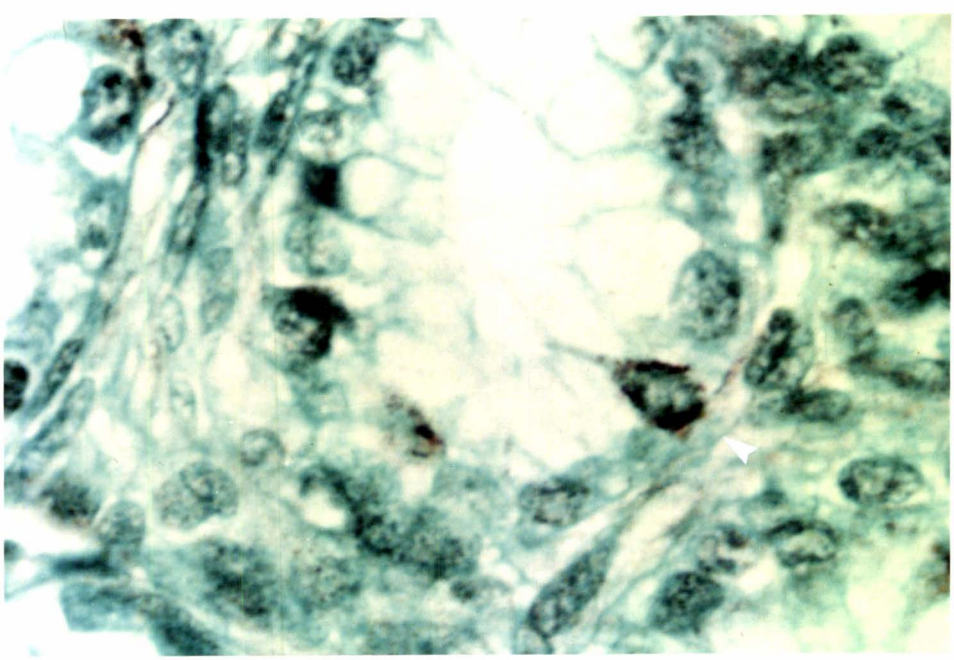
Figure 4.10: Argyrophilic cell (black), of the 'closed' type, with basal cytoplasmic cytoplasmic process (arrow) near base of gastric gland. Abomasal body; De Grandi method; magnification x 1850.

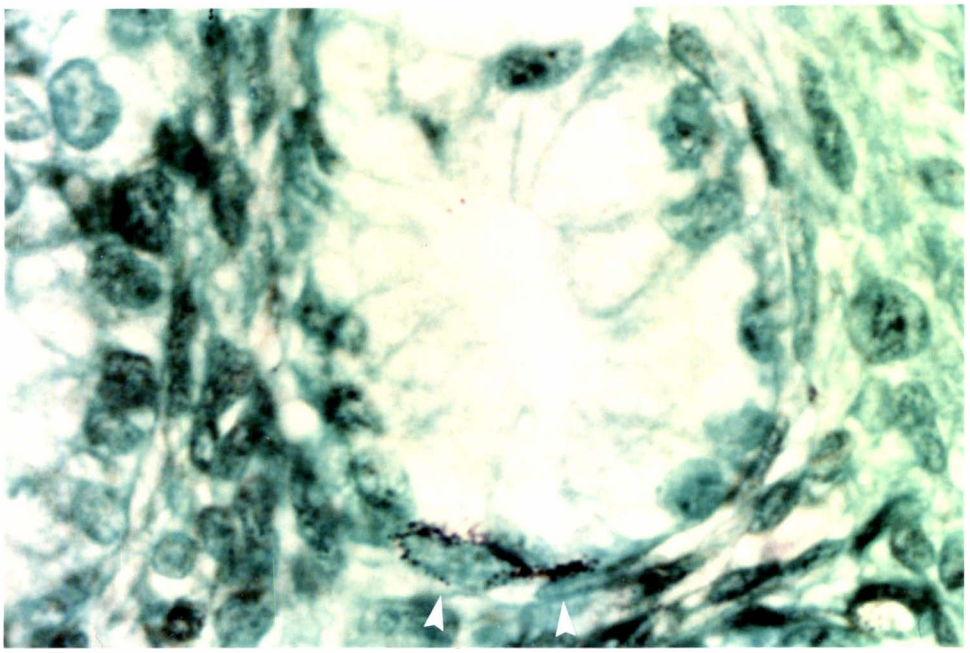
Figure 4.11: Group of argyrophilic cells (black), of the 'closed' type, near base of gastric gland. Abomasal body; De Grandi method; magnification x 1850.

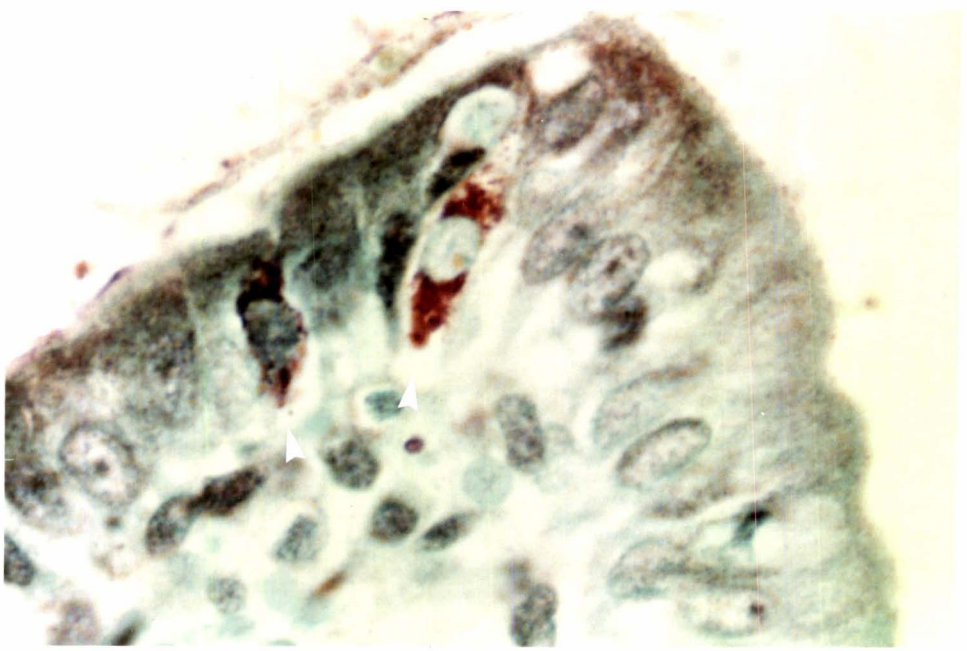
Figure 4.12: 'Open' type argyrophilic cell (arrow) opening in to base of antral gland. Abomasal antrum; De Grandi method; magnification x 1850.











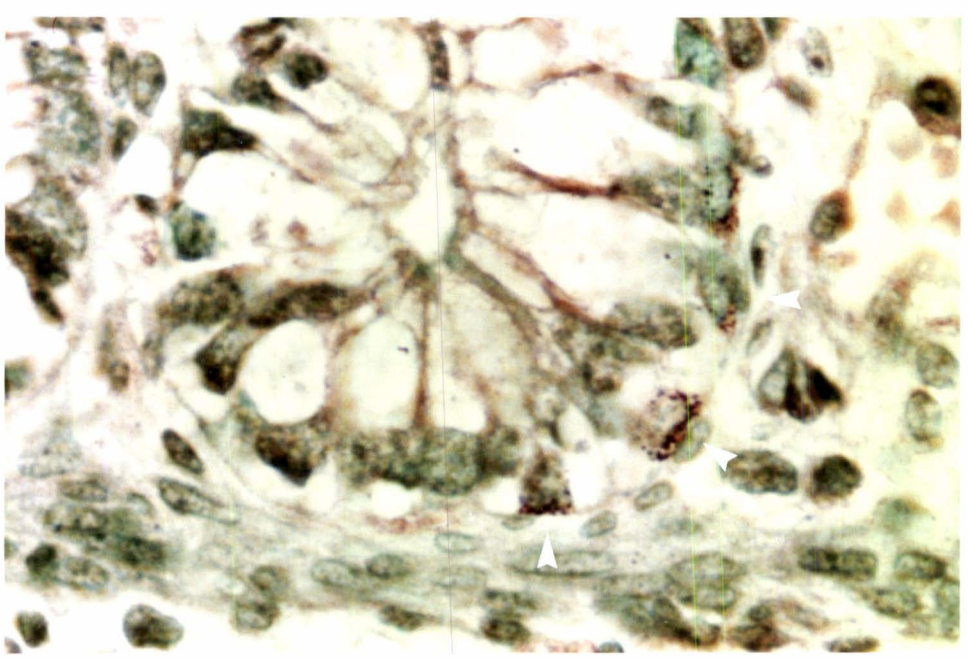


Figure 4.13: 'Closed' type argyrophilic cell (arrows) with basal cytoplasmic process, near base of antral gland. Abomasal antrum; De Grandi method; magnification x 1850.

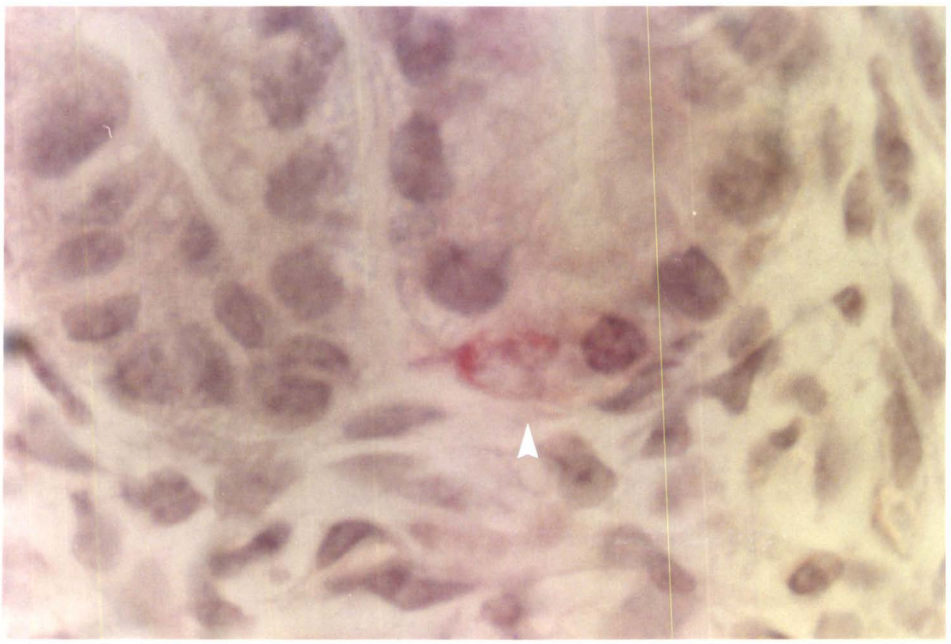
Figure 4.14: Argyrophilic cells (arrows) near tip of duodenal villus. Proximal duodenum; De Grandi method; magnification x 1850.

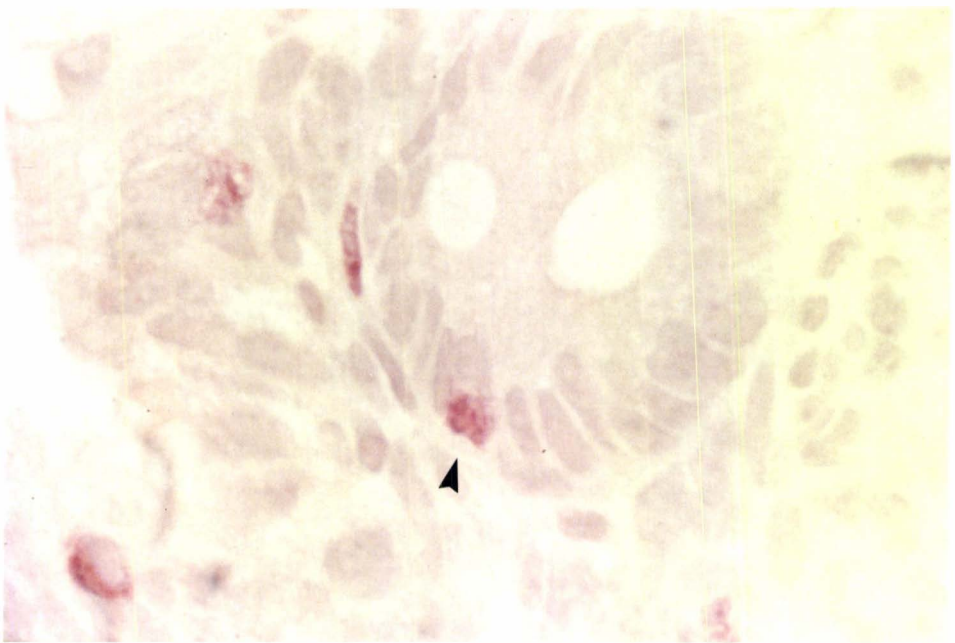
Figure 4.15: 'Closed' type argyrophilic cells (arrows) near base of intestinal crypts. Caecum; De Grandi method; magnification x 1850.

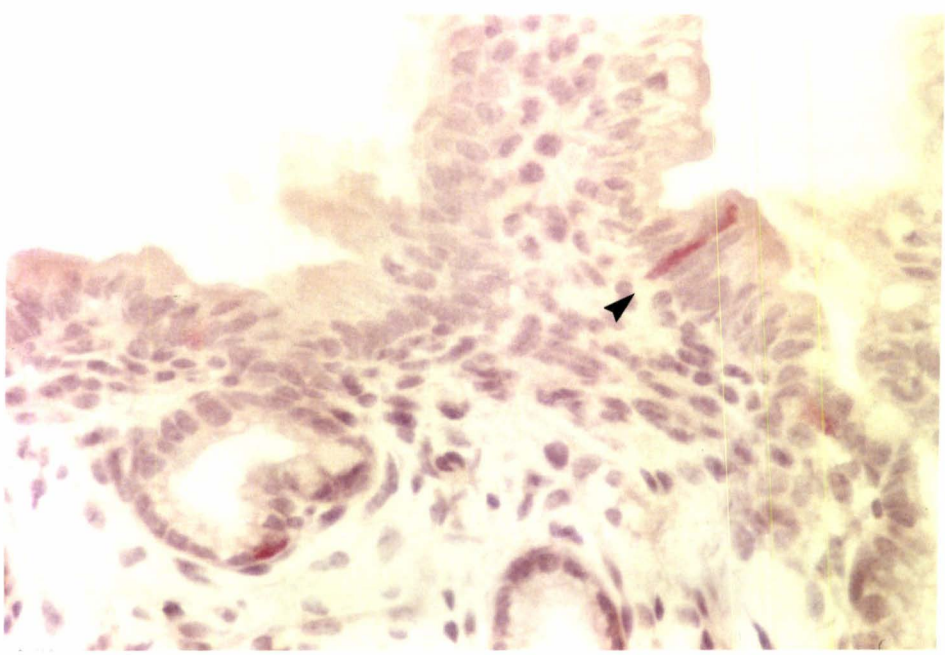
Figure 4.16: Poorly stained 'closed' type EC cell (arrow) near base of gastric gland. Abomasal body; fast garnet method; magnification x 1850.

Figure 4.17: 'Open' type EC cell (arrow) with intensely stained infranuclear cytoplasm near base of developing intestinal crypt. The other diazonium positive cells are CTMC's. Proximal duodenum; fast garnet method; magnification x 1850

Figure 4.18: Columnar type EC cell (arrow) with well stained granules throughout its cytoplasm, near base of developing villus. Proximal duodenum; fast garnet method; magnification x 740.







Such cells were found in the epithelium near the mucosal surface.

(b) Small, slender, teardrop shaped cells with intensely staining supranuclear cytoplasm were observed in the intestinal villi of the caecum and terminal ileum (Fig. 4.19). These cells were located close to the basal lamina or close to the luminal surface of the epithelium.

(c) CTMC's were variable in both size and shape and, as judged from their staining characteristics with fast garnet (Fig. 4.17), contained 5-HT. Similar cells occurred in small numbers in the abomasal and caecal mucosae.

Foetal G cells were of the 'open' type and a variety of shapes was observed in both abomasal antrum (Fig. 4.20) and proximal duodenum (Fig. 4.21). Cytoplasmic granules were well stained and scattered throughout the entire cytoplasm. G cells were most common near the mucosal surface, very few being found near the base of antral glands or intestinal crypts.

Few immunoreactive S and D cells were stained in foetuses. Foetal S cells (Fig. 4.22) were similar in appearance to those described in Section 3.3.3 and shown in Fig. 3.46. The morphology of D cells in foetuses was similar to that found in 6 month old lambs. Both 'open' (Fig. 4.23) and 'closed' (Fig. 4.24) cell types were identified. A detailed description of D cells in 6 month old lambs is given in Section 6.3.2.

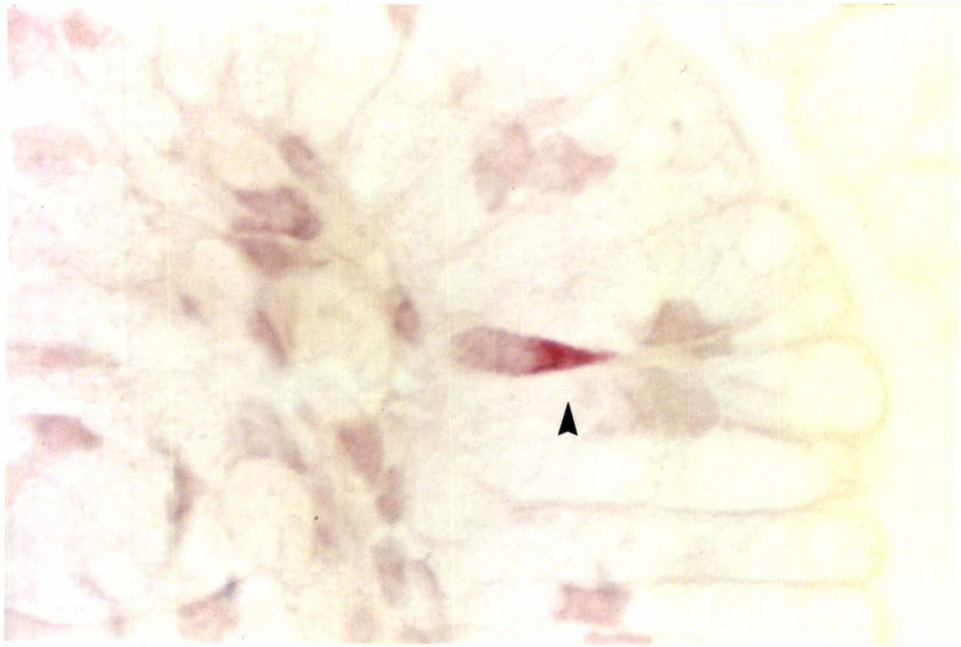
4.4 Discussion

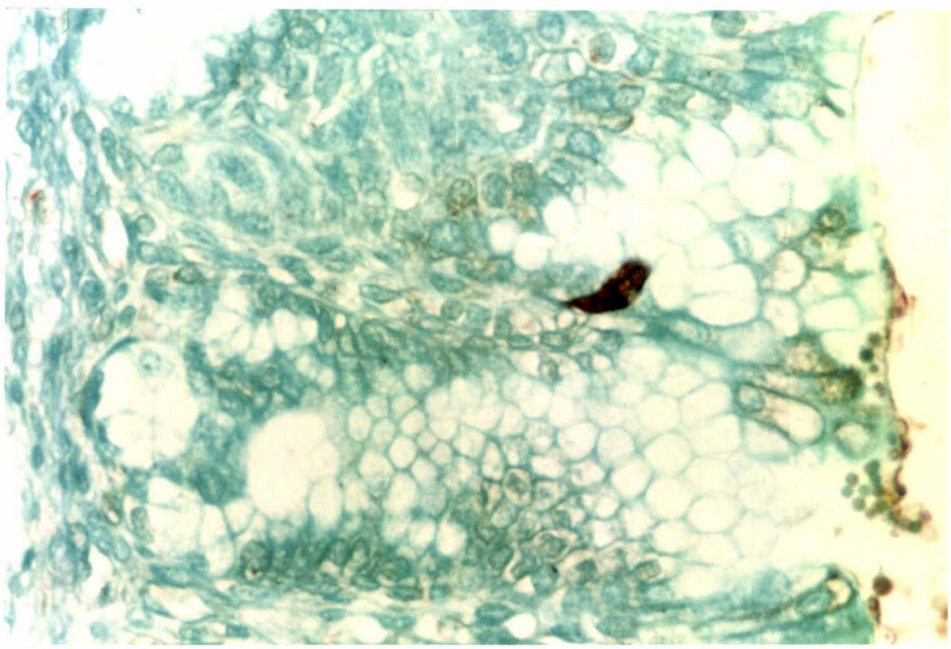
Great care was taken in the present study to ensure that the sites from which tissue samples were taken, were similar to those sampled in the adult animals. In this regard, the pylorus, caudal flexure, duodenojejunal flexure and the ileocaecal junction were used as important landmarks.

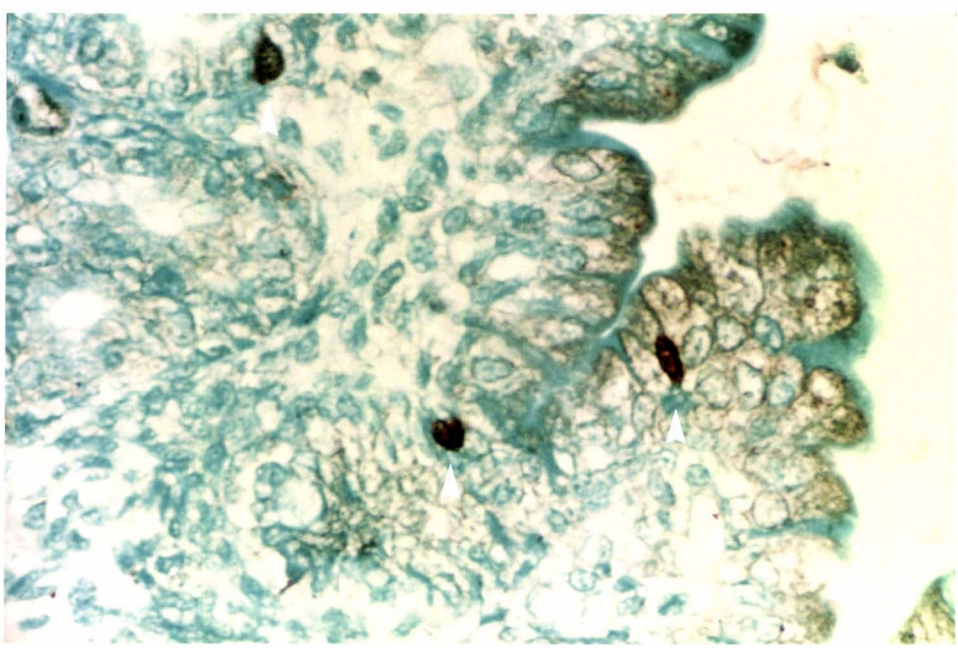
Figure 4.19: Diazonium positive cell (arrow) with intensely stained supranuclear cytoplasm, in intestinal villus. Ileum; fast garnet method; magnification x 1850.

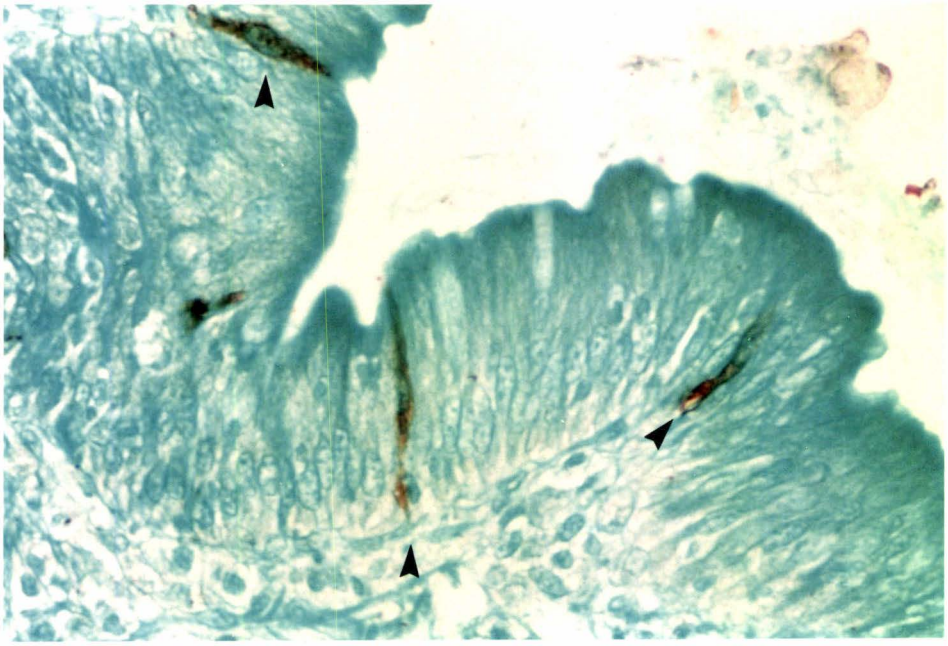
Figure 4.20: G cell (black) in abomasal gastric mucosa near base of developing gastric pit. Abomasal antrum; gastrin antiserum As'74/PAP method; magnification x 740.

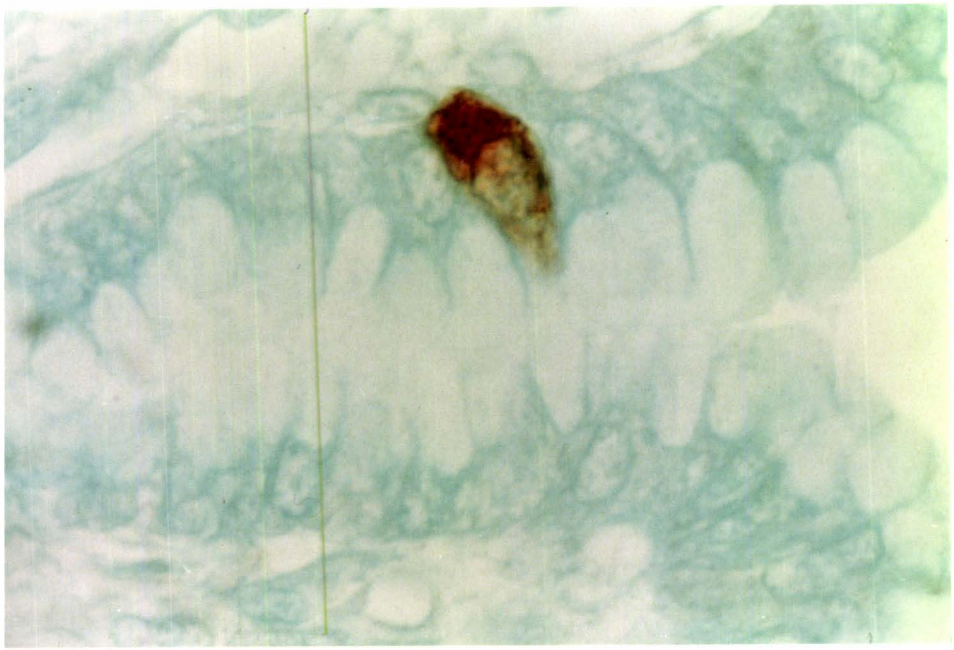
Figure 4.21: G cells (arrows) near mucosal surface in duodenal crypts. Proximal duodenum; gastrin antiserum As'74/PAP method; magnification x 740.











