BIOCHEMICAL AND STRUCTURAL CHANGES IN RESPONSE TO ABOMASAL NEMATODE INFECTIONS IN RUMINANTS

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A thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Four methods for estimating plasma pepsinogen concentrations were compared to determine the most suitable assay for routine use. Three of the methods were colorimetric assays which used glycine-buffered albumin, albumin, or haemoglobin as their substrates. The fourth method was a radial diffusion assay which used casein in agarose as its substrate. All four methods gave reproducible results when replicates were assayed on the same day. However, the first method had the least day-to-day variation and was the only method which gave a linear relationship with different concentrations of pepsinogen in a plasma pool.

Two artificial pepsinogen containing solutions were created, the first was an entirely aqueous solution of porcine pepsinogen and the second was plasma with an identical pepsinogen concentration, achieved by adding an identical amount of porcine pepsinogen to ovine plasma instead of water. The ovine plasma chosen had zero intrinsic peptic activity. When the peptic activities of the two solutions were compared the activity of pepsinogen in plasma was significantly reduced. This was due either to an interference in enzyme reaction rates by the presence of plasma proteins, or to a more specific interference with activated pepsin by an inhibitor, as yet unknown, present in plasma. Either way this phenomenon represented a potentially significant source of variation when recording plasma pepsinogen concentrations by assays measuring proteolytic activity.

The effects of excretory/secretory products (ES) of *Ostertagia circumcincta* on the release of pepsinogen and on smooth muscle function in vitro was investigated. ES was prepared by culture of *O. circumcincta* adults. Methods to monitor pepsinogen release from isolated, intact mucosal sheets and isometric contractions in abomasal smooth muscle were successfully developed and were both adequately responsive with the muscarinic agonist, carbachol. The addition of ES to tissues consistently resulted in the release of pepsinogen from mucosae and in smooth muscle contraction, but only in tissues obtained from animals with a history of previous exposure to helminth parasites. Tissues from known parasite-naive animals did not respond to the presence of ES. These responses were therefore considered to be hypersensitivity responses to antigens present in ES.

The distribution of pepsinogen in abomasal tissues was investigated immunohistochemically and biochemically in the abomasa of parasite-naive cattle and sheep, and in animals with

ostertagiasis and haemonchosis. In the parasite-naive animal the fundic chief cell was the major source of pepsinogen, as indicated by intensity of staining. Mucous neck cells also contained pepsinogen, but in lesser amounts. The pepsinogen content of surface mucous cells was variable in the bovine however this cell type did not contain pepsinogen in the normal ovine. Ostertagiasis was associated with focal hyperplasia around parasitised gastric glands and increased tissue pepsinogen content was detected within nodules. Normal epithelial cells, including chief cells, were replaced by hyperplastic mucous-type cells. These cells contained pepsinogen and hyperplasia resulted in an expanded population of cells producing the zymogen. Individual cells of the hyperplastic population contained less pepsinogen than did mature chief cells, but their greater numbers accounted for the increase in tissue pepsinogen content. In haemonchosis hyperplasia was generalised, and increased numbers of cells were positive for pepsinogen. However, overall tissue pepsinogen content was not increased by infection and it was observed that chief cell content was again reduced. Thus helminth infections were associated with an expanded zymogenic population and the inference was that pepsinogen secretion in infected animals was at least maintained in infection, if not increased. Some sheep were shown to produce little or no pepsinogen, but the consequences and significance of this finding remain unknown.

The role of the mucosal growth regulator, Transforming Growth Factor-alpha (TGF-α), in the development of hyperplasia in ostertagiasis was investigated. The immunohistochemical localisation of TGF-α in various gastrointestinal tissues of parasite-naive sheep was determined and compared to that of the related peptide, Epidermal Growth Factor (EGF). In normal fundus TGF-α immunoreactivity was confined to the differentiated, non-proliferative compartment, to parietal cells and to surface mucous cells, and the distribution of EGF was identical. In other gastrointestinal tissues, such as oesophageal mucosa, the distribution of the two growth factors was not the same and this was taken as evidence that the antisera used were able to recognise distinct peptides, i.e. the ovine equivalents of TGF-α and EGF. Within the nodular tissue obtained from animals with ostertagiasis, the greater numbers of mucous-type cells were associated with an increase of intensity of staining in the superficial mucosa, but the concomitant depletion of parietal cell number was associated with reductions of staining in deeper gland structures. Given the known effects of TGF-α these results strongly suggested that TGF-α may be important in the development of hyperplasia in infections with *Ostertagia* spp. parasites.

These studies have investigated the causes of elevated plasma pepsinogen concentrations that occur in animals infected with parasites of the genera *Ostertagia* and *Haemonchus*, and have shown that variations in plasma pepsinogen concentration between animals may be due to a number of causes. Amongst these causes inaccuracies inherent to the assay methods used may be important. Further these studies have shown that complementary to previous hypotheses concerning the mechanisms behind elevated plasma pepsinogen concentrations, such as changes in mucosal permeability, and the direct stimulation of pepsinogen secretion by parasites, must be added hypotheses concerning mucosal hypersensitivity responses to antigens released by parasites stimulating pepsinogen secretion indirectly and changes in the populations of cells secreting pepsinogen, which may even result in an enhanced zymogenic population.

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ABBREVIATIONS

A	absorption
AMP	Adenosine monophosphate
&	and
ANOVA	analysis of variance
AUC	area under the curve (plot profiles of staining intensity)
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumin
CCK	Cholecystokinin
C. elegans	*Caenorhabditis elegans*
cm	centimetre
CP	crude protein
CV	coefficient of variation
°C	degrees Centigrade
DAB	Diaminobenzidine
Δ	change in following parameter over specified time
DM	dry matter
ECF	Eosinophil Chemotaxis Factor
$ED_{50/100}$	effective dose for 50 or 100 per cent of the maximal effect
EGF	Epidermal Growth Factor
EGF-R	receptor for EGF peptide family
EL_4	larva arrested at the early fourth-larval stage
epg	eggs per gram (of faeces)
ES	excretory/secretory products
FEC	faecal egg count
5-HT	5-hydroxytryptamine
Fig.	figure
g	gram
g	unit gravity (10^{-11} Newtons)
GI	gastrointestinal
G-17/-34	Gastrin of 17 and 34 amino acid forms
H. contortus	*Haemonchus contortus*
H&E	Haematoxylin and Eosin
hr/hrs	hour/ hours
IgA/E/G/M	immunoglobulins of subclasses A, E, G and M
I_{max}	value of highest recorded staining intensity
ISA	intermittant spiking activity
iU	international unit (of peptic activity)
kD	kilodalton
kg	kilogram
L	litre
$L_{3/4/5}$	third/fourth/fifth-larval stages
LDH	Lactate dehydrogenase
log	logarithm
M	molar
MAPC	Migrating Action Potential Complex
µl	microlitre
µM	micromolar
ml	millilitre

mM	millimolar
min/mins	minute/ minutes
MMC	Migrating Myoelectric Complex
MW	molecular weight
n	number
NAD/NADH	Nicotine adenine dinucleotide/ reduced NAD
ng	nanogram
nm	nanometre
No.	number
NS	not significant ($p>0.05$)
O. circumcincta/ ostertagi	*Ostertagia circumcincta/ ostertagi*
O.D.	optical density
p	probability
PAS	Periodic Acid Schiff
PBS	Phosphate buffered saline
PCV	packed cell volume
PG	pepsinogen
PGD_2	Prostaglandin D_2
pH	negative log of the hydrogen ion concentration
Pmax	position of the point of maximal staining intensity with respect to the level of the mucosa
RSA	regular spiking activity
RSER	rough-surfaced endoplasmic reticulum
SD	standard deviation about the mean
SE	standard error of the mean
S-E	Secretory-Excretory (organ system of nematodes)
sig. diff.	significantly different ($p<0.05$)
spp.	species
T. spiralis	*Trichinella spiralis*
T. axei	*Trichostrongylus axei*
T. colubriformis	*Trichostrongylus colubriformis*
TGF-α	Transforming Growth Factor-alpha
Th1/2	T helper cells, subtypes 1 and 2
TP	Total protein
u	unit of peptic activity (as quoted by Sigma Chemical Co.)
U/L	units per litre (international units for assay of LDH concentration).
VGF	Vaccinia Virus Growth Factor
VIP	Vasoactive Intestinal Polypeptide
v/v	volume per volume
w/v	weight per volume
Z-E	Zollinger-Ellison

ACKNOWLEDGEMENTS

This work was generously funded by the Ian Sutherland Scholarship and by the Perry Foundation and the Leverhulme Trust.

The author would like to thank Quintin McKellar for the supervision of this work, for his enthusiasm and impeccable example to a young researcher.

I also wish to acknowledge the assistance and advice given by Drs. Andrea Nolan and Mike Stear, and Dr. Brian Morris for his help in conducting the image analysis. I must also thank Mssrs. Ian Gibson, Peter Baxter, and Dr. Ken Bairden and other staff of the Departments of Veterinary Clinical Studies, Parasitology and Pharmacology at Glasgow for technical assistance.

Finally, to fellow researchers and friends; Peter Graham, Hafid Benchaoui, Elizabeth Welsh, Robert McCormack, Mick Park, Julie Ewald, James McCormick and Fraser Hendrie. Cheers.

DECLARATION

The contents of this thesis are the work of the author, except for the following;

All processing and staining of fixed tissues for histological and immunohistological examination were carried out by Jane Irvine and Alma Dick, of the Department of Veterinary Pathology, University of Glasgow Veterinary School. The infection and regular sampling protocols for the Scottish Blackface lambs (section 6.5) and Hampshire Down lambs (Sections 7.2 and 7.3) were performed by staff of the Department of Veterinary Clinical Studies, University of Glasgow Veterinary School, and were part of larger studies carried out by, amongst others, Drs. Mike Stear and Dawn Wallace and by Mick Park.

This thesis has not been submitted previously to any university for the award of any degree. The following publications, published abstracts are based on the work contained in this thesis:

Scott, I. & McKellar, Q.A. (1995) The effects of excretory/secretory products of *Ostertagia circumcincta* on pepsinogen secretion and smooth muscle contraction in abomasal tissues derived from previously infected sheep and in parasite-naive animals. *Proceedings of the 15th International Conference of the World Association for the Advancement of Veterinary Parasitology* (abstract D5).

Scott, I., McKellar, Q.A., Irvine, J. & Dick, A. (1995) The immunohistochemical localisation of pepsinogen in the abomasal mucosa of sheep infected with *Haemonchus contortus* and in parasite-naive controls. *Proceedings of the 15th International Conference of the World Association for the Advancement of Veterinary Parasitology* (abstract C5).

Scott, I., McKellar, Q.A., Irvine, J. & Dick, A. (1995) The role of the mucosal growth factors, Transforming Growth Factor-alpha and Epidermal Growth Factor in the pathogenesis of infection of sheep with *Ostertagia circumcincta*. *Proceedings of the 15th International Conference of the World Association for the Advancement of Veterinary Parasitology* (abstract C16).

Scott, I., Stear, M.J. & McKellar, Q.A. (1995) Comparison of four methods for the determination of plasma pepsinogen concentration. *Research in Veterinary Science* **59**, 234-237.

Signed **Date**

16th September 1996.

1: GENERAL INTRODUCTION

The nematodes and other Metazoans are thought to have evolved, under anoxic conditions, in the sulphide-rich layer that is present at the bottom of all major bodies of water and is still rich in nematode species today (Bryant, 1982). Vertebrate ancestors, the chordates, were oceanic bottom-dwellers, almost certainly mud-sifters, and were likely to contact the sulphide layer. The redox potential, the availability of oxygen and carbon dioxide, and the pH are very similar in the marine sulphide layer and the gastrointestinal tract of vertebrate hosts (Bryant, 1982). Nematodes, like many organisms, developed the ability to detoxify oxygen, yet they have retained their great capacity for anaerobiosis.

Of all described nematodes (Phylum: *Nemathelminthes*; Class: *Nematoda*), 40 per cent are parasites of animals, mostly of vertebrates, and the majority of parasitic species belong to one large sub-class, the *Secernentea* (Anderson, 1984). Nematodes are one of the most successful animal groups and, in the course of evolving, may have made the transition from a free-living existence to a parasitic one many times. It is interesting therefore to consider the potentially long co-evolution of nematodes and their vertebrate hosts.

The compartmentalisation of the forestomach of ruminants is an evolutionary adaptation to enable utilisation of the bacterial products of the breakdown of tough plant matter otherwise indigestible in the mammalian gut. The final chamber, the abomasum, is analogous to the true stomach of monogastric animals. Conditions within the abomasum and stomach are unique to these organs; secretion of hydrochloric acid begins the breakdown of foodstuffs by hydrolysis and assists the acid-stable enzyme pepsin in the breakdown of protein. Host tissues are by no means resistant to these destructive elements and secrete copious mucus and bicarbonate which act as a barrier.

The hostility of conditions prevalent in the gastric compartment would seem an adequate deterrent against parasitism of this organ, yet numerous viral, bacterial and multicellular pathogens are accomplished colonists. In some instances an initial injury is required, allowing opportunistic spread of the invading organism, for example in Braxy in sheep where consumption of frozen pasture or fodder can predispose to overgrowth of the bacterium

Clostridium septicum (Barker et al, 1993a). Other infectious agents are quite capable of penetrating the uncompromised gastric environment and surviving and these include many species of nematode parasites that afflict a wide variety of hosts (Urquhart et al, 1987). Amongst monogastric host animals, *Trichostrongylus axei* and *Habronema* spp. are gastric parasites of horses (Leland et al, 1961; Barker et al, 1993b), *Hyostrongylus rubidus* affects pigs (Stockdale, 1974), and *Ollulanus tricuspis* (Hargis et al, 1983; Reindel, 1987) and *Gnathostoma* spp. (Kirkpatrick, 1987) are parasites of carnivores. The nematode *Nochtia nochti* causes gastric adenomatous tumours in the stomach of Javanese monkeys (Bonne and Sandground, 1939). In ruminant hosts, abomasal nematodes are of great economic importance and constrain animal production world-wide. Important nematode parasites of ruminant abomasa include *Ostertagia* spp., *Trichostrongylus* spp. and *Haemonchus* spp.

1.1: Nematode parasites of ruminants.

The predominant pathogenic nematodes of the ruminant gastrointestinal tract are all representatives of the family *Trichostrongylidae*. In temperate areas of the world the cattle parasite *Ostertagia ostertagi* is the most economically important parasite (Armour, 1970), accounting for considerable losses in animal production. The disease in cattle is, principally, a parasitic abomasitis. In sheep, parasitic gastroenteritis is generally attributed to a mixed spectrum of parasites, including; *Trichostrongylus, Cooperia* and *Nematodirus* genera. *Ostertagia circumcincta* (*Teladorsagia circumcincta*) is often the predominant and most pathogenic nematode species present (Armour et al, 1966). In sheep with mixed infections, the disease may be attributed to the abomasitis caused by *O. circumcincta* (and to a lesser extent *Trichostrongylus axei*) and enteritis caused by several enteric parasites, such as *Trichostrongylus colubriformis*. *Haemonchus* spp. parasites have a widespread geographical distribution, but are of greatest importance as abomasal parasites of ruminants in tropical and subtropical areas (Allonby, 1973).

Parasitic infection is, generally, of greatest importance in young ruminants. The adverse effects of parasitism on productivity are legion. Reductions of liveweight gain in young growing stock can be as high as 60 to 100 per cent (Sykes and Coop, 1976, 1977; Abbott et al, 1986; Fox et al, 1989a). These changes are accompanied by alterations in body composition; changes in soft tissue components and in bone (Sykes et al, 1977; Wilson and Field, 1983; Holmes, 1986). The quantity and quality of wool production in sheep can be affected (Steel et

al, 1982). Other production parameters such as milk production and reproductive performance may also be affected. Numerous studies have reported reduced milk production in infected animals (Bliss and Todd, 1973; 1976; Leyva et al 1982), however milk production is necessarily an activity of mature animals that may be more resistant to parasites (Parkins and Holmes, 1989). Ovulation rates have been shown to be consistently lower in sheep infected with *O. circumcincta*, but this did not translate into reduced reproductive performance (Jeffcoate et al, 1988). Mortality in heavy infections and the premature culling of affected stock are obvious and further sources of economic loss and are welfare concerns. Added to productivity losses are the costs of anthelmintic treatments and the cost and inconvenience of pasture and stock management to minimise exposure to infection.

1.2: The life cycle of *Ostertagia* spp. affecting bovine and ovine hosts.

The life cycle is direct, following a typical trichostrongylid pattern. Development involves progression from egg to sexually active adult via five larval stages; transitions are accomplished by moulting. Unembryonated eggs passed in the faeces of infected animals hatch and develop to the infective third larval stage (L_3) in two weeks given optimal conditions (Rose, 1961; Pandey, 1972 a, b; Young et al, 1980; Gibson, 1981). With adequate moisture the L_3 migrate onto the herbage and are consumed by grazing animals. Third stage larvae, retaining the cuticle of the second stage, exsheath in the rumen and may penetrate the gastric glands within 6 hours of ingestion (Osborne et al, 1960). After two further moults L_5 emerge onto the abomasal surface and become adult. The life cycle is slightly faster in sheep (Armour et al, 1966), but eggs generally appear in the faeces of both bovine and ovine hosts 21 days after initial infection. In certain instances (vide infra) development can arrest at the early stage of L_4 (EL_4), to resume at up to six months later.

1:3: Inhibited or arrested larval development (Hypobiosis).

Seasonal and immunological mechanisms of inhibition of the larval development of *Ostertagia* spp. have been proposed (Anderson, 1988; Eysker, 1993). Inhibition occurs at a precise stage in early parasitic development (EL_4). Hypobiotic larvae have lowered metabolic rates and there is cessation of growth. Winter inhibition, seen in colder climates such as northern Europe, has been associated with the chilling of larvae prior to ingestion. The climatic factors responsible for the summer/dry season inhibition of warmer climes are less

well known. There can be marked variations in the susceptibility of different parasitic strains to become inhibited (Armour et al, 1967; Borgsteede and Eysker, 1987). In field trials, towards the end of the grazing season, the extent of inhibition was greater in permanently grazed stock than in previously naive, tracer animals, demonstrating a potential influence of host resistance (Eysker, 1993). At least in northern Europe the onset of adverse climatic conditions favouring inhibition is often at the same time as host resistance is building. Factors or stimuli causing the resumption of development are unknown. The time course of inhibition may be set by the parasites themselves (Armour and Bruce, 1974). The synchronous development of large numbers of inhibited larvae giving rise to Type II ostertagiasis (vide infra), and the low prevalence, although this may be due in part to efficient prophylaxis, and variable onset of this condition point to the involvement of specific host factors.

1.4: Parasitic gastritis.

At the turn of the last century and in the early parts of this century reports of the biology of, and disease seen in association with, trichostrongyle parasites of ruminants were sporadic, but reflected the world-wide distribution of these parasites (Ostertag, 1890; Stiles, 1900-1901; Gardner, 1911; Ransom, 1911; Ackert and Moldoon, 1920, Stewart and Crofton, 1941). Abomasal parasitism was seen as a significant cause of diarrhoea, anorexia, weight loss/failure to thrive and mortality in affected animals.

It was not until the late 1950's that studies began to relate aspects of the biology of parasites to disease and to distinguish between separate disease syndromes. Douvres (1956) described, in detail, the morphology of the parasitic stages of *O. ostertagi*, and the concept of larval inhibition and the delayed onset of disease was noted (Martin et al, 1957).

In 1965, Anderson et al classified bovine ostertagiasis on the basis of three phases of disease: Type I, Pre-type II and Type II. Of these only Types I and II were clinically apparent. Type I disease was as seen in calves at grass for the first time, from late July until the end of the grazing season and arose from the development of sufficient parasite burden to cause disease, with minimal inhibition of larval development. The disease is generally a high morbidity, low mortality scour with accompanying anorexia and weight loss (Armour et al, 1973). Large, clinically inapparent populations of inhibited larvae characterise the Pre-type II phase. Up to 80 per cent of the total worm population may be inhibited (Armour, 1970) and animals appear

healthy unless the numbers of emergent larvae, having resumed development, are sufficient to produce clinical signs. Type II disease has been described by some workers as atypical gastritis (Martin et al, 1957), and arises with the resumption of larval development in outwintered or housed stock after their first grazing season. The synchronous resumption of development by many larvae can be catastrophic, with very rapid loss of condition in affected animals. Type II is predominantly of low morbidity, but potentially high mortality (Armour et al, 1973).

It is generally accepted that similar disease classifications apply to ovine ostertagiasis (Reid and Murray, 1973), Although type II disease in sheep is not necessarily associated with high mortality.

1.5: Pathogenesis and pathophysiology of infections with *Ostertagia* spp.

Several key studies elucidated the pathogenesis of ostertagiasis. Gross and histopathological changes were demonstrated as a consequence of single experimental infections in cattle (Ross and Dow, 1965; Ritchie et al, 1966) and sheep (Armour et al, 1966) and experimentally induced lesions were entirely consistent with those found in the field (Anderson et al, 1965). Studies of the secretory changes in the abomasum, as well as changes in biochemical, serological and haematological parameters, were also made (Anderson et al, 1965; Ross and Todd, 1965; Jennings et al, 1966). Murray et al (1970) identified the key lesion of bovine Type I ostertagiasis as a hyperplastic gastritis and defined the progression of a single experimental infection in three phases;

Phase 1 (from the point of infection to the seventeenth day after infection); during this phase developing larvae occupied the glands and cellular changes were more or less confined to the parasitised gland.

Phase 2 (day 17 to day 35 after infection); more severe lesions were associated with the maturation and emergence of adults - changes initially confined to the parasitised gland had spread.

Phase 3 (day 35 after infection onwards); changes following the loss of the worm burden - essentially the return of the mucosa to normal.

In phase 1, the normal, mature fundic glandular epithelium, consisting principally of parietal cells, mucous neck cells and chief cells, was replaced by a poorly differentiated epithelium. Initially stretched by the physical presence of the larvae, this epithelium became hyperplastic and cells were eventually recognisable as tall, mucus producing cells. The chief, grossly-apparent lesion of phase 1 was a pale nodule, with an umbilicated centre. Nodules grew in size as the parasites enlarged and in heavy infection nodules coalesced (the "Morocco-leather" effect; Ritchie et al, 1966).

Phase 2 was characterised by the loss of differentiation and hyperplasia of cells in the surrounding, non-parasitised glands. Emergence of the parasites was seen as critical in the spread of these changes. Phase 2 was also characterised by considerable epithelial cytolysis and loss of superficial epithelium. Initially epithelial loss simply enhanced the umbilicated appearance of each nodule, but where larger areas were lost it was observable grossly as "thumb-print" lesions and diphtheresis (Ritchie et al, 1966). In sheep, hyperplastic changes spread out from the central parasitised gland as early as day 8 (Armour et al, 1966), in association with the emergence of young adults, so that disease progression was observed to be faster in sheep.

Ritchie et al (1965) and Murray et al (1970) reported exponential worm loss between days 16 and 35 in cattle, but phase 2 was still dominated by the continued emergence of the remaining parasites and their associated pathology. With eventual worm loss phase 3 was characterised by a gradual return to normal appearance and function, and the return of recognisable parietal, mucous neck and chief cells.

The hyperplastic nature of lesions was underlined by the presence of more mitotic figures than normal. Murray et al (1970) described the hyperplastic cell population ultrastructurally as cells showing none of the features of mature epithelial cells. The cytoplasm and often quite large nucleus were relatively electron-lucent and rough-surfaced endoplasmic reticulum (RSER) was conspicuously absent. However a sizeable Golgi complex often encircled the nucleus. As cell differentiation proceeded RSER accumulated and secretory granules appeared towards the apex of cells which were themselves increasingly columnar. The granules were positive for Periodic Acid Schiff (PAS) in light-microscopical studies, but generally heterogeneous in terms of electron densities and reflected differentiation of cells to a mucus-secretory type.

Pathological changes of the Pre-type II phase were considered to be minimal (Anderson et al, 1965). Changes were confined to larval-occupied glands, with mucous cell replacement of the gland lining; mucous cell metaplasia. Areas of cytolysis were uncommon and rarely extensive. The switch to Type II disease can be dramatic, with the sequential development of numerous larvae, and hyperplasia in these circumstances has been shown to be marked (Murray, 1969; Snider et al, 1983) and the confluence of hyperplastic nodules depleted the mucosa of functionally differentiated cells and "thumb-print" lesions and areas of diphtheresis were extensive.

Mucous cell hyperplasia invariably results in derangements of abomasal secretory function in both Type I and Type II disease. When much of the abomasum is affected the ability to acidify the abomasal contents can be lost. Abomasal pH rises as the functional activity of parietal cells is lost. This has been implicated in the development of a rise in plasma pepsinogen, since pepsinogen is not converted to pepsin when pH is elevated (Jennings et al, 1966). Pepsinogen has also been thought to leak into the plasma through the impaired cell junctions that have been demonstrated between hyperplastic and immature cells (Murray, 1969, Murray et al, 1970). Loss of junctional integrity has also been considered responsible for the hypoalbuminaemia of infection. Murray (1969) demonstrated, in ultrastructural studies of Type II bovine ostertagiasis with associated severe hypoproteinaemia, that epithelial cells were uncharacteristically separated by spaces filled with electron-dense material. The electron-dense material was thought to be either plasma proteins leaking towards the abomasal lumen or pepsinogen travelling in the opposite direction, or perhaps both.

1.5.1 Enhanced epithelial permeability and hypoalbuminaemia.

Ostertagiasis is well characterised as a protein-losing gastropathy (Murray, 1969; Holmes and Maclean, 1971). Protein loss probably occurs via a paracellular mechanism; through the widened tight junctions existing between hyperplastic epithelial cells. In studies conducted by Murray (1969) between 50 and 90 per cent of the zonulae occludentes, a component of the tripartite junctional complex normally existing between cells (Farquhar and Palade, 1963; Murray, 1970), were partially or completely separated. This separation was not fully explained and existed between quite mature, better differentiated cells where the contentions, that cells were pulled apart or that the junctions were incomplete due to immaturity, are less likely. Areas of significant cytolysis and epithelial shedding are also likely to contribute to

plasma losses. Inflammatory changes in the microvasculature may also contribute (Murray, 1969).

Significant Nitrogen losses, reflecting overall protein loss, accrue due to the loss of plasma, epithelial cytolysis, the increased exfoliative rate of a hyperplastic mucosa and enhanced mucus production and secretion. Following oral inoculation of 9 to 13 month old lambs with 300,000 or 900,000 *O. circumcincta* larvae mean serum albumin concentration declined similarly in both infected groups after 1 week of infection and remained lower than that of uninfected controls for at least four weeks (Holmes and Maclean, 1971). Faecal plasma losses and catabolic rates of albumin were also estimated. These values rose between the fifth and ninth days after infection and reached peaks at about one to two weeks, but declined to near normal values by the fourth week. Although faecal plasma losses were similar in both groups, albumin turnover was more marked in animals given the 900,000 larval dose and only these animals developed clinical signs. Taylor et al (1989) also demonstrated significant loss of albumin into the gastrointestinal tract of *Ostertagia*-infected calves, but detected no rise in faecal nitrogen. Lost protein may therefore be broken down and reabsorbed more distally in the gut. Urinary Nitrogen was, however, increased suggesting failure to utilise what was reabsorbed. Infected animals respond with increased de novo synthesis of albumin, but production may not match loss and plasma albumin remains lowered (Symons, 1989; Fox, 1993).

1.5.2: Changes in abomasal pH.

Abomasal pH becomes elevated as hyperplastic changes become confluent. Elevation of abomasal pH in the bovine is therefore associated with the emergence of *O. ostertagi* from the mucosa at about 20 days after infection (Jennings et al, 1966) and in sheep elevation of pH may arise earlier (Armour et al, 1966), from day eight. As hydrogen ion concentration falls Na^+ increases, whilst K^+ and Cl^- fall.

The loss of mature parietal cells is not the sole cause of reduced acid secretion. Remaining and recognisable parietal cells may show morphological changes indicative of reduced, even negligible, parietal cell activity (Murray et al, 1970; McLeay et al, 1973). Features of normal parietal cells include extensive intracellular canaliculi lined by numerous microvilli (Murray, 1970). In the ultrastructural studies of Murray et al (1970) these features were often reduced,

even absent. Such changes are similar to those seen in parietal cells subjected to strong chemical inhibition (Vial and Orrego, 1963). Stringfellow and Madden (1979) showed reductions of staining specific for the parietal cell enzyme carbonic anhydrase following the oral inoculation of 3 month old calves with *O. ostertagi* L_3. Parietal cells in the vicinity of parasitised glands showed reduced staining and this reduction became widespread throughout the abomasum from 26 days following initial infection.

Bueno et al (1982a) suggested that elevation of abomasal pH in sheep infected with *H. contortus* was due, at least initially, to increased secretion of bicarbonate, but that eventually parietal cell inactivity predominated. Similar early changes in HCO_3^- secretion may occur in ostertagiasis (Dakkak and Khallaayoune, 1984).

The bacteriostatic effect of the abomasal environment is likely to be diminished by elevated pH. Jennings et al (1966) detected increased numbers of viable bacteria in the abomasa of infected calves once the pH had risen to 7.0, and equated the severity of diarrhoea to the duration of the elevation of pH.

The exact mechanisms of the loss of mature parietal cells and of the inhibition of the activity of remaining parietal cells are unknown. It is possible that an inhibitory factor, that may facilitate parietal cell loss as well as inhibition of hydrogen ion secretion, is released from either the parasites themselves or from damaged host tissues. This inhibitory factor may potentially have different effects related to its concentration. Close to its proposed site of production in the parasitised gland, higher concentrations may cause the loss of recognisable parietal cells, but at a greater distance and with lower concentrations, effects may manifest solely as the inhibition of parietal cell activity. It has been considered that parietal cell loss is due to the replacement of the normal gland lining by metaplastic mucous cells in response to the stretching of the gland by the motile parasite, it is unlikely that this itself would account for the inactivity of distant parietal cells.

Acid secretion in ostertagiasis was studied further by McLeay et al (1973), who monitored secretion from the main body of the abomasum in sheep in the presence of infection with *O. circumcincta*, whilst simultaneously measuring secretion from surgically separated fundic mucosal pouches that were free of infection. Pouch hypersecretion of acid occurred in the face of the normal hyposecretion in the infected abomasum. The inhibition of parietal cell activity

in the main body of the abomasum did not affect pouch activity, thus the putative inhibitory factor was not active systemically, only locally. Pouch hypersecretion was seen, rather, as evidence of a second mechanism operating in infected animals. An increase in the plasma concentration of the gut hormone gastrin (vide infra) was likely to be responsible.

The hypothesis that acid pH is deleterious to parasite survival, and that the worms would actively seek to increase abomasal pH, is attractive. It was perhaps not surprising that *O. ostertagi* parasites rapidly died in vitro at highly acidic pH (Eiler et al, 1981). Further, Eiler et al (1981) inhibited gastric acid production in rats in-vivo with a somatic extract of adult *O. ostertagi*. There are also rather scant reports of small molecular weight peptides, isolated from "in-vitro products of *Ostertagia*", which inhibited the respiration of isolated gastric glands in-vitro and enhanced mucus production and secretion in rat stomachs in-vivo (Rikihisa and Hammerberg, 1982). Since mitochondria occupy 30 to 40 per cent of parietal cell volume, in comparison to just 5 per cent in chief cells, O_2 utilisation by gastric glands is highly correlated with acid production (Soll and Berglindh, 1987). Excretory/secretory (ES) products of *Ostertagia ostertagi* did not alter basal acid production, as measured by aminopyrine accumulation in isolated and dispersed, bovine gastric glands (McKellar et al, 1990a), but basal accumulation of aminopyrine was significantly reduced by cimetidine, omeprazole and thiocyanate. However some parietal cell inhibitors, e.g., Epidermal Growth Factor (Finke et al, 1985), do not alter basal secretory rates but do suppress stimulation by, e.g., histamine. The results of in-vitro experiments must be interpreted carefully as the conditions used for culture of worms may be vastly different from those prevalent in the abomasum.

In experiments, where parasites were transplanted directly into the uncompromised abomasal environment of previously parasite-naive animals, worms became established and were viable for a number of days (as evinced by positive faecal egg counts)(Anderson et al, 1985; McKellar et al 1986; McKellar et al, 1987). Worms may survive by exploiting the mucus layer, where conditions are markedly different from the highly acidic lumen; indeed, during necropsy, the majority of worms can be found closely adherent to the abomasal surface (Eysker and Kooyman, 1993).

1.5.3: Hypergastrinaemia.

Acid hypersecretion, occurring in separated fundic pouches in the presence of infection of sheep with *O. circumcincta* in the main part of the abomasum, could be abolished by antrectomy (Anderson et al, 1981). The abomasal antrum is the major gastrointestinal source of gastrin (in sheep: Reynolds et al, 1979; and in cattle: Fox et al, 1993). Specialised endocrine cells, the G-cells, store and secrete gastrin in response to changes in lumenal pH, lumenal amino acids and fatty acids, and a presumed neural input (Titchen and Anderson, 1977). Hypergastrinaemia has been demonstrated directly by the radioimmunoassay of plasma gastrin in experimental infections of both cattle and sheep (Anderson et al, 1981; Fox et al, 1987) and in field studies of naturally-arising infections in grazing cattle (Entrocasso et al, 1986a). In normal animals circulating gastrin is a 17 amino acid polypeptide (G-17), however the gastrin form associated with parasite-induced hypergastrinaemia is the 34 amino acid form (G-34)(Anderson et al, 1988; Fox et al, 1993). Incomplete post-translational processing of gastrin precursors (progastrins) probably accounts for this difference. G-cells may store gastrin as G-34 or even as larger forms and the sudden release of stored gastrin in response to the stimulus of infection may prevent the completion of polypeptide splitting to the mature G-17. Elevated plasma gastrin occurs at the expense of G-cell stores, which are depleted, and it is likely that G-cell numbers are not changed, at least in the case of the lifespan of single experimental infections (Fox et al, 1993).

In cattle, hypergastrinaemia may well be caused solely by elevations of lumenal pH (Fox et al, 1987; Fox et al, 1993). However in studies of infections in sheep (Anderson et al, 1981) mild hypergastrinaemia occurred in advance of any elevation in pH. When pH was markedly elevated at the time of parasite emergence then plasma gastrin was further increased. This favoured, therefore, an alternative hypothesis; that parasites stimulated gastrin release themselves. Indeed gastrin rapidly returns to normal following anthelmintic treatment and worm removal (Anderson et al, 1981). Cellular changes in infected abomasa spread to surrounding glands earlier in sheep than in cattle. It could therefore be argued that the putative inhibitory factor for parietal cell activity (vide supra) was released earlier in sheep and initially exerted incomplete control over parietal cells, hence early increases in circulating gastrin were able to maintain the acidity of the abomasum. This assumes that the stimulus for enhanced gastrin release is subtle and that initial reductions of parietal cell activity do not translate as significant or detectable increases in abomasal pH. Eventually inhibition of

parietal cells becomes complete, or as in cattle the onset of inhibition is rapid and immediately total, so that abomasal pH can no longer be controlled and hypergastrinaemia becomes massive. As yet there is insufficient evidence to confirm either hypothesis.

Plasma gastrin became elevated within 24 hours of the direct transplantation of larval and adult stages of *O. circumcincta* into the abomasa of previously naive sheep (similar changes were observed with transplant of mainly adult populations)(Anderson et al, 1985). Elevation of plasma pepsinogen and reduced feed intake were associated with hypergastrinaemia. Reduced feed intake has also been seen with experimentally induced hypergastrinaemia consequent to parietal cell inhibition with omeprazole (Fox et al, 1989b). There may therefore be a close link between hypergastrinaemia and the anorexia of parasitic gastritis with *Ostertagia* spp. in ruminants.

The mechanisms of hypergastrinaemia in cattle and sheep in response to infection with *Ostertagia* spp., whilst superficially similar, may still prove to be different.

1.5.4: Plasma pepsinogen.

The mechanism of elevation of plasma pepsinogen concentration has been hotly debated. The "leak" theory expounded initially, whilst attractive, could not account for elevated blood zymogen at all times. Holmes and Maclean (1971) demonstrated plasma loss as a transient feature, but elevation of plasma pepsinogen was prolonged. In experiments where epithelial permeability was assessed using a marker of similar molecular weight to pepsinogen - Horseradish peroxidase - permeability was shown to be similar in infected calves and in uninfected controls (Stringfellow and Madden, 1979). Given that, after 26 days of infection, plasma pepsinogen in these animals was raised, that chief cells were denuded of granules and that abomasal fluid had no peptic activity, these workers argued that pepsinogen secretion had occurred directly into the plasma and that the half-life of pepsinogen in plasma was long. Plasma pepsinogen declined rapidly in sheep infected with *O. circumcincta* following anthelmintic treatment (Anderson et al, 1981), and in calves with patent infections of *O. ostertagi*, derived by larval inoculation or by transplantation of adults, plasma pepsinogen was elevated, but declined rapidly following antimuscarinic therapy with atropine, reaching lowest levels within four hours of treatment (Mostofa and McKellar, 1989). Where plasma pepsinogen had been elevated by the experimental hypergastrinaemia induced by repeat

treatments with omeprazole (Fox et al, 1989b), plasma pepsinogen concentrations declined to approximate those of placebo treated animals within days of the cessation of omeprazole treatments. Thus the half-life of pepsinogen appears to be relatively short and hypergastrinaemia itself may result in elevated plasma pepsinogen concentration, presumably without affecting abomasal permeability. Gastrin is a known stimulant of pepsinogen secretion by chief cells in-vitro and in-vivo (Hersey, 1987). Hypergastrinaemia may therefore contribute to the elevated plasma pepsinogen concentrations associated with ostertagiasis, but this can only be of significance if a sufficient cell population is synthesising the zymogen and is still responsive.

Estimations of abomasal peptic activity have indicated reduced output (Ross and Todd, 1965). In calves fitted with abomasal cannulae and infected with *O. ostertagi*, abomasal peptic activities were maintained (Jennings et al, 1966), although levels varied widely. The dry matter content of abomasal contents has been shown to be reduced from 7 to 8 per cent to less than 3 per cent in sheep infected with *O. circumcincta* (Dakkak and Khallaayoune, 1984). Fluid loss or secretion into the abomasum may result in lower enzyme concentrations. Interpretation of lumenal peptic activities should therefore be made with caution; the stability of pepsinogen/pepsin in abomasal fluid has not been adequately researched and other factors such as the total volume of all secretions must be taken into account.

Elevated plasma pepsinogen in sheep in response to transplant of mixed parasite populations (Anderson et al, 1985) and in calves transplanted with adult parasites (McKellar et al, 1986; McKellar et al, 1987) could be interpreted as a consequence of direct or indirect stimulation of chief cells by parasites. A parasite pro-secretory factor was associated with excretory/secretory (ES) products of both *O. ostertagi* and *O. circumcincta* and stimulated pepsinogen release from dispersed bovine and ovine abomasal glands (McKellar et al, 1990b).

Plasma pepsinogen is thus potentially influenced by many factors. A tripartite mechanism of enhanced permeability, which may only be a transient feature, stimulation of remaining chief cells by host factors, such as gastrin, and stimulation of the same cells by parasites, could best explain the wide range of responses seen.

1.5.6: Gastrointestinal motility and the onset of diarrhoea.

There is little information on changes of gastrointestinal motility in infections with *Ostertagia* spp. The rate of passage of digesta of calves infected with *O. ostertagi* parasites was reduced and part of this reduction could be explained by the reduced feed intake of these animals (Fox et al, 1989a). In sheep infected with *T. axei* and *Chabertia ovina* (Bueno et al, 1975) abomasal emptying rates were reduced within 4 to 6 hours after infection. Diarrhoea occurred after about 23 days, but the major changes in motility actually preceded it. The electrical activity most responsible for propulsion in ruminant gut - the Migrating Myoelectric Complex (MMC)(Gregory, 1985) - was severely disrupted and reticular and abomasal activity also declined. After recovery from diarrhoea MMC's returned to normal. In studies of sheep infected with *H. contortus* (Bueno et al, 1982a), infected animals did not develop diarrhoea, but the water content of faeces did increase. The duration of the abomasal antral motor cycle was reduced by infection, the frequency of duodenal MMC's was increased and the rate of duodenal flow was also increased. It was suggested that reduced duodenal acidification, associated with a less acid abomasal outflow in infected animals, may have reduced inhibition of duodenal activity. Motor disturbances were recorded within 4 hours of an intra-venous injection of a larval extract of *H. contortus* (Bueno et al, 1982b), and this suggests a direct chemical mechanism of stimulation of muscle activity by parasites.

Many parasites, including *O. circumcincta*, secrete acetylcholinesterase (Ogilvie et al, 1973; Douch et al, 1988; Oppermann and Chang, 1992). Acetylcholinesterase could potentially modulate intestinal muscle activity by destroying acetylcholine - the biological holdfast. However *Ostertagia* spp. do not secrete as much enzyme as other nematodes such as *Trichostrongylus* spp. (McKeand, J., personal communication). Recent work favours the hypothesis that secretion of acetylcholinesterase has important anti-inflammatory, and immune-modulatory effects (vide infra).

Increased abomasal pH in calves infected with *O. ostertagi* was associated with the onset and severity of diarrhoea (Jennings et al, 1966). Presumably the bacteriostatic effect of the mucosa was lost in these animals since increased numbers of viable rumen microflora were detectable in the abomasa. The potential consequences of bacteria and protozoa surviving the abomasum has not been adequately researched.

In diarrhoeic animals water turnover can be increased (Holmes and Bremner, 1971). Surprisingly, in some studies in *O. ostertagi* infected calves there were increases in both faecal and urinary water losses (Parkins et al, 1982). However in *Cooperia pectinata* infected calves urine losses were considerably reduced and total water loss was less in infected animals when compared to controls (Bremner, 1982). Water intake and retention are often increased in parasitised cattle and sheep, the latter arising due to increased tissue water and expanded plasma volume (Parkins et al, 1982; Entrocasso et al, 1986 b).

At present our knowledge of the likely causes of diarrhoea in parasitised ruminants is speculative and poor in general, perhaps more so than for any other aspect of the pathophysiology of trichostrongyle infections.

1.6: Immunity to ostertagiasis and the acquisition of host resistance.

Acquired, immune-mediated resistance to ostertagiasis develops slowly, and generally is most evident after the second grazing period under natural conditions (Holtenius et al, 1983; Entrocasso et al, 1986a). This immunity is usually not sterile. Single monospecific experimental infections of calves with *O. ostertagi* did not protect against subsequent challenge (Herlich and Tromba, 1982); only after sustained or repeated infection can effects attributable to acquired resistance be detected (Michel et al, 1973; Smith et al, 1983; Klesius, 1988).

In contrast to many viral and bacterial infections, where B cell memory may be sufficient for the provision of sterile immunity, responses to protozoal and helminth pathogens involve a complex T cell response (Reiner, 1994). Present understanding of T cell responses involves the Th1/Th2 dichotomy. Naive T helper cell precursors are stimulated via cytokines to develop either phenotype in response to input from a variety of other cells (e.g. T and B cells, macrophages, mast cells and eosinophils). Such activation can predict the efficacy of subsequent immune responses. Th1 responses, associated with Interferon-γ expression, confer greater resistance to protozoal infection, whereas Th2 responses are linked to helminth expulsion via an effector arm linked by Interleukins 4, 5 and 9. The resistance or susceptibility of certain strains of mice to *Trichuris muris* infection, has been shown to be due to whether responses are driven primarily by Th1 (susceptible) or Th2 (resistant) pathways (Else et al,

1992). Thus the quality of T cell recall may be just as important as other factors, such as the immunogenicity of a particular protein.

Manifestations of acquired immunity to many trichostrongyloses include; reduced magnitudes of pathological change and reduced pathophysiological reactions, lower worm burdens, decreased egg output, shorter adult worms and the absence of vulval flaps in female worms, implying impaired worm development (Klesius, 1988, 1993). Reduced faecal egg counts may be achieved in some instances, not by reduced worm burden, but by the reduced fecundity of individual female worms (Wallace et al, 1995). Effects may be manifest therefore as interference in worm development rather than worm expulsion.

Inflammatory cells are important in ostertagiasis, and reactions are characterised by the infiltration of numerous eosinophils into the mucosa and circulating eosinophilia (Ross and Dow, 1964; Ritchie et al, 1966; Wiggin and Gibbs, 1987; Snider et al, 1988). Other infiltrating cell types include lymphocytes, plasma cells, mast cells and globule leukocytes. Increases in the weights of local lymph nodes also occur with increased mitotic activity in germinal centres (Curtain and Anderson, 1971; Gasbarre, 1986; Wiggin and Gibbs, 1990).

Recent work has shown that of sheep infected with *O. circumcincta*, those that had higher mast cell numbers also had higher globule leukocyte and eosinophil numbers and had higher numbers of plasma cells and greater parasite-specific antibody responses (Stear et al, 1995b). The same workers argued that resistance to *O. circumcincta*, was due at least in part to local mucosal antibody responses, which regulate individual worm development and fecundity, and to local immediate hypersensitivty responses which regulate worm burdens. This resistance develops in three phases. During the first phase animals are susceptible and host effector mechanisms are developing. In the second phase antibody-mediated responses are manifest and suppress egg production. The generation of globule leukocytes, and of immediate hypersensitivity, is more prolonged and either prevents the establishment of incoming larvae, or expels established worms. The third phase is therefore associated with lower worm burdens and continued reduction of the fecundity of worms that do establish.

1.6.1: Antibody production.

Studies of antibody in infected animals show that antibody responses develop slowly and to a variety of somatic and metabolic (ES) antigens from different parasitic stages (reviewed by Klesius, 1993). The greatest antibody responses accompany the transition of L_4 to adults (Kloosterman et al, 1984; Entrocasso et al, 1986a). Studies have shown increases in IgG1, IgM and IgA (Jensen and Nansen, 1978, Smith et al, 1983, 1984; Canals and Gasbarre, 1990). Different studies have differed in the predominant antibody detected, but IgG2 levels are usually low. Canals and Gasbarre (1990) showed a peak in IgG1 after 35 days of a primary infection and an anamnestic response to a challenge infection. The kinetics of an IgA response were similar, but lower levels were recorded. Jensen and Nansen (1978) showed that antibody levels of a number of subclasses were correlated with worm burdens.

As a cautionary note plasma IgA responses may not correlate well with local mucosal IgA responses (Sinski et al, 1995) and an association has been observed between the numbers of fourth-larval stages of *O. circumcincta* and the local IgA and IgG1 responses in the mucosae of infected sheep (Stear et al, 1995b). Smith and coworkers (1995) showed that worm length was associated with peak IgA responses in gastric lymph and sheep with higher IgA responses had shorter worms.

Immunoglobulin E is recognised as an important isotype elevated by helminth parasites. Levels of IgE paralleled worm burden in lightly infected calves but in heavily afflicted animals IgE was inversely proportional to burden (Thatcher et al, 1989; Baker and Gershwin, 1993). Baker and Gershwin (1993) suggested that lowered IgE may be due to binding of IgE to mast cells and subsequent mast cell turnover. One function of IgE is the initiation of immediate hypersensitivity reactions (anaphylaxis) by binding to mast cells (Ward and Wagland, 1990).

Ostertagia specific antibody present in calf sera prior to turnout, when exposure to parasites has not occurred, may be ascribable to maternally-derived antibody, but clearly is not protective (Entrocasso et al, 1986a; Gasbarre et al, 1993).

1.6.2: Cellular immunity.

Eosinophils may act as immune effector cells in responses to helminth parasitism (Rothwell and Dineen, 1972; Dawkins et al, 1989). Grove et al, (1977) showed antibody and complement dependant killing of *Trichinella spiralis* larvae. Klesius (1993) refers to studies contained in the thesis of Washburn (1984), who was said to have demonstrated binding of eosinophils to *O. ostertagi* larvae, but their consequent actions, and potential for parasite damage, were unknown. There is some doubt as to whether eosinophils have direct effector activity against *Ostertagia* spp. (Huntley et al, 1995).

Hypersensitivity is a likely component of the cellular response to Type I ostertagiasis (Snider et al, 1985a; Wiggin and Gibbs, 1987, 1990; Cross et al, 1987; Klesius, 1988, 1993), and both eosinophils and mast cells may be involved. Both immediate (Cross et al, 1987) and delayed (Snider et al, 1985a) hypersensitivity reactions may occur. Eosinophils produce hydrogen peroxide, in a respiratory burst akin to that of neutrophils, and this as well as eosinophil major basic protein may account for the eosinophil as a potent effector of cell damage. Mast cells contain eosinophil chemotaxins and a variety of potent mediators of inflammation (Wasserman, 1979; Paul et al, 1993) and are considered to be the precursors of globule leukocytes (Huntley et al, 1984). Globule leukocytes are situated intraepithelially and have been associated with the acquisition of resistance to infection (Jarrett et al, 1967; Miller et al, 1983; McClure et al, 1992). These cells were also thought to be responsible for an anti-parasitic activity detected in the abomasal mucus layer (Douch et al, 1986).

1.6.3: Modulation of immune responses by *Ostertagia* spp. parasites.

There is a wealth of accumulated evidence suggesting that modulation of immune responses by parasites occurs in ostertagiasis (Klesius, 1993). Modulation is thought to occur in five areas:

1. Transient and non-specific suppression of lymphocyte reactivity. This suppression has been seen in both naturally and experimentally infected animals. Lymphocyte reactivities to larval (L_3) antigen and stimulation with phytohaemagglutinin or concanavalin-A have been seen to be suppressed (Klesius et al, 1984; Cross et al, 1986; Snider et al, 1986; Wiggin and

Gibbs, 1990). Suppression occurs in the pre-patent period, associated with the maturation of L_4 through to the adult stage (Klesius et al, 1984; Cross et al, 1986).

2. Suppression of antibody. Antibody responses in parasitised animals develop slowly. Soluble larval extracts reduced the antibody responses of mice to *O. ostertagi* antigen and to Keyhole limpet haemocyanin and sheep erythrocytes (Cross and Klesius, 1989).

3. Eosinophil chemotaxis. There are two potential mechanisms of eosinophil chemotaxis in infection. *Ostertagia* ES contains Eosinophil Chemotaxis Factor (ECF) - a lectin - which may be released by larvae into the abomasal tissues (Washburn and Klesius, 1984; Klesius et al, 1985, Klesius et al, 1989). Eosinophil chemotaxis also occurs after intradermal injection of ECF-lectin. Mast cell activation may also result in release of eosinophil chemotaxins (Marom and Casale, 1983). Both mechanisms may explain the accumulation of these immune cells around parasitised glands. Any advantage this may confer on the parasite is unknown.

4. Enhanced lymphocyte blastogenesis. Larval extracts stimulated blastogenesis in lymphocytes from parasite-naive calves (Cross et al, 1986; Wiggin and Gibbs, 1990) and abomasal lymph nodes from infected animals are larger and show increased mitoses compared to those from uninfected control animals (Curtain and Anderson, 1971; Gasbarre, 1986; Wiggin and Gibbs, 1990).

5. The secretion of acetylcholinesterase may also be an important mechanism of immune-modulation for nematode parasites. This enzyme could prevent the cholinergic stimulation of leukocytes. This process is thought to be of greater importance for those parasites that establish long-lasting infections with little associated pathology (Pritchard, 1993), and may therefore be of little importance in ostertagiasis.

Gastrointestinal nematode parasites may initially occupy immunologically privileged sites in the host, separated from host immune cells by epithelial barriers. Immune responses may rely therefore on diffusion/uptake of ES products or upon inflammation and tissue damage. Immune modulation by soluble factors released by parasites may form the first interactions between the host and parasite. Tissue and parasite damage due to hypersensitivity reactions, with associated release of parasite antigens, and the eventual development of antibody to

immunomodulatory factors may enable more effective responses to parasites to develop that are capable of expelling the parasite, or curtailing its development/activity.

1.6.4: Hypersensitivity reactions.

Following challenge exposure of previously exposed animals, that have some degree of acquired immunity, certain pathophysiological reactions have been demonstrated. Amongst these is the rapid elevation of plasma pepsinogen, seen in immune grazing sheep (Anderson, 1973), in immune dairy cows (Armour et al, 1979) and in immune sheep challenged after housing (Yakoob et al, 1983). Yakoob et al (1983) demonstrated concomitant elevation of plasma pepsinogen concentration and mucosal permeability, the latter evinced by albumin loss into the gut. Hypersensitivity responses are thought to be the likely cause, although treatment with the mast cell stabilising drug, sodium cromoglycate did not affect the magnitude of the pepsinogen response in immune ewes challenged with *O. circumcincta* (McKellar and Bogan, 1987). Hertzberg et al (1995) demonstrated rapid increases in both plasma pepsinogen and plasma gastrin in animals previously exposed to *Ostertagia leptospicularis* and given challenge infections, although abomasal pH was not measured. Hypersensitivity reactions were seen as the likely cause.

A syndrome of oedematous ostertagiasis has been described in two to three year old cattle (Raynaud and Bouchet, 1976), where the abomasal folds were grossly oedematous at necropsy, it is likely that this syndrome is a consequence of the larval challenge of immune animals.

The continual triggering of hypersensitivity responses in immune grazing animals, ingesting larvae may have detrimental effects on production (Anderson, 1973).

1.7: Comparative pathology.

In man three syndromes of hyperplastic gastropathy are recognised (Komorowski and Caya, 1991); all are relatively rare. The syndromes have been classified on the basis of either gastric acid hypersecretion or hyposecretion, the existence of normal gastrin levels or of hypergastrinaemia, and the pathological changes seen. The gastric glandular mucosa comprises two parts: the pit (foveolus) and the gland itself (containing parietal, chief and

mucous neck cells), and relative structural changes in the pit and gland characterise each syndrome. Each of the three syndromes represent morphologically unique hyperplastic gastropathies, accompanied by specific clinical and biochemical features:

1. Zollinger-Ellison (Z-E) syndrome arises due to neoplasia of gastrin-producing G-cells usually of the pancreas (McGuigan, 1983). Zollinger-Ellison syndrome is characterised as a hyperplastic, hypersecretory gastropathy with recurrent/intractable ulcer disease. Acid hypersecretion is secondary to hypergastrinaemia. Glandular hyperplasia and parietal cell hyperplasia occur in the face of a normal foveolar compartment, and glandular changes are entirely consistent with those seen in experimentally induced hypergastrinaemias (Waldum et al, 1991).

2. Hyperplastic, hypersecretory gastropathy (HHG), without hypergastrinaemia. A gastropathy of unknown aetiology that is clinically and pathologically similar to Z-E syndrome, but gastrin secretion is normal.

3. Hyperplastic, hyposecretory gastropathy (Menetrier's Disease). Giant thickening of the mucosal folds is achieved by massive foveolar hyperplasia and gland atrophy. There is acid hyposecretion, even achlorhydria, and reduced acid secretory responses to histamine/pentagastrin stimulation. The hypergastrinaemia accompanying Menetrier's disease occurs secondarily to elevated gastric pH. Consequent to the presence of enlarged pits there is often mucus hypersecretion. Menetrier's disease is seen in three distinct forms. Menetrier's disease accompanies gastric cytomegalovirus infections in children (Coad and Shah, 1986; Oderda et al, 1990; Occena et al, 1993). In adults it is generally idiopathic, "a gastropathy associated with massive foveolar hyperplasia but minimal inflammation" (Wolfsen et al, 1993), but a distinct form does exist that is associated with an underlying lymphocytic gastritis (Haot et al, 1991; Wolfsen et al, 1993). The cellular and biochemical features of Menetrier's disease are very similar to those seen in ostertagiasis, especially Type II disease (Snider et al, 1983). Diarrhoea is not a consistent feature of Menetrier's disease although features such as reduced acid secretion are common to both Menetrier's and ostertagiasis. Rather the principal clinical signs of Menetrier's disease are weight loss, epigastric pain and vomiting. It is often difficult to distinguish the three human hyperplastic gastropathies on clinical signs alone (Komorowski and Caya, 1991), and enlarged mucosal folds per se are generally asymptomatic.

All hyperplastic gastropathies may be accompanied by loss of plasma and cellular protein, as well as blood, into the gastrointestinal tract. Plasma protein loss is thought to occur in an identical manner as for ostertagiasis, i.e. through the widened tight junctions between hyperplastic epithelial cells (Kelly et al, 1982).

Recent work has implicated overproduction of the peptide growth factor Transforming Growth Factor-alpha (TGF-α) in the development of Menetrier's disease (Dempsey et al, 1994). Transforming Growth Factor-α has potent mitogenic effects for gastric epithelial cells, affects end-stage differentiation of gastric epithelial cells, inhibits acid production and enhances mucus secretion (Rhodes et al, 1986; Derynck, 1988; Dempsey et al, 1994, Bockman et al, 1995; Lee et al, 1995).

Foveolar hyperplasia and gland atrophy are frequently seen in gastric responses to a wide variety of insults (Barker et al, 1993c). Chronic trauma due to foreign bodies may be the triggering stimulus, as may some chemical irritants (Lushbaugh, 1947). Foveolar hyperplasia can often be found at the margins of ulcers (Barker et al, 1993c). Chronic abomasal involvement in mucosal disease or herpes rhinotracheitis, which are both viral diseases of cattle, (and some other viruses) can result in similar pathological changes. An autosomal recessive gene may be responsible for the hyperplastic changes seen in an in-bred mouse strain (Stewart and Andervont, 1938; Andervont, 1939a,b; Stewart, 1941). Intracellular, *Campylobacter*-like organisms were associated with this kind of pathology in a Beagle dog (Leblanc et al, 1993), but no direct cause could be demonstrated for the condition arising in Basenji dogs (Van Kruiningen, 1977) or in occasional case reports in Boxers and Bull Terriers (van der Gaag et al, 1974, 1976).

The commonest, specific causes of pathological change leading to foveolar hyperplasia and gland atrophy are the helminth parasites of either the stomach/abomasum itself or even those of other tissues. Hyperplastic lesions similar to those seen in ostertagiasis have been associated with many nematode infections including: *T. axei* infections of cattle (Ross et al, 1967; Ross et al, 1968; Snider et al, 1985b) and horses (Leland et al, 1961), *H. rubidus* in pigs (Stockdale, 1974), habronemiasis in horses (Nair and Damodaran, 1969), and the massive hyperplasia resulting in large adenomatous plaques in the stomachs of primates caused by the nematode *N. nochti* (Bonne and Sandground, 1939). Following intestinal infection of sheep (and minimal abomasal involvement) with *T. colubriformis* (Barker and Titchen, 1982)

abomasal acid secretion was reduced and there were mild signs of hyperplasia of primarily mucus producing cells. Ultrastructural features of inactivity were seen in parietal cells, which were also less prominent in light microscopical study. Infection with *T. colubriformis* is not the only non-gastric helminthosis to result in hyperplastic change. Development of the metacestode *Taenia taeniaformis* in the livers of rats resulted in massive hyperplasia in the stomach and small intestine (Cook and Williams, 1981). Stomach changes closely resembled Menetrier's disease and hypergastrinaemia developed as a consequence of the loss of parietal cells and acid secretory activity (Cook et al, 1981). Hyperplasia also occurred in the uninfected partners of parabiotic pairs (surgically prepared rat pairs sharing blood supply). In antrectomized rats hyperplasia still developed, but hypergastrinaemia did not.

Gastric mucosal hyperplasia (specifically foveolar hyperplasia and gland atrophy) develops as a consequence of usually chronic insult. The commonality of cell changes in response to a variety of helminth parasites, viruses, chemical irritants and genetic factors suggests that hyperplasia is primarily a host response, although the possibility exists that parasites might seek to initiate, or enhance such a response, ostensibly to improve their survival in the gastric environment. Transforming Growth Factor-α has been identified as a possible mediator of this common pathway, but the precise stimuli for its increased expression, or enhanced effects, are largely unknown.

1.8: The excretory/secretory system of nematodes.

Nematodes possess what has been termed a Secretory-Excretory (S-E) system (Bird and Bird, 1991). The Secernentean S-E system, although differing widely between different taxonomic groups, generally comprises an H-shaped tubular canal system with the two lateral canals running in the lateral cords. The S-E cell nucleus is commonly associated with the connecting transverse canal and the lateral canals are extensions of this cell. There is also an A-shaped bi-nucleate S-E gland cell, the nuclei situated in opposing arms. The lateral canals and the S-E gland cell may connect at a sinus or ampulla, from here a duct leads to the exterior, and is formed by a duct cell and a pore cell. The S-E pore generally opens in an anterior, mid-ventral position on the worm. The S-E systems of *Caenorhabditis elegans* and *H. contortus* are quite similar and the accessory S-E gland cells are packed with electron-dense granules (Nelson et al, 1983; Wharton and Sommerville, 1984). The ampulla and S-E cells are thought to be important for osmoregulation), since worms quickly fill with fluid following laser ablation of

the ampulla, S-E cell, or duct and pore cells, but not following ablation of the gland cell (Bird and Bird, 1991).

Lee (1970) demonstrated histochemically the presence of various enzymes, esterases, acetylcholinesterases and aminopeptidases, in the gland cell. When *T. colubriformis* worms were placed on substrate gels protease activity was demonstrable, but restricted to the area around the S-E pore (McLaren et al, 1974). S-E gland activity may therefore have a role in feeding. The gland cell in *H. contortus* decreased in volume in response to CO_2 stimulation, and might therefore be involved in exsheathment (Davey and Sommerville, 1982). S-E gland function may also include the secretion of protective or lubricating glycoproteins that may bind to the nematode cuticle (Bird and Bird, 1991). Any substance released in vitro or in vivo from parasites could be from a number of sources; the S-E system itself, the mouth, anus, reproductive organs, or a number of other structures with external communication. The term Excretory/Secretory (ES) products for in vitro released substances is therefore somewhat misleading. However there is some evidence to suggest that products are released by parasites to exert effects externally. The nervous co-ordination and control of the S-E system of nematodes is poorly understood. The precise spectrum of secretions, their chemical natures and functions, is unknown. The control of secretion may be vastly different in vivo, in the natural worm habitat, than in in vitro worm cultures.

1.9: Study objectives.

The studies presented in this thesis were intended to cover four main areas;

1. An evaluation of four different methods for assay of plasma pepsinogen concentration, ostensibly to find a method suitable for use in the subsequent studies reported in this thesis.

2. The effects of Excretory/secretory (ES) products of *O. circumcincta* on pepsinogen secretion and gastrointestinal smooth muscle activity were assessed. In vitro methods were used in the study of substances present in ES capable of stimulating pepsinogen secretion and gastrointestinal smooth muscle activity. Responses were compared in parasite-naive and previously exposed animals to assess whether they were the result of hypersensitivity to antigens present in ES.

3. The fate of cells producing pepsinogen in the abomasa of ruminant animals with abomasal nematode infection was determined. Immunohistochemical techniques for the localisation of pepsinogen in bovine and ovine mucosae were used to compare the staining patterns in normal, parasite-naive animals and those with ostertagiasis and haemonchosis. Changes observed in the distribution of pepsinogen were then compared to the cellular changes observed in conventionally stained histological sections.

4. The role of Transforming Growth Factor-alpha in the pathogenesis of ostertagiasis was investigated. The potential involvement of TGF-α in the development of hyperplasia was investigated initially by an examination of the distribution of the growth factor in normal and parasite-affected mucosae. These studies utilised immunohistochemical techniques to compare the distributions of both TGF-α and the related peptide Epidermal Growth Factor in the same tissues.

2: GENERAL MATERIALS AND METHODS.

2.1: Chemicals.

All chemicals used in the studies reported in this thesis were obtained from Sigma Chemical Co. (Poole, England), unless otherwise stated.

2.2: The estimation of the pepsinogen concentrations of plasma and other samples.

Pepsinogen concentrations were assayed using a modification of the method of Paynter (1992) which will be discussed fully in Section 3.2.

2.3: The measurement of tissue pepsinogen concentration.

Tissue pepsinogen concentrations were estimated by measuring the total peptic activity of tissue homogenates. Basson et al (1990) extracted pepsinogen from intact guinea pig gastric mucosa by homogenisation and freeze thawing in 0.1 per cent Triton X-100, a non-ionic surfactant. A similar method was used to extract pepsinogen from ovine abomasal mucosa before assay of pepsinogen concentration by the modified method of Paynter (1992).

Small sections of abomasal mucosa were cut using a cylindrical punch, thus all sections were of identical area. Tissues obtained in this way were generally less than 0.2 g in weight of wet tissue. The tissues were gently washed clean of gastric contents and blotted dry and the wet weight of individual tissues was recorded. The tissues were then thoroughly homogenised in up to 10 ml of detergent solution. The detergent solution was composed of 0.1 per cent Triton X-100 (BDH Chemicals, Poole, England), in 0.01 M Hydrochloric acid, pH 2.0. Pepsinogen concentration was then determined in the supernatant produced after centrifugation (2,000 *g* for 30 mins). This method was not assessed for its reliability, precision or accuracy, but an assessment of the short term stability of peptic activity in solution, using this technique, was made. Five abomasal mucosal pieces (of identical area) from one sheep were homogenised separately and the individual homogenates were then incubated at 37°C in a water bath for 2 hours. Aliquots of each homogenate were removed at 0, 30 mins, and at 1 and 2 hours and the

peptic activity measured following centrifugation. The results in terms of total tissue pepsinogen per mucosal cut at each time point and the mean values of all five tissues are displayed in Table 2.1.

The results were analysed by one-way analysis of variance, to test for the effect of time, and this was not found to be significant ($p=0.846$) although the mean values did increase slightly with time. These preparations were therefore considered adequately stable over two hours and since the degree of homogenisation was equivalent for all tissues the method was considered reliable.

Table 2.1: Total pepsinogen content (iU) of homogenates of individual mucosal pieces over time.

Time (mins)	Tissue 1	2	3	4	5	mean±S.E.
0	2937.86	2297.87	2268.80	2966.93	4683.06	**3030.92** ±439.35
30	4201.80	1816.94	2697.12	3151.30	3577.18	**3088.84** ±403.27
60	3922.15	2228.46	3298.19	2941.61	4100.44	**3298.19** ±339.45
120	4309.57	2304.39	2992.79	3770.83	3980.40	**3471.62** ±363.47

2.4: Assay of Lactate Dehydrogenase.

Lactate dehydrogenase (LDH) concentration in the mucosal bathing fluid of intact abomasal mucosae, maintained in vitro, was measured using a commercial assay kit (Sigma Diagnostics, catalogue no. 288-10, procedure no.288-UV). The kit was based on the interconversion of lactate and pyruvate, a reaction catalysed by Lactate dehydrogenase:

$$\text{Lactate} + \text{NAD} \Leftrightarrow \text{Pyruvate} + \text{NADH} + H^+$$

The oxidation of lactate to pyruvate is accompanied by the reduction of Nicotinamide adenine dinucleotide (NAD) and the formation of NADH results in an increase in absorbance at 340 nm and this increase is directly proportional to the LDH concentration.

The test reagent contained 50 mM Lactate and 7 mM NAD, buffered to pH 8.9. The reagent was warmed to 30°C and 1.0 ml was pipetted into a clean cuvet before the addition of 50 µl of the sample to be assayed. The contents of the cuvet were then mixed by inversion and then placed into the cuvet compartment (warmed to 30°C) of the spectrophotometer (Pye-Unicam Sp8-500). The absorbance at 340 nm was read after 30 seconds and then again after a further minute. The initial value was subtracted from the final value to give the change in absorbance in one minute. Results were expressed as U/L, given that one unit of LDH activity would catalyse the formation of one micromole of NADH per minute under assay conditions.

2.5: Assay of total protein concentration.

The determination of protein concentrations was carried out using a modification (Maizels et al, 1991) of the method of Bradford (1976). The presence of phenol red in the incubation medium used for parasite culture in the present studies was incompatible with the use of other methods of protein estimation (such as those using Bicinchoninic acid or Folin and Ciocalteu's reagent). Bradford's method utilises the binding of dye (Coomassie brilliant blue) to protein and the concomitant change in dye peak absorption from 465 nm to 595 nm.

Bradford's reagent was made by dissolving 100 mg of Coomassie brilliant blue in 50 ml of 95 per cent ethanol, and this was further dissolved in 100 ml of 30 per cent perchloric acid and then made up to 1 litre with distilled water. Final reagent composition was therefore as follows; 0.01 per cent Coomassie brilliant blue, 4.7 per cent ethanol and 3 per cent perchloric acid.

A 1 mg/ml bovine serum albumin (fraction V) solution was prepared with distilled water, which was then diluted to provide standards in the range 0 µg/ml to 1 mg/ml. Five protein standards (0, 1, 10, 100 µg/ml and 1 mg/ml) were treated in a manner identical to the

unknown samples. One hundred microlitres of sample/standard was added to 0.9 ml of Bradford's reagent and the mixture was then vortexed. Optical densities were measured after 5 minutes at 595 nm using a Pye-Unicam Sp8-500 spectrophotometer and using 1 ml plastic cuvets. Plastic cuvets were not supposed to bind die (Maizels et al, 1991), but some degree of die retention was noted so that cuvets were rinsed with methanol and then water after each reading. Optical densities from the standards were used to construct a standard line from which the protein concentration (in μg/ml BSA equivalents) could be calculated.

2.6: Faecal egg counts.

Except where otherwise stated faecal egg counts were measured by a modification of the McMaster method (MAFF/ADAS, 1984b). Faecal samples were collected directly from the rectum of each animal, and counts were done that same day. Two grams of faeces was placed in a plastic 50 ml tube and water was added to make a total volume of 25 ml. The tubes were then shaken vigorously. Twenty five millilitres of saturated salt solution was then added and shaken, and the mixture was then passed through a 250 μm mesh screen (Endecotts Ltd., London, England). The strained fluid was kept, whereas the debris materials were discarded. The filtrate was shaken and sufficient volume was withdrawn using a Pasteur pipette to fill one chamber of a McMaster egg counting slide (Gelman Hawksley Ltd., Northampton, England). A second volume was then withdrawn to fill the second chamber. In the half saturated salt solution strongyle eggs floated to occupy a position just below the coverslip, debris sinking. By focusing a dissecting microscope just below the coverslip only eggs were in focus. The total number of eggs counted in the two squares of the McMaster slide represented the eggs present in 0.3 ml of filtrate (2 × 0.15 cm × 1.0 cm × 1.0 cm). This figure multiplied by 83.33 gave the faecal egg count per gram of faeces.

In many instances a faecal egg count was done to confirm the absence of eggs (as in parasite-naive animals) to facilitate this the entire area of the McMaster slide was examined.

2.7: The preparation of *Ostertagia circumcincta* ES products.

Parasite naive and, in some cases, previously exposed sheep, of predominantly the Scottish Blackface breed, and generally 6 to 9 months old, were infected with *O. circumcincta* L_3 to allow the harvest of adult worm populations. Worms were also collected from sheep with

naturally acquired, patent infections of predominantly *O. circumcincta.* These sheep had grazed pasture in south-west Strathclyde, where nematode infections have been shown to be predominantly of *O. circumcincta* (Stear et al, 1995a).

Experimental infection of sheep was accomplished by the oral administration of infective *O. circumcincta* L_3 maintained in an aqueous suspension (usually 15,000-25,000 larvae per sheep). Donor sheep were killed at approximately 21 days post-infection when faecal egg counts confirmed the patency of infection and thus the presence of adult parasites in the abomasal lumen. At necropsy abomasa were collected and transported back to the laboratory. The abomasa were then opened along the greater curvature and the loose contents emptied into a 2 gallon container, many parasites were seen closely applied to the mucosal surface at this stage, so abomasa were thoroughly rinsed with sterile Phosphate-buffered Saline (PBS, pH 7.4) and if necessary worms still stuck to the mucosa were picked off individually. The gut contents and saline were then passed through a bed of several gauze sheets, which were then folded around the remaining solid contents and tied off. The gauze bags were then immersed in warm (37°C) PBS and left for two hours until the majority of worms had migrated free of the bags.

The worms were washed several times in final RPMI-1640 culture medium (Gibco BRL) supplemented with 1 per cent Glucose, 500 U/ml Penicillin and 500 μg/ml Streptomycin - replaced by 10 μg/ml Gentamicin in later incubations - and 0.001 per cent Di-thiothreitol (an anti-oxidant). Worms were incubated in 20 ml of final culture medium, in sterile containers at 37°C. The presence of antibiotics was to reduce contamination of ES with products of bacterial metabolism and help prevent any subsequent bacterial overgrowth of the cultures. Preparations were monitored regularly for the motility of parasites, as an indication of viability, and were successfully incubated for 18 to 24 hours at which time the culture fluid was collected. If motility was still good the worms were then incubated for a further period in new medium. Collected medium was passed through a 0.22 μm, low protein binding filter (Millex - GV, 0.22μm, Millipore S.A., Molsheim, France) to eliminate any contaminating organisms. Total protein concentrations of ES batches were determined by the method quoted in section 2.5. Batches of ES were stored at -20°C, or in some instances -70°C. Worms were fixed in 20 millilitres of Brunnel's fixative, at the end of the incubations and worm numbers were estimated by counting the total number of worms present in five 1.0 ml aliquots. Each aliquot was added to a petri-dish (9 cm in diameter) which had been scored by equidistant

vertical and horizontal lines, dividing the base into 16 non-equal areas, and the number of worms was then calculated from the total worm numbers in all areas.

2.8: Statistical analyses.

All statistical analyses reported in this thesis, unless otherwise stated, were carried out using the SAS system (SAS Institute, Cary, North Carolina, U.S.A.) or Minitab (Releases 8.2 and 10, Minitab Inc., State College, Pennsylvania, U.S.A.). Reported mean values were arithmetic means, unless otherwise stated, and probability (p) values of less than 0.05 were considered as significant. Probability values of between 0.05 and 0.10 were considered to be of borderline significance.

2.9: Photomicrography.

Light microscopical examinations and the photomicrography of histological specimens were accomplished using an Olympus AHBT Research Photomicrographic Microscope System (Olympus, Tokyo, Japan), unless stated otherwise.

EXPERIMENTAL STUDIES - PART 1: PEPSINOGEN ASSAYS

3: THE ASSAY OF PEPSINOGEN CONCENTRATION.

3.1 Introduction:

The measurement of plasma pepsinogen concentration has been recommended as an aid in the diagnosis of parasitic gastritis of ruminants since the technique was first applied by Anderson et al (1965) and Ritchie et al (1966) to cattle infected with *Ostertagia ostertagi*. A large number of methods for pepsinogen estimation have now been published and this probably reflects a degree of dissatisfaction with existing techniques since the assays are generally laborious and cumbersome (Berghen et al 1993). Plasma pepsinogen concentration is however still considered to be a valuable diagnostic parameter in abomasal nematode infection (Hilderson et al 1989).

The majority of assays estimate plasma pepsinogen concentration by measuring the total, acid-stable, proteolytic activity of plasma. Pepsinogen becomes converted to active pepsin at acid pH (1-4) and peptic digestion of protein substrates yields small peptide fragments, which can be measured spectrophotometrically by their ability to reduce complexed metal ions resulting in colour changes. Comparison is usually made to standards containing the phenolic amino acid tyrosine and results are then expressed as International Units (1 iU = 1μmol tyrosine released/min/litre of plasma). The results are influenced by variations in temperature, assay pH and incubation times, as well as by variations in substrate and enzyme concentration. The method first employed in veterinary studies of abomasal parasitism, that of Edwards et al (1960), utilised an albumin substrate and a 24 hour incubation. A modified method has recently been described which uses a glycine-buffered albumin substrate and requires only a three hour incubation period (Paynter, 1992). Simpler assay methods have also been developed based on the radial diffusion of pepsinogen into acidified and opaque substrate gels where peptic activity clears the gel. The dimensions of the clear zone are dependant on the original pepsinogen concentration and comparison has to be made to active pepsinogen standards (Thode-Jensen, 1977; Ishaque and Bardhan, 1978).

The following studies were conducted to determine the most appropriate assay method for routine use and included a limited assessment of the biochemistry of pepsinogen in different experimental situations, namely a comparison of the activities of pepsinogen dissolved in plasma and in entirely aqueous solution.

3.2: A comparison of four methods for the determination of plasma pepsinogen concentration.

In the present method development four methods were compared to determine the most appropriate for routine use. Three methods were based on the enzymatic digestion of an acidified substrate in solution and used tyrosine standards, whilst the fourth was a radial diffusion method in which peptic activity was measured in an agarose gel containing casein and compared to activated pepsinogen standards.

For each method an assessment was made of the precision (variability) of each method, based on the measurement of within-assay (intra-assay) variation, and the reproducibility of the methods, based on an examination of between-assay (inter-assay, or day-to-day) variation. In addition, the relationship between observed differences of pepsinogen concentration and actual differences of the quantities of pepsinogen present, was also examined.

3.2.1: Materials and methods.

3.2.1.1: Method 1.

This method was based on the method of Paynter (1992) and used glycine-buffered albumin as a substrate (3.2 per cent Bovine serum albumin (BSA), fraction V in 1 per cent glycine buffer, adjusted to pH 1.6 with 1M hydrochloric acid). Duplicate tubes containing 0.5 ml of plasma added to 0.75 ml of substrate were prepared for each sample. Both tubes were incubated at 37°C, one for thirty minutes, the other for an additional three hours (total incubation time 210 minutes). Undigested substrate was precipitated by the addition to each tube of 2.5 ml of 10 per cent (v/v) perchloric acid (BDH Chemicals, Poole, England). The undigested substrate was then separated with Whatman No. 50 filter papers (Maidstone, England). No controls were included to assess the rate of non-enzymatic hydrolysis of substrate, as this was assumed to be negligible in method 1 (Paynter, 1992).

A stock solution of L-tyrosine (10 mM) was prepared in 2 per cent (v/v) perchloric acid. From the stock solution five tyrosine standards were prepared at concentrations of 0.0, 0.9, 1.8, 3.6 and 5.4 millimolar, being diluted from the stock with 2 per cent perchlorate. Standards were added to substrate, instead of plasma, and were processed in an identical manner to samples, each standard tube was incubated for only thirty minutes before the precipitation of substrate. The tyrosine standards were then equivalent to plasma with pepsinogen concentrations of 0, 5, 10, 20 and 30 iU (Paynter, 1992).

The concentration of acid soluble peptide fragments in test solutions was estimated with a solution of bicinchoninic acid (BCA) and copper sulphate (50 parts of 10 per cent (w/v) BCA solution to 1 part of a 4 per cent (w/v) Cu(ii)SO_4-pentahydrate solution), 100 µl of filtrate was added to 2.0 ml of the colour reagent and incubated at 37°C for 30 minutes. The optical density was then measured with a Pye-Unicam sp8-500 spectrophotometer at 560 nm, blanked with distilled water. Optical density measurements were converted to iU using the standard curve prepared from the tyrosine standards.

Paynter (1992) had reported that reaction rates were only linear after the first thirty minutes of incubation, hence pepsinogen concentration in this method was estimated by subtracting the concentration of tyrosine-like substances present after thirty minutes of incubation from that present after 210 minutes.

3.2.1.2: Method 2.

This method was based on the method of Edwards et al (1960) and used 0.667 per cent bovine serum albumin, fraction V, at pH 1.5. As with method 1 duplicate tubes were required per sample, with 0.5 ml of sample added to 2.5 ml of substrate. One tube was not incubated, the substrate immediately precipitated with 5.0 ml of 4 per cent (w/v) trichloroacetic acid then separated using Whatman No. 44 filter paper and the filtrate was stored overnight at 4°C. The remaining tube was incubated at 37°C for 24 hours before the precipitation and filtration of undigested substrate.

This method required the incubation of substrate blanks (BSA-blanks) to assess the rate of non-enzymatic hydrolysis of substrate. Half a millilitre of distilled water, instead of plasma,

was therefore added to each of two tubes containing substrate, and one tube was immediately precipitated, whilst the other was incubated for twenty four hours.

The concentration of acid soluble peptide fragments was estimated using Folin and Ciocalteu's reagent (BDH Chemicals, Poole, England), 0.5 ml of filtrate was added to 4.0 ml of 0.25M sodium hydroxide and 0.75 ml of diluted colour reagent (Folin and Ciocalteu's reagent diluted 1:2 with distilled water) and incubated at room temperature for 30 minutes before the measurement of optical density at 725 nm. A reagent blank, which was 0.5 ml distilled water added to 4.0 ml NaOH and 0.75 ml colour reagent, was treated similarly. The optical density of the reagent blank was subsequently subtracted from all other spectrophotometer readings.

Comparison was made to standards containing 0.0, 0.1 and 0.2 mM L-tyrosine (diluted using distilled water from a 10mM stock solution originally prepared in 0.1M HCl). All other spectrophotometer readings were then converted into micromoles of tyrosine. Pepsinogen concentration was finally calculated by the following formula;

Pepsinogen concentration (iU) = [(A-B)-(C-D)] multiplied by 22.24

Where A was the amount of tyrosine equivalents (in μmols) released in the 24 hour incubated samples, B was the amount present in unincubated samples, C the amount in the incubated BSA-blank and D that of the unincubated BSA-blank. The figure of 22.24 was a correction factor to convert tyrosine released by the activity of 0.03125 ml of plasma in 24 hours to that which would be released by a litre of plasma in one minute (i.e. to international units).

3.2.1.3: Method 3.

This method was based on that published by MAFF and ADAS (1984a). A 2 per cent solution of horse haemoglobin (pH 1.7) was used as the substrate. Half a millilitre of plasma was added to each of two duplicate tubes containing 2.5 ml of haemoglobin substrate, but to one at the start of a three hour incubation at 37°C and to the other plasma was added at the end of the three hours, substrate having been incubated alone in this tube, and both tubes were precipitated with 2.5 ml of 10 per cent (w/v) trichloroacetic acid. Separation of precipitated substrate was achieved using Whatman No. 50 filter papers.

Folin and Ciocalteu's reagent was used in colour development and activity compared to standards with 0.0 and 0.138 millimolar tyrosine (equivalent to 0 and 50 μg of tyrosine per 2.0 ml). Two millilitres of filtrate/standard were added to 4.0 ml of 0.5M NaOH and 1.2 ml of Folin and Ciocalteu's reagent (again diluted 1:2 with distilled water). For a reagent blank 2.0 ml of water was substituted for the filtrate/standard. Incubation of the colour reaction was at room temperature for 20 minutes and the optical density (O.D.) was read at 560 nm. The first step in the calculation of pepsinogen concentration was the determination of the tyrosine (in μg) present in each 2.0 ml filtrate, T;

$$T = \frac{(\text{OD incubated} - \text{OD nonincubated})}{\text{OD standard}} \times 50$$

To convert T to iU;

iU = [T (μg) × Total incubation volume(5.5 ml)] divided by [plasma volume (0.5 ml) × filtrate volume (2.0 ml) × incubation time (180 mins)× MW tyrosine(181.2)]

3.2.1.4: Method 4.

This method was based on the radial diffusion of pepsinogen into an acidic and opaque substrate gel (Thode-Jensen, 1977; Mostofa, 1989a). Two grams of agarose (type 1-A) was dissolved by boiling in 100 ml of Tris[1] buffer (final concentration 0.05M TrisHCl, 0.107M Tris base, pH 8.2), the solution was then cooled to 50°C. Half a gram of casein was dissolved in a further 100 ml Tris buffer at 50°C and then the agarose and casein solutions were mixed and poured onto a 530 cm^2 microbiological assay plate (A/S NUNC, Kamstrup, Denmark) and allowed to cool. After the gels had solidified the plates were soaked in glycine buffer containing barium chloride (26 mM glycine, 25 mM sodium chloride, 60 mM barium chloride, pH 2.4) until the gels became uniformly opaque, after approximately three hours.

Wells were cut into the gel (7.72 mm diameter) and 0.5 ml of plasma or pepsinogen standard added, the plates were then incubated at 37°C for 24 hours. Peptic activity cleared the gel and the dimensions of the clear zone were proportional to the original pepsinogen concentration (see Fig. 3.1). Measurement of zone size was done with an electronic, digital calliper (Max-

[1] Tris - Tris[hydroxymethyl]aminomethane

Cal 0-6", CP. Instruments, Bishop Stortford, England) and recorded on a printer/calculator (QC-Printer, CP. Instruments). Pepsinogen standards were prepared by dissolving porcine pepsinogen in water at concentrations of 0.15, 0.3, 0.6, 1.2 ng/ml.

3.2.1.5: Samples.

Uninfected sheep were bled by jugular venepuncture into heparinised containers. After centrifugation at 2,000*g* for 10 minutes the plasma was pooled to form a stock with low intrinsic plasma pepsinogen concentration. The stock was divided into five aliquots and porcine pepsinogen was added to produce five spiked pools with 0, 0.15, 0.30, 0.60 and 0.90 ng/ml of added pepsinogen. These values were chosen to produce concentrations similar to those found in infected animals. The aliquots were then sub-divided and stored at -20°C.

3.2.1.6: Experimental design.

In experiment one, the five spiked pools were each tested five times with each of the four methods to give a total of 100 tests. The data were then analysed to compare the variability (precision) of the different tests. The data were also compared against the standards to determine if the relationships were linear. In experiment two, the five spiked pools were each tested with each of the four methods on four consecutive days to give a total of 80 tests. The data were then analysed to determine the extent of day-to-day variation in the different tests.

3.2.1.7: Statistical analyses.

Bartlett's test for the homogeneity of variance (Steel and Torrie, 1980) was applied to the data in experiment one (day one data) to test for differences among the spiked pools measured by the same method. One-way analysis of variance was applied separately to each method to determine the proportion of the total variance that was due to differences among pools and to differences within pools. The model fitted was **pepsinogen = pool**. Type III sums of squares were used to calculate the proportion of variance attributable to each effect. The accuracy of each method was assessed by multiple regression where the observed value was fitted as covariate and the squared value was fitted to test for departures from linearity (Snedecor and Cochran, 1980). Differences between days were assessed in a two-way mixed model analysis of variance with day fitted as a random effect and the spiked pool fitted as a fixed effect, the

model was **pepsinogen = day + pool**. Intra and inter-assay coefficients of variation were calculated for purposes of comparison. Coefficients of variation were calculated as the ratio of the standard deviation to the mean, multiplied by 100.

3.2.2: Results.

Appendices 1A to 1D contain the assay results for each replicate of each plasma standard on each day. Table 3.1 gives the mean and standard error for each method separately for each spiked plasma pool. Although methods 1, 2 and 3 purport to measure plasma pepsinogen concentrations in international units (one iU = 1μmol tyrosine liberated/ min/ litre of plasma), they are clearly not giving the same results. In particular method 1 gives values approximately five-fold greater than method 2.

Bartlett's test for the homogeneity of variance showed no significant differences in the amount of variation between different pools measured by the same method. therefore the methods do not show increased variability as the plasma pepsinogen concentration rises. However variance was significantly different between methods for pools 1 ($p<0.005$), 3 ($p<0.01$), 4 ($p<0.025$) and 5 ($p<0.025$). In separate analyses method 1 was not significantly different from either methods 2 or 3 for any pool, but demonstrated less variance than method 4 for plasma pools 3,4 and 5 ($p<0.025$, 0.025 and 0.05 respectively).

One-way analyses of variance for each method showed that differences between the spiked pools accounted for the vast majority of variation in each test when replicates were tested on the same day. For methods 1, 2, 3 and 4, variation among the pools accounted for 99.3, 99.6, 98.7 and 93.1 per cent of the variation; variation within pools was only 0.7, 0.4, 1.3 and 6.9 per cent of the total. Coefficients of intra-assay variation were also low. For methods 1, 2, 3 and 4 they were only 5.6, 2.9, 7.9 and 13.7 per cent respectively.

Regression analysis showed that, as expected, each method was highly associated with differences in the plasma pools ($p<0.0001$ for each method). For method 1 there was no evidence for a non-linear relationship; the effect of fitting the squared covariate was not significant ($p=0.68$). In contrast fitting the squared covariate was of borderline significance ($0.05<p<0.10$) for methods 2, 3 and 4. Given the relatively small number of tests ($n=25$) for each method, these results suggest, but do not prove, that methods 2, 3 and 4 do not give

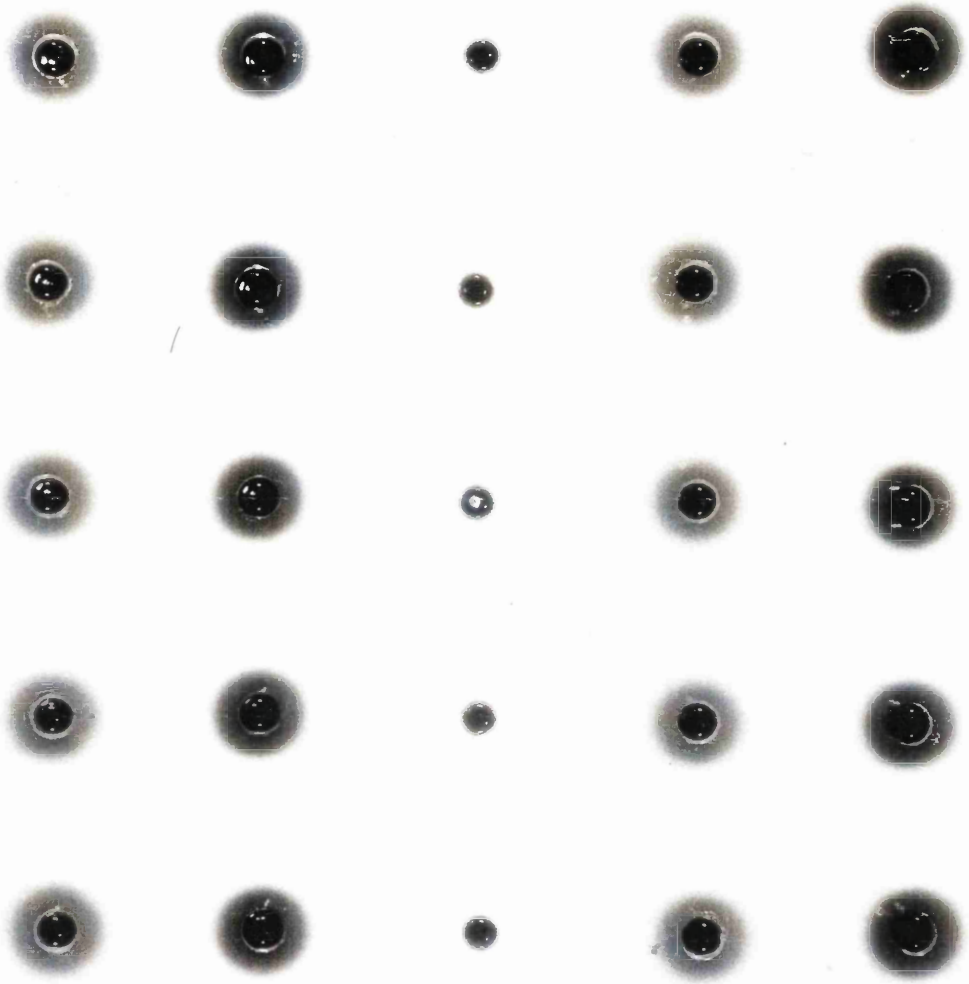
linear responses. Regression lines fitted by the method of least-squares for each method were 0.23 + 0.03(x) for method 1, 0.30 + 0.16(x) + 0.0037 (x^2) for method 2, 0.14 + 0.087(x) + 0.0076 (x^2) for method 3 and 0.40 + 0.90(x) - 0.13(x^2) for method 4. If confirmed, these results would suggest that methods 2, 3 and 4 would need correction factors if pepsinogen concentrations are to be estimated accurately, especially in animals with relatively high values.

Variation among days was tested separately for each assay in a two-way analysis of variance. To avoid possible problems with non-linear relationships, the spiked plasma pools were fitted as a fixed effect rather than as a covariate. The results showed that the variation among days was highly significant for methods 2, 3 and 4 ($p<0.0001$) but of only borderline significance for method 1 ($p=0.048$). Day-to-day variation accounted for 0.4 per cent of the variation in method 1 and 0.7, 8.2 and 30 per cent of the variation in methods 2, 3 and 4 respectively. For comparison the inter-assay coefficient of variation were 9.8, 9.3, 15.6 and 36.8 per cent for methods 1, 2, 3 and 4.

Table 3.1: Mean pepsinogen values (± S.E.) for each method when five replicates were compared on one day at each of five concentrations.

Amount of Pepsinogen added	**Method 1**	**Method 2**	**Method 3**	**Method 4**
ng/ml	iU	iU	iU	ng/ml
0.00	6.39 (0.26)	1.83 (0.02)	1.61 (0.10)	0.54 (0.04)
0.15	11.88 (0.47)	2.66 (0.05)	3.01 (0.16)	0.64 (0.03)
0.30	15.12 (0.26)	3.53 (0.04)	3.57 (0.09)	0.92 (0.07)
0.60	24.03 (0.29)	5.10 (0.07)	5.97 (0.12)	1.47 (0.08)
0.90	32.66 (0.54)	6.57 (0.07)	7.18 (0.10)	1.95 (0.11)

Fig. 3.1: The appearance of a casein-agarose plate at the end of the incubation period.



3.2.3: Discussion.

3.2.3.1: The comparison of the four methods.

A comparison of four methods for estimating plasma pepsinogen concentrations showed that all four methods gave reliable results when replicates were assayed by the same person on the same day. All the assay methods showed day-to-day variation but the method of Paynter (1992)(method 1) showed less variation than the other three methods. Paynter's method was the only method which showed a linear relationship with the spiked plasma pools and this method appears to be the method of choice for routine plasma pepsinogen determination.

The observation that all methods gave acceptable levels of variation within days, but not between days suggests that more effort is needed to minimise day-to-day variation. Possibly the use of a buffered substrate for method 1 was responsible for the reduced day-to-day variation with this method. Another possibility is to incorporate internal standards such as plasma pools of high and low activity.

The addition of porcine pepsinogen to simulate naturally elevated concentrations in ovine plasma allowed an assessment of method linearity. Graded dilutions of naturally high plasma could have been used, however this would have diluted other plasma proteins which at acid pH contribute to the total substrate present. Porcine pepsinogen is active over a similar pH range as ruminant group I pepsinogens and is essentially an equivalent protease (Mostofa, 1989b).

An assessment of method specificity for pepsinogen was not made. All four methods estimated plasma pepsinogen concentration from the total acid-stable, proteolytic activity in plasma and may be equally specific. In humans, the correlation between total proteolytic activity and pepsinogen measured by immunoassay is good (Hirschowitz, 1984). This is likely to be the same in ruminants were pepsinogen subtypes are elevated equally in parasitic infection and peptic activity has been shown to be confined to peaks, separable by gel filtration, representing only pepsinogen subtypes (Mosofa et al, 1990a, 1990b). Immunological methods (Axelsson et al, 1983, Turner and Shanks, 1982) and a recently developed colourimetric assay which uses *p*-nitroanilides as substrates (Heinova et al, 1992) may be more specific, although none of these methods has been fully assessed for routine use.

Although the results were expressed in international units for Paynter's method, and for methods 2 and 3, the results were quite different and care must be exercised to avoid confusion between the results obtained by different methods. Using method 1 Paynter (1992) established that plasma pepsinogen concentrations of up to 5.0 iU were indicative of minimal and insignificant abomasal damage. This is in comparison with studies using the assay method of Edwards et al (1960) where plasma pepsinogen concentrations of greater than 3.0 iU are considered indicative of severe clinical ostertagiasis (Armour, 1970). The five-fold differences between the results measured by methods 1 and 2 would mean that a plasma pepsinogen value of 3.0 iU, measured by method 2 (Edwards et al, 1960) would be equivalent to a value of approximately 15.00 iU when measured by method 1, and this latter value was found by Paynter (1992) to be indicative of severe abomasal damage in animals with ostertagiasis.

The main role for the estimation of plasma pepsinogens in ruminants lies in the detection of subclinical or clinical infections with abomasal nematodes such as *O. ostertagi* in cattle and *O. circumcincta* in sheep. As pointed out by Michel (1978) while plasma pepsinogen concentration cannot establish categorically whether an animal is suffering from ostertagiasis (especially subclinical infection), it is distinctly useful when considered alongside other pieces of evidence. The value of plasma pepsinogen concentration will also depend upon the proportion of the variation between tests that is due to variation in the test itself and the proportion that is due to variation in the ability of animals to respond to the same level of infection; as well as the proportion that is due to variation among animals in the level of infection. Methods with the lowest intrinsic variability are likely to be the most useful.

In conclusion all methods tested gave acceptable levels of variation when replicates were tested on the same day, but method 1 showed less variation when replicates were tested on different days and was the only test which showed a linear relationship with plasma pepsinogen concentrations in spiked plasma pools. Method 1 was simple and quick, but differed from the published method of Paynter (1992) who further simplified the assay procedure by using centrifugation to separate undigested substrate. Suitable equipment was unavailable at the time this study was carried out thereby necessitating the filtration step. Overall the method developed by Paynter was therefore the method of choice for routine estimations of plasma pepsinogen concentration.

3.2.3.2: Final modifications to Paynter's method for the routine assay of plasma pepsinogen in the laboratory.

The plasma pepsinogen assay was required to assay pepsinogen in a number of experiments reported in this thesis; the assay of pepsinogen in the plasma of experimental animals, the assay of pepsinogen in the secretions of in vitro abomasal glands or intact abomasal mucosa, and finally, the assay of pepsinogen in abomasal mucosal homogenates. To facilitate these measurements and speed the assay procedure, a number of modifications to the method quoted earlier (section 3.2.1.1) were made. As reported earlier, Paynter (1992) separated undigested substrate by centrifugation and not by filtration. This step was adopted in the present studies when suitable equipment became available. Following the arrest of the peptic reaction and the precipitation of undigested substrate, samples were centrifuged at 10,000*g* in a bench-top microcentrifuge (MSE). The use of the centrifugal separation of substrate also allowed the considerable reduction of reagent volumes (ten-fold). Instead of 500 µl of plasma, 50 µl was added to 75 µl of BSA substrate and the reaction stopped with 250 µl of perchlorate. This volume reduction allowed the use of 1.5 ml, conical Eppendorfs for the reaction step. Following the addition of sample and substrate to the Eppendorf, and before incubation, the tubes were vortexed briefly and then flash spun in the centrifuge, this ensured that the entire mixture returned to the base of the Eppendorf and would therefore be under the surface when the tubes were placed in the 37°C waterbath.

The colour reaction step using BCA/ Copper sulphate was transferred from test tubes to 98 well, flat bottomed, microtitre plates (Greiner Labortechnik Ltd., Dursley, England). It was found that the colour reagent was very sensitive to dust and other contaminants, giving erroneously high optical densities, and the use of multiple replicates permitted the elimination of any abnormally high data suspected to result from contamination. With four wells used for the addition of supernatant from each Eppendorf, a 98 well plate was sufficient for 6 tyrosine standards (with the inclusion of a 9 mM tyrosine standard, equivalent to 50 iU, in addition to the five listed earlier) and nine samples (Fig. 3.2).

Fig. 3.2: Set up of microtitre plate to measure 6 standards and 9 samples.

	Tyrosine Standards (iU)						Samples					
							1		2		3	
	0	5	10	20	30	50	30	210	30	210	30	210
	1	2	3	4	5	6	7	8	9	10	11	12
A	O	O	O	O	O	O	O	O	O	O	O	O
B	O	O	O	O	O	O	O	O	O	O	O	O
C	O	O	O	O	O	O	O	O	O	O	O	O
D	O	O	O	O	O	O	O	O	O	O	O	O
E	O	O	O	O	O	O	O	O	O	O	O	O
F	O	O	O	O	O	O	O	O	O	O	O	O
G	O	O	O	O	O	O	O	O	O	O	O	O
H	O	O	O	O	O	O	O	O	O	O	O	O
	4		5		6		7		8		9	
	Samples											

[30 and 210 refer to the duplicate tubes per sample incubated for 30 and 210 minutes respectively]

Each well contained 200 µl of the BCA and $CuSO_4$ mixture to which 10 µl of supernatant was added. Plates were then covered with adhesive plate sealers (Greiner Labortechnik Ltd.) and were then incubated at either 37°C for 30 minutes or at room temperature for 2 hours. Plates were then read using a Dynatech MR 5000 microplate reader, measuring at 562 nm and blanked on air.

Active pepsinogen standards may reduce inter-assay variation, by compensating for fluctuations in assay pH, temperature, etc. In some instances therefore the six tyrosine standards were replaced by three pepsinogen standards that were processed in an identical manner as were samples, i.e. with duplicate tubes per sample, one incubated for 30 minutes the other for 210 minutes. The pepsinogen standards then occupied the six rows of wells, used ordinarily in the plate for the colour reaction, for the tyrosine standards. The standard curve for the pepsinogen standards was prepared from the differences between the optical densities of the 210 and 30 minute incubated duplicates of each standard.

For the majority of pepsinogen assays, pepsinogen concentrations were compared in a small number of samples that were processed together in a single day, hence inter-assay variation was of no importance. However when a large number of samples had to be analysed over several days, active pepsinogen standards were used to reduce this variation. A single set of tyrosine standards could be run on one of the days to allow the conversion of the results to iU, if so desired. If data sets from an experiment did not require direct comparison with those from other experiments then conversion to iU was not necessary. Aqueous pepsinogen standards were not used in the assay of plasma samples.

Pepsinogen concentrations, in in vitro studies of pepsinogen secretion, were rarely within the range of values seen in plasma and the technique was further adjusted to allow measurements over a greater concentration range. This resulted in two methods that could be used independently according to the range of concentrations found:

1. The first method involved reagent quantities and procedures unchanged from those listed earlier in this section. Three pepsinogen standards were obtained by dissolving porcine pepsinogen in water at concentrations of 0.00, 0.75 and 1.50 ng/ml.

2. The modified method used porcine pepsinogen standards of 0.00, 1.50 and 3.00 ng/ml. To allow for higher concentrations of pepsinogen the volume of the standard/sample added to the substrate was reduced to 20 μl (from 50 μl) and the volume of the BSA substrate increased to 100 μl (from 75 μl). The volume of perchlorate used to stop the reaction was kept at 0.25 ml and the assay procedure was otherwise identical.

To ascertain the appropriate standard range for each separate experiment a small, representative number of samples were processed, along with standards in the 0.00 to 3.00 ng/ml range, and using the reagent volumes of the modified method procedure. Single tubes were used per sample or standard and incubated for three hours before the addition of perchlorate and subsequent centrifugation. The quantity of pelleted, undigested substrate was assessed visually, and compared to the standards, to decide the approximate concentration range covered by the samples. Samples with lower concentrations could then be analysed with the original method (standard range 0.0 to 1.5 ng/ml), or if the concentrations were outwith the higher standard range (0.0 to 3.0 ng/ml) then samples could be diluted accordingly.

For routine use of the pepsinogen assay BSA/glycine substrate as well as tyrosine and pepsinogen standards were prepared fresh each month, then refrigerated at 0 to 4°C. Pepsinogen standards were prepared from a stock solution of porcine pepsinogen (30 ng/ml) maintained at -20°C. From repeat experiments it was calculated that 1 ng/ml of porcine pepsinogen had peptic activity equivalent to 35.50 ± 1.70 iU (mean ± S.E., from 21 separate experiments performed over two years - see Appendix 2). It was more usual to quote the activities of the porcine pepsinogen standards using the peptic activity reported by the manufacturers (Sigma Chemical Co.). One unit (u) of peptic activity, measured as trichloroacetic acid-soluble products using haemoglobin as substrate, was that which produced a change in optical density at 280 nm of 0.001 per minute at pH 2.0 at 37°C. One nanogram of porcine pepsinogen was therefore equal to approximately 3.2 units. Quoting the peptic activity of pepsinogen standards in this way (u/ml rather than ng/ml) allowed for variation in batches of pepsinogen where the peptic activities of identical masses of compound might vary. Based on the multiple comparisons of pepsinogen and tyrosine standards quoted earlier (Appendix 2) one u/ml was equivalent to 11.0 ± 0.50 iU (mean value ± S.E., based on 21 measurements).

3.3: Assessment of Method 1 (Paynter, 1992) using plasma with naturally elevated pepsinogen concentrations.

To ensure adequate assay performance in all experimental situations, the precision/reliability of Paynter's method was determined with plasma obtained from sheep with plasma pepsinogen concentrations elevated in response to infection, rather than by the addition of exogenous zymogen.

3.3.1: Materials and methods.

Plasma was obtained from a group of 11, six month old Scottish Blackface lambs some of which (8/11) had been infected with an oral dose of 15,000 *O. circumcincta*. Animals were killed by electrical stunning followed by exsanguination, blood was collected as the animals were bled out and processed as described in section 2.2.5. Plasma was stored in aliquots at -20°C until used.

Five replicates per animal were analysed simultaneously on day one to ascertain the degree of intra-assay variation and single replicates were performed on four subsequent days to

establish inter-assay variance. Data were analysed by Bartlett's test (day one data only) and by a two-way analysis of variance using a General Linear Model (Minitab, Release 8, Minitab Inc., State College, PA). The model used was **plasma pepsinogen = animal + day**.

3.3.2: Results.

Plasma pepsinogen concentrations ranged from 0.35 iU to 13.58 iU (mean values of the five replicate analyses of plasma from individual sheep performed on day one - see Appendix 3). Bartlett's test detected no significant inequalities of variance between the variances recorded for the replicate estimates of individuals ($0.25<p<0.50$). Analysis of variance showed that differences between the pepsinogen concentrations recorded for different sheep were highly significant ($p<0.001$) and that between day variation was also significant ($0.025<p<0.05$).

3.3.3: Discussion.

Similar results were obtained with the naturally elevated plasma pepsinogen samples as had been obtained with the spiked pools tested previously. As shown for the spiked pools, within-assay variation was not significant when compared to the differences that existed between the pepsinogen concentrations of individual animals and day-to-day variation was marginally significant. Pepsinogen concentrations were high given the relatively light level of infection and this also suggests that method 1 is likely to give higher results than other methods.

3.4: Comparison of peptic activity in the presence and absence of plasma.

A problem encountered with the casein-agarose, radial diffusion assay in the method comparison study was the poor clarity of zone border. This feature may have led to inaccurate measurement of the zone diameter and might therefore account for the higher degree of intra-assay variation seen with this method. This was only a problem for the zones associated with the plasma samples, the aqueous pepsinogen standards produced well defined clear zones and therefore there may be some interference due to the presence of plasma. This phenomenon was reported by Thode-Jensen (1977), but was not seen as any hindrance to the routine use of the assay.

The present experiment was performed to compare the zones produced in the casein-agarose method by different types of pepsinogen-containing sample, and thus to further investigate the poor performance of this assay in the method comparison study.

3.4.1: Materials and methods.

Plasma was obtained from a parasite-naive, 9 month old Hampshire Down lamb that had zero intrinsic plasma pepsinogen activity (assayed by Paynter's method and confirmed in the radial diffusion assay). Added to this plasma was a known amount of porcine pepsinogen (1.5 ng/ml) and a second, entirely aqueous, solution was made with an identical concentration of porcine pepsinogen. Both solutions and the original plasma were then added to wells cut in a casein-agarose plate and incubated according to the method previously described (section 3.2.1.4). All solutions were also assayed by Paynter's method. Zone dimensions were calculated as the diameter of the cleared zone minus the well diameter.

3.4.2: Results.

All results for pepsinogen concentration, zone diameters for the plate method and optical densities for the colourimetric assay, are given in Appendix 4. Despite identical pepsinogen concentrations the zones produced by the pepsinogen/water solution (Fig. 3.3) were larger (mean (± S.E.) zone dimension 6.60 (0.08) mm, n = 10) than those produced by the pepsinogen/plasma solution (mean 5.80 (0.12) mm, n = 10) and this difference was significant when analysed by a two-sample T-test ($p<0.001$). Referring to Fig. 3.3 it can also be appreciated that the aqueous zones (wells 1, 3 and 5) were clearer and more sharply defined than the plasma zones (wells 2, 4 and 6). The original, blank plasma was added to well 7 and there was no proteolytic activity associated with it (mean 0.00 (0.00) mm, n=5).

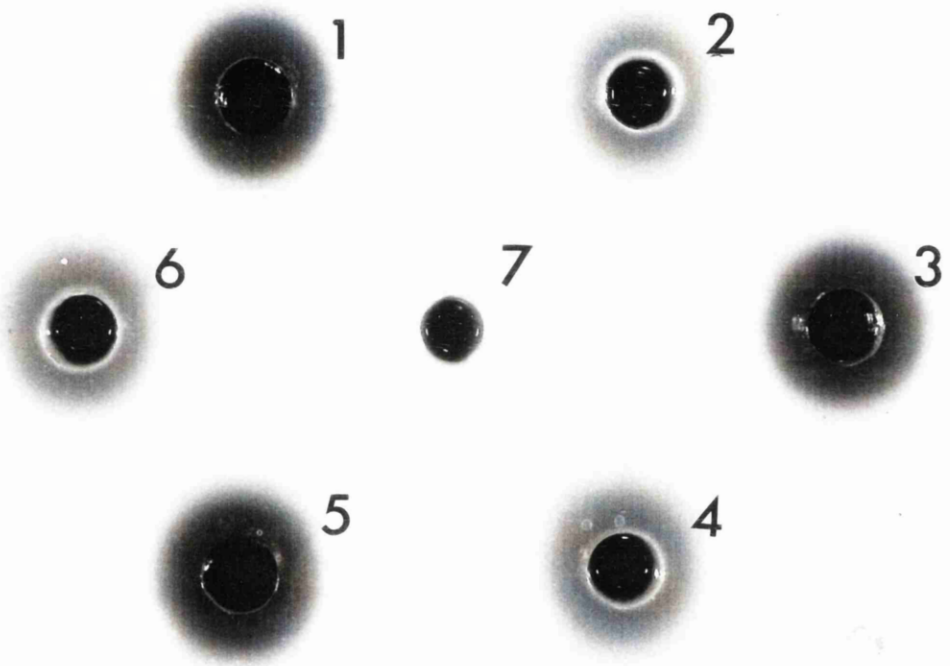
Assayed by Paynter's method replicates of the pepsinogen/plasma samples again gave lower results than the pepsinogen/water solution (mean (± S.E.) O.D. at 562nm of 0.19 (0.005), n=10, versus a mean of 0.28 (0.01), n=10), this difference was again significant by two-sample T-test ($p<0.001$).

3.4.3: Discussion.

Both methods clearly demonstrated that the presence of plasma affected the activity of pepsinogen, and reduced peptic activity significantly. This study also demonstrated that the use of aqueous porcine pepsinogen standards to assay plasma pepsinogen concentration by either method, is inappropriate. Entirely aqueous standards may yet have a role in assay of the peptic activity of experimental solutions, such as tissue culture supernatants, which are uncontaminated with plasma. These findings together with the lack of clarity of zone border in the casein-agarose assay are probably sufficient grounds for the abandonment of the radial diffusion method for routine assay of plasma samples at least for diagnostic or experimental purposes.

Thode-Jensen (1977) reported that "the lysed zones were not quite as clear and well-defined" for plasma, or for solutions supplemented with albumin, when compared to pure pepsinogen and pepsin standards. However the addition of albumin at concentrations of zero to five per cent did not alter peptic activities (zone-sizes) when compared to entirely aqueous pepsinogen at the same concentration. Some factor present in plasma may be inhibitory to pepsinogen, but at present this factor remains unidentified. At acid pH plasma proteins contribute to the total substrate present, but increasing substrate concentration would be expected to increase peptic activity, at least until the substrate was in excess. Thus indirect assays of plasma pepsinogen concentration that measure total peptic activity may also be affected by the concentration of any possible inhibitory factor and this represents a further and potentially significant source of assay variation.

Fig. 3.3: Comparison of the zones produced by proteolytic activity in a casein-agarose gel, by plasma and by an aqueous solution, both of identical pepsinogen concentration. Well 7 contained plasma with an intrinsic pepsinogen concentration of zero and wells 2,4 and 6 contained the same plasma fortified with 1.5 ng/ml of porcine pepsinogen. Wells 1,3 and 5 contained distilled water with 1.5 ng/ml added porcine pepsinogen.



EXPERIMENTAL STUDIES - PART 2: IN VITRO METHODS.

4: PEPSINOGEN SECRETION.

4.1: Introduction.

Continual exocrine secretion into the abomasum in ruminants reflects the relatively uninterrupted passage of digesta along the gastrointestinal tract and thus differs from the intermittent secretion which occurs in monogastric species. Apart from short quiescent periods, of 15-20 minutes duration which occur every 1 to 2 hours, abomasal outflow is effectively continuous (Bueno and Fioramonti, 1979). McLeay et al (1973) demonstrated continuous output of both pepsin and acid from separated pouches of abomasal fundus, which was reduced, but not abolished, by a period of restricted food intake. Basal secretion of both acid and pepsinogen occurs in man and is thought to be cholinergically mediated, and can be inhibited by very small doses of atropine (Hirschowitz et al, 1984). However basal secretion does not occur in dogs or cats (Hirschowitz, 1984). Total daily abomasal exocrine secretions have been estimated at 5 litres for an adult sheep and 30 litres for a 2-year old heifer (Hill, 1968).

Neural and hormonal stimuli (endocrine and paracrine) are undoubtedly involved in the maintenance of abomasal secretion. Vago-vagal reflexes in response to chemical and physical stimuli are likely to be involved (Titchen and Anderson, 1977) and the vagus operates in the psychic response to teasing with food and in the buccal response to the physical presence of food. Local neural reflex arcs may also operate. Gastric distension in dogs causes increased acid output that is partially abolished by vagotomy, due to loss of vago-vagal reflexes, and completely blocked by atropine, via blockade of local cholinergic reflexes (Debas et al, 1974). Local responses may also be controlled by enteric hormone release.

Cells of the abomasum which secrete pepsinogen include; the chief or zymogenic cells, the mucous neck cells and possibly even mucous cells lining the gastric pits (Andren et al, 1982; Yamada et al, 1988; Cybulski and Andren, 1990). The tissue distribution of pepsinogen and the existence of different pepsinogen subtypes are discussed in section 7.1.2.

Although in many circumstances pepsinogen and acid secretion occur in concert, this is not universal (vide infra). A great many endogenous and exogenous substances have been identified as secretagogues (stimulants) for pepsinogen secretion in vivo and in vitro, but not all are likely to be important in the physiological regulation of secretion. Cholinergic stimulation of pepsinogen secretion occurs in intact animals (Samloff, 1971a) and in vitro, in dispersed gastric glands (Koelz et al, 1982; McKellar et al, 1990b) and in intact mucosal sheets (Basson et al, 1990). Cholinergic activity reflects the endogenous neural control, which occurs via vagal innervation of the mucosa, or via internal plexi existing within it. In intact animals vagal stimulation results in secretion of aqueous fluid and gastrin and a number of other effects which might lead to the stimulation of pepsinogen secretion, however there is also ample evidence for a direct action on chief cells (Koelz et al, 1982; Kaskebar et al, 1983; Sanders et al, 1983). Beta-adrenergic agonists stimulate pepsinogen release (Koelz et al, 1982), but do not always affect acid secretion. Isoprenaline did not stimulate acid secretion in isolated rabbit gastric glands (Hersey et al, 1983b), but did in whole rat stomach in vitro, in the latter situation it was thought to be acting via atypical β-adrenoceptors (Canfield and Paraskeva, 1992). Chief cell adrenergic stimulation is direct and can be inhibited by propranolol, but not by atropine or the histamine receptor antagonist cimetidine. $Alpha_2$-adrenoceptor agonists inhibit pepsinogen release indirectly (Tazi-Saad et al, 1992). The influence of adrenergic stimulation in the intact animal is not as well defined. Histamine has variable action depending on the species investigated. Acidification of the gastric lumen in response to histamine may also result in increased pepsinogen output due to local cholinergic reflex (Johnson, 1972) and further adds to the perceived complexity of the controlling mechanisms of gastric secretion.

Several enteric, peptide hormones are reported stimulants of pepsinogen secretion. There is increasing evidence that many endocrine molecules also serve as neurotransmitters. Members of the peptide family which includes gastrin and cholecystokinin (CCK) have been reported as secretagogues, but CCK is generally more potent in this respect (Hersey et al, 1983a; Hersey, 1987). The effects of gastrin on chief cells may be largely indirect. Guinea pig chief cells possess two receptors for CCK/gastrin-related peptides, one has greater affinity for CCK and produces a marked response, the other binds gastrin and provides a weaker stimulus to secretion (Cherner et al, 1988). Whilst gastrin also stimulates acid production, CCK may be an important negative regulator of acid and an inhibitor of postprandial release of gastrin in humans (Schmidt et al, 1994). Other peptides reported to stimulate pepsinogen secretion

include the Vasoactive Intestinal Polypeptide (VIP)/secretin family (Raufman et al, 1983, 1984) and bombesin-like peptides which act by causing gastrin release (Hirschowitz and Molina, 1983). Glucagon inhibits acid and pepsinogen secretion (Konturek et al, 1975), as does somatostatin (Barros et al, 1978; Hirst et al, 1982; Vagne et al, 1982).

The coupling of stimulus and secretion is achieved by one of two intracellular mechanisms induced by the binding of agonist to membrane receptors (Hersey, 1987). Receptors for secretin/VIP-like peptides and β-adrenergic agents interact with adenylate cyclase and cause increased levels of cyclic adenosine monophosphate (cyclic AMP), whereas cholinergic agonists and CCK-like peptides cause increases in intracellular Ca^{2+} via production of inositol triphosphate. Ultimately both mechanisms cause elevations of intracellular Ca^{2+}, since forskolin, which activates adenylate cyclase directly, stimulates a slow, transient rise in Ca^{2+} (Chew, 1986). Calcium may be important in granule exocytosis (Hersey et al, 1984).

Secretion of pepsinogen by chief and other cells in response to stimulus involves the prompt release of stored granules, the synthesis and sustained secretion of new pepsinogen and the uptake of amino acids and the metabolic activity to support the synthetic process (Hirschowitz, 1984; Hersey, 1987). Thus secretion can be considered as biphasic with an initial burst due to degranulation followed by sustained secretion not involving granule formation. The exact mechanism of degranulation is unknown, but is thought to be similar to exocytosis in other cells.

Historically, studies of pepsinogen secretion involved studies of the intact animal, e.g. dogs fitted with gastric fistulae or pouches, or in studies of anaesthetised animals. Advantages of these methods include the retention of neural and humoral regulation, and the ability to study the main secretory stimulus for monogastric animals, food itself. Disadvantages are the very complexity of the regulatory mechanisms themselves due to the plasma clearance and metabolism of agents under investigation, and the potential for stimuli to act through indirect pathways. Recently much work has involved enzymatically dispersed gastric glands and it was in such a preparation that McKellar et al (1990b) demonstrated pepsinogen release in response to *Ostertagia* spp. excretory/secretory products (ES) in bovine and ovine glands. Advantages of methods employing dispersed glands include the loss of external neurohumoral control, but the retention of glandular architecture. The disadvantage of this, and of the less commonly applied technique utilising intact mucosal preparations, is the presence of a diverse

number of cell types, especially in the latter preparations. This may be advantageous in some instances, as pepsinogen and acid secretion might both be studied using the same preparation. Their advantages over other methods, in terms of the ease of preparation, probably account for their popularity.

The following work was intended to develop and investigate two methods for the in vitro monitoring of pepsinogen secretion (by dispersed and isolated gastric glands and by intact preparations of mucosal sheets), to assess the utility and responsivity of each method with the aim of using one method in later studies of the effects of parasite ES on pepsinogen secretion. Assessment would be made of the viability of preparations made by either method and their responsivity, in terms of pepsinogen secretion, to stimulation with the cholinergic agonist carbachol.

4.2: Pepsinogen Secretion by Isolated Gastric Glands.

Dispersed, isolated gastric glands have been used to study secretion of acid and pepsinogen in a variety of mammalian species (Berglindh and Obrink, 1976; Koelz et al, 1982; Hersey, 1987; Soll and Berglindh, 1987). Their preparation requires a less thorough enzymatic digestion than is involved in the liberation of single cells. Dispersed glands have only ever been used for short term cultures, the duration measured in hours rather than days and do not provide immortal cell lines that are characteristic of single cell culture methods.

Previous work has demonstrated pepsinogen secretion in response to ES products from *Ostertagia* spp. parasites using isolated bovine and ovine gastric glands (McKellar et al, 1990b) and the same method was used in the present studies. The responsivity of dispersed gastric glands was checked using the cholinergic agonist carbachol. Carbachol was used at either multiple, increasing doses or as a single dose calculated to give maximal stimulation.

4.2.1: Materials and methods.

4.2.1.1: Preparation of ovine dispersed gastric glands.

The abomasa of sheep were collected at Sandyford Abattoir, Paisley, Scotland and no more than 15 minutes elapsed between the time of death of donor sheep and the collection of

tissues. Abomasa were opened along the greater curvature and the mucosa was then washed with Hank's balanced salt solution (Gibco BRL, Life Technologies, Ltd., Paisley, Scotland.) supplemented with 25 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid) pH 7.3, 0.2 per cent (w/v) bovine serum albumin (fraction V) and 0.2 per cent (w/v) glucose, gassed with 100 per cent Oxygen. The mucosal folds were then removed from the main bulk of the abomasal wall and these were transported back to the laboratory in the same supplemented medium on ice (0-4°C).

Mucosal pieces were then minced finely and placed in Leibovitz medium (Gibco BRL) with identical supplementation as that added to the Hank's balanced salt solution (vide supra). The solution was gassed prior to the addition of minced mucosa with 100 per cent Oxygen, and vigorous gassing was continued throughout a subsequent 10 minute incubation at 37°C, this also served to stir the mucosal fragments. After 10 minutes the contents were allowed to settle, mucosal fragments quickly sank, whereas fatty debris and any other floating materials were decanted and discarded. The mucosal pieces were then resuspended in supplemented Leibovitz medium further supplemented with 0.1 per cent (w/v) collagenase, Type 1A and 0.1 per cent (w/v) Soyabean trypsin inhibitor, Type 1-S, gassed with 100 per cent Oxygen. The mucosal fragments were then incubated for 45 minutes at 37°C. At the end of this period the suspension was passed through a fine nylon mesh and the glands in the filtrate were resuspended in Leibovitz medium, supplemented only with 0.2 per cent (w/v) albumin and 0.2 per cent (w/v) glucose. In the only significant departure from the method employed by McKellar et al (1990b) the gland suspension was not centrifuged, but glands were allowed to sediment instead, this is thought to be a less deleterious procedure for glands than centrifugation (Soll and Berglindh, 1987). In order to clean the glands old medium was discarded following sedimentation and glands were then resuspended in 30 to 50 ml of fresh, oxygenated Leibovitz medium, supplemented with 0.2 per cent albumin and 0.2 per cent glucose. Washing was repeated at least five times. Yield of glands varied between preparations so that the volumes available for subsequent incubations varied accordingly.

4.2.1.2: Gland counts and assessment of viability.

Counts of glands were made using a Modified Fuchs Rosenthal Haemocytometer, and viability was assessed using a Trypan Blue Dye Exclusion test (Berglindh and Obrink, 1976). Equal volumes of a 0.4 per cent (w/v) solution of Trypan Blue and of glandular suspension

were mixed and then immediately examined by light microscope. Living cells are able to prevent dye uptake, whereas dead cells stain blue strongly. Viability was assessed at the start and finish of each experiment.

4.2.1.3: Carbachol dose response studies.

Dose response curves for pepsinogen secretion in response to carbachol were prepared using 5 doses of carbachol (at final concentrations of 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M carbachol, 0.1 micromolar to 1 millimolar) and a control dose (distilled water). Glandular suspensions were prepared from the fundic mucosa of six animals killed at Sandyford abattoir. The experiments covered a period of six non-consecutive days, in which glands from each sheep were processed in a single day.

Carbachol (carbamylcholine chloride) was prepared as a 0.1 molar stock solution using distilled water. One to five millilitres of glands were aliquoted into capped vessels, the exact volume depended on the total yield from each animal. Glands were then pre-incubated in a shaking water bath at 37°C for 30 minutes, at the end of which either a dose of carbachol or an equivalent volume of distilled water (vehicle for carbachol) was added. Multiple aliquots of glands from each animal were set up to receive each carbachol/vehicle dose. Agent/vehicle addition was never in excess of 1 per cent of total volume. Glands were then incubated for a further 60 minutes. Small samples (0.1 to 0.2 ml) were taken at the end of the pre-incubation period and at the end of the experiment. Samples were then diluted 1:4 with distilled water and centrifuged at 10,000 *g* in a bench-top Eppendorf centrifuge (MSE) to pellet glands/cells. Supernatants were then stored at -20°C to await analysis. Assay of pepsinogen concentration in supernatants was performed using the modified pepsinogen assay (section 3.2).

The pepsinogen concentrations in the pre-samples were subtracted from the final sample concentrations and then a mean value was obtained for each separate dose for each animal. To compare the mean values obtained from all six sheep, where the individual pepsinogen concentrations involved varied widely, data were expressed as an increase over secretion in control samples, and then as a percentage of the maximal response obtained in each animal. Non-normal data distribution required analysis by non-parametric tests. The pepsinogen concentrations obtained in response to different doses of carbachol were therefore compared using a Kruskall-Wallis test.

4.2.1.4: Studies involving supramaximal doses of carbachol.

Other experiments simply tested pepsinogen secretion in response to a dose of carbachol greater than that likely to be required to give maximal stimulation (extrapolated from other studies), versus basal unstimulated secretion. Glands were prepared from the abomasa of two sheep, both obtained from Sandyford abattoir. Different yields of glands meant that the total number of gland aliquots per treatment was ten with the first sheep, but six with the second. The one-tenth molar stock solution of carbachol was again used. The final concentration of carbachol in the culture medium for half the gland aliquots from each sheep was 1 mM, the other half remained unstimulated, with only an equivalent volume of carbachol vehicle (distilled water) added. Pepsinogen concentration was measured in the culture supernatants at the end of the thirty minute equilibration period, prior to the addition of carbachol/vehicle, and after a further thirty minutes. The pepsinogen concentration of the pre-samples (0 minutes) was then subtracted from that of the final samples (30 minutes). The mean pepsinogen concentrations in control and carbachol-treated glands for each sheep were compared by two-sample T-test.

4.2.2: Results.

4.2.2.1: Results of assessments of viability.

Gland counts per millilitre of final incubation medium are given in Appendices 5A and 5B. Because of the three-dimensional nature of glands, cells on the far side of glands could not be seen, it was therefore felt that absolute counts of viable and non-viable cells were inappropriate. Viability was, however, empirically very good when glands were prepared by the above method, an estimate of viability indicated that 70 per cent of all cells were viable and remained so during subsequent experiments (agreeing with the studies of McKellar et al, 1990b). Fig. 4.1 shows cross sections of dispersed glands harvested prior to the commencement of an experiment, following fixation in Brunnel's Fixative. Paraffin-embedded glands were sectioned at 2 μm thickness and stained with Haematoxylin and Eosin. Parietal and chief cells can be seen in normal apposition to each other. The apical cytoplasm of the chief cells is filled with eosinophilic granules. Chief cell nuclei show quite dense chromatin, but otherwise cells appear viable.

4.2.2.2: Results of dose response studies.

Pepsinogen concentration data from gland preparations from each animal are shown in Appendix 5A. Fig. 4.2 shows the overall mean results of all six separate dose response experiments. Although the gland preparations showed increased pepsinogen secretion at higher carbachol concentrations, and the curve approximates the sigmoid shape typical of other dose response experiments, the very high degree of variation encountered (illustrated by the extent of the standard error bars) meant that differences in pepsinogen secretion in response to different carbachol doses were not necessarily greater than that which might simply arise between preparations treated identically. The Kruskall-Wallis test showed that differences in secretion between the different carbachol doses were not significant ($p>0.150$).

4.2.2.3: Results of supramaximal stimulation studies.

The pepsinogen concentrations in control and carbachol-treated gland preparations are shown in full in Appendix 5B. Fig. 4.3 details the results from the two experiments and shows that each failed to produce a significant response to stimulation with a 1 millimolar concentration of carbachol, and in one (Fig. 4.3a) mean secretion in the presence of carbachol was in fact less than that of controls. Pepsinogen concentrations in control and carbachol-treated glands were not significantly different in either experiment ($p>0.05$, by Student's two-sample T-Test).

4.2.3: Discussion.

Dispersed abomasal glands prepared by the above method were inadequately responsive to carbachol, despite apparently good viability of the glands. Other workers have shown that significant responses to stimulation occur rapidly, within 30 minutes (Hersey et al, 1983a,b; McKellar et al, 1990b) and it is unlikely that insufficient time had been allowed for the generation of responses in the present studies. Possibly the doses of carbachol were too low to provoke responses or ovine glands in general do not respond, or respond only weakly, to carbachol stimulation. Both these arguments are unlikely given the wealth of information obtained from gastric gland studies from other species and the one study of bovine and ovine glands where cholinergic stimulation was successful (McKellar et al, 1990b). The major difference between the method used by McKellar et al (1990b) and the method adopted here

was the use of abattoir-derived abomasal tissue that had been transported in cold media. Cold transport/storage (0-4°C) of gastrointestinal muscle in parallel studies did not abolish responsivity (results not shown), however cold storage of gastric glands at the same temperatures has been associated with the subsequent poor performance of glands, with failure of acid secretion in particular (Soll and Berglindh, 1987). Other workers (Sack and Spenney, 1982) have shown that the exact culture conditions are crucial to the magnitude of responses obtained using dispersed gastric glands, at least for monitoring acid secretion via the intracellular accumulation of aminopyrine. The same workers listed a variety of factors, which included the shape of the incubation vessel, and the rate and direction of shaking, important in the determination of the magnitude of responses. Further work would therefore be required to enable the routine use of dispersed abomasal glands. Eventually this method was abandoned in favour of a more straightforward technique which utilised intact mucosal sheets (section 4.3).

Fig. 4.1: Cross sections of dispersed abomasal glands. (H&E) viewed with oil immersion and ×250 magnification.

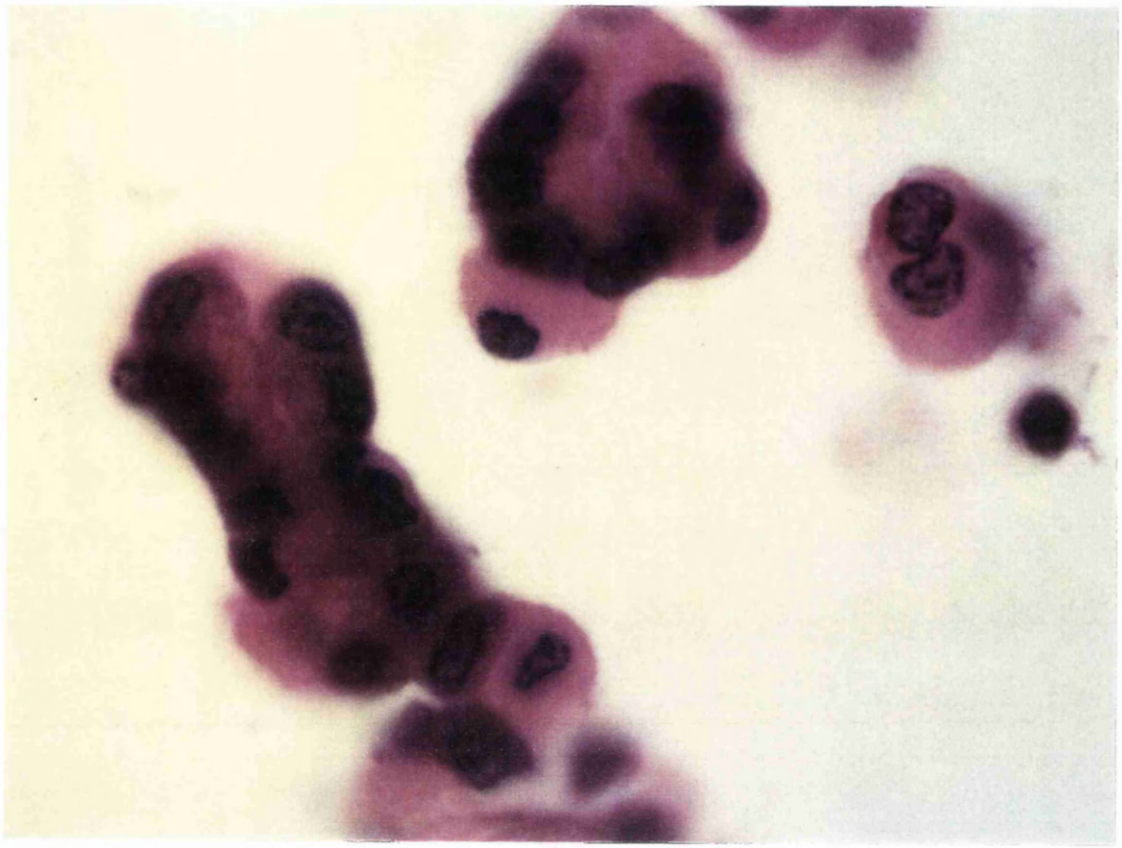
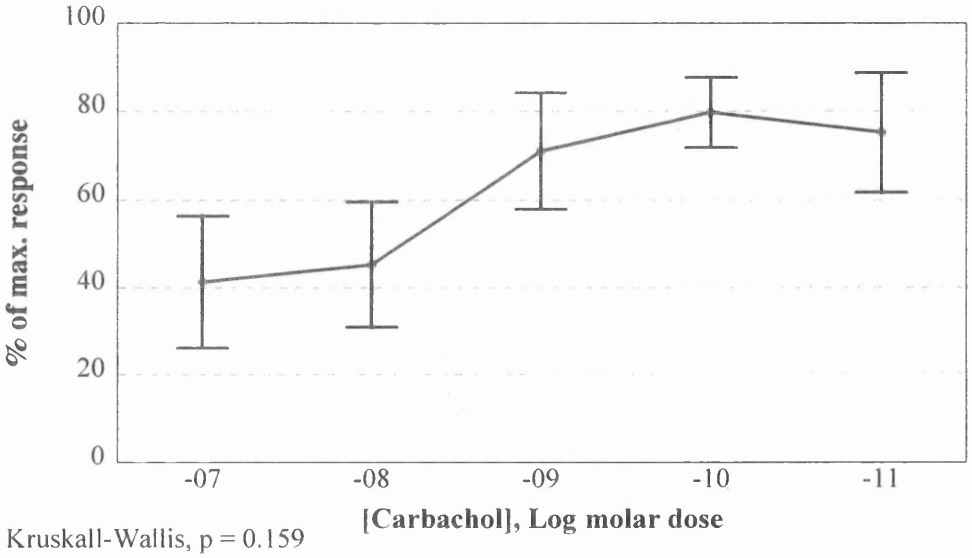


Fig. 4.2: Pepsinogen release from dispersed abomasal glands in response to Carbachol.

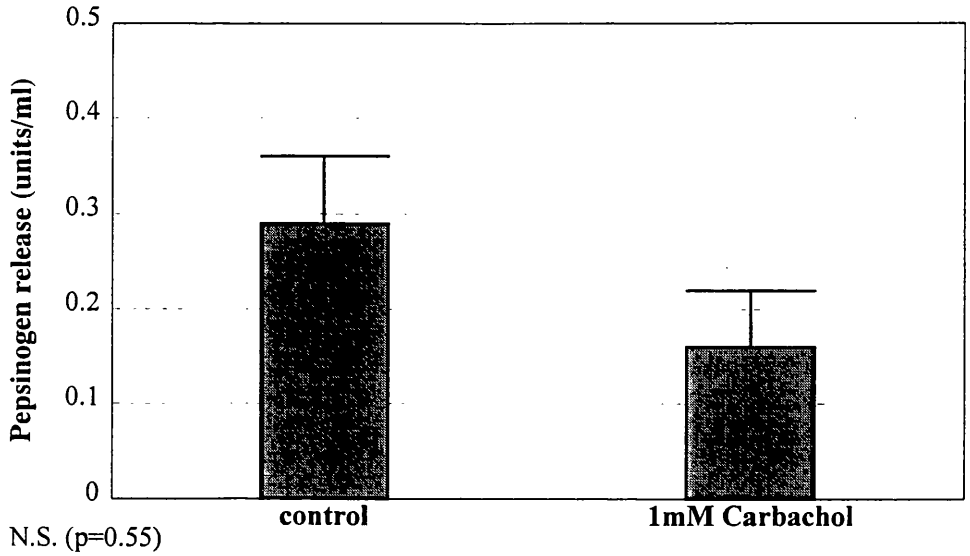
Data was expressed as the ratio of stimulated secretion to control secretion in each experiment and then as a percentage of the maximal response obtained in each experiment, and is displayed as the overall mean, +/- SE, of experiments in glands derived from 6 animals.



Kruskall-Wallis, p = 0.159

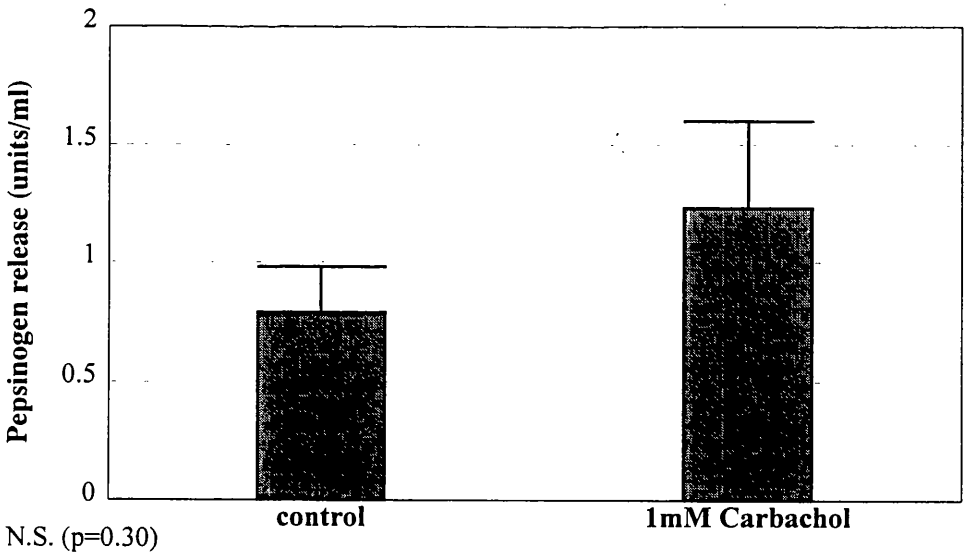
Fig. 4.3: Failure of maximal doses of carbachol to elicit significant increases of pepsinogen release from dispersed abomasal glands in two separate experiments.

3a: mean data +/- SE, of 10 preparations per treatment.



N.S. (p=0.55)

3b: 6 preparations per treatment.



N.S. (p=0.30)

4.3: Pepsinogen secretion by intact mucosal sheets.

Intact mucosal sheets mounted in Ussing chambers have been used extensively in recent years and can be utilised to monitor the secretion of both pepsinogen and acid simultaneously (Forte et al, 1975; Finke et al, 1985; Rhodes et al, 1986; Basson et al, 1990; Patel and Spraggs, 1992). The preparation of intact gastric mucosal sheets involves minimal processing of tissue, with no requirement for enzymatic digestion. An Ussing chamber is one where luminal and submucosal surfaces of the mucosal sheets face separate compartments and are therefore bathed by fluids that do not communicate except for a potential exchange through the tissue itself. Measurements of transmucosal potential difference as well as those of acid and pepsinogen secretion can be made using these preparations if suitable equipment is available. Luminal pH has generally been measured by pH-Stat technique involving auto-titration with sodium hydroxide. Pepsinogen secretion can be measured simply by assaying the pepsinogen concentration of samples of luminal fluid, collected at specific intervals.

The initial studies reported in this section were to establish the length of time required for the full development of secretory responses to stimulation and thus identify an appropriate sampling interval for routine experimentation. The method was further validated by establishing dose response curves for increasing concentrations of carbachol, these latter studies assumed that the twenty minute sampling interval used by other workers (Basson et al, 1990) was appropriate.

4.3.1: Materials and methods.

4.3.1.1: Preparation of intact mucosal sheets to monitor pepsinogen secretion.

Ovine abomasa were obtained from the abattoir, as described previously (section 4.2.1) and opened along the greater curvature and the contents discarded. The mucosa was then rinsed with warm (approximately 30°C) Ringer's solution (122 mM NaCl, 25 mM $NaHCO_3$, 5 mM KCl, 1.3 mM $MgSO_4$, 2.0 mM $CaCl_2$, 1.0 mM KH_2PO_4 and 20 mM glucose), that had been gassed with 95 per cent O_2 and 5 per cent CO_2. The abomasa were then placed mucosal side down and a volume of Ringer's solution (approximately 50 ml per site) was injected between the muscle layer and the mucosa to form a blister. The muscle coat was then cut away and the

freed mucosa was collected and placed in warm Ringer's for transport back to the laboratory. Mucosal pieces were later trimmed to a suitable size for mounting in tissue chambers.

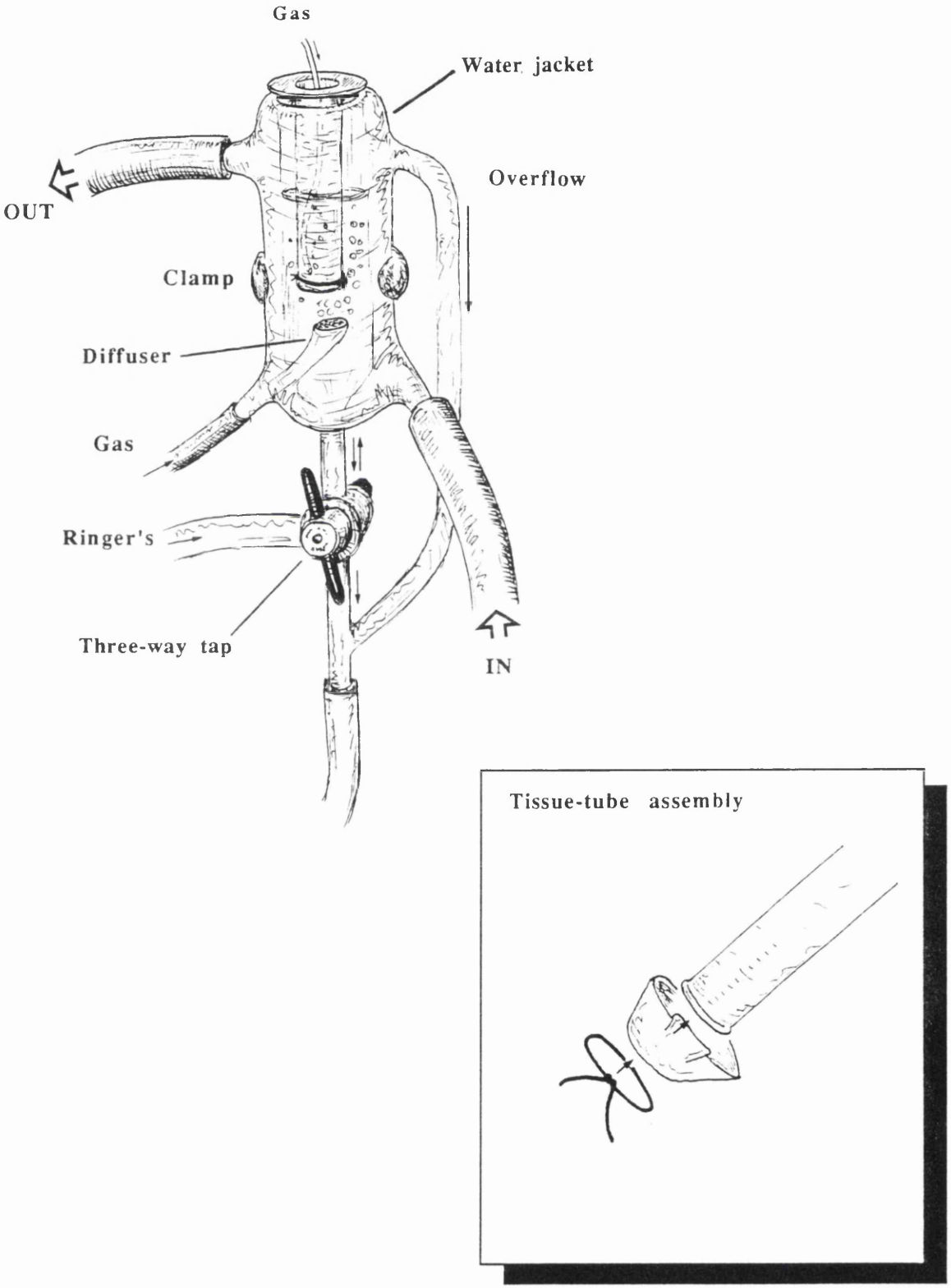
The tissue chambers themselves were hand-crafted from 2.5 ml polystyrene syringes (see Fig. 4.4). The plunger was removed and the tip of the syringe was cut away using a small hacksaw. With a fine file a groove was cut behind the tip, to give a raised lip, and the cut edges were smoothed. To seal the smoothed surfaces the tip was immersed briefly in acetone and then allowed to dry.

The mucosa was tied in place over the chamber and secured by strong thread, with the luminal surface facing into the chamber, having an exposed surface area of 0.64 cm^2. The tissue-chamber assembly was then placed in a 15 ml tissue bath and the submucosal surface was bathed with 10 ml of Ringer's bubbled vigorously with 95 per cent O_2, 5 per cent CO_2. The luminal surface was bathed with 2.0 ml Ringer's supplemented with 0.3 per cent Casein Acid Hydrolysate, and gassed with the same mixture as the submucosal fluid. The gas flow rate on the luminal side was kept high to better stir the bathing Ringer's. The full experimental apparatus is illustrated in Fig. 4.4.

Both luminal and submucosal solutions were maintained at pH 7.4 by the presence of bicarbonate and 5 per cent CO_2 in the gas mixture. Circulating water in the bath jackets was maintained at 37°C. The presence of casein hydrolysate in the luminal solution has been used by others (Basson et al, 1990) to improve the recovery of pepsinogen. It acts by saturating protein binding sites and acting as an alternative substrate to prevent autodigestion of pepsinogen if activated pepsin is present. Basson et al (1990) recorded a 30 per cent increase in yield of peptic activity in this way.

For at least the first two hours after set-up, tissues were allowed to equilibrate, with no additions of agonists/ES in this period. Luminal and submucosal solutions were replaced every 20 minutes, and old solutions were discarded.

Fig. 4.4: Experimental set-up for the in vitro monitoring of pepsinogen release from intact mucosal sheets. The inset details the tissue-tube assembly where the mucosa is stretched over the tube end and secured behind a raised lip with strong thread.



4.3.1.2: Time course of the response to cholinergic stimulation.

To confirm the appropriateness of the twenty minute sampling interval, tissues were obtained from three animals at Sandyford abattoir and pepsinogen release in response to cholinergic stimulation was closely monitored. Tissues were allowed to equilibrate for two hours before the experiments were begun. The submucosal bathing fluid was then replaced at 0, 15 and 30 minutes and then at twenty minute intervals until the end of the experiment. Thirty minutes after the end of the equilibration period and immediately after the submucosal bathing fluid had been changed, a dose of carbachol was added to the submucosal Ringer's. The dose was extrapolated from other studies and calculated to give a final bath concentration of 0.1 millimolar, i.e. a likely supramaximal dose of carbachol. The carbachol dose was repeated 20 minutes later when the mucosal fluid was again replaced. After a further twenty minutes the mucosal Ringer's was replaced for the last time, but this time with no further additions. Tissues were therefore exposed to carbachol for a total period of forty minutes. Throughout the experiment the luminal fluid (2.0 ml) was collected and replaced at five minute intervals. Immediately after collection of luminal fluid, the samples were acidified to a pH of approximately 5.0 with the addition of 20 µl of 1 molar hydrochloric acid, since pepsinogen and pepsin are stable at pH 5.0, but the activation of pepsinogen to pepsin at this pH will be minimal (Hirschowitz, 1984). Samples were stored at -20°C until the assay of pepsinogen concentration could be performed using the modification of Paynter's method (section 3.2). The volume of carbachol added to the submucosal bathing fluid did not exceed 1.0 per cent of total bath volume (i.e. 100 µl). The experiments used the same 0.1 molar carbachol stock solution as used earlier (section 4.2.1.3) Five mucosal tissue preparations were set up for each of two of the animals (sheep 2 and 3). Three preparations were set up for the third animal (sheep 1), however one tissue was ruptured accidentally in the course of the experiment and luminal fluid could only therefore be collected from the remaining two intact tissues.

4.3.1.3: Carbachol Dose Response studies.

Dose response studies were carried out in tissues from five animals (five tissues from each animal) and pepsinogen release was measured in response to increasing concentrations of carbachol. All solutions were replaced and the luminal fluid samples collected at twenty minute intervals. After the first two time points, during which tissues remained untreated, carbachol was added initially to give a final bath concentration of 10^{-9} M (1 nanomolar). The

concentration of carbachol in the mucosal bathing solution was then increased in ten-fold increments, in each subsequent twenty minute interval, to reach a maximum of 10^{-5} M (10 micromolar). Tissues were therefore exposed to each concentration of carbachol for twenty minutes. The 0.1 molar stock solution of carbachol (see section 4.2.1.3) was diluted to produce a series of working concentrations so that the volume of each necessary to achieve the required bath concentration would not exceed 100 µl. An examination of the pepsinogen released during the first two, untreated time points was used to confirm whether basal secretion was steady-state. Luminal samples were again acidified by the addition of 20 µl of hydrochloric acid prior to their storage at -20°C.

4.3.1.4: Statistical analysis of pepsinogen release data.

From the pepsinogen concentrations present in the luminal samples, pepsinogen secretion was calculated as the total pepsinogen released per 5 or 20 minute period. For the time course data separate one-way analyses of variance (ANOVA) were performed for each animal, this was to indicate whether any time points were associated with greater mean pepsinogen release than others. Subsequent to ANOVA, Dunnett's test was used to compare mean pepsinogen release for the treated timepoints (i.e. the response to carbachol) to a control mean, in this case the last untreated time point. Dunnett's test was also used to confirm that pepsinogen release during all untreated time points at the beginning of the experiments did not vary significantly and that steady-state conditions had indeed been reached by the end of the equilibration period. For the dose response data mean values were calculated for each animal for each time point, these data were then expressed as the ratio of the secretion in each time interval to that of the last untreated interval. This was to allow the comparison of the results from all five animals where the individual pepsinogen concentrations varied widely. The ratio data was then analysed by one-way ANOVA and Dunnett's test. The above analyses were preceded by Bartlett's test for homogeneity of variance, and if variance was significantly heterogeneous then data were $\log_{10}$-transformed, then resubmitted for Bartlett's test. Differences of the degree of variance were generally not detected after this transformation, and ANOVA and Dunnett's test could then be applied to the transformed data.

4.3.2: Results.

4.3.2.1: Results of investigations into the time course of the response to cholinergic stimulation.

The full results of experiments in tissues from the three animals are given in Appendices 6A to 6C. The results expressed as the mean pepsinogen release over basal for each animal over time are shown in Fig. 4.5. Pepsinogen release was at a steady rate during the first thirty minutes of the experiments in all animals. In two animals pepsinogen release became maximal within twenty minutes of exposure to carbachol. Pepsinogen concentrations in the luminal fluid rose rapidly in the first five minutes to become maximal by 10 minutes in sheep 3 and 15 minutes in sheep 1. In the remaining animal maximal pepsinogen release was only achieved 25 minutes after carbachol addition. Based on Analysis of Variance and Dunnett's test of $\log_{10}$-transformed data, pepsinogen secretion in the first thirty minutes in tissues from all three animals did not vary significantly ($p>0.05$). In tissues from sheep 1 pepsinogen release was significantly greater than basal ($p<0.05$) from the 35th minute until the 60th, and, in sheep 3, from the 35th minute until the end of the experiment (80 minutes). In sheep 2 pepsinogen release was significantly greater than basal only from the 41st minute, and remained so until the end of the experiment (75 minutes).

4.3.2.2: Results of dose response studies.

The full results for the dose response studies are given in Appendices 7A to 7E. The overall mean results are expressed in Fig. 4.6, and ED_{50} and ED_{100} values, which were assessed by visual examination of individual dose response curves, are given in Table 4.1. The dose response curve shows a typical sigmoid shape. Based on Dunnett's test of $\log_{10}$-transformed data from all six experiments, only the responses to 10^{-6} and 10^{-5} M carbachol were significantly greater than basal ($p<0.05$). Maximal stimulation of pepsinogen release was generally achieved by carbachol concentrations of at least 3 micromolar.

Fig. 4.5: The time course of the response to cholinergic stimulation. Mean pepsinogen release, ± S.E., (data expressed as increases above basal) from tissues from three animals, n = 5 tissues per animal (sheep 2 and 3) and n = 2 (sheep 1). (The mucosal bathing fluid was changed and carbachol was added at points as indicated by arrows on the horizontal axis).

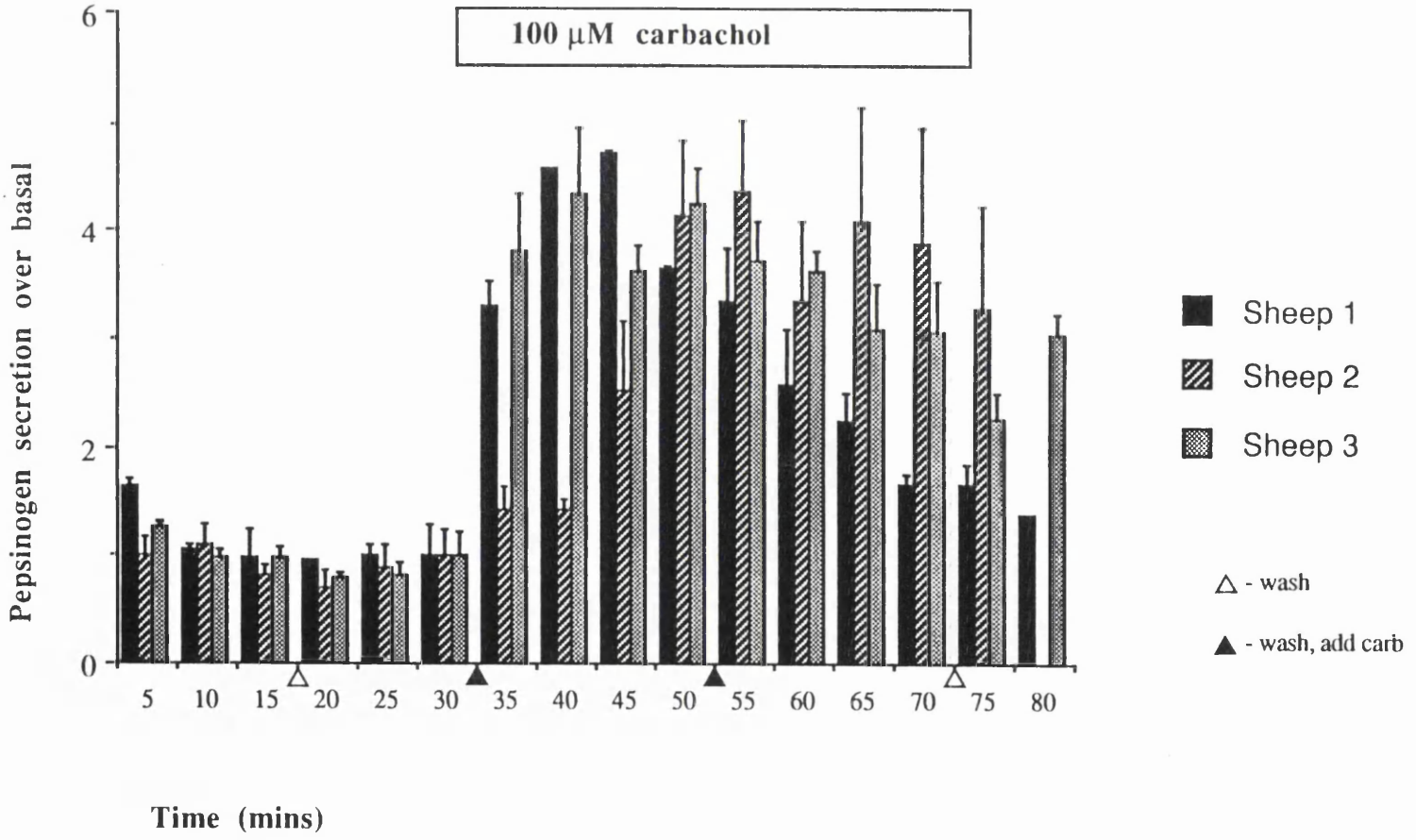
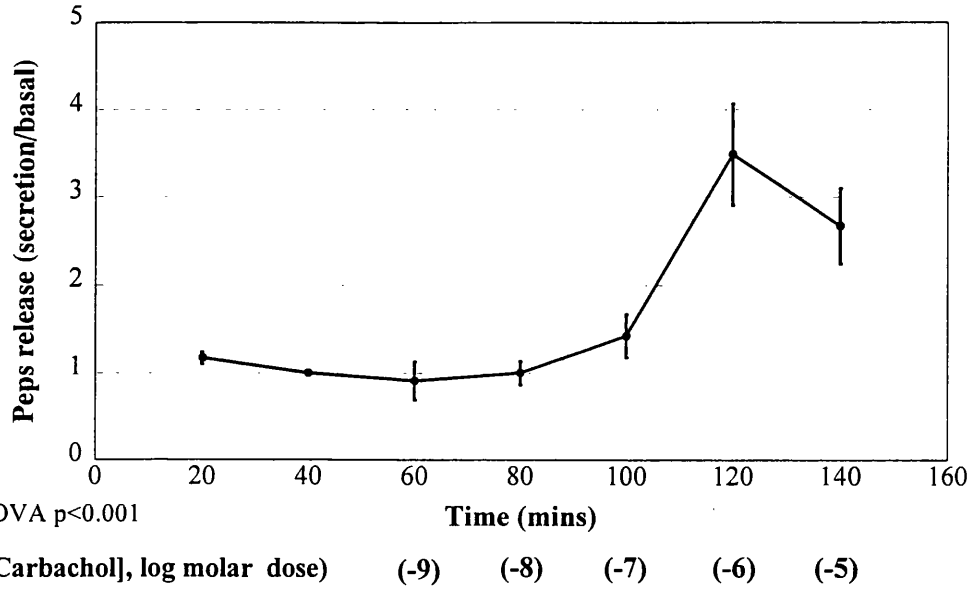


Fig. 4.6: Carbachol Dose Response Curve, showing pepsinogen release from mucosal preparations in response to increasing doses of drug

(mean values, +/- SE, of 5 separate experiments on different animals, each comprising 5 tissues from a single animal)



ANOVA p<0.001

([Carbachol], log molar dose) (-9) (-8) (-7) (-6) (-5)

Table 4.1: Concentrations giving 50 per cent and 100 per cent of the maximal response to Carbachol in studies of mucosal pepsinogen release in individual animals, based on visual examination of individual dose response curves. (molar concentrations).

	ED_{50}	ED_{100}
Animal 1	1.0×10^{-7}	1.0×10^{-6}
2	1.2×10^{-6}	1.0×10^{-5}
3	3.0×10^{-7}	1.0×10^{-6}
4	2.0×10^{-7}	1.0×10^{-6}
5	6.0×10^{-8}	1.0×10^{-6}
Mean (± S.E.)	3.0×10^{-7} (1.0×10^{-7})	2.8×10^{-6} (1.7×10^{-6})

4.3.3: Discussion.

A twenty minute sampling interval would have been adequate for two of the three animals in the time course experiments. The slower rise to peak pepsinogen output in tissues from sheep 2 is important. Tissues from this animal appeared grossly thicker than those of other animals, although an examination of mucosal wet weights revealed that they were not significantly heavier than those of sheep 1 and 3 ($p>0.08$, by balanced ANOVA, fitting sheep as a random effect). In other experiments, exceptionally thick mucosae often failed to give increases in secretion in response to cholinergic or other stimulation (results not shown). In these cases it is possible that secreted pepsinogen was unable to diffuse, or was delayed from diffusing, out of the mucosa. Alternatively, agonists may not have adequately penetrated into the tissues, or a failure of diffusion of oxygen or electrolytes may have caused significant tissue degeneration. It is likely that in most cases twenty minute sampling intervals will allow responses to stimulation to be fully expressed. Abomasa were selected from lighter, smaller sheep at the abattoir for use in subsequent experiments.

Pepsinogen release declined more rapidly in tissues from sheep 1, this might have been due to the gradual depletion of pepsinogen granules from cells. Since the tissues were bathed only in electrolytes, it is unlikely that much de novo synthesis of pepsinogen occurred in these preparations. Because of the potential for decline of pepsinogen granule content in intact mucosae, these preparations are unsuitable for repeat experiments testing more than one treatment.

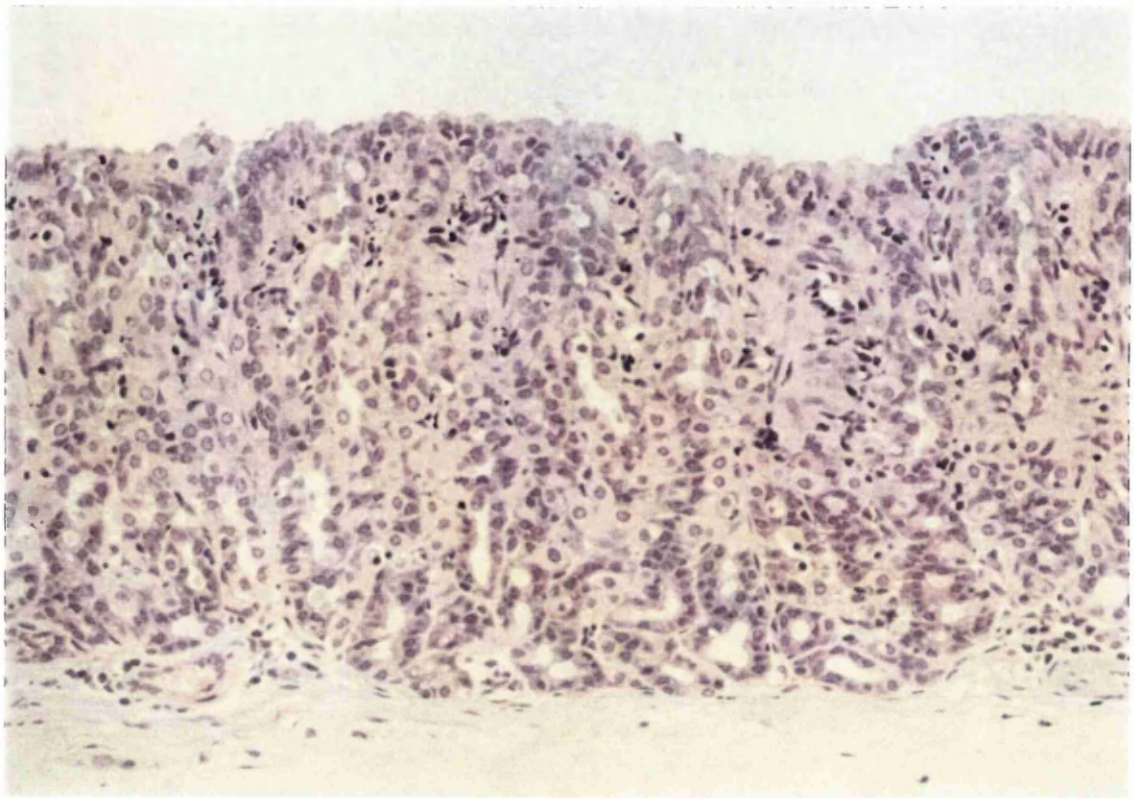
From the dose response studies any dose of carbachol giving a concentration of at least 3 micromolar can be considered to be supramaximal, that is greater than the ED_{100}. Maximal responses to carbachol were generally in the order of three to five times greater than basal secretion. The magnitudes of the responses were substantially less than those reported by Basson et al (1990), who recorded increases of up to forty times basal rates in response to cholinergic stimulation using intact guinea pig mucosae. The same workers reported that basal secretion in guinea pig mucosa was at the rate of 0.3 per cent of the total mucosal pepsinogen content per hour. This compared with 0.4 to 3.0 per cent per hour in isolated rabbit gastric glands, and guinea pig gastric glands and chief cells (Kaskebar et al, 1983; Raufman et al, 1984; Berger and Raufman, 1985). Shirakawa and Hirschowitz (1986) reported that basal pepsinogen secretion from frog oesophageal mucosa mounted in Ussing

chambers was between 16 and 19 per cent of the total pepsinogen content. In the present studies the total pepsinogen content remaining in the mucosae at the end of experiments was measured in mucosal homogenates from some experiments. From these values the total pepsinogen content present at the start of the experiment could be calculated (i.e. by adding the total of all pepsinogen released during the experiment). Basal secretion was then expressed as a percentage of the total present at the start of the experiment. In this manner, from a series of ten experiments, basal secretion was calculated as 7.06 ± 1.76 per cent of total pepsinogen content (mean value ± S.E.) with a range of 1.34 to 16.01 per cent. These values were recorded in tissues giving maximal responses to stimulation of 4.30 ± 0.56 times basal (mean value ± S.E.) with a range of 2.50 to 8.03. Thus basal secretion was higher than in the studies of Basson et al (1990), and maximal responses in comparison were perhaps correspondingly lower. In the present studies it was thought that tissues with higher basal secretory rates generally had lower maximal responses to stimulation, when this was calculated as an increase over basal, but this has not been confirmed.

Fig. 4.7 shows a section of an intact mucosal preparation, placed in fixative at the end of an experiment. The architecture of the pits and glands is well preserved, some glands appear slightly dilated. The muscularis mucosa can be seen, intact at the base of the tissue. Most epithelial cells appeared viable with nuclei containing chromatin apparently normal in appearance. Chief cells contained few granules, supporting the theory that they had depleted during these experiments, and parietal cells showed less cytoplasm than normal, with some containing cytoplasmic vacuoles. In the lamina propria, there was evidence of some tissue destruction with the presence of some pyknotic nuclei, with fragments of dense chromatin. The above findings are consistent with the maintenance of the viability of the chief cell elements of the preparations over the length of incubation normally used.

The intact mucosal sheet preparation was simpler in comparison to the dispersed gastric gland method, but was also the only one of the two methods to prove responsive to cholinergic stimulation, and as such was preferred for use in the subsequent studies of the effects of ES (section 6).

Fig. 4.7: Photomicrograph of a section of an intact mucosal sheet preparation, placed in fixative at the end of the experimental incubation. (original magnification × 50).



5: GASTROINTESTINAL SMOOTH MUSCLE.

5.1: Introduction.

The control of visceral smooth muscle function and motility is no less complex than that of gastrointestinal secretion and involves many of the same regulatory components including the sympathetic and parasympathetic branches of the peripheral nervous system, local nerves of the enteric nervous system and endocrine/paracrine hormonal input. Visceral smooth muscle activity in the gastrointestinal (GI) tract is also regulated by the muscle cells themselves. Gastrointestinal smooth muscle cells have a characteristically oscillatory membrane potential, giving rise to slow waves in membrane potential. Slow waves are the likely result of variability in the activity of sodium/potassium pumps in muscle cell membranes (Grundy, 1985a), and do not themselves result in contractions. They do however elevate resting membrane potential nearer the threshold required for the generation of major depolarisations, action or spike potentials. The overall result is the rhythmic contraction of GI smooth muscle due to spontaneous waves of depolarisation (Caprilli et al, 1982). Smooth muscle cells act in co-ordination with each other and there are recognised pacemaker areas where the frequency of slow waves is greatest. In the small intestine the longitudinal muscle of the proximal duodenum fulfils this function (Ruckebusch, 1981), communicating with the circular muscle and generating a regular cycle of activity, the Migrating Myoelectric Complex (MMC)(Ruckebusch, 1970). Phase I of the MMC lacks contractile activity, and slow waves are the predominant myoelectric activity. In phase II intermittent spiking activity occurs (ISA) as spike potentials become superimposed on the slow waves, these are accompanied by irregular contractions and the frequency of spike potentials increases until the onset of regular spiking activity (RSA) or phase III of the MMC. Migrating myoelectric complexes are propagated aborally and this is associated with the function of the MMC in monogastric animals as an interdigestive housekeeper (Szurszewski, 1969). In monogastric animals the passage of digesta is associated with supervening peristaltic contractions and intestinal contents are mixed by a variety of other activities of smooth muscle such as segmenting contractions. In the ruminant, however, the MMC is uninterrupted by feeding and serves to maintain an almost continuous flow of digesta along the GI tract (Grivel and Ruckebusch, 1972).

Innervation of the GI tract is both intrinsic (via the enteric nervous system) and extrinsic (the autonomic nervous system). Axons from ganglia of the intramural (Auerbach's) plexus, situated between the circular and longitudinal muscle layers, penetrate into both muscle layers. Input to these ganglia include autonomic efferents and axons from chemoreceptors and mechanoreceptors in the muscles and mucosa. A further plexus (Meissner's) situated in the submucosa is mainly involved with secretion and the relay of sensory information from the mucosa. The majority of neurones in the enteric nervous system are interneurones which integrate and assimilate sensory input and mechanical output.

Autonomic control of gastrointestinal smooth muscle is via parasympathetic and sympathetic supply. While it is considered that parasympathetic effects are generally stimulatory for contractile activity and that the sympathetic system has opposite effects, it is probably wrong to view them as simply mutually antagonistic (Gershon and Erde, 1981; Grundy, 1985b). The parasympathetic innervation to the proximal GI tract originates in the brain stem and the preganglionic fibres are contained in the vagus nerve and acetylcholine is the neurotransmitter in virtually all of these fibres (Gershon and Erde, 1981). The preganglionic fibres innervate local ganglia in the GI tissues and the postganglionic fibres may have excitatory or inhibitory effect. Sympathetic innervation of the most proximal parts of the GI tract is via postganglionic and noradrenergic fibres from the cervical and coeliac prevertebral ganglia. Most sympathetic nerves innervate pre-synaptic α_2-adrenoceptors on cholinergic neurones of the intramural plexus, sympathetic activity thereby preventing the release of acetylcholine (Gershon and Erde, 1981; Grundy, 1985c; Mamber and Gershon, 1979). Sympathetic innervation of sphincter areas is however direct and this direct stimulation of visceral smooth muscle cells generally results in hyperpolarisation of the cell membranes, preventing the formation of action potentials. The importance of extrinsic control of GI smooth muscle is dependant on the activity of brain stem nuclei and that of other central areas, and the baseline activity of vagal efferents in ruminants appears to be lower than that of monogastric species (Clark, 1990). In addition to this there is a discrepancy between the number of vagal efferents and the number of enteric ganglion cells such that many intramural cell bodies receive no direct vagal innervation (Weyns et al, 1987).