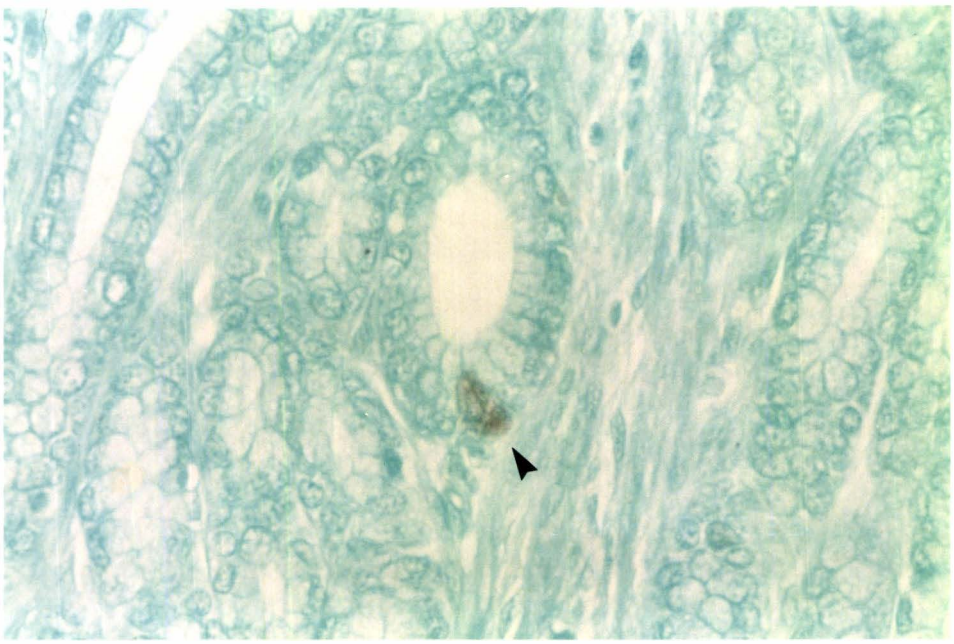


Figure 4.22: Immunoreactive S cells (arrows) near mucosal surface in developing duodenum. Proximal duodenum; secretin antiserum/PAP method; magnification x 740.

Figure 4.23: 'Open' type D cell (brown) in middle region of abomasal antral gland. Abomasal antrum; somatostatin antiserum/PAP method; magnification x 1850.

Figure 4.24: Poorly stained 'closed' type D cell (arrow) in abomasal antral gland. Abomasal antrum; somatostatin antiserum/PAP method; magnification x 740.

Within the GI tract of the ovine foetus, endocrine cells were most numerous in the abomasal and proximal duodenal mucosa. In these regions argyrophilic cell densities were only about 50% of those found in adult animals (Section 3.3.4). However, the patterns of distribution of argyrophilic, as well as EC, G and S cells, were similar to those described for adults. A more detailed comparison with adults and other age groups is presented in Section 8.2.

Comparisons of distribution patterns of argyrophilic and EC cells in ovine foetuses support the conclusion that the bulk of argyrophilic cells found in the proximal duodenum were EC cells. It is clear, however, that the majority of argyrophilic cells found in the abomasal body were not EC cells, or at least did not contain identifiable quantities of 5-HT, as few endocrine cells in this region were stained by the fast garnet technique.

Most likely the endocrine cells represented by the large concentration of argyrophilic cells in the abomasal body were ECL cells. Although ECL cells have not been specifically identified in sheep, results from adult animals (Section 3.4.1) suggest they form a large proportion of the endocrine cells in this region of the GI tract. In humans, morphologically 'mature' ECL cells, identified on the basis of their ultrastructural characteristics, have been found in the mucosa of the body region of the stomach in foetuses as early as the 63rd day of gestation (Track et al., 1979).

Comparisons of argyrophilic and G cell densities in the abomasal antral region of these foetal lambs, indicate that G cells were not argyrophilic. A similar conclusion was reached with regard to adult animals (Section 3.4.1).

Although the evidence is not definitive, comparison of the difference between antral argyrophilic and EC cell densities, with D cell densities, suggest that D cells are not argyrophilic. Similar comparisons for the proximal duodenum lead to the same conclusion. Similar logic leads to the conclusion that in the proximal duodenum S cells also are non-argyrophilic. Thus it appears that there is a population of argyrophilic cells in the ileum and caecum of ovine

foetuses, which are not EC, G, S or D cells. The endocrine cell types most likely to be represented by this population of argyrophilic cells are D_1 and L cells. Specific identification and distribution of these cell types has not been established in either foetal lambs or adult sheep. Because the secretory product(s) of D_1 cells has not yet been identified (Solcia et al., 1982), D_1 cells cannot be identified by immunohistochemical techniques. Their presence could be established, however, on the basis of their ultrastructural characteristics.

Although L cells have not yet been identified in sheep, GLI, a product of L cells, has been detected in GI mucosal extracts of 55 day old foetal lambs (Bassett and Madill, 1978). Highest tissue concentrations were shown to occur in the terminal ileum. L cells have been identified in human foetuses in both the small (Moxey and Trier, 1977) and large (Lehy and Cristina, 1979) intestines.

Somatostatin immunoreactive (D) cells were found in all abomasal and intestinal samples investigated and had their greatest density in the abomasal and duodenal mucosa. This is the first time D cells have been reported in the ovine GI tract. Many workers have reported the presence of D cells in the stomach (Track et al., 1979; Chayvialle et al., 1980) and intestine (Moxey and Trier, 1977; Lehy and Cristina, 1979; Track et al., 1979; Chayvialle et al., 1980) of human foetuses, and in foetal rats, rabbits, cats, dogs and pigs (Alumets et al., 1977).

The 5-HT containing cells (Fig. 4.17) found in the ileal and caecal epithelium are of unknown identity. They do not have the typical appearance of EC cells found in other parts of the ovine foetal GI tract: they differ in cell size and shape, and the distribution of their cytoplasmic granules. While it is possible that they represent foetal MMC's, they differ in cell shape and distribution of cytoplasmic granules, from the MMC's described for adult sheep in the previous chapter. Further, in human adults, MMC's contain little or no 5-HT (Bienenstock et al., 1982). This raises the possibility that there are 5-HT containing endocrine cells other than EC cells in the foetal ovine GI tract mucosa. Such a hypothesis is supported by evidence that in human and rat foetuses, endocrine cells other than EC cells (e.g. G, I and S cells) contain 5-HT (Larsson and Jorgensen, 1978). These workers

demonstrated that in human and rat foetuses and neonates, some GI tract endocrine cells are not fully differentiated until after birth and that before reaching maturity they may produce more than one biologically active product. In particular they demonstrated that 25 - 40% of duodenal G cells also stored 5-HT, but antral G cells were never observed to contain 5-HT. This difference between antral and duodenal G cells suggests that in the foetus there are sub-populations of G cells which differ in their metabolic capabilities. Whether this is in any way linked to the morphologically distinct IG (Buchan et al., 1979), TG (Larsson et al., 1981) and antral G cells found in the adult is not known. Larsson and Jorgensen (1978) also demonstrated 5-HT in immunoreactive S and I cells in the duodena of both human and rat foetuses. The hypothesis that ovine foetal G, S and I cells contain 5-HT, could be tested by double or sequential immunohistochemical staining techniques, or by the use of thin serial sections.

G cells were detected in mucosal samples of the abomasal antrum and duodenum of 100 - 110 day old foetuses. This result contrasts with that of Lichtenberger et al., (1981) who reported ovine antral G cells were initially seen between 120 - 130 days gestation. The reason for this discrepancy is not readily apparent, but may be due to different techniques used to demonstrate G cells: Lichtenberger et al., (1981) used the indirect immunoperoxidase method, which is less sensitive than the PAP technique used in the present study. In this regard it is relevant to note that Lichtenberger et al., (1981) were able to extract immunoreactive gastrin from the abomasa of foetuses at 103 days gestation, and detect by RIA, gastrin in serum of foetuses at 107 days gestation, while not being able to demonstrate G cells until 120 - 130 days foetal age.

Comparison of foetal and adult (see Section 3.3.4) G cell densities indicates a considerable increase in antral G cell density occurs between 100 - 110 days gestation and maturity. Lichtenberger et al., (1981) postulated the increase they observed in antral G cell numbers between the last 2 - 3 weeks of gestation and the first two weeks of life was attributable to elongation of the antral glands, and not an increase in endocrine cell density. This concept is not in agreement with results presented in subsequent chapters of this thesis (see Sections 5.3.1 and 6.3.1).

In the present experiment it was also established that G cells were present in the duodena of 100 - 110 day old foetal lambs. Duodenal G cell densities were lower than for adults (23 ± 4 vs 30 ± 4 c.mm^{-2}). This is in contrast to results for other species (Larsson and Jorgensen, 1978) in which duodenal G cell densities were higher in foetuses than adults. In sheep, the ratio of antral to duodenal G cells changed with age from 2.2:1 in foetuses to 5.2:1 in adults. This result suggests that in foetuses, duodenal G cells form a greater proportion of total G cell numbers than in adults.

As yet, the physiological function(s) of the secretory products of endocrine cells in foetuses have not been elucidated. It is probable, however, that some GI hormones, for example gastrin (see Johnson, 1980), CCK (Rothman and Wells, 1967) and secretin (Pansu et al., 1974), are important in the growth and maturation of the foetal GI tract.

Clearly, the mechanisms regulating the digestive functions of the GI tract must be present and functional soon after birth, when the survival of the neonate depends upon absorption of nutrients from the digestive tract. Some gut functions are known to occur before birth, for example, swallowing of amniotic fluid by foetal lambs has been reported (Bradley and Mistretta, 1973; Pearson and Mellor, 1976; Harding et al., 1984a, b). Thus, the foetal gut is exposed to ingested fluids and presumably absorption and secretion occur across the mucosal wall. Also, foetal lambs pass faecal pellets and indeed may re-ingest them, indicating that movement of gut contents along the tract is established before birth (M.P. Gurnsey, unpublished observations).

Results of this present study demonstrate that several of the endocrine cell types found in adult sheep also occur in the foetus, and further, such foetal endocrine cells contain identifiable quantities of secretory products(s). These observations suggest that foetal GI tract function may, at least in part, be regulated by the secretory products of GI endocrine cells.

CHAPTER 5

THE IDENTIFICATION AND DISTRIBUTION OF ENDOCRINE CELLS IN THE GI TRACT OF NEONATAL LAMBS

5.1 Introduction

Although EC cells (Tehver, 1930), immunoreactive G cells (Lichtenberger et al., 1981) and endocrine cells demonstrating substance P-like immunoreactivity (Harrison and Wathuta, 1982b) have been identified in the gut mucosa of newborn lambs, their densities have not been quantified (see Chapter 1).

Evidence from other species indicates that the endocrine cell distribution in the neonates, like that in foetuses (see Section 4.3.2), may differ markedly from that found in adults (see Section 1.8). In rat and human foetuses and newborn, endocrine cell frequency in the duodenum has been shown to be higher than in adults (Larsson and Jorgensen, 1978). Whether this is also true in newborn lambs has yet to be established.

The purpose of the present experiment was to examine the endocrine cell types, their morphology and distribution in the GI tract of neonatal (pre-ruminant) lambs.

5.2 Methods

Experimental animals were five two week old Romney-cross lambs (Table 2.1) from the same flock as that which provided the foetuses (see Chapter 4). The lambs were separated from their dams approximately 24 hours after birth and reared indoors. They were housed on wheat straw bedding and fed a cold milk mix (Ancalf Quikmix, N.Z. Co-op Dairy Co., Hamilton) ad libitum.

Tissue samples were collected as described in Section 2.2. Care was taken to ensure that the sites sampled corresponded as closely as possible to those previously sampled in the adults.

All sites mentioned in Table 2.2 were sampled but because the GI tracts of lambs are considerably shorter than those of adults, the duodenal samples were taken from sites 0 - 2, 8 - 10 and 20 - 22 cm from the pylorus, while the ileal site was 3 - 5 cm proximal, and the colonic site 8 - 10 cm distal to the ileocaecal junction. Tissue samples were prepared, fixed and processed as outlined in Sections 2.3 and 2.5.

Two histochemical staining techniques were used: De Grandi (Table 2.7) and fast garnet (Table 2.11). The PAP immunohistochemical method (Table 2.19) was used with primary antisera to gastrin, secretin, glucagon and somatostatin (Table 2.20).

5.3 Results

5.3.1 Distribution of Endocrine Cells

Argyrophilic cells were most common in the proximal duodenum (237 $\pm$ 21 c.mm^{-2}) with progressively decreasing numbers in the more distal intestinal samples (Fig. 5.1). Substantial numbers (102 $\pm$ 20 c.mm^{-2}) of argyrophilic cells were also present in the mucosa of the body region of the abomasum, whereas their density in the antral region was low (26 $\pm$ 4 c.mm^{-2}).

Although most argyrophilic cells in the mucosa of the abomasal body occurred singly, occasionally small clusters of 3 - 5 cells were observed.

In both small and large intestinal samples, argyrophilic cells, although most common in the lower third of crypts, occurred throughout the epithelium, including the villi. In the duodenum a substantial proportion (up to 15%) of argyrophilic cells were found in the upper third of the crypts. In contrast, in the ileum and large intestine less than 5% occurred in the upper third of the mucosa.

Argyrophilic cells were also present in the glands of Brunner, but their density was always less than 3 c.mm^{-2}.

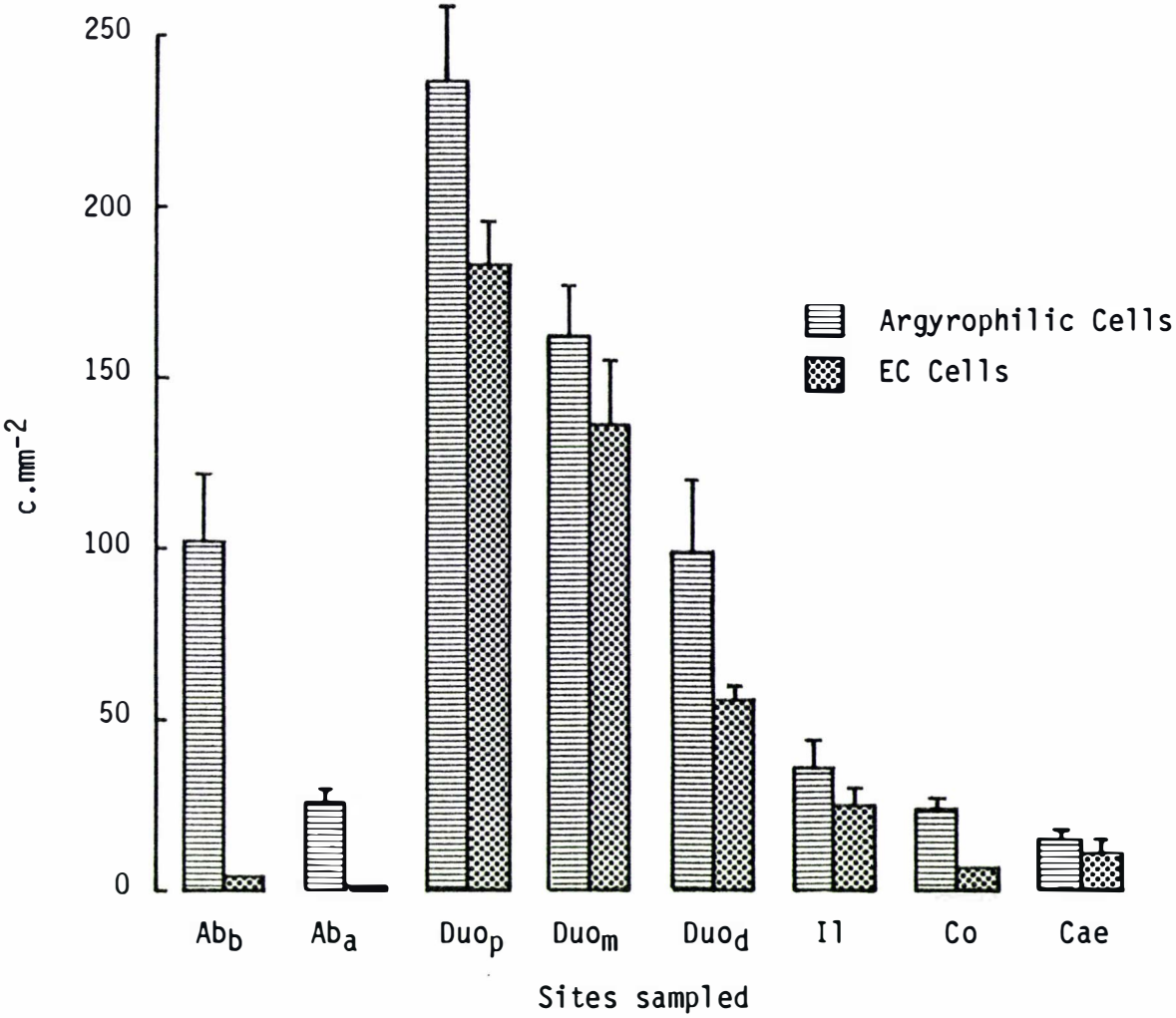


Figure 5.1: Distribution (mean + SEM) of argyrophilic and EC cells in the GI tract of 2 week old lambs (n = 5).

Analysis of variance revealed that at each site EC cell densities were significantly ($P<0.001$) lower than those of argyrophilic cells (Table 5.1, component C(i)). Despite that overall difference, the pattern of EC cell distribution in both small and large intestines was generally similar to that for argyrophilic cells: EC cells were most common in the proximal duodenum (187 ± 13 c.mm^{-2}) and successively less frequent in more distal sites (Fig. 5.1). On the other hand, EC cells were rare (<5 c.mm^{-2}) in both the abomasal body and antrum. Also the abomasal distribution of EC cells was significantly ($P<0.001$) different from that of argyrophilic cells (Table 5.1, interaction component D(ii)): the abomasal body to antral ratio of EC cell densities was 6.3:1 while that for argyrophilic cells was 3.9:1.

Immunoreactive G cells were found only in the abomasal antrum and duodenum (Fig. 5.2). Their densities were highest in the mucosa of the antrum (94 ± 17 c.mm^{-2}) and proximal duodenum (54 ± 9 c.mm^{-2}) and thence declined progressively along the duodenum. Within the glands of Brunner, G cells were always rare (<5 c.mm^{-2}).

G cell distribution within the mucosa varied according to site. In antral mucosal samples G cells were found scattered throughout the epithelium but the majority were located in the mid-gland region, whereas in duodenal samples most were found in the upper third of the crypts.

S cells were most common in the duodenum, particularly the proximal portion (60 ± 16 c.mm^{-2}), with only a few cells (<6 c.mm^{-2}) found in the ileum (Fig. 5.2). No S cells were identified in the abomasum, colon or caecum.

Immunoreactive D cells were found at all abomasal and intestinal sites examined (Fig. 5.2). They were common in the abomasal body (44 ± 8 c.mm^{-2}) and antrum (56 ± 17 c.mm^{-2}). In the intestine they were most common in the proximal duodenum (44 ± 11 c.mm^{-2}) and density declined progressively in the more distal samples. In both colon and caecum, D cells were rare (<1 c.mm^{-2}), indeed in half the slides examined they were absent from these sites. A few (always <4 c.mm^{-2}) D cells were found in the glands of Brunner.

Table 5.1: Summary of analysis of variance of data for staining methods which demonstrate argyrophilic, EC, G, S and D cells

Source of Variation		DF	Variance Ratios
A. Animals		4	2.95*
B. Sites		7	
(i)	Ab_b vs Ab_a	1	6.42*
(ii)	Duo_p vs Duo_m	1	49.72***
(iii)	Duo_p + Duo_m vs Duo_d	1	155.53***
(iv)	Duo vs Il	1	437.59***
(v)	Ab vs SI	1	183.30***
(vi)	Ab + SI vs LI	1	579.46***
(vii)	Co vs Cae	1	0.09
C. Staining methods		4	
(i)	DG vs FG	1	129.24***
(ii)	Gas vs Sec	1	15.39***
(iii)	Gas + Sec vs Som	1	9.98**
(iv)	Histo vs Immuno	1	558.31***
Non-orthogonal contrasts			
(v)	DG vs Gas	1	411.73***
(vi)	DG vs Sec	1	586.34***
(vii)	DG vs Som	1	380.89***
D. Interaction: sites x staining methods		28	
(i)	Ab_b vs Ab_a x DG vs FG	1	18.05***
(ii)	Ab vs SI x DG vs FG	1	62.64***
(iii)	Ab + SI vs LI x DG vs FG	1	9.22**
(iv)	Ab_b vs Ab_a x Gas vs Sec	1	92.54***
(v)	Ab vs SI x Gas vs Sec	1	73.54***
(vi)	Ab + SI vs LI x Gas vs Sec	1	5.13*
(vii)	Ab_b vs Ab_a x Gas + Sec vs Som	1	20.00***
(viii)	Duo vs Il x Gas + Sec vs Som	1	6.56*
(ix)	Ab + SI vs LI x Gas + Sec vs Som	1	100.73***
(x)	Ab_b vs Ab_a x Histo vs Immuno	1	110.80***

Table 5.1 continued

(xi)	Duo_p + Duo_m vs Duo_d x Histo vs Immuno	1	29.49***
(xii)	Duo vs Il x Histo vs Immuno	1	26.64***
(xiii)	Ab vs SI x Histo vs Immuno	1	224.86***
(xiv)	Ab + SI vs LI x Histo vs Immuno	1	10.83**
	Remainder	14	0.81
	Non-orthogonal contrasts		
(xv)	Ab_b vs Ab_a x DG vs Gas	1	225.59***
(xvi)	Duo_p + Duo_m vs Duo_d x DG vs Gas	1	9.86**
(xvii)	Duo vs LI x DG vs Gas	1	5.14*
(xviii)	Ab vs SI x DG vs Gas	1	50.61***
(xix)	Ab + SI vs LI x DG vs Gas	1	7.74**
(xx)	Ab_b vs Ab_a x DG vs Sec	1	29.16***
(xxi)	Duo_p + Duo_m vs Duo_d x DG vs Sec	1	9.04**
(xxii)	Duo vs Il x DG vs Sec	1	15.74***
(xxiii)	Ab + SI vs LI x DG vs Sec	1	25.34***
(xxiv)	Ab_b vs Ab_a x DG vs Som	1	40.16***
(xxv)	Duo_p + Duo_m vs Duo_d x DG vs Som	1	7.69**
(xxvi)	Duo vs Il x DG vs Som	1	28.46***
(xxvii)	Ab vs SI x DG vs Som	1	132.64***
(xxviii)	Ab + SI vs LI x DG vs Som	1	9.76**
	Residual mean square	156	1.17

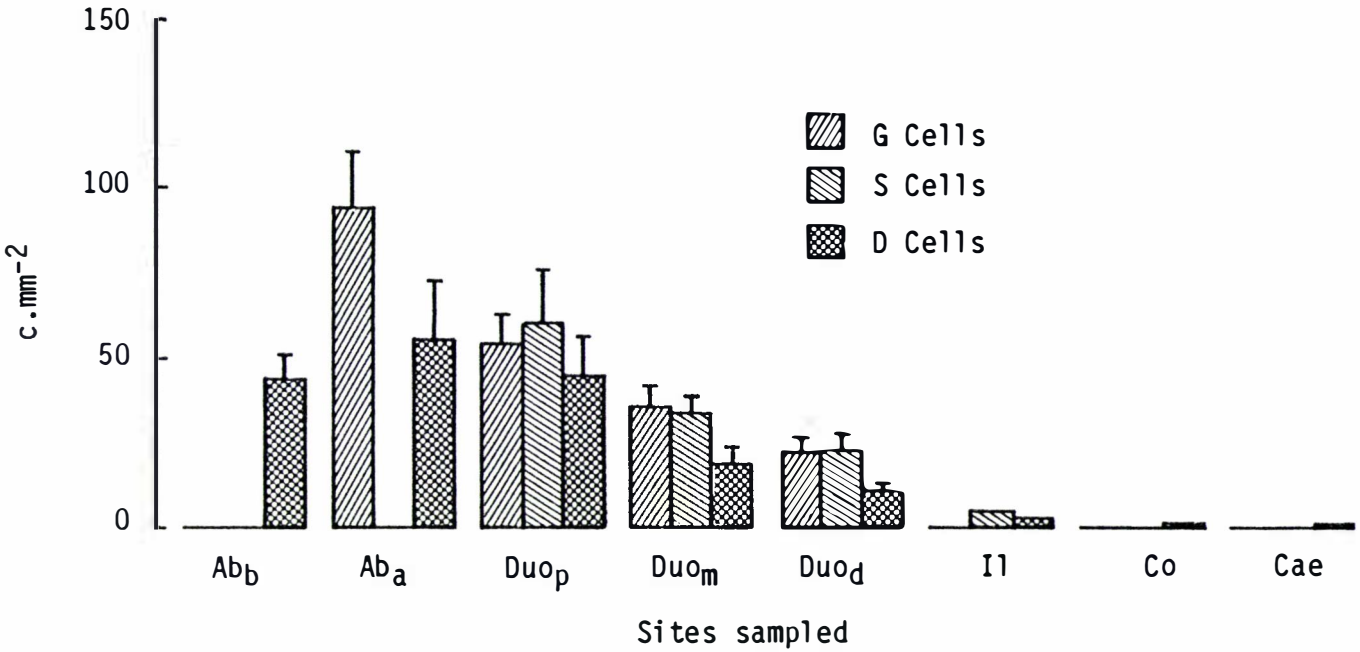


Figure 5.2: Distribution (mean + SEM) of immunoreactive G, S and D cells in the GI tract of 2 week old lambs (n = 5).