A striking feature of the enteric nervous system is the abundance of different neurotransmitters involved in signalling and control of visceral smooth muscle activity. In addition to the classical neurotransmitters acetylcholine and noradrenaline, there is ample

evidence that other substances, such as 5-hydroxytryptamine (5-HT, also known as serotonin), many peptides such as VIP, gastrin and CCK, and a variety of other agents such as Adenosine triphosphate and substance P, are present in enteric neurones. Many peptides such as gastrin and CCK function as part of the endocrine regulation of muscle function in addition to their neurotransmitter roles.

Parasites are known to cause anatomical and functional changes in the smooth muscle layers of the gut wall (Castro, 1989). Many parasitic infections have been shown to cause increases of intestinal muscle mass and other studies have demonstrated a non-specific increase in responsiveness of muscle to cholinergic and serotonergic stimuli in rat jejunum following *Trichinella spiralis* infection (Vermillion & Collins, 1988). Similarly in rats with primary nippostrongylosis an increase in muscle mass was accompanied by hyper-responsivity to 5-HT and to electrical stimulation (Farmer and Laniyonu, 1984). In sheep experimentally given concurrent *T. axei* and *C. ovina* infection, disturbances of the MMC, accompanied by the onset of diarrhoea, were detected 3 weeks after initial infection (Bueno et al, 1975), similar findings were found in *T.spiralis* parasitised rats (Castro et al, 1976) and dogs (Schanbacher et al, 1978). Unusual myoelectric activity was seen in *T. spiralis* infected rats as normal MMC frequency became reduced (Palmer et al, 1984). An abnormal but still highly organised electrical event was rapidly propagated aborally along the intestine; referred to as the Migrating Action Potential Complex (MAPC). The MAPC may be associated with more rapid intestinal transit and attempts by the host to eliminate pathogens.

Morphological and functional changes in smooth muscle function in response to infection may be the result of inflammatory reactions, but some workers have postulated the existence of chemical factors released by parasites with direct effects on motility. The intra-venous administration to sheep of an extract of crushed *H. contortus* larvae immediately reduced reticular contractions, followed by a prolonged reduction in abomasal and duodenal motility (Bueno et al, 1982a). Excretory/secretory products from *Nippostrongylus brasiliensis* have been shown to reduce spontaneous contractions in isolated sections of rat intestine (Foster et al, 1994) and a direct effect of ES from the dog heartworm (*Dirofilaria immitis*) on blood vasculature, the depression of endothelium dependant arterial relaxation, has been attributed to a filarial prostaglandin, prostaglandin D_2 (Kaiser et al, 1992). Intestinal transit was prolonged in calves with infections of *O. ostertagi* (Fox et al, 1989a).

Anamnestic and stimulus-specific responses occur as a result of challenge infections of immune animals with parasites. Secondary infection of rats with *T. spiralis* larvae resulted in myoelectric disturbances within 15 minutes of larval administration (Palmer and Castro, 1986). Changes occurring included the appearance of MAPC's and were associated with the rapid expulsion of larvae. Changes could not be elicited by the administration of killed larvae, larval ES or by inoculation with a heterologous parasite such as the coccidian *Eimeria nieschulzi*. Similar mechanisms may have been operating in studies carried out by Stewart (1953, 1955) into the self-cure phenomenon of ovine haemonchosis. Direct intra-abomasal inoculation of sheep already infected with *H. contortus* larvae resulted in increased muscle activity within ten minutes. These responses have been seen as the in vivo equivalents of an in vitro phenomenon, the Schultz-Dale reaction (Schultz, 1910; Dale, 1913; Chand and Eyre, 1978). The Schultz-Dale phenomenon is the contraction elicited in in vitro maintained smooth muscle preparations in response to antigenic stimulation, and only occurs in tissues from animals that have previously been actively or passively sensitised to the antigen. The central event of the Schultz-Dale reaction is thought to be the degranulation of mast cells in the presence of antigen. Antigen cross-linking of bound immunoglobulin, of subclasses IgE and IgG1, themselves already attached via FcεRI receptors at the mast cell surface, stimulates the exocytotic release of chemical mediators from mast cell granules (Plaut et al, 1989; Bissonnette and Befus, 1993; Perdue and McKay, 1993). Mast cell granules in a variety of species are thought to contain numerous mediators including histamine, 5-HT, leukotrienes, platelet-activating factor, prostaglandin D_2, a variety of proteases and nitric oxide (Bissonnette and Befus, 1993; Schwartz, 1984; Tamura et al, 1987). Chand and Eyre (1978) recognised several characteristics of the contractile reaction typical of the Schultz-Dale phenomenon in smooth muscle;

1. The response is anamnestic and stimulus specific, and tissues are sensitive to small doses of antigen. Antigen has no direct action on smooth muscle cells.

2. A latent period exists between the addition of antigen and the onset of the contractile response. This period is of variable length and may be due to the requirement for a build up of released products to effective concentrations.

3. Tachyphylaxis occurs, i.e. the diminishment of the response to a repeated yet identical antigen dose, without affecting the responses to other agents. Tissues recover if

rested for a period of hours and it is thought that tachyphylaxis is due to the depletion of mast cell granules and that time is required for their repletion.

Mast cells are not the only cells mediating manifestations of gastrointestinal anaphylaxis. Neurones of the enteric nervous system have also been implicated. In studies of ionic current changes in immune guinea pig colonic epithelium in response to *T. spiralis* antigen (Wang et al, 1991) mast cell products were thought to stimulate cholinergic nerve activity directly. Frieling et al (1994) also reached the conclusion that signalling between mucosal mast cells and the enteric nervous system was involved in colonic anaphylactic reactions to sensitising antigens. Other studies have shown that in nematode-sensitised animals many mast cells migrate to lie in close proximity to enteric neurones (Stead et al, 1987, 1991).

An investigation of the effects of ES on smooth muscle activity would be an invaluable adjunct to the studies of pepsinogen secretion. The following work was performed to develop a method for the in vitro maintenance of gut smooth muscle, and to test muscle preparations with cholinergic, histaminergic and serotonergic stimulation.

5.2: Isolated smooth muscle preparations.

The following studies were to assess the utility and responsivity of smooth muscle preparations.

5.2.1: Materials and methods.

5.2.1.1: Tissue collection.

The ballooning method used to separate abomasal mucosa from the underlying muscle during the preparation of intact mucosal sheets served a dual purpose since muscle could also then be collected. Muscle cuts were transported back to the laboratory in the same, warmed Ringer's as were the mucosal pieces. The muscle was then transferred to cold (0-4°C) Krebs-Henseleit solution (95 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4 and 11 mM glucose) and stored refrigerated until used. The larger smooth muscle pieces were later pinned to a cork board kept moist by drenching with cold Krebs, the muscle layers were not separated, but by cutting thin strips (up to one millimetre

in width by half to one centimetre in length) in line with the fibres of one muscle layer, the effects of that layer could be studied in isolation. Although the circular muscle layer of the abomasum was thicker the longitudinal layer was generally used as this tended to give less spontaneous activity. Thread was tied securely to each end of the muscle strips and these were then placed in 10 ml jacketed water baths (kept at 37°C) containing Krebs-Henseleit solution bubbled with the 95 per cent O_2 and 5 per cent CO_2 mixture.

5.2.1.2: Isometric measurements of muscle tension.

Tissues were anchored distally to secured metal hooks via the thread. The proximal end of each tissue was attached to an isometric force-transducer (FTO3, Grass Instrument Co., Quincey, Mass., U.S.A.), itself mounted on a sliding rack to allow the height of the transducer, and hence the resting tension in the tissue to be altered. The transducer fed to a Low Level D.C. Pre-amplifier (7P1G, Grass Instrument Co.) which in turn led to a Polygraph D.C. Driver Amplifier (7DAG, Grass Instrument Co.). Amplifier output was recorded as displacement of an ink trace, recorded on chart paper. The system was calibrated by the suspension of 1 to 5 gram weights from the transducer arm, and pen displacement was shown to be in direct relationship to the mass of the suspended weights. The experimental set-up is represented schematically in Fig. 5.1. Tissues were left for 20 minutes before being pretensioned by stretching the muscle strips until a tension equivalent to one gram was registered. The muscles invariably relaxed after initial stretch so that they were retensioned until resting tension was steady at one gram.

5.2.1.3: Carbachol Dose Response studies.

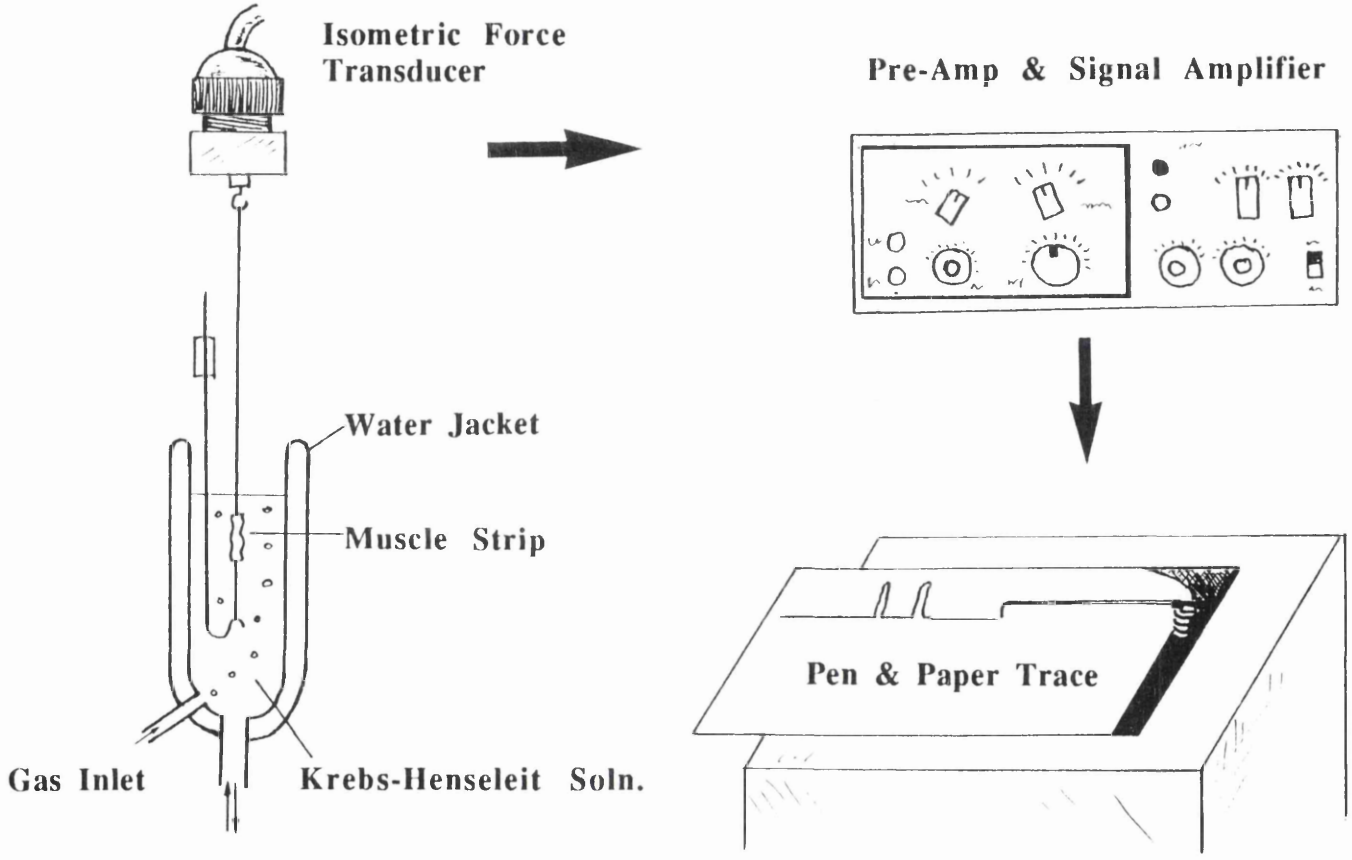
When tissues had been stable at one gram of resting tension for at least twenty minutes, the experiments were begun. Cumulative dose response studies to cholinergic stimulation with carbachol were carried out in muscle strips from four animals. Four muscle strips were tested from each of sheep 1 and 2, three from sheep 3 and six from sheep 4. Unlike dose response studies performed with the mucosal sheets, the bathing fluid was not changed between individual doses of carbachol. Instead the response to each dose was monitored on the trace and when the response to the previous dose had reached a plateau, the next dose was added. Six doses of carbachol were employed, in ten-fold increments of concentration from 1 nanomolar to 0.1 millimolar (10^{-9} M to 10^{-4} M). A similar 0.1 molar carbachol stock solution

and working dilutions as used in section 4.2.1 were prepared for these studies, and the volume of each dose added to the baths did not exceed 100 µl. Data were expressed as increases in grams tension above basal and then as the percentage of the maximal response to carbachol obtained in each tissue. The mean values from each animal were compared by a Kruskall-Wallis test (non-parametric data) to see if responses to any doses of carbachol were greater than others.

5.2.1.4: Studies of histaminergic and serotonergic stimulation.

Similar studies as those of the previous section were performed with longitudinal smooth muscle strips for the addition of histamine and serotonin (5-HT). Both chemicals were prepared as stock solutions in distilled water, both at concentrations of 10 millimolar, from which working, ten-fold dilutions were prepared to facilitate the generation of dose response curves. Investigations measured the responses to cumulative drug doses in tissues from three animals for histamine (sheep 1 to 3) and from two of the three animals for serotonin (sheep 2 and 3). Two tissues were obtained from sheep 1, and four muscle strips were obtained from both sheep 2 and 3. Both agonists were added initially at 1 nanomolar concentrations and increased in ten-fold increments to concentrations of 10 micromolar (serotonin) and 100 micromolar (histamine). The conduct of these experiments was as for section 5.2.1.3, but the responsivity of each muscle strip was checked with a 0.1 millimolar dose of carbachol at the beginning and end of each experiment. Responses to doses of histamine and serotonin were expressed as increases in grams tension above basal and then were expressed as a percentage of the maximal response of each tissue to the final 0.1 millimolar dose of carbachol. A mean was then taken for the response of all tissues from each animal to each dose and a final, overall mean value was obtained to compare all animals. Because of the low number of animals in this study the data were not subjected to further statistical analysis.

Fig. 5.1: Diagram of the experimental set-up to monitor isometric contractions in gastrointestinal smooth muscle.



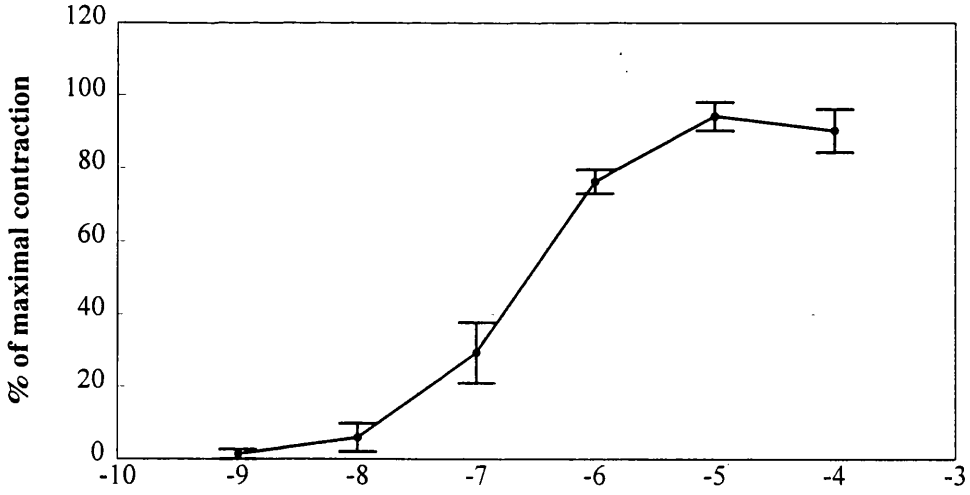
5.2.2: Results.

5.2.2.1: Results of Carbachol Dose response experiments.

Responses to carbachol addition developed rapidly, and reached the plateau of contraction within a few minutes. Full data from these experiments are recorded in Appendices 8A to 8D. The overall mean values of the responses from the four animals can be seen in Fig. 5.2. Maximal responses were obtained at carbachol concentrations of close to 10 micromolar. Responses to the different doses were significantly different ($p<0.002$). The ED_{50} and ED_{100} values for the responses to carbachol are shown in Table 5.1. These values were obtained by the visual examination of the dose response curves for tissues from individual animals. From this data doses of at least 100 micromolar carbachol were likely to give supramaximal stimulation.

Fig. 5.2: Mean carbachol dose response curve of tissues from four sheep.

(mean, +/- SE).



Kruskall-Wallis, $p<0.002$

Table 5.1: Carbachol concentrations giving 50 per cent and 100 per cent of the maximal response in dose response studies with abomasal smooth muscle. (molar concentrations).

	ED_{50}	ED_{100}
Animal 1	3.8×10^{-7}	1×10^{-4}
2	4.3×10^{-7}	1×10^{-5}
3	1.0×10^{-7}	1×10^{-5}
4	2.3×10^{-7}	1×10^{-4}
Mean (± S.E.)	2.8×10^{-7} (7×10^{-8})	5.5×10^{-5} (2.6×10^{-5})

5.2.2.2: Results of histaminergic and serotonergic stimulation.

When responses to either agonist were seen these usually developed as rapidly as responses to carbachol. Full data from these experiments are shown in Appendices 9A to 9E. The results from these experiments are shown in Fig. 5.3. Tissues from two animals (sheep 1 and 3) did not respond to histamine at any concentration, tissues from the third sheep only responded to high concentrations (10 and 100 μM). Responses to both agonists were considerably less than those to carbachol. The response of the tissues from sheep 2 to 100 μM histamine cannot be considered maximal as this would have required that the response to a higher histamine dose be no greater. The responses to serotonin concentrations of 1 and 10 μM were similar in tissues from both sheep tested, but were slightly greater to the higher dose, it is therefore likely that the response to 10 micromolar serotonin is at least close to maximal.

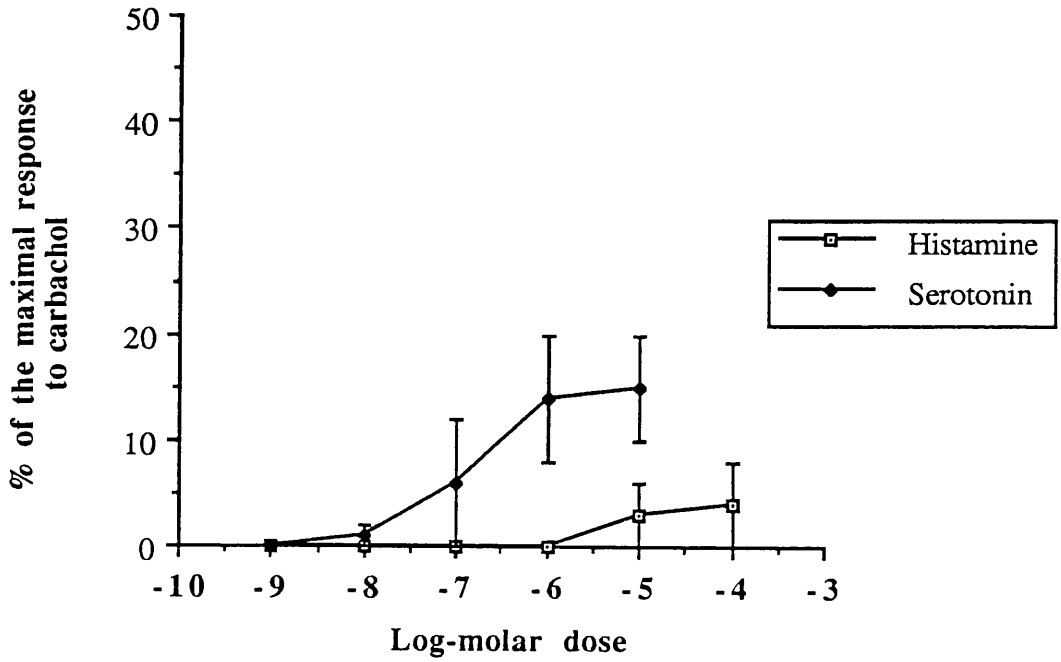
5.2.3: Discussion.

Abomasal longitudinal smooth muscle preparations proved consistently responsive to cholinergic stimulation. Unlike the intact mucosal sheet method where granule pepsinogen content was likely to deplete with time, smooth muscle preparations gave repeatable

responses to repeated agonist doses (results not shown). That carbachol was a consistent stimulus of smooth muscle contraction supports the hypothesis that cholinergic stimulation of abomasal smooth muscle is an important component of the physiological control of gastrointestinal motility in the normal ovine. The results for stimulation with histamine and serotonin suggest that these agents may not be important in the physiological control of ovine abomasal smooth muscle function. A significant advantage of smooth muscle preparations over the intact mucosal sheet studies was the immediacy of the results, contractions to stimulation were seen immediately in the pen trace. For example, when tissues were unresponsive to even carbachol they could be discarded and new tissues set up in their stead. In this way the success rate for this technique, the frequency with which this technique yielded meaningful data, was much higher. There was no need for a time-consuming second assay, such as the pepsinogen assay of luminal samples.

Fig. 5.3: Histamine and Serotonin dose response curves,

mean values, ± S.E., n = 3 animals (histamine), n = 2 animals (serotonin.



6: THE EFFECTS OF ES ON PEPSINOGEN SECRETION AND SMOOTH MUSCLE FUNCTION.

6.1: Introduction.

The potential influence of excretory/secretory products of *Ostertagia* spp. on pepsinogen secretion and smooth muscle function has already been discussed (sections 1.5 & 5.1), and was investigated in this series of experiments using the in vitro methods developed previously, and described in sections 4 and 5. Samples of ES prepared from cultured adult *O. circumcincta* parasites were tested on mucosal tissues and smooth muscle preparations obtained from the abomasa of animals that had prior experience of infection and also on tissues from animals that were known to be parasite-naive. This would determine whether responses to ES were the result of the direct stimulation of glandular secretion or of smooth muscle contraction by ES, or whether responses were hypersensitivity reactions due to the substances present in ES acting as antigens. Given the prevailing animal husbandry systems in the West of Scotland, most sheep in commercial flocks are kept outdoors, and will consequently have grazed nematode-contaminated pastures. Such animals will therefore have developed some degree of immunity to parasitism by nematode genera. *Ostertagia circumcincta* has been shown to be the predominant nematode species associated with parasitic gastroenteritis in sheep in this region of Britain (Stear et al, 1995a). Parasite-naive individuals would only be those that had been reared since birth in conditions likely to eliminate exposure to helminth parasites.

Further experiments would investigate some of the mechanisms of tissue responses to ES. 1) Comparison would be made between the time courses of pepsinogen release and smooth muscle responses to ES and to those due to cholinergic stimulation. 2) Release of the enzyme Lactate dehydrogenase is considered to be an indication of the rates of cell damage in gastrointestinal tissue culture (Koelz et al, 1982) and monitoring the release of LDH would therefore ascertain whether pepsinogen release in response to stimulation was due to; non-cytotoxic degranulation of chief cells, or due to accelerated rates of cell death. 3) An examination would also be made of the role of cyclooxygenase pathways in the generation of

responses to ES, by comparing responses in the presence and absence of Indomethacin (vide infra).

6.2: Responses to ES in tissues obtained from sheep with a history of likely exposure to ostertagiasis.

Experiments in this section tested the actions of adult *Ostertagia circumcincta* ES on tissues from animals that had experienced exposure to the parasites prior to their deaths.

The non-steroidal, anti-inflammatory drug Indomethacin has been used by some workers to enhance the overall responsivity of intact gastric mucosal preparations to stimulation (Reeves and Stables, 1985; Patel and Spraggs, 1992), and the ability of Indomethacin to enhance the responsivity of ovine abomasal mucosal preparations was to be investigated in the present studies. By blocking the production of prostanoids by cyclooxygenase, Indomethacin inhibits responses to agents which act by prostanoid pathways. Such a pathway is known to be involved in the physiological response of gastrointestinal smooth muscle to Epidermal Growth Factor (Hollenberg, 1994). Indomethacin might therefore interfere with the mechanism of any response to parasite ES, and this potential was therefore investigated. Comparison was made of the responses to ES in mucosal sheet and smooth muscle preparations treated with Indomethacin at doses similar to those used by Patel and Spraggs (1992), against those obtained in control tissues not given Indomethacin.

The release of Lactate dehydrogenase (LDH) has been used as an index of cell damage in studies of pepsinogen secretion from isolated gastric glands (Koelz et al, 1982). Measurement of LDH concentrations in the luminal collection fluid from mucosal preparations under basal conditions was therefore used to assess the viability of preparations in the present studies. Observation of changes in LDH concentrations following the addition of ES or carbachol was used to confirm that pepsinogen release in response to stimulation was not due to cell damage.

6.2.1: Materials and methods.

Adult parasite ES was prepared using the methods outlined in section 2.7

Tissues were obtained from sheep killed at Sandyford Abattoir. The ages, sexes, and exact histories, of these animals were not ascertained. The majority of animals killed at Sandyford Abattoir were of the Scottish Blackface breed or its crosses, and were either fattened lambs, born in the previous spring, or surplus stock, such as cast ewes. Intact abomasal mucosal preparations and smooth muscle strips were set up as described previously (sections 4.3. & 5.2). Four or five mucosal preparations and four smooth muscle strips were generally obtained from each animal. In some instances the responses to ES were examined in both tissue sets, set up from the same animal, but this was not so in every case, tissues being collected from some animals for study in only one system.

For the intact mucosal sheet preparations the mucosal bathing fluid was changed every twenty minutes and the luminal solution was collected at the same time to allow the determination of the rates of pepsinogen secretion. Experimental design allowed two twenty minute time periods at the start of each experiment (0 to 40 minutes), with no additions to the baths, to assess whether basal, unstimulated secretion was stable. After this time agents were added, immediately post-washing, to the submucosal bathing fluid. The responses to ES were investigated using a standard dose of any ES batch of 100 µl. Tissues were exposed to ES for twenty minutes (40 to 60 minutes). To ensure the viability of mucosae in these studies all experiments were completed with a final and supramaximal dose of carbachol (100 µM) given at 60 minutes. Experiments were therefore completed after a total of 80 minutes. The carbachol dose served as a positive control to confirm that if there was no response to ES then this was a valid result and not due to a more general failure of tissues to secrete. Acidified luminal samples were stored at -20°C until assay of their pepsinogen concentrations could be performed using the modified method reported earlier (section 3.2).

Experiments involving abomasal smooth muscle strips were conducted in the following way. After the muscle strips had equilibrated to one gram of resting tension, the tissues were given a supramaximal dose of carbachol (final bath concentration of 100 µM) to check that they were responsive. Tissues were then washed, several times, with fresh Krebs and left until the tension developed had returned to basal levels. Tissues were later tested with ES and the paper trace observed to see if contractions resulted. In common with all other agent additions, ES was added at the rate of one per cent or less of the total bath volume (i.e. a maximum of 100 µl per 10 ml baths). Tissues were washed and finally tested with carbachol (supramaximal dose) to ensure that they were still responsive.

The effect of the addition of blank RPMI culture medium on pepsinogen secretion from mucosal sheets and smooth muscle activity was investigated in tissues from four sheep. Equilibrated tissues in both in vitro systems were tested with the addition of 100 µl of blank medium, instead of the dose of ES. Tissues were then given an ES dose and then experiments were completed with a supramaximal dose of carbachol.

Dose response curves were prepared using smooth muscle strips involving serial, ten-fold dilutions of ES (1 in 10,000 to undiluted, raw ES). Dilutions were prepared using distilled water and were added to baths at the rate of 100 µl per 10 ml baths. Tissues from a number of animals (six) were tested in this way, although not all were tested with all ES dilutions. Following ES addition ample time was allowed for the full generation of responses before the next dose was given. Because experiments were performed at different times, different batches of ES were used in individual experiments, this was due to the depletion of the different ES batches with time.

Some experiments in both mucosal sheets and smooth muscle strips were performed in the presence of 3 micromolar Indomethacin. Indomethacin was prepared as a 100 millimolar stock solution in absolute alcohol. Intact mucosal sheets were bathed throughout the experiment with Ringer's solution supplemented with 3 micromolar Indomethacin. Smooth muscle strips were preincubated in the presence of Indomethacin before the addition of ES. Using only smooth muscle preparations the effect of Indomethacin was examined by pre-incubating half the tissues in one experiment (4/8 muscle strips from one animal) with 3 µM Indomethacin, the other half were given the vehicle for Indomethacin (absolute alcohol) at an equivalent dose volume. A dose of ES was then added and the responses to ES in the presence and absence of Indomethacin were compared by a two-sample Student's T-test.

In an initial experiment to assess LDH release, mucosal preparations from one animal were exposed to ES and then carbachol. The luminal fluid was collected in this experiment at the end of each twenty minute period, as per normal, but was not immediately acidified by the addition of hydrochloric acid. Instead assay of LDH was performed soon after sample collection, samples were then acidified to await pepsinogen assay. Assay of LDH concentration was performed using the method detailed in section 2.4.

Pepsinogen secretion for individual mucosal sheets was calculated as the total pepsinogen released in each twenty minute interval. To see if the addition of ES or blank medium in each experiment resulted in greater pepsinogen release, untransformed or $\log_{10}$-transformed data were compared by one-way ANOVA, followed by Dunnett's test. As for the cholinergic studies reported earlier (section 4.3), Dunnett's test determined whether pepsinogen release in the time period when tissues were exposed to ES (or blank medium) was greater than that in the preceding interval when tissues were unstimulated. The mean response to ES per animal was finally expressed as the ratio of the response to basal secretion, i.e. stimulated secretion divided by basal secretion.

For the smooth muscle strips, tension was monitored on the paper trace for the immediate period after the addition of any agents. If the tissues maintained zero spontaneous activity then the pen trace showed no deflections over time and the method was therefore sensitive to very small tension changes in response to agent addition, but if there was any spontaneous muscle activity then responses had to be of sufficient strength to exceed the magnitude of the spontaneous activity. Responses were measured as the distance the pen was deflected beyond the basal line and were then calculated as increases in grams of tension above basal activity. Responses to ES were finally expressed as a percentage of the maximal response obtained to carbachol in the same tissue. Statistical analysis was generally not performed on the data from the studies of smooth muscle, as comparisons would be being made between the tension developed in response to the agent (a positive number) and the basal activity of tissues, which had been arbitrarily set at zero. An appropriate method of statistical analysis would involve the construction of a confidence interval for basal activity, and responses to agents would need to exceed this to be significant, but this would be too cumbersome for routine use without the ability to analyse traces by computer. The mean responses of each animal to ES in the dose response experiments, expressed as the percentage of the maximal response to carbachol, and then as a percentage of the maximal response to ES, were however analysed by a Kruskall-Wallis test, to see if the responses to the different ES dilutions were different.

6.2.2: Results.

The numbers and source of worms used in the preparation of batches of ES, the times of duration of incubations and the final protein concentrations of each batch are given in Table 6.1.

No effect in either in vitro system was observed in response to the addition of blank medium (the relevant data are recorded in Appendices 10A to D). The mean response of all four animals to the addition of blank medium was 0.84 (± 0.04), expressed as an increase of pepsinogen secretion over basal. A figure of 1.0 would indicate pepsinogen release the same as basal, and the figure of less than one indicates that pepsinogen release in the presence of blank medium was in fact less than basal. The addition of blank medium to smooth muscle preparations did not cause any alteration in recorded muscle tension.

The addition of ES consistently resulted in increased pepsinogen release from mucosal sheet preparations and ES consistently stimulated smooth muscle contractions. The results of all experiments testing the effects of ES on pepsinogen secretion and smooth muscle function are summarised in Table 6.2 (full data is listed in Appendices 10A to R, and Appendices 11A to Q). Not included in the list of experiments for pepsinogen release from isolated abomasal mucosal preparations are those experiments were basal, unstimulated secretion did not achieve a steady-state (i.e. pepsinogen release in the first two untreated time points in the experiment were significantly different when analysed by Dunnett's test), and also those experiments were tissues, that did not respond to ES, also failed to respond to the carbachol dose given at the end of each experiment.

The results from a typical experiment examining pepsinogen release in response to the addition of blank medium, an ES batch and then the carbachol control dose, are shown in Fig. 6.1. Pepsinogen release did not increase in response to the addition of blank medium, yet the responses to ES and then to carbachol were significantly greater than basal pepsinogen release.

Table 6.1: ES batches, worm numbers and culture incubation times.

Date	Worms	Number	culture	time	medium	Protein conc. (μg/ml)
27/10/93	*O. circumcincta*	2500	1st	24 hrs	RPMI + Pen/Strep	7.00
			2nd	18 hrs	RPMI + Pen/Strep	8.40
28/10/93	*O. circumcincta*	3500	1st	24 hrs	RPMI + Pen/Strep	N.D.
03/11/93	Nat. infection	2250	1st	24 hrs	RPMI + Pen/Strep	9.80
			2nd	21 hrs	RPMI + Pen/Strep	8.20
05/10/94	*O. circumcincta*	1300	1st	24 hrs	RPMI + Gentamicin	3.80
			2nd	18 hrs	RPMI + Gentamicin	2.80
			3rd	24 hrs	RPMI + Gentamicin	3.20
06/10/94	*O. circumcincta*	3250	1st	24 hrs	RPMI + Gentamicin	5.60

N.D. - Analysis not done (insufficient medium remaining for protein concentration estimation).

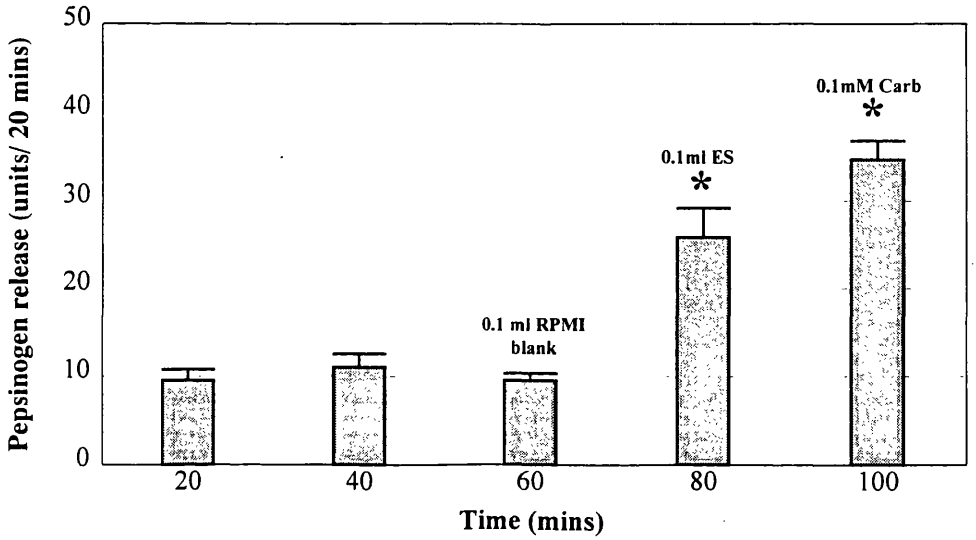
Table 6.2: The magnitudes of responses to ES in studies of mucosal pepsinogen secretion and of smooth muscle contraction. (mean data (± S.E.), n refers to the number of animals from which tissues were obtained and tested with each ES batch).

Batch	Pepsinogen release (increase over basal)	n	Smooth muscle contraction (% maximal response to carbachol)	n
27/10-1	1.80 (0.30)	4	35.00 (12.10) 85.00*	5 1
27/10-2	2.53 (0.58)	5		
28/10-1	2.88	1	8.00 (0.70)	2
03/11-1	2.25 (1.18)	3	0.00	1
05/10-1	6.40* (4.90)	2	17.00* (9.20)	2
06/10-1	1.54 (0.20)	3	85.00*(27.80)	6

* - Experiments performed in the presence of 0.3 μM Indomethacin

Fig. 6.1: Pepsinogen release from intact mucosal sheets in response to the addition of blank medium, an ES batch and carbachol.

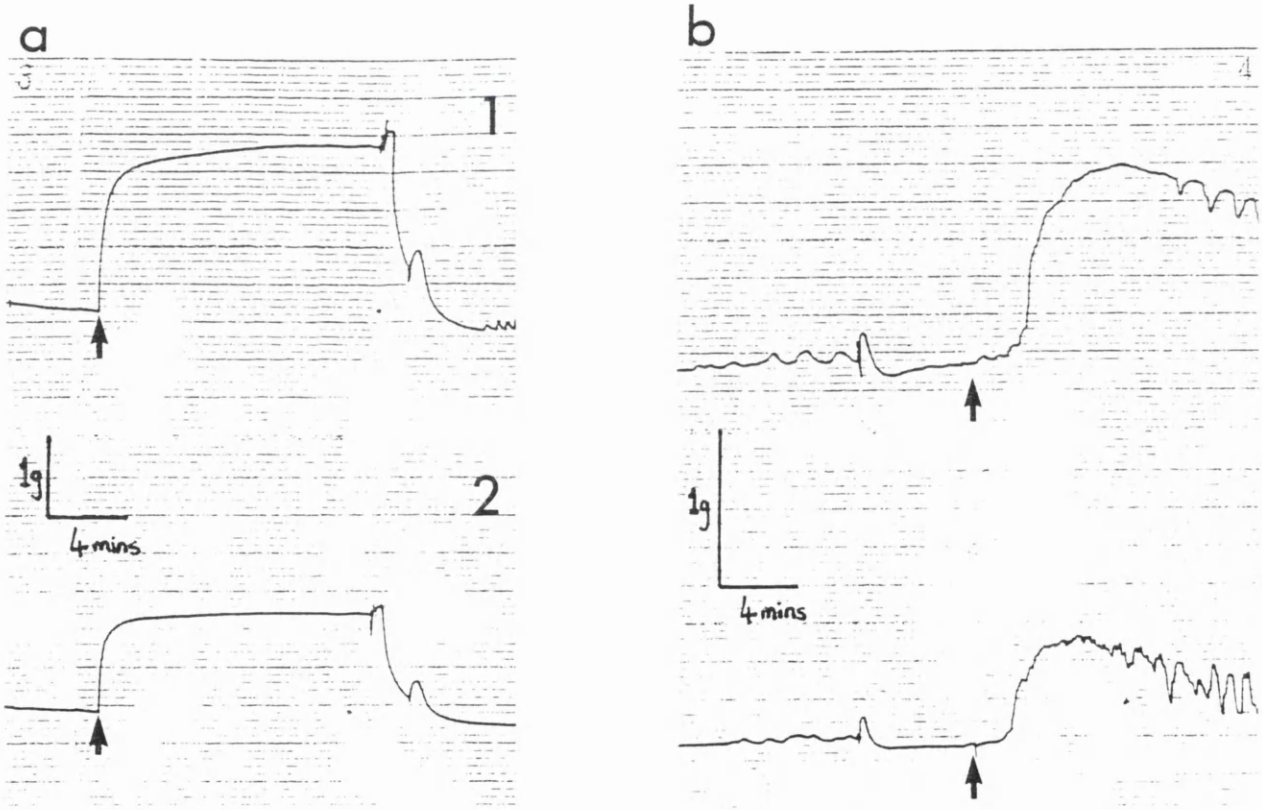
(mean +/-SE, 5 tissues from one animal).



ANOVA p<0.001, * sig. diff from basal (t40) by Dunnett's test.

Fig. 6.2 details a trace typical of experiments involving ES addition to smooth muscle strips. Muscle strips contracted to ES after a lag-period of variable length, the maximal tension developed was typically less than that seen in response to carbachol, and the tissues gradually relaxed and returned to basal tension, whereas tension was generally maintained in carbachol-contracted tissues until they were washed.

Fig. 6.2: Comparison of the responses to Carbachol (a) and ES (b) in abomasal smooth muscle strips. Time of agent addition is indicated by a solid arrow. Note the delay in onset of the response to ES and that the response to carbachol is immediate and larger (note change of vertical scale between a and b).



Despite the use of different ES batches in the preparation of dose response curves for ES, the results were sufficiently similar to allow the direct comparison of all experiments. The results are shown in Fig. 6.3 (full data is shown in Appendices 11A, B, F, J, N and P). Responses to different ES doses were significantly different by Kruskall-Wallis test ($p<0.02$), yet the responses to undiluted ES and to the one in ten dilution were not significantly different in a separate Mann-Whitney U-test ($p=0.08$).

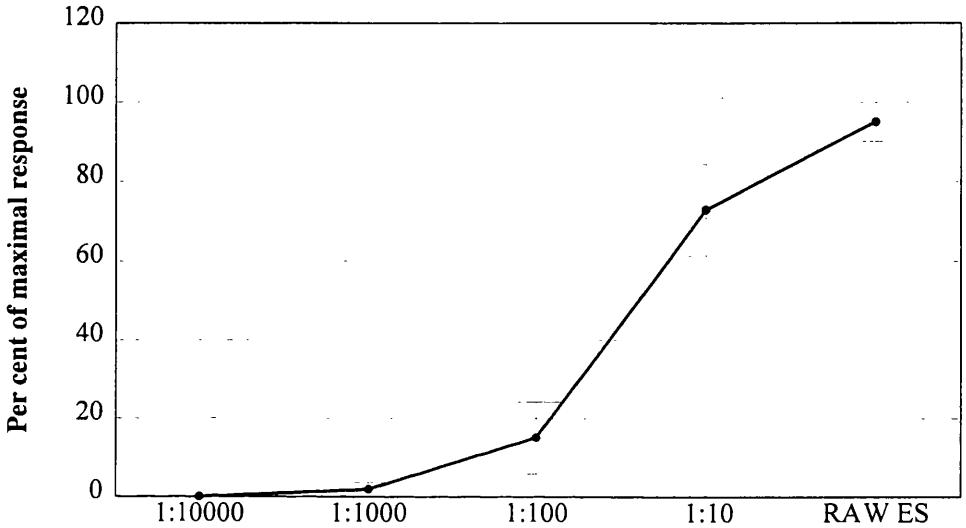
The addition of the vehicle for Indomethacin, absolute ethanol, had no effect on baseline smooth muscle tension. The addition of Indomethacin was occasionally associated with a slow decline in the amount of spontaneous activity exhibited by smooth muscles, but mostly Indomethacin had no effect on basal or spontaneous activity. In tissues from one animal the presence of Indomethacin, added approximately 5 minutes before the addition of ES, was associated with significantly greater responses to ES ($p<0.02$, by two-sample T-test). The results from this experiment are shown in Fig. 6.4. (full data is shown in Appendix 11H). In contrast there was no difference between the contractile responses to a supramaximal dose of carbachol in the presence and absence of Indomethacin. Tissues were stimulated with 100 μM carbachol and once the response had fully developed, the tissues were washed. The tension declined to baseline levels and the tissues were then treated with 0.3 μM Indomethacin, five minutes before the carbachol dose was repeated. The mean (± S.E.) response to carbachol, in terms of grams tension per gram of tissue wet weight, before the addition of Indomethacin was 802.00 (61.00), and after Indomethacin, 741.00 (45.00). These values were not significantly different by two-sample T-test ($p>0.40$). There is insufficient data to examine the responses to ES, in terms of pepsinogen release from intact mucosal sheets, with or without Indomethacin, but Indomethacin did not prevent responses to ES occurring in this system.

The results of an experiment investigating LDH release, mean (± S.E.) values for both pepsinogen release and LDH release, are shown in Fig. 6.5 (full data in Appendix 12). Basal rates of pepsinogen release were widely different in the individual tissues and for this reason data were expressed as pepsinogen release over basal (secretion in the second twenty minute period) for each tissue. Non-normal data distribution required non-parametric analysis. Pepsinogen release during the first two, unstimulated time intervals was acceptably constant and all tissues responded to ES with a small increase in release of the zymogen (median response; 1.50 times basal, n=5). Pepsinogen release did not rise further in response to carbachol, but declined slightly. In contrast LDH release remained fairly constant and at low

levels throughout. A Kruskall-Wallis test showed that pepsinogen release was significantly different ($p<0.01$) at some time points. The same test applied to untransformed data for LDH release detected no significant differences ($p>0.50$).

Fig. 6.3: Dose response curve showing smooth muscle contraction to serially diluted and raw ES batches.

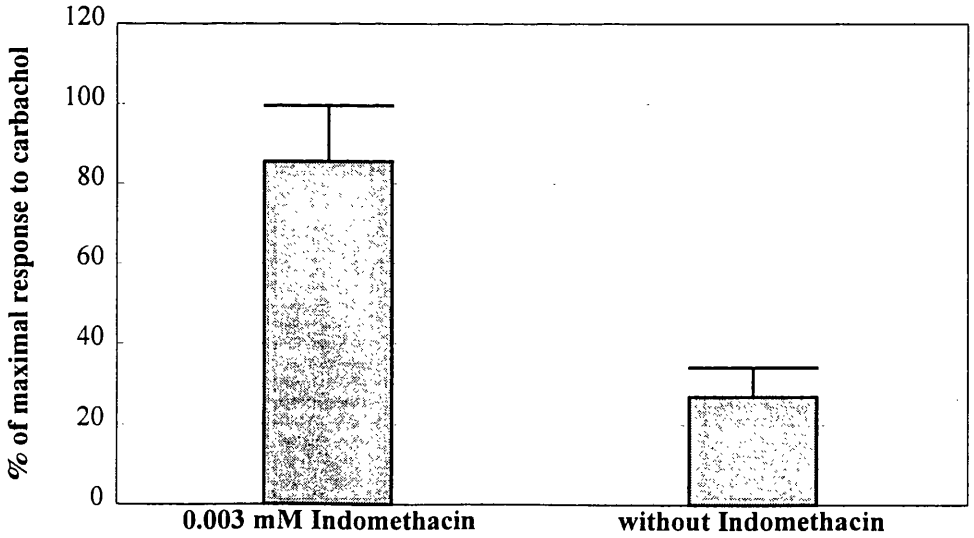
(mean data, +/- SE, tissues from n=1 animal (ES 1:10,000), 2 (ES 1:1,000), 4 (ES 1:100), 6 (ES 1:10 & ES raw).



Kruskall-wallis, $p<0.02$

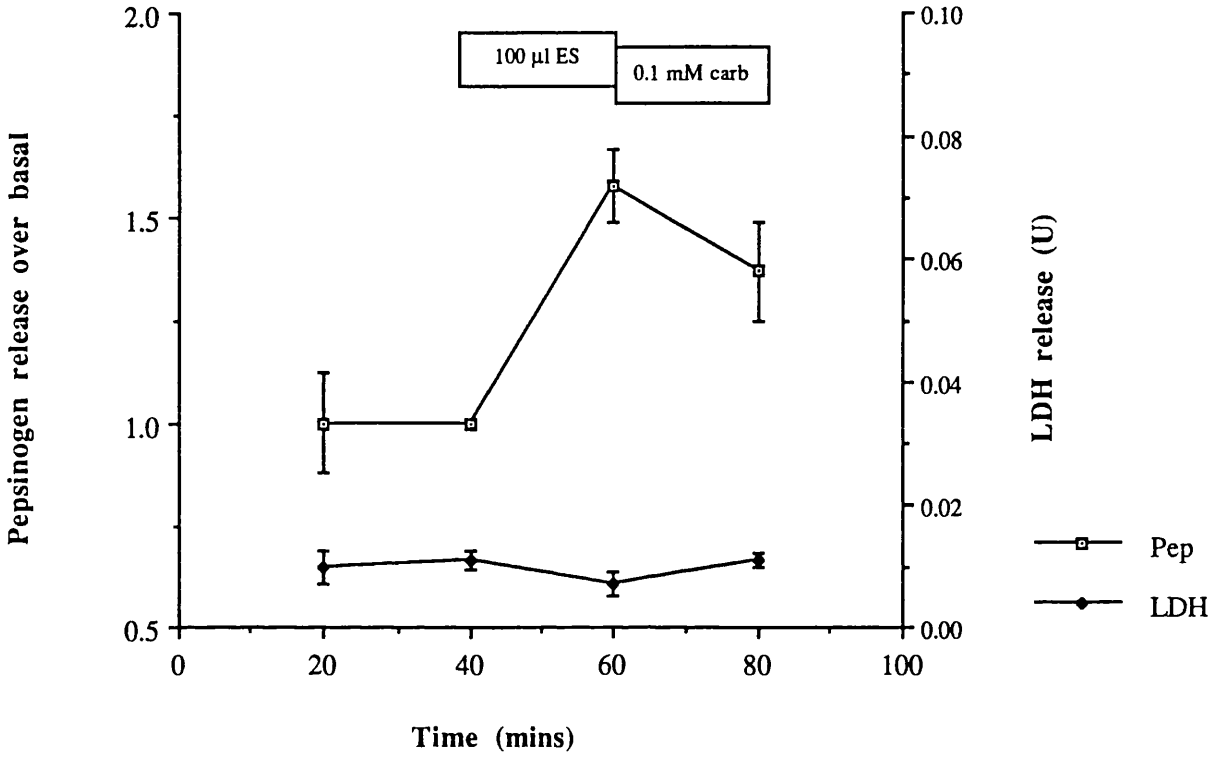
Fig. 6.4: Comparison of the response to ES in smooth muscle strips in the presence and absence of Indomethacin.

(mean +/- SE, 4 muscle strips per treatment).



two-sample T-test, $p<0.02$

Fig. 6.5: Comparison of pepsinogen and LDH release from mucosal preparations from one animal in response to the addition of ES and then Carbachol, mean values (± S.E.), n = 5 tissues (the durations of exposure to ES and then carbachol are indicated by bars).



6.2.3: Discussion.

The addition of ES repeatedly resulted in increased secretion of pepsinogen from intact mucosal sheets and the stimulation of contraction in smooth muscle preparations. It was not possible to say from these results whether this reflected a direct stimulation of host cells by pharmacologically active substances present in ES or whether the effects were simply manifestations of hypersensitivity reactions in animals that were previously exposed to *O. circumcincta* parasites. The lag period of the response to ES in smooth muscle strips suggested that the mechanism of effect of ES relied upon the formation or release of a second chemical, and this is a characteristic of the Schultz-Dale response (see section 5.1). However it is equally a characteristic of physiological responses to agents such as Epidermal Growth Factor (Hollenberg, 1994). The response to ES in terms of pepsinogen release from mucosal tissues, was less than the response to the following carbachol dose (Fig. 6.1), and generally ES responses were less than those to carbachol. A better comparison of the mucosal secretory responses to ES and carbachol would have required that pepsinogen secretion be allowed to

return to basal levels before the addition of the carbachol dose, but here the gradual depletion of pepsinogen content of the tissues might lessen the response to carbachol. A more correct method to compare the two responses would be to treat half of a larger number of tissues with ES and the other half with carbachol, but to be of sufficient value such an experiment would need larger numbers of tissues than could be easily processed together in the present studies. If the responses to ES in mucosal preparations are genuinely smaller than those to carbachol then this agrees with the results of the smooth muscle studies, but this is not to say that the mechanism of the response in both in vitro systems is the same.

The magnitude of the contractions to ES in Indomethacin-treated tissues was greater than in control smooth muscle strips, and Indomethacin did not block the development of responses in intact mucosal preparations. Indomethacin occasionally reduced the extent of spontaneous muscle activity in muscle tissues, but this was not consistently observed. Prostanoid molecules released in response to ES stimulation might have antagonised the development of the smooth muscle contractile response, and the inhibition of their production by Indomethacin might then have allowed the generation of a stronger contraction. Indomethacin had no effect on the response to carbachol, but the dose of carbachol used was supramaximal, and therefore in excess of the minimal effective dose required for a maximal response (the $E.D._{100}$). Indomethacin may have shifted the dose response curve for carbachol (as well as ES) to the left, resulting in a lower concentration for the $E.D._{50}$, but with the response to supramaximal carbachol concentrations remaining unchanged. Further work is clearly needed to not only confirm these results, but also to assess the effects of Indomethacin in intact mucosal preparations and to assess the physiological role of prostanoids in ovine abomasal mucosa and smooth muscle tissues.

Pepsinogen secretion in response to ES appeared to be unrelated to the release of LDH and any potential loss of viability of mucosal preparations. The one experiment investigating LDH release involved tissues that eventually proved poorly responsive to ES and carbachol. The concentration of LDH was calculated from the change in absorption at 340 nm (ΔA_{340}) per minute of the kit reagent to which the sample had been added. The range of ΔA_{340} min^{-1} values in this experiment were low, from 0.000 to 0.004 and were likely to be close to the limit of detection for this assay. The limit of detection was not assessed in the present studies and is not listed by the kit manufacturers. It is possible that LDH was not measurable in the buffering system of the luminal Ringer's solution used.

6.3: The mechanism of the response to ES: the release of Lactate dehydrogenase.

To assess LDH release by mucosal sheet preparations tissues were monitored under steady-state conditions and in response to ES and carbachol, but at the end of the experiment the total remaining tissue LDH and pepsinogen were measured, using this data the rates of release of both LDH and pepsinogen were expressed as percentages of the totals present in tissues at the time.

6.3.1: Materials and methods.

Six mucosal sheet tissue preparations, all from the same animal, were set up, three in group one and three in group two. At the end of the equilibration period the collection of luminal fluid every twenty minutes was begun. After forty minutes ES (100 μl) was added to group one tissues, but not group two tissues. After a further twenty minutes all tissues received a supramaximal dose of carbachol (final bath concentration of 100 μM). To complete the experiment the preparations were disassembled and the mucosal sheets were then homogenised in 4.0 ml of fresh luminal Ringer's and then centrifuged (see section 2.3 for fuller details). In the present experiment Ringer's replaced the Triton X-100 buffer normally used in the preparation of homogenates for the assay of tissue pepsinogen. The concentration of LDH in the luminal samples was assayed and also that of the mucosal homogenate supernatants, after which all were acidified with hydrochloric acid prior to pepsinogen assay.

6.3.2: Results.

The results are shown in Table 6.3. (full data in Appendix 13). The addition of ES did not result in any significant increase in pepsinogen release ($p>0.05$, by ANOVA and Dunnett's test of $\log_{10}$-transformed data). During the period when group one tissues were exposed to ES, mean pepsinogen release increased by 1.30 times basal, whereas in unstimulated, group two tissues during the same period, pepsinogen release also increased slightly over the preceding rate, by 1.10 times basal. All tissues subsequently responded to carbachol, and did so to a similar extent. The mean response of all tissues to carbachol was 8.40 times basal release rates. LDH concentrations were very low at all time points examined during the experiment, the ΔA_{340} min^{-1} values ranged from -0.001 to +0.002, yet considerable activity was recorded

for the mucosal homogenates where the mean ΔA_{340} min^{-1} value of all tissues was 0.231. There were no significant differences in LDH release for any time points.

From the amount of pepsinogen released in the second, unstimulated incubation period, basal pepsinogen release for all tissues was calculated as 8.50 peptic units per hour. Taking the figure of 201.40 peptic units as the mean total pepsinogen still present in the tissues at the end of the experiment and adding the mean amounts released during the experiment, it was calculated that 210.50 units would have been present 20 minutes after the start of the experiment. Based on these figures it was possible to calculate the percentage of the total pepsinogen present that would be secreted at basal rates in one hour, as 4.03 per cent. The same calculation was done for LDH release and the figure obtained was 1.50 per cent.

6.3.3: Discussion.

Values for LDH release were again very low and close to a theoretical limit of detection. However, LDH was measurable in this system as the analysis of mucosal homogenates clearly showed. Hourly basal pepsinogen release exceeded that of LDH release, which implied that pepsinogen secretion by tissues under steady-state conditions, and in response to carbachol at least, was not due to significant tissue damage or degeneration.

Table 6.3: Mean (± S.E.) pepsinogen and LDH release from mucosal tissues: basal release and the responses to the addition of ES and carbachol.

	Group 1		Group 2	
Time (mins)	**Pep (u)**	**LDH (U)**	**Pep**	**LDH**
20	3.02 (0.40)	0.005 (0.002)	2.64 (0.43)	0.005 (0.002)
40	2.70 (0.55)	0.007 (0.004)	2.96 (0.24)	0.009 (0.002)
60	3.56 (0.13)	0.002 (0.002)	3.34 (0.66)	0.005 (0.005)
80	25.73* (1.81)	0.002 (0.002)	21.85* (2.44)	0.005 (0.002)

Table 6.3 (continued from previous page) - Notes

Group 1; given ES, batch 27/10/93 (2nd culture), after 40 minutes.

Group 2; controls, not given ES.

All tissues given 100 μM carbachol after 60 minutes.

* - Sig. different from basal, t40 ($p<0.05$), by ANOVA and Dunnett's test, on log_{10}-transformed data.

6.4: The influence of the Non-steroidal Anti-inflammatory Drug, Indomethacin, on mucosal responsivity.

The effect of Indomethacin on the responsivity of intact mucosal sheets was further investigated in tissues obtained from one animal. The rates of basal secretion of pepsinogen and LDH were monitored in tissues exposed to Indomethacin and in tissues not exposed to the drug, the responses to ES and carbachol were also monitored.

6.4.1: Materials and methods.

Six mucosal tissue preparations, all from one animal, were set up, three of which were bathed, during a three hour equilibration period and during the experiment proper, with Ringer's solution supplemented with 3 μM Indomethacin, the others in normal Ringer's. After the forty minute period without additions to the baths all tissues received 100 μl of ES (Batch 27/10/93 - 2nd culture). At the end of the subsequent twenty minute period the tissues were washed and then left for a further twenty minutes before carbachol (final bath concentration of 100 μM) was added. The luminal fluid samples, collected as usual every twenty minutes, were not immediately acidified, but were analysed for LDH activity. At the end of the experiment all tissues were homogenised in 4.0 ml of luminal Ringer's solution, to allow the total tissue LDH to be measured. Acidified samples and homogenate supernatants were then frozen until pepsinogen concentration was assayed. Data for Indomethacin-treated and control tissues were analysed separately by ANOVA and Dunnett's test, after log_{10}-transformation.

6.4.2: Results.

The results for pepsinogen release with time are shown in Fig. 6.6. (full data in Appendix 14). Based on ANOVA and Dunnett's tests the responses to carbachol were significantly greater

than basal (secretion in the second, twenty minute time interval) in both tissue sets, however only the response to ES in Indomethacin-treated tissues was significantly greater than basal. Basal secretion was at a greater rate in control tissues than in Indomethacin-treated tissues. The hourly rate of basal secretion for control tissues was calculated as 7.94 ± 0.21 (mean ± S.E.) per cent of the total pepsinogen present and this figure was significantly greater ($p<0.001$, by two sample T-test) than that obtained for Indomethacin-treated tissues, i.e. 3.74 ± 0.14 per cent.

As in previous investigations the ΔA_{340} min^{-1} values for estimates of LDH in the sampled luminal collection fluid were close to zero, values ranged from -0.001 to 0.002, the values for mucosal homogenates were again much larger, and the mean (± S.E.) value for the total LDH contained in all six tissues was calculated as 1.76 (0.12) U. Assuming a maximum ΔA_{340} min^{-1} value of 0.002 for the analysis of samples from the second twenty minute period, giving total hourly basal release of LDH of 0.040 U, hourly LDH release would still only be 2.30 per cent of the total LDH contained in tissues, a figure smaller than both rates of pepsinogen release. There were no differences in LDH release between the two tissue groups (control and Indomethacin-treated) and no differences at any time point. The results for release of LDH with time are shown in Table 6.4.

6.4.3: Discussion.

In the present experiment basal release of pepsinogen exceeded the release of LDH and basal pepsinogen secretion again appears to be a physiological property of intact mucosal preparations that is unrelated to rates of cell death/damage.

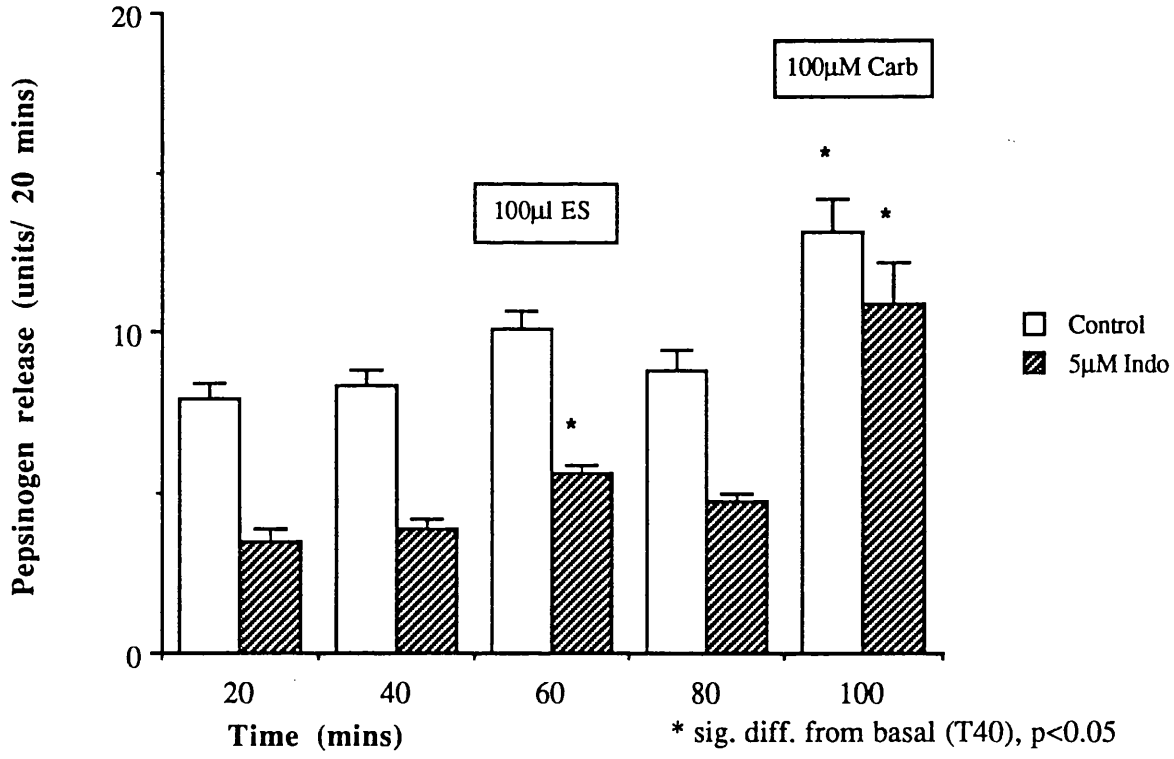
Treatment with Indomethacin lowered basal pepsinogen secretion. The assumption must be that cyclooxygenase products contributed to the maintenance of higher basal rates of secretion. In general, if responses to agents are expressed as the ratio of the secretion in response to the agent, to basal secretion (x/y), then preparations with lower basal rates of secretion (y) will give greater responses, even when the actual difference between the magnitude of responses and basal secretion (x-y) remains the same. This was the case in the present experiment, but the differences in the responses to ES expressed as increases over basal between control and Indomethacin-treated tissues were still not significantly different.

Indomethacin treatment may indeed enhance the responsivity of mucosal preparations, but further work is needed to confirm these findings.

Table 6.4: Mean (± S.E.) values for LDH release per twenty minute periods in Indomethacin-treated and control tissues (n = 3 tissues per treatment).

Time (mins)	Control tissue LDH (U)	Indo-treated LDH
20	0.005 (0.002)	0.005 (0.002)
40	0.000 (0.000)	0.002 (0.002)
60	0.007 (0.004)	0.002 (0.002)
80	0.005 (0.005)	0.002 (0.002)
100	0.002 (0.002)	0.002 (0.002)

Fig. 6.6: Comparison of the pepsinogen released from mucosal tissues from one animal in the presence and absence of 3 μM Indomethacin, mean values (± S.E.), n = 3 tissues (the durations of exposure to ES and carbachol are indicated by bars).



6.5: The time course of the response to ES in intact mucosal preparations.

Initial experiments of the effects of ES on smooth muscle function had shown that there was generally a delay, or lag period, between the addition of ES and any increase in recorded muscle tension. Responses to carbachol in the same tissues were however more immediate. It was proposed to see if a similar lag period existed between the addition of ES to intact mucosal preparations and the release of pepsinogen.

6.5.1: Materials and methods.

The time course of the pepsinogen release response to ES was investigated in tissues from two animals. Tissues were maintained in the presence of 3 μM Indomethacin throughout the experiments. Tissues were left unstimulated for thirty minutes, with the mucosal bathing fluid replaced every fifteen minutes, but the luminal fluid was collected every ten minutes. At the end of the thirty minutes and after the mucosal bathing fluid had been changed, ES (100 μl) was added to the tissues. The dose of ES was repeated after a further twenty minutes. After the initial thirty minutes the mucosal bathing fluid was changed at twenty minute intervals, but the luminal solution was still collected at ten minute intervals. Tissues were in the presence of ES for forty minutes, at the end of which the tissues were washed and then left untreated for the next ten minutes before the experiments were completed with supramaximal doses of carbachol (100 micromolar). Tissues from the two animals were not tested with the same ES batch, the 27/10/93 (2nd. culture) batch being used with tissues from sheep 1 and the 05/10/94 (1st. culture) batch used with tissues from sheep 2. Data were finally expressed as the mean pepsinogen released over basal (30 minutes) for each time point for each animal.

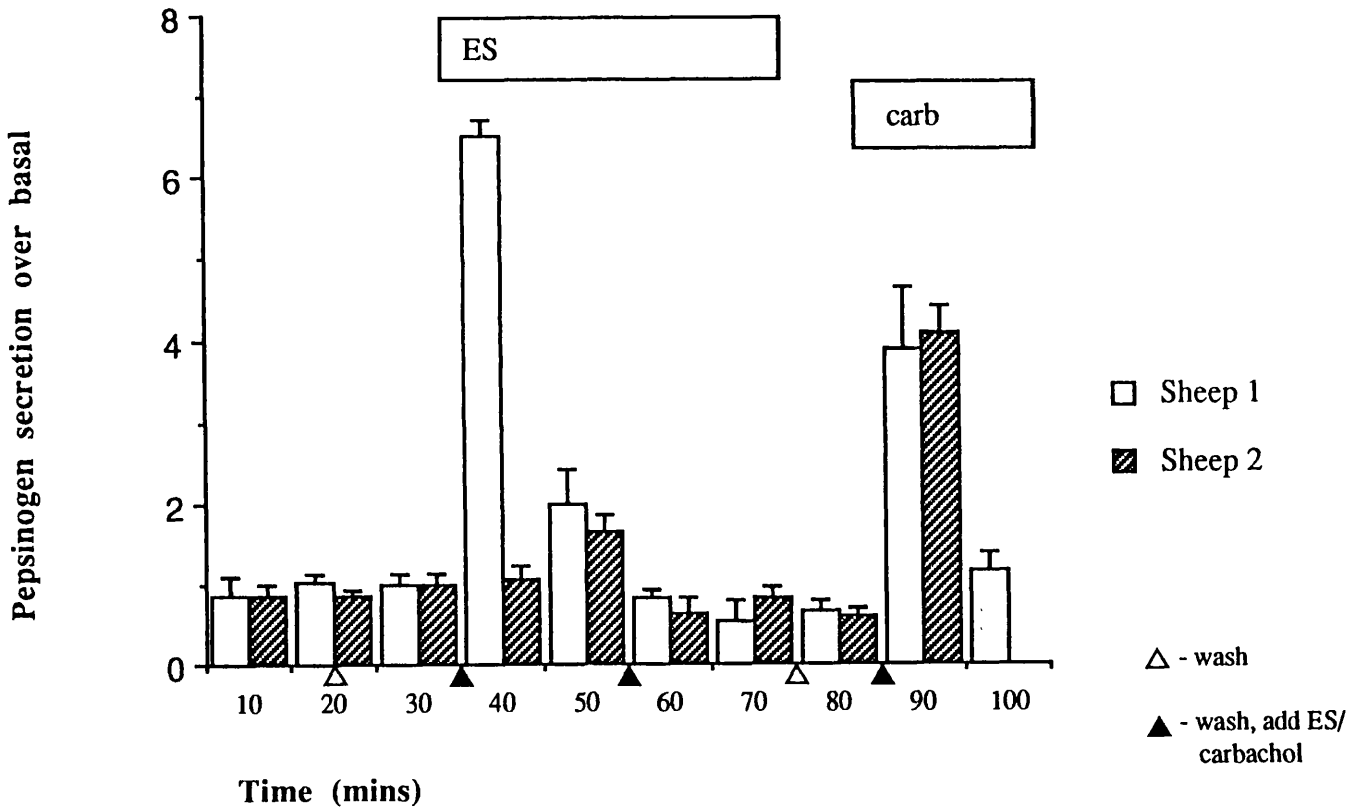
6.5.2: Results.

The results are shown in Fig. 6.7. (full data in Appendices 15A and B). The mean response to ES in sheep one tissues was large and immediate, occurring in the first ten minutes after ES addition. In sheep two however the response was delayed to the second ten minute interval and the magnitude of the response was much less. The response to carbachol in both animals was rapid, occurring within ten minutes, but declined as rapidly as the response to ES in sheep one tissues.

6.5.3: Discussion.

The two sets of tissues from the two animals behaved quite differently in their responses to the addition of ES, but behaved similarly with carbachol. Either the different animals differed in their ability to respond to the same stimulus (the addition of ES) or the different batches of ES used meant that the stimuli were not directly comparable. However as smooth muscle contractions generally occurred within five minutes of the addition of ES, the response in tissues of sheep 1 might still have been delayed. Earlier experiments examining the time course of the responses to only carbachol have shown that the intact mucosal preparations are variable in the speed with which responses develop, yet in smooth muscle studies responses to carbachol were always immediate. In the present study, in the tissues of sheep 2, the response to ES was delayed, but that to carbachol given subsequently was immediate, there is therefore some evidence that, like the responses in smooth muscle strips, a lag-period may be a feature of the mucosal tissue responses to ES.

Fig. 6.7: Time course of the response to ES in tissues from two animals, (mean response (± S.E.), n = 3 tissues, sheep 1 and n = 5 tissues, sheep 2 (the durations of exposure to ES and carbachol are indicated by bars)).



6.6: Responses to ES in tissues obtained from parasite-naive animals.

To test the hypothesis that responses to ES, observed previously in both in vitro systems, were immune manifestations, analogous to the Schultz-Dale response, mucosal and smooth muscle tissues were obtained from the abomasa of parasite-naive animals and were tested with ES.

6.6.1: Materials and methods.

6.6.1.1: Animals.

A total of eight parasite-naive animals were used. The first four were pure-bred Hampshire Down female lambs of approximately six months of age (animals 1 to 4). These animals had been reared indoors in conditions likely to eliminate the risk of exposure to nematodes. Faeces was collected from the rectum of each animal for faecal egg counts to confirm the abscence of patent infection. The four further parasite-naive animals were Scottish Blackface lambs of mixed sex, all were of less than one week of age (animals 5 to 8). The lambs had been orphaned soon after birth, and fed milk-replacer until they were killed. All eight animals were killed by either electrical stunning, or captive bolt, followed by exsanguination, abomasal mucosal and smooth muscle tissues were then collected.

6.6.1.2: Experimental procedure.

The experiments involving the intact mucosal preparations and smooth muscle strips of the parasite-naive animals in this section were conducted in an identical manner as the experiments involving tissues obtained from animals killed at Sandyford Abattoir (section 5.2). In studies of pepsinogen secretion from intact mucosal sheets, tissues were stimulated with ES (100 µl) in the third twenty minute period and then the preparations were checked with the carbachol control dose in the fourth period. Muscle strips that had equilibrated to resting tension levels and then checked by a supramaximal carbachol dose were given the standard dose of ES and then ample time was allowed to see if any contractions resulted, all tissues were then checked at the end of the experiments with a final dose of carbachol. The responses of longitudinal and circular smooth muscle from the abomasum of sheep 1 were tested, and tissues from sheep 1 and 2 were examined for the response to ES in the presence

and absence of 3 micromolar Indomethacin. Indomethacin was not used in any of the experiments involving the intact mucosal preparations.

The experiments in this section used a newly collected batch of ES (Batch 06/10/94). The only exception to the procedure adopted for the experiments using the mucosal preparations was that involving tissues from animal 1. The tissues of animal one, the mucosae and the muscle strips, were tested with the new ES batch on the same day as the ES had been harvested, and because this new batch had not been tested before all the tissues from animal 1 were also given a second dose of ES (Batch 27/10/94 - 1st. culture) that had already been shown to stimulate responses in tissues from animals from the abattoir population. The mucosal preparations from animal 1 were left unstimulated for a full hour before the addition of the new ES batch at the start of the fourth twenty minute time period, after a further twenty minutes all the tissues were then given the second ES batch, and were finally tested with carbachol in the sixth and last twenty minute period. Because of the depletion of the new ES batch experiments involving the mucosal preparations of the last animal tested (animal 8) and the muscle strips of the last two animals (7 and 8) had to be accomplished using a further ES batch (05/10/94 - 1st culture).

The data from the intact mucosal experiments for individual animals were analysed by ANOVA and Dunnett's test following appropriate transformations. The responses of all muscle strips to ES were expressed as the percentage of the maximal responses to carbachol occurring in the same tissues.

6.6.2: Results.

The results of the experiments involving the mucosal preparations and smooth muscle strips of all animals are given in Tables 6.5 and 6.6 respectively (full data in Appendices 16 A to H and 17 A to H). The intact mucosal preparations from sheep 1 did not respond to either ES batch and sheep 2 tissues did not respond to the new ES batch. Tissues from both sheep 1 and 2 did respond however to carbachol. The responses to carbachol in sheep 1 and 2 tissues were 5.59 and 3.87 times basal respectively. The data from the experiments with the mucosal tissues of animals 3 and 4 were rejected because tissues from these animals failed to achieve a steady basal secretory rate and did not respond to either ES or carbachol. The addition of ES to the mucosal tissues from the lambs did not result in any significant increase in

pepsinogen release. The mean (± S.E.) value for all lambs of pepsinogen release in the presence of ES was 1.00 (0.13) times basal release (release in the second twenty minute period). All tissues were however highly responsive to the subsequent carbachol dose, the mean response being 15.70 (5.90) times basal. The 95 per cent confidence interval for the mean response of mucosal tissues of all parasite-naive animals to ES (calculated from the ratios of secretion in response to ES to basal secretion), was 0.78 to 1.22. The figure of 1.00 clearly lies within this range so that the mean response to ES is not significantly different from basal, unstimulated secretion. In comparison the 95 per cent confidence interval for the mean response of all mucosal tissues from previously-exposed animals (Table 6.2) to various ES batches was 1.39 to 3.80.

Smooth muscle strips from all eight animals were responsive to cholinergic stimulation. However none responded to any ES batch tested. Circular smooth muscle strips from animal 1 also did not respond to ES and the presence of Indomethacin had no effect on the response to ES in circular muscle of sheep 1 and in longitudinal muscle from sheep 2. The addition of ES did not affect the frequency or the amplitude of the spontaneous contractile activity manifest in tissues from sheep 4, the same tissues did subsequently respond to carbachol (100μM), see Fig. 6.8. In comparison the 95 per cent confidence interval for the mean response of all muscle strips from previously-exposed animals (Table 6.2) to ES was 19 to 73 per cent of the responses to carbachol occurring in the same tissues.

Table 6.5: Mean (±S.E.) pepsinogen release (u/20 minute period) from intact mucosal sheets derived from the parasite-naive animals. Pepsinogen expressed as the total units released per twenty minute time intervals.

	Animal							
(Animal 1)	**1**	**2**	**5**	**6**	**7**	**8**	(Animals 2-8)	
Time (mins)							**Time (mins)**	
20	1.02 (0.14)	9.10 (1.92)	3.87 (1.02)	0.90 (0.33)	1.58 (0.50)	9.20 (1.73)		**20**
40	0.98 (0.12)	9.33 (1.96)	3.97 (1.47)	1.03 (0.52)	1.32 (0.42)	7.42 (1.04)		**40**
60	0.84 (0.18)	8.53 (1.25)	2.93 (0.53)	1.23 (0.45)	1.70 (0.52)	6.38 (0.74)	**(ES-1)**	**60**
80 (ES-1)	0.86 (0.07)	36.10* (5.51)	23.01* (5.94)	26.98* (4.01)	33.73* (4.89)	38.83* (0.79)	**(carb)**	**80**
100 (ES-2)	0.62 (0.17)							
120 (carb)	4.70* (0.75)							
ES-1	06/10/94	06/10/94	06/10/94	06/10/94	06/10/94	05/10/94 (1st.)		
ES-2	27/10/93 (1st)							
tissue no.	5	5	5	5	3	6		
ANOVA	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**	**p<0.001**		

Notes;
ANOVA; results of one-way analysis of variance performed on $\log_{10}$-transformed data (Animals 1 and 6; $\log_{10}(x+1)$-transformed data).
* - pepsinogen release significantly different from basal (last untreated time interval), when analysed by Dunnett's test ($p<0.05$).

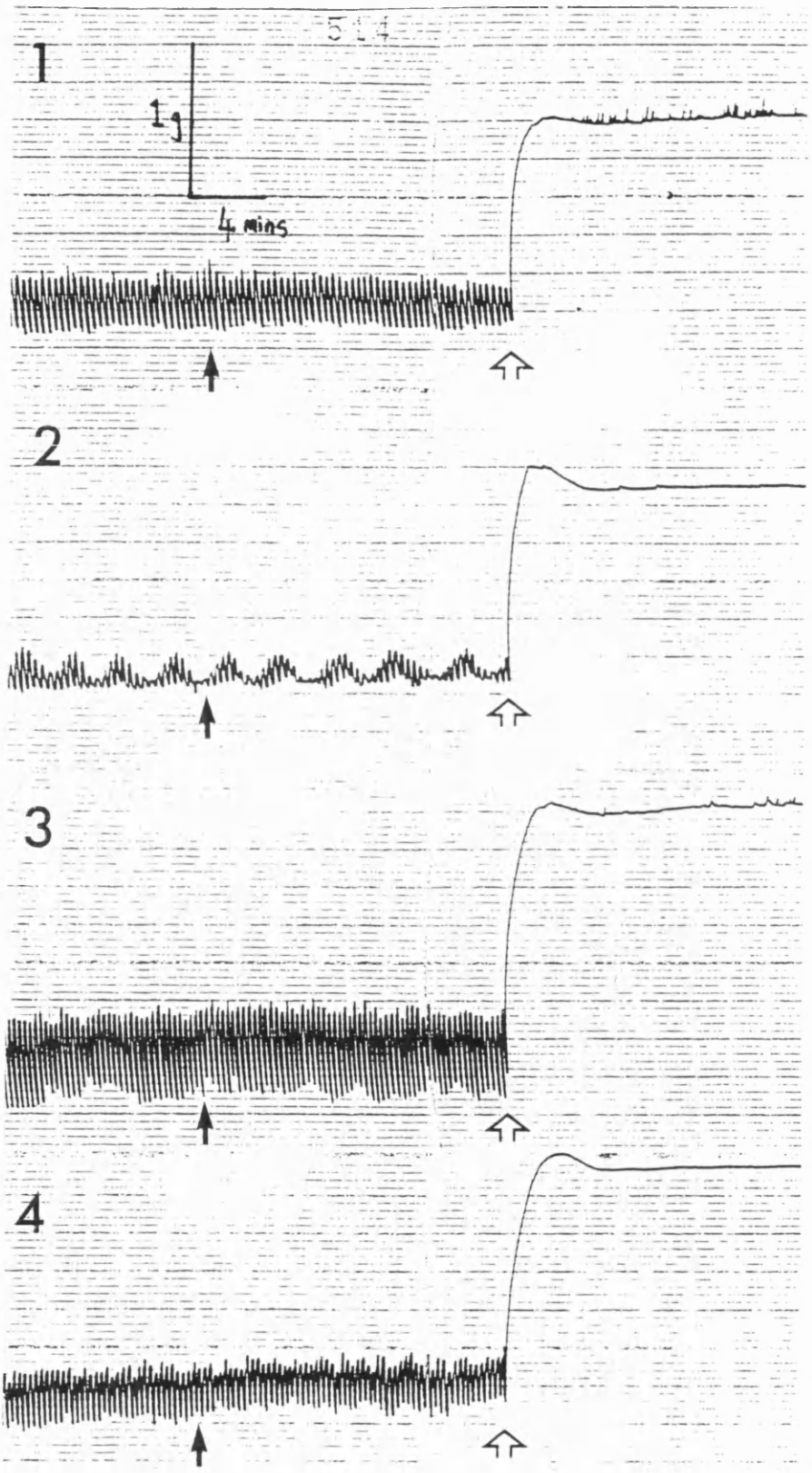
Table 6.6: Mean (± S.E.) responses of smooth muscle strips from parasite-naive animals to various ES batches. Responses expressed as a percentage of the response to 0.1 millimolar carbachol in the same tissues (see Appendices 17A to H).

Animal	Tissue type	± Indomethacin (3μM)	ES batch	no. of tissues	Response
1	long.	-	06/10/94	4	0.00 (0.00)
		-	27/10/93 (1st)	4	0.00 (0.00)
		-	05/10/94 (2nd)	4	0.00 (0.00)
	circ.	-	06/10/94	4	0.00 (0.00)
		-	05/10/94 (1st)	4	0.00 (0.00)
		+	06/10/94	4	0.00 (0.00)
2	long.	-	06/10/94	6	0.00 (0.00)
		+		4	0.00 (0.00)
3	long.	-	06/10/94	4	0.00 (0.00)
4	long.	-	06/10/94	4	0.00 (0.00)
5	long.	-	06/10/94	4	0.00 (0.00)
6	long.	-	06/10/94	4	0.00 (0.00)
7	long.	-	05/10/94 (1st.)	4	0.00 (0.00)
8	long.	-	05/10/94 (1st.)	4	0.00 (0.00)

Notes;

long. - longitudinal smooth muscle, circ. - circular smooth muscle.

Fig 6.8: The lack of effect of ES on the spontaneous muscle activity in tissues from a parasite-naive Hampshire, (point of ES addition indicated by solid arrows, that of carbachol, by open arrows).



6.6.3: Discussion.

The most important finding of the present studies was the complete lack of effect, in both in vitro systems, of *O. circumcincta* ES on tissues from parasite-naive animals. All batches of ES tested in parasite-naive animals were shown to elicit responses in tissues from animals from the abattoir population, animals that were likely to have been previously exposed to abomasal parasitism. Therefore the simplest and likeliest explanation of the results of the present studies is that responses to ES in tissues from animals previously exposed to parasites, were hypersensitivity responses to antigens present in ES. The mechanism of the response may have involved mast cell degranulation in response to antigen cross-linking of immunoglobulin bound to mast cell membranes, and the release of bioactive compounds from the mast cells. The excretory/secretory products of adult *O. circumcincta* have been shown to contain a number of different molecular weight substances (Wedrychowicz, 1986) many of which reacted with immune sera, and thus many substances present in ES could be responsible for the hypersensitivity responses seen.

The nature of the bioactive substances released by mast cells in sheep are not well known and in the present studies neither histamine nor serotonin were effective at stimulating smooth muscle contraction at likely physiological concentrations. The non-steroidal anti-inflammatory drug, Indomethacin, actually enhanced the response to ES, so it is unlikely that the responses to ES are due to the production of a prostaglandin by mast cells or other immune cells. Investigations into the mechanism of response will not be straightforward. The mast cell stabilising drugs Quercetin (Fewtrell and Gomperts, 1977) and Cromoglycate (Altounyan, 1967), may prevent mast cell degranulation by interfering with intracellular Ca^{2+} and these agents will therefore also interfere with smooth muscle responses to stimulation which are sensitive to changes in calcium (Fanning et al, 1983) and may also interfere with the exocytosis of pepsinogen granule contents.

Using dispersed bovine and ovine gastric glands obtained from parasite-naive animals, McKellar et al (1990b) demonstrated stimulation of pepsinogen release in response to *Ostertagia* spp. ES. Worm culture in the study of McKellar et al (1990b) used a different method from that used in the present experiments, since worms were cultured in distilled water for thirty minutes and antibiotics were not included. Some bacteria, for example *Escherechia coli*, are known to liberate toxins capable of stimulating mammalian tissues (De

Jong and Vaandrager, 1986) and their presence in worm cultures would complicate any subsequent conclusions about the effects of ES. McKellar et al (1990b) achieved partial block of the response to ES with the antimuscarinic drug, atropine, and therefore postulated that the agent was cholinergic in nature. It would be expected that both in vitro preparations in the present studies would have responded to such an agent if it had been present, and in sufficient concentration. The worm culture technique employed in the present studies was chosen to maximise the yield from the available worms by culturing worms for at least 24 hours. It is possible that the culture conditions in the present studies may have prevented the release of the agent that was previously present in ES from worms maintained in distilled water. The stimuli for the release of any ES component from *Ostertagia* spp. worms remain unknown.