A cells were demonstrated in pancreatic islets but not in the exocrine acini nor the mucosa of the GI tract. D cells also were observed in the islet tissue of the pancreas, but not in the exocrine acini. No immunoreactive G or S cells were identified in either the endocrine or exocrine portions of the pancreas.

No endocrine cells could be demonstrated in mucosal samples from the reticulum or ventral rumen.

5.3.2 Morphology and Mucosal Location of Endocrine Cells

Table 5.2 summarizes the morphological characteristics of endocrine cells identified in the GI tract of neonatal lambs. It also lists, for each endocrine cell type, the sites at which they were found and their predominant location within the epithelium.

A few argyrophilic cells had basal cytoplasmic processes, some of which terminated close to parietal cells (Fig. 5.3). Others terminated close to cells which could not be positively identified.

Within the epithelium of the abomasal antrum, 'closed' type argyrophilic cells (Fig. 5.4) were more common than 'open' type cells. The 'closed' type cells were most numerous in the deeper parts of the antral glands, whereas 'open' type cells were found scattered throughout the depth of the epithelium.

In the intestinal mucosa all argyrophilic cells were of the 'open' type (Fig. 5.5), while in the glands of Brunner both 'open' (Fig. 5.6) and 'closed' (Fig. 5.7) type argyrophilic cells were observed.

Argyrophilic cells found in the ileum and large intestine had noticeably fewer cytoplasmic granules than duodenal argyrophilic cells, although their granules were of similar staining intensity.

The cytoplasmic granules of 'closed' type EC cells were very lightly stained while those of 'open' type EC cells were intensely stained.

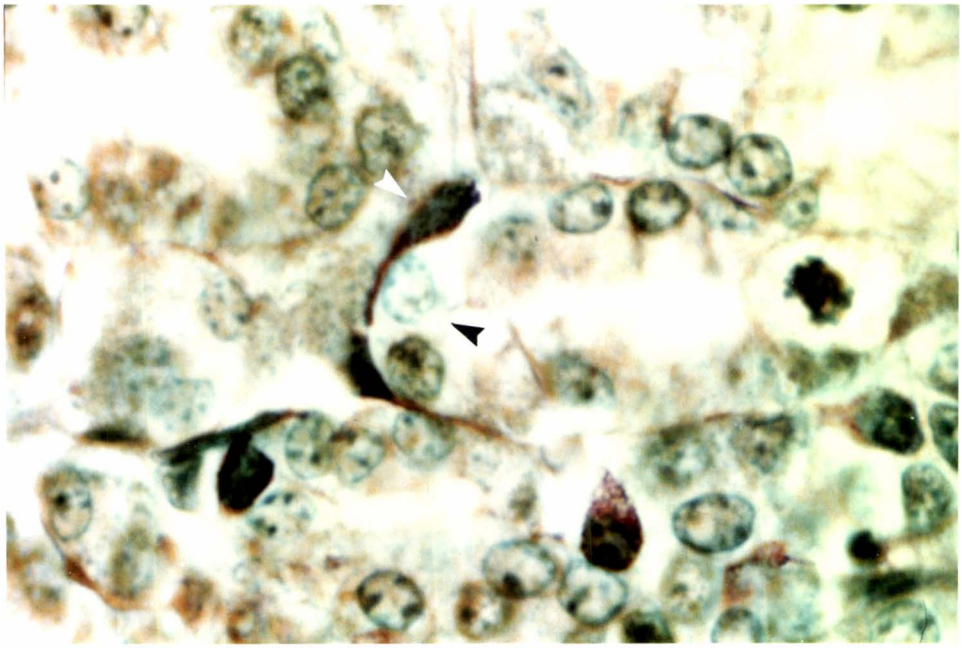
Table 5.2: Summary of morphological characteristics and distribution of endocrine cells in the GI tract of 2 week old lambs

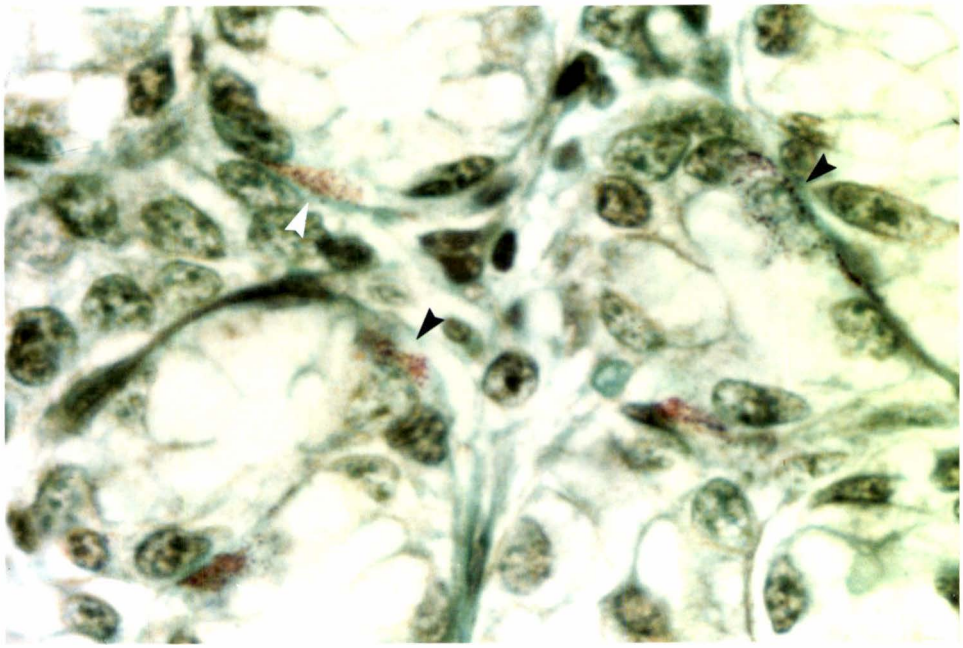
Endocrine cell	Cell type	Cell shape	Cell granules	Distribution	Predominant location in mucosa
Argyrophilic	'open'	pyramidal	basal	Ab_a Intestinal mucosa Glands of Brunner	scattered lower 1/3 deep
	'closed'	variable	scattered	Ab_a Ab_b Glands of Brunner	lower 1/3 lower 2/3 deep
EC	'closed'	oval	scattered	Ab_a, Ab_b	lower 1/3
	'open'	pyramidal	basal	Intestinal mucosa Glands of Brunner	lower 1/3 deep
G	'open'	pyramidal/ teardrop	basal	Ab_a Duo Glands of Brunner	middle 1/3 upper 1/3 deep
S	'open'	pyramidal	basal	Small intestine	upper 1/3
D	'open'	pyramidal	basal	Ab_a Intestinal mucosa Glands of Brunner	middle 1/3 scattered deep
	'closed'	variable	scattered	Ab_a, Ab_b Glands of Brunner	lower 2/3 deep

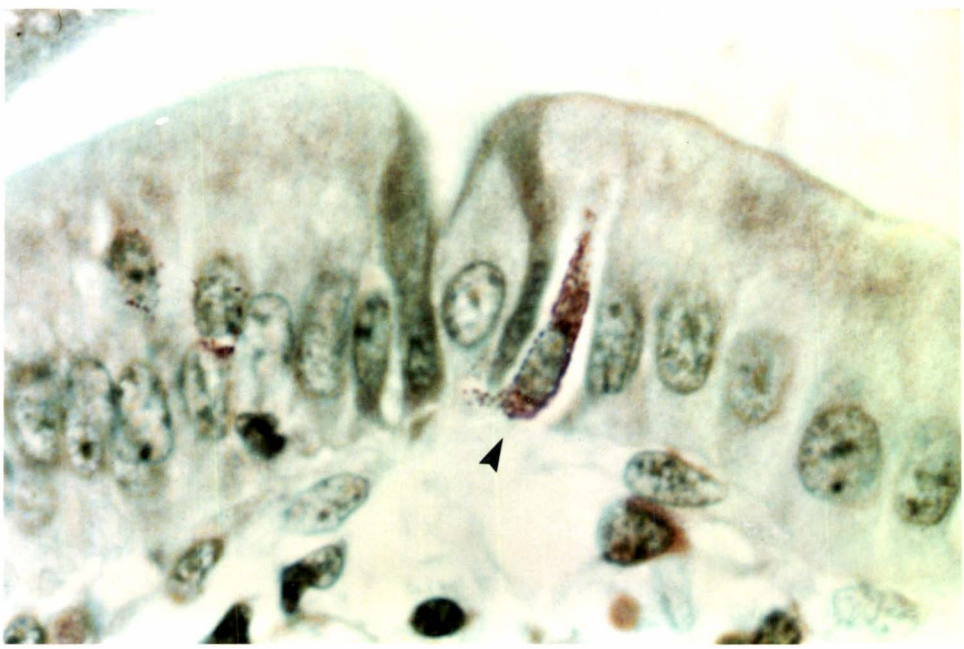
Figure 5.3: Argyrophilic cell (white arrow) with basal cytoplasmic process terminating close to parietal cell (black arrow). Abomasal body; De Grandi method; magnification x 1850.

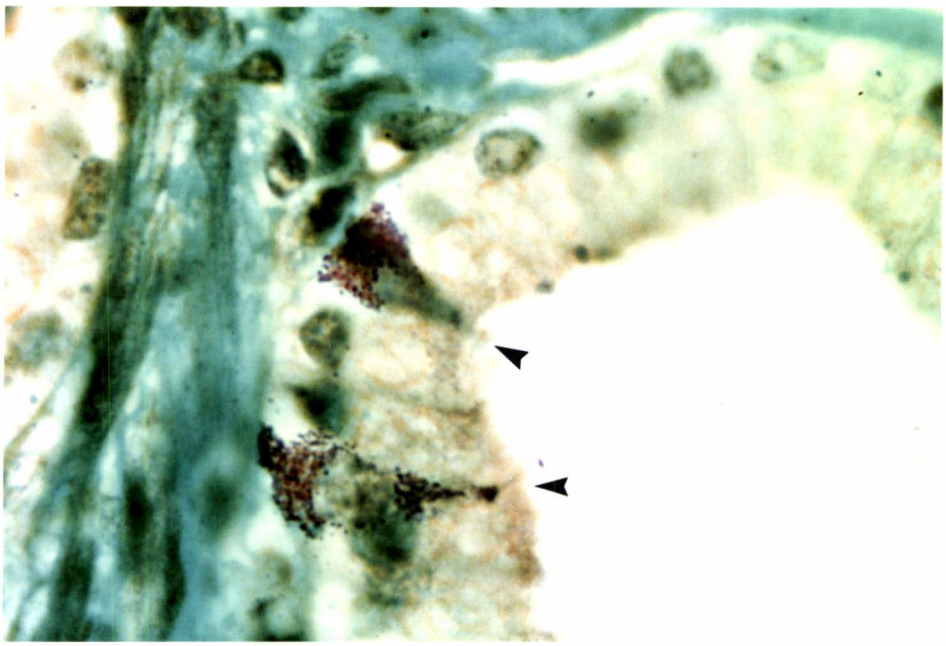
Figure 5.4: 'Closed' type argyrophilic cells (arrows), with fine black or brown cytoplasmic granules, in abomasal antral glands. Abomasal antrum; De Grandi method; magnification x 1850.

Figure 5.5: 'Open' type argyrophilic cell (arrow) near base of intestinal crypt. Proximal duodenum; De Grandi method; magnification x 1850.









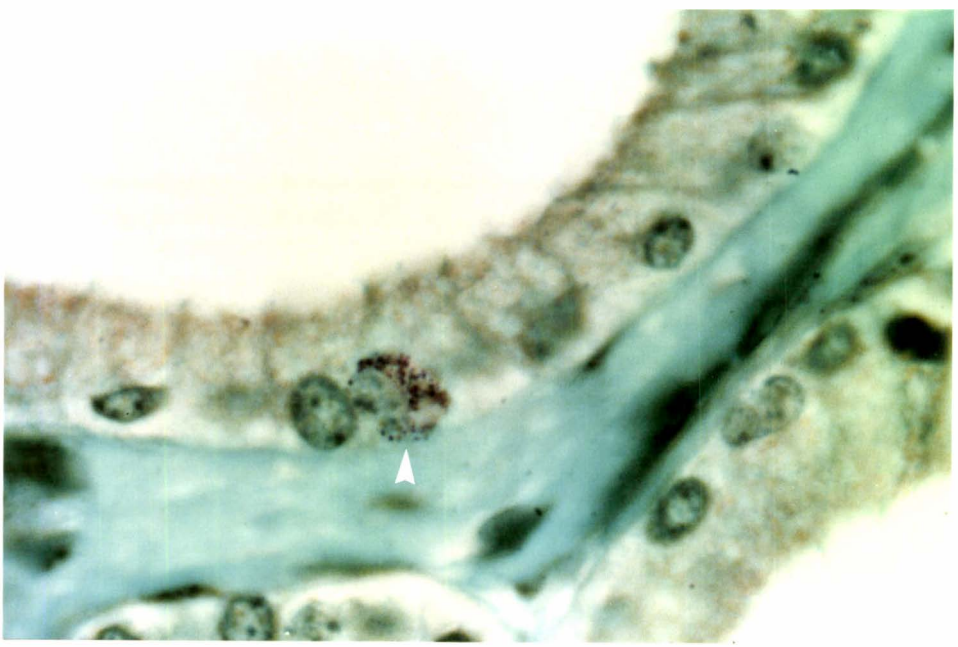


Figure 5.6: 'Open' type argyrophilic cells (arrows), with intensely stained cytoplasmic granules, in Brunner's gland. Mid-duodenum; De Grandi method; magnification x 1850.

Figure 5.7: 'Closed' type argyrophilic cell (arrow) in Brunner's gland. Mid-duodenum; De Grandi method; magnification x 1850.

Although intestinal EC cells were most common in the lower third of the crypts of Lieberkuhn, a few cells were found near the mucosal surface and in villi.

Immunoreactive G cells were of the 'open' type and although cell shape was variable those cells that were cut in true cross section, were pyramidal or teardrop in shape. Although well stained granules were found throughout the cytoplasm, the concentration of granules frequently was higher in the infranuclear region of the cell.

Morphologically, S cells were similar to the G cells described above: they were of the 'open' type, frequently pyramidal in shape and with well stained cytoplasmic granules, particularly in the infranuclear region.

D cells generally occurred singly in the epithelium. Although in antral mucosa they were most common in the middle third of the glands, they were found scattered throughout the epithelium. In this region 'open' type cells predominated. The morphology of D cells in two week old lambs did not differ noticeably from those of 6 month old lambs. A detailed description of D cells in the latter age group is given in Section 6.3.2.

5.4 Discussion

The mucosa of the GI tract of young lambs contains considerable numbers of endocrine cells, particularly in the abomasum and duodenum. While the distribution patterns of endocrine cells in young lambs were similar to those described for adult sheep (Chapter 3) and 100 - 110 day old foetuses (Chapter 4), for some sampling sites differences in cell densities between the age groups were observed. The possible significance of these differences are discussed in Chapter 8.

Between 100 - 110days of gestation and the second week after birth there was a 3-fold increase in argyrophilic cell density in proximal duodenal samples. Argyrophilic cell densities at this site in neonatal lambs were also considerably higher than in adult sheep. This latter

finding is in accord with that of Larsson who reported that in human* and rat‡ foetuses and neonates the frequency of endocrine cells in the duodenum was generally higher than in adults.

Comparison of argyrophilic and EC cell densities and their patterns of distribution indicate that, as in adult animals and foetuses, most duodenal argyrophilic cells were EC cells.

Although no data for EC cell densities were presented, Larsson and Jorgensen (1978) stated that in both human and rat foetuses and neonates EC cell frequencies may appear to be higher than in adults, because 'non EC' (in particular they suggested G, I and S) cells may contain measurable quantities of 5-HT.

In young lambs, however, EC cell densities were lower than in adult sheep. This does not preclude the possibility however, that a proportion of cells that contained 5-HT were not true EC cells but were immature G, I or S cells similar to those observed by Larsson and Jorgensen (1978).

It is clear from comparisons of EC and G cell densities that antral G cells in lambs did not contain 5-HT. This conclusion is in accord with the findings of Larsson and Jorgensen (1978) who reported that in both the human and rat, antral gastrin cells did not contain 5-HT at any stage of development. Definitive evidence to demonstrate the presence or otherwise of 5-HT in duodenal G, S and I cells could be obtained by combining ultrastructural studies or double staining techniques, with the usual diazonium methods, to demonstrate 5-HT.

G cell frequency in the abomasal antrum changed markedly with age, increasing from 50 $\pm$ 16 c.mm^{-2} in the foetuses to 94 $\pm$ 17 c.mm^{-2} in lambs and 155 $\pm$ 11 c.mm^{-2} in the adults. These data indicate that the increase, reported by Lichtenberger *et al.*, (1981), in G cell numbers in the ovine antrum throughout late pregnancy and the first two weeks of life is at least in part due to an increase in G cell densities.

* (Larsson and Jorgensen, 1978)
‡ (Larsson, 1977; Larsson and Jorgensen, 1978)

The duodenal G cell density more than doubled between the last 5 - 6 weeks of pregnancy and the 2nd week of life to reach a level of 54 ± 9 c.mm^{-2}, and then declined to 30 ± 4 c.mm^{-2} in the adult. These results suggest that G cell density in the duodenum reaches a peak shortly after birth and then declines. These results support the observations of Larsson and Jorgensen (1978) who found that G cell frequencies were higher in the duodena of human and rat neonates than in adults. Further, they demonstrated that this postnatal decrease in G cell frequency coincided with an increase in I cells. They were also able to demonstrate, using gastrin-specific and CCK-specific antisera, that in neonatal rats and humans some duodenal endocrine cells contained both gastrin and CCK. Such observations led these workers to suggest that some foetal and neonatal endocrine cells were not fully mature and were capable of synthesizing several different biologically active peptides and amines. Whether such observations also hold true for the sheep and thus provide an explanation for the postnatal decline in duodenal G cell densities remains to be established. This hypothesis cannot be confirmed until I cell frequencies in neonatal and adult sheep are determined. To date, CCK secreting cells have not been definitively demonstrated in the ovine GI tract.

The dramatic decrease in serum gastrin levels that occurs several weeks after birth (Lichtenberger *et al.*, 1981; Shulkes and Hardy, 1982) may be due to a decrease in G cell density or to a decrease in the rate of gastrin secretion. It seems unlikely, however, that the changes, described above, in G cell density of the duodenum could account for this phenomenon: (a) the change observed was not large, and (b) there was a much larger increase in G cell density in the abomasal antrum over the same period. It is more likely that the falls in serum gastrin levels reported by Lichtenberger *et al.*, (1981) and Shulkes and Hardy (1982) were due to changes in the secretory activity of the G cells.

Data presented here (Fig. 5.2) and in Sections 3.3.4 and 4.3.2, demonstrate that there was an increase in duodenal S cell density late in foetal development, and that by the 2nd week of life S cell density (60 ± 16 c.mm^{-2}) in the proximal duodenum was greater than that found in adults (32 ± 11 c.mm^{-2}). Similar observations were made by Larsson (1977) in the rat.

Because D cell density was considerably higher in the abomasal antral mucosa (56 ± 17 $c.mm^{-2}$) than argyrophilic cell density (26 ± 4 $c.mm^{-2}$) and further, because the distribution of D cells within the abomasum differed from that of argyrophilic cells, it is concluded that abomasal D cells in the young lamb are not argyrophilic. Similarly, comparison of D and EC cell densities and distributions in the abomasum indicate that the abomasal D cells do not contain measurable quantities of 5-HT in young lambs. Whether these conclusions may be extended to include all D cells of the GI tract of young lambs was not conclusively established.

Two different types of D cell were observed: (a) 'open', and (b) 'closed'. 'Open' type D cells which predominate in the abomasal antrum and intestine are thought to respond to intraluminal stimuli such as pH (Kobayashi and Fujita, 1974), whereas 'closed' type D cells respond to circulating hormones (Larsson, 1980) or nervous stimuli (Schubert *et al.*, 1982).

It has been suggested that both 'open' and 'closed' type D cells have a local regulatory role: that they modulate the function of other cells nearby (e.g. antral G cells: Helmstaedtler *et al.*, 1977; Larsson *et al.*, 1979, and parietal cells: Larsson *et al.*, 1979) by the release of somatostatin into the interstitial space close to the 'target' cells. In this respect exogenous somatostatin has been shown to inhibit the secretion of acid by parietal cells (Bloom *et al.*, 1974) and the release of gastrin from G cells (Konturek *et al.*, 1976*a*).

In the present studies some D cells were observed to have basal cytoplasmic processes some of which, in the case of D cells found in the abomasal body region, were shown to terminate close to parietal cells. In most instances, however, the identity of the cell on or near which the process terminated could not be ascertained. Further study with double immunohistochemical staining techniques could be used to identify the 'target' cells.

Argyrophilic, EC, G and D cells were identified in glands of Brunner of 2 week old lambs. The densities of these cells were low, less than 4 $c.mm^{-2}$ of gland. G cells were always of the 'open' type but

both 'open' and 'closed' type argyrophilic, EC and D cells were observed. The most common endocrine cells found in the glands were G and D cells and there were no obvious differences in cell densities between the lambs and adult animals. Although the majority of endocrine cells found here were located deep within the glands, the fact that 'open' type cells were found indicates that stimuli originating in the lumen may play a part in modulating the secretory activity of these cells. The functions of endocrine cells in the glands of Brunner have not been elucidated in any species. Possible roles include local regulation of the secretory activity of the glands and regulation of the growth and maintenance of the gland mucosa (see Johnson, 1980).

CHAPTER 6

THE IDENTIFICATION AND DISTRIBUTION OF ENDOCRINE CELLS IN THE GI TRACT OF 6 MONTH OLD LAMBS

6.1 Introduction

The aims of this study were to investigate the types, morphology and distribution of endocrine cells in the GI tract of 6 month old (young ruminant) lambs. As well as being of value in assessing the effects of the change from the pre-ruminant to ruminant state, on endocrine cell densities, the data so derived is used in Chapter 8 to establish the pattern of development of such cells in sheep, from foetus through to adulthood.

In addition to the staining methods employed in previous chapters (see Section 2.6), the fast black method was used. In EC cells, when both 5-HT and tryptamine are present, but one or both are in low concentrations, the colour contrasts with fast garnet staining (i.e. red-orange-yellow) become difficult to distinguish. Fast black reputedly has the advantage of better tinctorial resolution of 5-HT and tryptamine (Section 1.8.1). Thus the fast black method was used in addition to the fast garnet method, to determine whether 5-HT was present in all ovine EC cells. The fast black and fast garnet methods were compared for sensitivity and colour resolution of stained cell contents.

6.2 Methods

Seven 6 month old Romney-cross lambs (Table 2.1) were used in this study. They were housed indoors approximately 24 hrs before sample collection, and food, but not water, withheld.

Tissue samples from sites listed in Table 2.2 were collected, prepared, fixed and processed in the manner described in Sections 2.3 and 2.5.

Three histochemical staining techniques were used: De Grandi (Table 2.7), fast garnet (Table 2.11) and fast black (Table 2.12). The

PAP immunohistochemical method (Table 2.19) was used with the following primary antisera: gastrin, secretin, glucagon and somatostatin (Table 2.20).

6.3 Results

6.3.1 Distribution of Endocrine Cells

Argyrophilic cells (Fig. 6.1) were most common in the duodenum, particularly the proximal (236 ± 40 $c.mm^{-2}$) and middle (203 ± 37 $c.mm^{-2}$) regions. Substantial numbers of argyrophilic cells were also found in the abomasal body (117 ± 15 $c.mm^{-2}$) and distal duodenum (105 ± 15 $c.mm^{-2}$), while fewer (<33 $c.mm^{-2}$) occurred in the abomasal antrum, ileum and large intestine.

The highest concentrations of EC cells (Fig. 6.1), as demonstrated by the fast garnet technique, were found in the proximal, mid and distal duodenum (185 ± 21, 100 ± 15 and 50 ± 9 $c.mm^{-2}$, respectively) with substantially lower concentrations in the abomasum, ileum and large intestine (all <5 $c.mm^{-2}$).

Immunoreactive G cells (Fig. 6.2) were most numerous in the abomasal antral mucosa (133 ± 11 $c.mm^{-2}$). Lesser numbers of G cells were found in the duodenum (proximal duodenum: 46 ± 5 $c.mm^{-2}$), where they decreased in density with increasing distance from the pylorus. G cells were absent from the mucosa of the abomasal body region, ileum and large intestine.

S cells were confined in their distribution to the small intestine (Fig. 6.2), the greatest density occurring in the proximal duodenum (58 ± 5 $c.mm^{-2}$). The density of S cells in the small intestine decreased distally.

D cells were found in mucosal samples of all regions of the GI tract aboral to the omasum (Fig. 6.2); although they were rare (<2 $c.mm^{-2}$) in the colon and caecum. Greatest concentrations of D cells were found in the abomasal antrum (20 ± 2 $c.mm^{-2}$) and proximal duodenum (17 ± 3 $c.mm^{-2}$). D cells were also identified in the islets of Langerhans. Although they were found scattered throughout the islets, they were most numerous near the periphery of each islet.

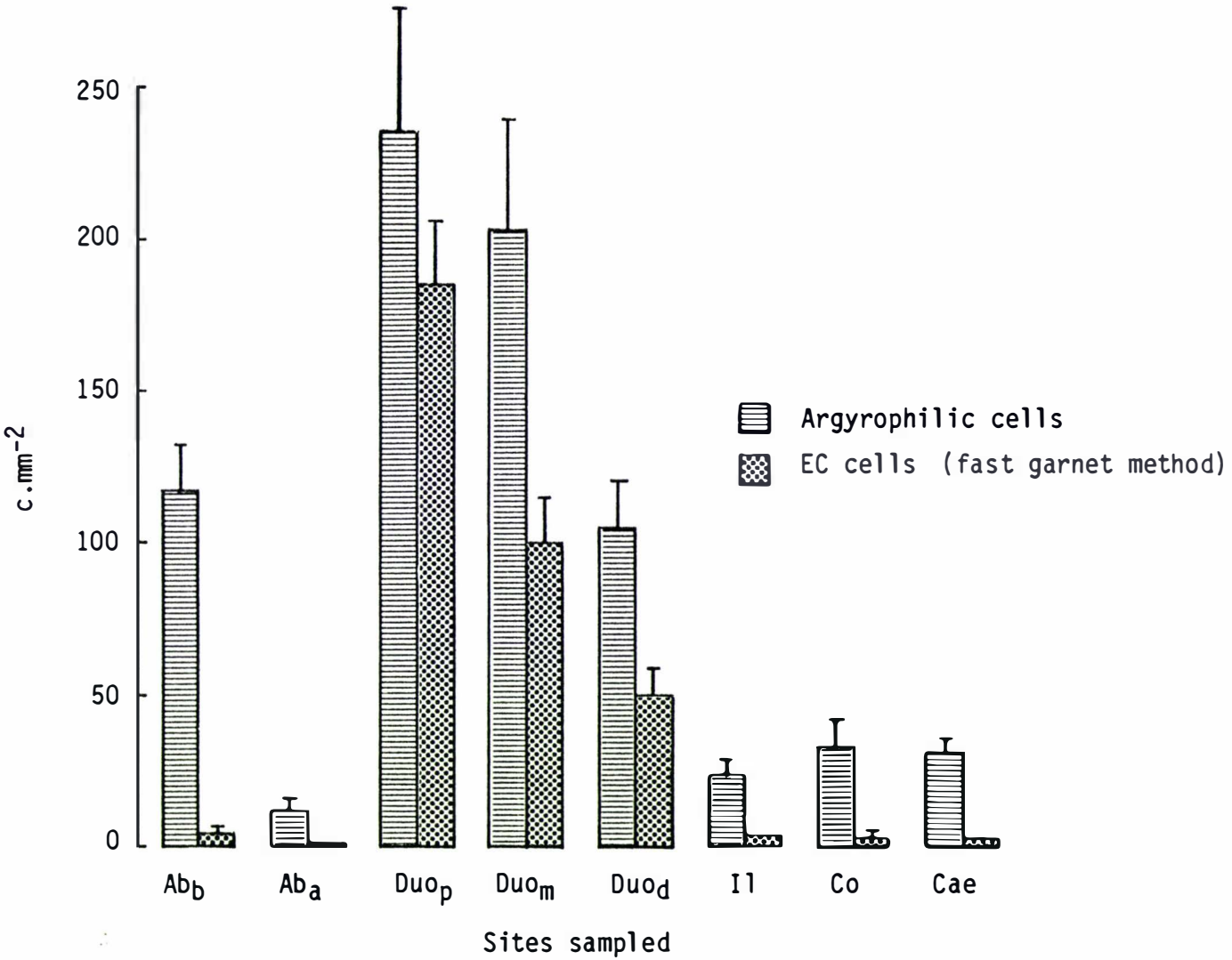


Figure 6.1: Mean density (+ SEM) of argyrophilic and EC cells in the GI tract of 6 month old sheep (n = 7).

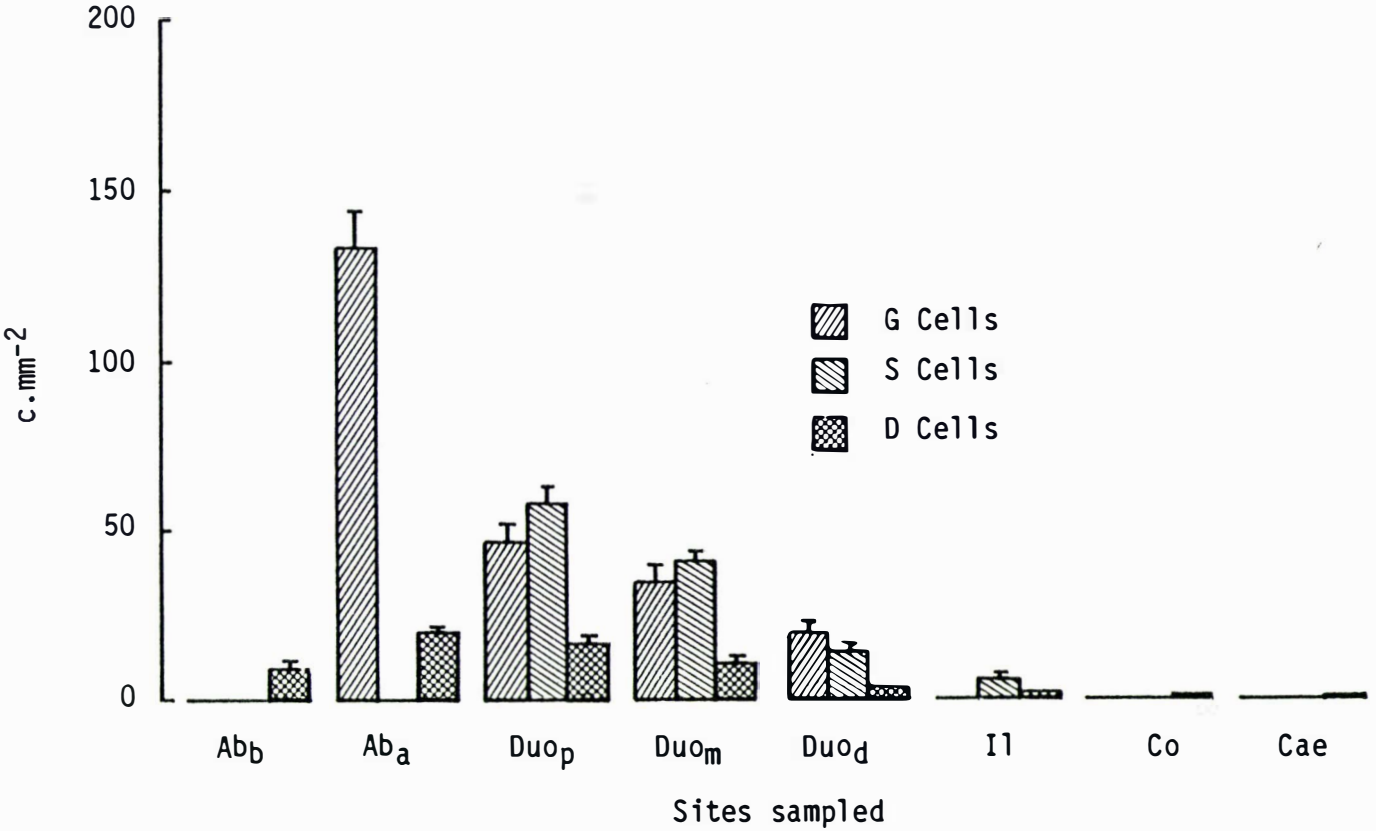


Figure 6.2: Mean density (+ SEM) of gastrin, secretin and somatostatin containing cells in the GI tract of 6 month old sheep (n = 7).

A cells were identified in islets of Langerhans, particularly near the periphery of each islet. No cells binding with a pancreatic glucagon antiserum were found in the abomasum or intestines.

Analysis of variance (Table 6.1) demonstrated a highly significant ($P<0.001$) overall difference between argyrophilic and EC cell densities (Table 6.1, component C(i)); argyrophilic cell densities were always higher (Fig. 6.1). This figure clearly demonstrates that although EC cells may make up the bulk of the argyrophilic cell population in the upper small intestine, this was not true for the abomasum, ileum or large intestine. In these regions, EC cell densities were only 4 to 12% of argyrophilic cell densities at the same sites. The highly significant ($P<0.001$) interaction term for these two staining methods within the abomasum (Table 6.1, interaction component, D(i)) largely reflects the difference in argyrophilic cell densities in the body and antral regions of this organ. Argyrophilic cell densities in the abomasal body were nearly 10 times higher than in the antral region, whereas for EC cells the difference was 4 fold.

Analysis of variance also demonstrated that argyrophilic cell densities were significantly higher ($P<0.001$) than those of G cells (Table 6.1, component C(v)). The significant ($P<0.001$) interaction term for the comparison of densities of these two cell types within the abomasum (Table 6.1, interaction component, D(xviii)) reflects differences in distribution of argyrophilic and G cells in this organ. Argyrophilic cells were common in the body region of the abomasum (Fig. 6.1) while G cells were not found at this site (Fig. 6.2). In the abomasal antrum, however, G cell densities were more than 10 times higher than argyrophilic cell densities.

The analysis of variance demonstrates that, overall, argyrophilic cell densities were significantly ($P<0.001$) higher than S cell densities (Table 6.1, component C(vi)). It also highlights the difference in distribution of G and S cells in the abomasum (Table 6.1, interaction component D(iv)) and small intestine (Table 6.1, interaction component D(v)): S cells were absent from the abomasum while G cells did not occur in the ileum.

Table 6.1: Summary of analysis of variance of data for staining methods which demonstrate argyrophilic, EC,* G, S and D cells

Source of Variation		DF	Variance Ratios
A. Animals		6	7.81***
B. Sites		7	
(i)	Ab_b vs Ab_a	1	10.78**
(ii)	Duo_p vs Duo_m	1	42.45***
(iii)	Duo_p + Duo_m vs Duo_d	1	232.64***
(iv)	Duo vs Il	1	696.65***
(v)	Ab vs SI	1	255.93***
(vi)	Ab + SI vs LI	1	599.82***
(vii)	Co vs Cae	1	0.06
C. Staining methods		4	
(i)	DG vs FG	1	388.77***
(ii)	Gas vs Sec	1	30.21***
(iii)	Gas + Sec vs Som	1	24.67***
(iv)	Histo vs Immuno	1	786.45***
Non-orthogonal contrasts			
(v)	DG vs Gas	1	565.34***
(vi)	DG vs Sec	1	857.04***
(vii)	DG vs Som	1	950.41***
D. Interaction: sites x staining methods		28	
(i)	Ab_b vs Ab_a x DG vs FG	1	83.56***
(ii)	Duo_p vs Duo_m x DG vs FG	1	10.27**
(iii)	Ab vs SI x DG vs FG	1	29.36***
(iv)	Ab_b vs Ab_a x Gas vs Sec	1	190.26***
(v)	Duo vs Il x Gas vs Sec	1	4.58*
(vi)	Ab vs SI x Gas vs Sec	1	153.91***
(vii)	Ab + SI vs LI x Gas vs Sec	1	10.07**
(viii)	Ab_b vs Ab_a x Gas + Sec vs Som	1	33.96***
(ix)	Duo vs Il x Gas + Sec vs Som	1	19.21***
(x)	Ab vs SI x Gas + Sec vs Som	1	27.62***

* EC cells stained by fast garnet method.

Table 6.1 continued

(xi)	Ab + SI vs LI x Gas + Sec vs Som	1	14.10***
(xii)	Ab_b vs Ab_a x Histo vs Immuno	1	261.63***
(xiii)	Duo_p vs Duo_m x Histo vs Immuno	1	7.28**
(xiv)	Duo_p + Duo_m vs Duo_d x Histo vs Immuno	1	27.62***
(xv)	Duo vs Il x Histo vs Immuno	1	147.72***
(xvi)	Ab vs SI x Histo vs Immuno	1	251.14***
(xvii)	Ab + SI vs LI x Histo vs Immuno	1	18.22***
	Remainder	11	0.65
	Non-orthogonal contrasts		
(xviii)	Ab_b vs Ab_a x DG vs Gas	1	556.52***
(xix)	Duo_p + Duo_m vs Duo_d x DG vs Gas	1	12.53***
(xx)	Duo vs LI x DG vs Gas	1	29.90***
(xxi)	Ab vs SI x DG vs Gas	1	148.99***
(xxii)	Ab_b vs Ab_a x DG vs Sec	1	95.98***
(xxiii)	Duo vs Il x DG vs Sec	1	57.88***
(xxiv)	Ab + SI vs LI x DG vs Sec	1	22.60***
(xxv)	Ab_b vs Ab_a x DG vs Som	1	135.65***
(xxvi)	Duo_p + Duo_m vs Duo_d x DG vs Som	1	16.20***
(xxvii)	Duo vs Il x DG vs Som	1	106.79***
(xxviii)	Ab vs SI x DG vs Som	1	111.39***
(xxiv)	Ab + SI vs LI x DG vs Som	1	23.63***
	Residual mean square	234	1.46

Overall, D cell densities were significantly ($P<0.001$) lower than argyrophilic cell densities (Table 6.1, component C(vii)). The differences in distribution of these two cell types was particularly marked in the abomasum (Table 6.1, interaction component D(xxv)) where D cell densities were greatest in the antrum while argyrophilic cell densities were highest in the body of the abomasum.

6.3.2 Endocrine Cell Morphology

The morphological characteristics of endocrine cells and their distribution within the mucosa of the GI tract of 6 month old lambs were similar to those described for adults in Chapter 3 and are summarized in Table 6.2. For this reason no repetitive description of the morphology of EC, G and S cells is given in this section.

Some argyrophilic cells, particularly in the body region of the abomasum, possessed single basal cytoplasmic processes which in a few cases were identified as terminating close to parietal cells. Although both 'open' and 'closed' type argyrophilic cells were found in the antral region of the abomasum, the latter type was more common. In the glands of Brunner these two cell types were equally common. Although intestinal argyrophilic cells were most common in the lower third of the crypts of Lieberkuhn, they were also observed in the villi.

The D cells found in the abomasal body were all of the 'closed' type (Fig. 6.3) while in the antrum both 'open' (Fig. 6.4) and 'closed' (Fig. 6.5) types were identified. In both regions a small number of D cells were observed to have basal cytoplasmic processes (Fig. 6.5). In the abomasal body region some of these processes terminated close to parietal cells, however, it was not always possible to identify the 'target' cell. D cells found in the crypts of Lieberkuhn were always of the 'open' type and were pyramidal or teardrop in shape (Fig. 6.6). A few D cells were found near the tips of villi in the duodenum where they typically appeared as slender columnar cells (Fig. 6.7). Occasionally D cells of both the 'open' and 'closed' types were observed in the glands of Brunner.

Table 6.2: Summary of morphological characteristics and distibution of endocrine cells in the GI tract of 6 month old lambs

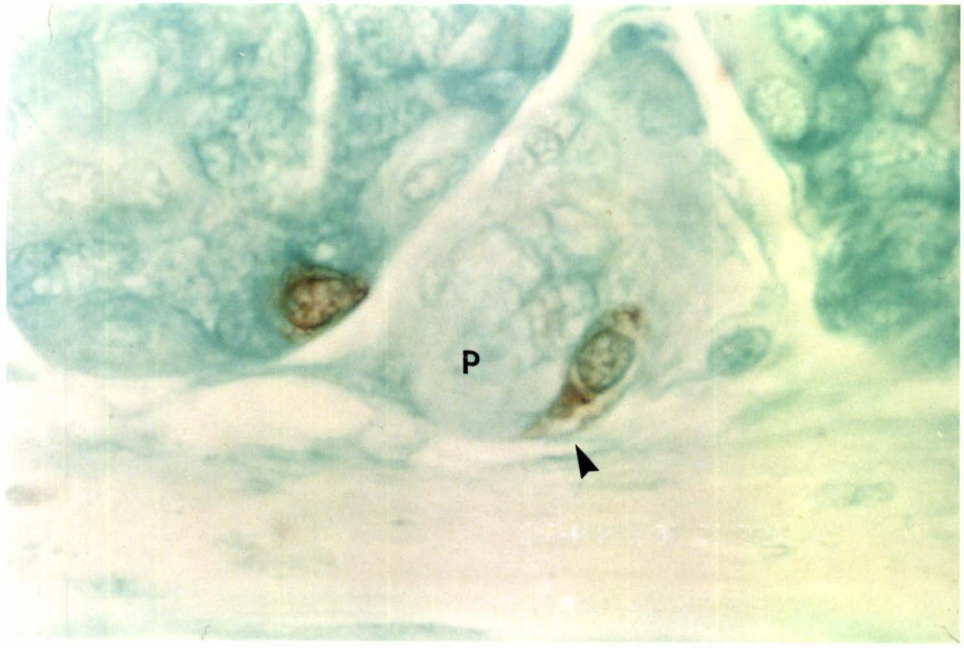
Endocrine cell	Cell type	Cell shape	Cell granules	Distribution	Predominant location in mucosa
Argyrophilic	'open'	pyramidal	basal	Ab_a Intestinal mucosa Glands of Brunner	scattered lower 1/3 deep
	'closed'	variable	scattered	Ab_a Ab_b Glands of Brunner	mid-lower 1/3's scattered deep
EC*	'closed'	oval	scattered	Ab_a, Ab_b	lower 1/3
	'open'	pyramidal	basal	Intestinal mucosa Glands of Brunner	lower 1/3 deep
G	'open'	teardrop/ pyramidal	basal	Ab_a Duo Glands of Brunner	middle 1/3 upper 1/3 deep
S	'open'	pyramidal	basal	Small intestine	upper 1/3
D	'open'	pyramidal	basal	Ab_a Intestinal mucosa Glands of Brunner	scattered scattered deep
	'closed'	variable	scattered	Ab_a, Ab_b Glands of Brunner	lower 1/3 deep

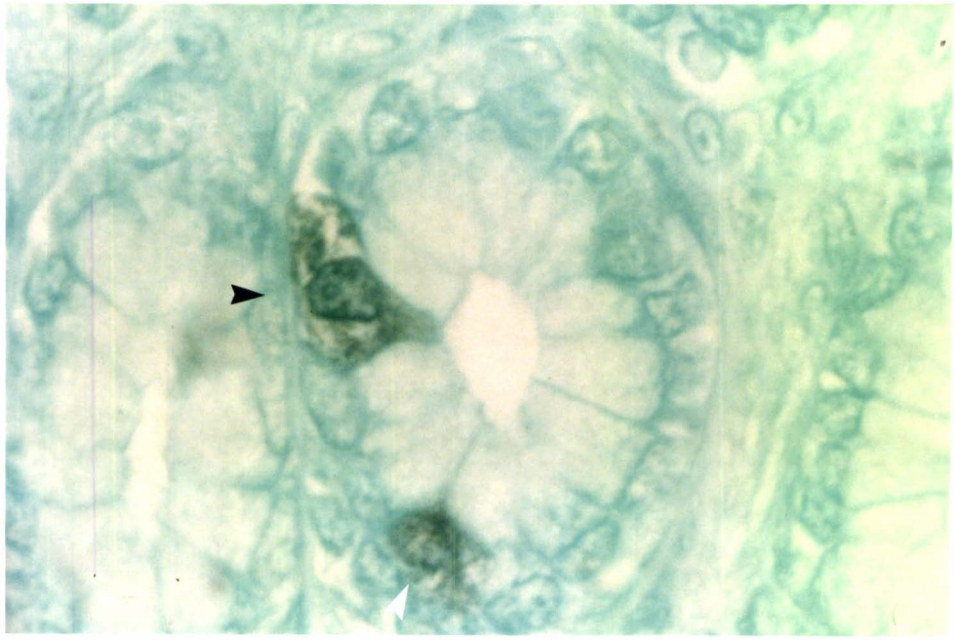
* Information pertaining to EC cells was derived from sections stained with fast garnet.

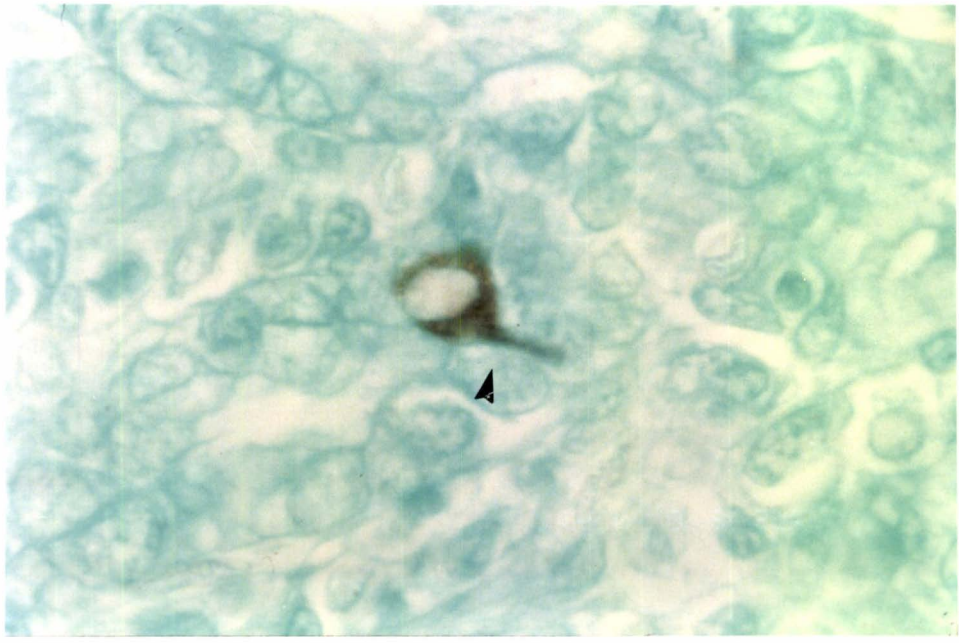
Figure 6.3: 'Closed' type D cells (brown) in abomasal gastric glands. One D cell (arrow) has a basal cytoplasmic process terminating near an adjacent parietal cell (P). Abomasal body; somatostatin antiserum/PAP method; magnification x 1850.

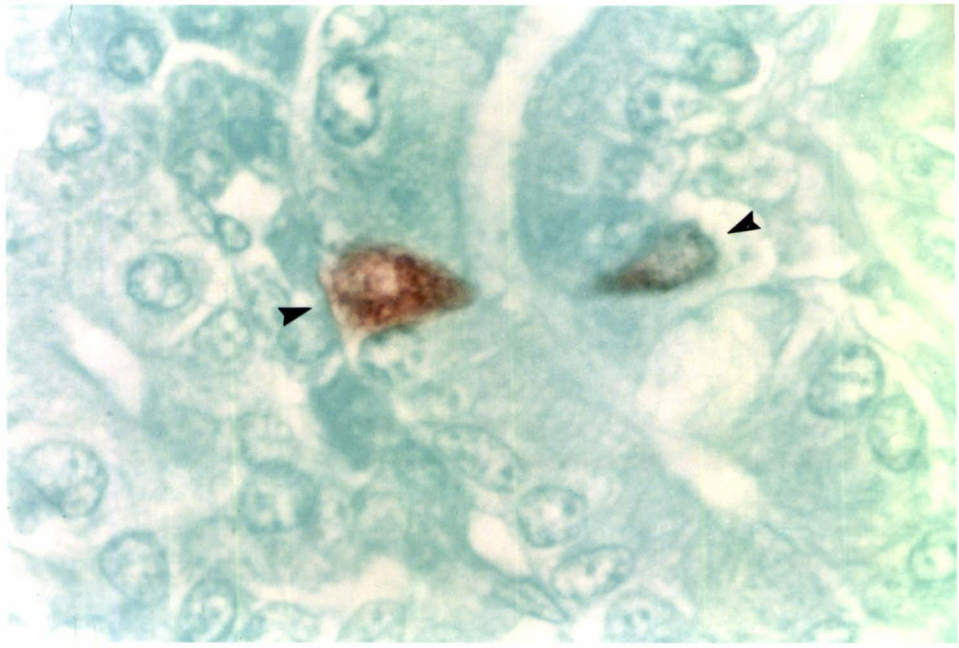
Figure 6.4: 'Open' (black arrow) and 'closed' (white arrow) type D cells in abomasal antral gland. Abomasal antrum; somatostatin antiserum/PAP method; magnification x 1850.

Figure 6.5: 'Closed' type D cell with basal cytoplasmic process (arrow) in abomasal antral gland. Abomasal antrum; somatostatin antiserum/PAP method; magnification x 1850.


P







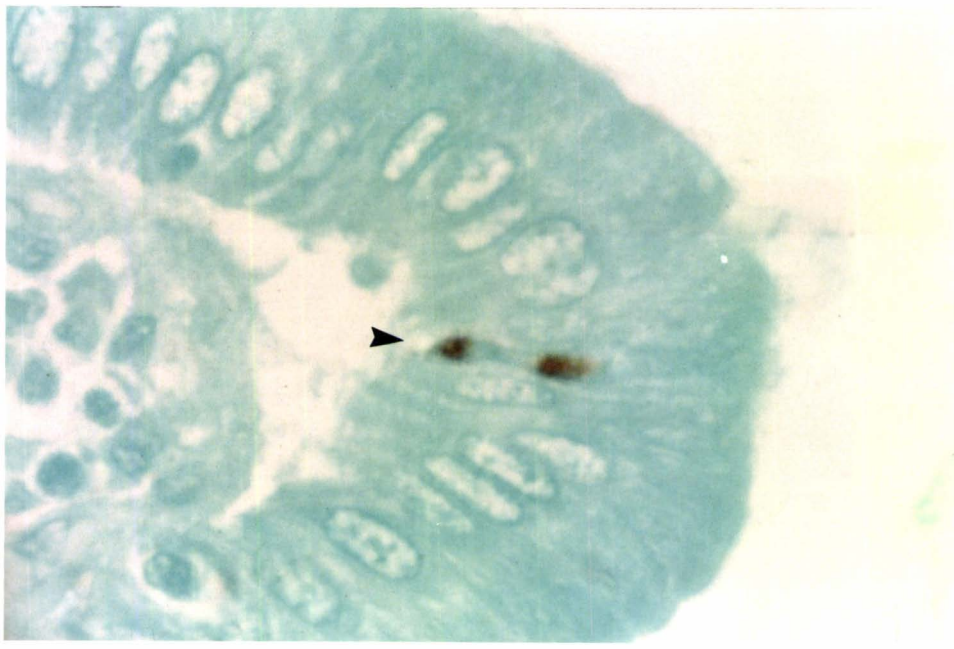


Figure 6.6: Pyramidal and teardrop shaped D cells (arrows) in duodenal crypt. Proximal duodenum; somatostatin antiserum/PAP method; magnification x 1850.

Figure 6.7: Columnar shaped, 'open' type D cell (arrow), near tip of intestinal villus. Proximal duodenum; somatostatin antiserum/PAP method; magnification x 1850.

6.3.3 Comparison of Diazonium Techniques

Cell frequency results obtained using the fast black and fast garnet techniques are given in Table 6.3.

Table 6.3: EC cell densities (Mean ± SEM) in the gut mucosa of 6 month old sheep (n = 7)

Site	Staining Method	
	Fast Garnet	Fast Black
Ab_b	4.7 ± 1.3	0.8 ± 0.4
Ab_a	0.8 ± 0.4	0.0
Duo_p	185.8 ± 21.3	180.0 ± 14.0
Duo_m	100.2 ± 15.5	102.9 ± 10.9
Duo_d	50.1 ± 9.0	53.7 ± 6.3
Il	3.1 ± 1.0	2.2 ± 0.5
Co	2.9 ± 1.5	1.3 ± 0.5
Cae	2.4 ± 1.1	1.0 ± 0.5

Analysis of variance (Table 6.4) of this data revealed no overall significant difference between the two techniques nor any significant component in the sites x staining methods interaction. These statistical results indicate that these two diazonium techniques stained essentially the same population of cells.

Within the analysis of variance, components of the sampling sites main effect showed again that EC cell densities were low in the abomasum, highest in the proximal duodenum, then decreased distally.

Intestinal EC cells were intensely stained by both diazonium methods. In contrast, abomasal EC cells were poorly stained being particularly difficult to identify in sections stained by the fast black method. This may have been due in part to a masking effect caused by the red-brown background staining observed in these sections.

Table 6.4: Summary of analysis of variance of data for the fast garnet and fast black methods of demonstrating EC cells.