EXPERIMENTAL STUDIES - PART 3: PEPSINOGEN IMMUNOHISTOCHEMISTRY.

7: THE IMMUNOHISTOCHEMICAL LOCALISATION OF PEPSINOGEN IN RUMINANT ABOMASA AND THE EFFECTS OF OSTERTAGIASIS.

7.1: Introduction.

7.1.1: Abomasal cell populations.

Exocrine secretion in the ruminant forestomachs is confined to the fundic and pyloric areas of the abomasal mucosa. The surface area of the fundic mucosa is increased by the presence of numerous folds, lying roughly in-line with the longitudinal axis of the abomasum. Fundic mucosa is the site of the majority of abomasal acid and peptic secretory activity. Cells of both fundic and pyloric areas are situated in one of two structures (represented schematically in Fig. 7.1). Invaginated from the mucosal surface are the pits (foveoli), at the base of which arise usually two or three, blind-ending tubular glands which penetrate deeper into the mucosa. The pit-gland junction is called the isthmus. The glands can be subdivided into neck, adjacent to the isthmus, and base regions. Pits in the fundus are shallow and glands are long and straight, whereas in pyloric mucosa the pits are often deeper with correspondingly shorter glands that are coiled. The greatest diversity of cells is found in fundic mucosa. Murray (1970) identified five main cell types in bovine fundic mucosa; the surface epithelial cells, lining the mucosal surface and the pits, the mucous neck cells, the parietal cells, the chief cells and the endocrine cells. In pyloric mucosa Murray (1970) noted four cell types, the surface epithelial cells, the pyloric gland cells, parietal cells, albeit in fewer numbers in pyloric areas, and the endocrine cells. Surface epithelial cells have also been called surface mucous cells (Cybulski and Andren, 1990; Karam and Leblond, 1993b).

Karam and Leblond (1992) distinguished eleven morphologically distinct cell types in mouse gastric corpus (equivalent to bovine fundus). In addition to those already described they identified other cells as more intermediate in nature from one type to another, and in further work traced the progression of all cell types from undifferentiated stem cells in the

isthmus (Karam, 1993; Karam and Leblond, 1993a,b,c,d). Karam and Leblond (1993a) identified three undifferentiated and granule free cell types present in the isthmus in mouse corpus. The first (subtype I) was identified as the pluripotential stem cell. This cell reproduces to renew itself and to give rise to two other granule free, precursor cells (subtypes II and III), and the granule-free pre-parietal cell (variant 1). Granule-free cells of subtypes II and III were distinguishable by the type of pro-secretory vesicles present adjacent to the golgi apparatus and were regarded as pre-pit cell precursors and pre-neck cell precursors respectively. In the mouse subtype II precursors may also give rise to a small number of variant 2 pre-parietal cells, named because of their possession of a small number of granules similar to those of pre-pit cells. Subtype III precursors give rise to pre-neck cells with granules similar to those of mucous neck cells and also give rise to variant 3 pre-parietal cells. Pre-neck cells migrate down the gland and become mucous neck cells and then zymogenic (chief) cells. Cells that have undergone some differentiation may still undergo mitotic cell division so that all cells of the pit/gland can be classified into one of four groups based on the frequency that each cell divides (Karam and Leblond, 1993a). Parietal and pre-parietal cells (all variants) do not divide. Zymogenic cells, pre-zymogenic cells (cells intermediate between mucous neck cells and zymogenic cells) and entero-endocrine cells divide only occasionally. Mucous neck cells and pit cells divide regularly, but at a low rate. Granule-free precursor cells and the pre-pit cell precursors and pre-neck cell precursors have the highest rates of cell divisions.

In the mouse pre-pit cells migrate towards the gastric lumen, and reach it as mature mucous cells in approximately 3 days (Karam and Leblond, 1993b). Initially a few secretory granules are scattered in the pre-pit cell cytoplasm, these granules increase in number and become aligned at the apical margin, thus marking the transition to mature pit cells. Granule number and size increase further in mid and higher pit levels, but cell organelles begin to show signs of degeneration, the mitochondria and nucleoli diminish in size and the number of free ribosomes decreases. By the time pit cells reach the free surface the cells produce fewer and smaller granules and degeneration of organelles is more advanced. The cells become spindle shaped, with the greatest cell volume, including the nucleus, situated apically, but also with fewer granules, and there are often spaces between the basal cytoplasm of neighbouring cells. Cells are eventually lost into the gastric lumen or are phagocytosed by a neighbouring cell that is itself eventually shed.

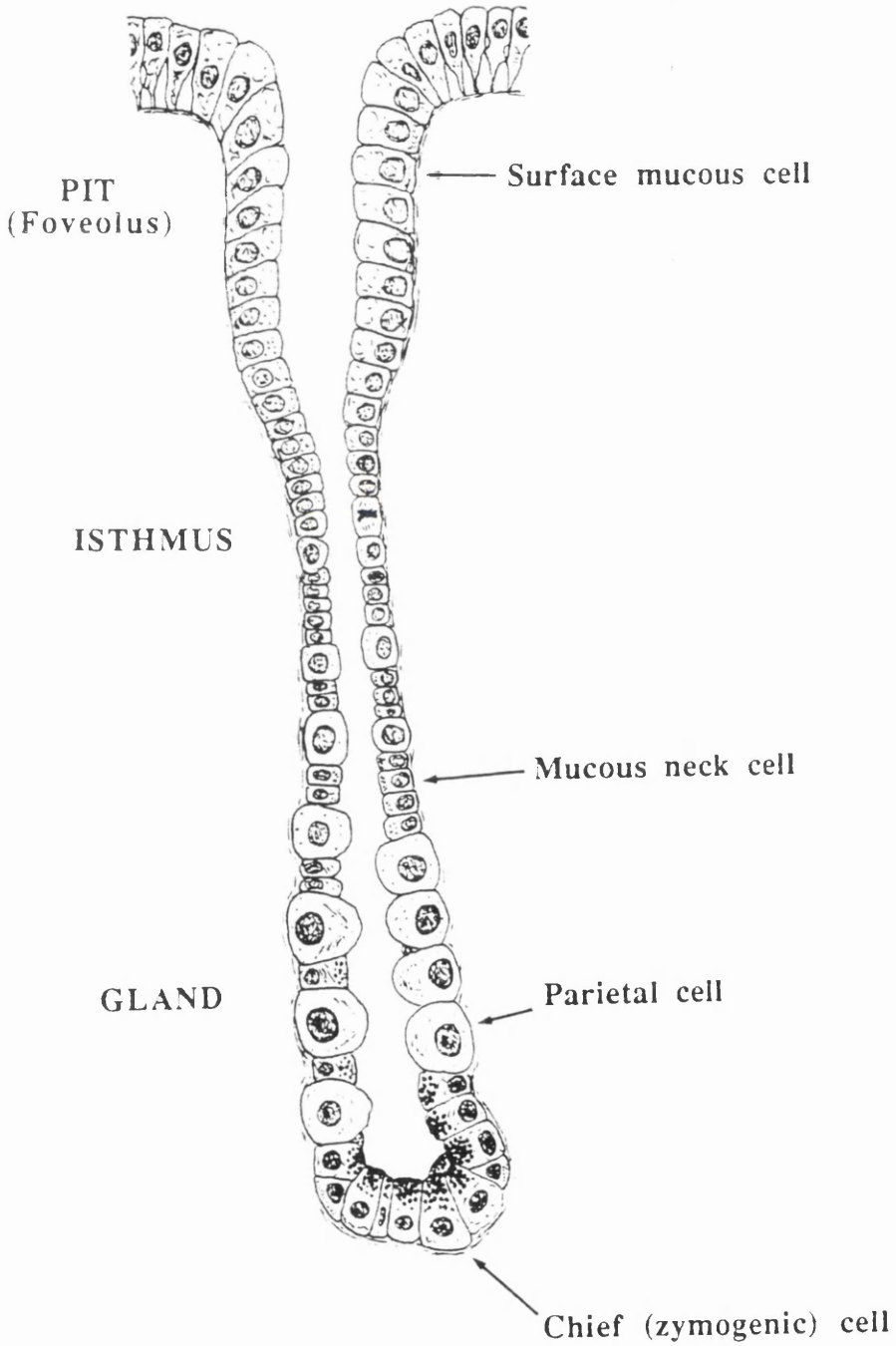
In mice, developing parietal cells migrate from the isthmus to both the pit and the gland. (Karam, 1993). The upward migration of pre-parietal cells presumably does not occur in the bovine as no parietal cells were present in the pits of bovine fundic mucosa (Murray, 1970). The average turnover time of parietal cells at different levels of the pit/gland units in the mouse was 54 days (Karam, 1993), that of parietal cells in the gland, 189 days. In the gland base 75 per cent of parietal cells were histologically normal, the remainder showed signs of degeneration and two per cent were dead (Karam, 1993). Parietal cell death occurs by necrosis, whereby parietal cells are shed from the gland base into the lumen, and by apoptosis, which is followed by phagocytosis of parietal cells by zymogenic cells, or an invading macrophage from the lamina propria (Karam, 1993).

Approximately two thirds of neck cells arise due to the migration and differentiation of pre-neck cell precursors, the remaining neck cells arising from their own mitoses (Karam and Leblond, 1993c). Turnover time for mucous neck cells has been calculated as between one and two weeks, at the end of which neck cells have become pre-zymogenic cells, which in turn become chief cells after a further month. The turnover time for chief cells in the gland base was calculated to be almost half a year. Dead chief cells are lost by the same mechanisms as parietal cells. The granules of neck, pre-zymogenic and chief cells illustrate the maturational changes taking place. The ultrastructural appearance of the granules varies with the fixation and post-fixation techniques used in tissue preparation (Murray, 1970; Karam and Leblond, 1992), but many studies show a similar pattern. The mucous neck cell granule has a core, usually situated eccentrically, and this feature is common to the bovine (Murray, 1970; Yamada et al, 1988), the guinea pig (Bouhours et al, 1981) and the mouse (Karam and Leblond, 1993c). The core represents pepsinogens (Yamada et al, 1988), the outer material being mucins. The granule of the chief cell is almost entirely pepsinogen, however a thin rim of mucin may remain (Bouhours et al, 1981). Karam and Leblond (1993c) showed a progression of granule appearance from neck cells to chief cells, characterised by gradually diminishing mucin content.

The granules of surface mucous cells may be more heterogeneous in appearance. Electron-dense and electron-lucent granules coexisted in mucous cells in the studies of Murray (1970). The appearance of pyloric gland cell granules was similar. Some surface mucous cell granules contain cores (Karam and Leblond, 1993b). In immunofluorescence studies of bovine fundus, surface mucous cells contained pepsinogen as well as the related zymogens

prochymosin and progastricsin (Cybulski and Andren, 1990). Pepsinogen was not demonstrated to be present in surface mucous cells of young calves by electron immunohistochemistry (Yamada et al, 1988).

Fig. 7.1: Schematic representation of a pit and gland from fundic mucosa.



7.1.2: Immunology of pepsinogens and their tissue distributions.

Up to seven, electrophoretically-distinct mammalian pepsinogens/pepsins have been identified (Hirschowitz, 1984; Hersey, 1987). The similarity of primary and tertiary structure of all the carboxyl (aspartyl) proteases, a group that includes pepsinogens, chymosin (the neo-natal, milk-clotting enzyme), renin and some cathepsins, implies that they may have evolved from a common ancestral peptide (Tang, 1979). Pepsinogens are further separable into two groups, pepsinogens of types I and II, on the basis of the commonality of major antigenic determinants, the pH optima for the proteolytic activity of the corresponding pepsins and the distribution of pepsinogens in tissues.

Using specific antibodies, pepsinogens of groups I and II (PG I and PG II) are distinct. Molecular weight estimates of pepsinogens from a wide range of species, including amphibians, fish and mammals, range from 29 to 65 kD. Much of this weight variation is accounted for by differences in the N-terminal sequence that is lost upon activation to pepsin (Foltman and Pederson, 1976; Ichihara et al, 1982; Schugerman et al, 1982), however there are differences in the catalytic peptide as well (Schugerman et al, 1982). Despite such differences the major antigenic determinants and the structure of the active site are consistent across species and indeed across phyla. One human pepsinogen gene encodes a 373 amino acid peptide with 82% homology to a porcine pepsinogen (Sogawa et al, 1983). The low molecular weight pepsinogen of frogs is immunologically indistinguishable from human PG I (Schugerman et al, 1982).

The distinction between pepsinogens of groups I and II is also present when their biochemistry and that of the corresponding pepsins is examined. The pH optima of the five human group I pepsins lie between pH 1.5 and 2.0 and for the two group II pepsins, the pH optimum is 3.2 (Samloff, 1983). Group I pepsinogens are more susceptible to denaturation in alkali than those of group II since denaturation occurs at pH 7.2 and pH 8.0 respectively, but they are less susceptible to heat (PG I, 16 per cent, after 15 minutes at 62 °C at pH 2.4, versus PG II, 80 per cent) (Samloff, 1983). Their peptide bond specificities with artificial dipeptide substrates and their rates of hydrolysis of different protein substrates also differ (Hirschowitz, 1984).

The distribution of PG I and PG II within tissues has been studied using immunofluorescent techniques. All studies have confirmed the original hypothesis of Langley (1881) that the chief cell of the gastric fundus is the major source of pepsinogen in mammals, but demonstrate that it is not the only cell type synthesising pepsinogen. Chief and mucous neck cells of the human fundic gastric gland were shown to contain both PG I and PG II (Samloff, 1971b, Samloff and Liebman, 1973). However cells of the cardiac and pyloric glands of the stomach and the Brunner's glands of the duodenum produced only PG II (Samloff and Liebman, 1973). The human prostate secretes PG II into the seminal fluid, although the significance of this is unknown (Samloff and Liebman, 1972). It is thought that all pepsinogens are filtered by the kidneys, but that PG II is reabsorbed, so that only PG I is present in urine, and PG II is consequently present in serum at a slightly higher level than its abundance in gastrointestinal and other tissues would suggest (Hirschowitz, 1984).

In the bovine, four group I pepsinogens, and one of group II, have been identified (Eckersall et al, 1987; Mostofa et al, 1990a). Only three pepsinogen subtypes, all group I, were identified in the sheep and goat (Mostofa et al, 1990a). The distributions of PG I and PG II in the bovine abomasum have recently been studied immunohistochemically and the pattern is similar to that observed in human gastric mucosa. In the milk-fed calf, prochymosin, the zymogenic precursor of chymosin, and PG I co-localised to mucous neck cells and chief cells. In concentrate fed calves and older cattle the distribution of pepsinogen remained similar whereas expression of prochymosin declined and the number of prochymosin producing cells was highly correlated to the level of milk-feeding (Andren et al, 1982). In further studies (Cybulski and Andren, 1990), prochymosin, PG I and PG II all co-localised to similar cell populations in both fundic and pyloric mucosae. Chymosin was the predominant zymogen present in 17 day old, suckling calves and was present in chief and mucous neck cells of the fundus and in pyloric gland cells. At this stage PG I was mostly confined to mucous neck cells. PG II immunostaining was minimal at 17 days, but by 45 days prominent staining could be seen in surface mucous cells of fundic and pyloric mucosa and in deep cells of pyloric glands. Calves were still milk fed to some degree at 45 days. In 180 day old, entirely concentrate-fed calves chymosin-specific staining had declined with slight staining only present in mucous neck cells and in pyloric glands. Type I pepsinogens were widely distributed in many epithelial cells, including surface mucous cells in the pits of both fundic and pyloric mucosae and PG II was prominent in surface mucous cells and in pyloric glands. The presence of PG I in pyloric mucosa was the one significant departure from the pattern

observed in humans (Samloff, 1971; Samloff and Liebman, 1972). In electron immunohistochemical studies of the mucosae of 1 month old, milk-fed calves, PG I and prochymosin were demonstrable in the same individual granules of chief and mucous neck cells and in cells intermediate in appearance between the two (Yamada et al, 1988).

There are no reports of the immunohistochemical localisation of pepsinogens in sheep, or of the distribution of pepsinogens in any ruminant animals infected with abomasal nematode parasites. In studies of Haematoxylin and Eosin stained sections, Stringfellow and Madden (1979) noted that chief cells of calf abomasa were entirely depleted of granules by 26 days after infection with *O. ostertagi* larvae, yet plasma pepsinogen concentrations were still high. They argued that pepsinogen was no longer being synthesised in the abomasum, indeed abomasal pepsin concentrations were zero at this time, and hypothesised that pepsinogen was retained in the circulation due to a long elimination half-life. However the half-life of pepsinogen is likely to be short (vide supra) and there may have been some continuing source of pepsinogen in these calves. Prolonged stimulation of pepsinogen secretion may not involve granule formation (see section 4.1), however Stringfellow and Madden (1979) reported that chief cells showing reduced granule content, had degenerative features and did not give the appearance of cells actively secreting pepsinogen.

Mucous cell hyperplasia is a recognised feature of the pathology of abomasal and gastric helminthoses and cell types other than chief cells can potentially provide a vast reserve of cells capable of producing pepsinogens if production by chief cells ceases. The following studies were initiated to assess the distribution of pepsinogen in normal, parasite-naive cattle and sheep, using a commercially available and polyclonal, anti-bovine PG I antiserum. It was assumed that, using this antiserum, bovine and ovine PG I would cross react and cross-reaction was assessed by a parallel study of the staining patterns of bovine and ovine abomasal mucosa. It was further proposed to investigate the distribution of PG I in parasitised animals; to ascertain whether changes in the mucosal cell population due to parasitism would result in changes in the number of cells producing pepsinogen, or changes in the zymogen content of individual cells. Biochemical studies of the peptic activity present in mucosal tissues were designed to support the immunohistochemical findings.

7.2: Preliminary investigations of bovine and ovine mucosae.

The following studies were primarily to test the suitability of using antibody raised against the bovine zymogen in the detection of its ovine counterpart, and to compare the staining patterns of abomasal mucosae from parasite-naive cattle and sheep for pepsinogen. It was further proposed to assess whether changes in the immunohistochemical localisation of pepsinogen, in response to parasitic infections of cattle and sheep, were readily apparent.

In the in vitro studies of abomasal secretion in section 5.6, tissues from lambs less than one week old were used. Tissues from these animals responded to cholinergic stimulation by releasing material with pepsin-like activity, i.e. acid stable proteolytic activity. Only small amounts of pepsinogen are produced in the abomasa of young calves (Andren et al, 1980), but significant amounts of pepsinogen have been detected in the mucosae of three day old lambs (Baudys et al, 1988). The cellular source of pepsinogen in the neonatal animal was therefore investigated using abomasal tissues from neonatal lambs.

7.2.1: Materials and methods.

7.2.1.1: Animals.

Sections of abomasal fundic mucosa were taken from four, six month old Holstein-Friesian calves and sections of fundic and pyloric mucosae were collected from two, nine month old Scottish Blackface lambs, and three Hampshire Down lambs of approximately the same age. Both the calves and lambs had been reared indoors since birth in conditions likely to minimise helminth exposure. Fundic and pyloric tissues were also obtained from seven, one week old Scottish Blackface lambs. The lambs had been fed artificial milk-replacer by stomach tube for the short period until they were killed.

Tissues were also obtained from cattle and sheep which had been infected with abomasal parasites, although faecal egg counts and total worm burdens were not assessed. These animals were; a group of eight yearling cattle, which were known to have patent, but sub-clinical infections of *O. ostertagi* following single, experimental, larval infections, and two, six month old Scottish Blackface lambs that had been grazing infected pasture for a number of months and were killed for humane reasons when clinical signs of gastroenteritis became

severe. Animals were killed by captive bolt (calves), or electrical stunning (sheep), followed by exsanguination. Tissues were collected as soon after death as possible and placed in Brunnel's fixative.

7.2.1.2: Histological methods.

Tissue sections, were prepared for pepsinogen immunostaining, and were also stained with Haematoxylin and Eosin (H&E), and Periodic Acid Schiff (PAS). Immunolocalisation of pepsinogen was determined on 2 μm paraffin sections of abomasal mucosae, previously fixed using Brunnel's fixative (Laboratory Supplies and Instruments Limited, Antrim, Northern Ireland). Endogenous peroxidase was blocked with 0.5 per cent hydrogen peroxide in methanol for thirty minutes, and sections were then treated with 0.1 per cent trypsin solution at 37°C for a further thirty minutes. Pepsinogen was visualised using a commercial antiserum from rabbits immunised with highly purified bovine pepsinogen type I (Chris Hansen's Laboratory Limited, Reading, England) and the appropriate Vectastain, Avidin Biotinylated enzyme Complex (ABC) kit (Catalogue number, PK-4001, Vector Laboratories Limited, Peterborough, England). The primary antiserum, used at a one in ten thousand dilution, was incubated on the sections overnight at 4°C, after which the secondary, biotinylated and peroxidase complexed antibody was incubated on the sections for a further 30 minutes. Bound peroxidase was then visualised using 0.1 per cent diaminobenzidine tetrahydrochloride (BDH Chemicals Limited, Poole, England). Sections were counterstained with Haematoxylin. Pepsinogen was localised as a brown stain, against a blue background. Two types of negative staining control were performed, the first by omitting the primary antiserum and the second by replacing the primary antiserum with normal (non-immune) rabbit serum (Catalogue number, S-5000, Vector Laboratories Limited).

In addition to the two negative-staining controls. Negative tissue controls were also obtained; tissues were taken from two sheep, from sub-mandibular salivary gland, trachea and large intestine and were stained immunohistochemically for pepsinogen.

7.2.2: Results.

Examination of parasite-naive calf abomasal fundus in H&E stained sections (Fig. 7.2) showed that pit and gland architecture was normal. Inflammatory cell infiltrates were

minimal and the few intra-mucosal lymphoid follicles present were discrete and appeared wholly non-reactive (i.e. there were no germinal centres). Pepsinogen-specific staining could be detected in epithelial cells at all levels of the mucosae of all calves with the exception of parietal cells (Fig. 7.3). Staining in chief cells was strongest and these cells bore numerous dark staining granules in their apical cytoplasm. The basal cytoplasm of the chief cells was a uniform light brown. The apical granularity exhibited by mucous neck and surface mucous cells was considerably finer and paler than that of chief cells. The histological appearance of all the parasite-naive sheep in H&E stained sections was similar to that of the calves. The pits appeared slightly shallower and the glands were correspondingly longer (Fig. 7.4). In PAS-stained sections mucins were confined to the neck of the fundic glands and to cells lining the pits (Fig. 7.5). Examination of multiple sections of sheep mucosa from each animal showed that the immunostaining for pepsinogen was consistent, qualitatively and quantitatively, from section to section. In the ovine fundus the immunostaining pattern for pepsinogen was similar to that in the calf tissues, but with one major difference, in that the surface mucous cells of the sheep were free of stain (Fig. 7.6). The pyloric mucosae of the sheep, examined in H&E stained sections, were unremarkable (Fig. 7.7), and the pepsinogen distribution was similar in all animals, with staining confined to deeper cells of the pyloric glands (Fig. 7.8).

Examination of H&E stained sections of the neonatal lamb fundic mucosa revealed a shallow mucosa in which the pits were short and lined by mucous cells with a voluminous mucus cap, which was foamy in appearance (Fig. 7.9). A major difference between these animals and those of other age groups lay in the population of the neck regions of the fundic glands, the majority of cells in this region resembling the surface mucous cells. Cells recognisable as chief and parietal cells were confined to deeper gland areas. Parietal cells were generally few in number and appeared to have lower cytoplasmic volume than parietal cells in tissues from older animals. Some chief cells had accumulated eosinophilic granules in their apical cytoplasm, but many had not. Pits in the pyloric mucosa were deep and lined by tall mucous cells with voluminous apical mucus (Fig. 7.10). The very short glands of the pylorus were lined by more cuboidal mucous cells and the gland lumen was often wide. Numerous mitotic figures were in evidence in the pyloric mucosa, but were rarer in the fundus. Immunohistochemical studies (Figs. 7.11 and 7.12) localised pepsinogen to the chief cells of the fundus, with a further group of cells staining at the level of the isthmus and lower pit. The staining of the cytoplasm of chief cells was still strongest despite their apparent lack of

much granular material. In the pylorus, pepsinogen immunoreactivity was detected, albeit weakly, in the basal and also the more apical cytoplasm of some of the mucous cells of the pits.

Figures 7.13, 7.14 and 7.15 show sections of fundic mucosa from two of the eight cattle of the infected group. In Fig. 7.13 (higher magnification in Fig. 7.14) a collapsed gland left after larval emergence was noted, the overlying mucosa appears depressed in comparison to the hyperplastic edges of the nodule (arrows). Epithelial cells lining collapsed glands were irregular in height and width, varying from tall, columnar to low cuboidal. Cells also varied in the intensity of staining for pepsinogen, with some cells demonstrating considerable apical staining, whilst adjacent cells stained more weakly. Epithelial cells lining slightly dilated structures above the collapsed gland stained only weakly for pepsinogen. In comparison, at either side of the centre of the nodule (arrows), at mid-mucosal levels and towards the surface, hyperplastic and hypertrophied mucous cells stained more strongly for pepsinogen than cells at corresponding levels of adjacent, more-normal mucosa, cells which had more abundant apical cytoplasm with larger mucus caps. At deeper mucosal levels some glands adjacent to the central, formerly-parasitised gland were mildly dilated, and were lined by low cuboidal and poorly differentiated cells that stained weakly for pepsinogen. Parietal cells were not present in these glands, but were still prominent in other glands in close proximity to them. No parasitised gland could be seen in the section shown in Fig. 7.15, however the raised outline of a nodule was discernible at the mucosal surface and there were associated accumulations of lymphocytes in the lamina propria and sub-mucosa. Deeper gland structures appeared normal and parietal cells were plentiful across the lesion. More obvious changes existed closer to the surface where hyperplastic and hypertrophied mucous cells showed increased staining for pepsinogen in comparison to cells at this level in normal areas adjacent to the lesion. Fig. 7.16 shows a section of a parasitised gland from another animal from this group. The gland was occupied by a large worm that was seen to be partly emergent from the mucosa in a different section. There was minimal change around this gland when compared to more distant areas, and although adjacent glands showed mild dilatation, parietal cells were still present in them. The epithelial lining of the parasitised gland was irregular with some hyperplastic fronds, and pepsinogen-specific staining in the cells was poor or absent. Parasites themselves did not stain, except for slight accumulations of positive material lining what could well correspond to the worm's gut.

Figure 7.17 shows an area of massively thickened fundic mucosa from one of the infected sheep, stained by Periodic Acid Schiff (PAS). Mucins, identified as PAS-positive material (violet coloration), were present in cells at all levels of the mucosa, although many cells, especially those deeper within glands, contained only small amounts. At higher magnification few parietal cells could be seen in this area of mucosa. The same tissue stained for pepsinogen (Fig. 7.18) revealed a very mixed pattern, with many cells at the gland base showing less staining and possessing fewer granules than chief cells in other, histologically normal, parts of the mucosa. However, in other areas, pepsinogen-specific staining was detected in cells at all levels of the mucosa. Similar changes were seen in mucosal sections taken from the other infected sheep (results not shown), but were generally less marked.

Pepsinogen-specific staining was not observed in any of the control sections and no pepsinogen-specific staining could be detected in the salivary gland, tracheal or large intestinal tissues from the two animals examined.

7.2.3: Discussion.

The immunohistochemical localisation of type I pepsinogen in parasite-naive calf mucosa in the above studies agreed closely with the reported distributions of pepsinogen in fundic mucosa of calves in other studies (Cybulski and Andren, 1990). Assuming that the antiserum employed recognised ovine type I pepsinogen equally well, the pattern of distribution of pepsinogen in normal sheep may differ from that of cattle since the surface mucous cells in the ovine do not appear to contain pepsinogen. The concentrations of pepsinogen in sheep surface mucous cells may have been below the sensitivity of the antibody method, however in studies of bovine mucosa, the antiserum could be diluted from a normal working dilution of 1:10,000, to at least 1:80,000 and staining in surface mucous cells was still apparent (results not shown). Staining of ovine pyloric mucosa for pepsinogen produced a similar pattern to that reported by others for the bovine pylorus (Cybulski and Andren, 1990) and disagreed with studies in humans where PG I was not present in the pylorus (Samloff, 1971).

The quality of staining for pepsinogen in both cattle and sheep was excellent, with no staining detected in other non-epithelial cells. The anti-bovine pepsinogen antiserum recognised ovine pepsinogen well. The antiserum may have recognised epithelial components other than pepsinogen, but the distribution in the bovine was similar to that

reported for other studies, and in the sheep at least attempts to localise pepsinogen-specific immunoreactivity to a variety of non-abomasal tissues were entirely without success.

Fig. 7.2: Photomicrograph of a section of the fundic mucosa from a parasite-naive calf. (H&E stain, original magnification ×25).

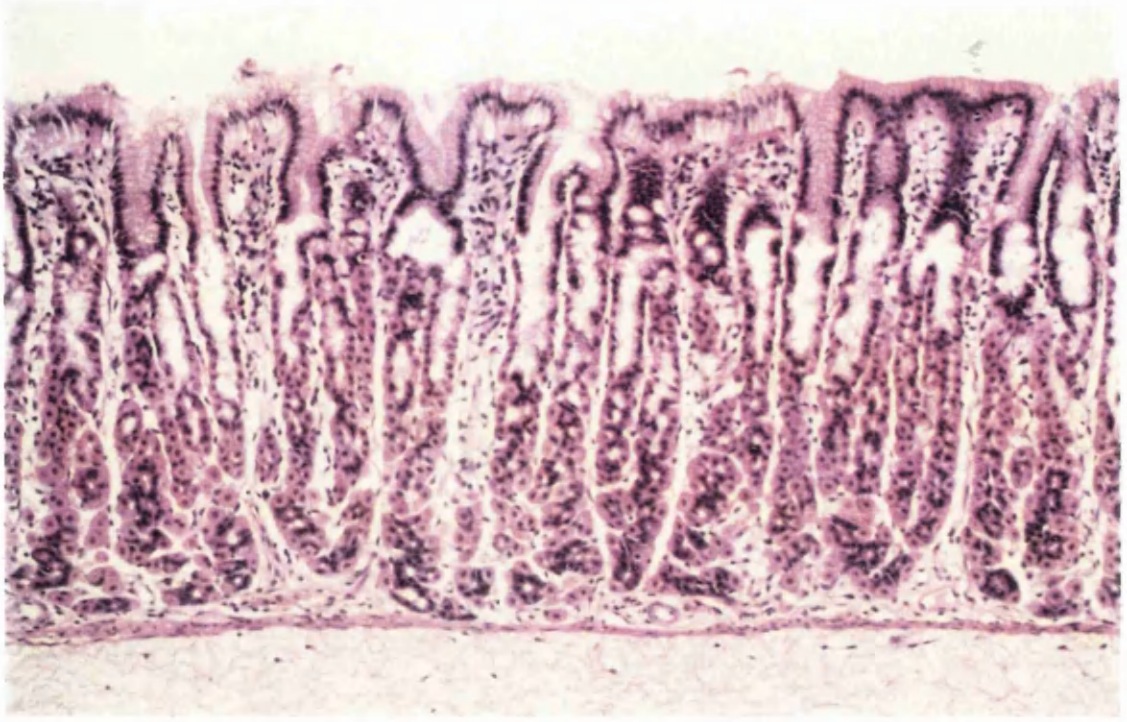


Fig. 7.3: Photomicrograph of parasite-naive calf fundic mucosa stained for pepsinogen. (Haematoxylin counterstain, × 25).

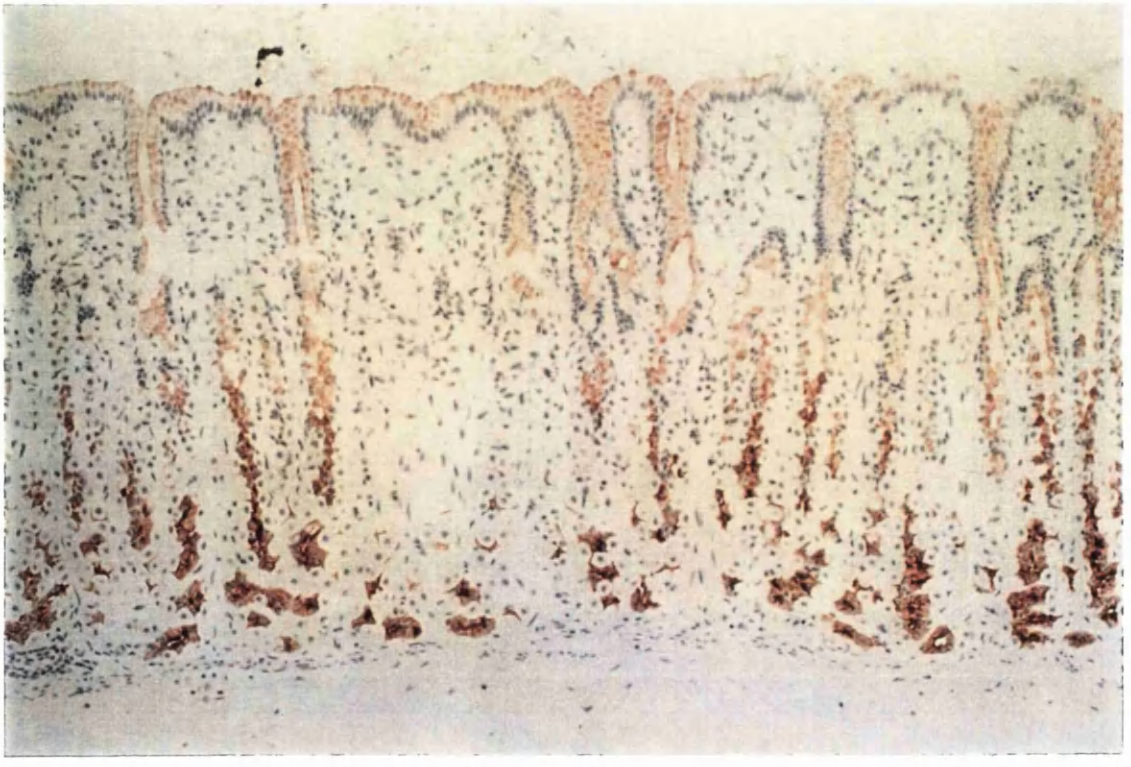


Fig. 7.4: Photomicrograph of a section of parasite-naive ovine fundus.
(H&E stain,× 25).

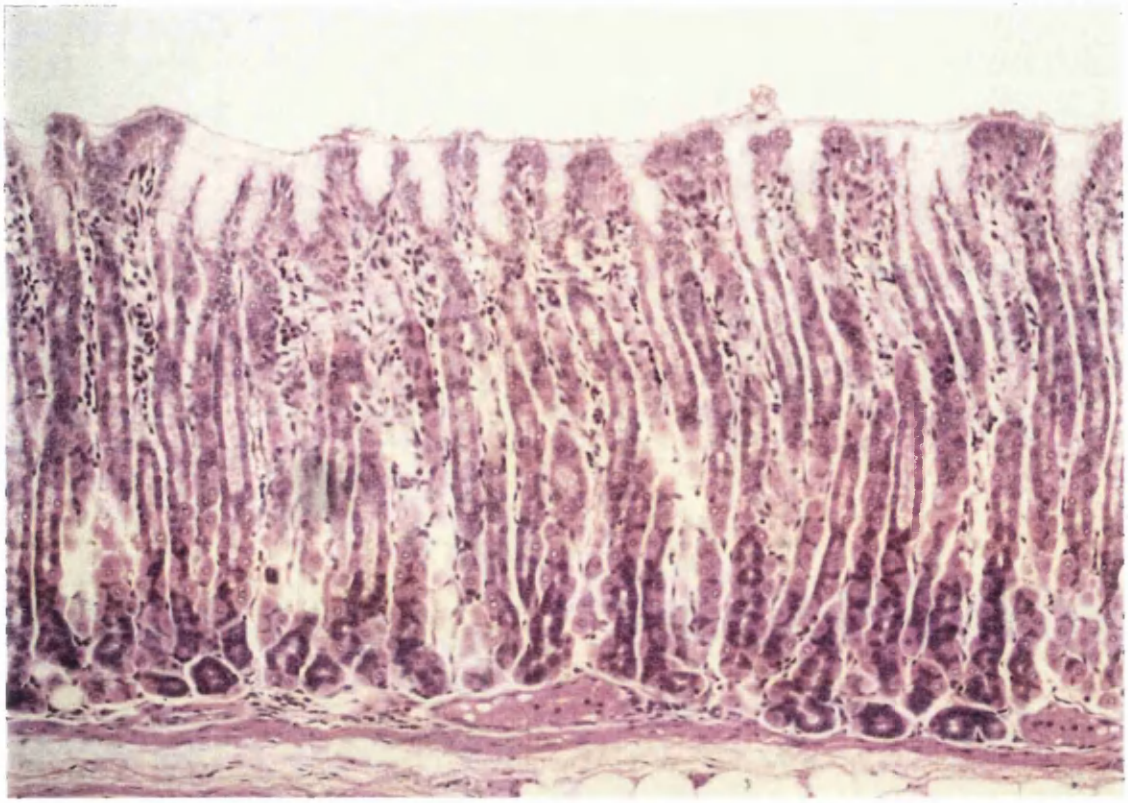


Fig. 7.5: Photomicrograph of parasite-naive ovine fundic mucosa. (PAS stain; mucins appear violet, × 25).

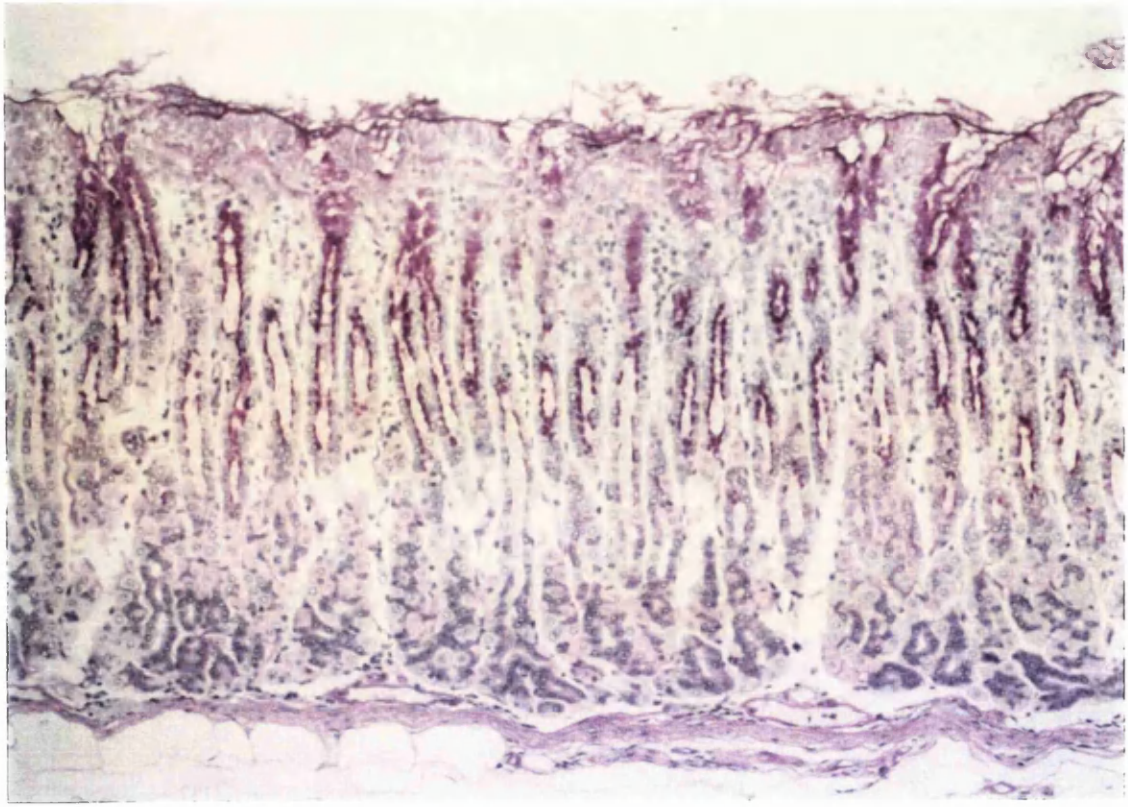


Fig. 7.6: Section of parasite-naive ovine fundic mucosa immunostained for pepsinogen. (Haematoxylin counterstain, × 25).

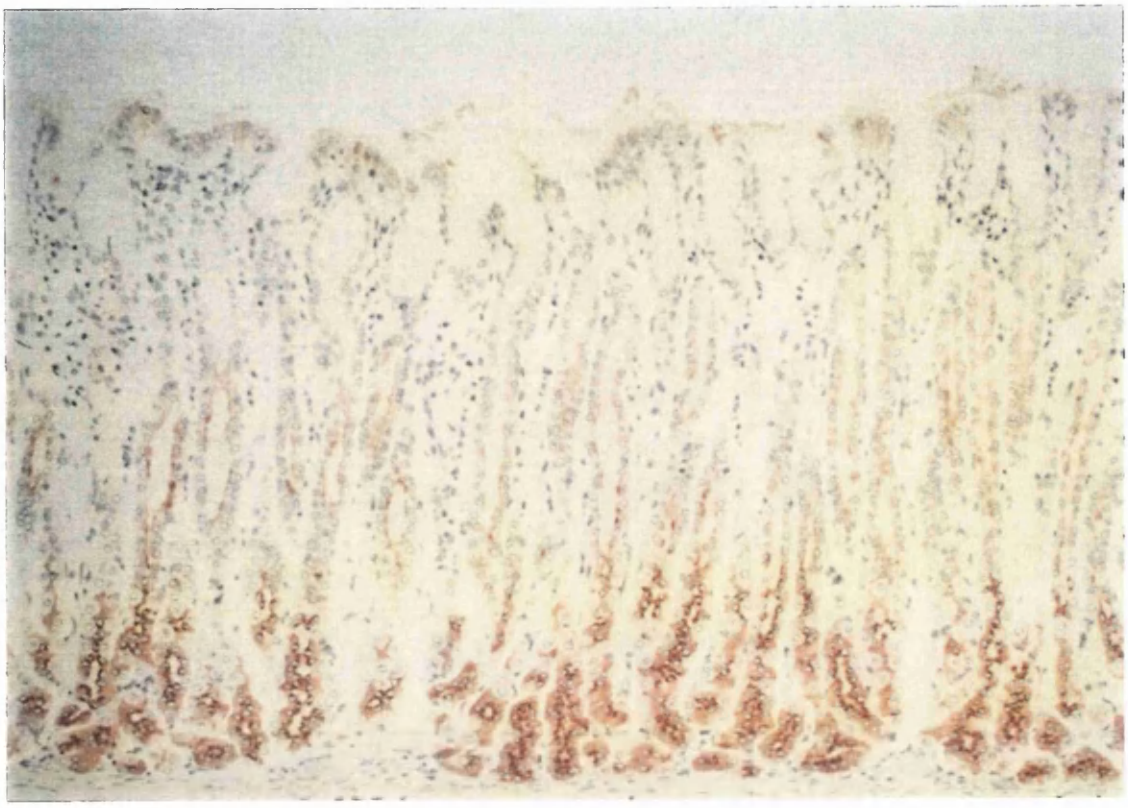


Fig. 7.7: Photomicrograph of a section of parasite-naive ovine pyloric mucosa. (H&E stain, × 25).

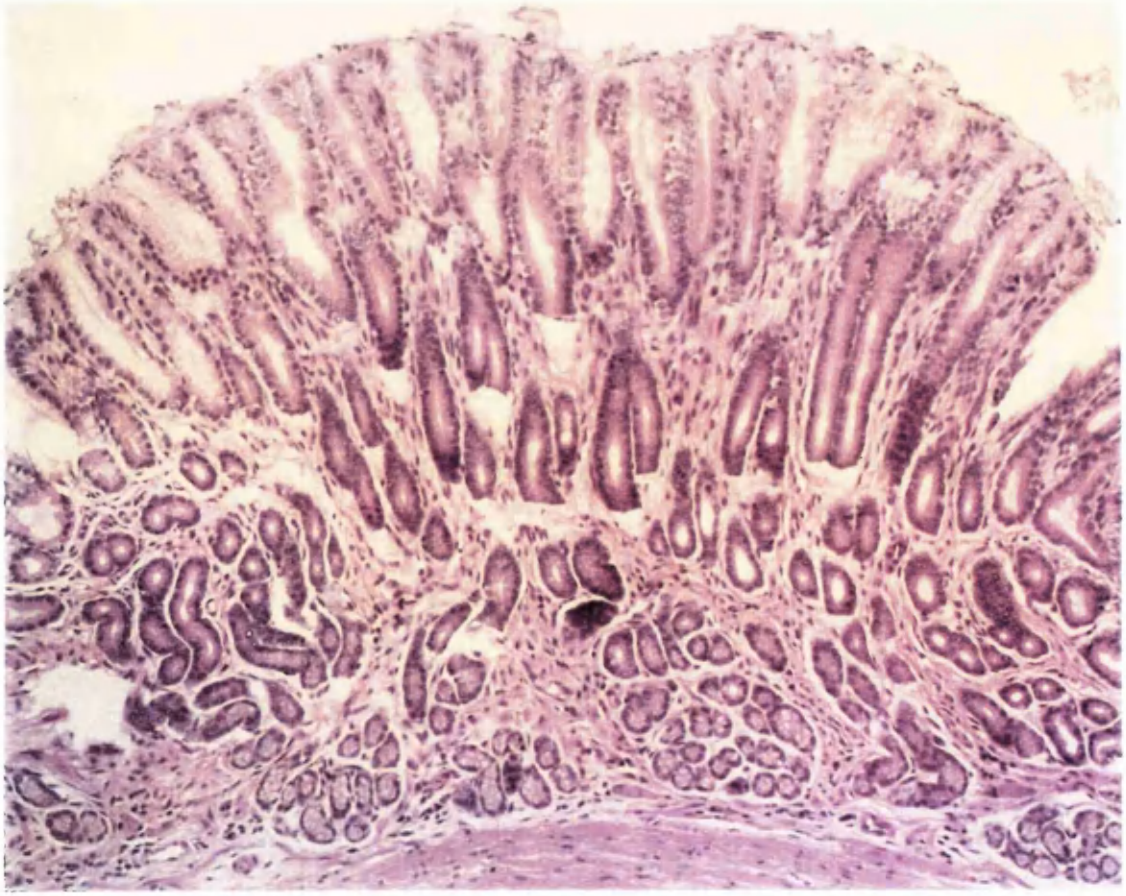


Fig. 7.8: Section of ovine pyloric mucosa immunostained for pepsinogen. (Haematoxylin counterstain, × 13.2)

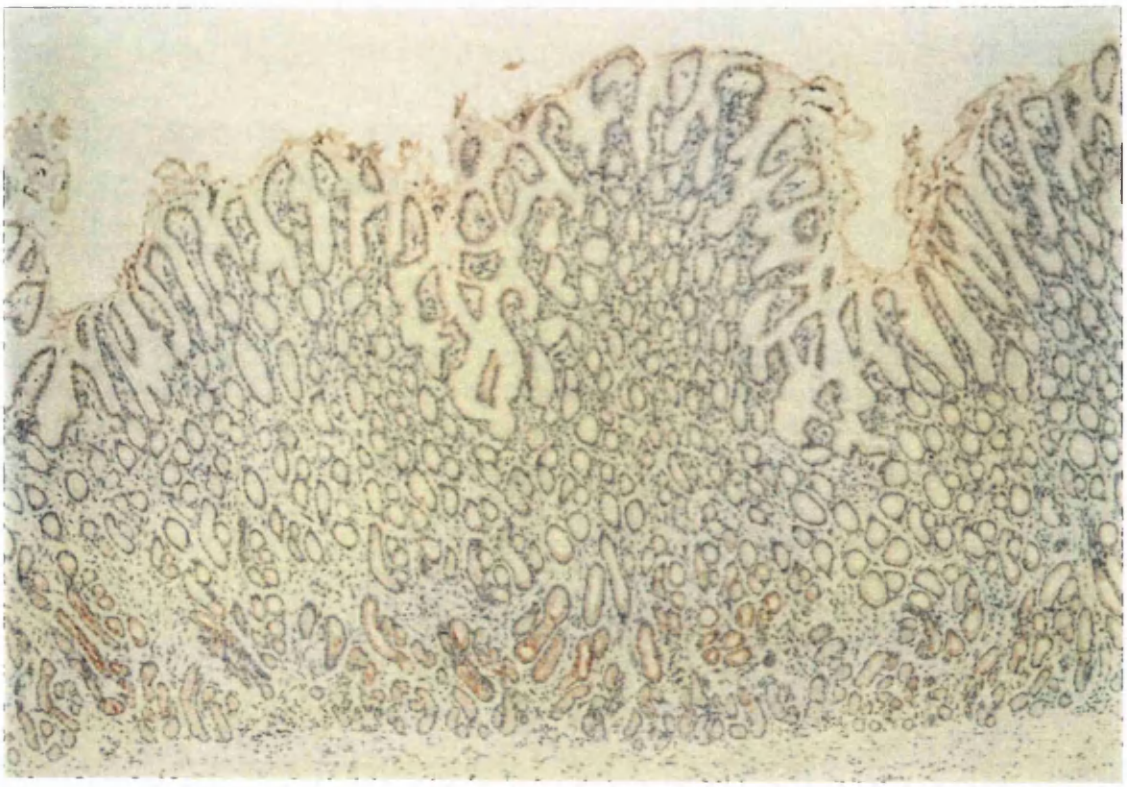


Fig. 7.9: Photomicrograph of the fundic mucosa of a lamb of less than one week of age. The upper gland levels are lined by cells bearing voluminous mucus caps (H&E stain, × 50).

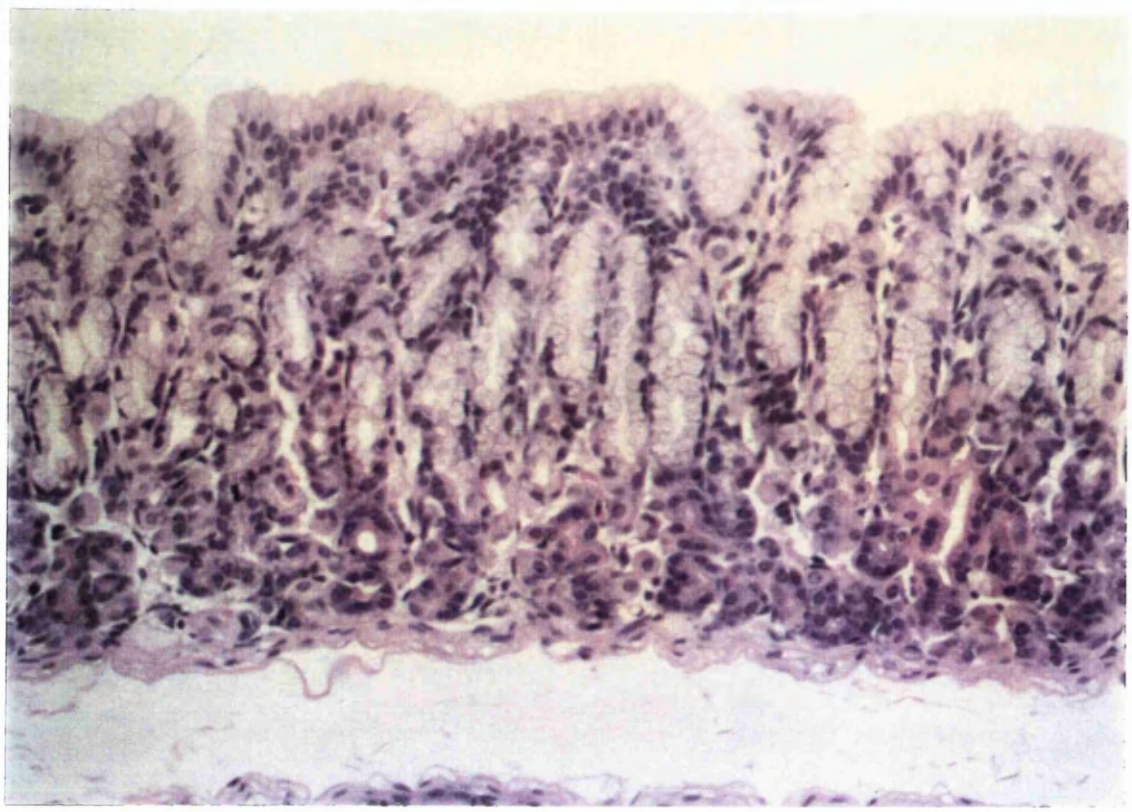


Fig. 7.10: Pyloric mucosa from a lamb of less than one week of age. (H&E stain, × 50).

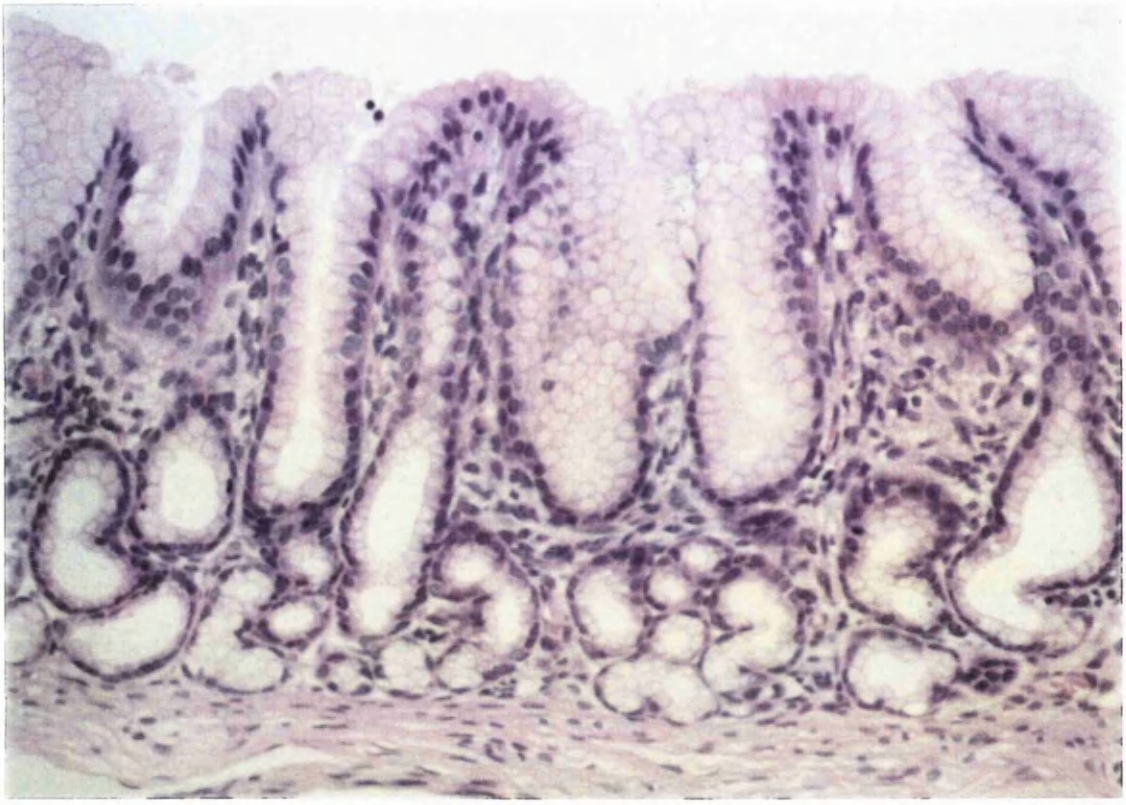


Fig. 7.11: The immunohistochemical localisation of pepsinogen in the fundic mucosa of a lamb. (Haematoxylin counterstain, × 50).

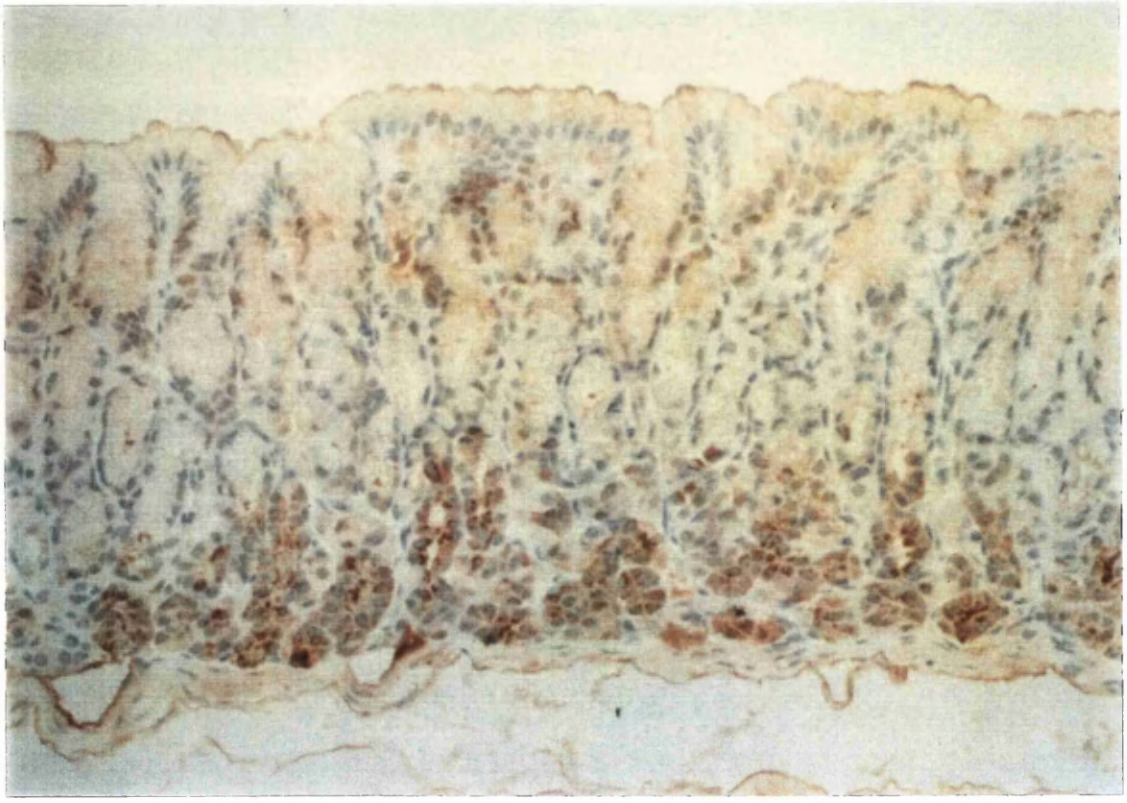


Fig. 7.12: Pyloric mucosa of a lamb of less than one week of age. (Immunostained for pepsinogen, × 50).

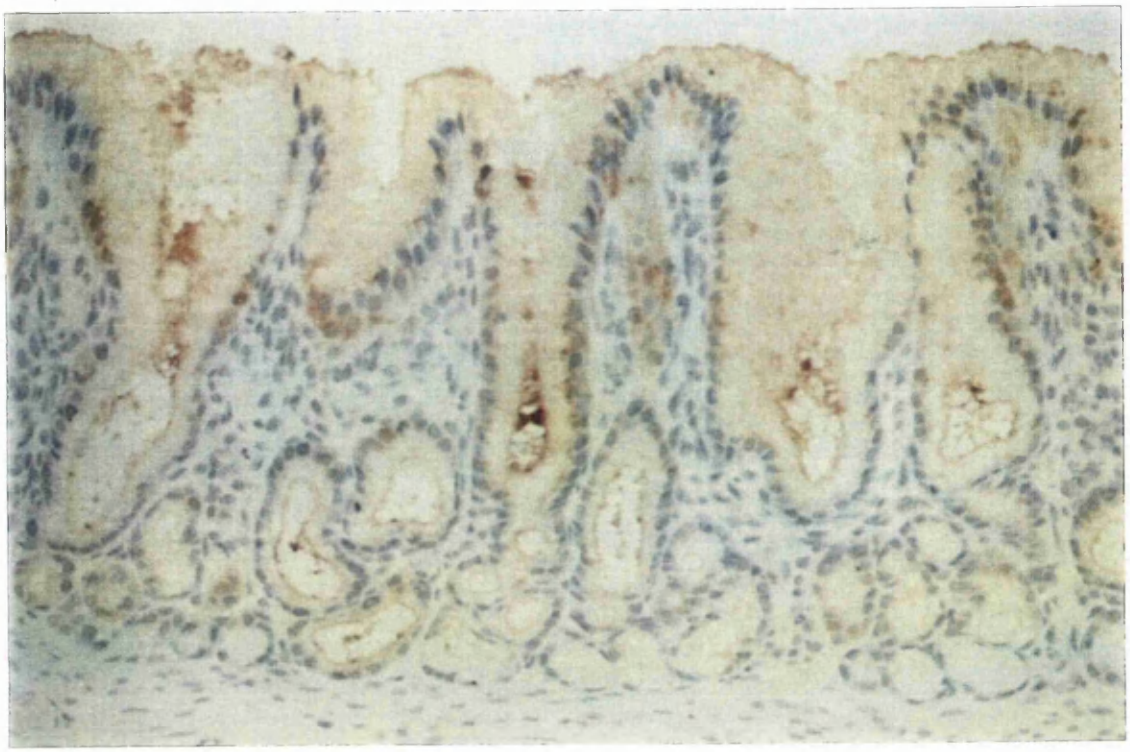


Fig. 7.13: The immunohistochemical localisation of pepsinogen in fundic mucosa of a calf with a patent *O. ostertagi* infection. Reduced staining can be seen immediately above the collapsed gland left after parasite emergence, but the hyperplastic edges of the nodule (arrows) show increased levels of staining (Haematoxylin counterstain, × 10).

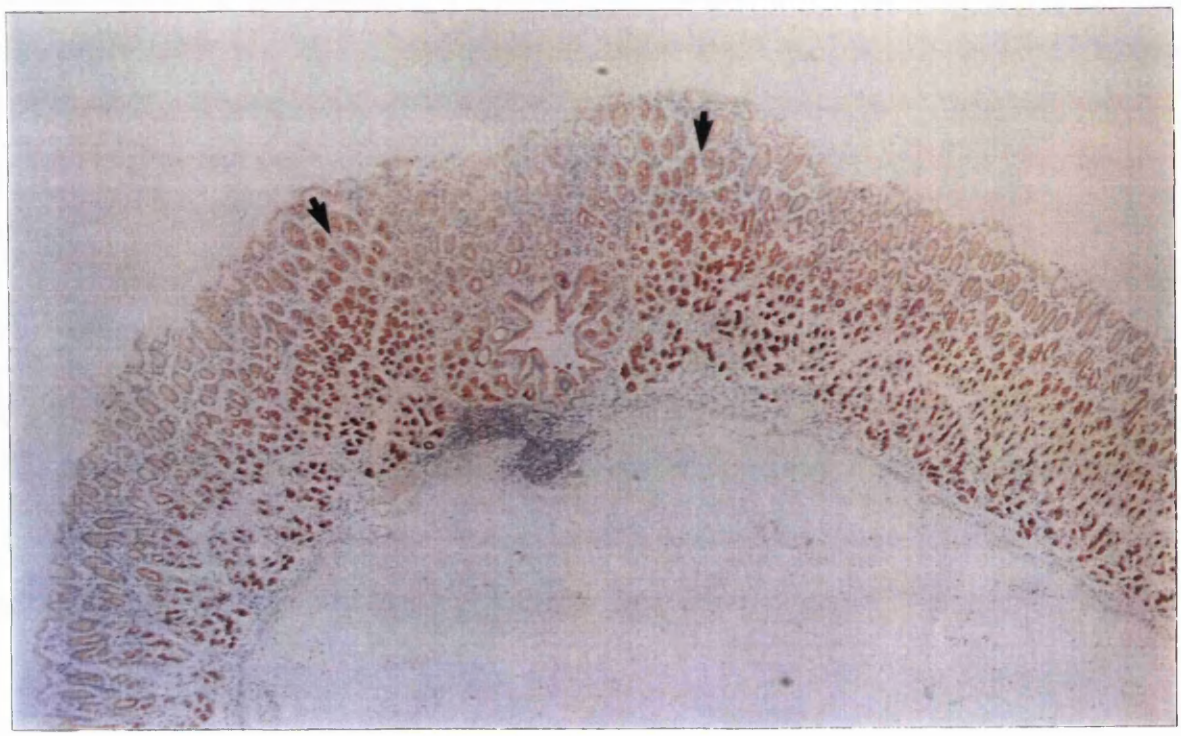


Fig. 7.14: Higher magnification of the collapsed nodule featured in Fig. 7.13. (Pepsinogen immunostain, × 40).

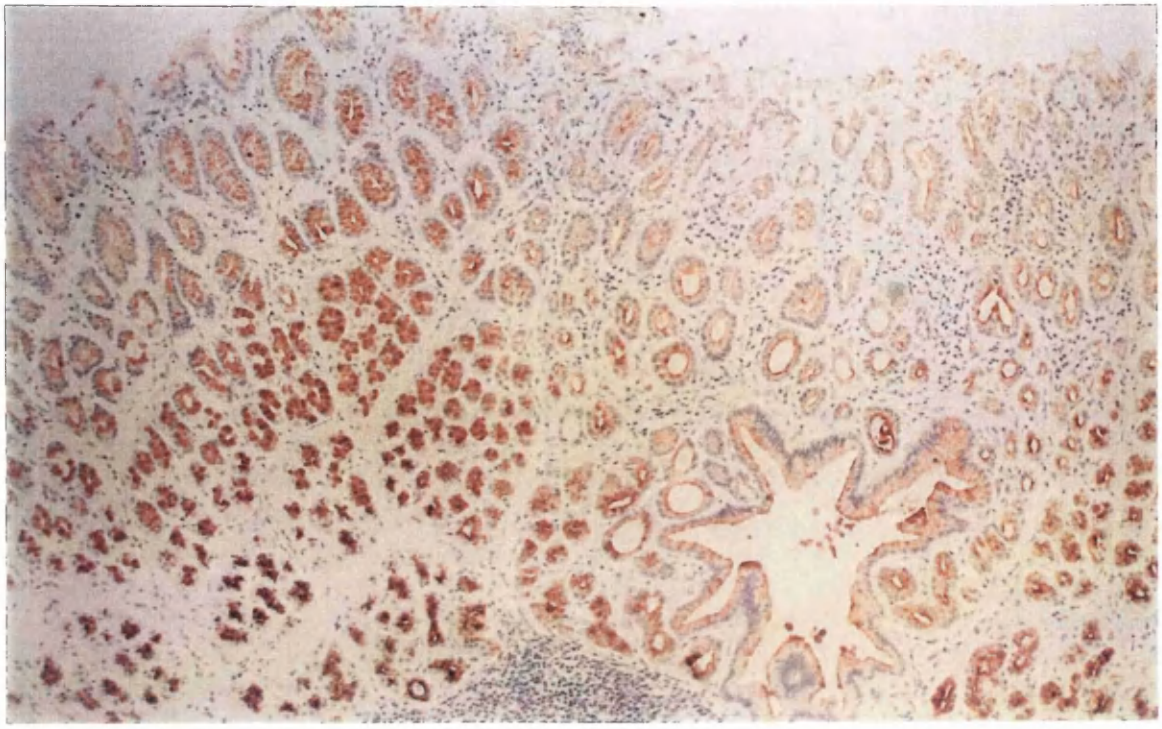


Fig. 7.15: Section of a presumed nodular lesion from a calf with a patent *O. ostertagi* infection. There is hyperplasia and hypertrophy of more superficial mucous cells and an apparent increase in pepsinogen-specific stain (× 10).

Fig. 7.16: Section of an area of mucosa from a calf of the same group as those featured in Figs. 7.13 and 7.15. Note the presence of a large parasite within the confines of a gland and that there is minimal change in the structures around it and consequently little change in the distribution of pepsinogen (× 10).

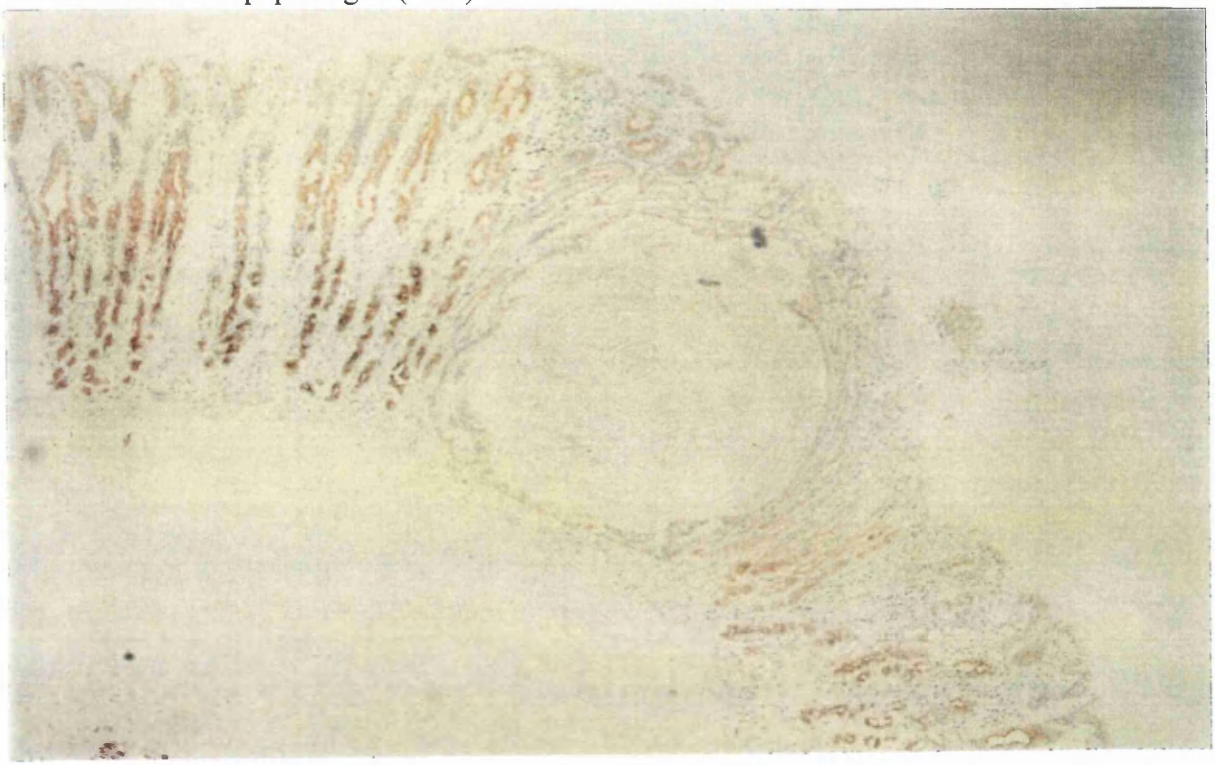


Fig. 7.17: Section of ovine fundic mucosa with massive hyperplasia due to parasitic gastritis. Note the presence of numerous parasite larvae (p) and the presence of PAS-positive material deep in the mucosa (arrows)(× 10).

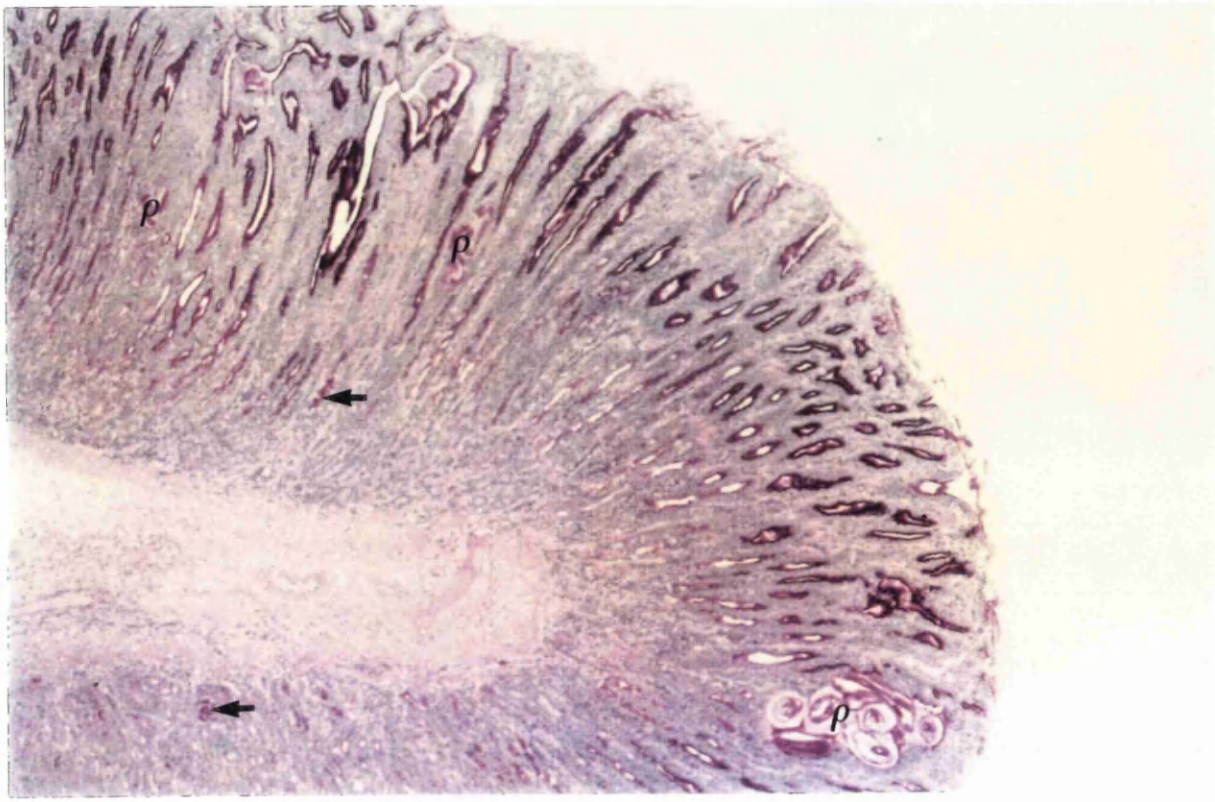


Fig. 7.18: The same tissue as in Fig. 7.17 immunostained for pepsinogen. A mixed pattern of staining is present with some deep glandular structures showing little stain (small arrows) and in other areas pepsinogen is present in numerous surface mucous cells (large arrows)(× 10).

These studies confirmed the presence of a PG I-like substance in the abomasa of young lambs. Despite the observed differences between the architecture of the lamb mucosae at a few days of age and those of older animals, the chief cell remained the major source of pepsinogen, as indicated by the intensity of staining. Although the exact control of pepsinogen secretion in the chief cells of neonatal animals may ultimately differ from that of older animals, it is however likely that cells of the neonate and adult respond similarly to many of the same secretagogues.

The preliminary results for infected animals strongly suggested that ostertagiasis resulted in changes in the pattern of distribution of pepsinogen in fundic mucosae, changes that were observable by immunohistochemical means. In the bovine examples studied the results were suggestive of increased amounts of pepsinogen present within parasitic nodules, but also indicated that the stage of infection might be important, with greater changes associated with older lesions. The mixed pattern observed in the parasitised ovine tissues might have resulted from differences in the maturation of the hyperplastic epithelial cells that had replaced the

normal gland components. The rapid amplification of very poorly differentiated cells may have resulted in the presence of large numbers of cells that possessed little synthetic machinery for the production of pepsinogen. These cells subsequently underwent some differentiation and a synthetic capability was then achieved, but it is possible that as cells differentiated to resemble fully mature mucous cells, which in the ovine do not contain pepsinogen, the ability to produce pepsinogen was finally lost.

7.3: Examination of tissue pepsinogen concentrations at multiple sites of the abomasa of sheep and cattle.

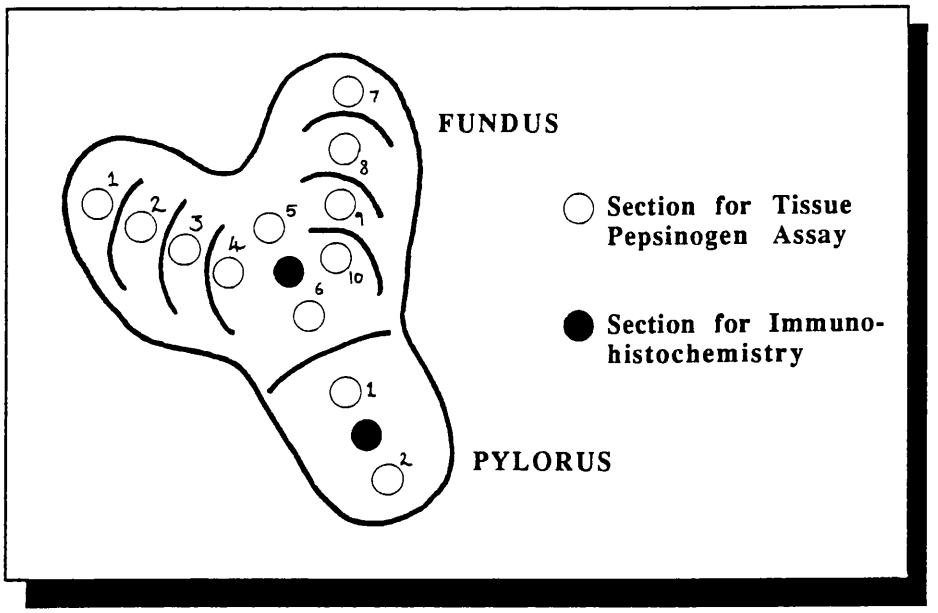
The degree of variation in pepsinogen concentration encountered within the abomasa of individual animals was assessed, to see whether tissue pepsinogen concentrations were consistent in different areas of the abomasum as had been suggested by the preliminary immunohistochemical studies.

7.3.1: Materials and methods.

Tissues were obtained from a group of five, six month old female calves, of mixed breeds (three Hereford-Friesian crosses, one Charolais cross and one Limousin cross) that had been reared indoors to minimise exposure to helminth infection. Ten sections of fundic and two sections of pyloric mucosa were collected from each animal for the measurement of mucosal wet weight and the assay of tissue pepsinogen. Sections were also taken for pepsinogen immunohistochemistry. Sections were taken in a similar pattern for all animals (Fig. 7.19), and cut using a 13.5 mm diameter punch biopsy. Sections were therefore of identical cross-sectional area, and fundic sections incorporated two mucosal layers, that is the opposing sides of the mucosal fold. Pyloric mucosal sections, consisting of one mucosal layer, had to be dissected free of the sub-mucosa, which was a relatively easy task. To allow a limited comparison with sheep, tissue was taken in a similar pattern from each of three Scottish Blackface, female lambs, all less than one year old. These animals had an incomplete history and may have been previously exposed to parasites. Sections were not taken for immunohistochemistry and due to a degree of difficulty in dissecting the pyloric mucosa free, the core punch was not used for the pyloric mucosal pieces and sections were therefore of non-standard area, and not directly comparable. Pepsinogen concentrations could however be compared, when expressed as peptic units per gram of tissue. Weighed tissues were

processed and tissue pepsinogen was assayed as described earlier (see section 2.3). From the values of pepsinogen concentration present in the supernatants of the processed tissue homogenates, the total pepsinogen present in each mucosal cut was calculated. Tissue pepsinogen concentrations were then expressed as peptic units per mucosal cut (all identical areas) or as peptic units per gram of tissue. The amount of variation in tissue pepsinogen concentration from site to site within the abomasa of individual animals was assessed by calculating the coefficient of variation (C.V.) for each animal. The C.V. was obtained by dividing the mean tissue pepsinogen concentration (iU/g) for each animal by the corresponding standard deviation and multiplied by 100 to obtain the per cent value. Tissue wet weights, total tissue pepsinogen values (iU/standard area) and tissue pepsinogen concentrations (iU/g) were compared by one-way analysis of variance to see if individual animals were different. The model **test parameter** (wet weight, tissue pepsinogen) = **animal**, was used.

Fig. 7.19: Schematic representation of the abomasal luminal surface, the abomasum was opened along the greater curvature and the approximate location and sequence of mucosal sections is shown.



7.3.2: Results.

None of the calves showed any evidence of any abomasal pathology, however a few parasitic nodules and adult parasites were seen in the abomasum of one sheep (No. 3). The results of tissue pepsinogen concentrations expressed as iU per gram of tissue (iU/g) are given in Table 7.1. The mean (± S.E.) values for fundic wet weights (g) and total tissue pepsinogens (iU per standard mucosal area) and individual values for pyloric measurements, are given in Table 7.2 (full data for fundic mucosal tissue total pepsinogen (iU/ standard area) and for fundic wet weights are contained in Appendices 18A and B). In all animals the pepsinogen content of pyloric mucosa was lower than that found in the fundus, mean values for cattle fundus ranged from 28,900 iU/g to 48,200 iU/g, whereas pyloric pepsinogen concentrations ranged from 800 iU/g to 10,000 iU/g. The sheep gave similar results. In all animals fundic pepsinogen concentrations were consistent between areas and variation calculated as coefficients of variation was generally low. The overall mean C.V. of all 8 animals was 19.5 per cent with individual values ranging from 14 to 25 per cent. For the three parameters of tissue wet weights, total tissue pepsinogen (iU/ area) and tissue pepsinogen concentration (iU/g), animals were highly significantly different from each other ($p<0.001$), i.e. within-animal variation was negligible in comparison to that already existing between animals. Immunohistochemical localisation of pepsinogen in the calf mucosae demonstrated no staining of the surface mucous cells, but otherwise staining was typical of that seen previously in parasite-naive animals.

7.3.3: Discussion.

Cuts of the fundic mucosal folds incorporated two mucosal layers, whereas those of the pylorus, only one. Assuming that tissue densities did not vary then tissue wet weights were directly proportional to the thickness of the mucosa, which would be affected by the vertical heights of the pits and glands. Some contribution to tissue weights would undoubtedly derive from submucosal structures, such as adipose tissue and larger calibre blood vessels, but judging from histological sections these features accounted for only a small proportion of total tissue mass and were fairly consistent from tissue to tissue. It is therefore likely that wet weights give a good indication of the size of the pit/gland units. The number of animals in this study was too small to allow a proper comparison of the pepsinogen concentrations of cattle and sheep, but both species contained pepsinogen concentrations of similar orders,

although there was the suggestion that there was greater variation between concentrations of the individual sheep. In comparison with earlier investigations of the distribution of pepsinogen in ruminants these results indicate similar staining patterns as well as similar tissue pepsinogen concentrations in both cattle and sheep.

Age is known to influence the pattern of pepsinogen staining in animals (Cybulski and Andren, 1990), but the possible effects of other factors, such as diet, are not known. Many factors may have accounted for the apparent presence of pepsinogen within the surface mucous cells in some cattle as reported earlier, and its absence in surface mucous cells of the calves in the present study. The present study has shown that abomasal fundic pepsinogen distribution is remarkably consistent from area to area in parasite-naive calves, and is likely to be the same in sheep. As a consequence, a single mucosal sample from a normal animal would give good information on the amount and distribution of pepsinogen present in the abomasum overall. The distribution of pathological changes during nematode parasitism may not be spread so evenly throughout the abomasum.

Table 7.1: Tissue pepsinogen concentrations (iU/g tissue wet weight) of multiple sites of the abomasa of cattle and sheep.

	Calf					Sheep		
Site	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**
Fundus								
1	37,754	72,749	37,088	30,236	26,241	8,635	26,394	101,732
2	67,764	56,442	33,682	27,459	22,576	14,186	28,401	71,662
3	50,331	59,964	40,956	23,984	30,017	11,318	17,301	97,500
4	62,243	66,851	39,766	30,339	19,056	11,641	28,589	80,428
5	57,536	66,048	34,902	34,519	32,049	9,775	21,977	96,671
6	36,228	55,546	43,902	34,406	37,359	8,076	28,931	100,529
7	36,406	53,239	32,726	32,058	21,244	6,855	37,481	72,240
8	56,026	46,160	47,432	36,382	29,252	10,343	31,932	62,810
9	38,072	54,995	41,389	34,693	38,569	9,831	29,898	95,303
10	39,976	81,015	49,050	49,109	32,302	12,543	26,463	99,858
Mean	**48,234**	**61,301**	**40,089**	**33,319**	**28,866**	**10,320**	**27,737**	**87,873**
(± SD)	(3,793)	(3,275)	(1,771)	(2,120)	(2,083)	(692)	(1,712)	(4,609)
C.V. %	**24.87**	**16.90**	**13.97**	**20.12**	**22.82**	**21.19**	**19.57**	**16.59**
Pylorus								
1	10,444	525	1,417	3,473	1,469	9,840	5,530	10,434
2	8,551	1,011	1,971	1,996	1,203	7,096	2,009	5,660

Table 7.2: Mean (± S.E.) tissue wet weights (g) and total tissue pepsinogen (iU per standard area) of multiple sites of the abomasa of cattle and sheep (values for pyloric mucosae are not mean values)

	Calf					Sheep		
	1	2	3	4	5	1	2	3
Wet wts								
Fundus	0.1231	0.1119	0.0933	0.0956	0.0988	0.0819	0.0929	0.0672
(± S.E.)	(0.0033)	(0.0065)	(0.0056)	(0.0033)	(0.0035)	(0.0035)	(0.0039)	(0.0020)
Pylorus								
1	0.0897	0.1208	0.1112	0.0747	0.0694	0.1282*	0.1527*	0.0363*
2	0.0947	0.1256	0.1223	0.1300	0.1309	0.1076*	0.1292*	0.1060*
Tot. Peps.								
Fundus	5875	6788	3710	3149	2857	844	2535	5887
(± S.E.)	(389)	(384)	(221)	(159)	(240)	(66)	(112)	(338)
Pylorus								
1	937	64	158	259	102	1261*	844*	379*
2	810	127	1464	259	157	763*	259*	600*

* - Ovine pyloric pieces were not cut using the core punch, therefore wet weights and total tissue pepsinogens are not directly comparable, however tissue pepsinogen concentrations (see previous table) are.