Source of Variation		DF	Variance Ratios
A. Animals		6	8.14***
B. Sites		7	
(i)	Duo_p vs Duo_m	1	81.87***
(ii)	Duo_p + Duo_m vs Duo_d	1	194.99***
(iii)	Duo vs Il	1	724.59***
(iv)	Ab vs SI	1	841.61***
(v)	Ab + SI vs LI	1	382.57***
	Remainder	2	0.59
C. Staining method: FG vs FB		1	0.38
D. Interaction: sites x methods		7	0.45
Residual Mean Square		90	0.85

6.3.4 Morphology of Diazonium Positive Cells

EC Cells

Both abomasal and intestinal (Fig. 6.8) EC cells, as demonstrated by the fast black technique, were similar in morphology to those fast garnet stained cells described in Section 3.3.3 and illustrated in figures 3.19 and 3.23. The cytoplasmic granules of all EC cells were stained black by the fast black method, with no evidence of any red staining granules.

Non-EC Cells

Two non-EC cell types, found within the epithelium of the glands of Brunner, were stained by the fast black technique:

(a) Round or oval shaped cells of the 'closed' type, lying deep within the glands, were found to have yellow staining cytoplasm containing large red stained granules (Fig. 6.9). These cells (thought to be MMC's) lay within the epithelium in contact with the basal lamina.

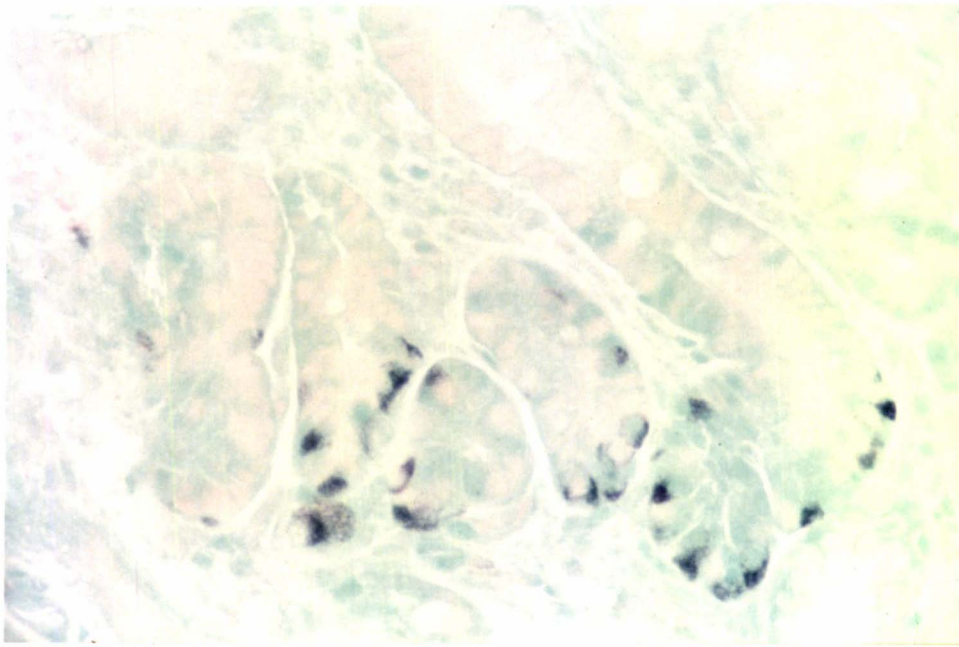
(b) Globular leucocytes (see Section 3.3.3) were found within the epithelium of the glands of Brunner, particularly in the ducts of the glands where the ducts passed through the muscularis mucosae (Fig. 6.10). These cells were large, frequently oval in shape, often with the nucleus located at one pole of the cell and the cytoplasm contained large, pale yellow staining granules. Such cells were found scattered throughout the depth of the epithelium.

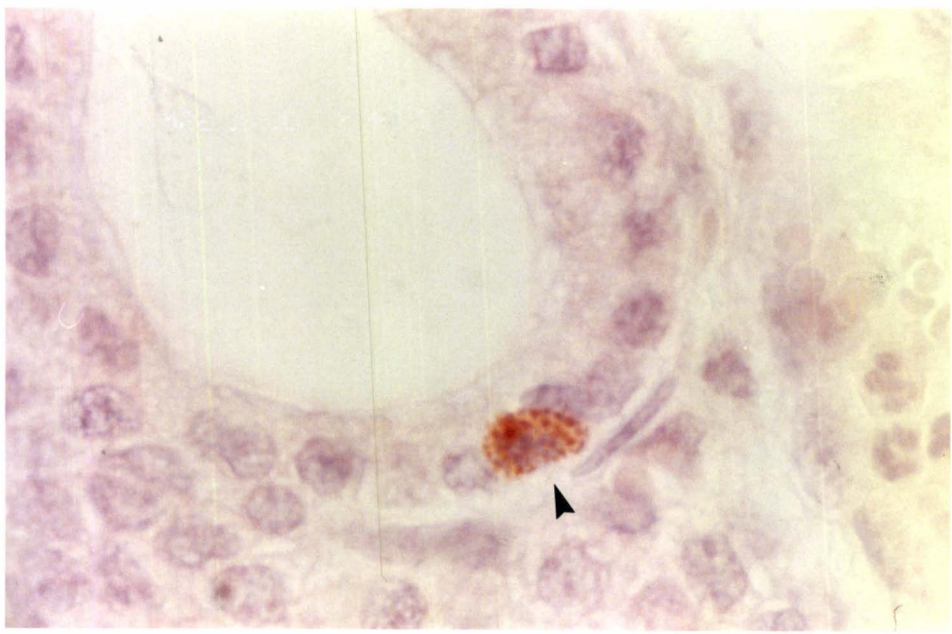
Two cell types, similar in both morphology and distribution to the MMC's and globular leucocytes described above, were observed in duodenal mucosal samples stained by the fast garnet technique. In contrast to the results described for fast black, the cytoplasm of MMC's was stained yellow by the fast garnet technique (i.e. there was no evidence of two types of granule within their cytoplasm).

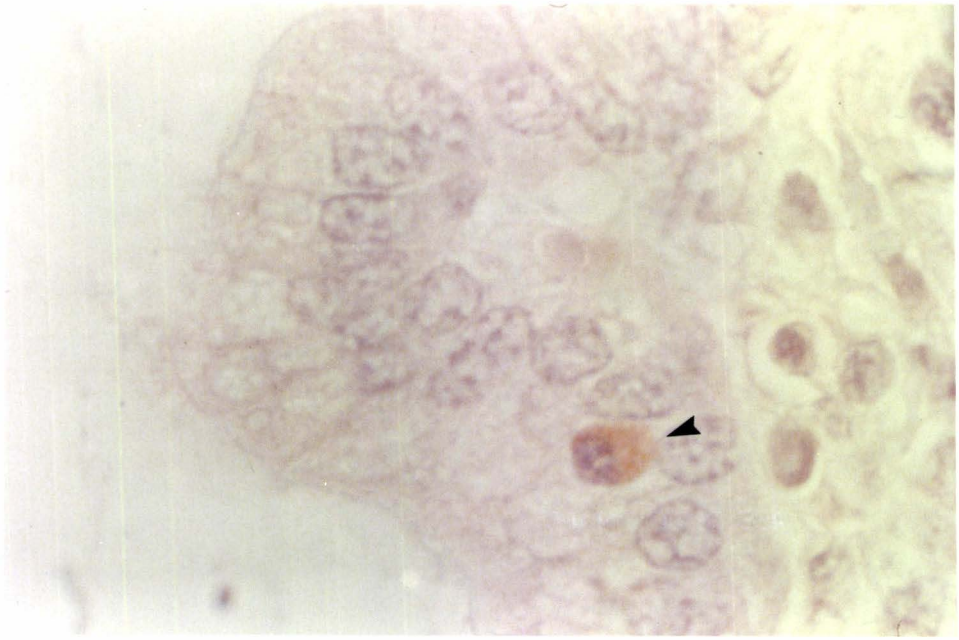
CTMC's stained by the fast black method (Fig. 6.11) were similar to the fast garnet stained CTMC's described in Section 3.3.3, and

Figure 6.8: EC cells (black), with stained granules concentrated in infranuclear cytoplasm, near base of duodenal crypts. Proximal duodenum; fast black method; magnification x 740.

Figure 6.9: 'Closed' type diazonium positive cell (arrow), possibly an MMC, in Brunner's gland. It's cytoplasm is stained yellow and contains large red-stained granules. Proximal duodenum; fast black method; magnification x 1850.







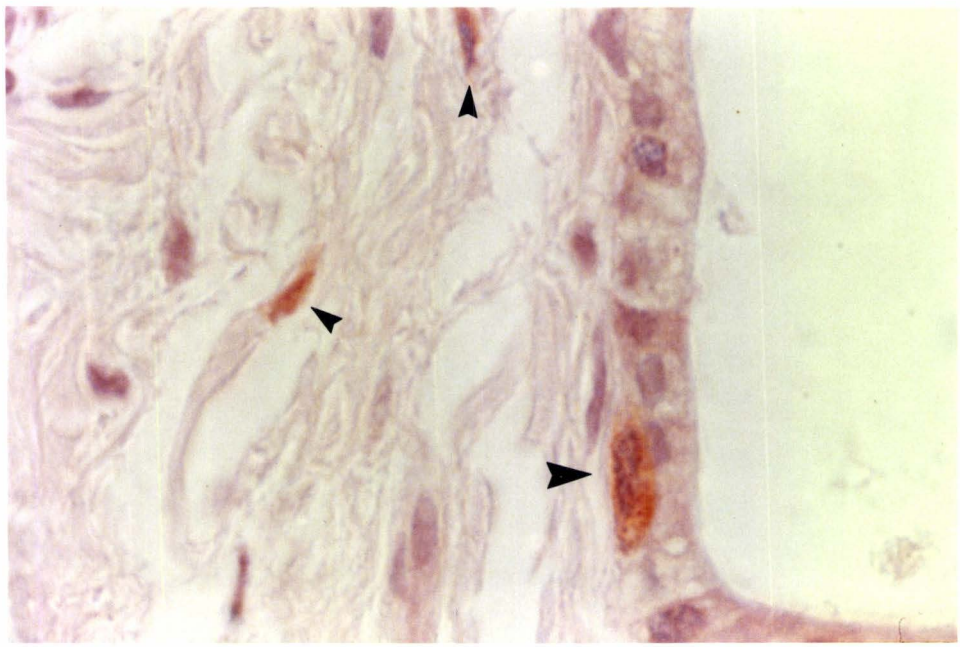


Figure 6.10: Globular leucocyte (arrow) within epithelium of duct of Brunner's gland. Proximal duodenum; fast black method; magnification x 1850.

Figure 6.11: CTMC's (arrows) within submucosal connective tissue surrounding Brunner's gland. Largest CTMC has yellow staining cytoplasm containing red-stained granules. Proximal duodenum; fast black method; magnification x 1850.

illustrated in figure 3.22. Such cells were found throughout the submucosal connective tissue at all sites sampled. In the upper part of the GI tract many such mast cells, when stained with fast black, were observed to have a yellow staining cytoplasm containing red storage granules. In contrast, in the lower parts of the tract, particularly the large intestine, the majority of CTMC's contained cytoplasmic granules that stained brown-black.

6.4. Discussion

Endocrine cell densities in the mucosa of the GI tract of 6 month old lambs revealed patterns of distribution similar to those described for adults (Section 3.3.4), foetuses (Section 4.3.2) and young lambs (Section 5.3.1). The duodenum contained the highest densities of argyrophilic, EC and S cells. Most of the argyrophilic cells were probably EC cells (Section 3.4.3). Intestinal endocrine cells were always found in greatest densities in the proximal duodenum and cell densities decreased along the length of the intestine. The abomasal body region was well endowed with argyrophilic cells, the bulk of which were considered to be ECL cells (Section 3.4.3), while the antral region contained the highest densities of G cells. Substantial numbers of G cells were also found in the duodenum. D cells were found at all abomasal and intestinal sites examined.

Although no valid statistical comparison could be made between the age groups, the results indicate that rate of increase in endocrine cell densities varies both between cell types and between sites. Detailed comparisons between age groups and discussion of the physiological importance these differences in endocrine cell densities are given in Section 8.3.

In the present experiment the fast black technique was found to be a useful method for distinguishing 5-HT and other 5-hydroxyindoles from tryptamine: the former produce a black precipitate while tryptamine produces a red one (Solcia et al., 1969b). Thus it was possible to demonstrate that EC cells contained 5-HT, or some closely related compound in their storage granules, but no tryptamine, as there was no evidence of red staining in their cytoplasm.

However, use of the fast black technique clearly demonstrated the existence within the epithelium of the glands of Brunner, of 'closed' type cells with storage granules containing tryptamine. It was not clear whether these cells were endocrine in nature or not. Because of the presence of cells of similar morphology and staining characteristics in the connective tissue surrounding these glands it seems more likely that the intraepithelial cells described represent MMC's (see Toledo et al., 1981).

This technique also clearly illustrated that CTMC's may contain variable amounts of 5-HT and related amines. Mast cells in the upper part of the GI tract contained predominantly red storage granules while in the lower part of the tract, particularly the large intestine, they contained brown-black granules. These results demonstrate that in different parts of the gut, mast cells may contain varying amounts of 5-HT and related compounds, or tryptamine.

Histochemical evidence presented in this chapter indicates that EC cells found in the abomasa of 6 month old lambs contain only small amounts of 5-HT. Thus some EC cells may escape detection by diazonium techniques and hence estimates of abomasal EC cell densities may be less than their true densities. In the abomasum, EC cells as demonstrated by the fast black technique, were more difficult to identify than those stained by the fast garnet method. Background staining may have been a factor as it was noted that whereas fast garnet was very specific, fast black produced a non-specific red-brown background stain which may have masked any slight positive staining of some abomasal EC cells. Thus it is possible that the fast black technique was slightly less sensitive than the fast garnet method. Such a difference would only become obvious in cells containing low concentrations of 5-HT. However, because the vast majority of EC cells were well stained by both techniques, an overall comparison of the two staining methods did not reveal any statistically significant difference.

In conclusion, the results presented here demonstrate that the distribution of endocrine cells in the GI tract of 6 month old lambs is similar to that found in other age groups. It is also clear that all ovine EC cells contain 5-HT or a closely related compound. However, the quantity of 5-HT stored depends on the site.

CHAPTER 7

EFFECTS OF TRICHOSTRONGYLUS COLUBRIFORMIS INFECTION ON THE DISTRIBUTION AND DENSITY OF ENDOCRINE CELLS IN THE GI TRACT OF LAMBS

7.1 Introduction

T. colubriformis is a helminth parasite which becomes established in the mucosa of the first 1-3 m of the small intestine (Barker, 1975a). It burrows into the mucosa causing damage to the epithelium, the degree of which is related to parasite numbers (Barker, 1975a). For a review of the clinical and pathophysiological effects of this parasite see Section 1.9.

Depression of food intake is a commonly observed consequence of trichostrongylosis in sheep. Recently Symons and Hennessy (1981) suggested the reduced food intake results from increased circulating levels of CCK. This duodenal hormone has been suggested as the mediator of the satiety response when food is placed in the stomach or intestines of experimental subjects (see Walsh, 1981). CCK-8, when injected in picomolar amounts into the cerebral ventricles, has been shown to depress food intake in sheep (Della Fera and Baile, 1979).

Barker and Titchen (1982) reported that in severe experimental trichostrongylosis there was a depression in abomasal acid secretion which was not fully accounted for by reduced food intake. This decreased secretion was associated with relatively inactive and poorly differentiated parietal cells, and may have been the result of a systemically acting gastric inhibitory factor released from the duodenum (Section 1.9).

Recently interest has been directed to the effects of nematode infections on the GI endocrine system of the sheep (Titchen, 1982), however, no data have been published on the effects of T. colubriformis on the distribution and density of gut endocrine cells.

The aim of this experiment was to investigate the effects of T. colubriformis infection in lambs, on the density and distribution of GI endocrine cells, with particular emphasis on the endocrine cells of the small intestine.

7.2 Materials and Methods

Eight Romney-cross lambs (Table 2.1) from the same flock as the foetuses (Chapter 4) and 2 week old lambs (Chapter 5), were raised to be free of nematodes according to the schedule shown in Table 7.1.

Table 7.1: Schedule for feeding and housing lambs raised to be nematode free.

Age	Schedule
24 hrs	Separated from dams, housed indoors on wheat straw. Fed cold milk mix* ad libitum.
3 weeks	Housed indoors in pens. Fed cold milk mix,* lucerne nuts and chaffed wheat straw ad libitum
13 - 14 weeks	Transferred to metabolism pens. Fed 600 g lucerne nuts and 50 g chaffed wheat straw once per day
24 - 25 weeks	Fed 800 g lucerne nuts and 100 g chaffed wheat straw once per day.

*12.5% (by weight) Ancalf (N.Z. Co-op Dairy Co., Hamilton) in cold water

The composition of the lucerne nuts fed from the 3rd week of age, is given in Table 7.2.

Table 7.2: Composition of lucerne nuts (F.R.M. Cockrem, pers. comm.).

Lucerne meal	60%
Barley meal	20%
Mill husk	20%
Sodium molybdate	0.4 g/100 kg
Sodium sulphate (Anhydrous)	4.4 g/kg

Salt blocks and water were available ad libitum throughout the experiment.

From 13 weeks of age, lambs were weighed weekly, each Monday between 11 am and noon.

At 24 weeks of age all lambs were shorn. Fleece weights were recorded and body weights were henceforth adjusted for the weight of wool removed.

Faecal worm egg counts were made 4 and 2 weeks before, and at approximately weekly intervals after, infection. Fresh faecal samples were collected from the anal canal and egg counts carried out using a modification of the McMaster slide technique (Gordon and Whitlock, 1939), either the same day or following storage overnight at 4°C. Results are expressed as epg of fresh faeces. The lower limit of detection for this technique is 50 epg.

All animals were drenched with 10 ml albendazole ("Valbazen" Smith, Kline and French (NZ) Ltd) 4 weeks before the experimental infection.

Third stage T. colubriformis larvae of ovine origin were obtained from Dr R.V. Brunsdon (Wallaceville Research Station, Upper Hutt, N.Z.) and stored at 4°C until required. On the day of infection a sample of larvae was examined microscopically to determine the number of live larvae present. Doses of 40,000 live larvae were suspended in tap water and administered to 4 of the sheep by ruminal intubation; the remaining 4 sheep served as non-infected controls.

Mucosal tissue samples were collected at postmortem, 9 weeks after the date of infection (i.e. at 44 - 47 weeks of age). Following stunning with a captive bolt gun, each sheep was exsanguinated, eviscerated and tissue samples collected from the sites listed in Table 7.3. Sample collection was completed within 20 minutes of stunning. Tissue samples were prepared and processed in the manner outlined in Section 2.3 and 2.5. Sections were stained using the methods employed in Chapters 4 and 5.

Table 7.3: Regions of the GI tract of infected and non-infected lambs from which mucosal samples were collected.

Region	Abbreviation	Site
Abomasal body	Ab_b	20-22 cm proximal to pylorus on parietal surface
Abomasal antrum	Ab_a	1.5-2.5 cm proximal to pylorus
Proximal duodenum	Duo_p	2-4 cm distal to pylorus
Middle duodenum	Duo_m	12-14 cm distal to pylorus
Distal duodenum	Duo_d	30-32 cm distal to pylorus
Proximal jejunum	Jej_p	100 cm distal to pylorus
Middle jejunum	Jej_m	approximately 700 cm distal to pylorus
Ileum	Il	10-12 cm proximal to ileocaecal junction

Worm counts were carried out on abomasal and small intestinal samples collected at postmortem. Following the removal of digesta, the mucosa was gently washed and the contents and washings mixed and made up to a known volume with water. Samples, which in the case of the abomasum constituted 10% and the small intestine 4%, of the total volume, were examined under a dissecting microscope for the presence of worms.

7.3 Results

7.3.1 Parasitology

Four weeks before experimental infection, small numbers (50 epg) of strongylate eggs were found in the faeces of 2 sheep (No's 28 and 29). Moderate numbers of coccidial oocysts were also found in faecal samples of all animals, including control animals. Following drenching with albendazole no strongylate eggs were found in the faeces of any animal when tested 2 weeks before and 2 weeks after experimental infection (Table 7.4). No coccidial oocytes were found in faecal samples after drenching.

Table 7.4: Numbers of Trichostrongylate eggs found in faeces of experimentally infected lambs.

Days before or after infection	Animal numbers			
	22	27	28	29
	Worm Egg Counts (epg)			
-28	0	0	50	50
-14	0	0	0	0
0	0	0	0	0
+14	0	0	0	0
+21	800	150	50	200
+28	350	0	0	0
+35	500	0	0	0
+46	500	0	0	0
+51	750	0	0	0
+58	400	0	0	0

Trichostrongylate eggs were identified in the faeces of all experimentally infected animals 3 weeks after infection (Table 7.4). At

4 weeks, however, and throughout the remainder of the experiment, eggs were found in the faeces of only one (No. 22) of these animals.

At postmortem, adult T. colubriformis worms were identified in the small intestinal contents of 2 experimentally infected animals (Nos 22 and 27; 1290 and 100 worms, respectively). There was no evidence of adult worms in the intestinal contents of sheep 28 and 29. No nematodes were identified in the abomasal contents of any of the infected animals.

At no time during the post infection period were trichostrongylate eggs found in the faeces of the control animals. No adult worms were found in the abomasal or intestinal contents of control animals at post-mortem.

7.3.2 Clinical Effects

The parasitic infection did not cause any noticeable depression of feed intake. Each sheep in the infected group consumed its daily ration of 800 g of lucerne nuts and 100 g chaffed wheaten straw. There was no evidence of watery diarrhoea or behavioural changes (see Section 1.9) indicative of parasitic infection.

Although the mean pre-infection body weight was higher for the control group (23.1 $\pm$ 1.7 kg) than for the infected group (20.8 $\pm$ 1.8 kg) and the mean body weight for the control group remained higher (Fig. 7.1) throughout the experiment, the differences were not significant ($P>0.05$). Liveweight gains for the infected and control animals were similar following infection (44.7 $\pm$ 6.7 and 45.8 $\pm$ 13.9 $g.day^{-1}$, respectively).

7.3.3 Histological Appearance of the Gut Mucosa

Histological evidence of epithelial damage included the finding of nematodes in 'tunnels' in the duodenal mucosa (Fig. 7.2) and infiltration of the lamina propria by lymphocytes in each of the infected animals. However, even in the most severely affected lamb (No. 22), mucosal damage was slight and confined to the duodenum. There were no signs of villus atrophy or discontinuities (Fig. 7.3) in the epithelium.

Figure 7.1: Body weights (mean + SEM) for control (n = 4) and infected lambs (n = 4). Dates when animals were shorn and drenched are indicated by open arrows 1 and 2, respectively. Infected lambs received T. colubriformis larvae by ruminal intubation at 35-36 weeks of age, as indicated (solid arrow).