7.4: The effects of natural infections of sheep, with predominantly *O. circumcincta*, on the distribution and quantity of abomasal tissue pepsinogen.

In the initial studies presented earlier in this thesis changes in pepsinogen distribution in the abomasa of animals with ostertagiasis were associated principally with the nodules caused by larval development and emergence and tissue pepsinogen content could have been increased in nodules as a consequence of the hyperplasia of immature, mucous-type cells. The following studies were designed to further investigate the nature of changes occurring in ovine tissues and to compare the pepsinogen content of nodular tissues to that of intervening areas of non-nodular mucosa in the same animals, and to assess the extent of any changes that might be present within infected mucosae.

7.4.1: Materials and methods.

7.4.1.1: Animals.

The sheep in this study had grazed pasture contaminated with parasitic nematode larvae on a commercial, upland farm in the west of Scotland. The group comprised twenty six, spring-born, male, Scottish Blackface lambs that were monitored monthly during the grazing season, from May to October, for faecal egg counts. With the exception of the last sampling date in October, the lambs were dosed with a broad-spectrum anthelmintic (Albendazole sulphoxide: Rycoben, Youngs Animal Health, Leyland, England), given at the time of sampling, at the recommended dose rate of 5 mg/kg body weight, based on the body weight of the heaviest lamb.

7.4.1.2: Procedure at necropsy.

The sheep were killed by exsanguination following electrical stunning. Blood was collected into heparinised containers, as the sheep were bled out, and samples were later centrifuged at 2,000 *g* in a Beckman GS-6R centrifuge for 10 minutes, plasma was then separated and stored at -20°C. Before the abomasal mucosa was washed (for the quantitation of parasite numbers), tissue from multiple sites was taken into Brunnel's fixative for immunohistochemical studies. Tissue was also collected from the first five animals killed for the estimation of the amounts of tissue pepsinogen present in nodular and non-nodular mucosa, cuts were made using the biopsy punch tool (see section 7.4.1) to take sections of identical area. Four sections containing parasitic nodules (the nodule comprising the majority of the whole cut, with a thin rim of normal mucosa) and four sections of the immediately adjacent, apparently normal mucosa, were taken from each animal. In one animal (Y27), because of a generalised thickening of much of the mucosa, areas relatively normal in appearance were too small to be cut separately from changed mucosa, changed tissue was however collected from this animal, and sections cut for histology incorporated some apparently normal mucosa.

7.4.1.3: Parasitological and biochemical examinations.

Faeces was collected from the rectum of each lamb at necropsy for faecal egg counts. Faecal egg counts were accomplished using a modification of the McMaster procedure (Gordon and Whitlock, 1939). Each egg counted represented 50 eggs per gram of faeces. Abomasal contents and washings were made up to 2 litres, from which ten, 4 ml samples were examined to estimate the size of the worm population at the abomasal surface. One half of the abomasum was digested in pepsin-hydrochloric acid for six hours at 42°C, after which the digest was made up to 2 litres from which ten, 4 ml samples were examined to estimate the numbers of the population of tissue-dwelling worm stages (Armour et al, 1966). Plasma pepsinogen was assayed using Paynter's method (1992).

7.4.1.4: Histological procedures.

Tissues, taken initially into Brunnel's fixative, were later processed for routine histological examination (H&E and PAS techniques) and for the immunohistochemical localisation of pepsinogen.

7.4.1.5: Assay of tissue pepsinogen.

The mucosal pieces were gently cleaned to remove remaining abomasal contents and were then blotted dry and the wet weights recorded. Tissue pepsinogen was measured using the method described earlier (section 2.3). The wet weights of mucosal pieces, the total pepsinogen content of the pieces and their pepsinogen concentrations were compared separately by two-way analyses of variance, to compare non-nodular mucosa with nodular tissues. The model fitted was **test parameter = sheep + mucosal type + sheep × mucosal type**, **sheep** was fitted as a random effect.

7.4.2: Results.

Faecal egg counts, abomasal parasite counts and plasma pepsinogen concentrations are detailed in Table 7.3. Faecal egg counts varied from 0 to 1700 epg, with only three animals shedding greater than a thousand eggs per gram of faeces. Total worm burdens varied from seven hundred to nearly twelve thousand, the majority of worms present at necropsy were

adult and more adult females were present than males in most animals. More worms were generally present at the fourth-larval stage than the fifth. Plasma pepsinogen values were mostly mid to high normal concentrations, based on the range for normal animals quoted by Paynter (1992). Fifteen of the twenty three animals, for which the parameter was examined, had plasma pepsinogen concentrations of 5 iU or less, that would be considered consistent with the absence of significant abomasal parasitism.

7.4.2.1: Gross pathology.

Adult worms were seen on the abomasal surface of all animals and a modest number of characteristic nodular lesions were scattered throughout the epithelium in all but three animals. These animals (Y27, Y166 and Y182) had extensive pathological change since the majority of the mucosal surface appeared thickened, was quite rough and pale in colour. In animals infected with *O. circumcincta* the increased cellularity of nodules causes them to appear paler than the surrounding mucosa and in very heavy infections the nodules can become confluent giving the appearance considered characteristic of Morocco leather. In the three exceptional animals the impression was not of a great abundance of individual nodules, but rather of a generalised mucosal thickening with few discrete nodular centres. In other animals the nodules were generally circular, except where two or three closely abutted each other and achieved a degree of confluence. Individual nodules were 5 millimetres across at most, were pale and well circumscribed. Oedema and haemorrhage were not observed in the abomasa of any of the sheep. One animal (B16) was mildly ascitic. The abomasal pathology of this animal was no worse than that of other sheep and no other changes were observed in the other visceral organs or elsewhere.

Table 7.3: Faecal egg counts, worm burdens and plasma pepsinogen concentrations at necropsy.

Lamb	FEC (epg)	Worms L4	L5	male	female	Total	Plasma Pep. (iU)
02	125	1300	0	750	1100	3150	4.50
Y27	275	1350	550	2750	3700	8350	13.30
Y43	225	1200	100	750	1450	3500	1.20
Y51	675	2200	100	1900	3500	7600	3.00
B60	475	2800	100	1700	2100	6700	1.40
Y6	50	2700	0	850	800	4350	5.00
B69	100	0	0	800	1150	1950	1.20
035	0	6200	400	800	700	8100	5.00
036	0	10400	0	550	550	11500	2.60
062	125	500	150	1400	1250	3300	2.40
B23	400	0	0	800	500	1300	6.83
B27	300	0	0	250	450	700	2.63
B37	200	3500	50	1050	1100	5700	2.89
Y165	1200	4600	0	900	2600	8100	6.30
Y166	100	900	0	300	500	1700	3.41
Y176	500	4600	0	1400	2100	8100	N.D.
Y179	500	2900	50	750	1200	4900	9.71
Y182	1200	7600	200	1600	2000	11400	7.35
B15	350	300	0	1350	1700	3350	2.63
B16	650	2300	100	2000	2900	7300	10.50
B20	250	1400	0	600	300	2300	6.10
B30	600	0	0	1550	2150	3700	0.73
B39	100	1500	0	700	1100	3300	5.61
B41	500	2800	0	500	1300	4600	N.D.
B46	1700	2300	0	1600	1800	5700	N.D.
Y192	650	700	0	1100	1300	4100	0.73

N.D. - not done, due to blood sample clotting.

7.4.2.2: Histopathology.

The appearance of the non-nodular areas of mucosa of most animals, in Haematoxylin and Eosin stained sections, was largely normal, but there was evidence of slight epithelial cytolysis at the superficial tissue margins. In some animals there was also moderate hyperplasia of mucous cells at the level of the upper gland and isthmus, even within the intervening areas of grossly normal mucosa. A cellular infiltrate of mainly lymphocytes and therefore typical of chronic inflammation was detected, but the level of infiltration within normal areas was slight. The distribution of intraepithelially situated globule leukocytes was sporadic with some areas heavily infiltrated by these cells. Parietal cells in these more-normal areas appeared histologically and numerically normal. Changes associated with the presence of infection were largely confined to nodular areas. Occasional worm larvae were seen within nodules, coiled within glands with the epithelium of the surrounding gland often floridly hyperplastic and moulded to the worm's outline. Some nodules contained more than one parasitic worm, occupying different glands, and at different stages of development. At the edges of nodules, parietal cell density appeared normal and changes were confined to mucous neck cell hyperplasia, so that the pits and glands were straight and tall and with little distinction between the two. The edges of many nodules were abrupt, with a step down to the adjacent normal mucosa.

At the centre of nodules mucous cells extended towards the mucosal base. Recognisable parietal cells and chief cells were absent at all levels. The enlarged pits were wide in many instances, almost cigar-shaped (Fig. 7.20). Mitotic figures were more numerous than normal, indicative of increased cell divisions, and occurred from just above the base of the glands to just below the mucosal surface. Cells lining the enlarged pits were variable in appearance. At the mucosal base poorly differentiated cells predominated. The basophilia, characteristic of the basal cytoplasm of chief cells, normally predominant at this level of the mucosa, was gone. Instead the cytoplasm was pale and eosinophilic, sometimes with an even paler, unstained area immediately adjacent to the nucleus. The nuclei themselves appeared larger than normal and were pale. Higher up the gland, cells were characterised by the presence of a mucus cap of variable size. In some nodules the majority of cells in the pit/glands possessed relatively small amounts of mucus, with significant amounts present only in the most superficial cells. This was the case in sections from sheep Y27. In contrast Fig. 7.20 shows a section of mucosa from animal Y51 and the mucus content of cells at all depths of the

mucosa was high. Superficial cells were flask-shaped with flattened nuclei at their bases and reduced cytoplasmic volume, and their mucin content appeared greater than that of normal pit cells. Nodules from other animals were generally at a mid-point between the extremes represented by animals Y27 and Y51, since hyperplasia was usually prominent, but the massive hypertrophy of mucous cells observed in the nodules present in sheep Y51 was not as marked.

Nodular areas were massively infiltrated by immune cells, sometimes grouped as large follicles below the mucosa, occasionally extending upwards through the muscularis into the lamina propria. Heavy immune cell infiltration within the mucosa appeared to spread glands further apart. Superficially there were often massive numbers of globule leukocytes situated intraepithelially. Eosinophils were an inconsistent feature, but were occasionally seen around parasitised glands and scattered within the lamina propria.

An examination of sections stained with PAS reinforced the differences in cell mucin content observed in H&E-stained sections. In the more grossly normal mucosa from animal Y27, the only difference from a normal mucosal appearance was the presence of PAS-positive material at deeper gland levels. This feature was even more prominent in the changed mucosa of Y27 (Fig. 7.21), but the quantity of mucus per cell remained small. The nodular mucosa from Y51 (Fig. 7.22) had considerable quantities of mucus contained in many cells at all levels, but the most basal cells in glands still contained only small amounts.

In both Y166 and Y182 the similarity of the gross changes reported in these animals to those described in Y27 earlier, was not repeated in the histopathological changes. Parietal cells were still evident in much of the mucosa, in all sections from both animals, in good numbers. Massive mucous cell hyperplasia and parietal cell loss were both apparent, but only as focal lesions around parasitised glands and were not generalised as in tissues from Y27. In intervening areas a lesser degree of mucous cell hyperplasia was apparent and the mucosae from these animals appeared correspondingly thicker. In sheep Y166 chief cells appeared abnormal (Fig. 7.23). The basophilia characteristic of the basal cytoplasm of normal chief cells was absent and the apical cytoplasm contained few granules. The cytoplasm was instead pale, and with a paler perinuclear area on the apical side of the nucleus. Mucus caps were not apparent, and in PAS-stained sections these cells were not PAS-positive.

Fig. 7.20: An area of extensive change within a nodule in the fundic mucosa of Y51. Large cigar-shaped pits extend the majority of the mucosal depth and are lined by more mature mucous cells characterised by clearer apical cytoplasm. Infiltration of the mucosa is mainly of intraepithelial globule leukocytes which can be visualised as highly eosinophilic cells. The sub-mucosa is filled with a large accumulation of lymphoid cells (H&E stain, × 25).

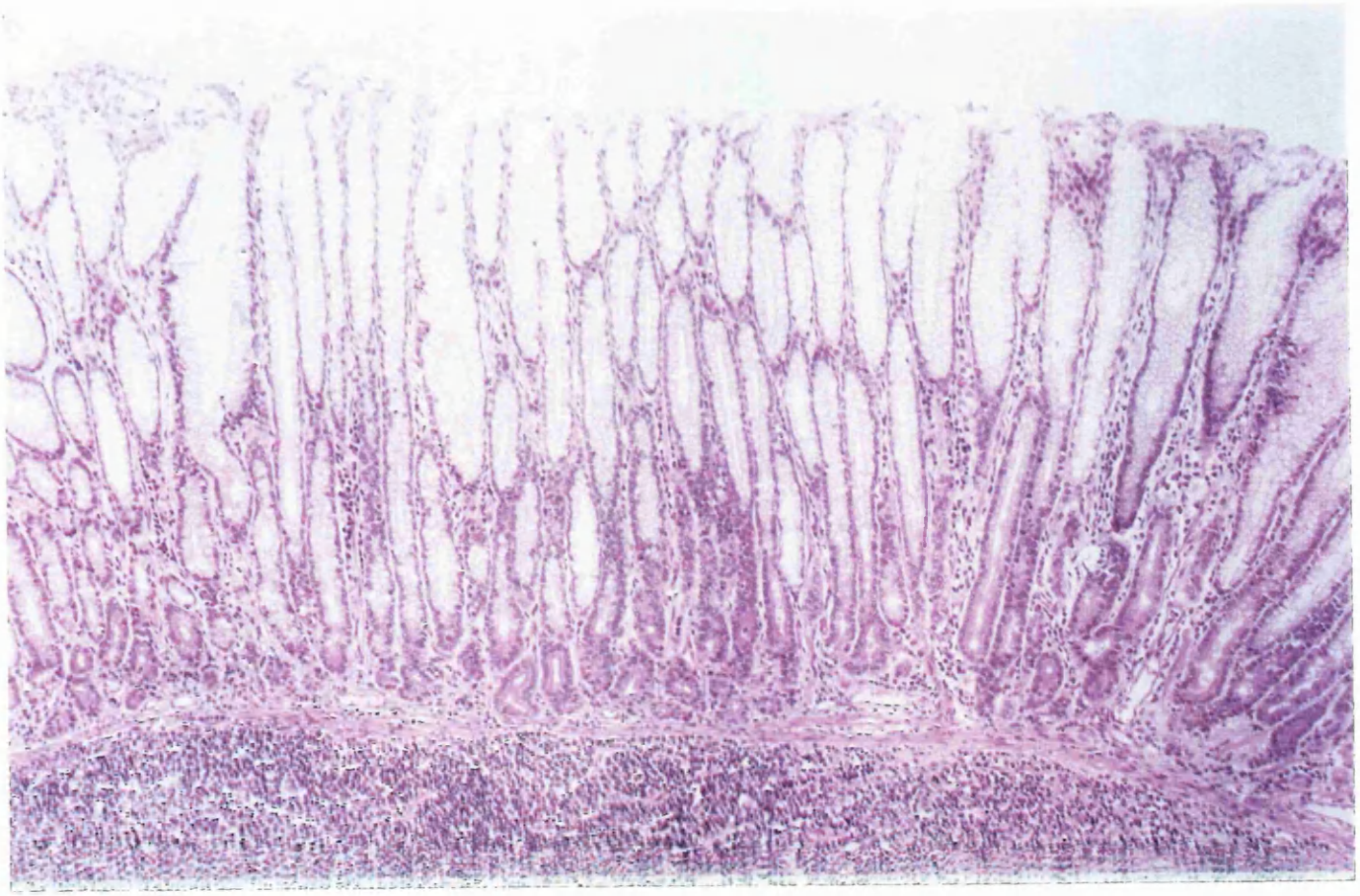


Fig. 7.21: Marked mucous cell hyperplasia within the abnormal epithelium of Y27. Parietal and chief cells are not discernible in the glands and are replaced by cells beginning to produce mucus. (PAS stain, × 25).

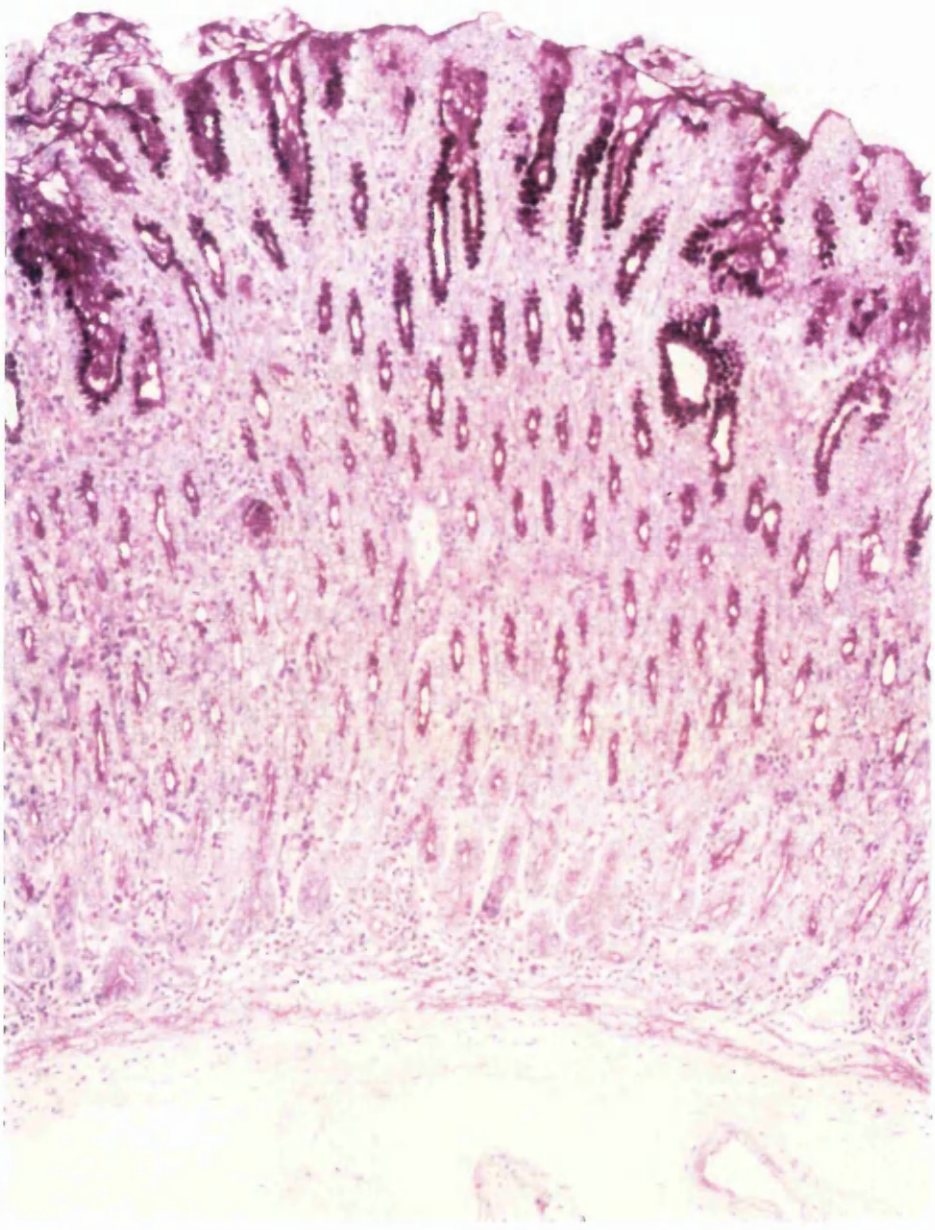


Fig. 7.22: Marked hypertrophy of mucous cells accompanying the hyperplasia associated with nodular lesions in the mucosa of Y51. (PAS stain, × 16).

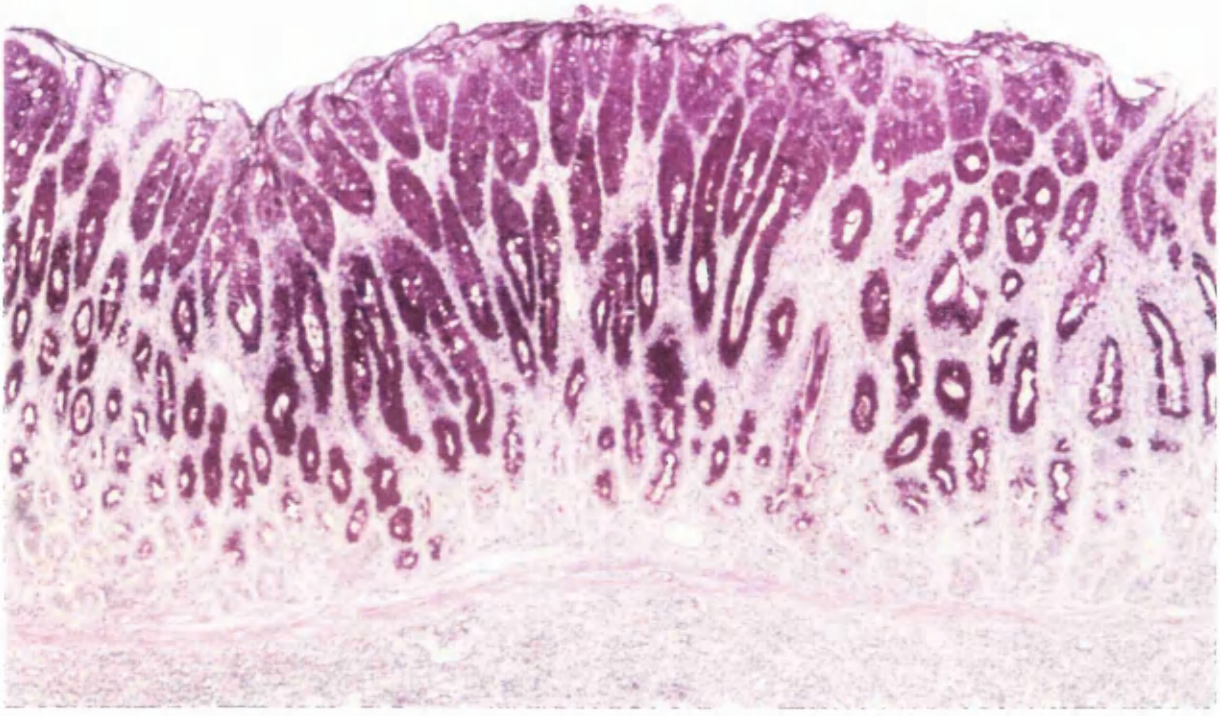
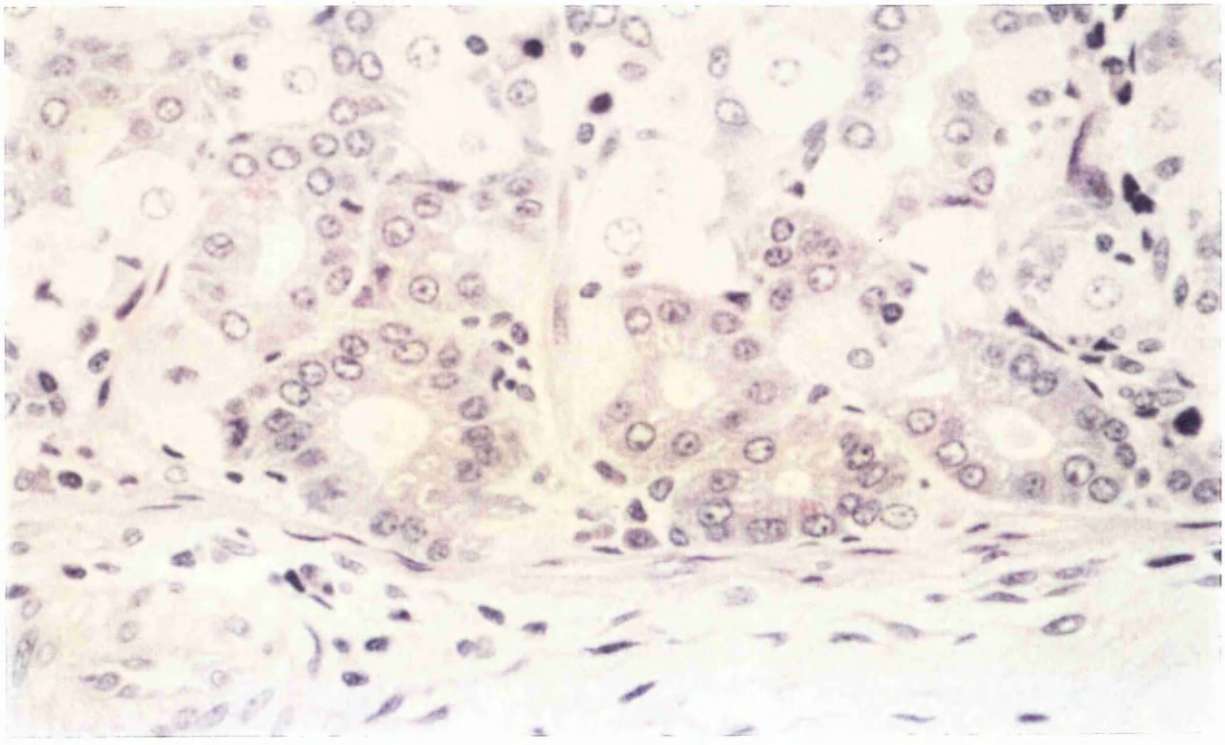


Fig. 7.23: The appearance of the abnormal chief cells lining the glands of Y166. (H&E stain, × 100).



7.4.2.3: Pepsinogen immunolocalisation.

The distribution of pepsinogen within grossly normal mucosae was largely the same as for parasite-naive animals. In some animals there was an enhanced degree of staining that could be attributed to the slight mucous cell hyperplasia identified at the upper gland/isthmus level. This was greatest in animal 02, where pepsinogen-specific staining was also demonstrable in the mucous cells of the pits even superficially. An examination of the basal portion of some glands in the mucosa of 02 also revealed a slight reduction in the pepsinogen content of the chief cells. The diminished content of chief cells was also apparent in animals Y27 and Y166. Chief cell pepsinogen content was reduced even in the relatively normal mucosa of Y27, in areas where parietal cells were still apparent, (Fig. 7.24). In the hyperplastic mucosa from animal Y27 no one level of the mucosa stained more intensely than any other (Fig. 7.25). The chief cells of Y166 that had appeared abnormal in H&E stained sections did stain for pepsinogen, but the granule content was reduced (Fig. 7.26).

Figures 7.27 and 7.28 show adjacent sections of the nodule shown previously in Fig. 7.20. Within nodules, pepsinogen immunostaining was prominent in the hyperplastic mucous-type cells lining the enlarged pit/glands, yet both photomicrographs illustrate the mixed nature of adjacent glands that was apparent. The cells lining some pit/glands were relatively free of pepsinogen immunostaining whereas adjacent structures were lined by cells strongly positive for pepsinogen. Fig. 7.28 shows that parasitised glands (p) were associated with increased pepsinogen content of surrounding structures, with an expanded population of cells positive for pepsinogen (Fig. 7.29). Deep within nodules the metaplastic cells replacing the normal chief cells at the base of glands did stain for pepsinogen (Fig. 7.30), but the marked granularity of chief cells was lost.

Two unusual features were detected in this study. In a section from one animal glandular elements at the centre of one nodule were present below the muscularis (Fig. 7.31). Cells lining this structure were weakly positive for pepsinogen and therefore assumed to be epithelial cells. In another section a direct communication was seen between the sub-mucosal structure and the mucosa. The second unusual feature seen in a section from another animal was the presence of a number of pepsinogen-positive cells scattered within the lamina

propria and submucosa. These cells were in an area of considerable mucosal damage with glandular degeneration and erosion of the overlying epithelium, surrounded by an inflammatory infiltrate of mostly lymphocytes which obliterated the muscularis in this area. The pepsinogen-positive cells were either scattered singly within the lamina propria and sub-mucosa (Fig. 7.32), or were accumulated into thin walled vessels in the sub-mucosa (Fig. 7.33). These cells were seen in consecutive sections taken from the same block of mucosa, but were not seen elsewhere in other sections of sub-mucosa from the same animal, or in similar sites in tissues from any of the other animals.

7.4.2.4: Mucosal wet weights and tissue pepsinogen concentrations.

Tissue wet weights and the tissue pepsinogen concentrations of the first five animals are shown in Table 7.4 (Full data is shown in Appendices 19A to C). Not surprisingly the mucosal cuts bearing nodules were significantly heavier ($p<0.05$) than the non-nodular areas. Tissues were generally of similar thickness in all animals and were similarly thickened by the presence of nodules (test for the effect of sheep on overall variation in wet weight, by ANOVA, $p=0.081$). Figure 7.34 shows the differences in tissue pepsinogen concentrations between nodular and non-nodular areas of mucosa and between individual animals and tissue pepsinogen (expressed as peptic units per standard area of mucosa) was found to be significantly greater in nodular mucosa ($p<0.05$), however there was no difference in pepsinogen content when calculated as peptic units per gram of wet tissue ($p=0.192$). The mucosal pepsinogen concentrations of the individual sheep were different ($p<0.02$, by ANOVA), but the effect of the presence of nodules was similar in all animals, as shown by Fig. 7.34, where the lines connecting the two mucosal types have similar gradients. The interactive terms (**sheep** × **mucosal type**) were not significant for all three test parameters ($p>0.10$, 0.50 and 0.25, for wet weights, total tissue pepsinogen and pepsinogen concentration respectively).

Fig. 7.24: Immunolocalisation of pepsinogen in the more normal mucosa of Y27. Pepsinogen is present in all cells of the tall and narrow pits and the content of chief cells appears reduced (× 25).

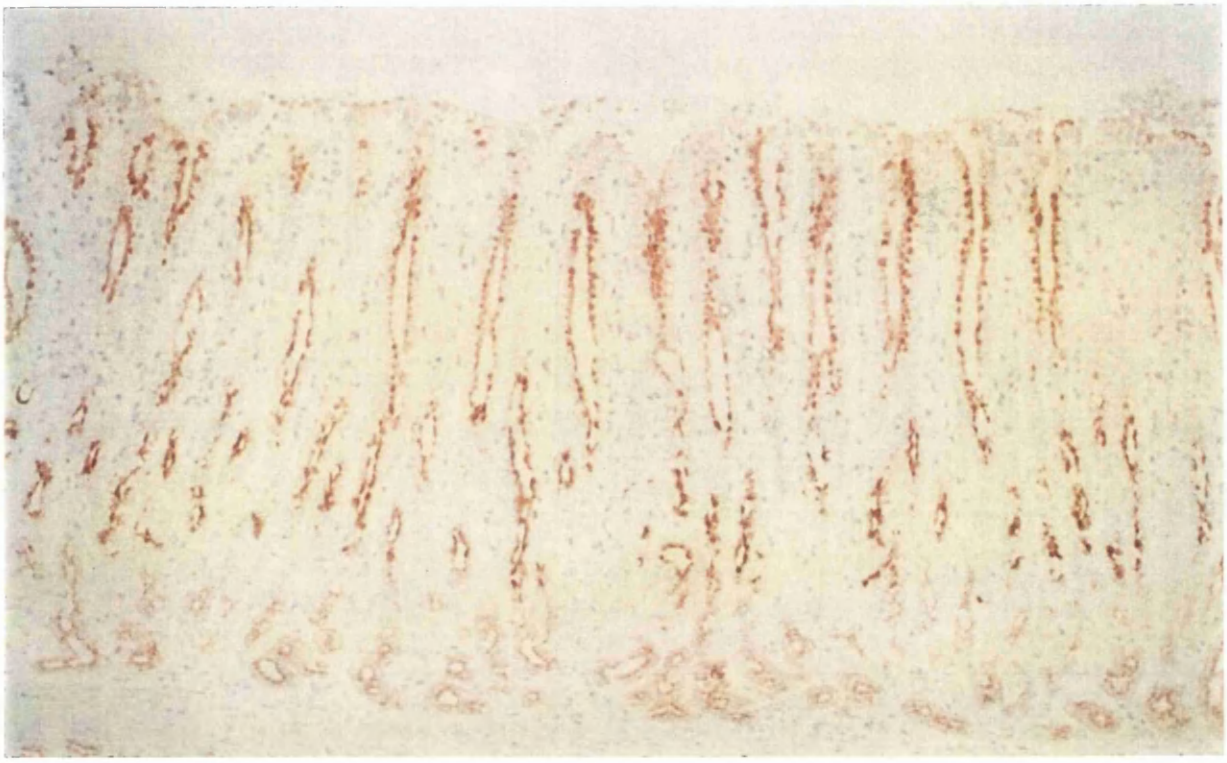


Fig. 7.25: Immunolocalisation of pepsinogen in the changed mucosa of Y27. No one level of the mucosa dominates in terms of the intensity of staining (× 10).

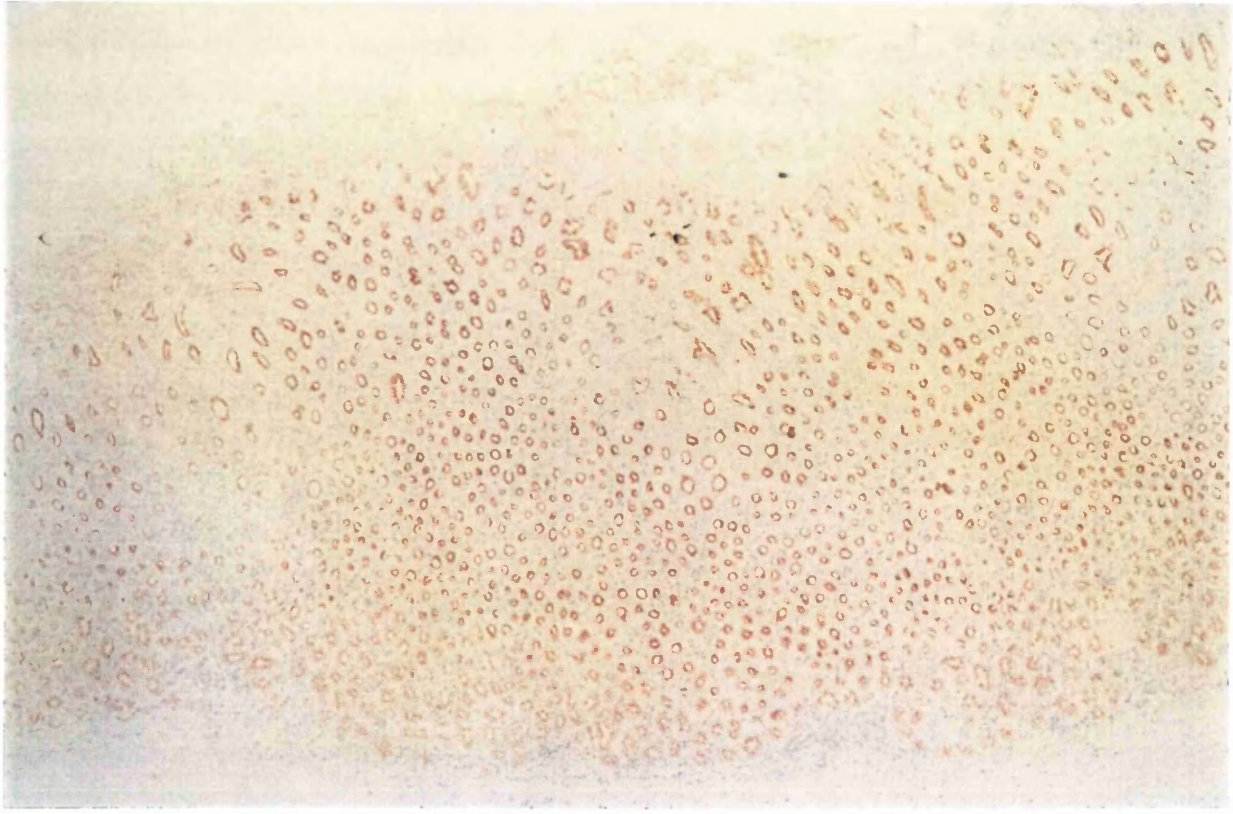


Fig. 7.26: The immunohistochemical localisation of pepsinogen to the abnormal chief cells of Y166. Note that pepsinogen-specific staining is still present, but that granule density appears less (× 100).

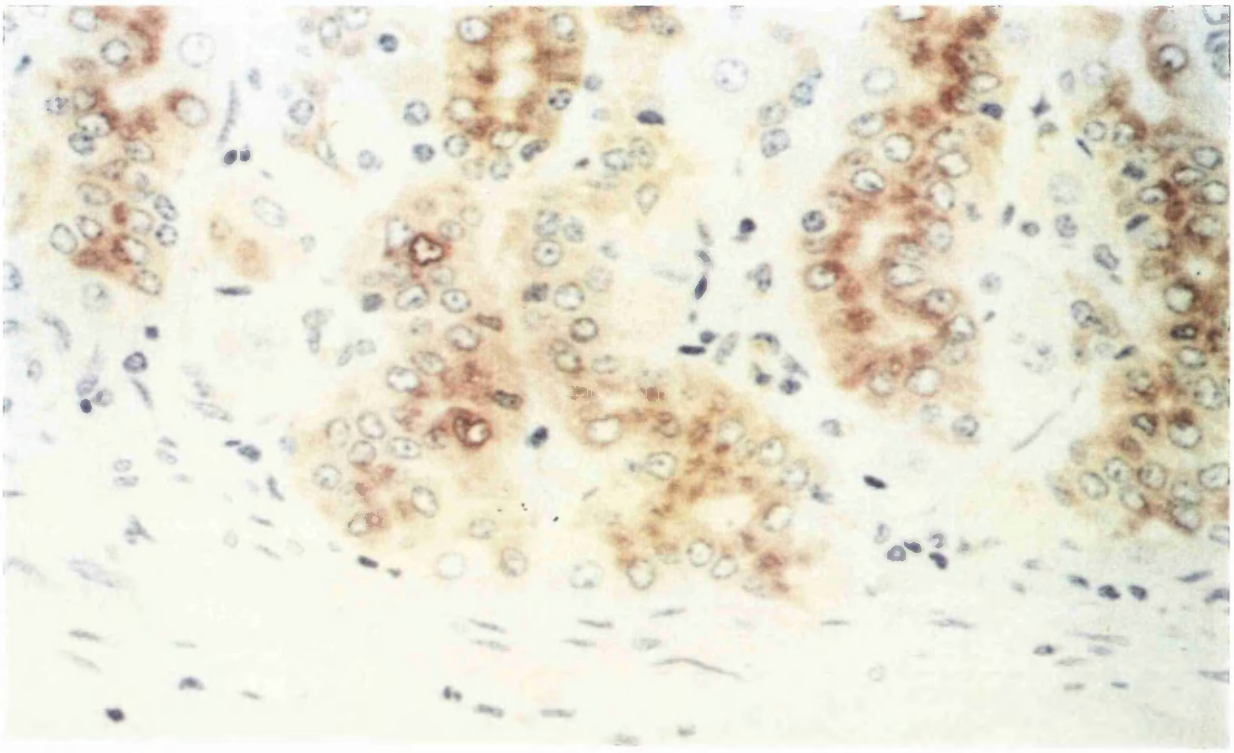


Fig. 7.27: Section of the edge of the nodule featured in Fig. 7.20. Pepsinogen immunostaining is prominent in hyperplastic mucosal cells at the stepped edge of the lesion (× 10).

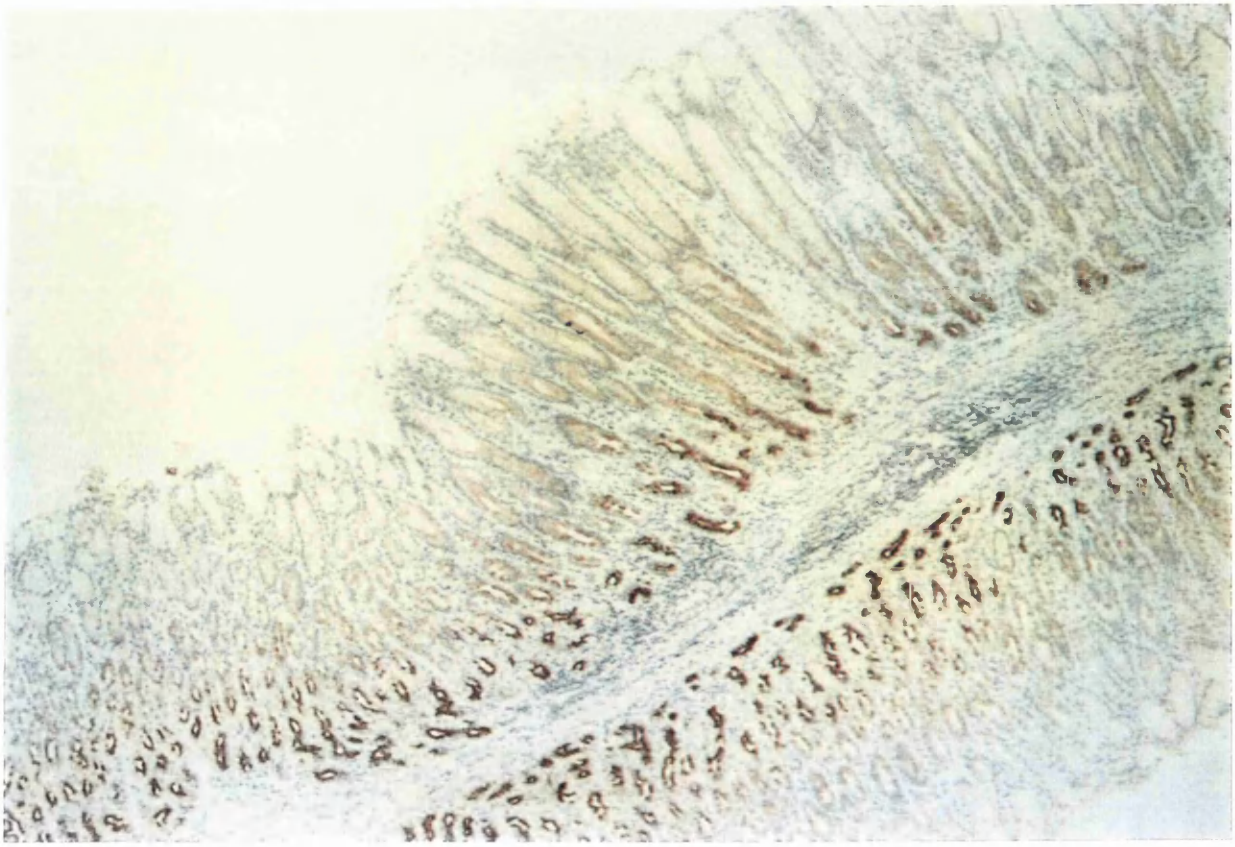


Fig. 7.28: Mid-section of the nodule featured in Fig. 7.20. Parasitised nodules (p) are associated with focal increases in pepsinogen immunostaining, adjacent glands may show depletion of stain (arrows)(× 10).

Fig. 7.29: Hyperplasia of mucous cells at a mid-mucosal level. (Pepsinogen immunostain, × 100).

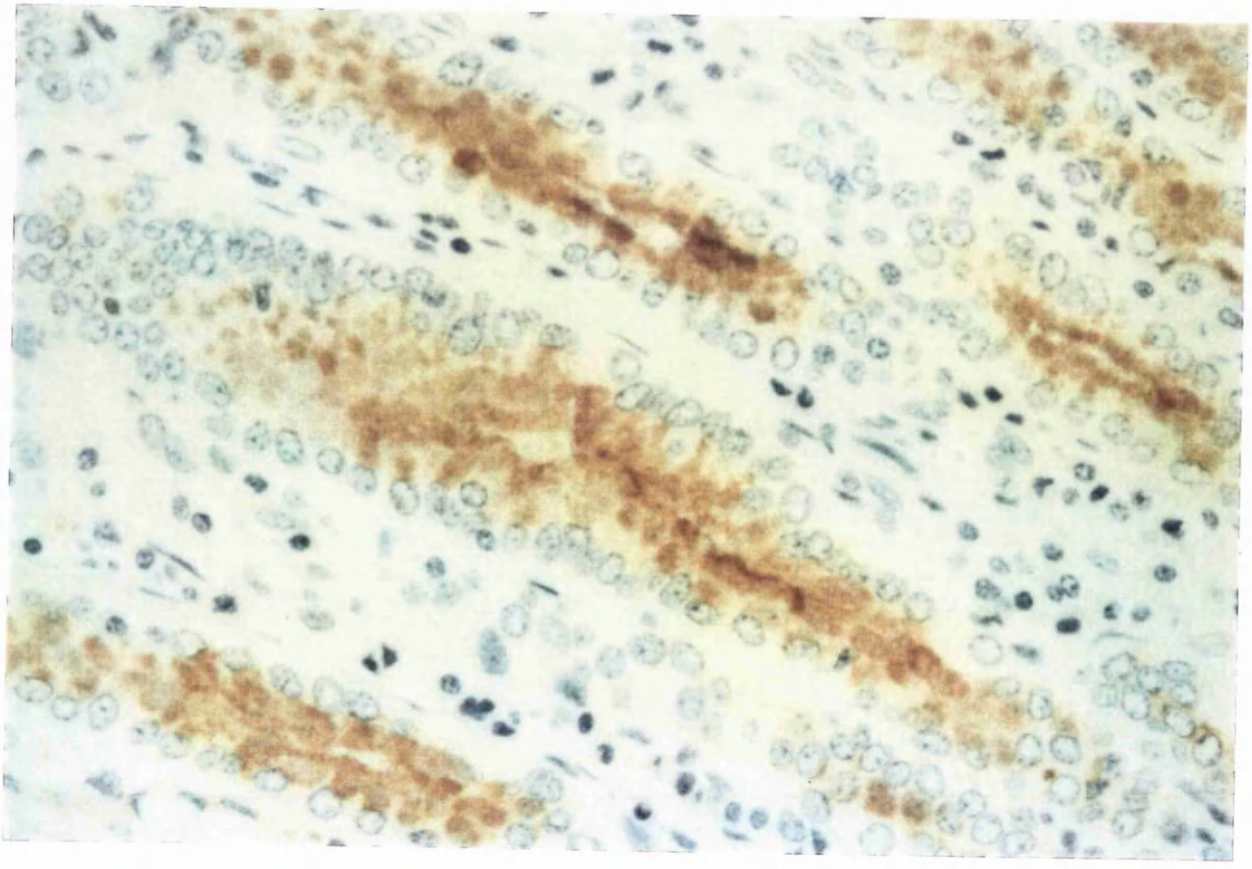


Fig. 7.30: Immunohistochemical appearance of the metaplastic cell population, replacing chief cells in deep mucosal areas within nodules. (Pepsinogen immunostain, × 100).

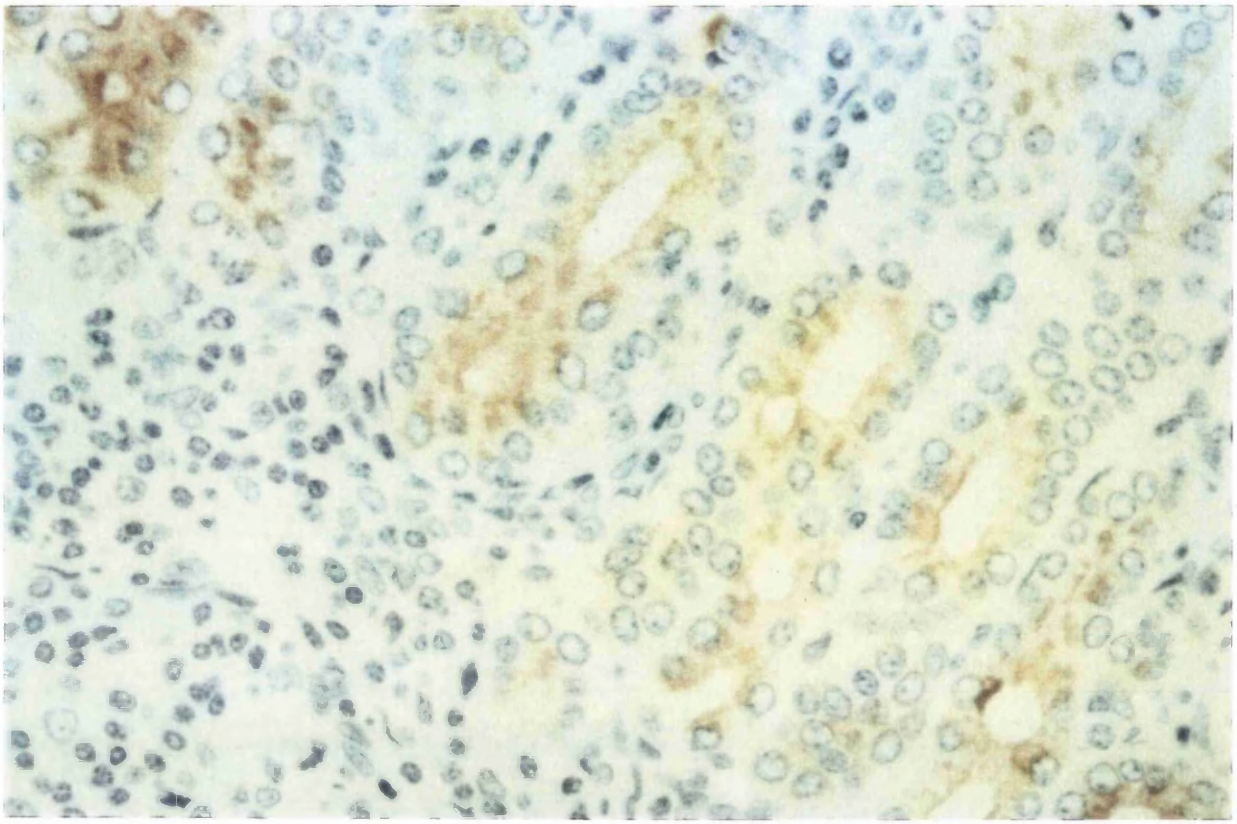


Fig. 7.31: Section showing glandular elements (with pepsinogen content) present in the sub-mucosa. (Pepsinogen immunostain, × 13.2).

Fig. 7.32: Unusual pepsinogen-positive cells scattered in the sub-mucosa below a destructive mucosal lesion. (Pepsinogen immunostain, × 50).

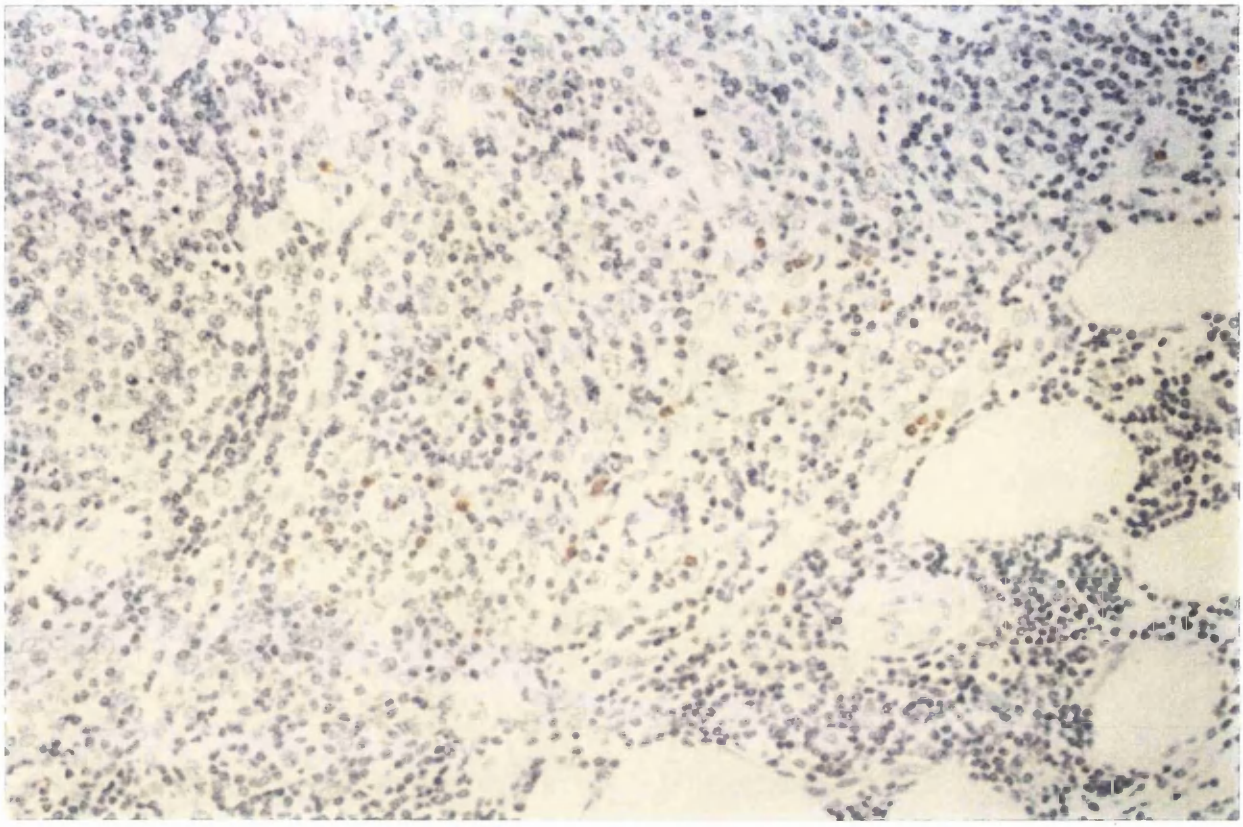


Fig. 7.33: Unusual pepsinogen-positive cells collected in a small calibre vessel in the sub-mucosa. (Pepsinogen immunostain, × 250).

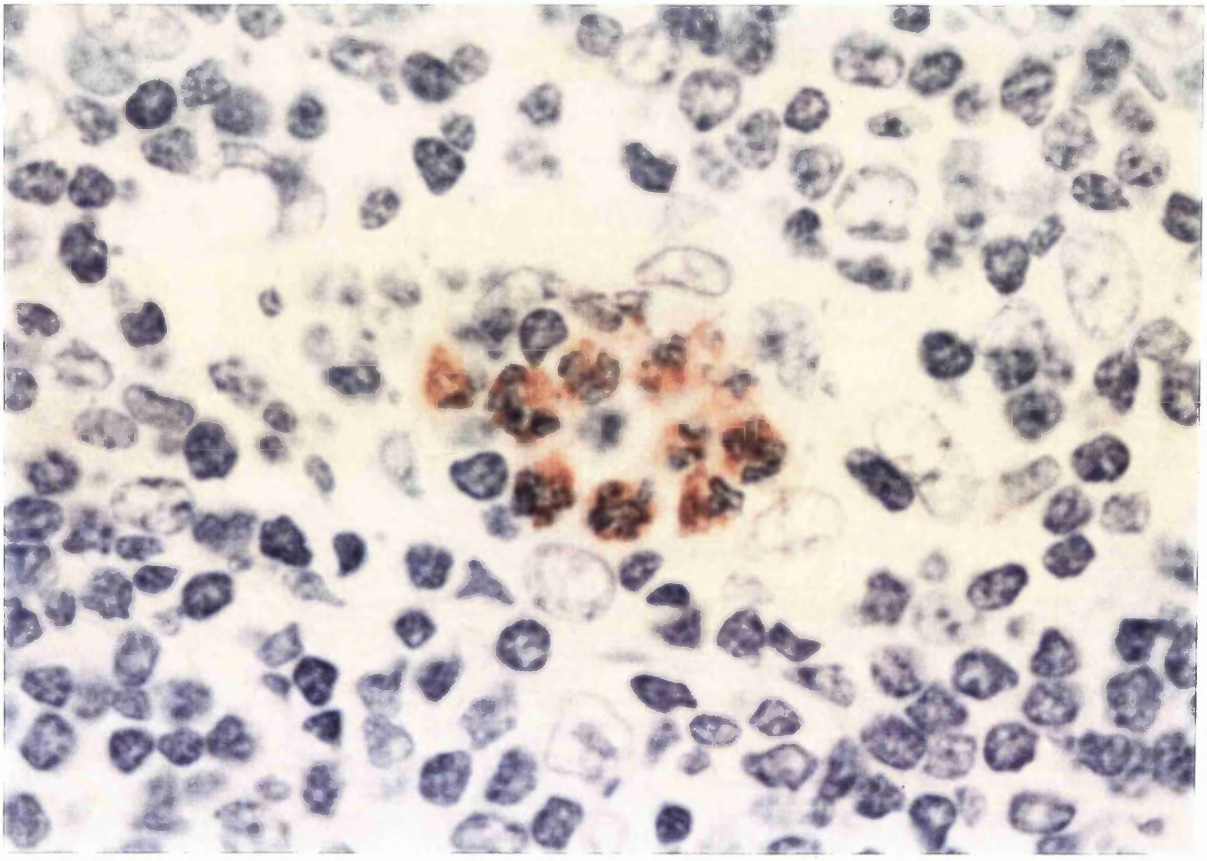
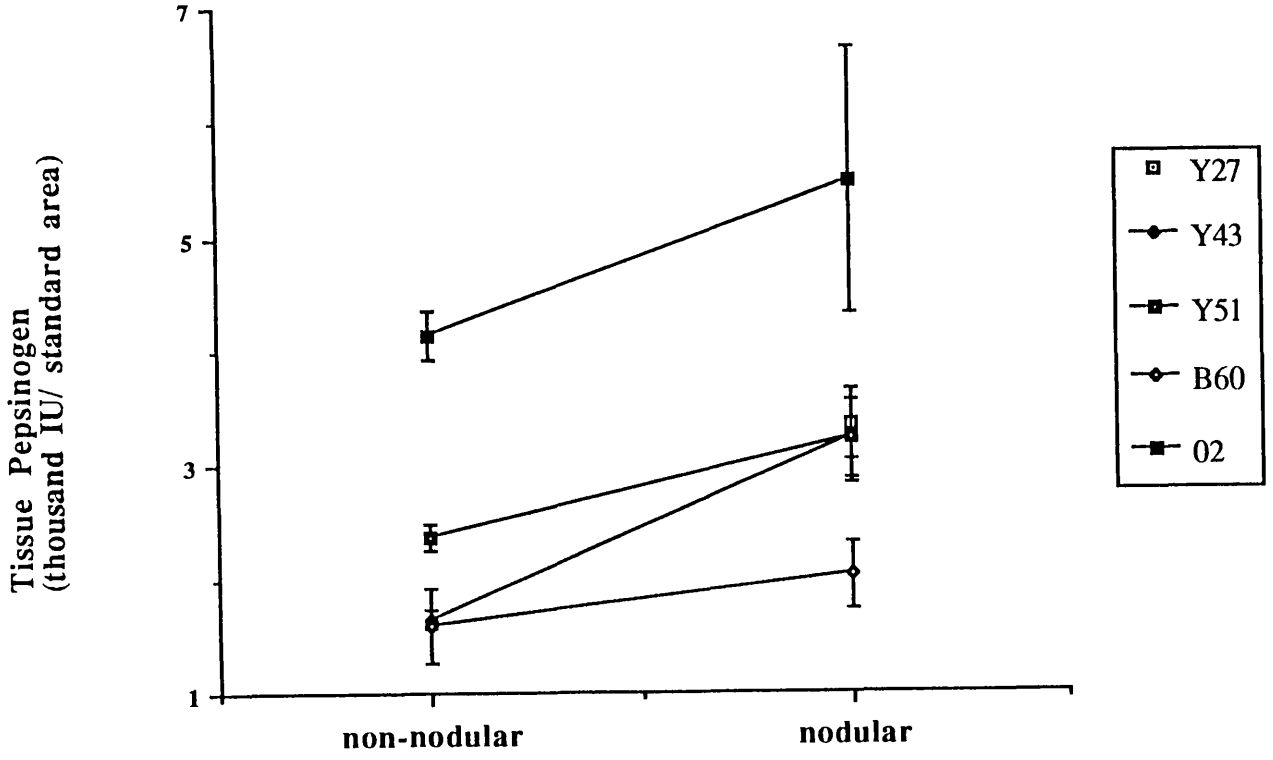


Table 7.4: Mean (± S.E.) values for mucosal wet weight and tissue pepsinogen concentrations of naturally infected lambs.

Animal	mucosal type	Wet weight (g)	Total Tissue Peps (iU/ area)	Tissue Peps (iU/g)
Y27	nodular	0.114 (0.006)	3,361 (304)	29,323 (2,478)
02	non-nodular	0.087 (0.004)	4,145 (220)	47, 928 (4,097)
	nodular	0.110 (0.008)	5,495 (1,163)	48,970 (9,229)
Y43	non-nodular	0.099 (0.008)	1,642 (82)	16,583 (660)
	nodular	0.124 (0.005)	3,246 (345)	26, 357 (3,825)
Y51	non-nodular	0.077 (0.003)	2,370 (126)	30,854 (1,604)
	nodular	0.089 (0.003)	3,250 (415)	37, 707 (5,404)
B60	non-nodular	0.090 (0.002)	1,590 (328)	17,722 (4,026)
	nodular	0.096 (0.004)	2,030 (304)	20, 397 (3,017)

Notes: means of observations of four mucosal cuts of each type per animal, except Y27, mean for eight mucosal cuts of predominantly changed mucosa.

Fig. 7.34: Comparison of tissue pepsinogen concentrations in nodular and non-nodular mucosa in sheep with natural infections, mean values (± S.E.) of four mucosal sections of each mucosal type from each animal, except for Y27; eight sections, all of nodular type.



7.4.3: Discussion.

By comparing tissue pepsinogen concentrations expressed as peptic units per area of mucosa, the pepsinogen content of parasitic nodules was higher than that of non-nodular mucosa. However when pepsinogen content, expressed as pepsinogen per gram of tissue, was compared the differences were lost. Thus the observed increase of tissue pepsinogen could simply reflect an overall increase in tissue mass, rather than an increase in the pepsinogen content of individual cells. However changes in the pepsinogen content of individual cells were observed immunohistochemically. Hyperplastic mucous cells within the nodules formed an expanded population of pepsinogen-producing cells, but judging by relative staining intensities these cells produced less pepsinogen individually than would normal chief cells. At the same time mature chief cells became less demonstrable within nodules. Both these effects were mirrored by similar changes in the 'normal' mucosal areas in some animals (such as 02, Y27 and Y166), but to a lesser extent. These findings are similar to the observations of Stringfellow and Madden (1981) who reported the depletion of chief cell granules following single experimental infections. There is some evidence to suggest that prolonged stimulation of pepsinogen release may not involve granule formation (see section 4.1), and the findings of the present studies could suggest hypersecretion of pepsinogen. The actual secretory rates of pepsinogen by mucosae and the peptic activities of the gastric contents of individual animals were not assessed, so it is not possible to confirm this hypothesis. However the assumption of Stringfellow and Madden (1981), on the basis of the appearance of chief cells, that pepsinogen production ceased in infected animals, ignored the potential reserve of pepsinogen producing cells that clearly exists within the abomasum.

At the time of necropsy the most severe lesions were in the mucosa of sheep Y27. The severity of the pathology in the abomasum of Y27, with generalised hyperplasia and subjectively lower parietal cell numbers, was not reflected in clinical illness, and all twenty six animals in this study were passing well-formed faeces at the time of necropsy. Severe abomasal pathology has been associated with type II disease in cattle (Snider et al, 1983), as waves of larvae develop, but although the numbers of L_4 in Y27 indicated the existence of an inhibited population, they were not greater than L_4 numbers present in other sheep.

Two unusual features were seen in tissues from this group of animals. The first feature was very similar in appearance to the gastric cystica profunda that have been associated with

Menetrier's disease in humans (Van Kruiningen, 1977) and in the stomachs of transgenic mice overexpressing TGF-α (Takagi et al, 1992). It is thought that the hyperplasia occurring in the pits/glands of the mucosa encourages the penetration of the muscularis mucosa. The muscularis must presumably need to be weakened for this to happen and in the example in this study the penetration of the muscularis by the inflammatory infiltrate might have been a pre-disposing lesion. The muscularis was also disrupted in association with the second unusual feature observed, but here the overlying mucosal lesion was destructive, not hyperplastic. The degeneration of chief cells in normal animals is thought to occur by necrosis and apoptosis (Karam and Leblond, 1993c). Necrotic chief cells are lost to the glandular lumen whereas those undergoing apoptosis are phagocytosed by other chief cells or by macrophages that invade the gland from the lamina propria. The pepsinogen-positive cells seen in the lamina propria and sub-mucosa in the mucosal lesion could be macrophages clearing up the tissue destruction in the epithelium then migrating back to the vascular systems. Destructive tissue lesions were uncommon in this study, most lesions were clearly hyperplastic, and this may account for the absence of similar cells in other sections and in other animals.

8: THE EFFECTS OF INFECTIONS OF SHEEP WITH *HAEMONCHUS CONTORTUS* ON THE DISTRIBUTION OF TISSUE PEPSINOGEN.

8.1: Introduction.

Haemonchus spp. parasites have a widespread distribution, but are more important as parasites of ruminant livestock in tropical and sub-tropical regions (Allonby, 1973). The pathology of haemonchosis in cattle and sheep has not been researched as extensively as that of ostertagiasis. The life cycle for *Haemonchus* spp. was first described by Ransom (1906), and was followed by a report detailing the precise morphology of the different developmental stages (Veglia, 1915). Like *Ostertagia* spp. the life cycle of *Haemonchus* spp. is direct, with development from ingested L_3 to adults taking place within the confines of the abomasum. Haemonchus larvae penetrate the gastric pits and undergo the moult to the fourth-larval stage. It is at this point, 48 to 60 hours after ingestion, that they leave the glands to become ambulatory on the mucosal surface (Veglia, 1915; Silverman and Patterson, 1960; Christie et al, 1967; Nicholls et al, 1985), thus the pathology of haemonchosis does not remain as focal as that of ostertagiasis. The moult to the fifth-larval stage occurs 10 days after initial infection (Veglia, 1915), and from this point the behaviour of *Haemonchus* spp. worms further differs from that of *Ostertagia* spp. The fifth-larval and adult stages of *Haemonchus* spp. possess a buccal lancet, that is used to penetrate mucosal blood vessels, allowing the worms to feed on the resulting haemorrhage. Females are prolific egg layers, eggs appearing after two to three weeks. Egg laying has been correlated to blood-feeding (Dargie, 1973) and severe blood loss may arise along with the onset of egg production (Whitlock, 1966).

Three clinical syndromes of ovine haemonchosis are recognised (Allonby, 1973). Hyperacute haemonchosis, characterised by low morbidity, has been associated with massive larval challenge and the simultaneous development of blood feeding stages. Up to 35,000 worms may be responsible for a severe haemorrhagic gastritis and a rapidly fatal severe anaemia and sudden death. The acute disease syndrome has been associated with smaller numbers of worms (up to ten thousand), a mixed population of different worm stages and a lower grade, but persistent, anaemia and hypoproteinaemia. Animals with acute disease may

show generalised oedema and 'bottle-jaw' (marked submandibular oedema). Animals may be lethargic, and in poor bodily condition, with wool loss. Agalactia may cause problems for suckling lambs. Untreated the condition is likely to prove fatal. In chronic haemonchosis smaller worm numbers (perhaps only a thousand) have been associated with chronic, low grade blood loss and abomasal dysfunction, leading to lethargy, marked weight loss and general poor performance. Anaemia and oedema may not be clinically apparent. The abomasal lesions may include severe and generalised hyperplasia and mucous-cell metaplasia. The outcome for affected animals may become particularly grave if subjected to drought conditions on poor pasture.

The presence of larvae within fundic gastric glands in the first hours of infections was associated with small nodules, 1 to 2 millimetres across (Charleston, 1965), generally with a central depression lying over the parasite-occupied gland. The edges of the nodules displayed mucous cell hyperplasia histologically. Nodules diminished by 6 days after a single infection, but from this time on the whole mucosa began to be thicker due to a generalised hyperplasia. Hyperplasia was of the foveolar compartment, with little effect on the glands themselves. In the studies of Hunter and MacKenzie (1982), following a single experimental infection, mucosal depth reached a maximum of close to twice normal, after the twelfth day of infection, and subsequently declined, but remained greater than normal at least until the thirty-fifth day. Generalised hyperplasia was associated with later larval and adult stages moving in and out of the mucosa, and with the onset of blood-feeding after approximately the tenth day of infection. The damage caused by later worm stages included the disruption of the epithelium at feeding sites and the appearance of honey-comb cells due to the loss of the contents of mucous cells on the mucosal surface. The presence of the parasites has been shown to provoke typical inflammatory responses involving eosinophils, mast cells and globule leukocytes, and lymphocytes (Salman and Duncan, 1984).

Abomasal function can be severely affected by haemonchosis. Doses of one million infective larvae resulted in elevated abomasal pH by the third day of infection (Christie et al, 1967). The mean abomasal pH of infected lambs rose to 5.5 by the third day of infection in the studies of Mapes and Coop (1970), after an identical dose of larvae, and the abomasal pH of lambs was still elevated after 19 days of infection. Mapes and Coop (1970) also demonstrated a rise in plasma pepsinogen concentration concomitant with the elevation in pH. Plasma pepsinogen concentrations returned to normal levels by the nineteenth day of

infection. Elevations of plasma gastrin have been demonstrated in groups of sheep given either fifty thousand or a million larvae (Nicholls et al, 1985), whereas abomasal pH only rose in those given the larger larval dose. The timing of the pH rise was similar to that found in other studies, with the pH rising, to 6.5, by day three of infection.

Plasma pepsinogen becomes elevated in both haemonchosis and ostertagiasis. Mucous cell hyperplasia is also a feature common to both infections, being generalised in haemonchosis after the decline of the initial hyperplastic nodules, but remaining focal for much longer in ostertagiasis as worms remain within the gastric glands for a longer period. Therefore, the following immunohistological and biochemical studies were designed to establish whether changes in abomasal cell populations (chief and mucous cells) associated with experimental *Haemonchus contortus* infection would affect the distribution of tissue pepsinogen, and whether infections were associated with an overall increase or decline in mucosal pepsinogen content. Experimental haemonchosis was studied in Hampshire Down lambs, a breed considered to be relatively parasite-susceptible (Loggins et al, 1965; Preston and Allonby, 1979). These animals were part of a larger study into the influence of nutrition and dietary protein/Nitrogen supplementation on the resistance of sheep to haemonchosis, carried out in collaboration with the Department of Veterinary Clinical Studies at Glasgow University Veterinary School. Two studies were performed over consecutive years. Tissues were obtained from animals killed in the first study to initially assess the histological and immunohistochemical changes due to haemonchosis.

8.2: Initial investigations of ovine haemonchosis.

8.2.1: Materials and methods.

8.2.1.1: Animals and experimental design.

Twenty four, five month old, Hampshire Down lambs, that had been reared in helminth-free conditions were allocated into four groups (two groups of four, two of eight) by stratified random sampling, according to body weight, haemoglobin type and sex. Lambs were fed one of two diets which differed in protein content, a basal diet (BD) containing 98g crude protein (CP) per kilogram of dry matter (DM), and a soyabean meal supplemented diet (SD) containing 173g CP/ kg DM (Wallace et al, 1995). The experimental design was such that

half of the lambs received the basal diet, the other half the supplemented diet. Of the twelve lambs in each dietary group, eight were infected with larvae of *H. contortus* (BDI and SDI groups). The others remained as uninfected controls (BDC and SDC groups). Two weeks after the lambs had been on the diets, animals (BDI and SDI groups) were given an initial infecting dose of 100 *H. contortus* third stage larvae per kilogram of body weight, and then a trickle infection regime of 200 L_3 three times a week (i.e. a total of 600 per week) for 10 weeks. All animals were housed on slatted floors and initially fed 1.4 kg fresh matter of the allocated diets divided into two equal daily feeds. The amount of food was increased fortnightly to maintain the same rate of feeding per kilogram body weight. The infective larvae were obtained from the Moredun Institute (Edinburgh, Scotland) and were inoculated into the lambs orally. All lambs were killed at the end of the ten week trickle infection period.

8.2.1.2: Procedure at, and subsequent to, necropsy.

Lambs were killed by exsanguination following electrical stunning. Blood was collected, as the sheep were bled out, into heparinised containers which were later centrifuged at 2,000 *g* in a Beckman GS-6R centrifuge for 10 minutes, plasma was separated and then stored at -20°C. Blood was also collected for haematological examination, into evacuated glass tubes containing ethylenediamine tetra-acetic acid (EDTA). Faeces was collected from the rectum of each animal to allow the determination of faecal egg counts (Gordon and Whitlock, 1939)(see section 7.4.1). Abomasa were opened along the greater curvature and their contents were collected and the mucosa gently washed and then one half of the mucosa was scraped. Worm burdens were determined using standard parasitological techniques (Armour et al, 1966)(see section 7.4.1). Mucosal tissue was taken from single sites of the fundus of the remaining half of the abomasum and placed in Brunnel's fixative for routine histology, and for pepsinogen immunohistochemistry (see section 7.2) Negative immunostaining controls included sections prepared omitting the primary, anti-pepsinogen antiserum, and those prepared with the substitution of the primary antiserum by non-immune rabbit serum. Total plasma protein and albumin were measured by continuous flow analysis (Standard Technicon Auto Analyser II). Plasma pepsinogen was assayed using Paynter's method (1992). Packed cell volumes were measured by microhaematocrit centrifugation.

Faecal egg counts at necropsy, for infected animals on the basal and supplemented diets were compared using a two-sample T-test. Total worm burdens were similarly compared with two-sample T-tests. All T-tests were preceded by Bartlett's test for homogeneity of variance. Data for total worm burdens were transformed by taking $\log_{10}$ (worm numbers + 1). Packed cell volumes, and the concentrations of total plasma protein, plasma albumin and plasma pepsinogen were compared by analyses of variance using general linear models. The model fitted was **test parameter = diet + infection + diet*infection**. When the interactive term, **diet*infection**, showed significance, F-statistics for **diet** and **infection** were calculated, using sequential sums of squares, with the interactive term as the denominator for the F-test, instead of the error term.

8.2.2: Results.

The total worm burdens, numbers of L_4, L_5, adult male and female worms, and faecal egg counts (mean values (± S.E.) for the four groups, BDI, BDC, SDI and SDC) are shown in Table 8.1 (full data is recorded in Appendix 20). The worms present in the BDC group were due to one individual animal (number 24) that had 200 adult males and 100 adult female worms at necropsy. The positive faecal egg count of the SDC group was due to two animals that had 100 (animal 15) and 50 (animal 30) strongyle eggs per gram. Mean (± S.E.) packed cell volumes, total proteins, plasma albumins, and plasma pepsinogen concentrations for all four groups are shown in Table 8.2 (full data is recorded in Appendix 21). Total worm burdens of both infected groups were not significantly different ($p=0.85$). Faecal egg counts were however significantly less in animals fed the supplemented diet ($p<0.02$). Plasma pepsinogen concentrations were significantly greater in infected animals ($p<0.02$) and there was no effect of diet ($p>0.50$). Packed cell volumes were similar in both control groups, and lower in infected animals, with the greatest drop in PCV recorded in the BDI group. For the analysis of variance for packed cell volumes the interactive term was significant ($p<0.05$), but the corrected F-statistics for **diet** and **infection** were not significant ($p=0.36$ and 0.28 respectively). Total plasma protein and albumin concentrations were affected by both diet and infection. Both were generally lower in animals fed the basal diet ($p<0.01$ and 0.001 for total protein and albumin respectively) and both parameters were reduced by infection ($p<0.01$ for both); the lowest values were recorded in animals of the BDI group. The interactive terms for both parameters were not significant ($p>0.05$).

Table 8.1: Mean (± S.E.) worm burdens and faecal egg counts of the groups of Hampshire Down lambs.

Group	Worms L_4	L_5	male	female	Total	FEC (epg)
BDC	0 (0)	0 (0)	50 (50)	25 (25)	75 (75)	0 (0)
BDI	94 (45)	300 (70)	687 (159)	631 (158)	1,713 (258)	25,638 (5,613)
SDC	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	37 (24)
SDI	106 (52)	219 (170)	1,163 (643)	781 (292)	1,794 (659)	7,450 (3,726)

Table 8.2: Mean (± S.E.) values for haematological and biochemical parameters of the groups of Hampshire Down lambs.

Group	PCV (per cent)	Total Protein (g/L)	Albumin (g/L)	Plasma Peps. (iU)
BDC	30 (0.70)	60 (1.80)	29 (0.70)	1.40 (1.10)
BDI	23 (1.10)	51 (1.60)	24 (1.20)	6.60 (1.30)
SDC	30 (1.00)	64 (0.90)	32 (0.50)	3.20 (1.40)
SDI	28 (0.70)	59 (1.70)	30 (070)	6.40 (1.40)

8.2.2.1: Gross pathology.

Abomasa from control animals were grossly normal. Haemorrhage and oedema were not observed in any of the abomasa from the infected Hampshire's. Changes were confined to an apparent increase in the thickness of mucosal folds in infected animals. When the mucosae were cut, the increase in mucosal fold thickness was apparent and could be seen to be due to increases in mucosal thickness and not an increase in sub-mucosal structures.

8.2.2.2: Histology.

The fundic mucosae of all control animals, examined in H&E stained sections, appeared normal. The pits were short and lined by surface mucous cells containing moderate amounts of mucus. The epithelium was continuous across the mucosal surface. Pits were seldom greater than one fifth to one quarter of the total depth of the mucosa. Pits were also quite wide. There appeared to be fewer pits than there were glands, with each pit associated with two or three glands at its base. All epithelial cells appeared normal. The height of the glands did seem to vary between animals and this factor appeared to be the greatest predictor of overall mucosal depth in the control animals. Mitotic figures were rare, as were cells of the inflammatory and immune systems. Globule leukocytes were not seen.

The changes detected in infected animals appeared similar in the two dietary groups, with the mucosae from infected animals appearing to be substantially thicker. There was also disruption and loss of surface epithelial elements. Increased mucosal depth was predominantly due to mucous cell hyperplasia at the mid-point of the depth of the mucosa. In PAS stained sections there was increased staining for mucins apparent in some animals at mid-levels of the mucosa (Fig. 8.1), with slight hypertrophy of the hyperplastic cells. Parietal cells were either spread thinly within this level, or were confined to deeper gland areas. Parietal cell numbers were not quantified in this study, but did not appear greatly affected in most instances. Pits became narrower and more tightly packed in the superficial mucosa, so that pit/gland junctions were no longer distinct, and there was an apparent increase in the number of pits and the pit/gland unit became very straight and tubular. In some animals there was a slight increase in size of the zymogenic unit (the most basal gland portion where chief cells usually are the most numerous cells), due to an apparent chief cell hyperplasia. These changes were most marked in the thickest tissue section examined, that from animal 17 (Fig. 8.2). Chief cells generally did not appear abnormal in the tissue from animal 17 however there was also an extensive area of foveolar hyperplasia and complete gland atrophy, with chief and parietal cells being completely replaced by metaplastic mucous cells. Examination of the tissue from this animal stained with PAS revealed that cells deep within this lesion were full of mucins (Fig. 8.3), even cells immediately adjacent to the muscularis.

Inflammatory cell infiltrates present within mucosae were generalised, with many lymphocytes and plasma cells. Eosinophils were seen much less frequently. Globule leukocytes were present in massive numbers in some animals, situated intraepithelially, and they often accumulated focally in large numbers. Globule leukocytes appeared to be present in more animals and in greater numbers in the supplemented diet group, but these differences were not quantified.

8.2.2.3: Immunohistochemical studies.

Pepsinogen-specific staining was strongest in chief cells in control animal fundus (Fig. 8.4), and the staining pattern was identical to those of other parasite-naive sheep previously reported (see section 7.2). The apparent pepsinogen content of mucous neck cells was more variable, but they generally stained less than chief cells. One animal, no. 22 (BDC group)

had very little pepsinogen-specific immunostaining present in any cell type, with only very small amounts present in chief cells (Fig. 8.5).

Several animals in both infected groups showed increases in the amount of staining present in the hyperplastic population from the middle of the mucosa towards the surface (Fig. 8.6), these changes were generalised, being present in all pit/glands of the mucosal section of each affected animal. In contrast the quantity of staining present in chief cells at the base of some glands appeared reduced. A reduction in the pepsinogen content of basal gland cells was most marked within the central lesion in the tissue from animal 17 described earlier (Fig. 8.7). Poorly staining cells at the base of glands corresponded to those that contained much PAS-positive material in other sections (Fig. 8.3). Changes did not appear to be influenced by diet.

Fig. 8.1: Mucous cell hyperplasia and hypertrophy at a mid-mucosal level of an animal from the SDI group, compare this to the appearance of a parasite-naive sheep (Fig. 6.5)(PAS stain, original magnification × 25).

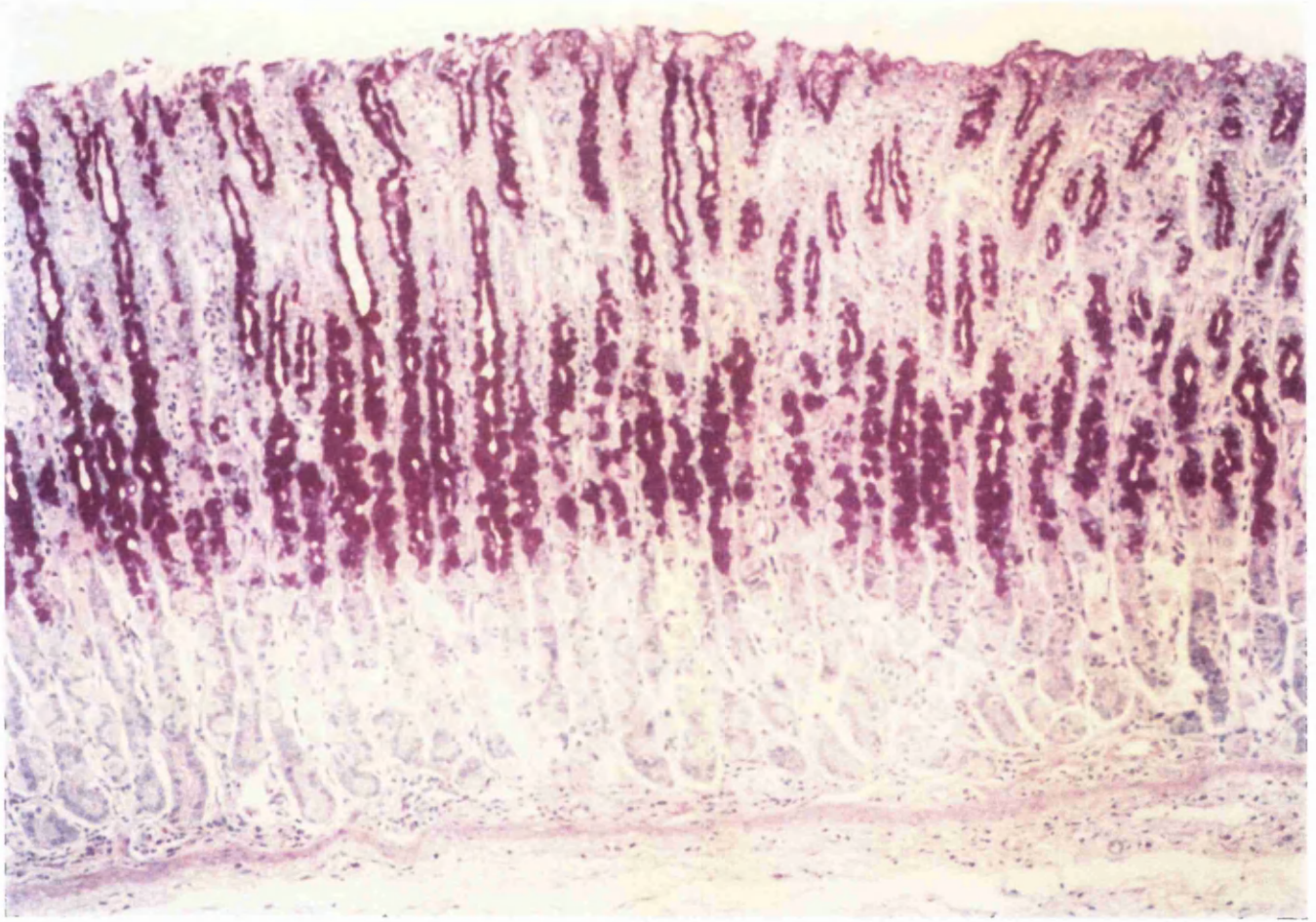


Fig. 8.2: Massive, generalised hyperplasia in the mucosa of animal 17 (BDI group). Note the elongation of zymogenic units at the bases of glands (arrows) and the extensive area of foveolar hyperplasia and complete gland atrophy (arrowheads). There is an extensive inflammatory cell infiltrate superficially (H&E stain, × 10).

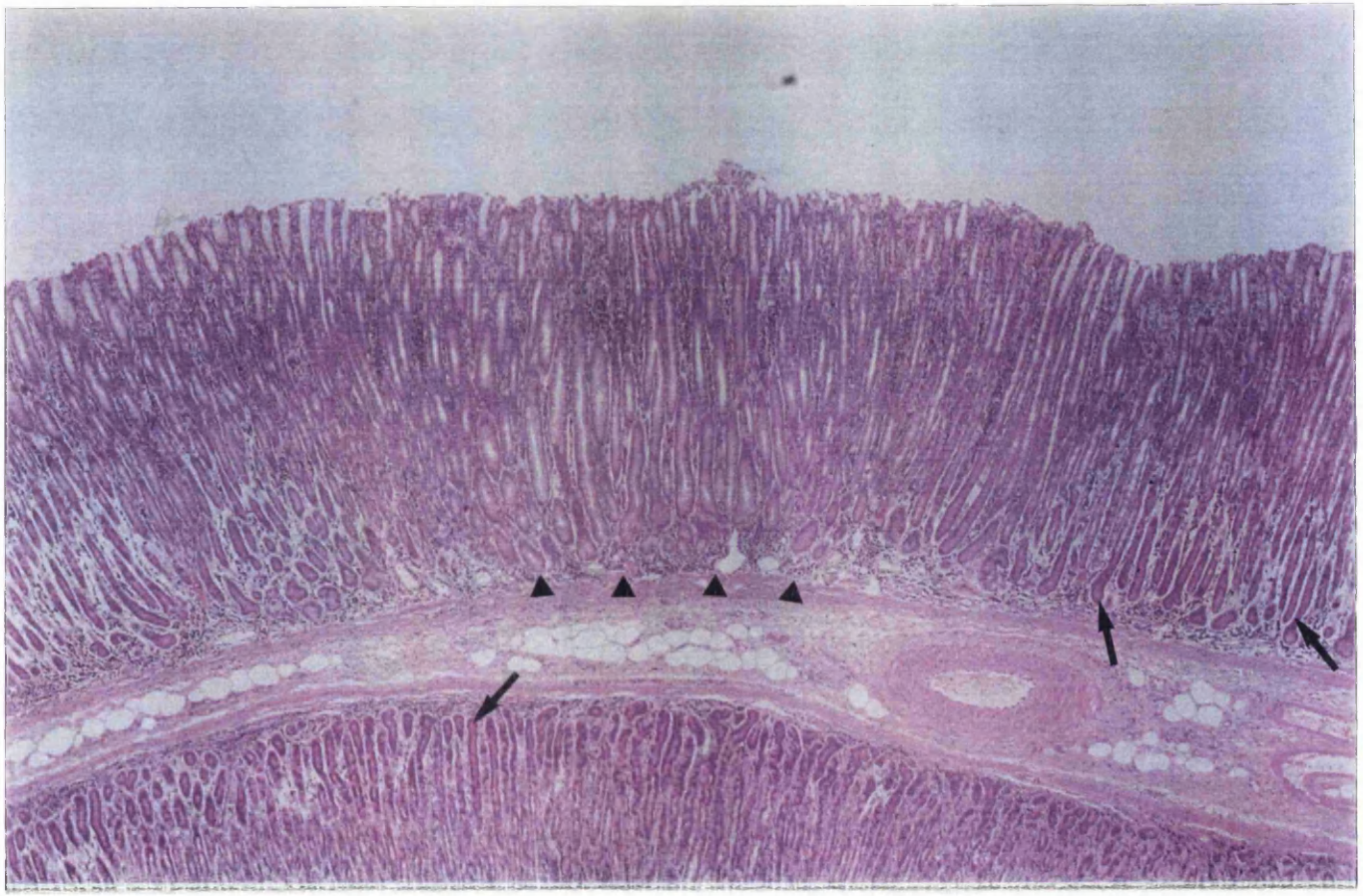


Fig. 8.3: Metaplastic mucous cells replacing normal chief cells in animal 17. (PAS stain, × 50).

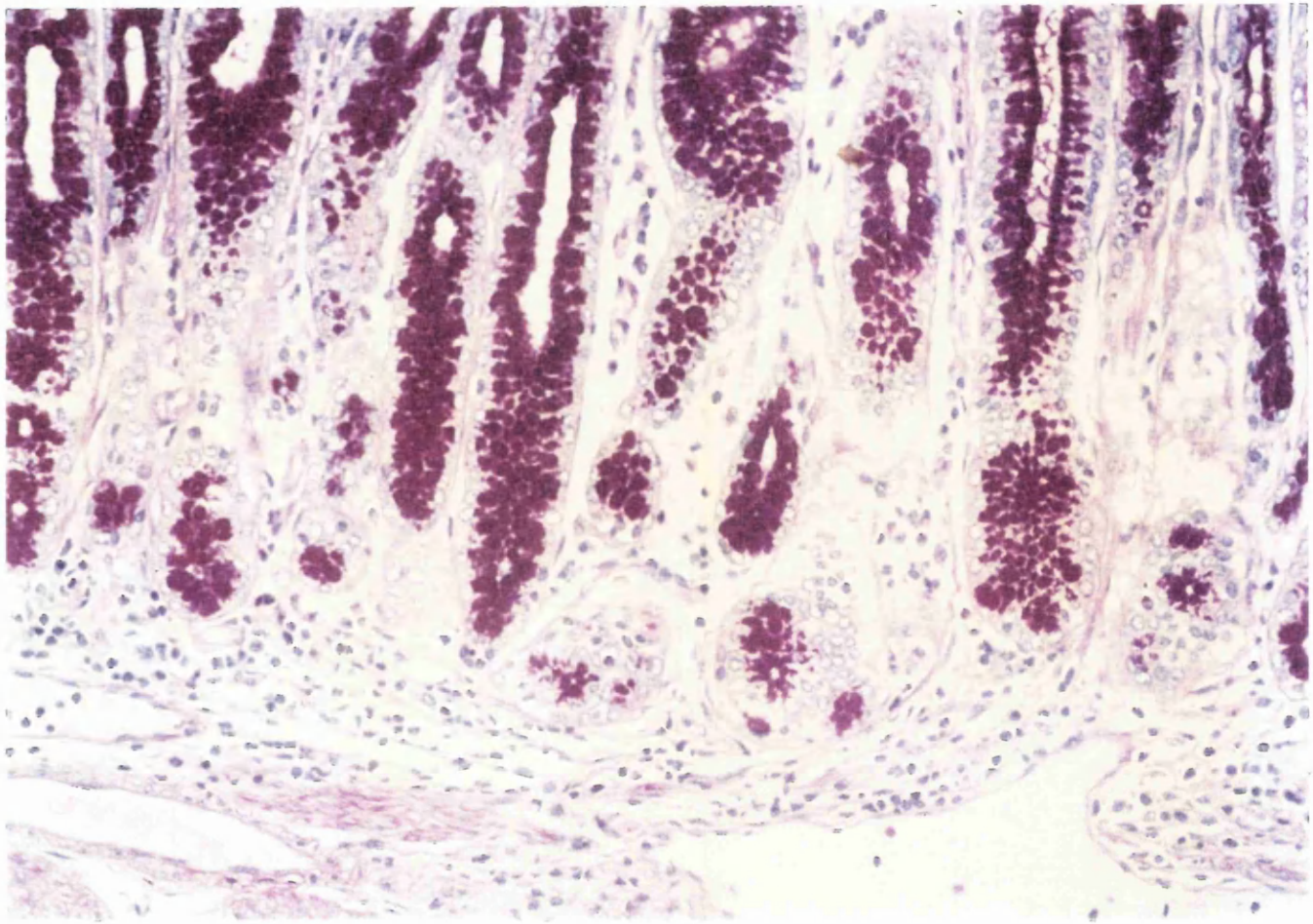


Fig. 8.4: The immunohistochemical localisation of pepsinogen to fundic mucosa of a control animal. (Pepsinogen immunostain, × 25).

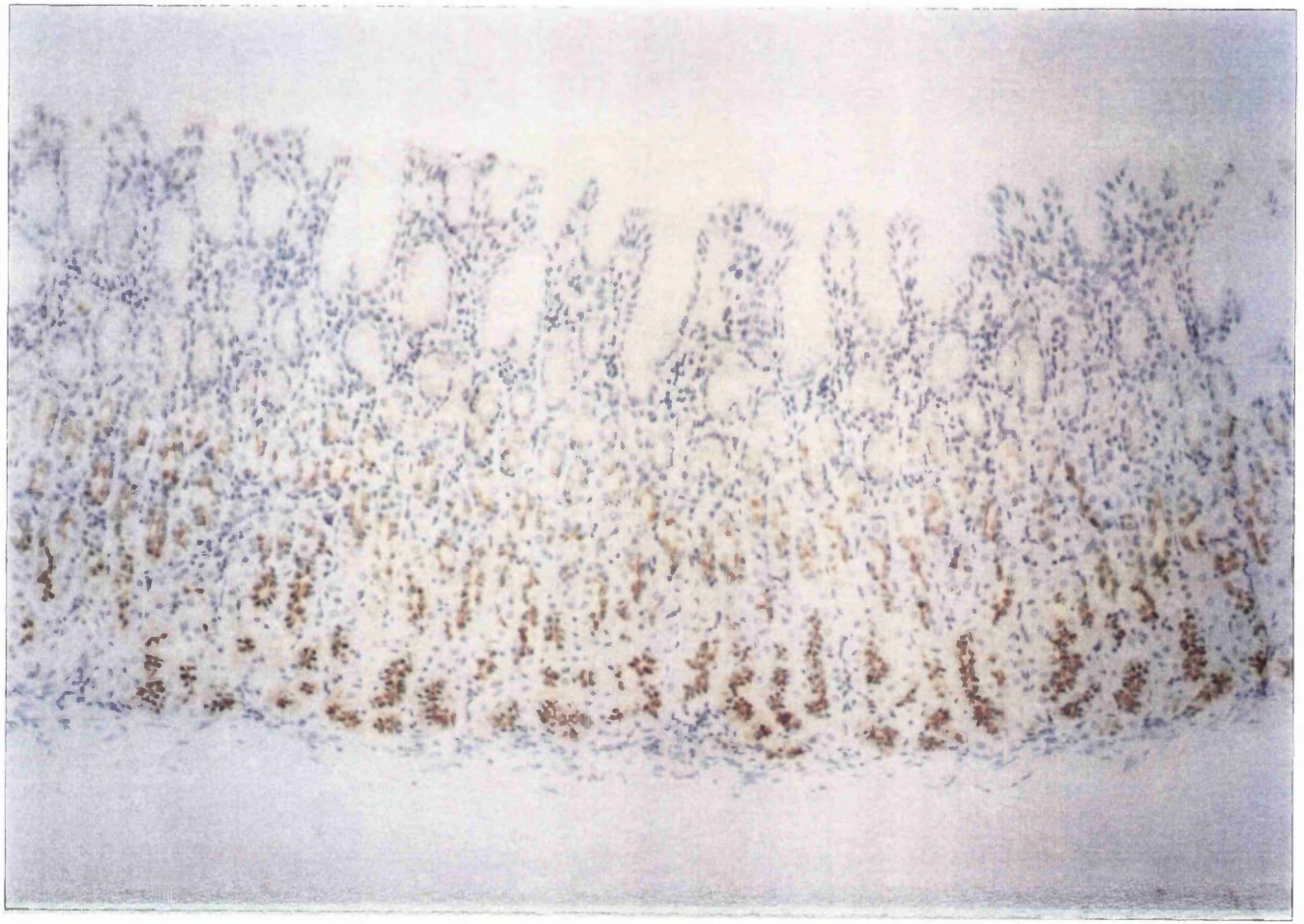


Fig. 8.5: Little pepsinogen-specific immunostaining in the mucosa of a control animal (BDC group). (Pepsinogen immunostain, × 25).

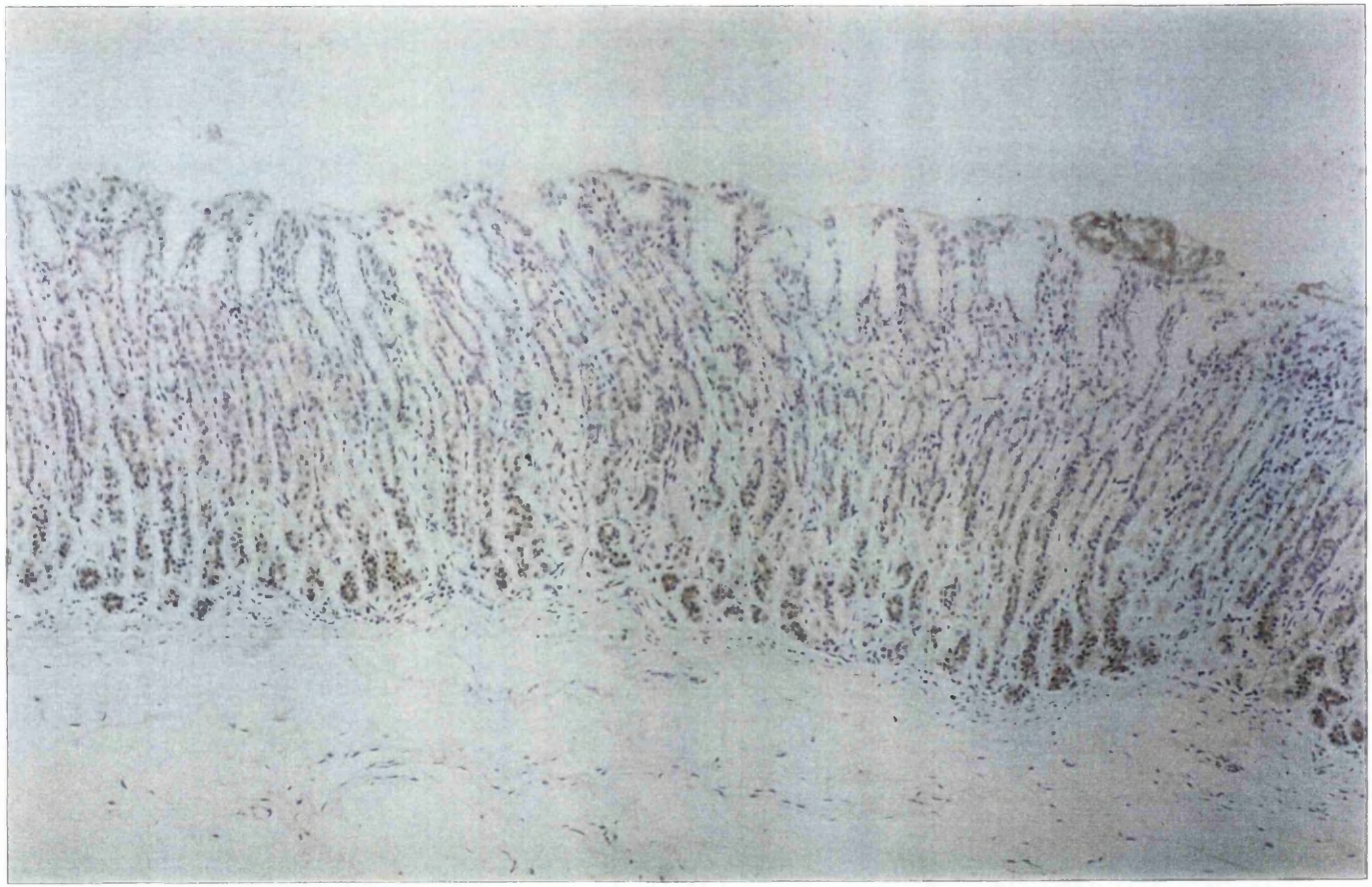


Fig. 8.6: Mucosa from an infected animal (BDI group) showing an enhanced population of cells positive for pepsinogen, including cells superficially. Note the reduced cytoplasmic volume and staining intensity of some chief cells (arrows). (Pepsinogen immunostain, × 25).

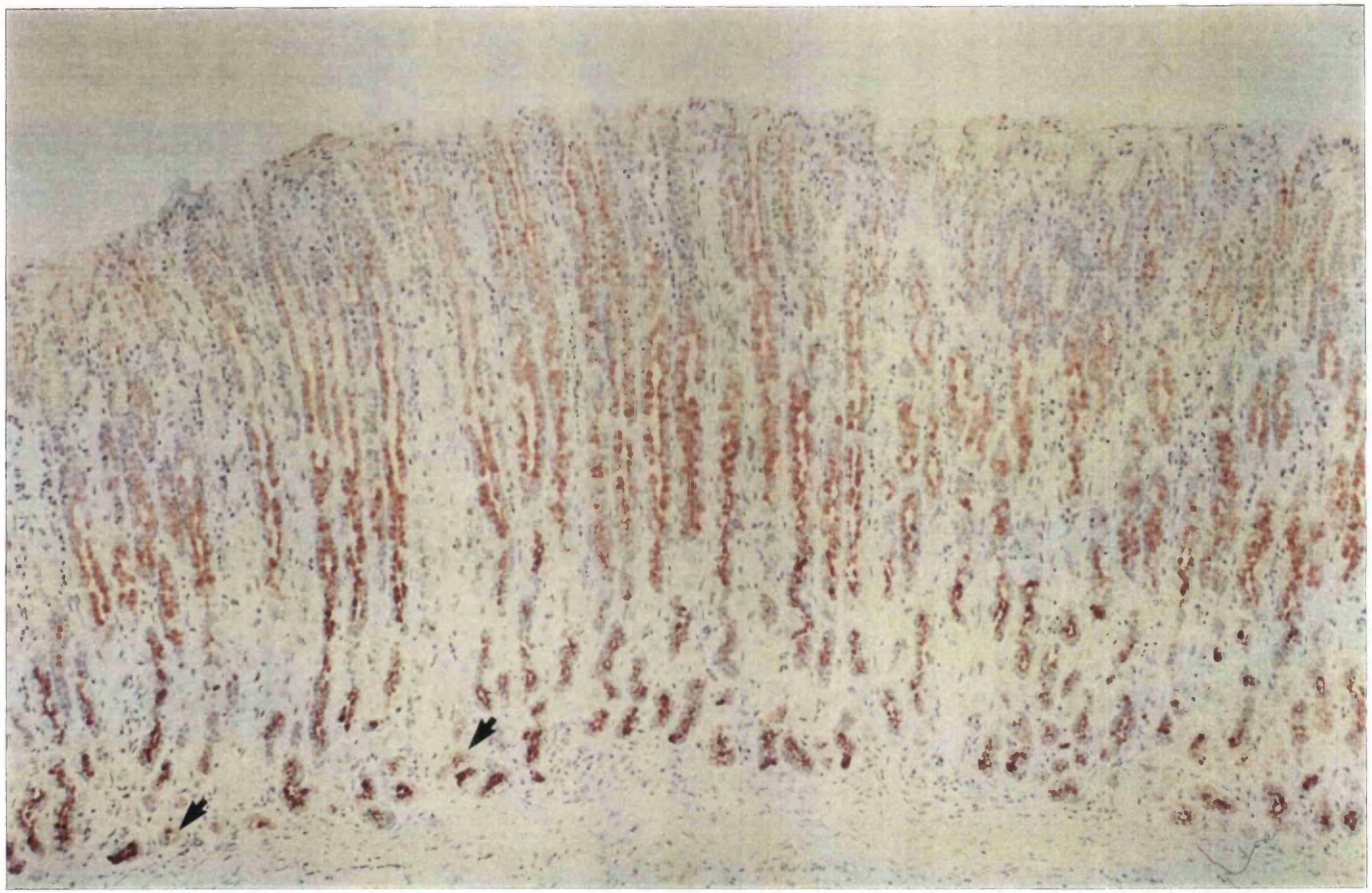
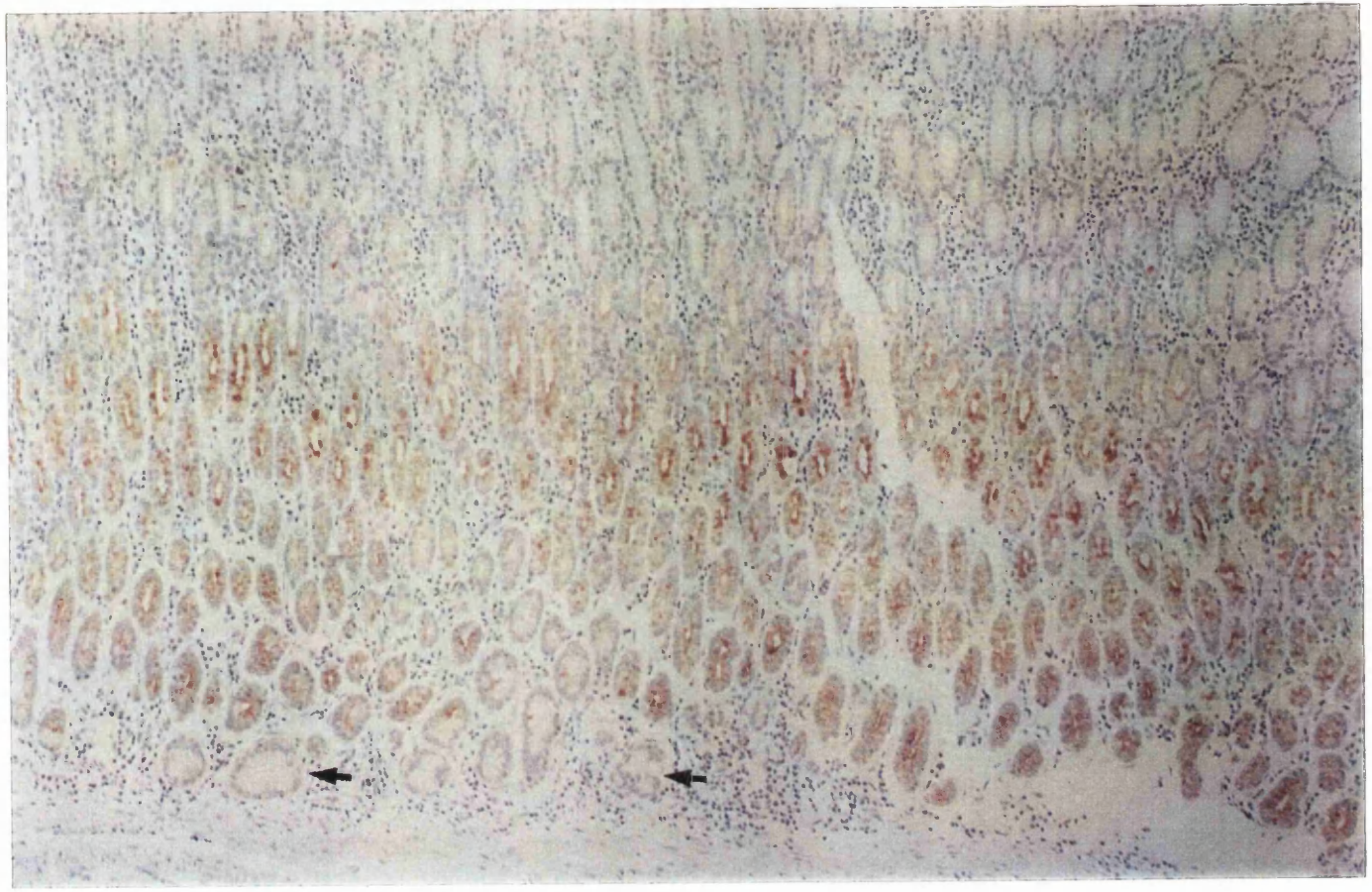


Fig. 8.7: Pepsinogen immunostaining at deep mucosal level of the tissue from animal 17. Cells that were strongly PAS-positive (Fig. 8.3) show little immunostaining (arrows). (Pepsinogen immunostain, × 40).



8.2.3: Discussion.

The presence of worms and eggs in control animals has not been explained, but might be the result of the accidental inoculation of an animal with larvae, or the contamination of samples occurring after necropsy.

Infections remained subclinical throughout the whole experiment, however faecal egg counts were significantly lower in animals given the supplementary ration, in contrast the numbers of worms present in infected animals was unaffected by diet. The presence of a small worm population in one animal of the BDC group (animal 24) and of low faecal egg counts in two animals of the SDC group (animals 15 and 30), have not been explained. Dietary supplementation reduced the magnitude of some pathophysiological reactions. Plasma total protein and plasma albumin concentrations were higher in infected animals fed the supplemented diet, and part of this effect could be explained by the higher concentrations found in control animals fed the same diet. Dietary protein supplementation may have enhanced immune responses against the parasites as adult female worms were shorter and contained fewer eggs in the supplemented sheep in comparison to the female worms present in animals fed the basal diet (Wallace et al, 1995). Plasma pepsinogen concentrations were increased in association with infection, but were unaffected by diet. The mean concentrations for both control groups (1.40 and 3.20 iU) were both below the 5.0 iU threshold established by Paynter (1992) as indicative of abomasal parasitism due to ostertagiasis. The means of both infected groups (6.60 and 6.40 iU) were above this figure and were compatible with moderate abomasal damage/dysfunction.

Infection resulted in fundic mucosal hyperplasia in animals fed both diets. Some tissues from infected animals were approximately twice as thick as those of control animals. Hyperplasia was not quantified, but did not appear to be affected by diet. Hyperplasia was predominantly of cells at the junction of the pits and glands, and as such parietal cell and chief cell numbers appeared little affected as both cells type were mainly confined to the base of glands. However in at least one animal hyperplasia was accompanied by total gland atrophy. This occurred in the animal with the greatest degree of apparent hyperplasia, and it is therefore possible that changes in this animal were more advanced.

Infection in many animals resulted in an increase in the total number of cells staining for pepsinogen. The hyperplastic cells stained less strongly for pepsinogen than chief cells, but the marked increases in their number could lead to increased mucosal content of pepsinogen, and could provide evidence of hypersecretion of pepsinogen occurring in infection. In the same animals the pepsinogen content of chief cells appeared reduced and this could also be evidence of hypersecretion, with pepsinogen being immediately secreted and not stored in granules. However the animal with the severest apparent hyperplasia also had an area where chief cells were entirely replaced by metaplastic mucus-producing cells, and the reduction in the pepsinogen staining of chief cells could reflect less advanced changes in cell phenotype to become more like mucous-type cells.

8.3: Further studies on the effects of *Haemonchus contortus* on pepsinogen production in abomasal tissues.

In the previous study animals infected with *H. contortus* had thicker abomasal mucosae, due to mucous cell hyperplasia. Pepsinogen immunohistochemistry revealed that the expansion of mucous-type cells was associated with an overall increase in the number of cells staining for pepsinogen. In contrast the pepsinogen content of some chief cells appeared reduced. However abomasal changes had been assessed only in single sections of fundus from each animal. The abomasal production of pepsinogen was therefore investigated further in a subsequent study of haemonchosis in Hampshire Down lambs, to assess whether previous histological and immunohistochemical findings were repeatable and quantifiable, whether changes affected the whole of the fundic mucosa, and whether the changes in the pattern of staining for pepsinogen were reflected in increases in the total tissue content of pepsinogen measured biochemically. Animals were again given one of two diets, a basal ration and a urea-, non-protein Nitrogen-supplemented ration, and some animals received a trickle infection of *H. contortus* infective, third stage larvae.

8.3.1:Materials and methods.

8.3.1.1: Animals and experimental design.

Twenty four, four month old Hampshire Down lambs were allocated into four groups as in the previous study (BDC, BDI, SDC and SDI groups, see section 8.2.1), and given either a

basal ration (88 g CP/ kg DM) or a urea supplemented diet (149 g CP/ kg DM). Feeding was initially at 1.4 kg fresh matter per day of the allocated diet, and feeding was increased at fortnightly intervals to maintain the same rate of feeding per kilogram of liveweight. The four lambs in both BDC and SDC groups remained uninfected, whereas after two weeks on the diets, those in the BDI and SDI groups (8 animals per group) received an identical trickle infection of *H. contortus* third stage larvae as had the lambs in the previous study (section 8.2.1), the trickle infection was given over the course of ten weeks, at the end of which all lambs were killed.

8.3.1.2: Procedure at, and subsequent to, necropsy.

Lambs were killed by exsanguination following electrical stunning. Faeces, whole blood and plasma were collected as outlined in section 8.2.1. Abomasal contents and washings were collected and one half of the abomasum was then prepared for the digest method to estimate the numbers of tissue dwelling larval stages. Fundic mucosal tissue samples were taken from the other abomasal half, using the biopsy punch (see section 7.3.1). Ten tissues were taken from the mucosal folds of each animal in a random pattern, five were immediately immersed in Brunnel's fixative, the other five were added to 10 ml plastic tubes and refrigerated (0-4°C) until they were later weighed and then homogenised to allow estimation of tissue pepsinogen concentration (section 2.3). Faecal egg counts, and worm burdens were ascertained using the procedures outlined in section 7.4.1. Blood biochemistry and haematological parameters were ascertained as described in section 8.2.1. Plasma pepsinogen concentration was measured using Paynter's method (1992).

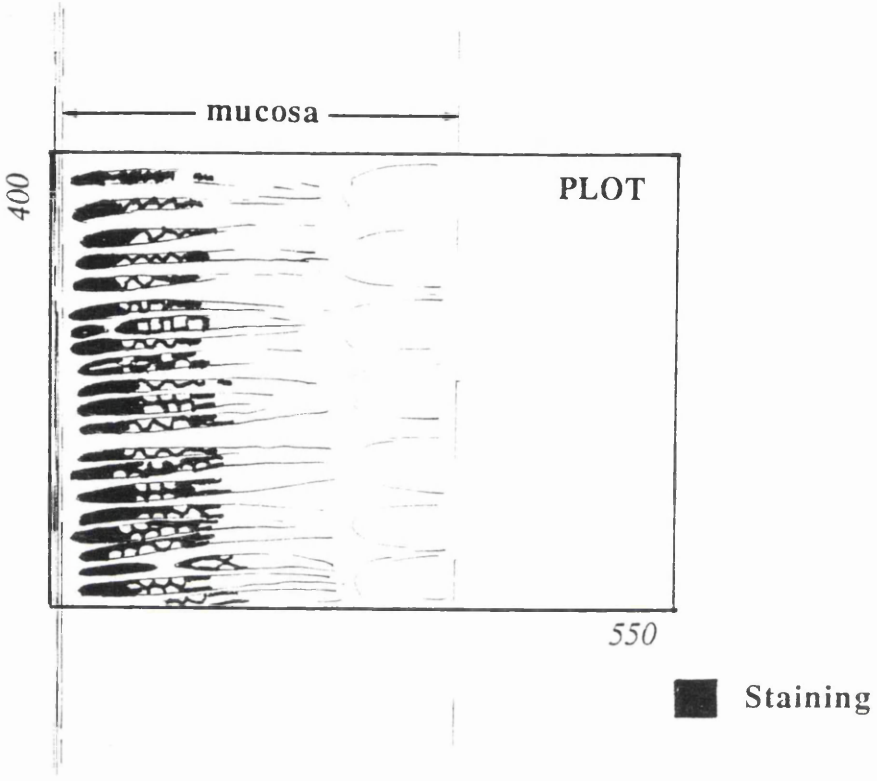
8.3.1.3: Immunohistochemistry and computer-aided image analysis.

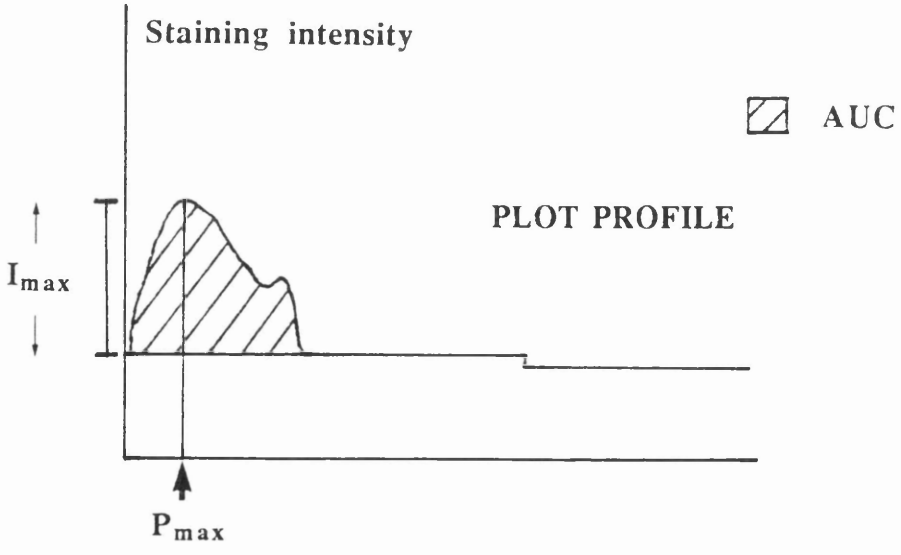
In addition to routine histological staining (H&E), the five fundic tissues were stained using the immunohistochemical procedures outlined in section 7.2.1. Stained sections were prepared with or without Haematoxylin counterstain. Negative controls included sections prepared omitting the primary, anti-pepsinogen antiserum, and those prepared with the substitution of the primary antiserum by non-immune rabbit serum. Those sections that were not counterstained were used in computer-aided image analysis in an attempt to quantify the amount and distribution of pepsinogen-specific staining within mucosae.

Image analysis was performed using the NIH Image (version 1.52) software package (National Institutes of Health, Research Services Branch, U.S.A.) and a Sony CCD video camera module (model XC-77CE) linked to an Olympus BH2 light microscope. The system utilised a captured monochrome image of each mucosal section and would therefore not distinguish between pepsinogen-specific diaminobenzidine (DAB) staining and the Haematoxylin counterstain, which was therefore omitted. Using a constant illumination setting and a constant magnification (×10 objective) the image of each mucosal section was projected onto the computer's television monitor. Pepsinogen-specific immunoreactivity was then measured using a standard plot size of 400 by 550 screen pixels. With the mucosa running from the top of the screen to the bottom, with the gland bases on the left and the luminal surface on the right, the plot was placed on the mucosal image so that the base of the plot was adjacent to the base of mucosal glands, in an area where gland bases were at equivalent depths in the mucosa, and the glands were seen in longitudinal section. The long side of the plot (550 pixels) was sufficient to encompass the total mucosal depth of all animals, from base to luminal surface. The width of the plot (400 pixels) was sufficient to allow approximately twenty adjacent glands to be scanned (see Fig. 8.8). The computer was then able to generate a plot profile that was in essence the average pixel densities of vertical columns of four hundred pixels at 550 points along the length of the scan. The data was then stored in numerical format in Minitab. Because of the apparent uniformity of the staining pattern across the whole of each mucosal cut, a single scan was performed for each of the five tissue sections and therefore the overall results represent approximately one hundred glands.

To allow easier data handling the 550 separate data points from each scan were condensed to 55 by taking the mean of every ten consecutive values. Then an overall mean value of the 5 scans from each animal was obtained. The background pixel density, i.e. the pixel density of non-stained areas, was subtracted, and from this data an "area under the curve" (AUC) for each animal was calculated by the summation of all data points, that therefore represented the total pepsinogen-specific immunoreactivity. Also calculated was the maximal staining intensity per animal (I_{max}), and its position in terms of depth of the mucosa (P_{max})(see Fig. 8.8).

Fig. 8.8: Schematic representation of the method used in the image analysis of pepsinogen immunostaining in fundic mucosal sections, and the forms of results obtained.





8.3.1.4: Statistical analyses.

Faecal egg counts at necropsy, for infected animals on the basal and supplemented diets were compared using a two-sample T-test. Total worm burdens were similarly compared with a two-sample T-test. All T-tests were preceded by Bartlett's test for homogeneity of variance. Packed cell volumes, and the concentrations of total plasma protein, plasma albumin and plasma pepsinogen for individual animals were compared using analyses of variance, using general linear models. The model fitted was **test parameter = diet + infection + diet*infection**. When the interactive term, **diet*infection**, showed significance, F-statistics for **diet** and **infection** were calculated with the interactive term as the denominator for the F-test. Tissue wet weights, tissue pepsinogen concentrations (both iU/area and iU/g), AUC's, I_{max} and P_{max} values were compared by a general linear model analysis of variance, using the same model. Because tissue pepsinogen concentration, measured as peptic units per mucosal area, and AUC values for the quantity of immunostaining, were essentially measures of the same thing, the two parameters were compared using a Pearson product moment correlation. The full image analysis data consisting of all data positions per animal were analysed with a multivariate mixed model analysis of variance using a general linear model. The model fitted was **staining intensity, positions 1 to 42 = diet + infection + diet*infection**. This analysis compared staining intensities at each position of the mucosa amongst the four groups, from the deepest (position 1) to the most superficial (position 42). Position 42 was the highest positive recorded value for any animal (see Appendices 25A to D).

8.3.2: Results.

Mean, total worm burdens, and the numbers of different parasite stages, as well as mean faecal egg counts, for each group are given in Table 8.3 (full data is shown in Appendix 22). Despite a lower mean faecal egg count in supplemented animals, the difference was not significant, by a two-sample T-test ($p=0.21$). Similarly total worm burdens in the two groups of infected animals were not significantly different ($p=0.90$). Mean (± S.E.) packed cell volumes, total protein, plasma albumin, and plasma pepsinogen concentrations for all four groups are shown in Table 8.4 (Full data is recorded in Appendix 23). Packed cell volumes were significantly lowered by infection ($p<0.001$), but were not affected by diet ($p=0.17$). Similarly total plasma proteins were reduced by infection ($p<0.02$), and were also unaffected

by diet ($p=0.14$). Albumin levels were affected by both diet ($p<0.01$) and infection status ($p<0.001$) with lowest values recorded in infected animals on the basal ration. Despite increases of plasma pepsinogen concentration in infected animals, the effect of infection was only of borderline significance ($p=0.061$), the effects of diet and of the interactive term were not significant ($p=0.648$ and 0.405 respectively).

Gross pathological changes, as in the previous study, were not readily discerned within abomasa or within the carcasses of any animal. On routine histological examination changes were confined to mucosal hyperplasia in a similar pattern as reported in the previous study (see section 8.2.2), and the presence of typical chronic inflammatory infiltrates. Hyperplasia did not appear as marked as in the previous study, and no tissue examined appeared to feature any significant parietal cell loss, although parietal cells were not quantified.

8.3.2.1: Tissue pepsinogen concentrations.

Mean group values for tissue wet weights and tissue pepsinogen concentrations are given in Table 8.5 (full data is shown in Appendices 24A, B and C). Tissues from infected animals were significantly heavier ($p<0.02$), and this change was unaffected by diet ($p=0.11$). The highest overall mean tissue pepsinogen concentrations (measured as iU/area and as iU/g) were recorded in the sheep of the SDC group, and were lowest in the infected animals on the same diet (SDI). In contrast the infected animals on the basal diet (BDI) had more pepsinogen than the control animals (BDC). This lack of parallelism in the pattern of effects is mirrored in the results for the analysis of variance. For tissue pepsinogen, as iU per mucosal area and as iU per gram of tissue, the interactive term (**diet*infection**) was either of borderline significance ($p=0.068$)(for iU/area data) or was significant ($p<0.02$)(for iU/g data), and following corrected F-tests, the effects of both diet and infection on their own were not significant. Individual variation between sheep was very great, values ranged from a mean (± S.E.) tissue pepsinogen of 172 (100) iU/area for one animal (SDI group) to 10,798 (744) iU/area for another (SDC).

Table 8.3: Mean (± S.E.) worm burdens and faecal egg counts of the groups of Hampshire Down lambs. Second study.

Group	Worms L_4	L_5	male	female	Total	FEC (epg)
BDC	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
BDI	44 (20)	256 (73)	1,156 (160)	806 (110)	2,262 (293)	9,688 (2,185)
SDC	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	288 (169)
SDI	112 (92)	112 (28)	1,144 (113)	944 (108)	2,312 (248)	6,469 (1,007)

Table 8.4: Mean (± S.E.) values for haematological and biochemical parameters of the groups of Hampshire Down lambs. Second Study.

Group	PCV (per cent)	Total Protein (g/L)	Albumin (g/L)	Plasma Peps. (iU)
BDC	33 (1.14)	65 (1.60)	23 (0.85)	1.84 (0.81)
BDI	24 (1.06)	55 (2.46)	18 (0.61)	4.08 (0.40)
SDC	33 (1.20)	68 (1.63)	25 (1.25)	2.14 (0.40)
SDI	27 (0.90)	58 (1.36)	21 (0.74)	3.04 (0.92)

Table 8.5: Mean (± S.E.) wet weights and tissue pepsinogen concentrations (expressed as iU per standard area and as iU per gram of wet weight of tissue) of the Hampshire Down lambs.

Group	Wet weight (g)	Tissue pepsinogen (iU/area)	concentration (iU/g)
BDC	0.0907 (0.0055)	4,179 (2,345)	42,732 (23,056)
BDI	0.1000 (0.0032)	5,207 (517)	51,719 (4,402)
SDC	0.0784 (0.0011)	6,733 (1,362)	86,748 (18,039)
SDI	0.0958 (0.0054)	3,289 (851)	31,977 (7,231)

8.3.2.2: Immunohistochemistry and image analysis.

Pepsinogen-specific immunoreactivity in control animals was similar to that reported for control animals in the previous study (section 8.2.2.3). The chief cells were the major site of staining and were characterised by the presence of large, dense granules packed within the apical cytoplasm (Fig. 8.9b). Lesser amounts could be detected within narrow populations of mucous neck cells. The changes seen in infected animals were similar to those of the previous study, but did not appear as marked, and although many animals featured an expanded population of cells staining for pepsinogen at a mid-mucosal level, staining was rarely continuous in cells to the luminal surface. The apparent repeatability of

immunostaining, both in the quantity and in the pattern of distribution, throughout the five mucosal sections from each animal, for both infected and control animals, was good. Chief cell content in some infected animals appeared lowered, and this was most obvious in cells from the animal with the overall greatest increase in mucosal mass, animal number 35 (Fig. 8.9d). Chief cells in this animal in H&E-stained sections, had paler basal cytoplasm than normal, with an even paler perinuclear area, and the eosinophilia of their apical cytoplasm was restricted (Fig. 8.9c). As reported in section 8.3.2.1, the quantity of tissue pepsinogen varied widely between animals, and this was appreciable in the immunohistochemical studies. The quantity of granules packing the apical cytoplasm of chief cells appeared to be the determining factor, and was offset against changes in the hyperplastic mucous cell layer in infected animals. Indeed the two animals with the lowest tissue pepsinogen concentrations, animals 15 (SDI) and 19 (BDC), with tissue pepsinogen values of 172 (100) iU/area and 312 (37) iU/area respectively, both had negligible immunostaining at any level of their mucosae (Figs. 8.9f and h). However in H&E-stained sections the chief cells of both animals could be seen to contain characteristic eosinophilic granules (Figs. 8.9e and g), but these were much less evident in the control animal (number 19).

Quantitative differences in the amount of pepsinogen-specific staining and its distribution were investigated using image analysis. The overall mean plot profiles for each animal are shown in Figs. 8.10a to d (full data is shown in Appendices 25A to D). The pattern of immunostaining in control animals appeared uniform, apart from animal number 19, with the greatest staining intensities recorded in the deepest parts of the mucosa, which would correspond to the chief cell population. Towards the middle of the mucosa reduced staining intensities correspond most likely to mucous neck cells. Greater individual staining intensities were recorded in the SDC group (Fig. 8.10b) in comparison to the BDC group, and this does agree with the results of the peptic activities of mucosal homogenates. Staining intensities were distributed in wider patterns in many infected animals, although some were similar to control animal patterns. When overall means for infected and control animals were plotted against each other (ignoring diet), the major difference was a much slower decline in staining intensity as the mucosa of infected animals was ascended (Fig. 8.11)(full data in Appendix 26). In the multivariate analysis of staining intensity in relation to mucosal position the following results were obtained (see Appendix 27). At position 1 (mucosal base) the interactive term (**diet*infection**) was significant ($p<0.05$), but was of borderline significance by position 2 ($0.05<p<0.10$) and was not significant by position 3 ($p>0.10$). The

effect of infection became of borderline significance by position 16, and was fully significant by position 21 and remained so for many of the other positions.

The mean results for AUC's, I_{max} and P_{max} for each of the four groups are shown in Table 8.6 (full data is shown in Appendix 28). The correlation of individual AUC and total tissue pepsinogen (iU/area) was positive, but poor (0.200), however certain features were consistent. Values for individual AUC's varied widely (from 0.55 to 9.64) and the lowest values, 0.55 and 0.65, as a consequence of negligible detectable immunostaining, were found in the two animals with the lowest values of tissue pepsinogen concentration, animals 15 and 19.

Analysis of variance of the AUC data showed that this parameter was not significantly affected by either diet or infection ($p>0.05$) and that the interactive term was also not significant. Similar analyses for I_{max} and P_{max} values data produced identical results.

Table 8.6: Group mean values (± S.E.) for AUC, I_{max} and P_{max} obtained following image analysis. Units are arbitrary, but relate ultimately to the pixel density of the scanned images.

Group	AUC	I_{max}	P_{max}
BDC	2.49 (0.72)	11.50 (3.28)	5.00 (1.68)
BDI	4.71 (0.98)	14.01 (2.09)	5.87 (1.66)
SDC	4.21 (0.64)	19.10 (2.94)	3.25 (0.63)
SDI	4.52 (0.69)	13.90 (2.05)	7.62 (1.88)

Fig. 8.9: Comparison of the histological appearance of chief cells, in H&E stained sections (a,c,e and g) and following pepsinogen immunohistochemistry (b,d,f and h), from a control animal (a and b), an infected animal (c and d) and in two animals with negligible immunoreactivity, animal 15 (e and f) and animal 19 (g and h).

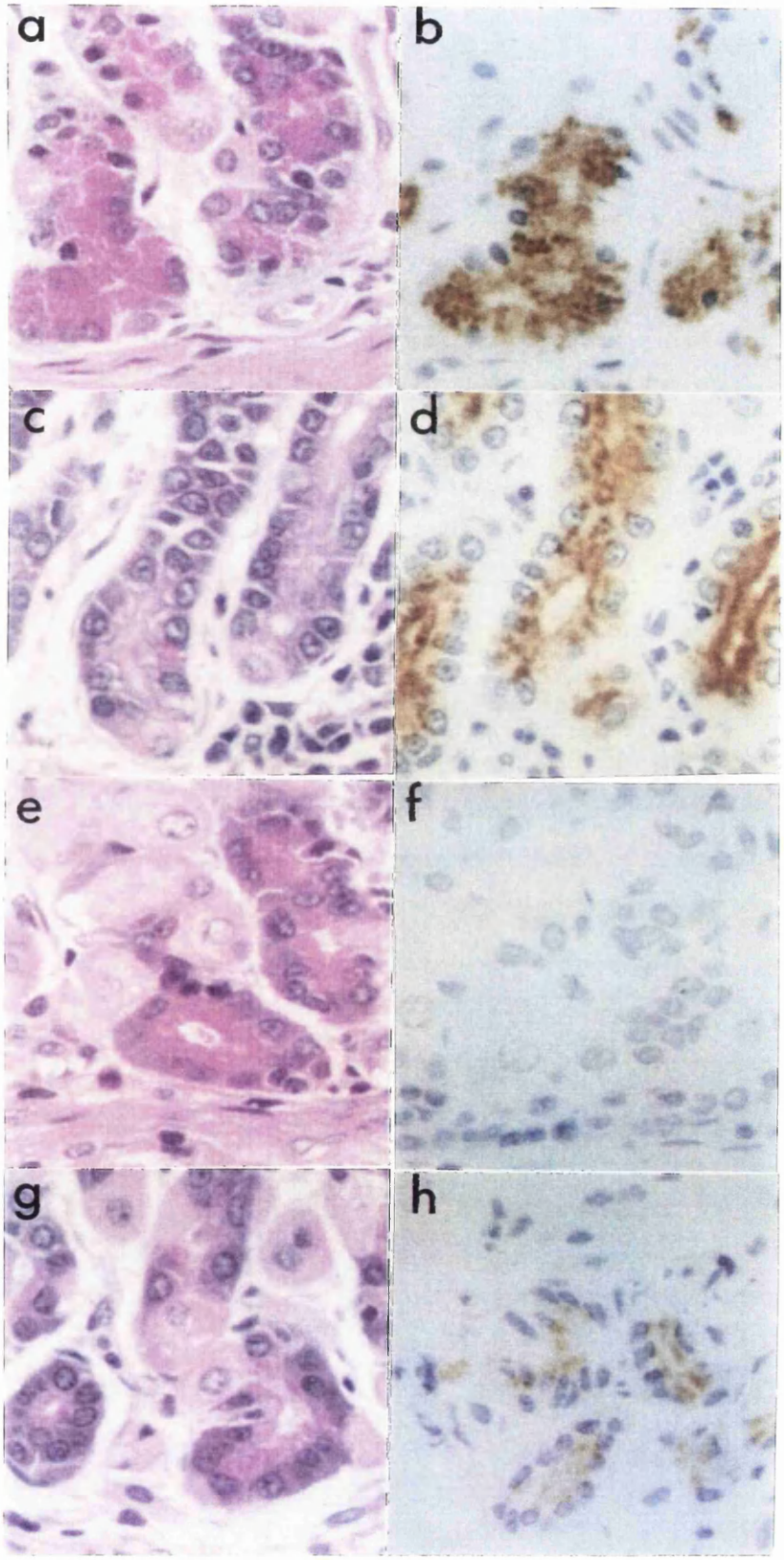


Fig. 8.10: Individual plot profiles for pepsinogen-specific staining intensities of each animal from each of the four groups, (each trace represents the mean obtained from the five scans for each animal).

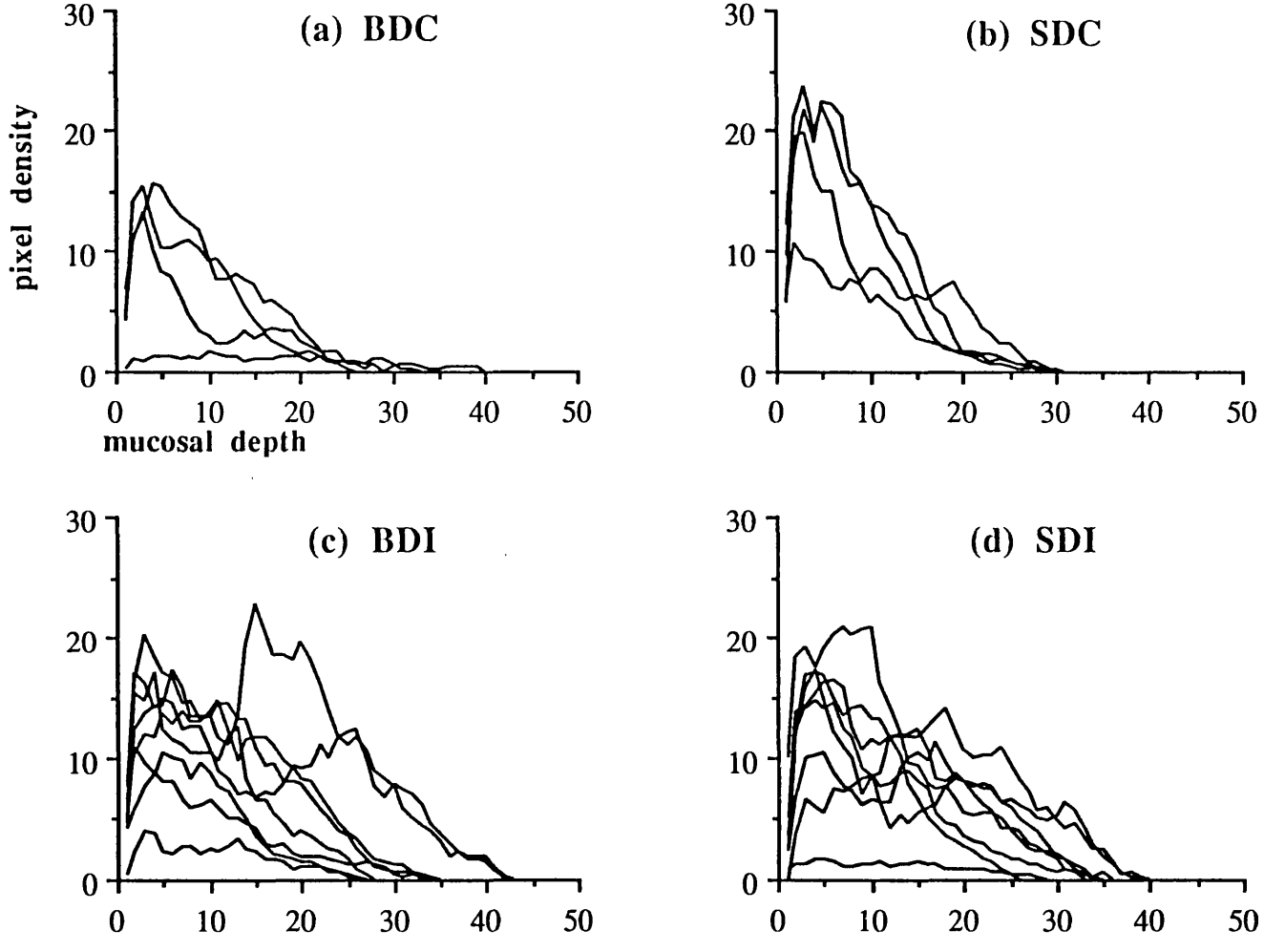
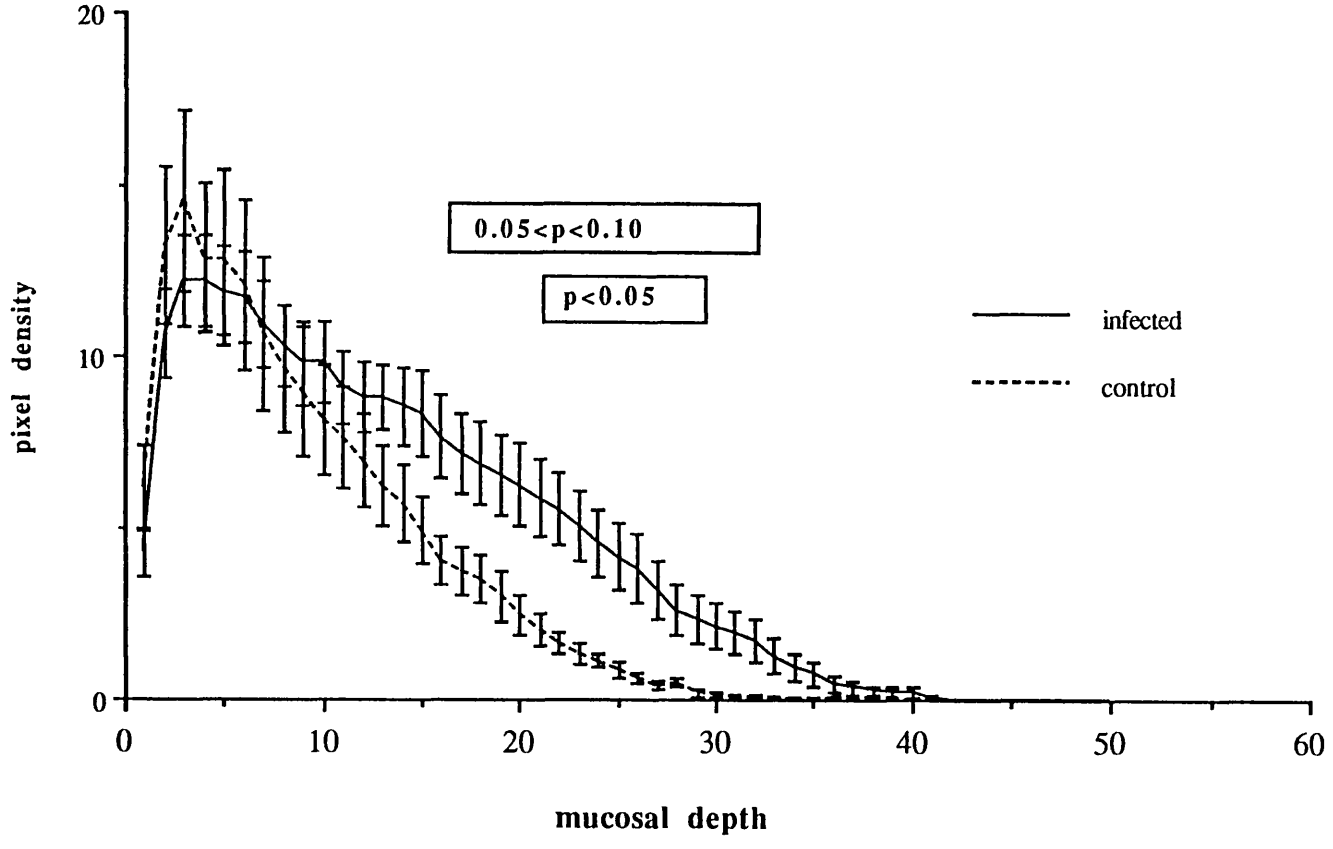


Fig. 8.11: Comparison of overall mean (± S.E.) plot profiles of control and infected animals.



8.3.3: Discussion.

Despite slightly higher worm burdens at the end of the trickle infection period in the present study, faecal egg counts at necropsy were generally not as high as in the previous year's study. Further, the affect of dietary non-protein Nitrogen-supplementation on the fecundity of female worms was not as marked in the latter infection series as it was in the former. Plasma pepsinogen concentrations were increased in response to infection, but to a much lower extent than in the previous study. Packed cell volumes, plasma total protein and plasma albumin concentrations were affected by the presence of infection, but only the reductions in plasma albumin concentrations due to infection were significantly ameliorated by feeding the supplementary ration.

The differences of the extent of mucosal hyperplasia and of the histopathological and immunohistochemical changes between the two studies have not been directly compared, but the overall impression was that changes were not as marked in the second infection series. The mucosal tissues of infected animals were significantly heavier, and this was seen to be due primarily to hyperplasia of mucous cells occurring at a mid-mucosal level. The hyperplastic mucous cells contained pepsinogen, and potentially represented an increase in the total number of cells in the fundus which were capable of secreting pepsinogen. This might be expected to have led to an increase in the total mucosal content of the zymogen, yet total tissue pepsinogen concentrations and the overall values of AUC's calculated from measurements of staining intensity were not significantly increased by infection. It was considered that in infected animals the pepsinogen content of chief cells might be reduced, and indeed the values for the I_{max} data, comparing the maximal staining intensity of any one level of mucosa and invariably corresponding to the position of the chief cell population, were lower for infected animals, but any difference was not significant. Increases in the number of cells producing the zymogen might therefore be offset by the reduction of chief cell pepsinogen content.

The influence of diet has been less thoroughly illuminated. Comparing only control animals dietary Nitrogen supplementation might have increased the demands for peptic digestion and this might explain the increased pepsinogen content of control animals fed the supplemented ration. Any increases related mostly to the enhanced pepsinogen content of chief cells.

Studies of the effects of infection and diet were hampered by the very high degree of variation shown by individual animals and it may be that some of the above results had more to do with the random assortment of widely different animals than with any real effects of diet or infection. To assess the affect of diet more completely, a larger number of uninfected animals could be used to overcome the high degree of variation between animals. This might also better elucidate the significance of animals that seem to produce very little pepsinogen. Abomasal secretion is effectively continuous and the relative absence of pepsinogen from the mucosa of some animals is therefore unlikely to result from factors such as differences from the time of death to an event such as feeding, but should represent real differences between animals in their production of the zymogen. Two animals in the present study had little tissue pepsinogen, one was infected, the other was a control animal, one was fed the supplemented ration, one the basal diet. There was good agreement between the results for the tissue pepsinogen content measured as both the peptic activity present in mucosal homogenates and as the quantity of immunostaining. This can be taken as further evidence of the specificity of the immunohistochemical techniques employed. Both animals also had low plasma pepsinogen concentrations. The biological significance of low tissue pepsinogen is unknown. Pepsinogen is known to be of greater importance in the digestion of proteins of animal origin, such as collagen, than plant material and more important in animals where mastication is limited or absent (Hersey, 1987), and low expression of pepsinogen may therefore be no hindrance to herbivorous animals. This work has not examined the secretory rates of the mucosae from these animals for pepsinogen. Pepsinogen secretion in these animals might bypass granule formation, yet in other animals a generalised cytoplasmic staining of basal cytoplasm implied that forms of pepsinogen are present in non-granular structures such as endoplasmic reticulum. Granules were not absent from the two animals with low mucosal pepsinogen concentrations, but stained poorly for pepsinogen. The exact content of such granules has not been examined.

Mucosal hyperplasia developed in animals by the end of the ten week trickle infection period. In other studies similar hyperplastic changes occurred by day twelve following a single experimental infection (Hunter and MacKenzie, 1982), and focal hyperplasia, associated with the initial larval occupation of the gastric glands, developed within days following a single larval inoculum (Charleston, 1965). Clearly the changes in the population of cells producing pepsinogen might have important implications for the development of increased plasma pepsinogen concentrations.

EXPERIMENTAL STUDIES - PART 4: GROWTH FACTOR IMMUNOHISTOCHEMISTRY.

9: THE ROLE OF TRANSFORMING GROWTH FACTOR-ALPHA IN THE PATHOGENESIS OF OSTERTAGIASIS.

9.1: Introduction.

Epidermal Growth Factor (EGF) was identified as a protein present in the secretions of the submaxillary salivary gland of the male mouse capable of causing precocious eyelid opening and incisor eruption (Cohen, 1960, 1962; Cohen and Elliott, 1963). Part of its biological activity was shown to be due to the direct stimulation of the proliferation and keratinisation of epidermal tissue. The primary structure of the mature 53 amino acid form of mouse EGF was later elucidated by Savage et al (1972). Separately, urogastrone was identified as a substance present in the urine of pregnant women that had a beneficial effect on experimentally-induced gastric ulcers (Sandweiss et al, 1938) and which inhibited gastric acid secretion (Culmer et al, 1939). The structure of urogastrone was reported by Gregory (1975) and shown to be identical to the reported structure of human EGF (Cohen and Carpenter, 1975). The discovery of Transforming Growth Factor-alpha (TGF-α) led from the observation that sarcoma virus-transformed cells showed reduced binding of exogenous EGF (Todaro et al, 1976; Todaro and De Larco, 1978). Subsequent experiments showed that substances present in media conditioned by virally-transformed fibroblasts competed with EGF in binding to the Epidermal Growth Factor receptor (EGF-R)(De Larco and Todaro, 1978). The observations that similar activities were produced by human tumour cells and by chemically transformed cells (Roberts et al, 1980; Todaro et al, 1980) confirmed that the substance was a product of cellular and not viral genes. The products of sarcoma virus-transformed cells were eventually separated into two subsets (Anzano et al, 1982, 1983), but only one component was shown to bind and act through EGF-R, and was identified as Transforming Growth Factor-alpha (Carpenter et al, 1983; Massague, 1983).

Epidermal Growth Factor and TGF-α have since been shown to be members of a large super-family of peptide growth factors that all bind to the Epidermal Growth Factor receptor.

Individual members of this group share a signature sequence of 36 to 37 amino acids with the following consensus sequence: $CX_7CX_{2-3}GXCX_{10}CXCX_3YXGXRC$[1] (Lee et al, 1995). Disulphide bonds formed between the first and third, second and fourth, and fifth and sixth cysteines of the consensus sequence result in a compact three-loop structure that is essential for high affinity binding to EGF-R. Other mammalian representatives of the group include amphiregulin (Shoyab et al, 1988), heparin-binding EGF (Higashiyama et al, 1991), and betacellulin (Sasada et al, 1993; Shing et al, 1993).

Pox family viruses encode EGF-R ligands, such as Vaccinia Virus Growth Factor (VGF)(Blomquist et al, 1984; Brown et al, 1985; Stroobant et al, 1985). Expressed in infected cells in the early stages of infection, secreted VGF may facilitate pox virus infection by causing other non-infected host cells to proliferate (Buller et al, 1988). A further member of the EGF-R ligand family is the product of the *lin-3* gene of the free-living nematode *Caenorhabditis elegans* (Hill and Sternberg, 1992). The *lin-3* gene product binds to the nematode EGF-R homologue *let-23*, and one of its roles is vulval development. The *glp-1* and *lin-12* gene products of *C. elegans* contain numerous EGF-like sequences (Yochem and Greenwald, 1989), as does the *Notch* gene of *Drosophila*, but none are likely to be ligands of EGF-R or its homologues.

The fully processed TGF-α peptide is fifty amino acids long and EGF and TGF-α share only 30 per cent homology in amino acid sequence (Marquardt et al, 1984). The activities of highly purified recombinant TGF-α and EGF have been compared, but not always using the peptides from the same species. Based on studies comparing TGF-α and EGF from the same species Ebner and Derynck (1991) demonstrated that both bind to EGF-R with similar affinities. In some bioassays TGF-α and EGF are approximately equipotent, for example in their abilities to stimulate DNA synthesis in a variety of cell lines (Schreiber et al, 1986) or eyelid opening in new-born mice (Smith et al, 1985). In other systems TGF-α appears to be the most potent (Derynck, 1988; Lee et al, 1995) and TGF-α has a more widespread distribution in tissues (vide infra). Both growth factors have been shown to regulate fundamental cell functions such as proliferation, migration, differentiation and gene expression, and may consequently be important in foetal development and tissue maintenance in adults. Transforming Growth Factor-alpha is a potent mitogen for epithelial

[1] - Amino acid abbreviations: C, cysteine, G, glycine, R, arginine, Y, tyrosine, X, unspecified amino acid.

and mesenchymal cells (Jhappan et al, 1990; Sandgren et al, 1990) and may be involved in activities as diverse as angiogenesis (Schreiber et al, 1986), wound healing (Schultz et al, 1987, 1992) and bone resorption (Ibbotson et al, 1985). Both TGF-α and EGF adversely affect hair growth (Moore et al, 1983; Tam, 1985) and EGF has been used to weaken hair shafts and allow the sheering of sheep by hand (Moore et al, 1982). Epidermal Growth Factor has been shown to stimulate cardiovascular and gastrointestinal smooth muscle contraction (Hollenberg, 1994), the physiological significance of which is unknown.

Binding of ligand induces dimerisation of the EGF-R and concomitant activation of the receptor's intrinsic tyrosine kinase. Tyrosine kinase activation is essential for the diverse effects of ligand binding, including rapid changes in intracellular Ca^{2+}, changes in intracellular pH, and activation of gene transcription and its ultimate effects on cell proliferation (Chen et al, 1987). The ligand/receptor complex is then internalised from the cell surface after which ligands may be treated differently. In mouse L cells and human keratinocytes, Ebner and Derynck (1991) demonstrated that whereas ligand and receptor were preferentially recycled to the cell surface from TGF-α/EGF-R complexes, they were degraded from complexes of EGF and EGF-R. This may help explain observed differences in biological activities in some systems, and prevention of receptor recycling may contribute to receptor down-regulation. An important observation of the control of TGF-α gene expression was that both EGF and TGF-α were able to enhance TGF-α production by human keratinocytes (Coffey et al, 1987). From this important discovery the concept of the autocrine function of growth factors was realised; cells producing the factors are potentially capable of not only affecting neighbouring cells (paracrine regulation) and distant cells via the blood stream (endocrine), but also themselves.

The localisation of Transforming Growth Factor-alpha in the tissues of normal adult mammals has been investigated. Normal tissues, including the skin, nervous system, lungs, endocrine organs, kidneys, haematopoietic cells and vascular tissues, express the factor in varying amounts. Expression of TGF-α in skin keratinocytes was confirmed by Coffey et al (1987), expression of EGF-R was however confined to basal cells of the epidermis (Nanney et al, 1984). Both TGF-α and EGF-R expression has been confirmed in adipocytes (Luetteke et al, 1993), and TGF-α or EGF may prevent adipocyte differentiation and limit the size of adipose tissues. Similarly TGF-α may limit the development of muscle and bone (Luetteke et al, 1993). The function and distribution of growth factors within the gastrointestinal tract,

especially in the gastric mucosa, may be of great importance in gastrointestinal parasitism. Transforming Growth Factor-alpha mRNA and protein were detected in greatest amounts in the parietal cells of human and guinea pig gastric mucosae (Beauchamp et al, 1989). The same workers detected little EGF mRNA. Thomas et al (1992) demonstrated that TGF-α immunostaining was confined to the differentiated, non-proliferative compartment of epithelia from human oesophagus, stomach, small intestine and colon. In the human oxyntic mucosa immunoreactivity was strongest in parietal cells and surface mucous cells. Epidermal Growth Factor immunostaining was confined to mucous neck cells of the oxyntic mucosa and the Brunner's glands of the duodenum. The receptor for TGF-α and EGF is assumed to be confined to the basolateral membranes of gastrointestinal cells of both the proliferative and differentiated compartments (Thompson et al, 1994). Beauchamp et al (1989) detected highest amounts of EGF-R mRNA in parietal cells of oxyntic mucosae, and smaller amounts in chief cell fractions.

It has been hypothesised that both TGF-α and EGF may serve in the maintenance of gastric mucosal integrity and in the response to injury, by promoting cell proliferation, migration and differentiation, and by inhibiting gastric acid secretion and enhancing mucus production. The re-epithelialisation of experimentally-induced gastric ulcers occurred at a rate faster than could be accounted for by mitosis alone (Hingson and Ito, 1971; Svanes et al, 1982). Epidermal Growth Factor stimulated DNA synthesis and mucosal growth in oxyntic mucosa (Johnson and Guthrie, 1980; Konturek et al, 1981, 1988) and both EGF and TGF-α have been shown to promote the migration of intestinal epithelial cells and keratinocytes (Blay and Brown, 1985; Barrandon and Green, 1987). Both EGF and TGF-α suppressed acid production by parietal cells in vivo and in vitro and were probably equipotent in this respect (Konturek et al, 1984; Finke et al, 1985; Rhodes et al, 1986; Gregory et al, 1988). Both growth factors enhanced mucous secretion (Yoshida et al, 1987; Kelly and Hunter, 1990; Romano et al, 1992). Epidermal Growth Factor protected against gastric ulcer formation at doses that did not reduce acid secretion (Konturek et al, 1981). Epidermal Growth Factor has been detected in saliva (Skinner et al, 1984; Konturek et al, 1988), as has TGF-α (Wu et al, 1993; Humphreys-Beher et al, 1994), and EGF was detected in milk (Beardmore and Richards, 1983; Koldovsky et al, 1991) and considerable interest has been focused on the function of growth factors in the gastrointestinal lumen. Some studies have shown that intraluminal EGF enhanced enteric mucosal growth and absorptive capacity (Ulshen et al, 1986; Opleta-Madsen et al, 1991), but in other studies oral EGF was absorbed in suckling

rats, but not in mature rats, as the ability to process luminal EGF had been lost (Thornburg et al, 1987; Rao et al, 1990; Thompson et al, 1994). Using intact mucosal preparations similar to the ones used in studies reported earlier in this thesis (section 4.3) both EGF and TGF-α have been shown to inhibit histamine-stimulated acid secretion when added to the serosal surface, but not when added to the luminal side (Finke et al, 1985; Rhodes et al, 1986). The gastric epithelium especially has been shown to be particularly impermeable to a wide variety of agents, presumably as further protection against secreted acid (Walsbren et al, 1994). However luminal EGF has been shown to be important in the maintenance of the gastric epithelium of mature animals, since sialoadenectomy (salivary gland removal) resulted in gastric mucosal atrophy in rats (Skinner et al, 1984) that could be reversed by the oral administration of EGF (Konturek et al, 1988), but the route of uptake of luminal growth factors remains unknown. Both TGF-α and EGF are relatively acid stable proteins (Cohen and Carpenter, 1975; Roberts et al, 1980), but EGF has been shown to be degraded by acid pH of less than four, to smaller forms (49 and 46 amino acid forms) that are less active (Playford et al, 1995).

The role of growth factors in disease is increasingly recognised. Deregulated expression of TGF-α and EGF-R has been widely observed in neoplasia in a variety of tissues (Derynck et al, 1987, Lee et al, 1995). Diffuse immunostaining for TGF-α was observed in pancreatic adenocarcinomas (Barton et al, 1991) and cultured pancreatic tumour cells produced and utilised TGF-α as an autocrine growth factor (Smith et al, 1987; Ohmura et al, 1990; Hofer et al, 1991). Transforming Growth Factor-alpha has also been implicated in the establishment of cutaneous paraneoplastic syndromes (Ellis et al, 1987). There is also evidence of the involvement of TGFα in non-neoplastic conditions; TGF-α mRNA and protein are increased in psoriasis (Gottlieb et al, 1988; Elder et al, 1989; Turbitt et al, 1990) and overexpression of TGF-α has been implicated in the pathogenesis of Menetrier's disease (Dempsey et al, 1992).

The pathological consequences of overexpression of TGF-α have been investigated using transgenic mouse models. Vassar and Fuchs (1991) increased its expression in skin using keratin promoters and observed increased epithelial thickness, reductions in hair follicle numbers and of hair growth, a propensity to develop benign papillomas, and overall changes similar to psoriasis. Sandgren et al (1990) and Jhappan et al (1990) increased TGF-α expression in multiple tissues by placing its expression under the control of the zinc-

inducible metallothionein (MT) promoter. Zinc-fed animals subsequently developed epithelial hyperplasia of many gastrointestinal tissues and frank neoplasia in the liver and mammary glands. The wet weights of the livers (in the absence of neoplasia), stomachs, and small and large intestines were two to three times normal, however the bodyweights of MT-TGF-α mice were reduced due to reductions in mesodermally-derived tissues such as fat, muscle and bone (Luetteke et al, 1993). In the pancreas of transgenic animals changes included hyperproliferation of connective tissue and acinoductular metaplasia, but there was no increase in the frequency of neoplasia in this organ. The changes in the stomachs of transgenic mice were also studied by Dempsey et al (1992) and Takagi et al (1992), and TGF-α overexpression resulted in many of the changes of Menetrier's disease of humans, changes which were also similar to those associated with ostertagiasis. The major changes were hyperplasia of surface mucous cells (foveolar hyperplasia), coupled with the reduction in the number and function of parietal cells. Overexpression of the transgene also blocked the maturation of zymogenic cells and reduced the amounts of pepsinogen mRNA (Bockman et al, 1995).

There is a strong argument to suggest a role for TGF-α in the development of abomasal mucous cell hyperplasia in animals with ostertagiasis, and in other helminth infections where gastric hyperplasia is prominent, such as infections of rats with *T. taeniaformis*. The following studies were carried out to confirm the presence of a TGF-α-like molecule in the gastrointestinal tissues of normal sheep and to investigate whether mucosal concentrations of TGF-α are altered by ostertagiasis, and whether such changes support the hypothesis that TGF-α is essential to the development of hyperplasia in parasitised abomasa.

9.2: A comparison of the immunohistochemical localisations of EGF and TGF-α in gastrointestinal tissues of parasite-naive sheep and in sheep with ostertagiasis.

Immunohistochemical techniques and light microscopy were used to localise TGF-α in tissues from parasite-naive and infected animals, and to compare the results with those obtained in immunohistochemical studies of EGF localisation in the same tissues. This would allow comparison to the published results of other workers on growth factor distributions to establish whether growth factor immunoreactivity was specific.

9.2.1: Materials and methods.

9.2.1.1: Animals.

Tissues were examined for TGF-α and EGF from a total of twenty one parasite-naive animals. Further tissues were obtained from multiple sites of the gastrointestinal tracts of three parasite-naive Hampshire Down lambs that were killed to provide tissues for investigations into the effects of ES products. Tissue was collected from the submandibular salivary gland, oesophagus, liver, abomasal fundus and pylorus, proximal duodenum and colon from all 3 animals. The immunohistochemical localisation of the two growth factors was also investigated in fundic and pyloric mucosal tissues of two parasite-naive Scottish Blackface lambs and in fundic mucosa from sixteen Hampshire Down lambs that were the parasite-naive controls in the studies of infection with *H. contortus*. The immunohistochemical localisation of both TGF-α and EGF was further investigated in the fundic mucosae of twenty six Scottish Blackface lambs that had natural infections of predominantly *O. circumcincta* (section 7.4). Oxyntic gland mucosa was also collected from two adult and helminth-free Sprague Dawley rats. The histological sections of rat stomach included the stratified squamous epithelium of the gastric cardia.

9.2.1.2: Immunohistochemical techniques.

The immunostaining of tissues in this section used the same Vectastain ABC kit and similar methods as for the studies of pepsinogen in sections 7 and 8. The primary antisera used were raised in rabbits against EGF from mouse submaxillary glands (ICN Pharmaceuticals, Inc., Thame, England) and against rat TGF-α (Peninsula Laboratories, Inc., St. Helens, England). The staining procedure was identical to that used for pepsinogen, but the trypsin-digestion step was omitted. Antisera were used at a 1 in 500 dilution for the anti-EGF sera and 1 in 1000 for the anti-TGF-α sera. Diaminobenzidine was again used as the chromogen and sections were counterstained with haematoxylin. Negative staining controls omitted the primary antisera or replaced them with non-immune rabbit serum (Vector Laboratories) at a 1 in 1000 dilution.

9.2.2: Results.

9.2.2.1: Tissues from parasite-naive animals.

The results for TGF-α and EGF immunoreactivity showed good similarity between animals in the staining patterns for the diverse gastrointestinal tissues examined, and the repeatability within each site, when this was examined, was good. No staining was present in any section where the primary antiserum had been omitted for either growth factor, but when serum from non-immune rabbits was substituted for the primary antiserum, there was a slight degree of non-specific reactivity (Fig. 9.1).

Strong immunostaining for both TGF-α and EGF was present in the cytoplasm of the striated ducts and larger, intralobular and interlobular ducts of submandibular salivary gland. The results for TGF-α are shown in Fig. 9.2. The cytoplasm of duct cells stained uniformly for both growth factors. Cells of the mucoserous acini did not stain. Cells lining the larger interlobular ducts bore plentiful apical cytoplasm, that was swollen in appearance (Fig. 9.3) and which stained intensely for both growth factors. Transforming Growth Factor-alpha was localised most strongly to suprabasal keratinocytes of the oesophageal epithelium. Staining appeared to be confined to the exterior of cells, but in the stratified squamous epithelium of rat cardia, TGF-α immunoreactivity was present throughout the cytoplasm of suprabasal cells. Staining of ovine oesophagus and rat cardia for EGF however demonstrated no immunoreactivity. In the duodenum the most intense immunoreactivity for TGF-α was in enterocytes on the flanks of the villi (Fig. 9.4). Enterocytes showed only weak immunoreactivity for EGF and the cells of the Brunner's glands did not stain for EGF (Fig. 9.5). In the large intestine TGF-α immunoreactivity was greatest in superficial enterocytes and EGF was again not detected.

The examination of the fundic mucosa of all parasite-naive animals revealed consistent patterns of immunoreactivities for both growth factors. Parietal cells in ovine fundus stained strongly for TGF-α (Figs. 9.6 and 9.7). Parietal cell cytoplasm stained uniformly. Chief cells bore only small amounts of predominantly apical immunoreactivity. Cells at the isthmus stained only slightly and the cytoplasm of surface mucous cells of the pits stained more strongly. The apical mucus cap of surface mucous cells did not stain. The distribution of EGF-specific staining was identical, but overall EGF immunoreactivity was paler than that

for TGF-α in most sections from the animals examined. The examination of rat oxyntic mucosa revealed an almost identical pattern for both growth factors. In pyloric mucosa TGF-α-specific staining was most prominent in the most superficial mucous cells of the pits and some cells of the pyloric glands stained strongly at various depths of the mucosa, but adjacent cells did not stain. The muscularis of pyloric mucosa was also stained strongly. Little EGF-specific staining could be detected in pyloric mucosa.

The results of TGF-α and EGF immunostaining in rat cardia, ovine oesophagus and ovine pylorus and large intestine, as well as the results of EGF immunostaining in ovine salivary gland and fundus are shown, for completeness, in Appendices 29-34.

Fig. 9.1: Negative staining control; ovine abomasal fundus stained with non-immune rabbit serum (× 25).

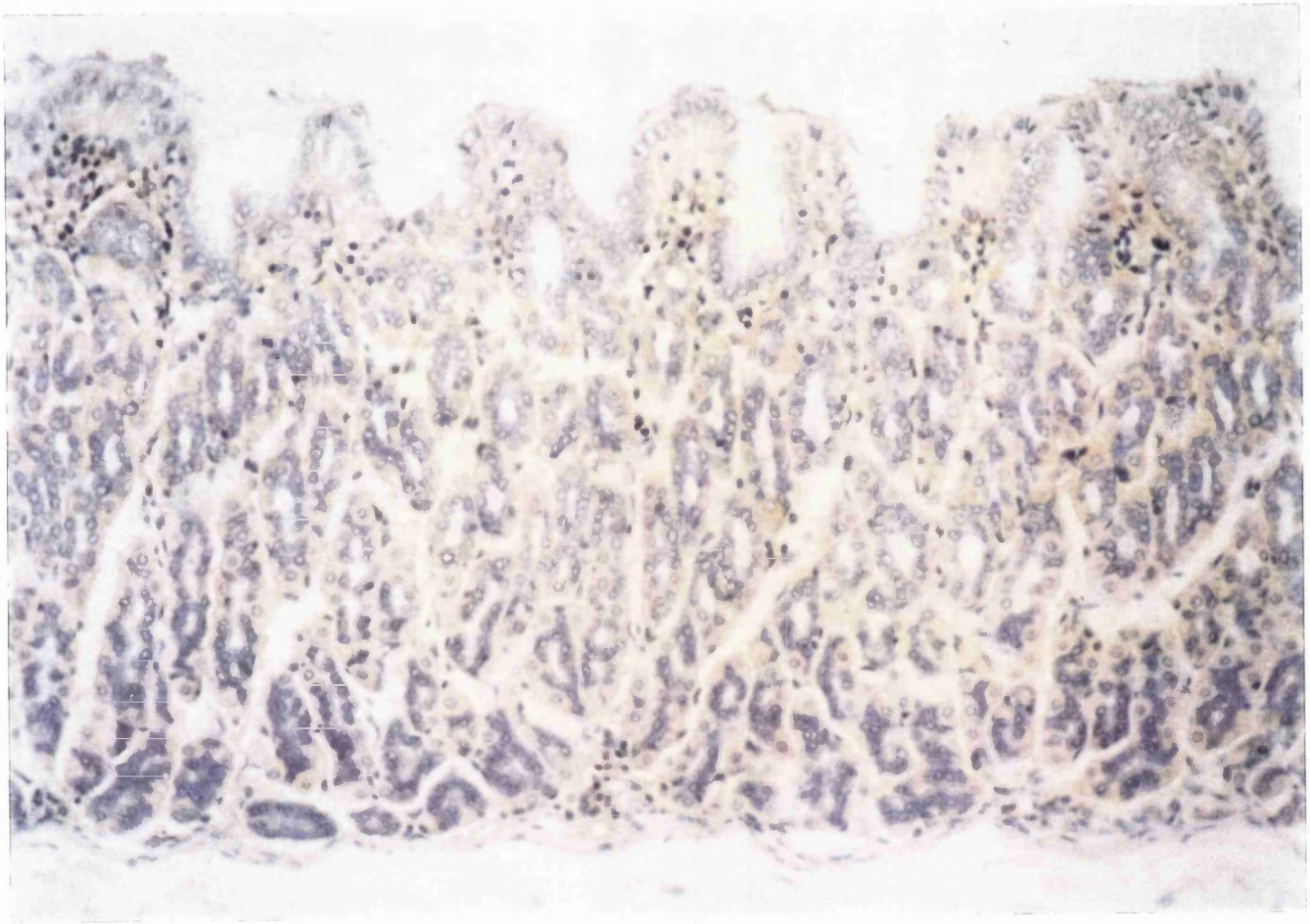


Fig. 9.2: Submandibular salivary gland. TGF-α immunoreactivity is confined to duct cells, whereas cells of the mucoserous acini do not stain. (Haematoxylin counterstain - original magnification × 25).