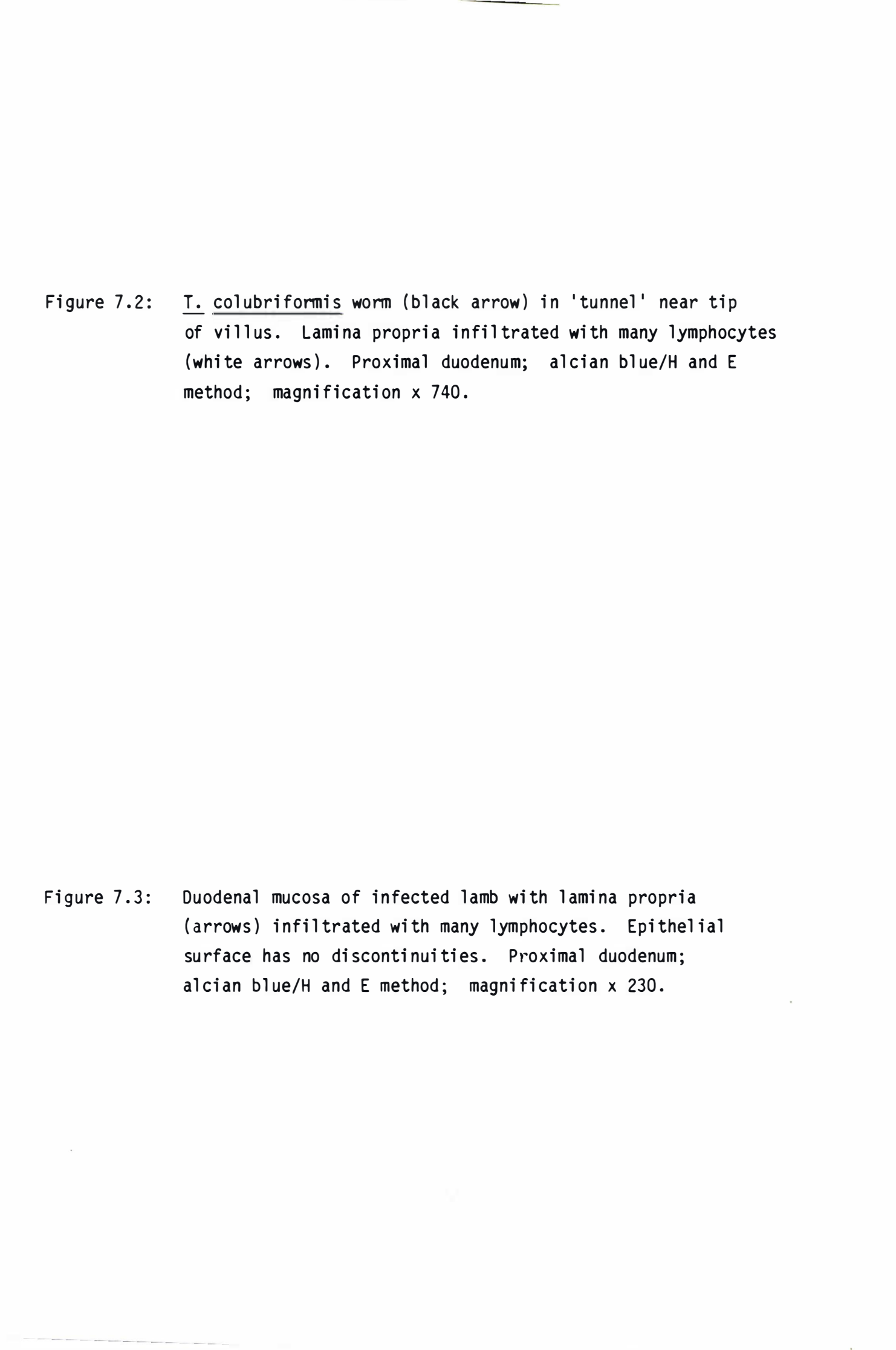
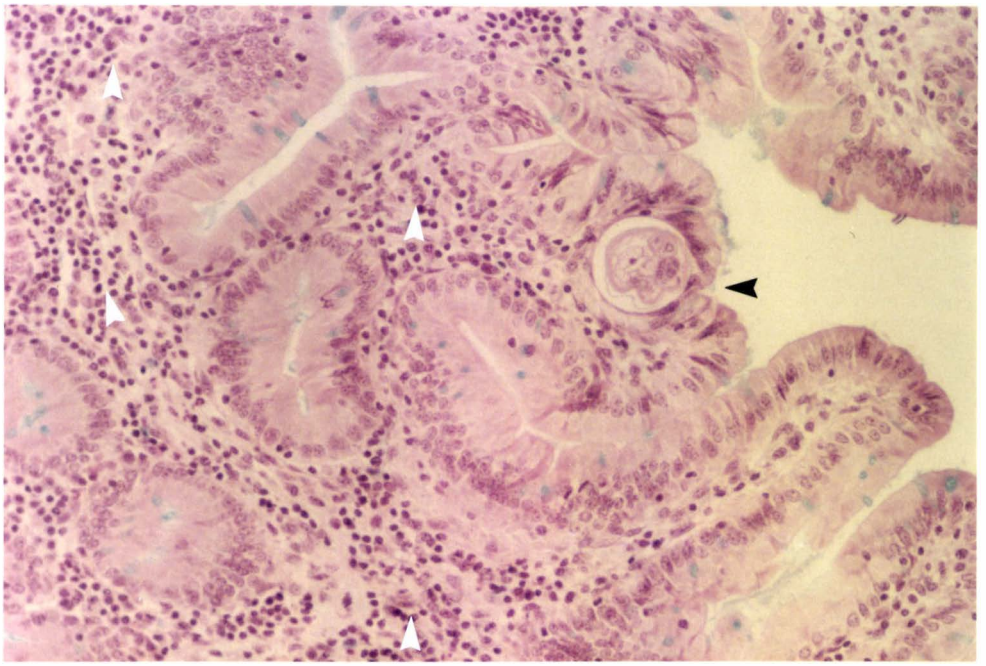
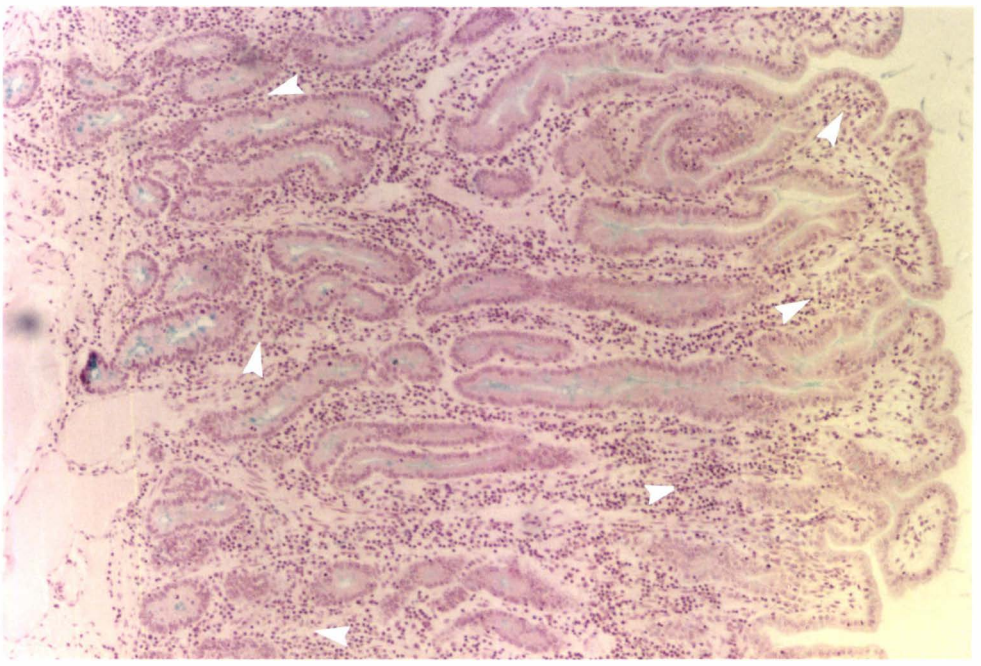


Figure 7.2: T. colubriformis worm (black arrow) in 'tunnel' near tip of villus. Lamina propria infiltrated with many lymphocytes (white arrows). Proximal duodenum; alcian blue/H and E method; magnification x 740.

Figure 7.3: Duodenal mucosa of infected lamb with lamina propria (arrows) infiltrated with many lymphocytes. Epithelial surface has no discontinuities. Proximal duodenum; alcian blue/H and E method; magnification x 230.





7.3.4 Effects on Endocrine Cell Densities and Distributions

Analysis of variance (Table 7.5) showed a significant ($P<0.001$) overall effect of *T. colubriformis* infection on argyrophilic cell densities which were consistently higher for the infected lambs at each site, with the exception of the abomasal antrum (Fig. 7.4). The greatest differences between infected and control animals occurred within the upper small intestine where the density of argyrophilic cells was from 28-175% (proximal duodenum 311 $\pm$ 11 *vs* 243 $\pm$ 17 c.mm^{-2}; proximal jejunum 110 $\pm$ 18 *vs* 41 $\pm$ 12 c.mm^{-2}) greater for the infected than the control group of animals. Within this group, the two animals (Nos 22 an 27) with the highest argyrophilic cell densities were those which had *T. colubriformis* worms in their upper small intestines. Compared with the small intestine, differences in abomasal argyrophilic cell densities of control and infected lambs, were small. This difference in effect of infection on abomasal and intestinal argyrophilic cell densities is reflected in the significant ($P<0.01$) component (Table 7.5, C(ii)) in the infection x sampling sites interaction.

The lack of any significant main effect or interaction components for other endocrine cell types indicates that EC (Fig. 7.5), G (Fig. 7.6), S (Fig. 7.7) and D (Fig. 7.8) cell distributions were similar in control and infected animals. G cells, which were most numerous in the abomasal antrum were absent from the abomasal body region. In the small intestine, EC, G and S cells were most numerous in the proximal duodenum and cell densities declined progressively along the length of the tract. G cells were not found in the mid-jejunum or ileum. Somatostatin containing D cells were found at all sites sampled, but were at their greatest density in the abomasal antrum and proximal duodenum.

Endocrine cell morphology was similar in both infected and control animals. Thus, all G, S and intestinal D cells, together with the majority of intestinal argyrophilic and EC cells, were of the 'open' type. All endocrine cells found in the body region of the abomasum (i.e. argyrophilic, EC and D cells) were of the 'closed' type, while in the antrum both 'open' and 'closed' type argyrophilic, EC and D cells were identified.

Table 7.5: Summary of analysis of variance of endocrine cell densities for argyrophilic, EC, G, S and D cells in infected and control lambs

	Source of Variation	DF	Variance Ratios for each Cell Type				
			Argyrophilic	EC	G	S	D
A.	Infected vs Control	1	29.19***	0.61	0.26	0.01	0.75
B.	Sites	7	137.98***	181.51***	178.22***	98.62***	51.73***
	(i) Ab_b vs Ab_a	1	274.37***	3.1	611.36***	0.00	22.05***
	(ii) Duo_p vs Duo_m	1	11.82**	36.61***	11.92**	7.08*	5.33*
	(iii) Duo_p + Duo_m vs Duo_d	1	54.75***	77.87***	29.70***	24.08***	28.79***
	(iv) Duo vs Jej_p	1	122.38***	118.28***	99.73***	40.83***	58.65***
	(v) Duo + Jej_p vs Jej_m	1	190.08***	154.70***	271.26***	111.59***	106.93***
	(vi) Duo + Jej vs Il	1	190.54***	378.14***	180.84***	133.81***	56.25***
	(vii) Ab vs SI	1	121.76***	498.17***	40.69***	374.75***	83.93***
C.	Interaction: Infected vs Control x Sites	7		0.17	0.05	0.12	0.47
	(i) Duo + Jej_p vs Jej_m x Infected vs Control	1	5.46*				
	(ii) Ab vs SI x Infected vs Control	1	7.82**				
	Remainder	5	0.77				
	Residual mean square	48	1.33	0.93	0.74	0.75	0.36

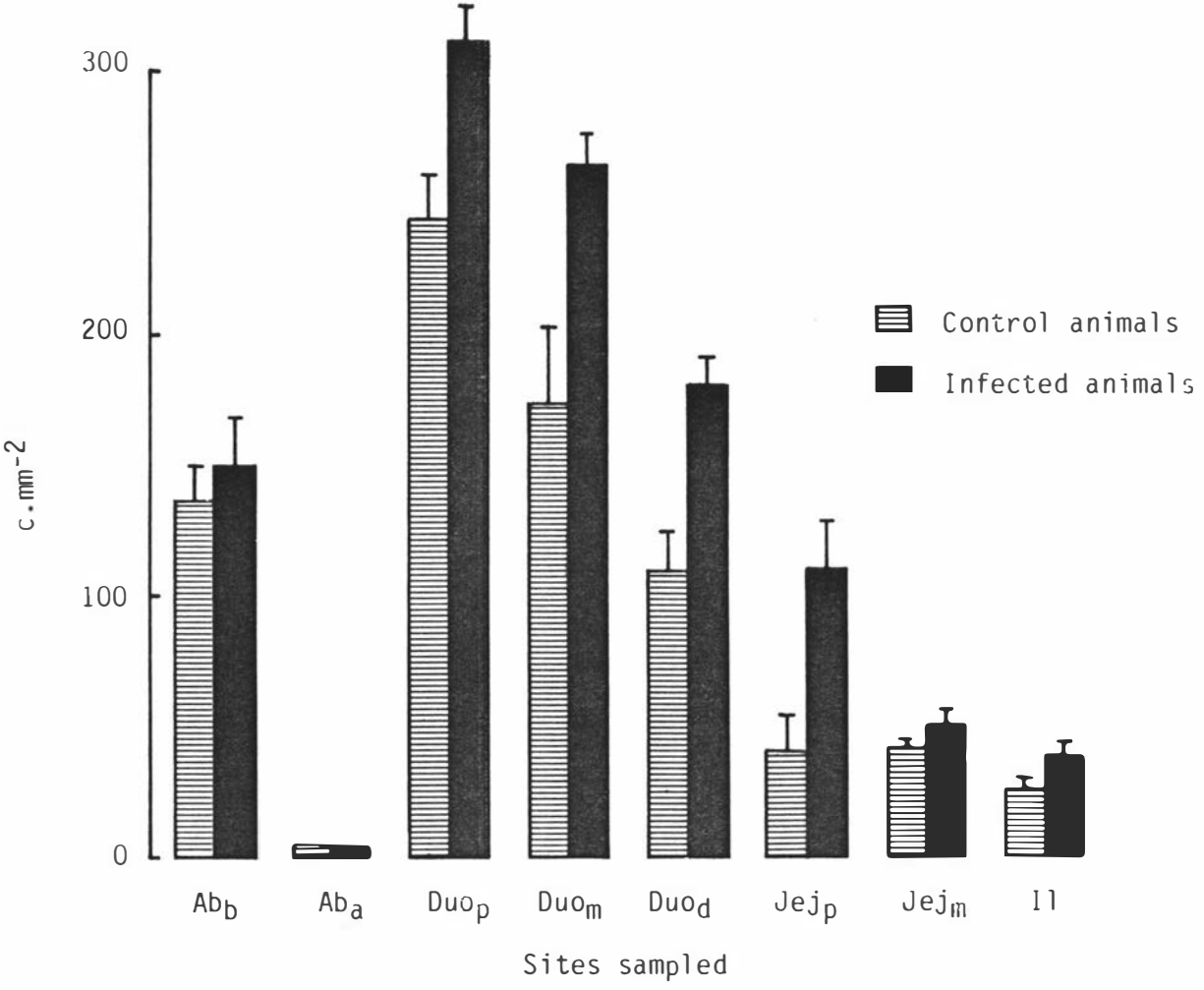


Figure 7.4: Distribution (mean + SEM) of argyrophilic cells in the GI tract of infected (n = 4) and control lambs (n = 4)

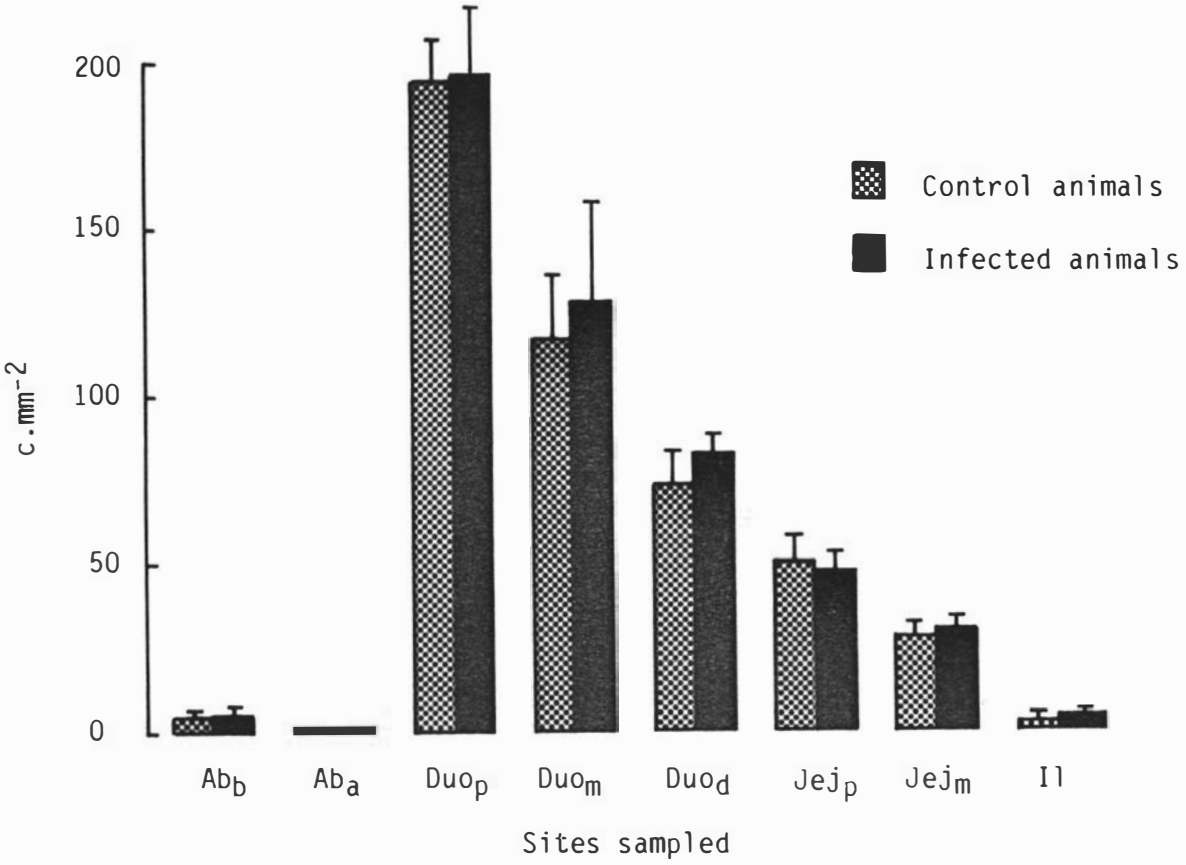


Figure 7.5: Distribution (mean + SEM) of EC cells in the GI tract of infected (n = 4) and control lambs (n = 4).

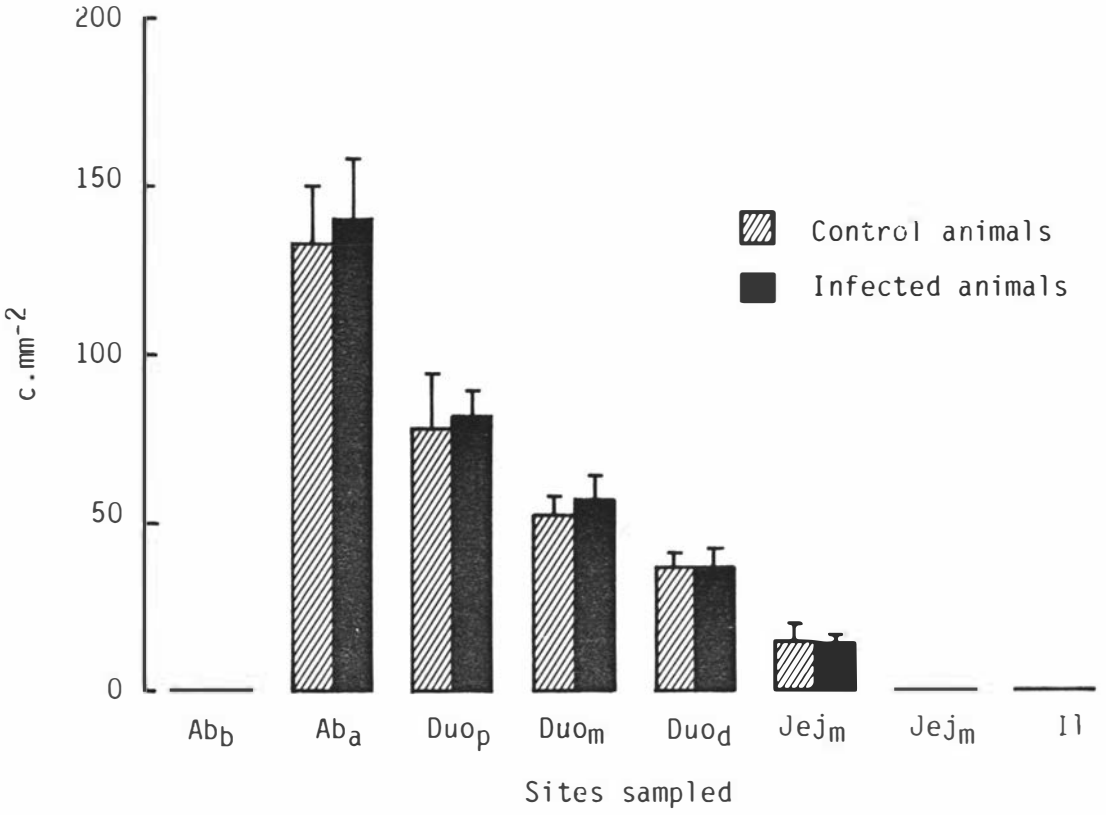


Figure 7.6: Distribution (mean + SEM) of immunoreactive G cells in the GI tract of infected (n = 4) and control lambs (n = 4).

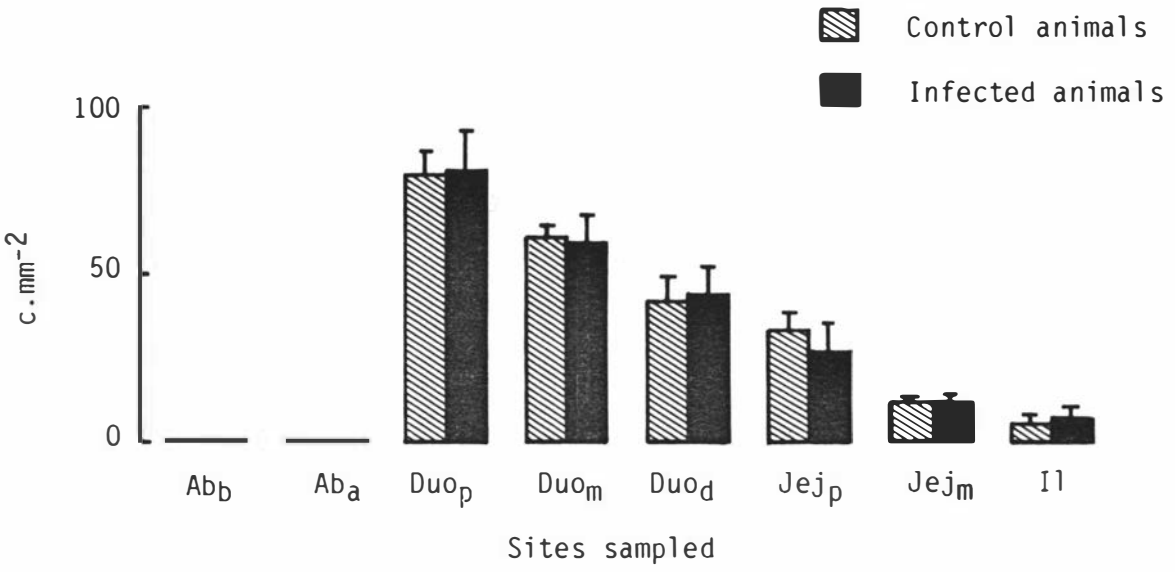


Figure 7.7: Distribution (mean + SEM) of immunoreactive S cells in the GI tract of infected (n = 4) and control lambs (n = 4).

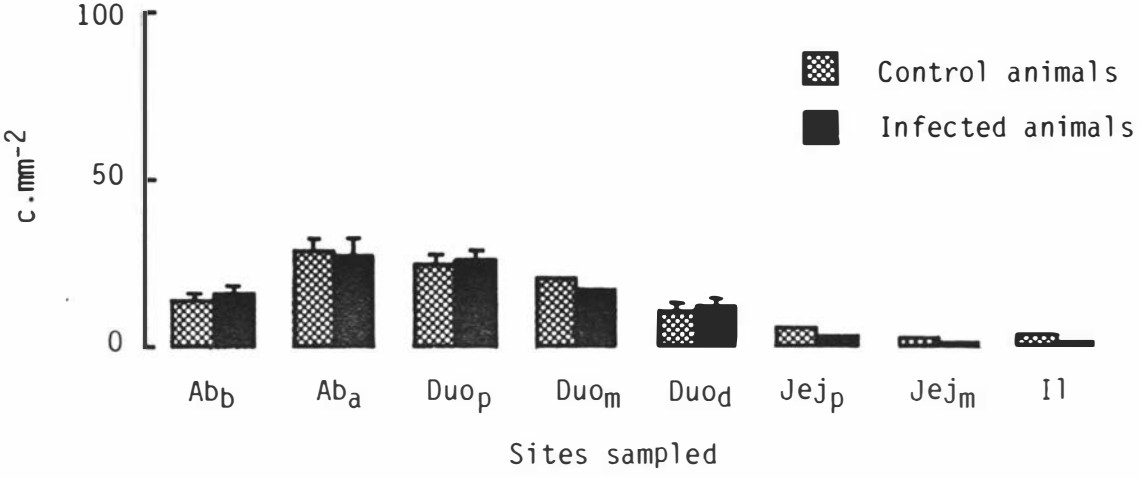


Figure 7.8: Distribution (mean + SEM) of immunoreactive D cells in the GI tract of infected (n = 4) and control lambs (n = 4).

7.4 Discussion

Evidence that T. colubriformis parasites had become established in the small intestine included the presence of nematode worms within the mucosa of each of the infected lambs. Strongylate eggs were present in the faeces of all the infected lambs three weeks after infection. Two lambs had adult worms in their intestinal contents at postmortem.

Egg and worm counts, however, for all infected lambs were very low compared with those reported by other workers (Horak et al., 1968; Fitzsimmons, 1969), indicating that the infection was very mild. Low egg counts probably cannot be attributed to inhibition of T. colubriformis larvae as arrested development of this nematode reportedly does not occur (Denham, 1969; Michel, 1974). Further, faecal egg counts are reported to be closely related to adult worm burdens (Gregg et al., 1978; Windon et al., 1980).

The lack of depression of feed intake, the absence of loss of body weight and the minor nature of the mucosal damage observed all confirm that the infection was very mild. Reasons for this are discussed below.

Despite the low level of infection, a significant increase in argyrophilic cell density was observed in the infected animals. This increase was marked in the duodenum and proximal jejunum, less so in the mid-jejunum and ileum. Thus, the greatest change in endocrine cell density occurred in that part of the GI tract where T. colubriformis worms become established and where greatest mucosal damage occurs (Barker, 1974). These changes in argyrophilic cell densities were most probably due to endocrine cell hyperplasia.

Alternatively, the increase in arygrophilic cell density may have been due to increased synthesis or storage of secretory products within the argyrophilic cells of the infected animals, thus resulting in increased staining intensity and a greater number of stained cells being visible on microscopic examination. Evidence that the content of secretory product within individual endocrine cells may vary has been provided by Forssman and Orci (1969) who demonstrated that the electron density of G cell granules increased in fasted cats and was reduced

following refeeding. The above explanation could be tested by an electron microscopic study of argyrophilic cells from control and infected animals, and examining the number and electron density of granules they contained.

The results of the present study clearly demonstrated that EC, G, S and D cell densities did not change as a result of infection. Further, it is unlikely that I cells contributed to the change in argyrophilic cell densities as, in other mammalian species, they are not argyrophilic (Table 2.2). The histochemical staining characteristics of ovine I cells, which have yet to be reported, could be established by use of double or sequential staining techniques.

Extrapolating from staining results and distribution studies in other species, the most likely cell types contributing to the change in duodenal argyrophilic cell density are D_1, K and L cells. Although the secretory product(s) of mammalian D_1 cells is unknown (Solcia et al., 1982), the secretory products of K and L cells have been shown to be GIP (Polak et al., 1973; Buffa et al., 1975) and GLI (see Sundby and Moody, 1980), respectively. What effect the disturbance of normal function of these endocrine cells might have in nematode infections, is unknown. Although the physiological functions of GIP have yet to be established it has been shown to inhibit gastric acid secretion in both dogs (Pederson and Brown, 1972) and man (Cleator and Gurlay, 1975), stimulates intestinal secretion in dogs (Barbezat and Grossman, 1971) and man (Helman and Barbezat, 1977) and is insulinotropic in man (Dupre et al., 1973), dogs (Pederson et al., 1975) and rats (Pederson and Brown, 1976). As yet no biological activity for any gut GLI has been clearly established however, some evidence of an insulinotropic effect of a gut GLI was reported by Gutman et al. (1973). Other reported effects of GLI are glycogenolysis (Valverde et al., 1970), binding to glucagon receptors (Rehfeld et al., 1973) and lipolysis (Horigome et al, 1977), however, the physiological importance of these effects remains to be established.