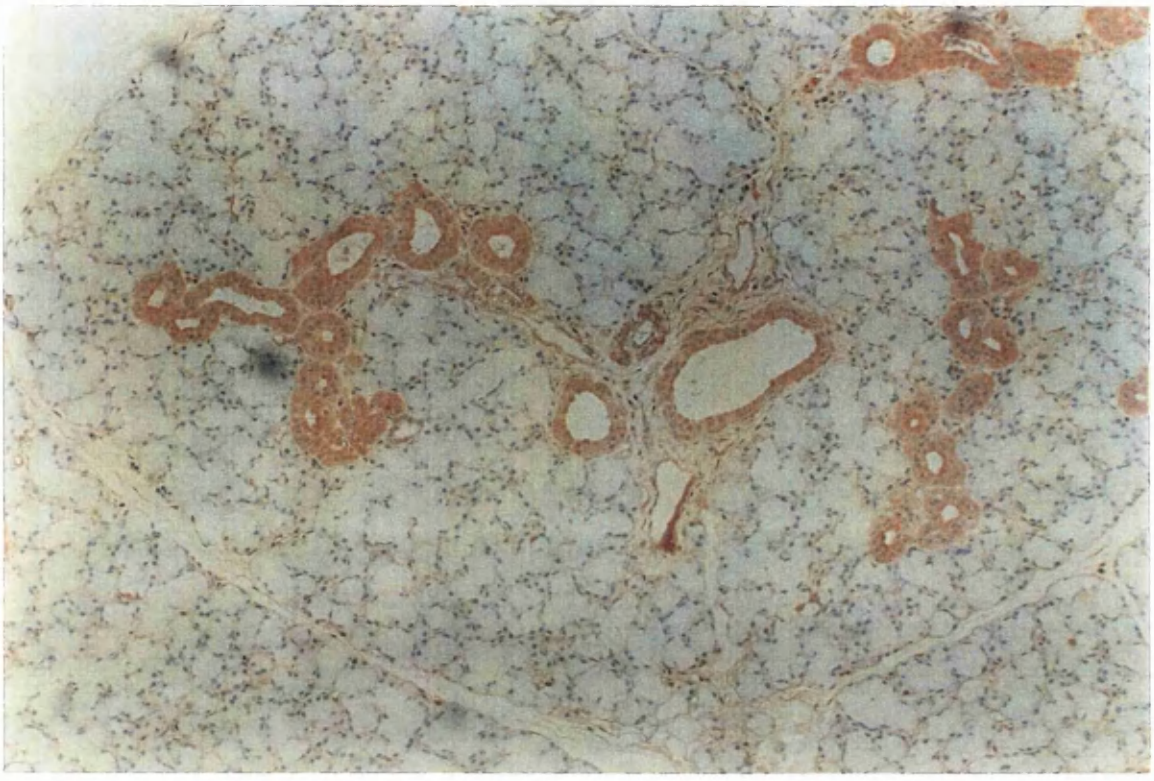


Fig. 9.3: Interlobular duct of submandibular salivary gland. The apical cytoplasm of duct cells appears swollen and strongly immunoreactive for TGF-α (arrows)(× 50).

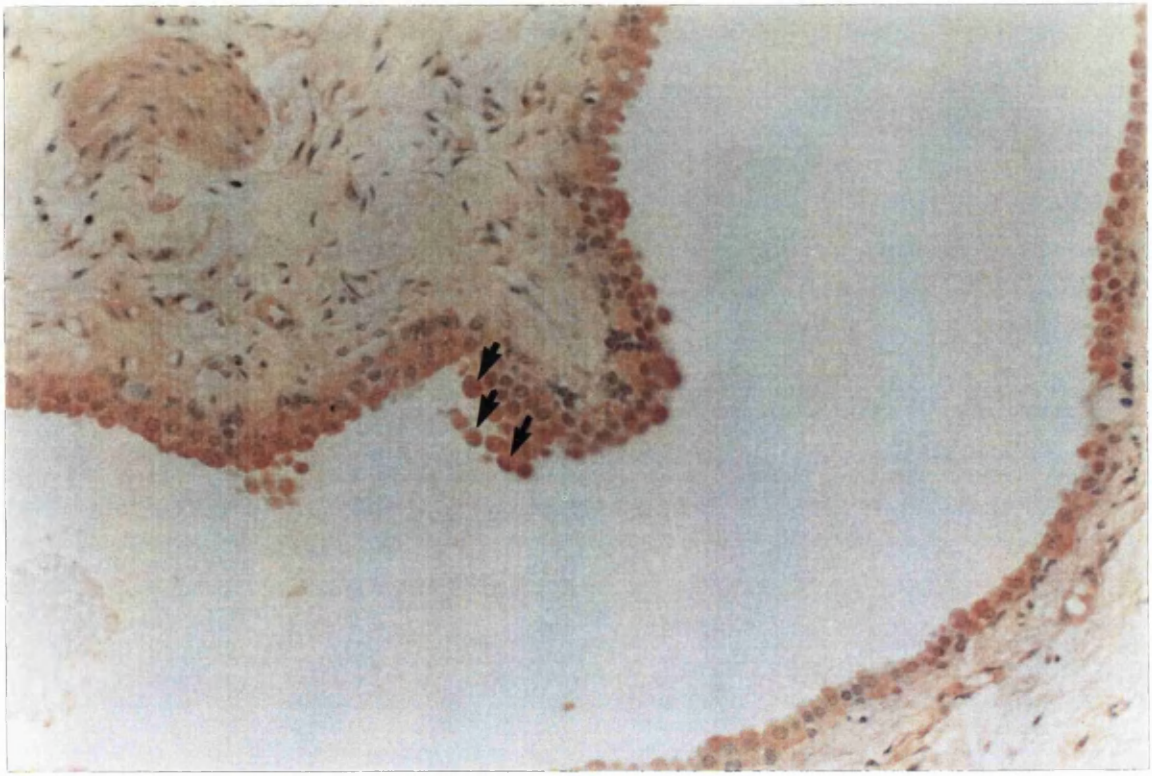


Fig. 9.4: Duodenum immunostained for TGF-α (× 10).

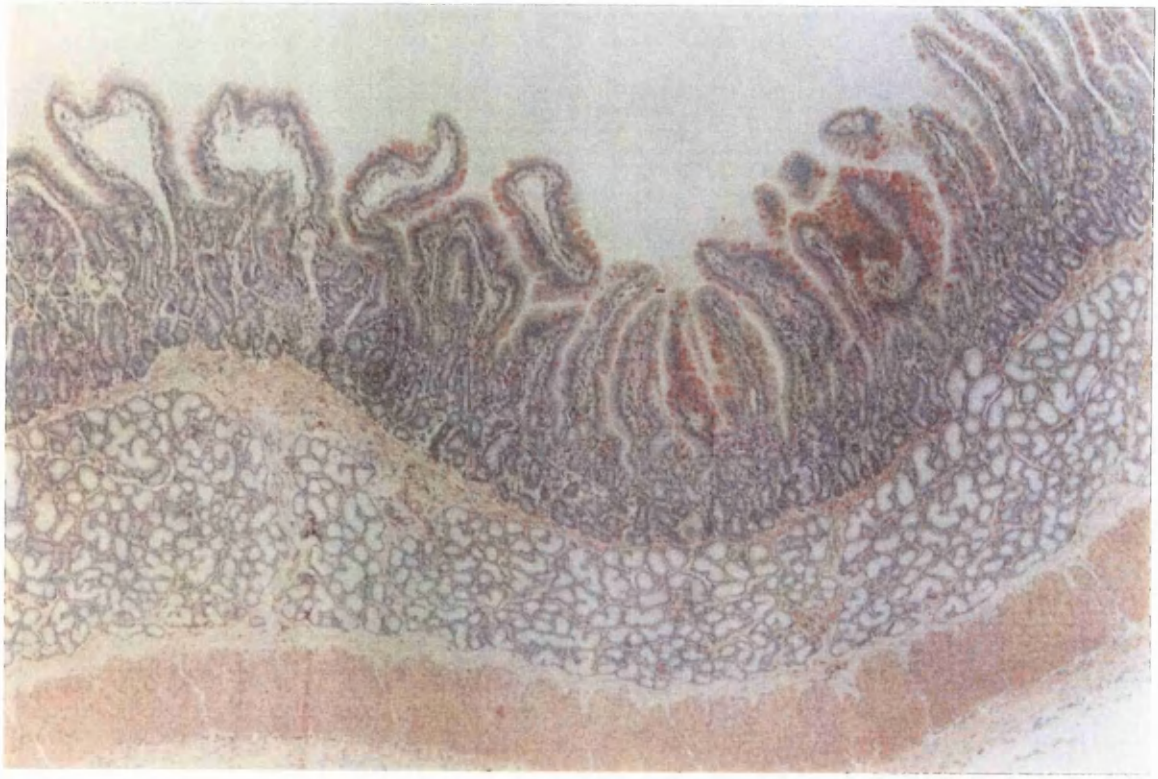


Fig. 9.5: Duodenum immunostained for EGF (× 10).

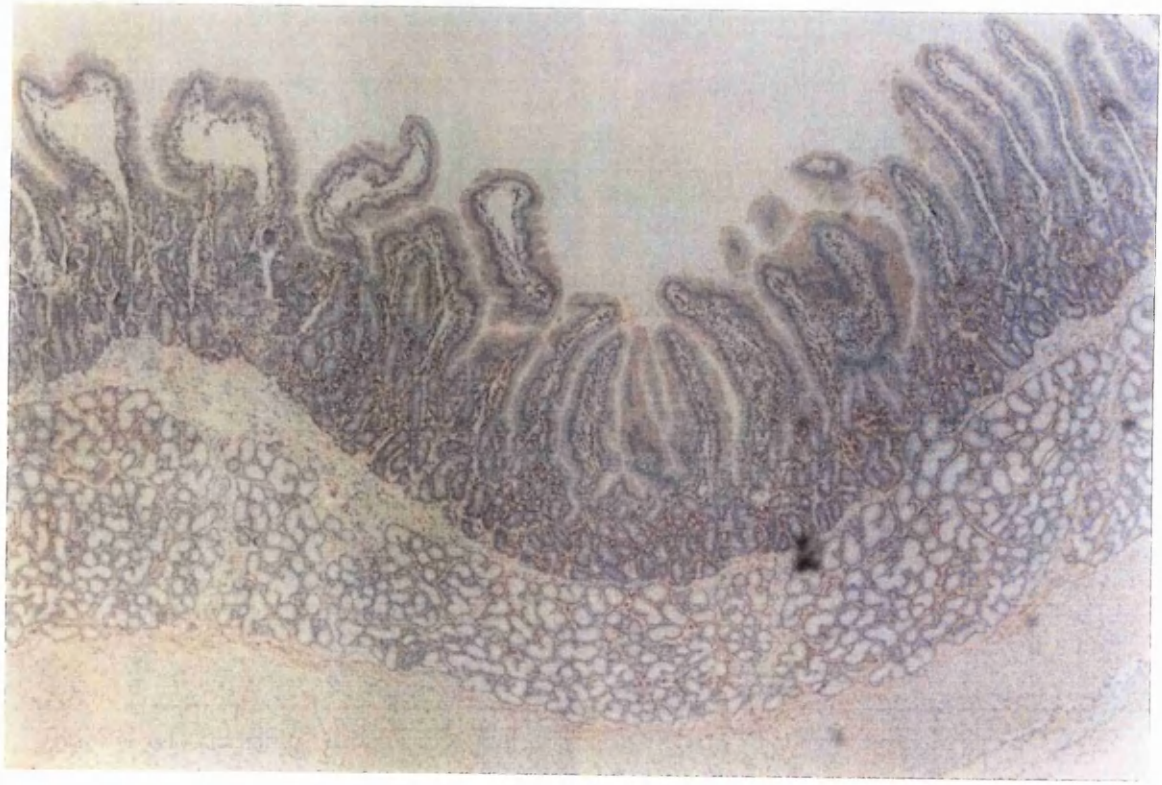


Fig. 9.6: Ovine abomasal fundic mucosa immunostained for TGF-α (× 50).

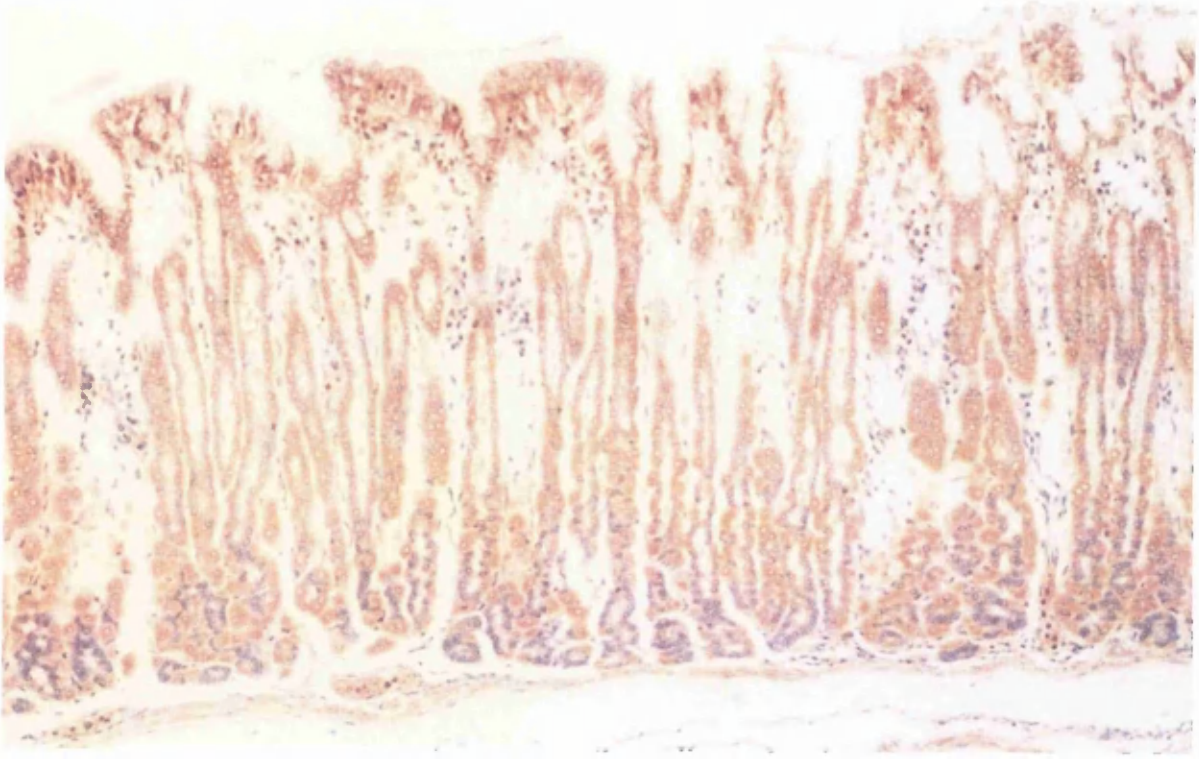
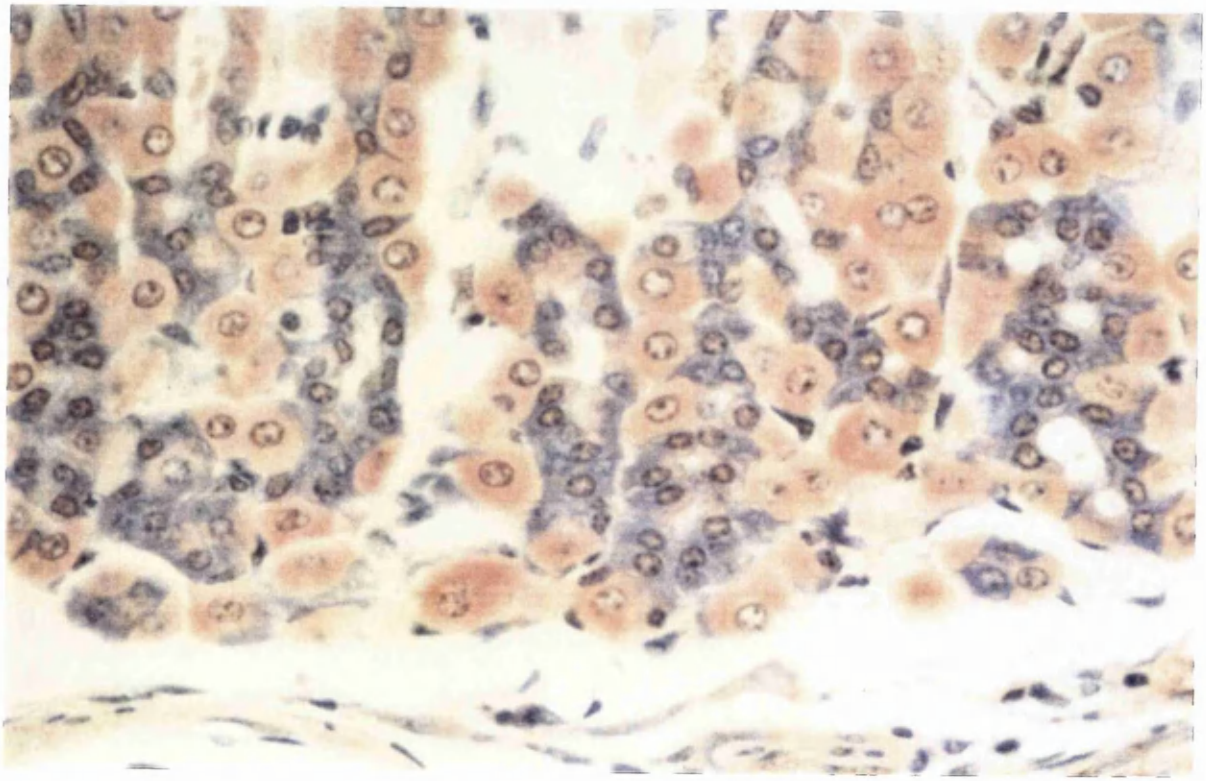


Fig. 9.7: Higher power view of the glandular cells of the abomasal fundus (× 100).



9.2.2.2: Growth factor immunoreactivity in tissues from animals with infections of predominantly *O. circumcincta.*

An examination of the negative staining controls prepared from infected animal tissues showed that, as before no staining was present in sections where the primary antiserum had been omitted. There was however a slight non-specific background staining present in some slides prepared with normal, non-immune rabbit serum and it was noticeable that parasites cut in section in these examples took up some stain (Figs. 9.8 and 9.9).

The most dramatic change in TGF-α immunoreactivity was present in sections from the fundic mucosae of infected animals in which there was an obvious increase in the size of the foveolar compartments within nodules. Figure 9.10 shows a section of the nodule from animal Y51 and featured previously in Fig. 7.20. Despite the expansion of the foveolar compartment to almost the base of the mucosa TGF-α immunoreactivity was strongest only in the outer half of the mucosa. In contrast to the appearance of parasite-naive fundus the number of mucous-type cells strongly positive for the growth factor was clearly increased. At higher magnification (Fig. 9.11) immunoreactivity was confined to the cytoplasm of surface mucous cells, but the mucus cap did not stain. The loss of parietal cells in this section led to a reduction in the apparent immunostaining of the deeper gland structures and the metaplastic cells within this area of the nodule remained free of staining even to the extreme base of the mucosa (Fig. 9.12). In other sections from other animals deep metaplastic cells showed weak cytoplasmic immunoreactivity (Fig. 9.13), but with a pale unstaining perinuclear area. Mitotic figures were numerous within nodules, but were mostly confined to unstained cells (Fig. 9.14). Mitotic figures were uncommon in the extreme base of the mucosa, but occurred in a broad band occupying approximately the middle third of the mucosa. The upper limit of this band was the area heavily immunoreactive for TGF-α and some mitoses occurred in cells that did seem to be positive for the growth factor (Fig. 9.15). The pattern of observed immunoreactivity for EGF in the same sections was similar to that for TGF-α, but was generally paler (results not shown). When larval and adult worm stages were identified within or lying on the surface of sections, immunoreactivity for both growth factors was observed in worm tissues.

Outside nodules the immunohistochemical appearance for both growth factors was nearly normal, but where pits were elongated in inter-nodular areas then the band of superficial,

mucous cell-related immunoreactivity was also wider. Recognisable parietal cells and chief cells appeared largely normal.

Occasional cells in the lamina propria were intensely immunoreactive for TGF-α, which, judging from their nuclear shape, may well be eosinophils (Fig. 9.16).

Fig. 9.8: Negative control; the appearance of nodular tissue stained using normal rabbit serum. Worms seen in section take up stain (Haematoxylin counterstain - original magnification ×25).

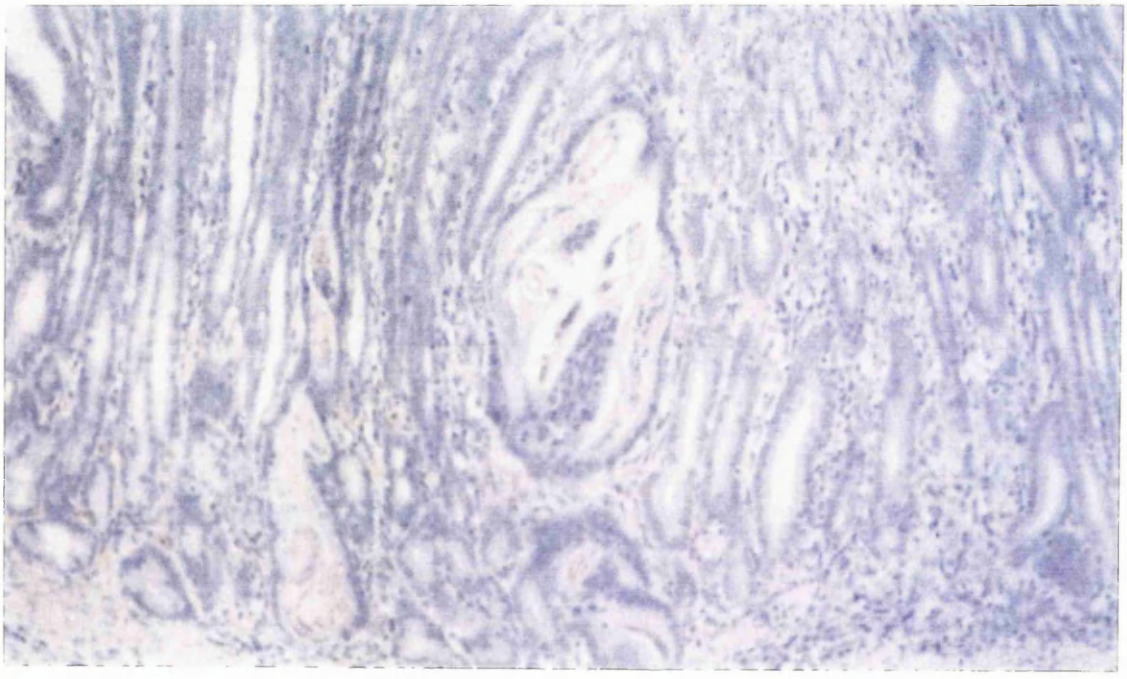


Fig. 9.9: Negative control; adult worm, free in lumen, can be seen to take up stain (normal rabbit serum - × 50).

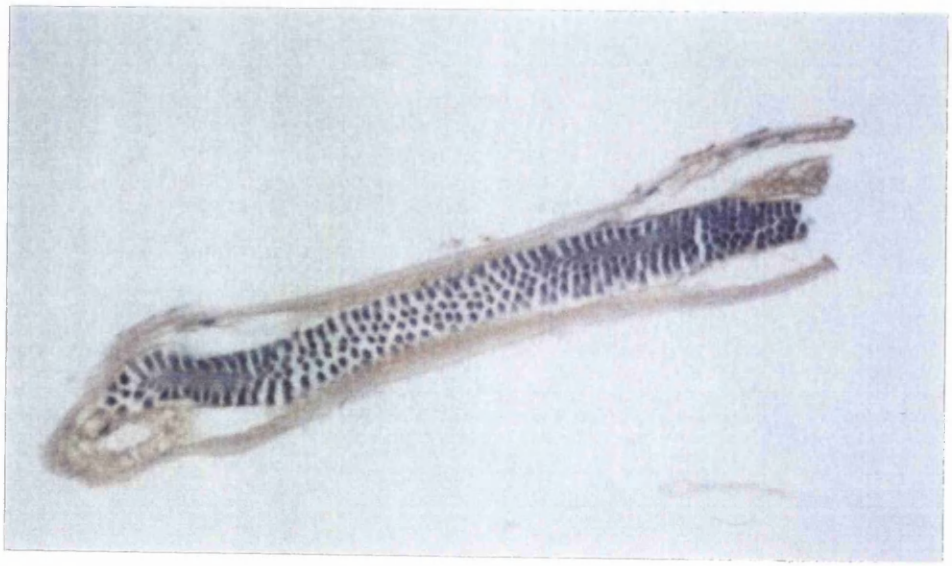


Fig. 9.10: The mucosa of animal Y51, immunostained for TGF-α (× 25).

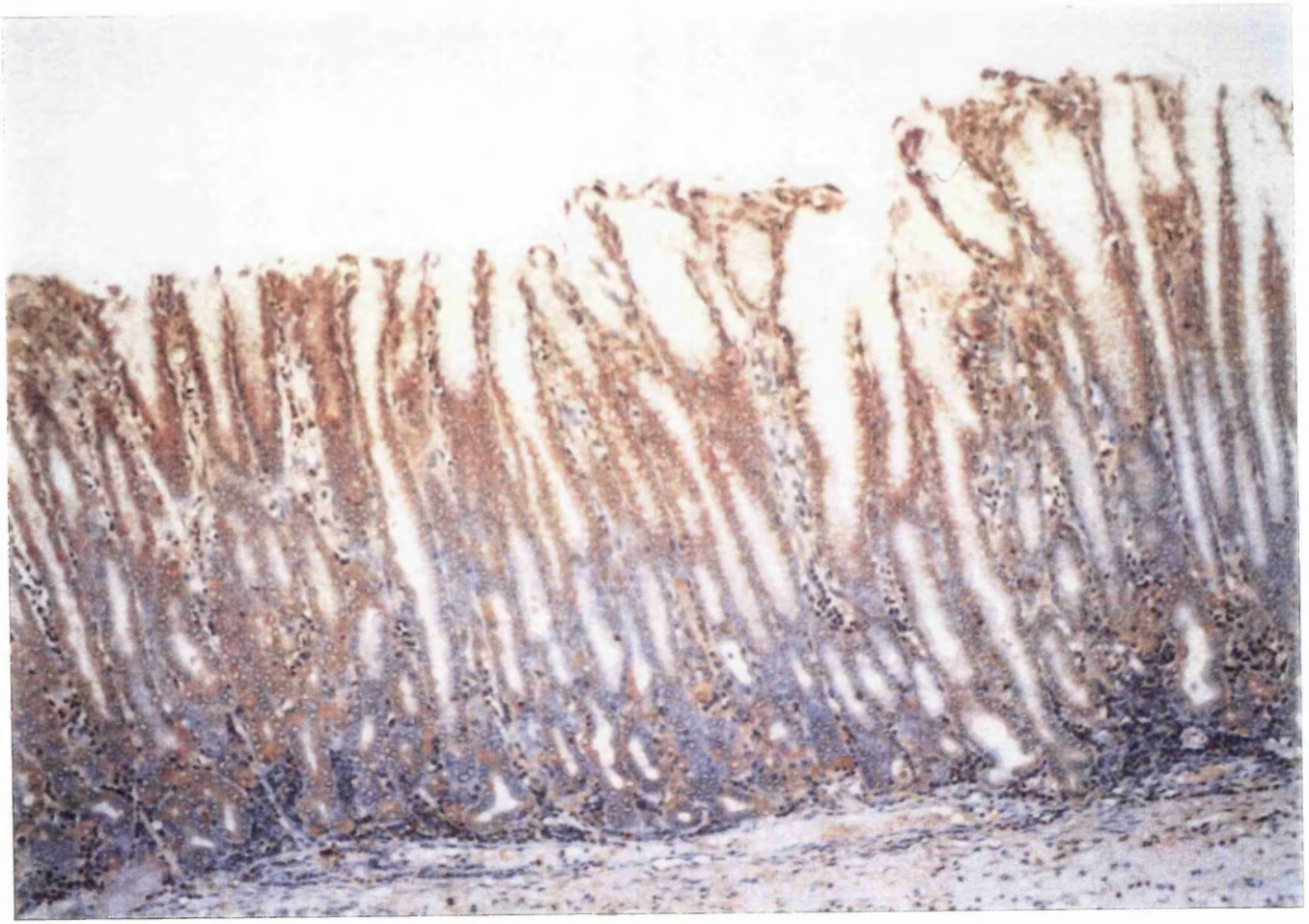


Fig. 9.11: Higher magnification of the tissue featured in Fig. 9.10, showing the increased size of the foveolar compartment (TGF-α immunostain - × 50).

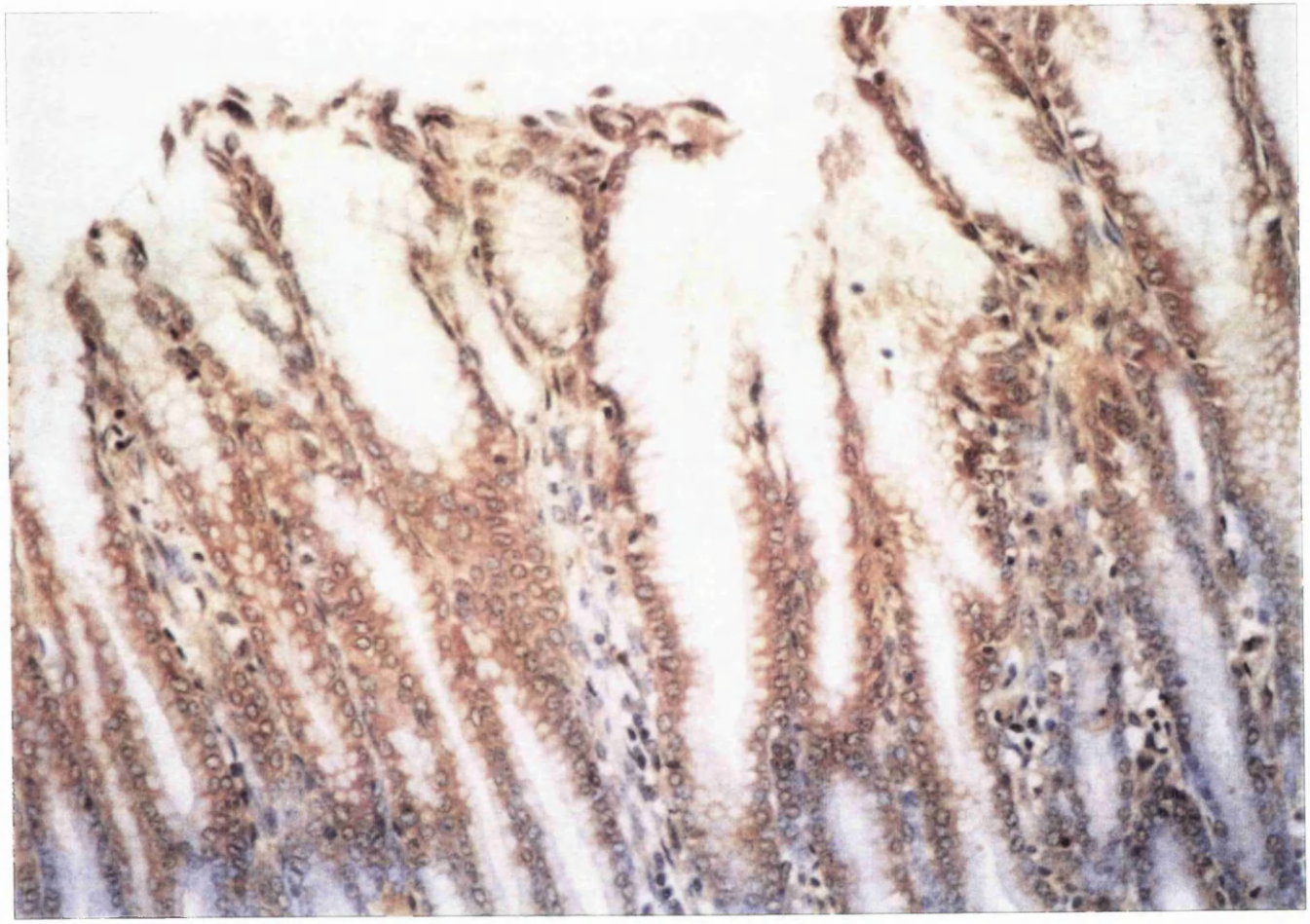


Fig. 9.12: Basal cells of the nodule featured in Fig. 8.10 show little immunoreactivity (TGF-α immunostain - × 100).

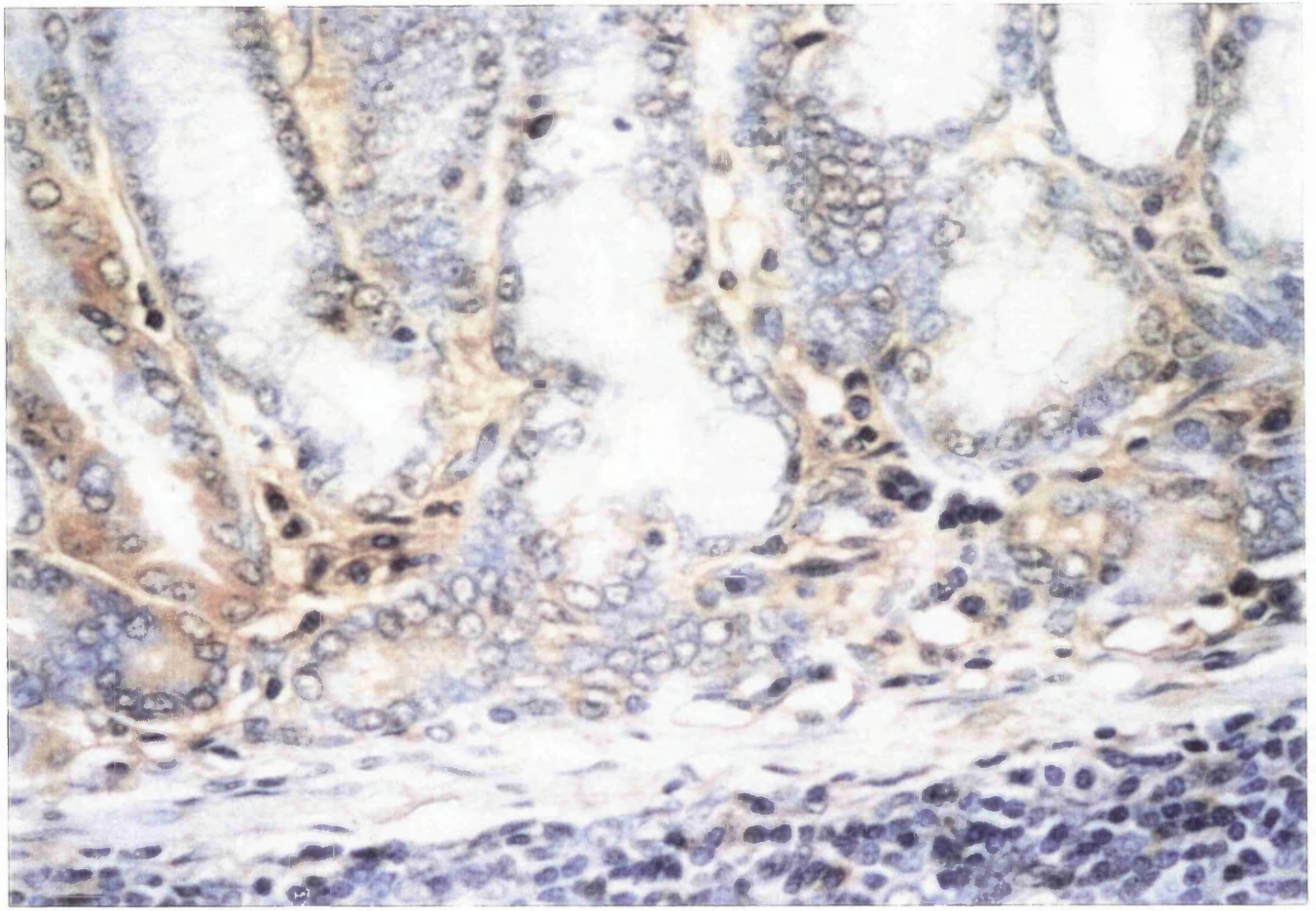


Fig. 9.13: Comparable cells as those seen in Fig. 9.12, from a nodule in animal B46, cells show weak cytoplasmic immunoreactivity (TGF-α immunostain - × 100).

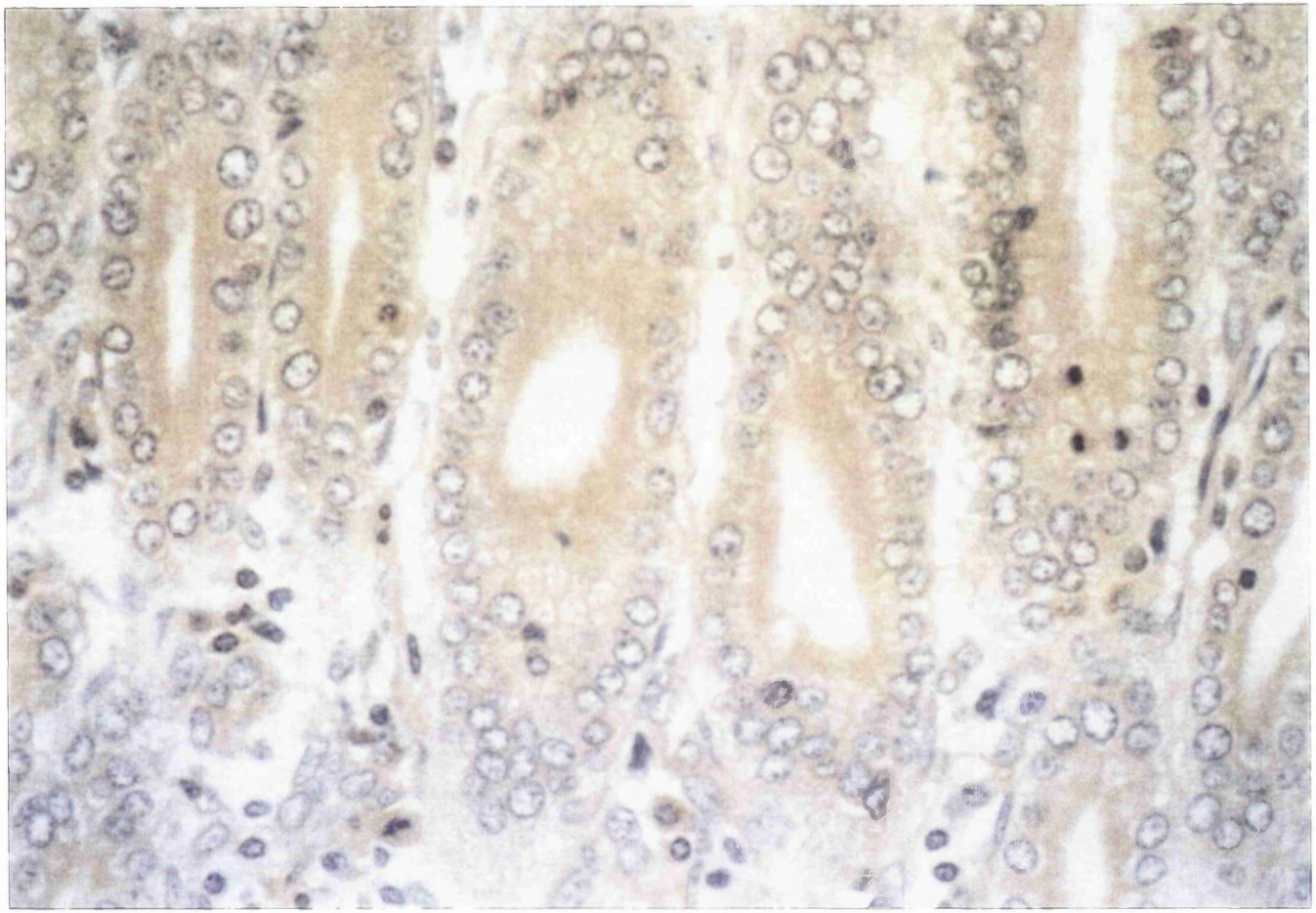


Fig. 9.14: A mitotic figure present within an expanded population of cells showing little immunoreactivity (TGF-α immunostain - × 100).

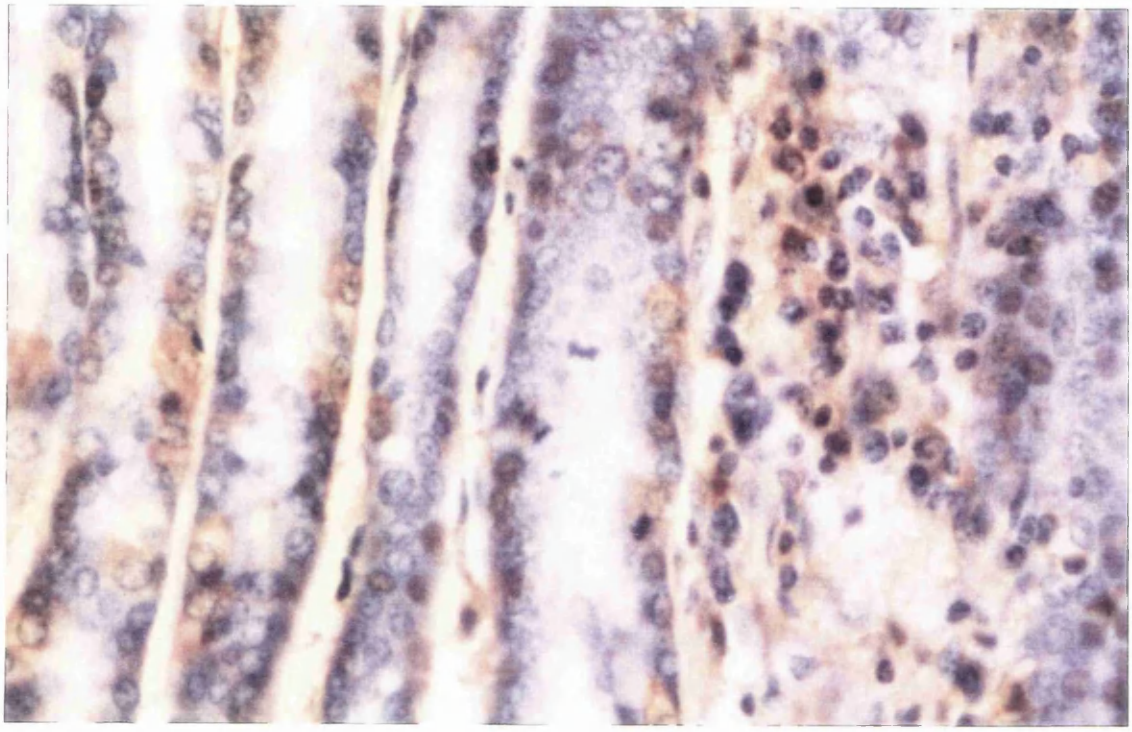


Fig. 9.15: A mitotic figure present at the boundary of immunoreactive and non-immunoreactive cells, from the same section as Fig. 9.14, the cytoplasm (arrow) is clearly immunoreactive (TGF-α immunostain - × 100).

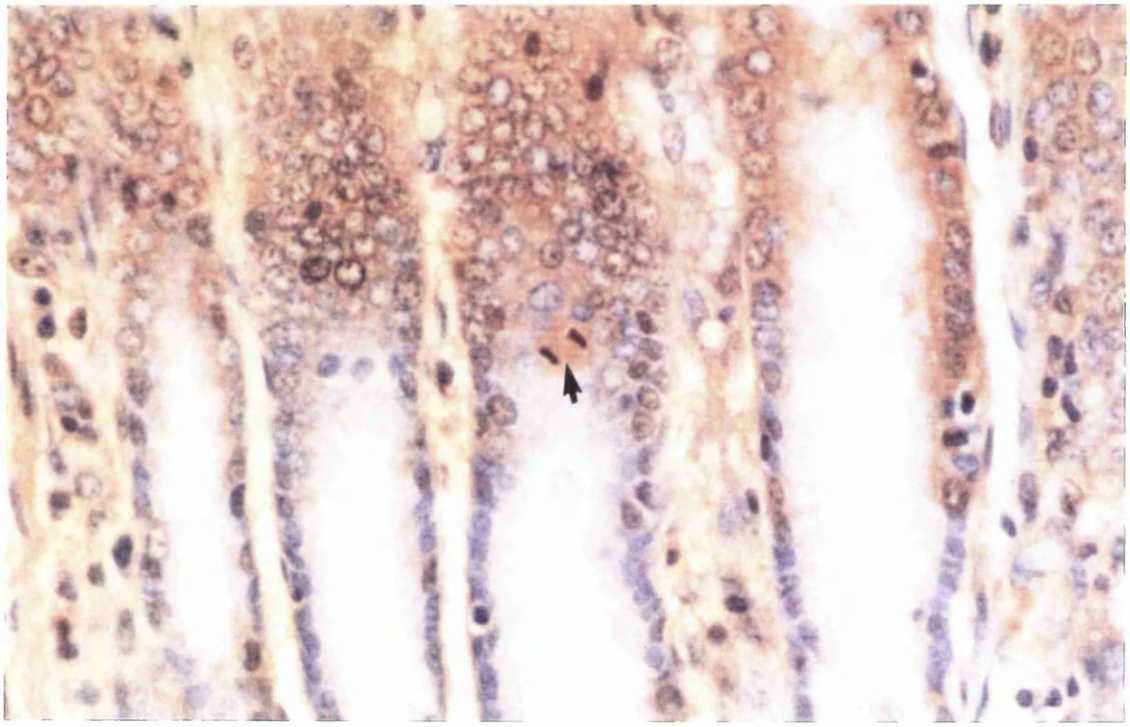
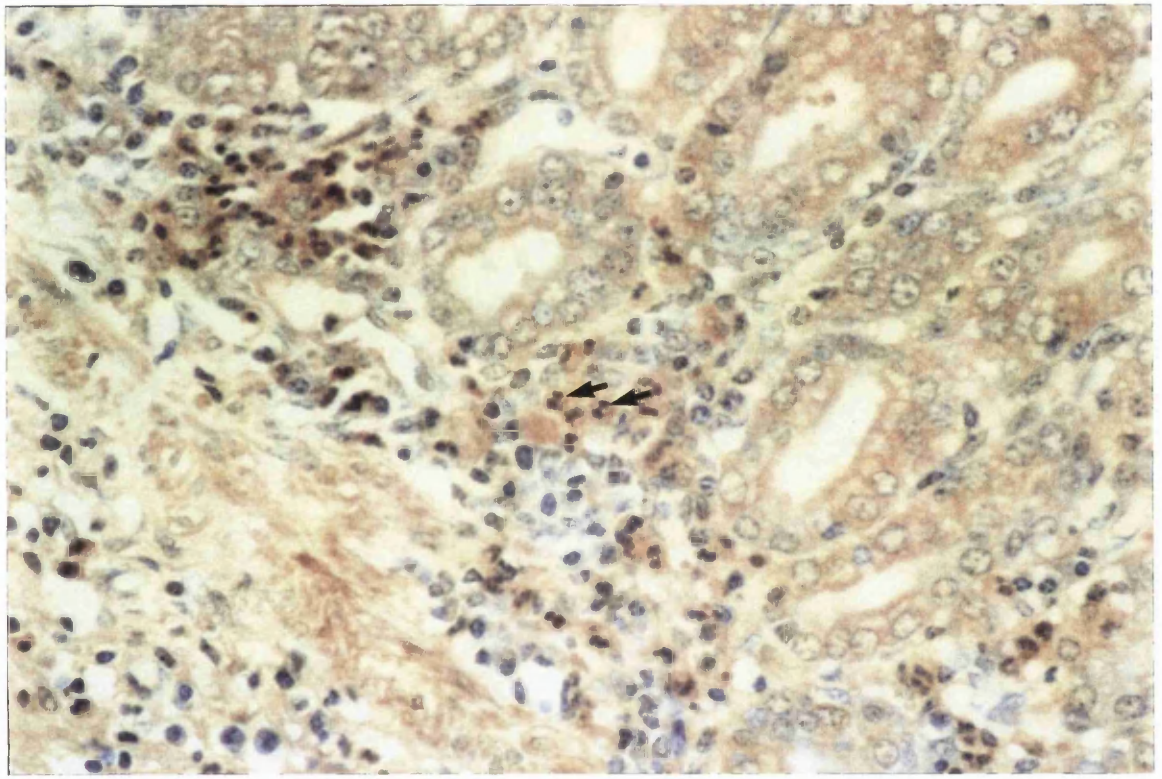


Fig. 9.16: Section of the grossly abnormal mucosa of animal Y27, immunostained for TGF-α, strong immunoreactivity can be seen in epithelial cells at deep mucosal level and in eosinophils (arrows) within the lamina propria (× 100).



9.2.3: Discussion.

The quality of staining in the present experiments was not as good as that observed for the immunolocalisation of pepsinogen (sections 7 and 8). Slight non-specific immunoreactivity was observed with non-immune rabbit serum and many cell types, including parietal cells and cells of the lamina propria and submucosa, were seen to take up stain, albeit weakly. Perhaps because chief cells showed strong basophilia with haematoxylin, any non-specific staining was masked and these cells appeared largely normal.

Nevertheless in the study of TGF-α immunoreactivity in gastrointestinal tissues from parasite-naive animals quite strong staining was seen in an identical pattern as reported in humans (Thomas et al 1992), with immunoreactivity confined to the non-proliferative compartments of many tissues. Staining was present in suprabasal, but not basal keratinocytes, in both rat cardia and sheep oesophagus; TGF-α immunoreactivity was also observed in superficial enterocytes in both duodenum and large intestine, but not in crypt cells. Transforming growth factor was therefore only present in cells that were differentiated to some degree. The results from fundic mucosa support this, with the growth factor present in mature surface mucous cells and parietal cells, but the reasons why chief cells (another end-stage cell) do not produce it in the same high concentrations remain unknown.

Strong TGF-α-positive immunoreactivity was observed in duct cells of submandibular salivary glands, adjacent to cells of the mucoserous acini that were completely free of stain. Parietal cell staining was also characteristically strong. Considered alongside the similarity between the patterns of TGF-α immunoreactivty in ovine tissues to the results reported in humans, and the differences with observed EGF-immunoreactivity in the same ovine tissues, there is therefore good evidence to suggest that immunoreactivty strongly reflected growth factor distributions and was not necessarily the result of non-specific adsorption of antisera. In some tissues EGF and TGF-α co-localised to the same cells (submandibular salivary gland and fundus), but the fact that in other tissues (rat cardia, ovine oesophagus, pylorus and large intestine) little EGF immunoreactivity was detected suggested that the two antisera recognised different epithelial components.

Unlike the situation in humans (Thomas et al, 1992) Brunner's glands of ovine duodenum showed little immunoreactivity for EGF. The presence of TGF-α and EGF in the ductal cells

of submandibular salivary glands was similar to the results obtained from mice, rats and humans (Gresick et al, 1985; Miettinen et al, 1993; Wu et al, 1993; Humphreys-Beher et al, 1994). That both growth factors might be secreted into the saliva was supported by the finding in the present study of dense immunoreactivity in duct cells of salivary glands. Concentrations of growth factors in saliva have been shown to be important in the maintenance of the mass of oxyntic mucosa and in its repair (see section 9.1), but in ruminants it is not known whether both growth factors can survive intact through the rumen, and this major difference in ruminant anatomy compared to monogastric animals might account for the anomalous presence of EGF in ovine fundic mucosal cells, and with the production of EGF in the abomasum, then its production in the duodenum is unnecessary.

The presence of either EGF or TGF-α in cells does not necessarily mean that that cell is producing the growth factor for itself, but could represent internalised ligand-receptor complexes, and thus to confirm expression of either growth factor by cells would require the demonstration of the respective mRNA's.

The reasons for the presence of both growth factors in many situations is poorly understood. Recent work has shown that a further member of the Epidermal Growth Factor-receptor ligand family, Heparin-Binding EGF, is also produced by parietal cells, but little is produced by surface mucous cells (Murayama et al, 1995). The differential timing of the production of each growth factor in response to injury, and subtly different spectra of activity and potencies in diverse epithelial and mesenchymal cell types may account for the continued expression of each growth factor by the same cells.

The sections from animals with ostertagiasis were similar in appearance, in terms of the presence of foveolar hyperplasia and gland atrophy, to examples of Menetrier's disease in humans (Dempsey et al, 1992) and to the stomachs of transgenic mice overexpressing TGF-α (Takagi et al, 1992), but changes were not as marked. In contrast to the immunohistochemical appearance of TGF-α in human examples of Menetrier's disease (Dempsey et al, 1992) the height of the TGF-α-positive band of mucous cells in the nodules of the sheep was less, but nevertheless greater than the normal pits of parasite-naive animals. Immature cells normally do not produce the growth factor and a large population of poorly differentiated cells associated with little immunoreactivity was present within nodules.

Transforming Growth Factor-alpha has recently been shown to be produced by eosinophils (Todd et al, 1991) and the growth factor-positive cells identified in the lamina propria could well represent members of this cell type. Growth factor immunoreactivity within worm tissues was probably a result of the non-specific adsorption of serum, even though nematodes do produce and utilise members of the EGF-R ligand family such as the *lin-3* gene product (Hill and Sternberg, 1992). It is equally difficult to interpret the findings of weak immunoreactivity within the deep metaplastic cells in some sections. This might genuinely reflect the production of the growth factor by cells under strong stimulation by high concentrations of the growth factor itself or might simply represent a higher non-specific reaction than normal. The use of monoclonal antibodies against growth factors, instead of polyclonal antisera, may reduce background staining, primarily because much higher dilutions can be used, and this could confirm or refute some of the above findings .

The immunohistochemical appearance of nodules from infected animals does not prove that TGF-α was the stimulus for hyperplasia. The staining pattern might simply reflect the cell changes that occurred due to other reasons, but given the known effects of TGF-α, it must be considered as a potential cause of hyperplasia. Transforming Growth Factor-alpha is a potent epithelial mitogen, it inhibits parietal cell activity, and prevents the differentiation of parietal and chief cells (Dempsey et al, 1992; Bockman et al, 1995). A number of potential hypotheses could explain the role of growth factors in the pathogenesis of ostertagiasis. The presence of a motile parasite within the confines of the gastric gland could trigger the production of growth factor by mucosal cells, or stretching the lining epithelial cells of the gland could allow the absorption of increased amounts of luminal growth factor. Alternatively, eosinophils, which are known to be attracted to abomasal mucosa in large numbers by the presence of parasites, by a number of mechanisms, could secrete growth factor. Whatever the initial cause of increases of growth factor concentration, its known ability to increase its own production could quickly account for a considerable increase in mucosal concentrations which could then effect the cell changes characteristic of ostertagiasis and inhibit parietal cell activity elsewhere. The hyperplastic response itself is unlikely to rid the animal of the initial stimulus, i.e. the parasite, but may even promote parasite survival, by generating a higher abomasal pH, and by enhancing mucus production.

10: GENERAL DISCUSSION.

Assay of plasma pepsinogen concentration undoubtedly has a role in the diagnosis and study of abomasal helminthoses, and convenient assay methods with lowest intrinsic variability will remain the most appropriate for routine use. Comparing four simple assay procedures for plasma pepsinogen estimation, the present studies demonstrated that a method, based on the work of Paynter (1992) and utilising a glycine-buffered, albumin substrate, was the best. The method was acceptably precise, was more proficient with a diverse range of enzyme concentrations and of all the methods had the lowest level of inter-assay variation (variation due to the performance of assays on different days). The present studies have also shown that two further, potential sources of variation exist and these may warrant further examination. Porcine pepsinogen added to ovine plasma produced a solution with lower peptic activity than an entirely aqueous solution of the same pepsinogen concentration, but it remains to be seen if the same effect would be observed with purified ovine pepsinogen. This finding raises the possibility that plasma might contain an inhibitory factor for pepsinogen which would influence estimations of plasma pepsinogen concentration based on measurements of the total, acid-stable proteolytic activity of samples. However, despite the putative existence of an inhibitory factor, work in humans has shown that the correlation of total plasma proteolytic activity and of plasma pepsinogen concentration measured by immunoassay is good (Hirschowitz, 1984). The second potential source of variation arises as a result of the intrinsic variability of animals to produce pepsinogen since some sheep were shown to produce relatively small amounts of pepsinogen and it is reasonable to argue that these animals might generate low plasma pepsinogen concentrations even in the face of a heavy abomasal helminth burden.

Excretory/secretory (ES) products of adult *O. circumcincta* stimulated abomasal glandular secretion and smooth muscle contraction, but these responses were identified as hypersensitivity responses that occurred only in animals with a history of previous exposure to helminth parasites. It is hypothesised that the responses resulted from mast cell degranulation following the crosslinking of immunoglobulins, present on sensitised mast cell surfaces, by antigens in ES. The in vitro contraction of smooth muscle from sensitised animals in response to antigen exposure is well characterised as the Schultz-Dale response

(section 6.1) and other experiments have demonstrated secretory and electrical responses in sensitised intestinal mucosae in vitro to antigens (Wang et al, 1991; Frieling et al, 1994), but the present studies are the first demonstrating enhanced zymogen secretion from the stomachs of sensitised animals. There is a known association between hypersensitivty responses and elevated plasma pepsinogen concentration, however this has previously been ascribed solely to mucosal permeability changes (Yakoob, et al, 1983). There is now clear evidence that part of the allergic response also involves increased release of pepsinogen from cellular sources.

Mucosal hypersensitivity responses have been considered to be important in the pathology of ostertagiasis (Wiggin and Gibbs, 1990). Mast cell degranulation and the release of numerous bioactive compounds can potentially cause permeability changes in mucosal vasculature, exacerbating fluid and protein losses, and may also contribute to the accumulation of eosinophils, via eosinophil chemotactic factors. The oxidative burst, characteristic of activated eosinophils, would almost certainly contribute to tissue damage. Mast cells and hypersensitivty reactions have also been associated with immune-mediated expulsion of certain helminth infections of the gastrointestinal tract (Miller, 1984; Nawa et al, 1994) and a knowledge of the antigenic components of ES involved in the responses demonstrated in the present studies may be of use in considering possible vaccine candidates.

Antiserum directed against bovine pepsinogen (PG I) recognised an ovine equivalent, presumably ovine PG I, in sheep fundic and pyloric mucosa. The immunohistochemical localisation of pepsinogen in the abomasa of parasite-naive cattle and sheep generally reflected patterns observed in other mammalian species (Samloff, 1971; Cybulski and Andren, 1990; Yamada et al, 1988), with the fundus being the major source of pepsinogen, and the chief cell the most intensely staining cell type. Pepsinogen was localised by immunohistochemistry to three cell types in normal bovine fundic mucosa; the chief cell, the mucous neck cell and the surface mucous cell, but the presence of pepsinogen in the bovine surface mucous cell was variable. Surface mucous cells in normal ovine fundic mucosa did not appear to contain pepsinogen.

Abomasal secretion is thought to be effectively continuous thus the granule content of chief (and other) cells should be relatively constant, assuming that the rate of granule loss is matched by the rate of production of new granules. A further consequence of the stability of

granule content is that differences in the pepsinogen content of the abomasa of different ruminant animals are likely to be due to definite phenotypic differences of pepsinogen production between animals and not the influence of events such as feeding, or other factors that might account for temporal changes in granule content in monogastric animals. The finding of considerable variation of tissue pepsinogen concentrations in the Hampshire Down lambs, in infected (*H. contortus*) and uninfected animals, is therefore significant, and suggests that the rates of pepsinogen secretion in the abomasa of individual animals were also markedly different. Although pepsinogen is more important in the digestion of animal and not plant protein the consequences for a herbivorous animal not producing pepsinogen are unknown.

Hyperplasia of abomasal epithelial cells in response to both ostertagiasis and haemonchosis was associated, on the basis of pepsinogen-specific immunoreactivity, with an expanded zymogenic population. This occurred in the face of an apparent decline in chief cell staining that was demonstrable in both infections. The secretory rates for pepsinogen by infected abomasa were not determined in the present studies, but on the basis of the immunohistochemical evidence it seems likely that secretion is at least maintained during infection, if not increased, as it is unlikely that the hyperplastic mucous cells producing pepsinogen are subsequently incapable of secreting it. The present studies have demonstrated the existence of a reserve capacity of the abomasum to continue producing pepsinogen, by the recruitment of relatively immature mucous-type cells arising as a result of hyperplasia. These cells may eventually further differentiate to assume the phenotype of mature mucous cells, which in the ovine do not produce pepsinogen normally, and pepsinogen production by these cells may then cease.

The present immunohistochemical studies did not determine the mechanism of pepsinogen secretion or the mechanism whereby pepsinogen gains entry to the blood stream. In the present studies all pepsinogen-specific immunoreactivity was intracellular, within epithelia and the surrounding lamina propria, confined to the cytoplasm of cells. Plasma pepsinogen has been shown to become increased in animals with experimentally induced hypergastrinaemia (Fox et al, 1989b). Gastrin was not thought to influence mucosal permeability, thus the mechanism of plasma pepsinogen elevation was attributed directly to the stimulation of pepsinogen secretion, both to the lumen and also in a retrograde manner to the blood stream. On the basis of the present and previous studies (Murray, 1970; Yamada et

al, 1988; Cybulski and Andren, 1990), the granules of pepsinogen-producing cells lie at their apical surfaces. Retrograde secretion may therefore not involve granule formation, although Murray (1970) did note the presence of some granules, situated atypically, at the basal membranes of mucous neck cells.

The decline of the pepsinogen content of chief cells in some infected animals was a consequence of either; accelerated granule loss by these cells in response to strong secretory stimuli, or a change of cell phenotype towards that of a non-zymogenic, perhaps mucous-type, cell. It is known that gastrin becomes elevated in infected animals, and this could then stimulate pepsinogen secretion and deplete granule number. More importantly perhaps, mucins (as PAS-positive material) were seen deeper in glands in infected animals than normal, so that it is more likely that the decline of chief cell staining is a consequence of phenotypic change. The histological appearance of chief cells in the present studies was very similar to observations of cells at the deep gland bases of the stomachs of transgenic mice overexpressing Transforming Growth Factor-alpha (Bockman et al, 1995). In these animals gland cells were prevented from maturing into either parietal or chief cells by the continuous presence of high concentrations of growth factor.

Foveolar hyperplasia and complete gland atrophy has been seen focally in both ostertagiasis and haemonchosis in the present studies, and is a generalised feature of Menetrier's disease of man. It has also been seen in transgenic mice overexpressing TGF-α (Dempsey et al, 1992; Takagi et al, 1992) and therefore represents a possible end-stage of disease induced by increased expression of, or stimulation by, TGF-α. It is therefore probable that TGF-α has a role in the development of the pathology of the gastric helminthoses where hyperplasia is prominent. The growth factor has been recognised as a potent inhibitor of parietal cell activity and may contribute to the inhibition of parietal cells seen in helminth-infected abomasa, which ultimately results in the disappearance of the cells altogether. The production of TGF-α in the mammalian gastrointestinal tract and indeed in other tissues is confined to the proliferative compartment, hence the strong immunoreactivity observed in surface mucous cells and in parietal cells of ovine fundic mucosa, but this does not explain the relative paucity of growth factor in normal chief cells, and it is likely that several factors are responsible for normal homeostasis in the abomasal epithelium. Gastrin is known to increase the numbers of parietal and zymogenic cells in situations where gastrin is elevated either artificially (Waldum et al, 1991) or by disease (McGuigan, 1983), and gastrin has been

shown to partially reverse changes in pancreatic tissues induced by TGF-α overexpression (Wang et al, 1993). The increase in gastrin in the mucosae of infected animals could stimulate the presence of pepsinogen within the hyperplastic cell population.

The presence of small concentrations of pepsinogen in the plasma of uninfected animals, can perhaps be taken as evidence of a continuous, but low grade, leak of the zymogen across the epithelial barrier. An identical mechanism might theoretically account for the uptake of luminal growth factors that is necessary for the continued maintenance of the gastric epithelial mass (see section 9.1). Hyperplastic lesions of the stomach/abomasum are associated with increased permeability of the gut wall, due to the poorly formed tight junctions that exist between the hyperplastic and immature epithelial cells, and this might increase growth factor uptake. Studies of tight junction width and of associated protein loss in humans with Menetrier's disease have shown that treatment with the anti-cholinergic agent propantheline bromide reduced tight junction width and concurrently reduced protein loss, whereas pentagastrin and bethanechol chloride (gastrin and acetylcholine analogues respectively) both increased protein loss, but had no effect on tight junction width (Kelly et al, 1982). Treatment of Menetrier's disease with what would be considered as primarily, anti-secretory agents, such as cimetidine (H_2-antagonist), is common (Kang et al, 1990; Yeaton and Frierson, 1993). That anti-muscarinic and other agents affect epithelial permeability could also help explain the reductions in plasma pepsinogen concentrations seen in sheep infected with *O. circumcincta* and given atropine (Mostofa and McKellar, 1989).

The results of the present work provide a more comprehensive description of some of the mechanisms that might operate in ostertagiasis, and, where similarities exist, in haemonchosis, than has previously been available. Ingested larvae penetrate the abomasal glands and stretch the lining epithelium. At this stage it may be of advantage to the larvae to reduce acid secretion within the glands, and larvae could do this in one of three ways. Firstly, the larvae might elaborate a chemical capable of directly inhibiting parietal cell activity, and some workers have demonstrated the presence of acid-inhibitory factors in the tissues or products of *Ostertagia* spp. parasites (Eiler et al, 1981; Rikihisa and Hammerberg, 1982). Secondly, damage to cells of the gland lining may result in the production and release locally of a factor, such as TGF-α, which is anti-secretory. Finally, the stretch and disruption of the gland lining could allow increased uptake of luminal growth factor. The initial hyperplasia

that occurs in ostertagiasis and haemonchosis does suggest that TGF-α might be operating at this early stage of infection and with worm growth there is the potential for the production of the growth factor by epithelial cells, or its uptake, to be increased. Parietal cell activity may consequently become inhibited and the differentiation of new parietal cells prevented. Hypergastrinaemia may then be a direct consequence of this inhibition, but the possibility of the direct stimulation of G-cells to release gastrin by parasites has not been excluded (Anderson et al, 1981).

Plasma pepsinogen concentrations become elevated due to a number of potential mechanisms which may include; an increase in mucosal permeability, which may only be a transient feature (Holmes and Maclean, 1971), the direct stimulation of pepsinogen secretion into the blood by hypergastrinaemia or other stimuli and possible hypersecretion by an enhanced zymogenic cell population. Yet the ability of the worms themselves to stimulate pepsinogen release cannot be discounted. Plasma pepsinogen concentrations rose rapidly in parasite-naive animals following the transplant of adult parasites (McKellar et al, 1986, 1987), and the present studies (section 6) do not rule out the production of a pro-secretory chemical by adult *O. circumcincta* in vivo. Hypersensitivity responses induced by parasite antigen may further assist in maintaining high plasma pepsinogen concentrations and may also affect mucosal permeability.

It is difficult to see how the mucosal hyperplastic response, whether initiated by a growth factor such as TGF-α, or by another as yet unidentified mechanism, can eliminate the parasites on its own. Mucous hypersecretion and hyposecretion of acid may rather favour parasite survival, and it is only the loss of the worm population, whether due to anthelmintic treatment, developing immunity, or the gradual senescence of adult worms with no further ingestion of infective larvae, that then eliminates the stimulus for the maintenance of the mucosa in the hyperplastic state, and allows the restoration of the normal homeostatic control of the abomasal cell population.

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11: APPENDICES.

Contents.

Appendix 1A: Replicate analyses of the 5 plasma standards by method 1.

(results in iU, figures in parentheses are mean values ± S.E. for day one data only).

Std.	Day 1	2	3	4	5
1	5.94 7.16 5.94 6.85 6.04 **(6.39 ± 0.26)**	7.42	6.33	6.34	5.47
2	10.64 13.08 10.95 12.16 12.57 **(11.88 ± 0.50)**	11.66	13.81	10.12	9.8
3	14.42 14.72 15.53 15.84 15.13 **(15.12 ± 0.26)**	11.75	16.63	13.65	16.87
4	22.98 24.30 24.50 23.89 24.50 **(24.03 ± 0.30)**	27.90	26.30	24.21	26.21
5	32.72 31.50 34.55 31.71 32.82 **(32.66 ± 0.54)**	27.28	36.24	31.77	35.47

Appendix IB: Replicate analyses of the 5 plasma standards by method 2.

(results in iU, figures in parentheses are mean values ± S.E. for day one data only).

Std.	Day 1	2	3	4	5
1	1.77 1.82 1.82 1.85 1.88 **(1.83 ± 0.02)**	1.77	1.61	1.29	1.78
2	2.65 2.73 2.72 2.47 2.75 **(2.66 ± 0.05)**	2.73	2.63	2.13	3.09
3	3.38 3.57 3.52 3.66 3.51 **(3.53 ± 0.04)**	3.52	3.42	3.29	4.03
4	4.93 5.29 5.14 4.97 5.16 **(5.10 ± 0.07)**	4.99	5.20	4.94	5.23
5	6.40 6.79 6.43 6.62 6.59 **(6.57 ± 0.07)**	6.60	6.76	6.37	7.17

Appendix 1C: Replicate analyses of the 5 plasma standards by method 3.

(results in iU, figures in parentheses are mean values ± S.E. for day one data only).

Std.	Day 1	2	3	4	5
1	1.26 1.85 1.52 1.63 1.77 **(1.61 ± 0.10)**	1.53	1.10	1.34	1.02
2	2.44 3.33 2.92 3.14 3.22 **(3.01 ± 0.16)**	2.22	2.13	2.43	1.47
3	3.07 3.36 3.62 3.33 3.40 **(3.57 ± 0.09)**	2.75	2.83	3.19	2.33
4	5.80 6.40 5.73 5.95 5.99 **(5.97 ± 0.12)**	4.05	4.35	4.97	3.76
5	7.06 7.32 7.28 6.84 7.39 **(7.18 ± 0.10)**	4.56	6.08	6.29	5.22

Appendix 1D: Replicate analyses of the 5 plasma standards by method 4.

(results in ng/ml, figures in parentheses are mean values ± S.E. for day one data only).

Std.	Day 1	2	3	4	5
1	0.55 0.67 0.57 0.44 0.46 **(0.54 ± 0.04)**	0.79	0.74	1.03	0.67
2	0.58 0.71 0.69 0.66 0.55 **(0.64 ± 0.03)**	1.49	0.89	1.21	0.71
3	0.93 1.19 0.81 0.81 0.84 **(0.92 ± 0.07)**	2.54	1.47	1.19	0.93
4	1.44 1.34 1.44 1.35 1.79 **(1.47 ± 0.08)**	2.75	1.75	1.95	1.44
5	2.04 2.07 1.71 2.23 1.68 **(1.95 ± 0.11)**	5.37	2.57	2.65	2.07

Appendix 2: The peptic activity of porcine pepsinogen standards with time; a comparison with tyrosine standards.

Date	1ng/ml is equivalent to: (iU)	1 u/ml is equivalent to: (iU)
12/03/93	48.03	15.37
07/09/93	43.03	13.77
12/09/93	31.72	10.15
30/11/93	31.37	10.04
08/12/93	33.19	10.62
10/01/94	31.31	10.02
25/01/94	29.75	9.52
18/02/94	32.00	10.24
08/03/94	45.72	14.63
09/03/94	42.25	13.52
08/04/94	29.75	9.52
12/04/94	33.09	10.59
19/04/94	29.28	9.37
21/04/94	29.87	9.56
22/04/94	42.81	13.70
28/04/94	26.53	8.49
04/05/94	49.37	15.80
10/05/94	30.94	9.90
18/08/94	23.87	7.64
12/09/95	22.87	7.32
13/01/95	38.59	12.35
Mean (± S.E.)	**34.50 (1.70)**	**11.00 (0.50)**

Appendix 3: Replicate analyses of plasma from the eleven sheep, experimentally infected with *O. circumcincta*, assayed for plasma pepsinogen concentration by Paynter's method.

(results in iU, figures in parentheses are mean values, ± S.E. for day one data only).

Sheep No.	DAI*	Day 1	2	3	4	5
1	NI	0.16 0.22 0.00 1.26 0.11 **(0.35 ± 0.23)**	0.56	2.80	2.19	0.81
2	28	1.10 0.88 1.15 0.88 2.47 **(1.30 ± 0.30)**	2.08	3.73	2.89	0.91
3	NI	3.35 2.75 5.11 2.64 2.75 **(3.32 ± 0.46)**	4.17	5.34	4.77	2.72
4	14	3.63 3.35 5.16 4.45 4.56 **(4.23 ± 0.33)**	7.78	8.05	6.25	5.95
5	NI	1.26 2.53 0.93 0.99 1.43 **(1.43 ± 0.29)**	0.76	1.61	1.00	0.71
6	7	5.60 2.80 3.40 2.20 4.78 **(3.76 ± 0.63)**	2.50	4.41	3.28	2.32

7	7	5.00 4.35 3.39 3.71 3.55 **(4.00 ± 0.30)**	4.37	5.17	4.61	4.04
8	14	5.56 5.08 5.56 8.71 7.42 **(6.47 ± 0.69)**	5.42	6.19	6.41	6.40
9	21	12.34 13.31 12.26 16.29 13.71 **(13.58 ± 0.73)**	11.74	11.86	12.58	10.59
10	28	6.05 6.69 5.08 4.68 4.84 **(5.47 ± 0.39)**	4.86	4.83	5.94	3.13
11	21	8.39 8.63 9.03 9.35 9.52 **(8.98 ± 0.0.21)**	6.87	6.19	13.00	8.78

* DAI - status at necropsy - days after infection with 15,000 O. circumcincta L3. NI - uninfected controls.

Appendix 4A: Zone diameters for blank plasma, and for plasma and water standards with identical concentrations of added porcine pepsinogen, figures are in mm, adjusted means are mean values for water and plasma standards minus the mean diameter of the plasma blank (equivalent to well diameter).

	1	2	3	4	5	6	7	8	9
Plasma blank	7.70	7.70	7.70	7.70	7.70				
Water Std.	14.75	14.32	14.18	14.08	14.33	14.22	14.73	14.37	14.02
Plasma Std.	14.31	13.50	13.60	13.09	13.32	13.74	13.77	13.28	12.99

Appendix 4B: Change in optical densities ($\Delta O.D._{560nm}$), over three hours of incubation, for blank plasma, and for plasma and water standards with identical concentrations of added porcine pepsinogen.

	1	2	3	4	5	6	7	8	9
Plasma blank	0.003	0.000	0.003	0.002					
Water Std.	0.306	0.315	0.271	0.266	0.283	0.328	0.250	0.289	0.232
Plasma Std.	0.167	0.152	0.205	0.187	0.198	0.184	0.186	0.196	0.203

Appendix 5A: Gland counts and pepsinogen secretion during exposure of isolated gastric glands to different doses of carbachol. Mean values for individual experiments expressed as increases over secretion in controls (figures in normal script) and then as a percentage of the maximum response in each animal (figures in bold script and parentheses). The overall mean (± S.E.) values of all experiments for pepsinogen secretion as a percentage of maximal response are shown in the last column.

	sheep **1**	**2**	**3**	**4**	**5**	**6**	**Mean ± S.E.**
gland count (glands/ml)	10,000	25,500	140,000	21,100	240,000	20,000	
no. replicates	3	3	5	3	5	5	**6**
[carb], 10^{-7} M	1.00 **(7.00)**	0.89 **(11.00)**	3.20 **(5.00)**	1.00 **(80.00)**	1.45 **(69.00)**	1.24 **(75.00)**	**(41.20 ± 15.10)**
10^{-6} M	0.00 **(0.00)**	1.48 **(18.00)**	17.50 **(29.00)**	1.12 **(89.00)**	1.60 **(76.00)**	0.98 **(59.00)**	**(45.20 ± 14.30)**
10^{-5} M	10.75 **(72.00)**	2.05 **(25.00)**	23.60 **(39.00)**	1.13 **(90.00)**	2.10 **(100.00)**	1.66 **(100.00)**	**(71.00 ± 13.10)**
10^{-4} M	8.50 **(57.00)**	8.12 **(100.00)**	51.00 **(85.00)**	1.25 **(100.00)**	1.20 **(57.00)**	1.32 **(79.00)**	**(79.70 ± 7.90)**
10^{-3} M	15.00 **(100.00)**	N.D.	60.00 **(100.00)**	1.12 **(89.00)**	0.70 **(33.00)**	0.90 **(54.00)**	**(75.20 ± 13.50)**

Appendix 5B: Gland counts and the pepsinogen concentrations released during two experiments investigating the effects of likely supramaximal doses of carbachol, pepsinogen concentrations are in u/ml, and represent the differences between pre-sample concentrations and those of samples collected after 30 minutes of incubation in the presence or absence (controls) of 1mM carbachol.

	sheep 1		**sheep 2**	
	control	1mM Carbachol	control	1mM Carbachol
gland counts				
(glands/ml)	8,000	8,000	18,000	18,000
replicate 1	0.70	0.06	0.93	1.50
2	0.32	0.19	0.75	1.54
3	0.31	0.32	0.00	0.00
4	0.33	0.13	0.66	2.66
5	0.00	0.24	0.99	0.89
6	0.04	0.57	1.41	0.82
7	0.23	0.01		
8	0.38	0.00		
9	0.58	0.00		
10	0.00	0.10		
Mean (± S.E.)	**0.29 (0.07)**	**0.16 (0.06)**	**0.79 (0.19)**	**1.23 (0.37)**

Appendix 6A: Pepsinogen release from individual tissues of, and mean values for, sheep 1 in time course of the cholinergic response experiments.

Time (min)	Tissue A	B	Mean (± S.E.) u/ml	Mean (± S.E.) Secretion/basal
5	5.93	6.39	**6.16 (0.23)**	1.64 (0.06)
10	4.13	3.88	**4.00 (0.12)**	1.06 (0.03)
15	2.67	4.68	**3.67 (1.00)**	0.98 (0.27)
20	3.66	3.53	**3.59 (0.06)**	0.95 (0.02)
25	3.49	4.13	**3.81 (0.32)**	1.01 (0.08)
30	4.82	2.70	**3.76 (1.06)**	1.00 (0.28)
35	13.26	11.62	**12.44 (0.82)***	3.31 (0.22)
40	17.27	17.30	**17.28 (0.01)***	4.60 (0.00)
45	17.73	17.85	**17.79 (0.06)***	4.73 (0.02)
50	13.88	13.55	**13.71 (0.16)***	3.65 (0.04)
55	10.82	14.49	**12.65 (1.84)***	3.36 (0.49)
60	7.76	11.64	**9.70 (1.94)***	2.58 (0.52)
65	9.46	7.40	**8.43 (1.03)***	2.24 (0.27)
70	6.60	5.95	**6.27 (0.32)**	1.67 (0.09)
75	6.94	5.63	**6.28 (0.65)**	1.67 (0.17)
80	5.24	5.25	**5.24 (0.00)**	1.39 (0.00)

Notes:

* - secretion significantly different from basal (t_{30}), based on ANOVA and Dunnett's test of $\log_{10}$-transformed data ($p<0.05$).

Appendix 6B: Pepsinogen release from individual tissues of, and mean values for, sheep 2 in time course of the cholinergic response experiments.

Time	Tissue A	B	C	D	E	Mean (±S.E.) u/ml	Mean (±S.E.) secn./basal
(min)5	1.64	2.51	2.90	1.21	1.64	**1.98 (0.31)**	1.01 (0.16)
10	2.96	2.35	3.00	1.06	1.48	**2.17 (0.39)**	1.10 (0.20)
15	2.12	1.34	2.05	1.31	1.32	**1.63 (0.19)**	0.83 (0.09)
20	1.55	1.14	1.89	0.24	2.02	**1.37 (0.32)**	0.70 (0.16)
25	2.17	0.91	2.32	0.67	2.75	**1.76 (0.41)**	0.90 (0.21)
30	2.01	0.86	2.66	0.91	3.38	**1.96 (0.49)**	1.00 (0.25)
35	2.22	2.24	3.34	2.11	4.10	**2.80 (0.39)**	1.43 (0.20)
40	2.55	2.48	3.39	2.59	3.12	**2.83 (0.18)**	1.44 (0.09)
45	2.72	2.86	9.49	4.89	4.93	**4.98 (1.22)**	2.54 (0.62)
50	6.99	7.54	13.32	5.42	7.48	**8.15 (1.35)***	4.15 (0.69)
55	9.29	7.25	12.83	4.89	8.83	**8.62 (1.30)***	4.39 (0.66)
60	5.53	5.99	12.05	3.15	6.14	**6.57 (1.47)***	3.34 (0.75)
65	8.19	6.09	15.20	2.42	8.41	**8.06 (2.08)***	4.10 (1.06)
70	7.65	6.79	14.85	1.84	7.19	**7.66 (2.08)***	3.90 (1.06)
75	6.55	5.77	13.40	1.69	4.76	**6.43 (1.93)***	3.27 (0.98)

Notes:

* - secretion significantly different from basal (t_{30}), based on ANOVA and Dunnett's test of $\log_{10}$-transformed data ($p<0.05$).

Appendix 6C: Pepsinogen release from individual tissues of, and mean values for, sheep 3 in time course of the cholinergic response experiments.

Time	Tissue A	B	C	D	E	Mean (±S.E.) u/ml	Mean (±S.E.) secn./basal
(min)**5**	1.44	1.57	1.86	1.60	1.69	**1.63 (0.07)**	1.26 (0.05)
10	1.64	1.07	1.11	1.37	1.15	**1.27 (0.11)**	0.98 (0.08)
15	1.49	1.07	1.50	1.37	0.90	**1.27 (0.12)**	0.98 (0.09)
20	1.05	0.72	1.21	1.15	1.00	**1.03 (0.08)**	0.79 (0.06)
25	1.51	0.67	1.11	1.15	0.95	**1.08 (0.14)**	0.83 (0.11)
30	2.29	0.67	1.21	1.04	1.27	**1.30 (0.27)**	1.00 (0.21)
35	4.71	4.14	7.42	3.34	5.13	**4.95 (0.69)***	3.82 (0.53)
40	7.50	5.40	7.17	3.13	4.97	**5.63 (0.79)***	4.35 (0.61)
45	5.17	4.72	5.56	3.81	4.29	**4.71 (0.31)***	3.63 (0.24)
50	6.27	5.49	6.57	4.96	4.34	**5.53 (0.41)***	4.27 (0.32)
55	5.40	3.90	6.26	5.01	3.60	**4.83 (0.50)***	3.73 (0.38)
60	4.31	4.13	5.54	5.07	4.49	**4.71 (0.26)***	3.63 (0.20)
65	3.13	3.94	5.83	4.44	2.72	**4.01 (0.54)***	3.09 (0.42)
70	3.13	3.26	6.32	4.01	3.17	**3.98 (0.61)***	3.07 (0.47)
75	3.33	2.19	3.97	2.70	2.55	**2.95 (0.31)***	2.27 (0.24)
80	3.74	3.49	4.36	3.41	2.96	**3.59 (0.23)***	3.05 (0.18)

Notes:

* - secretion significantly different from basal (t_{30}), based on ANOVA and Dunnett's test of $\log_{10}$-transformed data ($p<0.05$).

Appendix 7A: Pepsinogen release from individual tissues of, and mean values for, sheep 1 in carbachol dose response studies using intact mucosal sheets.

Time (min) [carb][1]	Tissue A	B	C	D	E	Mean (±S.E.) u/ml	Mean (±S.E.) secn./basal
20	5.19	4.06	3.68	4.20	6.91	**4.81 (0.58)**	1.01 (0.30)
40	8.66	9.87	3.36	5.04	3.21	**6.03 (1.37)**	1.00 (0.00)
60 **(-9)**	8.31	9.18	4.16	6.72	9.64	**7.60 (0.99)**	1.49 (0.38)
80 **(-8)**	8.66	7.79	3.20	5.71	4.66	**6.00 (1.00)**	1.06 (0.11)
100 **(-7)**	8.77	10.73	8.80	8.23	7.71	**8.85 (0.51)**	1.75 (0.33)
120 **(-6)**	30.13	20.95	16.32	10.59	21.85	**19.97 (3.23)***	3.87 (0.89)
140 **(-5)**	27.70	13.51	13.28	10.59	23.94	**17.80 (3.37)***	3.62 (1.06)

Notes:

* - secretion significantly different from basal (t_{40}), based on ANOVA and Dunnett's test of $\log_{10}$-transformed data ($p<0.05$).

[1] - Carbachol concentration, 10^n molar.

Appendix 7B: Pepsinogen release from individual tissues of, and mean values for, sheep 2 in carbachol dose response studies using intact mucosal sheets.

Time (min) [carb][1]	Tissue A	B	C	D	E	Mean (±S.E.) u/ml	Mean (±S.E.) secn./basal
20	5.59	5.20	4.39	3.55	5.80	**4.91 (0.42)**	1.13 (0.07)
40	5.33	5.30	3.95	2.58	5.10	**4.45 (0.53)**	1.00 (0.00)
60 **(-9)**	6.46	4.57	4.66	3.73	6.60	**5.20 (0.57)**	1.20 (0.10)
80 **(-8)**	4.89	4.75	1.61	2.93	3.70	**3.58 (0.61)**	0.82 (0.12)
100 **(-7)**	3.41	4.57	4.48	1.24	3.60	**3.46 (0.60)**	0.76 (0.11)
120 **(-6)**	7.42	12.97	6.46	6.04	9.30	**8.44 (1.26)**	1.93 (0.20)
140 **(-5)**	17.03	21.28	12.11	6.75	12.70	**13.97 (2.45)***	3.08 (0.27)

Notes:

* - secretion significantly different from basal (t_{40}), based on ANOVA and Dunnett's test of $\log_{10}$-transformed data ($p<0.05$).