CCK has been implicated in the control of satiety in sheep (Della-Fera and Baile, 1979) and animals with a simpler form of stomach. Exogenous CCK has been shown to depress food intake in rats (Gibbs

et al., 1973), mice (Strohmayer et al., 1976), monkeys (Gibbs et al., 1976), dogs (Sjodin, 1972), rabbits (Houpt and Anika, 1977), humans (Sturdevant and Goetz, 1976) and sheep (Baile and Grovum, 1974; Grovum 1977a, b; Grovum, 1981). Symons and Hennessy (1981) reported that plasma bioassayable CCK levels rose in sheep following infection with T. colubriformis, and that food consumption fell as plasma CCK concentration rose. They concluded that the anorexia commonly seen in severe trichostrongylosis was due to, or mediated by increased concentrations of circulating CCK. These workers did not, however, investigate whether altered plasma CCK levels were due to an increase in I cell numbers or to an increased rate of release of CCK. In the present experiment the absence of inappetence indicates, albeit indirectly, that plasma CCK concentrations were not raised in mild trichostrongylosis. It is suggested that I cell densities, although not investigated in this thesis, were probably unaltered by the mild experimental infection.

Barker and Titchen (1982) have recently demonstrated that in severe trichostrongylosis there were profound changes in abomasal function. A decrease in acid production appeared to be at least partly due to altered functions of parietal cells, many of which were relatively inactive or poorly differentiated. In the present experiment no changes in the histological appearance of parietal cells were found in the infected animals.

In view of these reports of abomasal dysfunction (Barker and Titchen, 1982) and anorexia (Horak et al., 1968) in severe infections with T. colubriformis, it would be of interest to examine densities of I, G, D, S and K cells in the duodena of sheep in which substantial numbers of nematodes were established.

Another parasitic disease of sheep which has been shown to cause disturbances in the GI endocrine system is ostertagiasis (for a review see Titchen, 1982). Ostertagia circumcincta, a parasitic worm which becomes established in the abomasum, causes gastritis. Severe infections with O. circumcincta cause reduced secretion of acid by the abomasum and many of the parietal cells are thought to be inactive (McLeay et al., 1973). Ostertagiasis is associated with profound hyper-

gastrinaemia (Anderson et al., 1981) and in this respect differs from trichostrongylosis (Titchen and Anderson, 1977). This hypergastrinaemia is thought to be due in part to the elevation in abomasal pH which occurs in ostertagiasis (Anderson et al., 1981). This is not the only cause, however, as plasma gastrin levels increase before abomasal pH. Reynolds et al., (1979a) demonstrated that abomasal mucosal gastrin concentrations were higher in infected than control animals, but that duodenal gastrin concentrations were lower in infected than control animals. Whether such changes in plasma and tissue gastrin levels were the result of changes in G cell densities or changes in G cell metabolism, have yet to be determined.

In view of the mild nature of the T. colubriformis infection, it is pertinent to ask whether the number of live larvae administered to the lambs was adequate. Gordon (1950) reported that single doses of 60,000 T. colubriformis larvae given to 10 month old sheep caused deaths between 17 and 34 days after dosing. Stewart and Gordon (1953) also found that single doses of 50,000-100,000 larvae given to 3-4 month old worm-free sheep proved fatal, whereas Gallagher (1963) found that 8 month old sheep survived single doses of 20,000 to 40,000 larvae while some sheep died following a dose of 50,000 larvae. Similarly, Gordon (1964) found that approximately 50% of forty 10 month old lambs died 17-118 days after a single dose of 50,000 larvae. In contrast, Kates and Turner (1953) were unable to produce any deaths within a 34 day period following single doses of either 50,000 or 100,000 larvae. Thus the weight of this evidence indicated that it was prudent to keep the dose below 50,000 larvae and for this reason a dose of 40,000 live larvae was chosen. The aim, after all, was to produce clinical signs of infection but not cause the death of the animals.

Age, previous exposure to parasites, plane of nutrition, genetic resistance and predisposing disease have been considered as possible factors determining whether a nematode infection becomes established (see Fitzsimmons, 1969; Windon et al., 1980).

Young lambs are more susceptible to helminth infections than mature sheep (see Windon et al., 1980). This is evidenced by the finding that lambs less than 12 weeks of age do not acquire significant resistance to

T. colubriformis larvae even after prolonged daily dosing with such larvae (Gibson and Parfitt, 1972; 1973; Chienjina and Sewell, 1974a, b). Similarly, young lambs are immunologically immature, compared with 6 month old sheep, when vaccinated with attenuated T. colubriformis larval vaccines (see Windon et al., 1980).

Previous exposure to T. colubriformis parasites has been shown to confer on the host resistance to subsequent infection with the same parasite (Gregg and Dineen, 1978; Gregg et al., 1978). This is an inadequate explanation for the 'poor take' seen in the present study as all the lambs were raised to be free of helminth parasites. Strongylate eggs were present, however, in the faeces of two of the lambs (Nos 28 and 29) before the experimental infection. Thus it is possible that previous exposure to strongylate worms may have contributed to the low level of infestation seen in the present study.

Plane of nutrition does not appear to be an important predisposing factor in the establishment of nematode infections. Gordon (1964) demonstrated there was no difference in the establishment of T. colubriformis in lambs fed ad libitum and those fed only half that ration. Similarly, plane of nutrition did not affect mortality of animals, however, sheep on lower intakes died sooner.

Genetic constitution is an important factor in the ability of animals to resist parasitic infections. Differences in susceptibility both between and within sheep breeds have been reported (see Windon et al., 1980) and resistance to trichostrongylosis has been shown to be heritable (Whitlock, 1955, 1958; Whitlock and Madsen, 1958). Windon et al. (1980) reported that when Merino lambs were vaccinated at 1 month of age with an attenuated T. colibriformis vaccine, about 40% developed resistance to infection by T. colubriformis larvae. This finding led to the concept that lambs may be segregated into 'responders' and 'non-responders' and that genetically determined factor(s) play an important role in determining the ability of young animals to mount an effective immunological response to an infection (Windon et al., 1980).

In view of the evidence cited above, the most important factors considered to be involved in the poor establishment of T. colubriformis

larvae in the duodenum of the lambs used in the present study were the age of the animals and their possible genetic resistance. Thus, it was considered that establishment of a T. colubriformis infection by reinfection of the same animals with similar numbers of larvae would have been unsuccessful.

Clearly it would be of considerable interest to repeat the present experiment using higher doses of T. colubriformis larvae in younger animals with no previous exposure to nematode infections. In such animals, a comparison of I cell densities in control and infected lambs, in which feed intake was significantly depressed, might establish whether there was a link in trichostrongylosis between I cell densities and inhibition of feeding.

In summary, the experiments described here demonstrate that even in quite mild nematode infections there may be considerable changes in endocrine cell densities. However, the specific identity of the endocrine cell type(s) affected by T. colubriformis infection remains to be established.

CHAPTER 8

GENERAL DISCUSSION AND CONCLUSIONS

The involvement of bioactive peptides and amines in the regulation of gastrointestinal function has long been recognized (see Sections 1.6 and 1.7). The major source of such compounds are the endocrine or endocrine-like cells of the GI mucosa (Solcia *et al.*, 1981). Seventeen morphologically distinct endocrine cell types have been identified in the GI mucosa of animals with a simpler form of stomach (see Table 1.1), however, there is little information available on the types and distribution of endocrine cells within the ovine GI tract (see Section 1.8.4).

In the present study, well established histochemical and immunohistochemical (see Chapter 2) methods were tested (see Section 3.3.1) to determine their suitability for the identification of endocrine cells in the ovine GI tract. The De Grandi, Sevier-Munger and fast garnet histochemical techniques, and the PAP immunohistochemical technique with antisera to gastrin, secretin, glucagon or somatostatin, all proved to be reliable. Histochemical methods which have been used in other mammalian species (see Tables 2.1 and 2.2), but found to be unsatisfactory in the sheep, were the Masson-Hamperl, chromaffin and lead-haematoxylin methods. Reasons for their failure in the sheep have been discussed in Section 3.4.2.

The reliable histochemical and immunohistochemical techniques were used to stain endocrine cells in mucosal samples from sheep of different ages (100-110 day old foetuses, 2 week and 6 month old lambs, and adults), and in lambs experimentally infected with the helminth parasite *T. colubriformis*. Sheep of these selected ages were used in order that the effects of age-related differences in gut function on endocrine cell distributions could be studied. Thus, foetuses were studied because it is generally thought that the GI tract plays little or no part in the nutrition of developing foetuses, instead their nutrients are derived from the maternal circulation and reach the foetus *via* the placenta. At birth this route is abruptly disrupted and the dietary needs of the neonate are met by food which is ingested, broken

down, and absorbed across the gut epithelium. In the case of 2 week old, pre-ruminant lambs, nutrients are derived from milk, while in ruminant sheep (6 month old and adults) they are chiefly derived from ingested plant material and the microflora of the ruminoreticulum. Lambs infected with T. colubriformis larvae were compared with controls in order to determine whether this nematode affects endocrine cell densities, and thus, whether any clinical effects of trichostrongylosis might be attributed to disturbance of the GI endocrine system.

8.1 Endocrine Cell Types Identified

A summary of the histochemical staining characteristics of ovine GI endocrine cells is shown in Table 8.1. In the present studies, EC, G, D, S and pancreatic A cells were specifically identified and evidence for the existence of a large population of ECL cells in the body, but not the antral region of the abomasum, has been presented (see Section 3.4.3).

It was concluded from data presented in Section 4.3, that ovine D cells were not argyrophilic, with either the De Grandi or Sevier-Munger techniques, nor argentaffinic. Similar results have been reported for cattle by Domeneghini and Castaldo (1981), however, these workers also found a small number of D cells in bovine cardiac glands that contained intensely argyrophilic secretory granules. Whether such a cell type exists in ovine cardiac glands remains to be determined.

Endocrine cells, other than ECL cells, that may form part of the argyrophilic, non-argentaffinic cell population of the abomasal body, include A, D_1 and X cells. Present results, however, demonstrated conclusively that A cells did not occur in the ovine GI tract, but were found only in pancreatic islets. This finding is supported by results of Rizzotti and co-workers who were unable to identify A cells in the GI tract of sheep, cattle or goats using histochemical techniques (Rizzotti et al., 1976a), or in the gastric glands of the ox using electron microscopy (Rizzotti et al., 1980). Such results are in accordance with earlier observations (Unger and Eisentraut, 1967) that extracts of bovine abomasum do not contain immunoreactive pancreatic glucagon.

Table 8.1: Summary of ovine GI tract and pancreatic endocrine cell types ('open' or 'closed') and their histochemical staining reactions

	Endocrine cell type					
Staining Methods	A	D	EC	ECL	G	S
De Grandi	nd	-	+	+	-	nd
Sevier-Munger	-	-	+	+	-	nd
Masson-Hamperl	-	-	+	-	-	nd
Chromaffin	-	-	+	-	-	nd
Fast Garnet	-	-	+	-	-	nd
Cell type		O/C	O/C	C	O	O

\+ = positive staining reaction
\- = negative staining reaction
O = 'open' cells
C = 'closed' cells
O/C = both 'open' and 'closed' cells
nd = not determined

In contrast, GLI has been detected in the GI tract and plasma of foetal and postnatal lambs, with highest concentrations occurring in the terminal ileum (Bassett and Madill, 1978). Rizzotti et al., (1979) using electron microscopy, identified endocrine cells in the rectal mucosa of the adult ox, that were morphologically similar to L cells (Solcia et al., 1981a). Similar cells had previously been identified, by histochemical techniques, in the small intestine, caecum, colon and rectum of calves (Rizzotti and Domeneghini, 1976b). It is therefore concluded that while glucagon (hence A cells) were absent from the ovine gut mucosa, GLI containing L cells probably occur in the terminal ileum and large intestine of sheep. This has been confirmed by using an antiserum which crossreacts with GLI (Bunnett, 1980).

Although in cattle D_1 and X cells have been identified by electron microscopy in cardiac (Domeneghini and Castaldo, 1981) and gastric (Rizzotti et al., 1980b) glands, and D_1 cells have been found in antral glands (Rizzotti et al., 1980a), neither of these cell types has yet been identified in ovine abomasa (Rizzotti et al., 1976a). The inability to identify these two cell types in the present study was due to the lack of any histochemical or immunohistochemical technique which specifically stains either of these types of cells. D_1 and X cells may be distinguished from other gut endocrine cells by the size and density of their secretory granules seen by electron microscopy (Solcia et al., 1981a). Thus, confirmation of the presence of D_1 and X cells in ovine gut mucosa awaits an electron microscopic study in this species.

An unexpected result of the present study was that ovine G cells were non-argyrophilic. This finding is at variance with the observations of Rizzotti et al., (1980b) who reported that bovine antral G cells were weakly reactive with the Grimelius technique. Further, in species with a simpler form of stomach, G cells generally have been described as argyrophilic (see Solcia et al., 1975a). Although McGuigan and Greider (1971) and Mitschke (1971) reported that porcine G cells were non-argyrophilic, Capella and Solcia (1972) demonstrated that they were slightly reactive to the Grimelius argyrophil method. The discrepancies between these results for pigs, have been ascribed by Solcia et al., (1975a) to difficulties with silver staining techniques, for as Creutzfeldt et al., (1974) stated, 'most silver staining methods

are non-predictable'. Inadequate technique, however, is not a satisfactory explanation for the failure to demonstrate argyrophilia in antral G cells in sheep as the De Grandi technique was found to be reliable (see Section 3.3.1). This latter technique differs slightly from the Grimelius argyrophilic method (see Table 2.10) which did not work when applied to gut mucosal samples from adult sheep (see Section 3.3.1). From the above it is therefore concluded that either ovine G cells differ from those of other species in that they are non-argyrophilic, or that the De Grandi technique is not as sensitive as the Grimelius method. There is no published evidence to support the latter contention.

In contrast, the observation that ovine G cells were not reactive with either the Sevier-Munger or Masson-Hamperl techniques (see Section 3.4.1) is in accord with similar findings for cattle (Rizzotti et al., 1980b), pigs (Capella and Solcia, 1972) and other mammals with a simpler form of stomach (see Solcia et al., 1975a).

Recent ultrastructural and immunohistochemical studies have established that there are three distinct types of G cells present in the duodenal mucosa of species with a simpler form of stomach. In addition to cells similar in morphology to antral G cells, IG (Buchan et al., 1979) and TG (Larsson and Rehfeld, 1979) cells have been described. Whether or not more than one type of G cell exists in the sheep remains to be established. The presence or otherwise of three types of G cells in the ovine duodenum could best be demonstrated using region specific antisera combined with electron microscopy. These three types of G cells may be distinguished from one another by the size and electron density of their secretory granules.

Evidence presented in this thesis is the first immunohistochemical demonstration of the occurrence of S cells in the small intestinal mucosa of the sheep. Results from other species indicate that S cells are consistently argyrophilic (see Solcia et al., 1975b). Confirmation of this and other histochemical staining characteristics of ovine S cells could best be established by double staining techniques or the use of thin, serial sections.

8.2 Distribution of Endocrine Cells

A summary of the distribution of endocrine cells in the ovine GI tract is given in Table 8.2.

No endocrine cells were identified in either the reticulum or ventral rumen. This is in agreement with the findings of Rizzotti et al., (1976a, b) who were unable to demonstrate any endocrine cells in the forestomachs of cattle using histochemical techniques similar to those described in this thesis. However, because some endocrine cell types may not be stained by the techniques used in the present studies (e.g. X, I and N cells), the presence or absence of endocrine cells in the ovine forestomach could best be established using a neuronal specific enolase antiserum. Such antisera have been shown to cross-react with all known GEP endocrine cells (Bishop et al., 1982).

In the present study, immunoreactive G cells were found only in the antral portion of the abomasum and the duodenum. This finding is in accordance both with the distribution of G cells described by Bunnett and Harrison (1979a) and Bunnett (1984), and the observation that the highest concentrations of immunoreactive gastrin in sheep occurs in the abomasal antrum, while little or no gastrin is found in the forestomach, body region of the abomasum or ileum (Reynolds et al., 1980; 1984). In contrast, it has been reported that in sheep appreciable amounts of gastrin-like activity occur in the forestomachs (Jury and McLeay, 1977) and in the body region of the abomasum (Anderson et al., 1962; Jury and McLeay, 1977). These workers used a bioassay to demonstrate gastric acid secretagogue activity and therefore would have been unable to distinguish between gastrin and other gastric acid secretagogues. It is possible that the secretagogue activity extracted from the forestomachs of sheep by Jury and McLeay (1977) was GRP (Bunnett, 1984), a peptide with the same C-terminal septapeptide as bombesin. Bombesin-like immunoreactivity has been found in many regions of the ovine alimentary tract (Harrison and Wathuta, 1982a), particularly the forestomach where it is found in the muscle layers, myenteric and submucosal plexuses and mucosa (Harrison and Wathuta, 1982a). Bombesin indirectly stimulates the secretion of gastric acid in humans, cats and dogs (Melchiorri, 1978) by releasing antral gastrin (Walsh and Grossman, 1975).

Table 8.2: Summary of the distribution of endocrine cells in the GI tract and pancreas of sheep

	Forestomach		Abomasum		Small Intestine		Large Intestine		
Cell Type	Reticulum	Rumen	Body	Antrum	Duodenum	Ileum	Colon	Caecum	Pancreas
A	-	-	-	-	-	-	-	-	+
D	-	-	+	+	+	+	+	+	+
EC	-	-	+	+	+	+	+	+	-
ECL	-	-	+	-	-	-	-	-	-
G	-	-	-	+	+	-	-	-	-
S	-	-	-	-	+	+	-	-	-

\+ = cells present
\- = no cells present

8.3 Age Group Comparisons

The present studies established that all the endocrine cell types observed in adult sheep also occurred at corresponding sites in the gut mucosa of 100-110 day old foetuses, but at lower densities. The foetal gut mucosa was less well developed than in the adult both in terms of its overall thickness and the degree of development of the crypts of Lieberkuhn. Thus, total numbers of endocrine cells in the foetal GI tract are substantially less than found in adult sheep, even on a per kg body weight basis.

Comparisons of argyrophilic, EC, G, S and D cell densities in the mucosa of the GI tract at various ages are illustrated in Figs 8.1 - 8.5. Some endocrine cell densities changed markedly with age. This was particularly so for argyrophilic and EC cell densities in the proximal duodenum, and G cell densities in the abomasal antrum. In these instances endocrine cell densities were substantially lower in foetuses than in adult animals. In contrast, at some sites endocrine cell densities changed little with increase in age (e.g. argyrophilic cell densities in the abomasal body). Such differences may be accounted for either by variations in the age at which particular endocrine cells first become identifiable, or by differences in the rate of development of specific endocrine cell types. These age group comparisons also clearly demonstrate that the pattern of change in cell density was not uniform along the length of the GI tract. Non-parallel development of GI endocrine cells has been described for humans (Larsson and Jorgensen, 1978) and pigs (Alumets et al., 1983).

From data presented in Section 4.3 it is clear that immunoreactive G cells were present in both antral and duodenal mucosa of 100 - 110 day old foetuses. Lichtenberger et al. (1981) were unable to find antral G cells in foetal lambs until they reached 120 - 130 days gestational age. This discrepancy in results is probably due to methodology: Lichtenberger and co-workers used an indirect immunoperoxidase technique as opposed to the more sensitive (Sternberger, 1979) PAP technique used in the current study.

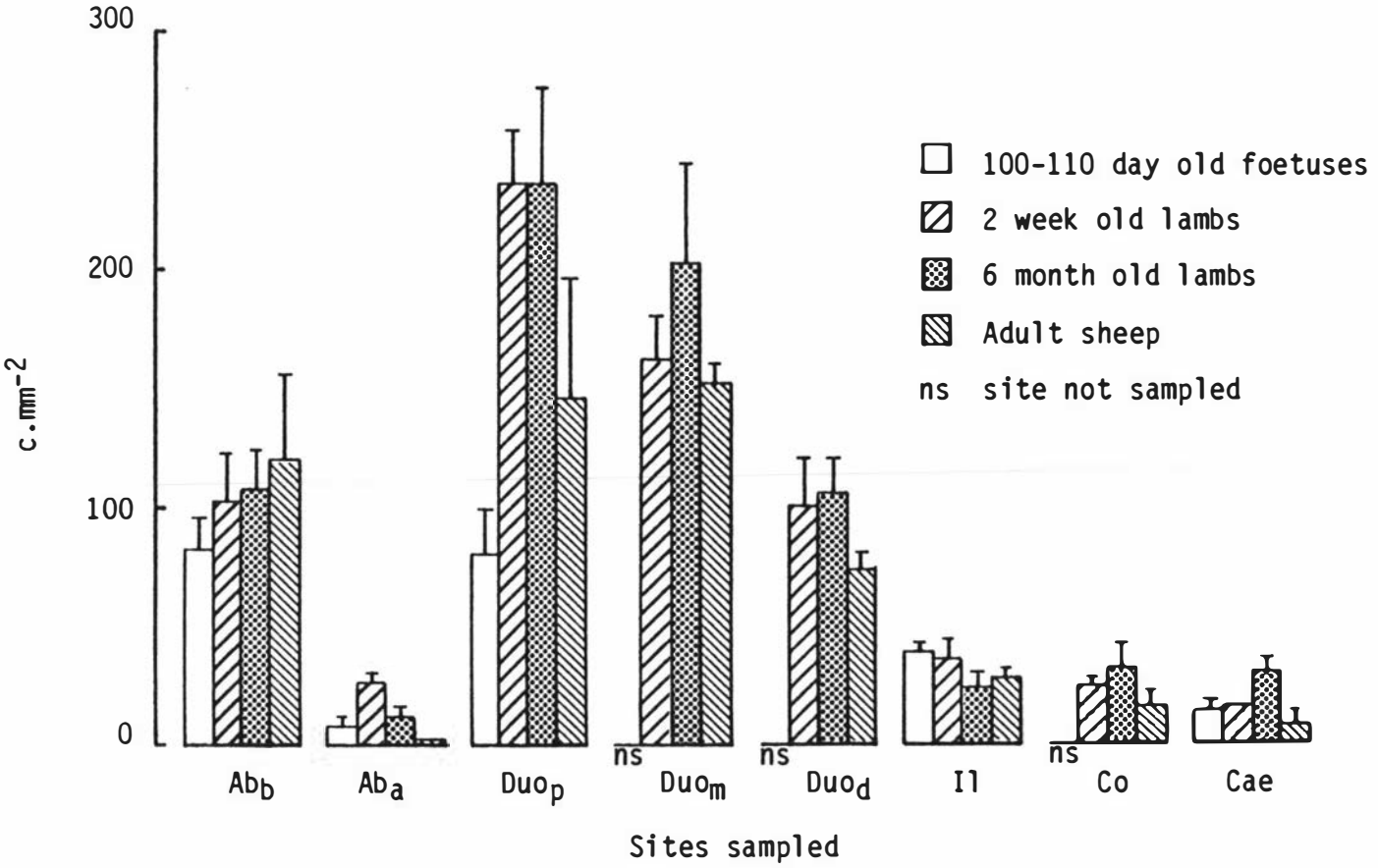


Figure 8.1: Argyrophilic cell densities (mean + SEM) in the GI tract of sheep at various ages.

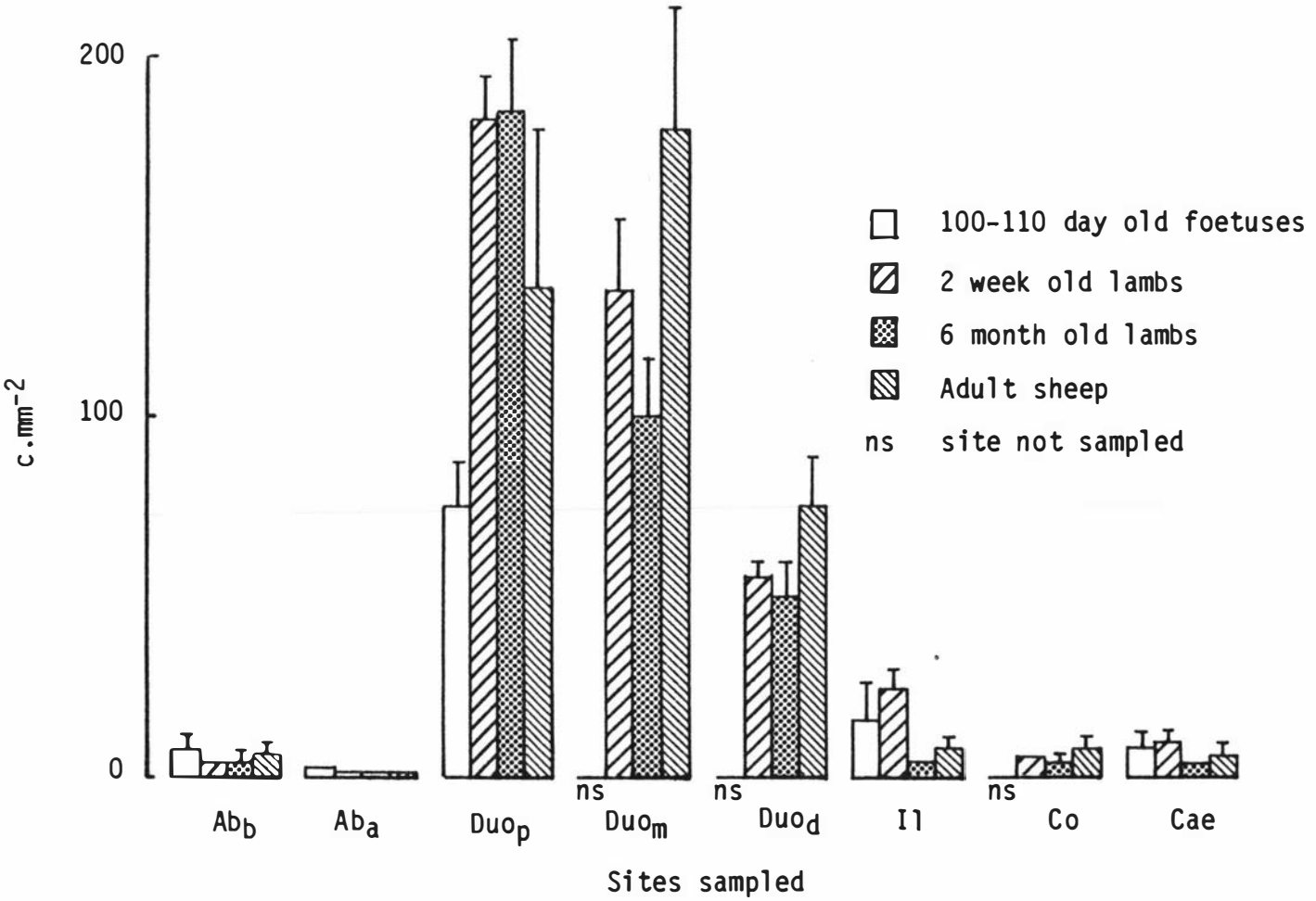


Figure 8.2: EC cell densities (mean + SEM) in the GI tract of sheep at various ages.

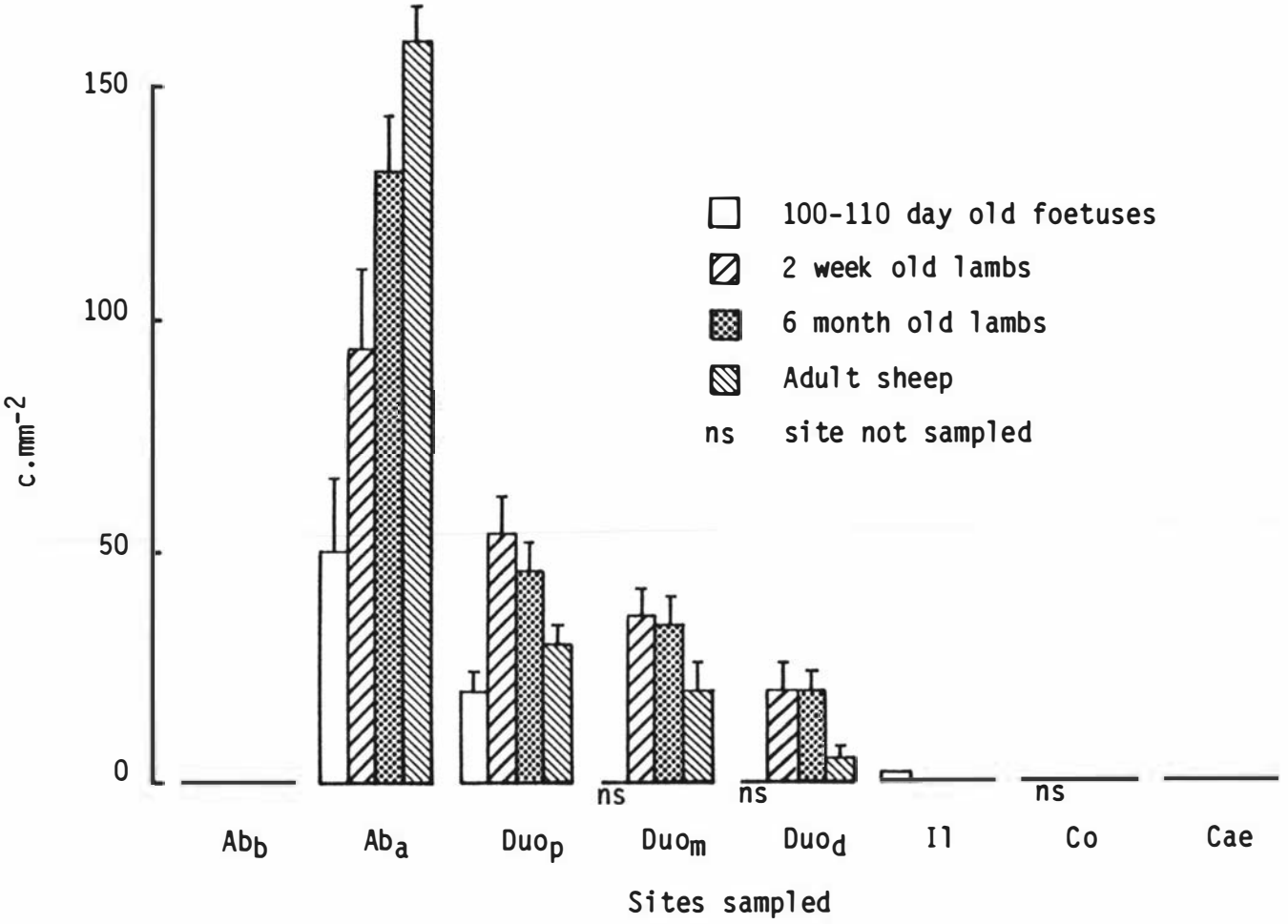


Figure 8.3: G cell densities (Mean + SEM) in the GI tract of sheep at various ages.

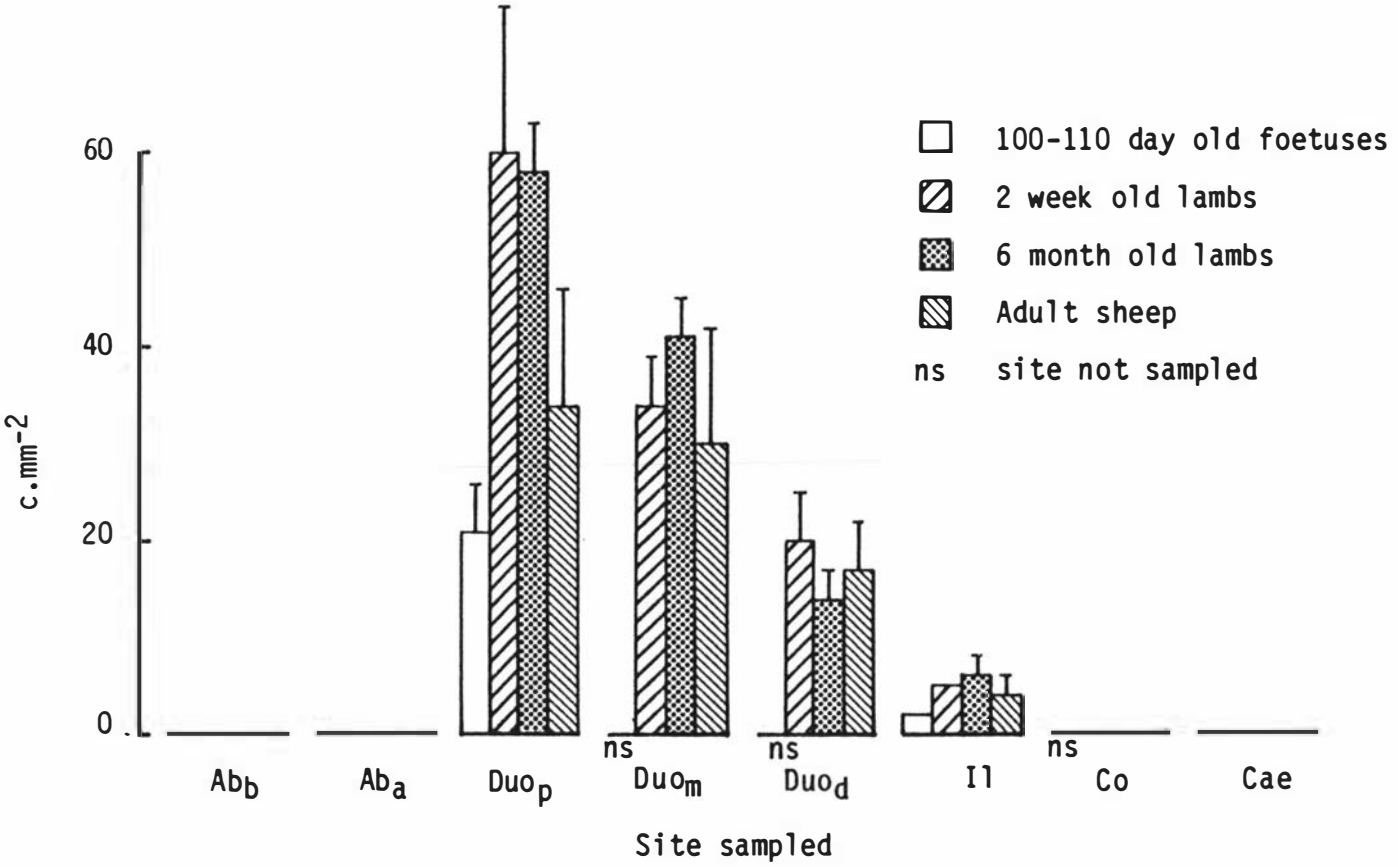


Figure 8.4: S cell densities (mean + SEM) in the GI tract of sheep at various ages.

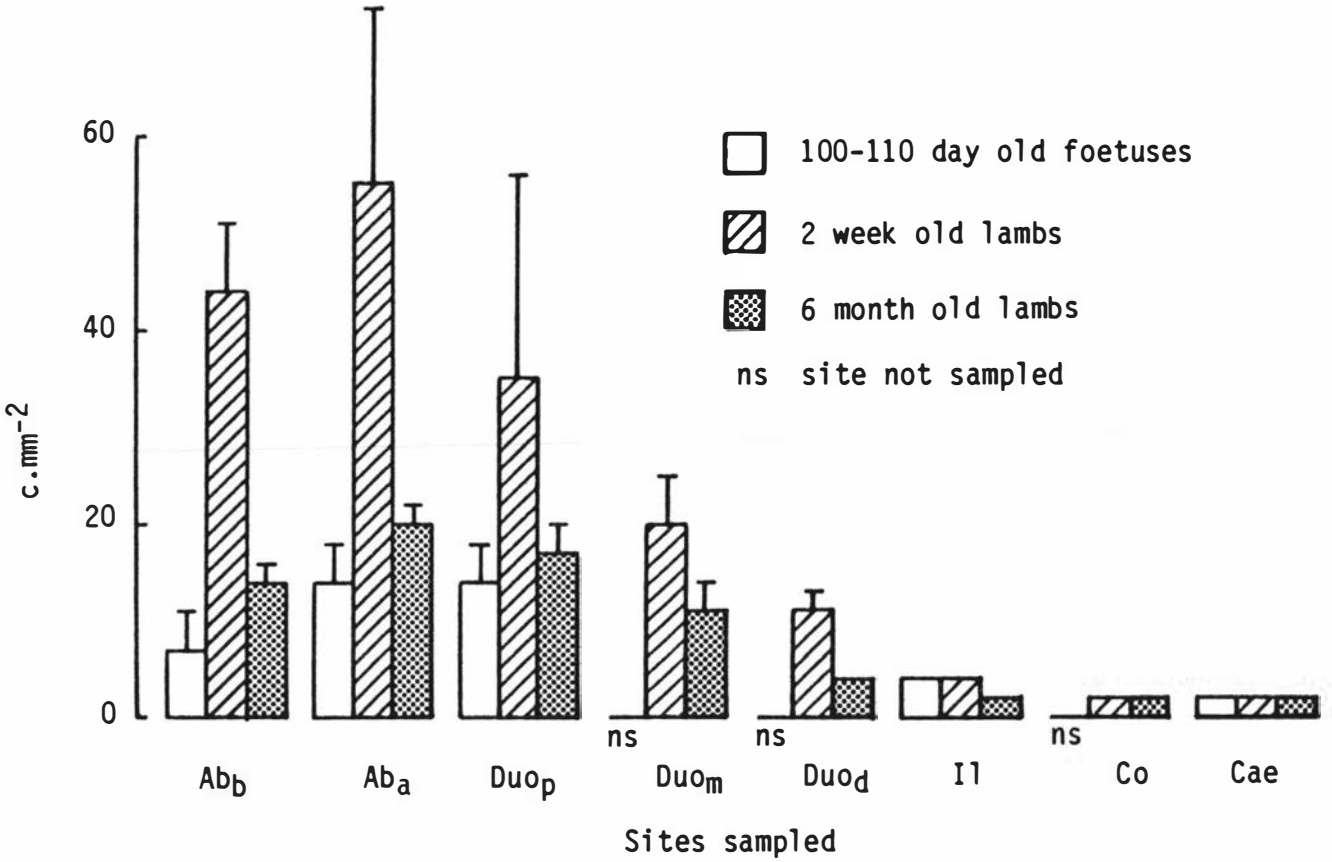


Figure 8.5: D cell densities (mean + SEM) in the GI tract of sheep at various ages.

Shulkes and Hardy (1982) detected immunoreactive gastrin in extracts of the ovine foetal abomasum as early as day 80. This gastrin obviously was of foetal origin as it has been shown that gastrin does not cross the placenta (Shulkes et al., 1982). Thus there is strong evidence, albeit indirect, that gastrin producing cells occur in the ovine foetus as early as day 80, even though G cells have not yet been demonstrated at such an early stage of life.

The changes in G cell densities illustrated in Fig. 8.3, and changes in circulating serum gastrin levels described by Lichtenberger et al., (1981) and Shulkes and Hardy (1982) are probably related to the development of digestive function of the GI tract in foetal and neonatal lambs. The present studies demonstrated that antral G cell densities increased with age while duodenal G cell densities declined from 2 weeks of age. The net result of such changes in density, together with increased mucosal thickness in the neonate was an increase in total (i.e. antral plus duodenal) G cell numbers with age. However, although circulating serum gastrin levels in the foetus rise in parallel with the increase in G cell density (Lichtenberger et al., 1981; Shulkes et al., 1981a), shortly after birth the gastrin levels decline and after several weeks stabilize at adult levels. Such observations indicate that the regulatory mechanisms for the release of gastrin in foetal and neonatal lambs differ. In adult sheep, acid produced by parietal cells inhibits gastrin release (see Chapter 1). However, parietal cells are uncommon in the abomasal mucosa of the foetal lamb, but increase dramatically during the first few hours of extrauterine life (Hill, 1956). This change in parietal cell numbers and the subsequent fall in abomasal pH, may in part, be responsible for the postnatal decline in plasma gastrin concentration, although altered sensitivity of regulatory receptors on the G cell may also contribute to the decrease in gastrin release.

Larsson and Jorgensen (1978) demonstrated a decline in duodenal G cell densities in humans and rats soon after birth. These workers attributed this decline and the subsequent increase in I cell densities at the same sites to a functional change in some "immature" endocrine cells.

Further, they demonstrated that some foetal duodenal endocrine cells contained both gastrin and CCK immunoreacivity. They concluded that the metabolic capabilities of such endocrine cells changed in emphasis from the production of gastrin to the elaboration of CCK early in the neonatal period (Larsson and Jorgensen, 1978). Whether similar changes account for the decrease in duodenal G cell densities observed in the present studies, remains to be established.

In view of the close anatomical relationship between antral G and D cells (Larsson, 1980) and the inhibitory role somatostatin plays in the regulation of acid secretion (see Section 1.6.1.7), antral G/D cell ratios have been calculated. These were 5.8 $\pm$ 2.9, 1.8 $\pm$ 0.4 and 6.6 $\pm$ 0.4 for foetuses, 2 week old and 6 month old lambs, respectively. G/D cell ratios for foetal and 6 month old lambs were similar to those reported for healthy human subjects (4.4: Arnold et al., 1982; 7.0: Crivelli et al., 1979). In 2 week old lambs, however, G/D cell ratios were much lower being comparable to the ratios (1.6 $\pm$ 0.2) found in human patients suffering from gastrinoma (Arnold et al., 1982).

The high G/D cell ratios in foetal lambs and the hypergastrinaemia reported to occur in late foetal and early neonatal life (Lichtenberger et al., 1981; Shulkes and Hardy, 1982), probably reflect the importance of gastrin as a trophic hormone (Johnson, 1980; Majumdar, 1984) during development and maturation of the GI tract. Gastrin has no role in regulation of abomasal acid secretion in foetal lambs as parietal cells are rare in foetuses (Hill, 1956) and hence they are hypo- or achlorhydric.

The low G/D cell ratio found in 2 week old lambs was largely due to the substantially higher antral D cell densities in these lambs compared to those for foetuses or 6 month old lambs (Fig. 8.5). It is possible that the rapid increase in parietal cell numbers following birth and the subsequent fall in antral pH stimulates D cell hyperplasia. This is in accord with the finding that in humans the antral G/D cell ratio depends on antral pH (Arnold et al., 1982). In neonatal lambs elevated D cell densities may play a role in lowering of plasma gastrin levels in the first few weeks of life, particularly during interdigestive periods.

8.4 Endocrine Cells and Paracrine Function

Some abomasal D cells and a few ECL cells were observed to have basal cytoplasmic processes which terminated close to parietal cells. These results indicate such cells function as paracrine cells, the secretory products of which are thought to modulate or regulate the function of other cells in their immediate vicinity (Pearse et al., 1977). Larsson et al., (1979) have presented histological evidence that somatostatin secreting D cells in the stomach function as paracrine cells. Further, somatostatin has been shown to inhibit gastric acid secretion by direct action on parietal cells (Konturek et al., 1976a).

Although the secretory product(s) of ovine ECL cells have not been identified, ECL cells of rats have been shown to elaborate histamine (Kubota et al., 1984). Histamine is thought to be the final common mediator for all secretagogues of gastric acid secretion (see Konturek, 1980a). Although the source of this histamine has not been clearly established, it is probable that it is released from ECL cells (Soll et al., 1981; Rubin and Schwartz, 1983) in the gastric gland epithelium in response to stimulation by acetycholine and/or gastrin (Rubin and Schwartz, 1983). With the techniques used in the present studies it was not possible to demonstrate whether either of these endocrine cell types had a close anatomical and hence functional relationship with any other cell type in the ovine abomasum. In particular it would be of interest to know whether D cells in sheep have a close asociation with G cells as has been reported by Larsson et al. (1979) for the human.

8.5 Effects of T. colubriformis infection

The hypothesis that some clinical effects of intestinal T. colubriformis infection were due to disturbance of the GI endocrine system (see Section 7.1) was tested by comparing endocrine cell distributions and densities in parasite-free and infected sheep.

Although the infection in this experiment was very mild, argyrophilic cell densities increased significantly in the duodena and proximal jejuna of infected animals. Although the identity of such cells was not

established, it was concluded that they were not EC, G, S or D cells and I cells probably also were unaffected by the infection. Thus, the most likely explanation for the changes in argyrophilic cell densities was that D_1, K or L cells increased in density.

Although the peptide secretory product of D_1 cells is not known, (Solcia et al., 1982), K and L cells have been shown to produce GIP (Buffa et al., 1975) and GLI (Ravazzola et al., 1979), respectively. At physiological levels, exogenous GIP stimulates intestinal secretion (Brown et al., 1970) and inhibits absorption of water and sodium in the jejunum (Helman and Barbezat, 1977). Exogenous glucagon inhibits intestinal absorption of water and electrolytes (Whalen, 1974) and inhibits intestinal motility (see Ruppin and Domschke, 1980). Thus it is possible that the watery diarrhoea characteristic of acute and severe infection by T. colubriformis may be due, at least in part, to disturbances in circulating levels of GIP and GLI.

Although in the present study, in which infection was mild, there was no evidence that densities of EC, G, S, D and I cells changed, this does not preclude the possibility that in severe trichostrongylosis they may alter.

In severe trichostrongylosis abomasal secretion of acid is greatly reduced probably because of reduced numbers of parietal cells, reduced stimuli for secretion, or inhibition of secretion (Barker and Titchen, 1982). These workers favoured the last hypothesis and suggested that the abomasal hyposecretion observed probably involved the release from the parasitized small intestine of a systemically acting gastric inhibitory factor. Hormones produced in the upper small intestine which might act as gastric inhibitory factors, particularly if they were secreted in excessive amounts, include secretin, GIP, VIP, glucagon and somatostatin (Konturek, 1980a; Barker and Titchen, 1982). The gastric inhibiting factor(s) released in trichostrongylosis may act directly on parietal cells or indirectly via ECL or abomasal D or G cells. In this regard it would be of interest to establish whether there were any changes in ECL, D or G cell densities that might indicate an involvement in parietal cell dysfunction.

Because of the role CCK may play as a mediator of the satiety response (see Walsh, 1981), it would be of interest to establish whether the anorexia so typical of more severe T. colubriformis infection was due to increased circulating levels of CCK. Such changes in CCK levels could be due either to increased secretory activity of I cells or to an increase in I cell density. The latter could be investigated either by electron microscopy or by use of a CCK specific antiserum.

In order to establish whether any of the changes postulated above or alterations in the density of any other endocrine cell type play a part in the clinical effects of trichostrongylosis, the parasite study described in this thesis should be repeated using higher doses of T. colubriformis larvae. Such a study would assist in establishing the role, if any, of hormonal factors in the disease syndrome.

8.6 Future Research

Extensions of the present work which might assist in furthering knowledge of gut function include:

(a) Application of a neuronal specific enolase antiserum to gut mucosal samples (see Bishop et al., 1982). None of the methods described in this thesis stain all gut endocrine cells and thus presently there are no good estimates of the total number of endocrine cells at any site in the ovine GI tract. Antisera to neuronal specific enolase are claimed (Bishop et al., 1982) to stain all GEP endocrine cells and could therefore be used to provide such data.

(b) Introduction of additional antisera to identify further specific types of endocrine cells. Ovine abomasal EC cells were difficult to identify because they stained poorly with the conventional histochemical techniques used in this study. Use of a specific antiserum would be of considerable assistance in identifying EC cells particularly when their content of secretory product is low. Use of an antiserum to histamine would permit positive identification of ECL cells for which no specific histochemical technique is available.

(c) Most CCK antisera produced by conventional immunological methods cross-react with gastrin. Thus CCK antisera to be used in future immunohistochemical studies should be screened for such cross-reactivity. Use of monoclonal antibody production technology should increase the probability of producing specific CCK antisera. In a similar manner, antisera specific for the C-terminal tetrapeptide of gastrin or the N-terminal portion of the gastrin molecule could be produced. Application of such specific antisera would establish the distributions and densities of I, TG, IG and antral G cells in the GI tact.

(d) The application of double staining techniques, or staining techniques combined with electron microscopy, would be useful in establishing whether close histological and hence physiological relationships existed between endocrine cell types and between endocrine cells and other mucosal cell types in the GI tract. In particular, such methods may be used to study the interrelationships between EC, ECL, G, D and parietal cells in a variety of physiological states.

(e) A study of ovine foetuses younger than the 100 - 110 day old foetuses used in this study might throw further light on the ontogeny of endocrine cells, and may assist in understanding the functions of these cells in foetal gut development.

(f) The present studies should be extended to include quantitative measurements of the peptide content of the mucosa and plasma. In the studies described here, no assessment was made of hormone content of individual endocrine cells. The techniques described may be modified such that there is a defined relationship between chromagen colour density and hormone content. In such circumstances estimates of hormone content could be made by densitometry (Sklarew, 1982).

Application of the data contained in this thesis and the extension of such studies as outlined above may assist in understanding nutritional and pathophysiological disorders in sheep. It is thought, for example,

that the failure of lambs to thrive when fed Lotus sp. containing high levels of tannins may in part be due to disturbances of the gut hormone system (T. Barry, pers. comm.). Circulating levels of GH and somatostatin are altered, however, the mechanisms involved are not understood. Knowledge of the changes that occur in the GEP endocrine system in such nutritional disorders may lead to a better understanding of the reasons for the failure of animals to thrive. Similarly, application of the techniques described in this thesis to animals suffering from other nematode infections (e.g. Ostertagia circumcincta) should lead to a better understanding of the disease syndrome. In ostertagiasis hypochlorhydria is accompanied by hypergastrinemia, however, it is not known if the latter is due to increased G cell numbers and/or increased release of gastrin. Other cell types involved in regulation of gastric acid secretion (e.g. ECL and D cells) may also be involved in abomasal dysfunction. Thus a study of endocrine cell densities in parasitized animals should aid in understanding the regulation of normal functions of the GI tract and some of the derrangements that occur in such disease states.

In conclusion, the specific experimental aims of this study have in large measure been achieved. Histochemical and immunohistochemical techniques suitable for use in sheep have been established. Using these techniques, argyrophilic, EC, G, S and D cell morphology and distributions were studied in animals ranging in age from 100 - 110 day old foetuses to mature adults. Finally, it was established that even in very mild trichostrongylosis changes in endocrine cell densities occurred indicating that hormonal imbalance may be responsible for some of the clinical effects of this disorder.

APPENDIX 1. List of chemicals and stains used in the present studies and their source of supply.

Chemical	Specification	Supplier	Catalogue No.
Acetic acid	glacial, 'AnalaR'	BDH	10001
Alcian Blue 8GX	C.I. 74240, 'GURR'	BDH	34089
Aluminium potassium sulphate	'AnalaR'	BDH	10009
Ammonium acetate	UNIVAR	Ajax	27
Ammonium hydroxide	'ARISTAR'	BDH	45200
Barbituric acid		Sigma	B0625
Barbital		Sigma	B0375
Bovine serum albumin (Fraction V powder)		Sigma	A4503
Chloral hydrate	GPR	BDH	27668
Chloroform	UNIVAR	Ajax	152
Citric acid	'AnalaR'	BDH	10081
Diaminobenzidine tetrahydrochloride		Sigma	D5637
Disodium hydrogen orthophosphate (anhydrous)	UNIVAR	Ajax	621
DPX	mountant	BDH	36029
Eosin Y	C.I. 45380, 'GURR'	BDH	34027
Ethyl alcohol	aboslute, GPR	BDH	28304
	95%	BDH	28303
Ethylenediaminetetra-acetic acid disodium salt	'AnalaR'	BDH	10093
Fast Black K	C.I. 37190, 'GURR'	Ajax	G12600
Fast Garnet GBC	C.I. 37201, 'GURR'	BDH	34118
Formalin	GPR	BDH	28421
Glutaraldehyde (25%)	UNILAB	Ajax	698
Haemalum (Mayer's)	'GURR'	BDH	35060
Haematoxylin	C.I. 75290, 'GURR'	BDH	34037
Hydrochloric acid	'AnalaR'	BDH	10125
Hydrogen peroxide (27%)	UNIVAR	Ajax	260
Hydroquinone	'AnalaR'	BDH	10312
Iodine	'AnalaR'	BDH	10135
Lead nitrate	GPR	BDH	29038

Light Green SF	C.I. 42095, 'GURR'	BDH	34043
Magnesium sulphate	'AnalaR'	BDH	10151
Mercuric chloride	'AnalaR'	BDH	10154
Picric acid	GPR	BDH	29554
Potassium chromate	'AnalaR'	BDH	10199
Potassium dichromate	'AnalaR'	BDH	10202
Potassium dihydrogen orthophosphate	UNIVAR	Ajax	391
Silver nitrate	UNIVAR	Ajax	449
Sodium acetate (anhydrous)	GPR	BDH	44472
Sodium carbonate (anhydrous)	'AnalaR'	BDH	10240
Sodium chloride	UNIVAR	Ajax	465
Sodium hydrogen carbonate	UNIVAR	Ajax	475
Sodium iodate	GPR	BDH	30171
Sodium sulphite (anhydrous)	'AnalaR'	BDH	10357
Sodium thiosulphate	'AnalaR'	BDH	10268
Thimerosal		Sigma	T5125
Thymol	GPR	BDH	30433
Toluidine Blue	C.I. 52040, 'GURR'	BDH	34077
Trasylol		Bayer	
Tris (hydroxymethyl) aminomethane	'AnalaR'	BDH	10315
Wax (Paraffin) m.p. 56°C	'GURR'	BDH	36107
Xanthydrol		H&W	52985
Xylene	GPR	BDH	30576

Suppliers:
Ajax Chemicals, Sydney, Australia
Bayer Pharmaceutical Co. Botany, N.S.W., Australia
BDH Chemicals Ltd, Poole, England
Hopkin & Williams, Chadwell Heath, Essex, England
Sigma Chemical Co., St Louis, USA

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