[1] - Carbachol concentration, 10^n molar.

Appendix 7C: Pepsinogen release from individual tissues of, and mean values for, sheep 3 in carbachol dose response studies using intact mucosal sheets.

Time (min) [carb][1]	Tissue A	B	C	D	E	Mean (±S.E.) u/ml	Mean (±S.E.) secn./basal
20	18.41	17.31	23.10	23.68	25.08	**21.52 (1.54)**	1.14 (0.09)
40	18.41	11.94	25.63	26.24	20.32	**20.51 (2.62)**	1.00 (0.00)
60 **(-9)**	12.70	8.66	3.38	11.20	13.97	**9.98 (1.87)**	0.54 (0.11)
80 **(-8)**	17.46	12.24	9.86	15.04	17.46	**14.41 (1.49)**	0.77 (0.12)
100 **(-7)**	22.86	15.82	4.51	19.84	20.63	**16.73 (3.26)**	0.92 (0.20)
120 **(-6)**	97.78	71.94	43.66	48.64	73.97	**67.20 (9.75)***	3.74 (0.86)
140 **(-5)**	51.43	43.58	30.42	27.20	49.52	**40.43 (4.94)**	2.24 (0.48)

Notes:

* - secretion significantly different from basal (t_{40}), based on ANOVA and Dunnett's test of $\log_{10}$-transformed data ($p<0.05$).

[1] - Carbachol concentration, 10^n molar.

Appendix 7D: Pepsinogen release from individual tissues of, and mean values for, sheep 4 in carbachol dose response studies using intact mucosal sheets.

Time (min) [carb][1]	Tissue A	B	C	D	E	Mean (±S.E.) u/ml	Mean (±S.E.) secn./basal
20	10.53	16.78	21.98	17.06	22.02	**17.67 (2.11)**	1.43 (0.08)
40	6.53	10.34	16.76	12.18	18.16	**12.79 (2.12)**	1.00 (0.00)
60 **(-9)**	1.26	1.17	5.59	5.89	5.87	**3.96 (1.12)**	0.29 (0.06)
80 **(-8)**	12.00	15.02	20.72	21.93	21.10	**18.15 (1.96)**	1.50 (0.14)
100 **(-7)**	16.21	17.76	18.92	24.97	19.63	**19.50 (1.49)**	1.69 (0.27)
120 **(-6)**	59.37	64.39	69.37	61.73	35.60	**58.09 (5.86)***	5.30 (1.18)
140 **(-5)**	45.89	30.83	40.00	25.99	26.42	**33.83 (3.93)***	3.20 (0.10)

Notes:

* - secretion significantly different from basal (t_{40}), based on ANOVA and Dunnett's test of $\log_{10}$-transformed data ($p<0.05$).

[1] - Carbachol concentration, 10^n molar.

Appendix 7E: Pepsinogen release from individual tissues of, and mean values for, sheep 5 in carbachol dose response studies using intact mucosal sheets.

Time (min) [carb][1]	Tissue A	B	C	D	E	Mean (±S.E.) u/ml	Mean (±S.E.) secn./basal
20	8.44	10.09	6.55	4.49	1.47	**6.21 (1.51)**	1.15 (0.22)
40	7.05	6.96	5.54	2.81	4.77	**5.43 (0.78)**	1.00 (0.00)
60 **(-9)**	6.35	5.04	5.93	5.96	1.93	**5.04 (0.81)**	1.04 (0.29)
80 **(-8)**	7.05	5.33	6.10	2.81	2.11	**4.68 (0.95)**	0.86 (0.12)
100 **(-7)**	21.08	9.39	8.36	8.21	5.96	**10.60 (2.68)**	2.00 (0.39)
120 **(-6)**	14.92	18.26	12.20	13.19	6.42	**13.00 (1.94)***	2.59 (0.56)
140 **(-5)**	8.44	7.88	5.08	6.46	2.57	**6.09 (1.06)**	1.22 (0.29)

Notes:

* - secretion significantly different from basal (t_{40}), based on ANOVA and Dunnett's test of $\log_{10}$-transformed data ($p<0.05$).

[1] - Carbachol concentration, 10^n molar.

Appendix 8A: Responses of individual muscle strips and mean values for sheep 1 in carbachol cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue (1.00 = maximum).

[carbachol] moles	Tissue A	B	C	D	Mean (± S.E.)
10^{-9}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-8}	0.00	0.03	0.04	0.03	0.02 (0.01)
10^{-7}	0.12	0.20	0.23	0.38	0.23 (0.05)
10^{-6}	0.50	0.67	0.68	0.88	0.68 (0.08)
10^{-5}	0.87	1.00	1.00	1.00	0.97 (0.03)
10^{-4}	1.00	1.00	0.91	1.00	0.97 (0.03)

Appendix 8B: Responses of individual muscle strips and mean values for sheep 2 in carbachol cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue (1.00 = maximum).

[carbachol] moles	Tissue A	B	C	D	Mean (± S.E.)
10^{-9}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-8}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-7}	0.11	0.08	0.04	0.17	0.10 (0.03)
10^{-6}	0.69	0.62	1.00	0.67	0.74 (0.09)
10^{-5}	1.00	1.00	0.88	1.00	0.97 (0.03)
10^{-4}	0.77	0.50	0.80	0.83	0.72 (0.08)

Appendix 8C: Responses of individual muscle strips and mean values for sheep 3 in carbachol cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue (1.00 = maximum).

[carbachol] moles	Tissue A	B	C	D	Mean (± S.E.)
10^{-9}	0.08	0.00	0.08	*	0.05 (0.03)
10^{-8}	0.19	0.14	0.19		0.17 (0.02)
10^{-7}	0.42	0.54	0.53		0.50 (0.04)
10^{-6}	0.77	0.91	0.83		0.84 (0.04)
10^{-5}	1.00	1.00	1.00		1.00 (0.00)
10^{-4}	1.00	0.82	0.97		0.93 (0.06)

*- Tissue not responsive.

Appendix 8D: Responses of individual muscle strips and mean values for sheep 4 in carbachol cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue (1.00 = maximum).

[carbachol] moles	Tissue A	B	C	D	E	F	Mean (± S.E.)
10^{-9}	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-8}	0.05	0.05	0.02	0.06	0.04	0.00	0.04 (0.01)
10^{-7}	0.34	0.36	0.26	0.41	0.43	0.25	0.34 (0.03)
10^{-6}	0.76	0.81	0.63	0.77	0.83	0.89	0.78 (0.03)
10^{-5}	0.94	1.00	0.95	1.00	1.00	1.00	0.82 (0.16)
10^{-4}	1.00	1.00	1.00	1.00	0.96	0.93	0.98 (0.01)

Appendix 9A: Responses of individual muscle strips and mean values for sheep 1 in histamine cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue to 100 micromolar carbachol (1.00 = maximum).

[histamine] moles	Tissue A	B	Mean (± S.E.)
10^{-9}	0.00	0.00	0.00 (0.00)
10^{-8}	0.00	0.00	0.00 (0.00)
10^{-7}	0.00	0.00	0.00 (0.00)
10^{-6}	0.00	0.00	0.00 (0.00)
10^{-5}	0.00	0.00	0.00 (0.00)
10^{-4}	0.00	0.00	0.00 (0.00)

Appendix 9B: Responses of individual muscle strips and mean values for sheep 2 in histamine cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue to 100 micromolar carbachol (1.00 = maximum).

[histamine] moles	Tissue A	B	C	D	Mean (± S.E.)
10^{-9}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-8}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-7}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-6}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-5}	0.02	0.02	0.01	0.00	0.01 (0.00)
10^{-4}	0.07	0.09	0.07	0.25	0.12 (0.04)

Appendix 9C: Responses of individual muscle strips and mean values for sheep 3 in histamine cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue to 100 micromolar carbachol (1.00 = maximum).

[histamine] moles	Tissue A	B	C	D	Mean (± S.E.)
10^{-9}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-8}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-7}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-6}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-5}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-4}	0.00	0.00	0.00	0.00	0.00 (0.00)

Appendix 9D: Responses of individual muscle strips and mean values for sheep 2 in serotonin cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue to 100 micromolar carbachol (1.00 = maximum).

[serotonin] moles	Tissue A	B	C	D	Mean (± S.E.)
10^{-9}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-8}	0.02	0.05	0.02	0.00	0.02 (0.01)
10^{-7}	0.12	0.18	0.09	0.08	0.12 (0.02)
10^{-6}	0.18	0.32	0.11	0.19	0.20 (0.04)
10^{-5}	0.18	0.32	0.12	0.21	0.21 (0.04)

Appendix 9E: Responses of individual muscle strips and mean values for sheep 3 in serotonin cumulative dose response experiments. Data as a proportion of the maximum response obtained in each tissue to 100 micromolar carbachol (1.00 = maximum).

[serotonin] moles	Tissue A	B	C	D	Mean (± S.E.)
10^{-9}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-8}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-7}	0.00	0.00	0.00	0.00	0.00 (0.00)
10^{-6}	0.05	0.10	0.04	0.12	0.08 (0.02)
10^{-5}	0.07	0.13	0.04	0.18	0.10 (0.03)

Appendix 10A: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	3.15	3.03	2.65	3.55	4.12	3.30 (0.25)
40 (Blank)	3.95	2.69	3.12	1.79	2.25	2.76 (0.37)
60 (ES)	3.91	2.99	4.33	4.59	2.88	3.74 (0.35)
80 (carb)	5.95	3.63	5.58	6.45	6.45	5.61* (0.52)

Notes;
Blank - 100 µl RPMI 1640 blank medium - mean response to blank is 0.84 times secretion in preceding twenty minutes (t_{20}).
ES - 100 µl Batch 27/10/93 (1st culture) - mean response to ES is 1.35 times response to Blank medium.
carb - 1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$).

Appendix 10B: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20			1.41	1.00	0.74	1.05 (0.97)
40 (Blank)			1.00	1.70	0.21	0.97 (0.43)
60 (ES)			1.21	0.53	1.70	1.15 (0.34)
80 (carb)			7.44	5.32	5.21	5.99* (0.73)

Notes;
Blank - 100 µl RPMI 1640 blank medium - mean response to blank is 0.92 times secretion in preceding twenty minutes (t_{20}).
ES - 100 µl Batch 03/11/93 (1st culture) - mean response to ES is 1.18 times response to Blank medium.
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$).

Appendix 10C: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20		0.00	0.00	0.36	0.00	0.10 (0.10)
40		0.32	0.00	0.00	0.00	0.10 (0.10)
60 (Blank)		0.16	0.07	0.00	0.00	0.10 (0.04)
80 (ES)		1.85	0.00	0.00	0.00	0.46 (0.46)
100 (carb)		2.58	6.14	5.27	6.35	5.08* (0.87)

Notes;
Blank - 100 µl RPMI 1640 blank medium - mean response to blank is 1.00 times secretion in preceding twenty minutes (t_{40}).
ES - 100 µl Batch 03/11/93 (1st culture) - mean response to ES is 4.60 times response to Blank medium.
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}(x+1)$ transformation.

Appendix 10D: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	6.45	7.61	13.75	9.69	9.89	9.50 (1.25)
40	9.93	7.87	16.87	11.09	9.18	11.00 (1.56)
60 (Blank)	9.81	6.97	11.41	10.61	8.89	9.50 (0.80)
80 (ES)	23.23	20.64	33.59	33.98	17.92	25.9* (3.30)
100 (carb)	26.71	35.94	38.75	37.42	34.69	34.7* (2.10)

Notes;
Blank - 100 µl RPMI 1640 blank medium - mean response to blank is 0.87 times secretion in preceding twenty minutes (t_{40}).
ES - 100 µl Batch 27/10/93 (2nd culture) - mean response to ES is 2.70 times response to Blank medium.
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t40) by ANOVA and Dunnett's test ($p<0.05$).

Appendix 10E: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	0.51	1.50	2.17	1.55		1.40 (0.34)
40	1.48	1.66	2.04	1.48		1.66 (0.13)
60 (ES)	2.32	2.24	2.58	2.24		2.35 (0.08)
80 (carb)	3.47	3.32	4.09	3.42		3.58* (0.17)

Notes;
ES - 100 µl Batch 27/10/93 (1st culture) - mean response to ES is 1.42 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$).

Appendix 10F: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	0.86	0.67	1.02	0.66		0.80 (0.10)
40	1.00	0.65	0.68	0.77		0.80 (0.10)
60 (ES)	1.17	2.12	1.41	1.10		1.44* (0.24)
80 (carb)	2.02	3.26	1.64	2.13		2.26* (0.35)

Notes;
ES - 100 µl Batch 27/10/93 (1st culture) - mean response to ES is 1.80 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}(x+1)$ transformation.

Appendix 10G: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	0.83	0.34	0.21	0.03	0.28	0.34 (0.13)
40	0.42	0.34	0.60	0.20	0.60	0.43 (0.10)
60 (ES)	1.10	1.82	2.44	1.44	3.08	1.98* (0.35)
80 (carb)	1.02	0.99	0.23	0.76	2.36	1.10 (0.35)

Notes;
ES - 100 µl Batch 27/10/93 (2nd culture) - mean response to ES is 4.60 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}(x+1)$ transformation.

Appendix 10H: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	15.55	8.20	11.82	17.88	11.34	13.0 (1.70)
40	16.68	5.65	12.42	11.21	8.95	11.0 (1.80)
60 (ES)	19.79	10.74	26.67	22.69	24.48	20.9* (2.80)
80 (carb)	61.62	36.36	47.58	51.04	47.46	48.8* (4.05)

Notes;
ES - 100 µl Batch 27/10/93 (2nd culture) - mean response to ES is 1.90 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10I: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	10.81	10.16	15.24	10.52	19.51	13.25 (1.82)
40	9.52	8.06	11.43	11.07	13.98	10.81 (0.99)
60 (ES)	11.45	13.52	19.81	11.76	15.00	14.31 (1.52)
80 (carb)	31.77	63.43	40.19	20.07	35.00	38.1* (7.14)

Notes;
ES - 100 µl Batch 27/10/93 (2nd culture) - mean response to ES is 1.32 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10J: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	4.96	5.64	5.99	5.00	7.30	5.80 (0.43)
40	5.49	6.85	6.89	5.08	11.43	7.15 (1.13)
60 (ES)	11.24	12.42	12.96	9.67	23.17	13.9* (3.39)
80 (carb)	20.88	24.11	25.09	18.61	37.46	25.2* (3.27)

Notes;
ES - 100 µl Batch 27/10/93 (2nd culture) - mean response to ES is 1.94 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10K: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	8.83	6.00	7.89	9.47	12.62	9.00 (1.09)
40	6.00	3.83	5.79	7.37	10.05	6.60 (1.03)
60 (ES)	22.17	8.50	15.44	13.16	35.93	19.0* (4.80)
80 (carb)	25.50	15.00	15.96	9.47	27.38	18.7* (3.40)

Notes;
ES - 100 µl Batch 28/10/93 (1st culture) - mean response to ES is 2.88 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10L: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	8.77	10.04	5.65	5.13	12.40	8.40 (3.04)
40	8.70	9.66	6.99	3.69	8.68	7.54 (2.36)
60 (ES)	8.33	9.37	7.81	6.47	4.79	7.35 (1.77)
80 (carb)	27.73	23.34	16.58	11.24	19.75	19.7* (6.30)

Notes;
ES - 100 µl Batch 03/11/93 (1st culture) - mean response to ES is 0.97 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10M: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	3.32	4.75	1.11	1.03	4.70	2.98 (0.82)
40	2.26	3.17	0.95	1.66	2.74	2.16 (0.39)
60 (ES)	6.79	8.38	2.37	5.77	4.70	5.60 (1.01)
80 (carb)	21.06	21.73	6.56	7.25	11.47	13.6* (3.29)

Notes;
ES - 100 µl Batch 27/10/93 (1st culture) - mean response to ES is 2.60 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10N: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	7.70	4.23	5.70	6.64	7.07	6.27 (0.60)
40	4.85	3.96	5.70	4.34	5.98	5.00 (0.39)
60 (ES)	5.52	5.29	6.32	5.84	6.47	5.89 (0.23)
80 (carb)	20.92	19.82	19.04	24.60	20.00	20.9* (1.00)

Notes;
ES - 100 µl Batch 06/10/94 (1st culture) - mean response to ES is 1.18 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10O: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	3.18	2.12	3.27	3.61	3.17	3.07 (0.25)
40	3.40	2.28	3.04	3.50	2.59	2.96 (0.23)
60 (ES)	3.96	3.23	4.91	7.28	3.04	4.84* (0.77)
80 (carb)	2.34	2.23	2.54	4.59	2.07	2.75 (0.46)

Notes;
ES - 100 µl Batch 06/10/94 (1st culture) - mean response to ES is 1.63 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10P: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	3.09	3.91	4.84	3.85	3.57	3.85 (0.29)
40	3.39	2.42	4.04	4.09	3.23	3.43 (0.31)
60 (ES)	5.24	4.75	7.80	8.45	6.70	6.59* (0.71)
80 (carb)	9.44	10.78	13.56	12.33	14.23	12.1* (0.88)

Notes;
ES - 100 µl Batch 06/10/94 (1st culture) - mean response to ES is 1.92 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10Q: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	4.28	3.88	3.72			3.96 (0.17)
40	2.24	2.74	5.88			3.62 (1.14)
60 (ES)	4.08	3.92	8.24			5.41 (1.31)
80 (carb)	37.96	34.12	33.72			27.1* (1.46)

Notes;
ES - 100 µl Batch 05/10/94 (1st culture) - mean response to ES is 1.49 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 10R: Individual and mean values of pepsinogen release (total units) for ES studies using intact mucosal preparations from abattoir-derived sheep.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	2.57	0.61	0.92			1.37 (0.13)
40	2.84	1.97	0.15			1.65 (0.79)
60 (ES)	18.64	9.85	17.86			15.4* (2.81)
80 (carb)	12.58	15.30	17.56			15.1* (1.44)

Notes;
ES - 100 µl Batch 05/10/94 (1st culture) - mean response to ES is 9.30 times basal secretion (t_{40})
carb - 0.1 mM carbachol
* significantly different from secretion in second period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$ transformation.

Appendix 11A: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES/10	0.32	0.32	0.30	0.34	0.32 (0.02)
100 µl ES raw	0.37	0.53	0.37	0.35	0.41 (0.08)

Notes;
ES - Batch 27/10/93 (1st culture).
ES/10 - 1 in 10 dilution of raw ES.

Appendix 11B: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES/100	0	0	0	0	0.00 (0.00)
100 µl ES/10	0.06	0	0	0	0.01 (0.03)
100 µl ES raw	0.20	0	0.02	0.09	0.08 (0.08)

Notes;
ES - Batch 28/10/93 (1st culture).
ES/100 - 1 in 100 dilution of raw ES.
ES/10 - 1 in 10 dilution of raw ES.

Appendix 11C: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES	*	0.19	0.09	0.00	0.09 (0.05)

Notes;
ES - Batch 28/10/93 (1st culture).
* - Tissue unresponsive to carbachol.

Appendix 11D: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES	0.04	0.29	0.09	*	0.14 (0.13)

Notes;
ES - Batch 28/10/93 (1st culture).
* - Tissue unresponsive to carbachol.

Appendix 11E: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES	0.00	0.00	0.00	0.00	0.00 (0.00)

Notes;
ES - Batch 03/11//93 (1st culture).

Appendix 11F: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	E	F	G	Mean (± S.E.)
100 µl ES/10	0.00	0.00	0.10	0.06	0.00	0.08	0.21	0.06 (0.08)
100 µl ES raw	0.07	0.00	0.20	0.25	0.06	0.10	0.25	0.13 (0.10)

Notes;
ES - Batch 27/10/93 (1st culture).
ES/10 - 1 in 10 dilution of raw ES.

Appendix 11G: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES	0.69	0.74	0.90		0.78 (0.11)

Notes;
ES - Batch 27/10/93 (1st culture).

Appendix 11H: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES + INDO*	0.73	0.60	1.23	0.86	0.85 (0.27)
100 µl ES - INDO	0.28	0.08	0.29	0.43	0.27 (0.14)

Notes;
ES - Batch 27/10/93 (1st culture).
* - presence or absence of 3 micromolar Indomethacin.

Appendix 11I: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES	0.52	0.63	0.83	1.07	0.76 (0.12)

Notes;
ES - Batch 06/10/94 (1st culture).
All tissues given 3 micromolar Indomethacin.

Appendix 11J: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES/100	0.00	0.00	0.11	0.14	0.06 (0.04)
100 µl ES/10	0.22	0.21	0.28	0.43	0.28 (0.05)
100 µl ES raw	0.22	0.21	0.28	0.49	0.30 (0.06)

Notes;
ES - Batch 27/10/93 (2nd culture).
ES/100 - 1 in 100 dilution of raw ES.
ES/10 - 1 in 10 dilution of raw ES.
All tissues given 3 micromolar Indomethacin.

Appendix 11K: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	Mean (± S.E.)
100 µl ES	0.66	0.70	0.68 (0.02)

Notes;
ES - Batch 06/10/94 (1st culture).
All tissues given 3 micromolar Indomethacin.

Appendix 11L: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	Mean (± S.E.)
100 µl ES	2.14	*	2.14 (0.00)

Notes;
ES - Batch 06/10/94 (1st culture).
All tissues given 3 micromolar Indomethacin.

* - Tissue not responsive to carbachol.

Appendix 11M: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES	0.96	1.00	0.92	1.13	1.00 (0.09)

Notes;
ES - Batch 06/10/94 (1st culture).
All tissues given 3 micromolar Indomethacin.

Appendix 11N: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES/ 10^{-4}	0.00	0.00	0.00	0.00	0.00 (0.00)
100 µl ES/ 10^{-3}	0.00	0.00	0.00	0.00	0.00 (0.00)
100 µl ES/ 10^{-2}	0.00	0.00	0.00	0.00	0.00 (0.00)
100 µl ES/ 10	0.31	0.17	0.15	0.29	0.23 (0.08)
100 µl ES	0.19	0.07	0.15	0.21	0.15 (0.06)

Notes;
ES - Batch 06/10/94 (1st culture).
All tissues given 3 micromolar Indomethacin.

Appendix 11O: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES	0.56	0.54	0.54	0.60	0.56 (0.03)

Notes;
ES - Batch 06/10/94 (1st culture).
All tissues given 3 micromolar Indomethacin.

Appendix 11P: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES/ 10^{3}	0.00	0.01	0.00	0.00	0.00 (0.00)
100 µl ES/ 10^{2}	0.01	0.04	0.02	0.06	0.03 (0.01)
100 µl ES/ 10	0.03	0.09	0.03	0.09	0.06 (0.02)
100 µl ES	0.04	0.11	0.06	0.12	0.08 (0.02)

Notes;
ES - Batch 05/10/94 (1st culture).
All tissues given 3 micromolar Indomethacin.

Appendix 11Q: Responses of smooth muscle strips, obtained from abattoir-derived sheep, to ES doses, data expressed as a proportion of the maximal response obtained to 0.1 mM carbachol.

ES dose	Tissue A	B	C	D	Mean (± S.E.)
100 µl ES	0.21	0.75	0.07	0.00	0.26 (0.17)

Notes;
ES - Batch 05/10/94 (1st culture).
All tissues given 3 micromolar Indomethacin.

Appendix 12: Pepsinogen and LDH release from intact mucosal preparations from one animal, pepsinogen release is expressed as the total units (u) released per 20 minutes and as pepsinogen release over basal (40 min)(figures in parentheses).

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
Peps						
20	1.59 (0.95)	3.77 (1.17)	2.36 (0.80)	3.45 (1.36)	1.51 (0.72)	(1.00 (0.12))
40	1.67 (1.00)	3.23 (1.00)	2.94 (1.00)	2.54 (1.00)	2.10 (1.00)	(1.00 (0.00))
60 (ES)	2.80 (1.68)	4.84 (1.50)	4.15 (1.41)	4.84 (1.90)	2.99 (1.42)	(1.58 (0.09))
80 (carb)	2.23 (1.33)	4.14 (1.28)	3.07 (1.04)	4.49 (1.77)	3.04 (1.45)	(1.37 (0.12))
LDH (U)						
20	0.000	0.010	0.014	0.017	0.010	0.010 (0.003)
40	0.007	0.014	0.014	0.007	0.014	0.011 (0.002)
60 (ES)	0.007	0.010	0.010	0.010	0.010	0.009 (0.001)
80 (carb)	0.014	0.007	0.010	0.010	0.014	0.011 (0.001)

ES - 100 µl Batch 27/10/93 (1st culture).

carb - 1 mM carbachol.

Appendix 13: Pepsinogen and LDH release from individual tissues in response to the addition of ES and carbachol. All data are expressed as the total units released per twenty minute period. Included are the total tissue content of pepsinogen and LDH at the end of the experiment.

	Group 1			Group 2		
Time	**Tissue**					
(min)	**A**	**B**	**C**	**D**	**E**	**F**
Peps (u)						
20	3.33	2.22	3.52	2.52	1.96	3.43
40	1.97	2.35	3.77	2.52	3.36	3.00
60 (ES)	3.83	3.46	3.40	4.65	2.52	2.86
80 (carb)	22.10	27.41	27.67	23.50	17.06	25.00
Tissue	**192.59**	**208.80**	**249.06**	**162.24**	**195.80**	**200.00**
LDH (U)						
20	0.007	0.000	0.007	0.000	0.007	0.007
40	0.000	0.007	0.014	0.007	0.007	0.014
60 (ES)	0.000	0.000	0.007	0.014	0.000	0.000
80 (carb)	0.000	0.007	0.000	0.007	0.000	0.007
Tissue	**1.44**	**1.58**	**1.51**	**1.52**	**1.58**	**1.70**

Group 1 tissues (tissues A to C), Group 2 tissues (tissues D to F).

ES - 100 µl Batch 27/10/93 (2nd culture) - given to group 1 tissues only.

carb - 0.1 mM carbachol, given to all six tissues.

Appendix 14: Pepsinogen and LDH release from individual tissues in response to the addition of ES and carbachol to tissues in the presence and absence of Indomethacin*. All data are expressed as the total units released per twenty minute period. Included are the total tissue content of pepsinogen and LDH at the end of the experiment. The mean (± S.E.) results for pepsinogen for groups 1 and 2 are given in parentheses.

	Group 1			Group 2		
Time	**Tissue**					
(min)	**A**	**B**	**C**	**D**	**E**	**F**
Peps (u)						
20	7.72	7.41	8.89 (8.0 (0.45))	4.32	3.13	3.17 (3.5 (0.40))
40	9.20	7.90	8.11 (8.4 (0.40))	4.00	3.35	4.40 (3.9 (0.31))
60 (ES)	9.96	11.20	9.30 (10.1 (0.60))	6.11	5.30	5.51 (5.6 (0.24))
80	9.29	9.66	7.70 (8.9 (0.60))	5.30	4.46	4.51 (4.8 (0.27))
100 (carb)	12.23	11.83	15.30 (13.1 (1.10))	13.03	11.09	8.63 (10.9 (1.27))
Tissue	**291.07**	**271.83**	**266.67**	**300.54**	**257.38**	**306.41**
LDH (U)						
20	0.007	0.007	0.000	0.007	0.000	0.007
40	0.000	0.000	0.000	0.000	0.000	0.007
60 (ES)	0.007	0.014	0.000	0.007	0.000	0.000
80	0.000	0.000	0.014	0.000	0.000	0.007
100 (carb)	0.000	0.000	0.007	0.000	0.000	0.007
Tissue	**2.296**	**1.836**	**1.553**	**1.688**	**1.728**	**1.458**

* - Indomethacin (3 micromolar) given to group 2 tissues only.

Group 1 tissues (tissues A to C), Group 2 tissues (tissues D to F).

ES - 100 µl Batch 27/10/93 (2nd culture), given to all six tissues.

carb - 0.1 mM carbachol, given to all six tissues.

Appendix 15A: Pepsinogen release from individual tissues from sheep 1 in time course of the response to ES experiment. Pepsinogen release expressed as the total units released per 10 minute period, and the mean values are also given as secretion over basal (figures in parentheses and bold script).

Time (min)	Tissue A	B	C	Mean (± S.E.)
10	4.32	4.44	1.76	3.51 (0.87) **(0.87 (0.22))**
20	4.64	4.60	3.23	4.16 (0.46) **(1.03 (0.11))**
30	2.88	4.60	4.63	4.04 (0.58) **(1.00 (0.14))**
40 (ES)	25.28	27.94	25.74	26.32 (0.82) **(6.52 (0.20))**
50	4.60	10.15	9.44	8.06 (1.74) **(2.00 (0.43))**
60 (ES)	3.49	2.35	4.07	3.30 (0.50) **(0.82 (0.12))**
70	2.06	3.97	0.37	2.13 (1.04) **(0.53 (0.26))**
80	1.90	3.82	2.41	2.71 (0.57) **(0.67 (0.14))**
90 (carb)	21.90	12.35	12.96	15.74 (3.09) **(3.90 (0.76))**
100	6.35	4.70	3.15	4.73 (0.92) **(1.17 (0.23))**

ES - 100µl Batch 27/10/93 (2nd culture), added to submucosal solutions in the 31st and 51st minutes. Tissues exposed to ES for forty minutes.

carb - 0.1 mM carbachol, added in 81st minute, tissues exposed to carbachol for twenty minutes.

Appendix 15B: Pepsinogen release from individual tissues from sheep 2 in time course of the response to ES experiment. Pepsinogen release expressed as the total units released per 10 minute period, and the mean values are also given as secretion over basal (figures in parentheses and bold script).

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
10	2.69	1.30	1.84	3.02	2.54	2.28 (0.31) **(0.86 (0.12))**
20	2.34	1.66	2.05	2.21	2.98	2.25 (0.22) **(0.85 (0.08))**
30	2.98	1.80	2.05	2.73	3.66	2.64 (0.33) **(1.00 (0.13))**
40 (ES)	3.90	2.74	1.34	2.66	3.43	2.81 (0.43) **(1.06 (0.16))**
50	3.90	2.67	6.01	4.65	4.78	4.40 (0.55) **(1.66 (0.21))**
60 (ES)	1.77	1.15	1.84	0.37	3.28	1.68 (0.48) **(0.64 (0.18))**
70	1.70	1.59	1.98	1.84	3.73	2.17 (0.40) **(0.82 (0.15))**
80	0.92	1.08	1.77	1.33	2.69	1.56 (0.32) **(0.59 (0.12))**
90 (carb)	8.58	9.67	12.93	9.89	13.06	10.83 (0.91) **(4.09 (0.34))**

ES - 100µl Batch 05/10/94 (1st culture), added to submucosal solutions in the 31st and 51st minutes. Tissues exposed to ES for forty minutes.

carb - 0.1 mM carbachol, added in 81st minute, tissues exposed to carbachol for ten minutes.

Appendix 16A: Pepsinogen release from individual tissues of parasite-naive animals in response to ES: animal 1. Pepsinogen release expressed as the total in each twenty minute period.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	0.82	0.58	1.32	1.06	1.31	1.02 (0.14)
40	1.18	0.79	0.99	0.64	1.31	0.98 (0.12)
60	1.47	0.99	0.51	0.54	0.70	0.84 (0.18)
80 (ES-1)	0.82	0.65	0.81	0.96	1.08	0.86 (0.07)
100 (ES-2)	0.55	0.36	0.79	0.22	1.18	0.62 (0.17)
120 (carb)	3.25	3.37	7.12	5.76	4.02	4.70*(0.75)

ES-1 - 100 µl Batch 06/10/94.

ES-2 - 100 µl Batch 27/10/93 (1st culture).

carb - 0.1 mM carbachol.

* significantly different from secretion in last untreated time period (t_{60}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}(x+1)$ transformation.

Appendix 16B: Pepsinogen release from individual tissues of parasite-naive animals in response to ES: animal 2. Pepsinogen release expressed as the total in each twenty minute period.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	4.84	5.05	15.16	10.54	9.89	9.10 (1.92)
40	5.68	5.89	12.69	15.27	7.10	9.33 (1.96)
60 (ES)	5.89	5.26	11.61	9.89	9.98	8.53 (1.25)
80 (carb)	31.58	20.84	46.88	50.54	30.66	36.1*(5.51)

ES - 100 µl Batch 06/10/94.

carb - 0.1 mM carbachol.

* significantly different from secretion in last untreated time period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$-transformation.

Appendix 16C: Pepsinogen release from individual tissues of parasite-naive animals in response to ES: animal 3. Pepsinogen release expressed as the total in each twenty minute period.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	7.67	7.10	8.70	9.60	8.18	8.25*(0.43)
40	6.60	6.01	5.87	4.64	4.24	5.47 (0.44)
60 (ES)	5.82	4.03	3.72	3.49	2.42	3.90 (0.55)
80 (carb)	3.59	3.29	2.59	4.17	5.17	3.76*(0.43)

ES - 100 µl Batch 06/10/94.

carb - 0.1 mM carbachol.

* significantly different from secretion in last untreated time period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$-transformation.

Appendix 16D: Pepsinogen release from individual tissues of parasite-naive animals in response to ES: animal 4. Pepsinogen release expressed as the total in each twenty minute period.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	4.12	5.30	4.12	2.66	4.72	4.18 (0.44)
40	3.37	3.91	2.82	1.75	4.23	3.22 (0.44)
60 (ES)	3.30	4.73	2.03	0.58	3.62	2.85 (0.71)
80 (carb)	2.12	3.42	2.89	2.29	2.72	2.69 (0.23)

ES - 100 µl Batch 06/10/94.

carb - 0.1 mM carbachol.

* significantly different from secretion in last untreated time period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$-transformation.

Appendix 16E: Pepsinogen release from individual tissues of parasite-naive animals in response to ES: animal 5. Pepsinogen release expressed as the total in each twenty minute period.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	1.55	1.23	5.09	6.04	5.43	3.87 (1.02)
40	1.37	1.23	5.46	8.99	2.82	3.97 (1.47)
60 (ES)	2.19	1.23	3.73	4.03	3.49	2.93 (0.53)
80 (carb)	13.97	9.77	34.41	39.87	17.05	23.0*(5.94)

ES - 100 µl Batch 06/10/94.

carb - 0.1 mM carbachol.

* significantly different from secretion in last untreated time period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$-transformation.

Appendix 16F: Pepsinogen release from individual tissues of parasite-naive animals in response to ES: animal 6. Pepsinogen release expressed as the total in each twenty minute period.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	0.57	0.57	0.00	1.65	1.69	0.90 (0.33)
40	1.00	0.28	0.28	3.03	0.56	1.03 (0.52)
60 (ES)	0.00	0.69	0.96	1.97	2.53	1.23 (0.45)
80 (carb)	32.03	12.69	25.79	27.89	36.48	27.0*(4.01)

ES - 100 µl Batch 06/10/94.

carb - 0.1 mM carbachol.

* significantly different from secretion in last untreated time period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}(x+1)$-transformation.

Appendix 16G: Pepsinogen release from individual tissues of parasite-naive animals in response to ES: animal 7. Pepsinogen release expressed as the total in each twenty minute period.

Time (min)	Tissue A	B	C	D	E	Mean (± S.E.)
20	2.49	1.48	0.77			1.58 (0.50)
40	2.15	0.84	0.97			1.32 (0.42)
60 (ES)	2.72	1.34	1.04			1.70 (0.52)
80 (carb)	35.21	24.61	41.37			38.8*(0.79)

ES - 100 µl Batch 06/10/94.

carb - 0.1 mM carbachol.

* significantly different from secretion in last untreated time period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$-transformation.

Appendix 16H: Pepsinogen release from individual tissues of parasite-naive animals in response to ES: animal 8. Pepsinogen release expressed as the total in each twenty minute period.

Time (min)	Tissue A	B	C	D	E	F	Mean (± S.E.)
20	13.85	13.33	11.03	5.03	3.71	8.77	9.20 (1.73)
40	11.41	5.53	6.22	5.69	5.83	9.85	7.42 (1.04)
60 (ES)	9.36	5.26	4.24	5.43	6.77	7.23	6.38 (0.74)
80 (carb)	40.39	40.64	38.94	36.56	40.15	36.31	38.8*(0.79)

ES - 100 µl Batch 05/10/94 (1st culture).

carb - 0.1 mM carbachol.

* significantly different from secretion in last untreated time period (t_{40}) by ANOVA and Dunnett's test ($p<0.05$) following $\log_{10}$-transformation.

Appendix 17A: Responses of individual muscle strips of parasite-naive animals to carbachol and ES: animal 1. Responses are expressed as grams of tension per gram of wet weight of tissue.

Treatment	Tissue A	B	C	D	Mean (± S.E.)
long. mm. carbachol	127.7	173.9	208.3	500.0	252.5 (84.1)
ES-1	0.00	0.00	0.00	0.00	0.00 (0.00)
ES-2	0.00	0.00	0.00	0.00	0.00 (0.00)
ES-4	0.00	0.00	0.00	0.00	0.00 (0.00)
circ. mm. carbachol	265.6	371.0	100.0	71.4	202.0 (70.7)
ES-1	0.00	0.00	0.00	0.00	0.00 (0.00)
ES-4	0.00	0.00	0.00	0.00	0.00 (0.00)
ES-1*	0.00	0.00	0.00	0.00	0.00 (0.00)

carbachol - 0.1 mM.

ES-1 - 100 µl Batch 06/10/94.

ES-2 - 27/10/93 (1st culture).

ES-3 - 05/10/94 (2nd culture).

ES-4 - 05/10/94 (1st culture).

* - tissues preincubated with 3 micromolar Indomethacin before addition of ES.

Appendix 17B: Responses of individual muscle strips of parasite-naive animals to carbachol and ES: animal 2. Responses are expressed as grams of tension per gram of wet weight of tissue.

Treatment	Tissue A	B	C	D	E	F	Mean (± S.E.)
carb	69.5	172.4	27.4	40.9	41.7	60.8	68.8 (21.6)
ES	0.00	0.00	0.00	0.00	0.00	0.00	0.00 (0.00)
ES*	0.00	0.00	0.00	0.00			0.00 (0.00)

carbachol - 0.1 mM.

ES - 100 µl Batch 06/10/94.

* - tissues preincubated with 3 micromolar Indomethacin before addition of ES.

Appendix 17C: Responses of individual muscle strips of parasite-naive animals to carbachol and ES: animal 3. Responses are expressed as grams of tension per gram of wet weight of tissue.

Treatment	Tissue A	B	C	D	Mean (± S.E.)
carbachol	181.0	224.4	188.3	246.8	210.1 (15.5)
ES	0.00	0.00	0.00	0.00	0.00 (0.00)

carbachol - 0.1 mM.

ES - 100 µl Batch 06/10/94.

Appendix 17D: Responses of individual muscle strips of parasite-naive animals to carbachol and ES: animal 4. Responses are expressed as grams of tension per gram of wet weight of tissue.

Treatment	Tissue A	B	C	D	Mean (± S.E.)
carbachol	50.33	155.56	186.27	155.34	136.9 (29.7)
ES	0.00	0.00	0.00	0.00	0.00 (0.00)

carbachol - 0.1 mM.

ES - 100 µl Batch 06/10/94.

Appendix 17E: Responses of individual muscle strips of parasite-naive animals to carbachol and ES: animal 5. Responses are expressed as grams of tension above basal.

Treatment	Tissue A	B	C	D	Mean (± S.E.)
carbachol	4.00	4.00	2.10	2.80	3.22 (0.47)
ES	0.00	0.00	0.00	0.00	0.00 (0.00)

carbachol - 0.1 mM.

ES - 100 µl Batch 06/10/94.

Appendix 17F: Responses of individual muscle strips of parasite-naive animals to carbachol and ES: animal 6. Responses are expressed as grams of tension above basal.

Treatment	Tissue A	B	C	D	Mean (± S.E.)
carbachol	1.00	1.20	1.25	0.60	1.01 (0.15)
ES	0.00	0.00	0.00	0.00	0.00 (0.00)

carbachol - 0.1 mM.

ES - 100 µl Batch 06/10/94.

Appendix 17G: Responses of individual muscle strips of parasite-naive animals to carbachol and ES: animal 7. Responses are expressed as grams of tension above basal.

Treatment	Tissue A	B	C	D	Mean (± S.E.)
carbachol	2.80	3.00	1.70	1.65	2.29 (0.36)
ES	0.00	0.00	0.00	0.00	0.00 (0.00)

carbachol - 0.1 mM.

ES - 100 µl Batch 05/10/94 (1st culture).

Appendix 17H: Responses of individual muscle strips of parasite-naive animals to carbachol and ES: animal 8. Responses are expressed as grams of tension above basal.

Treatment	Tissue A	B	C	D	Mean (± S.E.)
carbachol	1.85	1.55	0.40	0.74	1.13 (0.34)
ES	0.00	0.00	0.00	0.00	0.00 (0.00)

carbachol - 0.1 mM.

ES - 100 µl Batch 05/10/94 (1st culture).

Appendix 18A: Total tissue pepsinogen (iU/ standard area) of fundic mucosal pieces of cattle and sheep.

Site	Calf 1	2	3	4	5	Sheep 1	2	3
1	4319	8082	2974	2724	2965	753	2877	3245
2	7447	8336	4392	2771	2307	1228	2877	2673
3	6764	7130	3706	2511	2557	1060	1996	3588
4	7606	6685	3901	3243	1890	894	2553	3145
5	6876	6876	3169	2613	2557	976	2083	3673
6	4891	4633	4439	3141	4244	726	2436	3659
7	4478	7304	3299	3299	1946	502	2923	2709
8	6034	4764	4392	3456	2984	753	2826	2330
9	5208	6907	2483	3632	3660	670	2631	2830
10	5129	7146	4346	4096	3456	880	2143	4224

Appendix 18B: Tissue wet weights (g) of fundic mucosal pieces of cattle and sheep.

Site	Calf 1	2	3	4	5	Sheep 1	2	3
1	0.1144	0.1111	0.0802	0.0901	0.1130	0.0872	0.1090	0.0585
2	0.1099	0.1477	0.1304	0.1006	0.1022	0.0866	0.1013	0.0684
3	0.1344	0.1189	0.0905	0.1047	0.0852	0.0937	0.1154	0.0675
4	0.1222	0.1000	0.0981	0.1069	0.0992	0.0768	0.0893	0.0717
5	0.1195	0.1041	0.0908	0.0757	0.0798	0.0999	0.0948	0.0697
6	0.1350	0.0834	0.1011	0.0913	0.1136	0.0899	0.0842	0.0667
7	0.1230	0.1372	0.1008	0.1029	0.0916	0.0733	0.0780	0.0687
8	0.1077	0.1032	0.0926	0.0950	0.1020	0.0728	0.0885	0.0680
9	0.1368	0.1256	0.0600	0.1047	0.0949	0.0682	0.0880	0.0544
10	0.1283	0.0882	0.0886	0.0834	0.1070	0.0702	0.0810	0.0775

Appendix 19A: Tissue wet weights (g) of mucosal pieces from ovine fundus comparing nodular and non-nodular mucosa.

Sheep	Site 1	2	3	4
Y27 nodular	0.1019	0.1195	0.1235	0.1116
nodular	0.1201	0.1043	0.1131	0.1153
02 nodular	0.0982	0.1341	0.1023	0.1069
non-nodular	0.0859	0.0800	0.0981	0.0835
Y43 nodular	0.1095	0.1315	0.1249	0.1322
non-nodular	0.1227	0.0886	0.0939	0.0919
Y51 nodular	0.0860	0.0897	0.0809	0.0891
non-nodular	0.0755	0.0807	0.0685	0.0819
B60 nodular	0.1033	0.1025	0.0850	0.0916
non-nodular	0.0853	0.0933	0.0962	0.0869

Appendix 19B: Tissue pepsinogen (iU/ area) of mucosal pieces from ovine fundus comparing nodular and non-nodular mucosa.

Sheep	Site 1	2	3	4
Y27 nodular	2984	3505	3993	4301
nodular	2761	1731	3324	4163
02 nodular	3048	7615	7285	3940
non-nodular	4014	4768	3961	3770
Y43 nodular	4025	3568	2793	2538
non-nodular	1858	1572	1476	1625
Y51 nodular	2825	2283	4057	3781
non-nodular	2517	2634	2145	2145
B60 nodular	2814	1912	2007	1338
non-nodular	2475	945	1561	1338

Appendix 19C: Tissue pepsinogen (iU/g) of mucosal pieces from ovine fundus comparing nodular and non-nodular mucosa.

Sheep	Site 1	2	3	4
Y27 nodular	29,290	29,343	32,359	38,582
nodular	22,950	16,567	29,417	36,076
02 nodular	31,021	56,753	71,196	36,904
non-nodular	46,675	59,546	40,388	45,103
Y43 nodular	36,735	27,113	22,408	19,169
non-nodular	15,176	17,767	15,739	17,650
Y51 nodular	32,826	25,456	50,126	42,427
non-nodular	33,304	32,582	31,329	26,199
B60 nodular	27,240	16,036	23,640	14,666
non-nodular	29,078	10,078	16,280	15,452

Appendix 20: Individual faecal egg counts (epg) and worm burdens of sheep in the initial study of the effects of haemonchosis.

Animal	Group	FEC	L_4	L_5	Adults*	Total
14	**SDI**	3900	200	1400	2900/1300	5800
20		8500	0	100	1350/1400	2850
21		2550	0	50	700/550	1300
25		1450	0	0	400/500	900
28		50	0	0	0/0	0
32		200	200	0	100/100	400
33		2250	400	50	150/300	900
34		30700	50	150	1100/900	2200
15	**SDC**	100	0	0	0/0	0
19		0	0	0	0/0	0
29		0	0	0	0/0	0
30		50	0	0	0/0	0
16	**BDI**	29000	350	300	350/350	1350
17		16300	100	750	450/450	1750
18		27700	200	300	100/400	1000
26		62600	0	200	100/1100	1400
27		16200	0	300	1400/1500	3200
31		14300	0	150	750/300	1200
35		18400	100	100	350/250	800
36		20600	0	300	1100/700	2100
22	**BDC**	0	0	0	0/0	0
24		0	0	0	200/100	300
37		0	0	0	0/0	0
38		0	0	0	0/0	0

* - adult worm burdens are given as numbers of males/ number of females.

Appendix 21: Individual packed cell volumes (PCV), and the concentrations of total plasma protein (T.P.), plasma albumin and plasma pepsinogen (P.peps.) of sheep in the initial study of the effects of haemonchosis.

Animal	Group	PCV (per cent)	T.P. (g/L)	Albumin (g/L)	P. peps. (iU)
14	**SDI**	30	60	30	5.03
20		27	48	26	2.19
21		28	59	31	3.06
25		28	58	30	15.10
28		30	65	31	5.49
32		28	61	33	5.44
33		27	59	30	8.04
34		24	61	30	7.09
15	**SDC**	32	66	32	0.66
19		28	63	31	2.63
29		32	63	33	7.09
30		30	66	33	2.52
16	**BDI**	26	56	28	14.22
17		20	52	24	2.63
18		21	53	25	5.25
26		22	45	23	4.80
27		19	45	19	4.35
31		21	51	20	3.89
35		28	51	28	9.46
36		24	58	27	8.27
22	**BDC**	32	65	31	0.00
24		30	60	31	4.58
37		29	58	28	0.00
38		29	57	29	0.95

Appendix 22: Individual faecal egg counts (epg) and worm burdens of sheep in the second study of the effects of haemonchosis.

Animal	Group	FEC	L_4	L_5	Adults*	Total
10	**SDI**	6900	750	100	1350/950	3150
11		9200	100	0	1300/1100	2500
15		7500	50	150	1000/700	1900
17		8700	0	200	950/950	2100
20		5900	0	150	1100/950	2200
23		50	0	100	1750/1550	3400
33		7600	0	200	1000/850	2050
35		5900	0	0	700/500	1200
7	**SDC**	650	0	0	0/0	0
16		500	0	0	0/0	0
26		0	0	0	0/0	0
36		0	0	0	0/0	0
9	**BDI**	15600	50	500	1350/850	2750
21		6300	0	450	1350/1100	2900
25		3300	50	150	1350/550	2100
29		21500	0	250	350/250	850
32		10600	100	0	1050/1150	2300
37		4300	150	200	1400/1000	2750
41		6400	0	500	1750/950	3200
42		9500	0	0	650/600	1250
18	**BDC**	0	0	0	0/0	0
19		0	0	0	0/0	0
28		0	0	0	0/0	0
38		0	0	0	0/0	0

* - adult worm burdens are given as numbers of males/ number of females.

Appendix 23: Individual packed cell volumes (PCV), and the concentrations of total plasma protein (T.P.), plasma albumin and plasma pepsinogen (P.peps.) of sheep in the second study of the effects of haemonchosis.

Animal	Group	PCV (per cent)	T.P. (g/L)	Albumin (g/L)	P. peps. (iU)
10	**SDI**	25	58	19	3.05
11		27	53	23	0.97
15		29	58	24	0.97
17		27	58	23	1.79
20		26	57	20	2.07
23		26	60	21	3.45
33		24	83	18	9.08
35		32	65	21	2.94
7	**SDC**	34	64	25	1.66
16		29	68	22	1.38
26		35	68	28	3.20
36		33	72	24	2.34
9	**BDI**	21	53	16	4.30
21		28	60	19	3.87
25		27	60	19	5.21
29		21	44	15	4.67
32		22	48	17	5.87
37		24	63	19	2.73
41		22	62	20	2.99
42		27	53	18	2.99
18	**BDC**	30	66	22	2.49
19		31	60	23	0.97
28		35	67	25	0.13
38		34	66	21	3.77

Appendix 24A: Individual and mean (± S.E.) values for wet weights of mucosal cuts from the Hampshire's in the second haemonchosis study.

Sheep	Group	Tissue A	B	C	D	E	Mean (± S.E.)
10	**SDI**	0.0655	0.0851	0.1052	0.0812	0.0952	0.0864 (0.0070)
11		0.0968	0.0791	0.0892	0.0856	0.0762	0.0854 (0.0037)
15		0.0894	0.1061	0.1057	0.1020	0.1042	0.1015 (0.0031)
17		0.0745	0.0926	0.0917	0.0724	0.0845	0.0831 (0.0042)
20		0.0876	0.0767	0.0840	0.1065	0.0701	0.0850 (0.0062)
23		0.0981	0.1093	0.1041	0.1093	0.0988	0.1039 (0.0024)
33		0.0944	0.0840	0.0899	0.1014	0.0953	0.0930 (0.0029)
35		0.1069	0.1314	0.1220	0.1409	0.1413	0.1285 (0.0065)
7	**SDC**	0.0716	0.0775	0.0893	0.0710	0.0732	0.0765 (0.0034)
16		0.0739	0.0696	0.0870	0.0793	0.0834	0.0786 (0.0031)
26		0.0723	0.0998	0.0921	0.0785	0.0643	0.0857 (0.0056)
36		0.0885	0.0681	0.0852	0.0805	0.0640	0.0773 (0.0048)
9	**BDI**	0.0886	0.0790	0.0760	0.0902	0.0823	0.0832 (0.0027)
21		0.0923	0.0804	0.0976	0.0900	0.1011	0.0903 (0.0041)
25		0.1037	0.1056	0.1002	0.1013	0.1050	0.1032 (0.0010)
29		0.0890	0.0930	0.1128	0.1046	0.0990	0.0997 (0.0042)
32		0.1053	0.1107	0.1239	0.1133	0.1050	0.1116 (0.0035)
37		0.1026	0.1026	0.0964	0.0988	0.0883	0.0977 (0.0026)
41		0.0963	0.1032	0.0975	0.1034	0.1201	0.1041 (0.0043)
42		0.1215	0.1131	0.1113	0.0993	0.0958	0.1082 (0.0047)
18	**BDC**	0.0870	0.0950	0.1060	0.0795	0.0790	0.0893 (0.0051)
19		0.0670	0.0860	0.0803	0.0715	0.0738	0.0757 (0.0033)
28		0.0895	0.1113	0.1011	0.1008	0.0902	0.0986 (0.0046)
38		0.0937	0.1089	0.0999	0.0927	0.1018	0.0994 (0.0029)

Appendix 24B: Individual and mean (± S.E.) values for pepsinogen concentrations (iU/area) of mucosal cuts from the Hampshire's in the second haemonchosis study.

Sheep	Group	Tissue A	B	C	D	E	Mean (± S.E.)
10	**SDI**	3757	2837	2785	2950	3084	3083 (176)
11		1876	2216	1601	1748	1695	1827 (107)
15		0	8	94.3	223	537	172 (100)
17		4163	4488	5594	3322	4415	4396 (364)
20		3023	2403	3000	3770	8612	4162 (1133)
23		4147	3783	4077	3453	3508	3794 (142)
33		1106	571	938	857	1420	978 (140)
35		10011	12283	5578	8388	5911	7892 (1377)
7	**SDC**	11211	11415	13019	8790	9555	10798 (744)
16		3699	5389	7527	5937	5586	5628 (612)
26		5811	5151	7409	4472	4734	5516 (525)
36		5468	6579	4884	3440	4721	5018 (512)
9	**BDI**	2777	2748	3846	3935	3629	3387 (260)
21		4640	5036	8237	3823	5363	5420 (750)
25		7884	6477	8329	3770	8612	7014 (890)
29		4559	4323	6741	8106	4603	5667 (751)
32		4968	5059	7739	5565	7530	6172 (606)
37		4307	4362	3817	5761	4307	4511 (328)
41		2743	4106	2416	2963	2607	2967 (298)
42		7061	8096	7265	4706	5452	6516 (623)
18	**BDC**	1847	2078	1669	1944	1433	1794 (112)
19		375	252	419	288	225	312 (37)
28		5059	14662	11651	12256	10726	10871 (1592)
38		2947	4640	3757	3851	3595	3758 (271)

Appendix 24C: Individual and mean (± S.E.) values for pepsinogen concentrations (iU/g) of mucosal cuts from the Hampshire's in the second haemonchosis study.

Sheep	Group	Tissue A	B	C	D	E	Mean (± S.E.)
10	**SDI**	57360	33343	26474	36331	32392	37180 (5292)
11		19379	28022	17946	20415	22246	21602 (1751)
15		0	74	892	2183	5154	1661 (958)
17		55882	48467	61000	45886	52245	52696 (2679)
20		34515	31324	35713	25831	35693	32615 (1876)
23		42278	34614	39162	31593	35508	36631 (1857)
33		11712	6799	10433	8449	14901	10459 (1392)
35		93648	93475	45721	40313	41831	62998 (12509)
7	**SDC**	156578	147295	145787	123804	130535	140800 (5959)
16		50060	77433	86520	74866	66976	71171 (6131)
26		80356	51612	80449	56972	73629	68608 (6034)
36		61785	96605	57320	42734	73769	66443 (9029)
9	**BDI**	31345	34790	50607	43628	44091	40892 (3468)
21		50271	62632	84398	42473	53048	58565 (7218)
25		76023	61332	83124	37218	82018	67943 (8607)
29		51222	46484	59763	77498	46498	56293 (5829)
32		47175	45702	62466	49116	71713	55234 (5085)
37		41981	42517	39599	58314	48780	46238 (3379)
41		28485	39782	24776	28658	21706	28681 (3059)
42		58114	71581	65276	47387	56913	59854 (4087)
18	**BDC**	21231	21870	15745	24453	18141	20288 (1516)
19		5592	2925	5220	4031	3053	4164 (545)
28		56528	131730	115244	121591	118917	108802 (13352)
38		31456	42608	37608	41547	35311	37706 (2044)

Appendix 25A: Individual plot profiles for the image analysis of the pepsinogen immunohistochemistry of animals from the BDC group, results are mean values of the condensed data from five scans per animal.

Animal			
18	**19**	**28**	**38**
4.2	0.2	6.8	4.2
11.0	1.0	10.8	14.6
12.8	0.8	13.2	15.4
15.6	1.2	10.0	12.2
15.4	1.2	8.4	10.2
13.8	1.2	7.8	10.2
12.8	1.0	6.2	10.6
12.4	1.2	4.6	10.8
11.6	1.0	3.4	10.2
9.4	1.8	3.0	9.2
7.6	1.4	2.4	9.4
7.6	1.2	2.4	8.4
8.0	1.2	2.8	6.8
7.6	0.8	3.4	5.6
7.2	1.0	2.8	4.2
5.8	1.0	3.2	3.4
6.0	1.0	2.6	3.6
5.4	1.2	2.2	3.4
4.6	1.2	1.6	3.4
3.6	1.2	1.4	2.6
2.8	0.8	1.6	2.2
2.0	1.2	1.2	1.8
1.0	1.0	0.8	1.8
0.8	0.8	0.6	1.6
0.8	0.8	0.2	0.6
0.8	0.8	0.0	0.6
0.6	0.6		0.2
1.0	0.6		0.6
1.0	0.0		0.0
0.6	0.4		
0.2	0.6		
0.2	0.6		
0.0	0.4		
	0.2		
	0.2		
	0.4		
	0.4		
	0.4		
	0.4		
	0.0		

Appendix 25B: Individual plot profiles for the image analysis of the pepsinogen immunohistochemistry of animals from the BDI group, results are mean values of the condensed data from five scans per animal.

Animal							
10	**11**	**15**	**17**	**20**	**23**	**33**	**35**
5.0	3.6	0.8	2.4	10.0	3.6	2.4	0.0
13.8	12.4	1.2	6.8	18.2	12.2	11.8	3.8
14.0	16.8	1.2	10.0	19.2	14.2	15.8	6.6
14.6	17.0	1.6	10.2	17.4	15.4	17.2	6.0
14.0	16.8	1.6	10.4	19.0	16.2	14.8	5.6
14.4	14.8	1.4	8.8	20.2	16.4	12.2	7.4
12.4	13.4	1.0	7.6	20.8	15.8	10.8	7.2
11.8	14.0	1.0	7.2	20.2	12.6	10.0	7.6
9.2	14.2	1.2	6.2	20.6	10.6	7.0	8.4
8.6	13.2	1.2	6.6	20.8	11.4	8.6	8.6
7.6	13.2	1.4	6.4	16.2	11.0	6.4	8.8
7.8	11.8	1.0	6.4	14.2	11.6	4.2	11.8
8.8	10.2	1.2	9.2	12.2	11.8	5.4	12.0
9.0	7.8	1.2	10.0	9.6	11.6	4.6	12.0
8.0	6.6	1.4	10.4	9.4	11.6	5.6	12.4
7.4	5.2	1.2	9.8	7.8	12.6	6.0	10.6
5.6	4.4	1.2	11.2	7.4	13.2	6.2	8.4
4.6	3.6	0.8	9.6	7.6	14.0	7.6	8.0
4.4	3.2	0.8	8.2	6.6	12.2	8.2	8.8
3.6	2.8	0.8	8.0	5.6	10.6	8.0	8.0
3.2	2.4	0.8	7.0	5.4	10.0	7.8	7.6
3.0	1.8	0.8	6.2	5.6	10.2	7.4	7.8
2.4	1.4	0.6	5.8	5.6	10.2	7.4	7.6
2.2	0.8	0.6	4.2	5.0	10.8	6.4	6.4
1.8	0.4	0.4	4.2	4.0	9.4	6.0	6.6
1.6	0.0	0.4	4.0	3.4	8.2	5.2	6.2
1.2		0.4	3.0	2.6	7.2	4.4	5.8
1.2		0.2	2.8	2.4	5.8	4.0	5.0
0.8		0.0	2.2	2.4	6.0	3.0	4.8
0.6			1.6	2.2	4.8	2.0	5.4
0.8			0.8	1.6	4.2	1.8	6.4
0.6			0.6	0.8	4.6	1.2	5.8
0.0			0.6	0.4	3.4	0.8	4.2
			0.0	0.2	2.6	0.2	3.0
				0.6	2.6	0.0	2.0
				0.0	1.4		1.2
					0.4		0.6
					0.6		0.2
					0.2		0.2
					0.0		0.0

Appendix 25C: Individual plot profiles for the image analysis of the pepsinogen immunohistochemistry of animals from the SDC group, results are mean values of the condensed data from five scans per animal.

Animal			
7	16	26	36
9.6	5.8	6.6	12.2
17.6	19.4	10.6	21.0
21.6	19.8	9.4	23.6
19.0	16.2	9.2	19.8
22.4	15.0	8.6	22.0
22.2	14.8	7.0	20.0
21.0	10.6	6.8	16.8
16.6	9.0	7.6	15.4
15.8	7.4	7.2	15.6
13.8	8.6	5.8	14.0
13.6	8.6	6.4	12.2
13.0	7.6	5.6	10.2
11.4	6.2	4.8	9.0
11.2	6.0	3.8	7.6
9.6	6.4	2.8	5.8
6.8	6.0	2.6	3.8
5.4	6.4	2.4	2.8
4.6	7.0	2.2	2.0
2.8	7.4	1.8	1.6
1.8	6.2	1.6	1.4
1.4	5.0	1.6	1.2
1.2	3.6	1.4	0.8
0.8	3.2	1.4	0.6
1.0	2.4	1.2	0.6
0.8	2.4	0.8	0.4
0.6	1.6	0.6	0.2
0.6	0.8	0.4	0.2
0.4	0.8	0.2	0.4
0.2	0.4	0.0	0.0
0.2	0.0		
0.0			

Appendix 25D: Individual plot profiles for the image analysis of the pepsinogen immunohistochemistry of animals from the SDI group, results are mean values of the condensed data from five scans per animal.

Animal							
9	**21**	**25**	**29**	**32**	**37**	**41**	**42**
5.0	8.0	0.4	6.6	5.0	7.6	4.2	4.6
15.4	16.4	2.4	10.8	17.0	10.4	6.0	12.4
14.6	20.2	4.0	9.6	16.2	12.0	7.7	13.6
17.0	18.2	3.8	8.8	14.0	11.6	8.7	14.2
12.4	17.0	2.4	10.4	15.0	13.6	8.0	14.2
11.4	16.6	2.2	10.0	14.4	12.8	8.0	17.2
11.0	14.4	2.8	9.8	12.4	13.8	6.5	15.8
10.4	14.6	2.8	8.4	12.6	13.0	6.0	13.4
10.4	13.2	2.1	9.6	12.6	13.0	6.2	13.4
10.4	13.4	2.6	8.8	10.6	13.8	6.5	13.4
9.8	11.8	2.4	7.6	9.0	14.6	6.0	14.4
11.8	11.0	2.8	7.2	8.2	12.4	5.0	14.4
12.6	13.4	3.4	6.0	7.0	9.8	5.0	13.2
8.2	19.4	2.6	5.0	7.2	11.4	4.6	13.2
6.6	22.8	2.4	3.6	6.8	11.6	4.2	10.8
6.6	20.4	1.6	2.8	7.2	11.6	3.0	9.4
5.6	18.6	1.6	2.2	7.0	11.0	2.7	9.6
4.6	18.6	1.4	2.0	7.8	9.8	3.0	8.0
3.6	18.0	0.8	1.8	9.4	9.2	2.2	8.0
4.0	19.6	1.0	1.4	9.2	8.4	2.0	7.8
3.6	18.0	1.0	1.4	9.4	8.0	2.0	6.8
3.0	16.0	1.0	1.0	11.0	6.8	2.0	5.8
2.6	14.0	0.6	0.8	10.0	6.0	1.7	5.0
2.4	11.8	0.6	0.6	11.6	4.6	1.5	4.0
1.6	12.2	0.2	0.4	11.0	3.8	1.2	3.6
0.8	12.4	0.0	0.2	11.8	3.4	1.2	2.6
0.4	9.8		0.0	10.6	2.4	1.5	1.6
0.0	7.6			9.0	1.8	1.2	1.0
	6.8			8.4	1.4	1.0	0.8
	7.8			7.6	1.2	0.5	0.6
	7.4			6.0	1.2	0.2	0.6
	7.0			5.2	0.6	0.5	0.6
	6.2			4.4	0.4	0.2	0.0
	4.6			4.0	0.2	0.0	
	3.8			3.2	0.0		
	3.0			2.0			
	2.4			2.4			
	1.6			2.0			
	1.8			2.0			
	1.4			2.0			
	0.6			0.8			
	0.0			0.2			

Appendix 26: Overall mean (± S.E.) plot profiles for control (8 animals) and infected (16 animals) Hampshire Down lambs.

Position	Control	Infected
1	6.20 (1.28)	4.32 (0.70)
2	13.25 (2.26)	10.69 (1.31)
3	14.57 (2.61)	12.23 (1.34)
4	12.90 (2.16)	12.23 (1.30)
5	12.90 (2.56)	11.96 (1.30)
6	12.13 (2.47)	11.76 (1.31)
7	10.73 (2.23)	10.97 (1.27)
8	9.70 (1.85)	10.35 (1.19)
9	9.02 (1.89)	9.87 (1.21)
10	8.20 (1.59)	9.91 (1.17)
11	7.70 (1.51)	9.16 (1.06)
12	7.00 (1.37)	8.85 (1.03)
13	6.28 (1.17)	8.82 (0.94)
14	5.75 (1.12)	8.59 (1.13)
15	4.97 (0.99)	8.39 (1.29)
16	4.07 (0.69)	7.70 (1.21)
17	3.77 (0.69)	7.24 (1.17)
18	3.50 (0.71)	6.94 (1.18)
19	3.05 (0.74)	6.59 (1.16)
20	2.47 (0.60)	6.30 (1.20)
21	2.07 (0.47)	5.90 (1.12)
22	1.65 (0.31)	5.59 (1.07)
23	1.32 (0.30)	5.11 (1.00)
24	1.12 (0.22)	4.59 (0.98)
25	0.85 (0.23)	4.17 (0.97)
26	0.65 (0.17)	3.84 (1.00)
27	0.42 (0.10)	3.18 (0.86)
28	0.50 (0.11)	2.62 (0.72)
29	0.20 (0.12)	2.35 (0.68)
30	0.15 (0.08)	2.14 (0.68)
31	0.10 (0.08)	1.94 (0.64)
32	0.10 (0.05)	1.72 (0.60)
33	0.05 (0.05)	1.29 (0.51)
34	0.02 (0.02)	0.92 (0.40)
35	0.02 (0.02)	0.76 (0.33)
36	0.05 (0.05)	0.47 (0.23)
37	0.05 (0.05)	0.36 (0.20)
38	0.05 (0.05)	0.27 (0.16)
39	0.05 (0.05)	0.26 (0.16)
40	0.00 (0.00)	0.21 (0.15)
41	0.00 (0.00)	0.09 (0.06)
42	0.00 (0.00)	0.01 (0.01)

Appendix 27: Level of significance (probability) for factors influencing staining intensity at different mucosal positions. Figures in bold script are of borderline significance ($0.05<p<0.10$)$^{+}$ or are significant ($p<0.05$)*.

	Position 1	2	3	4	5
Model	**0.0449***	0.1606	0.2309	0.4437	0.2506
Diet	0.2271	0.1773	0.1272	0.1703	0.1819
Infection	0.1350	0.2814	0.3644	0.7790	0.7050
Diet*Inf.	**0.0151***	**0.0627^{+}**	0.1261	0.2230	0.1390

6	7	8	9	10	11
0.3101	0.4747	0.5630	0.5550	0.4436	0.3332
0.1115	0.1837	0.2282	0.3039	0.2603	0.2340
0.8834	0.9183	0.7636	0.7804	0.4040	0.4268
0.1463	0.2280	0.3038	0.2313	0.2481	0.1378

12	13	14	15	16	17
0.3646	0.2876	0.3634	0.3653	0.2942	0.3215
0.3016	0.3266	0.5788	0.6409	0.7523	0.8155
0.3016	0.1246	0.1384	0.1086	**0.0675^{+}**	**0.0717^{+}**
0.1930	0.3416	0.3491	0.5026	0.6552	0.7790

18	19	20	21	22	23
0.3382	0.3081	0.2485	0.1906	0.1466	0.1347
0.7939	0.8653	0.9573	0.9306	0.9331	0.8995
0.0767^{+}	**0.0659^{+}**	**0.0512^{+}**	**0.0360***	**0.0244***	**0.0214***
0.8250	0.8334	0.7282	0.7280	0.8368	0.9189

24	25	26	27	28	29
0.1696	0.1941	0.2406	0.2421	0.3045	0.2530
0.9299	0.9064	0.9412	0.9810	1.000	1.000
0.0289*	**0.0349***	**0.0469***	**0.0464***	**0.0633^{+}**	**0.0489***
0.8834	0.8271	0.8376	0.8903	0.9272	0.9233

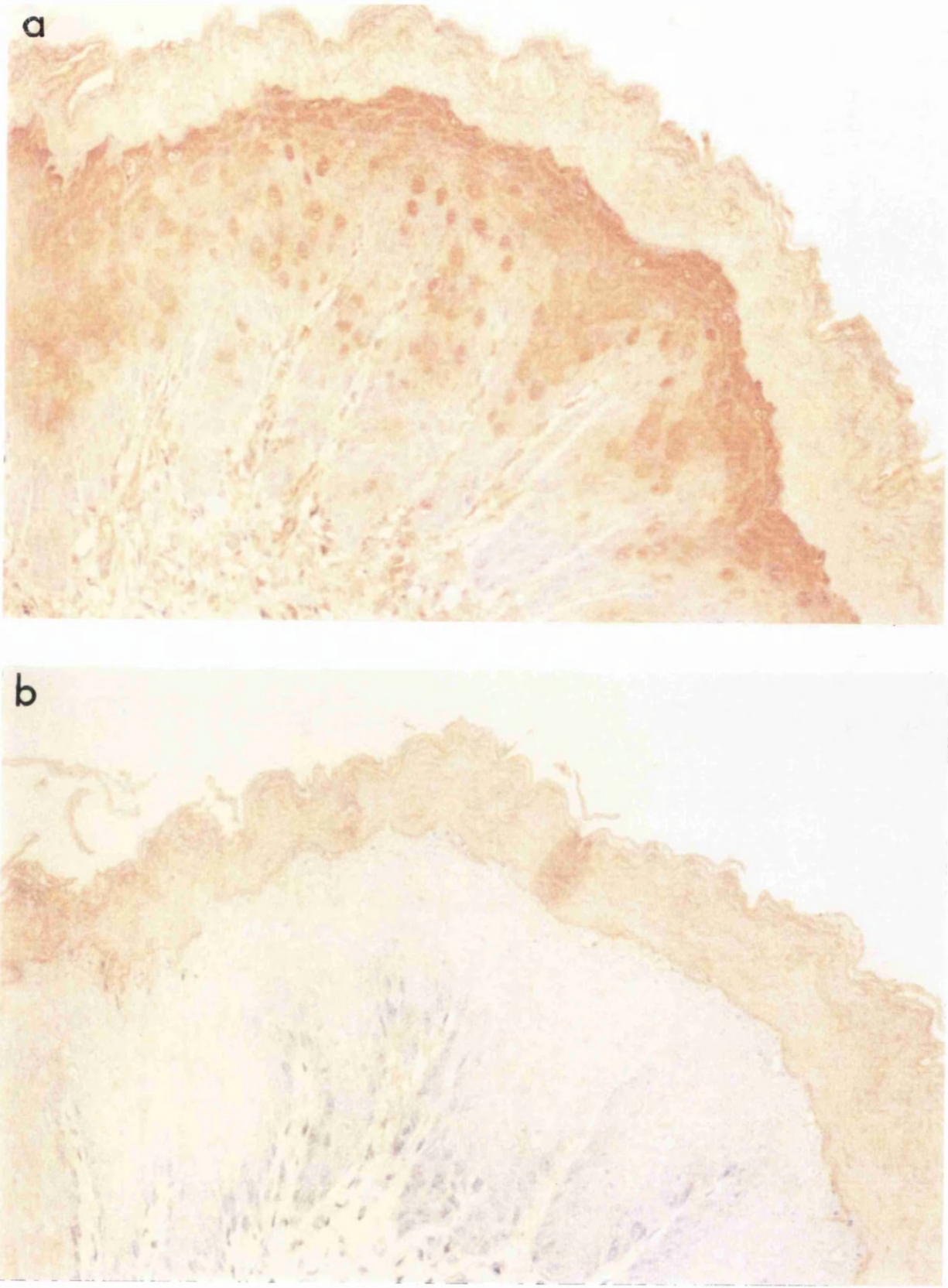
30	31	32	33	34	35
0.3086	0.3266	0.3840	0.4558	0.4953	0.5151
0.8704	0.9283	0.8970	0.8330	0.7439	0.7848
0.0651^{+}	**0.0702^{+}**	**0.0892^{+}**	0.1195	0.1517	0.1553
0.9759	0.9080	0.9294	0.9354	0.8063	0.8620

36	37	38	39	40	41	42
0.5553	0.3918	0.4279	0.3469	0.2657	0.2603	0.6012
0.5664	0.3381	0.3284	0.2661	0.3140	0.3107	0.4981
0.2297	0.2988	0.3284	0.3654	0.3140	0.3107	0.4981
0.7736	0.5294	0.5840	0.4870	0.3140	0.3107	0.4981

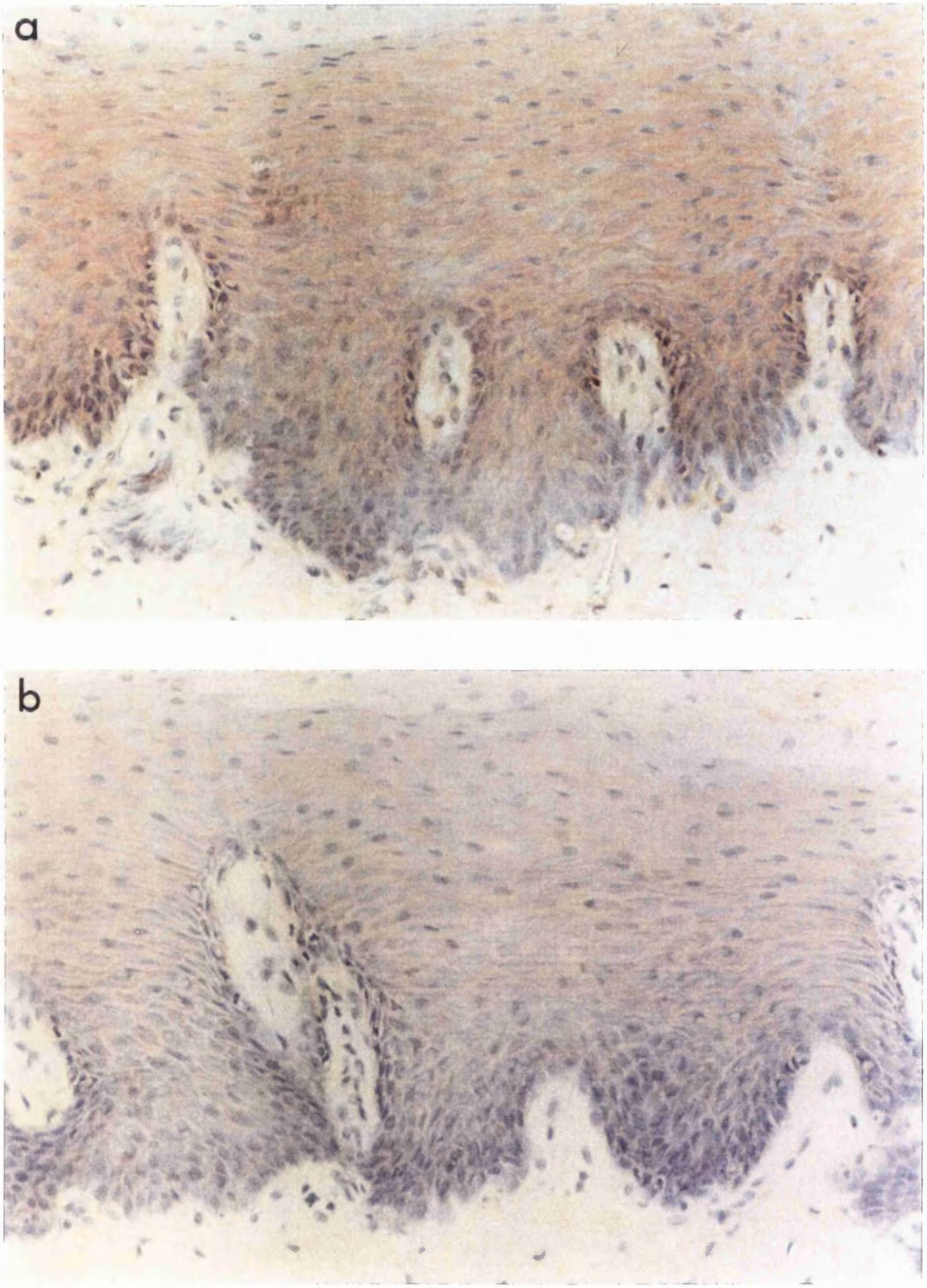
Appendix 28: Individual values of AUC, Imax and Pmax following image analysis of the pepsinogen immunohistochemistry for the second study of haemonchosis in Hampshire Down lambs.

Animal	Group	AUC	Imax	Pmax
10	**SDI**	4.08	14.6	4
11		4.24	17.0	4
15		0.55	1.6	4
17		4.17	11.2	17
20		6.50	20.8	7
23		7.02	16.4	6
33		4.61	17.2	4
35		4.98	12.4	15
7	**SDC**	5.34	22.4	5
16		4.29	19.8	3
26		2.41	10.6	2
36		4.82	23.6	3
9	**BDI**	4.12	17.0	4
21		9.64	22.8	15
25		0.98	4.0	3
29		2.74	10.8	2
32		6.86	17.0	2
37		5.46	14.6	11
41		2.40	8.7	4
42		5.48	17.2	6
18	**BDC**	3.88	15.6	4
19		0.65	1.8	10
28		2.07	13.2	3
38		3.36	15.4	3

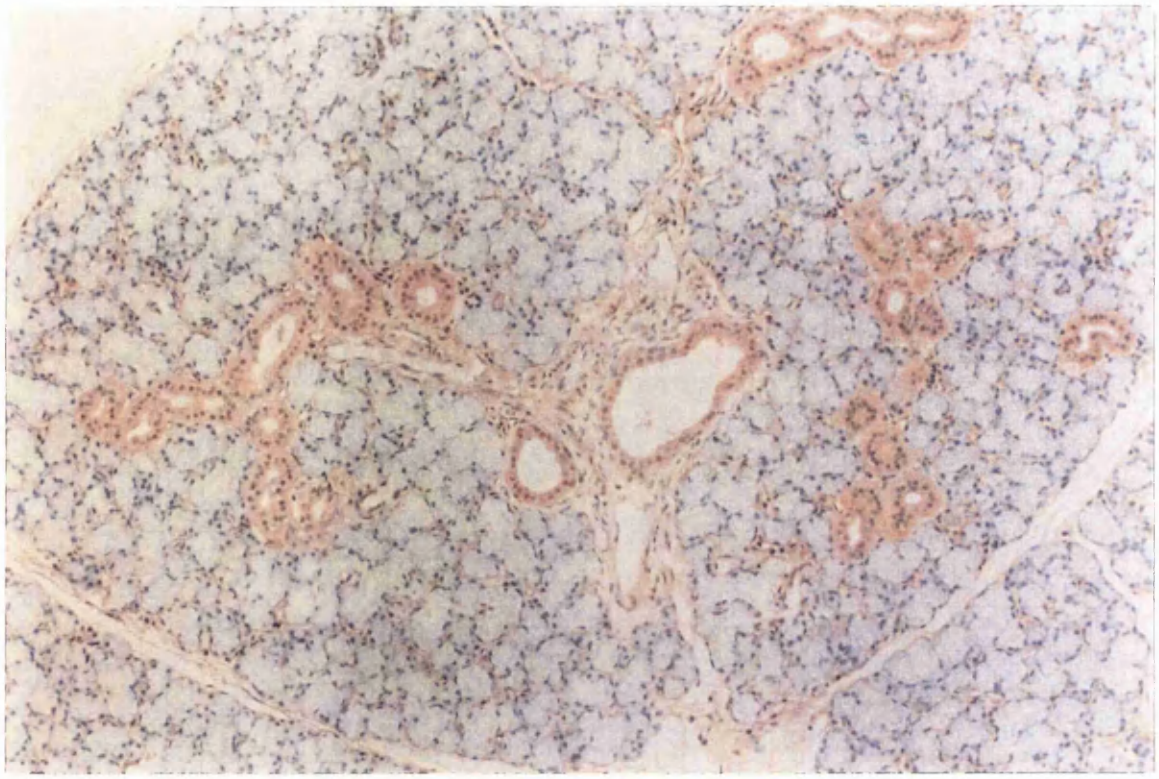
Appendix 29: TGF-α (a) and EGF (b) immunostaining in rat gastric cardia.



Appendix 30: TGF-α (a) and EGF (b) immunostaining in ovine oesophagus.

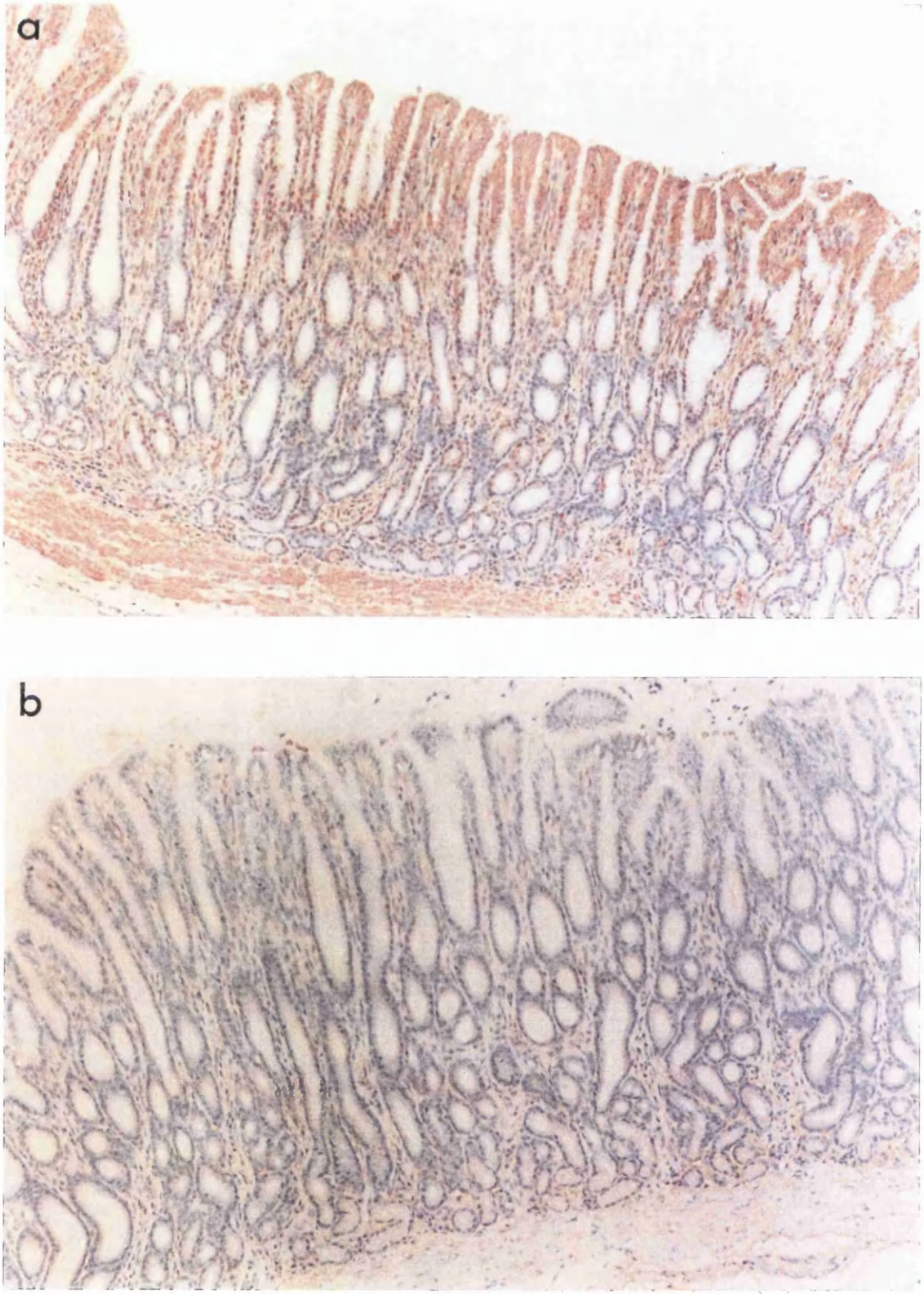


Appendix 31: EGF immunostaining in ovine sub-mandibular salivary gland.



Appendix 32: EGF immunostaining in ovine abomasal fundus.

Appendix 33: TGF-α (a) and EGF (b) immunostaining in ovine pylorus.



Appendix 34: TGF-α (a) and EGF (b) immunostaining in ovine colon.